

## Effect of TGF $\beta$ 1 and TGF $\beta$ 1-inhibitor on limbal stem cell properties

Sariya Jamjun<sup>1\*</sup>, Nipan Israsena<sup>2</sup>

<sup>1</sup> Department of Pharmacology, Faculty of Medicine

<sup>2</sup> Stem Cell and Cell Therapy Research Unit, Chulalongkorn University, Bangkok, Thailand.

\* Presenting Author

### Abstract

Corneal epithelium is constantly renewing itself from stem cells located at the limbus, the transition zone between the corneal and neighboring conjunctival epithelium. The maintenance of limbal stem cells (LSCs) is widely believed to be controlled by various cues present in their niches. The aim of the present study was to investigate the effect of TGF- $\beta$ , a key cytokine found during corneal wound healing, on cultivated LSCs. We found that when LSCs were grown at clonal density on 3T3 feeder, TGF- $\beta$ 1 treatment results in the clonal transition from cobblestone-like colonies to spindle-shaped colonies with migrating protrusions. Within spindle-shaped colonies there was marked reduction in putative stem cell marker  $\Delta$ Np63 $\alpha$  expression. In contrast, treatment with SB431542, TGF $\beta$ -inhibitor, promotes colony forming efficiency of LSCs as well as increase a proliferation rate of LSCs colonies. Our results suggest that TGF $\beta$  signaling induces limbal epithelial stem cells to transdifferentiate into fibroblast-like cells as described in epithelial-to-mesenchymal transition (EMT). TGF $\beta$ -inhibitor may be useful in promoting corneal wound healing and improve corneal transparency in patient with corneal injury.

**Keywords:** limbal stem cells, TGF $\beta$ , Epithelial-to-mesenchymal transition.

### Introduction

Stem cells of the corneal epithelial system are thought to be slow cycling cells localized to the epithelial basal layer of the limbus, the transitional zone between the cornea and the conjunctiva (Schlotzer-Schrehardt U. *et al.*, 2005). Upon activation by corneal wounding LSCs enter the cell-cycle and show high proliferative potential. It is still unclear which signals in the limbal niche play a role in regulating self-renewal and fate decision of limbal epithelial stem cells (Walker MR. *et al.*, 2009). One candidate pathway is the transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling. TGF- $\beta$  is known to play critical roles during corneal wound healing as well as control numerous cellular processes, such as proliferation, apoptosis, and differentiation (Goumans MJ., 2003). Both transforming growth factor-beta (TGF- $\beta$ ) receptors types I and II were reported to be weakly expressed in the central corneal epithelium of human eyes, but were presented at much higher levels in the basal cells of the limbus (Zieske JD *et al.*, 2001). Nevertheless, the effects of TGF- $\beta$ 1 on LSCs properties have not been fully clarified.

The aim of this study was to investigate the effect of TGF $\beta$ 1 and TGF $\beta$ -inhibitor (SB431542) on clonogenic potential and proliferation rate of cultured limbal stem cells.

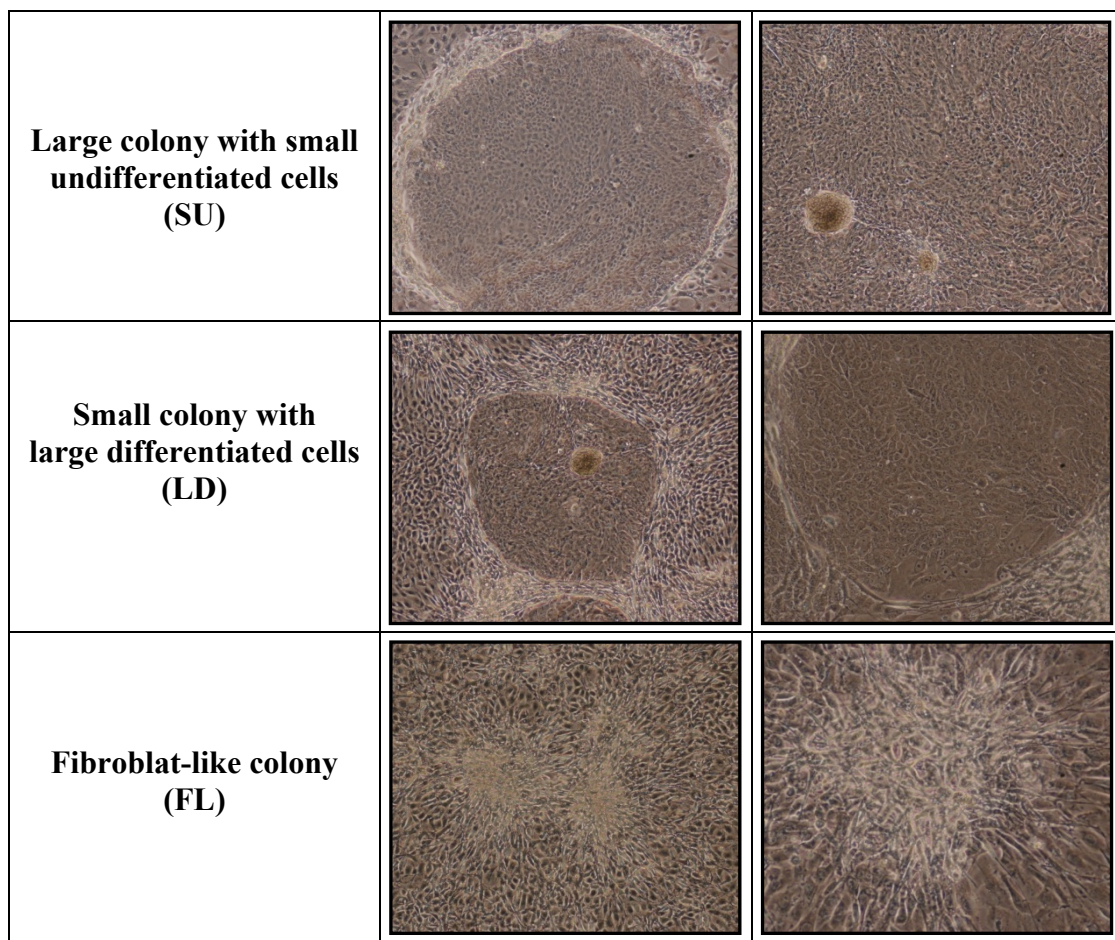
### Material and Methods

Human limbal rims taken from organ donors were provided by the Department of Ophthalmology of Thai Red Cross. After careful removal of excessive sclera and conjunctiva tissue, the limbal rings were exposed to dispase II (1.2 IU/mL in PBS) at 37°C under humidified 5% CO<sub>2</sub> for 1 hour. Following one rinse with DMEM containing 10% FBS The loosened tissues were cut into cubes of approximately 2x2 mm<sup>2</sup>. Limbal tissues were cultured on the laminin coated plate containing limbal complete media; 1:1 mixture of Dulbecco's

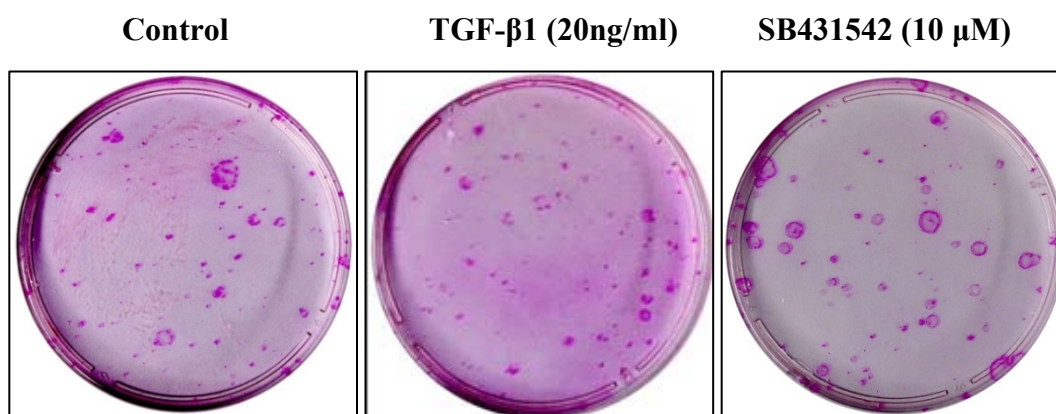
modified Eagle's medium (DMEM) and Ham's F12, supplemented with 10% fetal bovine serum USA grade (FBS), 1% Pen-Strep, 1.25 µg/mL amphotericin, 1% L-glutamine, 5 µg/mL insulin (Invitrogen), 20 ng/mL EGF (R&D), 0.5 µg/mL hydrocortisone, 2.5 mg/ml NaHCO<sub>3</sub>. Culture Media was changed every 1-2 days. After 5 days of cells growth from the explant, limbal epithelial cells were separated into single cells by 0.25% trypsin-0.02% EDTA for 2 minutes. For clonal analysis, single cells were inoculated onto multiwell plates/cultured dishes containing 3T3 feeder layers. TGF-β1 (R&D systems) or TGFβ-inhibitor SB431542 (Stemgent) were added at the first day of seeding. After 10 days clones were stained with rhodamine B then counted for determination of the colony-forming efficiency. Immunofluorescence staining with ΔNp63α antibody (Cell Signaling) was performed after 10 days of culture on 3T3 cells for putative LSCs identification and characterization.

## Results

Human limbal epithelium when cultured on mitomycin C (MMC)-treated 3T3 feeder cells give rise to three types of colonies: Large colonies consist mainly of small undifferentiated cells (SU), small colonies consist of large differentiated cells (LD), and abortive colonies (AB). When 20 ng/ml TGF-β1 were added to culture medium, all colonies were transformed into fibroblast-like colony (FL) that composed of more extended and elongated shape cells with migratory capacity (Fig. 1). After culture for 10 days, the migrating fibroblast-like cells covered an entire surface of the tissue culture plate. In contrast, 10 µM SB431542 (TGFβ-inhibitor) increases colony-forming efficiency (CFE) of limbal epithelium (Table. 1). Moreover, all of colonies cultured in the presence of SB431542 were large colonies with smooth perimeter, contained mainly small and rapidly growing cells (SU). Whereas in normal condition (untreat) had many of small colony containing large and terminally differentiated epithelium (LD), (Fig. 2). Immunofluorescence analysis showed that TGF-β1 treatment decreases a number of ΔNp63α-positive cells in their colonies.



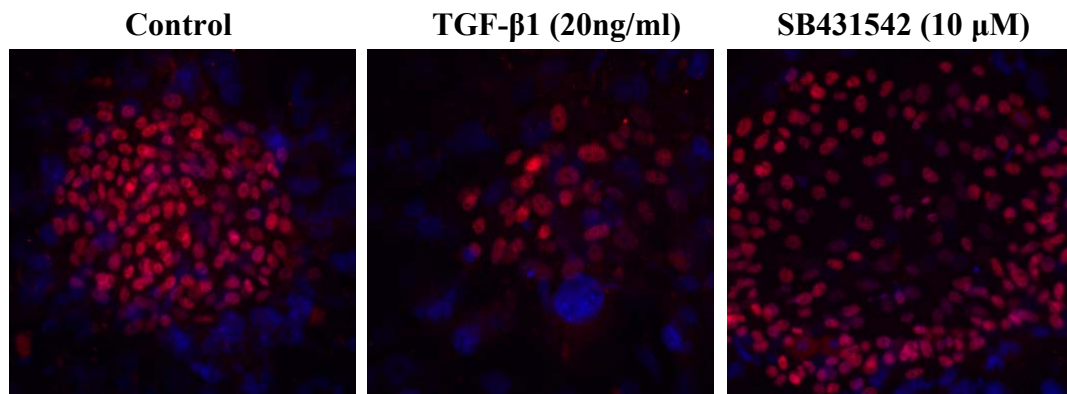
**Figure 1:** Morphology of human limbal epithelial stem cell colony formation. Both of two colony types (SU and LD) were observed in untreated condition (control). Culture with SB431542 treatment was found only SU-clones, whereas FL-clones were observed in 20 ng/ml TGF- $\beta$ 1 treatment. Magnification: (left) x4; (right) 10x.



**Figure 2:** Determination of the clonogenic ability. 300 limbal epithelial stem cells were plated onto inactivated 3T3 feeder layers. Dishes were stained 10 days later with rhodamine B. 10  $\mu$ M SB431542 treatment increases clonogenic ability and promotes limbal epithelial stem cell proliferation to form the large colony with a smooth perimeter.

**Table 1:** Clonogenicity of human limbal epithelial stem cells in each culture condition system were taken from the eye of a female, 69-yr-old organ donor. Colony-forming efficiency (CFE = number of colonies/number of cells seeded x100%). TGF- $\beta$ 1 treatment induces LSCs transition to fibroblast-like colony. There was more the large colony with small undifferentiated cells (SU) in 10 $\mu$ M SB431542 treatment significantly (\* p<0.008) when compared to untreated condition (control). And the cultivated human limbal clones in SB431542 treated condition were also formed more quickly.

| Clonogenicity of human limbal epithelial stem cells |                         |                          |                  |                       |
|-----------------------------------------------------|-------------------------|--------------------------|------------------|-----------------------|
| Colony type                                         | Culture system, (% CFE) |                          |                  |                       |
|                                                     | Untreat (control)       | TGF- $\beta$ 1 (20ng/ml) | SB (2 $\mu$ M)   | SB (10 $\mu$ M)       |
| Small colony with large differentiated cells (LD)   | 8.17 $\pm$ 0.73         | *<br>0.00                | *<br>00.00       | *<br>0.00             |
| Large colony with small undifferentiated cells (SU) | 17.5 $\pm$ 1.65         | *<br>0.00                | 22.72 $\pm$ 0.92 | *<br>26.44 $\pm$ 0.67 |
| Fibroblast-like colony (FL)                         | 0.00                    | *<br>6.94 $\pm$ 0.54     | 0.00             | 0.00                  |



**Figure 3.** Expression of  $\Delta$ Np63 $\alpha$  in LSCs colonies. To confirm the clone of limbal epithelial stem cells and progenitor cells. Note the reducton of  $\Delta$ Np63 $\alpha$  expression in fibroblast-like colonies observed from TGF- $\beta$ 1 treatment, whereas the size of  $\Delta$ Np63 $\alpha$ -positive colony increased considerably in SB431542 treatment. (Red fluorescence: Alexa Fluor 546-conjugated goat antirabbit IgG; blue fluorescence: nuclear counterstaining with Dap; magnification: X40)

### Discussion and Conclusion

In our experiment, we demonstrated that TGF- $\beta$ 1 treatment results in the clonal transition from limbal epithelial colony containing LSCs to migrating fibroblast-like colony corresponding with the reduction in the number of putative stem cells based on  $\Delta$ Np63 $\alpha$  expression. These results suggest that during injury, and perhaps in culture condition, TGF- $\beta$  may play a role in promoting LSCs differentiation and migration. Because the clinical success of cultivated LSCs therapy depends on whether they contained a sufficient number of stem cells essential for long-term epithelial renewal, our finding that SB431542 inhibits fibroblastic transformation and promote LSCs maintenance may be applied to improve the method for ex vivo expansion of LSCs used in clinical applications

### References

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