การเพาะเลี้ยงเซลล์ต้นกำเนิดของกระจกตาภายนอกร่างกายที่สามารถช่วยรักษาคุณสมบัติของเซลล์ต้นกำเนิดไว้ได้

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LIMBAL STEM CELLS MAINTENANCE IN THE IN VITRO CULTIVATION

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Limbal epithelial stem cells คือ เซลล์ตั้งต้นของกระจกตา จัดอยู่ในกลุ่มของ epithelial stem cells มีคุณสมบัติ เช่น แบ่งตัวช้า สามารถฟอร์มโคโลนีได้ เซลล์ต้นกำเนิดของกระจกตานี้ สามารถนำมาเพาะเลี้ยง และนำไปใช้รักษาผู้ป่วยโรคตา ที่มีสาเหตุมาจาก limbal stem cells deficiency ได้แต่ในการเพาะเลี้ยงเพิ่มจำนวนเซลล์ตั้งต้นดังกล่าวนั้นพบว่า เซลล์มักจะหยุดแบ่งตัว และสูญเสียคุณสมบัติเสมอ เมื่อทำการเพาะเลี้ยงไปได้เพียงไม่นาน จึงกลายเป็นอุปสรรคสำคัญในการนำไปใช้ ดังนั้นจึงสร้างระบบการเพาะเลี้ยง limbal epithelial stem cells ให้มีความใกล้เคียงกับ microenvironment ที่เซลล์ต้องการ โดยสร้างเซลล์พี่เลี้ยงให้มีการแสดงออกของ BMP antagonist ทั้ง Noggin และ Gremlin-1 และกระตุ้นการทำงานของ Wnt signaling ที่อยู่ในเซลล์ต้นกำเนิดของกระจกตาโดยใช้สารที่เรียกว่า GSK-3β inhibitor ซึ่งทั้งสองมีความสำคัญต่อการคงคุณสมบัติของเซลล์ต้นกำเนิดเมื่ออยู่ภายในร่างกาย จากการทดลองแสดงให้เห็นว่า BMP antagonist โดยเฉพาะ Gremlin-1 ที่แสดงออกอยู่ในเซลล์พี่เลี้ยงมีผลทำให้เกิดการแสดงออกของยีน $p27^{kip1}$ ซึ่งปกติทำหน้าที่ควบคุม cell cycle ของเซลล์นั้นเพิ่มสูงขึ้น ส่งผลให้เซลล์ต้นกำเนิดจากกระจกตาแบ่งตัวช้า และเมื่อใช้การกระตุ้นให้ Wnt signaling ทำงาน ควบคู่กันไปกับเซลล์พี่เลี้ยงดังกล่าว จะช่วยทำให้เซลล์ต้นกำเนิดของกระจกตาแบ่งตัวเพิ่มจำนวนได้ดีมากขึ้นโดยไปลดการแสดงออกของยีน $p27^{kip1}$ กล่าวโดยสรุปคือ เซลล์พี่เลี้ยงที่ทำให้มีการแสดงออกของยีน BMP antagonist และการกระตุ้น Wnt signaling สามารถช่วยคงคุณสมบัติและช่วยเพิ่มความสามารถในการแบ่งตัวของเซลล์ต้นกำเนิดของกระจกตาเมื่อทำการเพาะเลี้ยงอยู่ภายนอกร่างกายได้

สาขาวิชาวิทยาศาสตร์การแพทย์.....ลายมือชื่อนิสิต.....

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The maintenance of a healthy corneal epithelium under both normal and wound healing conditions is achieved by a population of stem cells (SC) located in the basal epithelium at the corneoscleral limbus. These cells, also called limbal epithelial stem cells (limbal SC) retain their self-renewal and maintain their proliferative capacity. Limbal stem cell can be expanded ex vivo on amniotic membrane and used for transplantation to the patients who suffer from limbal stem cell deficiency. However, this technique still has drawbacks; particularly stem cells lose their properties that limit the use for transplantation. Therefore, the studying about limbal stem cells niche and developing a culture system that similar to their niche are required for maintenance limbal stem cell in long term culture by activating Wnt signaling in limbal stem cells and creating Bone morphogenesis protein (BMP) antagonists expression feeder cells. These molecules have been showed a potential role in the proliferation and the maintenance of other epithelial stem cells in their niche. We founded BMP antagonist, Gremlin-1, prolonged cell cycle of limbal stem cell through down-regulation of cyclin-dependent kinase inhibitor, $p27^{kip1}$ and activation of Wnt signaling promoted proliferation of limbal stem cells.

Field of Study Medical Science.....Student's signature .....

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ABBREVIATIONS

SCs	Stem cells
AdSCs	Adult stem cells
LSCs	Limbal stem cells
LES	Limbal epithelial cells
mES	mouse embryonic stem cells
hES	human embryonic stem cells
SCs	Stem cells
HSCs	Hematopoietic stem cells
NSCs	Neural stem cells
EPCs	Epithelial stem cells
TAC	Transient amplifying cells
BMPs	Bone morphogenesis proteins
TGFβ	Transforming growth factor β
TCF-3	T-cell factors-3

Lef-1	Lymphoid enhancer factor-1
β-cat	β-catenin
BIO	6-bromoindirubin-3'-oxime
EGF	Epidermal growth factor

CHAPTER I

INTRODUCTION

Background and Rationale

Stem cells (SCs) have properties that can maintain their capacities for cell proliferation or self-renewal and pluripotence, which means they can differentiate into all cell types in our body and thus hold the promise tools for treating diseases. There are two major types of stem cells. The first one is embryonic stem cells (ESCs) which derived from blastocyst stage of fertilized egg. Another one is adult stem cells found in many adult tissues. Unfortunately, the use and study of embryonic stem cells are currently obstructed by ethical controversy. Adult stem cells offer a unique advantage in that they may be isolated, studied, or manipulated without harming the donor. Even so, there are still some problems concerning the markers for identify adult stem cells, undefined *in vitro* condition for cell manipulation, and the understanding of adult stem cells regulation within their environments.

All stem cell types require specific microenvironment or niche to provide growth factor, cytokines, or some signaling molecules for supporting stemness properties. For example, neural stem cells resided within subventricular zone in our brain close to blood vessel and surrounded by glial cells which translate signals and secrete some kind of growth factor or cytokines to promote stem cell maintenance. Haematopoietic stem cells (HSCs) attached to spindle-shaped N-cadherin+CD45+ osteoblastic cells lining the bone surface and functioning as a key component of the niche to support HSCs and that BMP signalling through its receptor controls the number of HSCs by regulating niche size (1). Next to those cells, epithelial stem cells are one of the most widely studied stem cell niches. Although the uniqueness of each epithelial stem cell niche in different organs makes it difficult to determine which exactly their microenvironment; they are likely to share many common aspect of regulation. The numbers of cell signaling factors sets up a microenvironment within the stem cell niche to promote adhesiveness of the stem cell, proliferation of the TA population, and

differentiation of the epithelium. One candidate is canonical Wnt signaling and its downstream transcription factors *L*ymphoid *e*nhancer *f*actor-1/*T*-cell *f*actor (Lef/Tcf) are intimately involved in the maintenance of the niche. In the presence of Wnt molecules, cytoplasmic level of unphosphorylated beta-catenin increase, allowing beta-catenin to enter the nucleus and interact with Lef/Tcf molecule, then regulate the expression of target genes that are important for cell proliferation, cell polarity, and cell fate decisions. (2,3). As for epithelium stem cell niche in the intestine, Wnt signaling is critical for either the maintenance of proliferation within the stem cell niche or inhibition of differentiation of the TA cells (4). Tcf3 and Lef1 are also thought to control differentiation of epidermal lineages, although by different mechanisms. Epidermal expression of dominant-negative mutations in Tcf3 or Lef-1 revealed that Lef-1 acts to determine hair follicle fate, whereas Tcf-3 acts independently of Wnt to maintain the stem cell compartment (5).

Another signaling pathway that also plays crucial roles in stem cell niche is bone morphogenetic proteins (BMPs). Bmps are members of the Tgf-beta superfamily. BMPs bind to specific serine/theronine kinase receptors, which transduce the signal to the cell nucleus through Smad proteins controlling the expression of target genes (6). BMPs, their antagonists and receptors are involved in regulating a large number of biological functions including cell proliferation, differentiation, cell fate decision, and apoptosis in many different types of cells and tissues during embryonic development and postnatal life. In epithelial stem cells, BMPs signaling in the control of cell differentiation and apoptosis in developing epidermis, as well as in the regulation of key steps of hair follicle development. Similarly, BMP4 was able to reduce hair-cell precursors, whereas the inhibition of endogenous BMP activity with Noggin had the opposite effect. (1,7). Furthermore, this signaling can inhibit self-renewal of intestinal epithelial stem cells self-renewal of intestine through suppression of Wnt-beta catenin signaling (8).

A number of recent studies have since disclosed that Limbal epithelium stem cells (LSC) share some features similar to adult stem cells. For instance, LSCs have the smallest cell size, are slow-cycling, label-retaining, and do not express markers destined for terminal differentiation such as cytokeratins 3 and 12, involucrin, and connexin 43. The maintenance of a healthy corneal epithelium under both normal and

wound healing conditions is achieved by a these population of stem cells (SC) located in the basal epithelium of the corneoscleral limbus. To dates, LSC can be expanded *ex vivo* and used for transplantation in patients who suffer from limbal stem cell deficiency. Several techniques have been optimized to make the culture systems more appropriate for the maintenance of LSC properties and to generate a greater numbers of cells. The culture system involves first separating the epithelial cells from the stroma by using dispase and then separating the epithelial cells from one another by using trypsin prior to seeding (9,10,11,12) either onto amniotic membrane which is the currently favoured substrate or onto a plastic tissue culture dish containing a feeder layer of growth-arrested 3T3 fibroblasts. Nevertheless, the techniques used to expand these cells are not enough for maintenance undifferentiated stage of LSC. Moreover, the cells often stop growing, become fully differentiate in about 2-3 weeks, and finally die. To solve this problem is to create *in vitro* environment that mimicked to LSCs niches in vivo.

Like epithelial stem cells, limbal stem cells are required unique microenvironments or niches. By definition, niche cells provide a sheltering environment that shields SCs from stimuli that may adversely promote differentiation and apoptosis. In this regard, LSC niche is located at the Palisades of Vogt which is highly pigmented because of melanocytes cells which function as the protector of LSCs from ultraviolate irradiation. The preferential expression of adhesion molecules such as N-cadherin, a member of the classic cadherin family mediated cell-to-cell adhesion via homophilic binding interactions and also involving with Wnt/Beta catenin cell-to-cell signaling, was founded in melanocyte and LSCs. This suggests that N-cadherin is an important cell-to-cell adhesion mediator between LESC and their niche. But the N-cadherin expression has been lost during the in vitro culture of those cells. Besides the cell-to-cell adhesion molecules, the communications between LSCs and niche cells through many cell signaling pathways such as Wnt/TCF and BMPs also help the LSCs to maintain their capacities. However, little is known about the cross-talk between LSCs and their niche. Hence, understanding of LSCs microenvironments is necessary. The knowledges gained from this study can be applied and used as tools for increase the potential of maintain LSCs in culture outside their niche. So in this paper, the researcher will study about how cell signalings via BMPs, Noggin, Gremlin-1 (BMPs antagonist), and Wnt/TCF

affect LSCs in culture and investigate whether those molecules can help to maintain the expanded LSCs and develop a protein transduction for culture LSCs. And another aim of this experiment is to use the expanded cells for clinical treatment in the future.

Research Questions

How and whether Wnt/Beta-catenin/TCF signaling and BMP signaling can help to maintain limbal stem cells capabilities when they are propagated in an *in vitro* culture?

Objectives

1. To study and understand the microenvironments of LSCs that are essential for *in vitro* LSCs maintenance of stem cell properties.
2. To find a defined technique that could help to maintain LSCs abilities to propagate *in vitro* cultivation.

Hypothesises

BMPs antagonists and the activation of Wnt signaling through could maintain undifferentiated state of limbal stem cells in the *in vitro* culture.

CHAPTER II

LITERATURE REVIEWS

Stem cells

Stem cells are defined as the cells that can divide themselves without losing their undifferentiated stage, so called **self-renewal**, and have a potential of infinite proliferation *in vitro*, include differentiated into all cell types in our body or **pluripotency**. From the properties of stem cells, these cells have led to many clinical applications. The classification of stem cells has divided into 2 major types which are embryonic stem cells (ESCs) derived from inner cells mass of blastocyst staged of fertilized eggs. The essential characteristics of embryonic stem cells also called pluripotent stem cell should include the following; (i) derivation from the pre-implantation or per-implantation embryo, (ii) prolonged undifferentiated proliferation, and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (13). Adult stem cells (AdSCs) or tissue-specific stem cells, such as Haematopoietic stem cells (HSCs), Neural stem cells (NSCs), including Epithelial stem cells (EPSCs) are nevertheless able to progress to differentiate along at least one (unipotent) and typically multiple (multipotent) lineages. Adult stem cells are participated in tissue homeostasis by regenerated the new cells replacing the damaged and apoptosis cells. In the same time, these cells are self-renewing maintained their own levels in tissue. Because of adult stem cells are specific in organs and tissues, they can be an alternative cells to generate patient-specific stem cells for using in therapeutic instead of the ESCs which currently obstructed by ethical controversy and cell reject problem. Even so there are still some problems about the markers for identify adult stem cells, undefined in vitro condition to manipulate the cells, and the understanding how adult stem cells regulated within their environments.

Limbal epithelial stem cells

To keep the tissue homeostasis of Ocular surface both in normal aging and injury in balance is processed by a cell population called limbal epithelial stem cells (LESCs). These LESCs are categorized as one of the tissue-specific stem cells and reside at the Palisades of Vogt of limbus between cornea and conjunctiva. There are some evidents showed that LESCs were found in this area. (14,15) For example, the study in the middle of 20th century showed that the injured corneal epithelium of rabbit eyes was rapidly replaced with the cells migrating from the limbus to cornea (16). And Schermer and the team (17) found that between conjunctiva and cornea recite distinct cell types whereas undifferentiated cells are mostly found in limbal basal. Latterly, Ebato and the teams (18) performed an experiment by cultured and compared the proliferative potential of cells from conjunctiva, cornea, and limbus. They suggested that the cells form limbus zone had more proliferative potential than the others. When LESCs responded to wound healing they divided into two daughter cells. One become cells with the same properties as the parent cells and the others become transit amplifying cells (TACs), which rapidly proliferate, differentiate, and migrate to the central of cornea, and renew the original-damaged corneal epithelial cells.

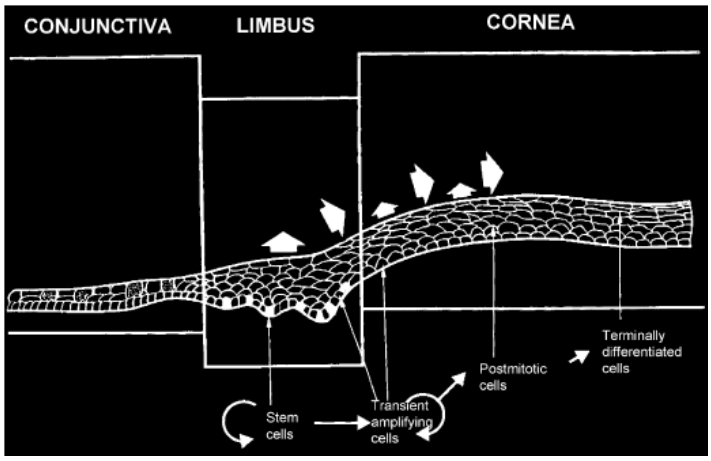

Figure 1: Concept of limbal location of corneal stem cells and transient amplifying cells. Stem cells(white) are located in the basal limbal epithelium at Palisades of Vogt. Transient amplifying cells occur in the basal epithelia of limbus and peripheral cornea. Post -mitotic and terminally differentiated cells make up the suprabasal and superficial layers. (19)

Limbal stem cells deficiency has three major consequences: conjunctivalization, corneal vascularization, and compromised epithelium causing the patient to lose their sight. The treatment of limbal deficiency employed utilized the human amniotic membrane which promoted new corneal epithelial cells migration; thus, replacing the damaged cells transplanted to the patients. Latterly in 1997, Pellegrini and the team (9) transplanted the limbal epithelial cells cultured on human amniotic membrane (hAM) until they became an epithelial sheet. The limbal epithelial cells were belonged to patient's family compatible limbal tissue or from cadaveric tissue.

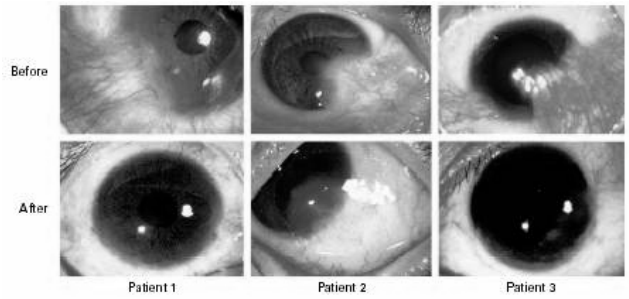

Figure 2: Serial Photographs of the Eye of Patient 3 before and after transplantation. 3 Patients who had patial and total unilateral limbal-epithelial stem cell deficiency were transplanted with biopsy limbal tissue explant cultured on human amniotic membrane. (20)

1. Limbal epithelial stem cells characteristic

Although at present there are no well proven biological markers of epithelial stem cells, there are several basic criteria that cells must fulfill in order to be considered stem cells. Structurally LESCs are small cell size and capable of unlimited **self-renewal** that upon division gives rise to progeny called transient amplifying or TA cells which have limited renewal capability. Furthermore, these stem cells have **slow-cycling**, which mean infrequently dividing in mature nontraumatic tissue and can be identified experimentally by label-retaining cells. This is done by long-term labeling of dividing cells and occasionally dividing stem cells with DNA precursor such as Bromodeoxyuridine (BrdU). Only the stem cells that cycle slowly retain the label. Immunofluorescence analysis by Vanessa Barbaro and co-workers in limbal sections revealed that C/EBPδ, and cyclin /cdk inhibitors p27Kip1, and p57Kip2 negatively regulate G1 progression were co expressed in the nucleus of quiescent limbal cells,

appeared in the cytoplasm at the G1–S transition, and were not expressed in actively proliferating cells (21). However, they have a **high proliferative potential**. Particularly, following wounding or *in vitro* culture, stem cells can be induced to divide more rapidly. Moreover, LESCs have the **clonogenic capacity** that enable single cells cultured in the presence of 3T3 feeder layer to form three types of colonies; **holoclones** a large colony containing 80-100 small cells thought to be stem cells ,smaller forming **meroclones** displaying TA cells, and **paraclones** endowed with terminally differentiated cells that can not be passaged. (22,23)

2. Limbal Epithelial Stem Cell-Associated Molecular Markers

A numbers of molecular markers have been proposed as potential limbal epithelial stem cells. Nevertheless, all methods presently used to distinguish LESCs are indirect, and an identification of a definitive marker remains elusive. To date, reliable molecular markers for LESCs can be classified as: (i) stem cell-associated markers, such as p63; and (ii) differentiation markers, such as K3 which distinguish stem cells from the more lineage committed cells.

2.1 Stem cell markers

2.1.1 Cytokeratin pair K5 and K14: Cytokeratins or Keratins are a group of cytoskeleton protein that forms intermediate filaments inside epithelial cells. The expressions are different in pattern during epithelial development and differentiation. At basal cells of corneal and limbal epithelia have been shown to express the cytokeratin pairs K5/14 and it represents the cells that still have proliferative potential.

2.1.2 Δ Np63α: p63 is p53 homologue that consistently express in basal layer of stratified epithelia and essential for epithelial development and differentiation. In ocular tissue truncated α isoform of p63 as known **Δ Np63α** is found to present in basal layer of limbus but absent in cornea epithelium and appeared in the resting and proliferating limbal stem cells where as other isoforms correlated with corneal epithelium and differentiated cells (24,25).

2.1.3 ATP binding cassette transporters or ABCG2: Otherwise, it known as breast cancer resistance gene 1 (BRCP1) and has been identified as marker of hematopoietic stem cells and a universal marker of stem cells (26). It has demonstrated that this protein is immunolocalized to the cell membrane and cytoplasm of some human limbal basal epithelial cells, but not in most limbal suprabasal cells and corneal epithelial cells. ABCG2-positive cells possess LSC-like characteristics, such as higher colony-forming efficiency than ABCG2-negative cells and greater expression in primary cultures of human limbal compared with corneal epithelium (27). ABCG2 expression is thought to be a common attribute of stem cells to protect them against drugs and toxin.

2.1.4 Nerve growth factor (NGF): Nerve growth factor a potential marker of limbal epithelial cells is a member of neurotrophin family. It has been identified as an essential growth factor supporting stem cell self-renewal outside the nervous system and was previously shown to stimulate corneal epithelial proliferation both in vivo and in vitro. The expression of NGF and its receptors TrkA in the corneal epithelium has been shown to preferentially localize to limbal basal epithelial cells. (28)

2.2 Differentiation markers

2.2.1 Connexin 43

Gap junction protein called Connexin 43 could serve as a negative marker of limbal stem cells. It has been found to express only in the corneal and limbal suprabasal epithelial cells. (29) The absence of gap junction could be a protective mechanism to make limbal stem cells less vulnerable to insult the affecting of other signaling outside their niche.

2.2.2 Cytokeratin K3/K12

Among numerous other keratins which are also present in a variety of other cell types, keratins K 3 and 12 are specifically expressed in corneal epithelial cells and are regarded as markers of corneal epithelial differentiation.

4. Ex vivo Limbal Culture method

Basically, there are two methods, namely the explant culture system and the suspension culture system, have been used to produce epithelial sheet containing LECs for transplantation.

4.1 Explant culture: During explant culture, limbal tissue is placed on human amniotic membrane both intact and the basement of removed epithelial cells amnion namely denuded amniotic membrane prior to use. Once confluent, air exposure may be introduced to promote cell differentiation. The preparation of the amniotic membrane effects the phenotype of limbal epithelial stem cells. There are several researches comparing characteristic of the cell outgrowth between intact and denuded membrane. They have demonstrated that the culture of LECs on amnion with an intact amniotic epithelium results in a more stem-cell-like phenotype than de-epithelialised amnion. The basement membrane of explant culture on this membrane appears healthy, expresses p63, and shows label-retaining DNA precursor. On denuded membrane, the cells grow faster than the intact one, present multi-layer and express differentiation markers such as cytokeratin pair K3 and 12. It has been proposed that denuded membrane is suitable to use in transplantation.

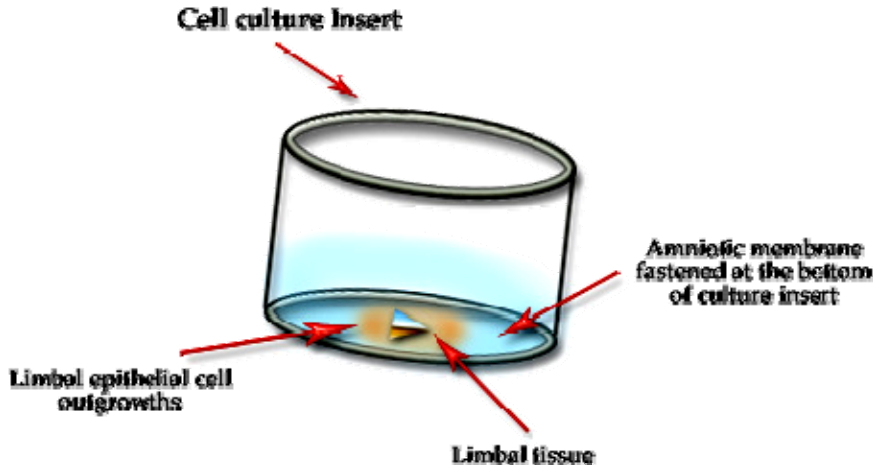

Figure 3: The explant culture system. Limbal tissue was cut into a small piece and placed on human amniotic membrane that fastened at the bottom of culture insert. The limbal epithelial cells were outgrowth within 10 days.

Additionally, the 3T3 cells co-culture with amniotic membrane system is variation of this technique. These primitive mouse embryonic fibroblasts are growth-arrested by irradiation or mitomycin c and are grown at the surface of cell culture well. According to high proliferative capacity and producing growth factors and matrix constituents that promotes epithelial growth features of 3T3, they have been used as feeder cells of epithelial cell culture especially skin and cornea

4.2. Cell suspension culture

Cell suspension culture system is similar to explant culture method except that the limbal tissue has to be digested their basement membrane with enzyme dispase II to seperate limbal epithelial stem cells and isolate them into single cells. This cell suspension is seeded onto either amniotic membrane or plastic cell culture dish that contains growth-arrested 3T3 feeder cells.

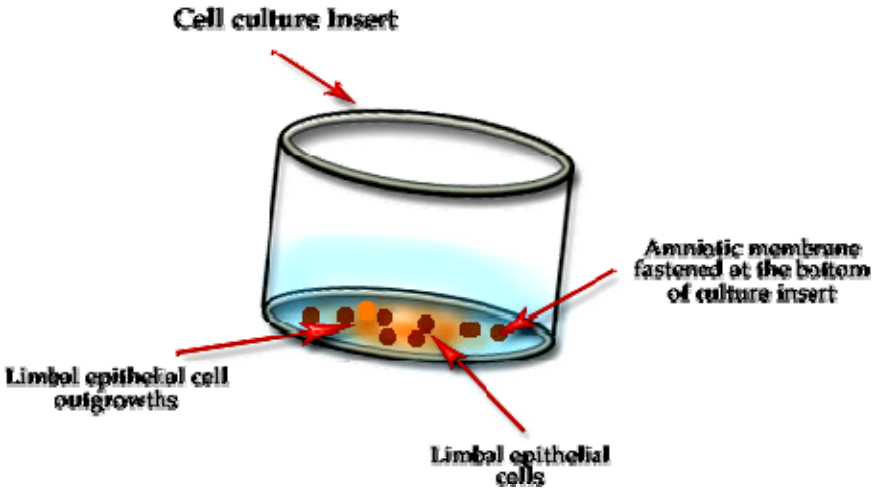

Figure 4: The cell suspension culture method. Limbal tissue was digested into single cells with Dispase II enzyme before they were seeded on human amniotic membrane.

Stem cell niches

The regulation of intrinsic factors such as transcription factors that play crucial roles in the maintenance of stem cells properties; both self-renew and differentiation, needs some unique extrinsic regulators to keep those factors work appropriately. Stem cells received extrinsic factors which are cell signaling, growth factors, and cytokines from neighbor cells and blood vessel. All these microenvironments are known as stem cell niche. The concept of stem cell niche came from the studies from Hematopoietic stem cells (HSCs) found that HSCs attached to osteoblastic cells which could maintain the quiescence stage of long-term HSCs. The studies from Drosophila reproductive organ made us understand more about the mechanism and how importance of microenvironment to stem cells. And it can be concluded that niche should compose of appropriate levels and types of signaling molecules derived from extracellular matrix such as fibronectin, collagen or released from the stromal cells. (30,31)

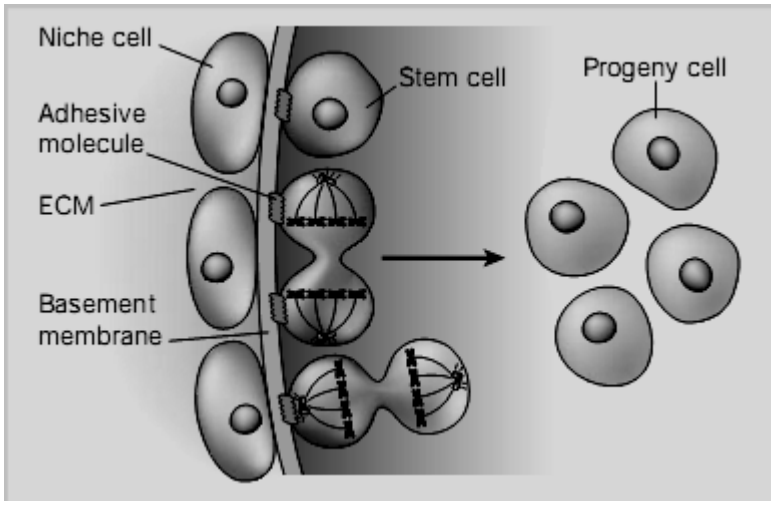

Figure 5: Niche structure. Niche of stem cell consists of many molecules such as extracellular matrix (ECM), adhesive molecules, and also niche cell underlying a basement membrane signal to stem cells to regulate stem cell behaviors; proliferation and differentiation (32).

1. Niche of Limbal epithelial stem cell

As previously mentioned in stem cell niche part that niche should compose of appropriate levels and types of signaling molecules which derived from extracellular matrix and neighbor cells. And in the term of stem cell niche, it has two dimensions: anatomy and function.

Anatomically, limbal epithelial stem cell niche is located at Palisades of Vogt which contains melanocytes which produce and transport melanin into epithelial cells so as to minimize damage caused by ultraviolet irradiation; and is infiltrated antigen-presenting Langerhan's cells; and suppressor T-lymphocytes. Limbal epithelial stem cells closely interact with cells in the underlying limbal stoma cells that expresse α9 integrin and n-cadherin without connexin 43. In addition, extra cellular matrixes such as laminin 1, 5; type 4 collagen also present in limbal basement membrane. Underneath the basement membrane is heavily innervated and vascularized. These suggested that the limbal basement membrane has an important role to modulate concentrations of growth factors, cytokines and signaling molecules that are released from surrounding cells and blood vessel for precise and efficient targeting onto limbal stem cells.

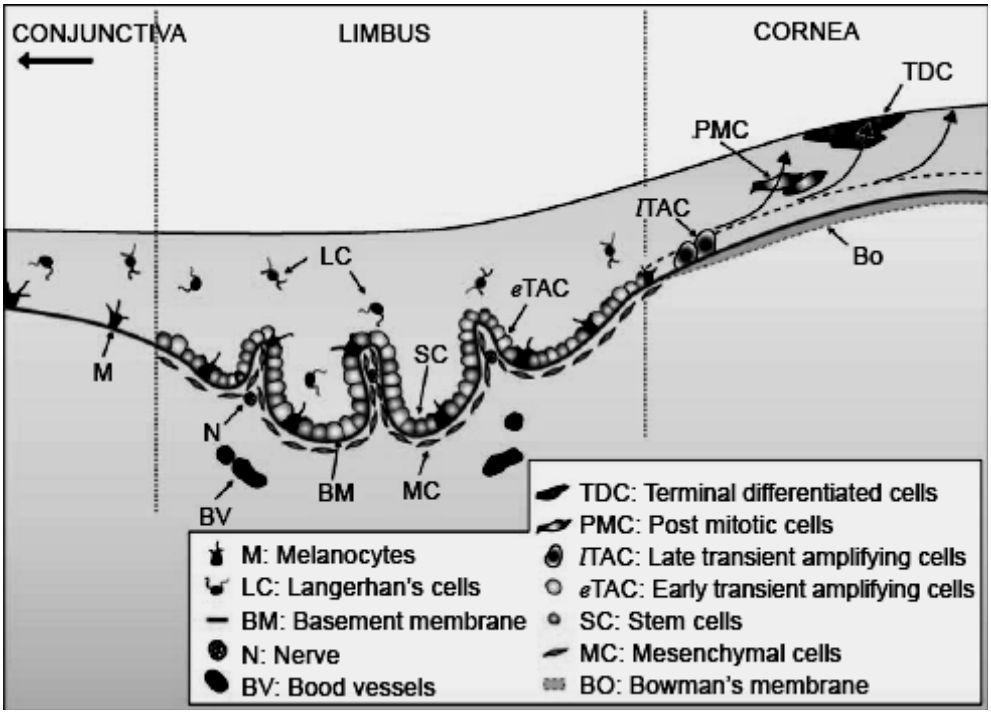

Figure 6: Hypothetical scheme of limbal stem cell niche. Limbal epithelial stem cells (SC) are located at the limbal basal layer. In this epithelial level, there are several other cell types in the vicinity such as the immediate progeny, i.e., early transient amplifying cells (*e*TAC), melanocytes (M), and Langerhan's cells (LC). It remains to be 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 (*l*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 (33).

Currently, the study (34) providing the first evidence for a limbal mesenchymal (niche) role in limbal SC differentiation during morphogenesis. It demonstrated that mouse null for expression of the *Dickkopf* (Dkk) family number Dkk2, one of the Wnt pathway inhibitors, was shown to lose the corneal fate decision on the ocular surface to epidermal differentiation. As a result, Wnt/beta-catenin pathway is upregulated in the limbal, but not corneal, mesenchyme, indicating that Dkk2 acts by inhibiting Wnt signaling in the limbal stroma. Furthermore, the data from Satoru Koyano et al. showed that Noggin, a member of BMP signaling antagonist, was synthesized and released by human amniotic membrane epithelium. These data supported by the culture condition with intact human amniotic membrane as a substrate could preserve limbal stem cells.

However, little is known about the communication between limbal SCs and their niche. Due to epithelial stem cells from epidermal systems have many features similar to epithelial lineages from the eyes, such as stratified epithelial phenotype, the expression of specific cytokeratin pairs, and clonogenicity, so niche of epidermal stem cell is used as a model to study about the regulation of cell signaling pathway in the niche (*35)*

2. Epithelial stem cells niches

In mammalian skin, epithelial stem cells are resided in the several location including at the basal layer of an interfollicular epidermis, sebaceous glands, and hair follicle (36). The epidermal stem cells are characterized as the cells that have capabilities of self-renewal, slow-cycling which shown by retaining nucleotide label, differentiating into multiple lineages, and *in vitro* colonies forming *(37).* To maintain tissue homeostasis, quiescent epithelial stem cells rapidly divide themselves and give rise to two daughter cells one of are their own cells and transient amplifying cells that move upwards toward the surface to above layer while actively differentiate into adult cells replacing the old-damaged cells (38). Beneath the epidermis is the supportive structure of the dermis containing blood vessels and extracellular matrixes providing some growth factor including signaling molecules that are participated in regulating the epidermal niche. Although there are more than one type of stem cells in skin system,

they are shown many of important cell signaling pathways in common such as BMP and Wnt signaling pathway

3. Bone morphogenesis protein signaling and theirs antagonist (BMP)

Bone morphogenetic proteins (BMPs) were originally identified as molecules that can induce ectopic bone and cartilage formation in rodents. They belong to TGF-b superfamily of secreted signaling proteins and play crucial roles during embryonic development; regulating cell proliferation differentiation and apoptosis and BMP transmitted its signal through two different kinds of Serine/Threonine receptor; TypeI and Type II which phosphorylated after ligand binding to the receptor and mediated phosphorylation of R-Smad (Smad 1,5,and 8). The phosphorylated R-Smads form a heterotrimeric complex with a common Smad4 (co-Smad). The Smad heterotrimeric complex translocates into the nucleus and cooperates with other transcription factors to modulate target gene expression. In addition to this “canonical” TGF-β signaling pathway, recent studies suggest that a mitogen-activated protein kinase (MAPK) pathway may mediate TGF- β signal transduction. These studies have shown that BMP signals can be transduced by TGF- β activated kinase 1 (TAK1), a MAP kinase kinase kinase (MAPKKK), and TAK1 binding protein 1. While the mechanism of the transduction of BMP signals by this BMP-TAK1 pathway is unclear, it is possible that activation of the BMP-TAK1 pathway leads to phosphorylation of transcription factors that then synergize with BMP-regulated Smads in the activation of BMP-target genes.

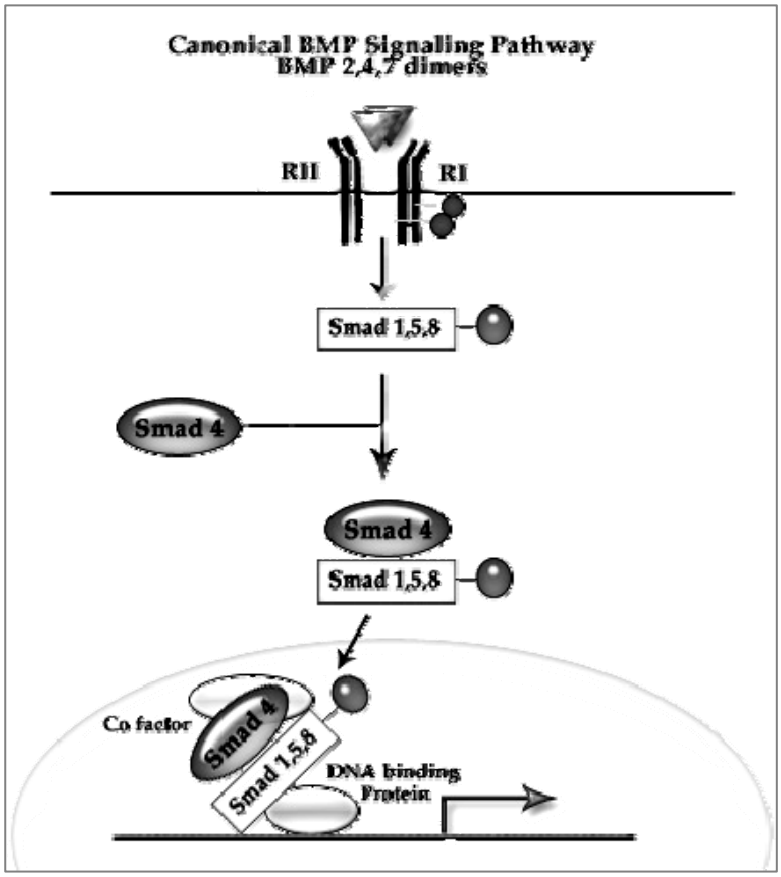

Figure 7: The "canonical" BMP–Smad pathway. BMPs molecule in dimers bind to their receptor complex leading to phosphorylation of type I receptor (RI) by type II receptor (RII) which in turn phosphorylates Smad 1, 5, 8 of R-Smad. The phosphorylated R-Smad then forms complex with co-Smad (Smad-4) and translocates into nucleus to stimulate transcription of target genes.

3.1 Noggin as a BMP antagonist

Noggin is secreted as a glycosylated protein, covalently linked homodimer of 64 kilodaltons (kDa). The primary structure of noggin consists of an acidic aminoterminal region and a CR carboxyterminal region containing a cystine knot. A central, highly basic heparin-binding segment retains noggin at the cell surface. Noggin inhibits BMP signaling by binding with various degrees of affinity BMP-2, -4, -5, -6 and -7, but not other members of the TGF β family of peptides and preventing them from the binding with their receptors. Noggin displays many functions in cell development. The absence of noggin expression leads to inappropriate development which results in a recessive lethal phenotype at birth which characterized by numerous defects: a shortened body axis caudal to the forelimbs with reduced size of the somites and neural tube, and excess bone and cartilage formation. In adult mice, high expression of noggin expression was observed in limited tissues and cells, such as osteoblasts, chondrocytes, and cranial suture in vertebrae and calvaria. The importance of the noggin gene was confirmed by human studies demonstrating that different heterozygous missense mutations of noggin coding sequence lead to proximal symphalangism and multiple synostosis syndromes (39).

3.2 Gremlin a BMPs antagonist

Gremlin is a member of *DAN/Cerberus Protein* another novel family of secreted proteins with antagonistic activities against BMPs, sharing a similar structural motif but nonidentical inductive activities. Although most of the members of the Dan family are expressed primarily during embryonic development and play a minor role in skeletogenesis, few of them, such as gremlin and sclerostin are important regulators of BMP activity in the adult skeleton. The gremlin gene was cloned from a Xenopus ovarian library for its axial patterning activities and encodes for a glycosylated homodimeric peptide of 28 kDa. Gremlin binds BMP-2, -4, and -7 with high affinity, but it does not interact with other members of the TGF β superfamily. Gremlin rat homolog, drm, overexpressed in osteoblastic tumor cell lines, up-regulates the protein levels of the cyclin-dependent kinase inhibitor p21 and induces apoptosis, indicating a possible role for gremlin/drm in the regulation of cell replication (40).

Accumulated evidence indicates that BMPs play an important role in the regulation of stem cell properties. However, their functions are different in the different stem cell compartments depending on time and dosages of the signal. BMPs have been shown to maintain cultured mES cells in an undifferentiated state by inhibit neural differentiaition. These data were supported by the treatment with an observation that treatment with BMP suppressed neural differentiation and in combination with LIF was sufficient to maintain self-renewal of mES cells in the absence of serum or feeder cells. (41) However, the effects of BMPs signaling on hES are contrast with these on mES. In hES cells, BMPs promote differentiation. Furthermore, treatment with the exogenous BMP antagonist Noggin prevented spontaneous differentiation into primitive endoderm differentiation.(24) Moreover, in the presence of FGF and noggin, hES cells can be maintained in a pluripotent state. During epidermal development, BMP effect on proliferation and differentiation is concentration dependence. Overexpression BMP4,6 inhibits epidermal proliferation while modulate expression stimulates proliferation of basal epidermal keratinocytes (42,43). In postnatal skin, BMP is involved in the control of differentiation. For example, BMPR-IA, IB and BMPR-II, BMP receptors, are seen only in suprabasal keratinocytes (44). BMP2,6 induce primary mouse keratinocytes differentiation in culture (45) Recently, by studying BMP signaling in the intestinal stem cell compartment, we found that BMP signaling also plays a direct role in restriction of stem cell activation and expansion through suppression of Wnt/Beta-catenin activity((8). Gene expression profile from previous study demonstrated that Noggin, Gremlin-1, Gremlin-2 were expressed by crypt which is intestinal epithelial stem cells niche. (46) Likewise, BMP receptor IA is found to be essential for the differentiation of progenitor cells of the inner root sheath and hair shaft. (47)

4. Wnt signaling

Wnts are powerful regulators of cell proliferation and differentiation, and their signaling pathway involves proteins that directly participate in both gene transcription and cell adhesion. The diversity of their functions is exemplified by mutations that lead to developmental abnormalities ranging from stem cell loss to kidney and reproductive tract defects. Signaling is initiated by Wnt ligand binding to two receptor molecules, Frizzled proteins and lipoprotein receptor–related proteins 5 and 6 (LRP-5/6). The classical pathway of Wnt signaling, β-catenin is a central player. Without Wnt activation, β-catenin that is connect to cadherin at plasma membrane binds to the destruction complex consisting of Serine/Threonine kinases, casein kinase I (CKI) and glycogen synthase-3β (GSK-3 β) that leads to β-catenin degradation. When Wnt receptor are engaged, GSK-3 β activity is inhibited, resulting in accumulation of cytoplasmic (signaling) β-catenin, which translocate to the nucleus and becomes available to bind the TCF/LEF family of transcription factors and to induce target gene expression. Thus, the key factors in β-catenin signaling are its stabilization and accumulation in the cytoplasm. In the absence of a Wnt signal, Tcf/Lef proteins repress target genes through a direct association with co-repressors such as Groucho. The interaction with β-catenin by disrupted Groucho/TLE protein transiently converts Tcf/Lef factors into transcriptional activators. (48) In sum, the canonical pathway translates a Wnt signal into the transient transcription of a Tcf/Lef target gene programme.

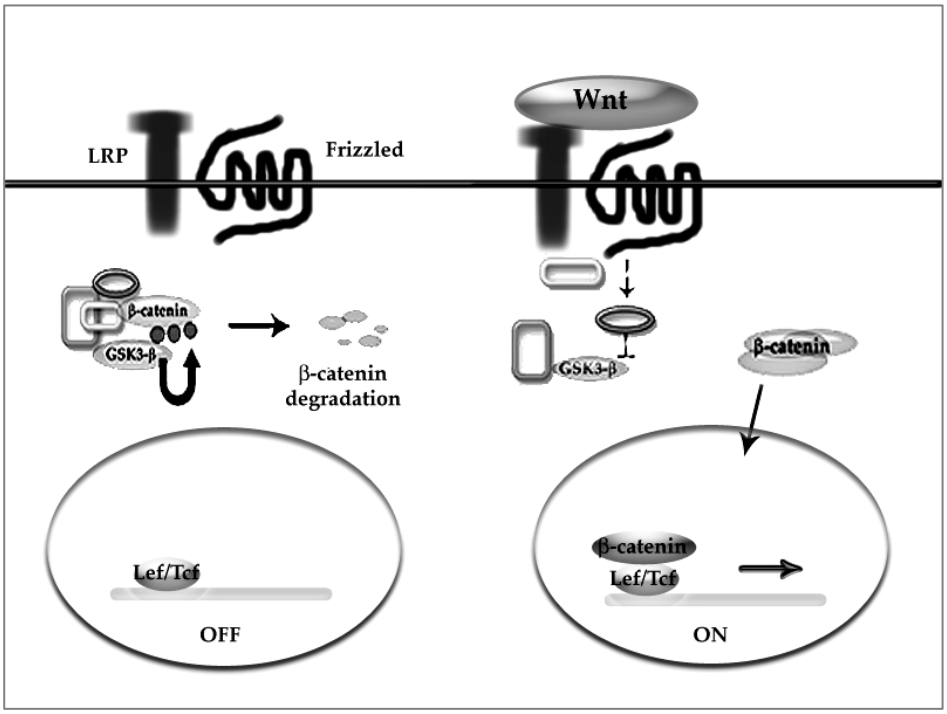

Figure 8: The canonical Wnt signalling pathway. In the absence of Wnt signaling (left panel), b-catenin is in a complex with axin, APC and GSK3-β, and gets phosphorylated and targeted for degradation. b-Catenin also exists in a cadherin bound form and regulates cell–cell adhesion. In the presence of Wnt signalling (right panel), b-catenin is uncoupled from the degradation complex and translocates to the nucleus, where its binds Lef/Tcf transcription factors, thus activating target genes.

2.2.1. T-cell specific transcription factors

The members of the TCF/LEF family of transcription factors, TCF-1 and LEF-1, were identified in screens for T cell-specific transcription factors. TCF-1 was identified by its ability to bind to the *CD3_* enhancer, whereas LEF-1 was found in a screen for proteins binding to the *TCR_* enhancer and a site in the HIV LTR. In more recent years, two additional family members were identified in mammals: *TCF-3* and *TCF-4*. Proteins of the TCF/LEF family contain an 80-amino-acid high mobility group (HMG) box binding DNA as monomers in a sequence-specific manner and also bending the DNA. By doing so, HMG boxes may coordinate the binding of other transcription factors. Because of this bending ability and the observation that TCF factors cannot directly activate transcription in reporter assays, it has been proposed that TCF/LEF family members primarily serve an architectural function. The TCF consensus recognition sequence is remarkably conserved between the family members and comprises AGATCAAAGGG. LEF-1 appears unique in that it contains a context-dependent activation domain (CAD) while the other TCF family members do not appear to contain a CAD domain.

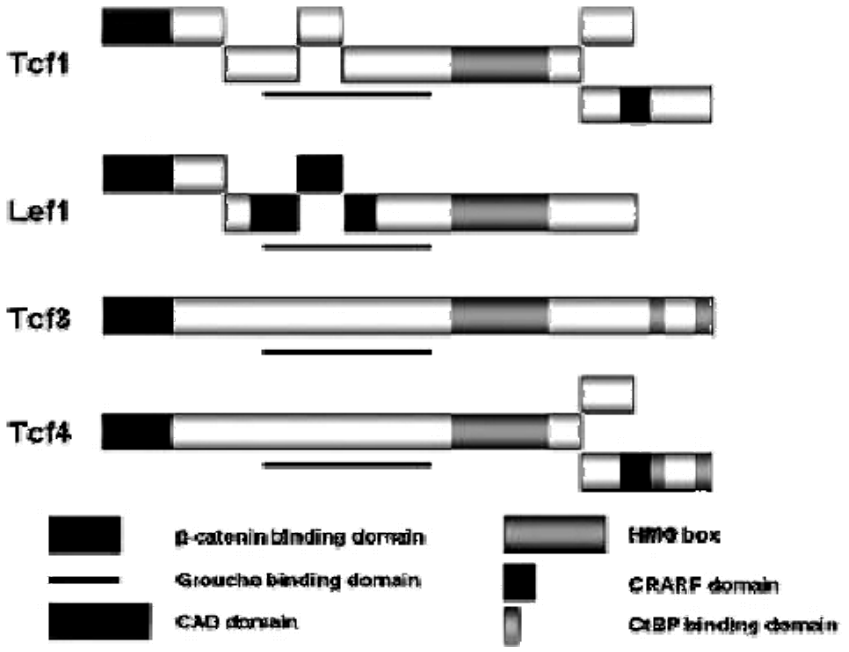

Figure 9: Schematic representation of Tcf splice variants and their most conserved domains. Short forms of Tcf-1 lack the N-terminal domain, which interacts with β-catenin. The most distinct regions of the Tcf family member is the C terminus, which contains the CRARF domain in the longer versions.

Canonical Wnts function not only acts as a maintenance signaling for quiescent state of stem cells but also promotes cell proliferation and determination. These are depended on the time of wnt acquiring, and status of a stem cell as well as its extracellular microenvironment, which may alter Wnt activity during development. Wnt/b-catenin signaling maintains 'stemness' in stem cells from different tissues and prevents them from differentiating. Treatment of embryonic stem cells with a synthetic pharmacological inhibitor of GSK3β activates the canonical Wnt pathway and sustains pluripotency and self-renewal (49). As in embryonic stem cells, Wnts regulate proliferation of intestinal stem cells skin stem cells and haematopoietic stem cells. In particular, treatment of haematopoietic stem cells with Wnt proteins and sustained expression of β-catenin promotes self-renewal in long-term cultures and increases the reconstitution of haematopoietic lineages in vivo. In addition to the above-mentioned, Wnt also influences lineage decisions in stem cells. For example, Wnt has been shown to regulate lineage decisions rather than stem cell maintenance in neural crest stem cells (NCSCs). Upon loss of β-catenin, sensory ganglia and melanocytes fail to develop in vivo. In the other hand, sustained β-catenin activity in NCSCs results in the formation of sensory neurons (including some at ectopic locations) at the expense of all other neural crest derivatives in vivo. Thus, unlike in other stem cells, canonical Wnt instructively promotes cell fate decisions rather than stem cell expansion in NCSCs In another study, in which neural stem cells derived from the telencephalon were cultured as neurospheres and challenged by Wnt signaling, it was reported that Wnt3A inhibits the maintenance of neural stem cells and promotes the differentiation into neuronal and astrocyte lineages. Likewise, in the skin, in vivo manipulation of genes encoding Wnt signaling components indicates an essential role of Wnt in fate decision processes of epidermal stem cells. In particular, β-catenin-deficient stem cells fail to differentiate into follicular keratinocytes and instead adopt an epidermal fate. And during wound healing the β-catenin-dependent Wnt pathway was active during wound repair but limited to the differentiated hair follicle shafts in the normal uninjured skin adjacent to the wound, not within the wound. The findings suggest that adult interfollicular epithelium is capable of responding to Wnt morphogenic signals necessary for restoring epithelial tissue patterning in the skin during wound repair (50).

CHAPTER III

MATERIALS AND METHODS

1. Materials and Reagents

1.1 Limbal complete media : 1:1 mixture of Dulbecco modified Eagle medium (DMEM) and Ham F-12 contain 10% fetal bovine serum USA grade, 1%Pen-Strep, 1% L-glutamine, 0.1mg/L Hydrocortisone, 2.5 mg/ml $NaHCO_3$, 10 ng/ml EGF (R&D) and 5 mg/L Insulin (Invitrogen)

1.2 1% Phosphate buffer saline without Ca^{2+} Mg^{2+}

1.3 Dispase II 1.2 Units

1.4 0·25%Trypsin/0·03%EDTA solution

1.5 Amphotericin B

1.6 Cell dissociation buffer enzyme-free Hanks'based

1.7 Cell scraper

1.8 Cell culture inserts

1.7 Human amniotic membrane:

1.8 Antibody: mouse anti-human cytokeratin 3/12; mouse anti-human phospho smad1,5,8; anti-BrdU; mouse anti-human ABCG2; mouse anti-human SOX2

2. Limbal epithelial cells culture

2.1 Human amniotic membrane

Preserved human amniotic membrane was kindly provided by the Department of Ophthalmology the Faculty of Medicine Chulalongkorn University. Before use, human amniotic membrane (hAM) was rinsed two times with PBS buffer and incubate in cell dissociation buffer enzyme-free Hanks'based for 2 hours at 37 $^{\circ}C$. After the incubation, the epithelial cells of hAM were removed by cell scraper. Then hAM was washed once with PBS buffer and fastened on to a culture insert. Store at room temperature until the membrane was completely dry.

2.2 Fibronectin coated 6 well plate

A 6 well plate is coated with 1 ml of 5 μg/ml fibronectin in PBS buffer store at room temperature for at least 4 hours and rinse once with PBS before use.

2.3 Limbal tissue explant culture

Corneoscleral tissues from human donor eyes, aged 20-70 year, were obtain from the Department of Ophthalmology of Thai Red Cross. The tissue was rinsed three times with 4 ml Phosphate Buffe (Mg and Ca free PBS) and 1.25ug/ml amphotericin B. After careful removal of excessive sclera and conjunctiva, the remaining tissue was placed in a culture dish and exposed for 1.30 hrs to DispaseII (1.2 U/ml in Mg and Ca free PBS) at 37°C under humidified 5% carbon dioxide. Following one rinse with DMEM containing 10%FBS, the tissue was cut into cubes of approximately 1x1.5x2.5 mm. Then put the cut tissue on the center of both hAM that fastened onto culture insert and the fibronectin coated 6 well plate. All tissues were cultured in limbal complete media containing 10 ng/ml of EGF and 5 mg/L of Insulin. Culture Media was changed every 1-2 days.

2.4 Limbal epithelial cells dissociation

Cultivated limbal epithelial stem cells from fibronectin were dissociated by cell dissociation buffer enzyme free Hank's based (GIBCO) and separated into single cells by 0.05%Trypsin-EDTA. Numbers of cells were counted under microscope low power field (10X).

2.5 Airlifing cultured limbal cells

Limbal cells cultured on both hAM and fibronectin coated plate for 14 days or the outgrowth size reached 0.8-1.0 cm in diameter.Then exposed to air by lowering the medium level (airlifting) for anther 10 to 14 days to promote corneal epithelial differentiation. After airlifting, the epithelial sheets were immunostained for corneal specific marker cytokeratin pairs 3 and 12.

3. Overexpression of the genes in 3T3

For creating microenvironment that similar to limbal stem cells niches in our eyes, we making feeder cells that expressed BMPs antagonist – noggin and gremlin-1 for limbal stem cells colony forming

3.1 Transfection

Noggin and Gremlin-1 plasmids from ORIGENE were cut with *EcoRI* and *XhoI* restriction enzyme and ligated with pcDNA3.1/myc-HIS B for transfection by FUGENE HD transfection kit (ROCHE) according to manufacturer's instruction . Briefly, 2 ug of plasmid DNA was transfected into 3T3 cells on 24 well. Only 3T3 cells that could grow in 600ug/ml Geneticin condition media would be selected to make stable cell lines for usage as feeder cells.

4. Gene expression analysis

4.1 RNA extraction

RNA was isolated by using Tri-reagent technique from the outgrowing limbal epithelial cells and BIO treated limbal epithelial cells after cultured for 14 days. In briefly, remove culture media, homogenize cells in TRI reagent and pass the cell lysate several times through a pipette. Store the homogenate for 5 minutes at room temperature. Next supplement the homogenate with BCP, cover the samples tightly and shake vigorously for 15 seconds and store the mixture at room temperature for 2-15 minutes. Then centrifuge the resulting mixture at 12,000 rpm for 15 minutes at 4^{o}C. After that, transfer the RNA that remains in the aqueous phase to a fresh tube. RNA was precipitated by mixing with isopropanol. Wash RNA samples with 75%ethanol and air-dry. Finally keep RNA samples in RNA storage solution at -20^{o}C.

4.2 Measurement total RNA concentration

Each RNA sample was aliquoted 5 ul for measuring total RNA concentration. Aliquot RNA had diluted into 20 times (fill up RNase-free water to 100 ul), total RNA measured by spectrophotometer at optical density 260 nm (OD_{260}) and 280 nm (OD_{280}). Calculation of total RNA was necessary for processing in reverse transcription polymerase chain reaction (RT-PCR). One unit of optical density at 260 nm equal 40 ug of total RNA per ml. Pure RNA had an OD_{260}/OD_{280} ratio of 1.6-1.9.

4.3 Complementary DNA (cDNA) synthesis

Synthesis of single-strand cDNA was carried out, as used total RNA 0.5 ug (maximum volume of RNA template not exceed 11.5 ul) for reverse-transcribed into cDNA by Thermoscripts™ Reverse Transcriptase Reagent (Invitrogen). RNA is transcribed at 65°c for 5 minutes, 55°c for 60 minutes and 85°c for 5 minutes. Complementary DNA was kept at -20°c until used for measuring gene expression by real-time PCR.

4.4 Quantiative Real-time Polymerase chain reaction (PCR)

The mRNA levels of TCF3, p21, $p27^{kip1}$ and the housekeeping gene 18s rRNA were measured by LightCycler® machine (Roche Molecular Biochemicals, Indianapolis, IN, USA). The principle of real-time PCR using SYBR was described previously. Each PCR reaction was set up for 20 ul reaction volume. PCR amplification included followed amplification step by heating at 95°C and immediately cool down to 60°C for 15 seconds repeated for 40 cycles for annealing and the extension step at 72°C for 1 min. After the processes were completed, the real-time PCR results were automatically reported by ABI 7500® software analyzed by relative quantification method (comparative Ct method).

5. Immunofluorescent staining

The immunofluorescent staining was performed to evaluate the expression and the location of different molecular markers that have been proposed to identified LESCs or differentiated cells. In brief, the outgrowth limbal epithelial cells on fibronectin plate were fixed with 4% formaldehyde at 4°C for 10 minutes and then blocked with 5% Goat serum in Ca and Mg free PBS for 45 minutes at room temperature to decreas nonspecific antibody interaction. The mouse anti-human ABCG2 (IgG2b ,1:20, 25ug/ml); MS anti-keratin K3/12 (IgG1, 1:100, 10ug/ml); MS anti-human TCF3/4 (IgG, 1:100, 5ug/ml); Rabbit anti-phospho SMAD1,5,8 (IgG, 1:100) were applied and incubated overnight at 4°C. Secondary antibody, Alexa Fluor 488 conjugated with goat anti-mouse IgG, IgG1, IgG2b; Alexa Fluor 546 conjugated with goat anti-rabbit IgG were then applied and inbubated in a dark chamber for 2 hour followed by nuclear-staining DAPI (2ug/ml in PBS) for 20 minutes. After washing with PBS, the cells were examined and photographed with fluorescent microscope (Nikon: Magnified 20X)

Chapter IV

Result

1. Ex vivo expansion of human limbal tissue on fibronectin coated plate

On fibronectin coated plate as shown in figure 2, the limbal cells began to grow frequently on day 7 of culture as comparable to the cultivated cells on denuded hAM. The limbal epithelial cells grew as a monolayer with small round shape and had about 1:1 nuclear cytoplasmic ratio at the first week. And some of the outgrowth cells on fibronectin plate became to differentiate in the second week with a size about 1.8-2 cm in diameter. Cultivated cells could be maintained on fibronectin plate for 6 weeks. And then, they displayed a large vacuole, shrink, and finally death.

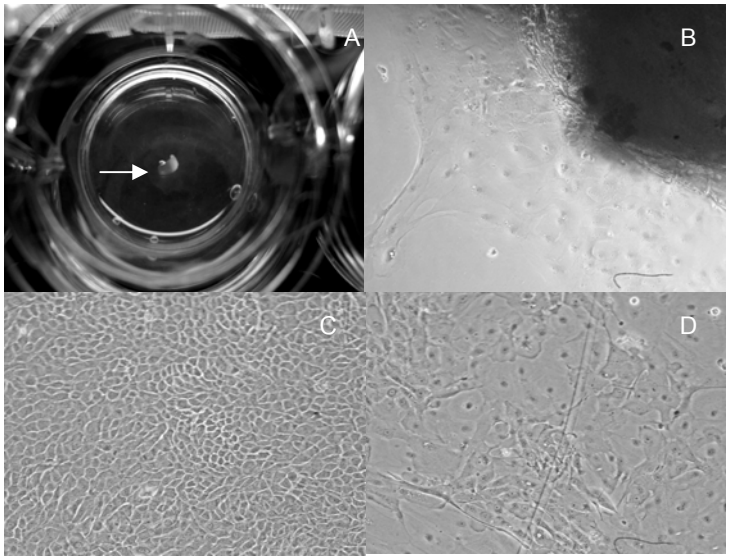

Figure 10: Human limbal tissue explant cultured on fibronectin coated 6 well plate. Human limbal explant cultured on fibronectin coated 6 well plate (A) the cell grew out after cultivated for 7 days (B) the cells were small and round and then differentiated into epithelial cells (C,D)

2. Limbal molecular markers.

ABCG2, the member of ATP binding cassette transporter, has been proposed as a universal marker of stem cells. The ABCG2 was immunodetected in the cell membrane and cytoplasm of limbal epithelial cells

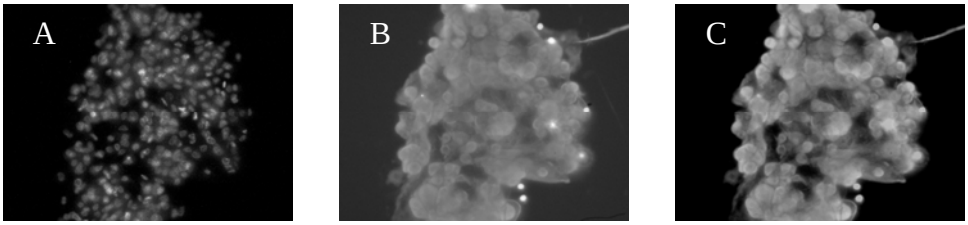

Figure 11: Staining pattern of cultivated cells with ABCG2 on week 1. The cultivated epithelial cells are strongly positive for ABCG2. (A) Nuclear Staining by DAPI (B) human LESCs cluster in fibronectin coated plate stained positive for ABCG2 (C) Merged

The limbal epithelial cells were promoted to differentiate by airlifting technique as describe in chapter III. After 10 to 14 days of airlifting, Cytokeratin pair K3 and K12 immunostaining showed that most of the limbal epithelial cells grown on fibronectin coated plate were positive.

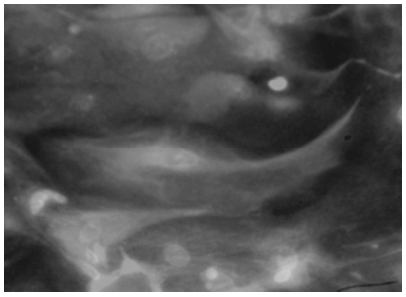

Figure 12: Cytokeratin pair K3 and K12 immunofluorescent staining in human limbal epithelial cells. Limbal epithelial cells on fibronectin plate strongly expressed cytokeratin pair K3/12 after exposed to the air for 7 days.

3. Clonogenic potential of cultivated limbal epithelial stem cells

To test the colony forming capacity of limbal epithelial cells outgrowth, LESCs grown on fibronectin coated plate were isolated into single cells and seeded on growth-arrested 3T3 feeder cells in 24 well about 5000 cells/well. At the first day, LESCs were floating and had many debris cells. Limbal typically formed colony in 10 days. We did not observe any colony. Moreover, LESCs were still floating and had vacuole. The cell membrane was rough and teared. The cells died in the next few days (Data not shown).

Stem cells are required specific microenvironment to govern and maintain their properties so we try to create microenvironment in culture that similar to LESC niche.

4. BMP signaling transduction in cultivated limbal epithelial cells

Accordingly, Bone morphogenesis protein signaling pathway plays important roles in the niche control of different types of stem cells including skin epithelial stem cells. Therefore, we investigated that whether BMPs signaling up-regulated in cultivated LESCs. The LECs outgrowths were stained with anti-human phosphorylated form of Smad 1, 5, 8 BMPs signal transducing molecules which represented the BMPs signaling pathway.

In the differentiated cells, phospho - Smad, was densely localized in the nucleus of the cells whereas undifferentiated cells with small and round shape phospho-Smad found densely in cytoplasm. Our data suggested that BMPs signaling pathway transduced their signals through phospho-Smad 1, 5 or 8 influenced to limbal stem cells differentiation.

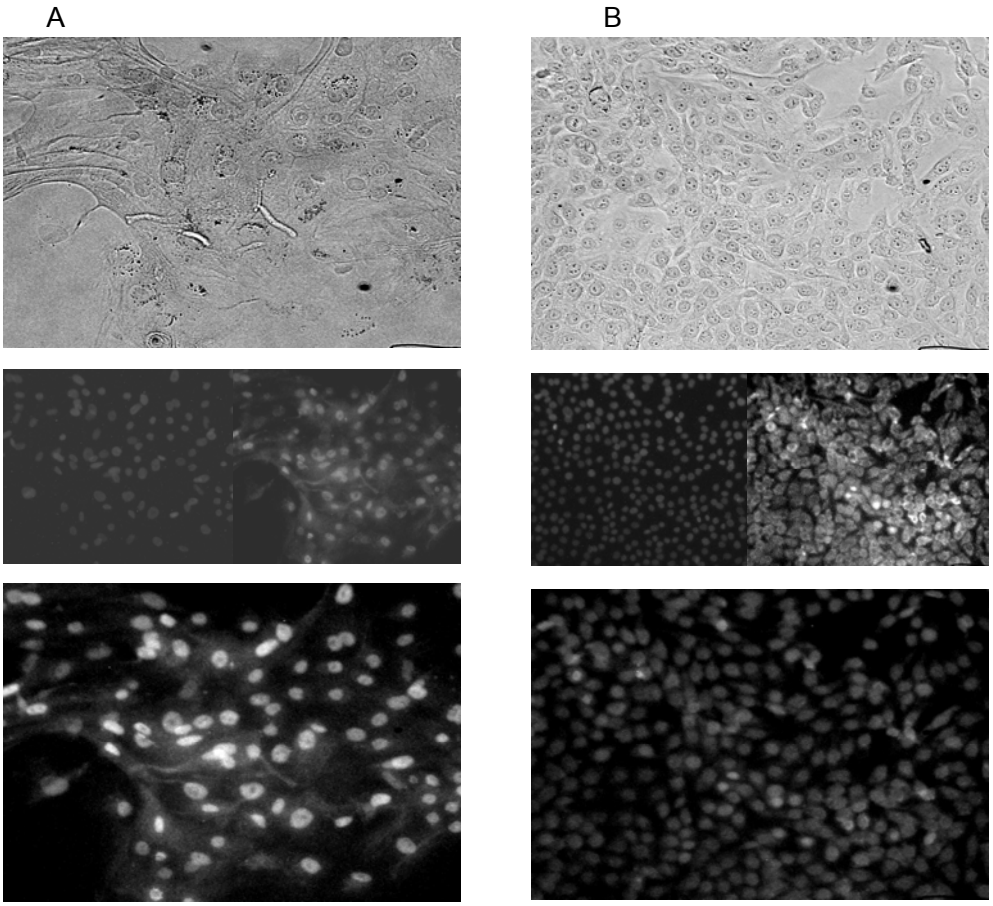

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Figure 13: Immunofluorescent staining of anti-human phospho-Smad in Human limbal epithelial cells. In differentiated cells Smad was positively stained in nucleus (A) while stained positively in cytoplasm of undifferentiated cells.

5. Transient expression of noggin in 3T3 feeder cells promotes limbal stem cells adherence and survival.

From above results, BMPs showed effect to limbal stem cells differentiation. Moreover there was a study demonstrated that Noggin was synthesized and released by amniotic membrane that use as substrate in ex vivo limbal tissue culture.(39) So transiently expressed noggin and gremlin-1 which are BMPs antagonist in 3T3 cells were made as feeder cells for LECs. And $7.5x10^4$ cells of LECs were seeded into each well of culture plate covered with these transiently expressed BMP antagonists 3T3 feeder cells to test whether these feeders could promote adherence and preserved limbal cells.

It could see that the limbal cells adhered well to 3T3 both BMPs antagonists expressed feeder cells after the cells were seeded for 1 day. Limbal colony could be seen at the 9 th day after. In noggin positive 3T3 feeder cells, there are 3.3 colonies on the average which less than control 3T3 feeder cells that had 11 colonies. But the size of colony in noggin+3T3 feeder cells was larger and contained more cells than the control ; 100-200 cells and 20-30 cells respectively.

Colony forming efficiency = Number of colonies / Number of cells x 100

Nog^+ = $3.3 / 7.5x10^4 \times 100$

= 0.004%

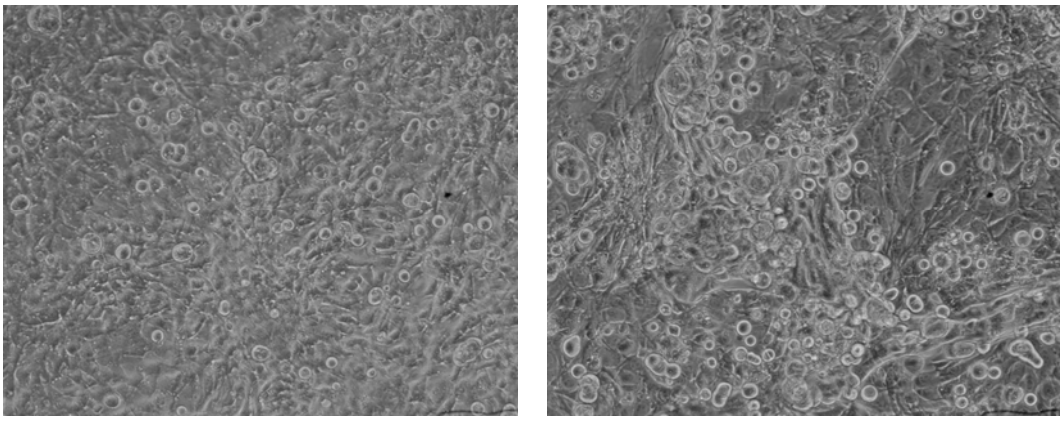
Nog- = $11 / 7.5x10^4 \times 100$

= 0.014%

Although the colony forming efficiency of control 3T3 feeder cells was higher than the test, the colonies on control feeder cells contained both limbal epithelial cells and undifferentiated cells in contrast to the colonies of 3T3 noggin positive feeder cells that most of the cells were small and round and still had the differentiated morphology. Nevertheless, it can not be concluded that these results are influential from BMPs antagonists since none of these limbal cells in each feeder cell types could survive to the second passage. Besides, there were 2 cell populations which were the

cells with and without noggin expression in the same plate due to the transfection efficiency. Hence the stable Noggin expression 3T3 feeder cells were made.

After the LECs were seeded to the stable noggin expression 3T3 feeder cells for 1 day, we founded that the limbal cells adhered well to 3T3 noggin expression feeder cells. In general we should see the colony within 9 days. But the cells could not be continued to proliferate.



Nog **Ctrl**

Figure 14: Human limbal epithelial stemc cells on noggin positive 3T3 feeders and control 3T3 feeder.

The result has variation due to the uncertainty of sample characteristic caused by the age of donors and sample collected date, these have an affect on the growth rate of limbal epithelial cells. Moreover, we can dissociate the cells only one time per one tissue sample so we decided to do the experiment by using rabbit limbal stem cells as our samples because the result will be more consistent than the human cadaveric samples.

6. Stable BMP antagonists expression 3T3 cells used as feeders layer in Rabbit limbal stem cells colony forming.

The morphology of rabbit limbal colonies

Morphology of rabbit limbal Colonies dissociated from fibronectin coated plate when supported by lethally mitomycin c treated feeder cells, rabbit limbal cells can give rise to macroscopic colonies within 9 days after inoculation. By 12 days, three colony types can be recognized:

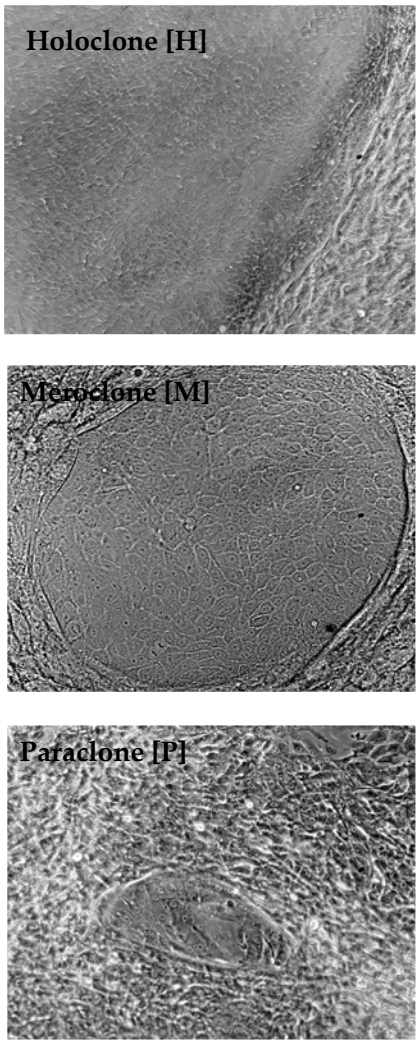


Figure 15: Morphology of rabbit limbal stem cell colony formation. A) Holoclone B) Meroclone C) Paraclone.

1). Large, with smooth perimeter: Such a colony is visible with 0.5-0.7 cm in diameter, rapidly growing and contains numerous of small cells concentrated near perimeter. Its perimeter is nearly circular. This type of colony grew so larger that the feeders are inserted. Colonies of this type are typically formed by the cells of a holoclone.

2). Small, highly irregular, and terminal： The colony area is less than 0.1 cm and the perimeter of the colony is drawn out into marked irregularities. All proliferation has ceased. The cells are large and flattened. This type is paraclone.

3). Wrinkled: This colony type grows progressively to macroscopic size but does not reach the same size as the typical colonies produced by holoclones. It has a wrinkled perimeter suggesting some kind of heterogeneity within such a colony will soon become terminal. It is typically formed by meroclones

Determination of colony numbers and colony forming efficiency

1. Control 3T3: Rabbit limbal cells give rise to colony in day 9 after inoculation. The colonies were count under low power field microscope. In this feeder, there are 13 colonies on the average: small =9; large = 5 colonies at the first. In the next two days the large colony numbers were increase to 8 colonies. Such colonies contain both holoclone and meroclone. And the others were paraclone which a little larger than the first date. The colony forming efficiency was about 0.32%.

2. Noggin expressed 3T3: The colonies were able to see at day 10 after seeded. There are 14 colonies on the average contained 11 of small colonies and 3 of large colonies. On day 12, the amount of large size colonies were increase to 8. Most of the large colonies had holoclone morphology. The colony size was about 0.3-0.7 cm in diameter. Colony forming efficiency was 0.36%.

3. Gremlin-1 expressed 3T3: Like Noggin experiment, rabbit limbal cells formed colonies on day 10. The average numbers of total colonies were 12 colonies divided to 7 and 5 colonies which are small size colonies and large size colonies, respectively. The large size colony numbers were increase to 10 on the next two days. There are 10 large colonies. The colony with large size about 5 mm in diameter had holoclone morphology. Colony forming efficiency was 0.32%.

Clonogenicity of rabbit limbal colony on each feeder type						
Feeder	Control		Noggin		Gremlin-1	
Type	Numbers	Std. Error	Numbers	Std. Error	Numbers	Std. Error
H	1.4	0.48	2.3	0.5	3.9	0.75
M	5.9	0.9	6.3	0.9	7	0.95
P	5.5	0.95	5.7	1.59	2.2	0.65
Total	12.8	1.64	14.3	2.36	12.9	1.54
CFE%	0.32	0.04	0.36	0.06	0.32	0.04

Table 1: Clonogenicity of rabbit limbal colony on each feeder types. Cultivated Rabbit limbal stem cells on control feeder formed colony earlier than other feeders. On both BMP antagonist feeder, we obtain more holoclone. The total numbers of colony and colony forming efficiency were not different in all experiments.

7. Rabbit limbal colony on day 14 real-time pcr result

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. Progression from G1 into S phase of the cell cycle is delayed by the action of two classes of Cyclin-dependent kinase inhibitors (CKIs) , the CIP/KIP family $p21^{cip1/waf1}$, $p27^{kip1}$, $p57^{kip2}$ and the inhibitor of kinase 4 (INK4). Previous evidence showed that $p27^{kip1}$ and $p57^{kip2}$ are localized in the nucleus of quiescent limbal cells but are not in actively proliferating cells. And p21 null mice exhibit neural stem cells and hematopoietic stem cells decrease in number as declined by loss of the ability to BrdU label-retaining DNA precursor. Therefore, 2 well of each experiment culture dish that had the most colony numbers were selected for RNA extraction and analyzed p21 and $p27^{kip1}$ expression by Quantitative Real-time PCR.

We detected slightly up-regulation of p21, $p57^{kip2}$ gene in rabbit limbal colony on both BMP antagonists feeder cells and the level of these genes were not different from control feeder. Furthermore, we noticed $p27^{kip1}$ up-regulate in limbal colony grown on BMP antagonists feeder cells. On Noggin feeder, p27kip1 was 4 folds up-regulated in rabbit limbal colony and 14 folds on Gremlin-1 feeder in comparable to control feeder. To confirm the results we compared the expression level of $p27^{kip1}$ gene of the tests with every 3T3 feeder types that we used. We founded p27kip1 level in both Noggin and Gremlin-1 expressed 3T3 feeders, was also up-regulation. Only in Gremlin-1 feeder that the up-regulation of $p27^{kip1}$ was significantly up-regulated this corresponded to the colony morphology and rate of proliferation. In Gremlin-1 feeder, limbal stem cells contained holoclone were slow proliferation noticed by some of holoclone colony sizes that were still small when compared to holoclone formed on other feeder types.

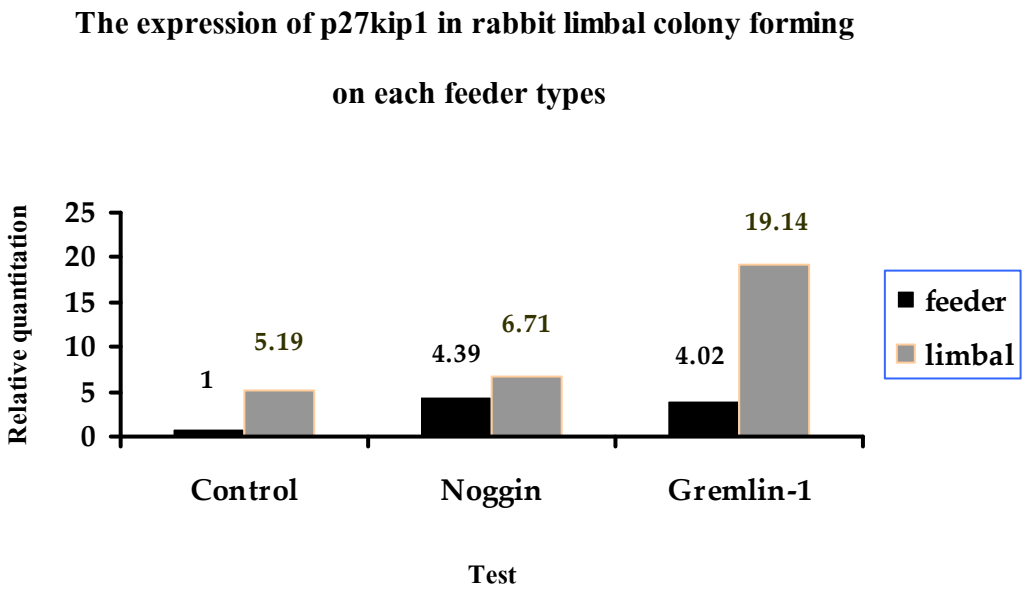


Figure 16: Relative quantitation real-time pcr result of $p27^{kip1}$ expression in rabbit limbal colony compared with feeder cells. The transcription level of $p27^{kip1}$ in rabbit limbal colony formed on Gremlin-1 feeder cells were the highest up-regulation. On noggin feeders, the level of $p27^{kip1}$ was slightly up-regulate.

Our results indicated that Noggin expressed feeder cells could help to maintain undifferentiated stage of rabbit limbal stem cells while Gremlin-1+ feeder cells could keep undifferentiated stage of limbal cells. In addition this feeder could also activate the transcription of p27kip1 gene and the colony displays slower proliferation.

8. The expression of TCF-3 in cultivated human limbal epithelial cells.

Wnt signaling is another signaling pathway that has a crucial role in stem cell maintenance. Beta-catenin the effectors of Wnt pathway accumulated in cytoplasm, translocated to nucleus and interacted with TCF/LEF transcription factors in the present of Wnt. These Beta-catenin/Tcf complexes involve in maintenance and growth of epithelial stem cells in many tissues. TCF3 and TCF4, a member of TCF transcription factor family which is the mediatiors of Wnt signaling were found in mammals. Furthermore, along with Wnt signaling tcf3 directs skin stem cells differentiation (41) Thus, cultivated limbal epithelial cells in fibronectin coated plate were stained with anti-human TCF3/4 antibody.

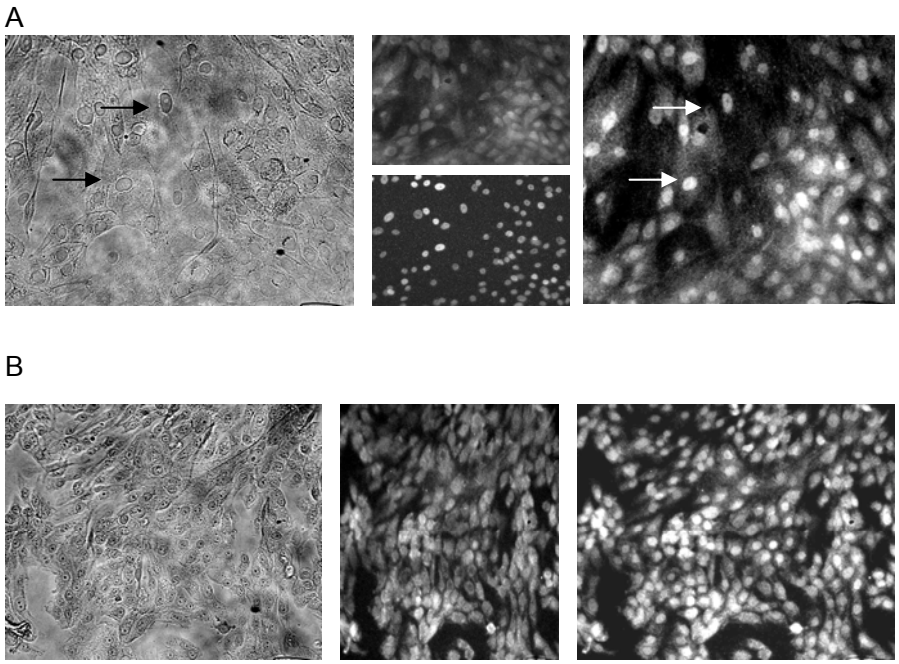


Figure 17: Immunofluorescent staining of TCF-3 in cultivated limbal epithelial cells on fibronectin coated plate. (A) limbal epithelial cells were positively stained with anti-TCF3 in their nucleus whereas (B) undifferentiated limbal epithelial cells were positively stained mostly in their cytoplasm.

Large cells that show epithelial morphology were strongly positive for TCF3/4 in their nucleus. In contrast, small cells found to express TCF3/4 in the nucleus of some cells. These data suggested that TCF3 has a role corresponded to limbal stem cell differentiation.

9. The Combination of Noggin or Gremlin-1 espression with GSK-3 inhibitor

In the absence of Wnt signal, beta-catenin resides in a large cytoplasmic complex consisting of the tumor suppressor APC, GSK-3β and Axin. In this destruction complex, beta-catenin is phosphorylated which finally lead to beta-catenin degradation. Chemical substance called GSK-3 inhibitor, 6-bromoindirubin-3'-oxime (BIO) has been reported to maintain the pluripotency of mouse and human embryonic stem cells through the activation Wnt/beta-catenin signaling. Hence, we added 20ng/ml of BIO in condition media of limbal stem cells cultured on BMPs antagonisms feeder layers.

Limbal stem cells formed colony within 9 days in all feeder types. The colony formation was earlier than without BIO experiment. The total numbers of colony were counted and compared with condition without BIO. The amounts of colony from all feeder types that added BIO are greater than without BIO condition as similar as colony forming efficiency which is 0.4-0.5%. There were three types colony morphology but most of colonies are smooth, large, and circular and high proliferate. The colonies grew so fast that they are almost full the plate within 5 days.

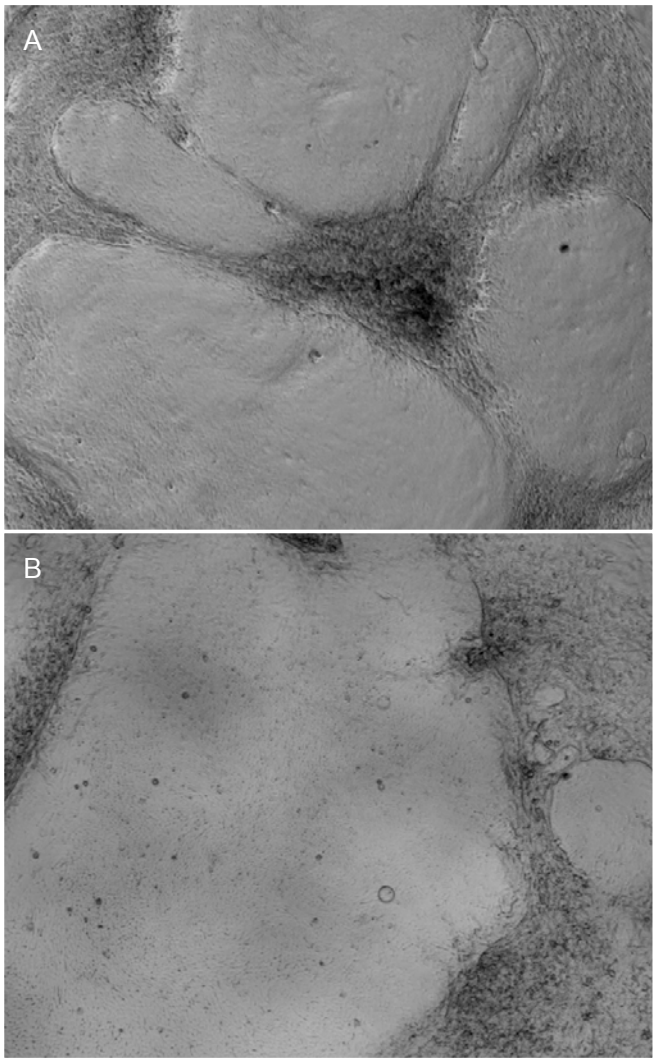


Figure 18: Rabbit limbal colony forming on BMP antagonists combined with BIO supplemented medium. (A) Rabbit limbal stem cells formed colony on Noggin feeder and (B) Gremlin-1 feeder. Such colonies were meroclone consisting of early state of TA cells which were increase proliferation when added BIO. The cells grew so quickly that full the plate within 5 days.

Clonogenicity of rabbit limbal colony on each feeder type combined with BIO						
Feeder	Control		Noggin		Gremlin-1	
Type	Numbers	Std. Error	Numbers	Std. Error	Numbers	Std. Error
H	2.2	0.63	2.7	0.83	3.6	1.03
M	7.2	0.89	9.6	1.98	10.9	1.27
P	8.3	1.41	7.1	1.65	5.4	0.78
Total	17.7	2.06	18.4	4.01	19.9	2.06
CFE%	0.44	0.51	0.46	0.1	0.49	0.05

Table 2: Clonogenicity of rabbit limbal colony on each feeder combined with BIO supplemented medium. We obtain more rabbit limbal colonies when cultured with BIO supplemented medium. And the cultivated rabbit limbal colonies in BIO condition medium formed more quickly than without BIO and also had more colony forming efficiencies. Most of the colonies were meroclone contained limbal TA cells which shown highly proliferate.

The changes of mRNA levels of TCF-3, p21 and $p27^{kip1}$ genes were determined on day 14 after BIO had been added. In the absence of BIO, TCF3 was down-regulated in the limbal colony formed on Noggin feeder. On Gremlin-1 feeder, the mRNA level of TCF3 did not differ from the control feeder. In the BIO condition, limbal colonies grown on control feeder showed down-regulation of TCF-3 level while in the combination with both BMP antagonist feeders; TCF-3 was 2 folds up-regulated. According to previous results that colonies of limbal stem cells grown on Noggin and Gremlin-1 feeder were large and high proliferation, these were shown by the decrease of $p27^{kip1}$ mRNA level. Interestingly, we detected the up-regulation of p21 in BIO combined with BMP antagonist feeder cell condition.

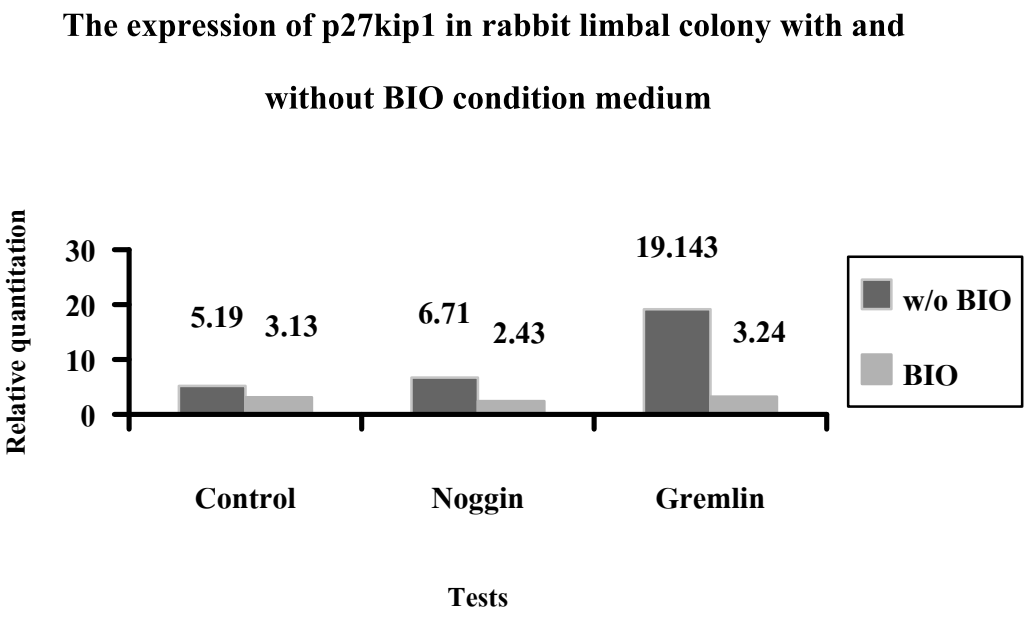


Figure 19: The transcription level of $p27^{kip1}$ compared between rabbit limbal colony cultivated with and without BIO supplemented medium on each feeder types. $P27^{kip1}$ was down-regulated in all rabbit limbal colonies that grown on every feeders that cultured with BIO condition medium.

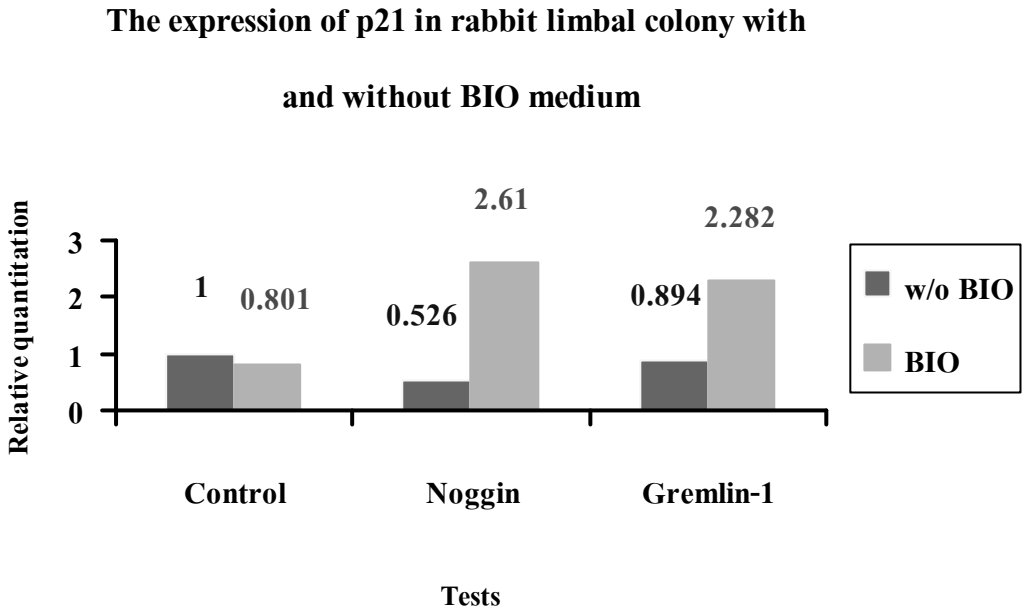


Figure 20: The transcription level of p21 compared between rabbit limbal colonies cultivated with and without BIO supplemented medium on each feeder types. Both Noggin and Gremlin-1 that combined with BIO showed higher up-regulation of p21 transcription.

These data suggested that BMP antagonist induced growth-arrested cell cycle of limbal stem cells possibly in the quiescent stage evidenced by the increase of cyclin-dependent kinase inhibitor, $p27^{kip1}$. And in the activation of beta-catenin/TCF-dependent Wnt signaling through BIO induced the transcripition of TCF3 genes and promoted limbal stem cells proliferation which shown by the reducing of p27kip1 mRNA level.

At the 7^{th} of the day after the colony forming, 2 well of each experiment culture dish that had the most colony numbers were selected for subculture to determine whether the limbal cells from these feeder types could be survived and formed colony again on the next passage. After the inoculation for 7 days, the cells only from Noggin+BIO and Gremlin + BIO conditions could generate 2-3 colonies on the average. However, all the colonies were paraclone consisted with differentiated cells.

10. Transplantation

10.1. Ex vivo expansion of human limbal tissue on denuded human amniotic membrane.

On denuded hAM as shown in figure 1, the epithelial outgrowth was able to see under microscope on the next 5-7 day after culture. The rate of epithelial outgrowth was slow for the first week but became rapid from then on and frequently reached a size about 2-2.5 cm in diameter in 3-4 weeks. The epithelial cells of the outgrowth during the first week was uniformly small cuboidal and with an area ratio of close to 1:1 between the nucleus and the cytoplasm. Cultivated cells were closely attached to each other and adhered well to hAM substrate. In the second week, Limbal epithelial cells grown on denuded hAM became layer and could be maintained for about 2 months.

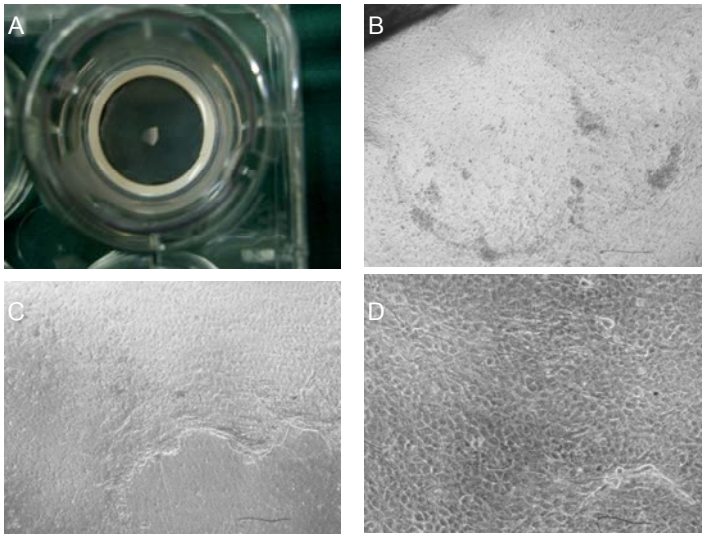


Figure 21: Human limbal tissue explant cultured on human amniotic membrane and the epithelial cell outgrowth. Human limbal tissue cultivated on denuded amniotic membrane (A) after 5 days the limbal epithelial cells grew out from limbal rim (B). In 3rd weeks, multilayer could be seen (C). The limbal epithelial cell observed under 10x power field microscope. (D)

The limbal epithelial cells were promoted to differentiate by airlifting technique as describe in chapter III before transplantation. After 10 to 14 days of airlifting, Cytokeratin pair 3 and 12 immunostaining. On denuded hAM, cytokeratin pair 3 and 12 was strongly expressed in the cells at the upper layer.

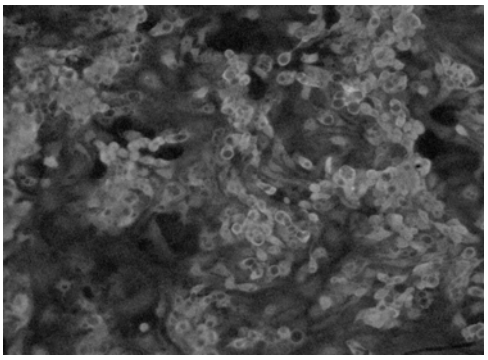


Figure 22: Cytokeratin pair K3 and K12 immunofluorescent staining in human limbal epithelial cells. Limbal epithelial cells on denuded hAM plate strongly expressed cytokeratin pair K3/12 after exposed to the air for 7 days.

10.2 Rabbit transplantation

The limbal tissues from autopsy were explanted on denuded human amniotic membrane. After the outgrowth cells were covered all hAM membrane surface that would be size 2-2.5 cm in diameter. The selected sample was carried to operation room for rabbit transplantation.

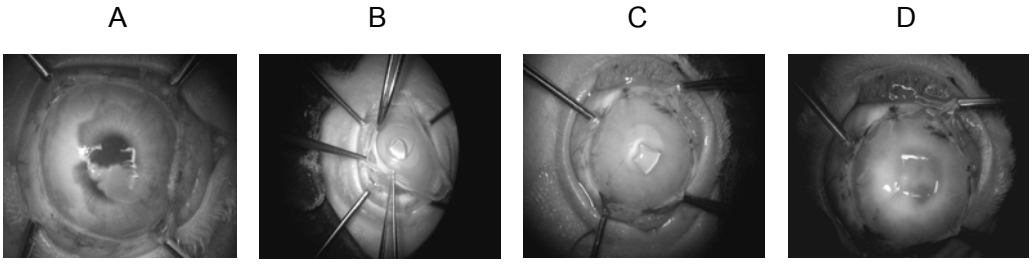


Figure 23: Rabbit transplantation A) Limbal deficiency can cause corneal epithelial cells defect. The area was shown in yellow. B,C) Transfering explant limbal culture on denuded hAM to the eye. D) After the tissue was removed and stained with fluorescein to confirm that limbal epithelial cells grew cover membrane surface. Our result showed negative staining (blue). That means, membrane surface was covered with limbal epithelial cells.

CHAPTER V

DISCUSSION AND CONCLUSION

Several studies have since disclosed that limbal epithelium stem cells (LESCs) share some features similar to epithelial stem cells one of adult stem cells. For instance, LSCs have the smallest cell size, are slow-cycling and hence label-retaining, and do not express markers destined for terminal differentiation such as cytokeratins 3 and 12, involucrin, and connexin 43. To dates, LSC can be used for reconstruction damaged corneal epithelium by transplantation to the patients who suffer from limbal stem cell deficiency. A number of techniques have been optimized to make the culture systems more appropriate for maintenance LSC properties and a greater numbers of cells. The culture systems both explant and suspension methods have used either amniotic membrane which is the currently favoured substrate or onto a plastic tissue culture dish containing a feeder layer of growth-arrested 3T3 fibroblasts. According to the culture method that used by D Meller, and co-leagues (51) we could expand limbal epithelial cells from cadaveric tissue on denuded human amniotic membrane and fibronectin coated plate. A few cells grown on fibronectin substrate expressed ABCG2 and showed label-retaining DNA precursor. Terminal differentiated state of limbal stem cells induced by airlifting technique from both substrates were strongly positive for cytokeratin pair K3 and 12. We can propagate limbal epithelial cells by using culture method described previously from several studies. However, limbal epithelial stem cells can not survive longer than 2 passages in culture. That means the culture system can not maintain limbal stem cell properties.

Stem cells need to be communicating with their niche to maintain their properties: self-renewal and fate determination in generating the progeny. Different studies have shown that signals such as Wnt/beta-catenin pathway, Notch pathway, and TGF-beta/BMP pathway play important roles in the niche control of different types of SCs. However, little is known about the regulation of limbal stem cells in their niche. The previously study (34) demonstrated that Wnt/beta-catenin pathway is up-regulated in the limbal niche and had effects on development. Furthermore, BMP signaling is another

signaling pathway that plays role in limbal stem cells. The data from Satoru Koyano et al. showed that noggin was synthesized and released by human amniotic membrane epithelium. These data supported by the culture condition that used intact human amniotic membrane as an appropriate substrate could preserve limbal stem cells. So we decided to create ex vivo environment that mimicked to LSCs niches in vivo.

We created 3T3 feeder cells that expressed Noggin and Gremlin-1, member of BMPs antagonists to test whether these molecules could maintain limbal stem cells in an in vitro culture. We observed human limbal epithelial cells dissociated from fibronectin coated plate adhered well to BMPs antagonist feeder cells after seeded. Limbal epithelial stem cells colony on BMPs antagonist feeder cells could be seen on day 10 -11 which slower than control. In addition, the morphology of colony in these feeders was quite similar to holoclone. Normally, the cell cycle profile of stem cells is characterized by a quiescence which arrests at G0/G1 phase. These cell cycle arresting was governed by the activities of cyclin-dependent kinase inhibitors (CKIs). Immunohistochemistry of limbal tissue showed that $p27^{kip1}$ and $p57^{kip2}$ were localized in the nucleus of quiescent limbal cells but were not in actively proliferating cells. In our experiment, BMP antagonists could possibly maintain undifferentiation stage by inducing cell-cycle arrested which shown by the up-regulation of $p27^{kip1}$ in rabbit limbal colonies that grown on both BMP antagonist feeders. The cells in these conditions had slower cell proliferation and displayed more stem cell-liked morphology. From immunocytochemistry we demonstrated that TCF-3 was corresponded to limbal stem cell differentiation supported by the down-regulation of TCF-3 in limbal colony formed on Noggin feeder.

Wnt signaling also has functions in maintenance of many stem cell types therefore we studied role of Wnt signaling in limbal stem cells. We activated Wnt signal through GSK-3 inhibitor (BIO) in limbal colony. In the presence of BIO, we obtained greater and faster colony formation. Moreover, limbal cells proliferation was increase shown by the decrease of p27kip1 regulation. Generally, 6-bromoindirubin-3'-oxime (BIO) or GSK-3 inhibitor has commonly used to maintain stem cells self-renewal through activation of Wnt/Beta-catenin signaling. However, there is study (52) showed that Wnt signaling and beta-catenin stabilization transiently activate Lef1/Tcf complexes and

promote their binding to target genes that promote TA cell conversion and proliferation to form the activated cells of the newly developing hair follicle. (53) In adult skin, quiescent multipotent progenitors express Tcf3 and commit to a hair cell fate in response to Wnt signaling. We also detected Tcf3 slightly up regulated in the combination of BIO and BMP antagonist feeder cells.

To summarizes, BMPs signaling showed to have crucial role in limbal stem cells differentiation. In the opposite, BMPs antagonisms such as Noggin and gremlin-1 help to maintain undifferentiated stage of limbal stem cells possibly by prolonged cell cycle progression through $p27^{kip1}$. Moreover BMPs antagonist 3T3 feeder cells could be useful as a new substrate for limbal epithelial stem cells in vitro cultivation. Furthermore, the activation of Wnt signaling through GSK-3 inhibitor increased limbal stem cell proliferation possibly through TCF3 and p21. However, these conclusions are indefinite because of mix population of cells. And the effects of TCF-3 remain unclear.

So in the future study, this experiment should be repeated but in feeder free culture system. And study about what exactly are the roles of TCF-3 in limbal stem cells by loss and gain of function.

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APPENDICES

APPENDIX A

TABLE OF NUMBERS OF RABBIT LIMBAL COLONY RAW DATA

Control feeder with BIO

Well	**Para**	**Mero**	**Holo**	**Total**	**CFE**
1	6	9	1	16	0.4
2	9	7	2	18	0.45
3	14	12	2	28	0.7
4	14	5	3	22	0.55
5	6	7	2	15	0.38
6	3	10	3	16	0.4
7	12	8	7	27	0.68
8	7	7	0	14	0.35
9	11	2	2	15	0.38
10	1	5	0	6	0.15
Total	83	72	22	177	
Average	**8.3**	**7.2**	**2.2**	**17.7**	**0.44**
STDV	4.47	2.82	1.99		0.16

Controlo feeder without BIO

Well	**Para**	**Mero**	**Holo**	**Total**	**CFE**
1	6	3	1	10	0.25
2	2	6	3	11	0.28
3	5	6	4	15	0.38
4	7	7	2	16	0.4
5	10	6	0	16	0.4
6	9	5	0	14	0.35
7	6	7	0	13	0.33
8	4	9	1	14	0.35
9	6	10	3	19	0.48
10	0	0	0	0	0
Ave	**5.5**	**5.9**	**1.4**	**12.8**	**0.32**
STDEV	2.99	2.85	1.51	5.18	0.13

Noggin feeder with BIO

Well	**Para**	**Mero**	**Holo**	**Total**	**CFE**
1	15	21	9	45	1.13
2	4	4	1	9	0.23
3	10	10	4	24	0.6
4	1	6	4	11	0.28
5	8	13	3	24	0.6
6	8	9	0	17	0.43
7	12	15	2	29	0.73
8	1	14	2	17	0.43
9	0	1	2	3	0.08
10	2	3	0	5	0.13
AVG	**6.1**	**9.6**	**2.7**	**18.4**	**0.46**
STADEV	5.24	6.26	2.63	12.7	0.32

Noggin feeder without BIO

Well	**Para**	**Mero**	**Holo**	**Total**	**CFE**
1	2	5	3	10	0.25
2	2	6	4	12	0.3
3	0	6	3	9	0.23
4	9	2	0	11	0.28
5	2	5	0	7	0.18
6	9	10	5	24	0.6
7	6	4	2	12	0.3
8	17	12	2	31	0.78
9	4	6	2	12	0.3
10	6	7	2	15	0.38
AVG	5.7	6.3	2.3	14.3	0.36
STDEV	5.01	2.87	1.57	7.45	0.19

Gremlin-1 feeder with BIO

Well	**Para**	**Mero**	**Holo**	**Total**	**CFE**
1	10	17	1	28	0.7
2	5	12	0	17	0.43
3	9	9	3	21	0.53
4	3	6	4	13	0.33
5	7	8	5	20	0.5
6	4	13	10	27	0.68
7	5	18	7	30	0.75
8	4	9	1	14	0.35
9	4	8	5	17	0.43
10	3	9	0	12	0.3
AVG	**5.4**	**10.9**	**3.6**	**19.9**	**0.5**
STDEV	2.46	4.01	3.27	6.51	0.16

Gremlin-1 feeder without BIO

Well	**Para**	**Mero**	**Holo**	**Total**	**CFE**
1	3	6	5	14	0.35
2	1	6	3	10	0.25
3	1	11	9	21	0.53
4	2	3	5	10	0.25
5	6	7	3	16	0.4
6		6	1	7	0.18
7	1	2	3	6	0.15
8	5	10	2	17	0.43
9	0	9	2	11	0.28
10	1	10	6	17	0.43
AVG	**2.22**	**7**	**3.9**	**12.9**	0.32
STDEV	2.05	3.02	2.38	4.86	0.12

APPENDIX B

REFERENCE SEQUENCES OF HUMAN NOGGIN

LOCUS NM_005450 1279 bp mRNA linear PRI 27-JUN-2007

DEFINITION Homo sapiens noggin (NOG), mRNA.

ACCESSION NM_005450

VERSION NM_005450.2 GI:38788425

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 121 cttccgcccc ggtgggagcc ggcgctgcgc gaagggctct cccggcggct catgctgccg
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 241 gccgcgggct cggcgtgctc tcctccgggg acgcgggacg aagcagcagc cccgggcgcg
 301 cgccagaggc atggagcgct gccccagcct aggggtcacc ctctacgccc tggtggtggt
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1261 atacgttaaa aaaaaaaaa
```

REFERENCE SEQUENCES OF HUMAN GREMLIN-1

LOCUS NM_013372 4175 bp mRNA linear PRI 27-JUL-2007

DEFINITION Homo sapiens gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis) (GREM1), mRNA.

ACCESSION NM_013372

VERSION NM_013372.5 GI:71164890

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