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Research article

Single mRNA detection of Wnt signaling pathway in the human limbus

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ABSTRACT

Limbal epithelial stem/progenitor cells (LSCs) are adult stem cells located at the limbus, tightly regulated by their close microenvironment. It has been shown that Wnt signaling pathway is crucial for LSCs regulation. Previous differential gene profiling studies confirmed the preferential expression of specific Wnt ligands (WNT2, WNT6, WNT11, WNT16) and Wnt inhibitors (DKK1, SFRP5, WIF1, FRZB) in the limbal region compared to the cornea. Among all frizzled receptors, frizzled7 (Fzd7) was found to be preferentially expressed in the basal limbal epithelium. However, the exact localization of Wnt signaling molecules-producing cells in the limbus remains unknown. The current study aims to evaluate the in situ spatial expression of these 4 Wnt ligands, 4 Wnt inhibitors, and Fzd7. Wnt ligands, DKK1, and Fzd7 expression were scattered within the limbal epithelium, at a higher abundance in the basal layer than the superficial layer. SFRP5 expression was diffuse among the limbal epithelium, whereas WIF1 and FRZB expression was clustered at the basal limbal epithelial layer corresponding to the areas of high levels of Fzd7 expression. Quantitation of the fluorescence intensity showed that all 4 Wnt ligands, 3 Wnt inhibitors (WIF1, DKK1, FRZB), and Fzd7 were highly expressed at the basal layer of the limbus, then in a decreasing gradient toward the superficial layer ($P < 0.05$). The expression levels of all 4 Wnt ligands, FRZB, and Fzd7 in the basal epithelial layer were higher in the limbus than the central cornea ($P < 0.05$). All 4 Wnt ligands, 4 Wnt inhibitors, and Fzd7 were also highly expressed in the limbal stroma immediately below the epithelium but not in the corneal stroma ($P < 0.05$). In addition, Fzd7 had a preferential expression in the superior limbus compared to other limbal quadrants ($P < 0.05$). Taken together, the unique expression patterns of the Wnt molecules in the limbus suggests the involvement of both paracrine and autocrine effects in LSCs regulation, and a fine balance between Wnt activators and inhibitors to govern LSC fate.

1. Introduction

The basal epithelium of the human limbus contains a distinct population of adult epithelial stem cells called limbal stem/progenitor cells (LSCs) (Davanger, 1971; Tseng, 1989). LSCs closely interact with their microenvironment, referred to as the stem cell niche, consisting of stromal cells, melanocytes, and extracellular matrix, in a highly vascularized and innervated stroma (Dziasko, 2016a; Scadden, 2006). These interactions confer dual functions on the limbus, i.e., formation of a barrier between the corneal and conjunctival epithelium, and renewal of corneal epithelial cells (Cotsarelis, 1989). Both functions are necessary to maintain homeostasis of the corneal epithelium, the integrity of the ocular surface, and visual function under physiologic conditions. Dysfunction or deficiency of LSCs and/or their niche can lead to limbal stem cell deficiency (LSCD), a condition that can cause corneal blindness (Bonnet, 2021d; Deng, 2019). Therapies of LSCD include surgical management when the LSC dysfunction cannot be medically treated and/or when the LSC pool needs to be restored (Deng, 2020b). Surgical techniques have been optimized to reduce the size of the donor's tissue required (Basu, 2016), and cell-based therapies from ex vivo LSC

Abbreviations: DKK1, dickkopf WNT Signaling Pathway Inhibitor 1; FRZB, frizzled-related protein; FZD, frizzled; ISH, in-situ hybridization; LSC, limbal stem cell; LSCD, limbal stem cell deficiency; SFRP5, secreted frizzled-related protein 5; WIF1, WNT inhibitory factor 1.

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expansion have been developed (Pellegrini, 1997; Rama, 2010). Direct and cultivated LSCs transplantation methods have been reported with high survival rates (Le, 2020), up to 76.6% 10 years after transplantation (Rama, 2010). In the scope of reducing the amount of donor tissue necessary for transplantation, enhancing the outcomes of transplantation from LSCs, and developing targeted therapies, it is important to identify the limbal niche factors involved in LSC regulation. Ultimately, understanding the mechanisms of regulation of the limbal niche will aid in the development of feeder layer-free substrates, mimicking the in vivo characteristics of the human limbus, while alleviating any xenobiotic risk (Levis, 2016; Robertson, 2021; Yazdani, 2019; Yazdanpanah, 2019).

Among the multiple and intertwined signaling pathways involved in LSC regulation (Bonnet, 2021b), Wnt signaling pathway activation has been reported in several studies (Han, 2014; Nakatsu, 2013; Vattulainen, 2019). We previously showed that 4 Wnt ligands, WNT2, WNT6, WNT11, and WNT16b, and 4 Wnt inhibitors, dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1), secreted frizzled-related protein 5 (SFRP5), frizzled-related protein (FRZB), and WNT inhibitory factor 1 (WIF1), are preferentially expressed in the limbus compared with the central cornea (Nakatsu, 2011). Activation of the Wnt/β-catenin pathway by small molecules (Gonzalez, 2019; Nakatsu, 2011; Zhang, 2020) or exogenous Wnt6 (Bonnet, 2021c) can also promote LSC proliferation in vitro. Moreover, frizzled7 (Fzd7), the putative stem cell marker and a main Wnt receptor in LSCs, plays a crucial role in the maintenance of the undifferentiated LSC phenotype (Mei, 2014b). This mounting evidence highlights the critical role of Wnt signaling in LSC regulation (Bonnet, 2021a; Bonnet, 2021b).

Wnt proteins are powerful morphogen growth factors with a short range of action (Alexandre, 2014) because of their palmitoylation (Clevers, 2012). This lipid group renders them hydrophobic and thus attracted to neighboring cell membranes or Wnt receptors. Upon secretion of Wnt proteins, the mechanisms of their transport to target cells remain unclear. Different models have been established to support their short range of action (Clevers, 2014). Some Wnt proteins may be incorporated into secretory vesicles that could operate over a short distance, through a paracrine signaling in the stem cell niche. For example in the intestinal niche, Paneth cells secrete Wnt ligands that bind to the intestinal stem cells receptors (Sato, 2011). In addition, interfollicular epidermis stem cells can produce their own Wnt ligands, driving their own self-renewal in an autocrine manner (Lim, 2013). In LSC culture models, the feeder layer, acting as a stem cell niche secreting Wnt ligands (Schrader et al., 2014), is critical for LSC proliferation in vitro, suggesting that paracrine signaling occurs in LSC proliferation in vitro. Although several transcriptome-profiling studies of the human limbus have been performed, the samples used in the studies contained multiple cell types and did not identify the types of Wnt-producing cells or their precise location (Collin, 2021; Dou, 2021; Kaplan, 2019; Li, 2021; Sun, 2021). Whether LSCs are capable of autocrine Wnt signaling in vivo still remains unknown, highlighting the need for more spatio-molecular data, to connect the limbal structure to its function of stem cell renewal.

Traditional histologic methods are limited by the lack of reliable antibodies and the number of markers that are detectable simultaneously; as a result, these methods lack the ability to characterize spatio-molecular details necessary to decipher the complex molecular and cellular structure of the LSC niche. Conversely, the recent development of small-molecule RNA fluorescent in situ hybridization (smFISH) methods allows for single-molecule in situ RNA localization and quantitation (Femino, 1998; Raj, 2008). Compared with traditional smFISH methods, the RNAscope in situ hybridization (ISH) assay could offer higher specificity and sensitivity in different tissue types (Nakajima, 2020; Yu, 2020).

In the present study we evaluated the spatial location of the 4 Wnt ligands and 4 Wnt inhibitors whose expression is upregulated in the limbus (Nakatsu, 2011), and we correlated their location with that of Fdz7 using the RNAscope ISH technology in the human limbus and central cornea. Wnt signaling pathway molecules were mostly preferentially expressed in the limbal stroma and at the basal epithelial layer of the limbus, and their expression was highly correlated with the localization of Fzd7. A gradient expression pattern was found within limbal epithelial layers. Overall, these results suggest that Wnt signaling in the limbus acts by both paracrine and autocrine signaling, providing new insights into the importance of the LSC niche molecular hierarchical structure to its functions.

2. Material and methods

2.1. Human corneoscleral tissue

Human corneoscleral tissues from 5 healthy donors (median age, 51 years; range, 22–74 years) were obtained with permission for research from Saving Sight (Kansas City, MO, n = 2 donors), EverSight (Clark, NJ, n = 2 donors), and CorneaGen (Orlando, FL, n = 1 donor). Criteria for study eligibility were the following: death-to-preservation time ≤8 h (range, 7h10 min to 8h), clear cornea with minimal epithelial exposure and no sloughing, no history of systemic or topical chemotherapy, no history of glaucoma medication use, and no history of ventilation. The use of postmortem human tissues was exempted by the University of California Institutional Review Board (IRB#12–000363). All tissues were preserved at 4 °C in Optisol-GS (Chiron Ophthalmics Inc., Irvine, CA). Under a stereomicroscope on dry ice, tissues were cut into 4 quadrants corresponding to the superior, inferior, nasal, and temporal areas. Each quadrant was embedded in a compound designed for optimal cutting temperature (Tissue-Plus, #4585, Fisher Scientific, Hampton, NH) on dry ice within 30 min of receiving the tissue and preserved at −80 °C. To prevent RNA damage, the tissues from each quadrant were sectioned by a cryostat (ThermoFisher Scientific, Waltham, MA) within a week of cryopreservation, sections were then placed overnight in −80 °C and used the following day. Experiments using human tissues adhered to the tenets of the Declaration of Helsinki.

2.2. In situ hybridization

The RNAscope Multiplex Fluorescent V2 Assay (Advanced Cell Diagnostics, Newark, CA) was performed on 12-μm fresh frozen sections according to the ACD protocol for fresh-frozen tissue without target retrieval (protocol #323100-USM). Each section was contained the limbal region and a section of the peripheral and central cornea. The following modifications to the protocol were made: slides underwent pretreatment for 1 h by baking at 60 °C, permeabilized by using Protease Plus (#322330, ACD, Newark, CA) for 15 min at 40 °C, and fixed in 4% paraformaldehyde (paraformaldehyde 16% aqueous solution, #C004, Electron Microscopy Science, Hatfield, PA, dilution 1:4) for 20 min. Cryosections were then hybridized with 3 mRNA probes per experiment (Supplemental Table 1).

A library of 9 probes, each composed of 20 Z-pairs complementary to sequences of genes involved in Wnt pathway regulation in the limbus was used (Mei, 2014b; Nakatsu, 2011). These probes were *WNT2*, *WNT6*, *WNT11*, *WNT16*, *DKK1*, *SFRP5*, *FRZB*, *WIF1* (Nakatsu, 2011), and a Wnt receptor *FZD7* (Mei, 2014b). FZD7 was combined with one Wnt ligand and one Wnt inhibitor, each of different channel, to avoid overlap during signal detection. The probes were amplified according to the manufacturer's instructions and labeled with the fluorophore Opal 520 (Akoya Biosciences, FP1487001KT; dilution ratio, 1:250), Opal 570 (Akoya Biosciences, FP1488001KT; dilution ratio, 1:1500), and Opal 690 (Akoya Biosciences, SKU FP1497001KT; dilution ratio, 1:1500). To confirm the mRNA integrity of tissue blocks, positive-control probes targeted against the human housekeeping genes RNA polymerase II subunit A (*POLR2A*), peptidylprolyl cis-trans isomerase B (*PPIB*), and hypoxanthine guanine phosphoribosyltransferase (*HPRT*), were used on one section in each individual experiment. To confirm signal specificity,

negative-control targeting against bacterial dihydrodipicolinate reductase from *B. subtilis* (*dapB*) gene, scrambled in the 3 channels, was used on one section in each individual experiment. (Supplemental Fig. 1).

2.3. Detection and quantitation of the fluorescence in situ hybridization signals

An upright confocal microscope was used to visualize the fluorescent ISH signals in the limbus and the central cornea at 40X magnification. Images were acquired by using the following parameters: 4 channels (405, 488, 570, 690 nm), sequential mode, 12 bits/pixel, numeric aperture of 0.95, dimension of 1600*640 pixels, and 12 *z*-stack scans acquired every 1 μm and processed by Maximal Projection on the Olympus FluoView FV1000 v. 4.2.2.9 software.

Quantitation of the spots among the limbal and corneal layers was performed with the surface and spot functions of Imaris software (V. 9.7.0, Imaris, Oxford Instruments, Oxon, UK). Epithelium was defined as the layer of positive nuclei counterstained with DAPI. Segmentation into basal, intermediate, and superficial layers was performed as follow: the basal layer was identified as the layer containing 1 or 2 cells at the junction with the stroma, presenting with very high cell density, having a vertical cell orientation, and possessing a small nucleus when compared with the rest of the epithelium (Romano, 2003). The superficial layer was defined as the most superficial layer of cells, having a larger nucleus and a horizontal cell orientation. The suprabasal layer was defined as the layer between the superficial and basal layers. The corresponding 3 areas were manually drawn and applied onto the 12 *z*-stacks for volume reconstruction (Supplemental Fig. 2A). Spots were detected by the spot function, which allowed for background subtraction and definition of the spot sizes (i.e., 1.63 μm for Opal 520, 2.01 μm for Opal 570, and 1.61 μm for Opal 647). Spot quality thresholds were set individually for each channel, and the same detection thresholds were used for quantitation consistency among the different experiments. The spots were automatically counted by Imaris processing software (Supplemental Figs. 2B–C).

2.4. Statistical analysis

To control for the thickness variations between limbal and corneal epithelia, the number of spots counted in each image was normalized to the epithelial volume. Analysis was performed by a biostatistician (C.H. T). Student's *t*-test and analysis of variance were performed using R software (www.r-project.org). A P value < 0.05 was considered to indicate statistically significant.

3. Results

3.1. Wnt signaling pathway molecules and LSC marker are differentially expressed in the limbus

Single-molecule RNAscope ISH enabled sensitive and specific in situ transcript detection in the human limbus (Fig. 1A-R) and central cornea (Fig. 1S–T). All 4 Wnt ligands, DKK1, and FZD7 appeared as single spots scattered predominantly in the basal limbal epithelium at a variable density (Fig. 1A-P), whereas SFRP5 was observed among all the limbal epithelial layers (Fig. 1I, M). FRZB and WIF1 were often present as very distinct clusters indicated by high-density signals at the basal layer of the limbus (Fig. 1H,K,L,O,P). These clusters colocalized with areas of FZD7 signals of high density in the basal limbal epithelium (Fig. 2).

Quantitation of these expression profiles retrieved high expression of all 4 Wnt ligands, 4 Wnt inhibitors, and FZD7 in the limbal stroma but not in the central cornea stroma (Supplemental Fig. 3), confirming the role of Wnt signaling in LSC regulation in the limbal niche. All 4 Wnt ligands, 3 inhibitors (WIF1, DKK1, FRZB), and FZD7 were preferentially expressed in the basal layer of the limbus (Fig. 3A–E, K), and the expression decreased in a gradient manner toward the superficial limbal epithelial layer. In the central cornea, expression of 1 Wnt ligand (WNT6), 3 Wnt inhibitors (WIF1, DKK1, SFRP5), and FZD7 was high in the basal layer. The expression level decreased toward the superficial epithelial layer in a gradient manner (Fig. 3F–K).

When compared to the central corneal basal layer, the basal layer of the limbus showed greater spatial expression of 4 Wnt ligands, FRZB, and FZD7 (Fig. 4). No expression in a cluster pattern was detected by any of the probes in the central cornea.

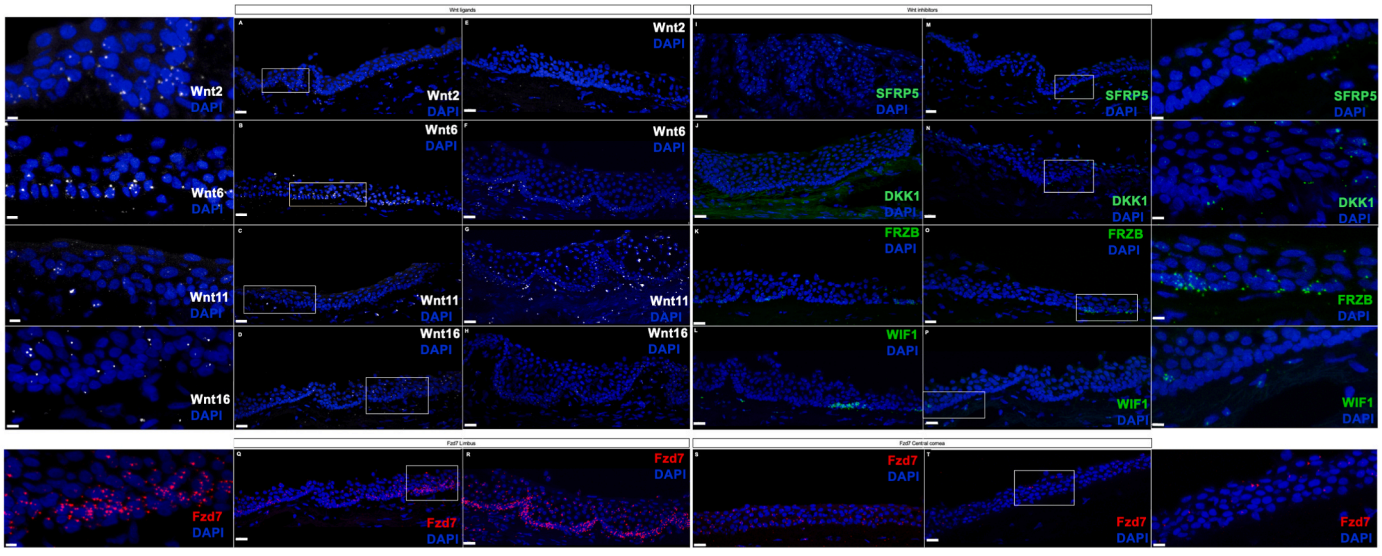

Fig. 1. Expression of Wnt signaling pathways ligands and inhibitors in the human limbus and of Frizzled7 in the human limbus and central cornea.
The assay allows specific and sensitive detection of the mRNA expression of Wnt signaling molecules. The mRNA expression of Wnt ligands (A–H) and some Wnt inhibitors (I–P) were preferentially expressed in the basal layer of the limbus. The expression of Fzd7, a putative LSC marker was preferential in the basal layer of the limbus (Q–R). The level of expression was low in the central cornea (S–T). Scale bar: 20 μm. High-magnification pictures represent the white box area in each picture. Scale bar: 5 μm.

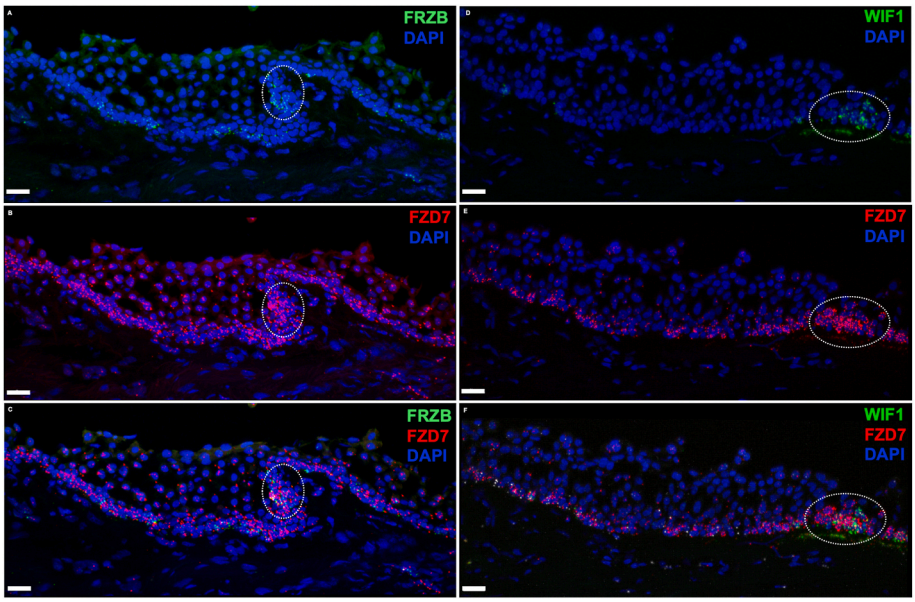

Fig. 2. High mRNA level of FRZB and WIF1 co-localized with high level of Fzd7 in the human limbus.
FRZB and WIF1 are expressed as clusters colocalizing with high-density Fzd7 signals (white circles).
A–C: Fzd7 and FRZB, E–F: Fzd7 and WIF1. Scale bar: 20 μm.

3.2. The LSC marker Fzd7 is preferentially expressed in the basal layer of the superior quadrant

Spatial expression profiles retrieved preferential expression of FZD7 in the basal layer of the superior quadrant compared to the other limbal quadrants (Fig. 5).

4. Discussion

In this study, we were able to provide a map of the expression patterns of Wnt signaling components previously shown to be preferentially expressed in the limbus by gene profiling. As summarized in Fig. 6, most of the Wnt ligands evaluated are expressed in a gradient manner in the basal limbal epithelial layer decreasing towards the superficial layers, whereas in the central cornea, only Wnt6 presents a decreasing gradient of expression. Conversely, most of the Wnt inhibitors present a decreasing gradient of expression towards the superficial layers in the central cornea, but not in the limbus.

4.1. Expression gradients

Wnt gradients have been reported in many epithelial tissue models such as the hair follicle (Sick, 2006), the intestine crypts (Clevers, 2013), the colon (Gregorieff, 2005), and the adrenal glands (Basham, 2019). Such models provide critical information on the function of some of these factors, where varying levels of Wnt/β-catenin signaling have different functional consequences for stem cell proliferation and maintenance. In the limbus, the Fzd7, a canonical and non-canonical Wnt receptor (Bonnet, 2021a) was found to co-localize with both Wnt signaling molecules involved in both canonical and non-canonical pathways: Wnt6 is a canonical and non-canonical activator in LSCs (Bonnet, 2021c); Wnt16 is a Wnt/β-catenin independent activator in LSCs (Zhao, 2022); Wnt2 and Wnt11, act as non-canonical ligands in many cell types (Fan, 2017); and DKK1 is a Wnt/β-catenin inhibitor (Bourhis, 2011). This observation suggests that both canonical and noncanonical Wnt pathways are tightly regulated by specific stromal and epithelial cells to maintain LSCs homeostasis. Varying temporal and spatial levels of canonical and non-canonical Wnt activation (Kestler, 2011) are likely intertwined and do not function in isolation or independently. Differential expression pattern between Wnt ligands, which are mostly expressed in the basal layer (Nakatsu, 2011), and Wnt inhibitors, which are more diffusely expressed within the epithelium, may create a highly regulated activation gradient from the basal towards the superficial limbal epithelial layer, similar to that described in the interfollicular epidermidis (Lim, 2013). In this scenario limbal epithelial cell would encounter an increased concentration of Wnt inhibitors as they differentiate and migrate out of the basal layer.

How gradients of secreted Wnt molecules regulate stem cell proliferation and how Wnt molecules travel to interact with their target cells is still unclear. Various modes of Wnt molecules secretion and signaling have been reported, such as extra-cellular vesicles and exosomes (Gross, 2012). For example, miR-6723-5p is upregulated in the limbal basal layers (Ruiz, 2022). miRNAs have also been reported in Wnt signaling regulation during embryogenesis (Nagai, 2019). They are promising therapeutic tools to treat corneal injuries and scars (Deng, 2020a).

Wnt signaling has also been shown to promote corneal wound healing in several animal models by activating metalloproteinase 12 (Lyu, 2005; 2006) and promoting proliferation and migration of corneal epithelial cells (Liang, 2022; Yang, 2020; Zhong, 2021). Understanding the individual molecular mechanisms involved in the limbus and the central cornea could lead to the development of targeted Wnt therapies, promoting both proliferation of LSCs and corneal wound healing.

4.2. Paracrine signaling

The importance of the limbal niche for LSC regulation has been extensively demonstrated over the past 30 years (Dziasko, 2016b; Dziasko, 2014; Levis, 2016; Seyed-Safi, 2020; Tseng, 2016). In ex-vivo LSCs expansion models, the presence of a feeder layer, either in direct contact or using a three-dimensional sandwich model, mimics the niche and supports the cell expansion by secreting soluble niche growth factors, cytokines, and through cell-cell interactions (Mei, 2014a). Moreover, the presence of native limbal stromal cells (Gonzalez, 2013) or of exogenous Wnt activator (Gonzalez, 2019) can increase the expansion efficiency of LSCs in culture. The current manuscript correlates the functions of the limbal stroma and Wnt signaling reported in ex-vivo LSCs proliferation with the preferential expression of Wnt molecules in limbal stromal cells.

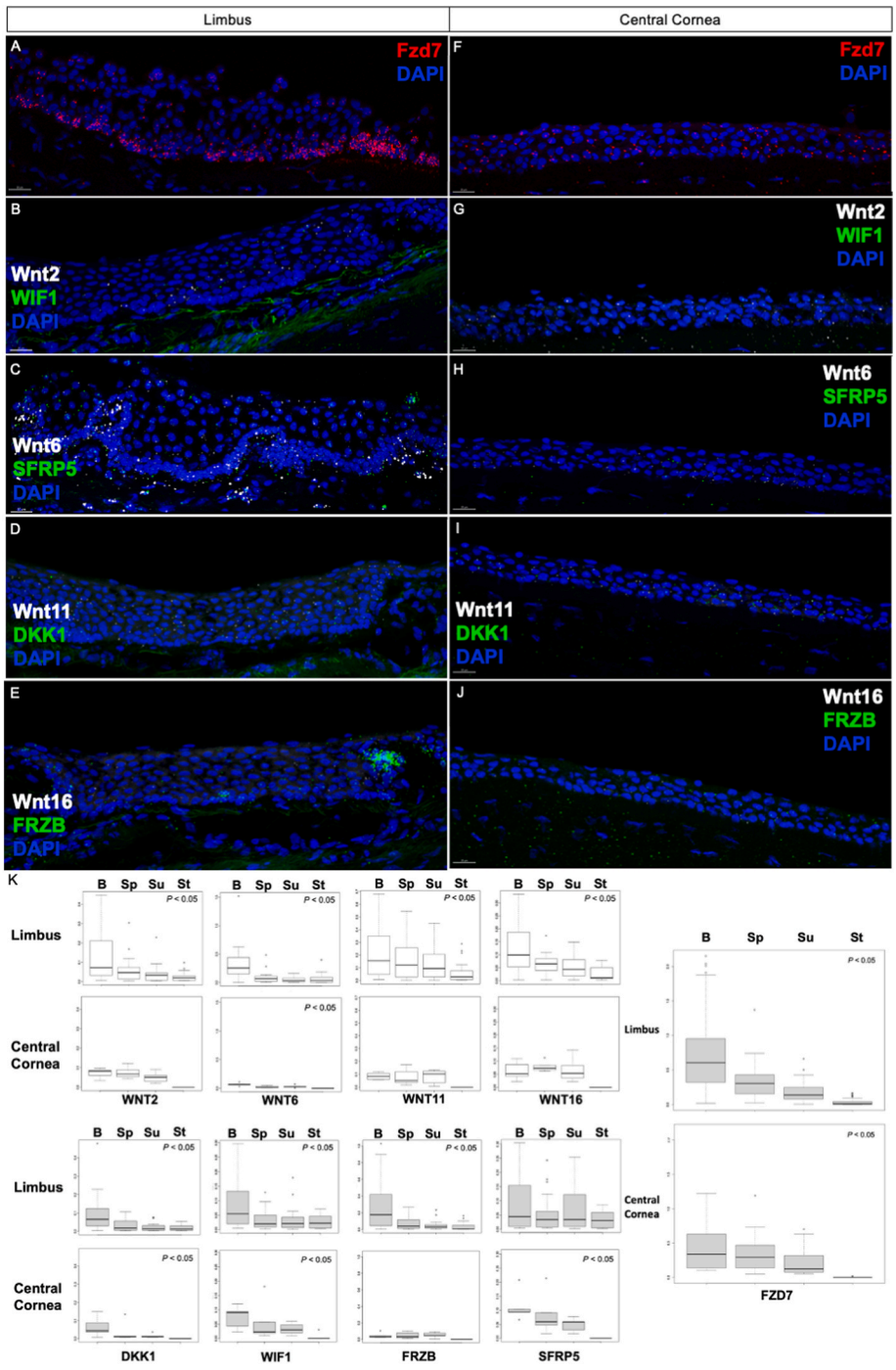

Fig. 3. Quantification of Wnt signaling molecules in the limbus and cornea.
A–E: Limbus, F–J: Central cornea Scale bar: 20 μm.
K: Box and whisker plots of the relative level of expression Wnt signaling pathway molecules in the limbus and the central cornea. All four Wnt ligands, WIF1, DKK1, FRZB, and FZD7 are expressed preferentially in the basal limbal epithelial layer, with a significant decrease of expression towards the superficial layer in a gradient manner. In the central cornea, the same gradient of expression is observed for WNT6, WIF1, DKK1, SFRP5, and FZD7. B = basal layer; Sp = suprabasal layer; Su = superficial layer; St = Stroma
Y-axis represents the number of dots detected normalized to the epithelial volume. N = 4 individual experiments performed in duplicates. Supplemental Table 2 details the quantification data.

4.3. Autocrine signaling

The high level of Wnt signaling molecules at the basal layer of the limbal epithelium suggest that LSCs can secrete their own mRNA encoding Wnt ligands and Wnt inhibitors, supporting an autocrine signaling in LSC homeostasis. Autocrine signaling has also been described in interfollicular epidermal stem cells (Lim, 2013), where interfollicular epidermal stem cells expressing *Axin2* can secrete their own Wnt4 and Wnt10a ligands to promote stem cell self-renewal and proliferation. The presence of both autocrine and paracrine signaling highlights the importance of fine tuning Wnt signaling pathway for LSC regulation. These redundant mechanisms (Fig. 6) are critical to avoid aberrant activation of Wnt signaling pathway, preventing ocular surface neoplasia development.

4.4. Fzd7 preferential expression in the superior limbus

Fzd7 mRNA expression is higher in the basal epithelial layer of the superior limbus, supporting the role of palisades-rich areas in LSC proliferation. Cultures of LSCs isolated from the superior and inferior limbus have better proliferation capacity than those isolated from the temporal and nasal quadrants (Dziasko, 2014; Meyer-Blazejewska et al., 2010).

The current study has several limitations. First, co-localization is limited to 3 markers due to the number of non-overlapping fluorescent molecules available. In the future, high-definition spatial

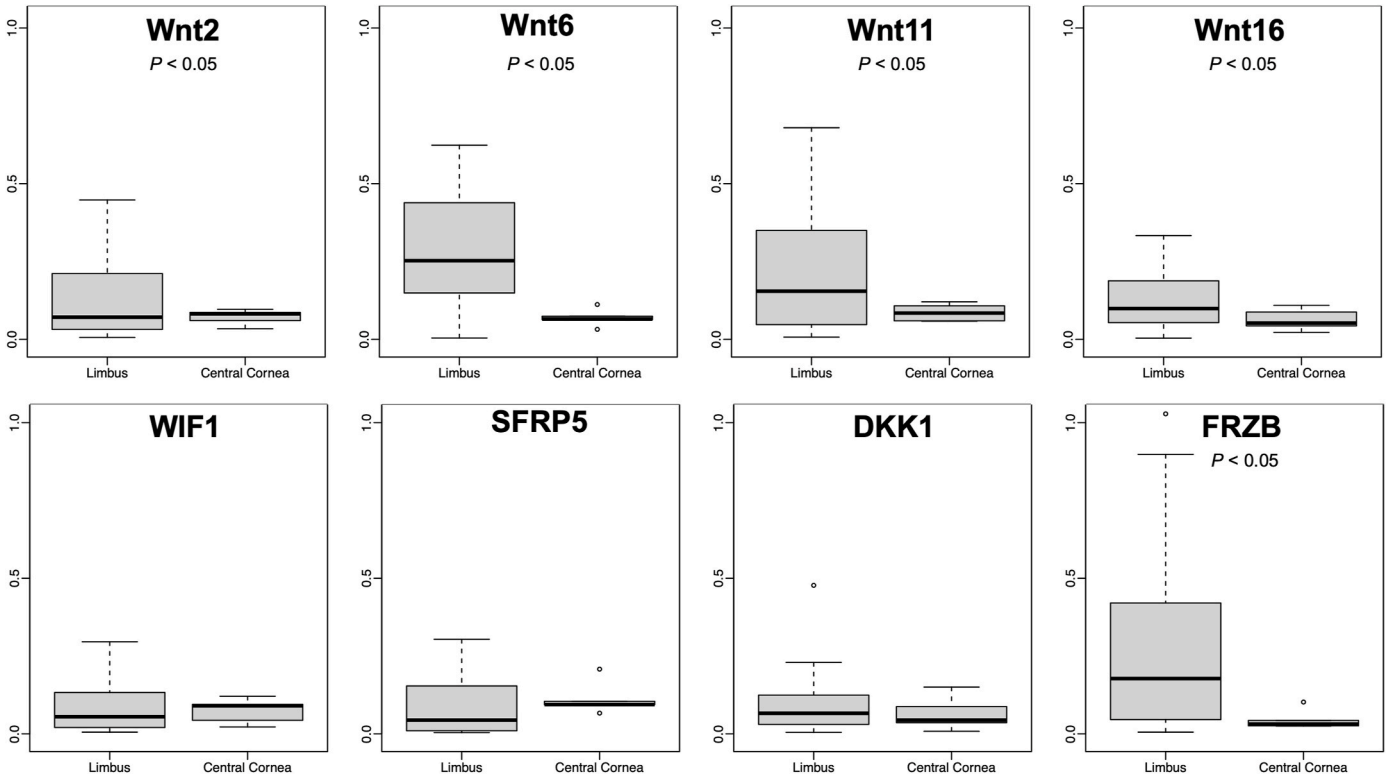

Fig. 4. Quantification of mRNA expression profiles of Wnt signaling molecules in the basal epithelial layer of the limbus and central cornea.
All 4 Wnt ligands, FRZB, and FZD7 present a preferential expression at the basal epithelial layer of the limbus compared with the basal epithelial layer of the central cornea.
Y-axis represents the number of dots detected normalized to the epithelial volume. N = 4 individual experiments performed in duplicates. Supplemental Table 3 details the quantification data.

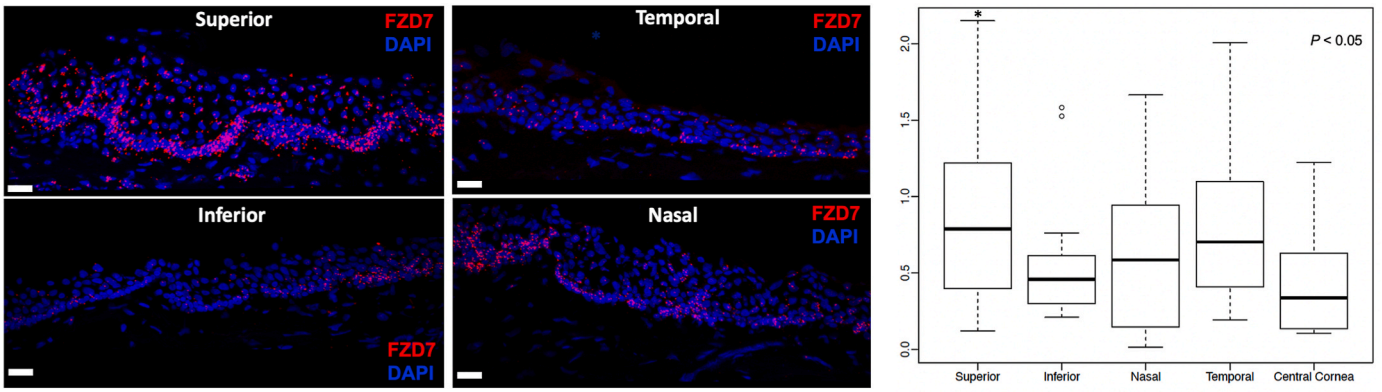

Fig. 5. The mRNA expression of Fzd7 in the basal limbal epithelial layer is highest in the superior region in the limbus.
A- Preferential mRNA expression of FZD7 in the superior quadrant. Scale bar: 20 μm.
B- Box and whisker plots of the relative level of FZD7 single transcripts detected in the basal layer of each quadrant. FZD7 was significantly more expressed in the basal layer of the superior quadrant compared with the three other quadrants ($P < 0.05$).
Y-axis represents the number of dots detected normalized to the epithelial volume. N = 4 individual experiments performed in duplicates. Supplemental Tables 2 and 3 detail the quantification data.

transcriptomics (Vickovic, 2019) and slide-seq technologies (Rodriques, 2019) are expected to improve our spatial understanding of limbal tissue organization. Second, because of the lack of specific antibodies available for the study of the Wnt signaling pathway in the limbus, the findings at the mRNA level could not be confirmed at the protein level. Finally, less than 5% of the limbus was explored, and it would be necessary to perform whole-mounted experiments to obtain a more complete overview when this is method becomes feasible.

4.5. *Conclusions*

In conclusion, several Wnt ligands and inhibitors that are preferentially detected in the limbus compared to the cornea are expressed in a decreasing gradient fashion from the basal limbal epithelial layer and the immediate underlying stroma toward the superficial limbal epithelial layer. As many of these Wnt signaling molecules mediate canonical and/or noncanonical pathways, colocalization of these molecules suggests an interwind and co-dependent relationship of these 2 pathways.

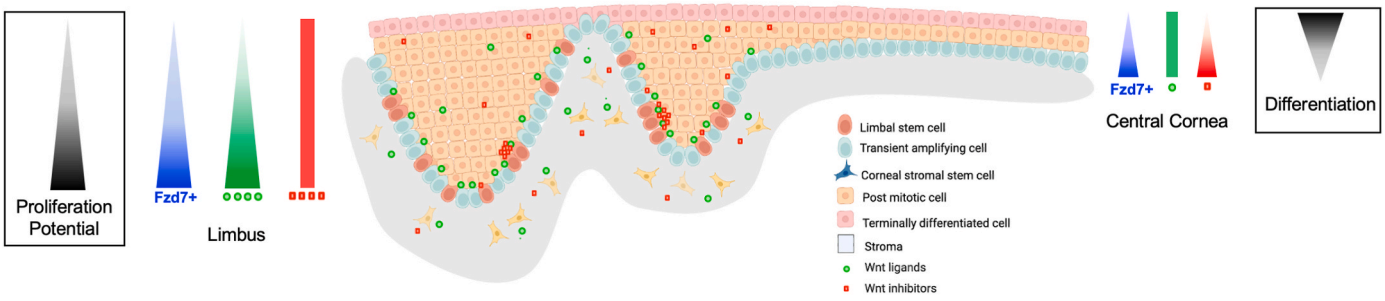

Fig. 6. Model of Wnt signaling expression in the human limbus.
Paracrine and autocrine signaling exist in the human limbus. These redundant mechanisms finely regulate Wnt canonical and non-canonical pathway.

In addition, both autocrine and paracrine effect likely exist in the LSC niche. Taken together, Wnt signaling is tightly controlled in the limbal stem cell niche involving both canonical and noncanonical pathways by a hierarchical expression gradient of Wnt signaling molecules and receptor.

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Declaration of competing interest

SXD is a consultant for Dompe US, Novartis US, and Claris Biotherapeutics, Inc.; other authors: none.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2022.109337.

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