CHAPTER IV

RESULT

Transforming growth factor-β (TGF-β) signaling has been known as a key signaling pathway in injury response during corneal wound healing. In addition, TGF-β also plays an important role in stem cells regulation by controlling numerous cellular processes, such as proliferation, apoptosis, and differentiation. However, the effects of TGF-β1 on limbal epithelial stem cell (LESC) properties have not been fully clarified. Therefore, our first aimed is to investigate the effect of TGF-β1 and its inhibitor on clonogenic potential and proliferation rate of the LESCs *in vitro*.

1. The effect of TGF-β1 and TGFβ-inhibitor (SB431542) on limbal epithelial stem cells properties.

1.1. Clonogenic potential of cultivated limbal epithelial stem cells on 3T3 feeders

To assess the effect of TGF-β and its inhibitor, human limbal epithelial cells were dissociated into single cell then 300 cells were seeded on MMC–treated 3T3 feeder cells. TGF-β1 and TGFβ-inhibitor (SB431542) were added at the first day of seeding. Colony forming efficiency and colony morphology were observed by using inverted microscope. In control condition, 16.7-26.1 percent of primary limbal epithelial cells seed on 3T3 can form colonies composed of differentiated epitheliums. In 20 ng/mL TGF-β1 treated group, there was a marked reduction in number of colony formed on 3T3, Moreover, all colonies found were transformed into fibroblast-like colonies that composed of more extended and elongated shape cells with migratory capacity. After 10 days of culture, the migrating fibroblast-like cells covered an entire surface of the tissue culture plate. In SB431542 treated group, colonies observed were mainly large colony with smooth perimeter, which contained mainly small and rapidly growing cells (Table 1). SB431542 treatment increased colony-forming efficiency (%CFE) of limbal epithelium (Table 2). However, an average size of colonies was similar between untreated and SB431542-treated group (Figure 6).

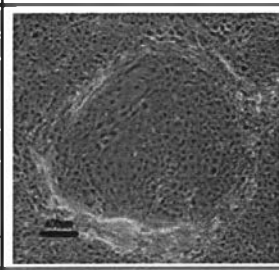
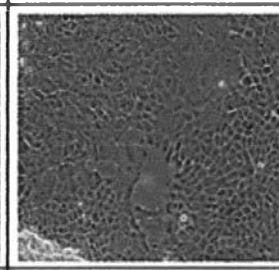
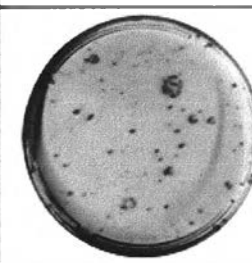
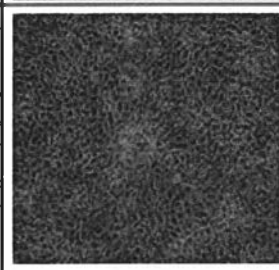
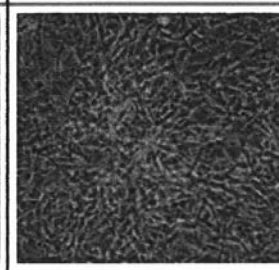
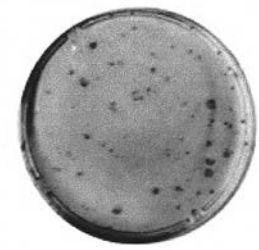
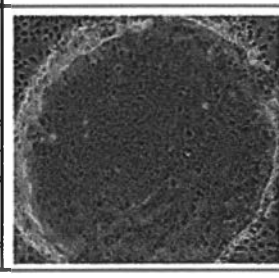
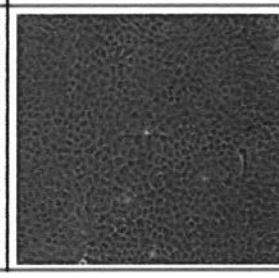
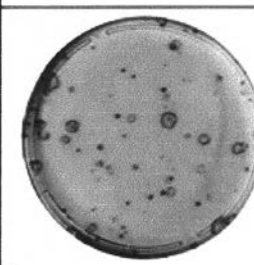
Culture System	Clonal Morphology		Clonogenic Capacity
	(X4)	(X10)	
Untreat (control)			
+ TGFβ1 (20ng/mL)			
+ SB431542 (10μM)			

Table 1. Clonal morphology and clonogenic capacity of LESCs. The same numbers of isolated LESCs, 300 cells were seeded on growth-arrested 3T3 feeders and cultured in the presence of TGF-β1 or TGFβ inhibitor (SB431542). Dishes were stained 10 days later with rhodamine B.

Colony forming efficiency of human limbal epithelial cells			
Sample (Sex/age) (n)	Culture system, (% CFE)		
	Untreat (control)	TGF-β1 (20ng/ml)	SB431542 (10μM)
LT33 (M/36) (n=3)	26.11 ± 1.29	*** 9.89 ± 0.77	* 31.00 ± 2.46
LT36 (F/69) (n=6)	25.67 ± 2.18	* 6.94 ± 0.54	26.44 ± 0.67
LT33 (M/36) (n=6)	18.08 ± 2.58	NA	** 28.92 ± 3.20
LT58 (M/32) (n=6)	16.77 ± 1.93	NA	** 23.63 ± 1.10

Table 2. Colony forming efficiency (%CFE) of LESCs cultured in TGF-β1 and TGFβ inhibitor (SB431542) treatment conditions. NA = data not available. *(*P<0.05, **P<0.001, ***P < 0.0001)*.

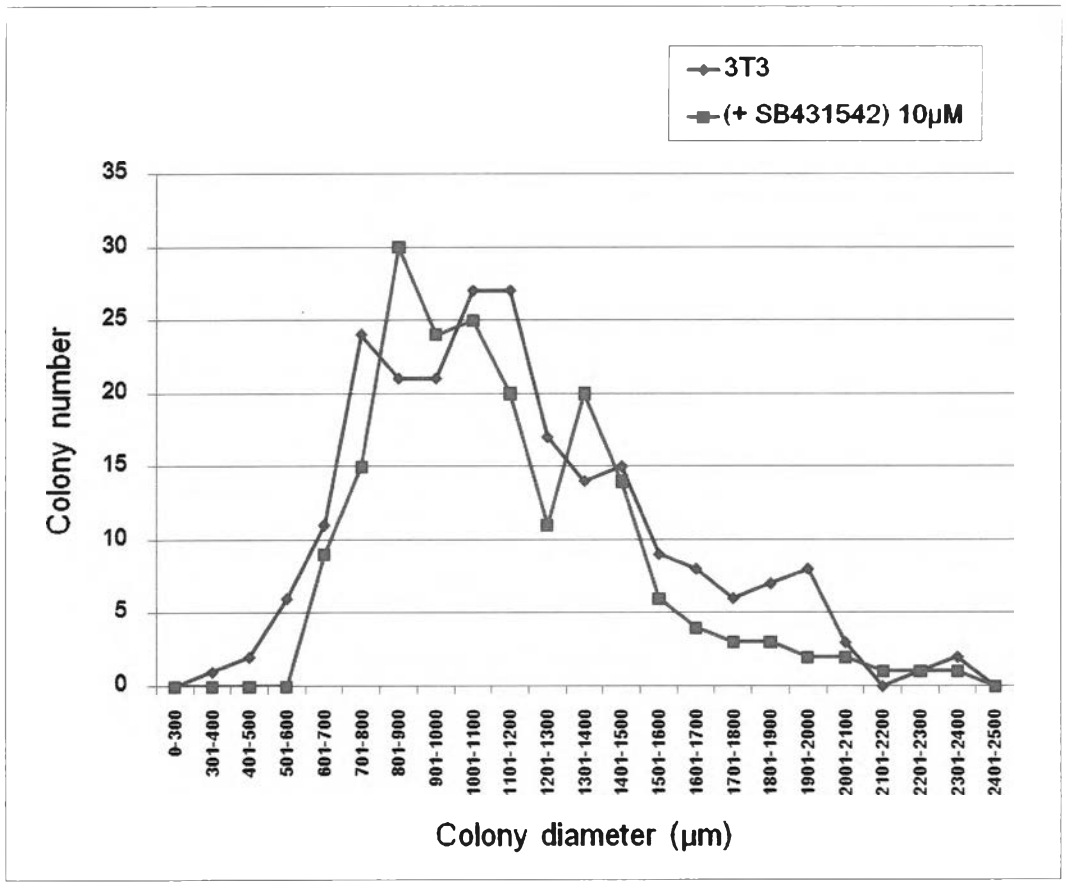


Figure 6. The correlation of colony number and colony diameter of LESC colonies cultured in untreated and SB431542-treated conditions.

1.2. Effects of TGF-β1 and TGFβ inhibitor (SB431542) on gene expression profile of the cultured limbal colonies

Next, we used immunofluorescence staining to further characterize colonies formed in the presence of TGF-β1 and TGFβ inhibitor (SB431542). We found that colony formed in TGF-β1 treated group contained lower number of ΔNp63α, LESCs-related marker, positive cells in their colonies (Figure 7). Whereas SB431542 treatment showed higher number of ΔNp63α-positive cells.

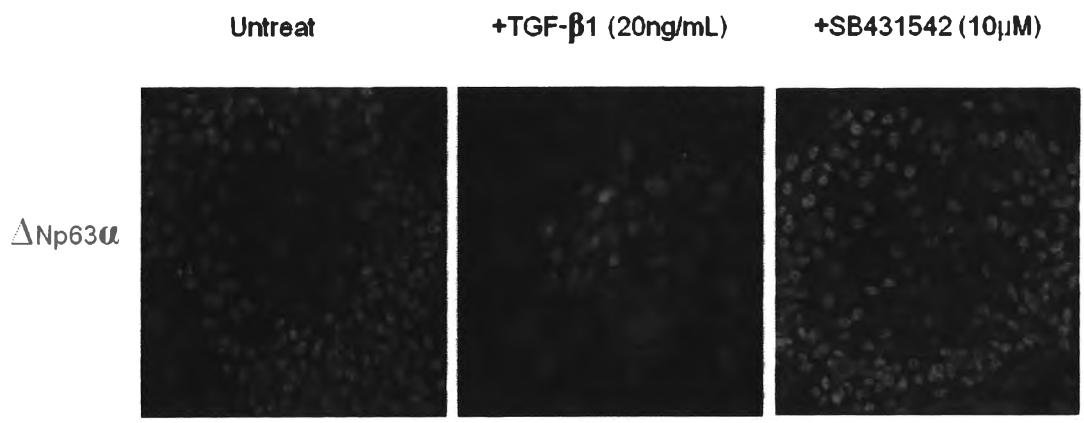


Figure 7. Expression of ΔNp63α in the culture LESC colonies. To confirm the clone of limbal epithelial stem cells and progenitor cells. Note the reducton of ΔNp63α expression in fibroblast-like colonies observed from TGF-β1 treatment, whereas the size of ΔNp63α-positive colony increased considerably in SB431542 treatment. Magnification: X40)

TGF-β has been known as strong EMT inducer in many cell types. Therefore, we hypothesized that TGF-β1 induce epithelial-to-mesenchymal transition (EMT) in LESCs that result in loss of epithelial stem cell while SB431542 inhibit this effect and maintain LESCs in undifferentiated state. So, we next evaluated the effect of SB431542 on epithelial-to-mesenchymal transition (EMT) related genes. For this purpose, limbal colonies in normal and SB431542 treated condition were picked at day 10. Total RNA were extracted and RT-PCR was performed. As expected, SB431542 showed its ability to inhibit EMT in limbal colony formation by suppressing mesenchymal cell markers expression such as N-cadherin, vimentin, fibronectin. In addition, reduction of mesenchymal markers was associated with up-regulation of epithelial cell adhesion molecule, E-cadherin (Figure 8) and stem cell marker, ABCG2 (Figure 9) and Id (inhibitor of differentiation/DNA binding) genes which were known to play an important role in inhibiting EMT process (Figure 9), Treatment with SB431542 also decreased $p21^{Cip1}$ expression level which may be accounted for a higher cell proliferation rate in this condition. Together with these data, our results demonstrated that TGF-β1 treatment induced the transformation of LESCs to fibroblast-like colony lead to

reduction of putative stem cells. Whereas SB431542 inhibits fibroblastic transformation and promotes LESCs proliferation.

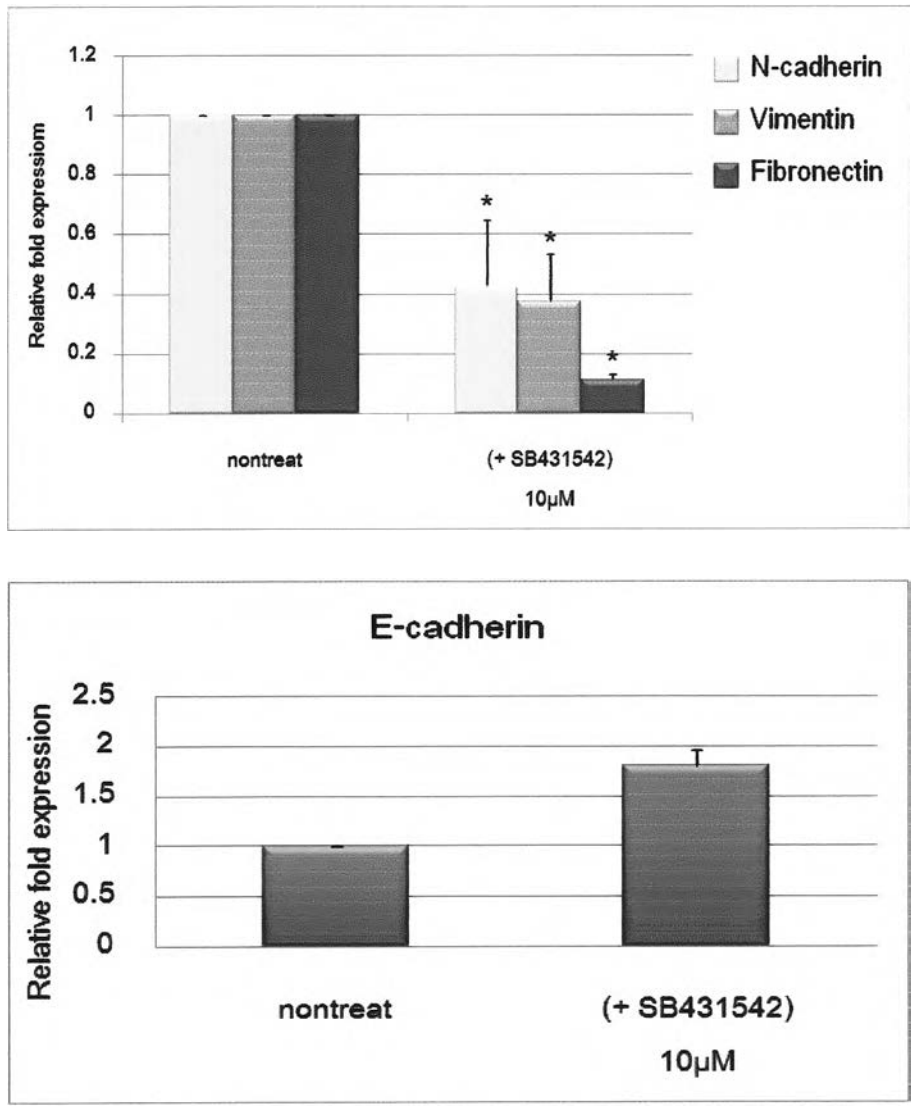


Figure 8. Quantitative Real-time PCR analysis of relative mRNA levels of EMT-related genes expression such as N-cadherin, vimentin, fibronectin , and the epithelial adhesion molecule E-cadherin in the cultured limbal colonies. Data was presented as average, (*$p < 0.05$, n = 3).

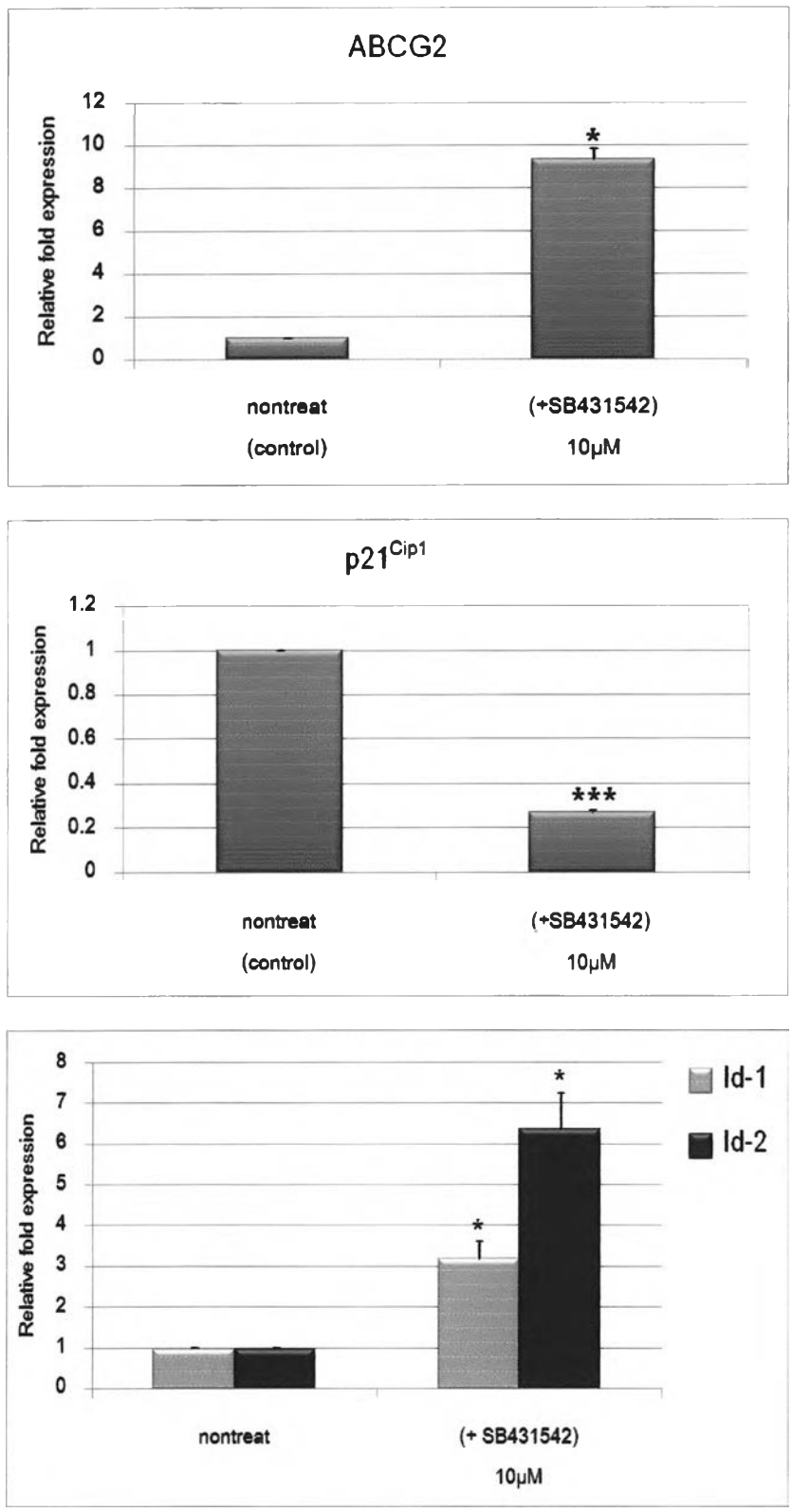
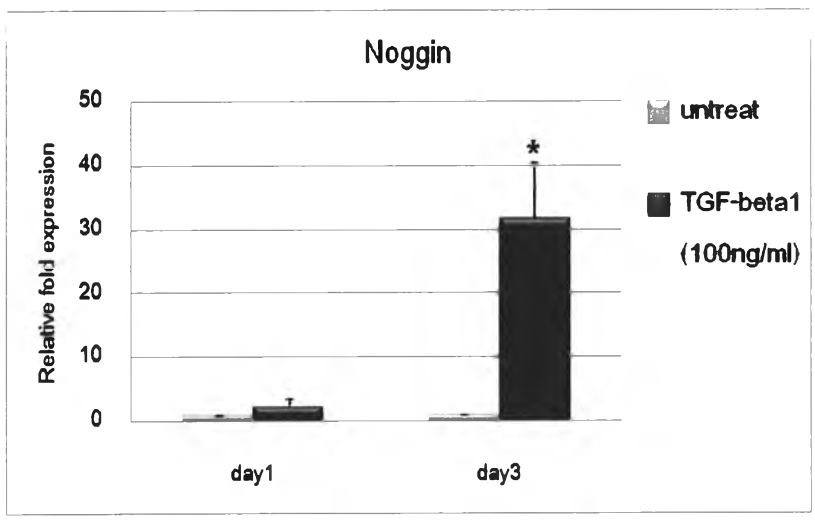


Figure 9. Quantitative Real-time PCR analysis of relative mRNA levels of ABCG2, $p21^{Cip1}$, Id-1 and Id-2 in the cultured limbal colonies. The relative gene expression was normalized to that of GAPDH in each sample. The data was presented as average, (*$P < 0.05$, ***$P < 0.0001$, n = 3).

Because of the complexity of TGF-β function, we further investigate an indirect effect of TGF-β1 on LESC niche by using limbal fibroblast isolated from stromal layer. In this study we focused on signaling cross-talk between TGF-β and BMP signaling. As TGF-β and BMP signaling are members of TGF-β signaling family and share common Smad protein to mediate their signaling. Then, we analyzed the transcription profile of BMP antagonists: noggin, gremlin, chordin and follistatin in the cultured limbal fibroblast under TGF-β1 treatment compared to untreated condition by using Real-time PCR. We found that BMP antagonists, especially noggin, were strongly up-regulated after TGF-β1 activation (Figure 10). Immunofluorescence analysis demonstrated that while in fresh, uninjured limbal tissue, Noggin was absented in limbal basal epithelium and was only slightly presented in the suprabasal limbal epithelium, in limbal tissue cultured with serum on laminin coated plate for 2 days showed up-regulation of noggin, expression both in limbal epithelium and limbal stomal cells (Figure 11). These data illustrated that TGF-β1 had an indirect effect on LESC niche by inducing BMP antagonists expression.



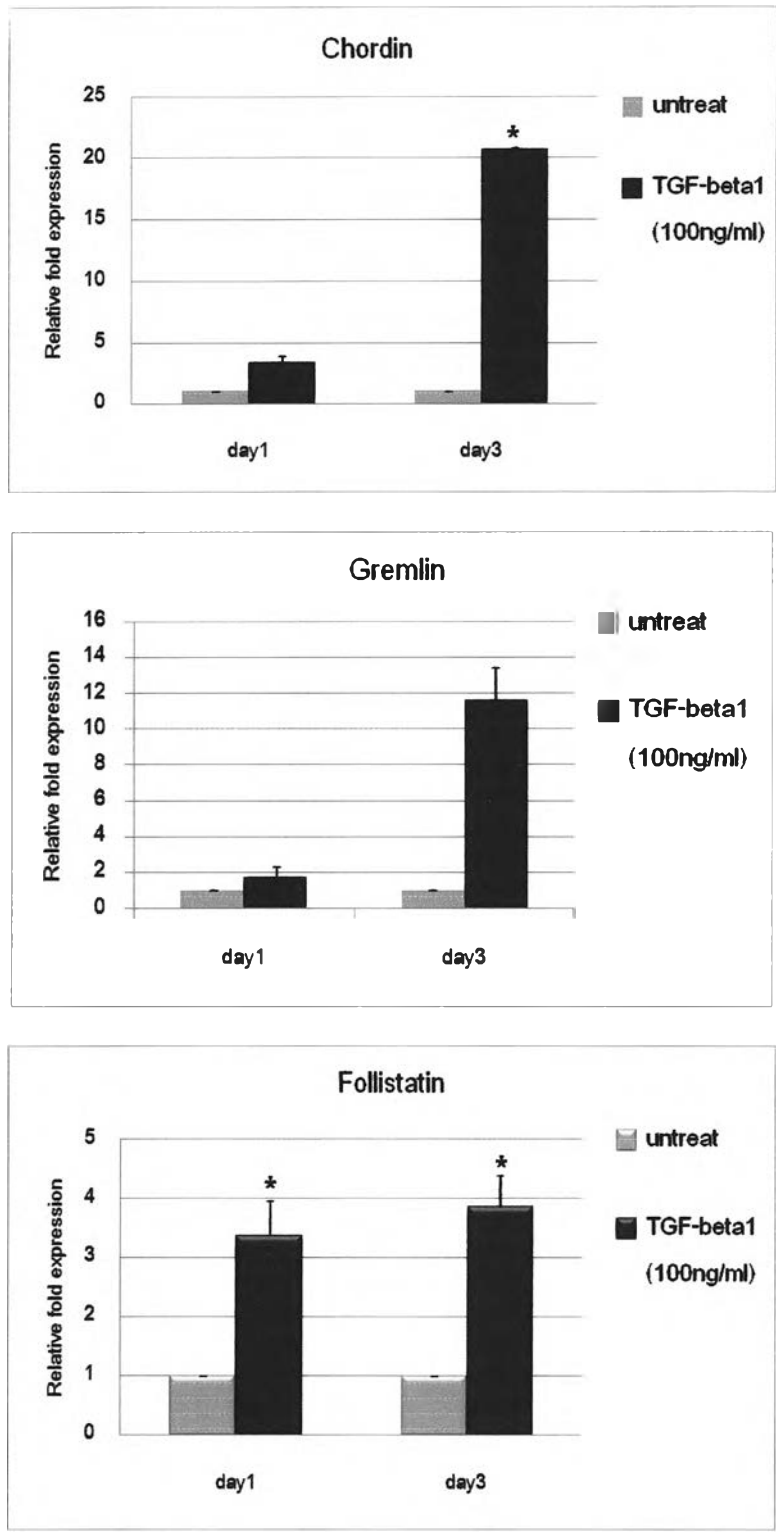


Figure 10. Quantitative Real-time PCR analysis of relative mRNA levels of BMP antagonists: noggin, gremlin, chordin, follistatin in the cultured limbal fibroblast treated with 100 ng/mL TGF-β1 as the injury activation for 1 and 3 days. The relative gene expression was normalized to that of GAPDH in each sample. The data was presented as average, (*$p < 0.05$, n = 3).

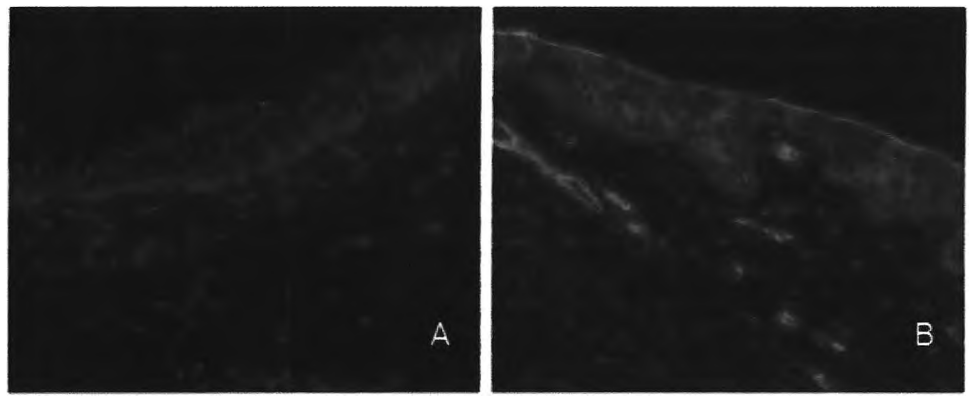


Figure 11. Immunofluorescent staining for proposed BMP antagonist (noggin) on paraffin sections of fresh (A) and cultured (B) limbal tissue. Alexa Fluor 546-conjugated goat anti-rabbit IgG; red fluorescence: nuclear counterstaining with DAPI (blue); magnification: X20.

BMP, as TGF-β, has been known as a cytokine that play a pivotal role in development. To evaluate the effect of BMP and BMP antagonist secreted from feeder cells that could be comparable to stromal niche for LESCs maintenance *in vitro* culture system. We generated 3T3 feeder cell line which overexpress BMP4 and BMP antagonist (noggin), named as 3T3-BMP4 and 3T3-Noggin respectively. These cells showed higher level of BMP4 mRNA (for 3T3-BMP4) and noggin protein expression (for 3T3-Noggin) than 3T3 cell line (Figure 12).

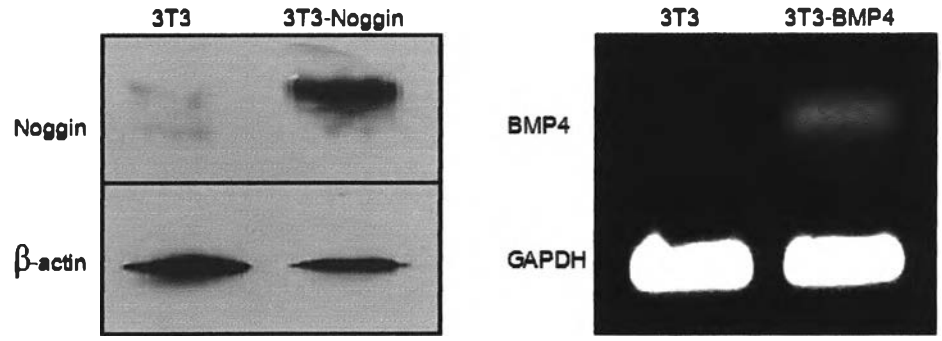


Figure 12. Western blot analysis of the expression of human noggin in protein extracts prepared from 3T3 and 3T3-Noggin, β-actin has been used as an internal control (left). Semi-quantitative RT-PCR analysis was performed human BMP4 transcription on 3T3 and 3T3-BMP4, GAPDH was used as an internal control (right). BMP4 and noggin-transduced 3T3 expressed high level of BMP4 mRNA and noggin protein respectively.

2. The effect of stable BMP4 and BMP antagonist (noggin) expression from 3T3 feeders on the cultured LESCs.

2.1. Clonogenic potential of cultivated limbal epithelial stem cells on each 3T3 feeder type.

Limbal epithelial cells were dissociated from laminin coated plate and seeded at a plating density of 300 cells/dish containing the MMC-treated 3T3 feeder layer. After 7 days of cultivation, colonies were stained with rhodamine B, identified under an inverted microscope, photographed and measured their diameters.

We found that 3T3-Noggin significantly increased colony-forming efficiency (%CFE), from 22.83% $\pm$ 2.05% (control) to 47.00% $\pm$ 3.33%. Moreover, LESCs cultured on 3T3-Noggin showed their ability to formed colony earlier than on 3T3 (control) and produced cobblestone-like colonies containing the cluster of small round cells and spindle-shaped cells at perimeter, while 3T3-BMP4 produced the large colony, which contained more packing epithelium with rapidly growing epithelium when compared to normal 3T3 feeders. Taken together, these data suggested that 3T3-BMP4 induced proliferation of limbal epithelial stem cells or progenitor cells, whereas 3T3-Noggin increased clonogenic ability of primary LESCs (Table 3-4, Figure 13).

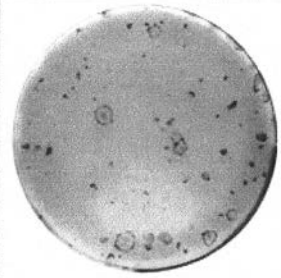
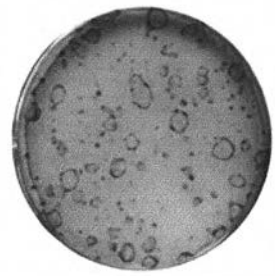
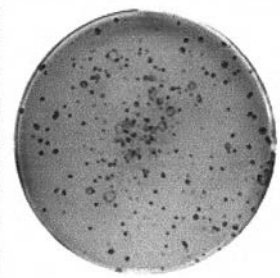
Feeder System	Clonogenic Capacity	%CFE (n=6)
3T3 (control)		22.83 ± 2.05
3T3-BMP4		** 31.10 ± 1.79
3T3-Noggin		*** 47.00 ± 3.33

Table 3. Determination of the clonogenic capacity of limbal colonies on each 3T3 feeder types. (**$P < 0.001$, ***$P < 0.0001$)

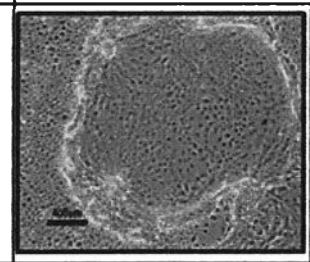
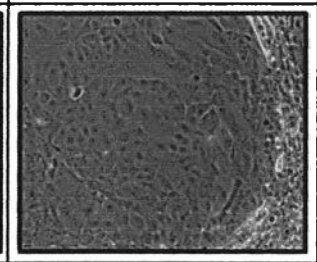
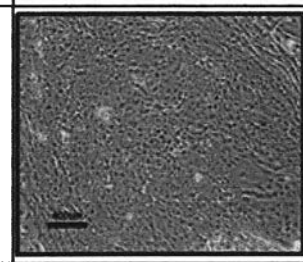
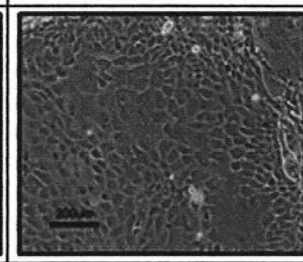
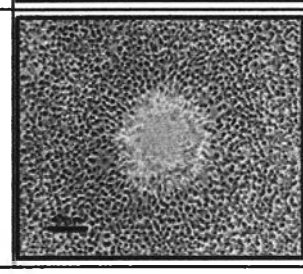
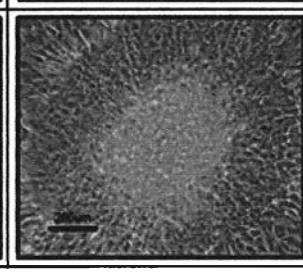
Feeder System	Clonal Morphology		Mean of Colony Diameter (μm; n=80)
	(X4)	(X10)	
3T3 (control)			478.40 ± 145.58
3T3-BMP4			*** 849.18 ± 285.57
3T3-Noggin			*** 301.90 ± 71.93

Table 4. Morphology and an average diameter of human LESCs colony formation on each feeder type. Magnification: X4 (scale bar: 400 μm.), X10 (scale bar: 200 μm.), ***$P < 0.0001$.

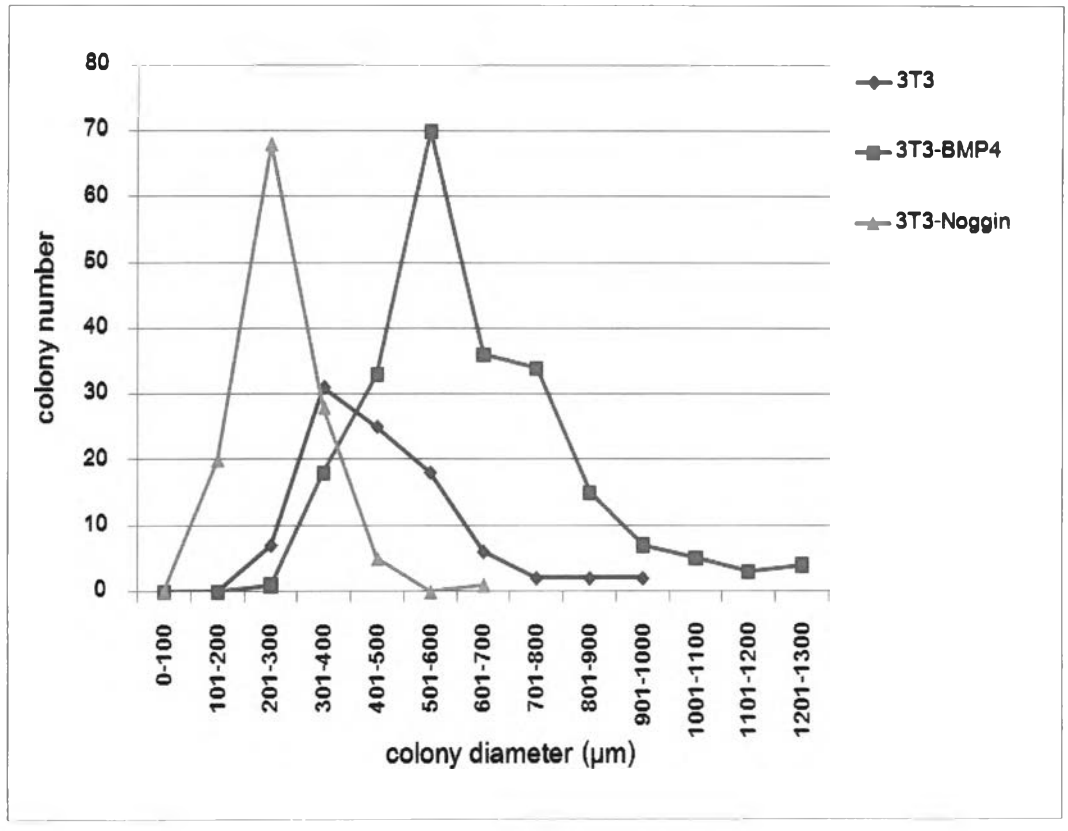


Figure 13. The correlation of colony number and colony diameter of LESC colonies cultured on each 3T3 feeder type.

Due to the phenotypic different of limbal colonies grown on 3T3-Noggin, we tested whether primary colony on 3T3-Noggin could form epithelial-like colony on 3T3 in secondary passage. So, the primary colonies on 3T3 and 3T3-Noggin were picked and dissociated into single cells by 0.05% trypsin-EDTA, then passaged to secondary colony forming on 3T3 feeders. After 7 days, we found the clonal transition of the primary cobblestone-like colony on 3T3-Noggin to the large epithelial-like colony on 3T3 feeders (Figure 14). Markedly, LESCs from primary clones on 3T3-Noggin have less clonogenic ability than 3T3-derived primary colonies when cultured on 3T3 feeders (Table 5).

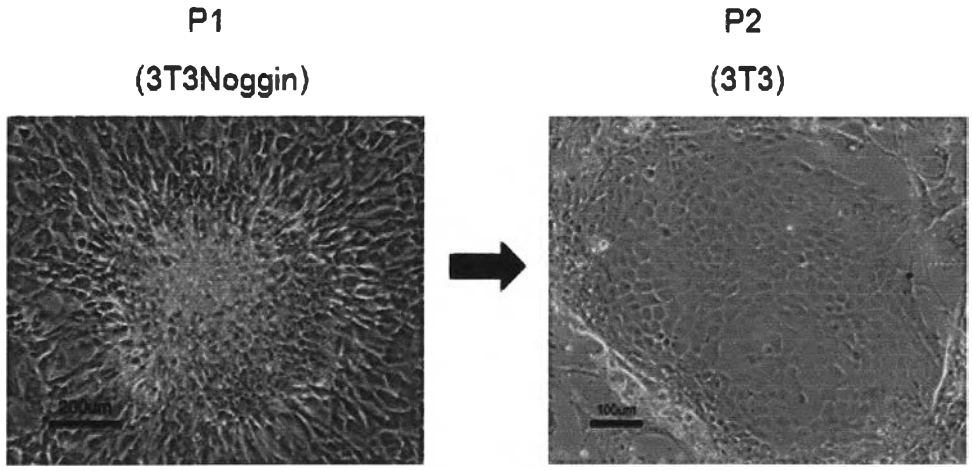


Figure 14. Morphology of primary limbal colony on 3T3-Noggin subcultivated into normal 3T3 feeders for secondary colony forming. Magnification: X10; scale bar: 200 μm (left), 400 μm (right).

Colony forming efficiency of secondary human limbal epithelial cells	
Feeder system of primary colonies, (% CFE)	
3T3	3T3-Noggin
18.70 ± 2.12	*** 4.23 ± 0.53

Table 5. Colony forming efficiency (%CFE) of secondary LESCs from primary clones on 3T3 and 3T3-Noggin. *(***P < 0.0001)*

2.2. Effects of BMP and noggin on gene expression profile of the cultured limbal epithelial cells

For putative LESCs identification and characterization in primary colony on each feeder system, Immunofluorescence analysis was preformed. The results showed that putative LESC-associated marker, ΔNp63α was abundantly expressed in both limbal colony types; the large flattened colony and the small clustered colony on 3T3 and 3T3-Noggin feeder systems respectively. (Figure 15)

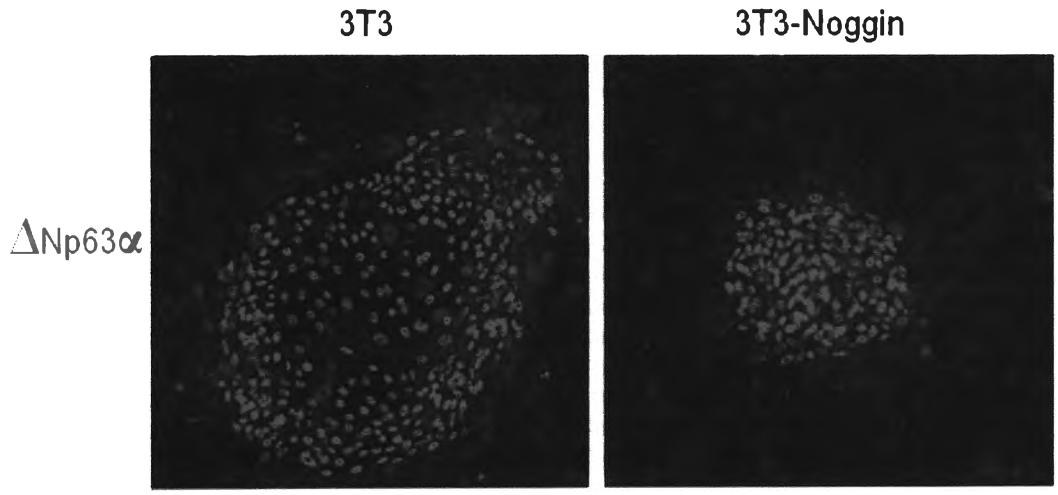


Figure 15. Immunofluorescent staining of LESC-associated marker, ΔNp63α in the large flattened colony and the small clustered colony on 3T3 (left) and 3T3-Noggin (right) feeder types respectively ; nuclear counterstaining with DAPI (blue); magnification: X10).

To assess the modulating effect of noggin on epithelial-to-mesenchymal transition (EMT) of LESCs, limbal colony on 3T3-Noggin were analysed for mesenchymal markers expression by Real-time PCR. As shown in Figure 16, noggin has partial ability to induce EMT in limbal colony formation, as evidenced from up-regulation of EMT-related genes expression, especially fibronectin, in the cultured limbal colonies on 3T3-Noggin feeders.

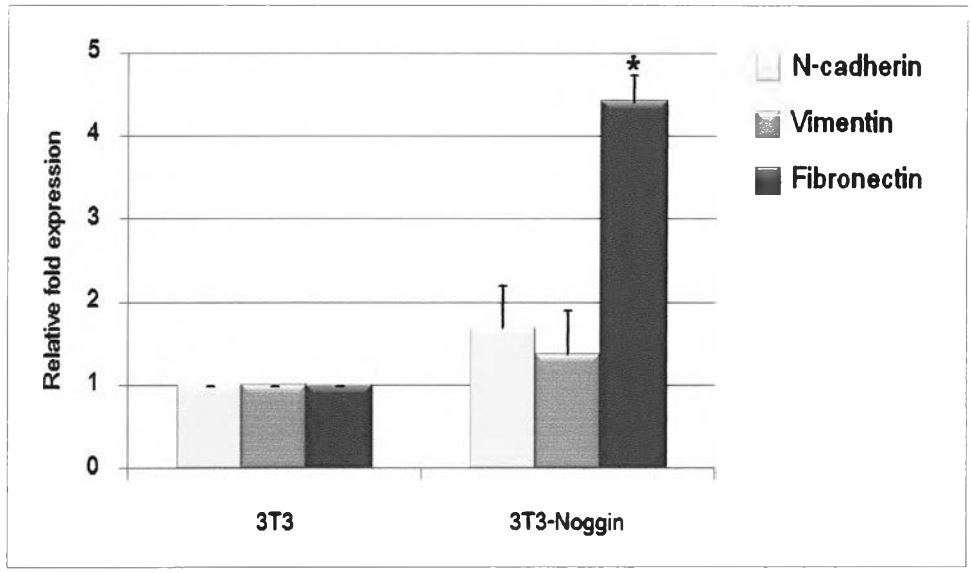
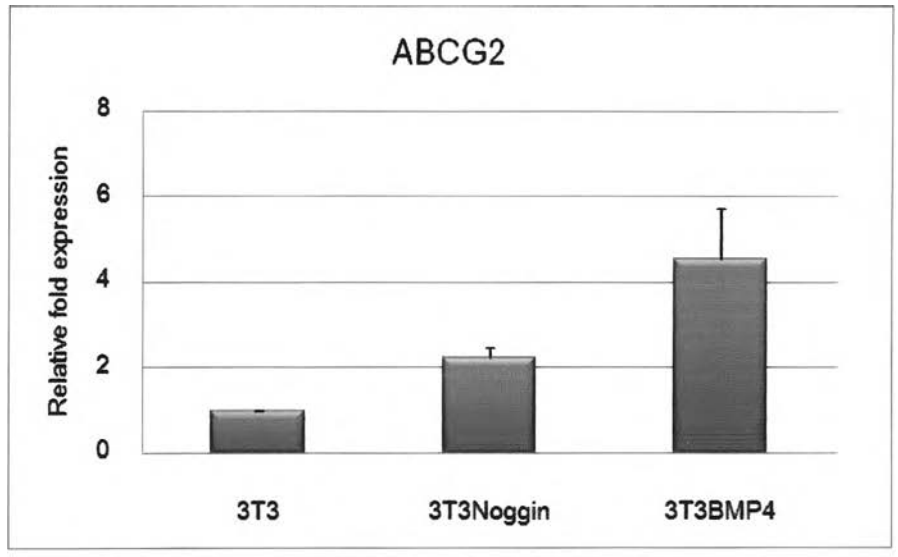
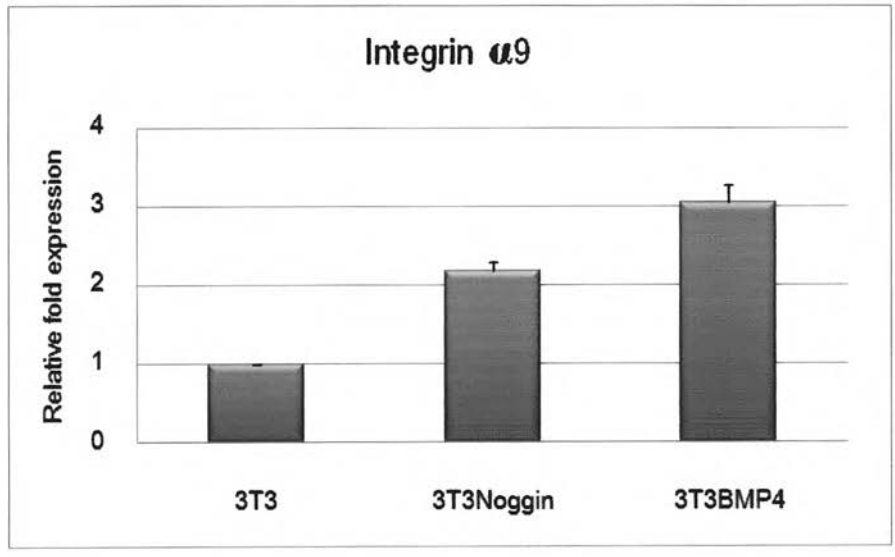


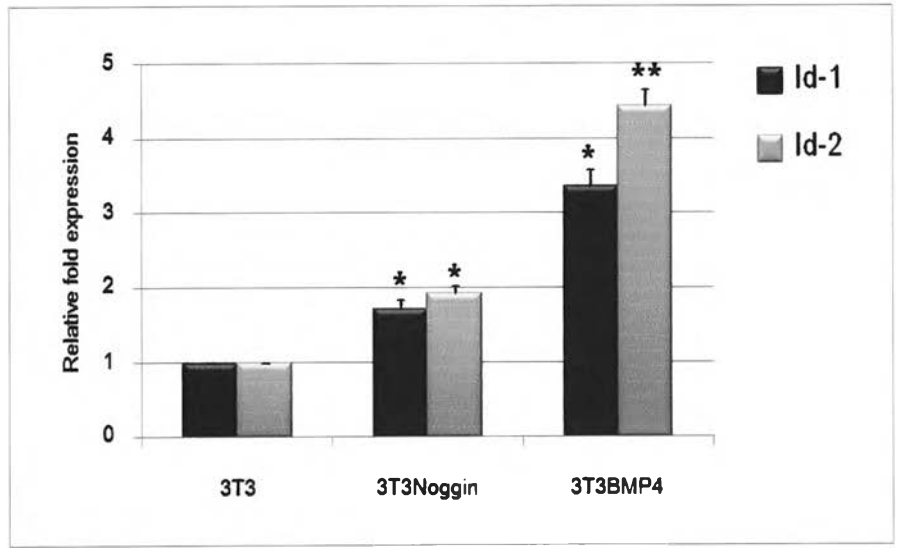
Figure 16. Quantitative Real-time PCR analysis of relative mRNA levels of EMT-related genes such as N-cadherin, vimentin and fibronectin in the cultured limbal colonies. Data was presented as average, ($*p < 0.05$, n = 3).

We further analyzed the gene expression profile of limbal colonies grown on 3T3, 3T3-Noggin, and 3T3-BMP4 by Quantitative Real-time RT-PCR.

We detected an up-regulation of putative LESCs markers: ABCG2 and Integrin α9 mRNA levels most prominently when LESCs were co-cultured with 3T3-BMP4. Interestingly, cyclin-dependtdent kinase inhibitors: $p16^{Ink4a}$ and $p57^{Kip2}$ was down-regulated in limbal colonies cultured on 3T3-Noggin and 3T3-BMP4 respectively, while Id-1 and Id-2 were up-regulated most prominently in limbal colonies on 3T3-BMP4 feeder system (Figure 17).


ABCG2
Relative fold expression
8
6
4
2
0
3T3
3T3Noggin
3T3BMP4


Integrin α9
Relative fold expression
4
3
2
1
0
3T3
3T3Noggin
3T3BMP4


Relative fold expression
5
4
3
2
1
0
*
*
*
**
Id-1
Id-2
3T3
3T3Noggin
3T3BMP4

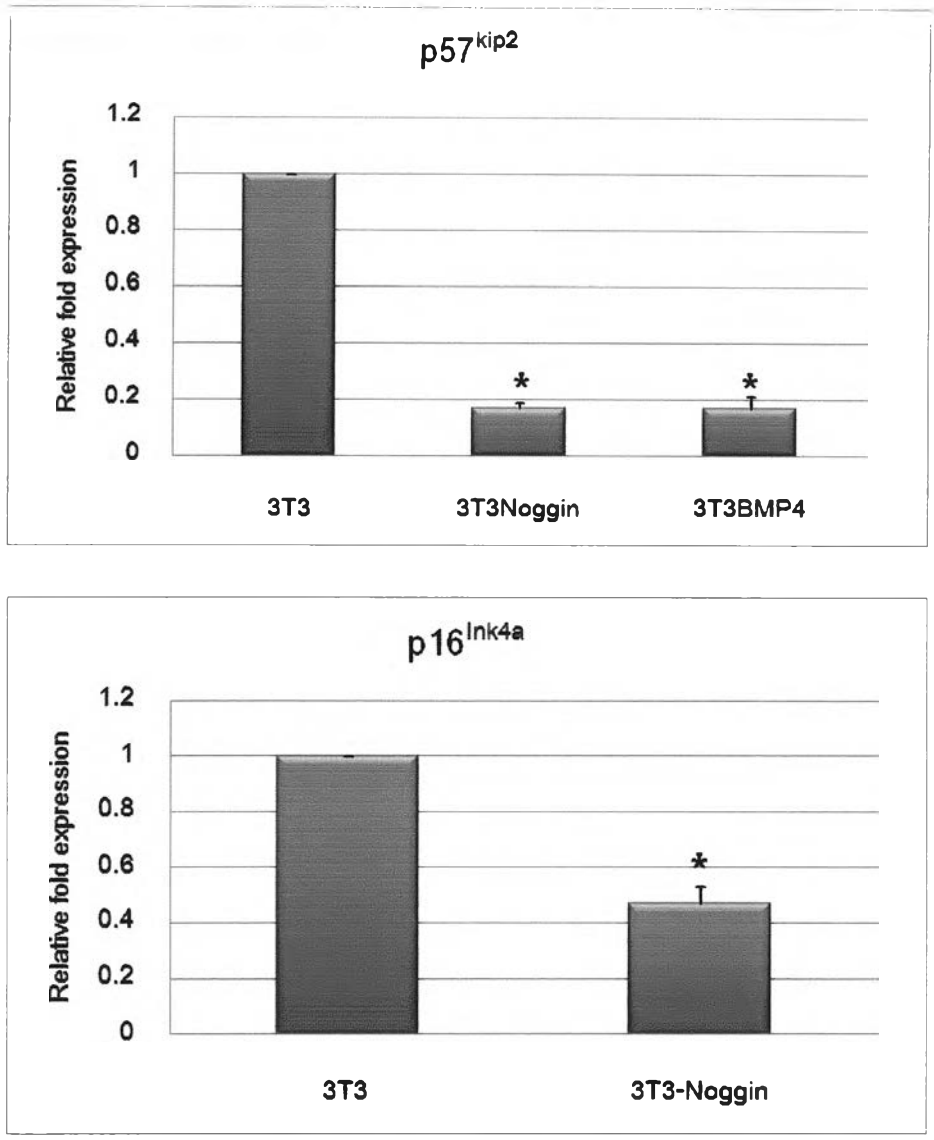


Figure 17. Quantitative Real-time PCR analysis of relative mRNA levels of ABCG2, Integrin- α9, Id-1, Id-2, $p57^{kip2}$ and $p16^{Ink4a}$ in the cultured limbal colonies on each feeder type. The relative gene expression was normalized to that of GAPDH in each sample. The data was presented as average, (*$P < 0.05$, **$P < 0.001$; n = 3).

These data showed the role of BMP signaling in LESCs maintenance. Both of BMP4 and Noggin overexpressed 3T3 feeders promoted proliferative capacity through down-regulation of $p57^{kip2}$. 3T3-Noggin feeder also increases clonogenic capacity in a primary culture of limbal epithelial cells through down-regulation of $p16^{Ink4a}$. BMP4 could maintain epithelial phenotype of LESCs/limbal epithelial progenitors via up-regulation of Id-1 and Id-2 and Noggin increased clonogenic capacity in a primary culture of limbal epithelium through down-regulation of $p16^{Ink4a}$.

In tissue morphogenesis, BMPs usually act as a gradient morphogen. Different concentration of BMPs could guide cells toward different fate. We, therefore, tested whether applying recombinant protein BMP2 and noggin in the culture media could affect limbal epithelial stem cells properties in a manner similar to feeder-derived proteins.

3. The effects of protein treatment of BMP2 and BMP antagonist (noggin) on human limbal epithelial stem cell properties.

3.1. Clonogenic potential of cultivated limbal epithelial stem cells on 3T3 feeders

To test the effect of BMP and Noggin applied in the culture media, human limbal epithelial cells were cultured on MMC–treated 3T3 feeder cells. BMP2 and noggin were added at the first day of seeding. As shown in Table 6, after culture for 10 days, LESCs in the control group formed large and smooth perimeter colonies contained rapidly growing epithelium, while cells cultured with 20 and 100 ng/mL BMP2 generated smaller colonies containing packed round cells in concentration dependent manner. Moreover, BMP2 treatments significantly reduce colony-forming efficiency (%CFE) of LESCs (Table 7). However, noggin treatment has no significantly affect to LESCs growth and proliferation, (Table 8).

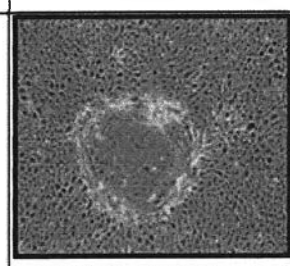
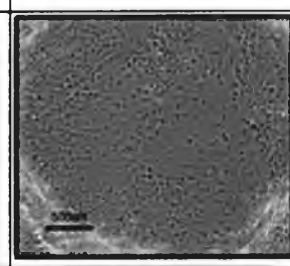
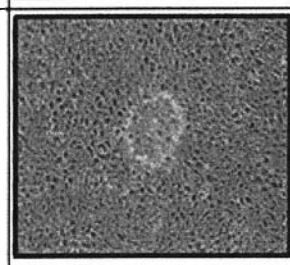
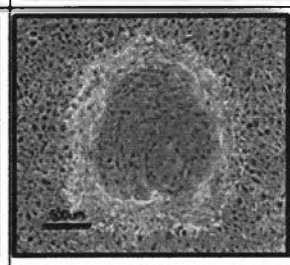
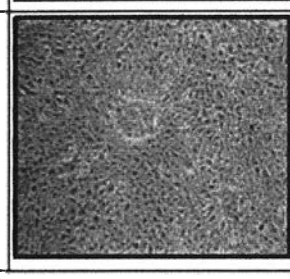
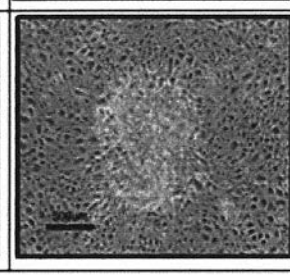
Culture System	Clonal Morphology		Mean of Colony Diameter (μm; n= 80)
	Day5	Day10	
untreat (control)			1020.60 ± 387.86
+BMP2 (20 ng/mL)			** 562.17 ± 297.92
+BMP2 (100 ng/mL)			** 356.62 ± 160.75

Table 6. The morphology and an average diameter of human limbal colonies cultured with and without BMP2 treatments. The size of an untreated colony (top) was larger than BMP2 treated colony after 5 days of culture and increased nearly fivefold at day 10, whereas the size of BMP2 treated colony increased slowly. Espacially 100 ng/mL BMP2 treatment condition, clonal morphology did not change or differentiate into flatted epithelial colony. Magnification: X4; Scale bar: 500 μm.

Colony forming efficiency of human limbal epithelial cells			
Sample (Sex/age) (n)	Culture system, (% CFE)		
	Untreat (control)	BMP2 (20 ng/mL)	BMP2 (100 ng/mL)
LT29 (F/28) (n=6)	29.83 ± 1.86	** 21.33 ± 1.39	** 8.25 ± 4.94
LT41 (F/18) (n=6)	35.78 ± 3.51	* 27.78 ± 0.66	** 16.33 ± 2.58

Table 7. Colony forming efficiency (%CFE) of human limbal epithelial cells in each culture condition. Samples were taken from the eye of a female, 28-yr-old and a female, 18-yr-old organ donors. *(*P < 0.005, **P < 0.0001).*

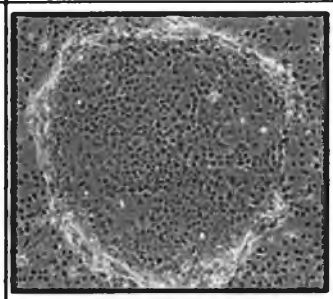
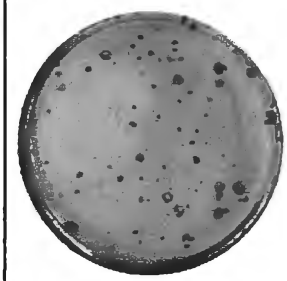
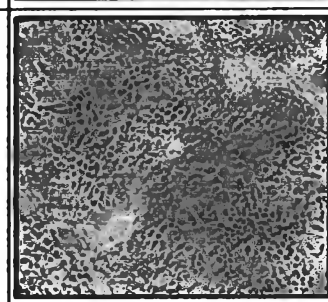
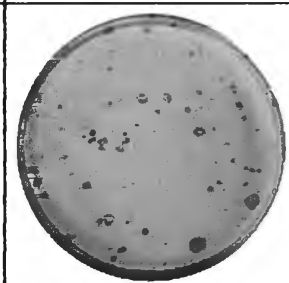
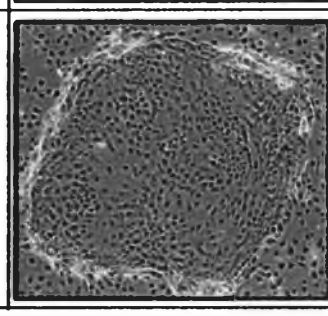
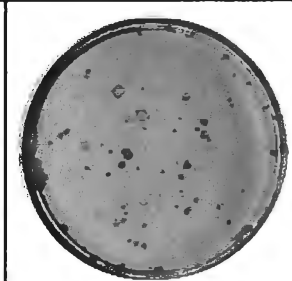
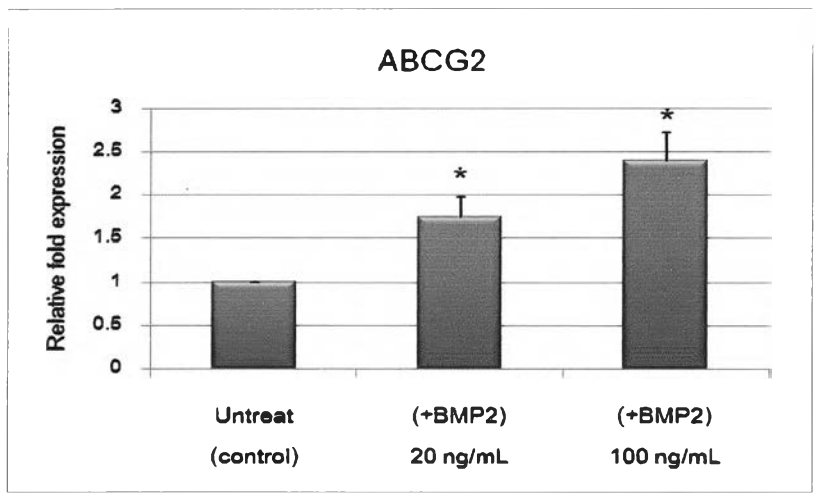
Culture System	Clonal Morphology	Clonogenic Capacity	% CFE (n=10)
untreat (control)			36.60 + 2.46
+ noggin (20 ng/mL)			* 28.90 + 5.45
+ noggin (100 ng/mL)			** 23.50 + 2.89

Table 8. Determination of clonal morphology and clonogenic capacity of the cultured limbal colonies in with and without noggin treatment conditions. LESCs (300 cells) were seeded on growth-arrested 3T3 feeders and cultured with/without noggin treatments. Dishes were stained 10 days later with rhodamine B. Both 20 ng/mL and 100 ng/mL noggin treatments gave the same clonal morphology as untreat condition (control); Magnification: X4. Unexpectingly, noggin treatment reduced colony-forming efficiency (%CFE). *(*P* < 0.005, ***P*< 0.0001*)*.

In the process of cell cycle regulation, cyclin-dependent kinase inhibitors (CDKI) are key factors in controlling cell cycle. Therefore, we further analyzed the gene expression profile in order to dissect the molecular mechanisms in cell cycle inhibition and LESCS maintenance of BMP2 protein treatment. Thus, limbal colonies of each experimental culture systems were picked for RNA extraction and analyzed ABCG2, $p27^{Kip1}$, $p57^{Kip2}$ and $p16^{Ink4a}$ expression.

Quantitative Real-time RT-PCR was performed on BMP2-treated and untreated limbal colonies after 10 days cultivation by using human gene specific primers (Appendix A). As shown in Figure 18, BMP2-treated colonies showed significantly higher level of ABCG2 and lower level of $p16^{Ink4a}$ compared to control group similar to what observed when LESCs were cultured on 3T3-BMP4. On the contrary, $p27^{Kip1}$ and $p57^{Kip2}$ were up-regulated in BMP2 treated colonies instead of down-regulated as previously seen when cultured with 3T3-BMP4 feeders. Our results indicated that all effects of BMP in gene expression were concentration dependent.



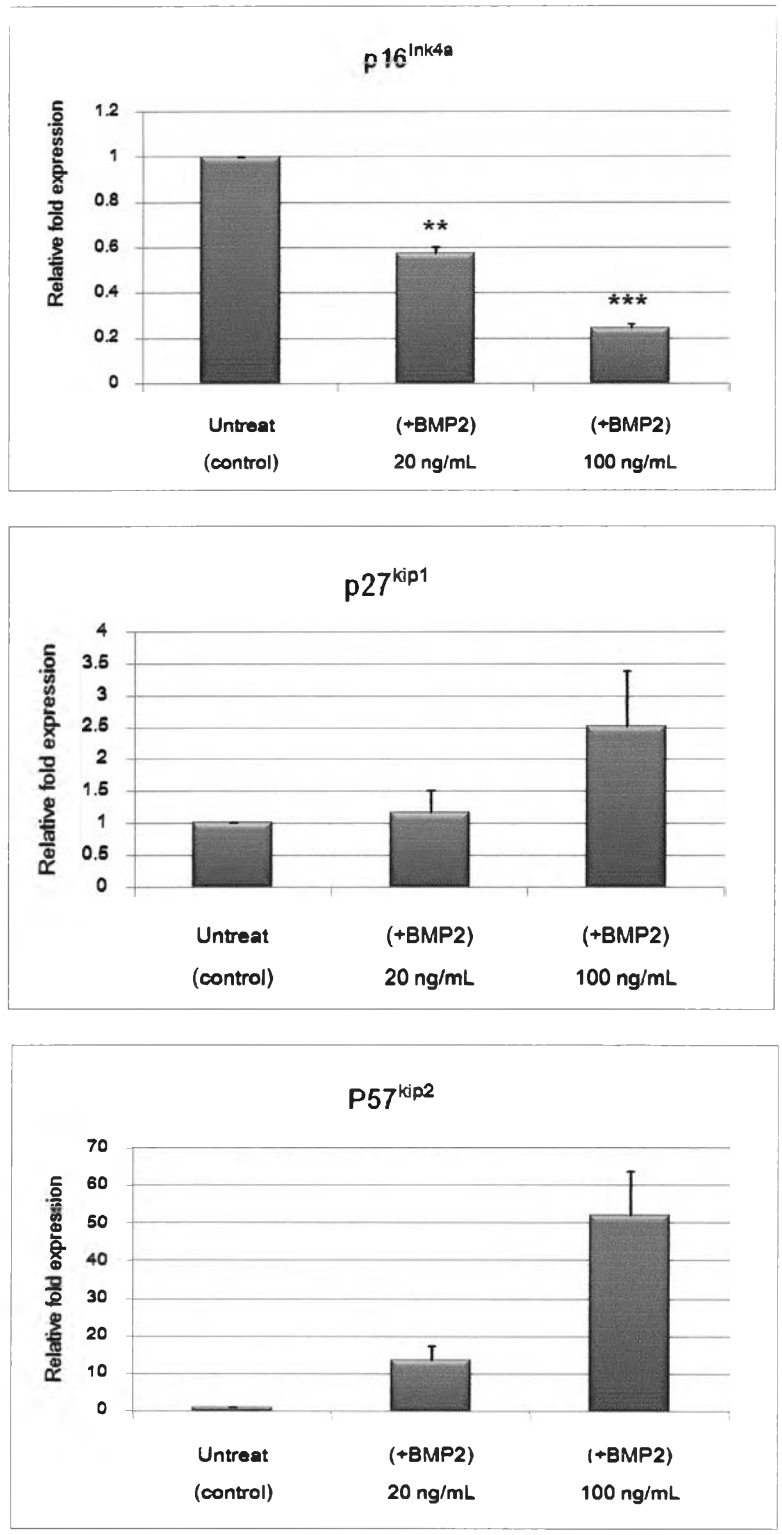


Figure 18. Quantitative Real-time PCR analysis of relative mRNA levels of ABCG2, $p16^{Ink4a}$, $p27^{Kip1}$ and $p57^{Kip2}$ in the cultured limbal colonies in with and without BMP2 treatments. The relative gene expression was normalized to that of GAPDH in each sample. (*P <0.05, **P < 0.001, ***P <0.0001).