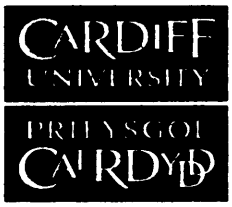

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NOTCH SIGNALLING AND ITS ROLE IN CORNEAL EPITHELIUM SURVIVAL AND DIFFERENTIATION

A thesis submitted to Cardiff University for the degree of

Doctor of Philosophy

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ABSTRACT

AIMS

To elucidate mechanism of corneal epithelial cell differentiation, proliferation and stratification is crucial for maintaining epithelial cell homeostasis, manipulating corneal wound healing and developing therapeutic strategy for treatment ocular surface diseases. The Notch signalling system regulates cell fate decisions and cell function. In human, there are four Notch receptors, Notch1 to 4, and five ligands, including Delta1, 3, 4, Jagged1, 2. Activation of Notch upon ligand binding is accompanied by proteolytic processing regulated by γ-secretase. This study aimed to determine whether the components of the Notch are expressed in the human corneal epithelial cells and the role of Notch signalling in corneal epithelial homeostasis, stratification and wound healing.

METHODS

Immunohistochemistry was employed for the localisation of the Notch receptors and their ligands in fresh human cornea and embryonic chicken cornea. Gene expression of Notch receptors and their ligands was determined using reverse transcriptase-polymerase chain reaction (RT-PCR) in cultured human corneal epithelial cells and keratocytes. Western Blotting analysis, immunocytochemistry in the presence or absence γ-secretase inhibitor and Jagged1 were used to correlate Notch with Ki67 (a marker of cell proliferation) and cytokeratin 3 (a marker of cell differentiation) expressions for a functional study of proliferation and differentiation in corneal epithelial cells. The co-culture model with amniotic membrane and an organ culture

model of intact rat cornea were used to investigate the function of Notch in corneal epithelial cell stratification. Also, an organ culture of wounded cornea was used to study the role of Notch in corneal epithelial and stromal wound healing.

RESULTS

Immunohistochemical results showed that Notch1 and Notch2 expressed throughout the corneal epithelium in superficial and suprabasal layers. Delta1 and Jagged1 appeared to be expressed throughout all cell layers of the corneal epithelium. The expression of activated Notch1, Notch2 and Ki67 was decreased and cytokeratin 3 was increased after the Notch pathway was blocked by a γ-secretase inhibitor. In contrast, activation of Notch pathway by Jagged1 induced the increase of the expression of activated Notch1, Notch2 and Ki67 and decreased the expression of cytokeratin 3. The corneal stratification was inhibited by activation of Notch. In wound healing study, Notch inhibited the wound repair at late stage. In addition, the expression pattern of Notch was exhibited in developmental embryonic chicken cornea.

CONCLUSIONS

Notch suppresses differentiation and stratification in corneal epithelium. Activation of Notch results the retardation of corneal wound repair. Notch signalling system plays a pivotal role in maintenance of corneal epithelial cell homeostasis and wound healing.

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

SIGNED.....(candidate)

Date 06-06-2006

STATEMENT 1

This thesis is the result of my investigations, expect where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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STATEMENT 2

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library been loan, and for the title and summary to be made available to outside organisations.

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LIST OF FIGURES

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Abbreviations

A_{260}	absorption at 260 nm
A_{280}	absorption at 280 nm
AM	amniotic membrane
BSA	bovine serum albumin
bp	base pair
°C	degrees centigrade
cDNA	complementary DNA
cm	centimeter
CK3	cytokeratin 3
CO_2	carbon dioxide
dH_2O	deionised water
ddH_2O	double deionised water
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
g	gram
HRP	horseradish peroxidase

Ig	immunoglobulin
µl	microlitre
µm	micrometer
µg	microgram
M	mol
MEM	minimal essential medium
mg	milligram
ml	millilitre
mm	millimeter
MMC	mitomycin C
mRNA	messenger RNA
NICD	Notch intracellular domain
OCT	optimal cutting temperature
OD	optical density
%	percentage
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	hydrogen ion concentration
PFA	paraformaldehyde
PS1	presenilin1
PS2	presenilin2
RNase	ribonuclease
RT	reverse transcription
γSI	γ-secretase inhibitor

SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	The standard error of the mean
T	time
T8	Trowell's T8 medium
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	hydroxymethyl aminomethane
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

CHAPTER ONE

INTRODUCTION

1.1. Cornea

1.1.1. Anatomical structures, physiology and function of cornea

The cornea is a clear, transparent avascular tissue and is located at the anterior of the eyeball, forming approximately one sixth of the outer tunic of the eye. It is unique among the stratified squamous epithelia of the body in that it provides an absolutely smooth, wet, apical surface that refracts and transmits light to the lens and retina (Maurice and Singh, 1985). The most important property of the cornea is that of transparency, but it is also a tough physical barrier to trauma and infection due to its vulnerable position. These functions are maintained due to a number of factors, including the regularity and smoothness of the covering epithelium, its avascularity and the regular arrangement of the extracellular and cellular components in the stroma, which are dependent upon the hydration, metabolism and nutrition of the stromal elements (Oyster, 1999a). In addition, assurance of these unique and vital functions is provided by a highly developed and extraordinarily dense sensory nerve system that can warn of potential danger and induce rapid protective response (Oyster, 1999a).

The tissue of the cornea appears simple in composition because it is composed only of an outer stratified squamous nonkeratinized epithelium, an inner dense connective tissue stroma with its resident fibroblast-like keratocytes and a monolayer cubical endothelium bordering the anterior chamber (see Fig. 1.1). The cornea, however, is highly ordered and complexly arranged. Its transparency, avascularity and highly ordered structure make it unique among all tissues of the body. Cells of all layers

interact with and influence each other's functions. Mediators (e.g. cytokines) expressed by one cell type can influence cells of adjacent layers.

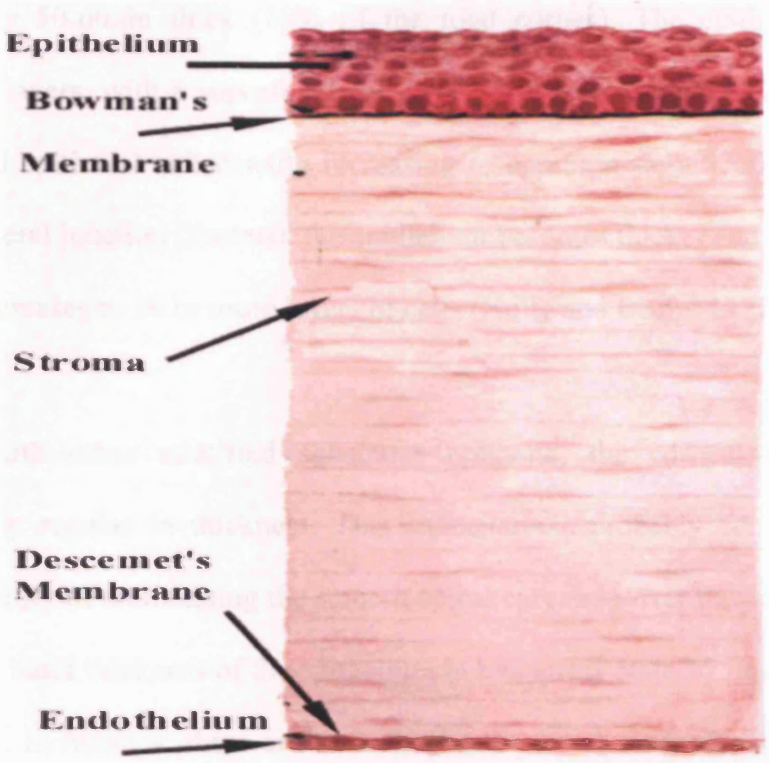


Fig. 1.1. Transverse section of a human cornea (copied from Forrester et al. 1999)

The highly specialised substructural organisation of the cornea comprises five differentiated layers (Fig. 1.1):

- Corneal epithelium
- Anterior limiting lamina (Bowman's layer)
- Corneal stroma (Substantia propria)
- Posterior limiting lamina (Descemet's membrane)
- Corneal endothelium.

Corneal epithelium

The corneal epithelium is a nonkeratinised, squamous, stratified epithelium of approximately 50-60μm thick (10% of the total cornea). The epithelium usually contains 5-6 layers, with a superficial cell density of approximately 1200 cells/mm^2 and the basal epithelial cell density increasing to approximately 5700cells/mm^2. At the corneoscleral junction (limbus), the epithelium becomes thicker and the number of cell layers increases to 10 or more layers of cells (Holly and Lemp, 1977).

Compared with other stratified squamous epithelia, the corneal epithelium is extraordinarily regular in thickness. This characteristic probably contributes to its unique capability of maintaining the smooth apical curvature over the corneal surface. The apical to basal thickness of the epithelium in humans is 50 to 52 μm (Maurice and Singh, 1985). In humans, there are three to four layers of outer flat squamous cells termed squames, one to three layers of midepithelial cells termed wing cells and a single layer of columnar basal cell (Oyster, 1999a).

The epithelium of the cornea is self-renewing. The basal cells are the only epithelial cells that undergo mitosis. As they divide, daughter cells begin their movement off the basement membrane and are pushed anteriorly. They thus change their shape, conforming to the contiguous wing cells, which are defined by the way they overlap onto the apices of the adjacent basal cells. As the cells continue to move anteriorly, they become the superficial cells, after which they disintegrate and are shed into the tear film in a process known as desquamation. The superficial cells represent the

terminal differentiation. The epithelium turns over about 5 to 7 days (Hanna et al., 1961).

The corneal epithelium has functions unique to it self and also functions that are common to other body epithelia. Several of its unique functions include light refraction, transmittance and survival over an avascular bed. The ability to refract light is brought about by its absolutely smooth, wet apical surface and its extraordinarily regular thickness.

In addition to its specialized functions, the corneal epithelium has the routine housekeeping functions of all epithelia that border the outside world. The layers of cells provide a barrier to fluid loss and pathogen entrance and resist abrasive pressure by tightly adhering to one another and to the underlying connective tissue stroma.

Bowman's Layer

Below the corneal epithelium lies the anterior limiting lamina (Bowman's membrane), which is essentially a modified acellular region of the stroma, discontinues at the limbus (Oyster, 1999a). It is approximately 8-12 μm thick and consists of fine, randomly arranged collagen fibrils that form an interface between the basal lamina of the epithelium and the subjacent lamellar stroma. Constituents of this layer contain several collagen types, including types I, V, and VII (Nakayasu, 1988; Gordon et al., 1994), and proteoglycans such as chondrioitin sulfate proteoglycan (Li et al., 1991).

Stroma

The corneal stroma is a layer of dense connective tissue that makes up approximately 90% of the cornea (Oyster, 1999a). It consists of keratocytes, extracellular matrices and nerve fibers. The cellular component (keratocytes) is only occupies 2-3% of the total stromal volume (Oyster, 1999a), with the remainder consisting of various extracellular matrices, mainly collagen and glycosaminoglycans. Type I is the major collagen component in human cornea, comprising some 68% of the dry weight (Maurice and Singh, 1985). The maintenance of corneal transparency is highly dependent on the regular spacing of the collagen fibrils (interfibrillary distance), that is regulated by proteoglycans, which form links between the collagen fibrils. Keratan sulfate proteoglycan and dermatan sulfate proteoglycan are the predominant proteoglycans within the corneal stroma (Oyster, 1999a).

The matrix components of the lamellar stroma are secreted and maintained by stromal fibroblasts, also known as keratocytes. These long, attenuated cells are arranged parallel to the corneal surface and are located between the collagen lamellae. The keratocyte cell body contains an elaborate rough endoplasmic reticulum and Golgi apparatus, reflecting its active synthetic function. Keratocytes extend slender cytoplasmic processes and can form gap junctions with neighbouring cells, resulting in a network of communicating cells (Oyster, 1999a).

In a normal healthy stroma there are no blood or lymphatic vessels, but in the anterior layers there are some sensory fibres which pass into the corneal epithelium suggesting potential route of between the epithelium and stroma under certain conditions, such as corneal wounding.

Descemet's membrane

The posterior limiting lamina (Descemet's membrane) lies between the posterior stroma and the corneal endothelium. In adults, this matrix consists of two layers. An anterior, banded layer is formed during fetal development and consists of highly organised collagen lamellae and proteoglycans. A posterior amorphous layer is synthesized after birth and is less organised than the fetal layer. Adult Descemet's membrane approximately 8-12 μm in thickness contains fibronectin, laminin, and type IV and VIII collagen, heparin sulphate, and dermatan sulphate proteoglycan. It is tightly adhered to the posterior surface of the corneal stroma and reflects any change in the shape of the stroma (Oyster, 1999a).

Endothelium

The endothelium is the single layer of cells located at the posterior surface of the cornea; it permits the passage of nutrients from the aqueous humor into the cornea (Tuft and Coster, 1990). The endothelium is responsible for maintaining the relatively low level of stromal hydration necessary for corneal transparency. Stromal hydration is controlled by the activity of ionic pumps in the plasma membrane of endothelium cells. The extracellular ion concentration produced by these pumps draws water from the stroma, thus maintaining the highly organised collagen lamellar structure required for transparency. The endothelium secretes the thick basal lamina, termed Descemet's membrane, which under lies it (Oyster, 1999a).

1.1.2. Tear film

Although the preocular tear film is not part of the cornea, it is intimately associated with the cornea anatomically and functionally. The tear fluid covers the corneal surface. Tear fluid protects the cornea from dehydration and maintains the smooth epithelial surface, this is essential in order for the epithelial cells to remain function. It consists of three layers (Holly and Lemp, 1977): a superficial lipid layer (about 0.1 μm), an aqueous layer (about 7 μm), and a mucin layer (0.02 to 0.05 μm). More than 98% of total tear volume is water.

The tear film contains many biologically important factors including electrolytes, glucose, immunoglobulins, lactoferrin, lysosome, albumin, oxygen, and a range of various biologically active substances such as histamine, prostaglandins, growth factors, and interleukins (Ohashi et al., 1989; van Setten et al., 1989). Therefore, the tear film serves not only as a lubricant and nutritional source for the corneal epithelium but also as the source of the regulatory factors for the maintenance and repair of the corneal epithelium (Nishida et al., 1983; Watanabe et al., 1987; Tsutsumi et al., 1988).

1.2. Corneal epithelial cell homeostasis

Maintenance of corneal structure is crucial for its physiologic functions in biodefence and as a refractive system. Therefore the cornea is equipped with a more active maintenance system: the active renewal of the corneal epithelium.

An understanding of normal corneal homeostasis is required to appreciate how changes in epithelial cell number occur during wound healing. This was recognised by Thoft and Friend in 1983 who formulated the X, Y, Z hypothesis of corneal epithelial maintenance (Fig. 1.2). This hypothesis stated that a constant number of corneal epithelial cells were maintained by:

Addition of new cells to the basal layer by proliferation of basal cells (X) plus the centripetal migration of new basal cells originating from limbal stem cells (Y) which was equal to the loss of cells from corneal surface (Z) (Thoft and Friend, 1983)

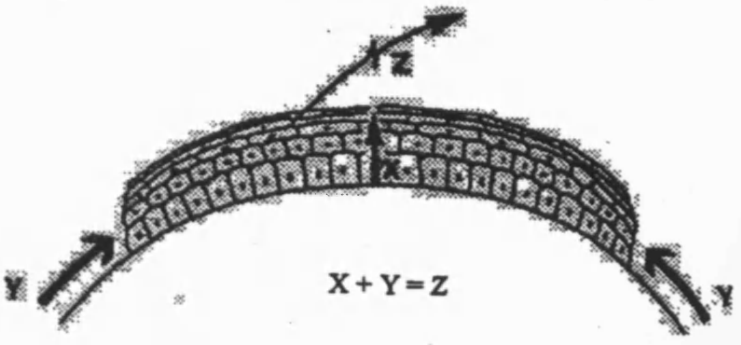


Fig. 1.2. X,Y,Z hypothesis of corneal epithelial maintenance (copied from Thoft et al. 1983)

Therefore a balance between cell loss and cell replacement is mandatory for maintenance of the corneal epithelium. It has now been established that the limbus is the germinative region of the corneal epithelium, containing stem cells in its basal layer (Bron, 1997).

1.2.1. Stem cells in tissue renewal

Stem cells are defined as relatively undifferentiated cells that have the capacity to self-renew and also generate one or more differentiated daughter cells (Boulton and Albon, 2004). Self-renewal is a term that has been used in connection with stem cells. Tissue lost can be replaced during normal wear and tear in tissues such as hair and epidermis. Wound healing in response to trauma can also be initiated. The ability to replace this tissue largely rests with a relatively small population of stem cells that have the capacity to self-renew and differentiate along specific molecular pathways throughout life. Hence stem cells are key to the maintenance of tissue integrity throughout the body, including the eye.

1.2.2. Corneal epithelial stem cells

Epithelial stem cells can undergo asymmetric cell division resulting in one daughter cell remaining undifferentiated to replenish the stem cell pool, while the other is destined to be a more differentiated transient amplifying cell that can further divide into differentiated cells of the corneal epithelium (Figure 1.3). It is also possible that local signals may influence similar daughter cells resulting from symmetric stem cell division to follow different cell fates (Morrison et al., 1997).

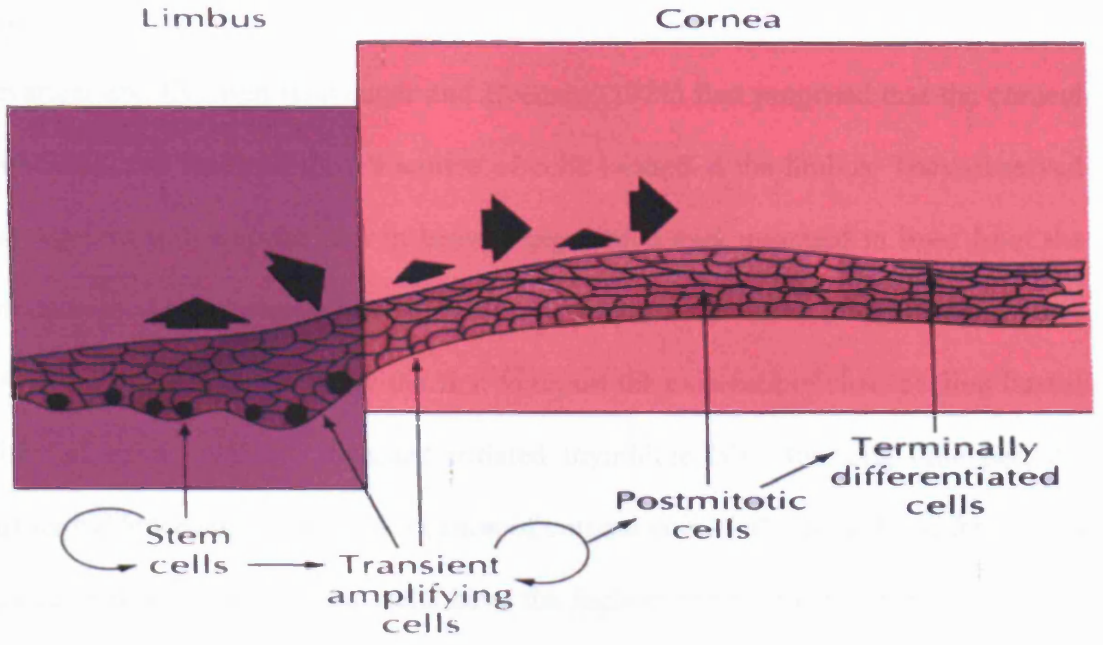


Fig. 1. 3. A model of the limbal location of corneal stem cells (copied from Kruse et al. 1994).

Stem cells have unique intrinsic characteristics that enable them to control cell replacement during homeostasis and tissue repair. These features distinguish stem cells from their more differentiated progeny, the transient amplifying cells. Intrinsic differences between the two populations are summarized in Table 1.1.

Table 1.1 Intrinsic differences between stem cells and transient amplifying cells

Stem cells	Transient amplifying cells
• Poor differentiated with primitive cytoplasm • Resistant to tumor promoters • High capacity for error-free self renewal • Slow cycling during homeostasis • Long life span	• Less primitive than stem cells • Susceptible to tumor promoters • Differentiate into post-mitotic and terminally differentiated cells • Divide more frequently • Limited proliferative potential

Davanger and Evensen (Davanger and Evensen, 1971) first proposed that the corneal epithelium was renewed from a source of cells located at the limbus. They observed that pigment in the epithelium in heavily pigmented eyes migrated in lines from the limbus to the central cornea in healed eccentric corneal epithelial defects. Cotsarelis et al (Cotsarelis et al., 1989) were the first to report the existence of slow-cycling limbal epithelial basal cells that retained tritiated thymidine label for long time periods. Further evidence supporting the location of corneal epithelial stem cells at the limbus includes cultured limbal basal cells have the highest proliferative capacity (Ebato et al., 1988; Lindberg et al., 1993; Pellegrini et al., 1999), surgical removal of the limbus results in delayed healing with noncorneal epithelium (Chen and Tseng, 1991; Huang and Tseng, 1991), and limbal transplants can regenerate corneal-like epithelium (Kenyon and Tseng, 1989).

1.2.3. The limbo-corneal epithelium

A more rigorous demonstration of the concept of the limbus as the germinal site for the corneal epithelium became possible following the recognition of the tissue specificity of cytokeratin pairs and their differentiation-dependent expressions (Moll et al., 1982). Along the basal cell axis, expression of these proteins started at the limbo-corneal margin, in concurrence with the stromal transition from vascular to avascular. From this margin to the corneal centre, all basal cells are uniformly positive for Keratin3 (K3) and/or Keratin12 (K12). The limbal stem cell hypothesis was cemented by subsequent work demonstrating the presence of slow cycling cells within the limbus and peak proliferative activity in the adjacent corneal periphery

(Cotsarelis et al., 1989; Lavker et al., 1991). From the percentage of radiolabeled thymidine retaining cells (i.e. slow cycling) found in the limbal zone, it was concluded that stem cells may represent as many as 10% of the total limbal population. Taken together, these results suggest that as cells move centripetally across the limbo-corneal (LC) demarcation, they concomitantly develop TA cell features and initiate de novo expression of K3 and/or K12.

The recognition that a small pool of slowly cycling cells within the limbus is able to regenerate the tissue on its own led to the re-evaluation of the belief that the conjunctival epithelium can slowly transdifferentiate into corneal epithelium. It has become clear that such a phenomenon does not really occur (Morrison et al., 1997). Rather, current evidence supports the notion that occasional recovery of corneal phenotype, observed following whole replacement of the corneal surface by conjunctival epithelium, is likely to represent repopulation from a small surviving pool of limbal stem cells (Kruse, 1994; Morrison et al., 1997).

1.3. Corneal epithelial cell stratification

At any point in time, a cell in the basal layer of a stratified epithelium may decrease its contact with the basement membrane and start its ascent towards the surface. This separation from the substratum inevitably implies loss of all proliferative capacity and the initial stage in the acquisition of features that may be unique to the intrastratal state and/or those features that ought to be expressed later on at the tissue surface. Proliferative pressure is not likely to account for centripetal migration. And migration may be in fact driven by enhanced rates of surface cell desquamation at the surface and also when coupled to the limbal strata distinct compaction and basal adherence,

such increased proliferation may be sufficient to force centripetal cell stream (Lavker et al., 1991).

It was established that after cell division, daughter cells either remain in the basal layer, where they may undergo additional rounds of cell division, or differentiate synchronously. When the latter occurs, the time between the previous cell division and differentiation is highly variable. These results clearly point to the absence of a direct link between division and differentiation. On the other hand, they may be indicative of a counting or timing mechanism where stratification for each individual basal cells is determined primarily by the number of division that has taken place, or the time that have elapsed, from the initial transition to the TA cell phenotype.

1.4. Regulators of epithelial cell homeostasis

1.4.1. Growth factors and cytokines

Human corneal epithelial and stromal cells, as well as the lacrimal gland, synthesise a number of growth factors or their receptors. Li and Tseng subdivided the corneal expression of 12 cytokines and their receptors into four functionally and anatomically different groups (Li and Tseng, 1995).

Type I cytokines included transforming growth factor-α (TGF-a), interleukin 1β (IL-1β) and platelet-derived growth factor-BB (PDGF-BB), which are expressed by epithelial cells to converse with fibroblasts. Type II cytokines include insulin-like growth factor type I (IGF-I), transforming growth factor-β1 (TGF-β1), TGF-β2, leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), which are cytokines crosstalking between the epithelium and fibroblasts. Type III cytokines

include keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), which are expressed by fibroblasts to stimulate epithelial proliferation and migration. Type IV cytokines include macrophage colony stimulating factor (M-CSF) and interleukin-8 (IL-8), which are expressed on epithelial cells and fibroblasts but their receptors are expressed on immune and inflammatory cells (Kruse and Tseng, 1991; Li and Tseng, 1995; Joyce and Zieske, 1997).

Among the various growth factors, epidermal growth factor (EGF), KGF and HGF are strong mitogens of corneal epithelial cells, and are considered to play an important role in epithelial wound healing. EGF has a growth promoting effect on both corneal epithelial cells and corneal stromal fibroblasts (Frati et al., 1972). Whereas KGF and HGF promote corneal epithelial cell proliferation, but not stromal fibroblast proliferation (Sotozono et al., 1994)

1.4.2. Extracellular matrix

The corneal extracellular matrix (ECM) components are major regulating factors in corneal epithelium. They could be pre-existing (native or degraded), newly synthesised, or deposited after occurrence of the wound to provide a provisional matrix. Two of the major matrix components are collagens and proteoglycans. Other major extracellular matrix components found in the basement membrane include laminins and entactin. These ECM components have been shown to regulate the corneal epithelial cell homeostasis and wound repair (Anderson, 1977; Buck, 1979; Beyer et al., 1990; Brazzell et al., 1991; Dua et al., 1994; Beales et al., 1999).

1.5 Stromal-epithelial cell interactions in the cornea

Stromal-epithelial interactions are key determinants of corneal function. Bi-directional communications occur in a highly coordinated manner between these corneal tissues during normal development, homeostasis and wound healing (Wilson, 1999; Wilson et al., 1999)

The epithelium, stroma and nerves participate in homeostasis of the anterior cornea and ocular surface. The lacrimal glands and tear film also contribute to the maintenance of surface smoothness and integrity important to function of the eye. Following injury, these components participate in an orchestrated response that efficiently restores corneal structure and function in most situations.

1.5.1. Stromal to epithelial interactions

The best characterised stromal to epithelial interactions in the cornea are mediated by the classical paracrine mediators Hepatocyte Growth Factor (HGF) and Keratinocyte Growth Factor (KGF). HGF and KGF are produced by the keratocytes to regular proliferation, motility, differentiation and possibly other functions of epithelial cells but not fibroblast (Wilson et al., 1993). Both HGF and KGF stimulated corneal epithelial cell proliferation, but only HGF stimulates migration (Wilson et al., 1994b; Wilson et al., 1994a; Honma et al., 1997). HGF markedly stimulated corneal epithelial cell motility and inhibited corneal epithelial cell terminal differentiation. Most recently, Carrington et al. (2005) suggest that neutralizing the effects of high concentrations of HGF may be a worth therapeutic intervention in corneal repair

(Carrington and Boulton, 2005). No effect of KGF on motility or terminal differentiation of corneal epithelial cells was detected (Wilson et al., 1999).

1.5.2. Epithelial to stromal interactions

Two pathways have been suggested for communication in this direction (Wilson, 1999). Epithelia to stroma interactions are mediated by cytokines, such as interleukin-1 (IL-1) and soluble Fas ligand that is released by corneal epithelial cells in response to injury. Other, yet to be identified, cytokine systems may be released from the unwounded corneal epithelium to regulate keratocytes viability and function. IL-1 appear to be a master regulator of corneal wound healing that modulates functions such as matrix metalloproteinase production, HGF and KGF production and apoptosis of keratocytes following injury (Wilson, 1999).

1.6. Epithelial cell homeostasis in corneal wound healing

1.6.1. General principles of wound healing

wound healing is generally characterised by a number of stages: platelet induced haemostasis, inflammation, proliferation, formation of granulation tissue, wound contraction, neovascularisation and tissue remodelling (Mast, 1997). Whilst well defined (classically in cutaneous healing), this is a dynamic process, in which there is considerable overlap between the stages, the modulators of each phase and the cellular components.

1.6.2. Corneal wound healing

The structure, position and function of the cornea, provide challenges in the process of wound healing not found in other tissues. The lack of blood supply, sequestration from the immune system, distal position (limbus) of the epithelial stem cell population and the need to retain transparency result in a specialised wound healing process. In some respects this is much simpler than cutaneous wound healing since the inflammatory response in the cornea is usually minimal, and there is no need to regenerate a vascular network within the tissue. However, corneal wound healing is complicated by the presence of tear fluid, which contains factors capable of regulating the healing response.

Corneal wounds can occur for a number of reasons, both accidental and elective. The type of wound, its extent and any underlying pathology can result in different clinical outcomes (Redbrake et al., 1997). Corneal wounds can be broadly divided into simple epithelial defects, those that penetrate into the underlying stroma, and those that puncture the endothelium, each increasing in severity and poor prognosis. Wounds involving the endothelium have added complications; presence of aqueous fluid, loss of endothelial pump activity and loss of ocular pressure, and will not be discussed further, as they are not the subject of this thesis.

1.6.2.1. Corneal epithelial wound healing

Clinically, the most obvious stage of wound healing occurs in the epithelium. The healing process can be divided into three stages: latent, migration and adhesion, and

proliferation phase (Fagerholm, 2000). These phases in vivo however form a dynamic and continuous process.

Latent phase

The latent phase lasts approximately 4-6 hours after insult (Dua et al., 1994). Polymorphonuclear (PMN) cells arrive by three hours after injury via the tear fluid (Robb and Kuwabara, 1962) and remove necrotic tissue and debris. This, along with rounding of epithelial cells at the wound edge (Pfister, 1975; Crosson et al., 1986), results in an apparent enlargement of the wound.

Migration and adhesion

The next phase starts with the completion of lamellipodia, filopodia, rearranging actin filaments and the migration of epithelial cells and lasts 24-36 hours. Cells increase their volume and flatten to cover a lager area (Fagerholm, 2000). Newly concentrated in the leading edge of migrating cells are actin filaments composed of proteins including fodrin, vinculin and ankyrin (Takahashi et al., 1992).

Focal contacts are formed between the migrating cell and the wound substrate surface (fibronectin, fibrin, fibrinogen and tenascin). Fibronectin is initially deposited from tears but is followed by deposition of cellular fibronectin secreted from the migrating cells (Gipson and Inatomi, 1995). Tenascin promotes the migration of epithelial cells (Tervo et al., 1991) and probably also derives from the tear film (Vesaluoma and Tervo, 1998). Fibrin and fibrinogen are deposited at the edge of the wounds

(Fujikawa et al., 1981; Phan et al., 1989), stimulating corneal epithelial cells to secrete plasminogen activator and collagenases. This cleaves cell adhesion at focal contacts, allowing the leading edge of the cell to move forward and new focal contacts to form (Gipson and Inatomi, 1995). The cell repeats this cycle of extending filopodia, attaching the actin cytoskeleton via focal adhesions to the wound substrate, moving the cell body through traction, and detaching focal adhesions until the epithelial defect is closed. However as the desmosomes between epithelial cells remain largely intact the epithelium moves laterally as a sheet away from the limbus (Dua et al., 1994; Messent et al., 2000).

Proliferation

The cells within the cornea exhibit a wide range of proliferative abilities. The change in proliferation profile of the corneal epithelium during wound healing starts shortly after insult. Within 8-12 hours of injury, basal cells distal to the wound area are stimulated to synchronously enter the cell cycle, however those undergoing migration across the epithelial defect do not proliferate (Chung et al., 1999). As seen in Figure 1. 4, the proliferative rate in the limbus, containing a mixture of slow cycling stem cells and more rapidly cycling transient amplifying cells, is somewhat lower than that seen in the rest of the cornea.

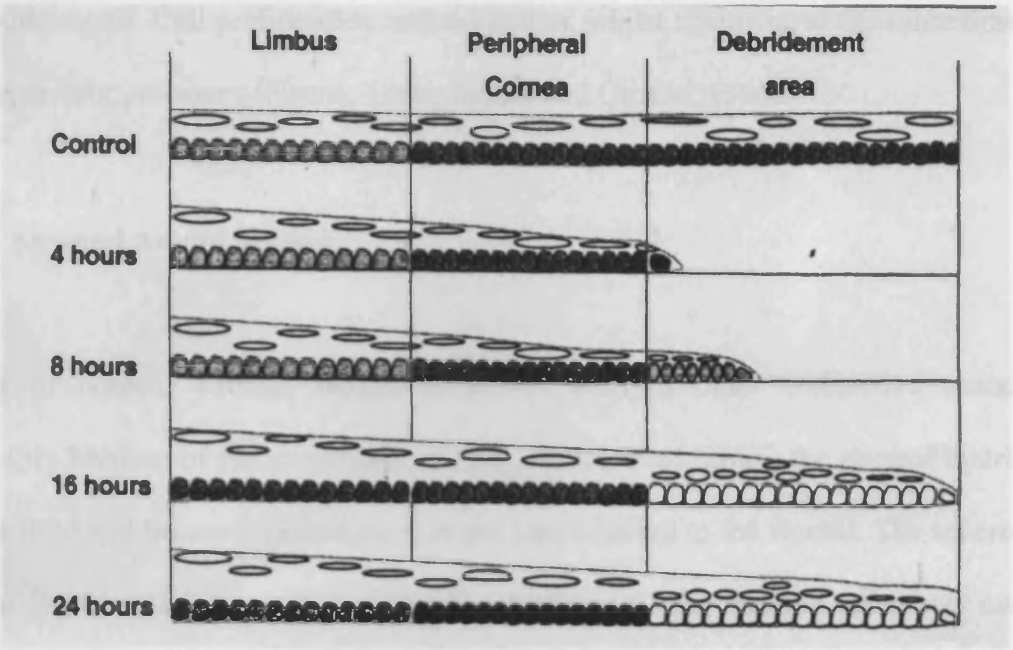


Fig. 1.4. Diagrammatic illustration of the relative rates of proliferation of corneal epithelial cells in unwounded corneas and in corneas following a 3-mm central epithelial debridement. Black indicates the highest rates of proliferation, grey indicates higher rates of proliferation, white indicates quiescent corneal epithelial cells (Copied from Nishida et al.1998).

These proliferative rates are greatly altered following wounding (Lavker et al., 1991; Chung et al., 1995; Messent et al., 2000). After a 3-mm central debridement wound, basal cells in the limbus and peripheral cornea increase their proliferative rate by several fold. The peak of S-phase cells occurs 28 hours after wounding (Lavker et al., 1991; Chung et al., 1995; Messent et al., 2000), with the limbus showing a broader peak beginning 16 hours after wounding (Chung et al., 1995). Intriguingly, the proliferative rate in the sheet of epithelium migrating over the wound area drops dramatically. These data indicate that the rate of proliferation is differentially regulated in different areas of the corneal epithelium both during normal homeostasis

and wound repair. Cell proliferation and migration, whilst occurring at the same time, are independent processes (Hanna, 1966; Zieske and Gipson, 1986).

1.6.2.2. Stromal wound healing

Healing of corneal stromal wound is slower than in other connective tissues presumably because of the avascularity. After an incisional injury, the stromal matrix imbibes fluid and becomes oedematous in the area adjacent to the wound. The severed acellular Bowman's layer and Descnmet's membrane (if the incision penetrates into the anterior chamber) do not heal, and the cut ends of the membranes remain retracted (Campos et al., 1993; Szerenyi et al., 1994; Wilson et al., 1994b; Wilson et al., 1994a; Gao et al., 1997; Kuo, 1997; Wilson and Kim, 1998; Wilson et al., 1999). The loss of keratocytes occurs immediately upon wounding and affects a zone extending up to 200μm from the exposed stroma (dependant on species) (Matsuda and Smelser, 1973; Nakayasu, 1988). It is hypothesised that this loss is intimately associated with the decreased integrity of the epithelial cell layer immediately overlying the keratocytes. Keratocytes under the wound undergo cell shrinkage, blebbing with the formation of membrane bound bodies, condensation of the chromatin and DNA fragmentation cobsistent with programmed cell death (otherwise termed apoptosis) (Gao et al., 1997; Helena et al., 1998; Wilson and Kim, 1998; Kim et al., 1999).

Stromal wound healing occurs via stromal keratocyte migration, proliferation, and deposition of extracellular matrix molecules, including collagen (specifically type III), adhesion proteins (e.g. fibronectin, laminin), and glycosaminoglycans. These processes are facilitated by a phenotypic change among quiescent keratocytes to

become active myofibroblasts, a task mediated by transforming growth factor beta (of presumptive epithelial origin).

1.7. The role of Notch signalling in epithelial cell homeostasis

The Notch signalling pathway is an essential gene encoding a signalling receptor that is required throughout development to regulate the spatial patterning, timing and outcomes of many different cell fate decisions in both vertebrate and invertebrate species (Artavanis-Tsakonas et al., 1999).

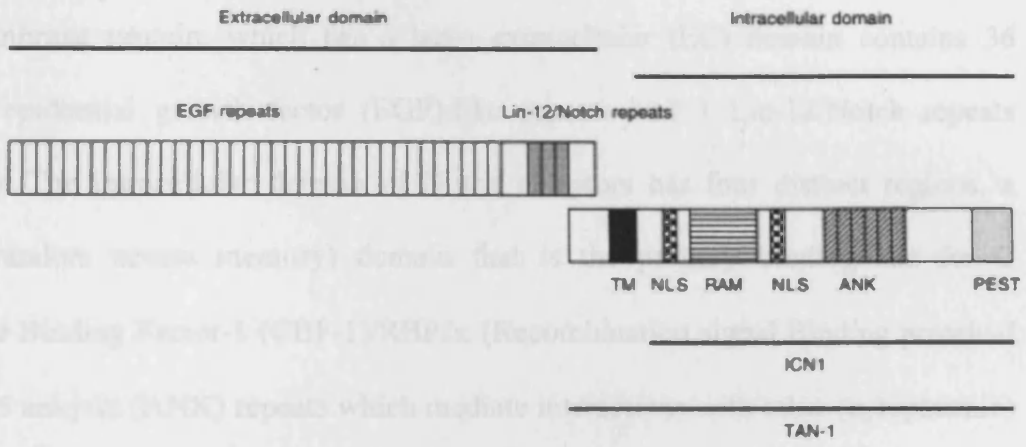


Fig. 1.5. Structure of human Notch1 receptor. EGF indicates epidermal growth factor: TM, transmembrane domain; NLS, nuclear localozation signal; ANK, ankyrin repeats; PEST, proline-glutamate-serine-threonine rich domain; ICN1, intracellular Notch1 (copied from Tamura et al. 1995).

Notch signalling controls an extraordinarily broad spectrum of cell fates and developmental processes resulted in a veritable explosion of Notch-related studies. These studies make it apparent that signals transmitted through the Notch receptors, in

combination with other cellular factors, influence differentiation, proliferation and apoptotic events at all stages of development (Artavanis-Tsakonas et al., 1999).

1.7.1. The elements of Notch signal

Notch receptors

The Notch gene, a -330 kDa human Notch receptor family consists of four members (Notch1-4), with Notch1 playing a widespread role in determination of cell fate during development (Larsson et al., 1994). A typical Notch receptor is a single-pass transmembrane protein, which has a large extracellular (EC) domain contains 36 tandem epidermal growth factor (EGF)-like repeats and 3 Lin-12/Notch repeats (Fig.1.5). The intracellular domain of Notch receptors has four distinct regions, a RAM (random access memory) domain that is the primary binding site for C promoter Binding Factor-1 (CBF-1)/RBPJκ (Recombination signal Binding protein-J kappa), 6 ankyrin (ANK) repeats which mediate interactions with other (cytoplasmic) proteins, a proline-glutamate-serine-threonine (PEST) domain which regulates protein turnover and 2 nuclear localization seqences (NLS) (Artavanis-Tsakonas et al., 1999). Notch1 and Notch2 have the highest homology with each other, while Notch3 and Notch4 are structurally slightly diverged from Notch1 and Notch2, both in the extracellular and intracellular domains (del Amo et al., 1993; Lardelli and Lendahl, 1993; Lardelli et al., 1996; Uyttendaele et al., 1996). Intracellular domain of Notch is known to act as transcriptional activator, is often called as activated Notch and widely used to investigate the function of Notch receptors and the mechanism of the Notch-mediated signalling.

Notch ligands

Notch ligands are divided into two subclasses, the Delta and the Serrate (in *Drosophila*) /Jagged (in vertebrates) families. These ligands are transmembrane proteins with a small intracellular (IC) domain, a large extracellular (EC) region comprising epidermal growth factor-like repeats (Lendahl, 1998). Mammalian ligands include two members of the Jagged family, Jagged 1 and 2, and three members of the Delta family (Delta1, Delta3, and Delta4, also known as Dll1, Dll3, and Dll4) (Gu et al., 1995; Gridley, 1997; Greenwald, 1998). Jagged cDNA encodes a protein with an apparent molecular mass of around -150 kDa that is localised to the cell surface (Lindsell et al., 1995) in tissues during embryonic development in the rat, including the eye, ear, kidney, pancreas, limb bud and skin also in other species. The -110 kDa normal Delta expression has been found in adrenal gland, placenta and neuroendocrine tumors such as neuroblastomas and pheochromocytomas (Laborda et al., 1993). In hematopoietic cells, Delta has been expressed broadly on hematopoietic cells and stromal cells (Han et al., 2000). Like Notch receptors, the ligands are also transmembrane proteins, and physically bind to the external domains of the Notch receptors. The extracellular region of Delta contains EGF-like repeats as well.

γ-secretase/Presenilins

γ –secretase is a membrane-bound protease which cleaves within the transmembrane domain of a number of substrate proteins, including the Notch family of receptors and the amyloid precursor protein (APP). Several new putative γ –secretase substrates

have recently been identified, including the CD44 cell-surface protein, E-cadherin and the low-density lipoprotain – receptor – related protein (LRP) (Fortini, 2002).

Two different proteins were shown to be essential for γ –secretase activity: presenilin (PS) and nicastrin. Presenilin, a ~46 kDa protein, is encoded by two highly homologous genes, PS1 and PS2, both contributing, independently, to γ –secretase activity.

Proteolytic cleavage of Notch and nuclear translocation of its intracellular domain have long been considered to be the crucial step in transduction of the signal. This theory is based on early studies documenting constitutive signalling from engineered Notch protein truncations (Fortini and Artavanis-Tsakonas, 1993; Greenwald, 1994), the identification of specific, cleaved products of endogenous mammalian Notch (Schroeter et al., 1998) and the demonstration that inhibiting Notch cleavage in transgenic mice results in reduced Notch signalling (Huppert et al., 2000).

1.7. 2. Notch receptor-ligand interactions

Cell aggregation studies of Drosophila cultured cells have revealed that receptor-ligand interactions are mediated by specific EGF repeats of the Notch receptor and the conserved extracellular region of the ligand (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). These observations reinforce the simple model in which a transmembrane ligand on one cell interacts with the receptor on a neighbouring cell. However, several observations that have attracted less attention indicate that Notch-ligand interactions are far more complex.

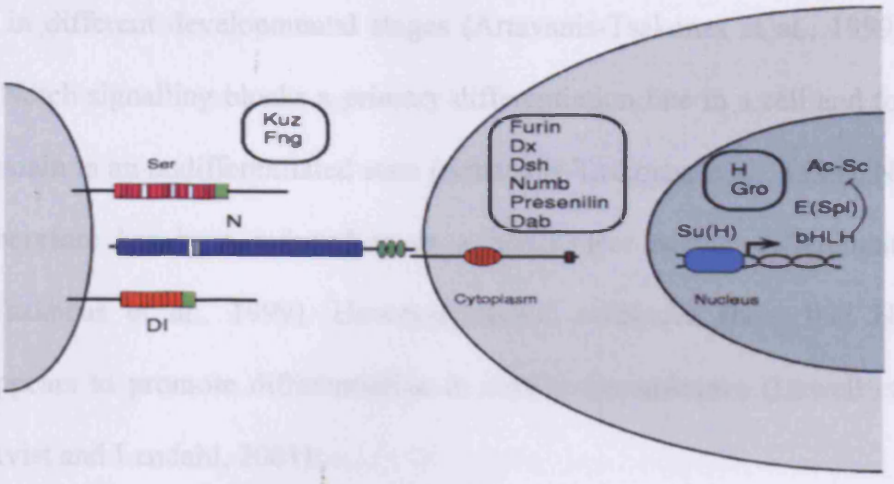


Fig. 1.6. Elements of Notch signaling (copied from Artavanis-Tsakonas et al. 1999).

The basic picture (Fig. 1.6) emerging from many different studies has the extracellular domain of the ligands, expressed on the surface of one cell, interacting with the extracellular domain of the Notch receptor on an adjacent cell. As a result of receptor activation, Su(H) binds to regulatory sequences of the *E(spl)* genes and up-regulates expression of their encoded bHLH proteins (Artavanis-Tsakonas et al., 1999). The bHLH factors, in turn, affect the regulation of downstream target genes. There is no doubt that this linear picture is only a skeleton, as each step is embellished with additional elements and features that modulated the activity and efficacy of the signals transmitted through the Notch receptor.

1.7.3. The function of Notch signal

Notch signalling regulates cell fate in many different tissues in a wide range of organisms including nervous system, vascular system, hematopoietic system, somites, muscle, skin and pancreas (Varnum-Finney et al., 1998; Artavanis-Tsakonas et al., 1999). The Notch signalling pathway plays different roles in different tissues even

same tissue in different developmental stages (Artavanis-Tsakonas et al., 1999). In most cases, Notch signalling blocks a primary differentiation fate in a cell and forces the cell to remain in an undifferentiated state (Artavanis-Tsakonas et al., 1999). Notch signalling therefore has been referred to as a 'gatekeeper against differentiation' (Artavanis-Tsakonas et al., 1999). However, recent evidences show that Notch signalling appears to promote differentiation in certain circumstance (Lowell et al., 2000; Lundkvist and Lendahl, 2001).

More recent studies revealed that apart from the well-documented involvement of Notch in differentiation, both proliferation and apoptotic events can be affected by Notch (Artavanis-Tsakonas et al., 1999). A link between proliferation events and Notch has been seen in several instances. In Drosophila, Notch, together with Wingless, induces cell cycle arrest within the so-called nonproliferative region (Johnston and Edgar, 1998). In contrast to cell cycle arrest, Notch activation can also induce proliferation (Cagan and Ready, 1989; Go et al., 1998). The elements mediating the nonautonomous effect of Notch on proliferation are unknown. The examination of receptor activation in different imaginal discs demonstrates that the ability of Notch to influence cell proliferation is the result of synergistic effects between Notch and other genes and depends on developmental context. For instance, the simultaneous expression of activated Notch and vestigial in the eye disc (Go et al., 1998), whereas other discs remain relatively unaffected.

It is now appreciated that Notch is widely expressed and functions in many tissues throughout vertebrate and invertebrate development. Notch seems to be able to participate in several key aspects of development, including patterning and lateral

inhibition. Lateral inhibition is a mechanism that explain how two identical adjacent cells can be induced in vivo to differentiate to different tissues during development. It occurs when one cell expressing Notch is stimulated by an adjacent cell expressing a Notch ligand. Activation of Notch in the first cell causes it to adopt expression of Notch ligand. The adjacent cell is then exposed to less Notch ligand and adopts a different differentiation program. This same mechanism is thought to contribute prominently to the development of 'boundaries' during development.

1.7.4. The Notch pathway in tissue development

1.7.4.1. Neurogenesis

In mammals, Notch activation is indispensable *in vivo* for the maintenance (self-renewal) of neural stem cells, which is the precursor of all neural and glial cells in the mammalian nervous system (Hitoshi et al., 2002). In the neural tube, nascent neurons express DSL ligands and inhibit the Notch-expressing progenitor populations (de la Pompa et al., 1997; Lewis, 1998). Notch signalling appears to control at least two other aspects of neural development: the differentiation of glial cells (Gaiano et al., 2000; Morrison et al., 2000). And neurite arborization, i.e. the length and organization of dendritic extensions from neurons (Redmond et al., 2000). Thus, Notch signalling regulates both stem-cell self-renewal and later events during neurogenesis.

In the past, Notch signalling has not been considered instructive because it was believed to have a nonspecific inhibitory effect on cellular differentiation. As such the Notch pathway was thought to passively influence cell fate by controlling the ability

of progenitors to respond to instructive developmental cues. However, recent data has suggested that Notch can play a more active role in promoting glial and perhaps stem cell identities. While intriguing, it is worth noting that this role is context dependent, both in vertebrates and in invertebrates, as Notch can either promote or inhibit gliogenesis, depending on the cell type being examined. Therefore, although in specific contexts the role of Notch might be termed instructive, it is currently not possible to define a uniform role for Notch with regard to glial fate.

1.7.4.2. Skin homeostasis and wound healing

In mammalian epidermis, keratinocytes progress through successive phenotypic stages as they migrate from the germinative basal layer to the skin surface. These continuous proliferative and differntiative processes result in tissue homeostasis. Studies on embryonic mice and rats have shown the involvement of the Notch pathway in epidermal differentiation (Del Amo et al., 1992; Thelu et al., 1998), as well as cutaneous appendage patterning (Kopan and Weintraub, 1993; Powell et al., 1998; Favier et al., 2000).

At the tissue resolution level, Jacques et al. (Thelu et al., 2002) observe continuous labelling in the basal layer, including putative stem cells and transit amplifying cells. Thus in the epidermis of human adult, Notch receptors and their ligands Delta/Jagged genes are transcribed in the proliferating area receiving the first differentiation signals. To a lesser extent, they are expressed in the suprabasal layers where differentiation is occurring. Lowell et al. (Lowell et al., 2000) found that Delta1 mRNA and protein

were restricted to the basal epidermal layer while immuno-restricted against Notch1 protein was patchy in the basal layer and fairly strong in the suprabasal layer.

In fact, Notch signalling controls cell choices in both invertebrates and vertebrates (Artavanis-Tsakonas et al., 1999) by inhibiting certain differentiation pathways, thereby permitting cells to either differentiate to an alternative pathway or to self-renew. Feed-back in the Notch pathway amplifies weak stochastic bias between adjacent cells so that even a subtle variation in the amount of signal would generate drastically distinct cell fate. In the epidermal basal layer, Notch receptors are present in transit amplifying cells (Lowell et al., 2000) and the cell-cell signalling system promotes both expansion and differentiation of these cells. Low Notch activity or even none may be permissive for over-growth of poorly differentiated cells, whereas high Notch activity would be expected to control growth and undergo a change of cells fate.

In skin wound space, proliferating cells, which are thought to be derived from the basal cell layer (Demarchez et al., 1987), migrated from the periphery to the centre of the wound, and progressively differentiated to constitute a pluristratified epidermis. The discrepancy between co-localisation of Notch-related transcripts in adult skin and specific pattern in most other systems may only be apparent, since at a cellular level, ligand and receptor gene expression may be cell-exclusive in the basal layer. Some cells, or clusters of cells, could express the ligands, and neighbouring cells that express only the Notch receptor may behave as responding cells. This was recently demonstrated by Lowell et al. (Lowell et al., 2000) for Delta1-Notch1 in keratinocyte cultures and neonatal foreskin. When keratinocytes enter into a pathological

proliferation status (basal cell carcinoma, psoriasis), they neither transcribe ligands nor receptors and the differentiating signal is absent. During healing, a rapid but temporary proliferation status of keratinocytes is required to cover the newly constituted granulocytic layer, and they no longer express ligands, nor receptor transcripts. Transcription is then progressively activated when the epidermis starts to become stratified and differentiated.

1.8. Notch in eye development

For vertebrate eye development, the areas that have received the most attention are the formation of the various components of the eye through inductive interactions and the determination and differentiation of retinal neurons. For example, the anterior neural plate has been found to be necessary for induction of the lens placode, whereas the optic vesicle and lens are thought to induce overlying surface ectoderm to form the cornea (Graw, 1996). The formation of the retina occurs after invagination of the optic vesicle, which creates a cup with an inner layer [presumptive neural retina (PNR)] and an outer layer [presumptive pigment epithelium (PPE)]. The peripheral margin of the optic cup differentiates into the ciliary body and iris (Bard and Ross, 1982). During these processes, cells become fated to be neural cell types, epithelial cell types, muscle cells, or other specialized cells peculiar to the eye. Within the neural retina, the cells differentiate into six different types of neurons and one type of glial cell from multipotent progenitor cells (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). Vertebrate homologs of Notch, Delta and Jagged have been identified in several species (Bettenhausen et al., 1995; Chitnis et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Lewis, 1996; Myat et al., 1996).

The structure and function of Notch have been found to be remarkably conserved. However each mammalian species has several homologs of Notch (Lardelli and Lendahl, 1993; Lardelli et al., 1994). The multiplicity of these molecules in vertebrates makes more complex functions.

The expression patterns of Notch1, Notch2, Delta and Jagged in the embryonic rat eye have been detected by Bao, Z. et al (Bao and Cepko, 1997). At rat E12.5 (embryonic day), Notch1 expression was barely detectable in the optic vesicle, a low level of Notch2 expression was seen in the PPE. Delta RNA was not detectable in the optic vesicle. In contrast, Jagged was found to be expressed at a high level in both the PNR and the lens placode. Jagged expression was limited to the dorsal half of these areas. At E15.5, the lens vesicle and optic cup are completely formed due to the invagination of the lens placode and optic vesicle, respectively. At this stage, Notch1 and Delta were expressed in many cells in the neural retina. The positive cells, however, appeared to be excluded from the periphery of the retina, the area corresponding to the presumptive ciliary body and iris. Interestingly, Jagged expression was limited to the presumptive ciliary body region and was not seen in the neural retina or presumptive iris. Jagged was also expressed at higher levels on the ventral side of the ciliary body region, in contrast to its dorsal-restricted expression pattern at E12.5. Notch2 was absent from the neural retina but was expressed in the optic stalk. Only Jagged was expressed in the lens, mostly in the equatorial region, and to a lesser extent in the anterior portion in the area of actively proliferating cells. At this stage, the posterior of the lens is filled by differentiating lens fiber cells.

Notch1 expression correlates well with neurogenesis in the retina, which occurs from E14 to P10 (postnatal day), with the peak around P0 (Alexiades and Cepko, 1996). With the progression of neurogenesis, the number of Notch1-positive cells decreased and eventually was reduced to zero when all cells were differentiated. Delta showed a pattern similar to that of Notch1 during retinal neurogenesis. Its expression eventually diminished when the cells had differentiated. Postnatal Notch2 expression appeared to be stronger in the ciliary body region in contrast to the E15.5 expression pattern. Jagged expression was confined to the ciliary body. Extending from the periphery of the optic cup, the ciliary body undergoes extensive folding in the neonatal period. At P0 and P5, a subset of inner nuclear layer cells and ganglion layer cells also appeared to express Jagged.

The spatial and temporal expression patterns of Notch1, Notch2, Delta and Jagged appear to define different domains in the developing eye. Notch2 expression is only in the non-neuronal tissues, including the pigment epithelium, optic stalk, and ciliary body, whereas Notch1 is expressed only in the neural retina. The domain of Delta expression is largely overlapping with that of Notch1. The spatial-temporal pattern of expression of Jagged is especially dynamic. Its expression is seen in the neural retina, ciliary body, and lens.

The expression patterns of Notch2 and Jagged suggest a role in the patterning of ocular tissues. Because very little is known about the mechanisms that define these nonretinal tissues as distinct from the contiguous retina, it will be of interest to investigate whether Notch2 plays an active role in patterning this region of the optic cup. The expression of Jagged in the developing eye also suggests a role in patterning.

Its expression was seen as early as E12.5 in the dorsal regions of the optic vesicle and lens placode. Patterning along the dorsal/ventral and anterior/posterior axes is evident within several laminae of the retina. The expression of Notch1 and Delta in the developing eye appears to be within undifferentiated progenitor cells of the neural retina. This is consistent with their expression in the other regions of developing CNS (Coffman et al., 1990; Weinmaster et al., 1991).

1.9. Aims of this study

The aim of this project is to investigate the expression and distribution of Notch signalling family in corneal epithelial cell and stromal keratocytes. The results obtained will then be related to cell proliferation and differentiation so as to explain the mechanism of Notch signalling in corneal homeostasis, stratification, corneal wound healing and corneal development.

The following studies will be undertaken:

1. To determine the expression of Notch receptors and their ligands *ex vivo* and in epithelial cell and keratocyte from gene and protein level.
2. To investigate the functions of Notch signalling pathway in epithelial cell proliferation and differentiation.
3. To investigate the function of Notch signalling in epithelial cell stratification.
4. To determine the mechanism of Notch signalling in corneal wound healing.
5. To investigate the distribution of Notch signalling in corneal embryonic development.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Materials and equipment

Chemical reagents – all chemical reagents, solutions and media used in these methods, are listed in Appendices I and II. All chemical reagents were of analytical grade. Cell culture solutions were prepared using deionised water. All materials used in cell culture procedures were autoclaved prior to use with the exception of disposable items, which were purchased as sterile. All growth media were sterilised by filtration through a 0.2 μm filter, unless made up under sterile conditions.

Routine maintenance of cell cultures was carried out in a Class II biological safety cabinet. Cell cultures were maintained at 37°C in CO_2 incubators within a humidified atmosphere containing 5% CO_2 and 95% air. The growth of cells was observed using an inverted microscope (Olympus IX 70) and digital images obtained using the Spot Advance image capture system (Image Solns, UK). The spectrophotometer was from GeneQuant II (Pharmacia Biotech, England) and the PCR machine was from GRI (England).

2.2 General methods

2.2.1. Primary cell isolation and culture techniques

2.2.1.1. Isolation of corneal epithelial cells

A total of 90 human donor corneoscleral rims (in tissue culture) were obtained from the Bristol Eye Bank and used in accordance with the tenets of the Declaration of Helsinki regarding the use and permission of human tissue for research. The mean age of donors was 62 ± 19 years.

The surface corneal epithelial cells of human corneoscleral rims were scraped off gently with a scalpel blade and the corneoscleral rims were cut into 1-2 mm segments. 1 segment was placed epithelial-side downwards in each well of a 24-well cell culture plate, containing cover-slips pre-coated with attachment factor (TCS Cellworks, 0.25 ml for each well). 0.5 ml fetal calf serum (FCS, Gibco) was added to each explant. The plates were left for 24 hours in standard incubator conditions at 37°C. The tissue was subsequently maintained in a growth medium based on that of Ebato et al. 1988 (Ebato et al., 1988) consisting of a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-10 medium (Gibco) supplemented with 5% FCS, 5% donor horse serum, 0.5% dimethylsulphoxide (DMSO), 5 μg/ml insulin, 2.5 μg/ml fungizone, 0.1 μg/ml choleratoxin, 1% antibiotics (Lab store, see Appendix II) and Glutamine (2mM). Explants were maintained at 37°C in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air and growth medium was changed every 2-3 days. When epithelial cell outgrowth was established, the explants were carefully detached (to prevent fibroblast contamination), and the cells allowed to reach confluence for subculture.

2.2.1.2. Isolation of corneal stromal keratocytes

For the keratocyte cultured, the corneal epithelial cells and endothelium were scraped off with a scalpel blade, corneal limbus was cut off. The stromal explants were placed onto each pre-scratched well of 6-well culture plates, 0.5 ml FCS was added to each explant. The plates were left 20-30 minutes before 2 ml of growth medium (DMEM supplemented with 20% FCS, 2.5 μg/ml fungizone, 1% antibiotics and glutamine)

was added to the each well. The plate was returned to the incubator and left for 2-3 days until keratocyte outgrowth was observed. Explants were then detached from the plate. Fresh medium was added every 2-3 days and the cells were allowed to reach confluence for subculture.

2.2.1.3. Maintenance and subculture of cells

The cells were cultured with appropriate culture medium in a standard incubator and the medium was changed every 2-3 days. Confluent cultures were subcultured as follows: each culture plate was washed twice with 5 ml sterile PBS (lab store, see Appendix II) which had been pre-warmed to 37°C in a water bath. After washing, 0.25 ml 0.25% trypsin - 0.02% EDTA solution was added to each well. The plate was incubated at 37°C in a standard incubator and observed at regular intervals using an inverted microscope until the cells had detached (this process usually took 2-5 minutes). Then the appropriate serum-containing growth medium was added to each well to inhibit further action of trypsin. The cell suspension was pelleted by centrifugation at 250g for 5 minutes. The supernatant was discarded and the cells were re-suspended in growth media and plated in 25 cm^2 culture flasks at a split ratio of 1:1 or 1:2.

2.2.2 Confirmation of cell purity

Two techniques were used to confirm cell purity: morphology and immunostaining.

2.2.2.1. Morphology

Morphology was assessed using an inverted microcope and cell appearance was recorded digitally. Human corneal epithelial cells can be identified by a characteristic 'cobble-stone' morphology (Pancholi et al., 1998).

The human corneal keratocytes were characterised on their distinctive fast growth characteristics and a typical fibroblast appearance (Pancholi et al., 1998).

2.2.2.2. Immunolabelling for confirmation

Cultures were fixed in 1% paraformaldehyde (PFA) (Appendix I) for 20 minutes and washed twice in PBS (5 minutes). Cells were incubated with 0.2% Triton-X-100 (v/v PBS) (20 minutes, room temperature) to permeabilise cell membranes and washed twice in PBS (10 minutes each). Non-specific binding sites were blocked by 5% milk/PBS at room temperature for 30 minutes and then the cells were incubated with the appropriate primary antibodies against vimentin (an intermediate filament protein) and cytokeratin 3 (an epithelial marker) using the reagents detailed in Table 2.1 for 2 hours at room temperature. Substitution of the primary antibodies with same species serum at same concentration acted as a negative control. The cells were washed with three changes of PBS for 10 minutes each and incubated for 2 hours in appropriate secondary antibody (see Table 2.1) before washing as above.

The cells were covered and mounted on microscope slides with Bis-benzimide (0.2%, Hoechst 33342, Sigma) in Hydromount, wrapped in foil and left in a refrigerator (4°C) overnight to solidify.

Table 2.1 Antibodies for confirming cell culture purity

Antigen	Primary antibody	Dilution	Secondary antibody	Dilution
Vimentin	Monoclonal mouse anti-Vimentin clone V9 (Sigma,UK)	1/100	AlexaFluor 488 donkey anti-mouse IgG antibody (Molecular Probes)	1/1000
Cytokeratin3	Monoclonal mouse anti-Cytokeratin 3. AE5 (ICN-UK)	1/100	AlexaFluor 488 donkey anti-mouse IgG antibody (Molecular Probes)	1/1000

Epithelial cells were characterised by positive immunostaining for both markers, whereas keratocytes stained positively for vimentin and negatively for cytokeratin 3 (CK3).

2.2.3. Cell storage in liquid nitrogen

For corneal cell storage, the freezing medium consisted of 10% DMSO and 90% FCS (v/v) was prepared. The cells were resuspended at 1×10^6 -10^7 cells/ml in the freezing medium. 1 ml aliquots of cells were dispensed to cryovials. The vials were put into an ampoule holder and the container was placed into a -80°C freezer. After reaching -80°C, vials were stored in liquid nitrogen.

For recovery of cultures, the frozen cells were thawed by directly plunging cryovials into a 37 °C water bath. Once thawing was completed, the cells were washed once with culture medium to remove DMSO. After centrifugation at 250g for 10 minutes, the cell pellet was resuspended and seeded into a 25cm^2 culture flask and cultured at 37 °C, 5% CO_2.

2.2.4. RNA extraction

2.2.4.1 The principle of RNA extraction

The quality and quantity of isolated RNA is of primary importance for the successful detection and analysis of RNA molecules with RT-PCR. The principles of successful isolation of RNA from cells require the following:

1. Efficient disruption of cellular structures;
2. Dissociation of nuclear-protein from nuclei acids;
3. Inactivation of ribonuclease (RNase) activity;

TRIzol reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). During sample homogenisation or lysis, TRIzol Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

2.2.4.2 RNA extraction

Cells were lysed directly in a 6 well plate by adding 1ml/well of TRIzol reagent and aspirating the cell lysate several times through a pipette. The lysate was transferred to an eppendorf tube. The samples were incubated for 5 minutes at room temperature and 0.2 ml of chloroform was added into each sample. After shaking vigorously by hand for 15 seconds, the mixture was incubated for 3 minutes at room temperature and separated by centrifugation at 12000g for 15 minutes at 4°C. Two phases were formed, the upper phase which contained RNA was the aqueous phase, the lower was organic phase. The aqueous phase was carefully transferred to a fresh Eppendorf tube, an equal volume of isopropanol was added and the tube was placed at room temperature for 10 minutes. RNA was pelleted by centrifugation at 12000g for 15 minutes at 4°C and the supernatant was discarded. Then the pellets were washed twice with 75% ethanol by vortexing and subsequently centrifuged for 5 minutes at 7500g. The ethanol was removed, the pellets were air dried for 5-10 minutes and dissolved in the appropriate volume of RNase-free water by passing the solution a few times through a pipette tip and incubation for 10 minutes at 55-60°C. The RNA samples were stored at –80°C.

2.2.4.3 Determination of RNA concentration and purity

A simple and accurate method was used to quantify the amount of nucleic acid in a preparation. This involved the measurement of the amount of ultraviolet light irradiation absorbed by the bases in a spectrophotometer. In this study, the concentration of extracted RNA was measured using a GeneQuant II

spectrophotometer. The optical density (OD) of RNA has its maximum absorbance wavelength at 260 nm. One OD unit is equivalent to 44 μg/ml of RNA. Contamination of RNA preparations by protein is demonstrated by measuring the OD at a wavelength of 280nm, since proteins typically have a maximum absorbance at this wavelength. A 260/280 absorbance ratio of 1.8-2.0 is appropriate for pure RNA. Contaminating protein will result in lower values of the 260/280 ratio. Tris-EDTA buffer (TE buffer) only was measured as the blank in the spectrophotometer. Then 1 μl of the RNA samples was diluted 100 times by TE buffer and pipetted into a cuvette. The data of wavelengths of 260nm, 280nm, 260nm/280nm and the concentration of RNA were read respectively. Only the samples in the range of 260/280 absorbance ratio of 1.8-2.0 were used for the studies reported in this thesis.

2.2.5. Reverse transcription and Polymerase Chain Reaction (RT-PCR)

2.2.5.1 Principle

RT-PCR combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method for analyzing gene expression. At first, reverse transcriptase catalyses the conversion of RNA to cDNA. Then PCR is carried out in a three-step process. First, the template cDNA that contains the target DNA to be amplified, is denatured by heating. Second, the solution is cooled in the presence of an excess of two single-stranded oligonucleotides that are complementary to the DNA sequences flanking the target DNA. Third, two new DNA strands that are identical to the template DNA strands can be synthesized with the presence of a heat-stable DNA polymerase and the four deoxyribonucleotides (Fig. 2.1).

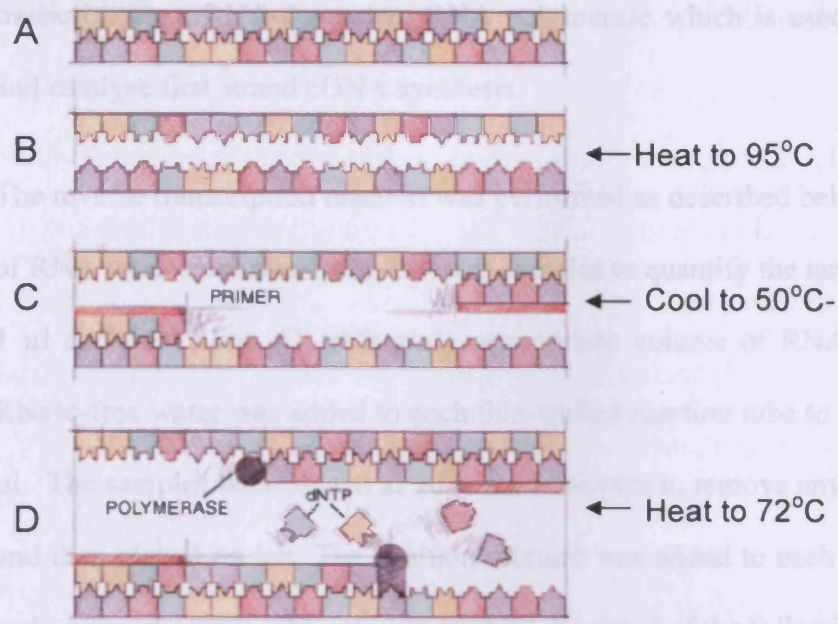


Fig. 2.1 Schematic representation of PCR. A. Double strands of cDNA B. DNA strands split C. Primers bind to the complementary DNA strands D. Taq DNA polymerase extends complementary DNA strands from the primers. (Copied from the manufacture of Abgene, UK)

The melting, annealing, and polymerisation cycle is repeated, the fragment of double-stranded DNA located between the primer sequences can be amplified over a millionfold. The fragment of DNA can be confirmed by running on an agarose gel. Ethidium bromide can combine with DNA and fluorescence detected under UV light, thus allowing the bands of DNA to be observed.

2.2.5.2 Reverse transcription

All the reverse transcription in this study was performed using the Reverse-iT™ First Strand Synthesis Kit (Abgene, UK). Reverse transcription reaction requires a reverse

transcriptase, a RNA-dependent DNA polymerase which is used to prime the RNA and catalyse first strand cDNA synthesis.

The reverse transcription reaction was performed as described below: Equal quantities of RNA were employed from different samples to quantify the target gene expression. 1 µl anchored oligo dT (0.5µg/µl), appropriate volume of RNA sample and sterile RNase-free water was added to each thin-walled reaction tube to a total volume of 13 µl. The samples were heated at 70°C for 5 minutes to remove any secondary structure and then placed on ice. The reaction mixture was added to each sample, mixed well and vortexed gently. The reaction mixture consisted of the following components:

Component	**Volume**
5X first Strand Synthesis buffer	4µl
dNTP mix- 5 mM each	2µl
RTase	1µl

After incubation at 47°C for 30 min, the RTase was inactivated by incubating at 75°C for 10 minutes. The cDNA was either used directly in amplification step or stored at -20°C for further use.

2.2.5.3 Polymerase Chain Reaction

The primers used for PCR were either from published papers or self-designed. For self-designed primers, mRNA sequences were obtained from the gene bank of the National Centre for Biotechnology Information (NCBI) and the design of the primer pair was according to the following principles:

1. Primers were 18-24 nucleotides in length
2. Primers selected had 40% to 60% GC
3. Primers were designed with G or C residues in the 5' and central regions so as to increase the primer's stability and confer hybridisation stability with the target sequence.
4. Complementary sequences at the 3' end of primer pairs were avoided to prevent amplification from the primers themselves to form primer-dimers.
5. A GC-rich 3' end was avoided
6. Mismatches at the 3' end was avoided
7. Sequences with the potential to form internal secondary structure were avoided.

Table 2.2 Primer sequences for RT-PCR analysis

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Product amplified (bp)
Notch1	Fw. 5'-GACATCACGGATCATATGGA-3' Rv. 5'-CTCGCATTGACCATTCAAAC-3'	50	666
Notch2	Fw. 5'-CCAGAATGGAGGTTCCTGTA-3' Rv. 5'-GTACCCAGGCCATCAACACA-3'	52	377
Notch3	Fw. 5'-CACTGAAGGCTCGTTCCA-3' Rv. 5'-GGTTGCTCTCGCATTCA-3'	50	202
Notch4	Fw. 5'-AGCCGATAAAGATGCCCA-3' Rv. 5'-ACCACAGTCAAGTTGAGG-3'	50	687
Delta1	Fw. 5'-AGACGGAGACCATGAACAAC-3' Rv. 5'-TCCTCGGATATGACGTACAC-3'	52	382
Delta3	Fw. 5'-GTGAATGCCGATGCCTAGAG Rv. 5'-GGTCCATCTGCACATGTCAC-3'	54	256
Delta4	Fw. 5'-TGACCACTTCGGCCACTATG-3' Rv. 5'-AGTTGGAGCCGGTGAAGTTG-3'	50	620
Jagged1	Fw. 5'-AGTCACTGGCACGGTTGTAG-3' Rv. 5'-TCGCTGTATCTGTCCACCTG-3'	54	227
Jagged2	Fw. 5'-GATTGGCGGCTATTACTGTG-3' Rv. 5'-AGGCAGTCGTCAATGTTCTC-3'	52	600
β-actin	Fw. 5'-CATCACCATTGGCAATGAGC-3' Rv. 5'-CGATCCACACGGAGTACTTG-3'	58	284
PS1	Fw. 5'-GCTCAGGAGAGAAATGAAACGC-3' Rv. 5'-CCTTCTGCCATATTCACCAACC-3'	54	80
PS2	Fw. 5'-CTTGCTGACTGTCTGGAACTT-3' Rv. 5'-CTCATTTCTCTCCTGGGCAGT-3'	52	46

All the primers (see Table 2.2) used in this thesis were synthesised by MWG BIOTECH (MWG Germany). 300-600 ng cDNA samples and 0.2-0.6 μM final concentration of each primer were added into PCR Master Mix (Abgene, UK).

The reaction mixture was placed in the thermal cycler and cycled as below:

Procedure	Incubation	Number of cycle
Initial denaturation	94°C, 2min	1 cycle
Denaturation	94°C, 20 sec	35 cycle
Annealing	50, 52, 54 °C, 30 sec	
Extension	72°C, 1min	
Final extension	72°C, 7 min	1 cycle

2.2.5.4 Agarose gel electrophoresis

2.2.5.4.1 Preparation of agarose gel

For making 1.2% agarose gel, 600 mg agarose was dissolved in 50 ml 1x TBE buffer (see appendix II) by heating in a microwave until the agarose had dissolved. The gel solution was left to cool to 55°C. Then 2.5μl (10 mg/ml) of ethidium bromide was added with mixing. The gel solution was carefully poured into a sealed gel holder, removing any air bubbles. The gel comb was then inserted into the gel and the gel was left to set for 30 minutes. The tapes were then removed from the gel holder and it was gently lowered into the electrophoresis chamber. TBE buffer was poured into the

electrophoresis chamber until it covered the surface of the gel and the comb was then removed.

2.2.5.4.2 Loading the samples and running the gel

2 µl of marker (1 Kb Plus DNA Ladder™, Invitrogen) was loaded into the gel. Equal volumes (5 µl-10 µl) of each PCR reaction were loaded onto the gel. The gel was run at 100 V until the dye front had travelled at least half way down. The gel was then viewed on a transilluminator under UV light. Positive bands were visualised and recorded electronically.

2.2.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

2.2.6.1. Principle

Protein electrophoresis is the method by which charged molecules will move in an electric field. Proteins will travel down a gel at various speeds, depending on their individual size, charge, solubility, and binding affinity. A common gel for separation of a protein mixture is the polyacrylamide gel, because these gels are chemically inert and readily formed by the polymerisation of acrylamide. Changing the concentration of acrylamide can vary the pore size of the gel. In sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), the protein mixture is denatured by heating to 100°C in the presence of excess SDS and a thiol reagent is employed to break disulfide bonds. SDS binds to the proteins and gives this complex a net negative

charge that is proportional to the mass of the protein. Thus the proteins can be separated on the basis of their size only.

Western blotting is an immunoassay technique that can detect very small quantities of a protein in a cell or body fluid. A mixture of protein is separated electrophoretically by SDS-PAGE, and the individual protein bands are transferred to nitrocellulose paper. A specific antibody is then used to probe for specific protein bands. The antibody to the protein bind (s) and the unbound antibody are washed away. The bound antibody is then detected by the addition of a second antibody against the immunoglobulin species that the first antibody was raised in. Conjugation of the secondary antibody to an enzyme allows the specific protein band to be visualised on addition of a substrate.

2.2.6.2 Sample preparation for SDS-PAGE and Western Blotting

The BCA protein assay kit (PIERCE, UK) was used to assay the total protein of each sample. The kit was composed of BCA protein assay reagent A, B and albumin standard. Samples of human corneal cells derived from different donors were prepared as described below:

The following steps should be performed on ice to protect protein activity. Culture medium was aspirated from cells in a confluent. 6-well cell culture plate then rinsed with PBS at room temperature for 10 minutes. Then PBS was removed and the cells were lysed by addition of 1 ml RIPA buffer (see Appendix II) to each well and shaking at 4°C for 30 minutes. A scraper was then used to detach any remaining

cellular material. The cell lysates were transferred to Eppendorf tubes and centrifuged at 12000g for 15 minutes at 4°C. The supernatant was transferred to a new microfuge tube and mixed with electrophoresis sample buffer (see appendix II, 1:2 v/v), boiled for 5 minutes to denature the protein and stored in a –20°C freezer. Samples, with equal protein concentrations, were loaded onto an 8% SDS-PAGE (see below).

2.2.6.3 Preparation of molecular weight markers

A wide range (7 – 395 kDa) molecular weight standard mixture (Sigma) was used in these studies. 1.5ml of 1x SDS-PAGE sample buffer (see Appendix II) was added into the lyophilised wide range marker, mixed by inversion, and vortexed five seconds to dissolve completely. The mixture was boiled in a water bath for one minute and stored at –20°C.

2.2.6.4 SDS-PAGE

The casting plates were swabbed with tissue paper soaked in 100% ethanol and allowed to air dry. The spacers and the two glass plates were assembled in the clamp. The clamp was tightened with the glass plates and spacers aligned on the casting stand. The sandwich was snapped onto the casting stand to seal the bottom of the assembly. Some 100% ethanol was applied to ensure it was water proof. The glass plate was marked with a marker pen to indicate the desired upper limit of the resolving gel. The 8% resolving gel solution (see Appendix II) was poured in the glass plate sandwich using a plastic pipette up to the marker line. The gel mixture was overlaid by 100% ethanol to exclude oxygen from the surface. After a clear line formed between the

resolving gel and the ethanol to indicate gel polymerization, ethanol was drained using tissue. 5% stacking gel (see Appendix II) mixture was prepared to overlay resolving gel. The comb was inserted. It is important that there were no air bubbles trapped beneath the comb.

After polymerisation of stack gel, the gel was transferred to the electrophoresis apparatus. 5µl molecular weight marker was loaded into left well and 15 µl of different samples were loaded into each of other wells. The electrophoresis apparatus was connected to the power pack. Gel was run at a constant current of 45 milliAmps per gel until the dye bands reached the gel bottom. This usually took 60-90 minutes.

2.2.6.5 Western Blotting

Immunoblotting was carried out essentially as described by Towbin et al. 1979 (Towbin et al., 1979). After electrophoresis, the proteins were transferred to nitrocellulose membrane in transfer buffer (see Appendix II) using a blotting apparatus (Biometra, Germany), set at 0.05 V, 0.65mA/cm^2 of gel for 1 hour 45minutes.

The nitrocellulose membranes were then blocked in 10% milk in TBS (Tris-buffered saline, see Appendix II) at 4°C, overnight. The next day, the membranes were incubated with the appropriate primary antibodies: Notch1, goat anti-human; Notch2, goat anti-human; AE5, mouse anti-human and Ki67, rabbit anti-human (See Table 2.3) diluted in TBS-T (0.05% Tween-20 in TBS) containing 3% milk for 2 hours at room

temperature. Then the membranes were washed at least 5 times with 5ml wash solution (TBS-T containing 3% milk), 5 minutes for each wash.

Table 2.3 the antibodies used in Western Blotting

Primary antibody	Dilution	Company
Notch 1	1/100	Santa Cruz
Notch 2	1/100	Santa Cruz
AE5	1/100	ICN-UK
Ki67	1/50	DAKO-Denmark

Second antibody	Dilution	Company
anti-goat HRP	1/2000	Santa Cruz
anti-rabbit HRP	1/2000	Santa Cruz
anti-mouse HRP	1/2000	Santa Cruz

After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-goat, anti-rabbit and anti-mouse. see Table 2.3) diluted in the same solution as primary antibodies for 1 hour at room temperature and washed 3 times for a minimum of 5 minutes with TBS-T and twice with TBS.

2.2.6.6 Detection of antibody-antigen complexes by enhanced chemiluminescence (ECL)

After the membranes were washed, equal volumes of reagent A (5ml) and reagent B (5ml) were mixed in a convenient sized container. Then the membranes were removed from TBS solutions and immersed in the ECL reagent mixture (Santa Cruz

Biotechnology) for 1 minute. After excess ECL solution was drained off from the membrane, the membrane was covered in Saran Wrap and place into an autoradiographic film cassette. In a darkroom, a piece of autoradiographic film (Kodak XOMAT-AR) was placed on top of the membrane. After an appropriate exposure time (1-2 minutes), the film was immersed into developer as soon as an image appeared, the film was rinsed in water and soaked in fixer for 5 minutes, rinsed again and left to air dry.

After scanning densitometry of the band using a scanner (EPSON expression 1680), and image capture (EPSON expression 1680), the relative band intensities were quantified by measuring same size of positive bands using Scion Image software. The data were recorded, the relative concentrations of target protein were analysed by PRISM software.

2.2.7. Immunofluorescent labelling of cells

2.2.7.1. Immunofluorescent labelling of cells for Notch receptors and their ligands

The standard method is same as in section 2.2.2.2. The coverslips containing cells were incubated in appropriate primary antibodies (see Table 2.4) and secondary antibodies (see Table 2.5).

Table 2.4 Primary antibodies used for immunolabelling

Primary antibody	Dilution in PBS	Company
Notch 1	1/100	Santa Cruz
Notch 2	1/100	Santa Cruz
Delta 1	1/100	Santa Cruz
Jagged1	1/100	Santa Cruz
AE5	1/100	ICN-UK
Ki67	1/50	DAKO-Denmark

Table 2.5 Secondary antibodies used for immunolabelling

Secondary antibody	Dilution	Abs(nm)	Em(nm)	Company
Alexa Fluor 488 donkey anti-goat IgG antibodies	1/1000	495	519	Molecular Probes
Alexa Fluor 488 donkey anti-rabbit IgG antibodies	1/1000	495	519	Molecular Probes
Alexa Fluor 488 donkey anti-mouse IgG antibodies	1/1000	495	519	Molecular Probes
Alexa Fluor 594 donkey anti-rabbit IgG antibodies	1/1000	578	603	Molecular Probes
Alexa Fluor 594 donkey anti-mouse IgG antibodies	1/1000	578	603	Molecular Probes
Alexa Fluor 594 donkey anti-goat IgG antibodies	1/1000	578	603	Molecular Probes

Abs. Approximate absorption. Em. Fluorescence emission maxima.

2.2.7.2. Notch receptors and ligands expression in relation to epithelial cell proliferation and differentiation

Ki67 is a nuclear protein that is expressed in proliferating cells and which is widely used in routine as a 'proliferation marker' to measure the growth fraction of cells (Schluter et al., 1993). AE5, cytokeratin 3 (CK3), has been regarded as a marker for 'corneal-type' differentiation (Cooper and Sun, 1986).

For double immunolabelling of Notch family with Ki67 or AE5, the standard method is also same as in section 2.2.2.2. The coverslips were incubated in appropriate primary antibodies (see Table 2.4) and secondary antibodies (see Table 2.5).

2.2.8. Quantification of positive immunolabelling in epithelial cells

After immunocytochemical staining, the cells were visualised and photographed by a inverted microscope (DMRAZ, Leica) in 5 areas from 3 coverslips (see Fig. 2.2). Positively stained cells were quantitated using Image Pro-Plus software. The rule of counting items on a picture was that all cells (including those touching the edges) should be counted. Total 15 areas (5 x 3 for each slide) were counted. The percentage of positive staining cells and the percentage of Hoechst-stained nuclei were recorded. The data were analysed by PRISM software.

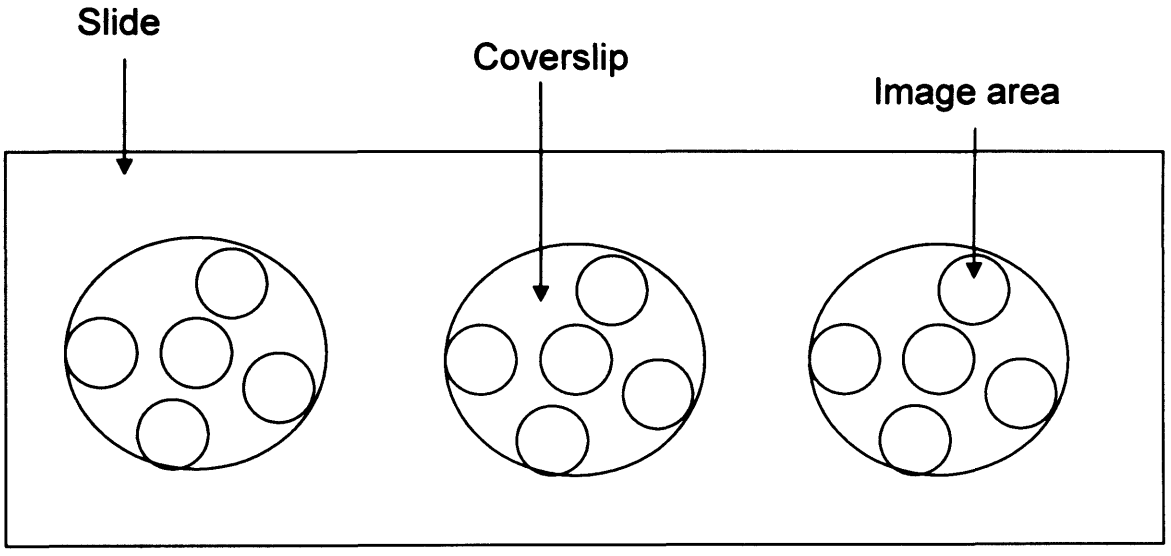


Fig. 2. 2. A cartoon showing the photographs from coverslips.

2.2.9. Histological Techniques

All human corneas were obtained from the Bristol Eye Bank and used in accordance with the tenets of the Declaration of Helsinki regarding the use and permission of human tissue for research.

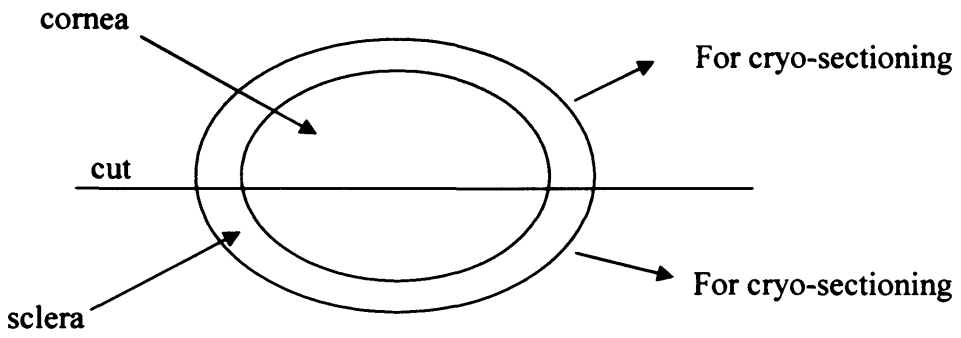


Fig. 2.3. A cartoon showing how the corneoscleral discs were bisected and prepared for cryo-sectioning.

The corneoscleral disc was excised from the rest of the globe using a surgical blade the lens and other sub-corneal tissue were then removed. Corneoscleral discs were bisected and prepared for cryo-sectioning as showed in Fig. 2.3.

2.2.9.1. Preparation of frozen sections

The corneal tissue was embedded cut-surface down in optimal cutting temperature (OCT) medium (Tissue Tek) and frozen in liquid nitrogen-cooled isopentane. The frozen tissue block was mounted on a chuck using OCT medium. Ice freeze was sprayed on to the block and chuck and they were left for 5 minutes. 8μm cryosections were cut using a microtome (Leica Microsystems CM3050s). The sections were

applied directly to Super Frost plus slides (BDH) and allowed to air-dry at room temperature for 2 hours, then wrapped in aluminium-foil and stored at –20°C.

2.2.9.2. Haematoxylin and Eosin staining

To examine corneal tissue morphology, sections were immersed in Harris' Haematoxylin (BDH, UK) for 5 minutes to stain all nuclei, washed in cold running tap water for 10 minutes, counter stained with Eosin for 2 minutes and then washed for 10 minutes in cold running tap water.

2.2.9.3. Immunolabelling techniques for frozen sections

Frozen sections were fixed in 100% acetone (BDH) for 10 minutes and allowed to dry for 10 minutes, then incubated for 20 minutes in 0.2% Triton X-100 (t-octylphenoxypolyethoxyethanol) in PBS to permeabilise cell membranes. Following this, sections were rinsed in 3 washes in PBS (10 minutes each). The sections were labelled and incubated in appropriate primary antibody (see Table 2.4) for 2 hours followed by three 10- minute washes with PBS. Substitution of the primary antibodies with same species serum (goat, mouse, rabbit) at the same concentration was used as a negative control. These sections were then incubated with the appropriate Alexa Fluor (Molecular Probes) secondary antibody (see Table 2.5) for 2 hours in the dark, and followed by three 10- minute washes with PBS.

The sections were mounted in Hydromount containing Bis-benzimide (10µl of 1mg/ml Bis-benzimide in 5ml Hydromount), and visualised under a DMRAZ microscope (Leica) equipped with a 100W mercury vapor lamp for illumination,

using filter with absorption 359nm and emission 461nm for bis-benzimide, filter with absorption 494nm and emission 518nm for Alexa Fluor 488, filter with absorption 570nm and emission 595nm for Alexa Fluor 594. Images were captured using Leica Qfluoro software and overlapped, positively stained cells were quantitated using Image Pro-Plus software. The rule of counting items on a picture was that all cells (including those touching the edges) should be counted. The percentage of positive staining cells and the percentage of Hoechst-stained nuclei were recorded. The data were analysed by PRISM software.

2.2.10. Corneal epithelial cell stratification

2.2.10.1. Preparation of Amniotic Membrane (AM)

In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human amniotic membranes were obtained at the time of routine Cesarean section (Queen's Medical Centre, University Hospital, Nottingham, UK). Under sterile conditions the membranes were washed with sterile phosphate-buffered saline (PBS) containing antibiotics and separated from the chorion into 5x5 cm squares and stored at -80°C in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) and glycerol (Sigma) at a ratio of 1:1 (vol/vol). Immediately before use, the amniotic membranes were thawed, washed three times with sterile PBS, then deprived of their amniotic epithelial cells by incubation with 0.5% EDTA (BDH) at 37°C for 3-6 hours to loosen the cellular adhesion, followed by gentle scraping with a cell scraper (Nalge Nunc International, Naperville, IL). With the scraped side uppermost, a horizontal cut was made in the top right corner to aid further orientation. Preliminary evaluation of

haematoxylin- stained as described in section 2.2.9.2, ethanol-fixed tissues confirmed that this protocol effectively removed epithelial cells from the amniotic membrane. Denuded AM was refrozen in 1:1 DMEM/Glycerol until required.

2.2.10.2. Mitomycin C treatment of 3T3 fibroblasts

Passage 149 3T3 fibroblasts (3T3 is code of a fibroblast cell line derived from mouse) were removed from the -80 °C freezer, thawed rapidly and the cell suspension was added to 10ml medium (DMEM + 10% FCS + 1% penicillin and 1% streptomycin). The cells were centrifuged, and the pellet was resuspended in fresh media (same as above) and seeded into 75 cm^2 flasks with a seeding density of 3.0×10^4 cells/cm^2. When the cells were sub-confluent (ie. 90% confluence of flask), the medium was removed and 4ml of 4μg/ml mitomycin C (MMC) in PBS (MMC aliquots stored at 200 μg/ml) was added. Then the cells were incubated for 2 hours at 37°C until cells lost their spindle shape and became round. After washing in PBS twice and addition of 0.05% Trypsin- EDTA for 2 minutes, followed by addition of 4 ml media and centrifugation at 250g for 5 minutes, the cells were resuspended in 6 ml media. 1ml of media was placed into each well of a 6-well plate with a density of 3.0×10^4 cells/cm^2. A further 1 ml of media was added to each well, and cells were incubated overnight before use.

2.2.10.3. Primary culture of corneal epithelial cells on AM

Denuded AM were thawed and rinsed in PBS. Orientation was achieved by a horizontal cut in the top right corner so that epithelial side was uppermost. The AM

was picked up by the top corner and placed onto a dry petri dish to remove as much liquid as possible. It was then spreaded out and placed onto a polycarbonate membrane culture insert (0.4 μm, Corning Incorporated), in a 6-well plate containing treated 3T3 fibroblasts and allowed to adhere for 2 minutes. 2 ml DMEM was added to the lower chamber and incubated overnight.

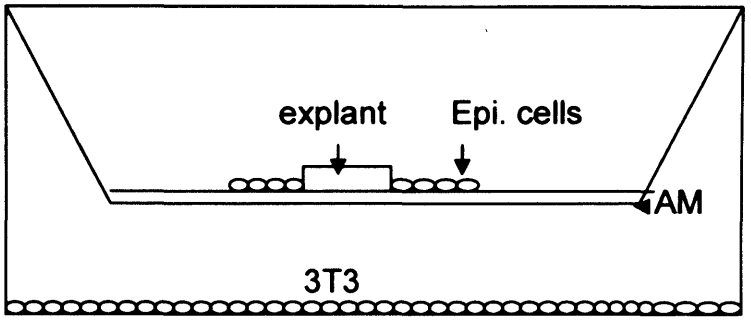


Fig. 2.4 Schematic diagram of corneal epithelial cell stratification model. Explant, human corneal explant; Epi. Cells, corneal epithelial cells; AM, amniotic membrane; 3T3, fibroblast cell line from mouse.

A human donor corneoscleral rim was cut into 6 pieces. The endothelium and approximately half the stromal depth was dissected from each segment, taking care not to disturb the limbal epithelia. Each piece (dabbed dry on tissue) was placed epithelial side down onto a prepared culture insert containing AM, with the limbal side pointing towards the centre. Each explant was allowed to adhere for 2 minutes, then 1.8 ml media was added to the lower chamber of the well. The plate was placed carefully into an incubator at 37°C. After 2 hours, the explant was examined to ensure it remained moist, then incubated for 1 or 2 days. The migration of epithelial cells was observed and the media was replaced every 2 days with 1.8 ml SHEM media (500ml

DMEM/F-12, 50ml FCS, 5ml penicillin, 250µl insulin, 50 µl cholera toxin, 5 µl EGF) (Fig. 2.4).

2.2.10.4. Setting up a epithelial stratification model

After 3 weeks, when the epithelial cells were confluent, they were air-lifted by addition of 1.6-1.8 ml media to lower chamber, thereby leaving epithelial cell surface exposed to air. SHEM media was changed every 2 days. After 2-4 days, the epithelial cells appeared stratified with a 'cobblestone' appearance.

Epithelial stratification in the air-lift model was maintained at different time points: 1 week, 2 weeks, 3 weeks (in submerged state) and 4 weeks (3 weeks submerged, followed by 1 week air-lift) were set up.

2.2.10.5. The effect of inhibition of Notch signalling in corneal epithelial stratification

2.2.10.5.1. The preparation of media

1mg γ-secretase inhibitor (Sigma) was dissolved in 56µl dimethyl sulfoxide (DMSO) to form a 25mM stock solution and stored at -20°C. 2µl of the inhibitor stock were added to 1 ml SHEM to form a final concentration of 50µM. Medium containing 0.2 % DMSO was used as a negative control.

2.2.10.5.2. Treatment with a γ-secretase inhibitor

When epithelial cell growth was observed (after 7 days), the SHEM was removed from wells containing the AM + corneal plant and SHEM with 0.2% FCS was added for 4 hours. Then the wells were rinsed with PBS twice, and SHEM containing either 0 or 50 μM γ-secretase inhibitor was added for 1 week, 2 weeks and 3 weeks (in submerged state) followed by 1 week air-lifted. Frozen blocks were taken at different time points as described above.

2.2.10.6. The effect of the activation of Notch signalling in the corneal epithelial stratification

2.2.10.6.1. The preparation of media

100 μg Recombinant Jagged1 (rJadded1, R&D SYSTEMS, 599-JG) was dissolved in 0.1ml PBS to prepare a working stock solution of 1mg/ml and stored at -20°C. For the experiment, 10 μg/ml of rJagged1 in SHEM was made up. The SHEM containing the same amount of PBS (1%) as used as a control.

2.2.10.6.2. The treatment of rJagged1

When the epithelial cell growth was established (after 7 days), the SHEM was removed from wells containing AM + corneal explant and SHEM containing 0.2%

FCS was added at 37°C for 4 hours. Then the wells were rinsed with PBS twice, the SHEM containing either 0 or 10 μg/ml rJagged1 was added at 37 °C for 1 week, 2 weeks and 3 weeks (in submerged state) followed by 1 week air-lifted.

2.2.10.7. Obtaining frozen sections from AM + corneal explant

The epithelial cells + AM were punched carefully with 6mm Biopsy trephine (Medisave, AWBP-60F) at different time points (week 1, week 2, 3 and week 4). The biopsies were frozen in isopentane cooled by liquid nitrogen in preparation for cryosectioning as described in section 2.2.9.1.

2.2.11. Notch signalling in corneal organ culture and wound healing

2.2.11.1. Choosing a species

All protocols in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with permission for research.

Due to difficulties in obtaining sufficient number of human corneas to carry out this study, species were chosen based on the following criteria:

- Availability and cost of tissue,
- Ease of performing the organ culture technique,
- Viability in organ culture. Morphological assessment of the corneal structure comparing 0 and 24 hours visualised following haematoxylin and eosin staining of frozen sections (see sections 2.2.9.2),

- Cross-reactivity of antibodies with tissue.

To determine which species could be used for subsequent experiments, corneal frozen sections were probed with antibodies against Notch 1, Notch 2, Delta 1, Jagged 1, Ki67 and cytokeratin 3 as described previously in sections 2.2.9.3. Results confirmed that the rat cornea was the most suitable species to use in a study to assess corneal organ culture model.

2.2.11.2. Setting up corneal organ culture and wound healing models

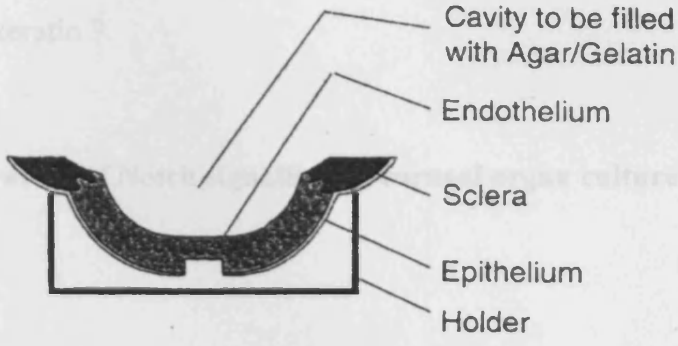
Adult rat eyes (Norwegian Brown, 6-8 weeks of age) were provided friendly (within 4 hours of slaughter and cleaned). The eyes were cleaned with four rinses in sterile 0.9% (w/v) sodium chloride (NaCl), followed by a 3 minute immersion in 5% polyvinylpyrrolidone iodine, with a 5 minute neutralisation in 2% sodium thiosulphate and finally rinsed in 0.9% (w/v) NaCl. The corneoscleral disc was excised from the rest of the globe using a surgical blade the lens and other sub-corneal tissue were then removed. One of two types of wound were created in the centre of each cornea: (a) a superficial scrape wound, in which the epithelium was removed from Bowman's membrane using a scalpel blade after light demarcation with a 3 mm trephine, and (b) an excisional trephine wound in which the epithelium and superficial stroma were excised using a scalpel blade after punching to a depth of approximately one-third of the thickness of the rat cornea (approximately 40μm epithelial plus 40μm stromal ablation) with a 3 mm trephine. Corneo-scleral rims, with approximately 5 mm of the limbal conjunctiva present, were then excised and rinsed in PBS.

All corneas (unwounded, epithelial and stromal wounded) were placed epithelial-side down into a sterile holder containing tissue culture medium (DMEM, Gibco) to prevent drying of the epithelium (Fig. 2.5a). Holder with suitable diameter was used to ensure that scleral rims would rest on the cup edges without damage to the corneal epithelium. The endothelial corneal concavity was then filled with serum-free DMEM containing 1% agar and 1% gelatin (Appendix I) at 37°C. This mixture was allowed to set for 1 minute. This mixture had been previously heated in a microwave oven (Sanyo) on a high setting for 10 minutes to melt the gelatin/agar matrix, and allowed to cool in a water bath at 45°C before placing in corneal cavity.

Corneas were then inverted and transferred to a 6-well plate and cultured at 37°C in a humidified 5% CO_2 incubator (Fig. 2.5b). Serum-free Trowell's T8 medium containing antibiotics, fungizone and glutamine (Appendix II) was added to each well. To moisten the epithelium, 100 µl of T8 medium was added dropwise onto the surface of the corneal epithelium daily. Medium levels were maintained to a level just below the limbal region (see Fig. 2.5b).

Sterile conditions were maintained throughout. The wounded corneas in triplicate were cultured for varying periods of time (up to 2 weeks). After healing intervals of 2, 4, 8, 16, 24, 48, 72 hours (epithelial wound), and 4, 8, 16, 24, 72 hours, 7 days, 14 days (stromal wound) the rat corneas snap were frozen and processed for cryosections (see section 2.2.9.1) and immunolocalisation of Notch family members, Ki67 and cytokeratin 3 (section 2.2.9.3).

a.



b.

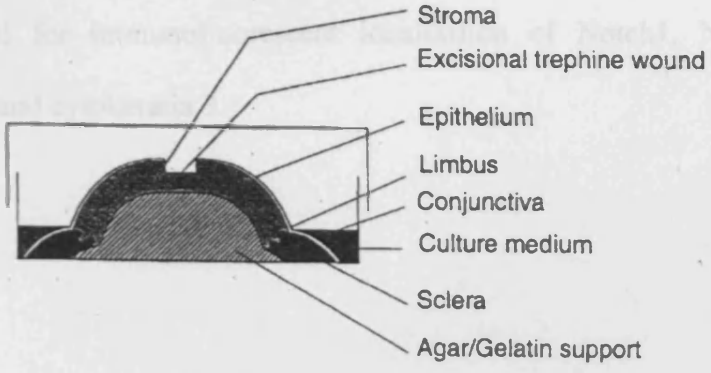


Fig. 2.5. Diagram representing organ culture model used in this study depicting an excisional trephine wound. (a). shows the stage after isolation of the corneo-scleral tissue; inversion of the tissue to allow the addition of medium containing 1% gelatin and 1% agar. (b). Once set the tissue is inverted again, placed in a 6-well plate and serum-free medium added to the level of the limbus.

2.2.11.3. The effect of the inhibition of Notch signalling in corneal organ culture and wound healing

Above corneas were treated with T8 media containing either 0 or 50 μM γ-secretase inhibitor as section 2.2.10.5.2. The cryosections of organ culture and wounded corneas were processed for immunofluorescent staining of Notch family members, Ki67 and cytokeratin 3.

2.2.11.4. Activation of Notch signalling in corneal organ culture and wound healing

Aat corneas (see above) were treated with T8 media containing either 0 or 10 μg/ml Jagged1 as section 2.2.10.6.2. The cryosections of unwounded and wounded corneas were processed for immunofluorescent localisation of Notch1, Notch2, Delta1, Jagged1, Ki67 and cytokeratin 3.

CHAPTER THREE

THE NOTCH SIGNALLING PATHWAY IN CORNEAL EPITHELIAL HOMESTASIS

3.1. Introduction

The corneal epithelium is a non-keratinised stratified squamous epithelium composed of 5-6 layers and is subject to a constant process of cell renewal and regeneration. The corneal epithelium exists in a state of dynamic equilibrium, with the superficial cells being constantly shed into the tear film, with a turnover period of 4-6 days (Hanna et al., 1961). To accomplish its self renewal process, the corneal epithelium and the epithelia of other self renewing tissues rely on the presence of stem and transient amplifying cells, which are the only cells with proliferative potential (Lavker and Sun, 1983; Morrison et al., 1997). Clinical and experimental evidence points to the corneal epithelial stem cells being located at the corneoscleral limbus (Dua and Azuara-Blanco, 2000).

As reviewed by Boulton and Albon (2004), not only do stem cells ensure that the corneal epithelium undergoes continual self-renewal, they are also responsible for epithelial tissue repair and regeneration throughout the life of the adult cornea. These stem cells are undifferentiated, slow-cycling cells that self-renew and produce transient amplifying cells which migrate to the corneal epithelium (Kinoshita et al., 1981; Tseng, 1989).

The cornea-specific cytokeratin 3 (CK3) has been used as a marker for differentiating epithelial cells and confirmation of epithelial cell purity (Schermer et al., 1986). In this study, AE5, a highly specific antibody against the 64 kDa CK3, has been used as a marker of differentiating epithelial cells.

Ki67, a nuclear antigen that is present in proliferating cells (Joyce et al., 1996), is a widely used biological marker to assess cell proliferation. Several studies have demonstrated that peripheral epithelial cells have a higher proliferative potential than those from limbal and central cornea (Ebato et al., 1988; Lavker et al., 1991).

The Notch pathway controls cell-fate specifications in all multicellular animals examined to date, ranging from sea urchins and nematodes to humans (Artavanis-Tsakonas et al., 1999). In higher vertebrates, multiple Notch homologues have been identified, including Notch1-4 in rodents and humans (del Amo et al., 1993; Lardelli and Lendahl, 1993; Lardelli et al., 1996; Uyttendaele et al., 1996). Multiple ligands for the Notch receptors are also identified in mammals (Jagged1/Serrate1, Jagged2/Serrate2, Delta1, Delta3, and Delta4). Delta1, Jagged1, and Jagged2 have been characterized as ligands for Notch1, Notch2, and Notch3 receptors (Jarriault et al., 1995; Lindsell et al., 1995; Luo et al., 1997; Shimizu et al., 1999; Shimizu et al., 2000). The Notch receptor undergoes a ligand-dependent extracellular cleavage that releases the extracellular domain leaving a membrane-tethered intracellular domain (Brou et al., 2000). The subsequent intramembrane cleavage by γ-secretase results in the translocation of the Notch intracellular domain to the nucleus where it influences the decision of cell fate (Greenwald, 1998; De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999; Mumm et al., 2000). It is generally believed that Jagged and Delta act as transmembrane proteins that interact with Notch receptors expressed on adjacent cells (Artavanis-Tsakonas et al., 1999).

A large number of both positive and negative modifiers of this pathway have been identified, and the eventual Notch pathway activity is dependent on the integration of

these multiple signals (Artavanis-Tsakonas et al., 1999). More recently, site-directed mutagenesis (Wolfe et al., 1999) and biochemical (Esler et al., 2000; Li et al., 2000) evidence has suggested that Presenilin (PS) is the catalytic component of γ–secretase. Presenilin, a ~46 kDa protein, is encoded by two highly homologous genes, PS1 and PS2, both contributing independently to γ–secretase activity. γ-Secretase inhibitor (S2188, Sigma) has been used in this thesis to block Notch signalling pathway by inhibition of PS1 activation, thus allowing the function of Notch signalling to be investigated.

Maintenance of corneal structure is crucial for its physiologic functions as a biodefence system and a refractive tissue (Klyce, 1972). Corneal homeostasis is dependent on the constant regulation of the epithelium via slow cycling stem cells at the limbus and centripetally directed transient amplifying cells (Kruse and Volcker, 1997). Cell proliferation, differentiation and stratification are considered to be essential in this process. However, the precise mechanisms which regulate these processes have not been fully elucidated, although cell-cell interaction is considered to be an important component.

Aim of this chapter is to investigate (1) expression of Notch family members in the cornea; (2) the role of the Notch signalling pathway in human corneal epithelial homeostasis.

3.2. Project design

A total of 6 human donor corneas (within 24 hours of cadaver time) and 40 donor corneoscleral rims were obtained from the Bristol Eye Bank and used in this study.

The mean age of donors was 62 ± 19 years. The use of human tissues was in accordance with the tenets of the Declaration of Helsinki and permission had been given for research.

3.2.1. Immunofluorescent localisation for Notch family members, Ki67 and CK3 from human cornea

6 human donor corneas were used for immunofluorescent staining. Images from corneal limbus, periphery and centre were captured using Leica Qfluoro software. The standard methods were the same as section 2.2.9.3.

3.2.2. Isolation and culture of human corneal epithelial cells and keratocytes

40 donor corneoscleral rims were used for the culture of corneal epithelial cells and stromal keratocytes. The standard methods were the same as described in section 2.2.1.1 and 2.2.1.2. Cells were used between passage 1-3 for all experiments.

3.2.3. RT-PCR and Western Blotting analysis of Notch receptors and their ligands in human corneal cells

When corneal cells were confluent, they were harvested and subject to RT-PCR and Western blot analysis as described in section 2.2.5 -2.2.6.

3.2.4. The effect of γ-secretase inhibition and Jagged1 activation on the Notch pathway and corneal epithelial cell homeostasis

3.2.4.1. Western Blotting analysis

A γ-secretase inhibitor (Appendix I) and Jagged1 (Appendix I) were used to evaluate the function of Notch signalling in the regulation of epithelial cell differentiation and proliferation. After reaching sub-confluence, epithelial cells were incubated in serum-free medium for one hour, then subjected to different treatments with γ-secretase inhibitor or Jagged1 (see section 2.2.10.5 and 2.2.10.6). The expressions of Notch1, Notch2, Ki67 and CK3 were semi-quantified following western blotting (see section 2.2.6). Concentrations of γ-secretase inhibitor (25μM and 50μM) and Jagged1 (5μg/ml and 10μg/ml) were chosen due to their previously reported respective inhibitory and stimulatory effect on the Notch pathway (Lindsell et al., 1995; Wolfe, 2001). Following scanning densitometry (scanner EPSON expression 1680) of immunopositive blots, Scion Image software was used to semi - quantitatively analyse changes in protein expression of Notch1, Notch2, Ki67 and CK3.

3.2.4.2. Immunofluorescent localisation of Notch receptors, Ki67 and CK3

Human corneal epithelial cells were cultured on coverslips, pre-coated with attachment factor, until sub-confluence was reached. Then the cells were treated with either γ-secretase inhibitor or recombinant Jagged1, in triplicate wells as described above. Immunolabelling and cell counting for Notch1, Notch2, Ki67, CK3 was performed as described in sections 2.2.2.2 and 2.2.8. The cells were visualised under a

Leica (DMRAZ) microscope and images were captured and analysed using Image Pro-Plus software (Image Solutions, UK).

3.2.5. Statistical analysis

All experiments were repeated at least three times and each treatment replicated a minimum of three times with each experiment. The results were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA of Western Blotting and cell population. $P< 0.05$ was considered statistically significant.

3.3. Results

3.3.1. The expression of Notch family members in human cornea

To identify the spatial localisation of Notch receptors and their ligands in human corneas, immunolocalisation was undertaken. The results showed that Notch1 and Notch2 were localised to the borders of the wing and superficial cells, in central, peripheral and limbal regions of the corneal epithelium. There was some positive staining in corneal stromal keratocytes. Labelling was not detected in any region of the epithelial basal cell layer (Fig. 3.1a, b, c, d, e, f). Delta1 and Jagged1 were localised to all layers of the central, peripheral, limbal epithelium and stromal keratocytes (Fig. 3.1g, h, i, j, k, l). In negative controls, immunoreactivity was not detected (Fig. 3.1m, n).

3.3.2. The localisation of corneal proliferation and differentiation

Ki67, a marker of actively cycling cells, was used to demonstrate the relative proliferative activity of limbal and corneal epithelial cells of human corneas.

Immunostaining of frozen sections of human corneas with anti-Ki67 antibody demonstrated staining in the central, peripheral and limbal regions of corneal basal epithelium (Fig. 3.2a, b, c).

Using a monoclonal antibody, AE5, that is highly specific for the 64 kDa corneal cytokeratin- CK3, the results showed that CK3 was suprabasally located in central, peripheral, as well as, the limbal region of the corneal epithelium (Fig. 3.2e, f, g). Basal cells of central and peripheral corneal epithelium were found to be CK3 positive, suggesting that these corneal 'basal' cells are in a more advanced state of differentiation than those in the basal layer of the limbus where CK3 immunoreactivity was not detected (Fig. 3.2e, f, g).

In negative controls, immunoreactivity was not detected in control epithelial cells processed with the same species of serum (Fig. 3.2d: rabbit; h: mouse) in replace of primary antibodies.

3.3.3. The gene and protein expression of Notch receptors and their ligands in human corneal epithelial cell and keratocytes

RT-PCR identified expression of the genes for the Notch receptors: Notch1 and Notch2 and their ligands: Delta1 and Jagged1 in human corneal epithelial cell (Fig. 3.3a-c) and keratocytes (Fig. 3.3e-g). Gene expression of Notch3, Notch4, Delta3, Delta4 and Jagged2 were not detected (Fig. 3.3a, b, c, e, f, g). As shown in Figure 3.3d, h, gene expression of PS1 and PS2 was detected in both epithelial cells and keratocytes.

Protein expression was confirmed by Western Blotting as demonstrated in Figure 3.4. Both active and inactive isoforms of Notch1 and Notch2 were identified in human corneal epithelial cells (Fig. 3.4A) and keratocytes (Fig.3.4B). Molecular weights of protein bands were 120kDa: active Notch1 (Notch1-120,), 300kDa: full-length Notch1 (Notch1-300), 85kDa: active Notch2 (Notch2-85), 285kDa: full-length Notch2 (Notch2-285), 80kDa: Delta1 and 160kDa: Jagged1.

3.3.4. Inhibition of the Notch signalling pathway decreased epithelial proliferation and increased differentiation

Exposure of cultured corneal epithelial cells to a γ-secretase inhibitor, which prevents cleavage of the intracellular domain of the Notch receptor, resulted in decreased expression of active Notch isoforms: Notch1-120 (Fig. 3.5A: black bars) and Notch2-85 (Fig. 3.5B: black bars), increased expression of full length Notch1-300 (Fig. 3.5A: white bars) and Notch2-285 proteins (Fig. 3.5B: white bars). The alterations in protein expression appeared to be dose-dependent manner, such that the changes increased with concentration of γ-secretase inhibitor from 0 to 25μM to 50μM. These differences were statistically significant for 25μM ($p<0.05$) and 50μM ($p<0.05$) of γ-secretase inhibitor treatment. The γ-secretase inhibitor also resulted in a significantly decreased expression of Ki67 (Fig. 3.5C, $p<0.05$) and increased expression of the epithelial differentiation marker CK3 (Fig. 3.5D, $p<0.05$). All effects were greatest following 50μM of γ-secretase inhibitor treatment.

3.3.5. Activation of the Notch signalling pathway increased epithelial cell proliferation and decreased differentiation

Incubation of cultured corneal epithelial cells with recombinant Jagged1, one of the Notch ligands, resulted in increased expression of the active forms: Notch1-120 (Fig. 3.6A: black bars) and Notch2-85 (Fig. 3.6B: black bars) with a corresponding decrease in the expression of full-length receptors: Notch1-300 (Fig. 3.6A: white bars) and Notch2-285 (Fig. 3.6B: white bars). These changes in Notch expression were accompanied by a significant increase in Ki67 (Fig. 3.6C) and decrease in CK3 (Fig. 3.6D). All effects were dose-dependent and all differences in expression were statistically significant after addition of both 5μg/ml ($p<0.05$) and 10μg/ml ($p<0.05$) of Jagged1.

3.3.6. Immunolabelling of epithelial cells after Notch inhibition and activation

Consistent with Western Blotting results, the epithelial cell immunolocalisation data demonstrated that following γ-secretase inhibition, the number of cells showing positive Notch1 and Notch2 immunoreactivity was decreased in cell nuclei with a concurrent increase in cell membrane expression (Fig. 3.7g, j). This was accompanied by a decreased number of cells expressing Ki67 immunoreactivity (Fig. 3.7h, k) and an increased intensity of CK3 expression (Fig. 3.7i, l).

Further confirmation of the role of the Notch signalling pathway was demonstrated since following Jagged1 treatment, the activation of epithelial cells increased the expression of Notch1 (Fig. 3.7m) and Notch2 (Fig. 3.7p) positive immunoreactivity in

cell nuclei, with a decreased expression of Notch1 and Notch2 in cell membrane related-immunoreactivity. This corresponded with an increased nuclear Ki67 (Fig. 3.7n, q) and a reduced CK3 cytoplasmic immunoreactivity (Fig. 3.7o, r).

3.3.7. The percentage of labelled cells after inhibition and activation of the Notch signalling pathway

Cell count data quantified and demonstrated the significance of the above changes in both Notch1 and Notch2 nuclear and membrane-bound expression in corneal epithelial cells following γ-secretase inhibition or Jagged1–induced activation. These data were demonstrated in Table 3.1 and Figures 3.8A, B, 3.9A, B.

Media		Notch1		Notch2		Ki67	CK3
		Nuclear	Membrane	Nuclear	Membrane		
Control		41.3±3.1	29.9±3.1	25.8±3.1	13.0±3.1	46.1±3.5	63.0±6.8
γ-SI (μM)	25	24.4±2.1	45.8±2.1	20.5±2.5	24.5±2.5	31.0±5.6	79.0±5.9
	50	12.5±2.4	56.7±2.4	13.3±3.5	33.4±3.5	21.2±4.5	90.1±6.8
Jagged1 (μg/ml)	5	64.1±5.6	18.8±4.1	40.5±5.5	8.5±2.5	67.0±5.8	39.0±7.0
	10	82.6±5.1	6.7±4.4	63.8±6.5	3.7±3.5	81.5±7.1	18.1±6.5

Table 3.1. The mean percentage of Notch1, Notch2, Ki67 and CK3 immunocytochemistry staining after the inhibition or activation of Notch signalling from human corneal epithelial cells. Total cells are 980 ± 60. Data are represented means ± SEM from three independent experiments.

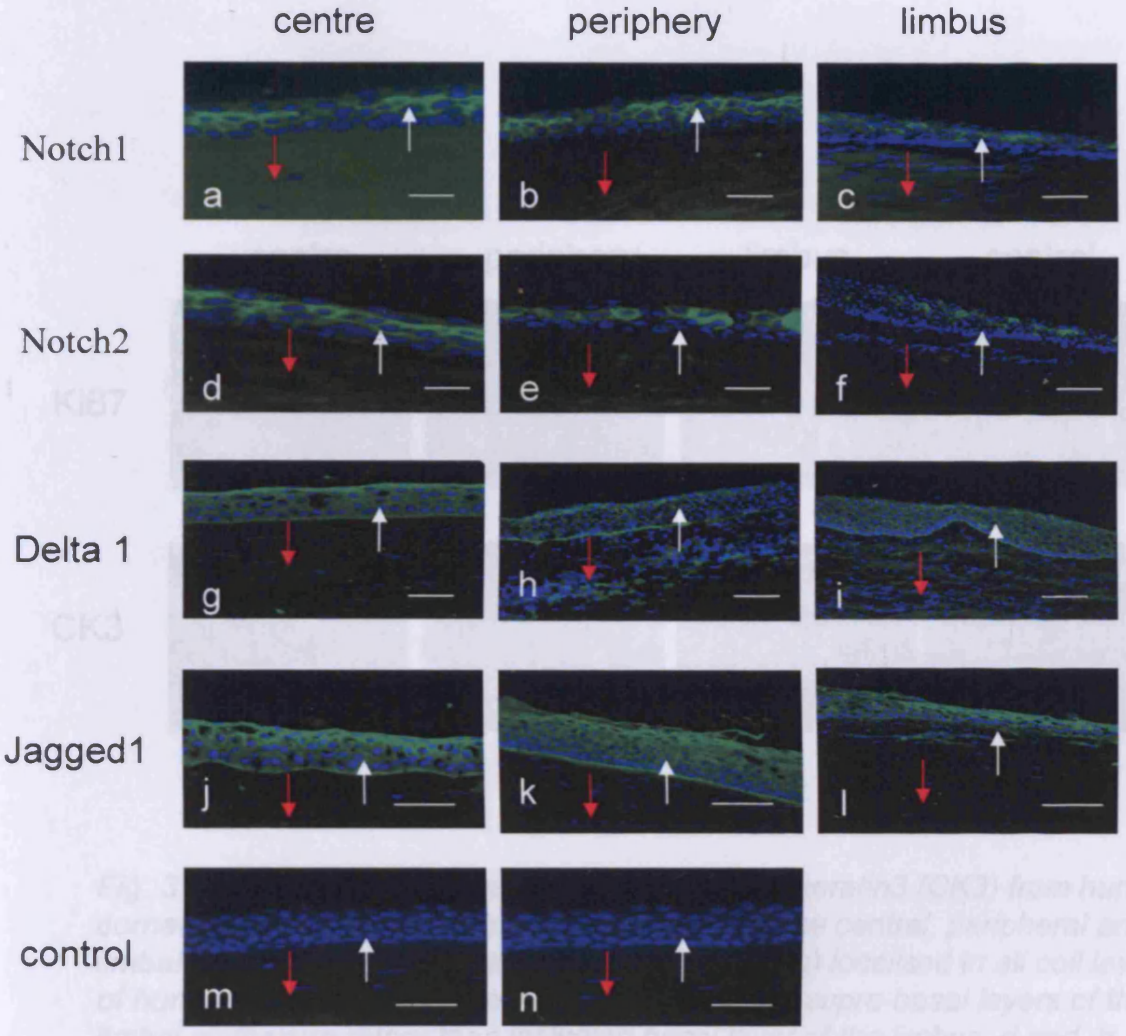


Fig. 3.1. The immunolocalisation of Notch family members from human cornea. Notch1(a-c) and Notch2 (d-f) were immunolocalised on all corneal epithelial suprabasal and superficial layers but not detected in the basal cell layer, in the all of the regions. Delta1 (g-i) and Jagged1 (i-l) appeared to express on all cell layers of the corneal epithelium. In negative control, immunoreactivity was not detected in corneal sections processed with same species and concentrations of serum (m: goat, n: rabbit) in replace of primary antibodies. Blue indicates Hoechst-labelled nuclei. White arrows indicate epithelial layers, red arrows indicate stromal layers. Bar=50μm

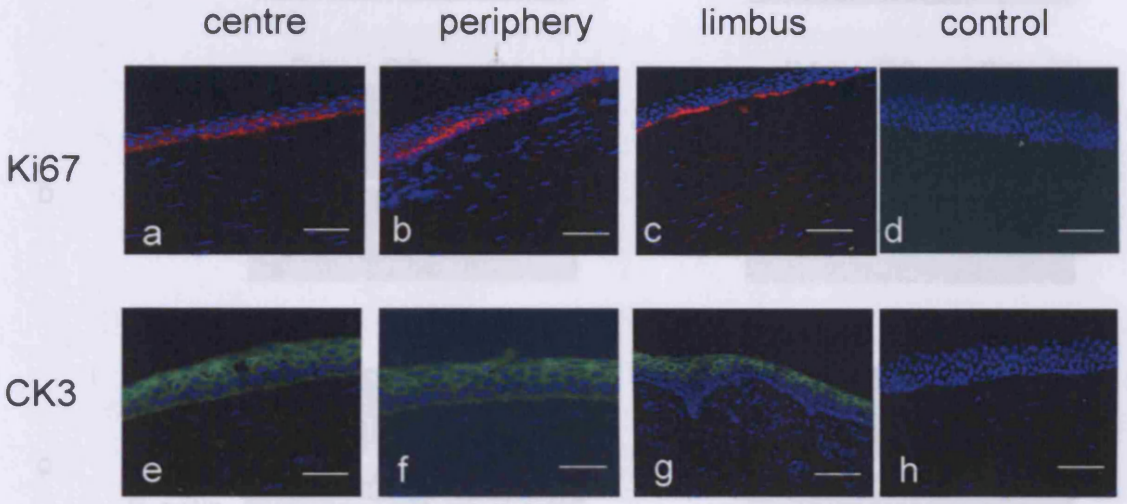


Fig. 3.2. The immunolocalisation of Ki67 and cytokeratin3 (CK3) from human cornea. Ki67 (a-c) was demonstrated staining in the central, peripheral and limbal region of corneal basal epithelium. CK3 (e-g) localised in all cell layers of human corneal epithelium, However only in the supra-basal layers of the limbal epithelium rather than including basal layer of the limbus. d and h are negative controls. Red is Ki67 positive staining, green is CK3 positive staining, blue indicates Hoechst-labelled nuclei. Bar=50μm

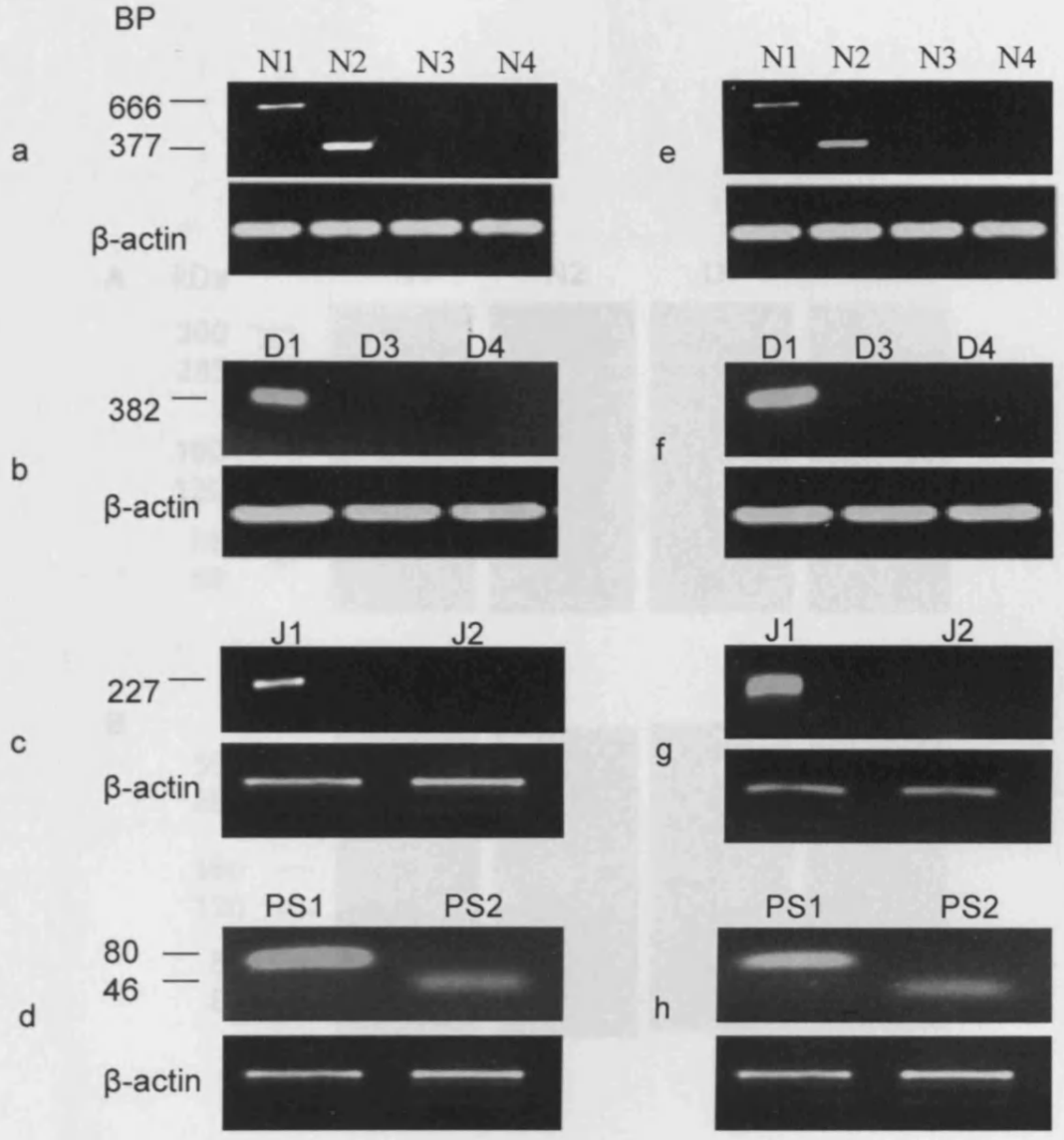


Fig. 3.3. Expression of Notch family members in cultured human corneal cells by RT-PCR. mRNA expression of Notch1, Notch2, Delta1, Jagged1, Presenilin1 (PS1) and Presenilin2 (PS2) was identified in human corneal epithelial cells (a-d) and keratocytes (e-h). In contrast, the mRNA expression of Notch3, Notch4, Delta3, Delta4 and Jagged2 was not found (a, b, c, e, f, g). β-actin was amplified as a positive control.

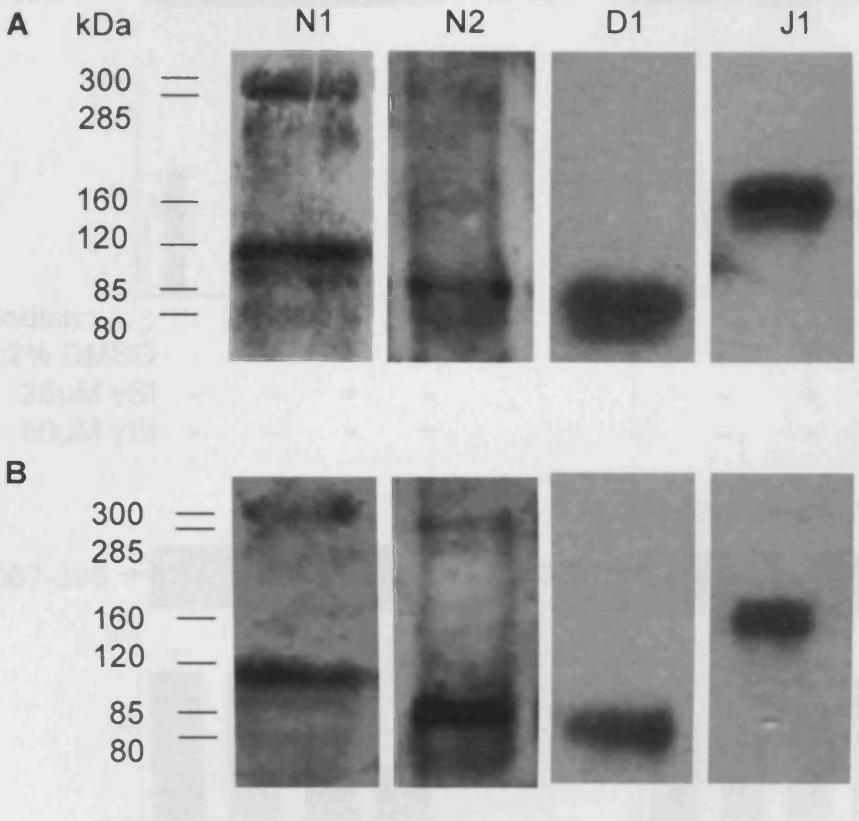


Fig. 3.4. Expression of Notch family members in cultured corneal cells by Western Blotting. A. Human corneal epithelial cells. B. Corneal stromal keratocytes. N1 (active Notch1-120 and full length Notch1-300), N2 (active Notch2-85 and full length Notch2-285), D1 (Delta1- 80), J1 (Jagged1-160).

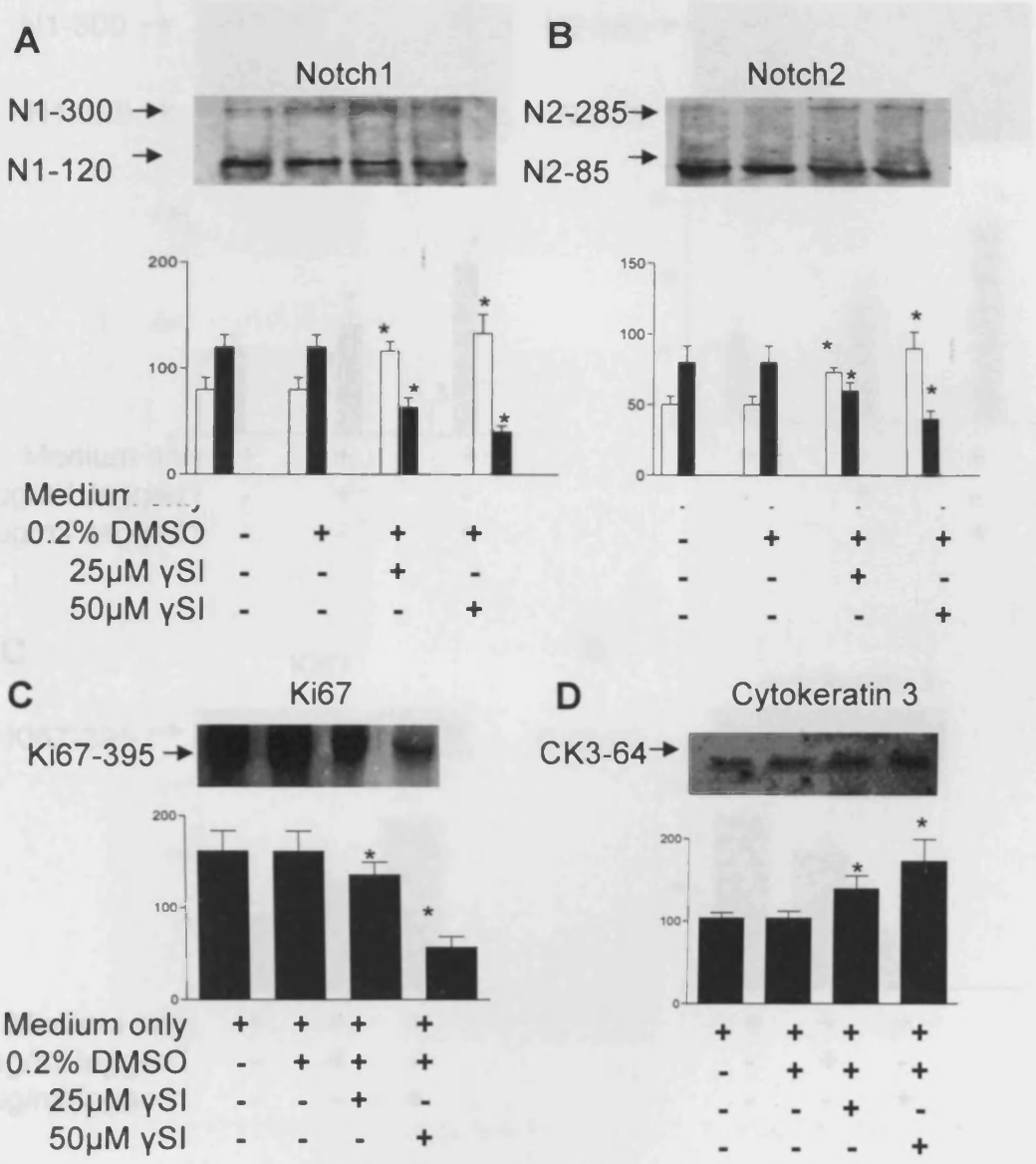


*Fig. 3.5. Western Blotting analysis of Notch receptors, Ki67 and CK3 after the treatment of γ–secretase inhibitor in cultured epithelial cells. A. Notch1 (full length N1-300: white bars, active N1-120: black bars); B. Notch2 (full length N2-285: white bars, active N2-85: black bars); C: Ki67; D: CK3. *p<0.05*

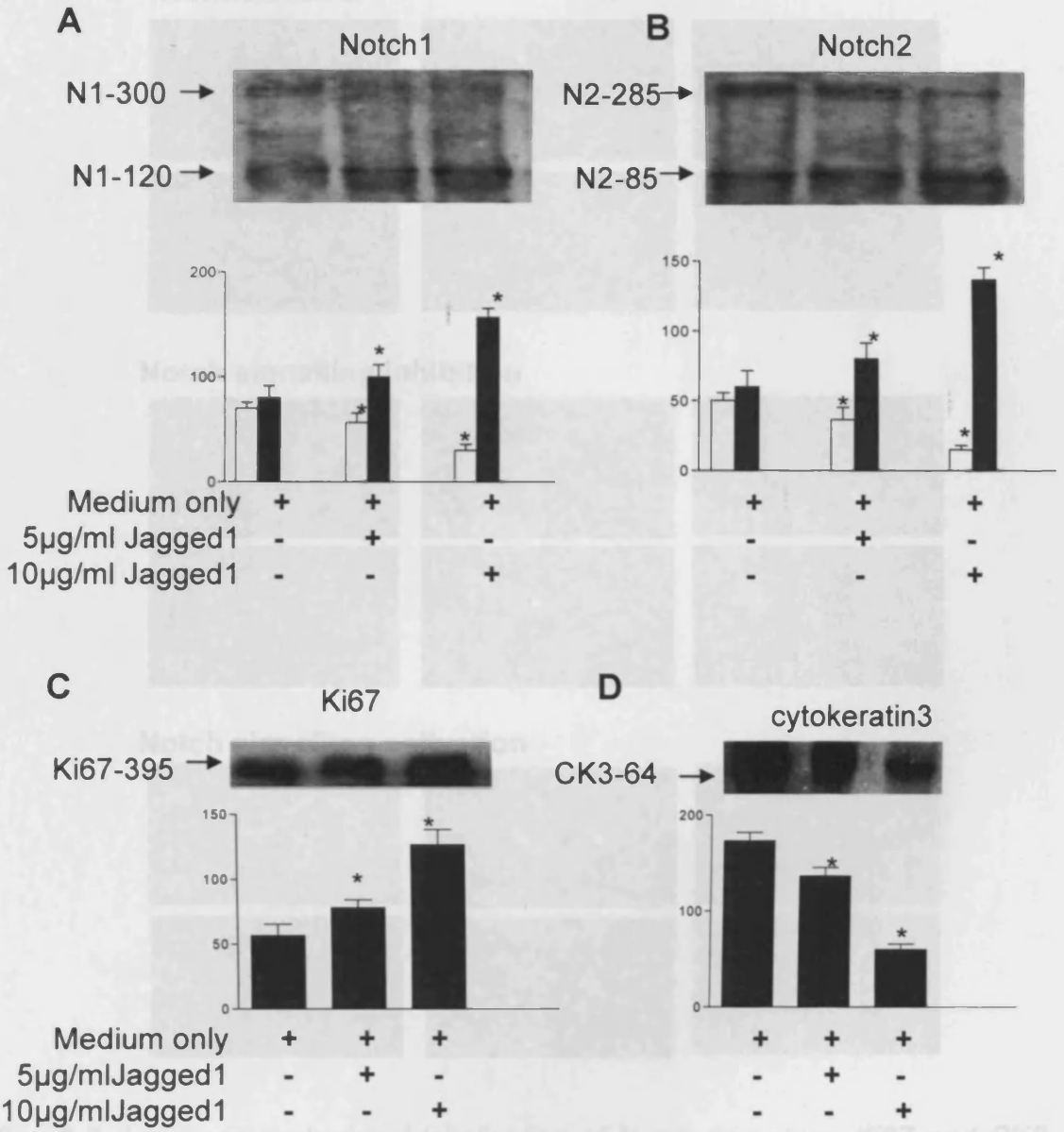


*Fig. 3.6. Western Blotting analysis of Notch receptors, Ki67 and CK3 after the treatment of Jagged1 in cultured epithelial cells. A. Notch1 (full length N1-300: white bars, active N1-120: black bars); B. Notch2 (full length N2-285: white bars, active N2-85: black bars); C: Ki67; D: CK3. * $p<0.05$*

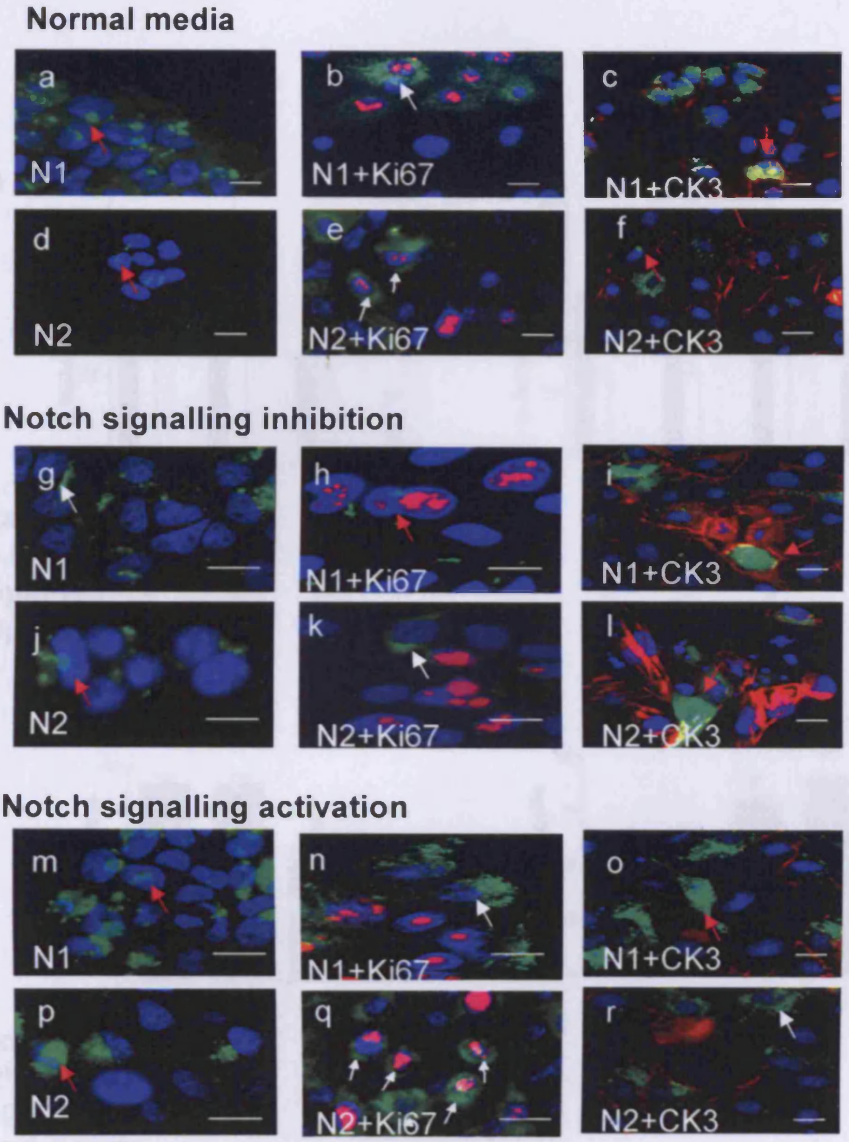


Fig. 3.7. Immunocytochemical localisation of Notch receptors, Ki67 and CK3 after the inhibition (γ–secretase inhibitor 50μM) or activation (Jagged1 10μg/ml) of Notch signalling in cultured epithelial cells. a-f: the expression of Notch1 and Notch2 with Ki67 or CK3 in normal media; g-l: the expression of Notch receptors with Ki67 or CK3 after signalling inhibition; m-r: the expression of Notch receptors with Ki67 or CK3 after signalling activation. Green is Notch1 and Notch2 positive, red is Ki67 and CK3 positive, blue is cell nuclear staining. White arrows indicate Notch1 or 2 positive staining in cell membrane, red arrows indicate Notch1 or 2 positive staining in cell nuclei. Bar=20μm

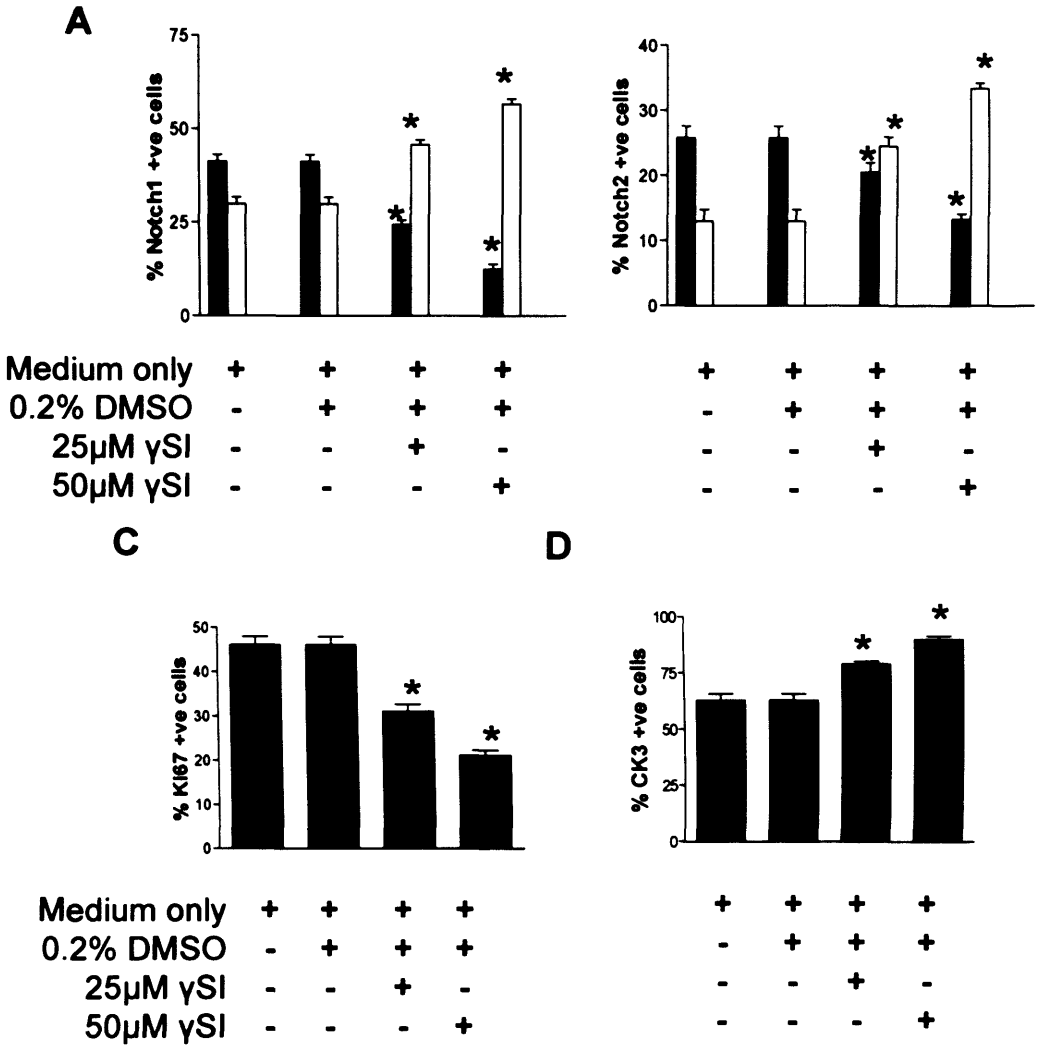


*Fig. 3.8. The percentage of labelled cells with Notch receptors, Ki67 and CK3 after the inhibition (γ-secretase inhibitor, γSI) of the Notch signalling in cultured epithelial cells. A. Notch1 (the expression of cell membrane: white bars, cell nuclei: black bars); B. Notch2 (cell membrane: white bars, cell nuclei: black bars); C: Ki67; D: CK3. *p<0.05*

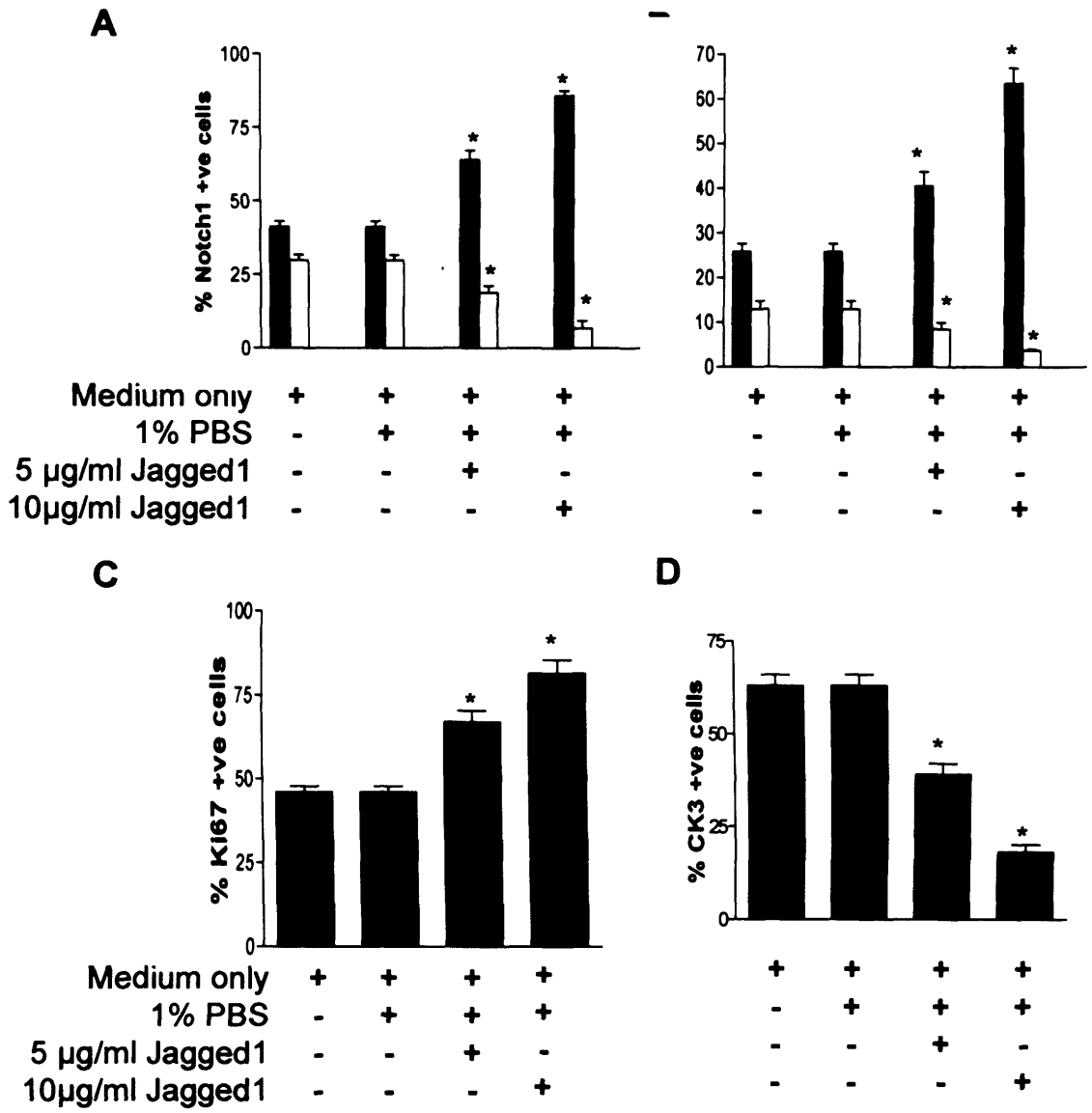


*Fig. 3.9. The percentage of labelled cells with Notch receptors, Ki67 and CK3 after the activation of the Notch signalling in cultured epithelial cells. A. Notch1 (the expression of cell membrane: white bars, cell nuclei: black bars); B. Notch2 (cell membrane: white bars, cell nuclei: black bars); C: Ki67; D: CK3. *p<0.05*

Corresponding changes in Ki67 and CK3 cell expression were also detailed (Fig. 3.8C, D, 3.9C, D). These differences were statistically significant after both γ-secretase inhibitor ($p<0.05$) and Jagged1 ($p<0.05$) treatment.

3.4. Discussion

Defining the cellular mechanisms responsible for the differentiation and self-renewal of corneal epithelial cells is a prerequisite to achieving a complete understanding of the process of corneal epithelial cell homeostasis. Despite various attempts aimed at defining the factors that regulate corneal epithelial cell homeostasis, the mechanisms involved in the determination of cell fate decisions during epithelial cell homeostasis remain ambiguous.

The cornea contains actively cycling cells (transient amplifying cells), slow-cycling stem cells (a subset of the limbal epithelial basal cells), terminally differentiated cells (limbal and corneal suprabasal cells), and quiescent cells (corneal keratocytes). These varying differential and proliferative states have been confirmed by the localisation of CK3 and Ki67 (Gerdes et al., 1984).

Our results showed that CK3 was localised to all cell layers of the human corneal epithelium, but only identified in the suprabasal cell layers of the limbal epithelium. These results are consistent with previous reports that the basal cell layer of the limbus contains a less-differentiated population of cells than that existing in the basal cell layer of the cornea , and that from basal layer to surface of the cornea, the epithelial cells become terminally differentiated (Schermer et al., 1986).

Immunostaining of human cornea with anti- Ki67 antibody demonstrated nuclear staining in the limbal, peripheral and central region of corneal basal epithelium. The number of labelled cells was in the order of peripheral > central > limbus, consistent with a previous report (Francesconi et al., 2000). In this study corneal epithelial proliferation *in vivo* and *vitro* was assessed using a monoclonal antibody to the Ki67 antigen (Delahunt et al., 1995). Ki67 is detected in all phases of the cell cycle in actively cycling cells, and is absent in noncycling cells (Gerdes et al., 1984). The cycling status of cells in the human cornea demonstrated Ki67 labelled proliferating cells in the limbal, central and peripherial region of the corneal basal epithelium. The corneal epithelium consistently had a higher percentage of labeled cells than did the limbal epithelium. This result indicated that Ki67 labelling can be used to assess proliferation during cell growth. Furthermore, double-labelling of Ki67 with other proteins of interest can provide valuable information regarding the conditions that stimulate cells to enter the cell cycle.

Notch signalling regulates cell fate in many different tissues in a wide range of organisms including the nervous system, vascular system, hematopoietic system, somites, muscle, skin and pancreas (Artavanis-Tsakonas et al., 1999; Song et al., 1999). The Notch signalling pathway plays different roles in different tissues, even within the same tissue at different developmental stages (Artavanis-Tsakonas et al., 1999). In most cases, Notch signalling blocks a primary differentiation fate in a cell and forces the cell to remain in an undifferentiated state (Artavanis-Tsakonas et al., 1999). However, recent evidence showed that Notch signalling also appears to promote differentiation in some circumstances (Fortini, 2001; Wolfe, 2001). In addition, apart from the well-documented involvement of Notch in differentiation,

both proliferation and apoptotic events can be affected by Notch signalling (Artavanis-Tsakonas et al., 1999).

In this study, the presence of Notch signalling protein components in the human corneal epithelium was demonstrated for the first time, suggesting that Notch may play an important role in the orchestration of human corneal homeostasis. Two Notch receptors (Notch1 and Notch2) and two Notch ligands (Delta1 and Jagged1) were identified in human corneal epithelial cells and keratocytes at both gene and protein level. This indicates that Notch signalling is likely to occur in human corneal epithelial cells and keratocytes where both ligand and receptor are expressed.

In the Notch downstream pathway, the remaining membrane-tethered Notch fragment is cleaved within its transmembrane domain by γ-secretase (De Strooper et al., 1999; Song et al., 1999; Struhl and Greenwald, 1999), leading to the release of the intracellular domain (NICD), which translocates into the nucleus to participate in the transcriptional activation of target genes (Jarriault et al., 1995; Fortini, 2001). Inhibition of γ-secretase will therefore prevent Notch signalling (De Strooper et al., 1999). Consistent with this, the results demonstrated that inhibition of the activation of Notch cleavage by γ-secretase, resulted in decreased expression of active Notch1 and Notch2 forms, accompanied by a significant reduction of cell proliferation and increased CK3 expression. These results have two implications: (a) that γ-secretase exists in human corneal epithelial cells and is involved in the mediation of Notch signalling and (b) that γ-secretase inhibition represses cell proliferation and promotes the differentiation of corneal epithelial cells, in a manner consistent with the downregulation of Notch signalling.

γ-secretase plays a role in other pathways, for example, γ-secretase mediates the intramembrane proteolysis of the amyloid precursor protein (APP) (Selkoe, 1999; De Strooper and Annaert, 2000; Steiner and Haass, 2000). Other putative γ-secretase substrates, recently identified, include the cell surface protein CD44, E-cadherin and the low density lipoprotein receptor– related protein (LRP) (Fortini, 2002). But there are no reports relative cornea have been found.

The presence of Delta1 and Jagged1 identified, in combination, with Notch1 and 2 in the human corneal epithelium (both at gene and protein level) suggests that Notch signalling is important in corneal epithelial cell homeostasis. Therefore in order to further elucidate the role of Notch in the regulation of corneal epithelial cell proliferation and differentiation, recombinant Jagged1, established as an effective activator of the Notch downstream pathway (Lindsell et al., 1995; Artavanis-Tsakonas et al., 1999) was used to challenge corneal epithelial cells. The results of this study confirmed a) the activation of Notch 1 and Notch2 by Jagged1 in corneal epithelial cells and b) that Jagged1 stimulation of Notch increased cell proliferation and decreased epithelial differentiation. Therefore a role for Notch signalling in the regulation of epithelial cell proliferation and differentiation in the human cornea was further supported.

A primary function of the Notch signalling pathway is to prevent differentiation via activating a transcriptional repressor (Artavanis-Tsakonas et al., 1999). However, our *in vitro* studies suggest that the proliferative event can also be affected by Notch signalling. A link between proliferation events and Notch has been reported

previously (Cagan and Ready, 1989; de Celis et al., 1998; Go et al., 1998), although the elements mediating the nonautonomous effect of Notch on cell proliferation are unknown. The proliferative effect of Notch signalling in different tissues may be the result of a synergistic effect between Notch and other proteins (e.g. cell growth factors) or other cell signalling pathway (Wnt signalling), as well as depending on the developmental context (Artavanis-Tsakonas et al., 1999).

Several proposals have also been raised with regard to the functional diversity among the Notch receptors, such that Notch2 functions as a negative regulator against Notch1 and Notch3 signals. Regarding the discrepancy, Shimizu et al. (2002) proposed that it may be due to a difference in the expression levels of other genes on this pathway, for example RBP-Jκ, a transcriptional factor bound with intracellular domain of Notch (Beatus et al., 1999; Beatus et al., 2001; Shimizu et al., 2002). In this study, the functions of Notch1 and Notch2 are clearly similar in corneal epithelial cells.

The balance between cell proliferation and differentiation is essential for maintaining epithelial homeostasis. Notch receptors and their ligands were shown to exist throughout the differentiated areas of the human corneal epithelium, in all regions of suprabasal and superficial layers, indicating a role for Notch in corneal epithelium differentiation. This is consistent with previous reports that Notch signalling regulates terminal differentiation (Frise et al., 1996; Capobianco et al., 1997; Bigas et al., 1998; Artavanis-Tsakonas et al., 1999; Gray et al., 1999). However, Notch protein expression was not identified in corneal and limbal basal cell layers, where proliferation of epithelial transient amplifying cells and stem cells occurs (Gerdes et

al., 1984). The mechanism for acquiring proliferative function of Notch may be reprogrammed or inhibited, and may be related to the specific location of basal cells and their contact with basement membrane. These localisation patterns suggested that the Notch pathway may involve a transitory stage where Notch is downregulated. The different proliferative effects of Notch *in vivo* and *vitro* reveal that the functional Notch signalling pathway may be affected by its interaction with other signalling pathways in the *in vivo* state (Artavanis-Tsakonas et al., 1999).

Taken together, Notch receptors and their ligands exist in epithelial cells of the human cornea. Notch receptors and their ligands appear to play a pivotal role in maintenance of corneal epithelial homeostasis via mediation of cell proliferation and differentiation.

CHAPTER FOUR

THE ROLE OF NOTCH IN HUMAN CORNEAL EPITHELIAL CELL STRATIFICATION

4.1. Introduction

The corneal epithelium is a non-keratinised, mucosal multilayer with a rapid self-renewing capacity. Renewal is essential to maintain a state of dynamic equilibrium with superficial cells constantly shed into the tears and replenished by proliferation, migration and differentiation of limbal epithelial cells. The complex multi-layered structure of the cornea allows it to fulfill its role, namely, transparency, refraction, photoprotection and protection of internal ocular structures from the external environment (Boulton and Albon, 2004). It is obviously important to establish a stratified epithelium for normal cornea function.

The amniotic membrane (AM), the innermost layer of the placental membrane, has been used as surgical material in a variety of fields (Dhall, 1984; Rennekampff et al., 1994). In ophthalmic applications, Kim and Tseng (1995) reported the transplantation of preserved human AM for corneal surface reconstruction in a rabbit model. These reports encouraged the use of preserved human AM for ocular surface reconstruction in patients with severe ocular surface diseases (Tsubota et al., 1996; Shimazaki et al., 1997; Tseng et al., 1998).

A variety of characteristics make AM ideally suited for use in ocular surface reconstruction. It has an anti-inflammatory effect (Kim et al., 2000; Solomon et al., 2001), antifibroblastic activity (Tseng et al., 1999), antimicrobial (Talmi et al., 1991), and antiangiogenic (Hao et al., 2000) properties, and very limited immunogenicity (Akle et al., 1981). In addition, it provides a healthy new substrate suitable for reepithelialisation by the corneal epithelium (Tsubota et al., 1999). Recently,

particular attention has been focused on the ex vivo expansion of corneal epithelial cells on various substrates, including preserved human AM (Tsai et al., 2000).

An increasingly popular surgical procedure for ocular surface reconstruction in individuals with severe thermal or chemical burns or serious ocular surface disorders, such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and recurrent pterygium (Lee and Tseng, 1997; Prabhasawat et al., 1997; Shimazaki et al., 1997; Tseng et al., 1998; Tsubota and Shimazaki, 1999), involves the use of preserved human AM as a biological drape to dress the bare stroma after the removal of abnormal conjunctival tissue. The results of ocular surface reconstruction with human AM are generally good. Moreover, it is reported that in ocular surface disorders with stem cell deficiencies, the use of limbal transplantation and keratoepithelioplasty in conjunction with AM transplantation is often highly successful (Trelford and Trelford-Sauder, 1979; Tseng et al., 1998). Thus, it seems evident that the combination of amniotic/corneal epithelial cell transplantation is a potentially powerful one.

Thus, AM has unique properties that can be helpful in epithelial stratification study (Connon et al., 2006). To monitor the development of a stratified epithelium formed in this study, morphologic and biochemical markers were used to demonstrate the degree of corneal epithelial stratification and tissue integrity. They are epithelial cell layers, cell type and Ki67, CK3, markers for corneal epithelial cell proliferation and differentiation.

For determination of mechanisms such as stratification, proliferation and differentiation, a rat corneal organ culture model was also used.

The evolutionarily conserved Notch signalling pathway controls cell fate in metazoans through local cell-cell interactions (Egan et al., 1998; Greenwald, 1998; Artavanis-Tsakonas et al., 1999). Notch signalling dictates cell fate and critically influences cell proliferation, differentiation, and apoptosis (Miele and Osborne, 1999) As showed in Chaper 3, Notch signalling system which is known to mediate cell fate decisions and has been shown to comprise an essential intercellular signalling system in tissue development and homeostasis. Notch family members including Notch receptors (Notch1 and Notch2) and their ligands (Delta1 and Jagged1) have been detected in human corneal epithelium, but the mechanisms that govern epithelial cell stratification have yet to be fully characterized. To investigate the Notch signalling pathway in corneal epithelial cell stratification, a γ-secretase inhibitor and recombinant Jagged1 have been used to evaluate the function of Notch signalling in the regulation of cell fate according to a previous report (Lindsell et al., 1995; De Strooper et al., 1999; Wolfe, 2001).

The aim in this study was to investigate the regulation of the Notch signalling pathway on human corneal epithelial cell stratification in a corneal air-lift cell culture model and rat corneal organ culture.

4.2. Project design

4.2.1. Setting up corneal epithelial cell stratification models

To investigate the mechanism of Notch signalling pathway in corneal epithelial cell stratification, the corneal epithelial cell stratification culture model was utilised with treatment of Notch inhibition (γ-secretase inhibitor 50μM) and activation (Jagged1 10μg/ml). In accordance with the tenets of the Declaration of Helsinki and with proper informed consent, human amniotic membranes were obtained at the time of Cesarean section. The standard method is the same as that described in section 2.2.10.

4.2.2. Setting up corneal organ culture model

To further confirm the regulation of Notch signalling in corneal stratification from corneal centre, periphery and limbus in corneal homeostasis, the rat corneal organ culture models have been set up with treatment of Notch inhibition (γ-secretase inhibitor 50μM) and activation (Jagged1 10μg/ml) as described in section 2.2.11.2-2.2.11.4.

4.2.3. The effect of Notch inhibition or activation on corneal epithelial cell stratification and organ culture models

After the treatment of inhibition and activation, the frozen sections were prepared from different time points (stratification:1 week, 2 weeks, 3 weeks and 4 weeks; organ culture: 0 day, 2 days, 7 days and 14 days; n=3 for each).

4.2.4. Haematoxylin and Eosin staining for detection of corneal stratification and epithelial cell morphology

To examine corneal stratification and epithelial cell morphology, sections from AM + cornea and rat cornea after the treatment of Notch inhibition or activation were stained with haematoxylin and eosin using the same methods as described in 2.2.9.2, and viewed using an inverted microscope (DMRAZ, Leica). The images from the stratification model and rat cornea were captured and analysed using the Spot Advance Image and Image Pro-Plus software (Image Solutions, UK). The data were analysed by PRISM software.

4.2.5. Immunohistochemical analysis for Notch1, Notch2, Delta1, Jagged1, Ki67 and cytokeratin 3 (CK3)

Immunohistochemistry was performed to identify specific features of Notch family members that are involved in the stratification of corneal epithelial cells. The immunolocalisation of Notch1, Notch2, Delta1, Jagged1, Ki67 and CK3 was performed as described in section 2.2.9.3 to determine the role of Notch receptors and ligands in cell proliferation and differentiation.

4.2.6. Statistical analysis

All experiments were repeated at least three times and the results are expressed as

mean ± SEM. Statistical analysis was performed using one-way ANOVA for cell population analysis, and Kruskal – Wallis Test for epithelial cell layer comparison, $P< 0.05$ was considered statistically significant.

4.3. Results

4.3.1. The role of Notch in the human corneal epithelial stratification model

4.3.1.1.Morphological assessment of corneal epithelial cells

Preliminary experiments on haematoxylin and eosin-stained, ethanol-fixed frozen section of AMs confirmed that this protocol effectively removed epithelial cells from the AM (Fig. 4.1)

Morphological examination of haematoxylin and eosin-stained sections of all time points were carried out to compare the number and type of epithelial cell layers and therefore to determine the degree of the stratification of corneal epithelium. After 1 week in culture, a confluent primary culture of flat epithelial cells had been established that covered the entire AM (Fig. 4.2a). At 2 weeks, the cultivated corneal epithelial cells showed 2-3 layers of 1 columnar basal layer, 0-1 wing layer and 1 flat or cuboid superficial layer (Fig. 4.2b). At 3 weeks, the corneal epithelial cells on AM showed the formation of 4 layers of stratified epithelial tissue. These appeared very similar to normal corneal epithelium (Fig. 4.2c): 1 columnar basal layer, 2 wing layers and 1 flat squamous layer. After 4 weeks, the cultured corneal epithelial cells formed 5-6 layers of well-stratified epithelium. It appeared healthy, and composed of

differentiated cells: 1 layer of basal columnar cells, 3-4 suprabasal cuboid wing cell layers, and 1 layer of flat squamous superficial cells (Fig. 4.2d).

After the treatment of γ-secretase inhibitor (Fig. 4.3b), there was an increase of the number of epithelial cell layers. By week 4, the superficial cells became more flatten and squamous. This result indicated that the cells became more differentiated due to inhibition of the Notch signalling pathway.

As shown in Figure 4.3c, there was a decrease of epithelial cell layers after the treatment of Jagged1. By week 4, the superficial and suprabasal cells appeared to be seen round and cuboidal. These results indicated that epithelial cells became less differentiated due to activation of the Notch.

4.3.1.2.Quantitation of the number of corneal epithelial cell layers

The statistical results of the number of corneal epithelial cell layers with different time points are shown in Figure 4.4. After treatment of γ-secretase inhibitor (50μM) or Jagged1 (10μg/ml), there was a significant increase or a significant decrease of cell layers, respectively.

4.3.1.3.Immunolocalisation of Notch1, Notch2, Delta1, Jagged1

The immunolocalisation of Notch receptors in corneal epithelial cell stratification culture was similar to that in the human corneal epithelium reported in Chapter 3. Notch1 and Notch 2 were localised to subrabasal and superficial layers with labelling absent in the basal cell layer until 4 weeks (Fig. 4.5a, b). The expression of Delta1 and

Jagged1 was also similar to that in human corneas. Immunolocalisation of Delta1 and Jagged1 was demonstrated throughout the epithelial cells until 4 weeks (Fig. 4.5c, d). In the negative control, immunoreactivity was not detected in epithelium processed with same species and concentrations of serum (goat, rabbit) in replace of primary antibodies. The green staining on AM indicated autoflurescence.

4.3.1.4.Immunolocalisation of cell proliferation and differentiation

Immunolocalisation of Ki67 in epithelial cell stratification appeared to be localised in the basal layer and suprabasal layers (Fig. 4.6a) and was similar to that seen for human corneas. There was no significant change of Ki67 expression after treatment of γ-secretase inhibitor (Fig. 4.6b) and Jagged1 (Fig. 4.6c). This result indicated that corneal epithelial cell proliferation might not have been regulated by the Notch signalling pathway in this model.

For immunolocalisation of CK3 in epithelial cell stratification, positive labelling was detected in all the cell layers, but there was a weak expression in the basal cell layer (Fig. 4.6d). These results were also similar to human corneas as reported in Chapter 3. After the inhibition of Notch signalling, there was no obvious change of the CK3 (Fig. 4.6e). However, the expression of CK3 decreased after addition of Jagged1. By week 4, only approximately 25% of the cells were expressing CK3 (Fig. 4.6f). This result indicated that Notch signalling pathway repressed corneal epithelial cell differentiation.

4.3.1.5.The percentage of Ki67 and CK3

As shown in Figure 4.7a, there was an increase of Ki67 expression from 1 week to peak expression at 3 weeks when approximately 50% of the cells were expressing Ki67. The expression of Ki67 then decreased to approximately 40% by 4 weeks. There was no statistical difference of Ki67 with Notch inhibition or activation compared to control.

The percentage of CK3 positive epithelial cells shown in Figure 4.7b, from 1 week onward was 95% to 100% positive epithelial cells with increasing time. After Notch inhibition, there was no statistically significant change of CK3 expressing cells over 4 weeks. However, activation of Notch signalling induced a significant decrease of CK3 expression to 25%. This result further confirmed that the Notch signalling pathway can repress corneal epithelial cell differentiation.

4.3.2. The role of Notch in rat corneal epithelial stratification

Rat corneas were cultured with control medium, treatment media including addition of γ-secretase inhibitor or Jagged1, and maintained for varying periods of time up to 2 weeks (0 to 14 days). Corneal cryosections were stained with haematoxylin and eosin for morphological analysis, immunolocalisation for detecting the distribution of Notch family members (Notch1, Notch2, Delta1, Jagged1), and immunostaining of Ki67 and CK3 to determine the proliferative and differentiated status of corneal epithelium during organ culture.

4.3.2.1.Morphological assessment of rat corneal epithelial cells

Figure 4.8 depicts the changes that occur during organ culture for central, peripheral and limbal regions of rat cornea for 0, 2, 7 and 14 days. At 0 day, the rat corneal epithelium had 5 layers in the centre (Fig. 4.8a), and the number of layers increased towards the periphery (Fig. 4.8e) until then reached a maximum of 7-8 cell layers at the limbus (Fig. 4.8i). The epithelium at the limbus was composed of a layer of column-shaped basal cells, 4-5 layers of suprabasal cuboid wing cells, and 1-2 flat superficial cells. After 2 days of organ culture the cornea lost its uniformity, and numbers of cell layers in central and peripheral regions reduced to 3-4 layers (Fig. 4.8b, f), and 4-5 layers in limbus (Fig. 4.8j) compared to day 0. There is an obvious drop of epithelial cell layers at 7 days with 1-2 layers in the centre (Fig. 4.8c), 2-3 layers in periphery (Fig. 4.8g) and 3-4 layers in limbus (Fig. 4.8k), with 1-2 flat superficial cells 1 layer wing cell and 1 layer cuboid basal cell. The number of cell layers continued to decrease, at 14 days it reached 1-2 layers in centre (Fig. 4.8d), 2-3 layers in periphery (Fig. 4.8h) and limbus (Fig. 4.8l) with 1-2 layers more flat squamous superficial cells sloughing off from corneal surface.

After the inhibition of Notch (Fig. 4.9), by day 14 the number of cell layers decreased at a reduced rate in corneal centre (Fig. 4.9d), periphery (Fig. 4.9e) and limbus (Fig. 4.9f). Epithelial superficial cells became flatter: 3-4 flattened superficial cells in the limbus (Fig. 4.9f). Differences of this kind in epithelial cell layers and type were even more obvious at 14 days of organ culture. The range of cell layers is between 4-5 layers in centre (Fig. 4.9d) and 6-7 layers in limbus (Fig. 4.9f). These results indicated there was more epithelial cell stratification after Notch inhibition.

After activation of Notch, the number of cell layers decreased quicker than in control medium (Fig. 4.9a-c) in the corneal centre (Fig. 4.9g), periphery (Fig. 4.9h) and limbus (Fig. 4.9i). The epithelial superficial cells were still flat and squamous, but the basal cells became round in the corneal centre, periphery (Fig.4.9g, h). The maximum difference was observed at 14 days of organ culture, the epithelium even reached 0-1 layer in the centre, periphery and limbus (Fig. 4.9g, h, i). These results demonstrated there was less epithelial cell stratification after activation of Notch.

4.3.2.2.The number of corneal epithelial cell layers

The number of epithelial cell layers in the corneal centre, periphery and limbus has been counted at 0 day, 2 days, 7 days and 14 days using Image Pro-Plus software (Image solutions, UK). Graphs in Figure 4.10 showed that there were significant increases of epithelial cell layers in the corneal centre (Fig. 4.10a), periphery (Fig. 4.10b) and limbus (Fig. 4.10c) compared to control after Notch inhibition, and significant decreases of cell layers in above three regions (Fig. 4.10a, b, c) due to induce Notch activation.

4.3.2.3.Immunolocalisation of Notch1, Notch2, Delta1, Jagged1

The immunolocalisation of Notch receptors in rat cornea was similar to that in the human epithelium demonstrated in Chapter 3. Notch1 and Notch 2 were localised in subrabasal and superficial layers with labelling absent in the basal cell layer (Fig. 4.11a-f). The expression of Delta1 and Jagged1 in rat cornea was also similar to that in human corneas. Immunolocalisation of Delta1 and Jagged1 was demonstrated

throughout the epithelial cell layers in corneal centre, periphery and limbus (Fig. 4.11g-l).

4.3.2.4.Immunolocalisation for cell proliferation and differentiation

Immunolocalisation of Ki67 in rat cornea revealed expression in cell nuclei of basal layer that was similar with human corneas and in the order of magnitude periphery>centre>limbus (Fig. 4.12a-c).

For immunolocalisation of CK3 in rat cornea, positive labelling was detected in the superficial and suprabasal cell layers and a weak expression in the corneal basal cell layer, but absent in limbal basal layer (Fig. 4.12d-f). These results were also similar with human corneas demonstrated in Chaper 3.

As shown in Figure 4.13, after the treatment of Notch inhibition, there were no obvious changes of Ki67 and CK3 in corneal limbus, periphery and centre (Fig. 4.13d, e, f). After activation of Notch signalling, in contrast to control medium (Fig. 4.13a, b, c) there was also no change of Ki67 in the corneal limbus, periphery and centre (Fig. 4.13g, h, i). However, there was a dramatic decrease of CK3 expression after Notch activation. By 7 days, only approximately 30% of epithelial cells were expressing CK3 in the corneal limbus and 50% of epithelial cells in the periphery and centre (Fig. 4.13g, h, i).

These results indicated that the Notch signalling pathway repressed corneal epithelial cell stratification due to inhibition of cell differentiation rather than cell proliferation in both corneal epithelial stratification and organ culture models.

4.3.2.5.The percentage of cells expressed Ki67 and CK3

Figure 4.14 shows that the percentage of Ki67 positive cells decreased in the corneal limbus, periphery and centre with organ culture. In the corneal limbal region (Fig. 4.14a), the percentage of Ki67 positive cells decreased from approximately 18% to 2% from 0 day to 14 days. For the peripheral region (Fig. 4.14b), the percentage of Ki67 positive cells decreased from approximately 38% to 5% during organ culture. In the corneal centre (Fig. 4.14c), Ki67 decreased from approximately 28% to 3% by 14 days. There were no statistically significant changes of Ki67 expression after Notch inhibition or activation compared control medium.

In Figure 4.15, there was no significant change in CK3 positive cells after Notch inhibition in corneal limbus, periphery and centre, but there was an obvious decrease of CK3 expression with Notch activation in the three corneal areas during organ culture. In the corneal limbus (Fig. 4.15a), the range of CK3 expression was from 70% to 75% with control medium since 0 day until 14 days. After Notch inhibition there was no obvious change of CK3 in contrast to control medium, but there was a significant decrease of CK3 with Notch activation, the percentage from 70% to approximately 25% by 14 days. In the corneal periphery (Fig. 4.15b), approximately 95% - 100% of epithelial cells expressing CK3 with control medium, there was no obvious difference in CK3 with Notch inhibition. However, there was a significantly decreased CK3 after Notch activation, the percentage was between 95% and 30% during culture time. Figure 4.15c shows the percentage of CK3 expressing at corneal centre, approximately 95% to 100% epithelial cells expressing CK3 with control medium, there was no obvious difference in CK3 staining with Notch inhibition.

However there was a significant change of CK3 after Notch activation, the percentage of CK3 expression decreased from 95% to 20% by 14 days.

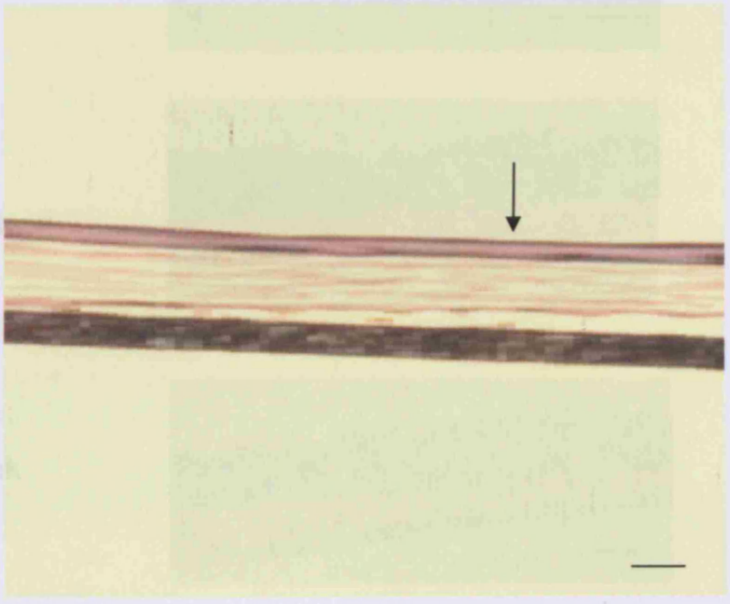


Fig 4.1 Human amniotic membrane after epithelial cell removal. The arrow shows smooth surface of membrane without epithelial cells. Bar=25 µm

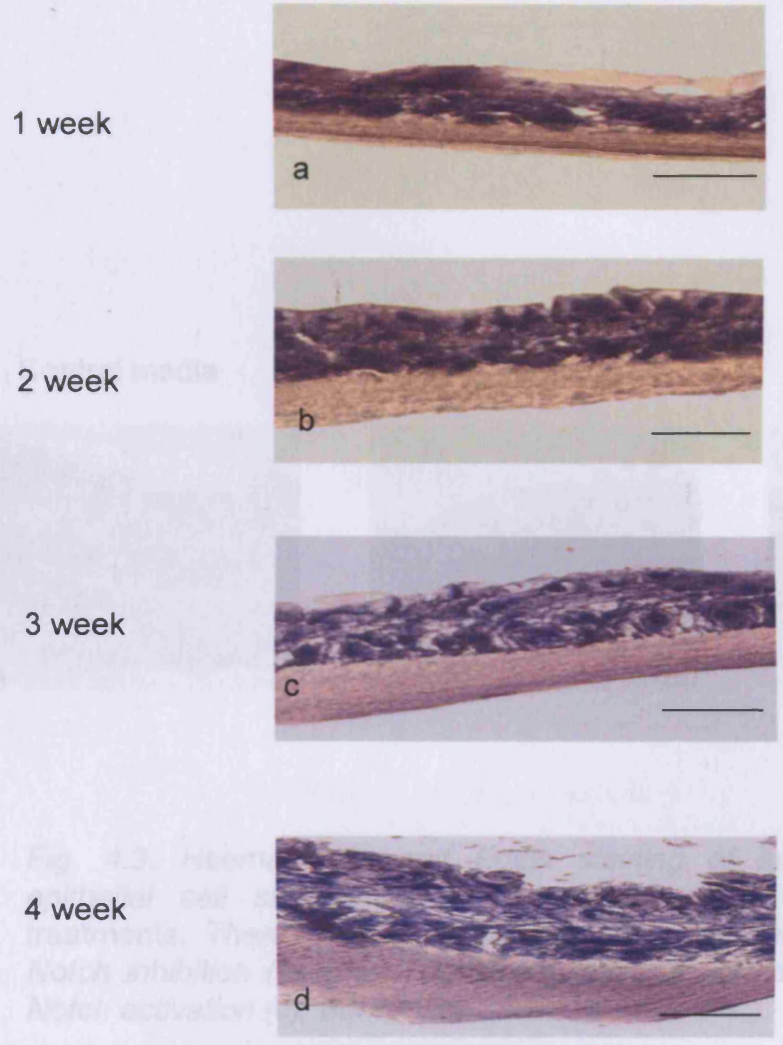


Fig. 4.2 Haematoxylin and Eosin staining of human corneal epithelial cell stratification culture at different time points. There was a confluent primary culture of flat epithelial cells had been established after 1 week (a); At 2 weeks, the epithelial cells became 2-3 layers (b); At 3 weeks, the corneal epithelial cells showed the formation of 4 layers (c); After 4 weeks, the cultured corneal epithelial cells formed 5-6 layers (d). Bar=40μm.

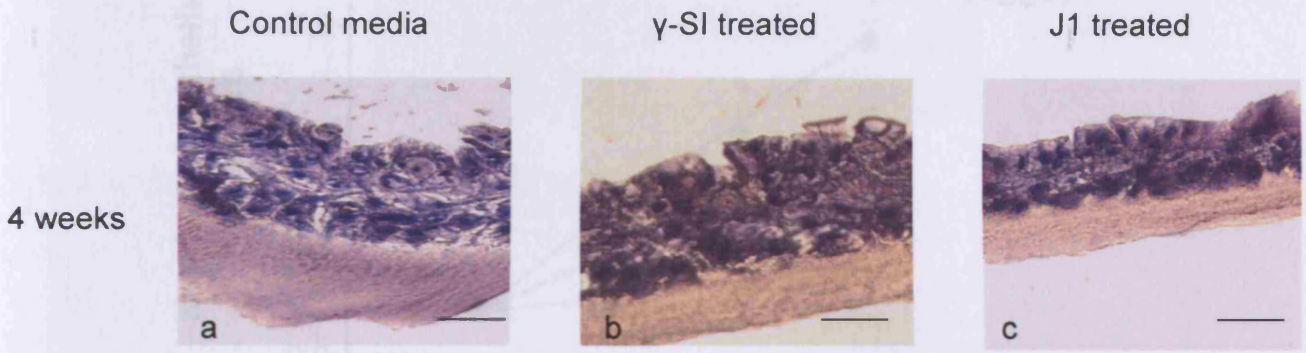


Fig. 4.3. Haematoxylin and Eosin staining of human corneal epithelial cell stratification culture at 4 weeks with different treatments. *There was a significant increase of cell layers after Notch inhibition (b) and a significant decrease of cell layers after Notch activation (c). Bar=50μm.*

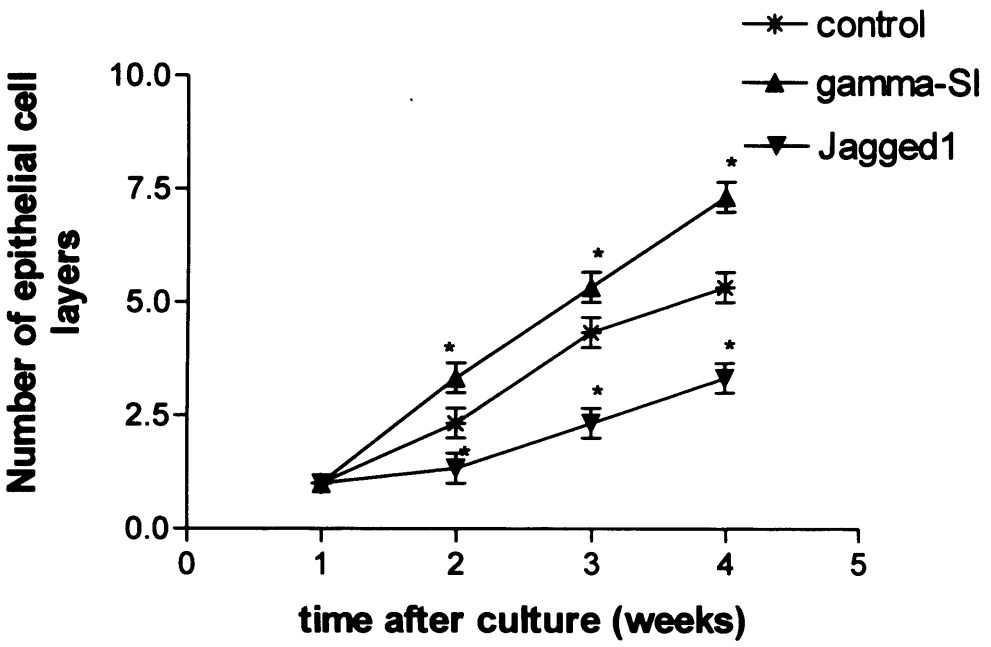


*Fig. 4.4 The number of corneal epithelial cell layers in corneal epithelial cell stratification culture after the treatment of γ – SI (50μM) and Jagged1 (10μg/ml). There were significant increase of cell layers after Notch inhibition and significant decrease of cell layers after Notch activation. *P<0.05.*

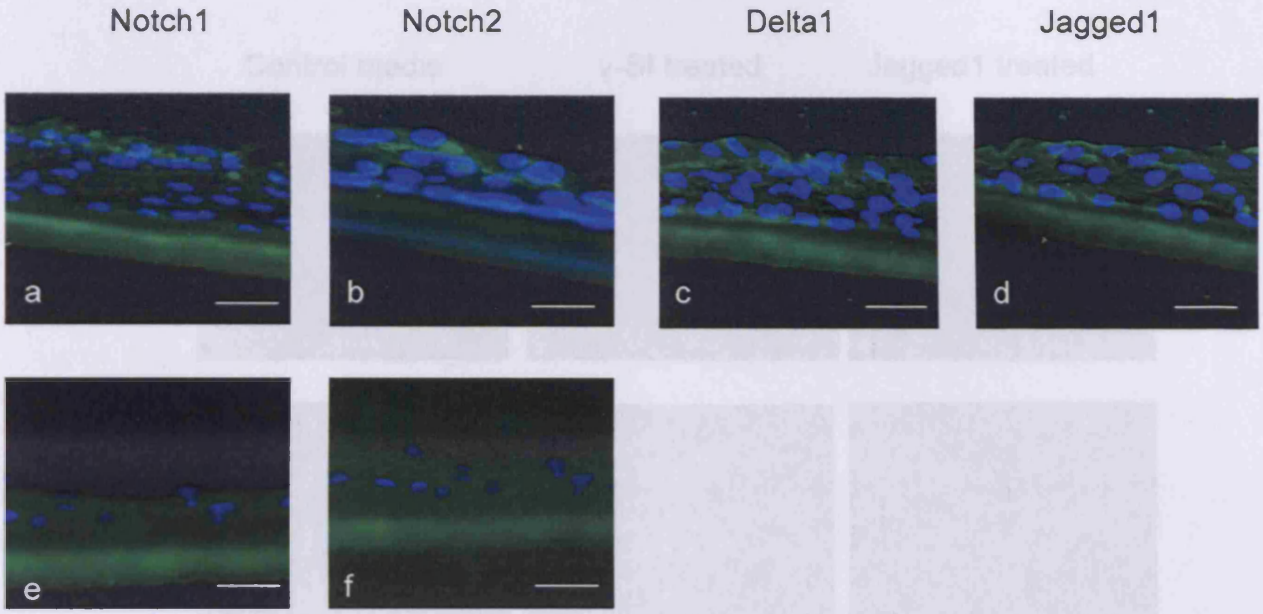


Fig.4.5. The immunolocalisation of Notch family members in corneal epithelial cell stratification culture (4 weeks). a, b: the expression of Notch1and Notch2 has appeared in superficial and suprabasal layers (green); c, d: Delta1 and Jagged1 appeared to be expressed in all the epithelial cell layers (green); e, f: negative control with goat, rabbit serum instead of primary antibodies. Blue indicates nuclear staining. Bar=50 μm

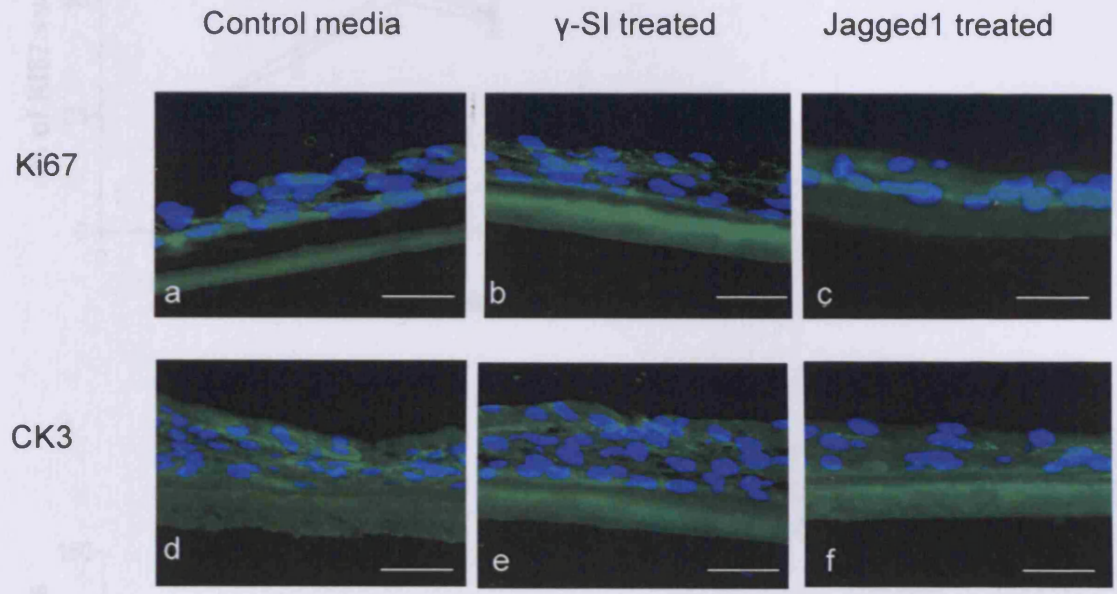
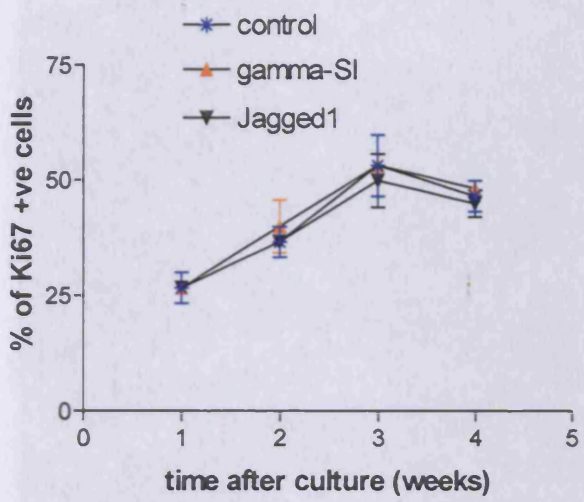


Fig. 4.6. Immunolocalisation of Ki67 and CK3 in corneal epithelial cell stratification culture (4 weeks). Cultures with control media (a,d), γ-SI treatment (b, e) and Jagged1 treatment (c, f) . There were no obvious changes of Ki67 (green) expression in basal cell layer after Notch inhibition or activation (b, c). The expression of CK3 (green) appeared to be seen in all cell layers (d, e, f). There was no obvious change of CK3 after Notch inhibition (e) but a significantly decreased CK3 after Notch activation (f). Bar=50μm

a



b

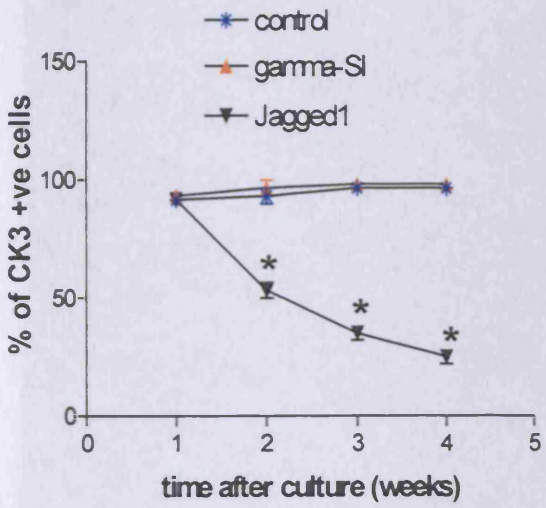


*Fig. 4.7. Percentage of Ki67 and CK3 positives in corneal epithelial cell stratification culture after the treatment of γ – SI (50μM) and Jagged1 (10μg/ml). (a) There was an increase of Ki67 expression since 1 week to peak expression by 3 weeks, this then decreased by 4 weeks, but no significant changes of Ki67 expression after Notch inhibition or activation compared to control. (b) There was no significant change of CK3 after Notch inhibition (γ – SI) but a significant decrease of CK3 after Notch activation (Jagged1) compared to control. *P< 0.05*

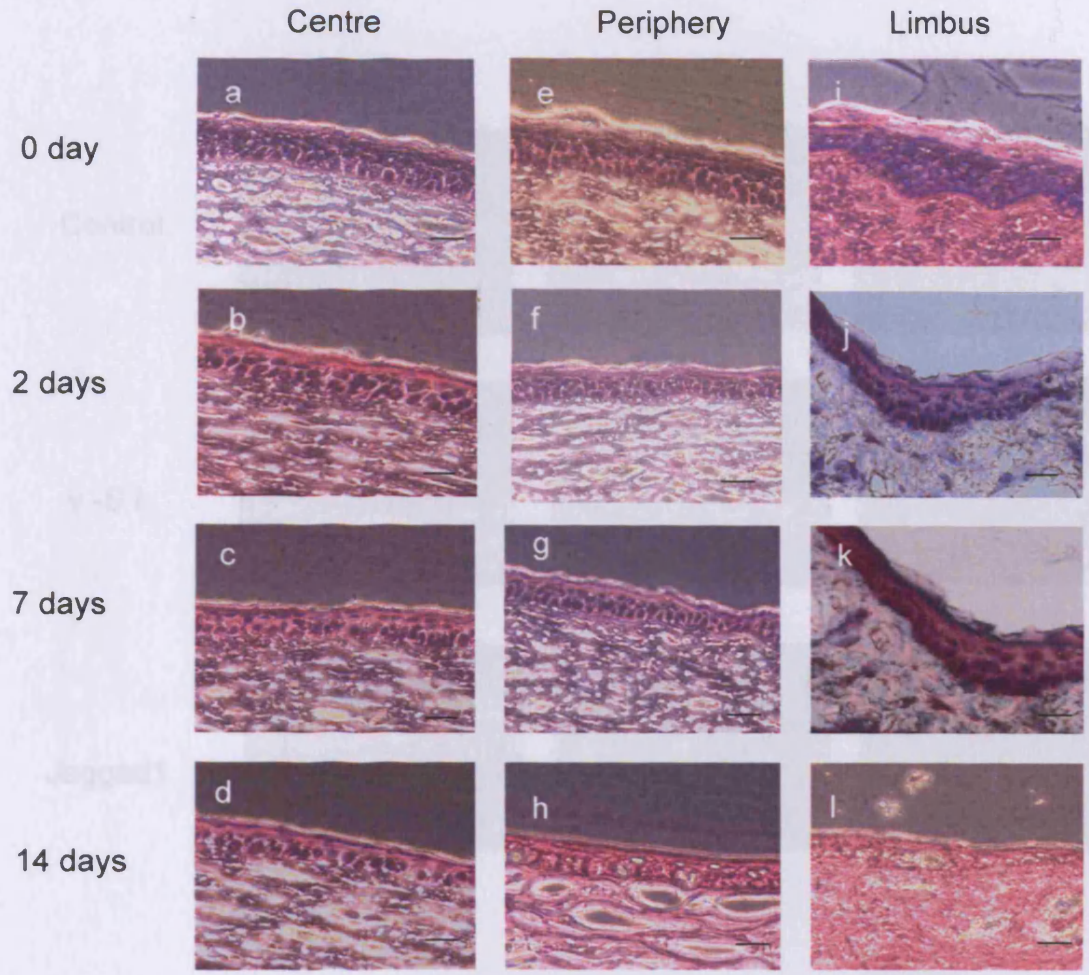


Fig. 4.8. Haematoxylin and Eosin staining of rat corneal epithelium in organ culture. There were significant decreases of cell layers in corneal centre (a-d), periphery (e-h) and limbus (i-l). Superficial cells became more flat and squamous with increased time. Bar=50μm

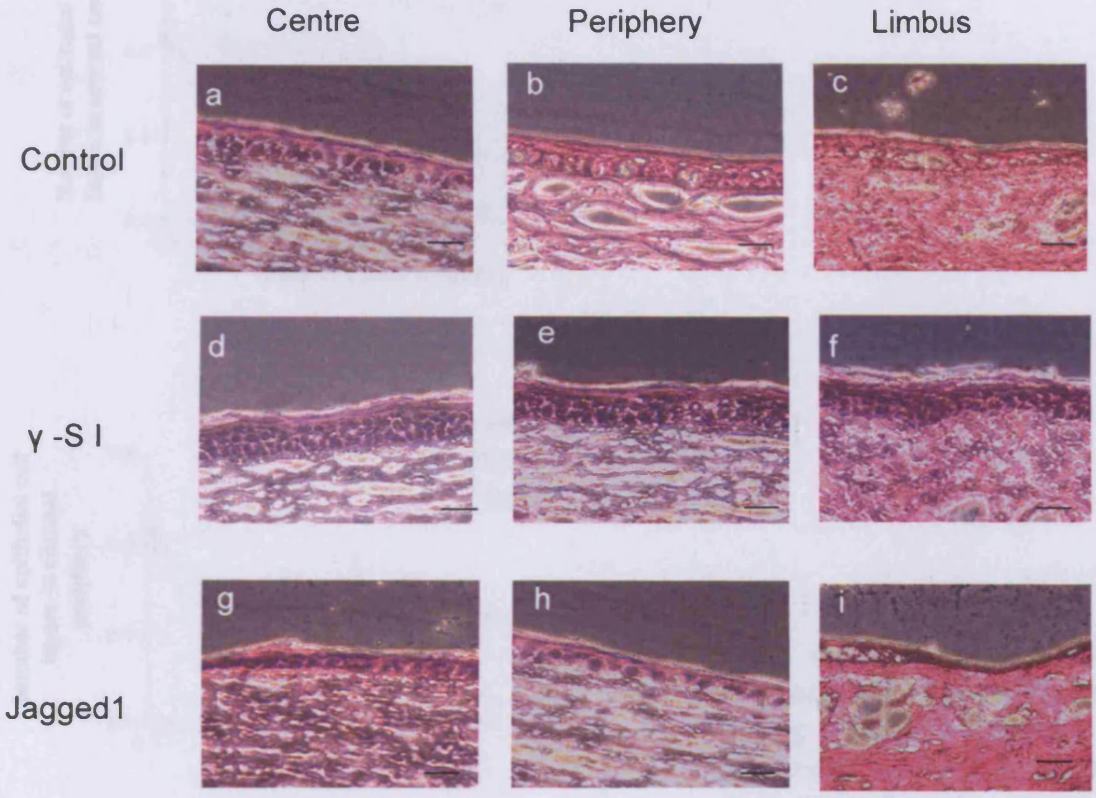
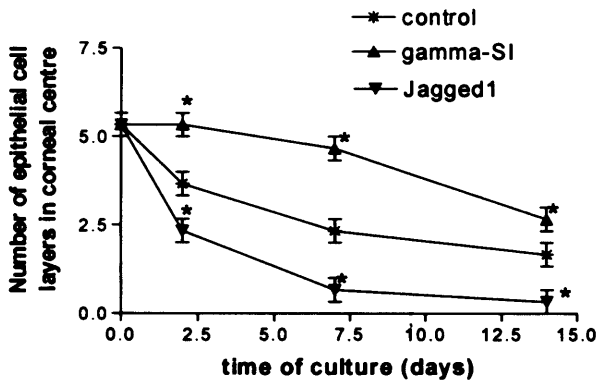


Fig. 4.9. Haematoxylin and Eosin staining of rat corneal epithelium in corneal organ culture (14 days) after different treatments. The number of cell layers decreased at a reduced rate in corneal centre (d), periphery (e) and limbus (f) after Notch inhibition (γ-secretase inhibitor) *compared to controls (a-c). However, the number of cell layers decreased quicker than in control medium after Notch activation (Jagged1; g, h, i). Bar=50μm*

a



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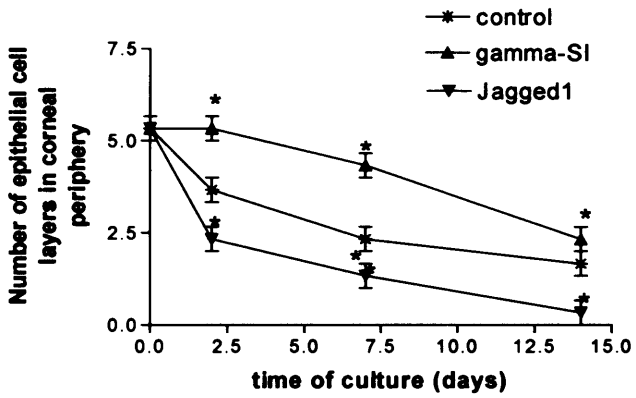

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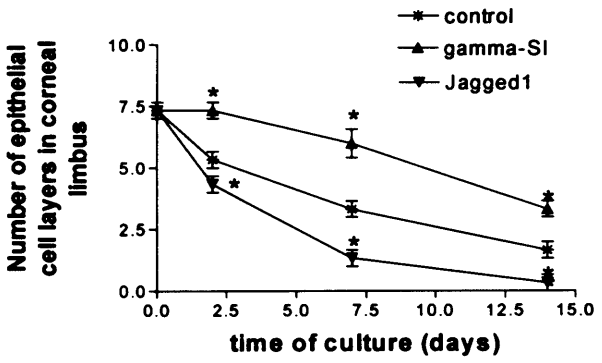


*Fig. 4.10. The number of epithelial cell layers in cornea during organ culture with treatment of Notch inhibition and activation. In contrast to control, there were significant increases of cell layers in corneal centre (a), periphery (b) and limbus (c) after Notch inhibition and significant decreases of cell layers in centre (a), periphery (b) and limbus (c) after Notch activation. *P<0.05.*

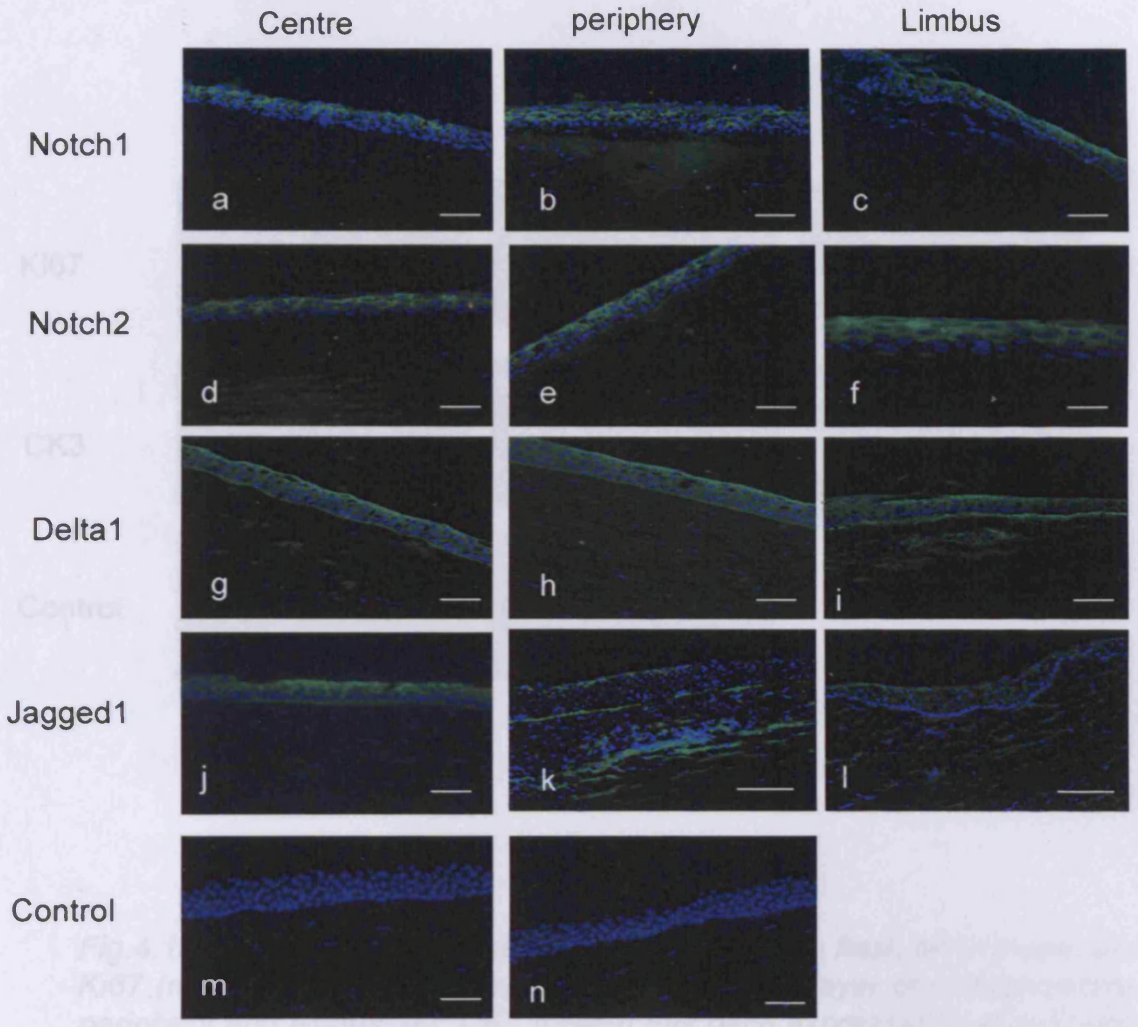


Fig.4.11. The immunolocalisation of Notch family members in rat corneas. a-f: Notch1 and Notch2 appeared to be expressed in corneal epithelial superficial and suprabasal layers but absent in basal layer; g-l: Delta1and Jagged1 have been detected in all epithelial cell layers. m, n: negative control with goat, rabbit serum instead of primary antibodies. Bar=50 μm

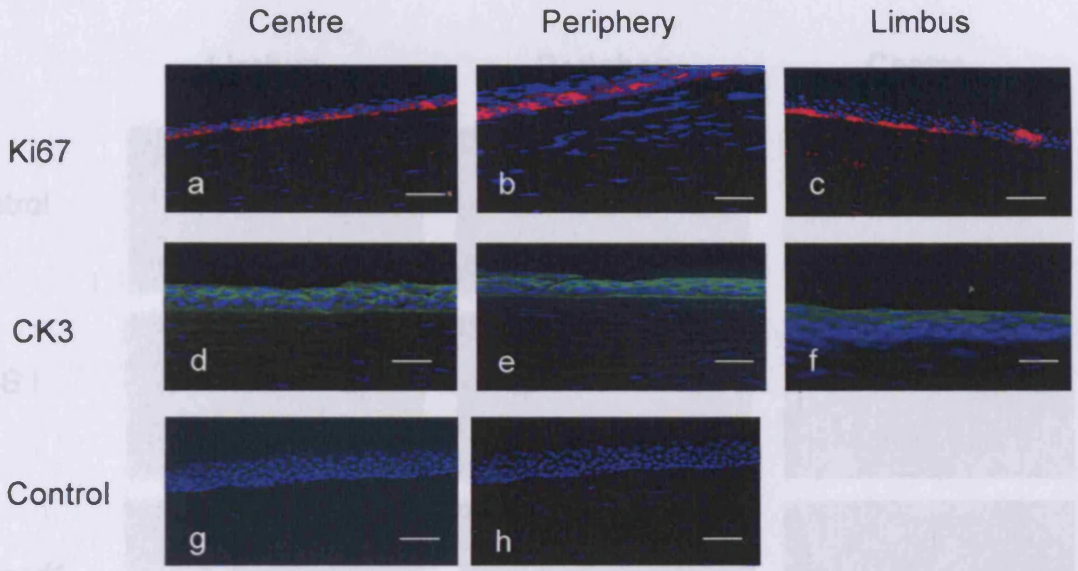


Fig.4.12. The immunolocalisation of Ki67 and CK3 in fresh rat corneas. a-c: Ki67 (red) appeared to be expressed in basal cell layer of corneal centre, periphery and limbus; d-f: CK3 (green) has been expressed in all epithelial cell layers but absent in limbal basal layer; g, h: negative control with rabbit, mouse serum instead of primary antibodies. Bar=50 μm

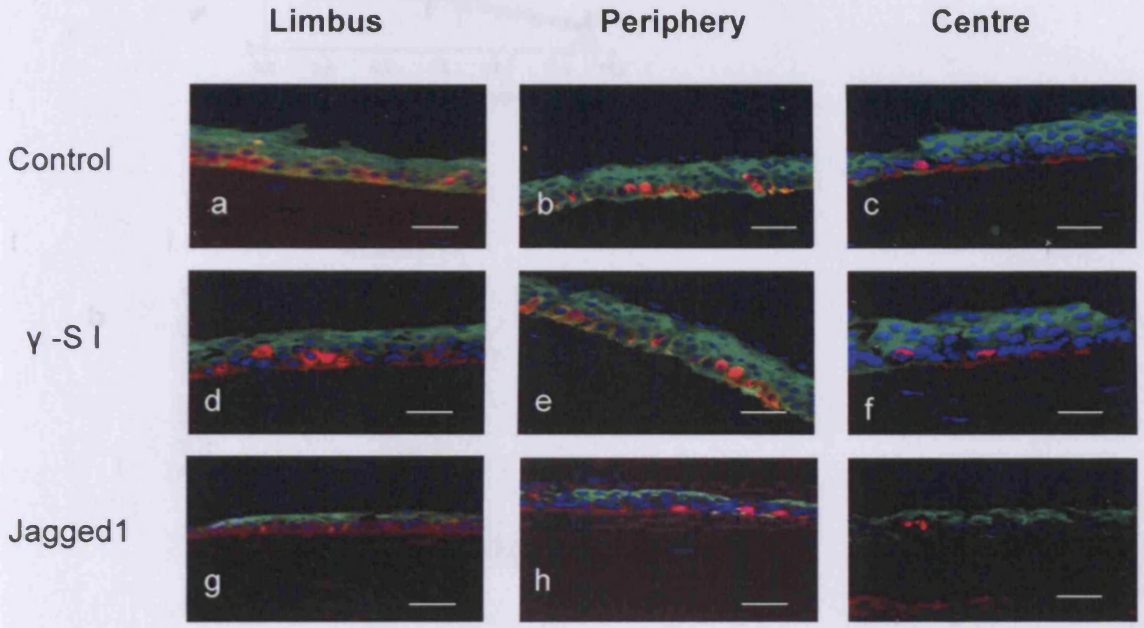
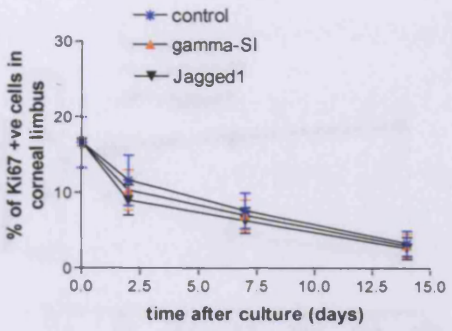


Fig.4.13. The immunolocalisation of Ki67 and CK3 in rat corneal organ culture (7 days) with different treatments. There were no obvious changes of Ki67 and CK3 in corneal limbus (d), periphery (e) and centre (f) after Notch inhibition. After Notch activation, there was also no change of Ki67 but a significant decrease of CK3 in corneal limbus (g), periphery (h) and centre (i) compared to control (a-c). Red: Ki67 positive. Green: CK3 positive. Blue: the staining of cell nuclei. Bar=50 μm

a



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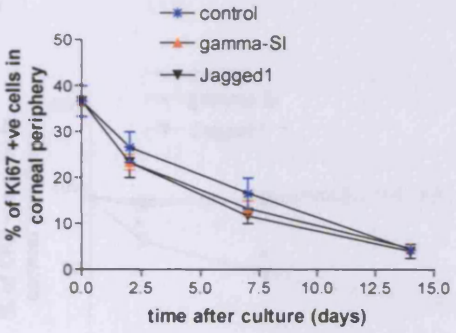

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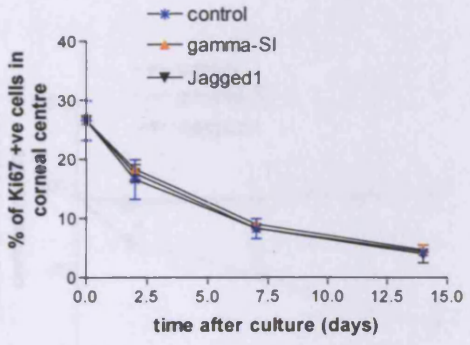


Fig. 4.14. Percentage of Ki67 positive cells in rat corneal organ culture with treatment of Notch inhibition or activation. There were no significant changes of Ki67 expression in limbus (a), periphery (b) and centre (c) in contrast to control.

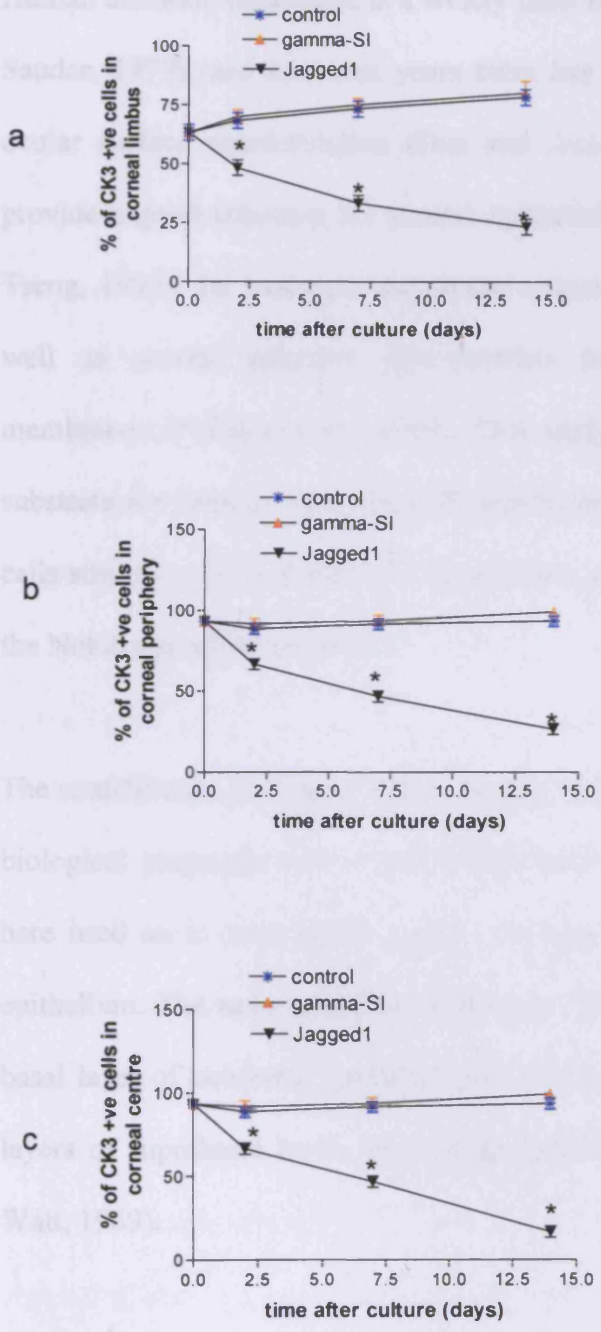


*Fig. 4.15. Percentage of CK3 positive cells in rat corneal organ culture with treatment of Notch inhibition or activation. There was no significant change of CK3 expression with Notch inhibition in limbus (a), periphery (b) and centre (c), but there were significant decreases of CK3 with Notch activation in corneal limbus (a), periphery (b) and centre (c) in contrast to control. *P<0.05.*

4.4. Discussion

Human amniotic membrane is a widely used surgical material (Trelford and Trelford-Sauder, 1979), and in recent years there has been a renewed interest in its use for ocular surface reconstruction (Dua and Azuara-Blanco, 1999). AM acts so as to provide a good substrate for normal epithelial migration and stratification (Kim and Tseng, 1995). Its basement membrane contains collagens (Modesti et al., 1989) as well as several adhesive glycoproteins found in corneal epithelial basement membranes (Fukuda et al., 1999). This study was designed to a) utilise AM as a substrate for corneal epithelial cells and b) investigate how limbal corneal epithelial cells stratify on AM at different time points, as a result of inhibition or activation of the Notch signalling pathway.

The stratification of corneal epithelial cells plays an important role in the physical and biological properties of corneal homeostasis and development. The data presented here used an in vitro model system to show the development of stratified corneal epithelium. The term 'stratified epithelium' refers to epithelial tissue comprised of a basal layer of columnar epithelial cells in contact with the basement membrane and layers of suprabasal layers containing flattened wing and squamous cells (Hall and Watt, 1989).

When corneal limbal explants were seeded onto AM, morphological and immunohistochemical examination of the resultant epithelium showed the formation of a stratified corneal epithelium. The Ki67 expression continually increased and induced an increase in the number of cell layers. At 3-4 weeks, the expression of Ki67

in the cultivated corneal epithelial cells appeared to peak at 50%, the cultures showed 5-6 layers of stratification which were composed of 1 basal columnar cell layer, 3-4 suprabasal cuboid wing cells, 1-2 flat squamous superficial cells, and demonstrated immunoreactivity for the cornea-specific cytokeratin 3, indicating that AM supported normal corneal epithelial cell differentiation and stratification.

The expression of Notch family members appeared to be seen in corneal epithelial cell stratification culture indicating Notch signalling may regulate epithelial cell stratification. As is already known γ-secretase inhibitors prevent the activation of the Notch signalling pathway. After the inhibition of the Notch pathway, an increased number of cell layers were observed compared with controls. Epithelial cells became more flat and squamous in the superficial layer and suprabasal layers. The percentage of CK3 expressing cells showed there was no significant change, also the expression of Ki67 has not shown statistical differences with Notch inhibition.

To further confirm the role of Notch in corneal epithelial cell stratification, addition of Jagged1- one ligand of Notch signalling, resulted in a significant decrease in the cell layers on AM and less flat superficial cells. The expression of CK3 appeared to become significantly decrease, but Ki67 expression did not show any changes with Notch activation. These results further verify that the Notch pathway plays a role in regulating human corneal epithelial cell stratification.

However, the important challenge is to identify what cell-fate decisions the Notch signalling machinery govern in corneal epithelial cell stratification during the process of corneal homeostasis? Are there any different patterns of the stratification in corneal

epithelial centre, periphery and limbus? How does this explain the decreased stratification with corneal organ culture? As shown in the results, rat corneal organ culture can be a good model for investigation of epithelial stratification in organ culture as Notch family members, cell proliferative and differentiating status in rat cornea have a similar distribution to human.

Investigation of morphological changes during corneal organ culture for different periods showed that there was reduction in the number of cell layers in corneal centre, periphery and limbus from 0 day to 14 days of organ culture. These results are comparable to that of Albon et al 2000 (Albon et al., 2000) in their human corneal organ culture storage model. In this study, after Notch inhibition the number of corneal epithelial cell layers decreased slowly with the superficial cells became more flat and squamous, and more sloughing cells from corneal surface; after the activation of Notch signalling, the decrease of cell layers became significantly quicker compared to control medium. These effects have been observed in every corneal region. There were no obvious changes of CK3 expression with Notch inhibition as almost all corneal epithelial cells had already expressed CK3, but there were still dramatic decreases of CK3 expression in every region by addition of Jagged1- activation of Notch. This is a similar result to the epithelial cell culture on AM. The expression of Ki67 showed no obvious changes after Notch inhibition or activation. These results further confirmed that Notch signalling can also regulate epithelial cell stratification in corneal organ culture by repressing corneal epithelial cell differentiation rather than cell proliferation.

Corneal epithelial renewal is a constant feature of the corneal epithelium in homeostasis. The cycling status of cells in rat cornea demonstrated Ki67 labelled proliferating cells in the central, peripheral and limbal regions of the corneal basal layer. The peripheral corneal epithelium showed more proliferative activity compared to the central and limbal regions. This is consistent with previous reports that the limbal epithelium contains a mixture of slow-cycling stem cells and more rapidly cycling transient amplifying cells (TACs) that enter the basal layer of the epithelium. As these TACs move upwards from the basal layer, they become postmiotic and differentiate and eventually slough off as flattened superficial cells from the epithelial surface (Ebato et al., 1988; Cotsarelis et al., 1989; Pellegrini et al., 1999). Corneal epithelial cell proliferation has been regulated by many growth factors e.g. EGF, KGF and HGF etc. (Gherardi et al., 1993; Werner et al., 1994). As shown in this study, a primary function of the Notch signalling pathway is to prevent epithelial cell stratification via inhibition of differentiation. Consequently, a balance between cell differentiation and proliferation is maintained by the interaction of Notch signalling with other growth factors and other signalling pathway in corneal epithelial cell stratification and homeostasis.

CHAPER FIVE

THE REGULATION OF NOTCH SIGNALLING IN CORNEAL WOUND HEALING

5.1. Introduction

Corneal wound healing, as in other parts of the body, is the end result of a sequence of events which are controlled by many factors. The human epithelium is arranged in 5-7 layers of cells and is approximately 50μm thick. It consists of a single layer of mitotically active columnar basal cells covered by a layer of wing cells that are usually 1-3 cell layers thick. These are overlaid by 2-3 outer flattened cell layers of squamous cells. The basal epithelial cells adhere to their basement membrane through a series of adhesion complexes.

If any portion of an epithelial cell is disrupted, the entire cell is usually lost, leaving a defect in the epithelial cell mantle. The most common type of corneal injury is mechanical. With this type of injury, both the cellular components and the extracellular components of the cornea are involved. Signals from the disrupted cells or signals generated by exposure of epithelial basement membrane to either the circulation or the tear film are sent to the nearly intact epithelial cells (Tuft et al., 1993). After injury, there is a latent phase during which the cell cytoskeleton and intercellular junctions are modulated (Crosson et al., 1986; Gipson et al., 1989; Takahashi et al., 1992; Gipson and Inatomi, 1995).

The initial migrating mass of epithelial cells in corneal wound healing is generally composed of one- to two- layers (Anderson, 1977; Trokel et al., 1983). The cells migrate until contact inhibition of migration is established by physical contact with adjacent cells. Once the injured surface has again been covered by epithelial cells and the corneal stroma is protected from the environment, the epithelial cell mass re-

establishes normal thickness (Nishida et al., 1983; Ebato et al., 1988; Cotsarelis et al., 1989). When normal thickness is re-established, the more highly differentiated cellular characteristics, such as the glycocalyx, the microvillae, and microplicae, are re-formed. Intercellular attachments are re-established, as are the basal surface contacts. The epithelium has returned to its steady state.

In contrast to healing skin epithelium, the corneal epithelium requires a much longer time to re-establish full-strength pre-injury characteristics, including basal anchoring complexes (Fujikawa et al., 1981; Gipson et al., 1989; Gipson, 1992). During this interval, the epithelium is vulnerable to injury from much weaker stimuli than the mature epithelium, and clinical problems such as recurrent erosion of the epithelium may develop.

The healing process is believed to involve many growth factors and cell signalling pathways. Of particular interest is that the Notch signalling system which is known to mediate cell fate decisions and has been shown to comprise an essential intercellular signalling system in tissue development and homeostasis via influencing cell adhesion, migration, differentiation and proliferation (Artavanis-Tsakonas et al., 1999).

Aim:

The aim of this chapter was to investigate the potential association between the Notch signalling pathway and corneal wound healing. Since limited availability of human eyes exists, the rat corneal organ culture model in this study was used to investigate

the role of Notch signalling in epithelial cell proliferation and differentiation in corneal wound healing.

5.2. Project design

5.2.1. Wound healing model and organ culture

5.2.1.1. Setting up corneal wound healing model

All protocols in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with permission for research. The rat cornea wound healing models (epithelial wound and stromal wound) were set up as described in section 2.2.11.

5.2.1.2. The effect of Notch signalling inhibition and activation in corneal wound healing

Adult rat corneas (Norwegian Brown, 6-8 weeks of age) were used in this study. Corneas were set up in organ culture in triplicate. When the rat corneas were wounded (epithelial wound and stromal wound), media containing either γ-secretase inhibitor (50μM) or rJagged1 (10μg/ml) was added during organ culture. Corneas were left for periods of time from 0 to 72 hours for epithelial wound, 0 to 14 days for stromal wound. The standard methods are same as described in sections 2.2.11.2 - 2.2.11.4.

5.2.2. Haematoxylin and Eosin staining

To examine corneal morphology after inhibition or activation of Notch, corneal frozen sections were stained with Haematoxylin and Eosin as described in section 2.2.9.2. The number of epithelial cell layers in the wound area was counted and images captured using Image Pro-plus software (Image Solutions, UK).

5.2.3. Immunolocalisation of Notch family members in corneal wound healing

To investigate the expression of Notch signalling in corneal wound healing, immunolocalisation of Notch receptors (Notch1 and Notch2) and their ligands (Delta1 and Jagged1) was performed as described in section 2.2.9.3.

5.2.4. Immunolocalisation of Ki67 and CK3 in corneal wound healing

To investigate of the regulation of Notch signalling in corneal epithelial cell proliferation and differentiation during corneal wound healing, the immunolocalisation of Ki67 and CK3 was performed as described in section 2.2.9.3. The positive immunostaining was quantified using Image Pro-Plus software and the percentage of positive epithelial cells was recorded.

5.2.5. Statistical analysis

All experiments were repeated at least three times and the results were expressed as

mean ± SEM. Statistical analysis was performed using one-way ANOVA for cell population analysis and Kruskal – Wallis Test for epithelial cell layer comparison, $P< 0.05$ was considered statistically significant.

5.3. Results

5.3.1. The role of Notch signalling in corneal epithelial wound healing

5.3.1.1. Morphological analysis of the corneal epithelium in healing corneas

The examination of the rat cornea by microscopy (DMRAZ, Leica) confirmed that the morphology of rat corneal epithelium was similar to that of human. Morphologic examination of all haematoxylin and eosin stained sections allowed analysis of epithelial cell layers and cell type.

In Figure 5.1, the central corneal epithelium had 5-6 layers (Fig. 5.1a), and the number of layers increased towards the periphery until it reached a maximum of 7-8 cell layers at the limbus (Fig. 5.1b). The epithelium at the limbus was composed of 1 layer of columnar-shaped basal cells, 4-5 layers of suprabasal cuboidal wing cells, and 1-2 flat superficial cells (Fig. 5.1b).

Following wounding, in control corneas (Fig. 5.2a-c) the epithelium was seen to start its migration 4 hours after wounding. After 24 hours, there were 1-2 cell layers with some thinning of the epithelial layers over the original wound edge but no obvious

stratification (Fig. 5.2b). By 72 hours, the wound had completely re-epithelialised with 3-4 cell layers that were composed of well stratified cells (Fig. 5.2c).

Inhibition of Notch signalling (Fig. 5.2d-f) did not appear to affect the initiation of migration which began at 4 hours after wounding (Fig. 5.2d). However the epithelium appeared to have more cell layers, and stratified flat squamous superficial cells were identified at later stages (Fig. 5.2e, f) of healing compared to the control. Also, activation of Notch signalling (Fig. 5.2g-i) did not affect the start of migration (Fig. 5.2g), but in contrast to controls, the epithelial cells showed less cell layers, and less flat squamous superficial cells in the later stages of wound repair (Fig. 5.2h, i).

5.3.1.2. The number of epithelial cell layers as a function of Notch inhibition / activation in wounded cornea

The number of epithelial cell layers in the wound area following epithelial wounding is shown in Figure 5.3. In control corneas, there was an increase in 4-5 layers from 0 to 72 hours. After Notch inhibition, cell stratification appeared to be accelerated, reaching 7-8 layers at 72 hours. However, there was a significant decreased in the number of epithelial cell layers (2-3 layers) at 72 hours after the activation of Notch.

5.3.1.3. Immunolocalisation of Notch1, Notch2, Delta1 and Jagged1

As shown in Figure 5.4, by 72 hours epithelial wounding the immunolocalisation of Notch receptors in corneal epithelial wounding was similar to that in the human corneal epithelium reported in Chapter 3. Notch1 and Notch 2 were localised to

subrabasal and superficial layers with labelling absent in the basal cell layer (Fig. 5.4a, b). The expression of Delta1 and Jagged1 was also similar to that in human corneas. Immunolocalisation of Delta1 and Jagged1 was observed throughout the epithelial cells (Fig. 5.4c, d).

5.3.1.4.The effect of Notch inhibition and activation on cell proliferation in corneal epithelial wounding

As seen in Figure 5.5, immunolocalisation of Ki67 in epithelial wounding appeared to be localised to the basal layer and was similar to that seen in human corneas (Chapter 3), but the level of proliferation cell was altered following epithelial wounding. After a central epithelial wound, basal epithelial cell proliferation in the limbus and peripheral cornea was increased. The peak of proliferation occurred 24 hours after wounding in corneal limbus (Fig. 5.5a-c), periphery (Fig. 5.5d-f) and centre (Fig. 5.5g-i). Proliferation in the sheet of epithelium that migrated over the wound area dropped dramatically and reached normal levels by 72 hours after wounding (Fig. 5.5c, f, i).

Graphs shown in Figure 5.6 depict an increased percentage of Ki67 in the corneal limbus (Fig. 5.6a) from 18% to a peak of 37% between 0 and 24 hours. This then decreased to a normal level of proliferation at 18% about 72 hours after wounding. In the peripheral region of the cornea (Fig. 5.6b), there was an increased level of Ki67 positive cells from 38% to a peak of 65% between 0 and 24 hours. This then decreased to a normal proliferation level of 38% at 72 hours. In the central (near wound) region (Fig. 5.6c), there was an increase in Ki67 positive cells from 28% to a

peak of 48% between 0 and 24 hours of wound repair. This decreased to 20% by 72 hours after wounding.

After the inhibition or activation of the Notch signalling pathway, the cellular expression of Ki67 in the corneal limbus (Fig. 5.7b, c), periphery (Fig. 5.7e, f), and centre (Fig. 5.7h, i) showed no obvious changes at any time points after corneal epithelial wounding when compared to control (Fig. 5.7a, d, g). Representative results (24 hours after wounding) are shown in Figure 5.7. This result suggests that corneal epithelial cell proliferation might not be regulated by the Notch signalling pathway in this model.

As shown in Figure 5.8, after Notch inhibition and activation, there was no significant changes in cellular expression of Ki67 in corneal limbal (Fig. 5.8a), peripheral (Fig. 5.8b) and central (Fig. 5.8c) regions during corneal epithelial wound healing.

5.3.1.5.The effect of Notch inhibition and activation on cell differentiation in corneal epithelial wounding

For immunolocalisation of CK3 in rat cornea following epithelial wounding, positive labelling was detected in all the cell layers (Fig. 5.9a-i), but absent in the limbal basal layer (Fig. 5.9a-c). These results were also similar to human corneas as reported in Chapter 3. As shown in Figure 5.10, there was no obvious change of the percentage of CK3 positive cells in rat corneal limbus (Fig. 5.10a), periphery (Fig. 5.10b), and centre (Fig. 5.10c) after epithelial wounding.

After the inhibition of Notch signalling pathway, the cellular expression of CK3 in the corneal limbus (Fig. 5.11b), periphery (Fig. 5.11e) and centre (Fig. 5.11h) showed no obvious changes in distribution, but superficial cells became larger, more flattened and squamous after epithelial wounding when compared to control (Fig. 5.11a, d, g). Representative results (72 hours after wounding) are shown in Figure 5.11. However, there was an obvious decrease of CK3 expression in the limbus (Fig. 5.11c), periphery (Fig. 5.11f), and centre (Fig. 5.11i) after the activation of Notch signalling: the expression of CK3 reduced and appeared even only in superficial cell layers. This result indicated that corneal epithelial cell differentiation had been repressed by the Notch signalling pathway in the process of corneal epithelial wound healing.

Figure 5.12, shows the quantitive assessment of CK3 expression after Notch inhibition and activation. There were no significant changes of CK3 expression in corneal limbus (Fig. 5.12a), periphery (Fig. 5.12b) and centre (Fig. 5.12c) after Notch inhibition. However, activation of Notch signalling induced a significant decrease of CK3 expression in corneal limbus (Fig. 5.12a, from 65% to 25%), periphery (Fig. 5.12b, from 90% to 25%), and centre (Fig. 5.12c, from 90% to 15%) during epithelial wound healing. This result further confirmed that the Notch signalling pathway can repress corneal epithelial cell differentiation.

5.3.2. The role of Notch in rat corneal stromal wound healing

Rat corneas were wounded and cultured with control medium or treatment media which included addition of γ-secretase inhibitor (50μM) or Jagged1 (10μg/ml), and maintained for varying periods of time up to 2 weeks (0 to 14 days). Corneal cryosections were stained with haematoxylin and eosin for morphological analysis,

immunolocalisation for detecting the distribution of Notch family members (Notch1, Notch2, Delta1, Jagged1), and immunostaining of Ki67 and CK3 to determine the proliferative and differentiation status of corneal epithelium during organ culture.

5.3.2.1. Morphological assessment in rat corneal stromal wounding

During stromal wound healing (deep of 80μm, Fig. 5.13), in control corneas (Fig. 5.13a-d), the epithelium was seen to start migration at 16 hours after wounding (Fig. 5.13a). By 72 hours, the wound has reepithelialised with 2-3 layers of epithelium (Fig. 5.13b). Hypercellularity was observed in the epithelium surrounding the wound by 7 days until 14 days (Fig. 5.13c, d).

Inhibition of Notch signalling (Fig. 5.13e-h), did not affect the migration start time (Fig. 5.13e), but the epithelium appeared more stratified in the late stages of wound healing (Fig. 5.13f, g, h). There were more cell layers with a larger number of flat squamous superficial cells. Activation of Notch signalling (Fig. 5.13i-l), also did not affect migration compared to control, but the epithelial cells showed less stratification with a lower number of cell layers and no obvious flat superficial cells were observed until 7 days after wounding (Fig. 5.13k).

The data analysis for epithelial cell layers at the wound area following a deep penetrating wound is shown in Figure. 5.14. In control corneas there was an increase in epithelial cell number up to 8-9 layers from 16 hours to 14 days. After Notch inhibition, cell stratification appeared quicker than control corneas reaching 12-13

layers by 14 days. In contrast to control, there was a significant decrease in epithelialisating cell layers after the activation of Notch.

5.3.2.2.Immunolocalisation of Notch1, Notch2, Delta1 and Jagged1

As shown in Figure 5.15, the immunolocalisation of Notch receptors in corneal stromal wounding was similar to that in the human corneal epithelium reported in Chapter 3. Notch1 and Notch 2 were localised to superficial and suprabasal layers with labelling absent in the basal cell layer (Fig. 5.15a, b). The expression of Delta1 and Jagged1 was also similar to that in human corneas. Immunolocalisation of Delta1 and Jagged1 was demonstrated throughout the epithelial cells (Fig. 5.15c, d).

5.3.2.3.The effect of Notch inhibition and activation on cell proliferation in corneal stromal wounding

In Figure 5.16, the graphs showed there was an increased percentage of Ki67 in the corneal limbus (Fig. 5.16a) from 18% to a peak of 38% between 0 and 72 hours. This then decreased to a normal level of proliferation at 18% by 14 days after wounding. In the peripheral region of the cornea (Fig. 5.16b), there was an increased level of Ki67 positive cells from 35% to a peak of 55% between 0 and 72 hours. This then decreased to a normal proliferation level of 35% at 14 days. In the central region (Fig. 5.16c), there was an increase in Ki67 positive cells from 25% to a peak of 36% between 0 and 72 hours of wound repair. This decreased to 25% by 14 days after wounding.

After Notch inhibition or activation, there was no significant change in cellular expression of Ki67 in corneal limbal (Fig. 5.17a), peripheral (Fig. 5.17b) and central (Fig. 5.17c) regions during corneal stromal wound healing. This result indicates that Notch signalling pathway might not regulate corneal epithelial cell proliferation during wound healing in the rat cornea.

5.3.2.4.The effect of Notch inhibition and activation on cell differentiation in corneal stromal wounding

After immunolocalisation of CK3 in rat cornea stromal wounding, positive labelling was counted. As shown in Figure 5.18, there was no obvious change of the percentage of CK3 positive cells in rat corneal limbus (Fig. 5.18a), periphery (Fig. 5.18b), and centre (Fig. 5.18c) after stromal wounding with an increased time.

As shown in Figure 5.19, there were no significant changes of CK3 expression in corneal limbus (Fig. 5.19a), periphery (Fig. 5.19b) and centre (Fig. 5.19c) after Notch inhibition. However, there was a significant decrease of CK3 expression in the corneal limbus (Fig. 5.19a, from 65% to 25%), periphery (Fig. 5.19b, from 90% to 25%), and centre (Fig. 5.19c, from 90% to 15%) after activation of Notch signalling during stromal wound healing. This result further confirmed that the Notch signalling pathway represses corneal epithelial cell differentiation.

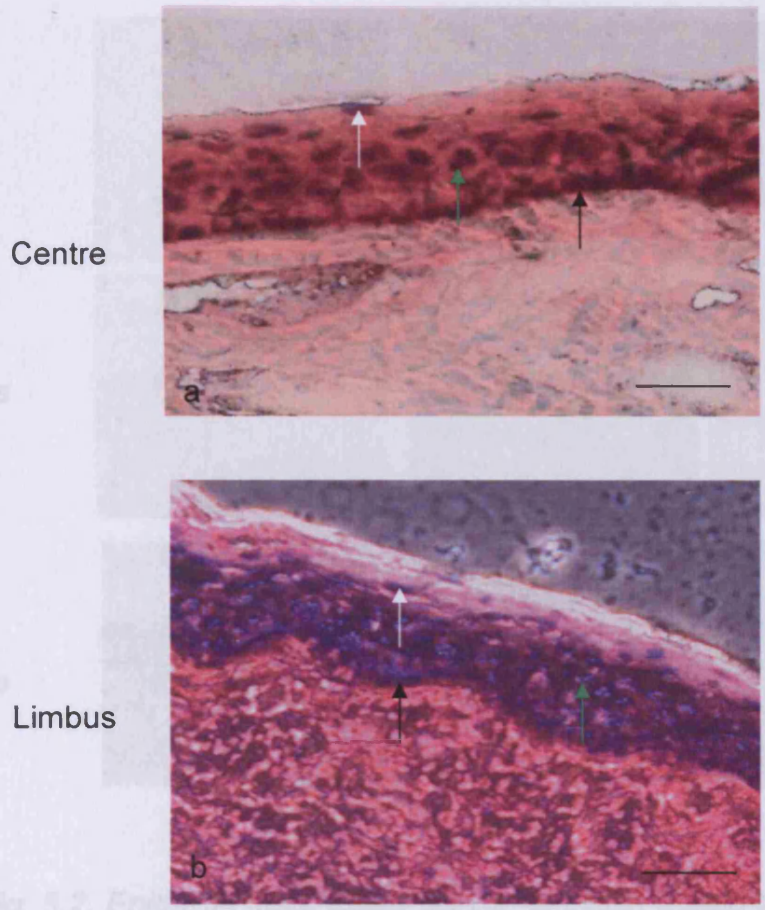


Fig.5.1 Epithelial morphology in unwounded rat cornea. (a) central corneal epithelium had 5-6 layers, and (b) limbal epithelium had 7-8 layers. The epithelium was composed of columnar basal cells (black arrows), suprabasal or wing cells (green arrows) and superficial flattened epithelia (white arrows). Bar=50 µm

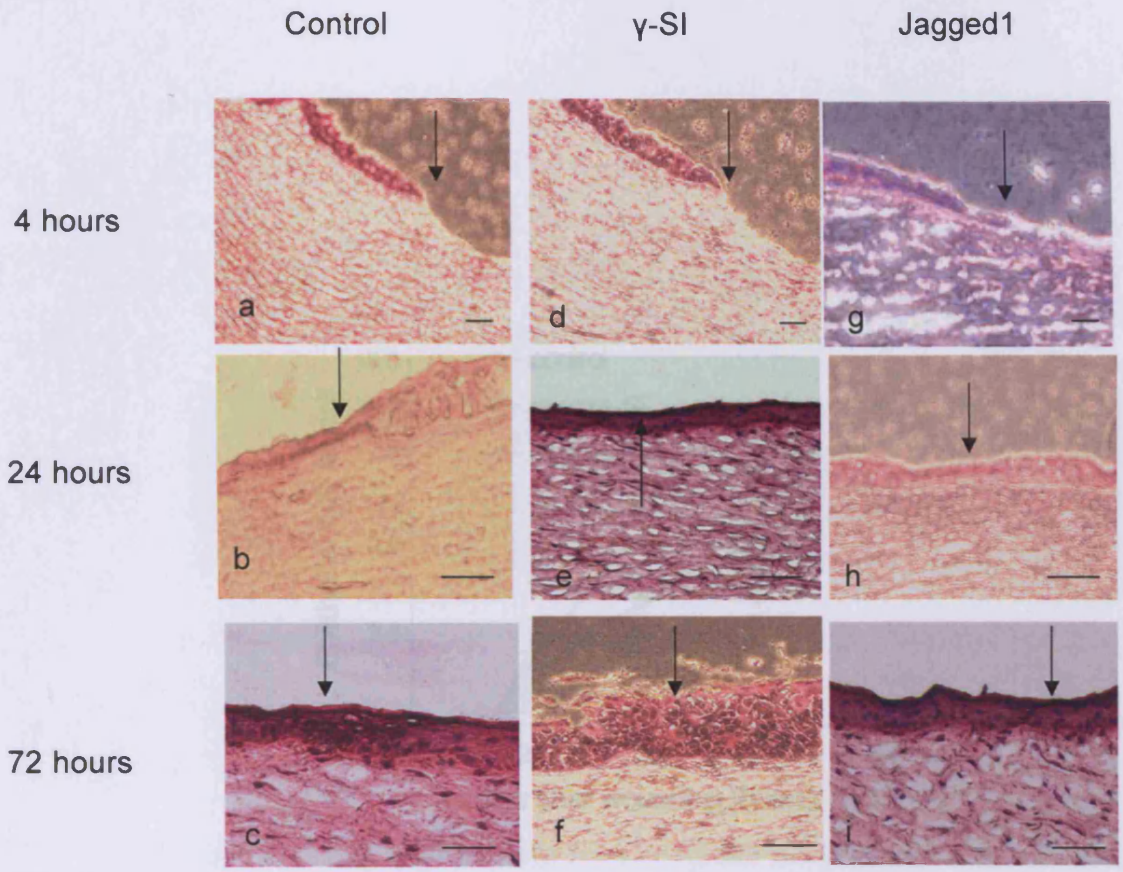


Fig. 5.2. Epithelial morphology during epithelial wound healing. In control corneas (a-c), the epithelium is seen to start its migration (black arrow) 4 hours post wounding (a) then some thining of the epithelial layers over the original wound edge (b, black arrow). By 72 hours, the wound has completely reepithelialised (c, black arrow). Inhibition of Notch signalling (d-f), the epithelium exhibited more stratified at wound healing late stage (e,f, black arrows). Activation of Notch (g-i), the epithelial cells showed less stratification in the late stages of wound healing (h,I, black arrows). Bar=50 μm

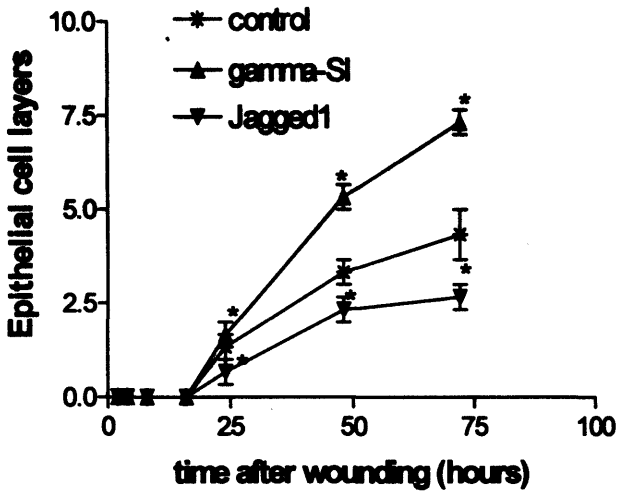


*Fig. 5. 3. The number of epithelial cell layers in the wound area in rat corneal epithelial wounding after treatment of γ-SI (γ-secretase inhibitor) and Jagged1 at different time points. There was a significant increase or decrease of cell layers after the Notch inhibition or activation, respectively. Each time point represents the mean of at least 3 corneas ± SEM. *P<0.05.*

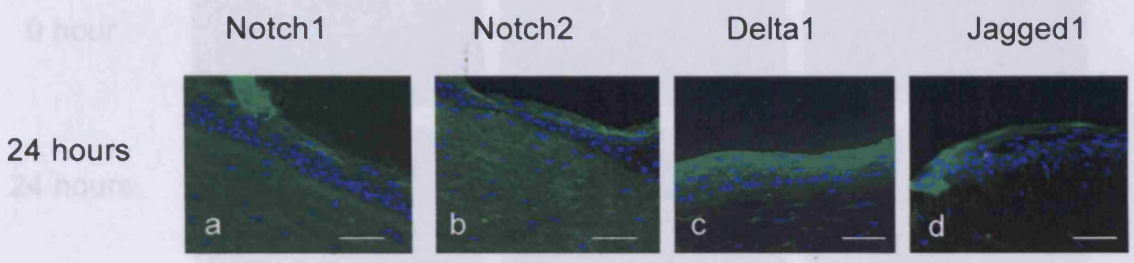


Fig. 5.4. Immunolocalisation of Notch family members in rat corneas following corneal epithelial wounding (72 hours). The expression of Notch1 and Notch2 was apparent in epithelial superficial and suprabasal layers after wounding (a, b); Delta1 and Jagged1 appeared to be expressed in all the epithelial cell layers after epithelial wounding (c, d). Bar=50 μm

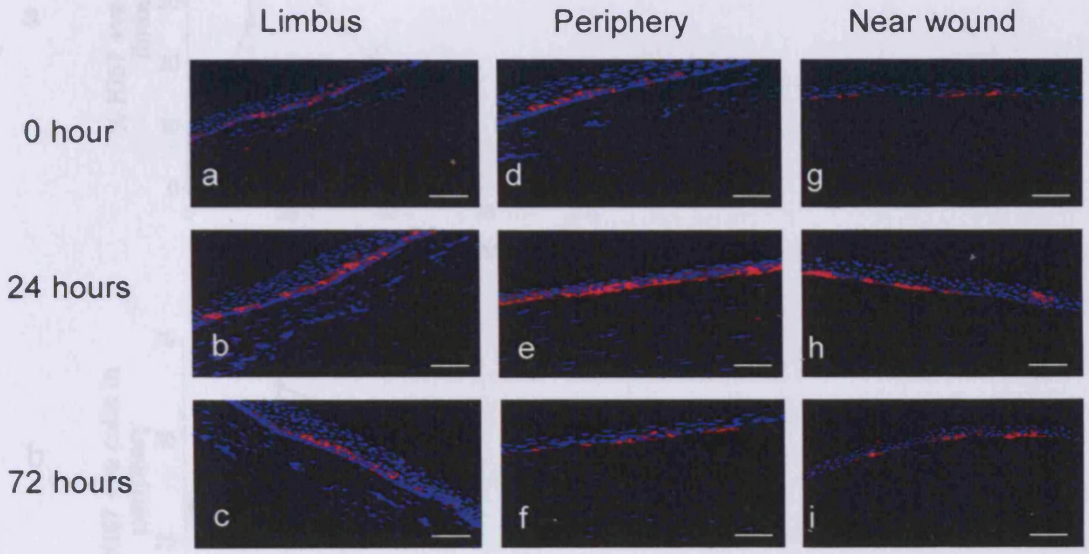
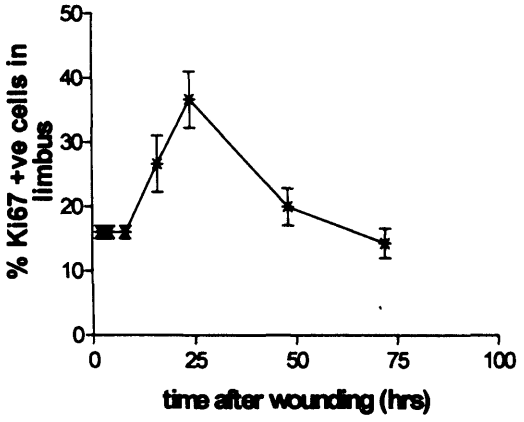
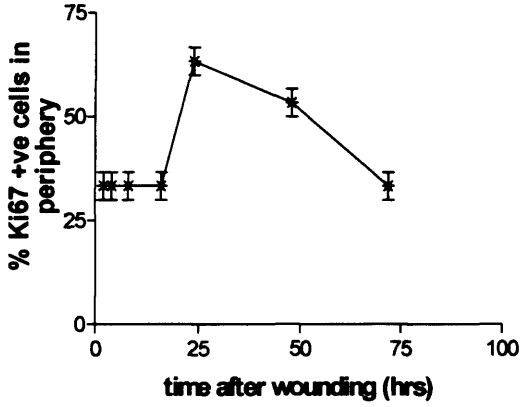


Fig. 5. 5. The immunolocalisation of Ki67 in rat corneas following epithelial wounding at different time points. Proliferation is shown in the limbus (a-c), periphery (d-f) and in central cornea (near wound, g-i). Basal epithelial cell proliferation in the limbal and peripheral corneas (red) was increased after wounding. The peak of proliferation occurred 24 hours after wounding (b, e, h), this then decreased to a normal proliferation level at 72 hours (c, f, i). Bar=50 μm

a



b



c

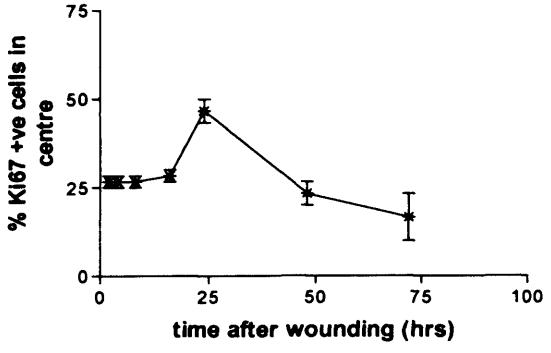


Fig. 5.6. Percentage of Ki67 positive cells in rat corneal epithelial cells during epithelial wound healing. There was an increased percentage of Ki67 in the corneal limbus (a), periphery (b) and centre (c) by 24 hours after wounding, these then decreased to normal proliferation levels at 72 hours.

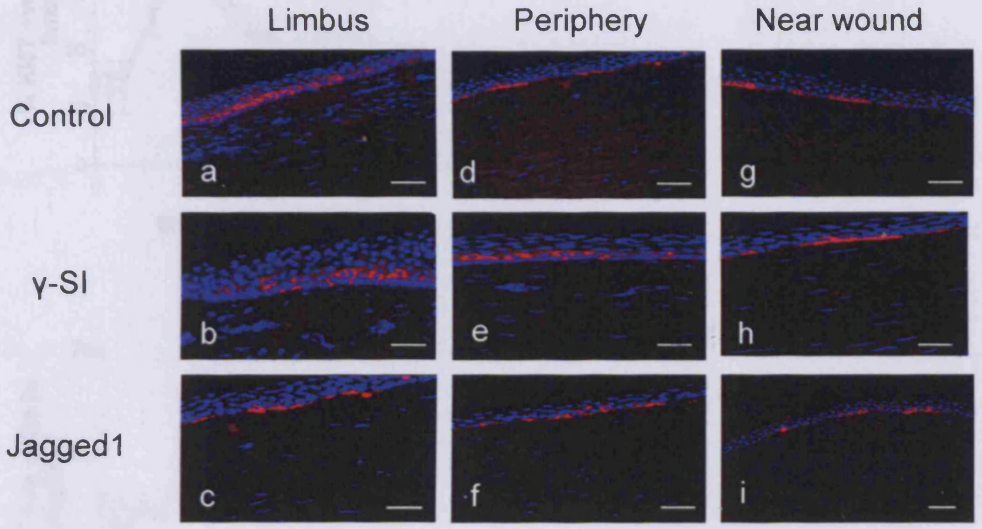
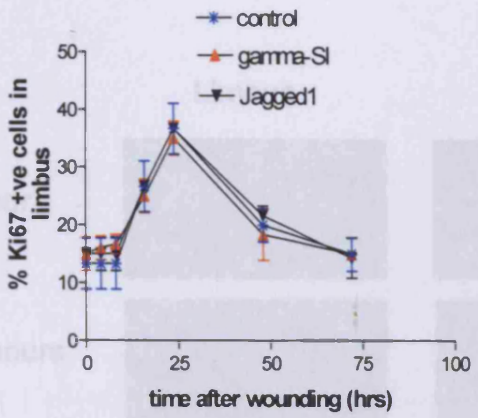


Fig. 5. 7. The immunolocalisation of Ki67 24 hours after corneal epithelial wounding following Notch inhibition (by γ-SI) and activation (by Jagged1). Proliferation is shown in the limbus (a-c), periphery (d-f) and in the central near wound (g-i). No changes in Ki67 expression in three corneal regions were identified following Notch inhibition (b, e, h) or activation (c, f, i) compared to control corneas (a, d, g). Bar=50 μm

a



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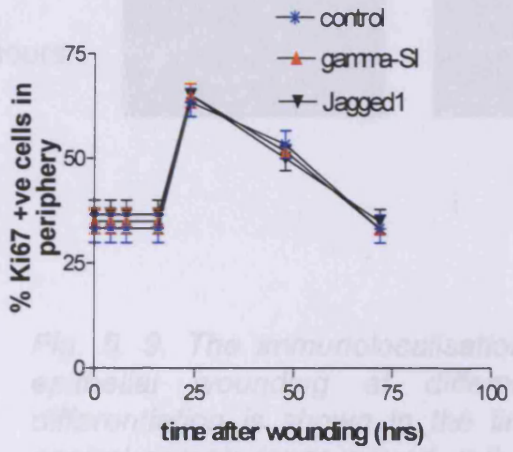

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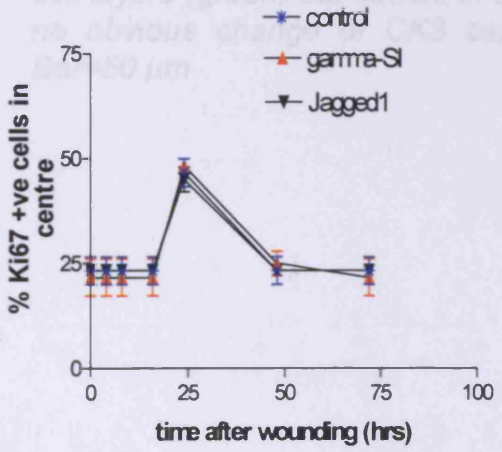


Fig. 5.8. Percentage of Ki67 positive cells in rat corneal epithelial cells after Notch inhibition (γ-secretase inhibitor, γSI) or activation (Jagged1) during epithelial wound healing. There were no significant changes of Ki67 expression in the corneal limbus (a), periphery (b) and centre (c).

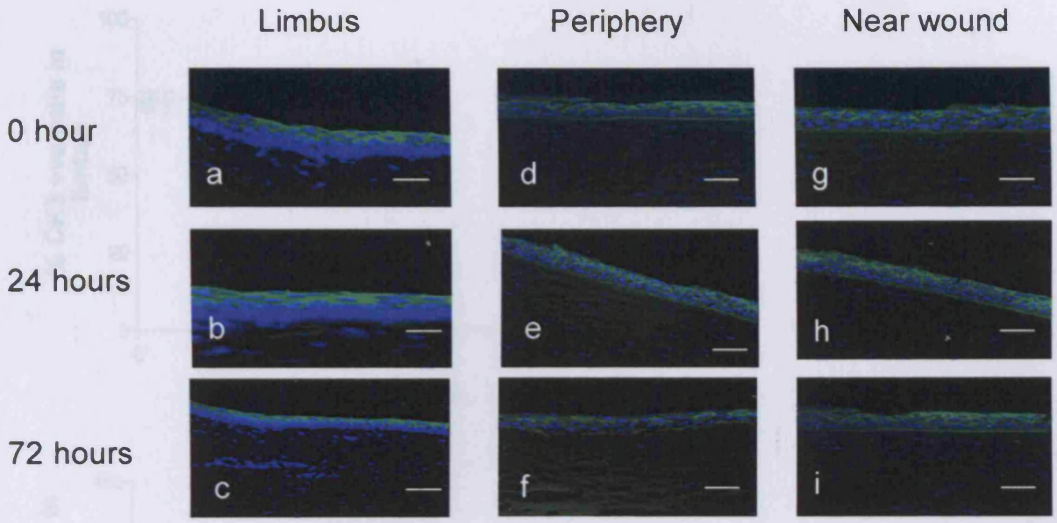
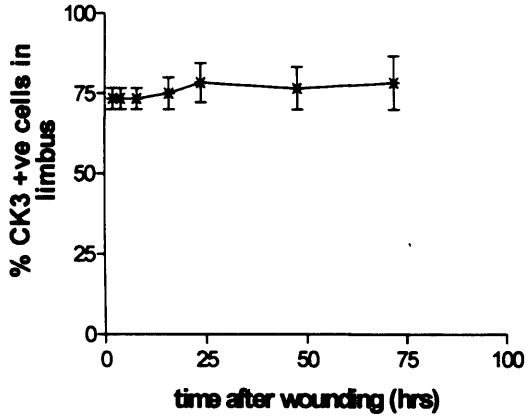


Fig. 5. 9. The immunolocalisation of CK3 in rat corneas following epithelial wounding at different time points. Epithelial cell differentiation is shown in the limbus (a-c), periphery (d-f) and in central cornea (near wound, g-i). CK3 is expressed in all epithelial cell layers (green) but absent in the limbal basal layer (a-c). There is no obvious change of CK3 expression after epithelial wounding. Bar=50 μm

a



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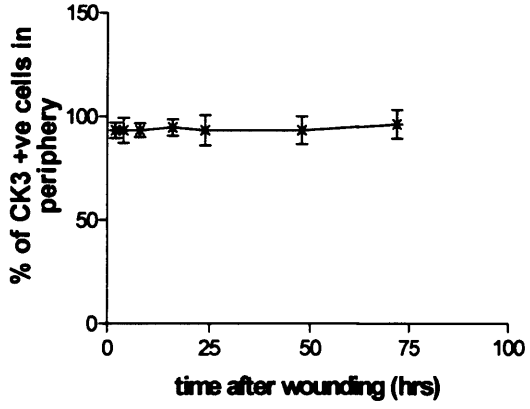

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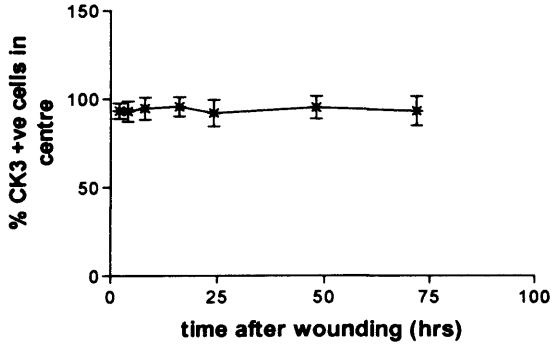


Fig. 5.10. Percentage of CK3 positive cells in rat corneal epithelial cells during epithelial wound healing. There was no obvious change of CK3 expression in the corneal limbus (a), periphery (b) and centre (c) after wounding over 72 hours.

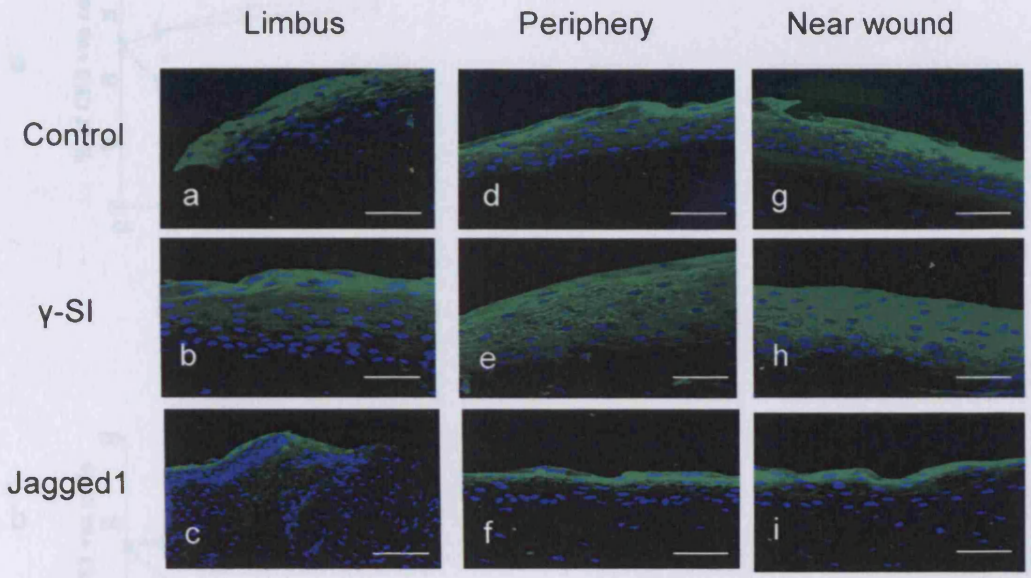
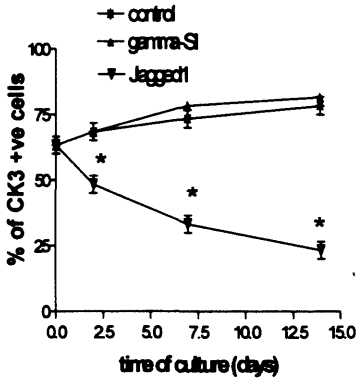


Fig. 5. 11. The immunolocalisation of CK3 in rat corneas 72 hours after epithelial wounding with treatment of Notch inhibition (γ-SI) and activation (Jagged1). Epithelial cell differentiation is shown in the limbus (a-c), periphery (d-f) and in central cornea (near wound, g-i). CK3 is expressed in all epithelial cell layers (green) but absent in the limbal basal layer (a-c). There was no change of CK3 expression after Notch inhibition (b, e, h) but a significant decrease of CK3 after Notch activation (c, f, i). Bar=50 μm

a



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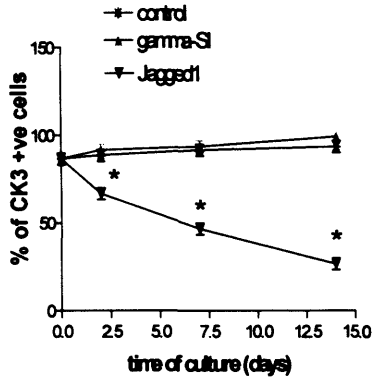

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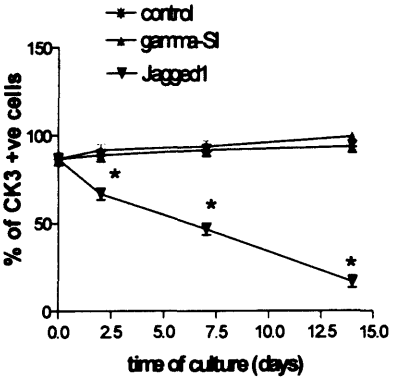

*Fig. 5.12. Percentage of CK3 positive cells in rat corneal epithelial wounding with treatment of Notch inhibition or activation. There were no significant changes of CK3 expression with Notch inhibition in limbus (a), periphery (b) and centre (c), but there were significant decreases of CK3 with Notch activation in corneal limbus, periphery and centre (a-c) in contrast to control. *P<0.05.*

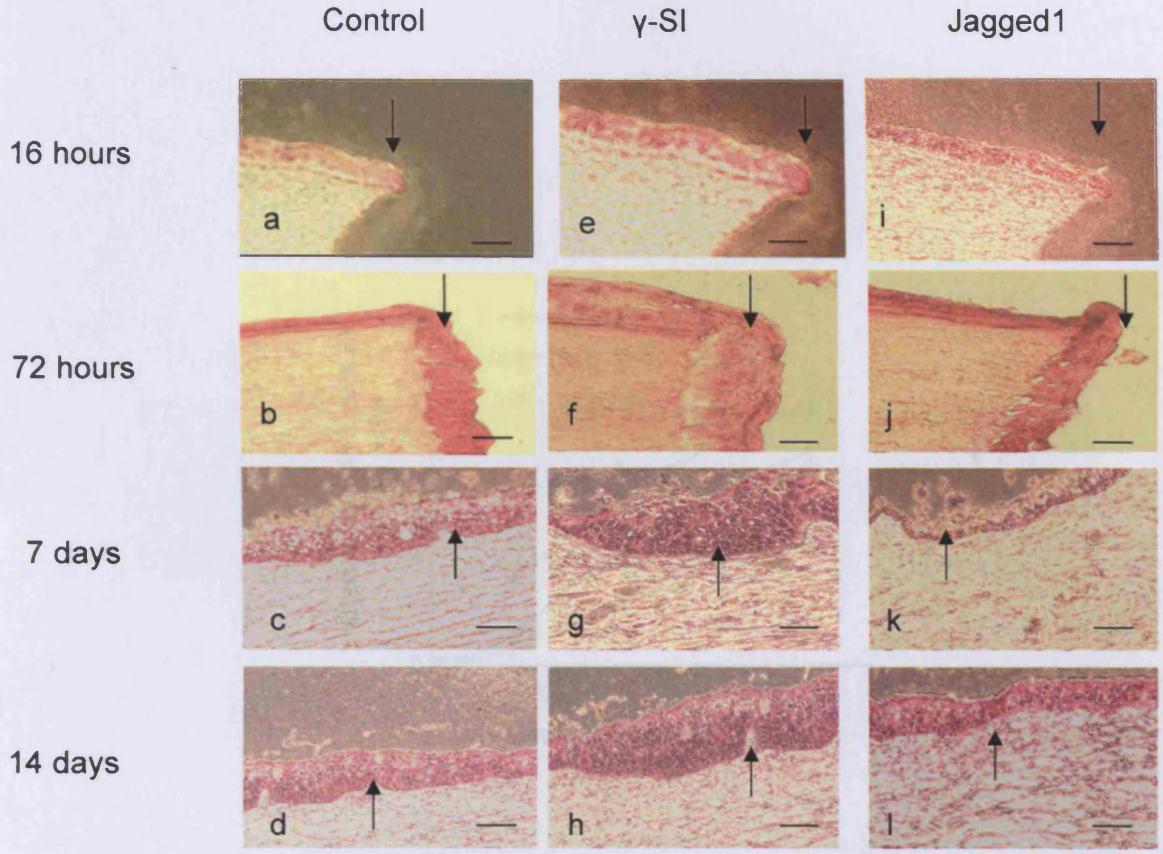

Fig. 5.13. Epithelial morphology during stromal wound healing. In control corneas (a-d), the epithelium is seen to start its migration (black arrow) 16 hours post wounding (a). By 72 hours, the wound has completely reepithelialised (b, black arrow). Hypercellularity (black arrows) was observed in the epithelium surrounding the wound after 7 days (c, d). Inhibition of Notch signalling (e- h), the epithelium exhibited initiation of migration (black arrow) by 16 hours post wounding (e), and was more stratified (black arrows) in the late stages of wound healing (g, h). Activation of Notch (i-l), the epithelial cells to start their migration (black arrow) at same time compared to control (i), but showed less stratification (black arrows) in wound healing late stages (k, l). Bar=50 μm

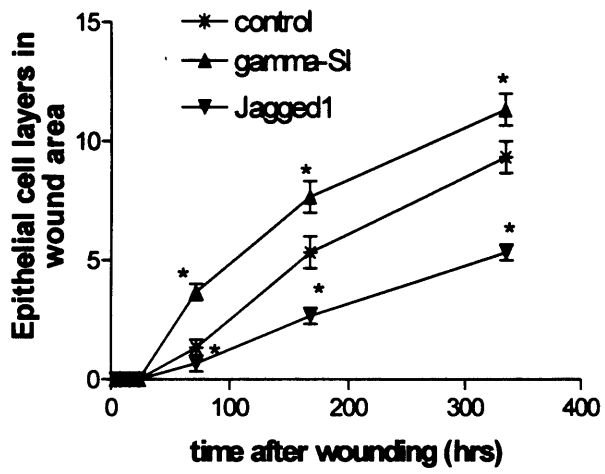

*Fig. 5. 14. The number of epithelial cell layers at the wound area in rat corneal stromal wounding after treatment of γ-SI (γ-secretase inhibitor) and Jagged1 at different time points. There was a significant increase or decrease of cell layers after Notch inhibition or activation, respectively. Each time point represents the mean of at least 3 corneas ± SEM. *P<0.05.*

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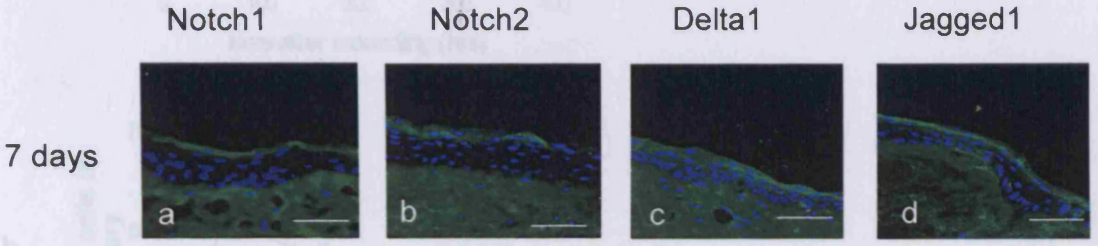
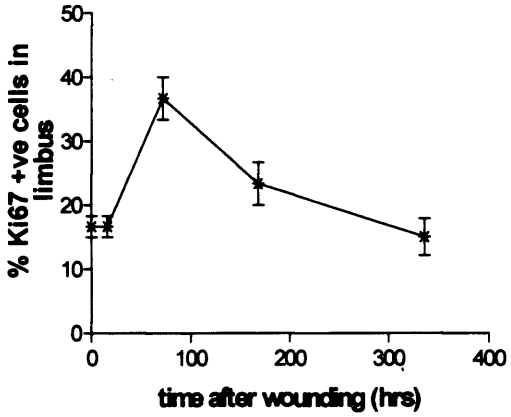


Fig. 5.15. Immunolocalisation of Notch family members in rat corneas following corneal stromal wounding (7 days). The expression of Notch1 and Notch2 was apparent in epithelial superficial and suprabasal layers after wounding (a, b); Delta1 and Jagged1 appeared to be expressed in all epithelial cell layers (c, d). Bar=50 μm

a



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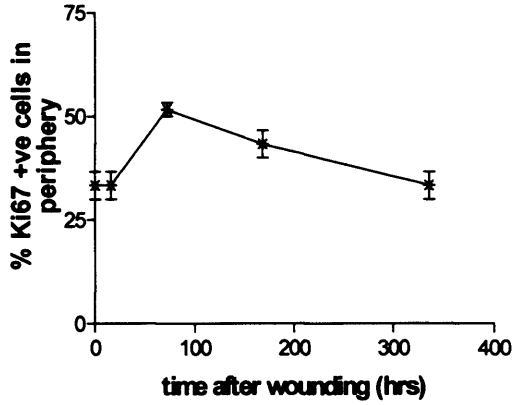

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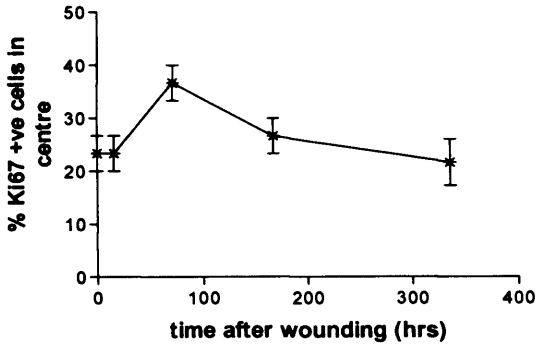
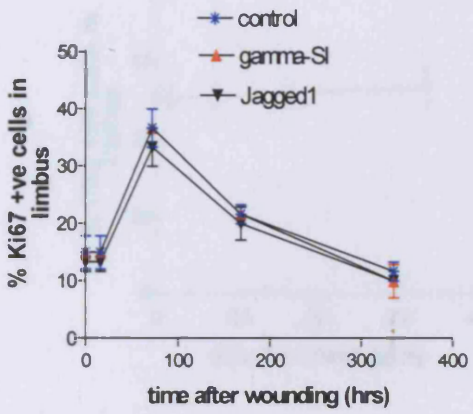


Fig. 5.16. Percentage of Ki67 positive cells in rat corneal epithelial cells during stromal wound healing. There was an increased percentage of Ki67 in the corneal limbus (a), periphery (b) and centre (c) by 72 hours after wounding, this then decreased to normal proliferation levels at 14 days.

a



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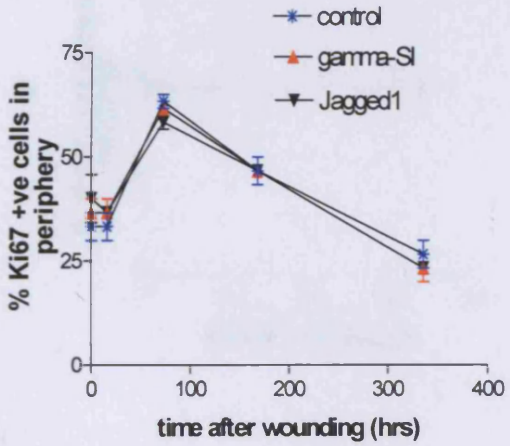

c

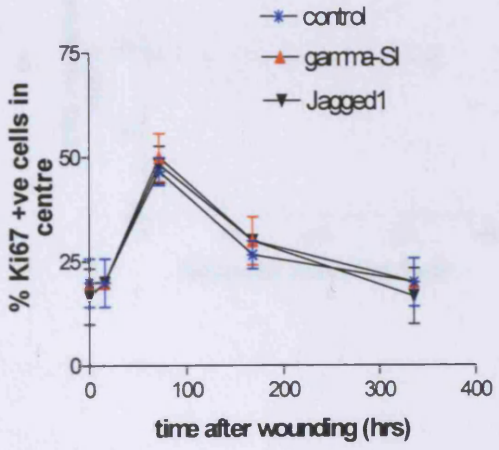

Fig. 5.17. Percentage of Ki67 positive cells in rat corneal epithelial cells after Notch inhibition (γ-secretase inhibitor) or activation (Jagged1) during stromal wound healing. There was no significant change of Ki67 expression in the corneal limbus (a), periphery (b) and centre (c).

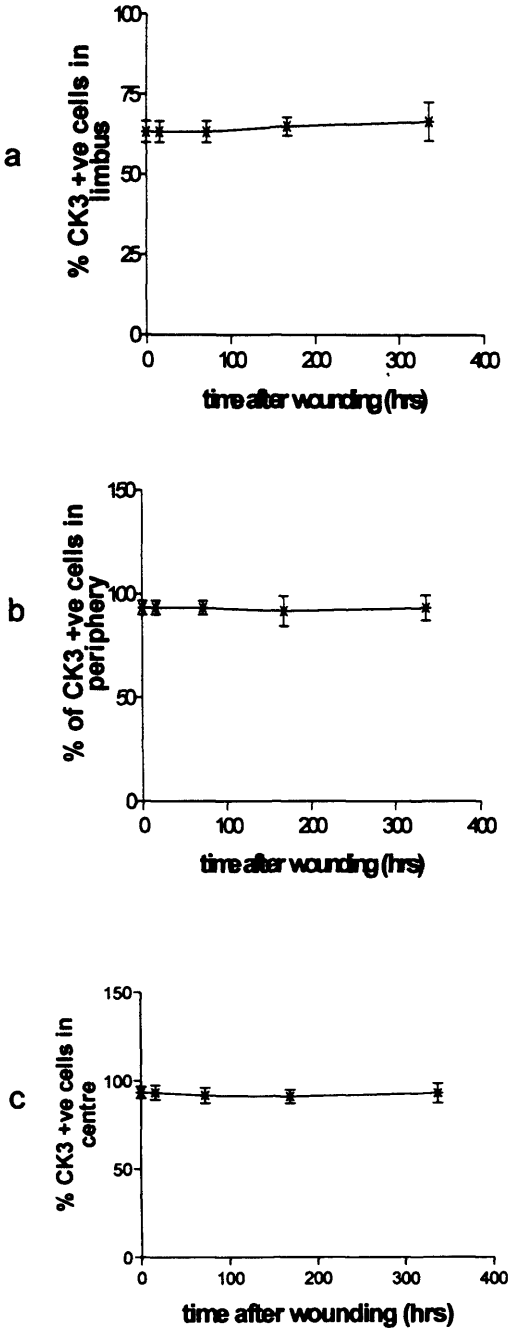

Fig. 5.18. Percentage of CK3 positive cells in rat corneal epithelial cells during stromal wound healing. There was no significant change of CK3 expression in the corneal limbus (a), periphery (b) and centre (c) after wounding up to 14 days.

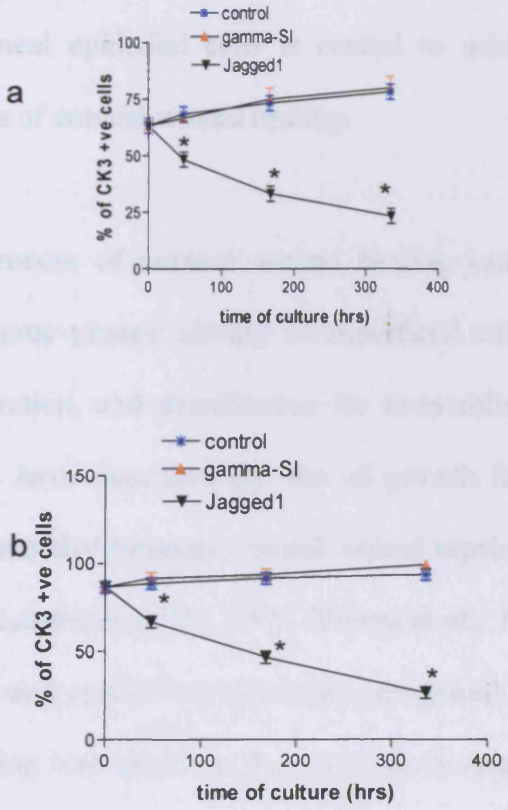

*Fig. 5.19. Percentage of CK3 positive cells in rat cornea following Notch inhibition or activation. There were no significant changes of CK3 expression with Notch inhibition (γ-secretase inhibitor) in limbus (a), periphery (b) and centre (c), but there were significant decreases of CK3 with Notch activation (Jagged1) in corneal limbus, periphery and centre (a-c) in contrast to control. *P<0.05.*

5.4. Discussion

Defining the cellular mechanisms responsible for the differentiation and proliferation of corneal epithelial cells is central to achieve a complete understanding of the process of corneal wound healing.

The process of corneal wound healing can be divided into several distinct but continuous phases: sliding of superficial cells to cover the denuded surface, cell proliferation, and stratification for re-establishment of multicellular layers. Several studies have described the use of growth factors that are synthesized by cells as molecules that promote corneal wound repair (Brazzell et al., 1991; Sotozono et al., 1995; Lambiase et al., 1998; Wilson et al., 1999). In addition, interactions between stroma and epithelium have been recognised as important for proper healing. Notch signalling was shown to be involved in epithelial cell homeostasis in the previous chapters. Thus in this chapter, the corneal organ culture model (Foreman et al., 1996) was used to mimic in vivo wound healing, which allowed investigation of the role of Notch signalling in corneal wound healing.

Notch, a transmembrane receptor member of the epidermal growth factor-like family of proteins, participates in cell-to-cell signalling to control cell fate in many different tissues in a wide range of organisms (Egan et al., 1998). Since Notch signalling is known to function as a developmental tool to direct cell fate and consequently organ formation and morphogenesis it is likely that Notch and its ligands play a role in regulating cell patterning and differentiation of the corneal epithelium. In corneal epithelial cells, despite various attempts aimed at identifying the factors that initiate

corneal wound healing, the mechanisms involved in the determination of cell fate decisions remain obscure. In this study, Notch signalling was demonstrated for the first time in corneal wound healing.

The major finding of this experiment is that the Notch signalling pathway is critical in the cornea in response to environmental stimuli such as injury. After Notch inhibition or activation, there was no change of Ki67 expression in corneal epithelial and stromal wound healing. However, there was a statistically significant decrease of CK3 expression in both types of injuries after activation of Notch. The results suggest that Notch signalling is involved in mediating epithelial cell differentiation including cell stratification and maturity, but does not appear to be involved in the regulation of epithelial cell proliferation in corneal wound healing. This is consistent with the previous results in Chapter 3 and Chapter 4 which showed that Notch signalling did not influence the epithelial cell proliferation in corneal tissue. The regulation of Notch signalling to cell proliferation may be reprogrammed or inhibited due to the specific location of basal cells and their contact with basement membrane.

Findings of particular note include: in early stages of corneal wound healing 4 hours after epithelial wounding, 16 hours after penetrating wounding, the epithelial cell shape and number of cell layers at the wound edge indicated that there was no change after Notch inhibition or activation. However, in the later stages (24 hours after epithelial wounding, 72 hours after stromal wounding), there was a significant promotion of epithelial cell stratification after inhibition of Notch signalling: a larger number of cell layers and more typical morphology of the mature (differentiated) epithelium. In contrast, significant repression of cell stratification via activation of

Notch signalling: a less number of cell layers and a typical morphology of epithelium. This was not surprising considering that the Notch pathway may reflect the inhibition of cell differentiation which starts at the later stage of wound repair, rather than cell proliferation which occurs in early stages of wound healing.

The cornea is a target tissue for all of the major families of growth factors. These include epidermal growth factors (EGF), fibroblast growth factors (FGF), platelet derived growth factors (PDGF), insulin like growth factors (IGF) and transforming growth factors beta (TGFβ). Also corneal wound healing is affected by many other factors including the size of the wound, its depth, causative agent and tear quality. Does the regulation of Notch interact with other growth factors? These will need to be investigated in the future.

The ability to modulate corneal wound healing to achieve better clinical outcomes would be beneficial in several situations. Results from in vitro experiments and various animal models of corneal wound healing suggest that it should be possible to modulate healing of epithelial or stromal with addition of potent growth factors, extracellular matrix components, and protease inhibitors (Coulter et al., 1980; Woost et al., 1985; Gallar et al., 1990; Woost et al., 1992). In this thesis, the results suggest that the Notch signalling pathway contributes to the molecular regulation of corneal wound healing. Characterisation of Notch in corneal wound repair will facilitate the development of strategies to manipulate corneal epithelial cell homeostasis in wound healing and in ocular surface diseases.

CHAPER SIX

THE EXPRESSION OF NOTCH FAMILY MEMBERS IN EMBRYONIC CORNEAL DEVELOPMENT

6.1. Introduction

The development of the human eye is a complex series of orderly events that begins with the fertilization of the ovum and continues until the early postnatal period. Although the tendency is to depict these changes in distinct stages, numerous events happen simultaneously. Not only may interrelations between ocular tissues influence their development, but one ocular tissue may induce the formation of another. Impairment or interruption of these events may result in congenital abnormalities of the eye. The earlier the disruption, the more severe the anomaly (Duke-Elder, 1963).

Embryogenesis begins with cell proliferation, cell movement, and changes in cell shape. Individual cells contain the entire genome of the organism, and therefore have the potential to become any one of the roughly 200 classes of cells in the body. At some time and place, however, embryonic cells make a commitment to a particular developmental path; this determination means that the cell's future options have become limited (Alberts, 1994). The next step is differentiation, which makes the commitment explicit; differentiation means that the cell begins to manufacture the proteins and intracellular organelles necessary for the lifestyle to which it has been committed. Also, the cell usually acquires a characteristic shape and structure that provide observable evidence of differentiation (Maclean, 1987).

Eye development begins 4 weeks after fertilisation. Once the surface ectoderm separates from the lens vesicle, it differentiates into a two-layered epithelium. This structure, which rests on a basal lamina, is the primitive cornea. By the end of week 6,

junctional complexes appear between cells. In week 7, mesenchymal cells derived from neural crest migrate forward from around the lens vesicle in three waves:

- First wave of cells migrates between the surface ectoderm and lens to form the corneal endothelium;
- Second wave migrates between the corneal epithelium and endothelium to form the stroma;
- Third wave migrates between the corneal endothelium and lens to form the iris stroma.

The corneal endothelium form as a two-cell layer of cuboidal cells. In week 8 these cells produce a basement membrane, Descemet's membrane (Maclean, 1987).

In month 3, fibroblats and collagen fibrils appear. The fibroblasts begin synthesis of the glycosaminoglycan ground substance. Keratan sulfate production becomes apparent in the cornea. Bowman's layer is first noted in month 4; it develops as an extension of filaments from the basal lamina of the epithelium. It is also around this time that tight junctions form between the apices of the endothelial cells. Further development results in enlargement of the cornea and dehydration of its stroma to form a transparent structure (Maclean, 1987).

Notch receptors and ligands are widely expressed during organogenesis in embryos. Although some Notch receptors appear to have genetically functions in some developmental contexts, e.g. hematopoiesis, neurogenesis, somitogenesis, and vasculogenesis (Artavanis-Tsakonas et al., 1999), we still have not known yet the role of Notch in the development of cornea, e.g. does the Notch signalling regulate the corneal epithelial cell lineages? To investigate the expression of Notch signalling

during corneal development and provide a context for our discussion of Notch signalling in human corneal homeostasis, a model from embryonic chick was set up at vary time points by embryonic day (see below) for this study.

6.2. Project design

6.2.1. The collection of embryonic chick

Embryonic chicken corneas were used as models for investigation of developing corneas in this study. At all times the ARVO statement for the use of animals in ophthalmic and vision research was adhered to, as were local rules.

Fertile white leghorn chicken eggs (Henry Steward and Co, PO Box 7, louth, lincolnshire, UK) were transferred to a 38°C incubator (Octagon 100, Brinsea, sandford, England) on embryonic day 0 (D0) for incubation. For corneal cryosection immunofluorescently staining, eggs were removed from the incubator at the appropriate age (D5, D8, D10, D12 and D20), the chicken corneas were cryostated and sectioned at 8μm on SuperFrost Plus slides, and stored at -20°C (see section 2.2.9.1).

6.2.2. Haematoxylin and Eosin staining

To examine corneal tissue morphology from embryonic chicken at different stages, haematoxylin and eosin staining have been accessed by the same method as section 2.2.9.2.

6.2.3. Immunoflurescent staining for Notch family members

After these collections, the immunolocalisation of Notch family members (Notch1, Notch2, Delta1 and Jagged1) have been processed by immunoflurescent staining as same method as section 2.2.9.3.

6.3. Results

6.3.1. Haematoxylin and Eosin staining for embryonic chicken at different stages

Haematoxylin and Eosin staining in embryonic chicken corneal epithelial cells showed that as early as 5 days of embryonic development (Fig. 6.1.), corneal limbus still attached with epithelium of lens, but the presumptive morphology of cornea was already formed in this time embryo. By D10 (Fig. 6.2), the epithelium was approximately 4 cells thick in corneal centre. Stromal fibroblasts were present 8 cells thick. On D12 (Fig. 6.3), in corneal centre the epithelia had continued to stratify becoming 5 cells thick and the stromal fibroblasts were present 10 cells thick. Endothelium was 2-3 cells thick. At developmental day 20 (Fig. 6.4), the embryonic chicken corneal epithelium was well-stratified (approximately 5 cells thick) with 10-12 cell layers of fibroblasts.

6.3.2. The expression of Notch family members in embryonic chicken corneas

To identify the temporal and spatial localisation of Notch receptors and their ligands

in embryonic chicken, immunolocalisation was undertaken. The results showed that as early as 8 days of embryonic development (D8), Notch1 and Notch2 were localised to all the presumptive corneal epithelium in central, peripheral and limbal regions of the cornea (Fig. 6.5, 6.6). Meanwhile, Delta1 and Jagged1 also were localised to all layers of the corneal epithelium (Fig. 6.7, 6.8).

By day 10, immunofluorescent localisation of Notch1 and Notch2 were demonstrated throughout the all corneal epithelium in central, peripheral and limbal regions of the cornea (Fig. 6.5, 6.6). Delta1 and Jagged1 also were localised to all layers of the corneal epithelium (Fig. 6.7, 6.8).

By day 12, immunolocalisation of Notch1 and Notch2 demonstrated their presence throughout the corneal epithelium in all suprabasal and superficial cells, but absent in the basal epithelial cell layer (Fig. 6.5, 6.6). Delta1 and Jagged1 were localised to all epithelial cells in all regions (Fig. 6.7, 6.8).

On D20, immunolocalisation of Notch1 and Notch2 still has been found throughout the corneal epithelium in all suprabasal and superficial cells, but absent in the basal epithelial cell layer (Fig. 6.5, 6.6). In contrast, Delta1 and Jagged1 were localised to all layers throughout all regions of the corneal epithelium (Fig. 6.7, 6.8). Negative control showed Hoeschst-positive blue nuclear staining (Fig. 6.5, 6.6, 6.7, 6.8) without immunoreactivity.

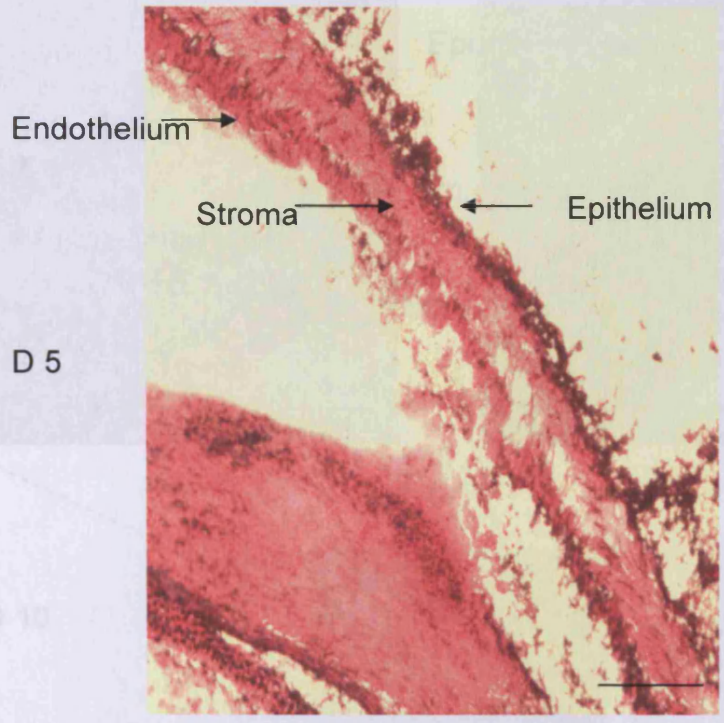

Fig. 6.1. Haematoxylin and Eosin staining for embryonic chicken at Day 5. Bar=50 μm

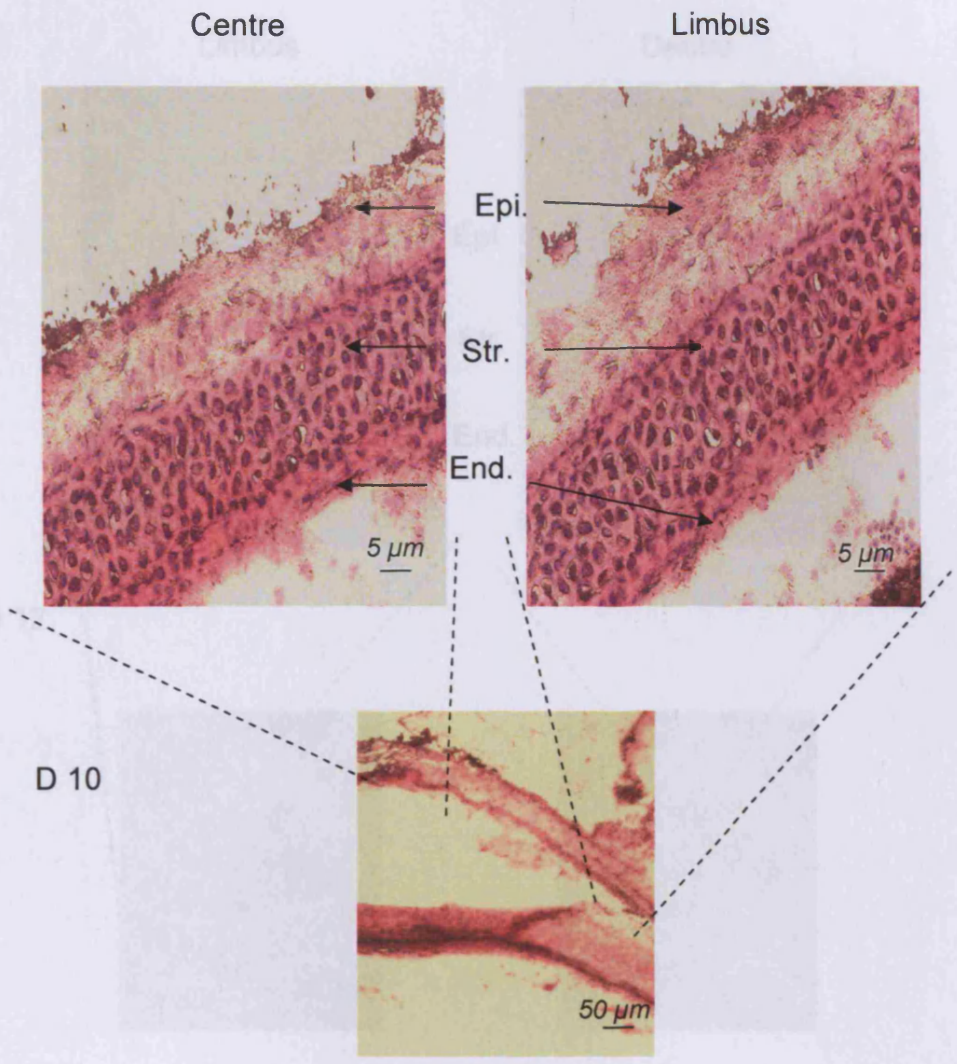

Fig. 6.2. Haematoxylin and Eosin staining for embryonic chicken at Day 10. Epi: epithelium; Str: stroma; End: endothelium.

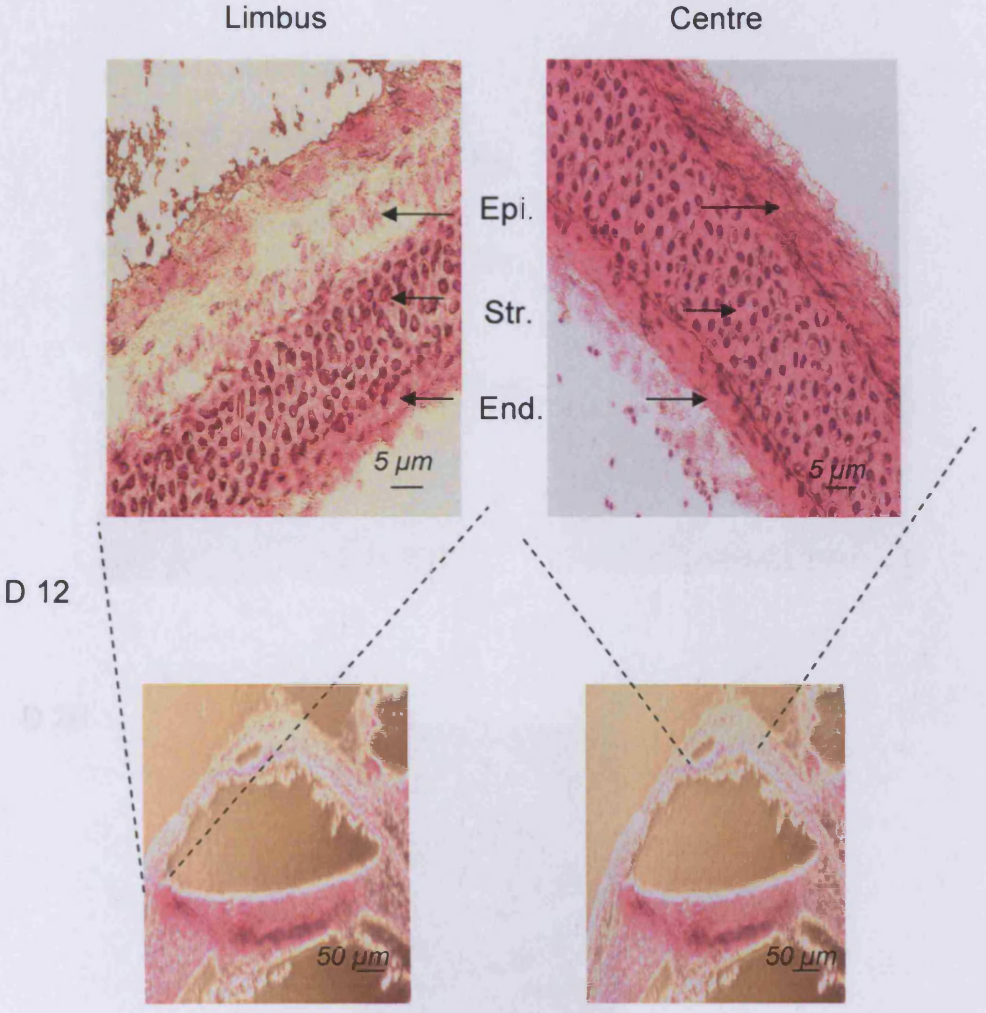

Fig. 6.3. Haematoxylin and Eosin staining for embryonic chicken at Day 12. Epi: epithelium; Str: stroma; End: endothelium.

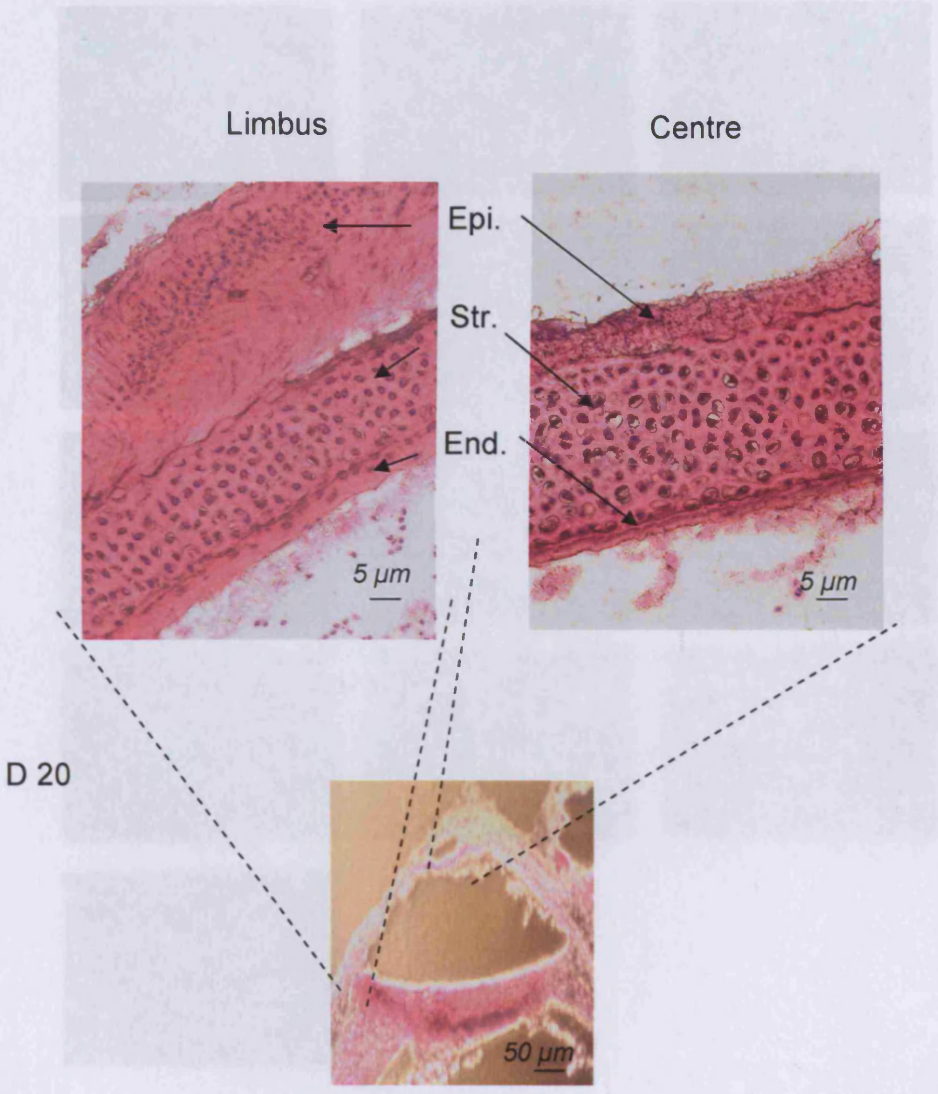

Fig. 6.4. Haematoxylin and Eosin staining for embryonic chicken at Day 20. Epi: epithelium; Str: stroma; End: endothelium.

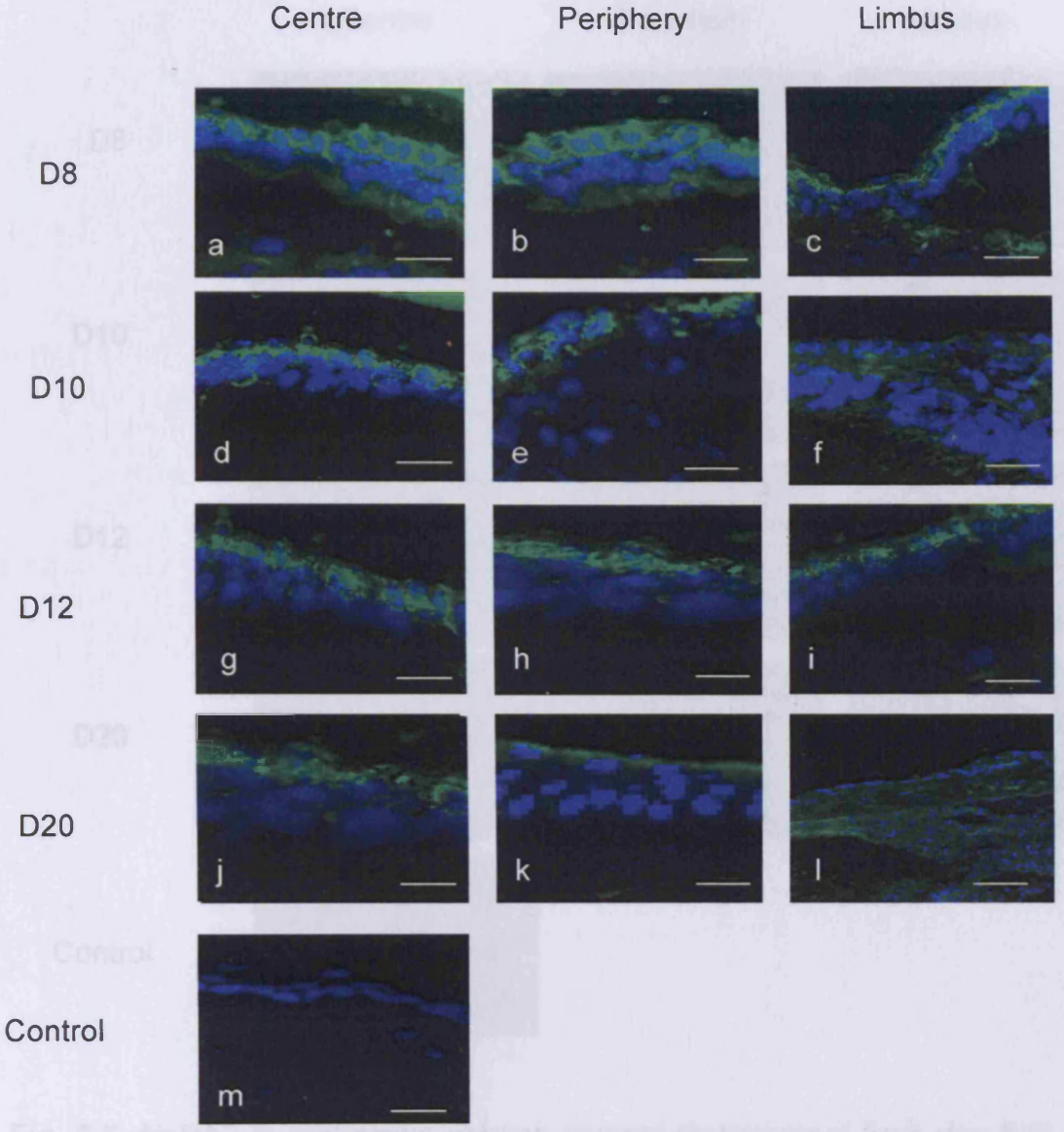

Fig. 6.5. Notch1 in embryonic chicken corneal development from day 8 to day 20. Notch1 was localised to all layers of the corneal epithelium at day 8 (D8, a-c) and day 10 (D10, d-f). After day 12 (g-l), Notch1 was demonstrated their presence throughout the corneal epithelium in all suprabasal and superficial cells, but absent in the basal epithelial cell layer in central, peripheral and limbal regions of the cornea. Green is positive staining, blue indicates cell nuclei. m is negative control. *Bar=50μM*

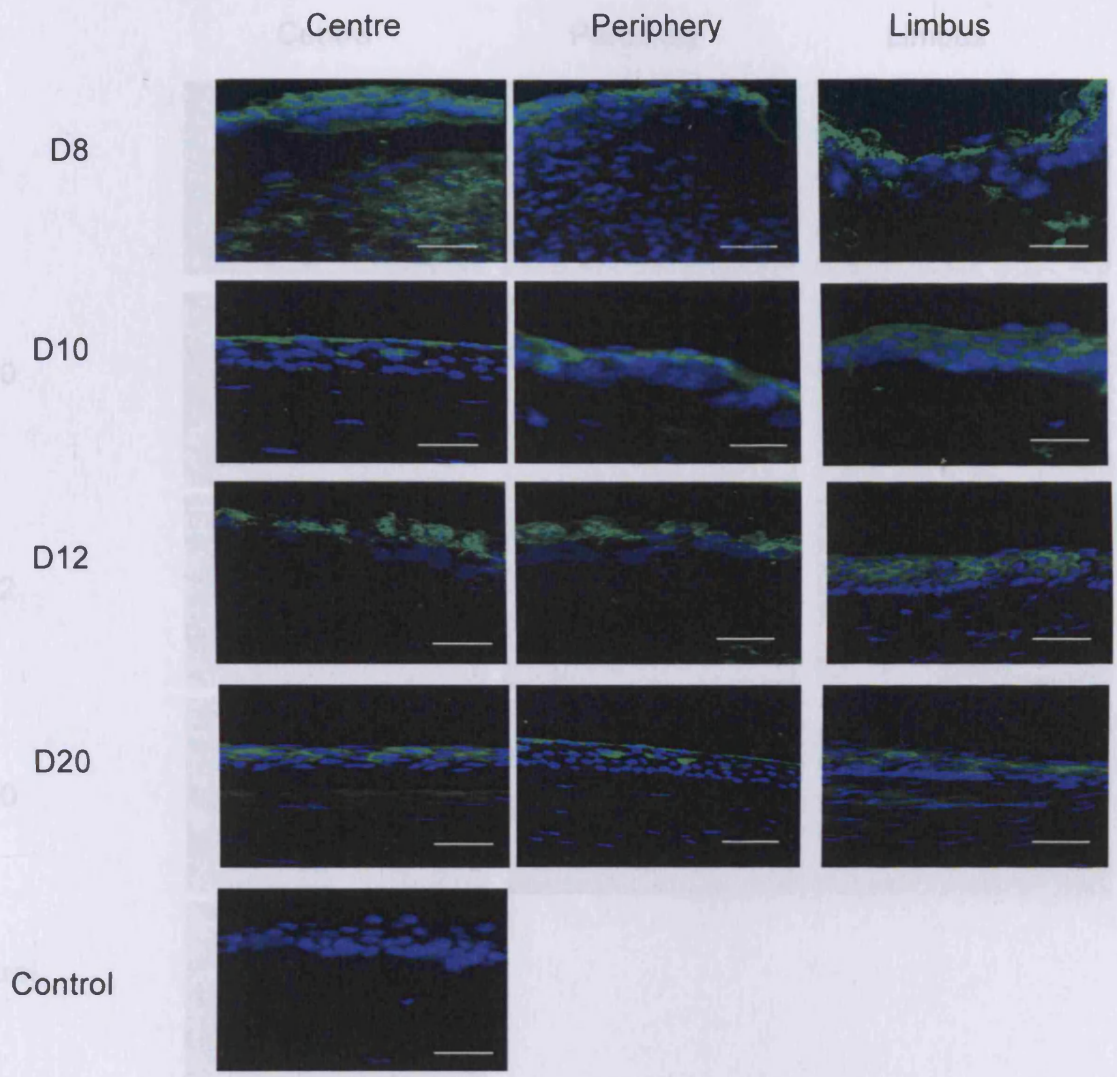

Fig. 6.6. Notch2 in embryonic chicken corneal development from day 8 to day 20. Notch2 appeared to be expressed in all layers of the corneal epithelium at day 8 (D8) and day 10 (D10). After day 12, Notch2 was demonstrated their presence throughout the corneal epithelium in all suprabasal and superficial cells, but absent in the basal epithelial cell layer in central, peripheral and limbal regions of the cornea. Green is positive staining, blue indicates cell nuclei. *Bar=50μm*

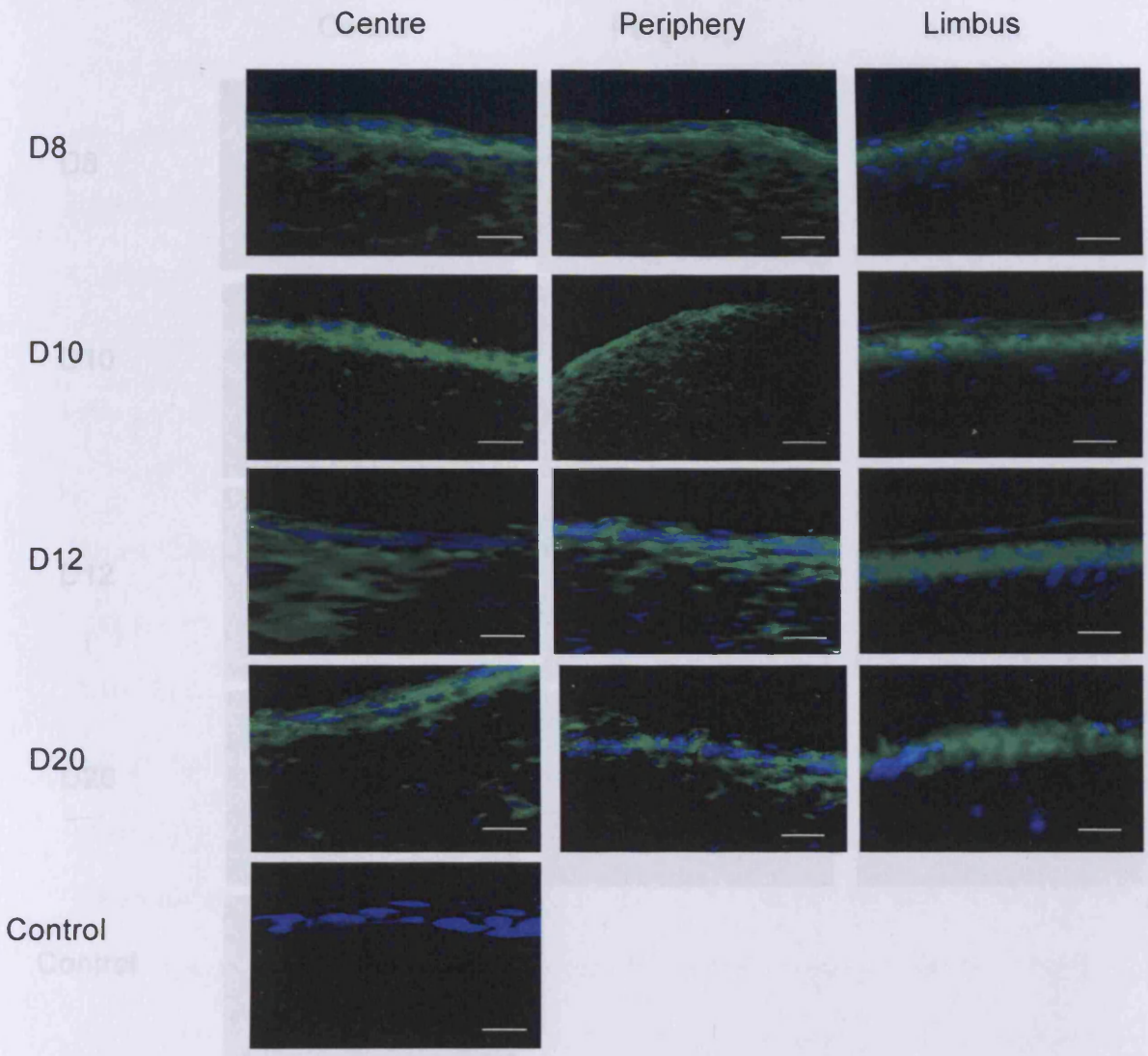

Fig. 6.7. Delta1 in embryonic chicken corneal development from day 8 to day 20. Delta1 was localised to all layers throughout all regions (centre, periphery and limbus) of the corneal epithelium. Bar=50 µm

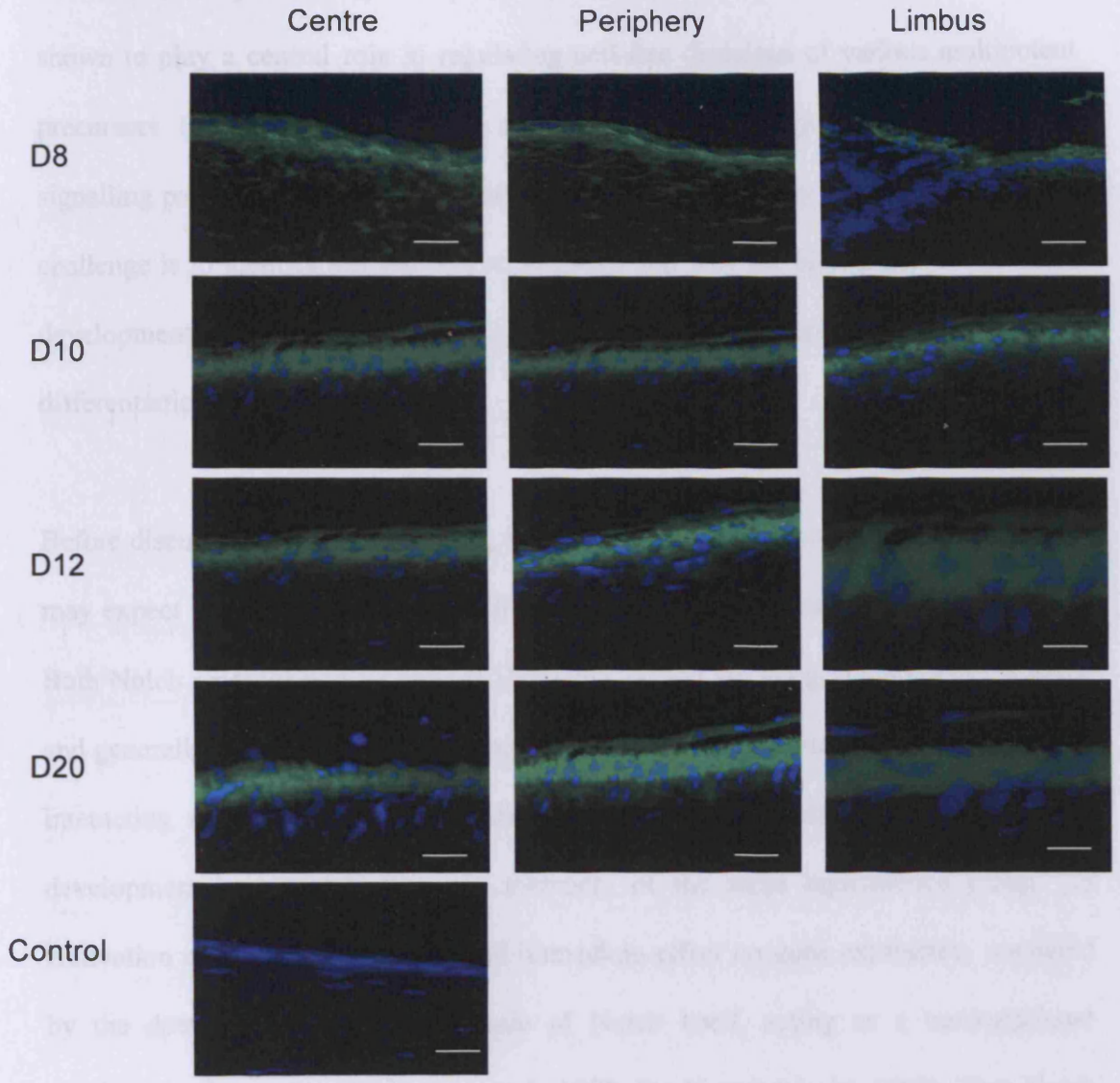

Fig. 6.8. Jagged1 in embryonic chicken corneal development from day 8 to day 20. Jagged1 was localised to all layers throughout all regions (centre, periphery and limbus) of the corneal epithelium. Bar=50 μm

6.4. Discussion

In other developmental systems, Notch-mediated cellular interactions have been shown to play a central role in regulating cell-fate decisions of various multipotent precursors (Artavanis-Tsakonas et al., 1999). When components of the Notch signalling pathway are genetically damaged or perturbed, many things go wrong. The challenge is to identify not just where, but how and why the effects occur. For tissue development, what part does it play in defining the spatial pattern of cell differentiation?

Before discussing specific examples, it is helpful to consider some principles that one may expect to apply to Notch signalling generally (Kimble and Simpson, 1997). (1) Both Notch receptor and its ligands Delta and Jagged are integral membrane proteins and generally transmit signals only between cells in direct contact. In many cases, the interacting neighbours are cells with a similar developmental history and similar developmental potential- they are members of the same equivalence group. (2) Activation of Notch has a direct and immediate effect on gene expression, mediated by the detached intracellular domain of Notch itself, acting as a transcriptional regulator in the nucleus (Schroeter et al., 1998; Struhl and Adachi, 1998). Thus Notch signalling can readily throw genetic switches that determine choices of cell fate. (3) Activation of Notch in a given cell frequently regulates production of Notch ligands by that cell. Because the level of Notch activation in the cell depends on the level of ligand expression in its neighbours, and this gives rise to feedback loops that correlate the fates of adjacent cells and control the fine detail of the spatial pattern of differentiation (Collier et al., 1996; Lewis, 1996) which had been served to amplify

the effect of so-called 'lateral inhibition'. Lateral inhibition is a mechanism that explains how two identical adjacent cells can be induced in vivo to differentiate to different tissues during development (Lewis, 1998).

However Notch signalling can also be responsible for the contrary phenomenon, lateral induction, activation of Notch promotes production of Notch ligands (de Celis and Bray, 1997), it will cause cell to make their fate choices cooperatively, preventing the occurrence of all-or-none behaviour and the formation of defined boundaries of gene expression (Artavanis-Tsakonas et al., 1999).

In vertebrates, the most thoroughly analysed example of Notch signalling is in control of neurogenesis. Notch signalling here operates between cells within an equivalence group, mediates lateral inhibition, and controls the commitment to differentiate. In the neural tube - the rudiment of the vertebrate central nervous system - neurons are generated from dividing precursors whose cell bodies lie in a proliferative zone close to the lumen of the tube. When a progenitor divides, its progeny have a choice. Each daughter can either remain a progenitor or become committed to differentiate as a neuron; in the latter case, it withdraws from the cell division cycle and migrates out into the mantle zone of the neuroepithelium, where it differentiates (Myat et al., 1996). Notch1 is expressed throughout the proliferative zone; Delta1 is expressed in the outer part of that zone, in a scattered subset of cells (Myat et al., 1996). From their location and their non-dividing character, these Delta-expressing cells can be identified as nascent neurons (Henrique et al., 1995). The lateral inhibition mediated by Delta-Notch signalling is the mechanism that regulates the choice between remaining as a progenitor and embarking on differentiation (Artavanis-Tsakonas et al., 1995). The

inhibition delivered from the differentiating progeny to their dividing progenitors acts homeostatically to limit the proportion of cells that differentiate, thereby maintaining a balanced mixture of the two classes of cells so that neurogenesis can continue (Henrique et al., 1995).

The chicken cornea develops in a series of discrete steps that have been reviewed previously (Coulombre, 1958; Hay, 1969). In brief, beginning at 3 days of incubation, an initial acellular extracellular matrix (termed the primary stroma) is deposited by the corneal epithelium on its basal surface. At 4 days, mesenchymal cells from the periphery migrate centrally on the undersurface of the primary stroma and form an epithelium that becomes confluent by 4.5 days. After 5 days, the ectodermal cells migrate and become corneal epithelial cells. Approximately a day later, day 6, the compact primary stroma rapidly swells, and is invaded by periocular mesenchymal cells. These cells differentiate into corneal fibroblasts and secrete elements of the secondary, or mature, corneal stroma. At 12 days, the secondary stroma begins to condense and ultimately becomes transparent and epithelial cells continue growth to 4-5 cell layers.

In this study immunohistochemical techniques were used. The results indicated that embryonic chicken corneal epithelium consistently contained high levels of Notch immunoreaction.

These results are consistent with the temporal and spatial expression of Notch described before which was hypothesized that Notch signalling may be involved in

regulating the proliferation and differentiation of corneal cells during development and that it may play a role in tissue maintenance in the adult cornea.

The most well characterised role of Notch signalling in embryonic chicken development is mediating the process of lateral inhibition, which refines the expression of Notch family members from corneal epithelial cells except corneal basal layer. In the early stages (before 10 days), the function of Notch signalling also regulates to basal epithelial cells where the corneal epithelial stem cells reside at. The expression of Delta1 and Jagged1 was also found in all epithelial cell layers, indicating the function of Notch signalling in corneal development.

This investigation significantly builds upon these earlier findings by correlating Notch family member expression with the actual development of multi-layered tissues and highlights the importance of precursor cells during this process. Moreover, a continued study of Notch signalling pathway in the developing corneal epithelium may ultimately lead to a better understanding of its molecular mechanisms and underlying function, potentially resulting in therapeutic treatments for corneal wound healing and disease.

CHAPER SEVEN

GENERAL DISCUSSION

Notch, a transmembrane receptor member of the epidermal growth factor-like family of proteins, participates in cell-to-cell signalling to control cell fate in many different tissues in a wide range of organisms (Egan et al., 1998).

Studies on embryonic mice and rats have shown the involvement of the Notch pathway in epidermal differentiation (Del Amo et al., 1992; Thelu et al., 1998). In the epidermal basal layer, Notch receptors are present in transit amplifying cells and the cell-cell signalling system promotes both expansion and differentiation of these cells (Lowell et al., 2000). Notch signalling also appears to control the differentiation of glial cells and later events during neurogenesis (Gaiano et al., 2000; Morrison et al., 2000).

The cornea is populated by a number of different cell types that communicate and cooperate in carrying out specific functions essential to multi-cellular organisms. The surface of the cornea is composed of a smooth continuous stratified epithelium and serves as the frontal barrier to the whole eyeball and maintains key optical features, such as refraction and transparency. To preserve these functions the corneal epithelium is in a constant state of cell renewal, which is in part dependent upon the integrity of basement membrane, cell-cell and cell-matrix interactions (Taliana et al., 2001; Connon et al., 2006). Defining the cellular mechanisms responsible for the differentiation and self-renewal of corneal epithelial cells is central to achieving a complete understanding of the process of corneal epithelial cell proliferation, differentiation and homeostasis.

7.1. Corneal epithelial cell proliferation

Epithelial cell proliferation can be regarded as a process involving a sequential pattern of (cyclic, repeating) changes in gene expression leading ultimately to the physical division of the cells. This is in contrast with cell growth, which involves an increase in cell size or mass. In this study corneal epithelial proliferation was assessed in vivo and vitro by immunolabelling for the Ki67 antigen (Delahunt et al., 1995). Ki67 is a marker for cell proliferation that is detected in all phases of the cell cycle in actively cycling cells, but is absent in no cycling cells (Gerdes et al., 1984). The cycling status of cells in the human cornea demonstrated Ki67 labelled proliferating cells in the limbal, central and peripheral region of the corneal basal epithelium and the corneal peripheral epithelium consistently had a higher percentage of labelled cells than did the limbal epithelium. No Ki67 staining was detected in either limbal or corneal suprabasal cells.

This is consistent with previous reports that the limbal epithelium contains a mixture of slow-cycling stem cells and more rapidly cycling transient amplifying cells. Transient amplifying cells have a limited regenerative capacity and migrate centripetally towards the central cornea. These cells are pushed upwards into the suprabasal corneal layers and at this stage they lose the ability to proliferate and become terminally differentiated (Cotsarelis et al., 1989; Lavker et al., 1991). The amount of staining is more intense in periphery compared to the central region. Ki67 was also localised in cultured epithelial cells and keratocytes from cornea. This result indicates that Ki67 labelling can be used to assess proliferation during cell growth. Furthermore, double-labelling of Ki67 with other proteins of interest can provide

valuable information regarding the conditions that stimulate cells to enter the cell cycle.

7.2. Corneal epithelial cell differentiation

Epithelial cell differentiation can be defined as a qualitative change in the cellular phenotype that is the consequence of the onset of the synthesis of new gene products, i.e. the non-cyclic changes in gene expression that lead ultimately to functional competence (Lajtha, 1979). All external surfaces of the body, including the cornea, are covered by stratified squamous epithelia. These cells synthesize tissue-restricted keratin intermediate filament proteins, and form a specialized submembrane structure. Cytokeratins comprise a diverse group of intermediate filament proteins (IFPs) that have been found to be useful markers of tissue differentiation (Eckert, 1989; Fuchs, 1990). The cornea-specific CK3 has been used as a marker for differentiating epithelial cells and confirmation of epithelial cell purity (Schermer et al., 1986). In this study, AE5, a highly specific antibody against 64 kDa CK3, has been used as a marker of differentiating epithelial cells.

The results of this thesis showed that CK3 localised in all cell layers of the human corneal epithelium, however, it was only identified in the supra-basal layers of the limbal epithelium and not observed in the basal layer of the limbus. These results are consistent with previous reports that the basal layer of the limbus contains a 'less-differentiated' population of cells than exists in the basal layer of the cornea, and that from basal layer to surface of the cornea the epithelial cells have become terminally differentiated (Schermer et al., 1986).

The fact that epithelial basal cells of the central cornea but not those of the limbus possess the 64 kDa keratin therefore indicates that corneal basal cells are in a more differentiated state than limbal basal cells. These results, coupled with the known centripetal migration of corneal epithelial cells strongly suggest that corneal epithelial stem cells are located in the limbus, and that corneal basal cells correspond to transient amplifying cells in the scheme that stem cells differentiate into transient amplifying cells which then become terminally differentiated cells (Schermer et al., 1986).

In corneal epithelial cells, despite various attempts aimed at identifying the factors that initiate corneal epithelial homeostasis and wound healing, the mechanisms involved in the determination of cell fate decisions remain obscure. In this study, for the first time Notch signalling protein components were identified in human corneal tissue as well as cultured epithelial and stromal keratocytes by immunostaining, RT-PCR and Western Blot analysis.

7.3. Notch receptors and ligands in cornea

Immunofluorescent localisation of Notch1 and Notch2 was demonstrated throughout the corneal epithelium in all suprabasal cells i.e. in the wing and superficial epithelial cells, in the central, peripheral and limbal regions. However, labelling was not detected in the epithelial basal layer where the epithelial cells were in varying proliferative states. In contrast, Notch ligands Delta1 and Jagged1 appeared to be expressed throughout all regions, and all cell layers of the corneal epithelium. These

results suggest that Notch signalling occurs through cell-cell interactions via transmembrane receptors and ligands, and that Notch may be involved in mediating epithelial cell differentiation including cell stratification and maturity which occurs at corneal epithelial superficial and suprabasal layers. This is consistent with previous reports that Notch signalling blocks terminal differentiation (Frise et al., 1996; Capobianco et al., 1997; Bigas et al., 1998; Artavanis-Tsakonas et al., 1999).

7.4. Notch signalling in the human corneal epithelial cells

The major finding of the present study is that two Notch receptors (Notch1, Notch2), their ligands Delta1, Jagged1 were identified in human corneal epithelial cells and keratocytes at both gene and protein levels. Western Blotting analysis confirmed the presence of Notch1, Notch2, Delta1 and Jagged1 in cultured epithelial cells. The position of the positive bands correspond with the molecular weight of Notch1-120 kDa and Notch2-85 kDa which are the molecular weight of intracellular domain of Notch1 and Notch2, Notch1-300 kDa and Notch2-285 kDa are full length of Notch1 and Notch2 (Fehon et al., 1990; Struhl and Adachi, 1998). These indicate that Western Blotting for both active and inactive isoforms of Notch1 and Notch2 can be used for investigation of Notch signalling function.

7.4a The role of Notch in corneal epithelial cell proliferation and differentiation

In the Notch signalling pathway, the remaining membrane-tethered Notch fragment is cleaved by γ-secretase within its transmembrane domain (De Strooper et al., 1999; Song et al., 1999; Struhl and Greenwald, 1999), which translocates into the nucleus,

where it participates in transcriptional activation of target genes (Jarriault et al., 1995; Fortini, 2002). Inhibition of γ-secretase prevents Notch signalling (De Strooper et al., 1999; Wolfe, 2001). A recombinant Jagged1 was used to induce of Notch signalling to confirm the function of Notch (Lindsell et al., 1995; Artavanis-Tsakonas et al., 1999). After addition of γ-secretase inhibitor, the treated epithelial cells analysed by Western Blotting and immunocytochemical staining. The results indicated that there was a significant decrease of Notch activation (Notch1-120, Notch2-85) accompanied by a significant reduction of Ki67 and an increased CK3 expression. In contrast to Notch inhibition, the expression of Notch1-120, Notch2-85 increased with an increased Ki67 and a decreased CK3 after addition of Jagged1. In agreement with Western Blotting analysis, the percentage of Notch1 and Notch2 expression in epithelial cell nuclei decreased with a decreased Ki67 and an increased CK3 after Notch inhibition. After Notch activation, the percentage of Notch receptors expression in cell nuclei increased with an increased Ki67 and a decreased CK3. These results have two implications: (1) Notch signalling is present in corneal epithelial cells regardless of whether they are actively differentiating or proliferating; (2) Notch pathway inhibits corneal epithelial cell differentiation, but promotes cell proliferation.

A primary function of the Notch signalling pathway is to prevent differentiation via activating a transcriptional repressor (Artavanis-Tsakonas et al., 1999). However, our *in vitro* studies suggest that the proliferative event can also be affected by Notch signalling. Important questions are: how to interpret the function of Notch signalling in proliferative cycling cells? why Notch does not appear to be expressed in proliferative area of corneal tissue?

A link between proliferation events and Notch has been reported previously (Cagan and Ready, 1989; de Celis et al., 1998; Go et al., 1998; Johnston and Edgar, 1998), although the elements mediating the nonautonomous effect of Notch on cell proliferation are unknown. The proliferative effect of Notch signalling in different tissues may be the result of a synergistic effect between Notch and other proteins and depends on the developmental context (Artavanis-Tsakonas et al., 1999). The mechanism for acquiring proliferative function of Notch in corneal tissue may be reprogrammed or inhibited and this may be related to the specific location of basal cells and their contact with the basement membrane. These localisation patterns suggested that the Notch pathway may involve a transitory stage where Notch is downregulated.

This suggests that the Notch pathway plays a pivotal role in corneal epithelial cell homeostasis and paves the way for understanding the process of corneal epithelium stratification and wound healing.

7.4b The role of Notch in corneal epithelial cell stratification

The process of corneal stratification occurs during corneal wound healing and corneal development to ensure that the tissue can resume or create its normal function in light refraction. Cell-cell junctions are an important feature of stratified epithelia, indicating functional communication within the tissue and suggesting the existence of a functional barrier. To monitor the development of a stratified epithelium formed in this study, morphologic and biochemical markers were used to demonstrate the degree of corneal epithelial stratification and tissue integrity. The term stratification in the

present study is used to describe the process by which corneal epithelial cells differentiate and develop distinctive sublayers that can be identified by morphologic and biochemical markers. This definition differs from the term differentiation, which refers to the process of epithelial cell programming for the expression of a characteristic phenotype (Watt, 1987).

The human amniotic membrane (AM) is known to be ideally suited for use in corneal epithelial stratification with a number of successful applications (Dhall, 1984; Rennekampff et al., 1994; Tsubota et al., 1996; Shimazaki et al., 1997; Tseng et al., 1998; Connon et al., 2006). The function of Notch signalling was investigated in human corneal epithelium stratification by using corneal limbal explants on AM, which were subjected to Notch signalling inhibition or activation. The results demonstrated there were significant changes of the number of epithelial cell layers and the percentage of CK3-a marker for cell differentiation, but there was no change in cell proliferation- Ki67 expression. These findings suggest that the Notch signalling repressed the ability of primary corneal epithelial cells to form a stratified epithelium via inhibiting cell differentiation rather than cell proliferation.

As reported previously, during eye organ culture storage, an obvious loss of epithelial cell layers was revealed (Albon et al., 2000). In this thesis, the number of epithelial cell layer decreased with a significant decrease of Ki67 expression during storage. It was apparent that the demand for tissue regeneration was not met, therefore proliferation disrupted homeostasis of corneal epithelium. However, the results also detected that the decrease of epithelial cell layers became significantly slower due to

inhibition of Notch. This provides a potential strategy for the maintenance of cornea storage with inhibition of Notch function.

7.4c The role of Notch in corneal wound healing

Notch-mediated cellular communications between cells of the cornea were very complex especially in the corneal wound healing process. For the regulation of Notch in corneal epithelial wound healing assays, an organ culture model (Foreman et al., 1996) was used to investigate Notch signalling in corneal wound healing with and without γ-secretase inhibitor or Jagged1 treatment. The results demonstrate that the Notch signalling repressed corneal wound healing at late stages via preventing epithelial cell differentiation and stratification.

As an application of Notch signalling in corneal wound repair, γ-secretase inhibitor therapy may be developed. The aims of the therapy are to promote corneal epithelial cell stratification, provide mature epithelium, prevent regression of re-epithelialisation of the cornea, and restore epithelial clarity.

7.4d The role of Notch in corneal development

The cornea develops from the ectoderm giving rise to the epithelium and neural crest cells which generates keratocytes, endothelial cells, and nerve cells (Oyster, 1999b). Cytokine- and growth factor-mediated communications are likely involved in directing neural crest cells to the appropriate location and regulating their proliferation, differentiation, and function in creating the adult cornea. The development of the Bowman's layer may involve interactions between the epithelium

and developing stroma (Oyster, 1999b). Little is known about the specific mediators involved. Since Notch signalling is known to function as a developmental tool to direct cell fate and consequently organ formation and morphogenesis it is likely that Notch and its ligands play a role in regulating cell patterning and differentiation of the corneal epithelium.

To reveal the expression of Notch family members in corneal development, embryonic chicken corneas at different stages were used for immunolocalisation of Notch members. The results demonstrated that the Notch receptors and their ligands appeared to be expressed in all epithelial cell layers including basal cell layer in early developmental stages (before day 10). This suggests that it is likely there are similar characteristics between different cell layers: superficial, suprabasal and basal layer, perhaps even in the epithelial stem cell niche in early stages of chicken cornea. After day 12 until day 20, Notch receptors were expressed in suprabasal and superficial layers of the epithelium, but were absent in the basal layer. This result is similar with this study from adult cornea. These suggest that (1) Notch signalling is required for the normal development of the cornea and may regulate both proliferation and differentiation of corneal epithelial cells at early developmental stage; (2) the expression of Notch in corneal epithelial cell may be reprogrammed at late developmental stage of chicken cornea, so the function of Notch signalling may be changed.

The ability of a cell to respond to Notch activation, as well as the outcome of that response, is dependent upon temporal as well as spatial cues. This suggests that the differentiated state or competency of a cell contributes greatly to its response.

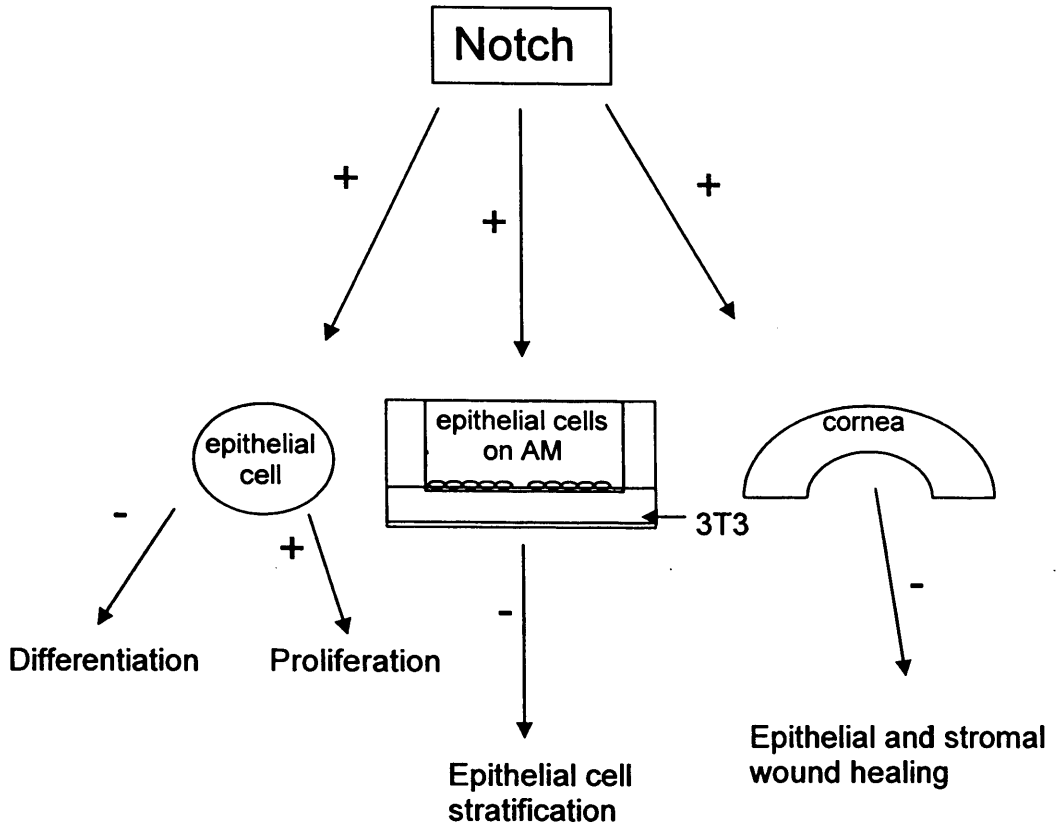

Fig. 7.1. Schematic diagram to illustrate the main findings of this thesis

Given this, it is perhaps not surprising that so many different domains of Notch receptors and ligands contribute to the overall flexibility of the system. Although many of the components are ubiquitously expressed, it is clear that differentially specialised cell types express tissue-specific components capable of modulating the Notch signal (Lewis, 1996, 1998). It would not be surprising, therefore, given the broad spectrum of cell types known to utilize Notch signals, to find many more cell-type specific regulators. Elucidating the multiple controlling elements in this complex signalling system promises to be one of the more challenging areas of future research in this field.

Figure 7.1 is schematic diagram to illustrate the main findings of this thesis.

7.5. Conclusion

The presence of Notch family members (Notch1, Notch2, Delta1 and Jagged1) in the corneal tissue suggests that they play pivotal role in corneal homeostasis, stratification and wound healing by inhibition of epithelial differentiation, rather than cell proliferation. These will facilitate the development of strategies to manipulate corneal epithelial cell homeostasis in corneal storage, wound healing, stem cell transplantation and ocular surface diseases.

Since Notch signalling is known to function as a developmental tool to direct cell fate and consequently organ formation and morphogenesis it is likely that Notch signalling plays a role in regulating cell patterning and differentiation in corneal development.

APPENDIX I—CHEMICAL REAGENTS AND SUPPLER

Acetone	BDH
Acrylamide	BIO-RAD
Agar	BDH
Agarose	BRL
Amphotericin B	Gibco
Attachment factor—ZHS-8949	TCS CellWorks
Anti-human AE5 antibody	ICN
Anti-human Ki67 antibody	DAKO
Anti-human Notch1 antibody	Santa Cruz
Anti-human Notch2 antibody	Santa Cruz
Anti-human Jagged1 antibody	Santa Cruz
Anti-human Delta1 antibody	Santa Cruz
Anti-goat IgG-HRP antibody	Santa Cruz
Anti-rabbit IgG-HRP antibody	Santa Cruz
Boric acid	Sigma
Bromophenol Blue	Sigma
Betadiene	SetonHealthcare
BSA	BDH
Chloroform	BDH
Chondrotin Sulfate	Sigma
Collagenase	Sigma
Developer	Kodak Ltd., UK
Dulbecco's Modified Eagle Medium	Gibco
Dimethylsulphoxide (DMSO)	Sigma
EDTA	BDH
Eagle's minimal Essential Medium	Gibco
Embedding molds	BDH
Embedding rings	BDH
Ethanol	BDH
Eosin	Gibco
Fixer	Kodak Ltd., UK
Fetal calf serum (FSC)	Gibco

Filter paper	BIO-RAD
Formaldehyde	BDH
Formalin	BDH
Fungizone	Sigma
Gelatin	Sigma
Glycerol	Sigma
Glutamine	Gibco
Haematoxylin (Mayers and Harris)	BDH
High Molecular Weight Marker	Sigma
Ham's F-10 Medium	Gibco
Hoechst H033258	Sigma
Hydrogen Chloride	BDH
Hydromount	BDH
Kanamycin Sulphate	Sigma
Labeled Donkey anti-Goat IgG antibody	Molecular Probes
Labeled Goat anti-Mouse IgG antibody	Molecular Probes
Labeled Donkey anti-Rabbit IgG antibody	Molecular Probes
Luminol reagent	Santa Cruz
MEM (Eagle's Mimimumal Essential Medium)	GibcoBRL
Microtome disposable blade	Lamb
Nitrocellulose membrane	BIO-RAD
OCT	Lamb
Proteinase K	Sigma
Pronase	BDK
Penicilin-G	Sigma
Paraffin wax	Lamb
Scalpel blades NO 22/23	Fisher
Sodium Chloride	BDH
Streptomycin Sulphate	Sigma
Super frost plus slides	BDH
Surgical blades	Fisher
Tissue-Tek OCT compound	Lamb
Trowells T8	Gibco
Tris (hydroxyemthyl)	BDH

Triton X-100	Sigma
Trypsin	Sigma
Tween	Sigma
TEMED	Sigma
X-ray film	Kodak Ltd, UK
Xylene	BDH

APPENDIX II –PREPARATION OF BUFFERS AND SOLUTIONS

Phosphate buffered saline (PBS) 10X (1 liter)

80g sodium chloride

14.24g disodium hydrogen orthophosphate

2g sodium dihydrogen orthophosphate

dd H2O adjust pH to 7.4

Antibiotics and Glutamine -- for 100ml

Streptomycin Sulphate	(Sigma #S-9137)	1mg
Kanamycin	(Sigma #K-4000)	1mg
Penicillin-G	(Sigma #PEN-NA)	600mg
L- Glutamine	(Sigma #G-5763)	1.46gm
ddH_2O		100ml

Fungizone 250 µg/ml

After thawing completely dispense aliquots (2ml) was dispensed into sterile bijoux and frozen at –20°C

ReddyMix™ PCR Master Mix

The addition of the template and primers (in a volume of 2.5 µl) resulted in a final reaction volume of 25 µl, containing:

0.625 units Taq DNA polymerase

The component of 20ml 8% acrylamide resolving gel

H_2O	9.3µl
30% acrylamide mix	5.3µl
1.5 tris (pH 8.8)	5.0µl
10% SDS (sodium dodecyl sulfate)	0.2µl
10% ammonium persulfate	0.2µl
TEMED	0.012µl

The component of 5ml 5% acrylamide stack gel

H_2O	3.4µl
30% acrylamide mix	0.83µl
1.5 tris (pH 8.8)	0.63µl
10% SDS	0.05µl
10% ammonium persulfate	0.05µl
TEMED	0.005µl

Buffer for western blotting

TBS (10X) (9% NaCl, 100mM Tris HCl, pH 7.4)

NaCl	90.00 g
Tris	12.11 g

Make up to just below 1 litre (900ml if using HCl 1M, 985ml if use HCl 11M) check and adjust pH, make up to 1 litre.

2X SDS-PAGE sample buffers (8ml)

Stacking buffer (0.5 M Tris-HCl pH 6.8)	2ml
Glycerol	1.6ml
10% SDS	3.2ml
2-mercaptoethanol	0.8ml
0.1 % (w/v) bromophenol blue in water	0.4ml

Store at -24 °C freezer

10x running buffer: 1 L

Trizma base (= 0.25 M)	30.3g
Glycine (= 1.92 M)	144g
SDS (= 1%)--add last	10g

Transfer buffer (1X) 1litre

3.0 g Trizma base (= 0.25 M)

14.4 g Glycine (= 1.92 M)

200ml Methanol

pH should be 8.3; do not adjust

RIPA buffer

Tris-HCl	50mM pH 7.4
NP-40	1%
Na-deoxycholate	0.25%
NaCl	150 mM

EDTA	1mM
PMSF	1mM
Aprotinin	1µl/ml each
Leupeptin	1µl/ml each
Pepstatin	1µl/ml each
Na_3VO_4 (stock in dark)	1mM
NaF (stock in dark)	1mM

Agar/Gelatin

2g Agar

2g Gelatin

200ml ddWater

Place agar, gelatine and water into a 300ml bottle and atuocalve at 120°C for 20min

Agar/Gelatin Support

200ml Agar/Gelatin

20ml 10x MEM

10ml 7.4% sodium bicarbonate

2ml Antibiotics

1ml Fungazone

Heat agar/molten mixture until molten using the microwave on low power. Filter

(0.2 µm) remaining solutions into cooled solution.

Trowells T8 Media (500ml)

500ml Trowells T8

5ml Antibiotics

5ml Fungazone

Filter (0.2μm) remaining solutions into the media bottle.

7.4% sodium bicarbonate

22.2g sodium bicarbonate

300ml ddWater

Mix until dissolved and store at room temperature

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