# CHAPTER II

# BACKGROUND AND RELATED LITERATURES

## 1. Stem Cells

Stem cells are cells at the origin of any cell lineages or migratory pathways in the tissue and they are the only permanent long-term residents of the tissue. Within adult replacing tissue of the body, the stem cells can be defined as a small subpopulation of the proliferating compartment, consisting of undifferentiated proliferative cells that maintain their population size when they divide, while at the same time producing progeny that enter a dividing transit population, together with differentiation events, resulting in the production of differentiated functional cells required of the tissue. The stem cells persist throughout the animal's lifetime in the tissue with a large division potential, these cells are the most efficient repopulators of the tissue following injury.

Stem cells are defined by the following three criteria. First, stem cells undergo self-renewing cell division that can produce at least one identical cell to maintain the stem cell pool. Second, stem cells have a high proliferative capacity for cell division that maintained through the lifetime of an organism. Third, stem cells undergo lineage commitment and differentiation; they generate more differentiated progenitors and ultimately terminally differentiated cells.

## 2. Limbal Epithelial Stem Cells

Throughout life, our corneal epitheliums on the front surface of the eye rely upon populations of stem cells/ progenitors to replenish themselves following normal wear and tear. At the corneo-scleral junction in an area known as the limbus, there is population of limbal epithelial stem cells (LESCs). The first experimental indication of the location of LESCs in the limbus was the pigment melanin movement from the limbus region towards an epithelial defect in rabbit corneas following wounding (9). Davenger and Evenson later observed a centripetal migration of pigment from limbus to central cornea in human and proposed that putative LESCs residing in the bottom of the epithelial papillae forming the limbal Palisades of Vogt (10, 11) that contain the pigment

melanin, which protects limbal basal cells against UV damage (13). For the reason that there is no consensus bona fide LESCs marker, the identification and characterization of LESCs have been based on the following criteria, which share common features with other adult somatic stem cells including :

1) Small size and cuboidal in shape that have a high nuclear to cytoplasmic ratio. (11, 14)
2) Slow cell cycling during homeostasis and the ability to retain labeled DNA precursors for long time periods, however in the event of injury or in vitro culture conditions they can become high proliferative potential that regenerate colony forming on feeder system (15, 16)
3) Asymmetric cell division for giving rise to a stem cell and a transient amplifying cell (TAC) which is the fast-cell-cycling and more differentiated cells. TAC can divide to form terminally differentiated cells that maintain or regenerate the tissue (17). However, in mammalian system, stem cells can also divide symmetrically to produce two stem cell daughters or two TACs (18).
4) Expression of putative LESC markers such as ABCG2, ΔNp63α, integrinα9, cytokeratin K19 that be used to identify LESCs in combination with the non-expression of cornea-specific differentiation markers such as cytokeratins K3/K12 and connexin 43 (12).

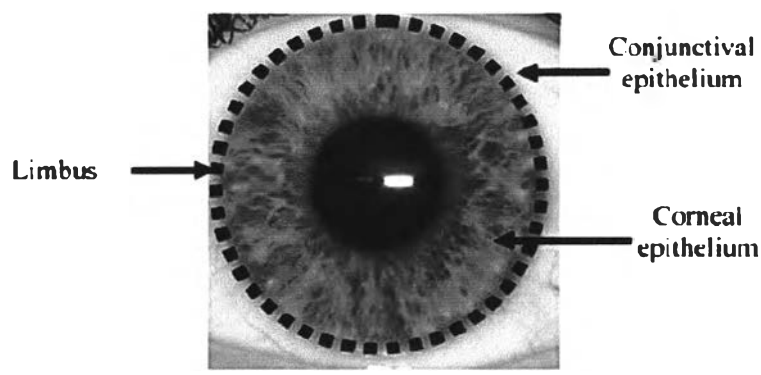


**Figure 1.** Location of the limbus. Limbal epithelial stem cells (LESC) reside in the limbal region of the cornea, along the dashed line (19).

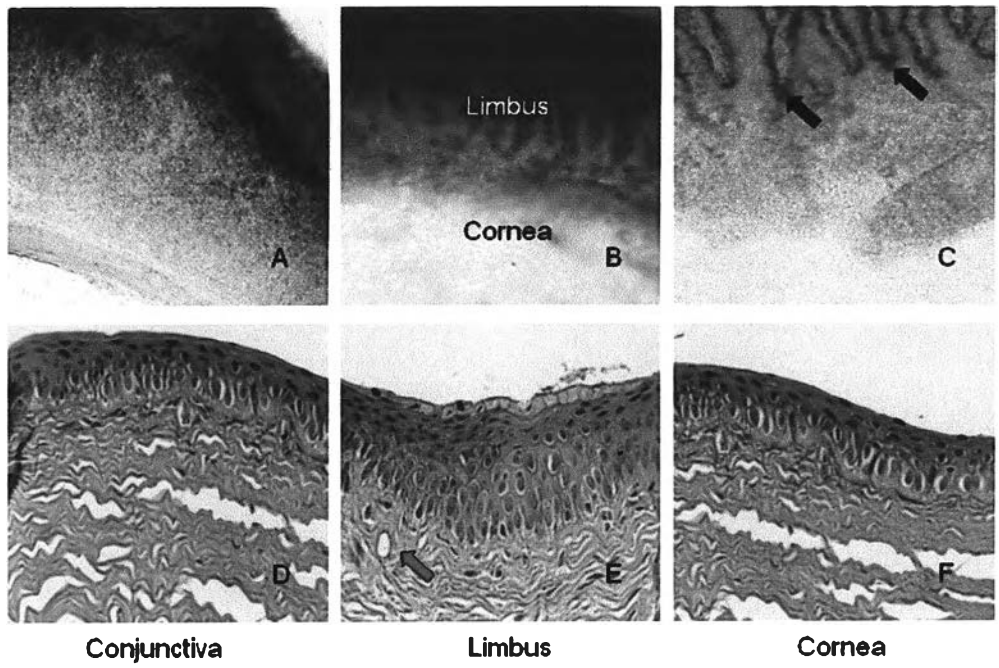


**Figure 2.** Light microscopic overview of the limbal explants showed the cluster of putative stem cells (LESCs) and putative early progenitors containing melanin granules (black arrows) at the bottom of the epithelial papillae forming the palisades of Vogt (A-C). H&E staining on human limbal paraffin sections showed a stratified epithelium, about five layers of corneal epithelia and 8-10 layers of limbal epithelia (D-F). Interestingly, the basal limbal epithelia connect to basement membrane with invagination, and the underneath stroma contains groups of limbal fibroblats and capillaries (E, red arrow shows blood vessel).

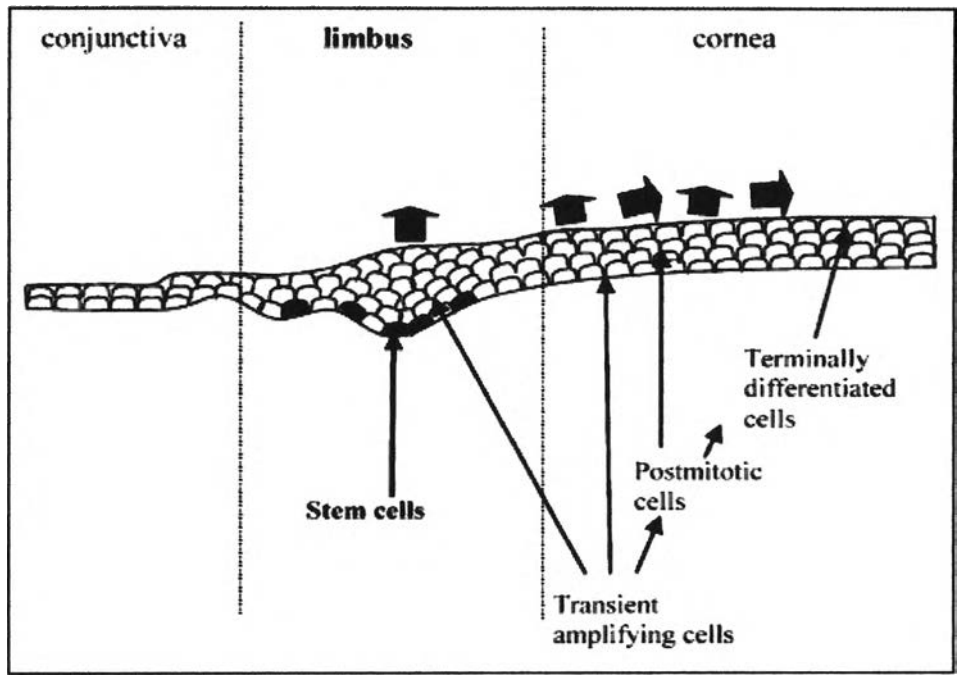


**Figure 3.** Limbal epithelial stem cells are normally located in the basal layers of the limbus and give rise to transient amplifying cells, which migrate towards the superficial layers of the corneal area ultimately forming the terminally differentiated epithelium (19).

## 3. Putative Limbal Epithelail Stem Cell-Associated Molecular Markers

The literature shows many attemps to prospectively identify LESCs using a specific marker. As yet no single or reliable molecular marker has been found. However, a combination of the expression and non-expression of putative LESC markers and differentiation markers seems to be allowed for specificity.

### 3.1. Putative LESC markers

**3.1.1. ATP-binding cassette transporter (ABCG2):** It has been proposed to be an universal marker for stem cells. Many types of organ-specific stem cells, including LESCs have been shown to exhibit a side population (SP) phenotype that is able to efflux DNA-binding dye Hoechst 33342 through ABCG2 (20). This protein has been immunolocalised to the cell membrane and cytoplasm of limbal basal cells and a few suprabasal cells (21). Furthermore, ABCG2-positive cells produce higher colony-forming efficiency (CFE) values in vitro than their negative cells (22).

**3.1.2. ΔNp63α**: p63 is transcription factor critical for maintaining the progenitor-cell populations, epithelial development and morphogenesis. It has been implicated in keratinocyte cell fate and maintenance of epithelial self-renewal. In vivo, p63 was located in basal epithelium of limbus and central cornea. However, limbal epithelial cells expressing high levels of nuclear p63 represent more stem like (23-25). ΔNp63α isoform is necessary for the maintenance of the proliferative potential of limbal stem cells and their ability to migrate over the cornea. Further work indicated that it was expressed in limbal epithelial cell derived holoclones, therefore ΔNp63α isoform may more specific for LESCs (26).

**3.1.3. Integrin α9**: Transmembrane glycoprotein that attach cells to extracellular matrix proteins or to ligands on other cells. It was reported to localize to the cell membranes and cytoplasm of limbal basal cells that absent in the central cornea and associated with TAC in wounded murine corneas (27), whereas some integrins such as Integrin-β1, β4 and α6 are more highly expressed overall in central corneal basal cells and the slow-cycling label-retaining cells. Integrin α6 are involved in hemidesmosome formation that showed a polarized distribution at the basal aspect of the basal cells (28)

**3.1.4. Cytokeratin K19**: A proliferative marker of keratinocytes in the skin which has been used to localize epidermal stem cells in hair follicles and human limbal epithelium. Colocalization if K19 and vimentin was particularly pronounced in basal limbal cells. These findings suggest that K19 seemed to localize specifically to TACs along the corneal-limbal borderline (12, 16).

**3.1.5. N-cadherin**: An important mediator of cell-cell adhesion via hemophilic binding interactions that play a key role in the long-term maintenance of haematopoietic stem cells quiescence by facilitating adhesion to osteoblasts in the bone marrow niche (29). Hayashi et al found expression of N-cadherin in subpopulation of limbal basal epithelial cells and in adjacent melanocytes that support the human LESC niche, suggesting N-cadherin plays a critical role in interactions between corneal epithelial stem/progenitor cells and their corresponding niche cells (30).

**3.1.6 CCAAT/enhancer binding protein δ (C/EBPδ)**: Transcription factor regulates cell cycle by inducing a $G_0/G_1$ arrest. Expression of C/EBPδ in a subset of LESCs both in vivo and in vitro has recently been implied to promote the self-renewal and induce mitotic quiescence through a positive regulation of $p27^{kip1}$ and $p57^{kip2}$ and increase the cell cycle length of LESCs (31).

### 3.2. Corneal epithelial differentiation markers

**3.2.1. Cytokeratin K3 and K12**: Cytoskeletol proteins that form intermediate filaments specifically in corneal epithelial cells, presented in all layers of the corneal epithelium and the suprabasal layers of the limbal epithelium (12).

**3.1.2 Connexin 43 (Cx43)**: Gap junction for cell-cell communication presented in the corneal epithelium. Incontrast, it has been reported to be absent from basal limbal epithelium in human, whereas suprabasal limbal cells showed slightly positive membrane staining. Gap junction-mediated intercellular communication has been linked to the regulation of cellular homeostasis, growth, development and differentiation. It means that the expression of Cx43 denotes the corneal TACs differentiation and that the absence of this gap junction may maintain their stemness in a distinct microenvironmental niche (32).

## 4. Limbal epithelial stem cell niche

Fundamentally every tissue of the adult organism has mechanism for maintenance homeostasis of the tissue and response to injury by a population of stem cells. Stem cells are maintained and supported by surrounding microenvironment, referred to the stem cell niche. The niche includes cellular and non-cellular components, consisting of intrinsic and extrinsic cellular mechanisms; such as niche cells, extracellular matrix molecules, growth factors, cytokines and diffusible signals. The niche is believed to maintain a balance of quiescence, self-renewal, and cell fate commitment (33). Within the limbal region of cornea, the LESC niche is expected to be located in the Palisades of Vogt (PV), which is highly pigmented with melanocytes in the limbal basal layer and is infiltrated with Langerhan's cells and T-lymphocytes. The melanin pigmentation is thought to shield LESCs from ultraviolet light damaging and the

reactive oxygen formation (34). The deep undulations of PV at the limbus provide the protection of LESCs with an environment from shearing forces (35). The basement membrane lining the LESCs niche contains papillae of stroma that project upward and fenestrated, suggesting that LESCs might closely interact with the underlying stromal cells (36). The LESCs interact with unique extracellular components, containing laminin-1,-5 and $\alpha 2\beta 2$ chains that not found in cornea. Furthermore, type IV collagen $\alpha 1$, $\alpha 2$ and $\alpha 5$ chains are located in the limbal region whereas $\alpha 3$ and $\alpha 5$ are found in the cornea. Recent study found patchy immunolocalisation of laminin $\alpha 3$ chain, BM40/SPARC and tenascin C, which co-localized with ABCG2/p63/K19-positive and K3/Cx43/integrin $\alpha 2$ negative cell clusters (37). Moreover, the basement membrane also help separate and modulate growth factors and cytokines concentration that released from limbal niche cells and involved in LESC regulation and function (38). Underneath the basement membrane, limbal stroma is vascularised and highly innervated unlike avascular cornea; therefore it is a potential source of nutrients and growth factors for LESCs.

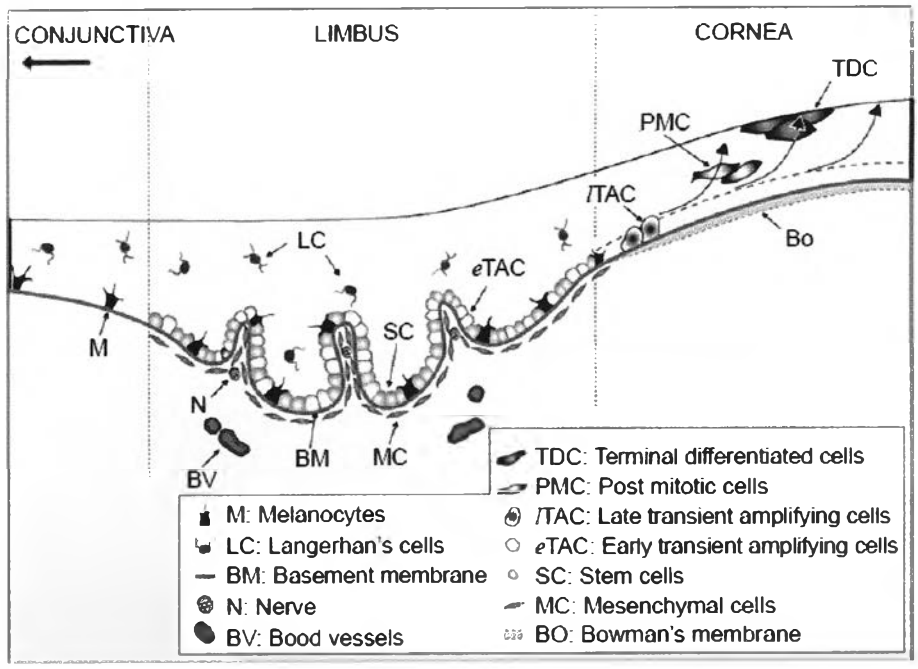


**Figure 4.** Hypothetical scheme of limbal stem cell niche. Limbal epithelial stem cells (SC) are located at the limbal basal layer. There are several other cell types in the surrounding such as early transient amplifying cells (eTAC), melanocytes (M), and Langerhan's cells (LC) that determined whether these cell types act as niche cells. It is believed that eTAC will be destined for progeny production by differentiating into late TACs (/TAC) located at the corneal basal layer, then into suprabasal post-mitotic cells (PMC), and finally into superficial terminally differentiated cells (TDC). The limbal basement membrane (BM) separating the epithelium from the underlying stroma has several unique components. The subjacent limbal stroma contains mesenchymal cells (MC), which may also serve as niche cells. Because the limbal stroma is highly innervated and vascularized, the respective role of nerves (N) and blood vessels (BV) in the niche remains to be defined (34).

## 5. Niche signaling in limbal epithelial stem cells

Currently, major developmental signaling pathways have been implicated in regulation of epithelial stem cells. Sonic hedgehog (Shh), Wnt/β-catenin, BMP/TGF-β and Notch signaling pathways have all being implicated in the stem cell niche (39).

### 5.1 Wnt/ β-catenin signaling

In the context of stem cells, Wnt signaling pathway and it's downstream transcription factors, Lymphoid enhancer factor-1/T-cell factor (Lef/Tcf) are involved in the maintenance of the niche. It has been implicated in cellular proliferation and in differentiation in the intestine and the skin. It is critical for either maintenance of proliferation within the stem cell niche or inhibition of differentiation of the TA cells. In addition, components of the Wnt signaling pathway, β-catenin, Lef-1, and Tcf-3 are expressed in the dermal papillae and the matrix cells, indicating that Wnt signals play an important role in hair follicle morphogenesis. Furthermore, c-Myc, a downstream target of Wnt signaling, can stimulate differentiation of epidermis in vitro (40). In vivo studies overexpressing c-Myc in the basal layer of the epidermis resulted in an increase in sebaceous gland, suggesting that Wnt signaling promotes sebocyte differentiation (41). Wnt signaling may also impact cellular adhesion and migration within the epidermal stem cell niche. Forced expression of c-Myc resulted in depletion of the stem cells within the epidermal stem cell niche as the animal aged, suggested that c-Myc acts to stimulate cells to exit the stem cell compartment by modulating the adhesiveness of the stem cell niche (42). However, different levels of Wnt signaling may result in different effects on stem cell behavior. In mice, Dkk2, a Wnt pathway inhibitor expressed in the corneal/limbal epithelium, is required to modulate Wnt activity in the limbal stromal region. Dkk2 leads to corneal epithelium formation and prevents the development of a stratified epidermis and skin appendages, these data show that Dkk2 is a key regulator of the corneal versus epidermal fate of the ocular surface epithelium. The expression of Pax6, a homeobox gene of profound importance for eye development and maintenance of the cornea, is also lost in the corneal epithelial cells of Dkk2 mutant, suggesting it is downstream of Dkk2 (43).

### 5.2. Notch signaling

Notch proteins are large transmembrane proteins. Four Notch genes have been identified in mammals (*Notch1-Notch4*), that interacts with neighboring cell surface ligands Delta and Jagged (*Delta 1, 3, and 4* and *Jagged 1 and 2*). This interaction triggers the proteolytic cleavage of the Notch receptor at the membrane proximal region

by the γ-secretase presenilin. In "canonical" pathway in mammalian cells, the Notch intracellular domain (NotchIC) translocates to the nucleus where it mostly associates with the recombination signal binding protein for the immunoglobulin kappa J region (RBPJκ). The NotchIC/RBPJκ complex transactivates downstrem targets such as Hairy/Enhancer of Split (*HES*) and Hairy/Enhancer of Split-related with YRPW motif (*HEY*) genes, which in turn affect numerous pathways involving cell-fate determination. HES proteins inhibit the activity of various basic helix-loop-helix transcriptional activators, including Math-1 and neurogenin 3.

The Notch signaling pathway is implicated in determining cellular differentiation in both the intestinal and epidermal stem cell niche. In human epidermis, expression of the Notch ligand DLL1 (Delta1) was confined to the basal layer, and was most highly expressed where the keratinocyte stem cells reside, which indicates a possible role for Notch/Delta signalling in stem-cell maintenance. Cells expressing high levels of Delta1 failed to respond to Delta signals from their neighbours. In contrast, wild-type keratinocytes those were in contact with neighbouring cells expressing Delta1 were stimulated to leave the stem-cell compartment and initiate terminal differentiation after a few rounds of division. It proposed that high Delta1 expression by epidermal stem cells has a protective effect on stem cells by blocking Notch signaling and enhanced cohesiveness of stem-cell clusters, which may interfere association to neighbouring cells; and signalling to cells at the edges of the clusters to differentiate (44). Inactivation of Notch1 in the epidermis resulted in depression of Wnt/β-catenin signaling in cells that normally undergo differentiation, this suggested that Notch1 acts to suppress pathways that normally promote proliferation (45). Further, in vitro overexpression of Delta in keratinocytes resulted in failure of cells response to neighboring cells and blocked these cells from undergoing terminal differentiation (46). This observation suggested that Notch signaling promotes epidermal differentiation by promoting migration of differentiating cells. In addition, p63 expression is suppressed by Notch1 activation in both mouse and human keratinocytes, whereas exogenous expression of ΔNp63α blocked Notch1-dependent growth arrest and differentiation in mouse keratinocytes by inhibiting p21 expression and maintaining integrins expression. Thus, a complex cross-

talk between Notch and p63 is involved in the balance between keratinocyte self-renewal and commitment to differentiation (47, 48). However, Notch-1 plays an active role in maintaining progenitor cells in an undifferentiated manner in several systems such as neural and hematopoietic systems. Recently, Notch-1 was found to be expressed mainly in limbal basal region where stem cells reside, whereas it was almost devoid in the central cornea (49). Hes1, a major target gene in Notch signaling, was observed mainly in the corneal epithelial stem/progenitor cells and plays a critical role in the regulation of corneal epithelial integrity. The absence of the bHLH factor gene *Hes1*, corneal development and morphogenesis is severely disturbed and cornea tissue revealed abnormal cell differentiation and less proliferative ability (50).

### 5.3. Bone Morphogenetic Protein (BMP) signaling

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, which includes TGF-βs, activins/inhibins, Nodal, myostatin and Mullerian inhibiting substance (MIS). They are secreted cytokines that regulate a broad array of cellular responses including proliferation, differentiation, migration and apoptosis. Mammals possess more than 20 BMP-related proteins. BMP-1 through BMP-7 is expressed in skeletal tissue, and BMP-2, 4, 6 are the most detectable BMPs in osteoblast cultures.

BMP signaling is initiated by ligand-induced heteromeric complex formation of specific type I and type II serine/threonine kinase receptors, both of which are required for signal transduction. Up on ligand binding of type II receptor, the type I receptor (BMPR-IA, BMPR-IB and ActR-IA, also called ALK3, ALK6 and ALK2 respectively) is phosphorylated by the type II receptor (BMPRII, ActRII and ActR-IIB) on specific serine and threonine residues in the juxtamembrane domain. The activated type I receptor initiates intracellular signaling through phosphorylation of receptor-regulated Smads (R-Smads) at two C-terminal serine residues. The recruitment of R-Smads to the receptor complex is mediated by auxiliary proteins, such as Smad anchor for receptor activation (SARA). Activated R-Smads form a complex with common partner Smads (Co-Smads), and this complex translocates to the nucleus to control the transcription of target genes by interacting with various transcriptional factors and transcriptional co-activators or co-

repressors (Figure 5). Eight Smad proteins (Smads 1 through 8) identified in mammals, Smad1, 5, 8 are BR-Smads activated by BMP type I receptors, whereas Smad2 and 3 are AR-Smads activated by TGF-βs, activin and Nodal type I receptors. Smad4 is the only Co-Smad in mammals, that shared by both BMP and TGF-β/activin signaling pathways. Smad6 and 7 function as inhibitory Smads (I-Smads), negative regulators of the TGF-β superfamily signaling, that compete with R-smads for activation by type I receptors and prevent complex formation between R-Smads and Co-smad (51).

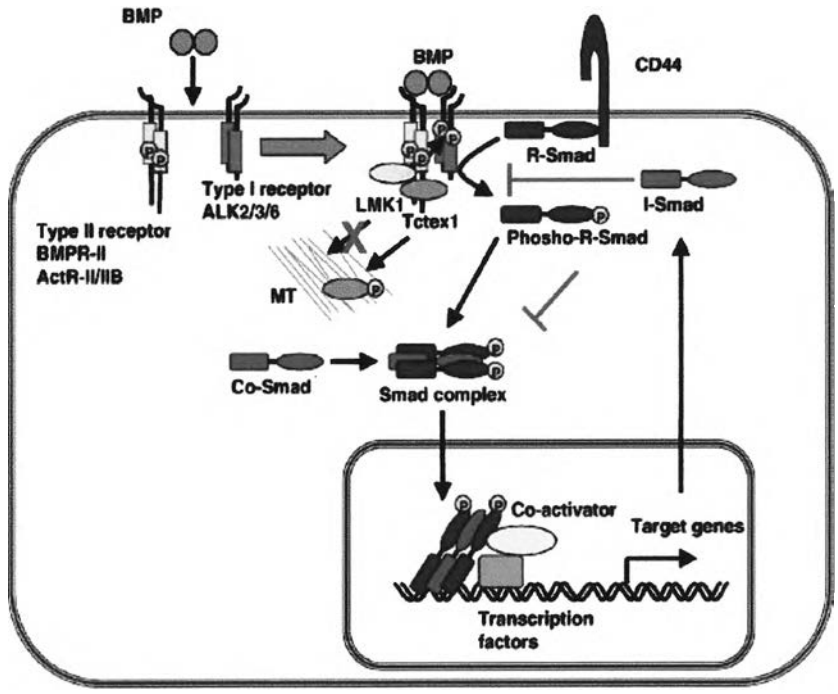


**Figure 5.** BMP signaling through Smad proteins. BMPs bind to two distinct serine/threonine kinase receptors, and induce phosphorylation of R-Smads. R-Smads form complexes with Co-Smad, and move into the nucleus, where they regulate transcription of target genes. One of the targets of Smad signaling is I-Smads, which suppress Smad signaling by interacting with activated type I receptors and inhibiting the complex formation between R-Smads and Co-Smad. The C-terminal region of BMPR-II physically interacts with some proteins, and regulate BMP signaling, e.g. through regulation of cytoskeletal protein functions. BR-Smads are anchored at the plasma membrane by CD44, a receptor for hyaluronan, MT: microtubule (51).

BMPs play a critical role in skin development, the control of epidermal homeostasis, hair follicle growth, and melanogenesis. Furthermore, BMPs are implicated in a variety of pathobiologic processes in skin, including wound healing, psoriasis, and carcinogenesis. BMP signaling regulates the wound-healing process by changing the proliferative activity and differentiation of epidermal keratinocytes and by altering the collagen-producing activity of dermal fibroblasts (52). The effect of BMPs depend on the concentration and distribution of BMPs and their antagonists, low or high dosages of BMPs always result in opposite cell fate decisions: either proliferation or differentiation. Different effects of BMPs are often mediated by distinct BMP receptors. Several models suggest that the proliferative effect is mainly mediated via BMP receptor-IA (BMPR-IA) that expressed in the basal layer, whereas apoptotic signaling is mainly mediated through the receptor BMPR-IB that expressed in the suprabasal layer of epidermis (53). BMP-4 functions in ectodermal and epidermal development by stimulating sox-$1^{+}$ neural precursors to undergo apoptosis while inducing epidermal differentiation through ΔNp63 gene activation (54). BMP signaling had been shown to inhibit anagen-phase induction of the hair follicle and restrict epithelial stem cells activation and expansion (55). In the bulge area, a niche in which epithelial stem cells reside, BMP signaling inhibits the Wnt/β-catenin signal partially through the PTEN-Akt pathway and may also through Smad-mediated signaling for controlling activation/arresting of stem cells (56). Upon constant expression of BMP, dynamic expression of Noggin, which is the most powerful BMP-2 and BMP-4 antagonist, plays a role in coordination with Wnt signaling to temporarily activate β-catenin that overriding BMP signaling and leading to initiation of a new hair follicle growth cycle (6). Correspondingly, whereas BMP4 is constantly expressed in the mesenchyme of intestinal microvilli, burst of Noggin expression in the villi stem cell niche acts transiently to lower BMP signaling, thus allowing stem cells to proliferate for epithelial renewal (57).

In human epidermal keratinocytes, BMP-2/6 signaling inhibits cell growth and triggers terminal differentiation involved in up-regulation of cyclin-dependent kinase inhibitors; $p57^{Kip2}$ and $p21^{Cip1}$ (58). Intriguingly, ΔNp63/BMP-7 signaling pathway modulates wound healing process through transcriptional regulation of extracellular matrix protein matrilin-2 (59). However, BMP signaling plays a synergistic role with Wnt

signaling in hair shaft differentiation. Further, BMP2/4/7 signaling plays an essential role in FGF-dependent secondary lens fiber differentiation/formation in mouse (60).

BMP-2, BMP4, and their receptors are expressed in the cells of the adult human cornea. The functions regulated by these cytokines may include keratocyte proliferation and apoptosis (61). Recently, the BMP family was correlated with the morphogenesis of digits and ocular development, and BMP-4 heterozygous null mice revealed skeletal abnormalities including polydactyly and ocular anterior segment abnormalities. Patients with a haplo-insufficiency of the BMP4 gene also had the congenital corneal opacity and feet polysyndactyly (62). Moreover, overexpression of BMP-7 is an effective strategy for treatment of ocular alkali burns. Exogenous BMP-7 gene expression accelerated cell proliferation in the healing epithelium and that might be attributed to the reduction of TGF-β that normally has a growth-suppressing effect on cells (63).

### 5.4. Transforming Growth Factor-β Signaling

In general, members of the TGF-β superfamily bind to heterotetrameric Receptor complexes consisting of type I and type II receptors that contain serine/threonine kinase domains. Five type II receptors have been identified so far. Seven type I receptors are designated activin receptor-like kinases (ALK-1 to ALK-7) and presently known in mammals (64). In most cells, TGF-β binds to ALK-5 receptor that activates Smad2 and Smad3. In a few other cells, TGF-β activates ALK-1 in the presence of functional ALK-5 resulting in phosphorylation of Smad1 and Smad5. The decision which type I receptor is activated is determined by receptor expression and/or ligand concentration. ALK-1 activation triggers proliferation and migration, whereas ALK-5 opposes these responses (65).

Transforming growth factor-β (TGF-β) is a multi-functional growth factor with several critical roles during normal wound healing. TGF-β regulates cell proliferation, extracellular matrix (ECM) synthesis and degradation, mesenchymal-epithelial interactions, inflammation, carcinogenesis and promotes connective tissue regeneration (66). There are three mammalian isoforms; TGF-β1, -2 and -3 which have been detected in corneal epithelium and stroma and they play distinct roles in wound repair (67). Among them, TGF-β1 and TGF-β2 are the predominant forms in the ocular surface and

play roles as negative modulators of corneal cell proliferation, and they induce G1-phase arrest in limbal basal cells through an autocrine or a paracrine mechanism (68, 69). Furthermore, TGF-β1 was found that may control the differentiation and proliferation of corneal epithelial cells through down regulation of various targets, including plasminogen activator inhibitor type 2 (PAI-2), transferrin, integrin α3, and cyclin D1 (70).

TGF-β also induces cell migration and inhibits proliferation of injured corneal epithelial cells, whereas it stimulates proliferation of normal corneal epithelial cells via effects on the MAPK family and Smad signaling (71). TGFβ -induced cell migration is correlated with the production of components of the extracellular matrix (ECM) proteins and expression of integrins and matrix metalloproteinases (MMPs) that cleave various types of ECM molecules including collagens, laminins, fibronectin as well as its ligands, the integrins (4). TGFβ receptor Types I and II had been found to present in human corneal epithelium and that both are preferentially localized in the limbus. Moreover, they are expressed at high levels in cell migrating to cover a corneal wound. However, cells migrating to cover the original wound appear to be inhibited from proliferating, the cell-cycle-dependent kinase inhibitor $p15^{INK4b}$ appears to be preferentially upregulated (72). Likewise, TGF-β1 and TGF-β2 treatments in primary cultured human limbal epithelial cells stimulated production of the CDKIs $p57^{Kip2}$ and $p15^{INK4b}$ and inhibited proliferation of these cells (73). Additionally, TGF-β1 is upregulated and involved in the response to corneal injury and the process of corneal stromal scarring. TGF-β1 over-activity is considered the primary pro-fibrotic factor that causes excessive ECM accumulation and scar. On the other hand, exogenous application of the antifibrogenic TGF-β3 has been associated with improved wound healing outcomes (74). TGF-β has been shown to inhibit epithelial cell cycle progression and promote apoptosis that contribute to the tumor suppressive role during carcinoma initiation and progression. TGF-β is also able to promote an epithelial-to-mesenchymal transition (EMT) that has been associated with increased tumor cell motility, invasion and metastasis (75).

## 6. Epithelial-to-Mesenchymal Transition (EMT)

EMT is necessary for embryonic development, tumor progression and organ fibrosis. During EMT, epithelial characteristics are lost and a mesenchymal phenotype is acquired. The cell morphology changes from a cuboidal to a fibroblastic shape and intercellular epithelial adhesion molecules such as E-cadherin and zonula occludentes protein (ZO-1) are replaced by mesenchymal cytoskeletal markers such as fibroblast-specific protein 1 (FSP1), vimentin, α-smooth muscle actin (α-SMA). Instead of interacting with the extracellular matrix (ECM) at the basal cell surface, the transdifferentiated cells acquire the ability to invade the ECM (76). In adults, EMT is reviewed involving resident epithelia in response to injury, as an additional source of myofibroblasts/fibroblasts which are essential for repair of injured tissue (77). Thus, the reverse program, termed the mesenchymal-to-epithelial transition (MET), also occurs both during embryonic development and during several pathological processes. Several transcription factors; these include Snail/Slug, Twist1, FOXC2, ZEB1 and ZEB2 had been found to be capable of repressing the transcription of E-cadherin and several polarity factors, including Crumbs3 and Lgl2, thereby causing the detachment of cell-cell adhesion that occurs during EMT (78). One additional transcriptional pathway regulating EMT is the β-catenin-mediated transcription program. The dissolution of E-cadherin from adherens junctions results in the release of its partner, β-catenin, into the cytosol, which then enters the nucleus where it can activate LEF/TCF-mediated transcription. Several studies suggest that β-catenin-mediated transcription can induce the expression of Slug and Twist1, thereby contributing to the EMT program (79).

## 7. Inhibitor of differentiation/DNA binding (Id)

Inhibitors of differentiation/DNA binding (Id) proteins are helix-loop-helix (HLH)-transcription factors, which lack a basic site-specific DNA binding domain. Id proteins act as dominant inhibitors of bHLH transcription factors by heterodimerization, thus inhibiting gene expression. Id proteins have been implicated in regulating a variety of cellular processes, including cellular growth, senescence, differentiation, apoptosis, angiogenesis, and neoplastic transformation. Id proteins may function as oncogenes, in

addition to inhibiting G1 cell cycle arrest and differentiation. Id genes have been shown to enhance cell cycle progression, and their overexpression can induce apoptosis in serum-deprived fibroblasts (80). The Id family of proteins (comprised of 4 members designated Id1–Id4) has been demonstrated to bind E-proteins or cell lineage-restricted bHLH transcription factors, leading to inhibit lineage-specific gene expression and differentiation. Transcriptional inhibition by Id proteins is mediated via inhibition of DNA binding of bHLH or other activator proteins at E boxes (CANNTG), N boxes (CACNAG), or Ets sites (GGAA/T) present in the promoter regions of regulated genes. While E-proteins activate transcription by binding to promoter E-boxes, Id proteins lacking basic DNA-binding domain form DNA binding incompetent heterodimers with E-proteins. This leads to the transcriptional repression of different cell cycle regulators, which implies important roles of Ids for cell fate decision of growth and differentiation. Expression of Id proteins is typically high in actively proliferating cells and is down-regulated as a prerequisite for exit from the cell cycle and terminal differentiation. Id proteins promote cell proliferation by regulating the expression of genes or activities of proteins involved in cell cycle such as the cyclin-dependent kinase inhibitors and the retinoblastoma tumor suppressor protein pRb (81, 82).

Id-1 has been shown to inhibit E-protein and Ets-protein-mediated activation of the cdk inhibitor p16/INK4a, leadting to extends the lifespan of primary human keratinocytes and activates telomerase activity (83). In addition, breast cancer cell lines that express Id-1 have been shown to possess more invasive phenotypes than those that did not (84). Id-1 has recently been proposed as a key regulator in tumour progression and found to be associated with activation of angiogenesis and EMT (85, 86). In a different manner, TGF-β induces apoptosis through repression of Id-2 and activation of hypoxia-inducing factor-1 (HIF-1) in gut epithelial cells (87), whereas Id-3 act as one of the downstream targets for ΔNp63α-mediated behaviours to restrain tumour invasion in human squamous cell carcinoma (88).

## 8. Cell cycle regulation

The cell cycle profile of stem cells is characterized by a predominance of cells in G0/G1 or slow-cycling, and as such removal of the inhibition of progression through G1 has been thought to be a rational approach to expand stem cells ex vivo. Cell division consists of two consecutive processes; mitosis (M) and interphase (including G1, S, and G2 phases). M, G1, S, and G2 form the cell cycle. In the process of cell cycle regulation, cyclin-dependent kinase inhibitors (CDKI) play an important role. Two major classes of CDKI have been identified: Cip/Kip and INK4. The Cip/Kip family contains the more general CDKI consisting of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which specifically inactivate G1 cyclinE/CDK2 and cyclinD/CDK4/6. Member of the INK4 family, including $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$, possess 4 ankyrin repeats and inhibit the G1 cyclinD/CDK4/6 complexes. Increased levels of these CDKIs result in a major inhibition of CDK activities associated with the early G1 phase progression, thus blocking the cell cycle prior to the G1 restriction point. Further, expression of $p16^{Ink4a}$ and $p19^{Arf}$ in normal hematopoietic stem cells resulted in proliferative arrest and p53-dependent cell death (89, 90). p53 protein is the product of a pivotal tumor-suppressor. The p53 protein integrates multiple cellular stress signals assessing cellular damages to trigger either cell-cycle arrest or programmed cell death (apoptosis). These effects are predominantly due to p53's ability to bind DNA through p53-responsive element (p53RE) and regulate the transcription of genes involved in these processes, such as $p21^{Cip1}$ (cell cycle arrest), Puma or Scotin (apoptosis). Thereby, p53 prevents proliferation of genetically abnormal cells and thus cancer formation (91).