CHAPTER V

DISCUSSION AND CONCLUSION

Stem cell niches are dynamic microenvironments that regulate stem cell activity to maintain tissue homeostasis and repair throughout the lifetime. Stem cells of the corneal epithelium have been believed to be localized to the Palisades of Vogt in the corneal limbus. Compared to other stem cells, relatively little is known about the limbal niche. While recent study which showed that central cornea of various mammals also contain some clonogenic keratocytes with long-term self renewal and the capacity to sustain serial transplantation (92) argue against the concept that the limbus is the sole niche for corneal stem cells (93), it is still generally agreed that stem cells from limbal region are the main source of regenerating corneal epithelium after a massive corneal injury. In response to injury cues, dormant LESCs are efficiently activated and produce numerous progenitors and mature cells. Nevertheless, signals involved in limbal stem cells maintenance and signals that activate LESC from quiescence state to produce progenitors and mature cells are not well understood. Since irreversible clonal evolution occurs during serial LESCs cultivation *in vitro*, understanding how limbal niche signals work could eventually lead to a way to improve the method for ex vivo expansion of LESCs for therapy.

A number of cytokines has been reported to be involved during corneal wound healing, including EGF, KGF, IGF and TGF-β. In this study, effects of TGF-β1 on LESCs and nearby niche cells were examined. It is known that transcripts of TGF-β1 is present in corneal epithelium and stroma, whereas the TGF-β receptors RI and RII were reported to be weakly expressed in the central corneal epithelium, but were present at much higher levels in the limbus (72). Although TGF-β has been identified as the potent inducer of myofibroblast transformation in corneal stromal cells during injury (94, 95), the effect of TGF-β on LESCs has not been studied.

In our experiment, we demonstrated that TGF-β1 treatment resulted in a reduction of colony forming efficiency of limbal epithelium as well as promoted the clonal transition from large epithelial-like colonies to colonies mainly consisted with

migrating fibroblast-like cells. The changes in cell phenotype and gene expression pattern detected by Real-time PCR were corresponding to what observed in epithelial-mesenchymal transition process (78). There was a marked reduction in the number of putative stem cells in each colonies based on ΔNP63α expression. In contrast, adding TGF-β inhibitor SB431542 inhibits fibroblastic transformation and slightly increase colony forming efficiency. Levels of genes associated with EMT in SB431542 treated group were even lower than those cultured with standard 3T3 cells, suggested that in the standard LESCs culture with 3T3 feeders, there were some TGF-β1 activity which promote EMT process in the LESCs which may be accounted for the loss in clonogenic potential after serial passage. Because the clinical success of cultivated LESCs therapy depends on whether they contain a sufficient number of stem cells essential for long-term epithelial renewal, our finding that SB431542 inhibits fibroblastic transformation and promote LESCs proliferation may be applied to improve the method for ex vivo expansion of LESCs and may also be used to promote corneal wound healing by applying directly.

The effect of TGF-β on limbal stem cells properties may be, in part, mediated by indirect effects. Our finding showed that there was an up-regulation in the levels of BMP antagonists; Noggin, Gremlin, Chordin, and Follistatin in limbal stromal fibroblsts upon TGF-β1 treatment. Since BMP antagonists have been reported to be expressed in stromal cells from many types of cancer and play an important role in tumor progression, we investigated the role of BMP antagonists on LESCs. We found that Noggin alone when highly expressed in 3T3 feeders can increase colony forming efficiency of limbal epithelials suggested that it may activate dormant LESCs to enter proliferative state. While phenotype and gene expression pattern of colonies grown on 3T3-Noggin showed fibroblastic change associated with EMT. Nevertheless, some cells within the colony retained the ability to generate epithelial-like colony when co-cultured with regular 3T3. In contrast, limbal epithelial cells when co-cultured with 3T3-BMP4 produced mainly mature epithelial-like colony. These results support the role of BMP in maintaining epithelial phenotype of LESCs as well as preventing EMT. It should be noted that while there has been a report that LESCs can be induced to acquire neural properties by treating them with FGF/EFG plus noggin in a serum free media (96), we

were unable to detect any change in the level of neural markers; nestin, beta-III tubulin and pluripotent markers Oct4 and sox2 within our system. Interestingly, while 3T3-BMP promoted limbal epithelial cells proliferation when compared to control 3T3, when BMP was applied to the media directly, it inhibited rate of epithelial cell proliferation in concentration-dependent manner. While colonies grown on 3T3-BMP4 expressed low level of $p57^{kip2}$, colonies on regular 3T3 treated with high dose BMP2 exhibited high level of $p57^{Kip2}$. This observation can be explained by the BMPs has U-shape dose-effect curve, only low and appropriate level of BMP could promote cell proliferation while high dose inhibit cell division Alternatively, 3T3-BMP4 cells may express different level of niche-associated molecules other than BMPs due to the autocrine effects. To test this hypothesis will require further studies.

Taken together we propose a model explaining the role of TGF-β on LESCs and limbal niche during injury (Figure 19). In this model, during corneal injury there is an up-regulation of TGF-β in the epithelium surrounding wound area. With the disruption of basement membrane, TGF-β activates limbal stromal cells to migrate to the wound area and secrete BMP antagonists. The combine effect of TGF-β and BMP antagonists together with EGF, another injury-associated molecule, force dormant LESCs entering into proliferative state. While some LESCs give raise to TACs which in turn promotes epithelial repair, LESCs and TACs that receive strong/sustain TGF-β and BMP antagonist will undergo EMT change. The EMT process promotes cell migration and basement membrane repair by increasing ECM production, reduces TGF-β passage to the stromal layer, thus reduces the BMP antagonists expression in the stromal fibroblasts. Based on our data, cells that undergo early EMT change may be reversed to epithelial fate by higher level of BMP signals but sustained EMT stimuli will also cause LESCs and TACs to lose an ability to generate corneal epithelium. BMP is very likely to be one of the key mediators that keep LESCs in the dormant state as well as promote epithelial gene expression program preventing irreversible EMT change during injury (Figure 20).

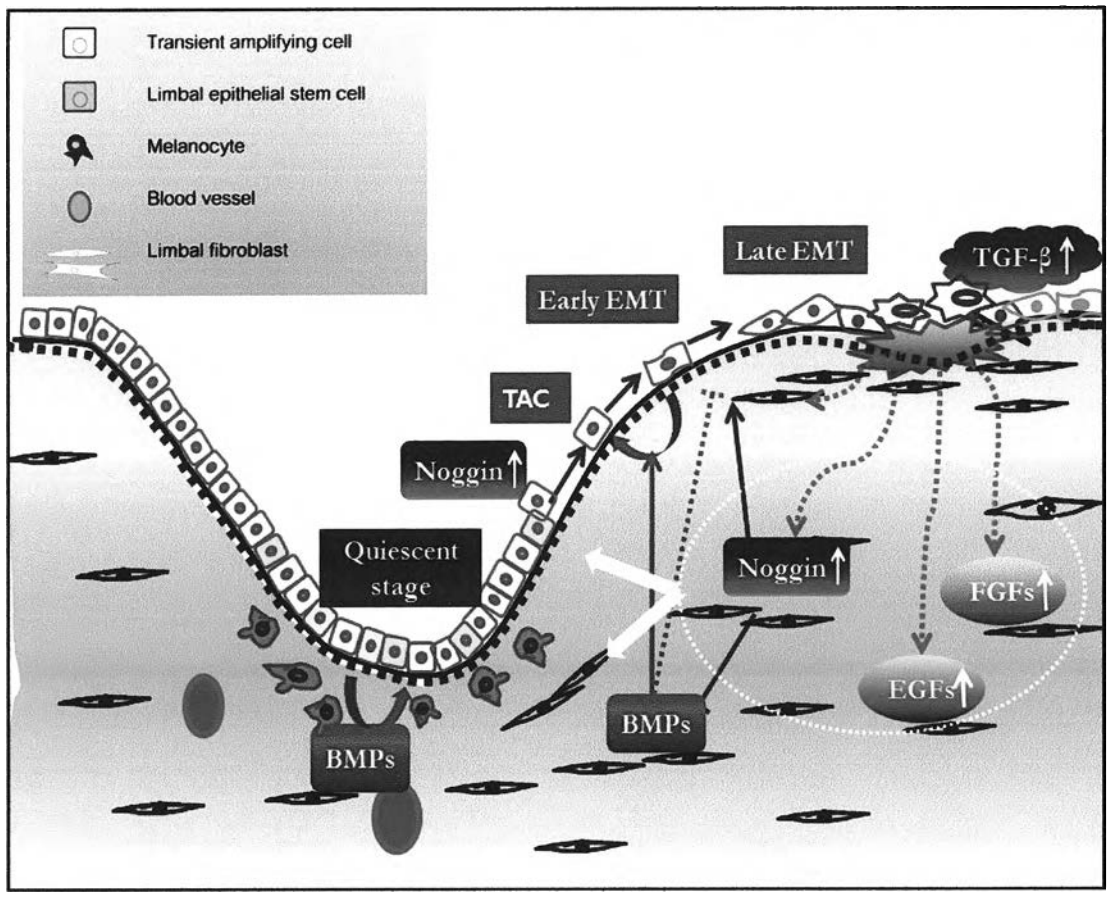

Figure 19. A model of TGF-β and BMP signaling in limbal epithelial stem cells and limbal niche during injury.

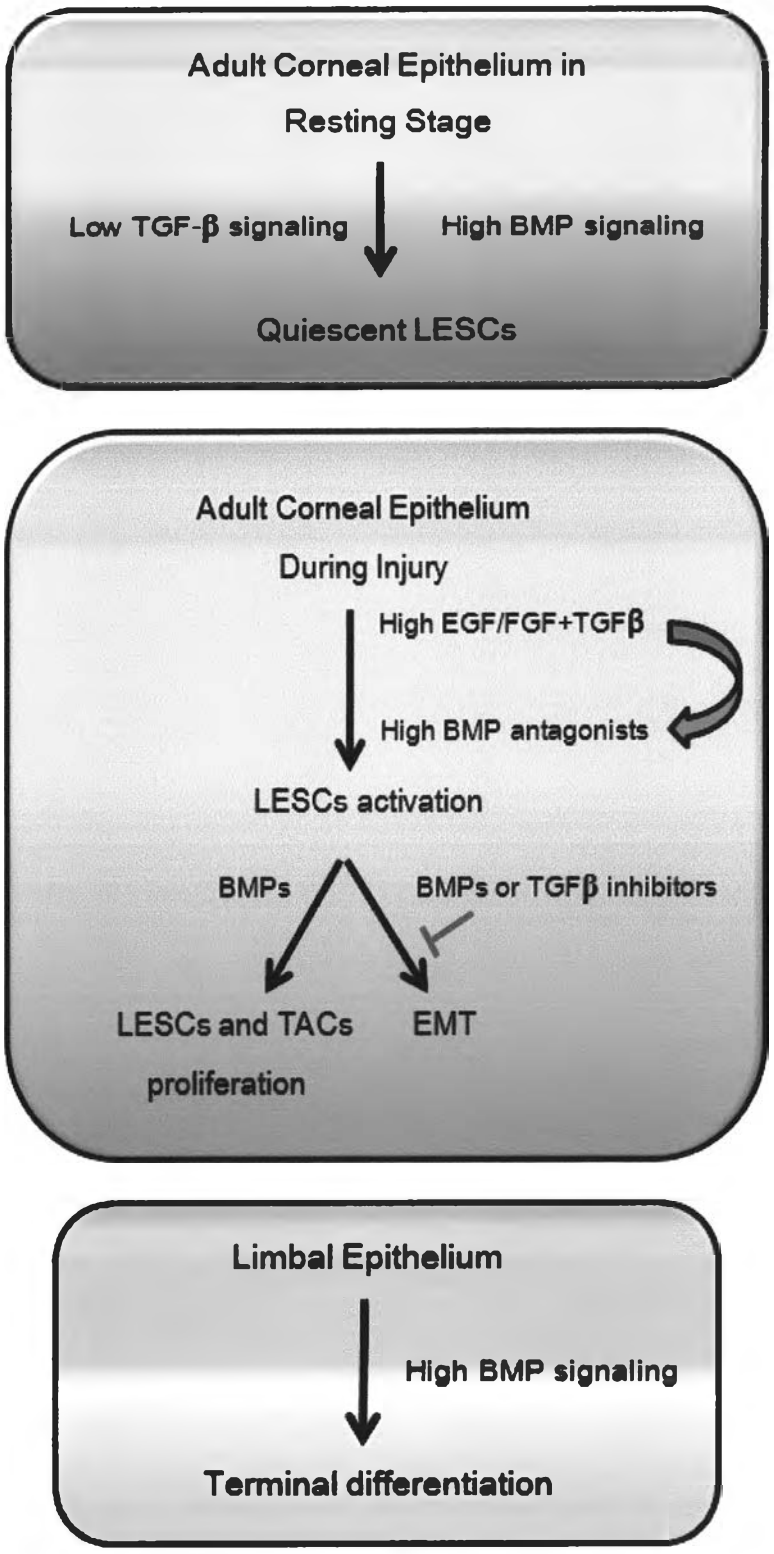

Figure 20. Mechanisms of TGF-β and BMPs in limbal epithelial stem cell fate regulation.