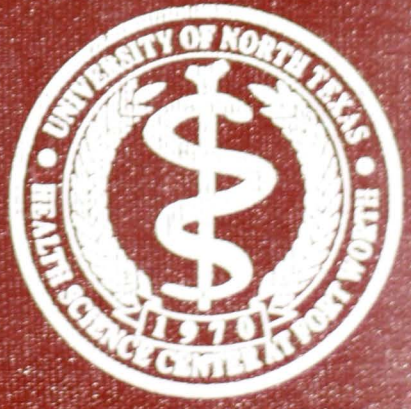
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Shankardas, Jwalitha.
Role of 14-3-3[sigma] in
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Abstract

Epithelial differentiation is a highly coordinated process that is essential for proper function of epithelia in their respective tissues. In the cornea epithelial differentiation is necessary to maintain transparency of the cornea, which is essential for vision. 14-3-3σ/stratifin is a proposed epithelial cell marker, which is up regulated in response to UV damage. This up-regulation causes the cells to arrest in G2/M phase so that DNA repair can take place. In a number of epithelial cancers 14-3-3σ has been shown to be down-regulated. The function of 14-3-3σ in the corneal epithelium and other stratified epithelial tissues other than the skin has not been studied. In the cornea there are no reports of hyperplasia so it would be a good model to study the role of this protein.

In this study, for the first time we have evaluated the expression of 14-3-3 family of proteins in the cornea. We have also shown the involvement of 14-3-3σ in the process of corneal epithelial differentiation *in vitro* using primary corneal epithelial cells and *in vivo* using the Er/+ mouse model. We have also been able to extend in vitro lifespan of corneal epithelial cells by down regulating 14-3-3σ expression. The down regulation also resulted in immortalization and generation of a corneal epithelial cell line. We have characterized and shown that this cell line is a suitable model to study signaling cascades involving corneal epithelial proliferation, differentiation and apoptosis. Preliminary data presented in this study also imply that ΔNp63 is an upstream regulator of 14-3-3σ expression.

ROLE OF 14-3-3σ IN CORNEAL EPITHELIAL DIFFERENTIATION

Jwalitha Shankardas, M.S

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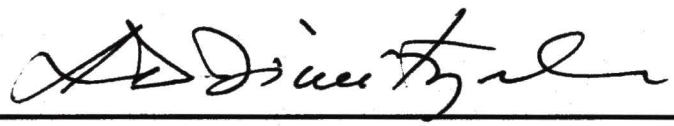

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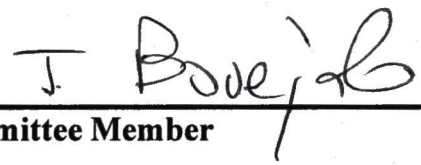

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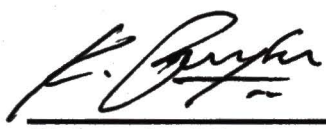

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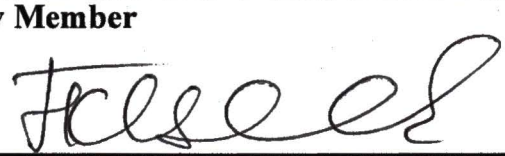

Dean, Graduate School of Biomedical Sciences

ROLE OF 14-3-3σ IN CORNEAL EPITHELIAL DIFFERENTIATION

DISSERTATION

Presented to the Graduate Council of the

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CHAPTER I

INTRODUCTION

Background

Ocular surface

The anterior surface of the eye, like the skin, is the primary defense compartment against a variety of chemical and microbial insults. Both survive well under normal conditions but in combat create hostile environments, both biological and chemical, that can exceed the protective capacity of the ocular surface and cause injuries of varying severity. The chief constituents of the ocular surface include the surface epithelium of the cornea, the conjunctiva, the underlying stroma and the tear fluid [Figure 1(1)].

The principle component that has a direct impact on the vision is the cornea. In addition to protective function cornea, which because of the transparency and focusing power is one of the key players in delivery of the image to the retina facilitating capture of light energy and its interpretation buy the brain.

The main function of the ocular surface is the maintenance of the clarity of the cornea, a protective barrier against external environment and also providing nutritional support, all of which are required for proper vision. The ocular surface epithelium is continuous and includes the corneal epithelium, the conjunctival epithelium and the lachrymal glandular epithelium. The tear film component of the ocular surface is maintained by secretions of the lachrymal gland, the conjunctival goblet cells, the conjunctival and corneal epithelial cells[1-11]. The epithelia of the ocular surface depend on

the tear fluid for nutrients that are required for regeneration, wound healing and for dilution of microbial and chemical insults. The proper function and maintenance of ocular surface is crucial for vision.

Epithelia/Ocular surface epithelia

Epithelial tissues cover the entire surface of the body. These epithelia are composed of cells that are closely packed and are present as single or multilayered compartments. This tissue is specialized to form the lining of all internal and external surfaces of the body. Epithelia are usually separated from the underlying connective tissue by the basement membrane. This is a specialized extracellular matrix that plays an important role in providing structural support for the epithelium, and also connects it to neighboring structures, and separates the cells from adjacent tissue components. Epithelial tissues are broadly classified into two types (1) Simple Epithelium and (2) Stratified epithelium.

Simple epithelium consists of a single layer of cells and can be further classified based on shape and function. Stratified epithelium is composed of multiple layers of epithelial cells. This type of epithelium is usually present in areas that need to withstand a substantial wear and tear. Stratified epithelia are broadly classified into keratinized (skin) and non-keratinized epithelia (oral cavity, reproductive tract, cornea and conjunctiva). One of the major functions of the epithelial tissues is to act as a barrier that protects the internal organs from environmental (external) insults such as microbial, physical and chemical injuries and maintain the hydration (prevent evaporative losses)[1-3].

The epithelia of the cornea and conjunctiva are non-keratinized squamous epithelia that are separated by a narrow transition zone known as the limbus. The corneal epithelium is transparent, multilayered and is devoid of goblet cells. The stroma underlying the corneal epithelium is highly organized and is devoid of any vasculature except in the limbus[1-3].

The conjunctival epithelium is not as organized as the epithelium of the cornea and consists of goblet cells that secrete mucins, which are important components of the protective film that covers the ocular surface [Figure 1(2)]. The conjunctival epithelium can be divided into the bulbar epithelium, the fornix and the eyelid (Palpebral) epithelium[1-3].

Figure 1 (1): Components of the ocular surface.[Modified from www.ncbi.ie]

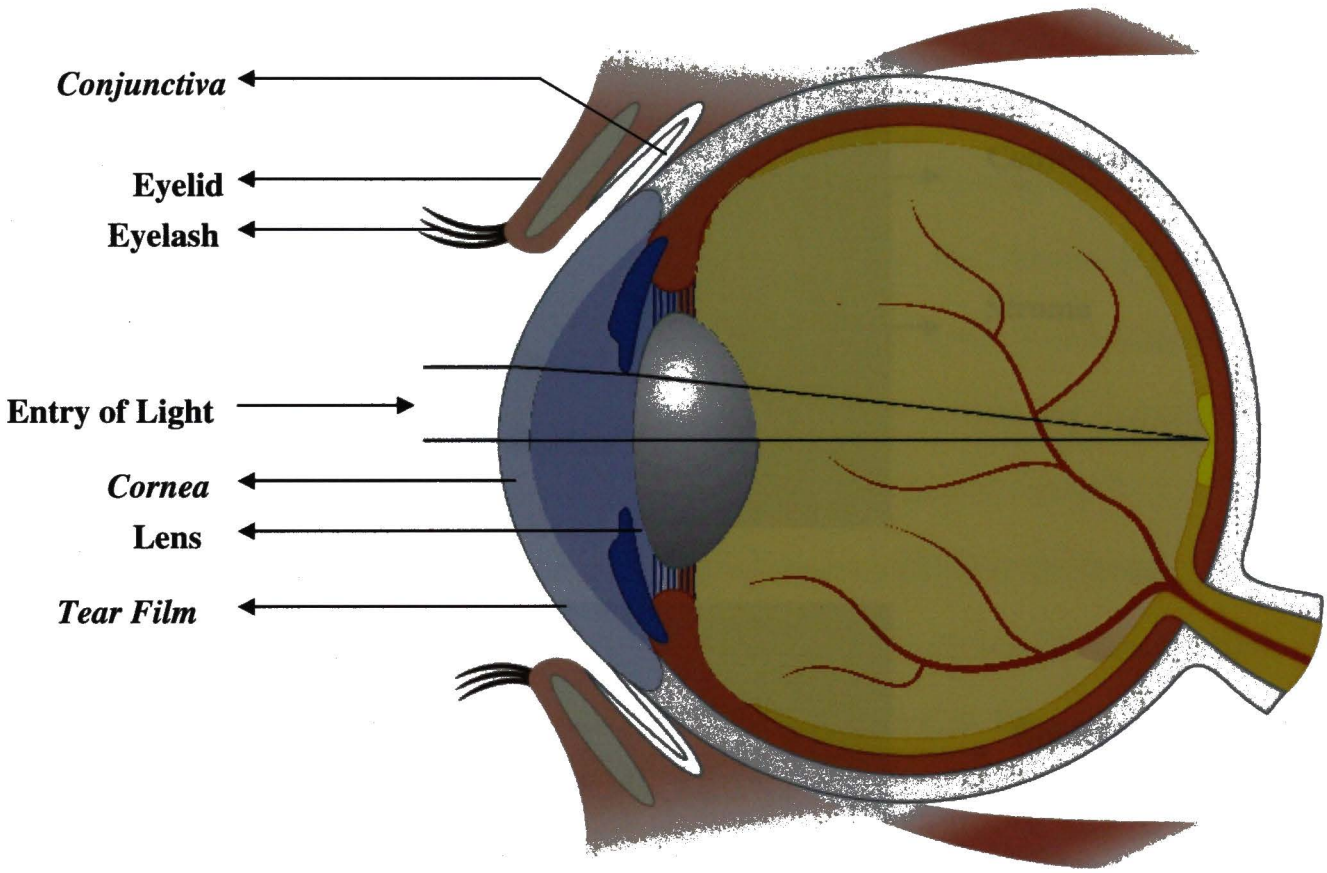
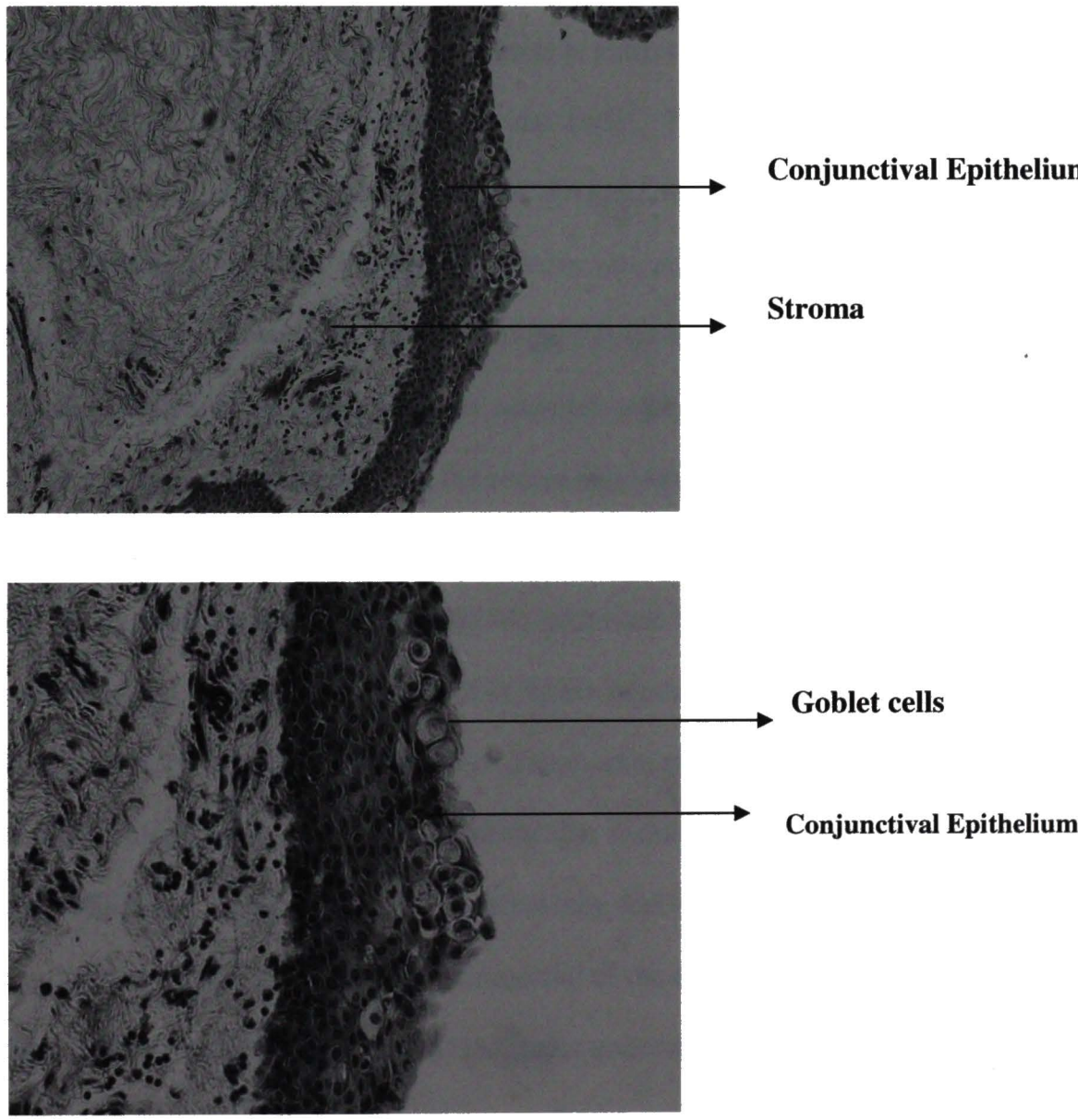


Figure 1 (2): H&E staining of the cross section of the human conjunctiva showing the presence of (a) stratified epithelium (Magnification of 10X), (b) 20X magnification of the conjunctival epithelium revealing the presence of goblet cells.



Structure of the cornea

The cornea is the transparent, dome-shaped structure that forms the outermost part of the anterior surface of the eye. It is an avascular transparent tissue which along with the tear fluid, is responsible for up to 80% of the focusing power of the eye[2]. The lack of vasculature in the cornea contributes to its optical transparency so that it allows the undisturbed entry and passage of light. The cornea is extremely sensitive because it has more nerve endings than any other organ in the body[1]. The human adult cornea is 0.5mm (millimeter) thick and is comprised of 5 layers that include the epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium[1]. The stratified epithelium that covers the outer surface of the cornea consists of 5-6 layers of epithelial cells. Bowman's membrane is an acellular region located just beneath the epithelium and it plays a role in protecting the cornea from mechanical injury[1-4].

The stroma is the thickest compartment and is situated beneath Bowman's. It is composed mostly of extracellular matrix (ECM) populated by corneal fibroblasts, also known as keratocytes. These keratocytes and ECM are organized in the stroma in such a way that preserves the optical transparency. Descemet's membrane lies between the stroma and the endothelium. The corneal endothelium maintains the hydration levels of the cornea, which is crucial for stromal transparency and therefore proper vision by controlling solute transport from the anterior chamber of the eye [Figure 1 (3)]. All three compartments are optically transparent and the major concern in homeostasis and repair is preservation of this transparency.

Corneal Epithelium

The corneal epithelium, because of its anatomical exposure, is in a constant state of cellular turnover based on renewal of basal cells and loss of its superficial cells. The cell proliferation and differentiation that maintain the stratified epithelial barrier and contribute to the homeostasis of the anterior ocular surface (including the tear film) is driven by the adult stem cell compartment located in the peripheral zone between the cornea and conjunctiva referred to as the limbus. Specifically, so called Pallisades of Voght are therefore the niche for self renewing population of cells that are critical to the corneal homeostasis and healing after injury[3-9]. The presence of these cells is easily visualized in the whole epithelial sheet obtained from African American donors because the cells are pigmented [(Figure 1(5)].

The limbus is different from the rest of the cornea as it has a rich supply of blood vessels in the underlying peripheral stroma. The cells of the limbus are capable of self-renewal symmetrical and asymmetrical cell division to produce fast dividing transient amplifying progenitor cells[10]. Damage to the limbal cornea (limbal insufficiency) eventually leads to blindness. The limbus is therefore the source of cells that are appropriate and have been shown to be useful in restoration of vision after blinding injuries[3-9]. A number of attempts have been made to determine specific markers for these cells [Table 1(1)].

In vivo the corneal epithelium regenerates throughout life but *in vitro* these cells have a very short life span. In keeping with a continuous and increasing interest in the useful proliferative potential of a number of adult cell types, studies have been conducted

with corneal and conjunctival epithelial cells that have resulted in the development of a number of models that are useful for studies of basic tissue biology studies and ophthalmic pharmaceuticals, and tissue engineering directed to development of 3D models and corneal replacements.

Figure 1 (3): H&E staining of the human cornea. (Magnification of 10X)

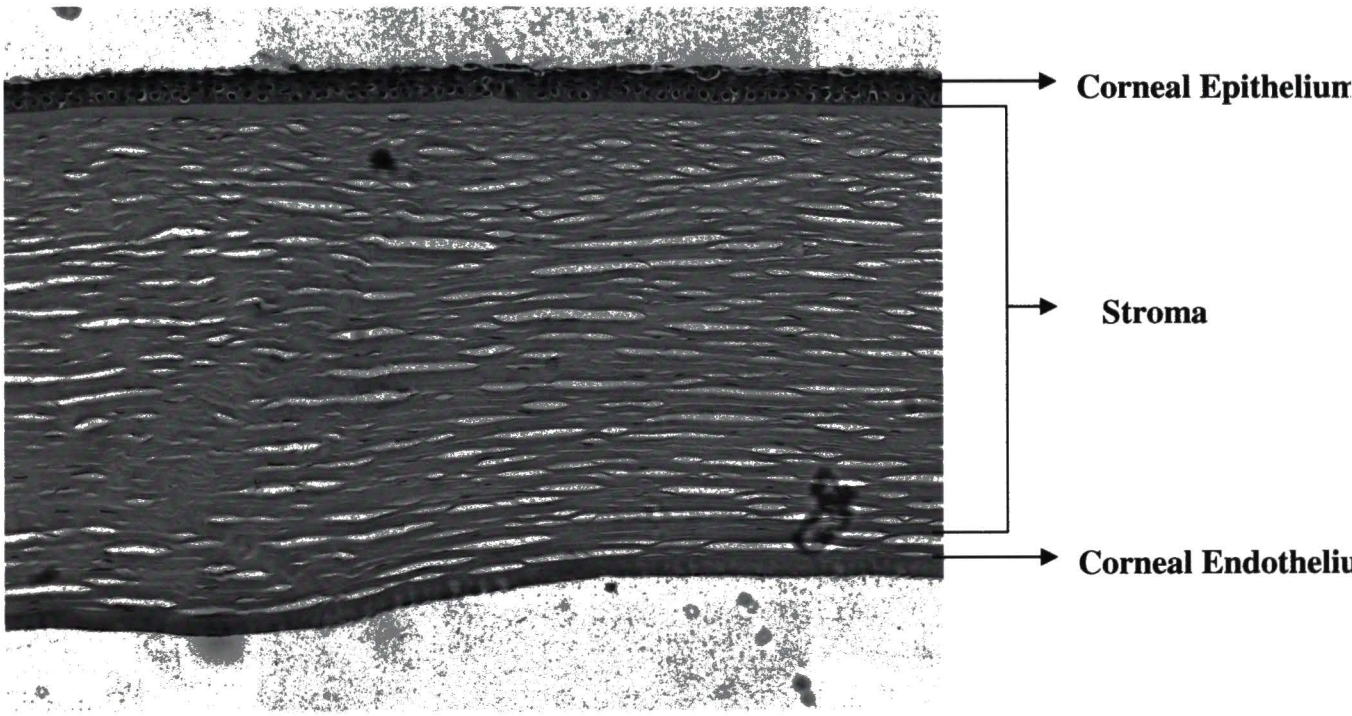


Figure 1 (4): H&E staining of the cross section of the human cornea showing the presence of stratified epithelium in (a) The central cornea and (b) The limbus.

(a)

(b)

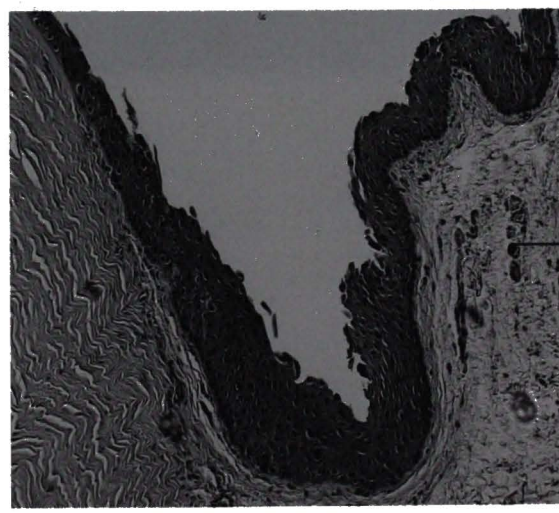


Figure 1 (5): Phase contrast image of the harvested whole epithelium from an African American donor revealing the presence of pigmented cells of the Palisades of Voght. [11]

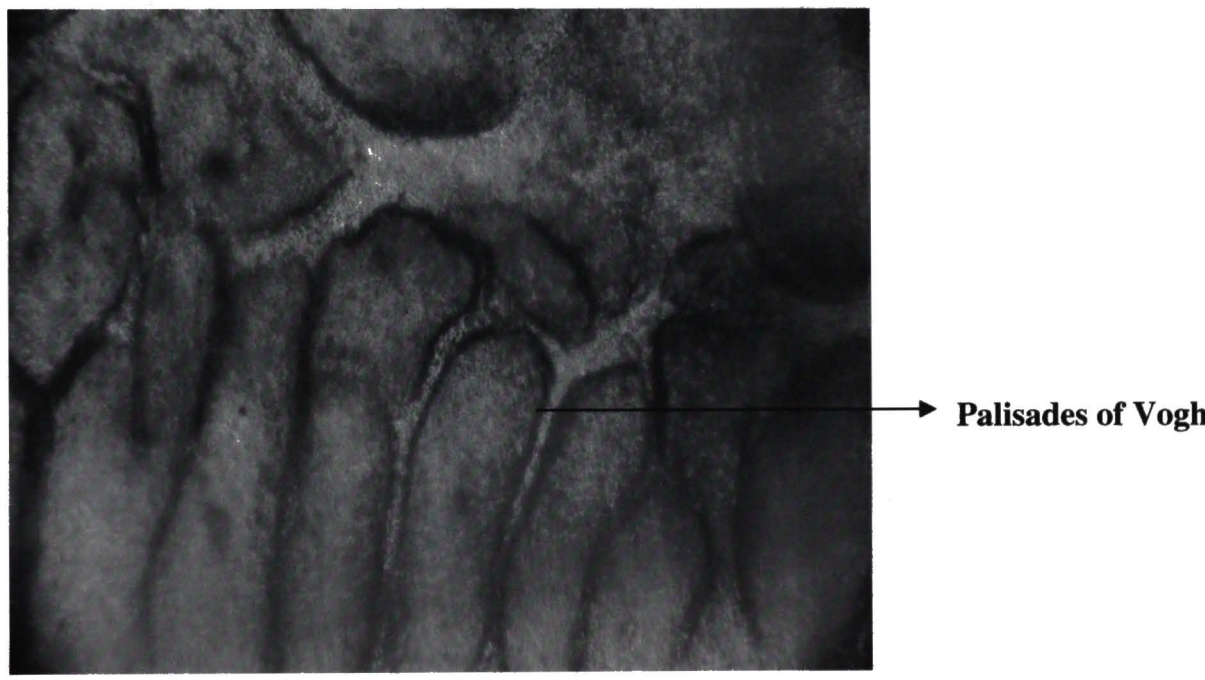


Table 1 (1): Putative stem cell markers of the corneal epithelium-Limbus.[12]

S.No	Marker	Reference
1	Keratin 19	Michel et.al 1996[13], Kruse EF et.al 2005[14].
2	Vimentin	Kruse EF et.al 2005[14],Kasper et.al 1988, 1992, Lauweryns et.al, 1993b[15, 16]
3	α Enolase	Kruse EF et.al 2005[14], Zieske et.al 1994[17], Zhao et.al, 2002[18]
4	ΔNp63	Kruse EF et.al 2005[14].
5	Metallothioein	Kruse EF et.al 2005[14], Lauweryns et.al, 1993b
6	P-Cadherin	Kruse EF et.al 2005[14].
7	β-Catenin	Kruse EF et.al 2005[14], Huelsken et.al, 2001[19, 20].
8	Integrin αv	Kruse EF et.al 2005[14].
9	Integrin β1	Kruse EF et.al 2005[14].
10	Integrin β2	Kruse EF et.al 2005[14].
11	ABCG2	Kruse EF et.al 2005[14],Zhou et.al,2002[18] LiDQ et.al,2005[21]

Need for cellular and tissue models/Strategy for extension of *in vitro* life span

The number of cell types with a useful *in vitro* lifespan is limited. Primary corneal epithelial cells like other somatic cells cease to proliferate *in vitro* after a limited number of passages. The primary cells when harvested from tissue, are a heterogeneous population of proliferating and differentiating cells, which makes the process of studying proliferation controlling factors, transition from proliferation to differentiation and differentiation inducers, using these cells very difficult. The primary cells are also difficult to work with due to variations in donor age, gender and the method of preservation of the tissue, most of which is eye bank tissue obtained from older donors. Cryo-preserved primary cultures are hard to revive. Due to the number of problems encountered while working with primary cells, there have been studies to develop cell lines. Cell lines differ from primary cells (wild type (WT) - somatic cells) in that they are easy to culture in standard serum containing and inexpensive media. Since they are immortal (self-renewing), cell lines are much simpler to incorporate into high throughput serial studies that generate results that are amenable to simple statistical analyses. Classically large or small T antigen of the Simian Virus strain (SV-40)[13], papilloma virus (HPV) envelope proteins E6 and E7 (alone or in combination) E6/E7 cells)[22] and adenoviral proteins (AE1 and BE1) have been used to immortalize human and animal cells. However, in the process of by-passing the mortality check-points 1 and 2 the expression of the oncoproteins p53 or pRb or both in resulting cell lines are abrogated[22]. The SV-40 large T-antigen binds to and inactivates the retinoblastoma protein (Rb) and p53 in these cells, as a result of which these cells do not respond to DNA damage and

have altered expression of cell cycle proteins[19]. The E6/E7 cells do not express p53 protein and have inactive Rb and hence these have altered cell cycle regulators and are immortal. These cells like any virally immortalized cells are heteroploid and hence are not the appropriate models to study differentiation/cell cycle control[22].

Since somatic cells undergo telomere shortening at each cell division, the strategy for the immortalization process involves activation of telomerase, the enzyme that in stem cells and cancer cells maintains telomere length[23-31]. Another strategy to generate cell lines was introduction of ectopic expression of human telomerase reverse transcriptase (hTERT) into normal human corneal epithelial cells, which resulted in generation of a corneal epithelial cell line with extended lifespan[32, 33]. The advantage of constitutive hTERT activity is that it arrests telomere shortening, promoting mitosis and delaying differentiation. Corneal cells have been immortalized using this strategy but in order to produce successful constitutive expression the cells were cultured on 3T3 feeder layers[33]. Although the feeder layers can be inactivated, there is the possibility of cross-contamination with feeder cells (usually 3T3 fibroblasts). In cases of cross-contamination, the environmental conditions determine the type of cell line that will prevail. This problem has also been encountered with feeder layer supported cultures of embryonic stem cells.

The alternative cellular model is the telomerase transformed cell line (hTERT CEC), which we generated by transfecting primary corneal epithelial cells cultured on collagen type IV (CIV) and not on feeder layers. These cells have uniform cobblestone morphology, which is characteristic of epithelial cells and have been successfully

cultured over passages 50[32]. Our lab has characterized these cells extensively and shown that they express all the cell cycle proteins (p53, p63, p16 and p21) similar to WT (primary) corneal epithelial cells[32]. These cells also express the epithelial specific keratins[32], and proliferation markers such as telomerase, Ki67 and PCNA. We have also shown that these cells not only express telomerase but also to have telomerase activity[32]. These cells have a population doubling time of 36 hrs and are diploid, do not grow in an anchorage independent manner like tumor cells as shown by the absence of colony formation in an anchorage independent growth assay, and undergo differentiation. They respond to adriamycin induced DNA damage by activating their p53 signaling cascade and this adriamycin treatment is not cytotoxic to the hTERT CEC cells[32].

Almost all cancer cells express telomerase activity; there is therefore a need to seek more selective or cell specific methods of extending lifespan of different somatic cell types *in vitro*. A more interesting family of proteins in this respect that has recently been studied with respect to cell cycle control is the 14-3-3 proteins, particularly 14-3-3σ which when suppressed has been shown to extend the proliferative potential of human skin and gingival keratinocytes[34].

14-3-3 proteins

14-3-3 (FTT) is a highly conserved, ubiquitously expressed family of acidic proteins originally discovered in the central nervous system (CNS). At least seven isoforms are known in mammals: β, γ, ε, σ, ζ, τ and η, each being a product of a separate gene[26]. FTT proteins form dimers which associate with over 200 different target

molecules.[35] These interactions take place because of the presence of the highly specific phosphoserine / phosphothreonine-binding sites on target molecules. [35, 36]There are however, phosphorylation independent targets for these proteins such as Bax and telomerase[37, 38]. This family of proteins function in a number of different ways: (1) they can bind and alter localization of target molecules, (2) they can act as adapter molecules for two or more different proteins, (3) they can bind to and inactivate target molecules by masking active sites, (4) they can bind to and prevent phosphorylation/de-phosphorylation of the binding partners. This family of proteins is therefore involved in activation of a number of downstream signaling cascades. Their role in cell function includes cell cycle control, response to DNA damage, apoptotic cell death and stress response signaling[39, 40]. The ability of this family of proteins to bind and regulate several oncogenic proteins points to a prominent role in cancer development and progression and it is in this context that a number of isoforms are being studied in detail[41-45].

14-3-3σ

The 14-3-3σ, the FTT isoform that is also known as **Stratifin** is a unique member of this family of proteins in that has been proposed to be expressed exclusively in epithelial cells in tissues that have been examined and is proposed to be marker of stratified epithelia[34]. These 14-3-3 proteins are also functional as dimers, and evidence to date supports the view that 14-3-3σ proteins tend to form homodimers to attain biological activity[46].

There are two major up-stream regulators of 14-3-3σ[26]. One is p53, tumor

suppressor gene, which in the event of DNA damage is up-regulated. p53 induces cell cycle arrest in order to allow DNA repair or apoptosis. In order to effect DNA repair or apoptosis, p53 induces cell cycle arrest at the G1/S phase. This G1/S arrest is mediated by p21. p53 also induces 14-3-3σ a mediated cell cycle arrest at the G2/M phase[47]. In the event of DNA damage p53 becomes de-phosphorylated and binds to the promoter region of 14-3-3σ protein and increases its transcription. This increased expression of 14-3-3σ leads to sequestration of cdc2/cyclin B in the cytosol preventing the entry of the cell into mitosis. Conversely 14-3-3σ may increase p53 expression by a positive feedback loop mechanism [48]. As far as 14-3-3σ is concerned, BRCA1 has been shown to act synergistically with p53 in response to DNA damage events[26, 49][Figure 1(6)]. Other members of the 14-3-3 family inhibit cell-cycle progression by interacting with the Cdc25 phosphatases while 14-3-3σ appears to induce a G2/M arrest by binding Cdc2–cyclin-B complexes and preventing their translocation to the nucleus and instead sequesters them in the cytoplasm[47, 50]. It has also been shown that binding to 14-3-3σ protects p53 from Mdm2-mediated ubiquitinylation, thereby stabilizing its level by preventing its degradation. 14-3-3σ binding also promotes p53 tetramerization, resulting in increased transcriptional activation[48].

Another up-stream regulator of 14-3-3σ is p63, a member of the p53 family which has been known to function as transcriptional regulator. The ΔNp63 isoform has been shown to act as a transcriptional repressor of 14-3-3σ gene[51]. This isoform is up-regulated in the proliferating cells of the epithelium and has been considered to be the regulator of the adult stem cell fate[52-54]. This has been mostly studied in the keratinocytes

of the skin, where the ΔNp63 is absent in differentiated cells, in which the expression of this p63 isoform is regulated by the IP3 kinase[55]. ΔNp63 isoform is expressed in the corneal epithelium, particularly in the limbus as shown by RT-PCR[56]. We have shown that the ΔNp63 isoform is localized to the nucleus in the basal cells of the limbus while it is localized in the cytosol in the superficial cells of the limbus and the central cornea[11]. In vitro the expression level of ΔNp63 is undetectable upon differentiation[11].

The expression of 14-3-3σ protein has been reported to be down-regulated in a number of cancers including breast cancer[49, 50, 57-60], prostate cancer[51, 61], liver[58] and cancer of the oral cavity[62]. This implies that this protein is a tumor suppressor oncogene. There are two well-known mechanisms via which the silencing of the 14-3-3σ gene occurs. One results from CpG methylation of the 14-3-3σ promoter[57, 58] and the other is by estrogen mediated finger protein (EFP) mediated ubiquitination and proteolytic degradation of the protein[59, 60, 63]. In certain types of cancers, such as, pancreatic[62, 64], lung[65], head and neck cancers[62, 66] however 14-3-3σprotein has been shown to be up regulated. Thus targeting 14-3-3σ as a strategy to attack cancer is quite complicated.

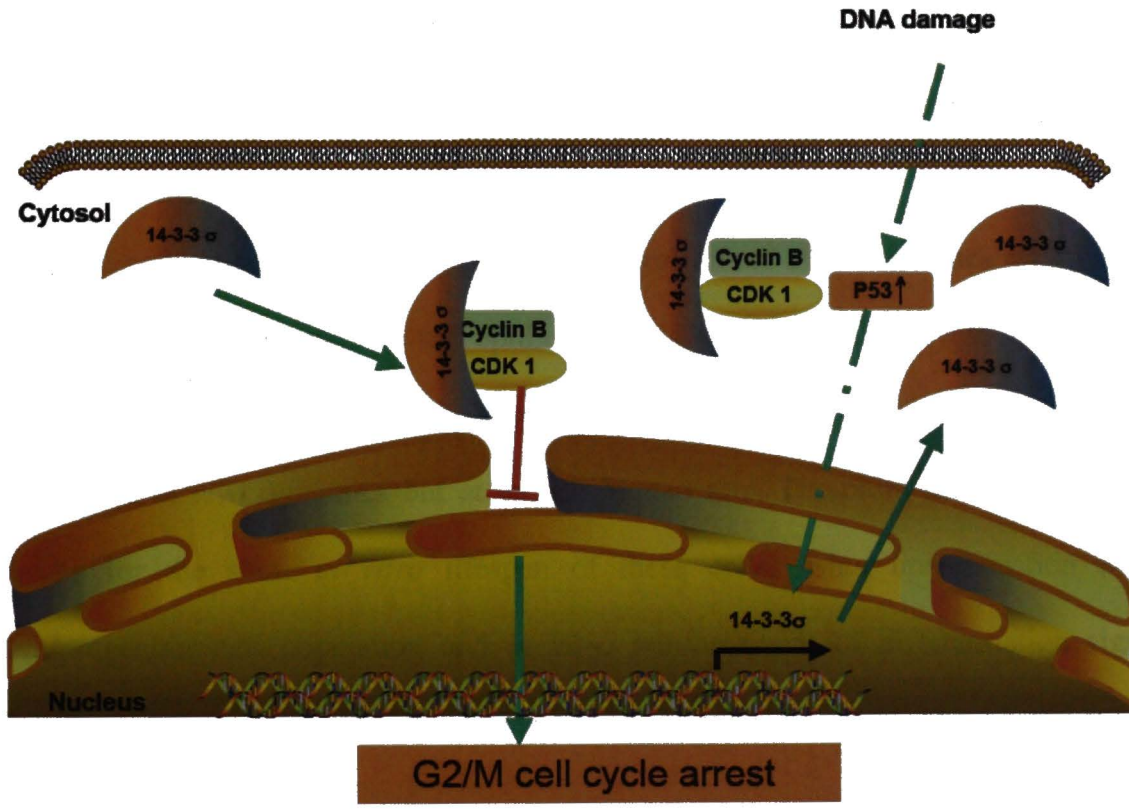
14-3-3σ also plays a role in apoptosis by interacting with bax and sequestering it in the cytoplasm in a phosphorylation independent manner. In the absence of 14-3-3σ activity, DNA damage is accompanied by translocation of bax to the mitochondria and initiation of apoptosis. [67]

14-3-3σ protein levels are significantly reduced or are negligible in a number of transformed cell lines and primary tumors of epithelial origin. [58, 68]It has been shown that

down-regulation of 14-3-3σ expression constitutes a single step that can immortalize primary keratinocytes. [34] This leads to the view that 14-3-3σ acts as a tumor suppressor, and a number of research groups are interested its potential role in cancer.

In the human epidermis and the epithelia of the oral cavity, 14-3-3σ is highly expressed in the suprabasal (differentiated) layers, while it is barely detectable in the basal layers.[34, 69] It has been shown that in primary keratinocytes and gingival keratinocytes, the down regulation of 14-3-3σ is accompanied by a down regulation of p16 and maintenance of telomerase activity.[34] The down-regulation of 14-3-3σ was accomplished by transfecting primary keratinocytes with LXSN retroviral vector (in packaging cell line) encoding the full-length 14-3-3σ antisense cDNA. [34] The role of 14-3-3σ in corneal proliferation and differentiation has not been studied.

Figure 1 (6): Schematic representation of the DNA damage response signaling cascade involving 14-3-3σ protein (Drawn using Science Slides 2006)



Differentiation *In vitro*

Primary cells when cultured *in vitro* undergo senescence after a limited period of time beyond which they no longer proliferate. I*n vivo*, cells however do not have to proliferative constantly, but are metabolically active and can be sustained in this state (senescence) for prolonged periods. This is seen in the case of fibroblasts which do senesce *in vitro* after a specific number of population doublings dependent on the age of the donor[70-73]. The Hayflick limit is therefore an *in vitro* phenomenon and does not apply to the ageing process *in vivo*, as older people continue to heal after an injury[74, 75]. Epithelial cells also have a short life span *in vitro*; this is also sometimes referred to as senescence. *In vivo* however the epithelial cells arrest the cell cycle and undergo differentiation. Consequently it could be proposed that epithelial cells behavior *in vitro* mimics the *in vivo* dynamics but at an accelerated rate. Therefore there could be two reasons for the short *in vitro* lifespan of these cells, using up/exhaustion of the stem/progenitor cell population and *in vitro* reinforcement of differentiation signals. Proliferation and differentiation are two mutually exclusive processes and proliferation precedes differentiation.

The alternative strategy for lifespan extension of epithelial cells is therefore the delay in differentiation. In stratified epithelia, differentiation is the maturation of epithelial cells directed toward the attainment of tissue function. In order to understand cell cycle regulation to extend proliferation it is important to understand the factors that influence differentiation. Differentiation when applied to stratified epithelia indicates maturation to provide tissue function at the expense of the loss of mitotic activity .The

important proteins that are commonly used as markers of proliferation and differentiation in epithelial cells are cytokeratins/keratins (CK). The suprabasal and superficial cells of the epithelium are non-mitotic, differentiated cells that establish cell-cell contacts characteristic of a tight barrier against environmental insults. Hence the differentiation of corneal epithelial cells is crucial for their function. Differentiation and cell proliferation are mutually exclusive processes and the process of differentiation takes place following a cell cycle arrest and changes in cytoskeleton that are required for stratification and formation of a tight barrier.

Keratin expression in the different layers of the corneal and conjunctival epithelium has been routinely used to classify them. Another important marker for terminal differentiation in the corneal epithelium is expression of involucrin.

Markers of Differentiation

The significance of keratins in the cornea epithelium

Keratins are cytoskeletal proteins that are broadly classified into type I and type II, which together account for about 75% of all the intermediate filaments (IF) in human tissues[76]. Keratins form heterodimers with at least one member of each family. Pairs of keratins (type I and type II) are expressed together in a tissue specific manner. These pairs of type I and type II keratins are expressed in epithelial tissues[76] , in such a way that their expression is differentiation dependant and developmentally regulated. Thus, these proteins have been used as markers of proliferation and differentiation in a number of

tissues including the skin and the eye. Keratins have a basic molecular structure comprising of a central alpha helix flanked by end domains of highly variable size and amino acid sequence. The head domains appear to play a critical role in regulating filament assembly. Since they contain phosphorylation sites (serine residues), the regulation of head domain phosphorylation makes the domains very important. These domains could also function to detect and react to signals that involve mobilization of the cytoskeleton (eg: cell motility, cell division and differentiation)[77].

Type I and Type II keratins under physiological conditions undergo polymerization. Keratin monomers of either type (I or II) are highly unstable and are rapidly degraded in the absence of the appropriate partner. Heteropolymerization of keratins is associated with the physical resilience of the filament network in tissues where they are particularly important, and is also a mechanism that balances the quantities of the two co-expressed keratins. [78] The Intermediate filaments produced by mismatched pairs of keratins are mechanically and structurally different from those formed by the natural pairs.[78]

All cytokeratins are subject to posttranslational modifications such as proteolysis, phosphorylation and glycosylation. Most of all the phosphorylation occurs on serine residues that are localized within the repeat sequences on the carboxyl terminus[79].

Keratins reinforce the cytoskeleton that keeps epithelial cells of the cornea intact when subject to physical forces (eg: shear due to blinking). Keratins make up a system of filaments that run through the cytoplasm of cells and are involved in both cell-cell and cell-substrate connections and communications (eg: Mechanosignaling). These

interactions create a 3 D network of functional complexes through out the epithelium, which is critical for the structural and functional characteristics and performance of the entire tissue.

In the cornea and other stratified epithelia, the basal proliferative layer of cells express K5 and K14 while the suprabasal and superficial cells express K3 and K12 pair [Figure 1(7)].[80] Basal layer cells appear to synthesize K5 and K14 pairs only as long as the cells are in contact with the basement membrane. When this contact is lost in response to a differentiation signal, there is a change in keratin synthesis and differentiation specific keratins K3 and K12 are synthesized.

K14 has been shown to interact with plectin, a major hemi-desmosomal protein and is therefore involved in cell-basement membrane adhesion plaques.[81] K12 deficient mice have been shown to have fragile corneal epithelium supporting the involvement of K12 in desmosomal plaques. [82, 83] These cell-cell contacts are essential for maintenance of an intact corneal epithelium that functions as a barrier to the external environment. Since the keratin pair expression varies in proliferating and differentiating cells, keratin expression has been used as marker of proliferation and differentiation. Absence of K12:K3 expression and organization have been shown to result in loss of corneal transparency which is crucial for vision.[84] This could be due to the fact that the "loose epithelium" is not able to prevent evaporative loss from the stroma.

Involucrin

Involucrin is a cell envelope protein and is a commonly used marker of terminal differentiation in corneal epithelial cells[85]. It is the corneal analogue of filaggrin in the skin epithelium. This protein is mostly cytosolic and it cross-links with other proteins and lipids to form an insoluble barrier below the plasma membrane.

Figure 1 (7): Schematic Representation of the expression of keratin pairs in the central cornea and the limbus.[Modified from Lavkar RM et.al, 2003]

***In vivo* models to study differentiation**

The process of differentiation operates globally during development. Present strategies of gene expression analysis during development frequently utilize gene knock out models to obtain useful i*n vivo* information about specific proteins and their role in development.

Two mutations namely the pupoid fetus (Pf)[86-92] and the repeated epilation (Er)[93-100] mutation result in a failure of epidermal differentiation (skin epithelial stratification) in homozygous mice. These two mouse models are therefore being used to study differentiation in skin. Skin, is an example of stratified epithelia similar to that in the cornea. However, the skin epithelium is keratinized so that the superficial differentiated layers consist of cornified enucleated epithelial cells. In contrast the epithelia of the ocular surface are squamous and non-keratinized. The skin epithelium also has been known to have a basal layer of proliferative epithelial cells which undergo differentiation to give rise to superficial differentiated layers.

The animal model that has been used for studying the role of 14-3-3σ (stratifin-SFN) in the skin is the Repeated-Epilation (Er) mouse model[101, 102]. This animal model was generated after gamma irradiation of a male mouse, which resulted in a single A-T pair insertion mutation at nucleotide 624 in the ORF (open reading frame) of the SFN gene. This mutation caused a frame shift, which resulted in 40 amino acid truncation of 14-3-3σ protein at the C-terminus. This mutation is lethal in Er/Er homozygous mice. These mice die at birth because of a fused oral cavity, which results in respiratory distress, have malformed digits and stumpy limbs and are characterized by abnormal

proliferation and lack of differentiation of the keratinocytes resulting in thickening of skin. The Er/+ heterozygous mice survive, but have abnormal hair loss as their hair cycle is extended by 1 week. These animals develop spontaneous tumors after 6 months of age and they eventually die primarily as a result of their tumors[101].

Immunofluorescence on skin sections of the Er/Er mice show K14 expression in all layers of the epithelium; these epithelial cells are highly proliferative in nature and this excessive proliferation results in the thickening of the skin. The superficial epidermal layers, which are normally differentiated and express loricrin in WT control mice, are absent in the Er/Er mice. Also, keratinocytes from the Er/Er mice were shown to be more proliferative and could be maintained in culture longer when compared to the WT control cells. The differentiation phenotype of the Er/Er keratinocytes was also shown to be rescued by over-expression of exogenous 14-3-3σ[101, 102].

Studies of differentiation of the keratinocytes in these mice show that the epidermis of the Er/Er mutant mice when transplanted into a normal mouse tend to normalize and undergo differentiation. These data suggest that environmental factors/local cytokine secretions play a major role in proliferation/differentiation of these epithelial cells[88, 89].

14-3-3 family of protein was discovered in the cerebrospinal fluid (CSF). Therefore it is to be expected that isoform independent analysis has shown the presence of 14-3-3 protein in the CSF with respect to diseases such as Creutzfeldt-Jakob disease (CJD), multiple sclerosis[103-106] and prion disease[106-111]. Functions of this 14-3-3 protein in the CSF have not been determined. 14-3-3σ is one of the members of the 14-3-3 family

of proteins that has been identified to be a keratinocyte derived anti-fibrogenic factor (KDAF) present in keratinocyte conditioned medium[112-119]. Co-culture experiments with keratinocytes and addition of recombinant 14-3-3 σ proteins has been shown to significantly increase the expression of matrix metalloproteinases (MMPs) in dermal fibroblasts. This secreted form of the protein has been shown to have collagenase stimulatory effects on dermal fibroblasts suggesting its involvement in epidermal-mesenchymal communictation[117]. This is one of the first demonstrations of a possible extracellular function for 14-3-3σ protein. However, the mechanism by which it initiates MMP up regulation in target cell is still obscure. Since this family of proteins lack ER signaling peptides and therefore cannot be secreted by the classical ER secretory pathway. The mechanism of secretion of this protein is also not known .

The effect of the Er mutation on the cornea and other stratified epithelia other than that of the oral cavity and the skin have not been studied.

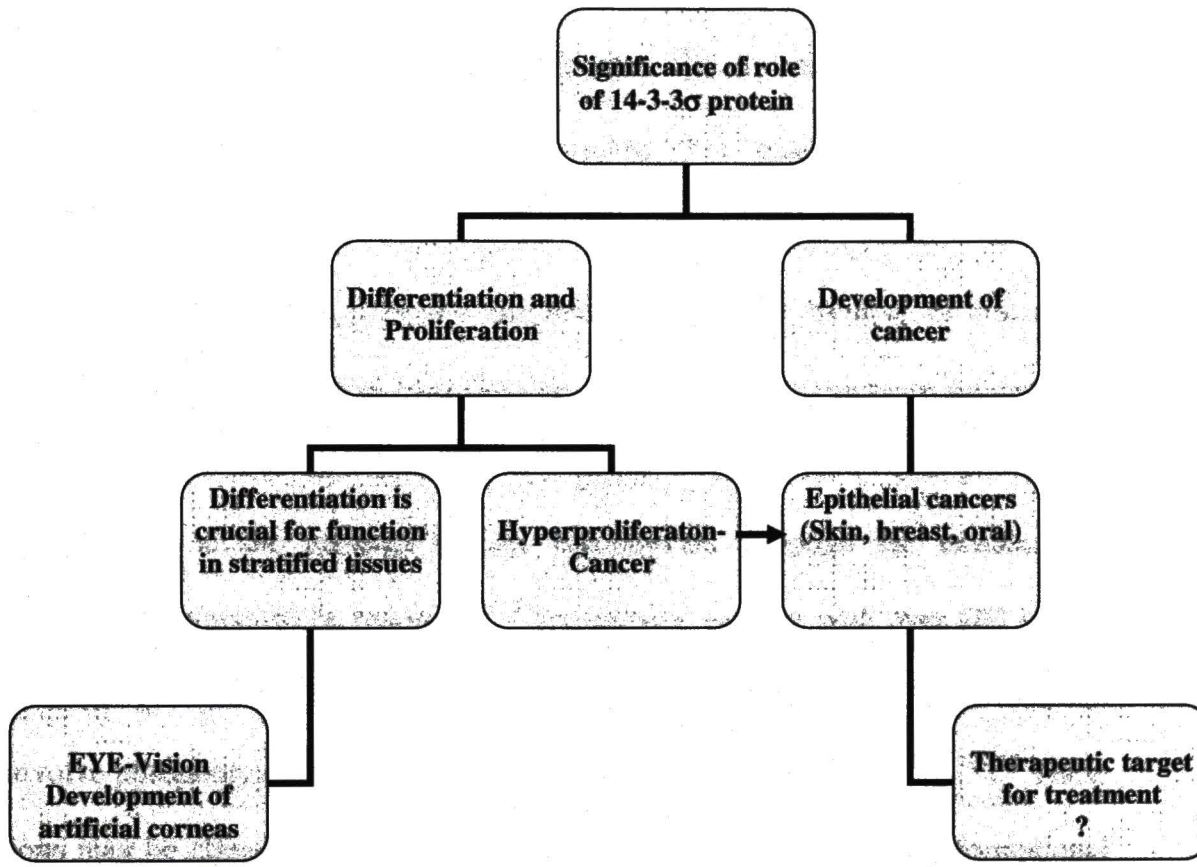
Impact/Significance:

Corneal epithelial differentiation is crucial for the function of the cornea as a barrier tissue that protects the inner eye. The various steps involved in corneal epithelial cell differentiation are poorly understood. This study is expected to help establish the role of 14-3-3σ protein as a novel player in differentiation. The switch in keratin expression corresponding to the cytoskeletal changes in differentiated cells and the signals and proteins involved are not well understood. This study will help clarify some of the questions associated with these issues. Corneal grafts are beginning to be studied as

candidates for corneal transplants in case of chemical or burn injuries and also in cases of partial and total limbal stem cell deficiencies. In the absence of methodologies that maintain "stemness" of limbal/corneal stem cells, specifically designed cell lines are possible global transplant material. Several epithelial cell lines are being studied for use as components of corneal equivalent. Differentiation of these cell lines will be crucial for appropriate maintenance of the transparency of the corneal graft. Maintaining proliferation by delaying differentiation may also be necessary for long-term maintenance of these corneal equivalents. This study could therefore provide vital information for construction of corneal equivalents.

Another major area where studies on 14-3-3σ will be significant is in the cancer field where this protein has been shown to be involved. Since down regulation of 14-3-3σ is observed in a number of cancers, this protein may be a therapeutic target in studies involved in the treatment of cancer.

Figure 1 (8): Significance of studies on the role of 14-3-3σ protein.



CHAPTER II

PRESENCE AND DISTRIBUTION OF 14-3-3 PROTEINS IN THE ANTERIOR SEGMENT OF THE HUMAN OCULAR SURFACE.

The 14-3-3 proteins, discovered in the CNS and cerebro-spinal fluid, in 1967, make up a family of highly conserved acidic molecules. [26]In mammals, seven isoforms (β ,γ, ε, σ, ζ, θ, η) are known, each being the product of a separate gene. Although phosphorylation is not considered to be required for their biological activity, 14-3-3 α, δ and τ are the phopshorylated β,γ and θ isoforms. [26, 27, 111]The number of eukaryotic cells/tissues in which these proteins have been detected continues to increase suggesting a ubiquitous expression and function. The biological activity of 14-3-3 proteins is predominantly due to the presence of the homodimers although the existence of heterodimers is beginning to be reported.[31] [30]The dimers interact with over 200 known target molecules phosphorylated at specific serine/threonine residues but interactions that do not involve phosphorylated partner are also beginning to be identified. [28, 112-115] These interactions may be classified on the basis of the following functions i) conformational change in the binding partner, ii) masking or exposing of the functional motifs that regulate the partners intracellular localization and iii) changes in phosphorylation state or stability of the target molecules.[31] The diversity of functions results from these interactions is growing and includes events associated with metabolism[26], cell cycle control[26, 34], apoptosis[30], protein trafficking[26], transcription[26], stress responses[116] and malignant transformation.[26, 30, 34, 116] However, this is still a new and rapidly developing

field of study, and many regulatory signaling pathways involving 14-3-3 proteins remain poorly understood or unknown. The biological activities of 14-3-3 isoforms that have so far been established concern exclusively intracellular events. The discovery that some isoforms are secreted and may have paracrine role suggest intriguing possibilities for extracellular 14-3-3 functions. [103-105, 107, 117]

Considering the scope of involvement of 14-3-3 proteins in the functions of living systems it is not surprising that studies cover areas of interest from cancer biology to embryonic development. Of particular interest in the former is the involvement of 14-3-3σ isoform in cell cycle regulation and its importance in a variety of cancers [breast cancer[118], carcinomas of the urinary bladder[119], ovaries[120], prostrate[120] and salivary glands[121]] supporting the view that this isoform is an oncogene.[122]

Specific Aim 1

To determine the expression of the 14-3-3 proteins in the human cornea, the conjunctiva and the primary cells comprising these tissues and the tear fluid.

Rationale

Our particular interest is in homeostasis, repair and pathologies of the tissues that comprise the anterior ocular surface. The epithelia in these dynamic barrier tissues are particularly tightly regulated and maintenance of tissue kinetics is achieved by balancing cell proliferation, migration and differentiation. The cell cycle regulation in these tissues may be considered to be a crossroad between proliferation and differentiation. A wide variety of intracellular and extracellular molecules are involved in these events but the

role of 14-3-3 proteins has not been studied.

Material and Methods

Cell Culture

Primary Corneal and Conjunctival epithelial cells. Epithelial sheets were obtained from Eye Bank corneas and conjunctivas (primarily bulbar) as previously described.[123, 38] Briefly, donor tissue was incubated in dispase (diluted with calcium free EpiLife medium to 12 Units/ml) at 4°C for 48 hrs. The epithelial sheets were dissociated into single cell suspension and then plated into collagen type IV coated 75 cm^2 vented flasks (tissue culture, TC) and cultured in serum free defined media (EpiLife, Cascade Biologicals) to 80% confluence. The epithelial cells are then subcultured by harvesting with trypsin/EDTA (Gibco Brl) neutralization of proteolytic activity with trypsin inhibitor (Sigma-Aldrich), and plating into freshly collagen IV coated TC flasks.

Conjunctival epithelial cells and corneal epithelial cell lines:

Corneal epithelial cell lines SV40[124] (a kind gift from Alcon Laboratories Inc) and E6 / E7[125] (a kind gift from Dr. V. S. Mootha; Dept. of Ophthalmology UT Southwestern Medical School, Dallas) immortalized, were thawed from frozen stock and cultured in serum free defined media (EpiLife, Cascade Biologicals) to 80% confluence. The epithelial cells are then subcultured by harvesting with trypsin/EDTA (Gibco Brl) neutralization of proteolytic activity with trypsin inhibitor (Sigma-Aldrich), and plating into freshly collagen IV coated TC flasks.

Corneal stromal fibroblasts

The epithelium was removed from the donor corneas as described above, and the residual

endothelium was peeled off as the descement membrane bearing residual endothelial cells. The denuded stromas were minced into 2mm cubes, which were allowed to adhere to tissue culture flasks. To these pieces medium (DMEM containing 10% fetal bovine serum - FBS from Atlanta Biologicals) was then carefully added. When the outgrowth of corneal fibroblasts was evident (within a week from set up), the pieces were removed and the culture process was continued. The cells were then subcultured into fresh flasks.

Corneal Endothelial cells:

E6/E7 transformed endothelial cells (a kind gift from Dr. V.S. Mootha, UT South Western medical center, Dallas) were thawed out from frozen stock and cultured in DMEM containing 10% serum. Primary endothelial cells were cultured from whole endothelial explants and cultured in DMEM containing 10% serum.

Indirect immunofluorescence:

Immunocytochemistry: Cells, cultured on glass coverslips (Fisherbrand), were rinsed in PBS, and fixed/permeabilized in methanol: acetone (1:1, 10 minutes at -20^{O}C). After re-hydration in PBS (0.256g/L $NaH_2PO_4\,H_2O$, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4; for 30 min), and distilled water washes (3x) the cells were blocked (overnight at 4°C) in PBS + 1% BSA. The specimens were then rinsed with PBS and distilled water (3x), and incubated with 1° antibody (4°C, overnight) diluted in 1XPBS. After rinsing in PBS (3x10min) containing Tween 20 (0.1%), cells were incubated with 2° antibody (1.5 hrs, RT) and rinsed in PBS +Tween 20 (0.1%, 3x10min). Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (30 min), stained with DAPI and mounted on glass slides (FluorSave™, Calbiochem, La Jolla, CA).

Immunohistochemistry

Donor corneas and conjunctiva were fixed in 4% formaldehyde and after the series of ethanol and xylene incubations, embedded in paraffin. Tissue sections (~10μ) were cut and deparaffinized. After re-hydration in PBS (0.256g/L NaH_2PO_4 H_2O, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4; for 30 min), distilled water wash (3x) and blocking (overnight at 4°C) in PBS + 1% BSA +1% horse serum, specimens were rinsed with PBS and distilled water (3x), and incubated with 1° antibody (4°C, overnight). After rinsing in PBS (3x10min) containing Tween 20 (0.1%), the tissue sections were incubated with 2° antibody (1 hr, RT) and rinsed in PBS +Tween 20 (0.1%, 3x10min). Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (1X10 min), and glass slides were mounted on the sections after DAPI staining (FluorSave™, Calbiochem, La Jolla, CA).

Antibodies: Primary antibodies were obtained from commercially available sources. 14-3-3 antibodies were purchased from Santa Cruz biotechnology and were used in the dilution recommended by the supplier [Table 2(i)].

Alexa Fluor 594nm goat anti-mouse, Alexa Fluor 594 goat-anti-rabbit and Alexa Fluor 594 donkey anti-goat (from Molecular Probes) secondary antibodies were used at dilutions of 1:1000. Negative controls in all experiments were specimens labeled with 2° antibody only and DAPI to show nuclei; these showed virtually no fluorescence.

Image Acquisition: Mounted specimens were examined on Olympus AX70 fluorescent microscope using SPOT Twain software. Electronic images were captured at a

magnification of 20X.

Western blot analysis: Cells harvested, whole epithelia, and endothelia were treated with lysis buffer [2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled water]. Genomic DNA was sheared by several passes through a 22-gauge needle, and samples stored at –20°C until needed. BCA protein assays (Pierce, Rockford, IL) of lysates were performed to determine the protein concentration and ensure equal loading of lanes. SDS PAGE was performed with 20µg protein/lane at room temperature using 12% Tris-Glycine, at 150V in Tris/glycine running buffer. Electro-blotting was carried overnight (4°C) at 10V in Tris/glycine buffer with 20% methanol onto nitrocellulose membranes (VWR international) and the transfer was confirmed by Ponceau Red (Sigma-Aldrich) staining of the membranes.

After de-staining in distilled water, membranes were blocked for 1 hour (RT) in blocking buffer (5% powdered milk and 1% BSA in PBS). Membranes were incubated at RT then at 4°C overnight, followed by 30 minutes at room temperature the following morning. Membranes were incubated in 1° antibody [Table2(i)] for 30 minutes (RT) incubated at 4°C overnight, and for 30 min. at RT the following morning. Membranes were then rinsed 3x10 minutes in PBS + 0.1% Tween-20 and incubated in 2° antibody for 1 hour (RT). After rinsing 3x 10 minutes in PBS + 0.1% Tween-20, membranes were developed (ECL chemiluminescence Amersham Biosciences, UK).

Table 2(i): Antibody Information. [IHC-Indirect Immunofluorescence, WB-Western Blot analysis]

Antibody	Source	Species	Dilution	
			IHC	WB
14-3-3σ	Santa Cruz	Goat	1:50	1:200
14-3-3ζ	Santa Cruz	Rabbit	1:50	1:200
14-3-3η	Santa Cruz	Goat	1:50	1:200
14-3-3θ	Santa Cruz	Rabbit	1:50	1:200
14-3-3γ	Santa Cruz	Rabbit	1:50	1:200
14-3-3ε	Santa Cruz	Rabbit	1:50	1:200
14-3-3σ	Lab Vision	Mouse	1:1000	1:1000
14-3-3β	Santa Cruz	Rabbit	1:50	1:200

Tear Fluid Collection

Tear washes were collected from 3 normal subjects as previously described.[126, 127] Sterile saline was instilled onto the donor ocular surface. Subjects were the asked to move their eyes without blinking. Tears were then collected using a micropipette. Cellular debris was removed by centrifugation. Approximately 20mg of protein was then directly loaded onto the gel for western blot analysis.

Detection of secreted 14-3-3 isoforms

Conditioned medium from corneal, conjuncival epithelia cells, E6/E7 and SV40 transformed corneal epithelial cells were collected after 48hrs of culture. The conditioned medium was centrifuged to remove any cellular debris. Strataclean resin (Stratagene) was used to pull down protein from the conditioned medium as per the manufacturer's instructions. Briefly, the conditioned medium was centrifuged at 10,000 rpm to pellet the resin. The resin was then re-suspended in 10ul of 1X sample loading buffer. The samples were loaded onto a gel and analyzed using western blot analysis as described earlier.

Results

We here describe the first systematic profiling of human cornea, conjunctiva, the component cells and some cell lines, by probing for the expression of all 7 isoforms using indirect immunofluorescence and confirming our findings with western blot analysis. We furthermore show the secretion of several isoforms into the conditioned media obtained from cultures of the corneal and conjunctival cells and in the human tear fluid from normal volunteers.

Expression of 14-3-3 isoforms in human cornea and conjunctiva

Using commercially available antibodies directed against 14-3-3 (β, ε, γ, η, θ, σ and ζ), we probed the tissue sections of paraffin embedded and frozen human cornea and conjunctiva for the expression of all the 14-3-3 isoforms. There were no differences in expression of these proteins between the results obtained for frozen sections and those from sections of paraffin embedded tissue. The collage of fluoromicrographs shows that six 14-3-3 isoforms (θ,γ, η, β,ζ and ε) are present in all corneal compartments of both the

central cornea and the limbus [Figure 2(1(A) a, c, d, e, f and g], while the expression of 14-3-3σ is confined to the epithelium [Figure 2(1(A)) b]. Specifically 14-3-3σ is strongly expressed in the superficial layers of the central corneal epithelium and the limbus epithelium.1 4-3-3β, 14-3-3γ and14-3-3η are strongly expressed in the superficial layers in the central cornea and uniformly expressed in all the layers of the limbus [Figure 2(1(A)) a, e, f]. 14-3-3ε and ζ are uniformly expressed in all the layers of the central cornea and the limbus [Figure 2(1(A)) c, g]. 14-3-3θ is uniformly expressed in all the layers but the level of expression in the limbus is higher in comparison to the central corneal epithelial cells [Figure 2(1(A)) d]. All the seven 14-3-3 isoforms are expressed in the human conjunctiva and 14-3-3σ is confined to the conjunctival epithelial cells [Figure 2(3)].

Expression of 14-3-3 isoforms in cultures cells of the cornea and conjunctiva

We then examined the expression in cultured primary cells of the cornea and conjunctiva and two human cornea cell lines. All the 14-3-3 isoforms are expressed in the primary corneal and conjunctival epithelial cells [Figure 2(1) B]. Corneal epithelial cells, E6/E7 transformed and SV-40 transformed also express all the seven 14-3-3 isoforms [Figure 2(1) B]. Corneal stromal fibroblasts and corneal endothelial cells express all the 14-3-3 isoforms except 14-3-3σ [Figure 2(5)]. Transformed endothelial cells (E6/E7) also show a similar 14-3-3 expression profile to the primary cells [Figure 2(5)].

14-3-3 isoforms secreted by primary corneal and conjunctival epithelial cells

Primary corneal and conjunctival epithelial cells when cultured in the serum free defined medium (EpiLife) were shown to secrete 14-3-3σ, 14-3-3γ and 14-3-3ζ into the culture

medium [Figure 2(6(A)]. Western blot analysis was used to show that transformed corneal epithelial cells also secrete 14-3-3σ, 14-3-3γ and 14-3-3ζ [Figure 2(6(A)]. Serum free culture medium treated in an identical fashion to the conditioned medium was used as a negative control.

Presence of 14-3-3 isoforms in the human tear fluid

We then examined tear fluid from normal volunteers for the presence of the 14-3-3 isofoms that were shown to be secreted into the culture medium of corneal and conjunctival epithelial cells. Western Blot analysis on tear samples from 3 donors revealed the presence of 14-3-3γ and 14-3-3ζ isoforms. 14-3-3σ isoform however was absent [Figure 2(6(B)].

Discussion

The ubiquitous functions of 14-3-3 family of proteins have been described in several tissues and the component cells but the distribution and signaling role studies have been limited initially to the CNS and more recently to skin. We have been interested for some time in the human ocular surface tissue.[34-37] The diversity of cellular events, specifically the balance between the cell cycle regulation and differentiation of the self-renewing epithelia, is a feature in homeostasis and repair in these tissues. We therefore consider that the diverse biological activities of the 14-3-3 family of proteins display the spectrum of biological activities and could play a significant role in the human cornea and conjunctiva. Our results for the first time show that all the 14-3-3 isoforms are expressed in the cornea and conjunctiva but that the expression of 14-3-3 σ, also known

as *stratifin,* is confined to the differentiated superficial layers of the corneal epithelium. This correlates well with the expression of stratifin in the epidermis a stratified epithelium of the skin.[25, 128] Although this 14-3-3 isoform is proposed to be associated with differentiation, its presence in stratified epithelia other than skin has not been determined and the connection with stratification (implying differentiation) has not been established.

The abrogation of 14-3-3 σ in primary keratinocytes has lead to immortalization[25, 129] and its expression is reduced in some cancers[120, 121, 130] but up-regulated in others[25, 32, 120, 130-132] In order to begin a detailed study of this proposed relationship with cell cycle control, we compared the expression profile of all the isoforms in the primary corneal epithelial cells with that in two immortalized cell lines in which the cell cycle arrest has been altered by E6/E7 and SV-40 transduction [Table 2(1)]. In this context, SV40 and E6/E7 immortalized cells showed levels of expression of all seven isoforms (including 14-3-3 σ) similar to those determined for the primary cells with the exception of the low levels of expression of 14-3-3σ. The isoform that was "differentially" expressed was 14-3-3 θ. It was barely detectable in the primary cells but expressed robustly by both the corneal epithelial cell lines. This 14-3-3 isoform has been shown to be involved in nuclear localization of telomerase,[29]an enzyme that has been established to play a well defined role in somatic cell proliferation and senescence. Furthermore, telomerase is constitutively expressed in a majority of cancers and has been shown to extend proliferative life-span *in vitro* when constitutively expressed in several types of somatic human cells.[133-136] The expression of 14-3-3 θ is also low in the wild type endothelial

cells, which in spite of having long telomeres, are not particularly mitotic.[50] This raises the question whether in each tissue compartment, particularly the epithelia, the role of 14-3-3 σ is combined with alternate mechanisms involving possibly other 14-3-3 isoforms (s), and that 14-3-3 θ might indirectly play such a role in the cell cycle regulation. Further credence to the possible tissue specificity is that the level of expression of 14-3-3 γ by the primary corneal epithelial cells was very low, but its expression by immortalized cells was uniform and definitive. Since the corneal epithelium is not predisposed to neoplasia and hyperproliferative epithelial cells either do not differentiate or do so with difficulty, the role of 14-3-3σ in corneal epithelial cell cycle appears to be more complex than its role in the differentiation of the epidermal (keratinocytes) homeostasis and pathologies.

We also examined the expression of 14-3-3 proteins in the conjunctival tissue. Similarly to their profile in the cornea, all the 14-3-3 isoforms were also expressed in the epithelium and stroma and the 14-3-3 σ expression was confined to the epithelium. Since we examined Eye Bank tissue and consequently had no information about the tissue orientation it was more difficult to arrive at the precise distribution within the epithelium that is possible in the highly organized corneal tissue. In contrast to the primary corneal epithelial cells a low expression of the 14-3-3 ζ isoform was evident but we did not have the access to SV40 and E6/E7 immortalized conjunctival epithelial cell lines to make a comparison between normal and hyperproliferative cells and compare these with the results that we obtained for the corneal epithelial cells. In the primary conjunctival epithelial cells 14-3-3 ζ expression was very low in contrast to its strong presence in the

primary corneal epithelial cells. These results suggest that the interaction of 14-3-3 isoforms with proliferation / differentiation machinery in the epithelial cells may exhibit some tissue specificity.

It has also been proposed that 14-3-3 σ is secreted in the culture medium during propagation of an SV40 immortalized corneal cell line.[137] We show for the first time that primary corneal and conjunctival epithelial cells secrete 14-3-3 σ into the culture medium as do E6/E7 and SV40 immortalized corneal epithelial cells. Western blot analysis of the "conditioned media" from the primary cells and cell lines showed the presence of 14-3-3 γ and ζ isoforms in addition to the 14-3-3σ isoform. Although the intracellular functions of 14-3-3 proteins has been studied and is beginning to be understood, their extracellular function is largely obscure. It has been shown that the 14-3-3 σ can activate fibroblasts to up-regulate secretion of matrix metalloproteinases (MMPSs) but the mechanisms of export from the secreting cells, and the nature of interaction with the cell membrane and subsequent import into the target cells, are not known.[105] MMP functions in tissue homeostasis are regulated by the appropriate tissue inhibitors of matrix metalloproteinases (TIMPs). This equilibrium is altered after injury and during disease processes where matrix degradation and reorganization (remodeling) are likely to take place.[138] Under normal conditions in the cornea the signaling via soluble ligands might not be expected under homeostatic conditions since remodeling either does not take place or it extremely slow. Injuries to the ocular surface alter this scenario and the signaling events originating from the epithelium and targeting the cellular component in the stroma

are escalated in intensity and scope. Consequently the possible paracrine role of 14-3-3σ might be confined to the wound healing / tissue repair process.

The interaction of the epithelia with the tear film compartment is also interesting. Corneal and conjunctival cells are contributors to the tear film composition together with other anterior segment tissues/cells. Conversely the tear fluid contributes to the homeostasis and proper functions of the ocular surface epithelia. We therefore examined the human tear fluid for the presence of 14-3-3 isoforms that are secreted *in vitro*. Although 14-3-3 σ could not be detected, the 14-3-3γ and ζ isoforms were present. In view of the fact that 14-3-3γ is predominantly expressed by the conjunctival epithelial cells while 14-3-3ζ is strongly expressed by the corneal epithelial cells it might be speculated that the contributors of these two isoforms to the tear fluid composition is due to the conjunctival and corneal epithelial cells respectively. It is unlikely that the two isoforms arise from lysed epithelial cells because 14-3-3σ which is strongly expressed in these cells is absent in the tear fluid.

In this study we have systematically examined the corneal and conjunctival tissue and report the expression of all the 14-3-3 isoforms and their distribution within the tissue the highlight of which is the specific presence of 14-3-3 σ in both the epithelia. We have also shown the presence of these proteins in the corneal and conjunctival primary cells and corneal cell lines and report the secretion of three isoforms: σ, γ and ζ. Surprisingly, of the three secreted isoforms only 14-3-3 γ and ζ were found to be present in tear fluid from healthy human donors.

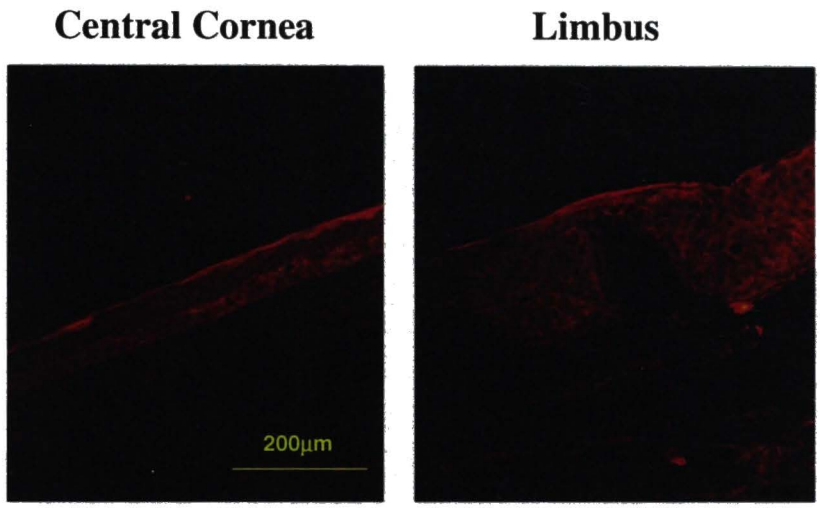
We were interested to note the suppressed expression of 14-3-3 θ the primary corneal epithelial cells and 14-3-3 ζ in the primary conjunctival epithelial cells and the functional implications of this differential expression. It is also interesting to speculate on the extracellular role of secreted isoforms and the origin and the function of the secreted isoforms present in the tear fluid.

Figure 2 (1):

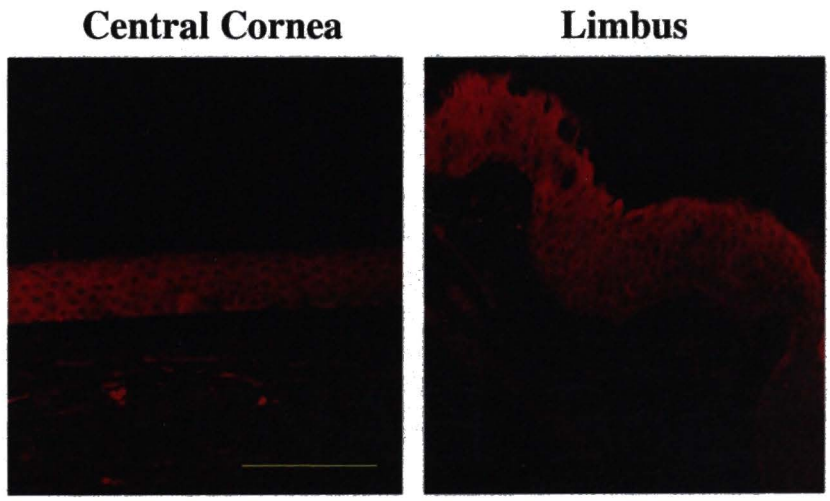
(A) Expression of 14-3-3 isoforms in the human cornea (Central and the limbus) as determined by Indirect Immunofluorescence.

(B) Expression profile of 14-3-3 isoforms in (a) Corneal epithelial cells WT, (b) Corneal epithelial cells E6/E7 transformed and (c) Corneal epithelial cells SV40 transformed, as determined by Western Blot analysis.

(A)

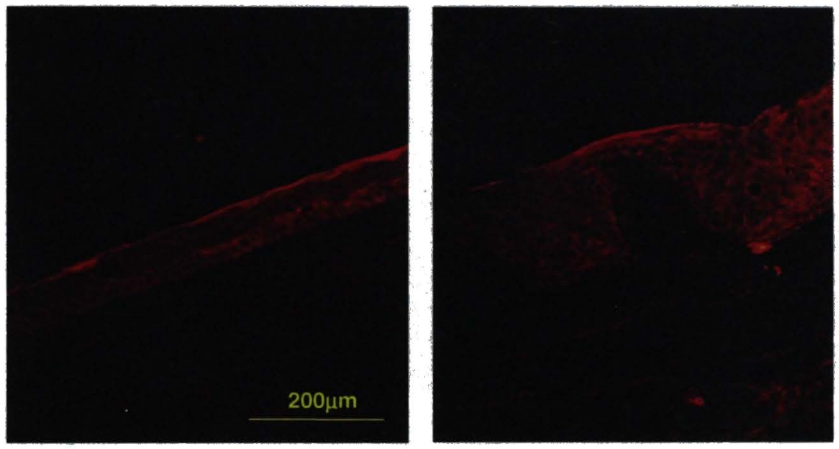


14-3-3β



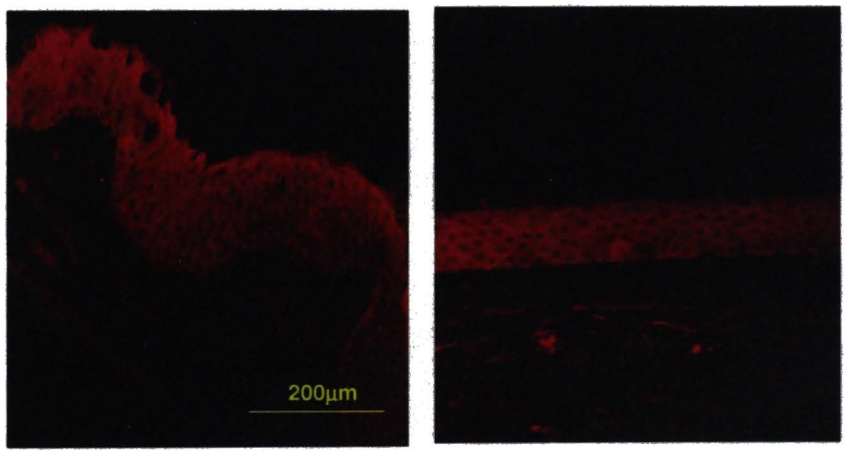
14-3-3ε

Central Cornea **Limbus**

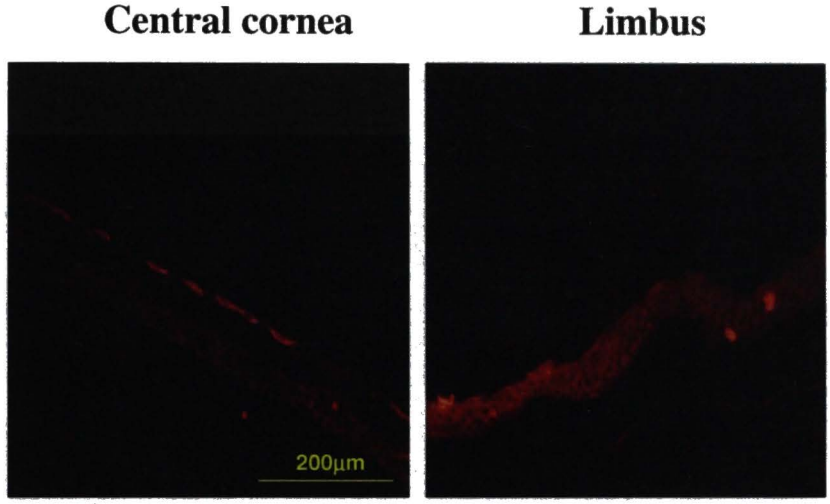


14-3-3σ

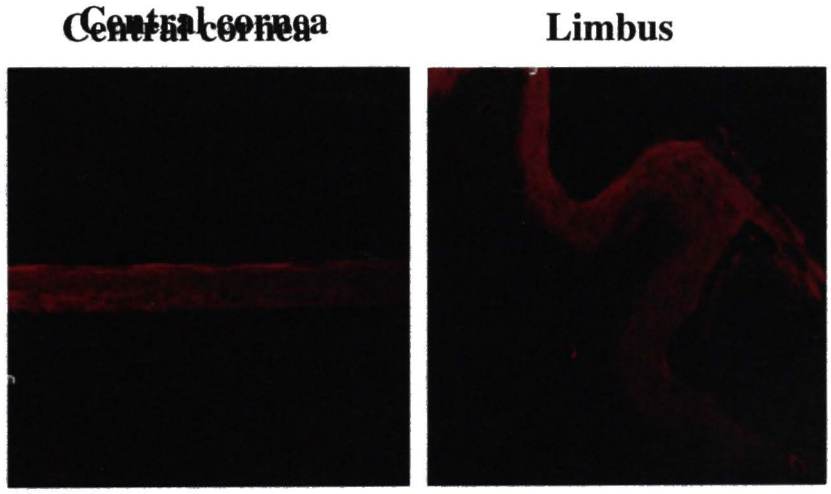
Central Cornea **Limbus**



14-3-3θ

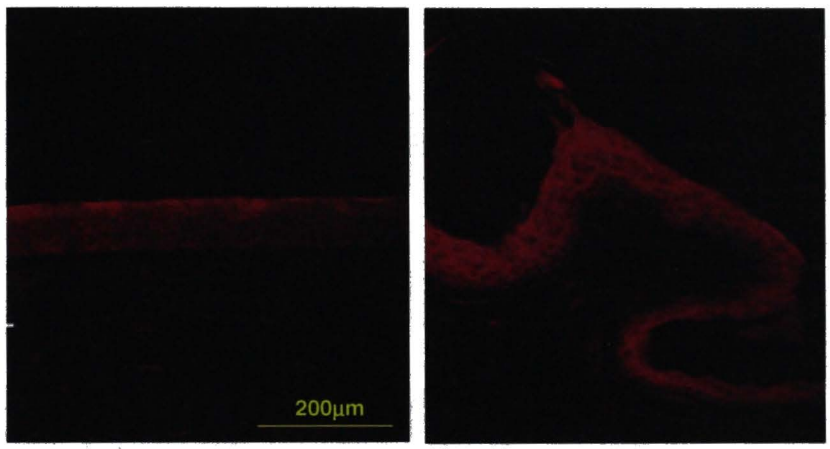


14-3-3γ



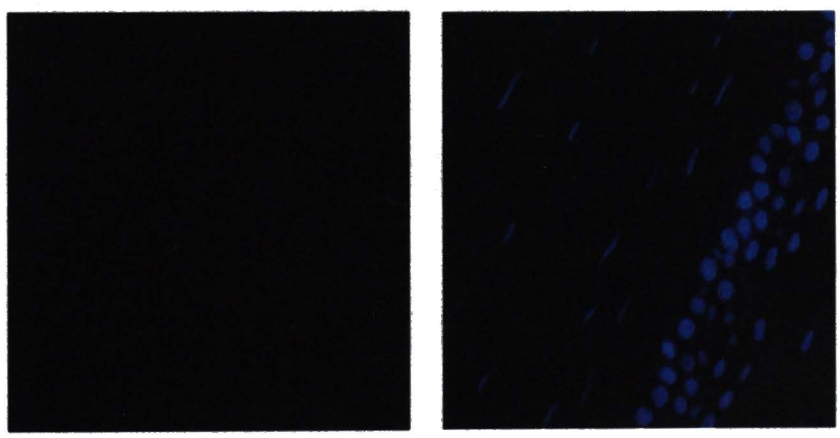
14-3-3ζ

Central cornea **Limbus**



14-3-3η

Central cornea **Limbus**



Antibody Control

(B)

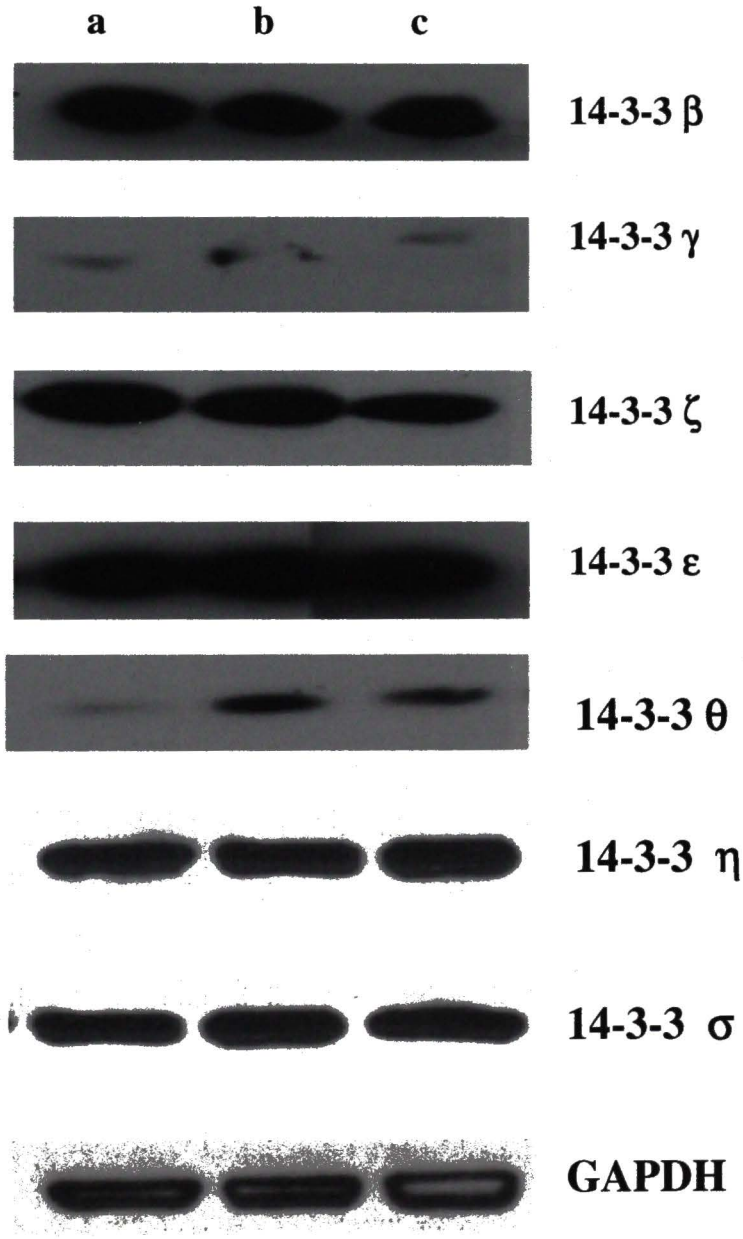
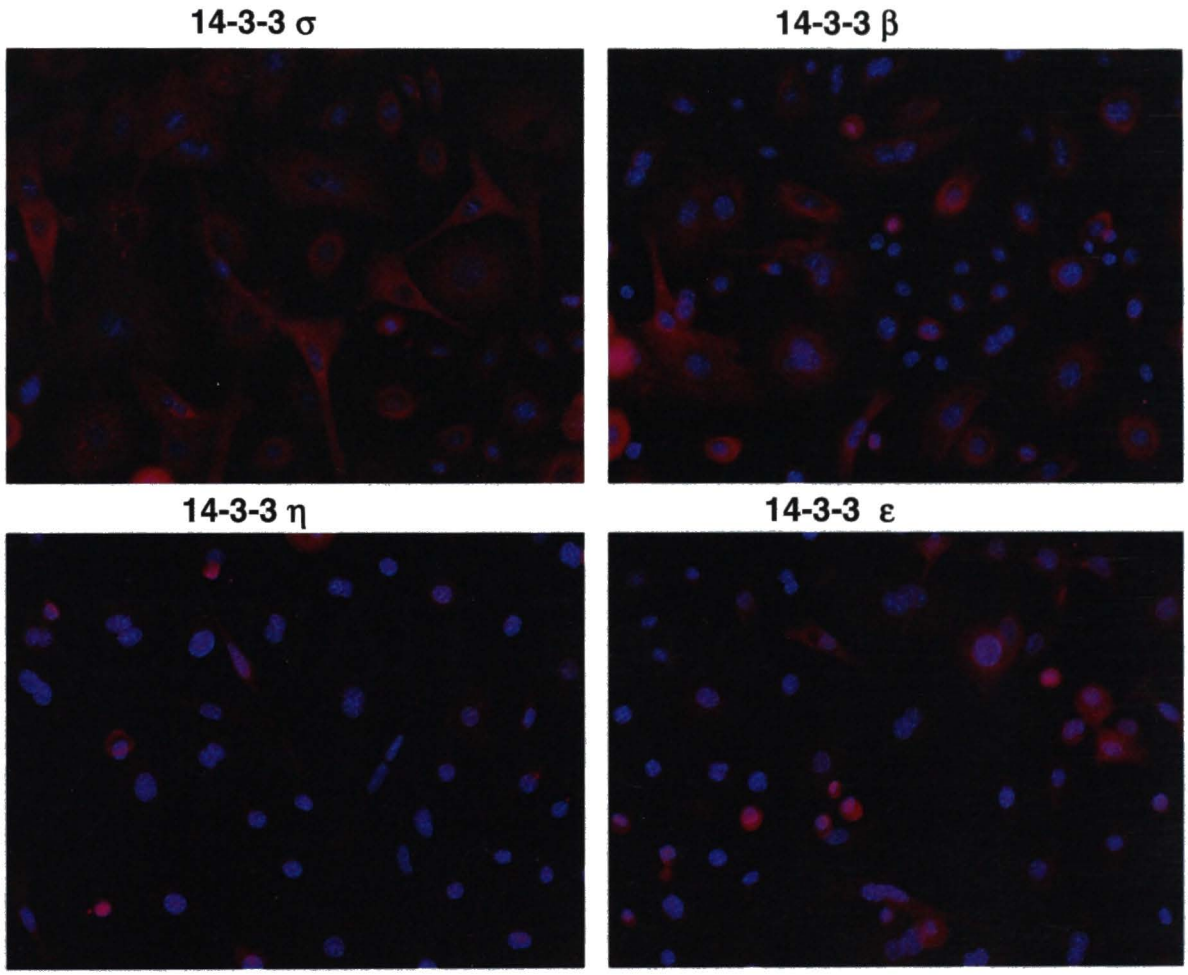


Figure 2 (2): Expression of 14-3-3 isoforms in corneal epithelial cells as determined by Indirect Immunofluorescence. [Magnification-20X]



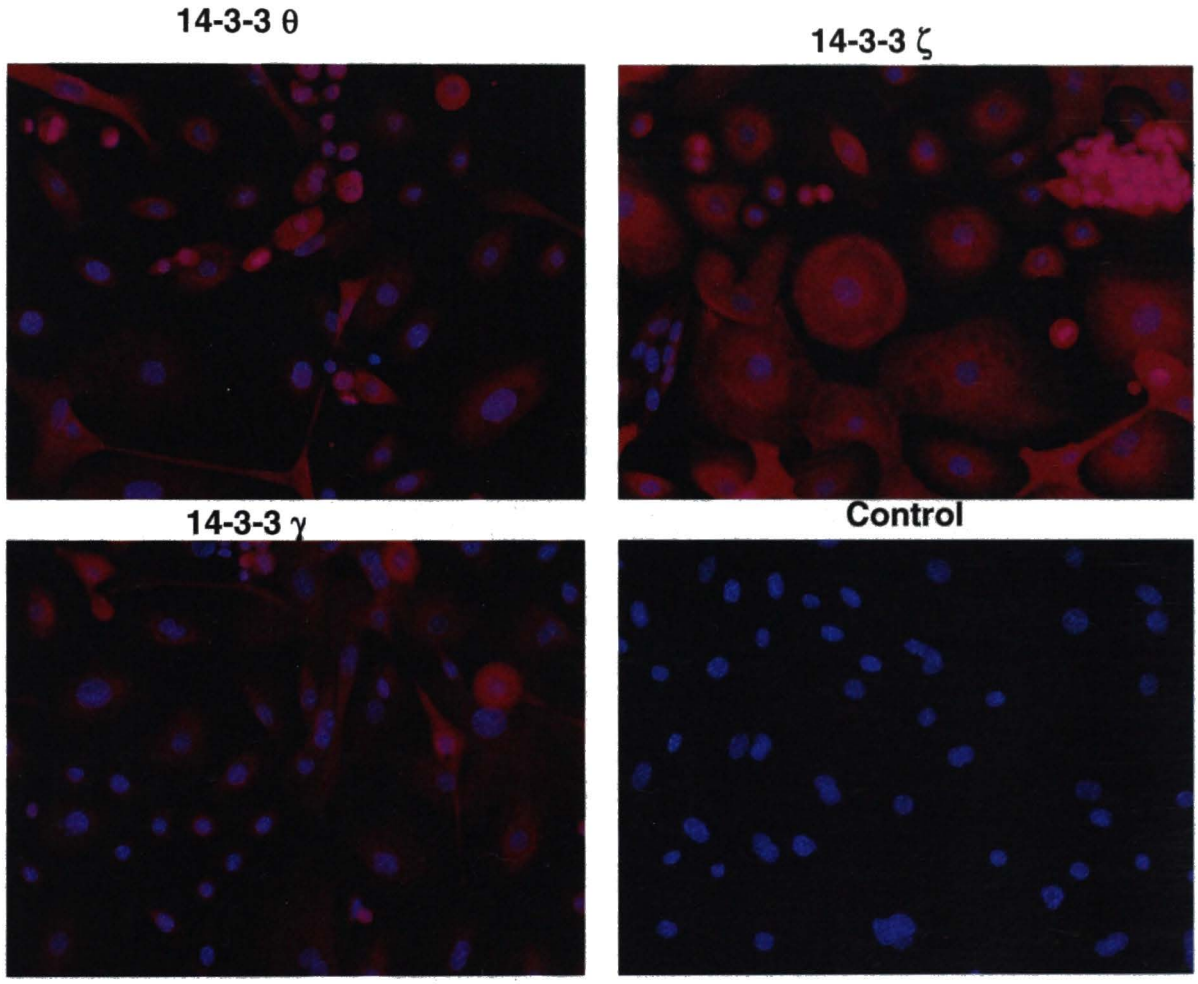
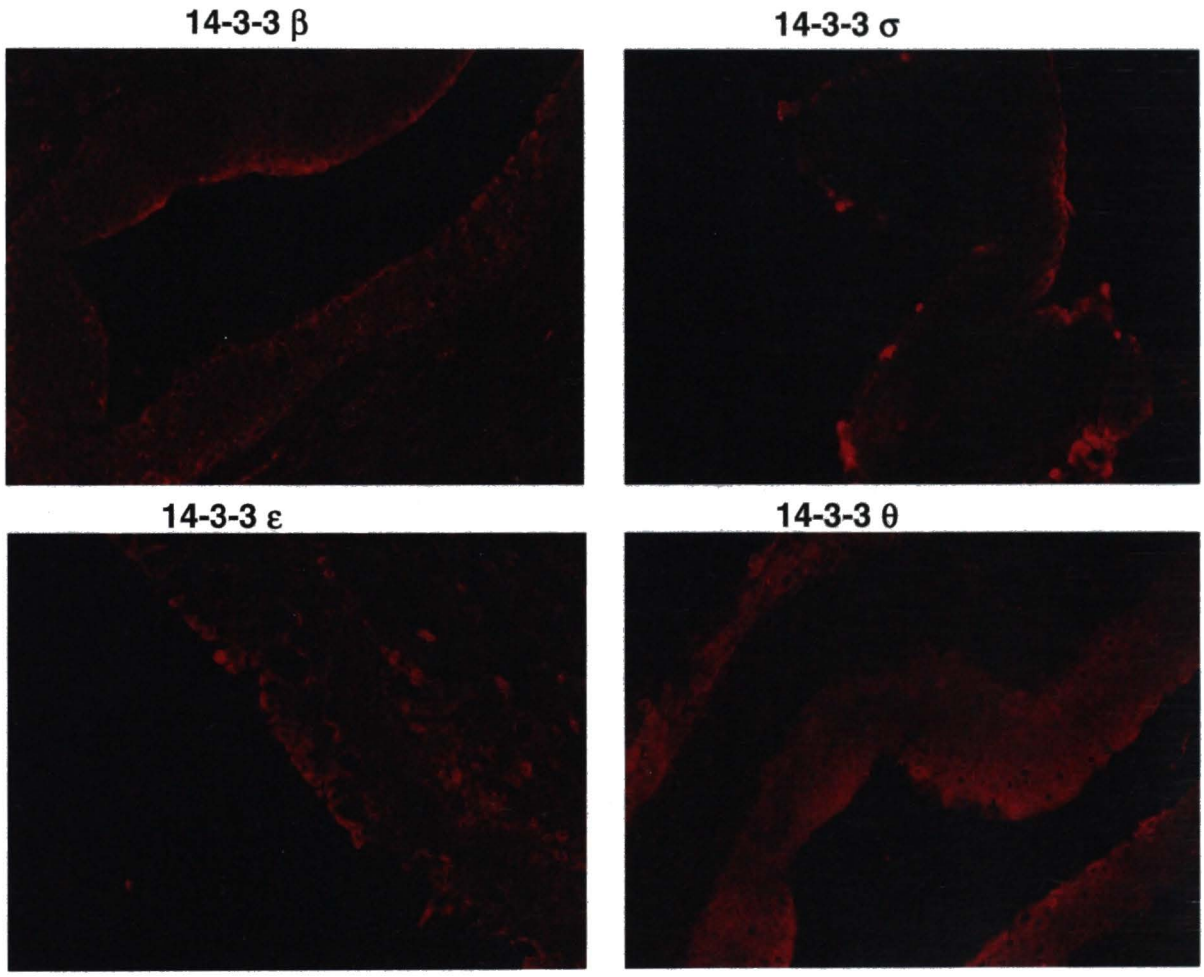

14-3-3 θ
14-3-3 ζ
14-3-3 γ
Control

Figure 2 (3): Expression of 14-3-3 isoforms in whole conjunctival tissue as determined by Indirect Immunofluoresence. [Magnification-20X]



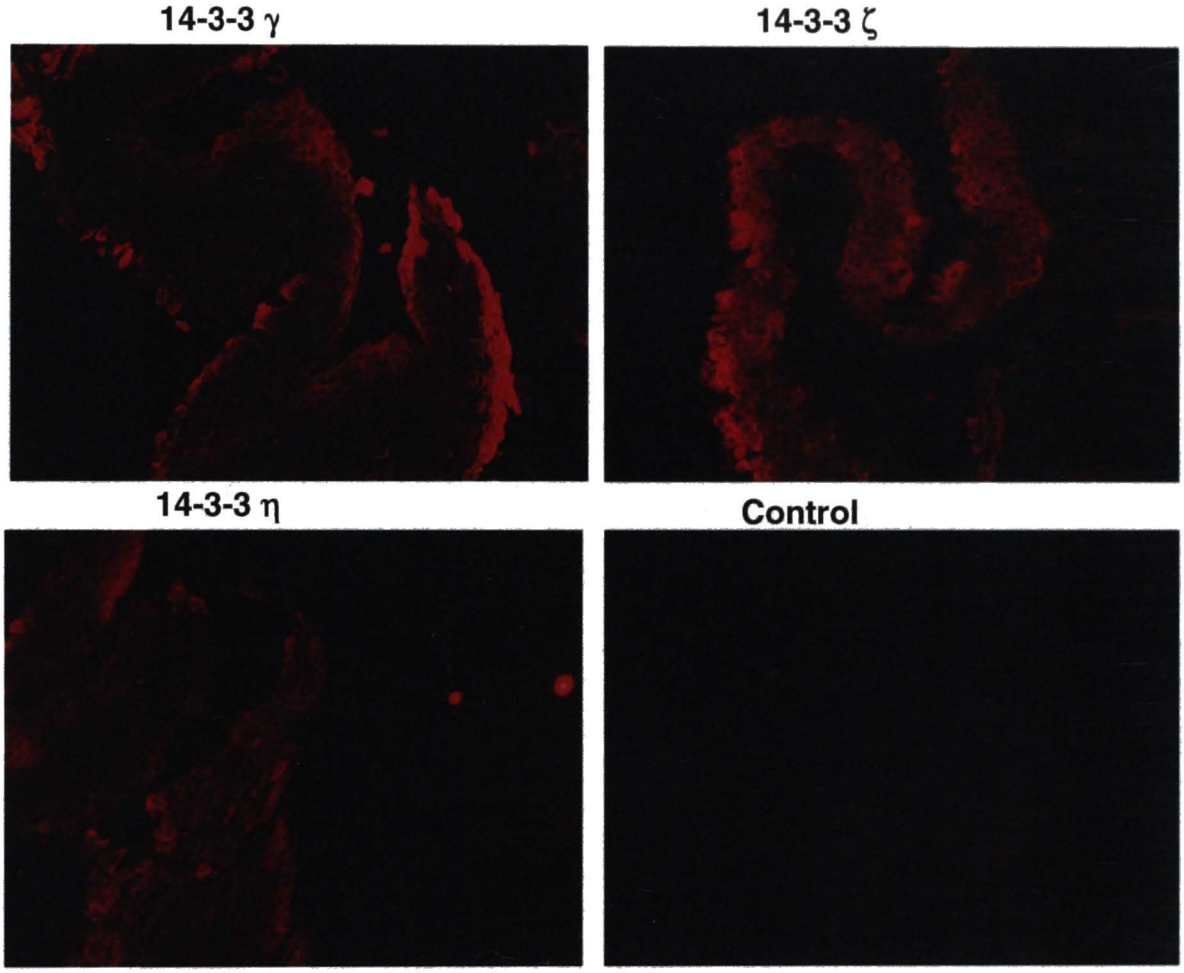
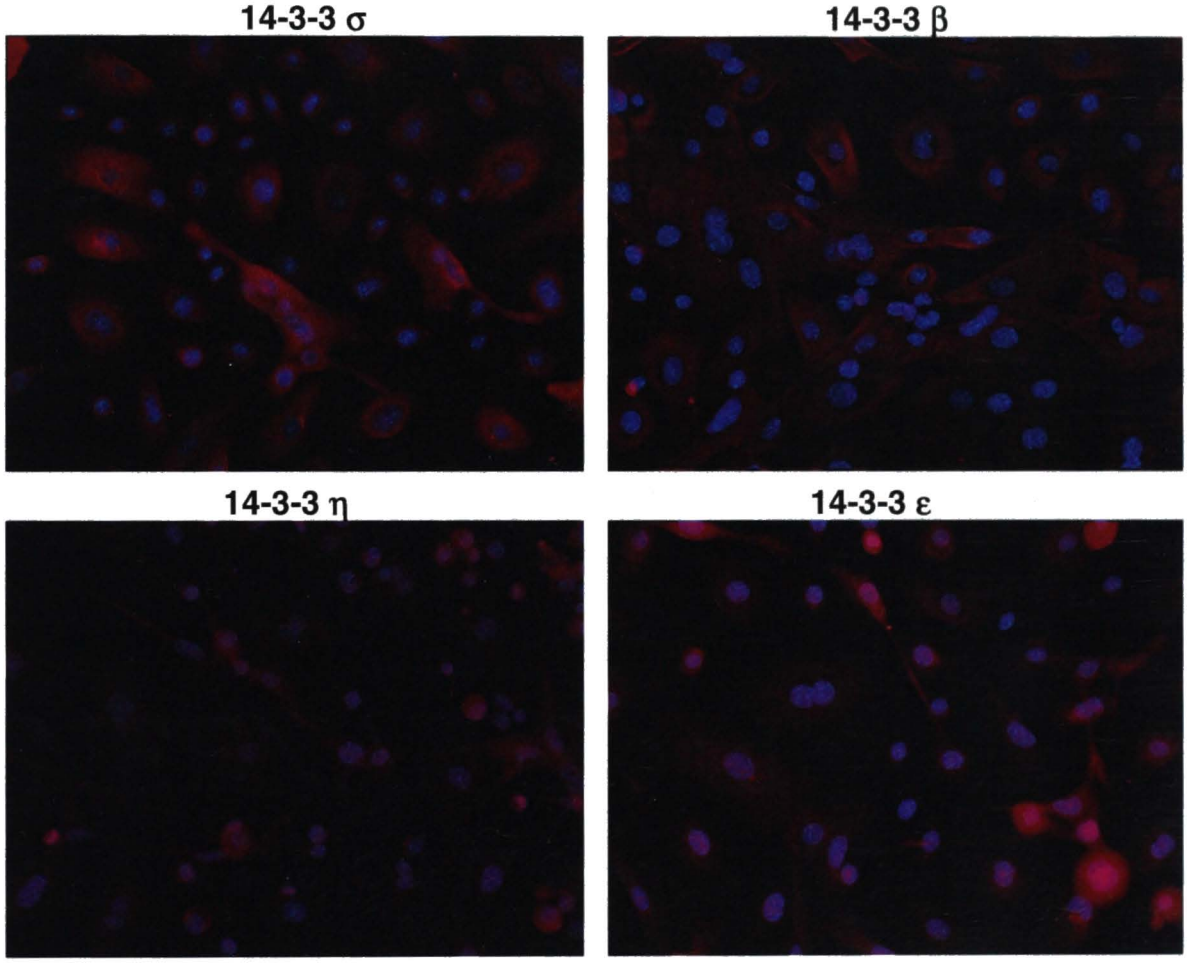

14-3-3 γ
14-3-3 ζ
14-3-3 η
Control

Figure 2 (4): Expression of 14-3-3 isoforms in conjunctival epithelial cells as determined by Indirect Immunofluorescence. [Magnification-20X]



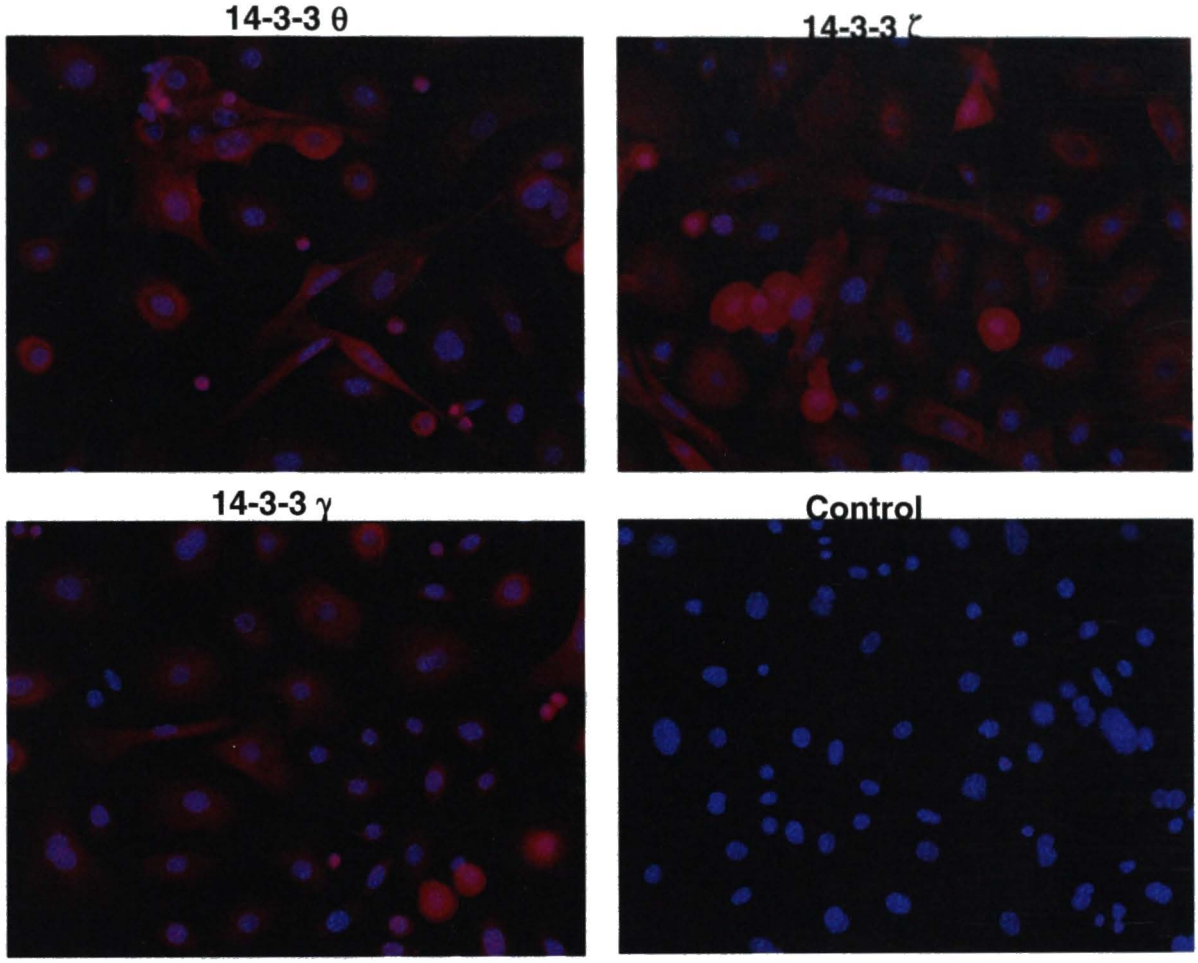

14-3-3 θ
14-3-3 ζ
14-3-3 γ
Control

Figure 2 (5): Expression profile of 14-3-3 isoforms in (a) Conjunctival Epithelial cells WT, (b) Corneal stromal fibroblasts WT, (c) Corneal endothelial cells WT and (d) Transformed corneal endothelial cells as determined by Western Blot analysis.

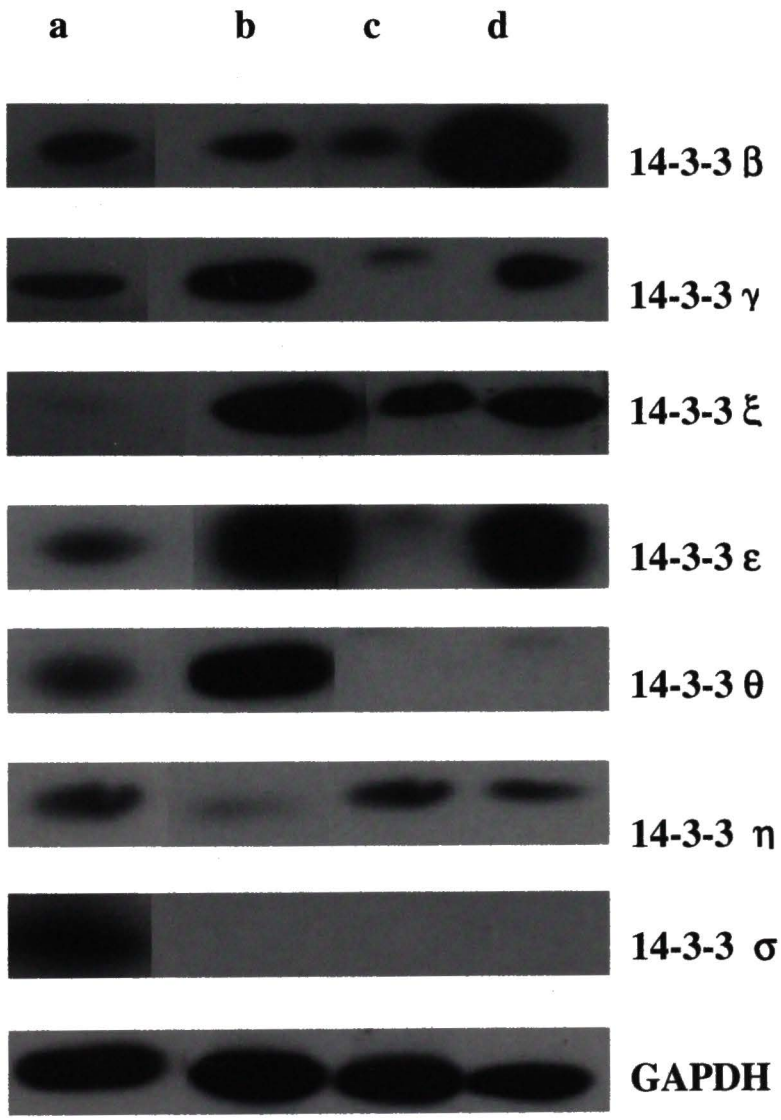
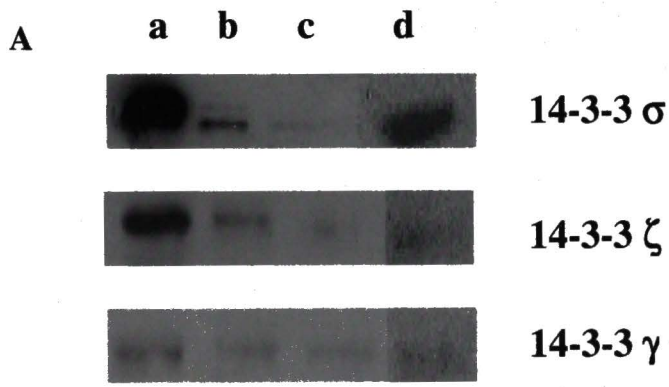


Figure 2 (6): Analysis of secreted isoforms.

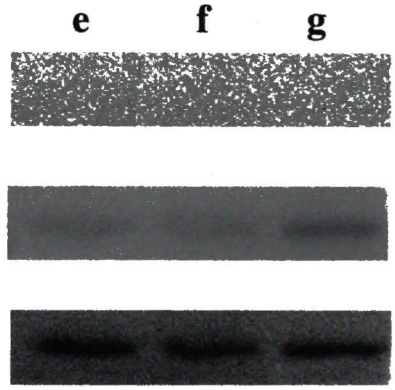
(A) Isoforms of 14-3-3 secreted by (a) Corneal epithelial cells WT (early passage), (b) Corneal epithelial cells E6/E7 transformed and (c) Corneal epithelial cells SV40 transformed as determined by Western Blot analysis and (d) Conjunctival epithelial cells.

(B) Isoforms present in human tears (e) T1 (f) T2, (g) T3.

(C) Ponceau stain of tear samples



B



C

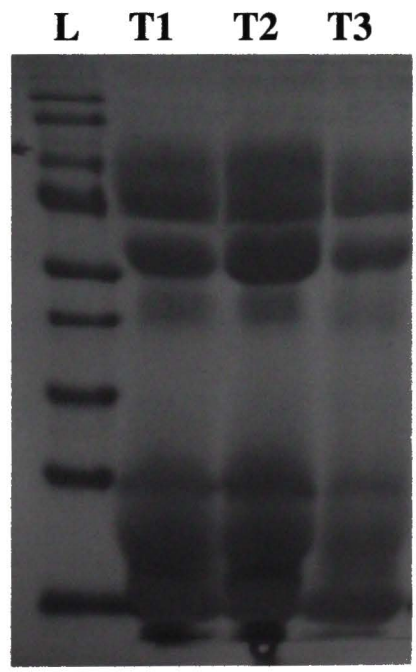


Table 2 (1): Summary of the 14-3-3 protein expression in the cornea and conjunctiva.

14-3-3 Isoforms	Corneal Epithelial cells			Conjunctival Epithelial cells	Corneal Stromal fibroblasts	Corneal Endothelial cells	
	WT	E6/E7	SV-40				
σ	++	++	++	++	-	-	-
β	++	++	++	++	+	+	++
γ	+	+	+	++	+	+	++
θ	+	++	++	+	+	+	+
ζ	++	++	++	+	+	+	+
η	+	+	+	+	+	+	+
ε	++	++	++	+	+	+	++

Expression levels as determined by western blot analysis. Densitometric value (after normalization) of 0.5 or greater:"++" (very high expression), value less than 0.5: "+" (positively expressed) and value of 0: "-" (negative-no expression).

CHAPTER III

ROLE OF 14-3-3σ IN NORMAL CORNEAL EPITHELIUM

14-3-3 σ has been proposed to be a tumor suppressor gene whose expression is up-regulated in response to UV damage.[38] p53, an upstream regulator of 14-3-3σ binds to its promoter and increases transcription. Increased levels of 14-3-3σ then arrests cell cycle progression in G2/M phase allowing DNA repair or apoptosis to take place.[58] 14-3-3 σ is a protein that is expressed in normal epithelial cells such as keratinocytes[25] and also cancer cell lines[57]. The role of this protein in normal ocular tissues has not been studied.

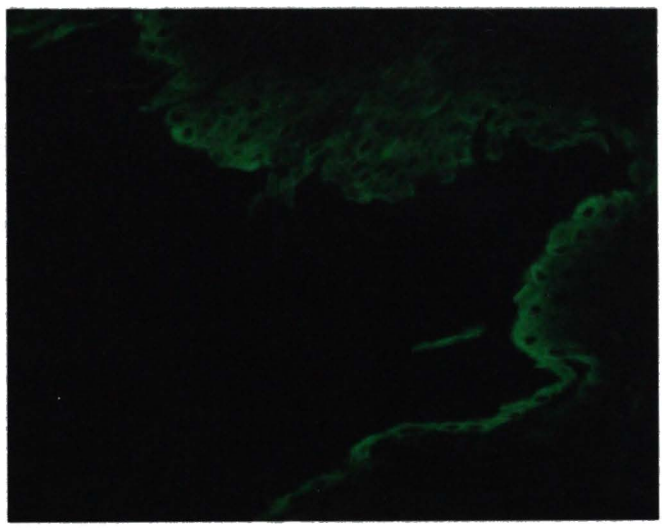
In the presence of high calcium (1mM) keratinocytes undergo differentiation as shown by the expression of fillagrin (a differentiation marker) and this is accompanied by an increase in 14-3-3 σ expression .[25] Down-regulation of 14-3-3 σ using antisense cDNA containing plasmid resulted in increased proliferation and extension of lifespan in primary human keratinocytes.[25] These data suggest that 14-3-3 σ alone can control cell cycle progression and since it is present in higher levels in differentiated cells it may play a role in supporting differentiation of epithelial cells.

Corneal epithelial cells are not predisposed to hyperproliferation and present a unique model to study proliferation and differentiation. Also, 14-3-3σ expression and its function in these cells have not been studied.

Figure 3 (1): Expression of 14-3-3σ in the cornea as determined by Indirect Immunofluorescence. (a) Central Cornea, (b) Limbus and (c) Control.

(Green: 14-3-3σ expression).

(a)



(b)

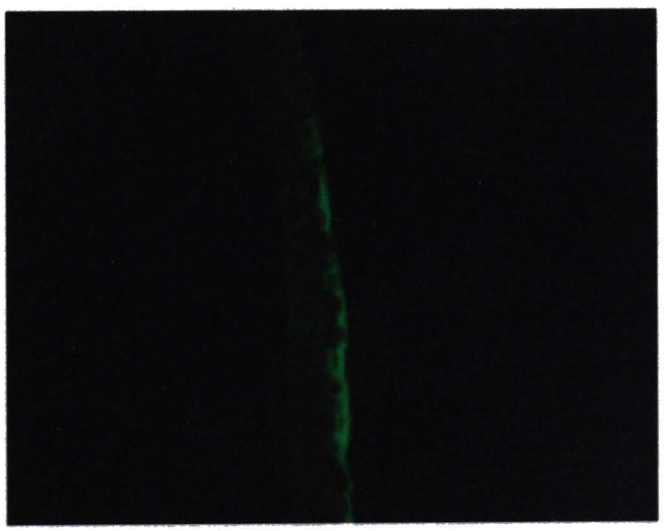
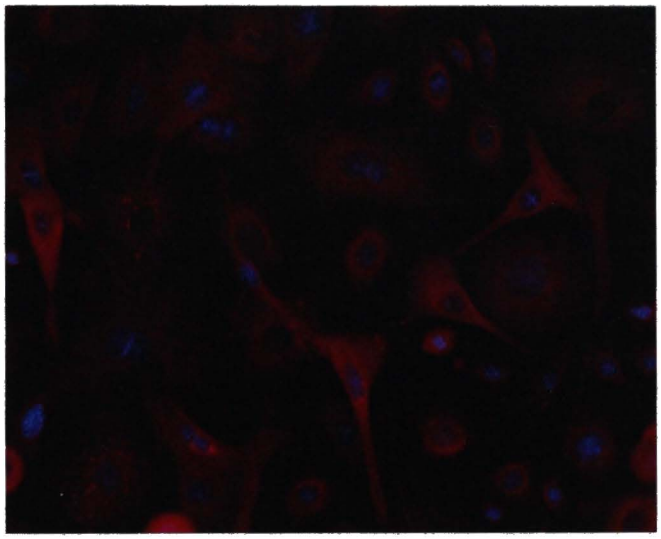
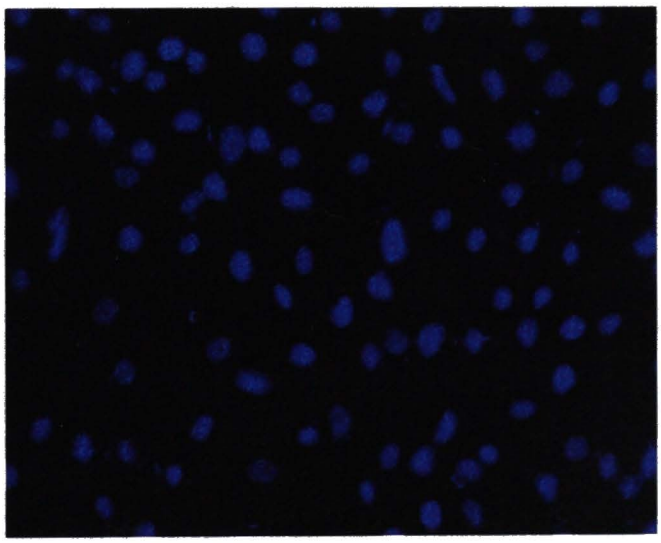


Figure 3 (2): Expression of 14-3-3σ in cultured corneal epithelial cells as determined by Indirect Immunofluorescence. (a) WT CEC and (b) Control

(a)



(b)



Specific Aim: 2

14-3-3 σ is involved in corneal epithelial cell cycle regulation (Differentiation and DNA damage response).

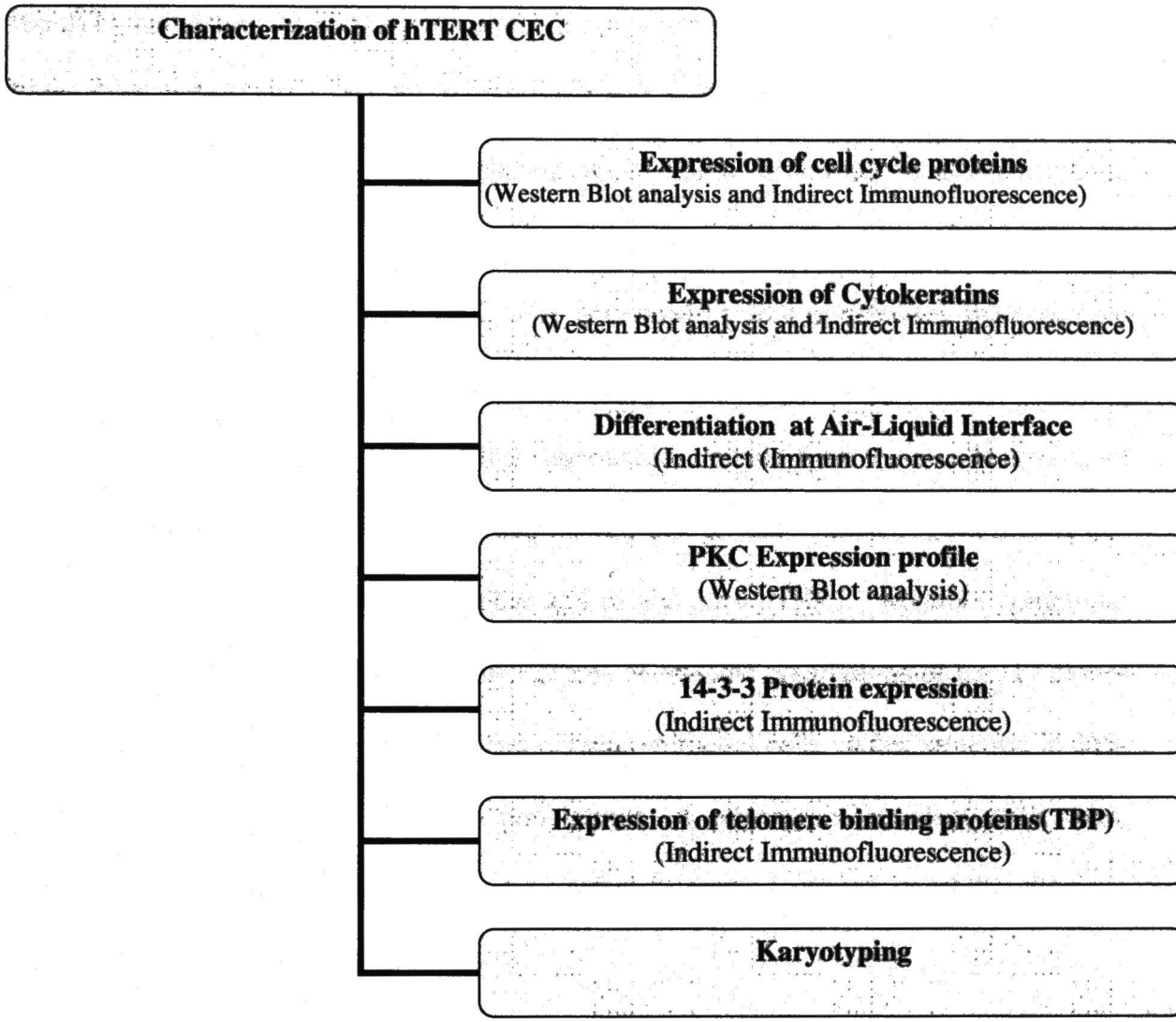
2(a) To show that hTERT CEC is a suitable model to study the role of 14-3-3σ.

Rationale

Harvested corneal epithelium is a heterogeneous mixture of proliferating and differentiating/differentiated cells. Under *in vitro* culture conditions the overall proliferation potential of these cells decrease and they begin to differentiate. This makes a variety of studies including that of differentiation difficult. It is therefore necessary to develop and use a cellular model that is very similar to the WT CEC.

Telomerase immortalization of cells (hTERT CEC) by ectopic expression of reverse transcriptase does not alter the expression of cell cycle regulators, unlike the traditionally generated cell lines (E6/E7 or SV-40 transformed). These have inactive pRb and/or p53 proteins. These cell lines are also heteroploid in nature and therefore may not serve as suitable models of WT corneal cells. The hTERT CEC cell line available from ATCC was developed using 3T3 feeder layers and hence there is a possibility of cell line cross contamination. However, the hTERT CEC we have generated have not been cultured on feeder layers avoiding the possibility of cross contamination. These cells therefore could be the most suitable cell line to study corneal epithelial differentiation.

Research Design



Materials and Methods

hTERT CEC generation

Corneal Epithelial Cells: Donor corneas were incubated in dispase solution (10 Units /ml), at 4°C for 2 days. The epithelial sheet was removed, dispersed into a single cell suspension, and plated into collagen type IV (4μg/cm^2) coated 25 cm^2 flasks. Epithelial

cells were cultured in EPI Life Medium (Cascade Biologicals), and subcultured (36-40pdl) by harvesting (at 80% confluence) with trypsin (0.025%) EDTA (0.27mM).

Retroviral Infection: EcoRI fragments from pGRN145, containing hTERT, was subcloned into the retroviral vector pBabe puro, in which the puromycin resistance gene is under the control of the SV40 promoter. Recombinant viruses of the empty pBabe puro vector and the wild type hTERT CEC were generated by first transfecting the ecotropic packaging cell line PE501 cells using Fugene (Roche Molecular Biochemicals, IN) and selecting with 4 μg/ml puromycin. Viral supernatants derived from these cells were used to infect the amphotrophic PA317 packaging cell line to generate clones containing un-rearranged proviral copies of pBabe puro and pBabe puro hTERT. Medium containing released viruses produced from confluent dishes of selected populations of PA317 clones were filtered (0.45 μm) and used to infect corneal epithelial cells. After selection in 350-400 ng/ml puromycin for 5 days, populations of puromycin resistant cells were maintained in their respective media and the selection applied after every 15-20 pdls.

Cell Culture

Corneal epithelial cell lines hTERT CEC and E7/hTERT (generated by our lab) immortalized CEC, were thawed from frozen stock and cultured in serum free defined media (EpiLife, Cascade Biologicals) to 80% confluence. The epithelial cells are then subcultured by harvesting with trypsin/EDTA (Gibco BRL, Carlsbad,CA) neutralization of proteolytic activity with trypsin inhibitor (Sigma-Aldrich, ST Luis,CA), and plating into freshly collagen IV coated TC flasks.

Construction of Corneal tissue equivalents (CTE)

The collagen solution was mixed thoroughly at 4°C as 8 parts collagen (3mg/ml, porcine Cellmatriix), 1 part 10X Ham's F-12 medium, and 1 part reconstitution buffer (0.05N NaOH, $NaHCO_3$, Hepes) and the pH was adjusted to 7.4. To this solution 350,000/ml keratocytes was suspended in this collagen mixture which was allowed to polymerize in wells of a 6 well plate at 37°C, 5% CO_2. The CTE were treated with Hams F-12(Gibco Brl) containing 5 % FBS (Atlanta Biologicals) until the cells were accommodated in the collagen. hTERT CEC, (3x106/cell type) were seeded on top of the CTE at after removal of medium and allowed to attach overnight. The CTE were carefully transferred into multiwell plate inserts (Costar). Hams F-12 containing 5% FBS was added below the insert and 200ul of Epilife medium was added on top of the epithelial cells. The CTE were cultured for 3 weeks after which they were fixed in 4% formalin and sectioned for immunohistochemistry.

Indirect Immunofluorescence

Immunocytochemistry. Approximately 15,000 WT CEC and hTERT CEC cells were plated on glass coverslips (12 cm^2, Thermofisher, Fischer Scientific, Pittsburgh, PA) and cultured in their respective media. When the culture has stabilized the coverslips were rinsed in PBS, and fixed/permeabilized in methanol: acetone (1:1, 10 minutes at -20^{o}C). After re-hydration in PBS (0.256g/L NaH_2PO_4 H_2O, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4; for 30 min), and distilled water washes (3x) the cells were blocked (overnight at 4°C) in PBS + 1% BSA. The cells were then rinsed with PBS and distilled water (3x), and incubated at 4°C, overnight, with 1° antibody diluted in PBS. After rinsing in PBS,

containing Tween 20 (0.1%; 3 x10min), cells were incubated with 2° antibody (1.5 hrs, RT) and rinsed in PBS +Tween 20 (0.1%, 3x10min). Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (30 min), stained with 4',6-diamino-2-phenylindole (DAPI) and mounted on glass slides (FluorSave™, Calbiochem, La Jolla, CA).

Immunohistochemistry

Donor corneas and CTE (hTERT) were fixed in 4% formaldehyde (4°C, 24 hrs), dehydrated through a series of ethanol and xylene incubations, and then embedded in paraffin. Embedded tissues were sectioned (~10μ) and sections deparaffinized by incubations in xylene and ethanol. After re-hydration (30min) in PBS (0.256g/L NaH_2PO_4 H_2O, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4), distilled water washes (3x) and blocking (overnight at 4°C) in PBS + 1% BSA +1%horse serum, specimens were rinsed with PBS and distilled water (3x), and incubated with 1° antibody (4°C, overnight). After rinsing in PBS (3x10min) containing Tween 20 (0.1%), the tissue sections were incubated with 2° antibody (1 hr, RT) and rinsed in PBS +Tween 20 (0.1%, 3x10min). Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (1X10 min), and glass slides were mounted on the sections after DAPI staining (FluorSave™, Calbiochem, La Jolla, CA).

Image Acquisition: Mounted specimens were examined on Olympus AX70 fluorescent microscope using SPOT Twain software.

Western Blot Analysis

Cultured hTERT CEC , E7 hTERT CEC and whole corneal epithelium were treated with lysis buffer [2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled

water] for 5 min. at room temperature. Hela cell lysate was used as a control. Genomic DNA was sheared by several passes through a 22-gauge needle, and samples stored at –20^{O}C until needed. BCA protein assays (Pierce, Rockford, IL) of lysates were performed to determine the protein concentration (and ensure equal loading of lanes). SDS PAGE was performed at room temperature (RT), loading 20µg protein/lane using 12% Tris-Glycine, at 150V in Tris/glycine as the running buffer. Protein bands were transferred onto nitrocellulose membranes (VWR International Irving, TX) by electro-blotting overnight (4°C) at 10V in Tris/glycine buffer with 20% methanol and confirming the transfer with Ponceau Red (Sigma-Aldrich, St Louis, MS) staining of the membranes. After de-staining in distilled water, membranes were blocked for 1 hour (RT) in blocking buffer (5% powdered milk and 1% BSA in PBS). Membranes were incubated with 1° antibody for 30 minutes (RT), then at 4°C overnight, and for 30 min. at RT the following morning. After rinsing in PBS + 0.1% Tween-20 (3x10 min.), the membranes were incubated with 2^{O} antibody for 1 hour (RT), rinsed 3x 10 minutes in PBS + 0.1% Tween-20, and finally developed (ECL Chemiluminescence Amersham Biosciences, UK).

Antibodies: Primary antibodies were obtained from commercially available sources and were used in the dilution recommended by the supplier [Table 3(i)] [Also refer to Chapter II, Table 2(i)]. Alexa Fluor 594nm goat anti-mouse, Alexa Fluor 594 goat-anti-rabbit and Alexa Fluor 594 donkey anti-goat (Molecular Probes / Invitrogen, San Diego, CA) secondary antibodies were used at dilutions of 1:1000. Negative controls in all experiments were specimens labeled with 2° antibody only and DAPI to show nuclei; these showed virtually no fluorescence.

Table 3(i): Antibody Information. [IHC-Indirect Immunofluorescence, WB-Western Blot analysis].

Antibody	Source	Species	Dilution	
			IHC	WB
p53	Oncogene	Mouse	1:100	1:350
p21	Oncogene	Mouse	1:100	1:300
p16	Novocastra	Mouse	1:30	1:300
p63	Lab vision	Mouse	1:1000	1:1000
AE1	ICN	Mouse	1:50	1:200
AE3	ICN	rabbit	1:50	1:200
AE5	ICN	rabbit	1:50	1:200
Cytokeratin 14	Santa cruz	Mouse	1:50	1:200
Cytokeratin 12	Santa Cruz	Rabbit	1:50	1:200
Cytokeratin 5	Santa Cruz	mouse	1:50	1:200
hTERT	Novocastra	Mouse	1:50	1:200
EGFR	Santa cruz	Rabbit	1:100	1:200
Involucrin	Novocastra	Mouse	1:100	1:200
Ki67	Serotec	mouse	1:50	ND
PCNA	Novocastra	Mouse	1:50	1:200
GAPDH	Chemicon	Mouse	1:500	1:1000

Population doubling

The length of time cells take to double in number is defined as the population doubling time (pdl). 100,000 cells [primary CEC (passage (P)2) and hTERT CEC] were seeded in 6 well plates (2 wells/cell type). After 5 hours the number of cells that did not attach were counted to determine the actual number of cells that were seeded per well. Cells were the harvested at 18, 24, 36 and 72 hrs and counted. The ratio of the number of cells seeded and final number of cell counted at different time points was used to determine the population doubling time (pdl).

Karyotyping

Karyotyping was performed according to a protocol described earlier [24, 139-142]. hTERT CEC were cultured in 6 well plates (3 wells/cell type) plates until they were 50% confluent. The cells were then treated with 10ug/ml colcemid (Sigma-Aldrich) in culture medium overnight at 37°C in the incubator. The cells were passaged as described earlier using Trypsin-EDTA (Invitrogen). The cell pellet was incubated in 500ul of 0.75M KCl at 37°C for not more than 25 minutes. The cells were then centrifuged at 4000 rpm and then the KCl was removed. Freshly prepared cold fixative (methanol:acetic acid, 1:1) was added to the cell pellet and gently pipetted to break it up the cell pellet . The cells were incubated at 20°C for 15 minutes, the centrifuged at 4000 rpm and to remove the fixative. Fresh fixative was once again added and cells were incubated at -20°C for another 15 minutes. The cells were the dropped onto slides (pre-cleaned with 100% ethanol and dried) (Fisher Scientific) from a height of 8 inches. The slides were finally stained with DAPI (Molecular probes) and chromosomes were counted under a

fluorescent microscope.

Results

Morphology and population doubling time

The hTERT CEC have cobblestone morphology similar to that of the WT CEC cells [Figure 3(3)]. These cells have a population doubling time (pdl) of 36 hrs while the WT CEC cells have a pdl of 48 hrs [Figure 3(4)].

Expression of Cell cycle proteins and cytokeratins

Cell cycle proteins such as p53, p21 and p16 are expressed in hTERT CEC cells as they are in primary WT CEC cells [Figure 3(5(a)) and 3(5(b)]. The hTERT CEC have a similar keratin expression profile to that of the WT CEC cells as detected by AE1, AE3 and AE5 antibodies [Figure 3(6)]. Cell cycle and keratin protein expression was determined by western blot analysis and indirect immunofluorescence.

Differentiation

The hTERT CEC cells were seeded on CTE and cultured in medium containing high calcium for 2 weeks. The differentiated epithelium expresses keratins (AE1, AE3 and AE5) in a similar fashion as seen in the WT corneal sections as shown by indirect immunofluorescence [Figure 3(9)]. In the corneal epithelium and CTE, AE1 is expressed at higher levels in the superficial layers of the epithelium and is expressed at lower levels in the basal epithelial layer [Figure 3(9)]. AE5, a corneal differentiation marker is expressed only in the superficial layers of the epithelium in both the cornea and the CTE

[Figure 3(9)]. AE3 is expressed in all the layers of the epithelium in the cornea and the CTE [Figure 3(9)].

PKC expression profile

PKC profile analysis in the hTERT CEC cells by western blot analysis reveals a similar expression profile to the WT CEC cells. Both hTERT CEC and WT CEC express PKC α, βI, βII and ζ [Figure 3(7)].

Expression profile of 14-3-3 proteins

The hTERT CEC express all the 14-3-3 isoforms that the WT CEC cells express. The 14-3-3θ expression is however higher in the hTERT CEC cells [Figure 3(8)].

Expression of telomere binding proteins (TBP)

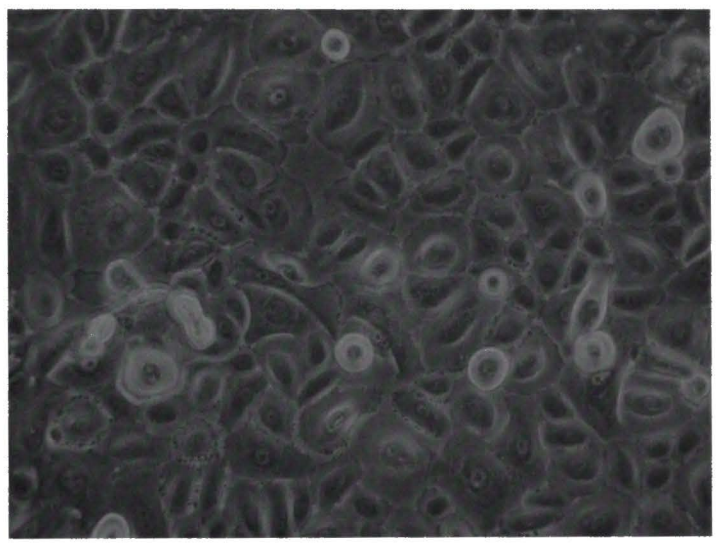
Analysis of the expression of TBPs revealed the presence of higher levels of Tankyrase and lower levels of TRF1 and Tin2 in hTERT CEC cells when compared to WT CEC cells [Figure 3(10)]. Tankyrase is a positive regulator of telomerase and TRF1 and Tin2 are negative regulators of telomerase.[143]

Karyotyping Analysis

Karyotyping analysis reveals the presence of a diploid number of chromosomes in the 20 hTERT CEC cells that were examined [Figure 3(11)].

Figure 3 (3): Phase contrast images of corneal epithelial cells. (a) WT CEC and (b) hTERT CEC showing similar cobblestone morphology. [Magnification-20X]

(a)



(b)

Figure 3 (4): Determination of population doubling time of hTERT cells. The time taken by the hTERT CEC cells to double is 36 hours. Primary WT CEC cells (Passage 2) double in approximately 48 hours.(Po-Initial population, Pf-Final population).

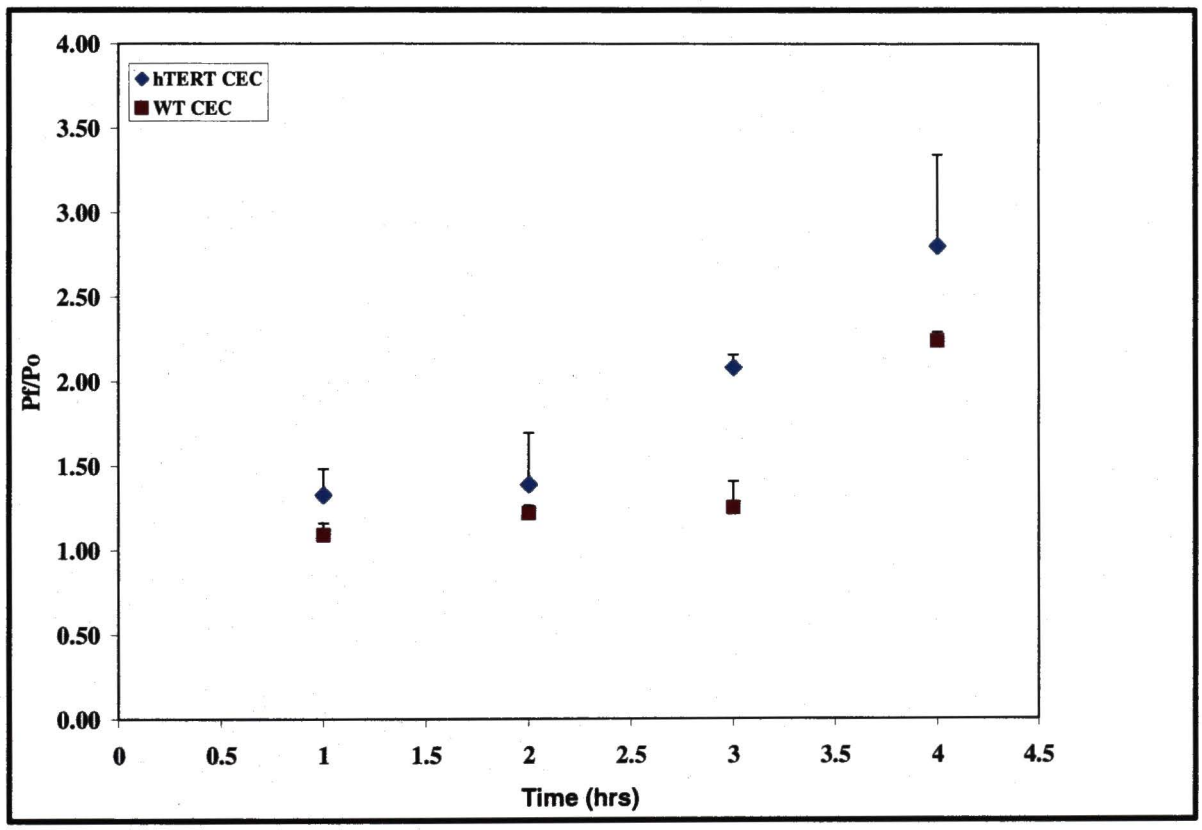


Figure 3 [5(a)]: Expression of hTERT, p53, p16 and p21 as determined by western blot analysis. GAPDH was used as a loading control.

1. Cepi E7/hTERT (+ ve control for p16 and hTERT)

2. Whole Corneal Epithelium

3. Hela cell lysate (+ ve control for p16 and - ve control for p53)

4. hTERT CEC.

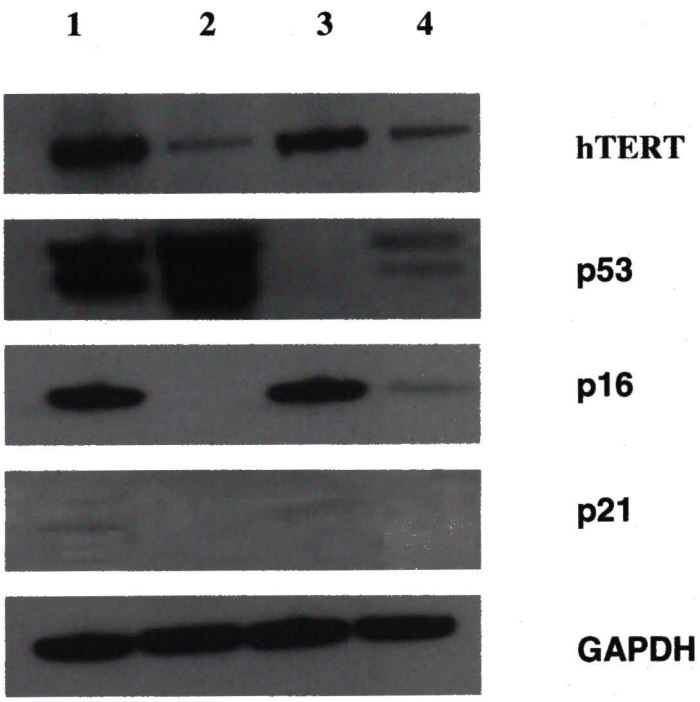
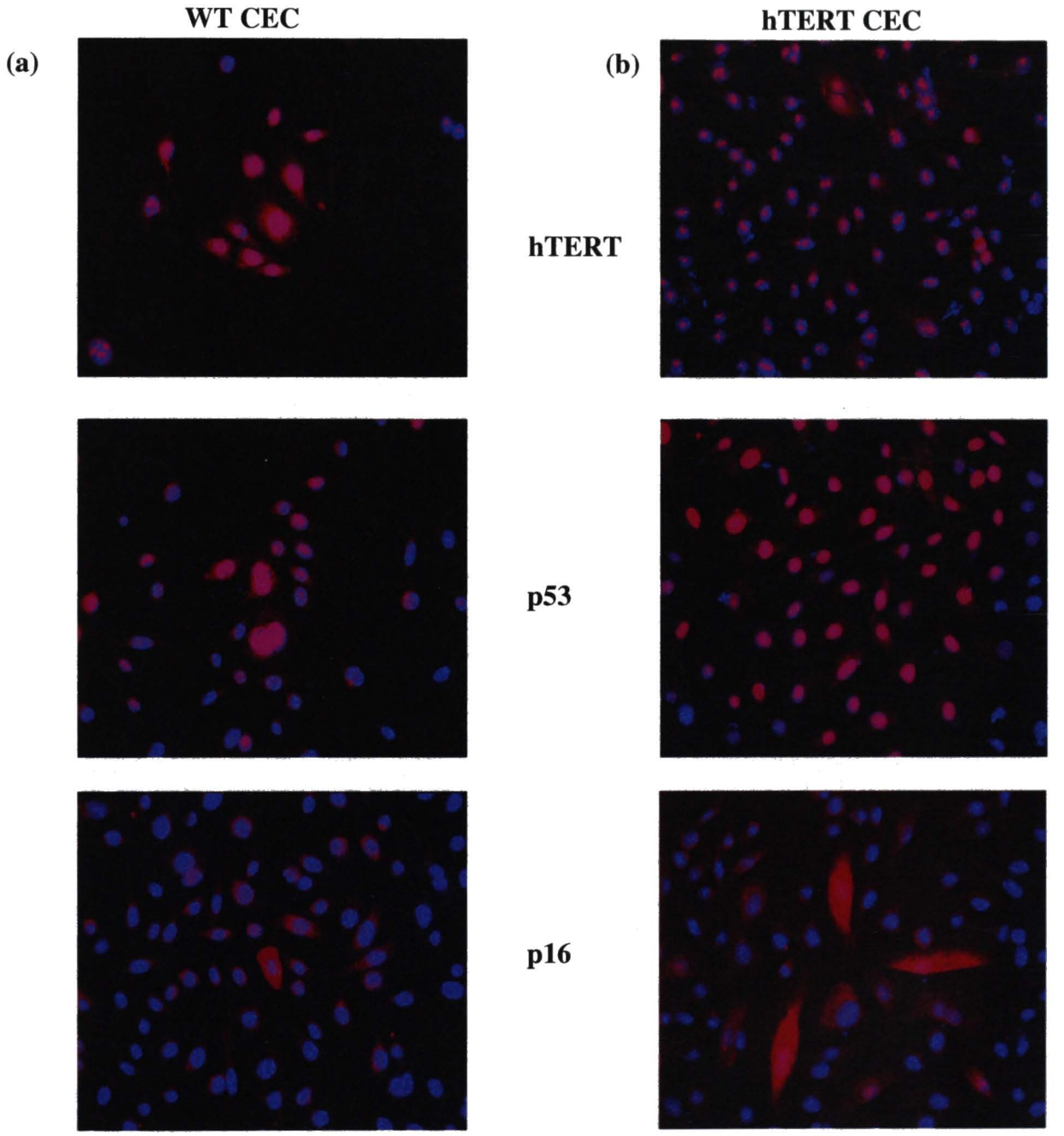


Figure 3 [5(b)]: Expression of hTERT, p53, p16 and p21 as determined by Indirect Immunofluorescence. a) WT CEC (P2) and (b) hTERT CEC. (DAPI-Nuclear stain, Magnification -20X)



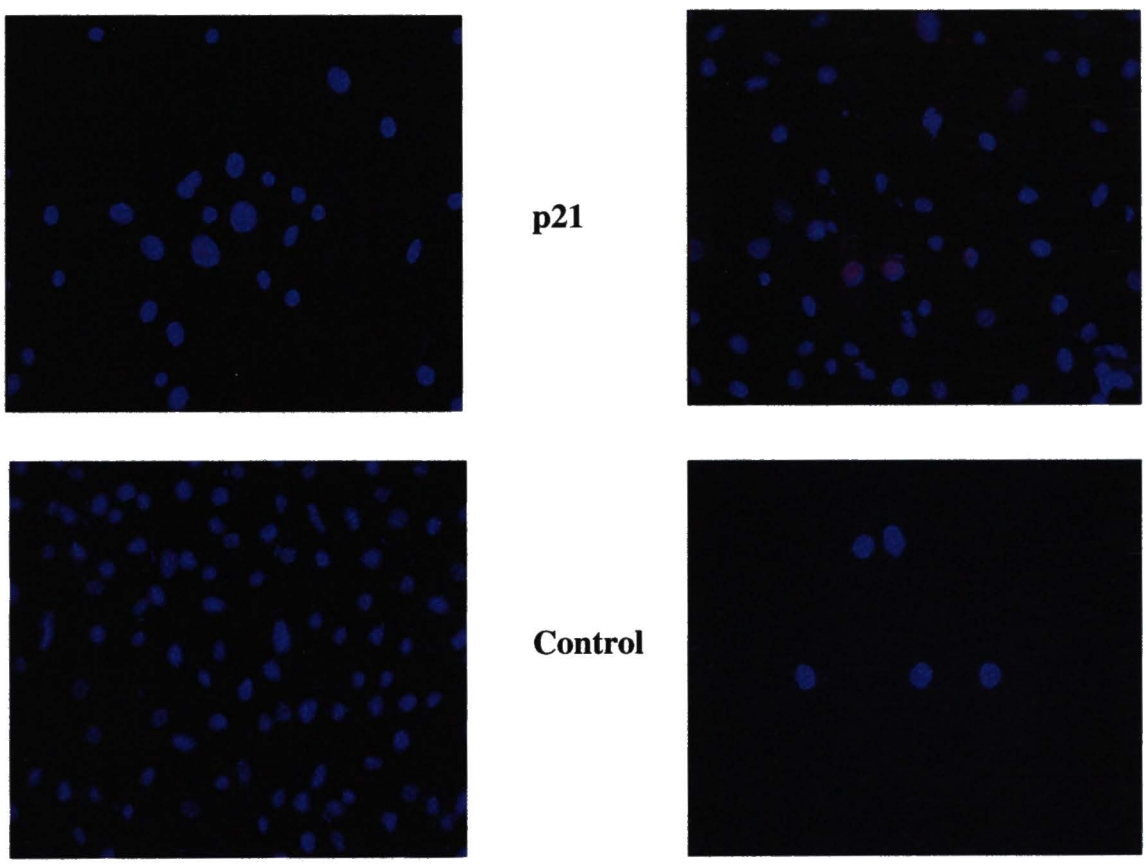

p21
Control

Figure 3 (6): Expression of Cytokeratins in corneal epithelial cells. (a) hTERT CEC and (b) WT CEC as determined by Indirect Immunofluorescence.

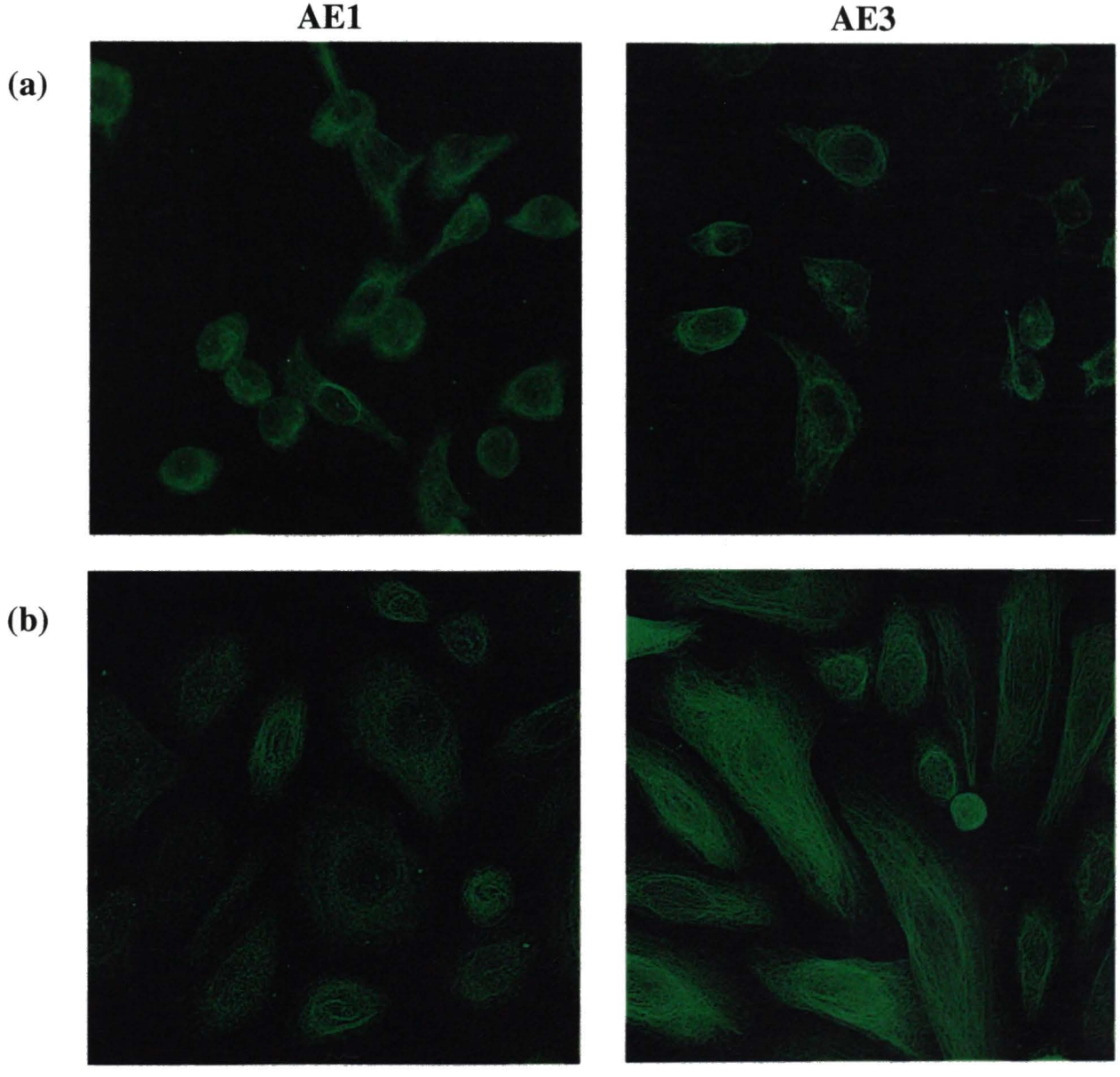


Figure 3 (7): PKC expression profile in corneal epithelial cells.(1) WT CEC and (2) hTERT CEC cells as determined by Western Blot analysis.

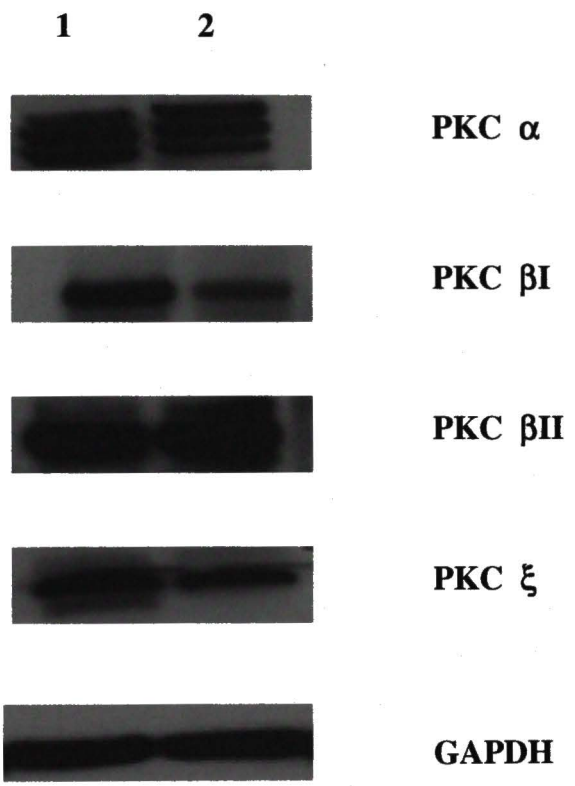


Figure 3 (8): Expression of 14-3-3 proteins in hTERT CEC cells as determined by Indirect Immunofluorescence.

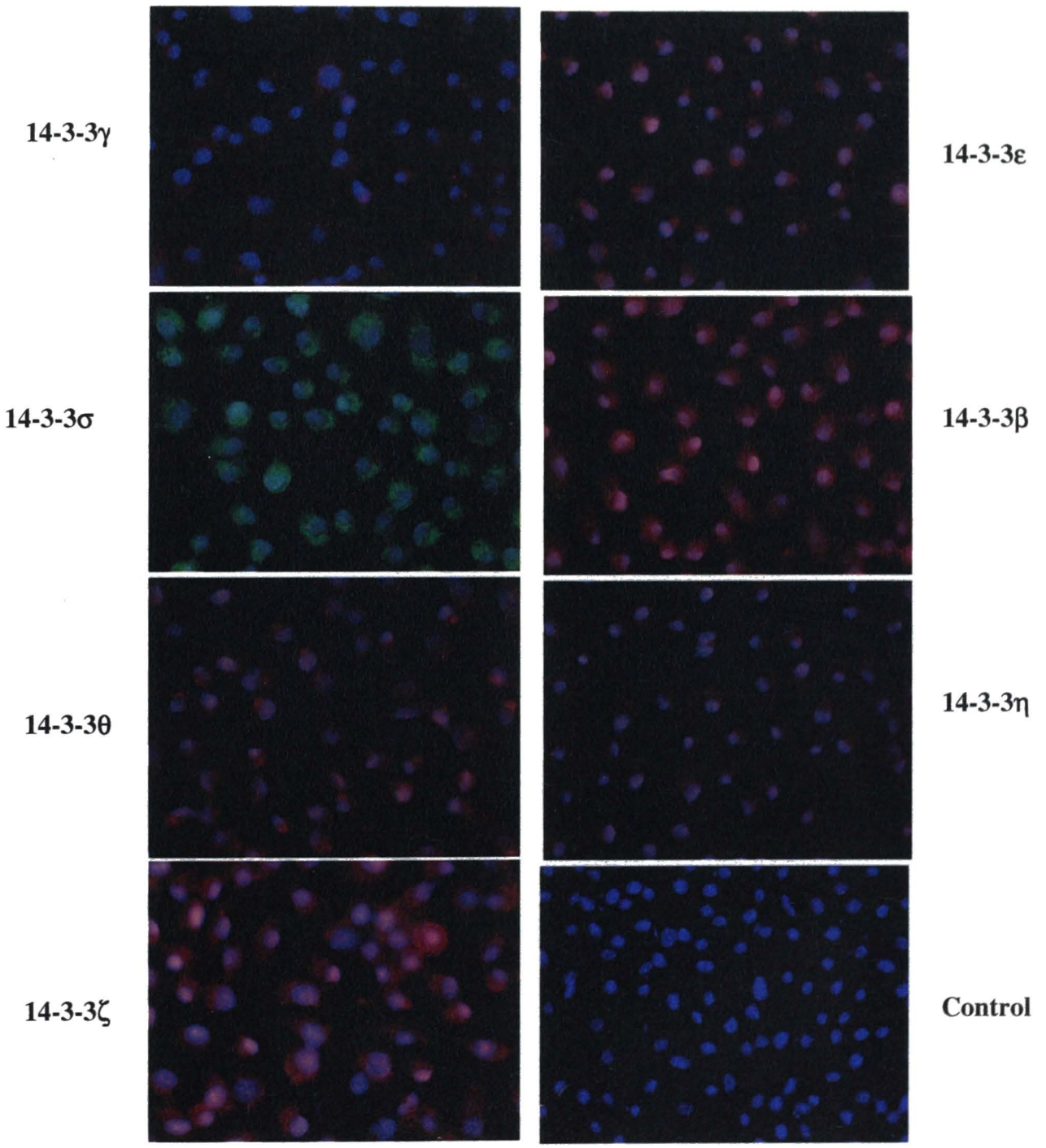


Figure 3 (9): Expression of keratins in corneal tissue equivalents(CTE). (a) Corneal constructs seeded with hTERT CEC submerged in medium containing high calcium and (b) Human Cornea as determined by Indirect Immunofluorescence. [Magnification-20X]

AE1-Acidic (Type I) keratins (green)

AE3-Basic (Type II) Keratins (green)

AE5-Keratin 3 (Corneal Differentiation marker-green)

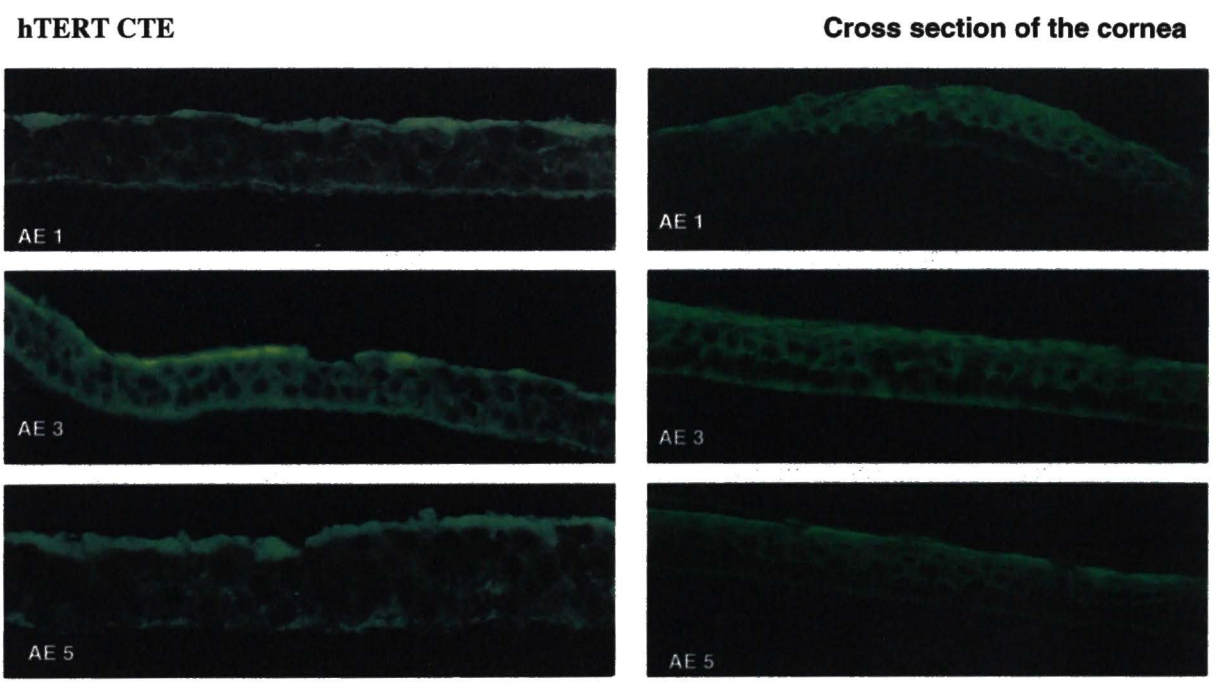
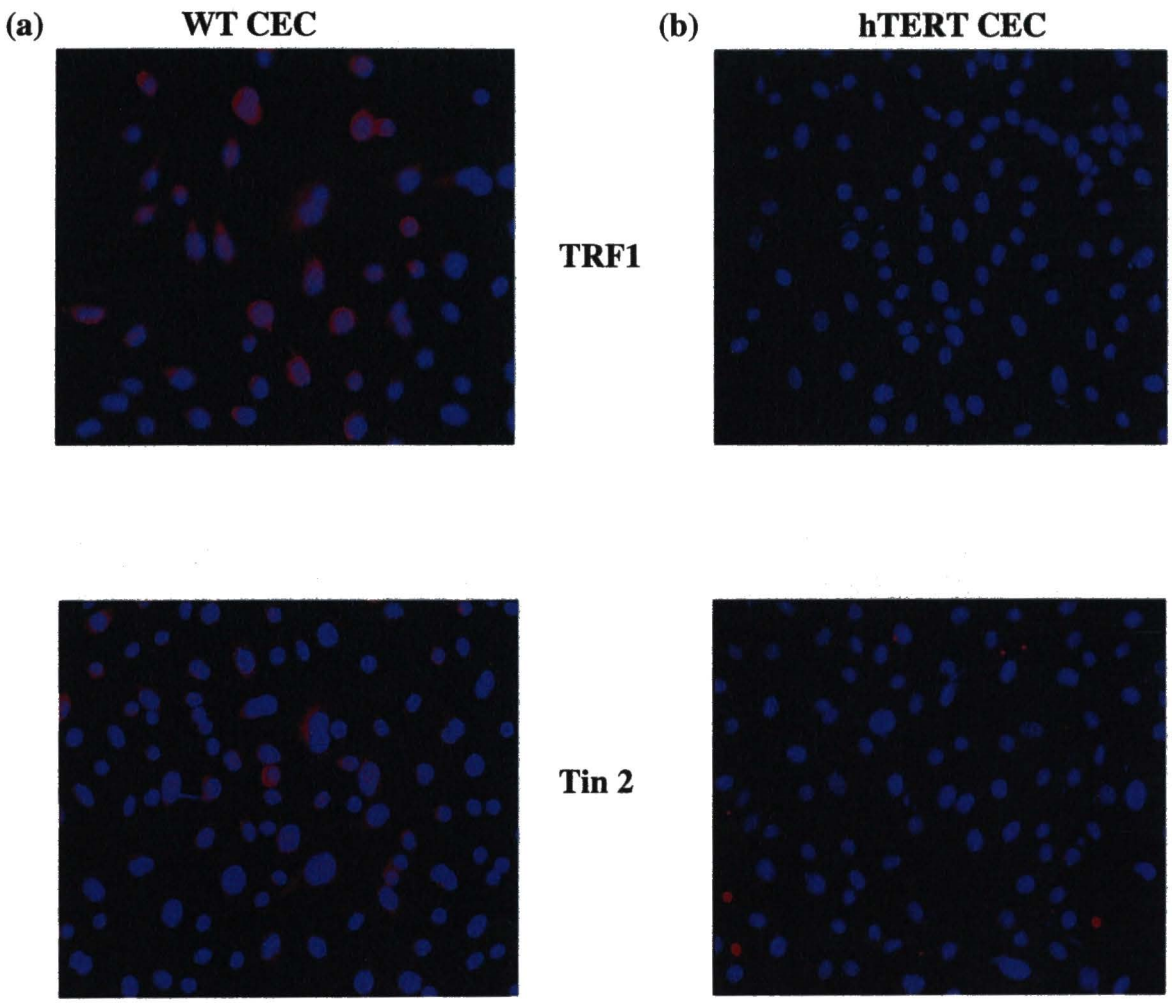
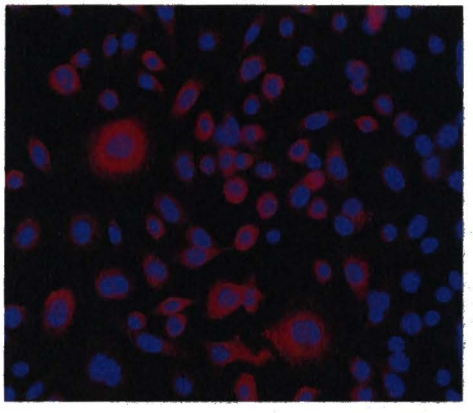
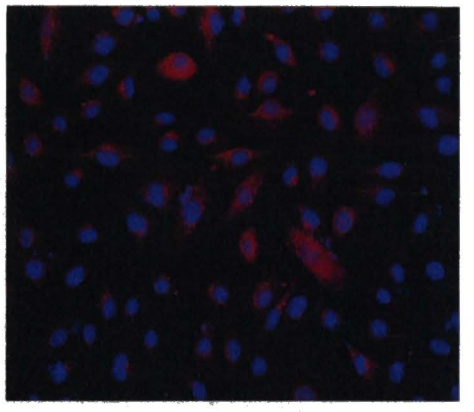


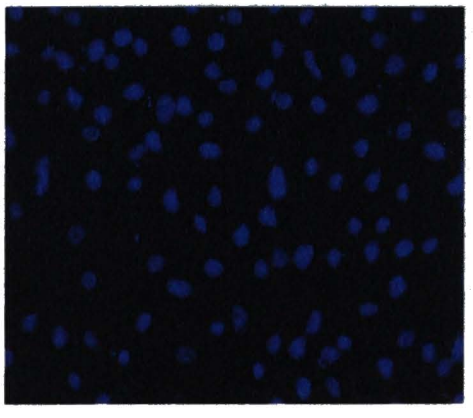
Figure 3 (10): Expression of TBPs; TRF-1, Tin 2 and Tankyrase as shown by western Blot analysis. (a) WT CEC and (b) hTERT CEC. [(DAPI-Nuclear stain), Magnification-20X]





Tankyrase





Control

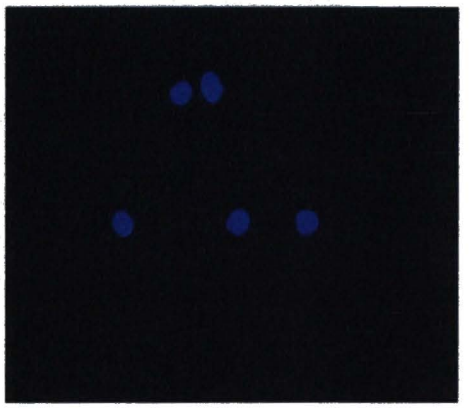


Figure 3 (11): Karyotyping analysis of hTERT CEC cells. Karyotyping hTERT CEC cells (n=20) reveals the presence of a diploid (46) number of chromosomes. (DAPI- stains the chromosomes).

Table 3 (1): Comprehensive summary of comparison of protein expression in WT CEC and hTERT CEC as determined by Indirect Immunofluorescence and Western Blot analysis.

Protein	WT CEC	hTERT CEC
AE1	+	+
AE3	+	+
AE5	++	+
p16	++	+
p21	-	-
p53	+	+
p63	+	+
Ki67	+	+
PCNA	+	+

Protein	WT CEC	hTERT CEC
K18	+	+
K19	+	+
14-3-3σ	++	+
14-3-3ε	+	+
14-3-3ζ	+	+
14-3-3η	+	+
14-3-3θ	+	++
14-3-3β	+	+
14-3-3γ	+	+

Specific Aim 2

14-3-3 σ is involved in corneal epithelial cell cycle regulation (Differentiation and DNA damage response).

2(b) To demonstrate that 14-3-3 σ is involved in corneal epithelial cell differentiation. {Determination of expression of differentiation markers-Involucrin and K12 and Proliferation marker-K14}

Rationale

We have for the first time shown the expression of all mammalian 14-3-3 proteins in the cornea.[144] Immunohistochemical analysis of sections of the human cornea reveals the presence of 14-3-3 σ isoform in the epithelium only [Figure 3(1)]. This protein is expressed in the cytosolic compartments. The expression levels of the 14-3-3 proteins vary between the different layers of the stratified corneal epithelium.

In keratinocytes 14-3-3σ has been studied as a down-stream mediator of the p63 protein. Studies have shown that in response to calcium induced differentiation signals there is a decrease in p63 protein and a corresponding increase in 14-3-3σ expression[42, 53]. This suggests the involvement of 14-3-3σ in differentiation in keratinocytes.

In the cornea similar to skin epithelium, the suprabasal and superficial layers of the epithelium have higher expression levels of 14-3-3 σ protein in comparison to the basal epithelial cells. The role of 14-3-3 σ in proliferation and differentiation of corneal epithelial cells has not been studied.

Research Design

To show that* 14-3-3 σ *expression in WT CEC increases in response to serum mediated differentiation signals.

Primary epithelial cells were cultured and lysates were collected over passage and lysates were examined for expression of 14-3-3 σ and differentiation marker (Involucrin) by western blot analysis. Primary WT CEC from the same donor were divided into two separate cultures and one was cultured in Epilife and the other was cultured in Epilife containing 10% FBS. Cells were also cultured on cover slips under the same experimental conditions. After 24 hours the cells were fixed for immunocytochemistry. hTERT CEC were used as hyperproliferative controls. The hTERT CEC were analyzed by western blot analysis after 2 weeks of culture and by immunocytochemistry after 1 week in culture under the same two sets of conditions.

Increase in* 14-3-3 σ *expression is associated with differentiation of corneal epithelial cells. {Expression of differentiation marker-Involucrin}

Since WT CEC are a heterogeneous population of proliferating and differentiating cells, it is very difficult to study differentiation. To overcome this difficulty hTERT CEC cells were used as they are a highly proliferative under normal culture conditions. hTERT CEC were transfected with a plasmid containing full length cDNA for 14-3-3 σ (MGC-5018, ATCC) to over-express the protein. Transfected cells were selected by antibiotic selection (ampicillin) and then lysed and analyzed by western blot analysis for differentiation marker-involucrin.

Materials and Methods

Cell Culture

WT CEC and hTERT CEC cultured in serum free defined media (EpiLife, Cascade Biologicals) to 80% confluence. The epithelial cells are then subcultured by harvesting with trypsin/EDTA (Gibco BRL, Carlsbad,CA) neutralization of proteolytic activity with trypsin inhibitor (Sigma-Aldrich, ST Luis,CA), and plating into TC flasks coated with collagen type IV (CIV) ($5\mu g/cm^2$).

Differentiation Experiments

WT CEC and hTERT CEC cells were cultured in defined media (EpiLife, Cascade Biologicals) containing 10% fetal bovine serum (Atlanta Biologicals) to induce differentiation. When the conventional cobblestone morphology of the WT CEC and hTERT CEC changed, the cells were lysed and were analyzed by western blot analysis.

Transfection of hTERT CEC

Fugene (Roche) was used to transfect hTERT CEC with a plasmid (MG5019, ATCC) containing the full-length cDNA for 14-3-3σ protein. The transfected clones were selected by antibiotic resistance (ampicillin, Sigma Aldrich) 48 hrs following transfection.

Western Blot Analysis

WT CEC cells were lysed 48 to 72hrs after culture in serum containing medium. The hTERT CEC were lysed after culture for 2 weeks in serum containing medium. The cell lysates were subjected to SDS PAGE gel electrophoresis as previously described.

Indirect Immunofluorescence

Approximately 15,000 WT CEC and hTERT CEC cells were plated on glass coverslips (12 cm^2, Thermofisher, Fischer Scientific, Pittsburgh, PA) and cultured in their respective media. WT CEC were fixed in methanol:acetone (1:1) 48 hrs after culture in serum containing medium. The hTERT CEC were fixed 7 days after culture in serum containing medium. The cells were then subject to indirect immunoflurescence analysis as previously described.

Results

14-3-3σ expression increases with passage

The number of proliferating cells decreases with passage as cells differentiate and detach from the growth surface (free floating). Increased differentiation with passage is shown by the increase in involucrin expression, a well established marker of corneal epithelial cell differentiation. A corresponding 7 fold increase in 14-3-3 σ protein [Figure 3(12)], from Passage (P)2 to P3 was determined by densitometric analysis [Figure 3(12)]. Early passage P2 is a population of cells that are proliferative and hence do not express involucrin and they also express very small amounts of 14-3-3 σ protein. This suggests that increase in 14-3-3σ expression is an indicator of differentiation.

Serum induces epithelial cell differentiation in WT CEC and hTERT CEC

The presence of serum results in corneal epithelial cell differentiation within 24 hours. This is shown by a change in cell morphology [Figure 3 (13(a))], and by increase in involucrin [Figure 3 (14(a) and 14(b))] and K12 expression [Figure 3 (15)], and a

corresponding decrease in K14 expression [Figure 3 (15)]. hTERT CEC cells show very little differentiation even after 10 days in culture in medium containing serum as shown by involucrin expression [Figure 3 (14(c))] .

In the presence of serum there is an increase in 14-3-3σ expression. This is shown by western blot analysis and indirect immunofluorescence [Figure 3 (14(a) and 14(b))]. This increase is detected after 24 hours in serum containing medium.

14-3-3σ expression also increases in hTERT CEC cells in response serum. But the increase is detected after 2 weeks culture in FBS containing medium [Figure 3 (14(a))].

Over-expression of 14-3-3σ induced differentiation

Transfection of hTERT CEC cells with MGC-5018 increased the expression of 14-3-3 σ as shown by western blot analysis [Figure 3 (16)]. This is also supported by the expression of involucrin [Figure 3 (16)].

Figure 3 (12): Expression of 14-3-3 σ and involucrin in WT CEC with increase in passage (P2 to P6) as shown by Western Blot analysis. GAPDH is used as a loading control. [Normalized densitometric ratios are indicated above the 14-3-3σ protein bands]

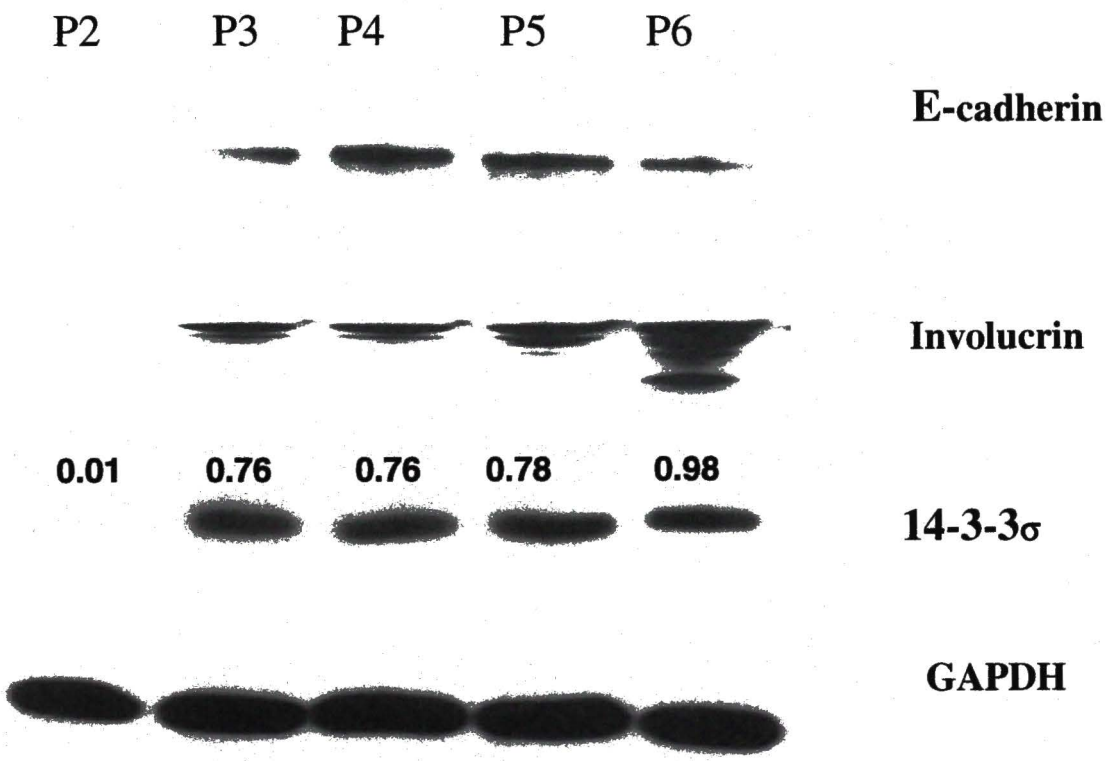
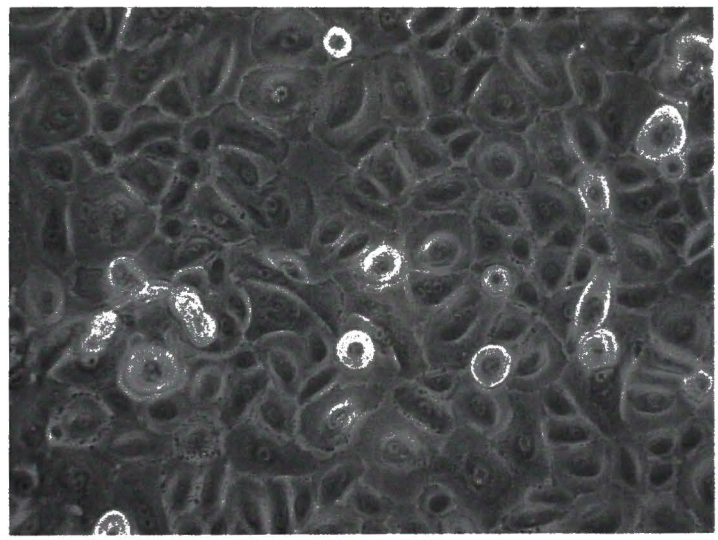


Figure 3 [13(a)]: Changes in WT CEC morphology under differentiation conditions.

(a) Normal culture conditions, (Magnification -20X)

(b) In the presence of serum.(Magnification-20X)

(a)



(b)

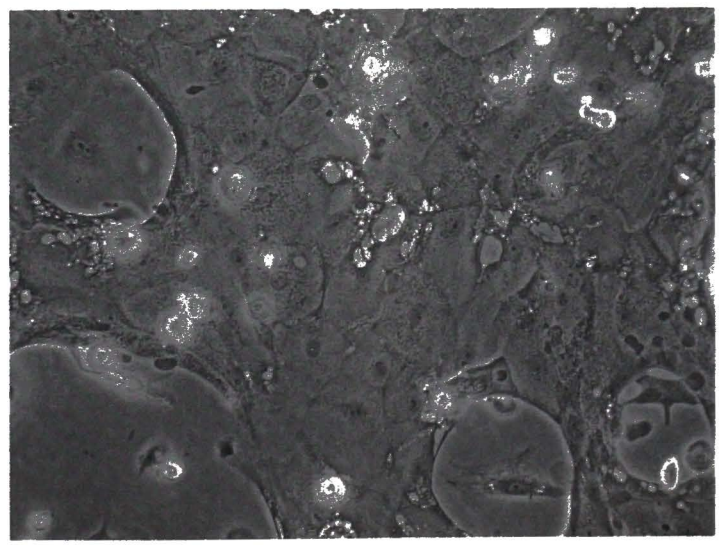


Figure 3 [13(b)]: Changes in hTERT CEC morphology under differentiation conditions. [Magnification of 20X].

(a) Normal culture conditions

(b) In the presence of serum for 24 hrs

(c) In the presence of serum for 48 hrs and

(d) In the presence of serum for 5 days

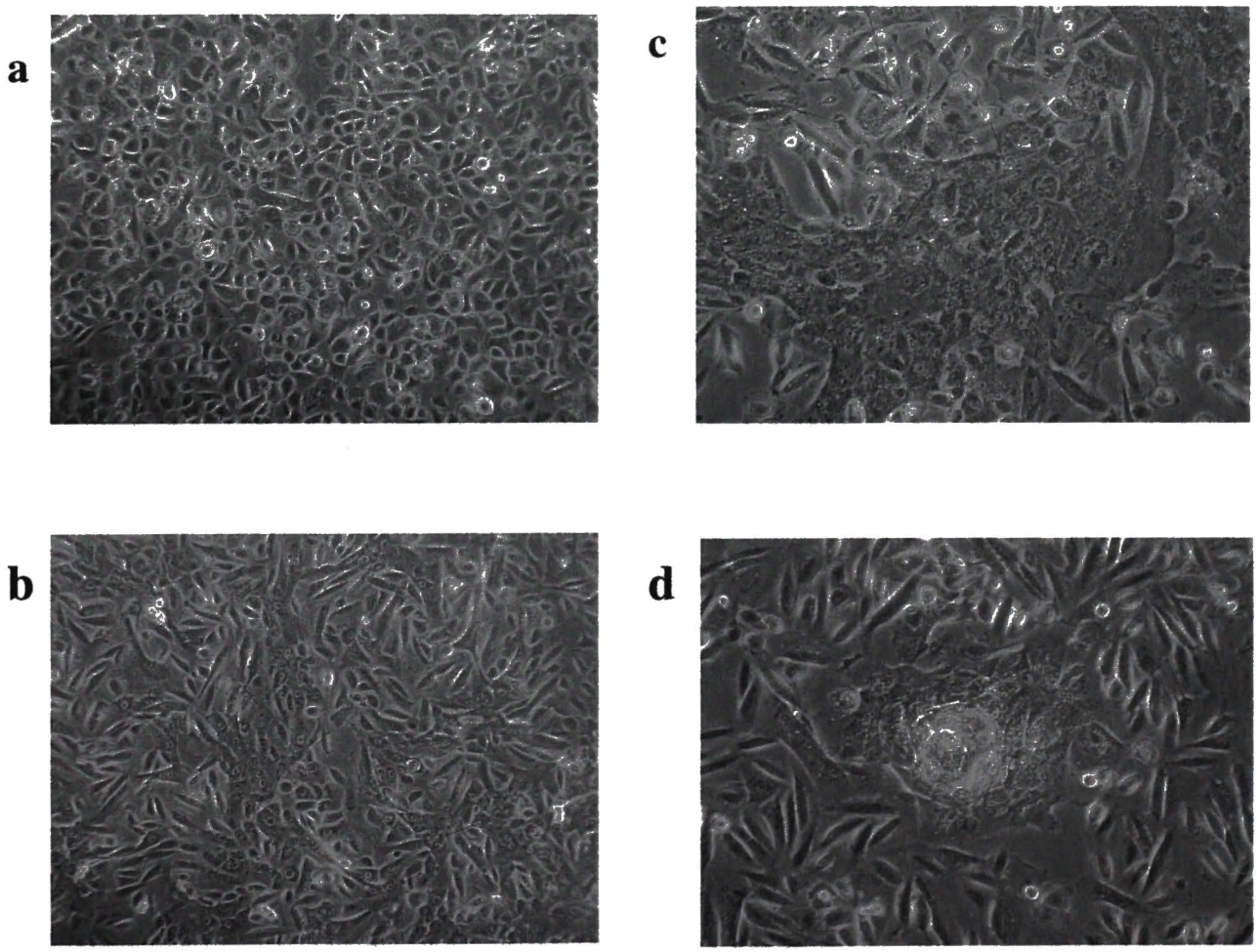


Figure 3 [14(a)]: Expression of 14-3-3σ and involucrin in WT CEC and hTERT CEC in response to serum as shown by Western Blot analysis. Normalized densitometry values for 14-3-3σ and involucrin expression are indicated.

1. WT (control/proliferating/normal conditions),

2. WT (In presence of serum),

3. hTERT CEC(control/proliferating/normal conditions) and

4. hTERT CEC(In presence of serum)

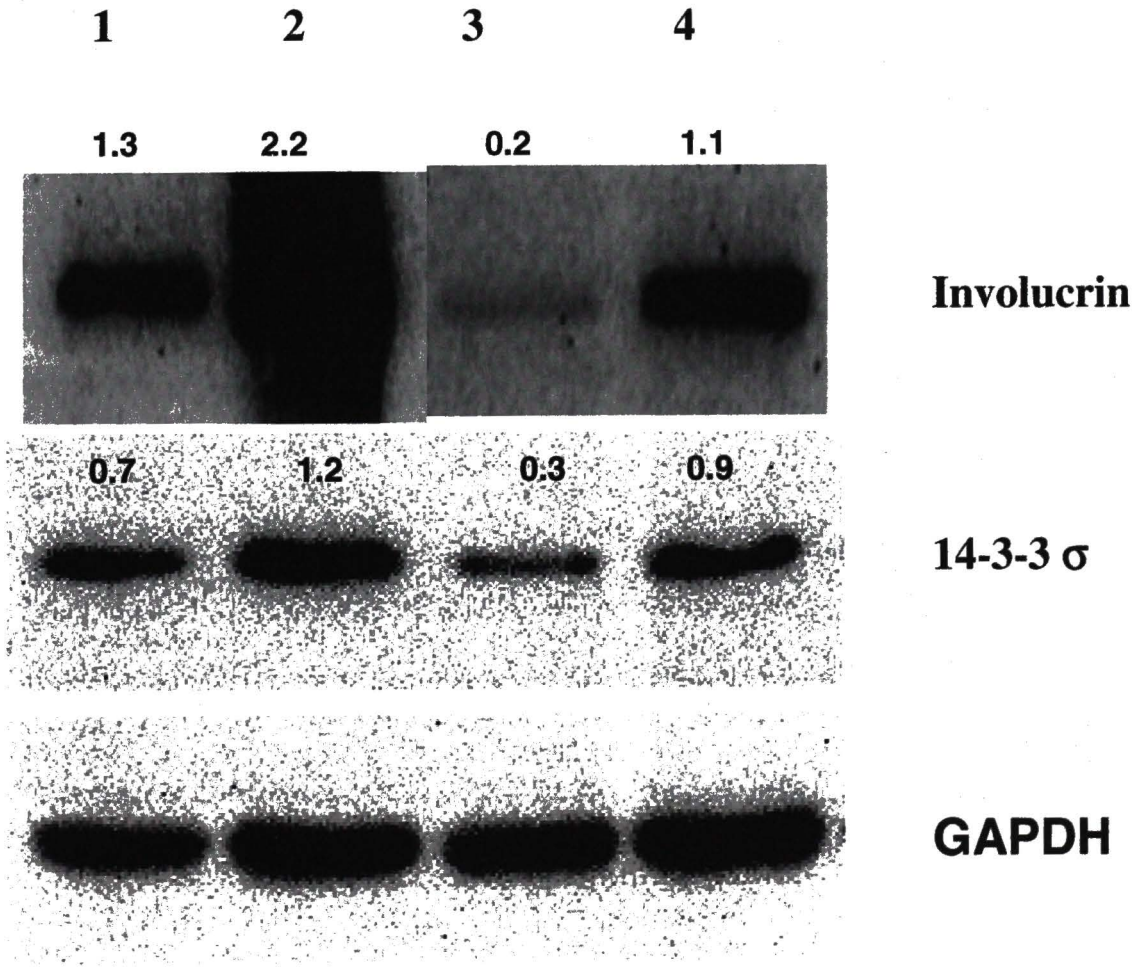


Figure 3[14(b)]: Expression of 14-3-3σ and involucrin in response to serum as shown by Indirect Immunofluorescence 48hs after introduction of serum shows an increase in expression of both the proteins.

(a and c) WT CEC cells under normal culture conditions,

(b and d) WT CEC cells in the presence of serum.

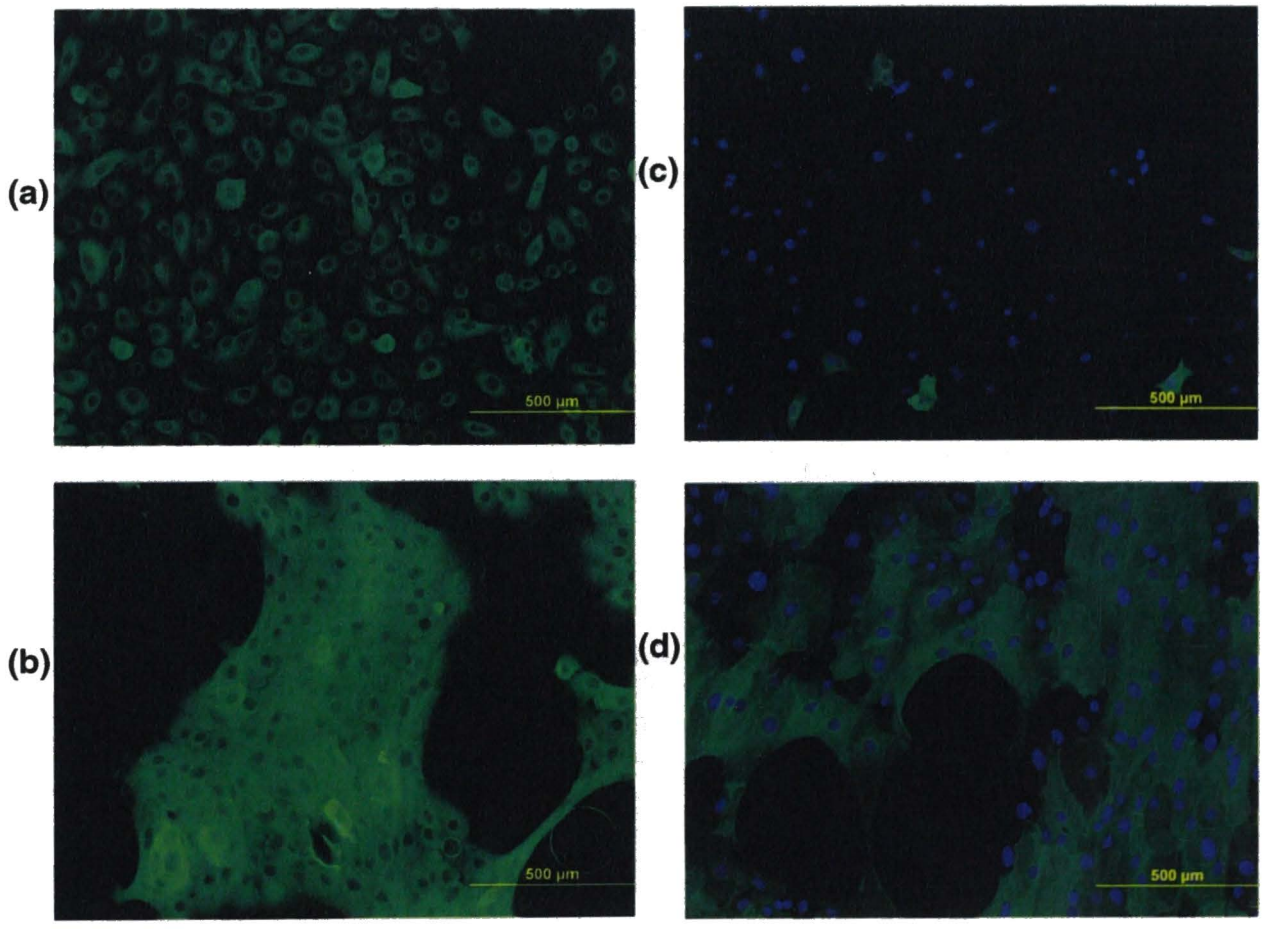


Figure 3 [14(c)]: Expression of 14-3-3 σ and involucrin in response to serum as shown by Indirect Immunofluorescence 1 week after introduction of serum shows an increase in expression of both proteins. (a and c) hTERT CEC cells under normal culture conditions, (b and d) hTERT CEC cells in the presence of serum.[Magnification -20X]

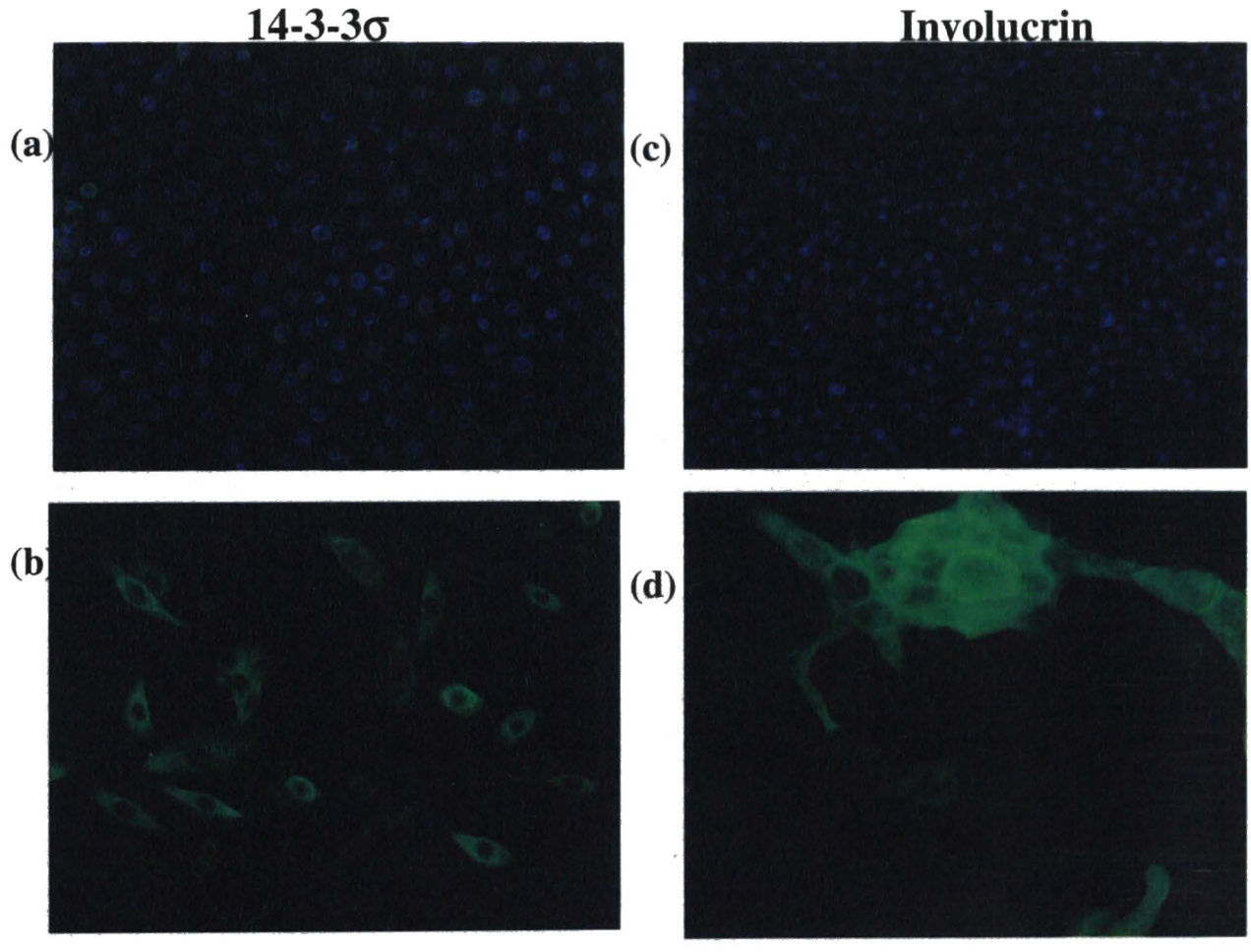


Figure 3 [15]: Expression of cytokeratins K12 and K14 in response to differentiation as shown by Indirect Immunofluorescence in WT CEC cells.

(a and c) WT CEC cells under normal proliferating conditions

(b and d) WT CEC cells 48hrs after the introduction of serum.

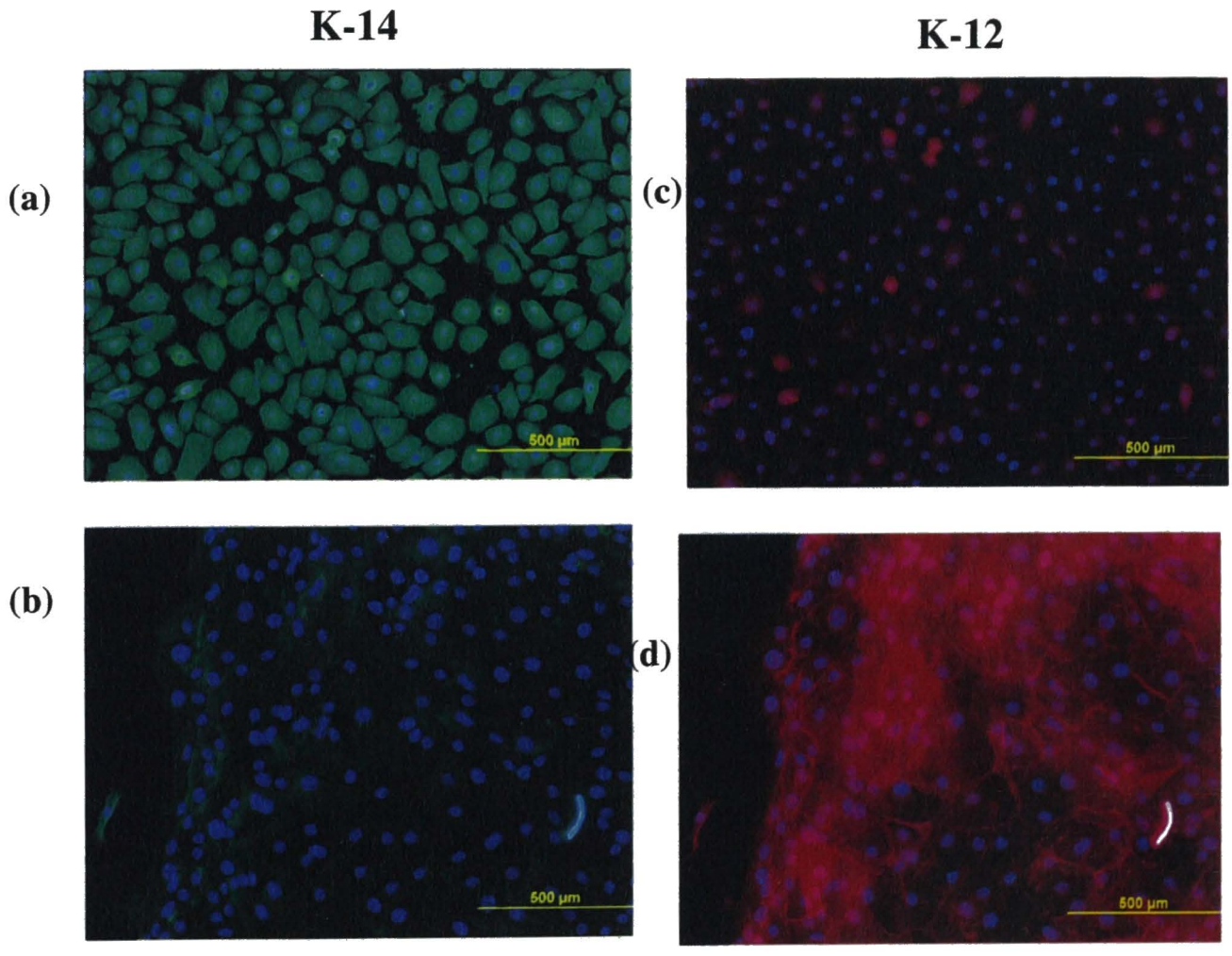


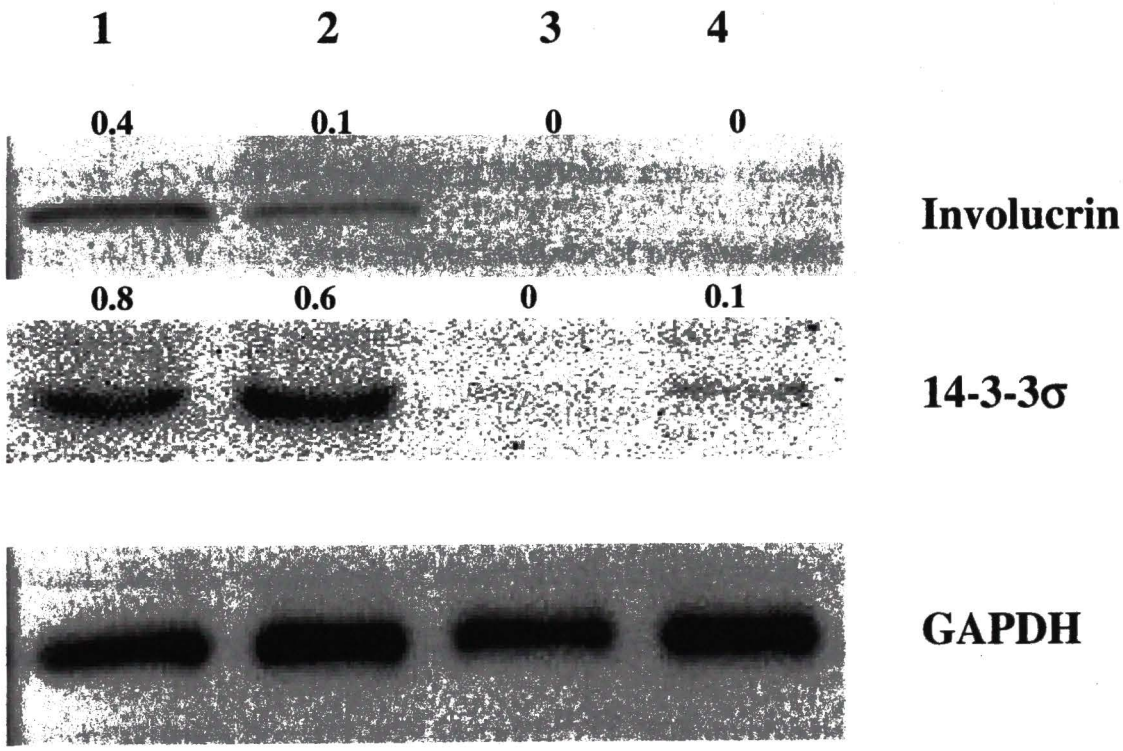
Figure 3 [16]: Effect of over expression of 14-3-3σ on involucrin expression in hTERT CEC cells as shown by Western Blot analysis. [Denitometric ratios after normalization for involucrin and 14-3-3σ are indicated above the protein bands]

1.hTERT CEC in medium containing serum,

2. hTERT CEC transfected with plasmid containing full length cDNA for 14-3-3 σ

3. hTERT CEC floaters in presence of serum,

4. hTERT CEC under normal proliferating culture conditions.



Specific Aim: 2

14-3-3 σ is involved in corneal epithelial cell cycle regulation (Differentiation and DNA damage response).

2(c) To show that the he expression of 14-3-3 σ induces cell cycle arrest in response to DNA damage (Adriamycin).

Rationale:

Under normal conditions p53 is maintained at low levels of expression by E3 ubiquitin ligase (MDM-2) mediated degradation. In response to DNA damage or stress there is up regulation of p53, which either arrests the cell cycle so that they can undergo DNA repair or undergo apoptosis. p21 is a downstream mediator of p53 mediated response. p21 is a CDK inhibitor (CDKI) that arrests the cells in G1/S phase of the cell cycle. 14-3-3 σ also a downstream mediator of p53 that arrest cells in G2/M phase of the cell cycle. 14-3-3 σ expression is induced in response to gamma irradiation and other DNA damaging events and results in G2/M cell cycle arrest. In breast cancer cells over-expression of 14-3-3 σ results in cell cycle arrest by sequestering cdc2 and cyclin B in the cytosol. Nuclear localization of these two proteins is crucial for entry into the M phase of the cell cycle [38].14-3-3 σ shares cyclin/cdc2 binding motifs with several cell cycle inhibitor proteins like p21, p27 and p57 [38, 145]. The involvement of 14-3-3 σ in response to DNA damage in corneal epithelial cells has not been studied.

Research Design and Methodology:

DNA damage in corneal epithelial cells causes a time dependent increase in* 14-3-3 σ *expression.

Primary CEC, hTERT CEC, SV-40 and E6/E7 transformed cells were treated with 0.2ug/ml adriamycin for 6, 16, 24, 48 and 72 hrs. After different treatment periods the cells were lysed and WB analysis was performed on the samples. p53, 14-3-3 σ and p21 expression was determined over time.

Materials and Methods

DNA Damage Experiments

WT CEC, hTERT CEC, SV-40 and E6/E7 transformed CEC at a density of 250,000 cells /well were cultured in 6 well plates. The cells were then treated with 0.2ug/ml of adriamycin (Pharmachemie BV, Harlem, Holland). The hTERT CEC and WT CEC were lysed 0, 6, 24, 48 and 72 hrs after adriamycin treatment. Western blot analysis was performed on the cell lysates as described earlier.

Cytotoxicity assay

The hTERT CEC, SV-40 CEC and E6/E7 CEC were plated at 4.0 x 10^3 per well (n=8) in 100µl of the appropriate medium in Costar 96 well plates. The perimeter wells were not utilized, but contained PBS to reduce evaporation. Background media controls (n=4) were also plated at the same volume and replaced with each feeding. After 24 hours, the media was removed and the cells were fed 150µl/well of the appropriate medium (Epilife and Epilife containing 0.2µg/ml adriamycin). Subsequently the cells were fed at 48 hr intervals until the assay was terminated.

SRB (Sulforhodamine B) Staining

SRB assay was performed to determine cell viability[146]. At time points of 0, 6hrs, 24hrs, 48hrs and 72hrs, the media was removed and cells were fixed with 10% Trichloroacetic acid (TCA) for 10 minutes at 4°C. The cells were washed 4 times with distilled water, and then stained for 10 minutes with SRB (1.0% in acetic acid). The plates were washed 4 times with 1.0% acetic acid and allowed to air dry. Prior to reading the plates, 200µl of 10mM Tris base (unbuffered) was added per well to solublize the dye. The plates were mixed for 5 minutes on a Lab Line Instruments Titer Plate Shaker and read at 564nm on a Molecular Devices SpectraMax 340 using Soft Pro Max acquisition software. Absorbances were exported into an Excel spreadsheet for further analysis. Cell densities were obtained by correcting absorbances for media controls and plotted.

Propidium Iodide (PI) Staining

The hTERT CEC cells were plated on coverslips (Fisherbrand) at a density of 10,000 cells/coverslip. The cells were then treated with adriamycin (0.2ug/ml) for 72 hrs. The cells were then rinsed in PBS, and fixed/permeabilized in methanol: acetone (1:1, 10 minutes at -20^{O}C). A solution of PI at a concentration of 3 µM was prepared by diluting the 1 mg/mL (1.5 mM) stock solution 1:500 in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 0.5 mM MgCl2, 0.1% Nonidet P-40). 1 mL of PI diluted in staining buffer was added to the cells that were fixed. The cells were incubated with PI for 15 minutes at room temperature. Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (30 min), and mounted on glass slides (FluorSave™, Calbiochem, La

Jolla, CA). Mounted specimens were examined on Olympus AX70 fluorescent microscope using SPOT Twain software.

Results

Adriamycin treatment induces increase in 14-3-3 σ expression in corneal epithelial cells.

In response to adriamycin treatment there was a time dependent increase in p53 over time. In WT CEC cells the p53 level is undetectable in control cells (untreated) and there is a significant increase in 6 hrs and the p53 level is maximum at 72 hrs. There was also a corresponding increase in p21 expression, which is one of the downstream regulators of p53. 14-3-3σ is also a downstream regulator of p53, and its expression in response to adriamycin induced DNA damage was also time dependent [Figure 3 (17)]. This data indicated the involvement of 14-3-3σ in DNA damage signaling in corneal epithelial cells.

Over time there is an increase in p53, 14-3-3 σ and p21 expression in hTERT CEC cells [Figure 3 (18)]. This indicates the hTERT CEC though very proliferative have an intact DNA damage signaling cascade.

Response of SV-40 and E6/E7 CEC to adriamycin treatment

In response to adriamycin treatment there is no change in p53 and p21 expression in the SV-40 CEC. The control E6/E7 CEC do not express p53. Following treatment with adriamycin, they still do not express p53, also there is no p21 expression in these cells.

Note

Adriamycin has been used widely for cancer therapy and hence has been shown to induce cell death in a number of cell lines. [147, 148]Adriamycin is not toxic to hTERT cells and does not induce cell death as shown by cytotoxicity assay and nuclear staining with propidium iodide. [Supplementary figure 3(A) and 3(B)].

Adriamycin is also not cytotoxic to SV-40 and E6/E7 CEC cells as shown by cytotoxicity assay. There is no difference between the control and adriamycin cells over time as shown [Supplementary Figure 3(C) and 3(D)].

Figure 3 [17]: Expression of p53, p21, 14-3-3 σ by WT CEC cells in the presence of 0.2μg/ml of adriamycin as shown by Western Blot analysis. [Normalized densitometry ratios for p53, p21 and 14-3-3σ are indicated]

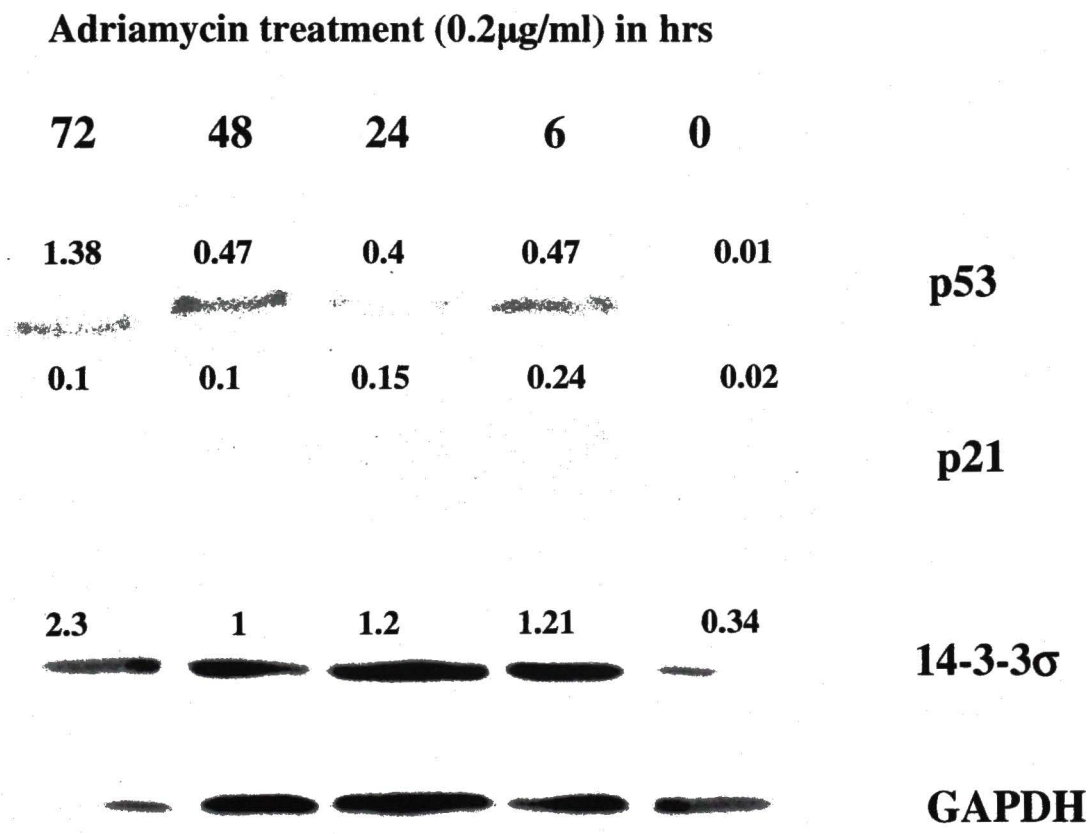


Figure 3 [18]: Expression of p53, p21, 14-3-3σ by hTERT CEC cells in the presence of 0.2μg/ml of adriamycin as shown by Western Blot analysis. [Normalized densitometry ratios for p53, p21 and 14-3-3σ are indicated]

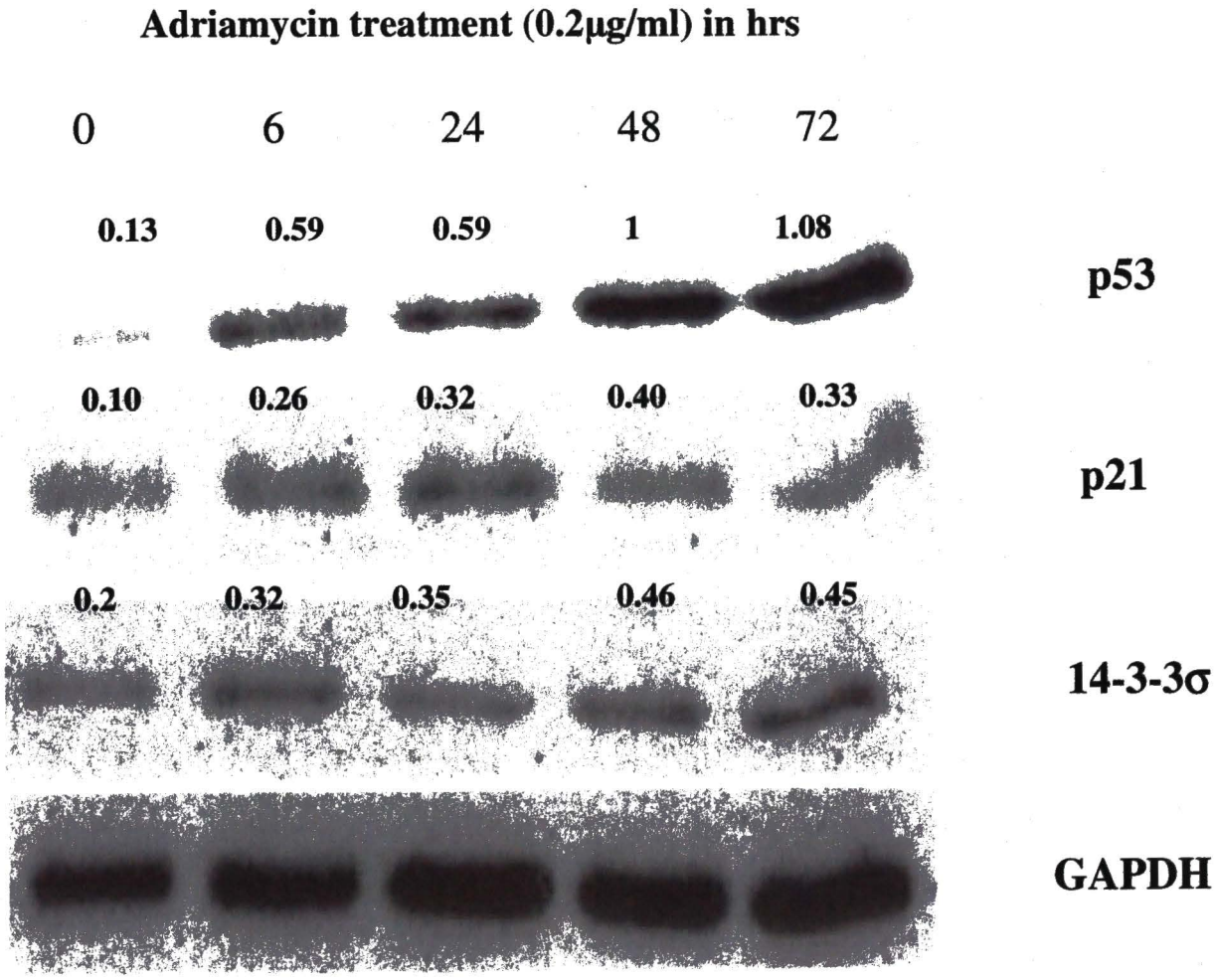


Figure 3[19]: Expression of p53 and p21 by SV-40 CEC cells in the presence of 0.2μg/ml of adriamycin as shown by Western Blot analysis.

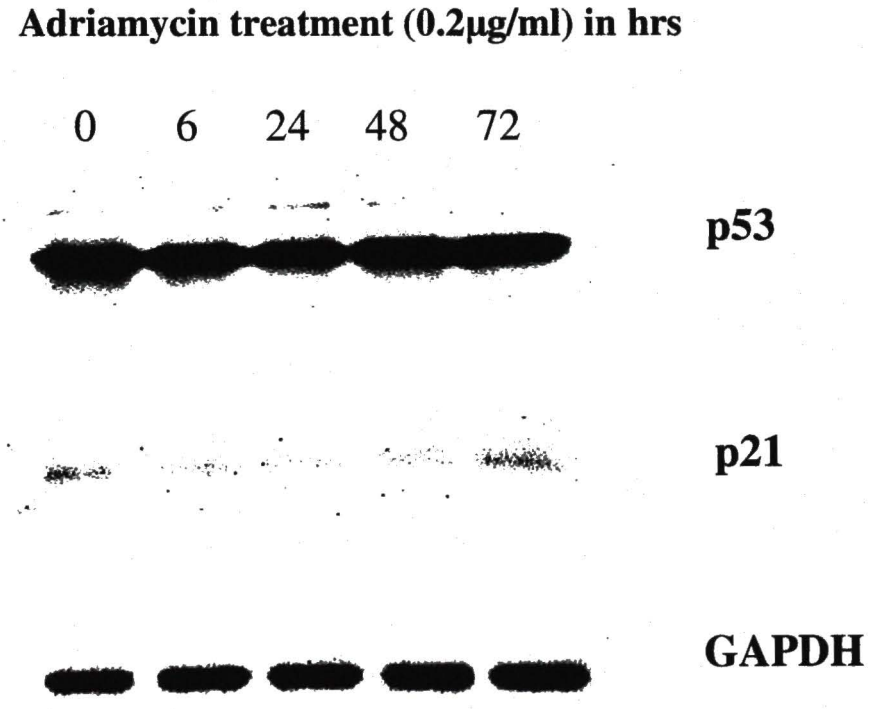
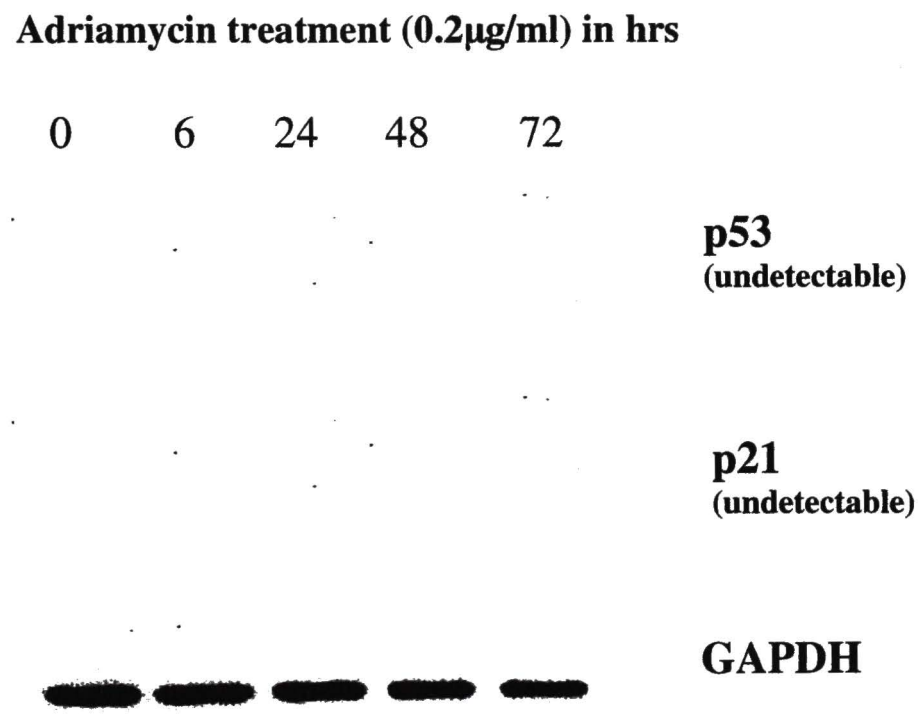


Figure 3 [20]: Expression of p53 and p21 by E6/E7 CEC cells in the presence of 0.2μg/ml of adriamycin as shown by Western Blot analysis.



Discussion

Over passage (P2-P3) there is an increase in 14-3-3 σ expression in WT CEC [Figure 3 (12)]. This is due to the fact that the harvested epithelial cell population is a heterogeneous population consisting of highly proliferating cells that includes a fraction of limbal stem cells and transient amplifying cells as well as differentiated, poorly mitotic cells. As seen *in vivo* this population of cells express little or undetectable levels of 14-3-3 σ [Figure 3 (1)]. *In vitro,* as these cells proliferate they begin to undergo differentiation indicated by an increase in 14-3-3 σ protein expression. This is also confirmed by the presence of involucrin (an accepted marker of terminal differentiation) that is expressed P3 higher [Figure 3 (12)].

14-3-3 σ is also up regulated in response to serum in the culture medium. Further more this induces corneal epithelial cell differentiation as shown by the increased expression of involucrin. There is corresponding increase in K12 and a decrease in K14 expression, which is a cytokeratin expression profile that is crucial for function of these epithelial cells [Figure 3 (15)]. This indicates the direct involvement of 14-3-3σ in corneal epithelial differentiation.

The hTERT CEC are similar to proliferating population of primary WT CEC and are a suitable model to study the aspects of corneal epithelial proliferation and differentiation. Over expression of 14-3-3σ in hTERT CEC cells results in differentiation as shown by the involucrin expression. hTERT CEC were used for these experiments because the WT CEC cells in vitro are already in the process of

differentiation . Hence a cell line in which the cell cycle regulators are intact is a better model to study both proliferation and differentiation events.

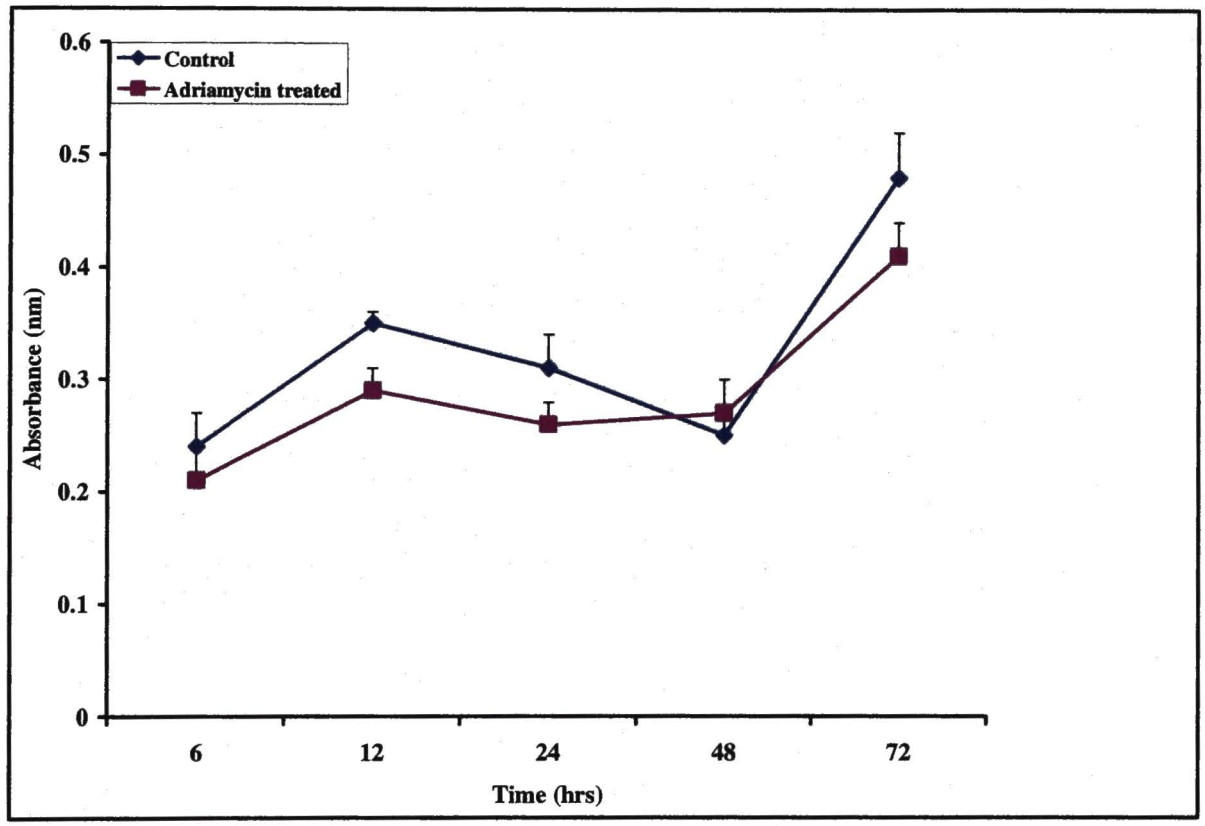
In response to DNA damage there is an increase in p53 protein expression, which in control cells (hTERT CEC) is minimal and in WT cells undetectable [Figure 3(17) and 3(18)]. Under normal conditions, p53 has a very short half-life, which could explain why such a small amount of protein is detected in the control cells. The Mdm2 protein, due to its ubiquitin ligase activity interacts with p53 protein and targets it for degradation[149-151]. Upon detection of DNA damage, the ATM kinase mediates the phosphorylation of the Mdm2 protein, which blocks its interaction with p53. Phosphorylation by ATM kinase and by other kinases also stabilizes and activates the p53 protein[152].

The p53 protein activates the transcription of cyclin-dependent kinase inhibitor, p21. The p21 protein then inactivates the CyclinE: Cdk2 complexes, and prevents entry of the cell into S phase, leading to G1/S cell cycle arrest. Another downstream regulator of p53 is 14-3-3σ. 14-3-3 σ inactivates the CyclinB: Cdk2 complexes, and prevents entry of the cell into M phase, leading to G2/M cell cycle arrest.[153] If the DNA damage is irreparable, the cell undergoes apoptosis.

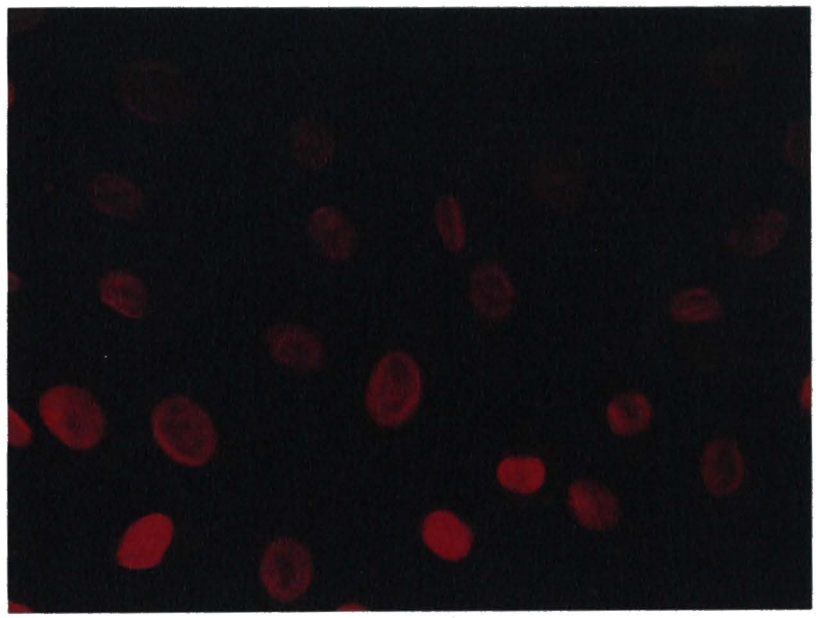
In WT and hTERT CEC cells there is an increase in p21 and 14-3-3σ [Figure 3(17) & 3(18)] suggesting that both the DNA damage pathways are functional in primary corneal epithelial cells. This also supports the fact that our hTERT CEC are a suitable cellular model for studies involving proliferation events operating in the corneal epithelium.

However, the DNA damage signaling pathway is impaired in the SV-40 and E6/E7 CEC cells [Figure 3(19) and 3(20)] as there is no activation of p53 and its down stream regulators. Hence these two cell lines are not suitable cellular models to study DNA damage and differentiation in corneal epithelial cells.

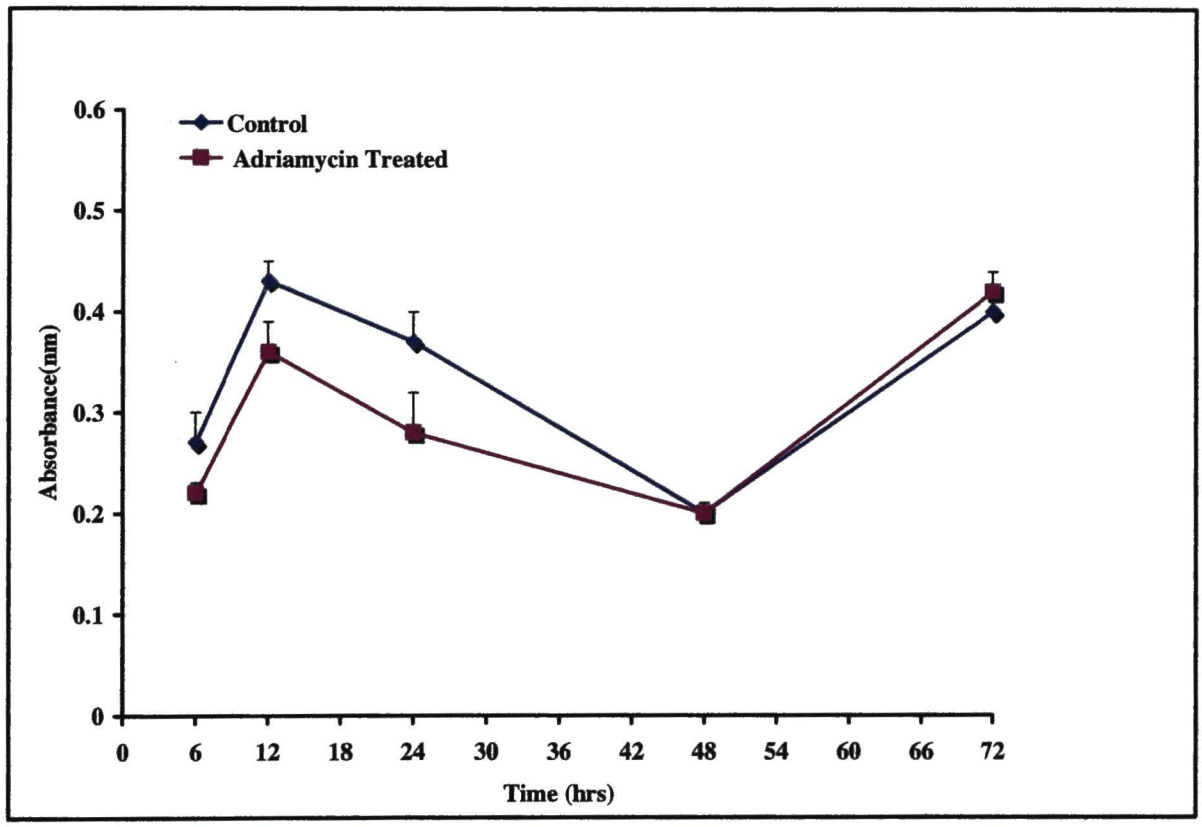
Supplementary Figure 3(A): Time dependent cytotoxicity of adriamycin on hTERT CEC. [Absorbance is proportional to protein concentration, which indicates cell viability]



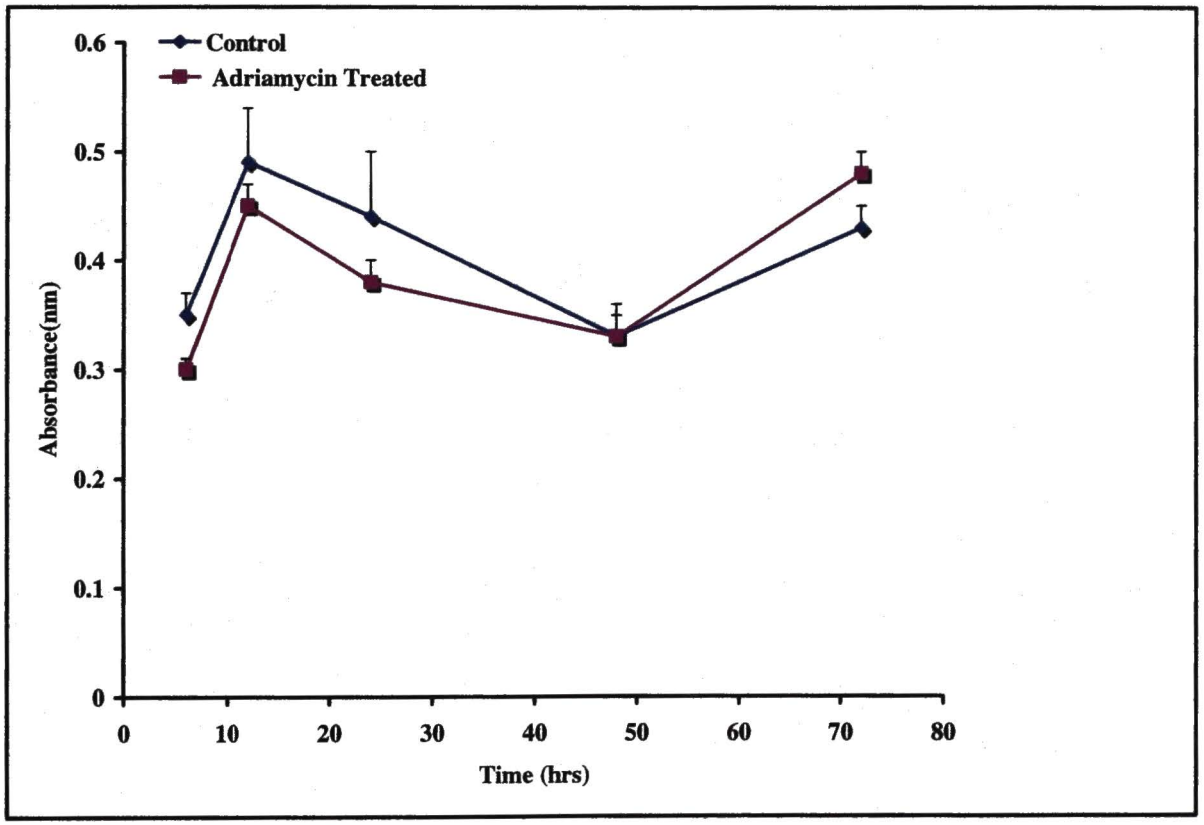
Supplementary Figure 3(B): Propidium iodide staining of hTERT CEC treated with adriamycin for 48hrs, shows intact nuclei. (Magnification -40X)



Supplementary Figure 3(C): Time dependent cytotoxicity of adriamycin on SV-40 CEC. [Absorbance is proportional to protein concentration, which indicates cell viability]



Supplementary Figure 3(D): Time dependent cytotoxicity of adriamycin on SV-40 CEC. [Absorbance is proportional to protein concentration, which indicates cell viability]



CHAPTER IV

EFFECTS OF DOWN-REGULATION OF 14-3-3σ IN CORNEAL EPITHELIAL CELLS

Primary human corneal cells like several other human somatic cells have a limited life span *in vitro*. As a result of this several immortalization strategies have been utilized to generate cell lines, which help overcome problems that limit the availability of primary cells, difficulties culturing them and also variability and reproducibility of results arising from donor related factors such as age and gender. The current immortalization strategies including SV-40 T-antigen, E6/E7 onco-proteins and more recently telomerase are not cell type specific but are in fact use global mechanisms used to immortalize cells from various tissues[16, 17, 19, 20, 154-157].

Extension of *in vitro* life span of human keratinocytes, as a result of 14-3-3 σ down-regulation was the first indication that the inhibition of expression of a single endogenous gene can result in immortalization. This is a more cell type specific immortalization strategy as 14-3-3 σ is a protein expressed specifically in epithelial cells. Since corneal epithelium is also a stratified epithelium like the skin, and we have shown a direct involvement of 14-3-3 σ in differentiation (Specific Aim 2), down-regulation of this protein should lead to extension of corneal epithelial cells *in vitro*.

Specific Aim 3

To show that down-regulation of 14-3-3σ extends in vitro lifespan of corneal epithelial cells.

Rationale

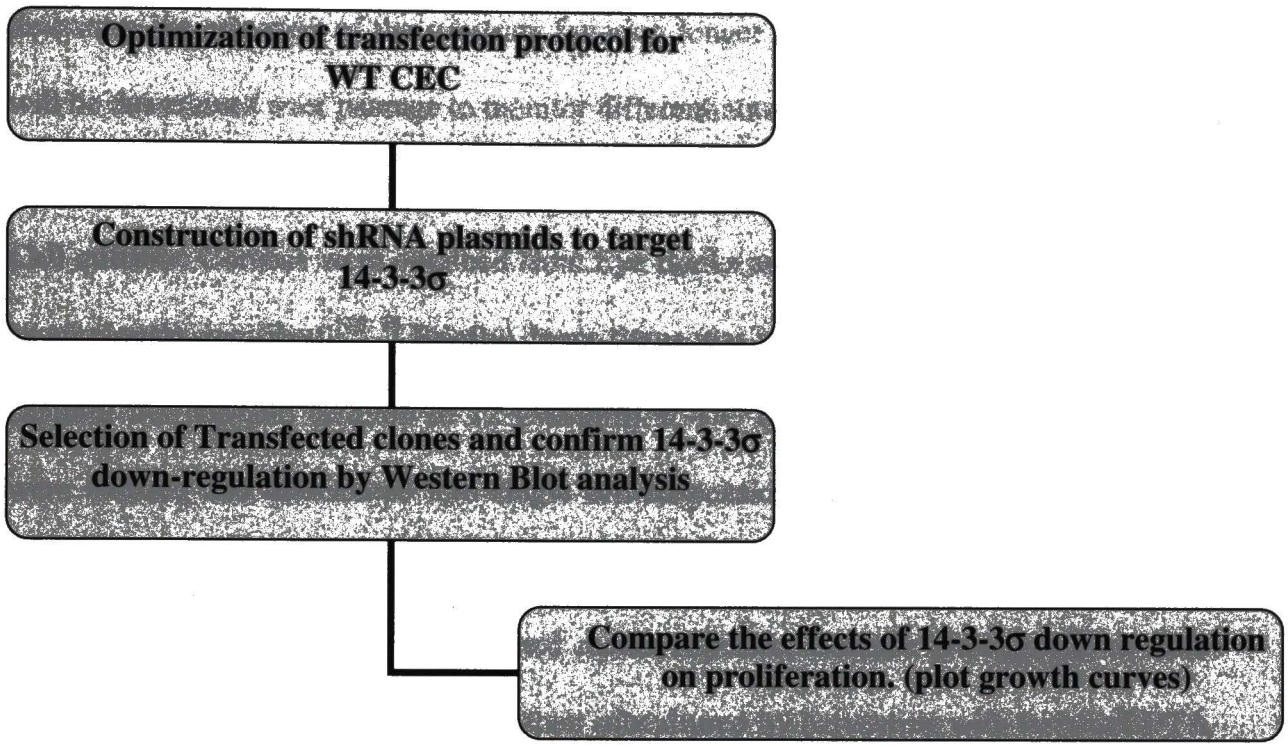
Primary skin keratinocytes and gingival keratinocytes normally stop proliferating after a limited number of passages. [25, 129] It has been shown in these two cell types that down-regulation of 14-3-3 σ achieved using full length antisense DNA results in abrogation of differentiation and maintenance of telomerase activity.[25, 129] These two Studies suggest that suppression of a single gene can result in immortalization. The effect of down-regulation of 14-3-3σ in corneal epithelial cells has not been previously studied.

Primary WT CEC are difficult to transfect. Conventional methods of transfection including Lipofectamine and Fugene transfection result in 95%-100% cell death. It is therefore crucial to establish transfection conditions (tranfection technique and culture conditions) in order to achieve higher transfection efficiency and ensure cell survival and proliferation after transfection. However, because the primary cells have a short *in vitro* life span selection of transfectants is relatively simple.

Preliminary experiments show that using Amaxa Nucleoporation primary WT CEC can be transfected within 5 hours as shown by the expression of GFP with commercially available positive control (Amaxa).

Research Design and Methodology

1. **Optimization of transfection protocol for primary corneal epithelial cells.** Nucleofection will be used to transfect the primary corneal epithelial cells. The right protocol and transfection efficiency will be determined as per manufacturer's instructions.
2. **shRNA plasmids will be constructed that would suppress 14-3-3 σ expression.**
3. **Transfected clones will be selected and decrease in 14-3-3 σ protein expression demonstrated.** Once the transfection protocol is optimized primary corneal epithelial cells will be transfected with the shRNA plasmids. Transfected clones will be selected using ampicillin (50-300ug/ml) although a natural selection is also effective. Western blot analysis will be performed on transfected cell lysates to confirm 14-3-3 σ down-regulation. Expression levels of 14-3-3 σ will be normalized with GAPDH expression.
4. **Calculations of pdl over passage.** After transfection the population doubling with passage will be calculated to generate a growth curve in and demonstrate the effects of 14-3-3 σ down regulation on proliferation.



Specific Aim 3:

To show that down-regulation of 14-3-3 σ extends in vitro lifespan of corneal epithelial cells.

3(a) To determine suitable transfection conditions to for transfecting WT CEC.

Research Design

Determine the right transfection protocol for nucleoporation. Amaxa's Nucleoporation apparatus will be used to transfect primary WT CEC. The manufacturer defined primary epithelial cell programs will be tested to determine the protocol that results in the highest transfection efficiency. The cells will be suspended in epithelial cell specific buffers and will be transfected in designed propritory cuvettes.

Determination of suitable substrates for culture. WT CEC cells will be cultured on plastic, fibronectin (FNC) and Collagen type IV (CIV). Growth curves will be plotted to

determine the selection of the substrate favoring longer *in vitro* lifespan. p16 expression will be determined over passage to monitor differentiation.

Materials and Methods:

Cell culture: Corneas were obtained from Eye Banks and epithelial sheets were isolated as previously described.[123, 38] Briefly, donor tissue was incubated in dispase (diluted with calcium free EpiLife medium to 12 Units/ml) at 4°C for 48 hrs. The epithelial sheets were isolated and dissociated into single cell suspension and the cells plated into CIV coated, FNC (Fisherbrand) coated or non-coated 75 cm^2 vented flasks (tissue culture, TC) and cultured in serum free defined media (EpiLife, Cascade Biologicals) to 80% confluence. The epithelial cells were then subcultured by harvesting with trypsin/EDTA (Gibco Brl) neutralization of proteolytic activity with trypsin inhibitor (Sigma-Aldrich), and plating into freshly coated (CIV or FNC) or non-coated TC flasks.

Transfection

Primary WT CEC of early passage (P2/P3) were transfected as per manufacturer's protocol. Briefly WT CEC were harvested as described earlier. The harvested cells were counted and re-suspended in epithelial buffer (Amaxa Inc. Gaithersburg, MD USA). The GFP plasmid (0.5-1μg, Amaxa Inc. Gaithersburg, MD USA) was added to the re-suspended cells. The cell suspension was then added to the electroporation cuvette (Amaxa Inc. Gaithersburg, MD USA), placed in the electroporation unit and then transfected. The cells were then very gently transferred into flasks and cultured in Epilife. Medium was changed 12 -24 hrs following transfection.

Western Blot analysis

Cells cultured on various substrates were harvested and at each passage, 50% of the harvested cells were lysed and 50% were re-seeded into fresh flasks. Cells harvested were treated with lysis buffer [2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled water]. Genomic DNA was sheared by several passes through a 22-gauge needle, and samples stored at –20°C until needed. BCA protein assays (Pierce, Rockford, IL) of lysates were performed to determine the protein concentration and ensure equal loading of lanes. SDS PAGE was performed with 20µg protein/lane at room temperature using 12% Tris-Glycine, at 150V in Tris/glycine running buffer. Electro-blotting was carried overnight (4°C) at 10V in Tris/glycine buffer with 20% methanol onto nitrocellulose membranes (VWR international) and the transfer was confirmed by Ponceau Red (Sigma-Aldrich) staining of the membranes. After de-staining in distilled water, membranes were blocked for 1 hour (RT) in blocking buffer (5% powdered milk and 1% BSA in PBS). Membranes were incubated at RT then at 4°C overnight, followed by 30 minutes at room temperature the following morning. Membranes were incubated in 1° antibody for 30 minutes (RT) incubated at 4°C overnight, and for 30 min. at RT the following morning. Membranes were then rinsed 3x10 minutes in PBS + 0.1% Tween-20 and incubated in 2° antibody for 1 hour (RT). After rinsing 3x 10 minutes in PBS + 0.1% Tween-20, membranes were developed (ECL chemiluminescence Amersham Biosciences, UK).

Results

Morphology and cell attachment on different substrates

Epithelial cells show better attachment on CIV coated flasks when compared to FNC and non-coated surfaces [Figure 4 (1)]. Cell morphology on day 8 shows the presence of a number of large cells, which is representative of differentiated cells when they are cultured on FNC and plastic [Figure 4 (1)].

Growth of primary WT CEC cells on different substrates/surfaces

Primary WT CEC grown on FNC and plastic divide and proliferate for a maximum of 4 to 5 passages and a pdl of 10 to 12. Cells cultured on CIV can be cultured for 6 to 7 passages and a pdl of 15 and higher (highest 26) [Figure 4 (2)].

Expression of p16 in cells cultured on different substrates

The expression of p16 in WT CEC cultured on plastic and FNC remain the same over passage indicating the presence of a significant percentage of differentiated/differentiating cells [Figure 4 (3)]. However, WT CEC cultured on CIV show low/undetectable levels of p16 at P2, but the level of p16 in these cells gradually increases with passage [Figure 4 (3)].

Transfection Efficiency using Amaxa Apparatus

Epithelial cell targeted nucleoporation programs S-05, T-13, T-20, T-23 and U-17 programs were used to transfect the WT CEC cells with GFP. The percentage of GFP expressing cells was determined 24 hrs after transfection. Programs T-13 and T-23

Transfection Efficiency using Amaxa Apparatus

Epithelial cell targeted nucleoporation programs S-05, T-13, T-20, T-23 and U-17 programs were used to transfect the WT CEC cells with GFP. The percentage of GFP expressing cells was determined 24 hrs after transfection. Programs T-13 and T-23 resulted in the highest transfection efficiency of approximately 25% [Figure 4(4)]. Program S-05 had a very poor transfection efficiency of <5%.

Discussion

WT CEC cells when cultured on CIV have better attachment efficiency [Figure 4 (1)]. Also, a large number of cells begin to differentiate to appear when cultured on plastic and FNC by day 8 compared to the cells cultured on CIV [Figure 4 (1)]. The WT CEC cells also can be cultured longer when cultures on CIV [Figure 4 (2)] and the p16 expression in these cells gradually increases over passage [Figure 4 (3)]. Lower p16 expression in these cells indicates fewer differentiated cells in the culture. Therefore for good transfection efficiency and better culture conditions following transfection would be to culture the WT CEC cells on CIV coated flasks.

Results of GFP transfection using the Amaxa Nucleofector system indicates that the highest transfection efficiency is achieved using the program T-23 [Figure 4(4)]. Hence, would be the most suitable program for transfecting WT CEC cells.

Figure 4 (1): Phase –Contrast micrographs to show the morphology and attachment of primary WT CEC on different substrates at Day 1 and Day 8. (White arrows indicate differentiated cells with large flat morphology)

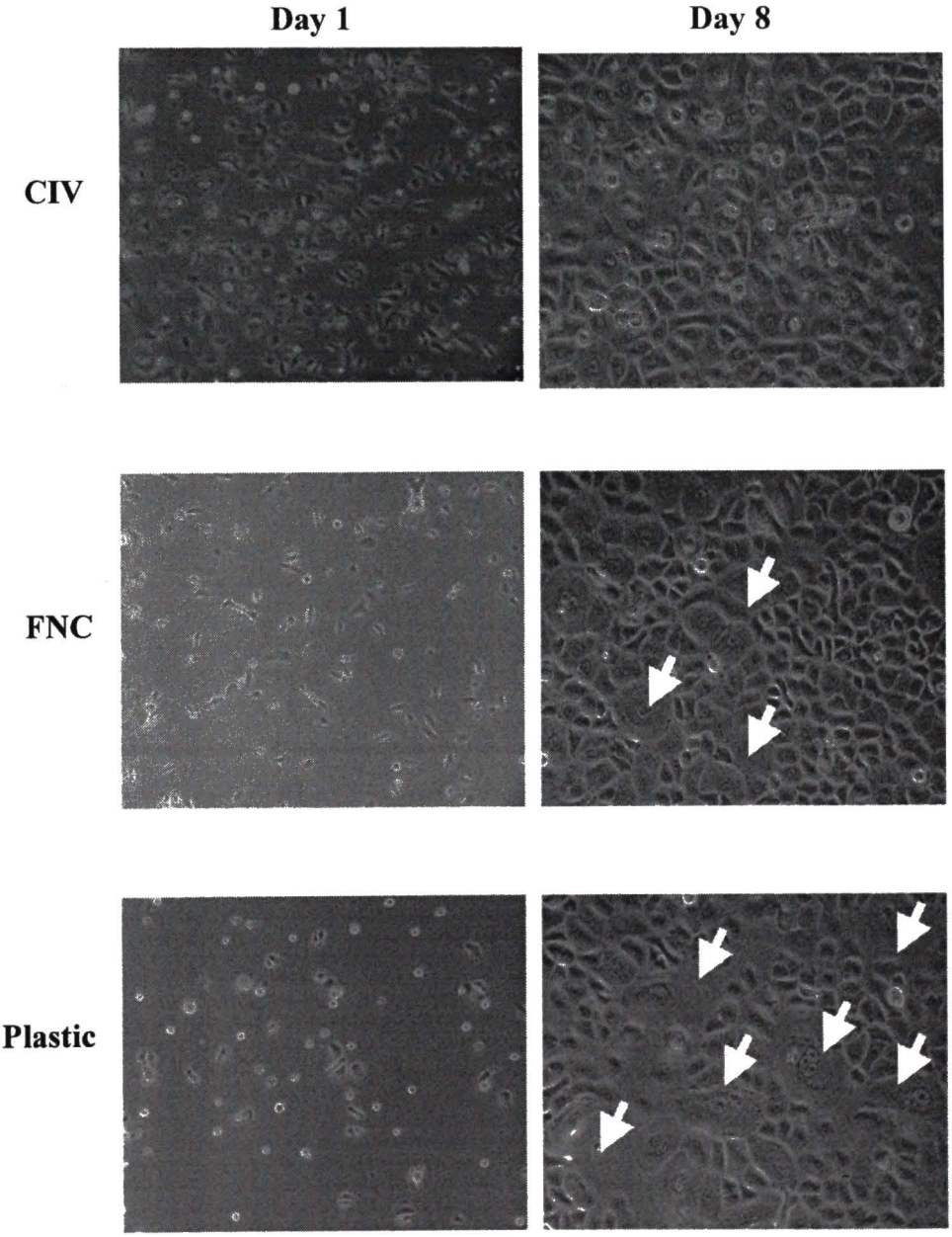


Figure 4 (2): Growth curve over passage of primary WT CEC on different substrates. Population doubling (Pdl) is plotted along the Y-axis and Passage on the X-axis.

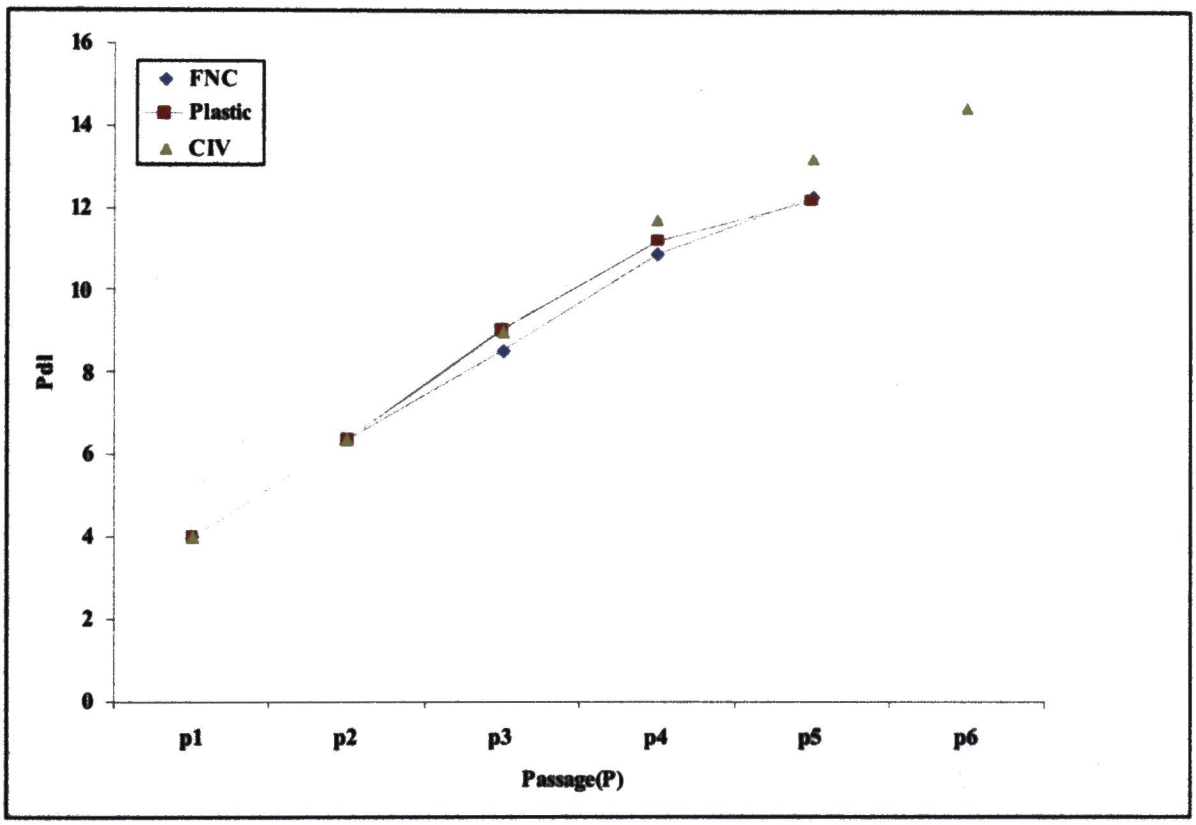


Figure 4 (3): Expression of p16 in primary WT CEC over passage following culture on different substrates as determined by Western Blot analysis (a) CIV, (b) FNC and (c) Plastic.

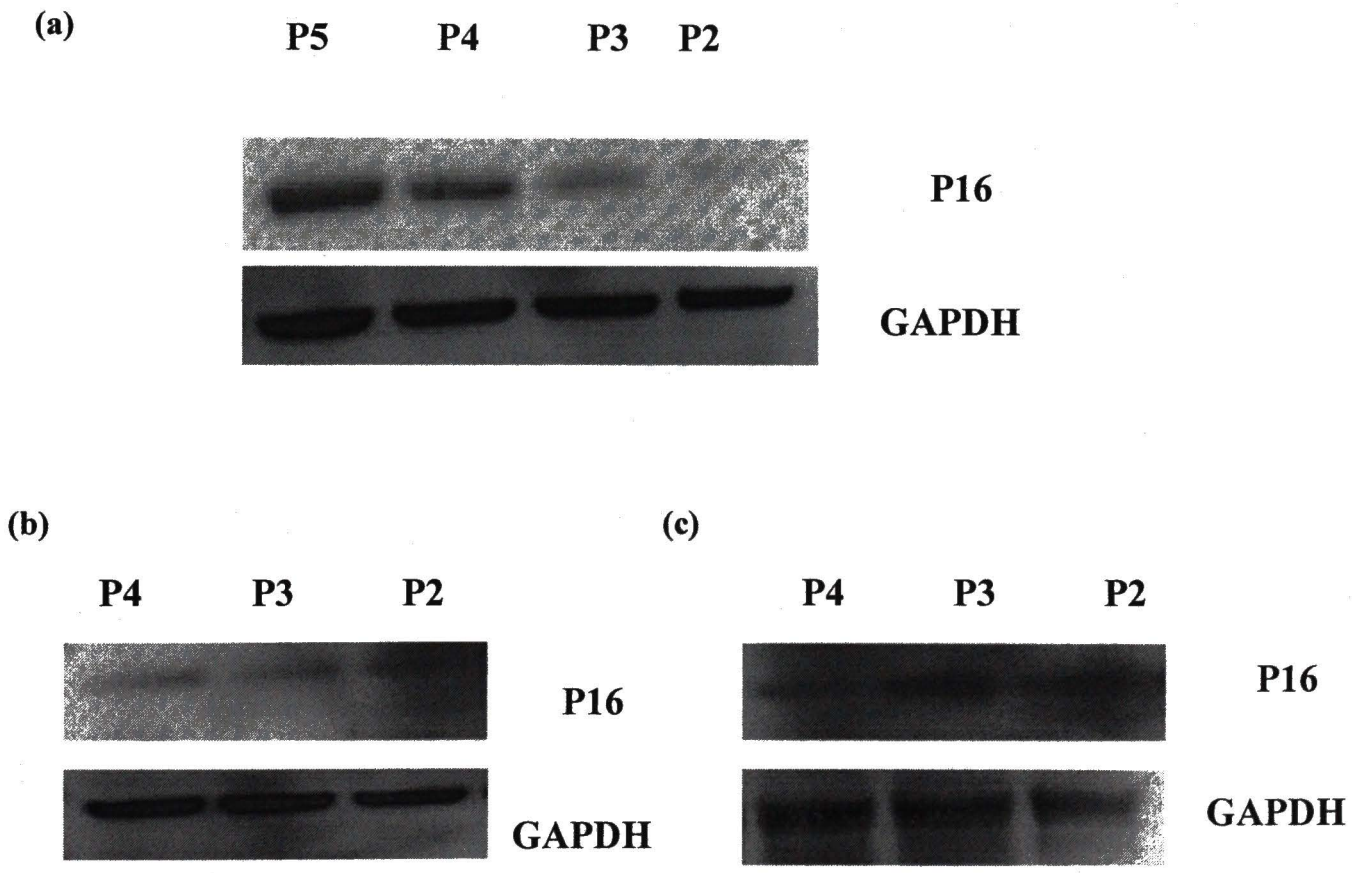
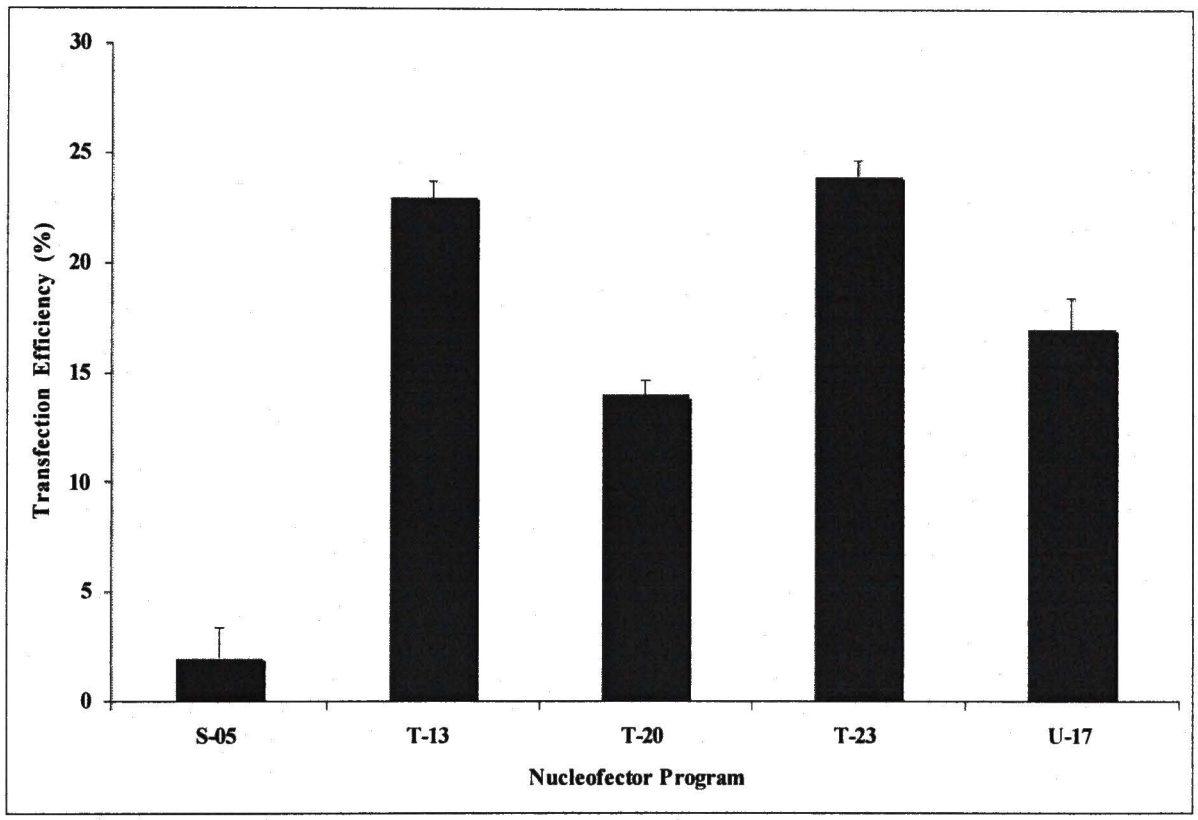


Figure 4 (4): Transfection efficiency of primary WT CEC cells after transfection using Amaxa electroporation system. (a)Amaxa Nucleoporation device and (b) Transfection Efficiency.

(a)

(b)


30
25
20
15
10
5
0
Transfection Efficiency (%)
S-05
T-13
T-20
T-23
U-17
Nucleofector Program

Specific Aim 3

To show that down-regulation of 14-3-3σ extends in vitro lifespan of corneal epithelial cells.

3(b) Down-regulation of 14-3-3σ extends *in vitro* lifespan of WT CEC.

Materials and Methods

shRNA plasmid sequences and construction

Three shRNA plasmid sequences were generated using the siDIRECT website (http://genomics.jp/sidirect/index.php?type=fc) (Table 4 (1)). These sequences were cloned into the pBluescript plasmid (Invitrogen) between the Not I and Hind III restriction sites.

Table 4(1): shRNA sequences as determined using siDIRECT.

Serial No.	Sequence
1	**FORWARD**: 5'-GTACCTC-GACATGGCAGCCTTCATGAAAGG-TCAAGAG-CCTTTCATGAAGGCTGCCATGTC-TTTTTGGAAA-3' **REVERSE**: 5'AGCTTTTCCAAAAA-GACATGGCAGCCTTCATGAAAGG-CTCTTGA-CCTTTCATGAAGGCTGCCATGTC-GAG-3'
2	**FORWARD:**5'-GTACCTC –TGGAGGGTGCTGTCCAGTATTGA-TCAAGAG- TCAATACTGGACAGCACCCTCCA- TTTTTGGAAA-3' **REVERSE:**5'AGCTTTTCCAAAAA-TGGAGGGTGCTGTCCAGTATTGA -CTCTTGA- TCAATACTGGACAGCACCCTCCA- GAG-3'
3	**FORWARD:**5'-GTACCTC-GGGTCTTCTACCTGAAGATGAAG-TCAAGAG-CTTCATCTTCAGGTAGAAGACCC- TTTTTGGAAA-3' **REVERSE:**5'AGCTTTTCCAAAAA-GGGTCTTCTACCTGAAGATGAAG-CTCTTGA-CTTCATCTTCAGGTAGAAGACCC- GAG-3'

Transfection Primary WT CEC were cultured on CIV coated flasks until confluent (as described earlier). The cells were harvested, counted and were then transfected with the shRNA plasmid and with an empty vector control using cells from the same donor in two

separate transfections. The transfected cells were then cultured in CIV coated flasks changing the medium every 24 hrs. When nearly confluent (95%), the cells were harvested and 50% of the cells were lysed for western blot analysis and the other 50% was re-seeded onto fresh CIV coated flask. Over passage the initial and final population of cells was determined at each passage so that the pdl could be calculated.

Western Blot Analysis

WT CEC transfected with shRNA plasmids and empty vector controls (evC) were analyzed for 14-3-3σ expression levels by western blot analysis. Cells were harvested and treated with lysis buffer [2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled water]. Genomic DNA was sheared by several passes through a 22-gauge needle, and samples stored at –20°C until needed. BCA protein assays (Pierce, Rockford, IL) of lysates were performed to determine the protein concentration and ensure equal loading of lanes. SDS PAGE was performed with 20μg protein/lane at room temperature using 12% Tris-Glycine, at 150V in Tris/glycine running buffer. Electro-blotting was carried overnight (4°C) at 10V in Tris/glycine buffer with 20% methanol onto nitrocellulose membranes (VWR international) and the transfer was confirmed by Ponceau Red (Sigma-Aldrich) staining of the membranes.

After de-staining in distilled water, membranes were blocked for 1 hour (RT) in blocking buffer (5% powdered milk and 1% BSA in PBS). Membranes were incubated at RT then at 4°C overnight, followed by 30 minutes at room temperature the following morning. Membranes were incubated in 1° antibody [Table 2 (i)] for 30 minutes (RT) incubated at 4°C overnight, and for 30 min. at RT the following morning. Membranes

were then rinsed 3x10 minutes in PBS + 0.1% Tween-20 and incubated in 2° antibody for 1 hour (RT). After rinsing in PBS + 0.1% Tween-20 (3x 10 minutes), membranes were developed (ECL chemiluminescence Amersham Biosciences, UK).

Antibodies

Listed in Chapter II, Table 2(i) and Chapter III, Table 3(i).

Results

Trasfection of WT CEC with shRNA plasmid (2) and (3) resulted in 100% cell death. The shRNA plasmid (1) did not result in 100% cell death and hence this plasmid was used in the transfection experiments. Other problems encountered during transfection included microbial and fibroblast contamination [Table 4(2)], as a result of which some cells were discarded and experiments aborted.

Proliferation of shRNA transfected cells versus control

Growth curve of pdl (Y-axis) against passage (X-axis) was plotted. The growth curve for all the empty vector transfected control cells were also plotted and the maximum passage these cells could be cultured was P8 to P13 (pdl of 13 to 35) [Figure 4(6) and Table 4(3)]. The cells of the same donors that were transfected with the shRNA plasmid survived longer in culture [Figure 4 (7) and Table 4 (3)].The cells from 74/M (FTT CEC) donor not only has an extended life span of over P11 but they immortalized and could be maintained in culture over P20 (pdl of 50).

Down-regulation of 14-3-3σ

Successful down-regulation of 14-3-3 σ is achieved using shRNA plasmid (1) as seen in the 50% down-regulation of protein expression from P3 to P6 in Cepi 74/M donor [Figure 4(5)]. In the control cells the 14-3-3 σ expression continues to increase over passage [Figure 4 (5)]. These cells also maintained constant levels of 14-3-3 σ expression over passage [Figure 4 (5)]. The intensity of the protein bands were normalized to loading control (GAPDH) using NIH Image J software.

Discussion

Transfection of primary WT CEC is very difficult when these are not cultured on feeder layers. The skin and gingival keratinocytes that were immortalized were both transfected using retroviral vectors and were also cultured on 3T3 fibroblast feeder layers. Despite culture on feeder layers of several transfections, only one or 2 donors resulted in immortalization. We were successfully able to transfect WT CEC cells with shRNA plasmids by electroporation using Amaxa's Nucleofection device. Culturing the cells in flasks coated with CIV instead of feeder layers also eliminates the possibility of cross-contamination with feeder layer cells (3T3 fibroblasts). However, of the 4 successful transfections only one resulted in an immortalized cell line (FTT CEC).

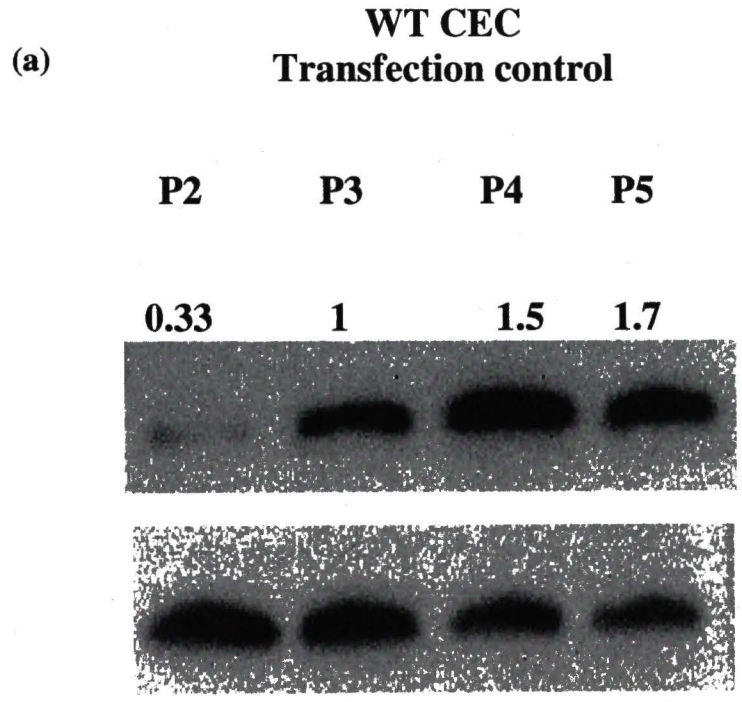
Table 4(2): Transfection results on cells from different donors.

S.No	Donor Information (Age/ gender)	Results
1	15/M	Fibroblast Contamination
2	53/M	Fibroblast Contamination
3	77/F	Fibroblast Contamination
4	17 /M	Extension of lifespan
5	39 /F	Extension of lifespan
6	59/F	Same as control
7	54 /M	Extension of lifespan
8	74 /M	Extension of lifespan and Immortalization
9	75/M	Microbial Contamination
10	65/F	Microbial Contamination
11	17/F	Microbial Contamination

Table 4(3): Passage and population doubling data on successful transfections along with their corresponding transfection controls.

S.No	Donor Information (age Y.O)	Control/shRNA plasmid (1)	Max Passage(P)/ Max Pdl
1	59	evC plasmid	6/9
		shRNA plasmid	6/10
2	74	evC plasmid	4/8
		shRNA plasmid	13/36
3	54	evC plasmid	3/5.6
		shRNA plasmid	8/13.5
4	17	evC plasmid	3/6.8
		shRNA plasmid	8/16.3
5	39	evC plasmid	6/9
		shRNA plasmid	9/17.6

Figure 4(5): Western Blot Analysis to determine 14-3-3σ expression over passage after transfection. (a) Cells transfected with control vector (evC plasmid) and (b) Cells transfected with shRNA (1) (Donor 74 year old / Male)



(b)

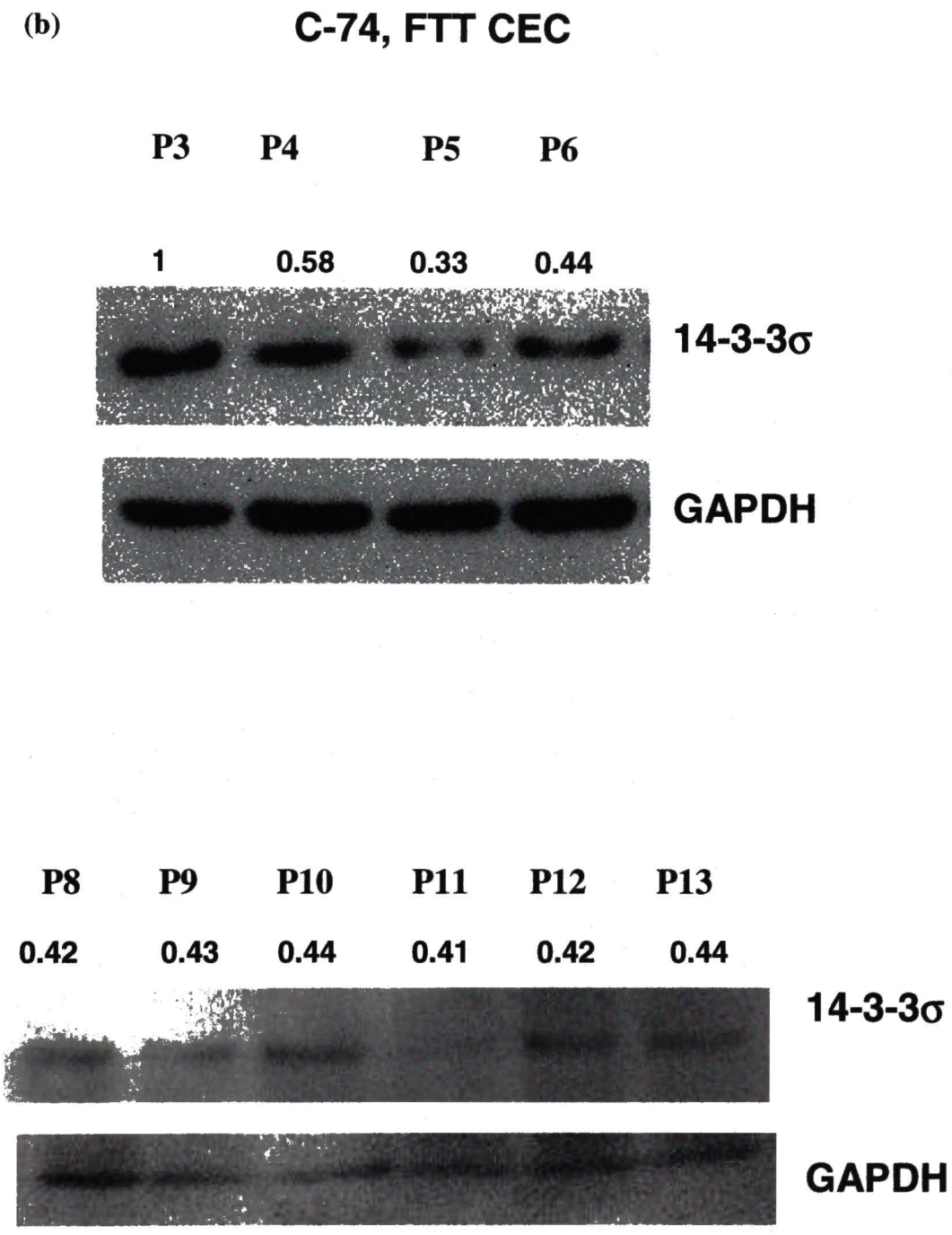

C-74, FTT CEC
P3
P4
P5
P6
1
0.58
0.33
0.44
14-3-3σ
GAPDH
P8
P9
P10
P11
P12
P13
0.42
0.43
0.44
0.41
0.42
0.44
14-3-3σ
GAPDH

Figure 4(6): Growth curve of primary WT CEC from different donors transfected with vector controls (evC) (evC1-74 Y.O, evC2-17Y.O, evC3-54 Y.O, evC4-39 Y.O and evC5-59 Y.O).

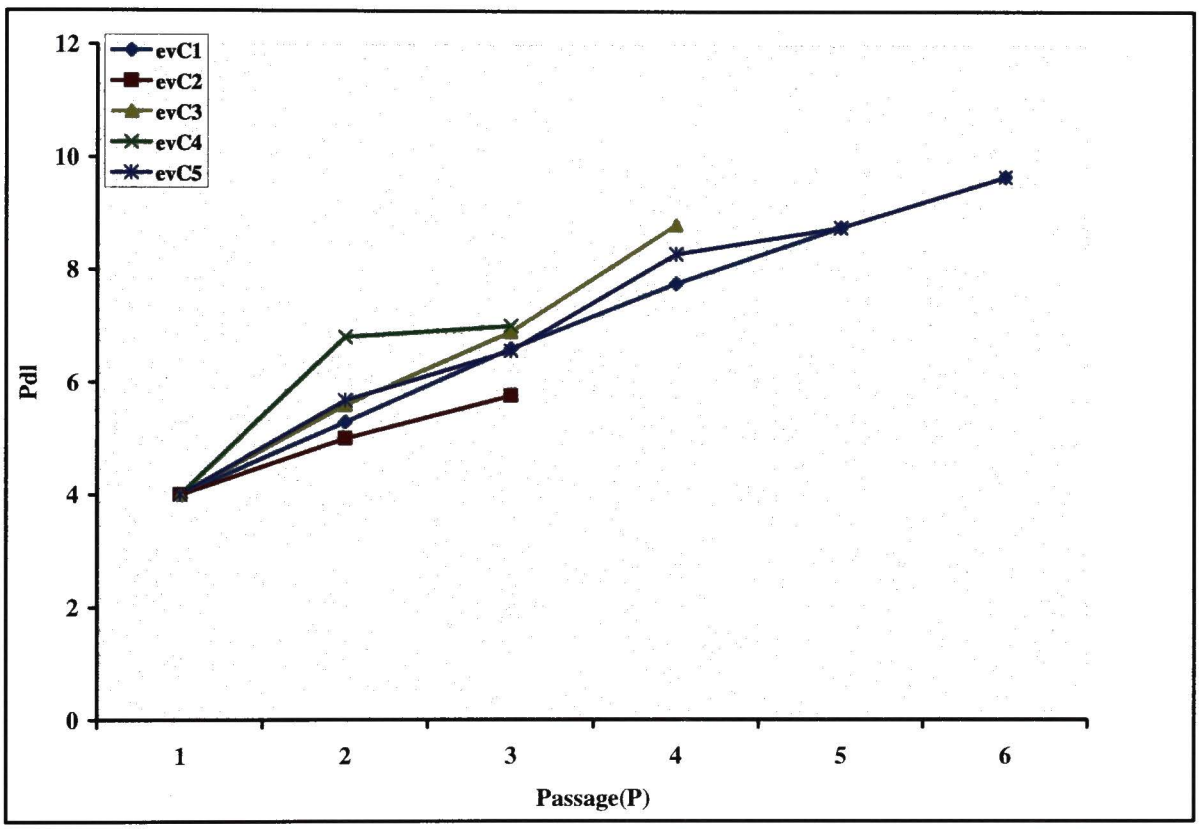
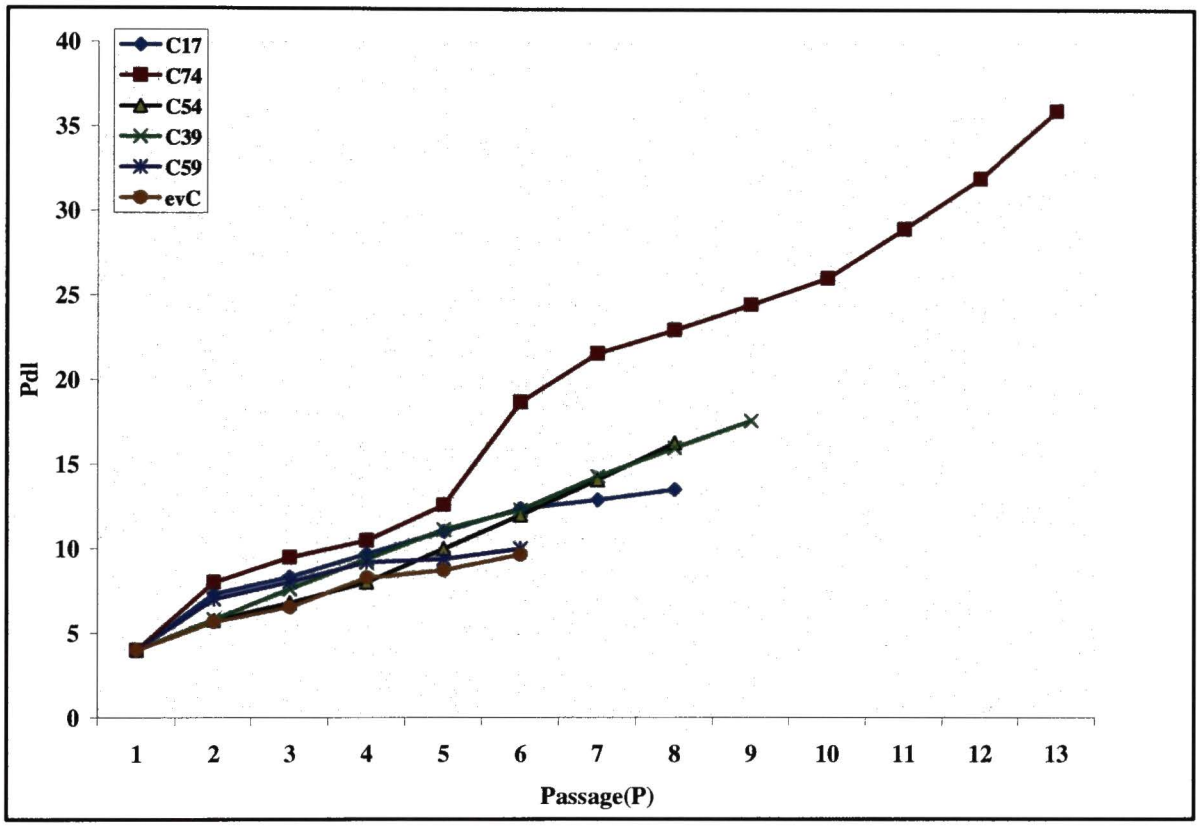


Figure 4(7): Growth curve of primary WT CEC cells from different donors transfected with shRNA (1) (C74, C17, C54, C59 and C39). [ev control—average vector control from Figure 4(6)]



CHAPTER V

CHARACTERIZATION OF FTT CEC CELLS

Down-regulation of 14-3-3σ has been reported in a number of cancers such as breast cancer, lung cancer[41, 48, 50, 56] and oral cancers[53, 158, 159] . Since 14-3-3 σ has been shown to be involved in cell cycle regulation, it is considered to be a plausible target for cancer therapy.

In keratinocytes, down-regulation of 14-3-3 σ using antisense strategy has been shown to result in extension of *in vitro* life span and immortalization. This was the first indication that the inhibition of a single endogenous gene can result in immortalization. Inadvertently this may also be considered the first step towards determining the role of 14-3-3 in normal human epithelial cells. Another more recent study showed immortalization of gingival keratinocytes by down-regulation of 14-3-3 σ[129]. However, the cell lines generated as a result of 14-3-3σ down-regulation have not been characterized very extensively, particularly with respect to their ability to differentiate.

The experiments from specific Aim 4(b) resulted in the generation of an immortal corneal epithelial cell line (FTT CEC). Characterization of these cells is necessary in order to be able to use them as a model to study the other signaling cascades involved in corneal epithelial proliferation, differentiation and apoptosis. Since down-regulation of 14-3-3σ has been implicated in cancer, these cells may also be a useful model in the area of cancer research.

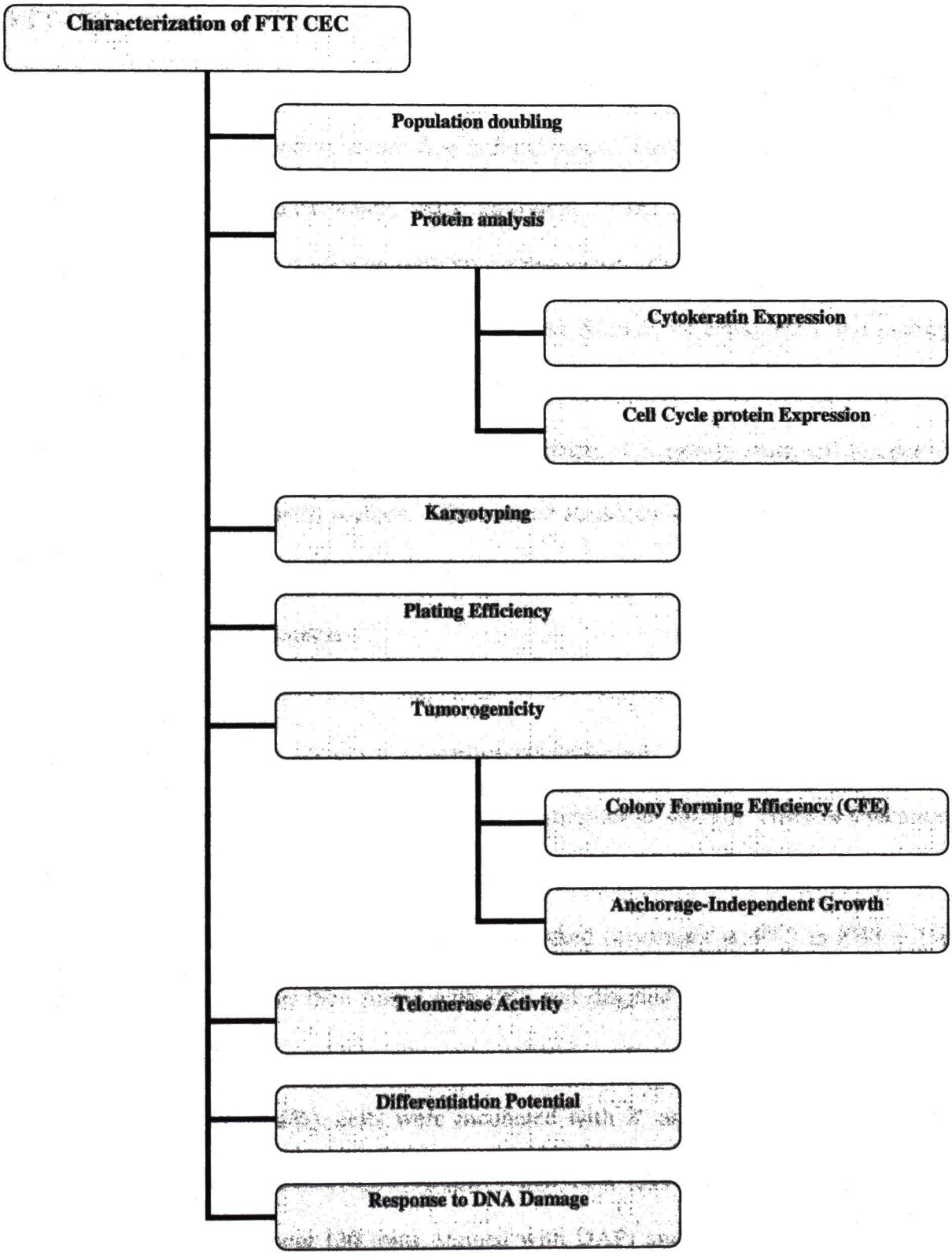
Specific Aim 4

To characterize FTT CEC cells those were generated as a result of down-regulation of 14-3-3σ.

Rationale:

Several corneal epithelial cell lines have been generated for *in vitro* studies. Common methods of generating cell lines include introduction of oncogenes such as SV-40 T antigen (large and small) and E6/E7 oncoproteins[13, 19, 20, 125, 157]. The expression of these oncogenes results in alteration of cell cycle components and produce cells that are unsuitable as models to study processes such as differentiation. These methods are also target global mechanisms of cell cycle disruption and they have been used to immortalize any cell type.

Immortalization as a result of down-regulation of 14-3-3σ has a potential to be epithelial cell specific, so it is important to characterize the resulting cells in order to study alterations in the expression of cell cycle proteins and impact on differentiation potential.


Characterization of FTT CEC
Population doubling
Protein analysis
Cytokeratin Expression
Cell Cycle protein Expression
Karyotyping
Plating Efficiency
Tumorogenicity
Colony Forming Efficiency (CFE)
Anchorage-Independent Growth
Telomerase Activity
Differentiation Potential
Response to DNA Damage

Materials and Methods

FTT CEC culture.

The cells that were generated in Specific Aim 4 following 14-3-3σ down-regulation using shRNA were cultured in serum free defined media (EpiLife, Cascade Biologicals, Portland, OR) until approximately 80% confluent. The epithelial cells are then subcultured by harvesting with trypsin/EDTA (Gibco BRL, Carlsbad, CA) neutralization of proteolytic activity with trypsin inhibitor (Sigma-Aldrich, St Luis, MS), and plating into freshly collagen IV coated TC flasks (BD Biosciences, San Jose, CA). Flasks were coated with murine collagen type IV (at a concentration of 5 μg/ml) using cell scraper to evenly coat the entire growth surface. The coated flasks are then allowed to dry under sterile conditions before use (or stored sterile at 4°C).

Indirect Immunofluorescence

Immunocytochemistry.

FTT CEC cells, cultured on glass coverslips (Fisherbrand), were rinsed in PBS, and fixed/permeabilized in methanol: acetone (1:1, 10 minutes at -20^{O}C). After re-hydration in PBS (0.256g/L NaH_2PO_4 H_2O, 1.19g/L Na_2HPO_4, 8.76g/L NaCl, pH 7.4; for 30 min), and distilled water washes (3x) the cells were blocked (overnight at 4°C) in PBS + 1% BSA. The specimens were then rinsed with PBS and distilled water (3x), and incubated with 1° antibody (4°C, overnight) diluted in 1XPBS. After rinsing in PBS (3x10min) containing Tween 20 (0.1%), cells were incubated with 2° antibody (1.5 hrs, RT) and rinsed in PBS +Tween 20 (0.1%, 3x10min). Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (30 min), stained with DAPI and mounted on glass slides

(FluorSave™, Calbiochem, La Jolla, CA).

Antibodies: Primary antibodies were obtained from commercially available sources [Refer to Chapter II, Table 2(i) and Chapter III, Table 3(i)]. Alexa Fluor 594nm goat anti-mouse, Alexa Fluor 594 goat-anti-rabbit and Alexa Fluor 594 donkey anti-goat secondary antibodies (from Molecular Probes) were used at dilutions of 1:1000. Negative controls in all experiments were specimens labeled with 2° antibody only and DAPI to show nuclei; these showed virtually no fluorescence.

Image Acquisition: Mounted specimens were examined on Olympus AX70 fluorescent microscope using SPOT Twain software. Electronic images were captured at a magnification of 20X.

Western blot analysis

FTT CEC cells were harvested and treated with lysis buffer [2.5ml 1M Tris buffer (pH = 7.0), 1g SDS, and 2.5g sucrose in 50ml distilled water]. Genomic DNA was sheared by several passes through a 22-gauge needle, and samples stored at –20°C until needed. BCA protein assays (Pierce, Rockford, IL) of lysates were performed to determine the protein concentration and ensure equal loading of lanes. SDS PAGE was performed with 20μg protein/lane at room temperature using 12% Tris-Glycine, at 150V in Tris/glycine running buffer. Electro-blotting was carried overnight (4°C) at 10V in Tris/glycine buffer with 20% methanol onto nitrocellulose membranes (VWR international) and the transfer was confirmed by Ponceau Red (Sigma-Aldrich) staining of the membranes. After de-staining in distilled water, membranes were blocked for 1 hour (RT) in blocking buffer (5% powdered milk and 1% BSA in PBS). Membranes were incubated at RT then

at 4°C overnight, followed by 30 minutes at room temperature the following morning. Membranes were incubated in 1° antibody for 30 minutes (RT) incubated at 4°C overnight, and for 30 min. at RT the following morning. Membranes were then rinsed 3x10 minutes in PBS + 0.1% Tween-20 and incubated in 2° antibody for 1 hour (RT). After rinsing in PBS + 0.1% Tween-20 (3x 10 minutes), membranes were developed (ECL chemiluminescence Amersham Biosciences, UK).

Population Doubling

100,000 cells (primary CEC, hTERT CEC and FTT CEC) were seeded in 6 well plates (2 wells/cell type). After 5 hours the number of cells that did not attach were counted to determine the actual number of cells that remained attached and were seeded per well. Cells were the harvested at 18, 24, 36 and 72 hrs and counted. The ratio of the number of cells seeded and final number of cell counted at different time points was used to determine the population doubling time (pdl).

Karyotyping

Karyotyping was performed using a previously described protocol [140-142, 160, 161]. Cells (hTERT CEC and FTT CEC) cells were cultured in 6 well plates (3 wells/cell type) until they were 50% confluent. The cells were then treated with colcemid (10ug/ml) (Sigma-Aldrich) in culture medium overnight at 37°C in the incubator. Cells were harvested as previously described earlier using Trypsin-EDTA (Invitrogen). The harvested cell pellet was incubated in 500ul of 0.75M KCl (Sigma-Aldrich) at 37C for not more than 25 minutes and pelleted by centrifugation at 4000rpm and to remove the KCl. The cell pellet was gently broken up in freshly prepared cold fixative (methanol:Acetic acid, 1:1). The

cell suspension was then incubated at 20°C for 15 minutes and then centrifuged at 4000 rpm and the fixative was removed from the pellet. Fresh fixative was once again added, the pellet gently broken up and the suspension was incubated at -20C for another 15 minutes. The cell suspension (10µl) was then dropped cells onto slides (pre-cleaned with 100% ethanol and dried) (Fisher Scientific) from a height of 8 inches. The slides were stained with DAPI (Molecular probes) and the chromosomes counted under fluorescent microscope.

TRAP Assay

Telomeric Repeat Amplification Protocol (TRAP) assay, a PCR based telomerase activity assay was performed on the FTT CEC cells as per the manufacturer's instructions (Intergen). Briefly 100,000 cells were lysed and PCR amplification was performed using the primers provided by the manufacturer. The PCR products were then analyzed by PAGE gel electrophoresis. Lysis buffer and CEC cells of P5 were used as negative controls, E6/E7, SV-40 immortalized cells, hTERT CEC and cell lysates provided by the manufacturer were used as positive controls.

Colony Forming Assay

Colony Forming Efficiency (CFE) was calculated using a modification of the protocol described earlier [139, 160]. Briefly, 500 cells were seeded into 3 wells of a 6 well plate. The number of floaters was counted the following day to determine the exact number of attached cells that had attached. After nine days of culture the cells were fixed in 10% TCA at 4°C for 10 minutes. The wells containing cells were then washed 3 times with distilled water and the colonies were stained using 0.4% SRB in 1% acetic acid for 10

minutes at RT. The cell were then washed four times with 1% acetic acid and allowed to air dry. The stained colonies were then counted under a dissection microscope.

Anchorage Dependent growth Assay

Anchorage independent growth assay was performed using a modification of a protocol described earlier [160]. hTERT CEC (10,000cells/ml) and FTT CEC (10,000cells/ml) in an aliquot of 0.4% agarose solution was poured onto a 0.8% agarose gel bed in 3 wells/sample of a 6 well plate. The cells were fed with medium once a week for a total of 3 weeks. Number of colonies (>5 cells) were counted under the microscope. WT CECs were used as a negative control.

To evaluate the effect of loss of anchorage dependence on cell viability, the cells were then incubated with 0.5% MTT (Sigma-aldrich) for 3 hours. Viable cells were counted under light microscope. Cell viability was calculated as a percentage of viable cells in the total number of cells counted. WT CEC were used as a negative control and MCF-7 cells were used as a positive control.

Results

Morphology

The FTT CEC cells have a cobblestone morphology which is characteristic of epithelial cells [Figure5 (1 c)]. The cells are uniform in size unlike WT CEC where there is a mixture of smaller proliferating and larger differentiating cells [Figure 5 (1a)].

Population Doubling

The FTT CEC cells have a pdl time of 24 hrs. The primary WT CEC (P2) cells have a pdl time of 48 hrs, while the hTERT CEC cells have a pdl time of 36 hrs [Figure 5(2))].

Protein Expression

The FTT CEC cells express all the 14-3-3 isoforms [Figure 5(3)] and also all the epithelial specific keratins detected by AE1, AE3 and AE5 antibodies [Figure 5 (4)]. These cells also express cell cycle associated proteins such as p53, telomerase and p63 [Figure 5 (5) and Table 5 (1)] and proliferation markers such as ki67 and PCNA [Figure 5 (5)].

Karyotyping

Karyotyping analysis revealed the presence of a diploid number of chromosomes [Figure 5 (6)]. The number of cells counted was 20.

Plating Efficiency (PE)

FTT CEC have a high plating efficiency of 80% while the WT CEC and hTERT CEC have a PE of 50-60% [Figure 5 (7)].

Tumorogenicity

Colony forming assay was done to determine colony forming efficiency (CFE). Primary WT CEC cells at early passage form colonies as they contain a heterogeneous population of proliferating cells (some stem cells and early progenitors). Less that 10% of WT CEC and 50—60% of hTERT CEC have colony forming capability [Figure 5 (8)]. More than 85% of the FTT CEC cells have colony forming capability [Figure 5 (8)].

In an anchorage in-dependent growth assay, the FTT CEC and the hTERT CEC cells do not form colonies. WT CEC cells were used as a negative control and MCF-7 cells were used as a positive control where 90% of the cells form colonies. An MTT stain also reveals that the cells (FTT CEC, hTERT CEC and WT CEC) were not viable under anchorage independent conditions [Figure 5 (9)].

Telomerase activity

Indirect immunofluorescence reveals the presence of telomerase- the protein [Figure 5 (4)] while TRAP assay indicates telomerase activity in the FTT CEC cells. The hTERT CEC cells were used as a positive control and the WT CEC cells (P5-large number of cells committed to differentiation) were used as a negative control [Figure 5 (10)].

Differentiation

The FTT CEC cells when subject to differentiation conditions such as the presence of serum (10%) in medium undergo differentiation as shown by change in cell morphology [Figure 5 (11)]. This is also supported by the expression of involucrin in differentiated cells and differentiated "floaters" [Figure 5 (12) and 5 (13)] which are detached differentiated cells. There is no involucrin expression in proliferating FTT CEC culture [Figure 5 (12) and 5 (14)]. A culture of WT CEC under normal conditions is a heterogeneous culture containing proliferating cells and differentiating cells which express involucrin. Involucrin is an accepted a differentiation marker that increases under differentiation conditions [Figure 5 (12)]. Differentiation may also be monitored by the expression of cytokeratins. Cytokeratin 14 is highly expressed under proliferating

conditions while there is no cytokeratin 12 expression. When subject to differentiation conditions cytokeratin 14 expression decreases and cytokeratin 12 expression increases [Figure 5 (15)].

Over-expression of 14-3-3 σ

Over-expression of 14-3-3σ in the FTT CEC cells by transfection with the MGC-5018 (contains full length cDNA for 14-3-3σ) plasmid, induces involucrin expression. FTT CEC cells under differentiation conditions were used as a positive control while the FTT CEC cells under proliferating conditions were used as a negative control for this experiment [Figure 6 (16)].

DNA Damage

In response to adriamycin there is no increase in p53 and p21 in FTT CEC cells [Figure 5 (17)]. A cytotoxicity assay was used to determine that adriamycin was toxic to FTT CEC [Figure 5 (18)]. This is confirmed by nuclear fragmentation in FTT CEC when they are exposed to adriamycin for 24 hrs [Figure 5 (19)].

Conclusions

The FTT cells that were generated as a result of 14-3-3σ down-regulation were characterized in order to determine the presence of any abnormalities and differences when they were compared with the WT CEC. These cells are very similar to the WT cells in expression of cell cycle proteins, cytoskeletal proteins and proliferation markers. The one noticeable difference is the absence of p16 in these cells, which the WT cells express

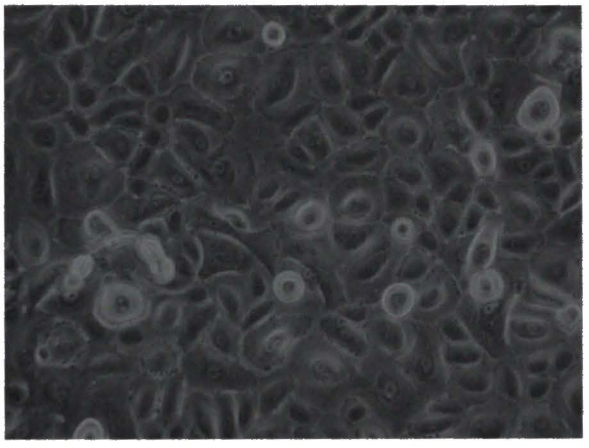
p16. This could be due to the fact that the FTT cells are highly proliferative and p16, a marker of differentiation may not be expressed.

Since they are inherently highly proliferative FTT CEC have a higher pdl than the WT CEC cells. Population doubling time of WT CEC cells also tends to vary depending on donor age, gender and also cell culture conditions. The FTT cells are diploid indicating that no chromosomal abnormalities were introduced by 14-3-3σ down regulation. These cells also have a higher plating efficiency and CFE indicating that they are highly proliferative in nature. They however do not divide in anchorage independent fashion as tumor cells indicating that they are not tumorogenic.

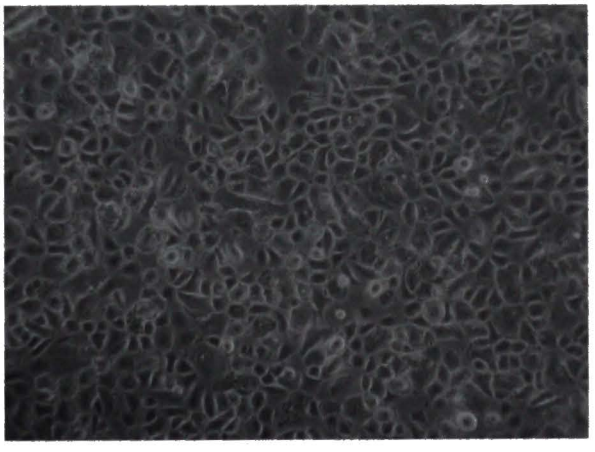
In response to DNA damage, these cells do not express higher levels of p53 or p21. They undergo apoptosis in 48 to 72 hrs when exposed to adriamycin which suggests that these cells are sensitive to DNA damage, and adriamycin is toxic to these cells. This information may be utilized in development of drugs to target cancers in which 14-3-3σ is down-regulated.

Figure 5 (1): Phase Contrast micrographs show the cobblestone morphology of WT CEC, hTERT CEC and FTT CEC cells. [Magnification -20X]

WT CEC



hTERT CEC



FTT CEC

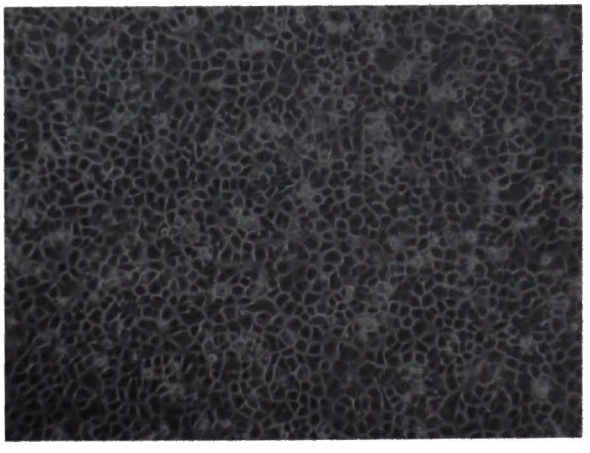


Figure 5 (2): FTT CEC population doubling time was compared to that of WT CEC and hTERT CEC cells. The FTT CEC cells double in 24 hrs.

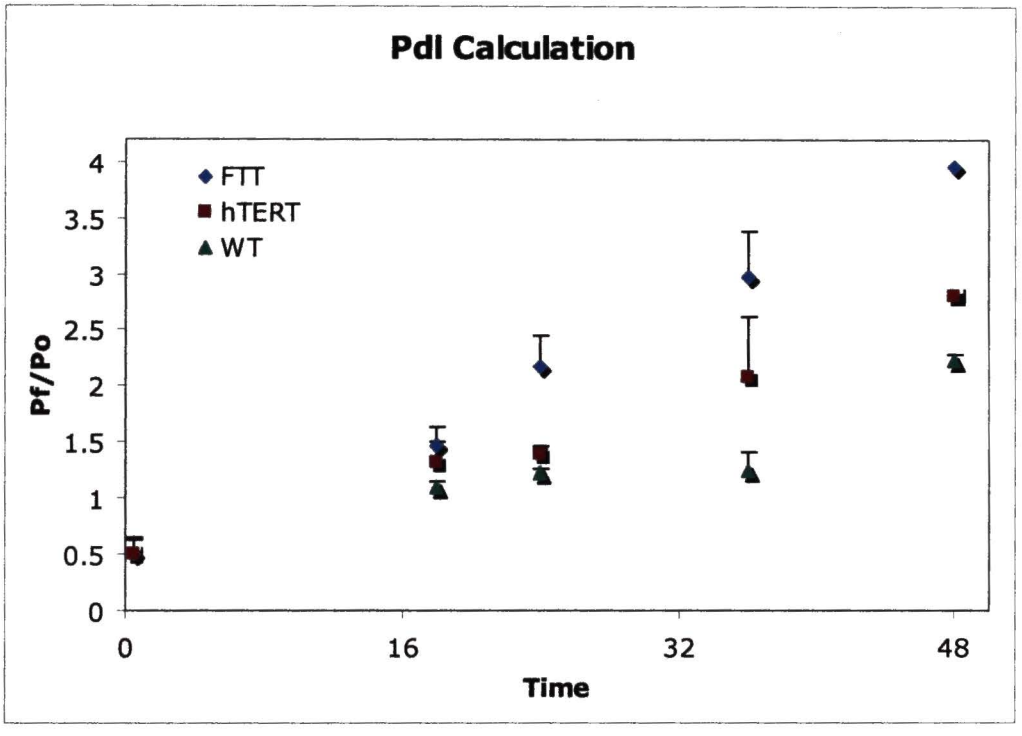
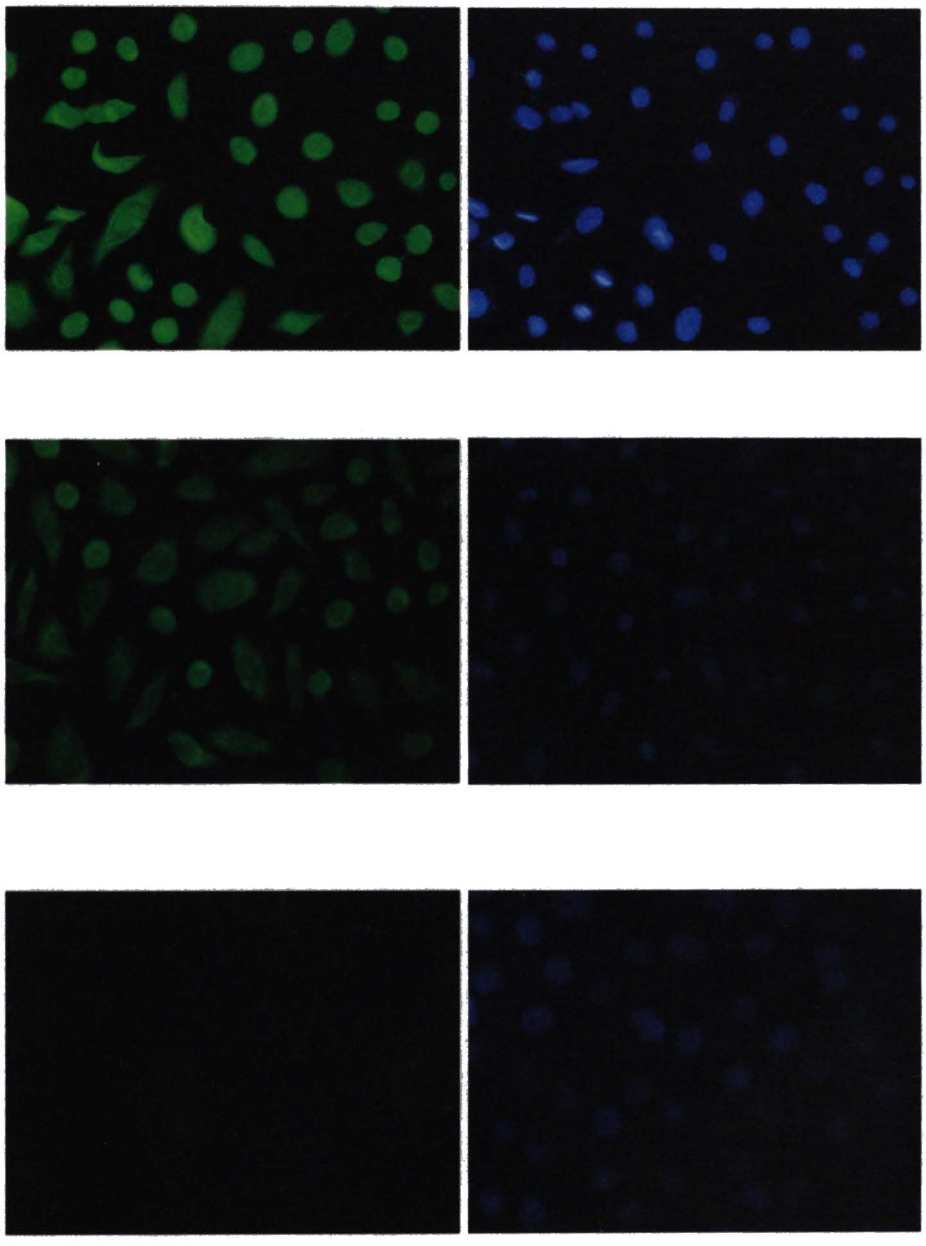


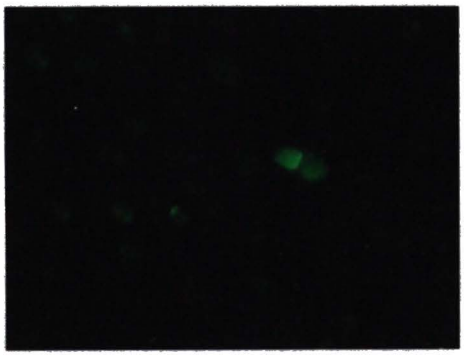
Figure 5 (3): Expression of 14-3-3 isoforms in FTT CEC cells as determined by Indirect Immunofluorescence.



14-3-3η

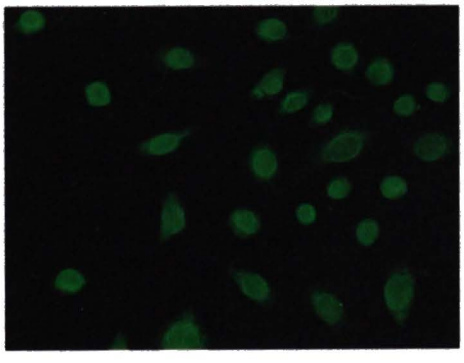
Dapi

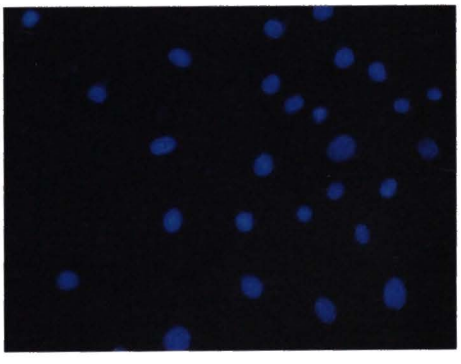
14-3-3ζ



Dapi

14-3-3θ





Dapi

Figure 5 (4): Indirect Immunofluorescence shows keratin expression in FTT CEC. AE1-Acidic (Type I) keratins (green), AE3-Basic (Type II) Keratins (green), AE5-Keratin 3 (Corneal Differentiation marker-green)

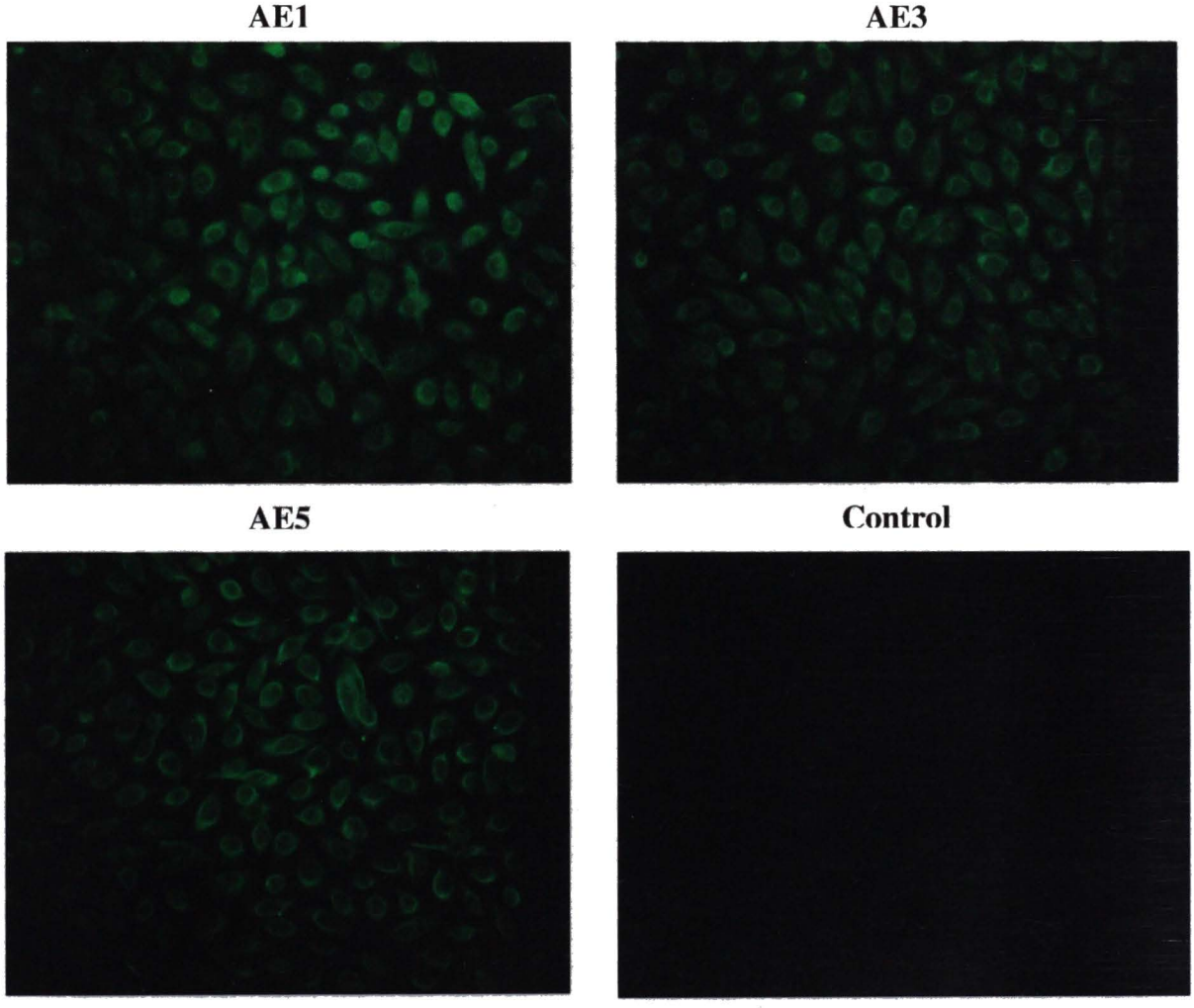
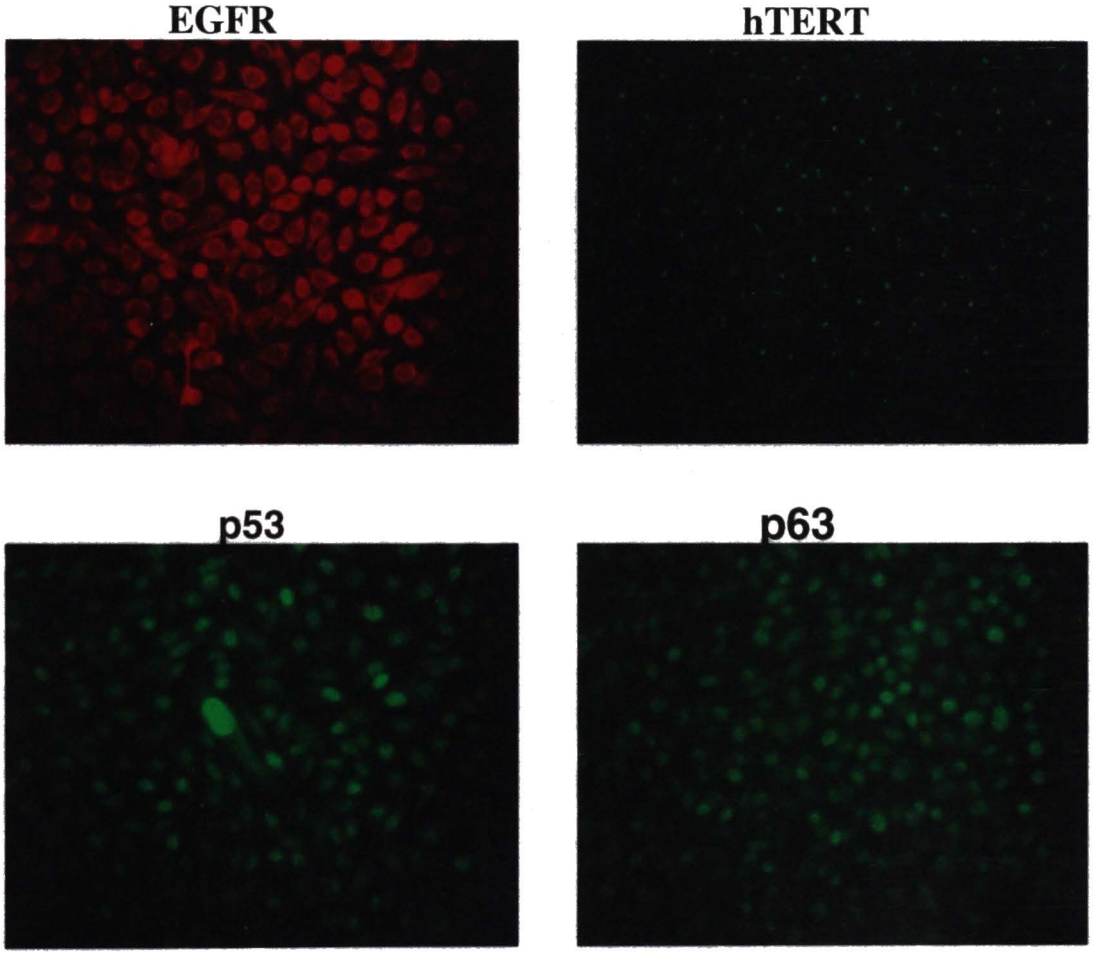


Figure 5 (5): Expression of cell cycle related proteins p63, p53, PCNA, ki67, hTERT and EGFR in FTT cells as determined by Indirect Immunofluorescence.



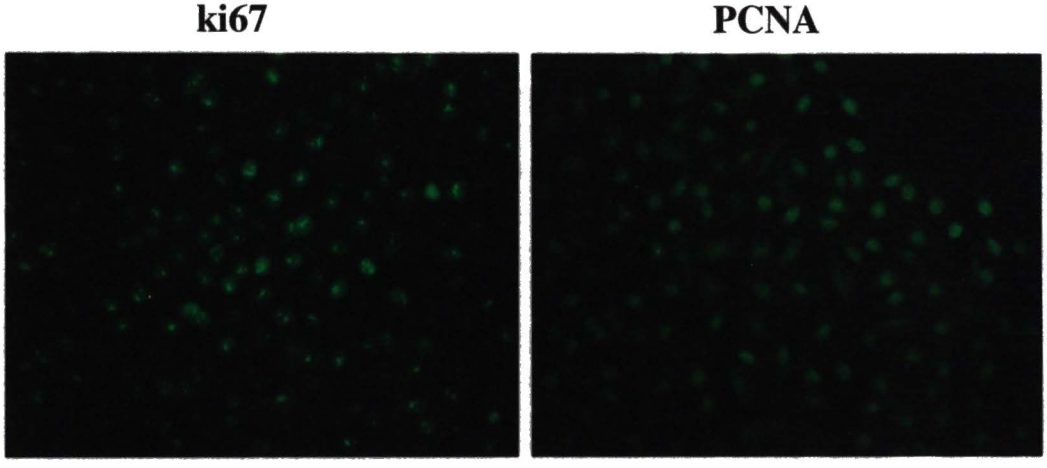

ki67
PCNA

Table 5 (1): Comprehensive summary of comparison of protein expression in WT CEC and hTERT CEC as determined by Indirect Immunofluorescence and Western Blot analysis.

Protein	WT CEC	hTERT CEC	FTT CEC
AE1	+	+	+
AE3	+	+	+
AE5	++	+	+
p16	++	+	-
p21	-	-	-
p53	+	+	+
p63	+	+	+
Ki67	+	+	+
PCNA	+	+	+

Expression levels as determined by western blot analysis. Densitometric value (after normalization) of 0.5 or greater:"++" (very high expression), value less than 0.5: "+" (positively expressed) and value of 0: "-" (negative-no expression).

Protein	WT CEC	hTERT CEC	FTT CEC
K18	+	+	+
K19	+	+	+
14-3-3σ	++	+	+
14-3-3ε	+	+	+
14-3-3ζ	+	+	+
14-3-3η	+	+	+
14-3-3θ	+	++	+
14-3-3β	+	+	+
14-3-3γ	+	+	+

Expression levels as determined by western blot analysis. Densitometric value (after normalization) of 0.5 or greater:"++" (very high expression), value less than 0.5: "+" (positively expressed) and value of 0: "-" (negative-no expression).

Figure 5 (6): Karyotyping of the FTT cells shows the presence of diploid number (46) of chromosomes. DAPI is used to stain chromosomes.

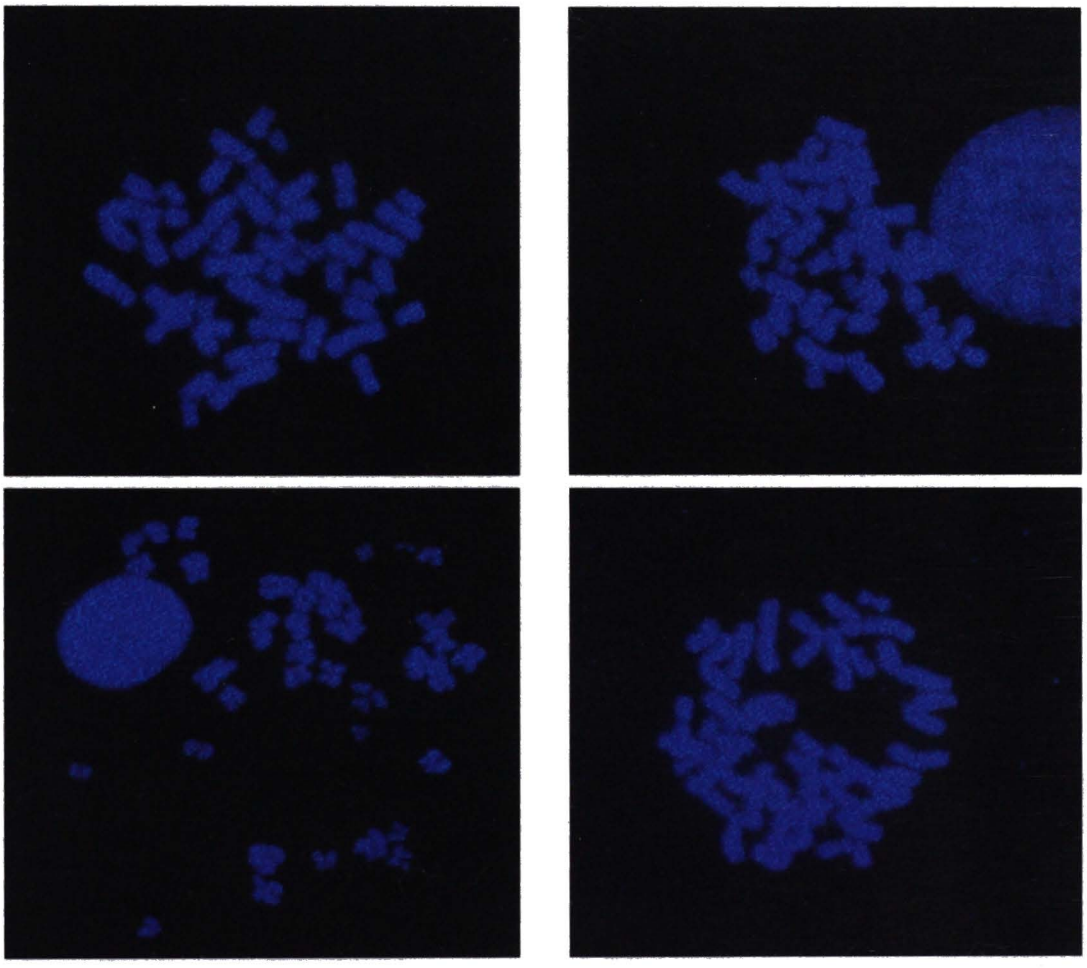


Figure 5 (7): Plating efficiency of the FTT cells was compared to WT CEC and hTERT CEC. There is no significant difference ($p>0.05$ using pairwise t-test) between the plating efficiency of WT CEC and hTERT CEC. However, there is significant difference ($p<0.05$ using pairwise t-test) between the plating efficiency of WT CEC and FTT CEC.

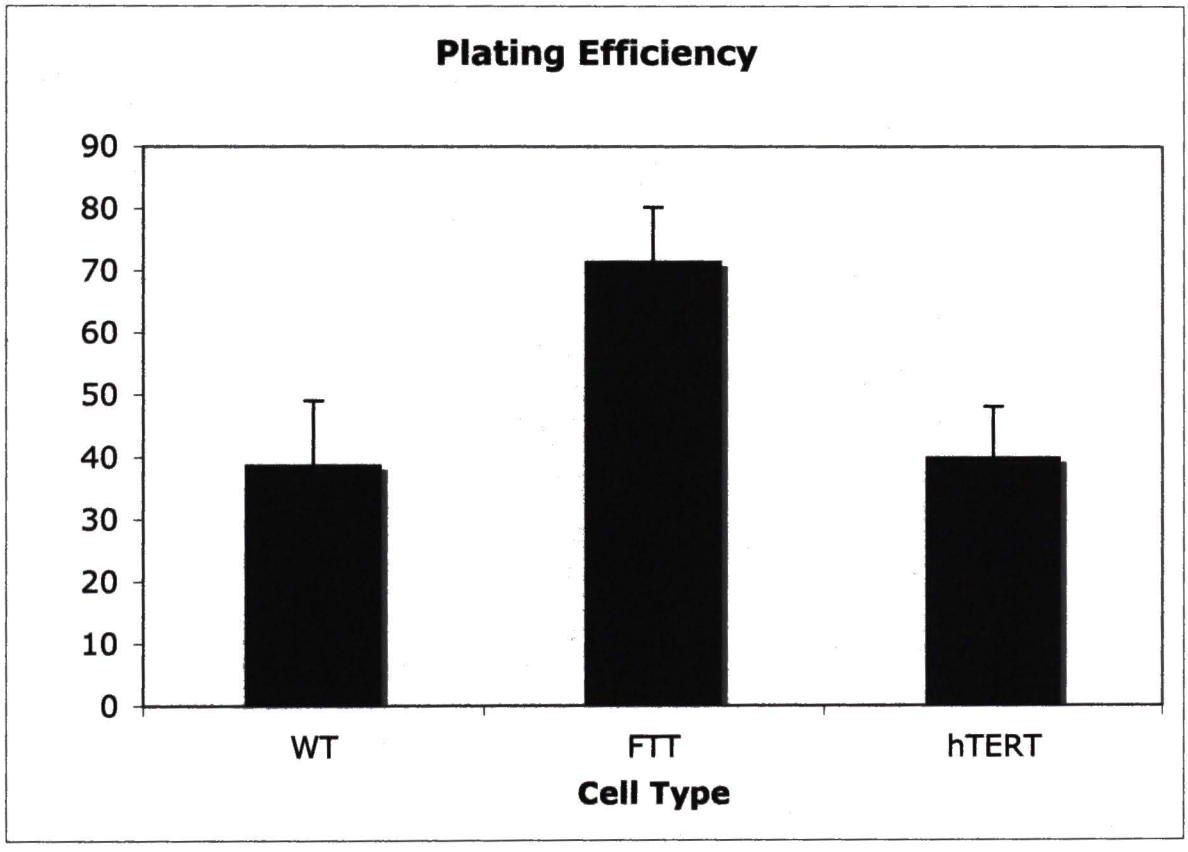
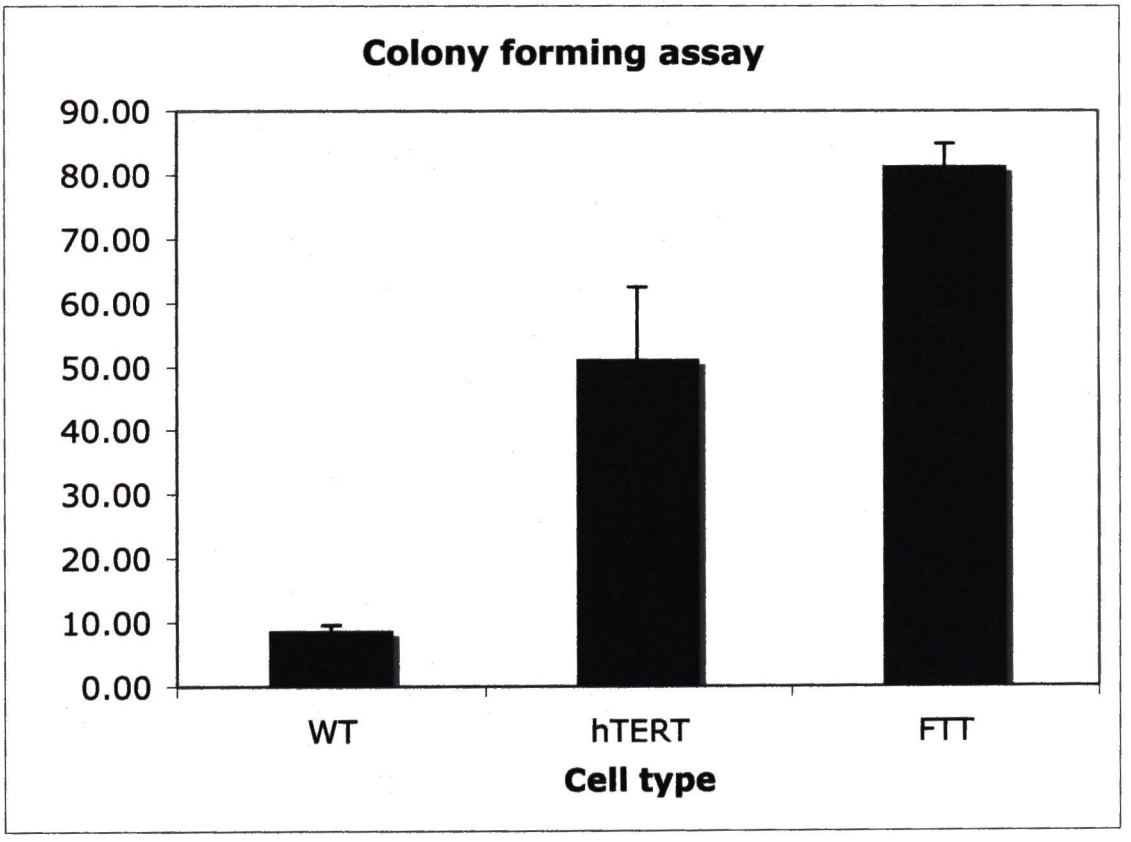
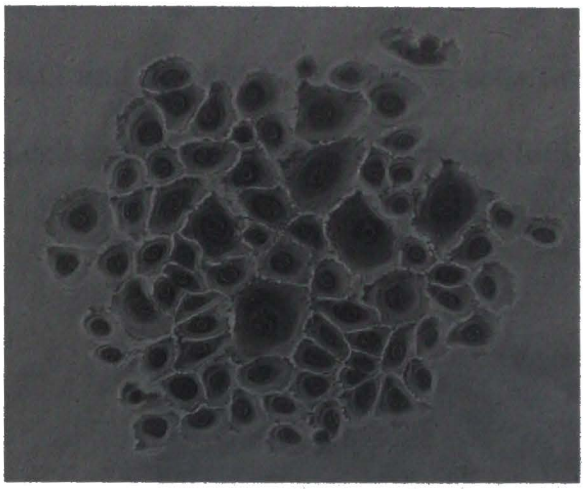


Figure 5 (8): Colony Forming Assay to determine the percentage of cells that formed colonies. (a) Colony forming efficiency of the FTT cells was compared to WT CEC (P2) and hTERT CEC. (b) Colonies were stained with Sulfrorhodamine B for visualization. There is significant difference ($p<0.05$ using pairwise t-test) between the colony forming efficiency of the WT CEC in comparison to both the FTT CEC and hTERT CEC cells.

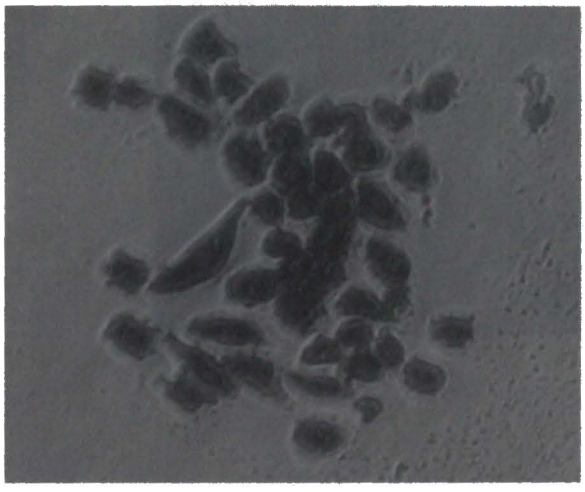
(a)



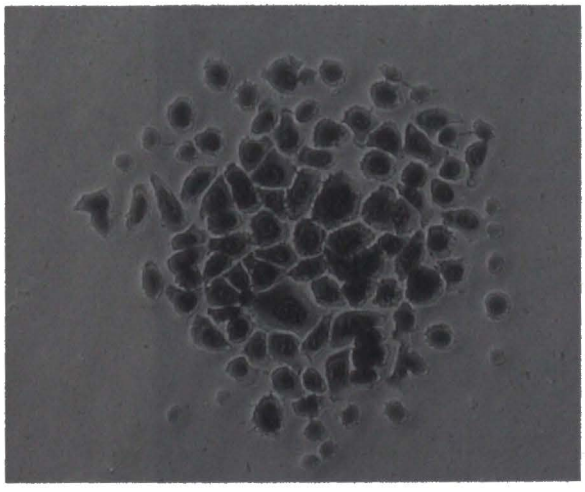
(b)



WT CEC



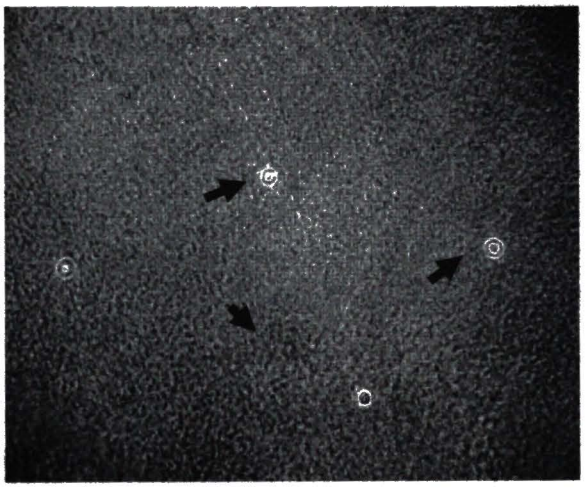
hTERT CEC



FTT CEC

Figure 5 (9): Anchorage independent growth assay. (a) WT CEC , (b) hTERT CEC, (c) FTT CEC and (d) MCF-7. [MCF-7 cells are the positive control shows the presence of cell growth in the form of colonies indicated by arrows.]

(a)



(b)

(c)

(d)

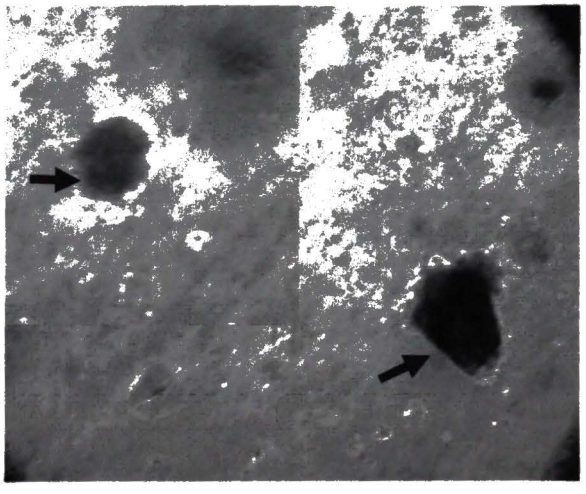
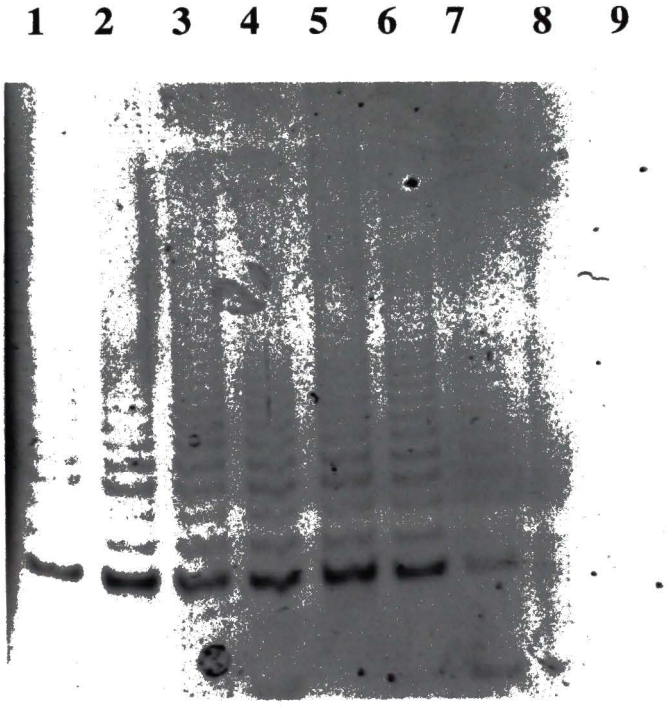


Figure 5 (10): TRAP assay was used to determine the telomerase activity in the FTT cells. hTERT CEC cells were used as a positive control and WT CEC were used as a negative control.



1&2	Positive control
3	hTERT CEC
4	hTERT CEC
5	FTT CEC
6	FTT CEC
7	WT CEC (P1)
8	WT CEC (P4)
9	Negative Control

Figure 5 (11): Changes in Morphology of FTT CEC cells when exposed to differentiation conditions. (a) Normal culture conditions control), (b) 24 hrs in differentiation medium, (c) 48hrs in differentiation medium and (d) 5 days in differentiation medium. [Magnification-20X]

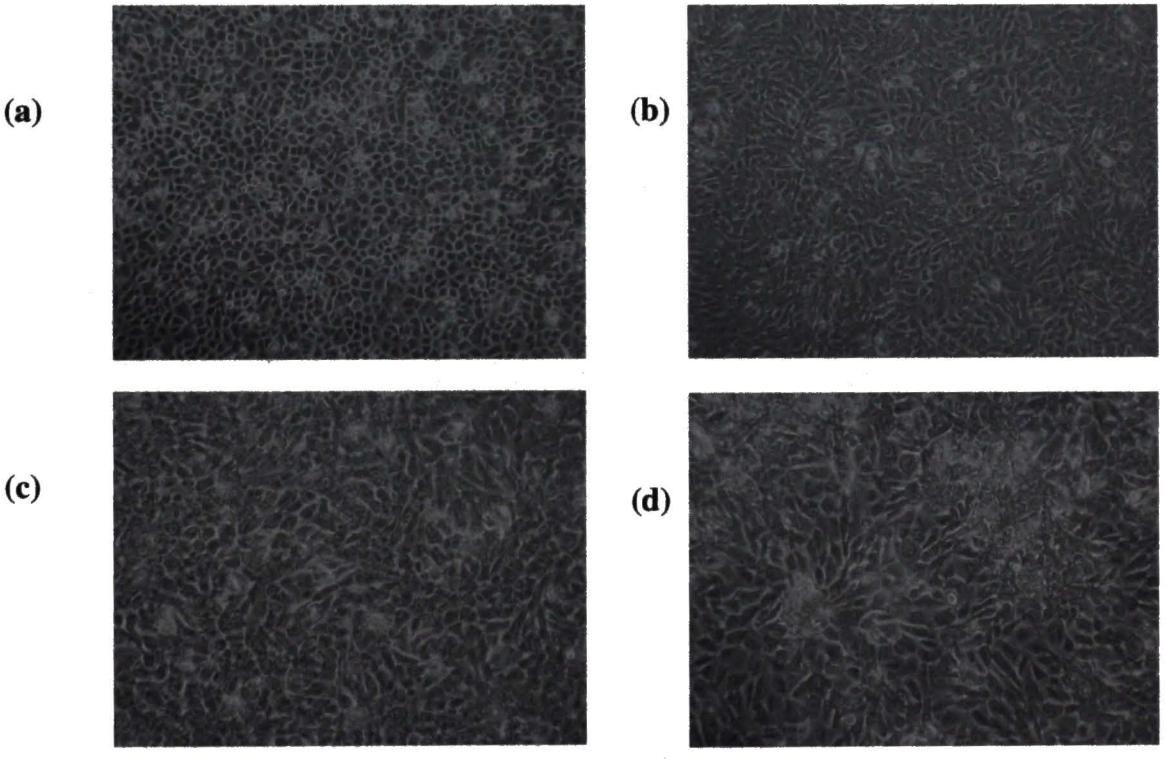
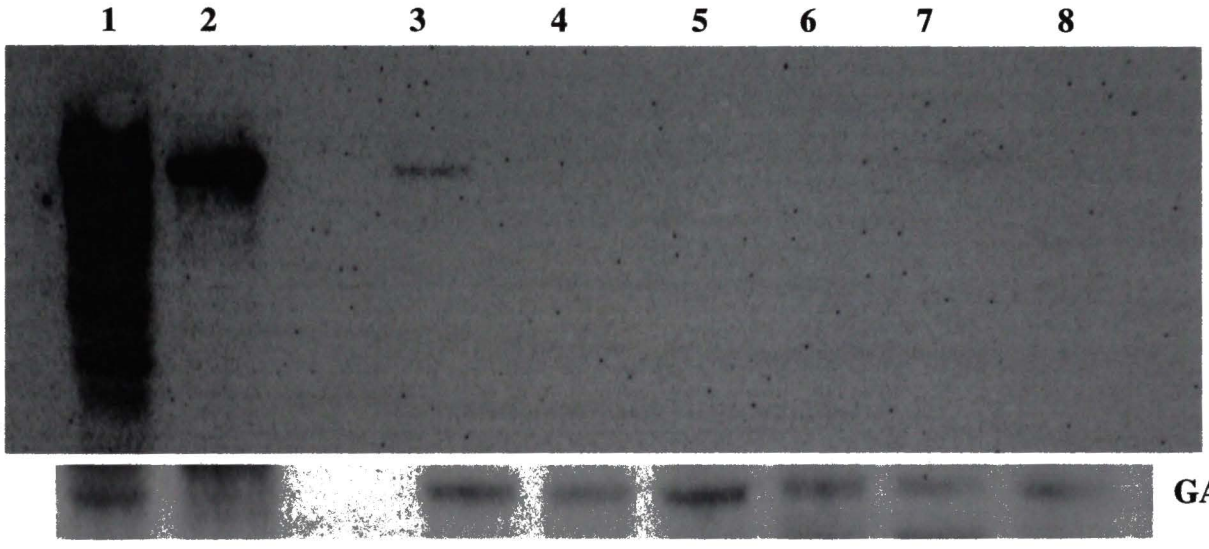


Figure 5 (12): Western Blot analysis to showing the expression of involucrin in FTT CEC cells under normal and differentiation conditions. GAPDH was used as a loading control.`



1. WT CEC Differentiation conditions(10% serum)

2. WT CEC Normal Culture conditions(Epilife)

3. FTT CEC Differentiation conditions(10% serum)

4. FTT CEC Differentiation conditions(5% serum)

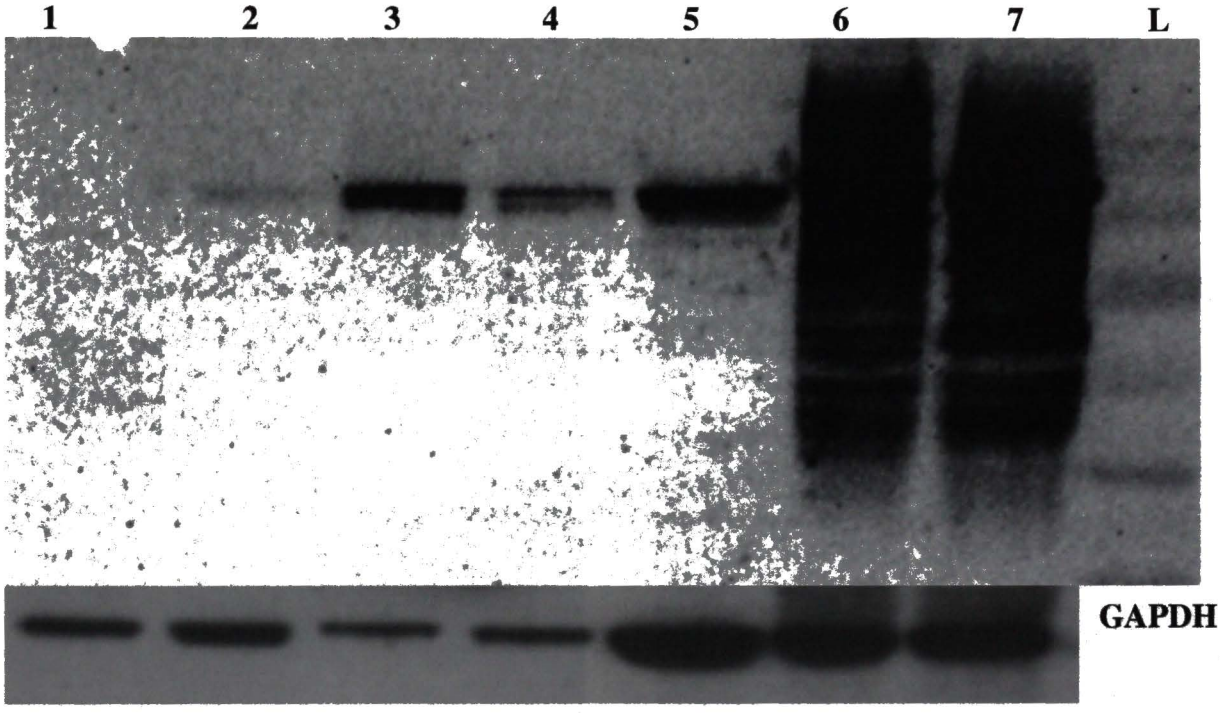
5. FTT CEC Differentiation conditions(1% serum)

6. FTT CEC Differentiation conditions (high calcium)

7. FTT CEC Normal Culture conditions

8. FTT CEC Control(-ve control)

Figure 5 (13): Western Blot analysis to determine the expression of involucrin in detached cells "floaters" under normal and differentiation conditions.



1. hTERT CEC floaters(differentiation conditions-10%FBS)

2. SV-40 floaters (differentiation conditions-10% FBS)

3. E6/E7 floaters (differentiation conditions-10% FBS)

4. FTT CEC floaters (differentiation conditions-10%FBS)

5. FTT CEC floaters (normal culture conditions-Epilife)

6. WT CEC floaters (differentiation conditions-10%FBS)

7. WT CEC floaters (normal culture conditions-Epilife)

Figure 5 (14): Indirect Immunofluorescence to determine the expression of involucrin in FTT CEC cells under (a) normal and (b) differentiation conditions (10 days, 10% serum).

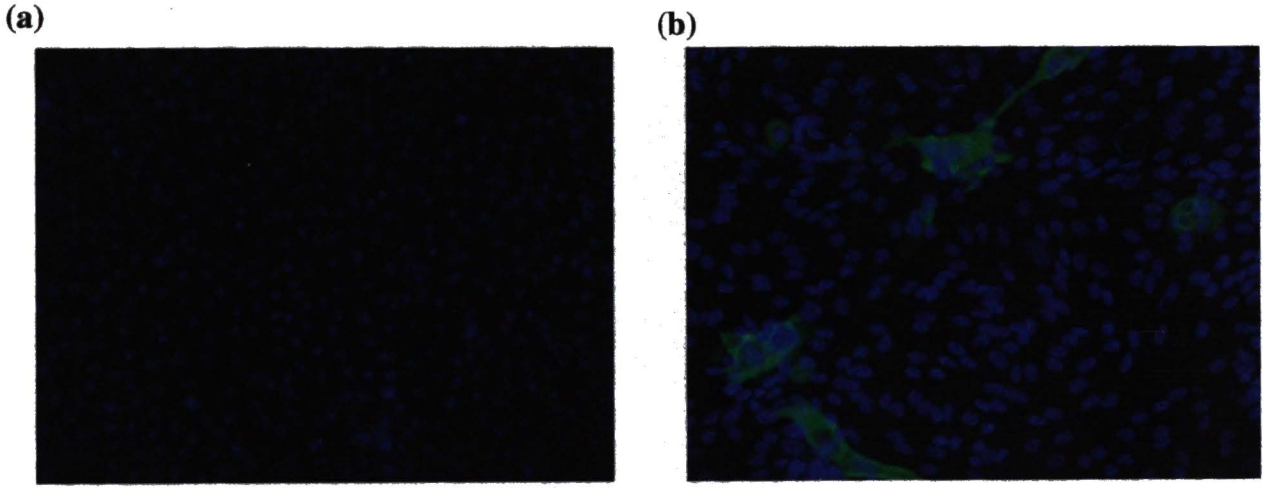


Figure 5 (15): Indirect Immunofluorescence to determine the expression of cytokeratin 14 (green) and cytokeratin 12 (red) in FTT CEC cells under (a)normal and (b) differentiation conditions (10 days, 10% serum).

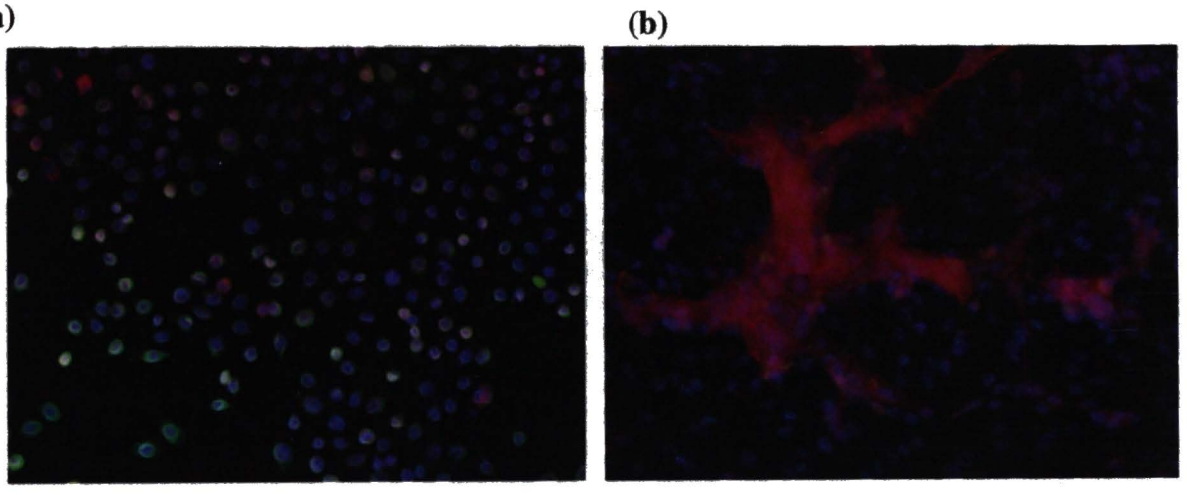
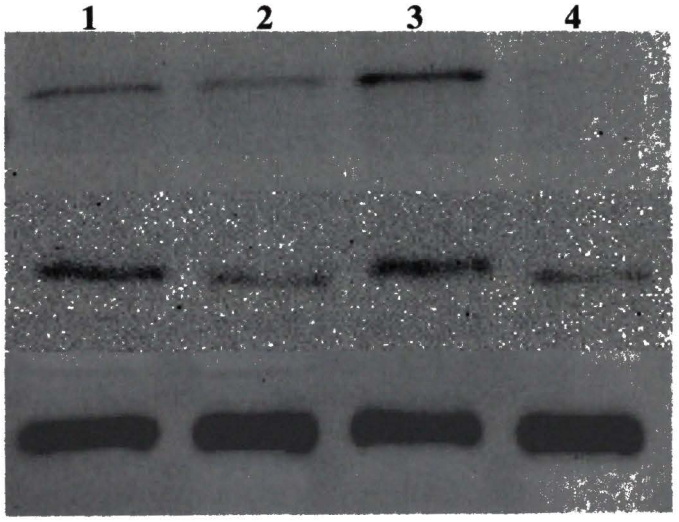


Figure 5 (16): Western Blot analysis to determine the expression of involucrin and 14-3-3σ in FTT CEC cells. GAPDH was used as a loading control.



1. **FTT CEC in 10%FBS (10 days)**
2. **FTT CEC floaters in 10%FBS (10 days)**
3. **Over-expression of 14-3-3σ in FTT CEC (MGC-5018)**
4. **FTT CEC Control (proliferating culture)**

Figure 5 (17): Western Blot analysis to determine the expression of p53, p21 and 14-3-3σ following DNA damage. GAPDH was used as a loading control.

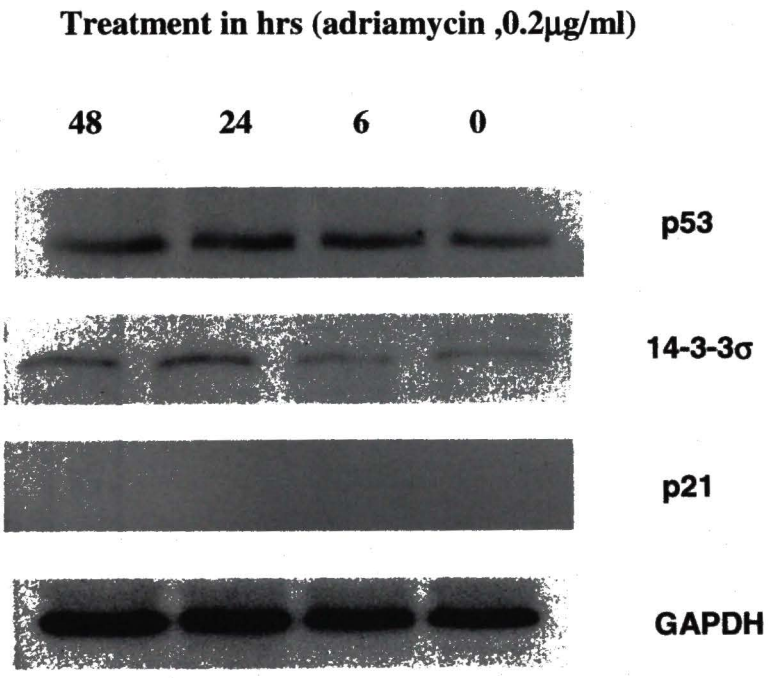


Figure 5 (18): The toxic effects of adriamycin on FTT CEC cells. [Absorbance is proportional to protein concentration which indicates cell viability].

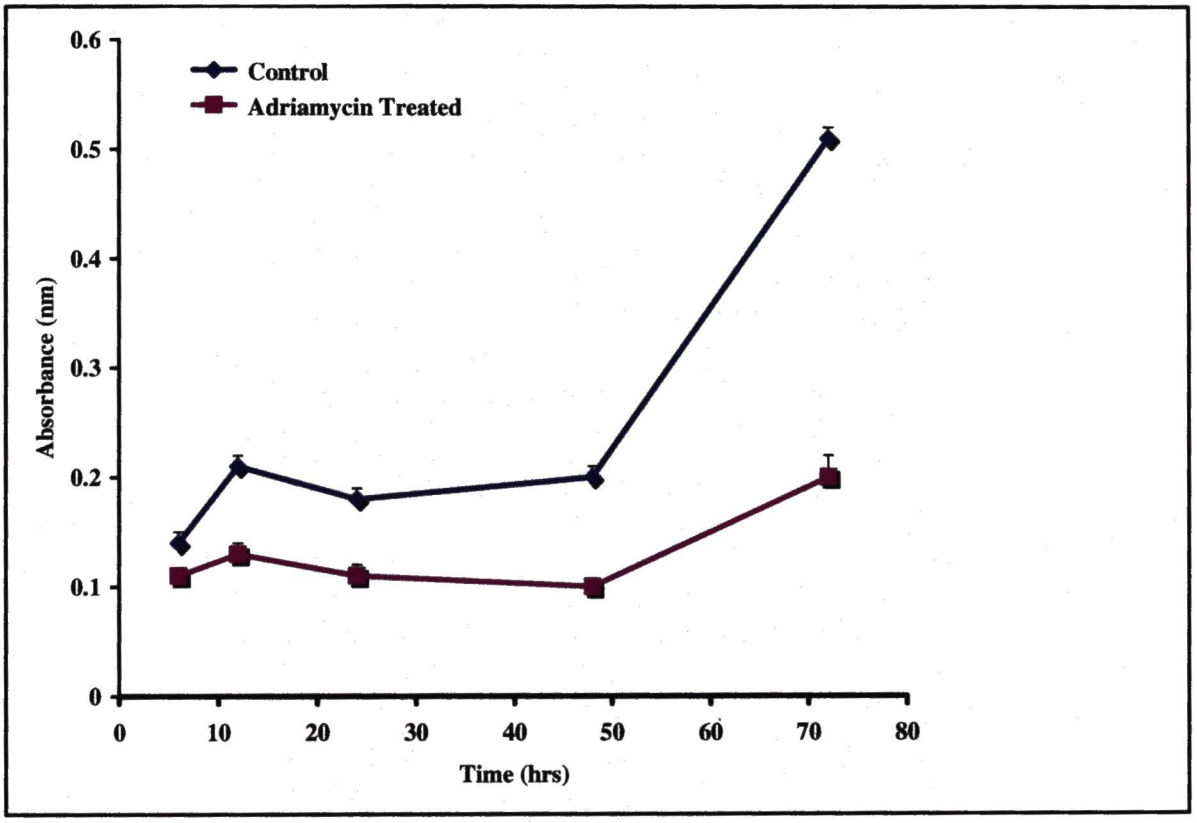
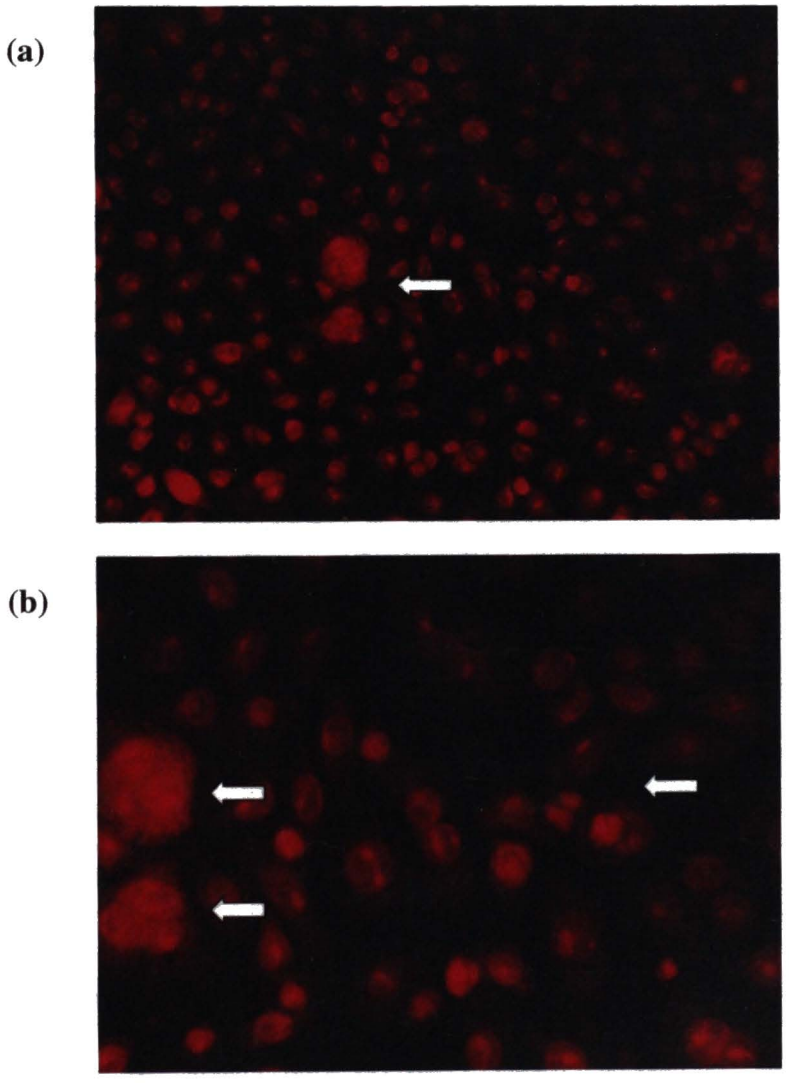


Figure 5 (19): Propidium Iodide (PI) stain of FTT CEC cells treated with 0.2μg/ml adriamycin for 24 hrs to show nuclear fragmentation (indicated by white arrows). (a) 20X magnification and (b) 40X magnification.



CHAPTER VI

EFFECTS OF 14-3-3σ DOWN-REGULATION *IN VIVO*

14-3-3σ, also known as HME1 or *Stratifin* has received particular attention recently because of its expression specificity in stratified epithelia [25, 26, 111]. In addition to its involvement in the regulation of expression of cell cycle regulating proteins (p53, p63 and BRAC1) and induction of G2/M cell cycle [38, 40, 162](interacting with Bax), 14-3-3σ also plays a role in initiation of apoptosis [26, 58]. 14-3-3σ protein levels are significantly reduced or are negligible in a number of transformed cell lines and primary tumors of epithelial cell origin [49, 59]. The fact that the suppression of 14-3-3σ expression alone immortalizes primary keratinocytes [25], lead to the proposed tumor suppressor function, and the view that loss of the expression may be crucial event in the progression of cancer.

Cell cycle regulation, apoptosis and differentiation are key processes in embryonic development and homeostasis in adult organisms. Current strategies utilize the gene knock out models to obtain information about proteins that may control these and other regulated processes. Although stratifin knock-out has yet to be achieved two mutations, the pupoid fetus (Pf)[80, 81] and the repeated epilation (ER)[92, 93], involve alterations in its expression. The ER mouse was obtained from gamma irradiation of a male mouse that resulted in a single A-T pair insertion mutation at nucleotide 624 in the open reading frame (ORF) of the SFN gene.[92]. Subsequent frame-shift truncated 14-3-3σ protein at the C-terminus by 40 amino acids. The Er/Er mice die at birth because the fused oral cavity results in respiratory distress; their skin is thickened due to abnormal

proliferation and lack of differentiation of the keratinocytes, and they also have incomplete limb and tail development. All these abnormalities indicate serious interference with the differentiation events during organogenesis of several organs. On the other hand, the Er/+ heterozygous mice survive but have abnormal hair loss, due to one-week extension of the hair cycle, and develop spontaneous skin tumors after 6 months of age. Er/Er but particularly Er/+ mice are a very useful model for studies of the role of stratifin in differentiation and cancer development.

The "skin thickening" in Er/Er mice was shown to be due to impaired differentiation. The epidermis was composed of highly proliferative keratinocytes expressing K14 in all layers (lack of differentiation). The expression of loricrin (epidermal differentiation marker) was absent in the epidermis of Er/Er mice but present in the superficial differentiated epidermal layers of the normal animals. Keratinocytes isolated from the Er/Er mouse skin have a longer *in vitro* lifespan then the cells derived from the skin of normal mice, but over-expression of exogenous stratifin rescues the Er/Er cells from hyperpoliferation[92, 93]. Similarly, the epidermis of the Er/Er mice when transplanted onto normal mice, was shown to "normalized" and undergo differentiation[80, 88] It is interesting to speculate on the identity of extracellular factors present in the skin of normal mice that restore differentiation of Er/Er epidermis and what the mechanism of this rescue might be. Although 14-3-3 proteins were first discovered in the central nervous system (CNS) and their presence in the cerebrospinal fluid (CSF) in Creutzfeldt-Jakob disease (CJD), multiple sclerosis (MS) and prion diseases has been observed ,the extracellular functions have not been determined. It has also been observed that stratifin

is present in keratinocyte conditioned medium [103, 107] The secreted protein (or recombinant protein or bacterial origin) increases expression of MMPs by dermal fibroblasts and thus appears to participate in epidermal-mesenchymal communication. The mechanism of this interaction is still obscure, since 14-3-3 proteins lack ER signaling peptides and are not subject to the classical secretary pathway and the re-entry mechanism into target cells is not known.

Rationale

The observations derived from studies of Er/Er and Er/+ models to date indicate that 50% reduction in the expression of stratifin during development is sufficient to alter skin and hair differentiation and induce tumor formation later in life (6 months and older). The effect of the Er mutation on the development of the cornea as well as other non-keratinized stratified epithelia, other than that of the oral cavity[88], have not been studied. Having previously shown that the expression of stratifin is confined to human corneal and conjunctival epithelium and primary human cells[144] we here describe our findings of the age related expression of stratifin in the corneas of Er/+ mouse when compared with age and gender matched controls.

Materials and Methods

Tissue Preparation and Immunohistochemistry

After euthanization, the eyes were carefully enucleated from the Er/+ mice and age and gender matched control animals, and then washed in PBS and fixed in neutral formalin (4%, room temperature for 48 hrs), washed and incubated in PBS overnight at 4°C. The whole globes were then dehydrated by incubation in series of ethanols (70%, 85%, 95%

100%) and xylenes (50% xylene:ethanol, 100% xylene) and then prepared for embedding by incubation in paraffin (50%, 100%). Paraffin embedded whole globes were cut in half and sectioned. Paraffin tissue sections (8μ) were deparaffinized and then washed in distilled water (3x) and blocked (overnight at 4°C) in PBS containing 1% BSA and 1% horse serum. The sections were treated with primary antibodies to 14-3-3 proteins β, γ, ε, σ, ζ, θ and η, (Santa Cruz) for 24 hrs at 4°C and washed thoroughly with PBS (3x10min) containing Tween 20 (0.1%). The sections were then incubated with secondary antibodies (Alexa Fluor 594, Molecular Probes) (1.5 hrs, RT) and rinsed in PBS containing Tween 20 (0.1%, 3x10min). The sections were also stained with DAPI (4',6-diamidino-2-phenylindole). Finally, the specimens were rinsed in PBS (3 x 10 min), distilled water (30 min), and mounted on glass slides (FluorSave™, Calbiochem, La Jolla, CA). Sections were also stained with hemotoxylyn and eosin.

Antibody Information

Listed in Chapter II, Table 2(i) and Chapter III, Table 3(i)

Image Acquisition

Mounted specimens were examined on Olympus AX70 fluorescent microscope using SPOT Twain software.

Results

Expression of 14-3-3 isoforms

All the 14-3-3 isoforms were found to be expressed in corneas of Er/+ (FVB) mice and their gender and age matched controls. Also in analogy to the human cornea, 14-3-3 σ

(Stratifin) is only expressed in the epithelium of both Er/+ and normal mice [Figure 6(1), 6(2) and 6(3)].

Expression of 14-3-3σ in the cornea

The corneas of Er/+ mice mature at the same rate as the corneas of age and gender matched normal control (e.g. single cell layer at 3 days of age, fully stratified at 1 month and 7 months). However, at 3 days, 1 month and 7 months of age expression of full length Stratifin is greatly reduced when compared with age and gender matched normal mice [Figure 6(4)].

Keratin Expression in the corneal epithelium

Expression level of "proliferation indicators", cytokeratins K5/K14, is high in the corneal epithelium of Er/+ mice. In contrast, "differentiation marker" cytokeratin K12 is not expressed in Er/+ mouse corneal epithelium but is present in the epithelium of normal mice. These results support our hypothesis that in analogy with Er/+ mouse skin, the effect of reduced expression of stratifin causes a similar delaying effect on differentiation of the corneal epithelium [Figure 6(5)].

Presence of tumors in the anterior surface of the eye

There appears to be a strain difference in the response to reduction in expression of full-length stratifin protein. Thus, in contrast to the FVB Er/+ mice, the B6 Er/+ mice at about six months of age develop what appear to be "ocular tumors" that interfere with eyelid closure [Figure 6 (6a)]. On preliminary analysis, these "tumors" are not the result of corneal or conjunctival epithelial hyper-proliferation. Instead they are part of the eyelid

and appear to be glandular tissue as shown in the H&E stained sections of the tumor [Figure 6 (6b)].

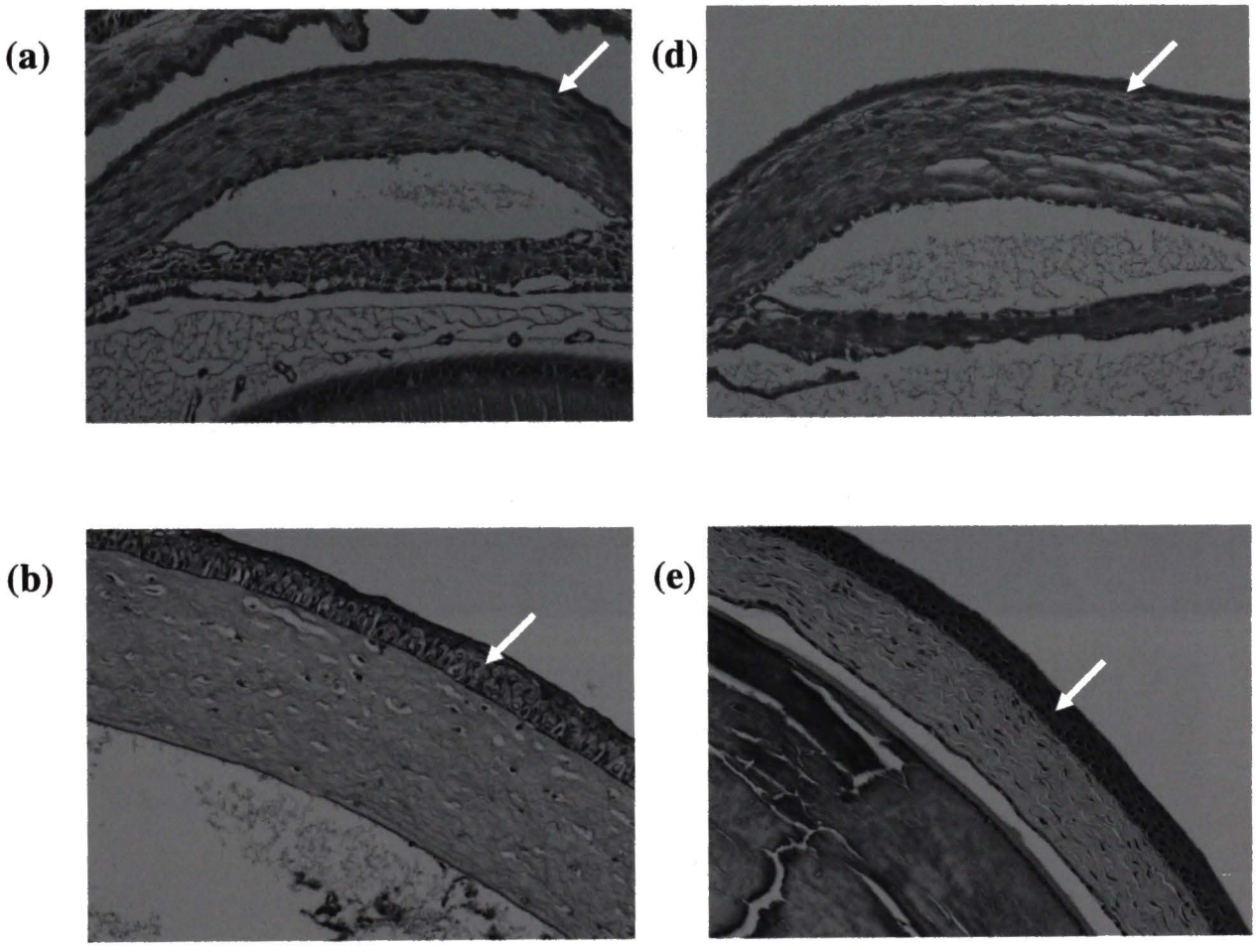
Conclusions

We have shown earlier that 14-3-3σ in directly involved in corneal epithelial cell differentiation. Down-regulation of 14-3-3 σ in WT CEC cells resulted in extension of *in vitro* life span of these cells and also resulted in immortalization of cells by RNAi suppression of 14-3-3σ expression only. Characterization of these cells showed that they are highly proliferative in nature and require much longer exposure to appropriate conditions to differentiate, than their wild type counterparts.

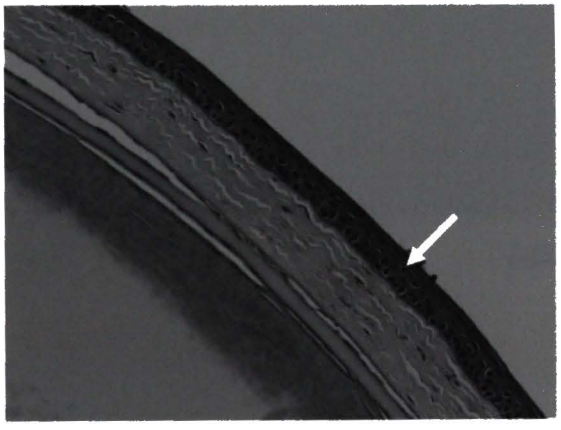
The Er/+ mouse model has lower levels of 14-3-3 σ and it could be expected that this down-regulation might lead to hyperproliferation and epithelial cancers. Skin tumors have been reported in these mice but the occurrence of the other cancers of epithelial origin has been studied. There are also no reports of corneal cancers in humans.

There were no differences in the corneas of the Er/+ mice and their WT controls on visual observation [Figure 6(1)]. Indirect immunofluorescence however, reveals higher levels of expression of keratins that are proliferation markers in the Er/+ mice (FVB strain) [Figure 6(6)]. The overall epithelium however appears to be intact. In the B6 strain of the Er/+ mice there were eye associated tumors which originally appeared to be related to the conjunctiva. Sectioning and H&E staining revealed these tumors in the eye to be of glandular origin [Figure 6(8)]. Hence, the down-regulation of 14-3-3σ in these animals may not be sufficient to cause corneal tumors, but may induce "expression" of auxiliary lachrymal glands.

Figure 6(1): H&E staining of the corneal sections obtained from Er/+ Mice. (a) 3 day, (b) 1 month and (c) 7 Month old Er/+ mice and their corresponding age matched controls (d), (e) and (f).(White arrows indicate the corneal epithelium)



(c)



(f)

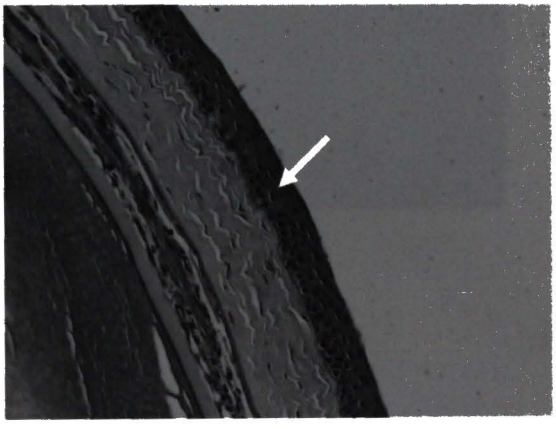
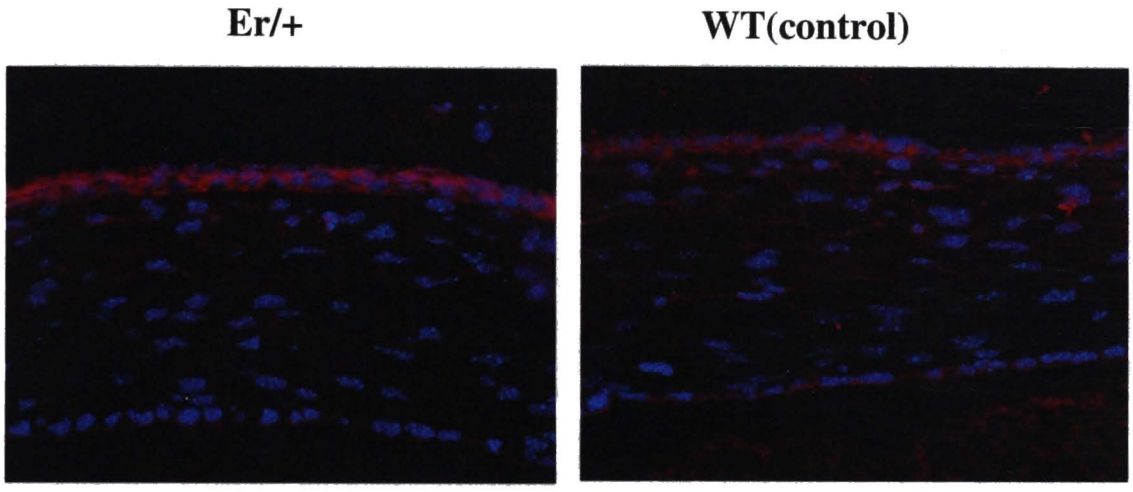
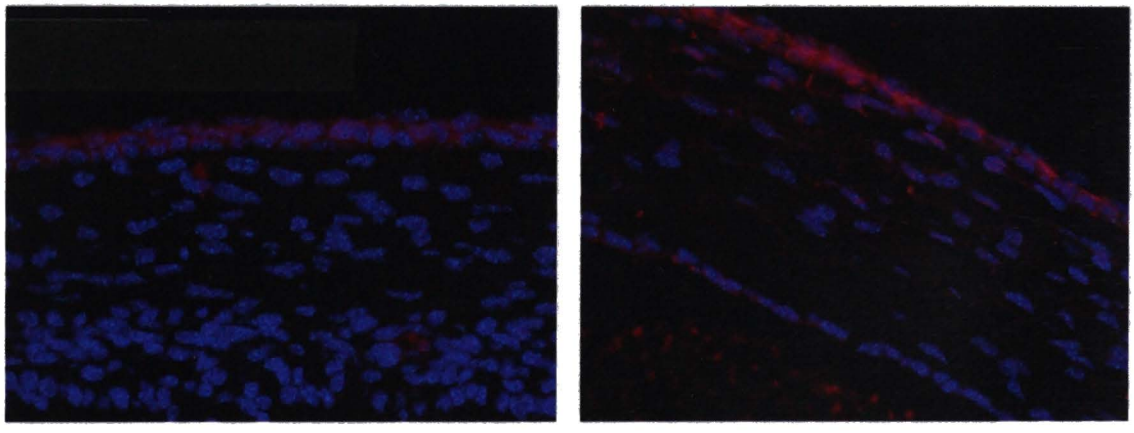


Figure 6(2): Expression of 14-3-3 isoforms in the corneal sections from 3 day old Er/+ mice and their age matched control as determined by Indirect Immunofluorescence.

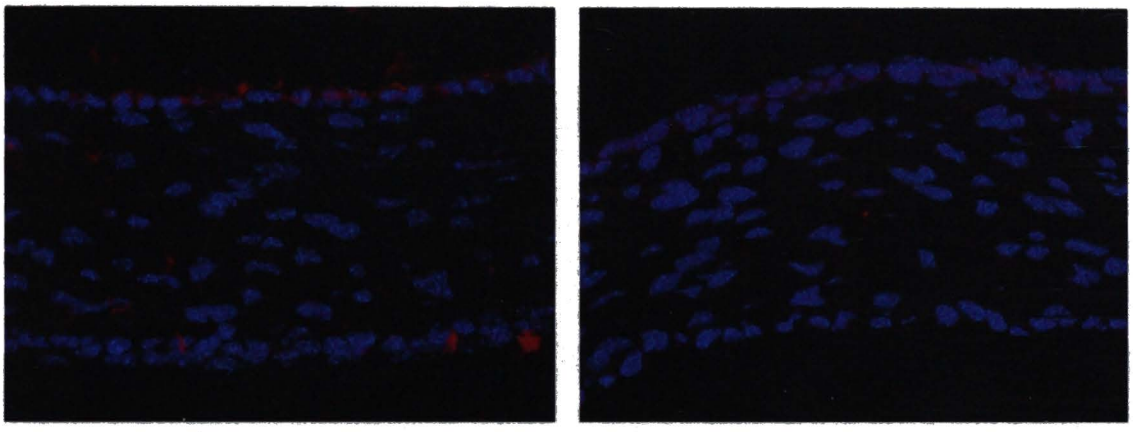


14-3-3β

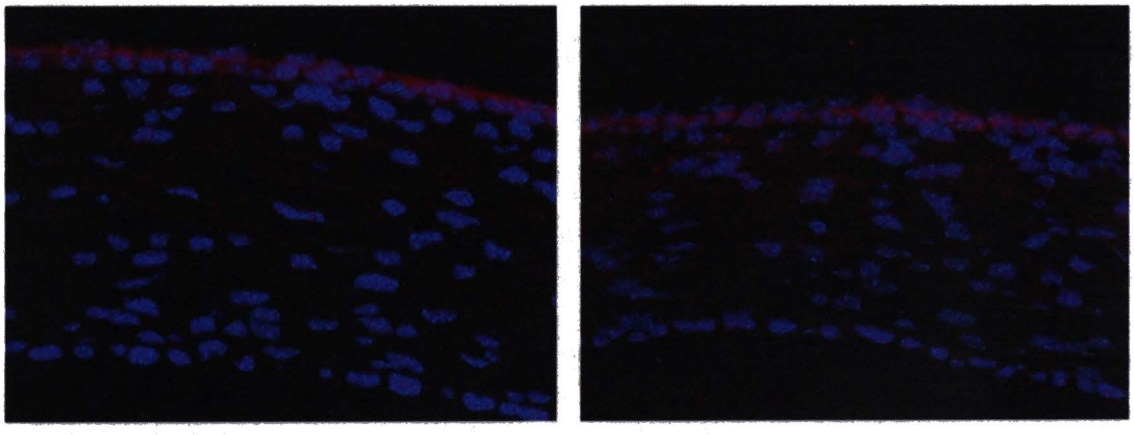


14-3-3ε

Er/+ WT(control)

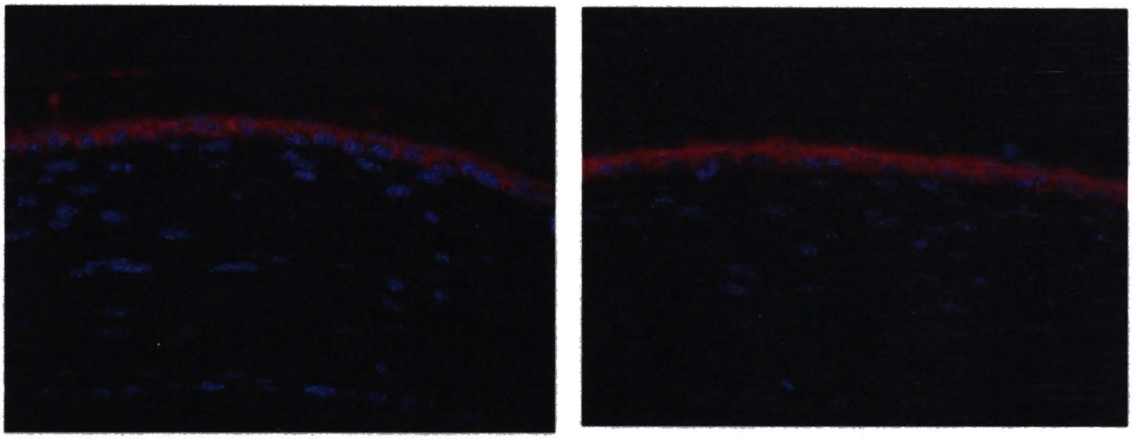


14-3-3η

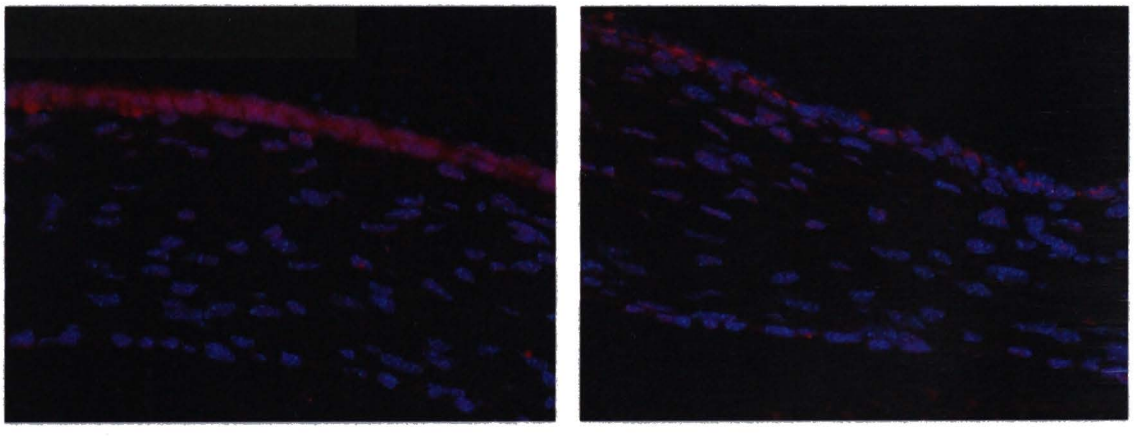


14-3-3γ

Er/+ WT (control)



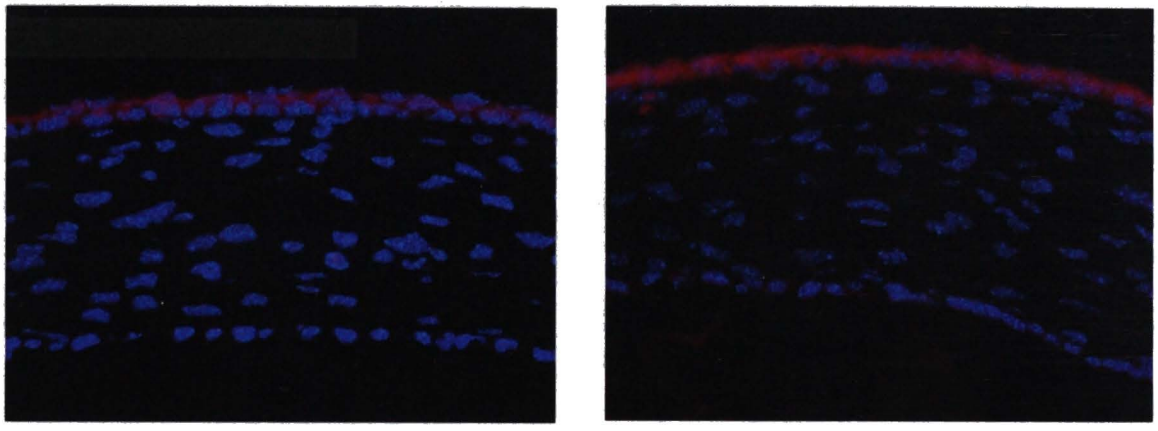
14-3-3σ



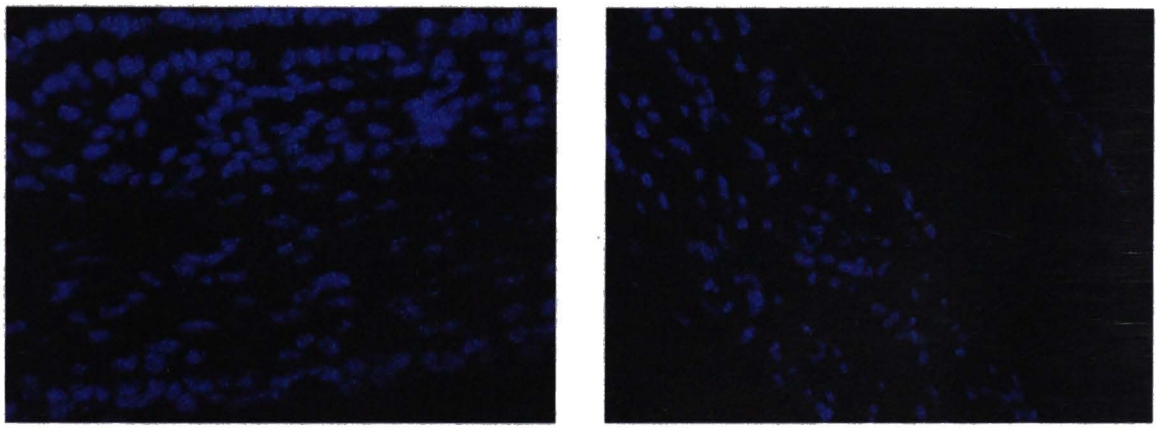
14-3-3θ

Er/+

WT(control)

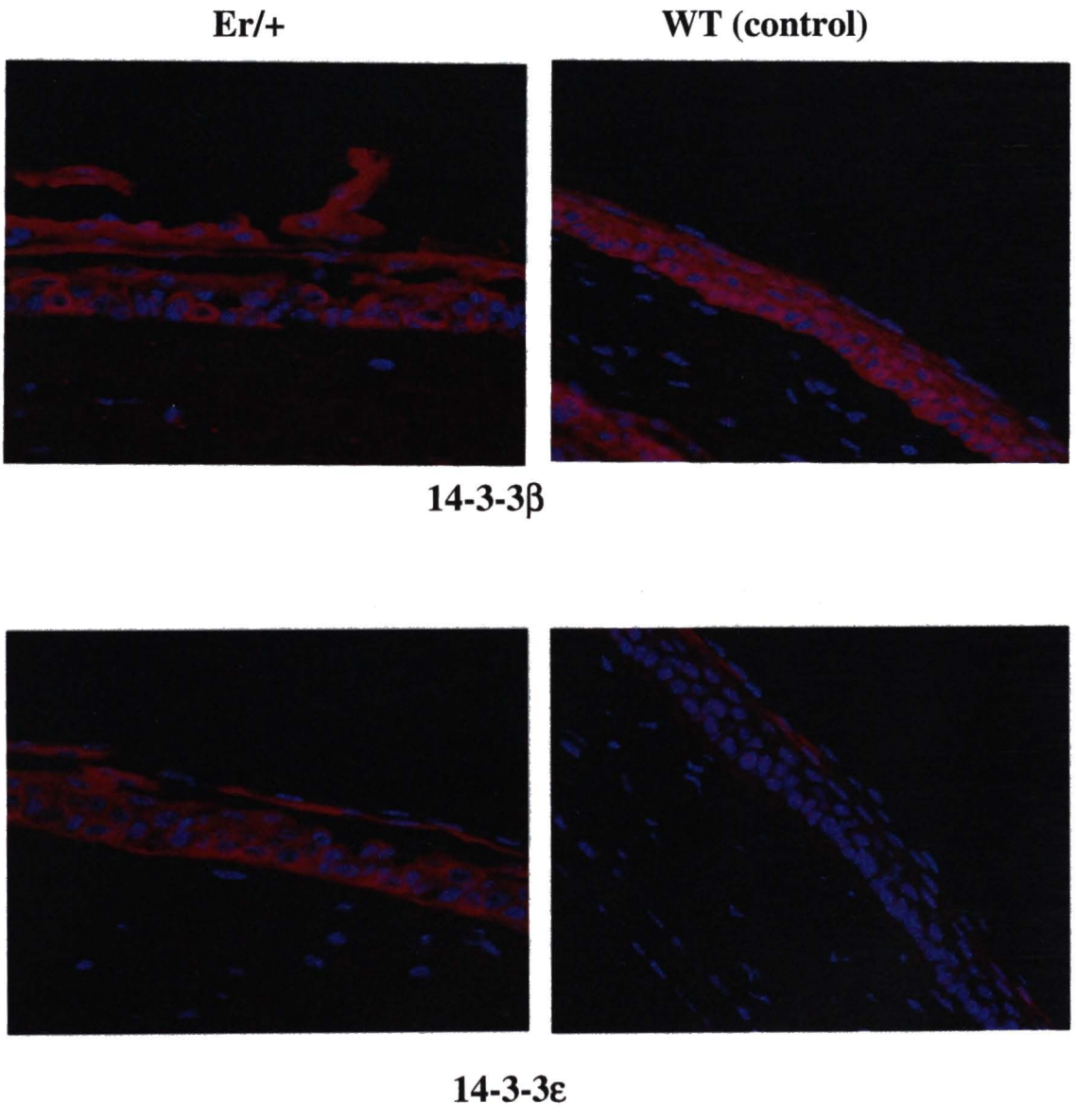


14-3-3ζ

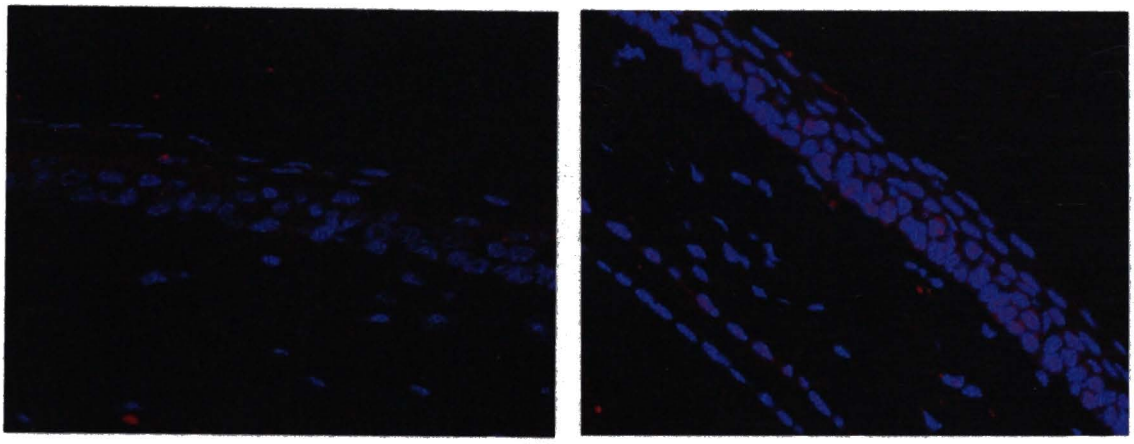


Control

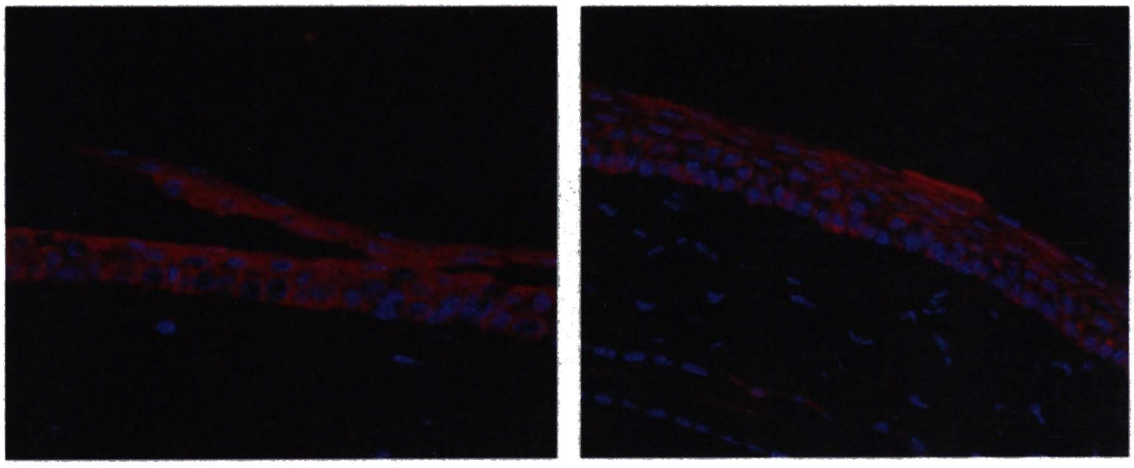
Figure 6(3): Expression of 14-3-3 isoforms in the corneal sections from 1 month old Er/+ mice and their age-matched control as determined by Indirect Immunofluorescence.



Er/+ WT (control)

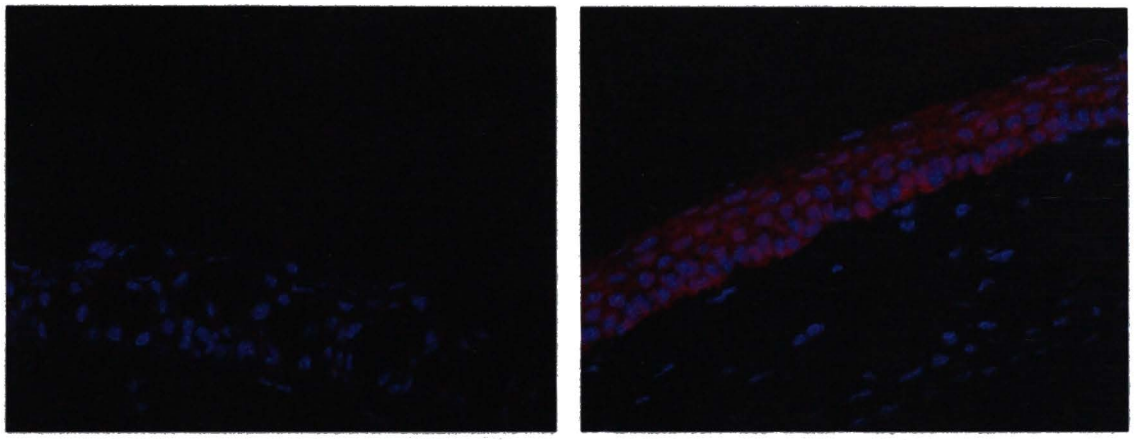


14-3-3η

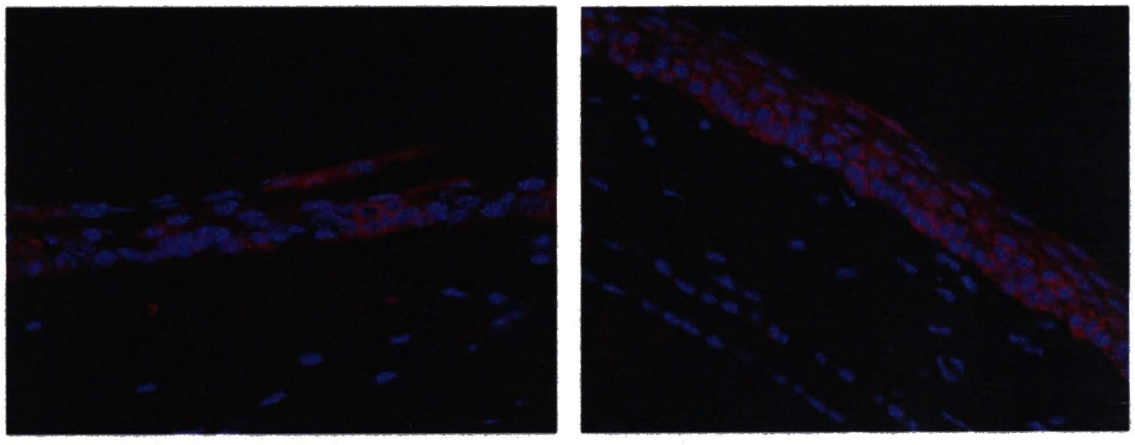


14-3-3γ

Er/+ WT (control)

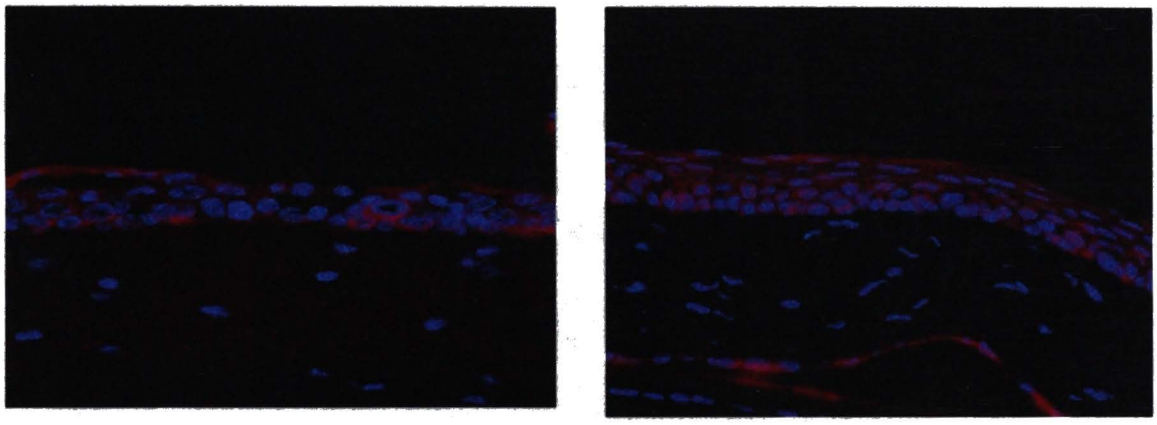


14-3-3σ

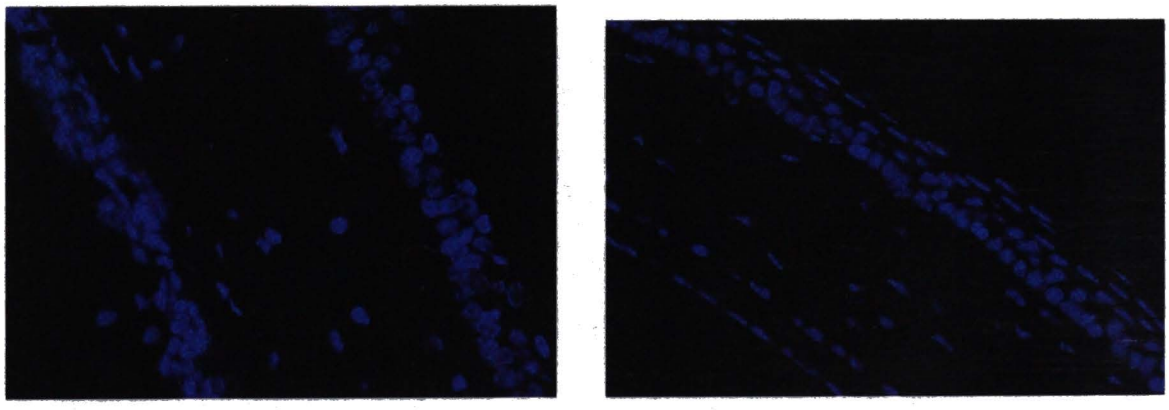


14-3-3θ

Er/+ WT (control)

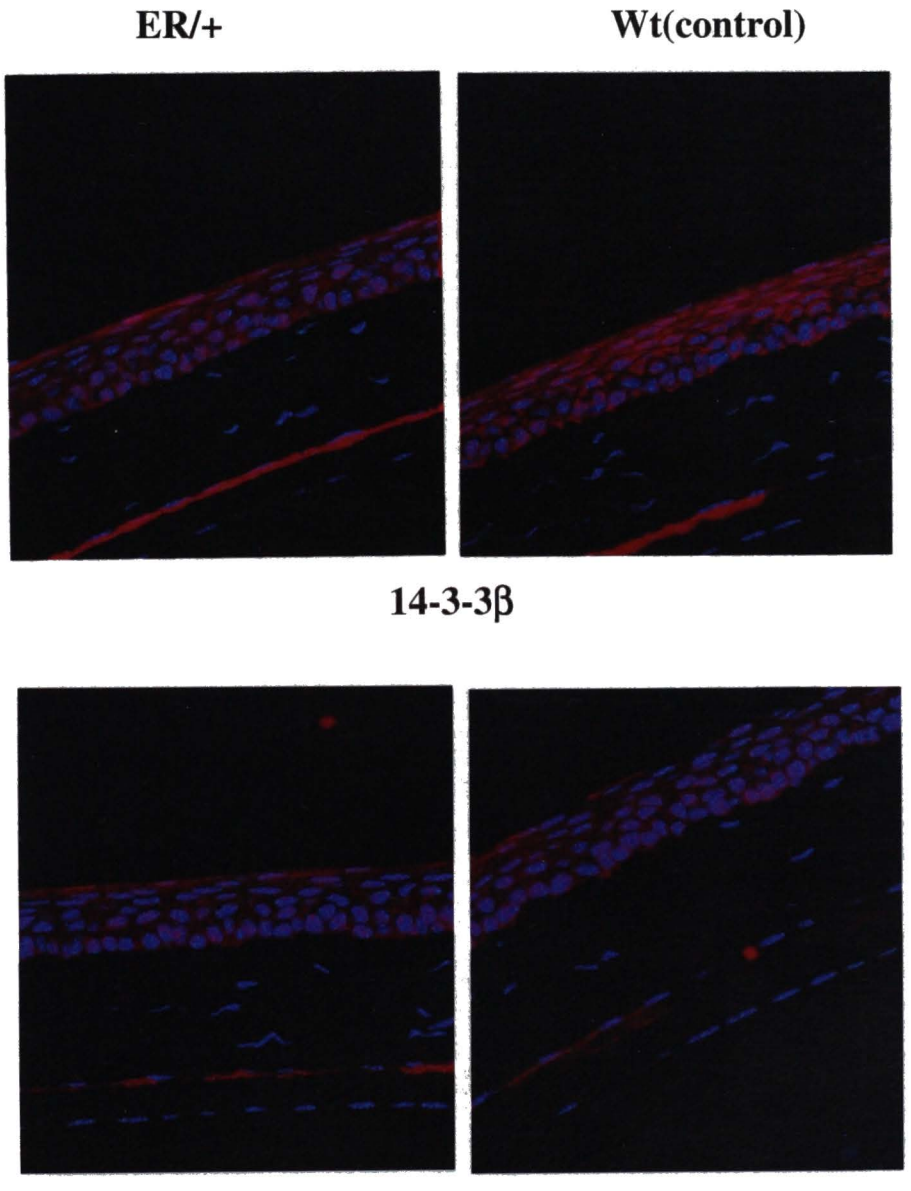


14-3-3ζ

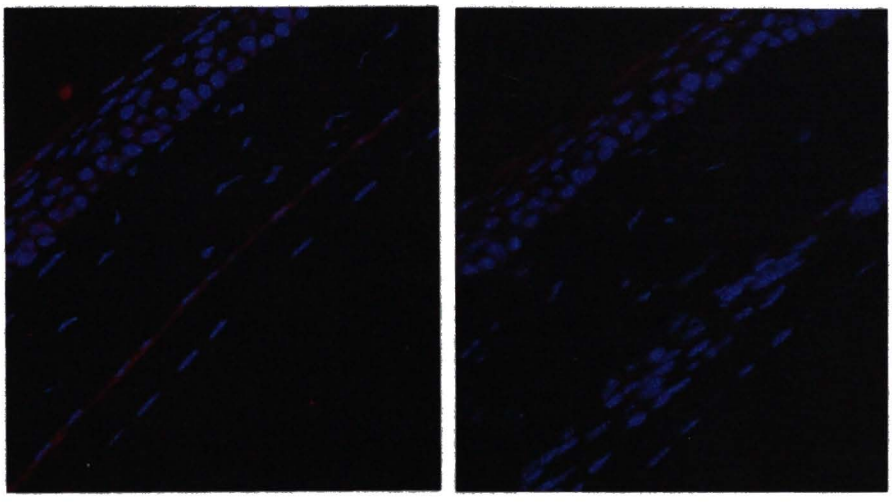


Control

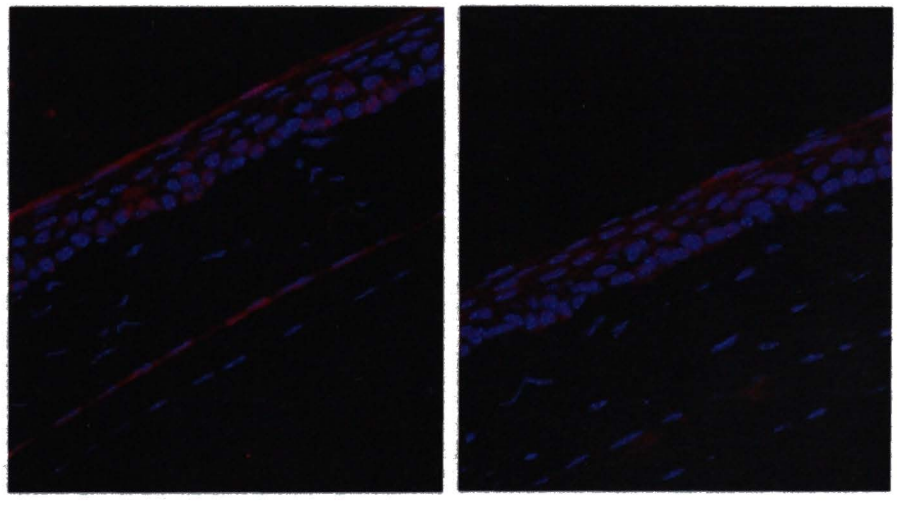
Figure 6 (4): Expression of 14-3-3 isoforms in the corneal sections from 7 month Er/+ mice and their age matched control as determined by Indirect Immunofluorescence.



ER/+ WT(control)

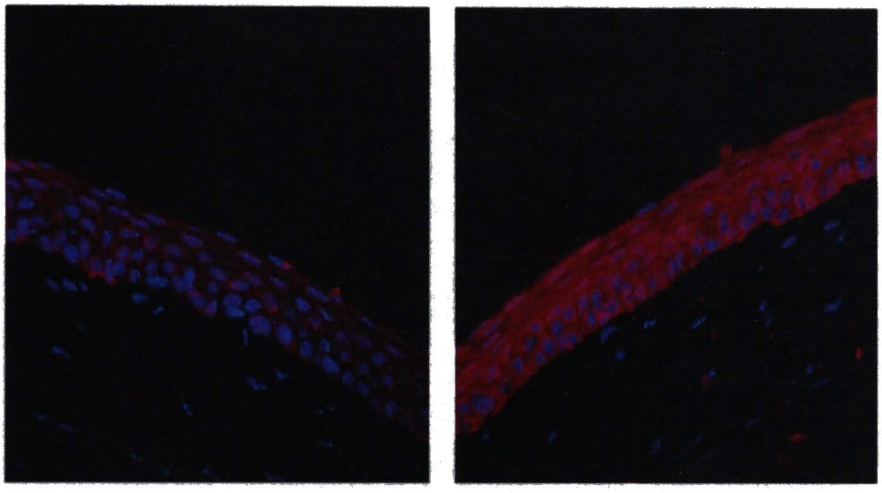


14-3-3η

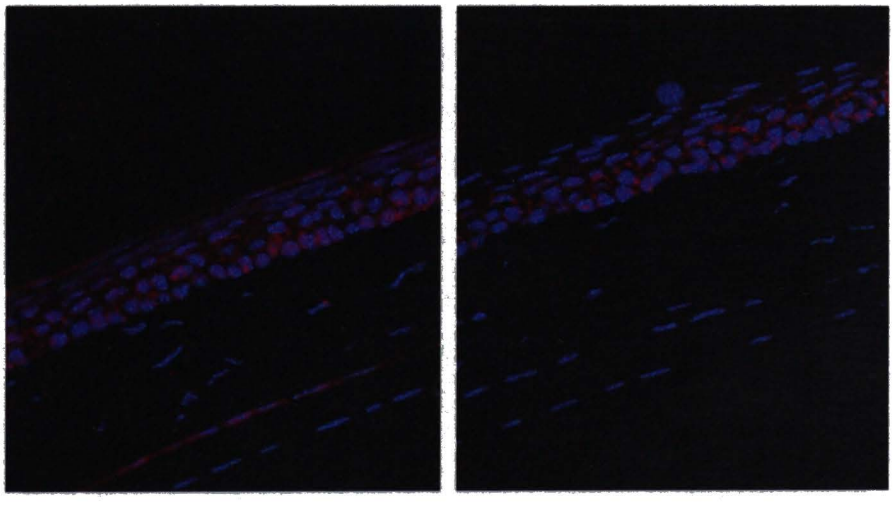


14-3-3γ

ER/+ WT(control)

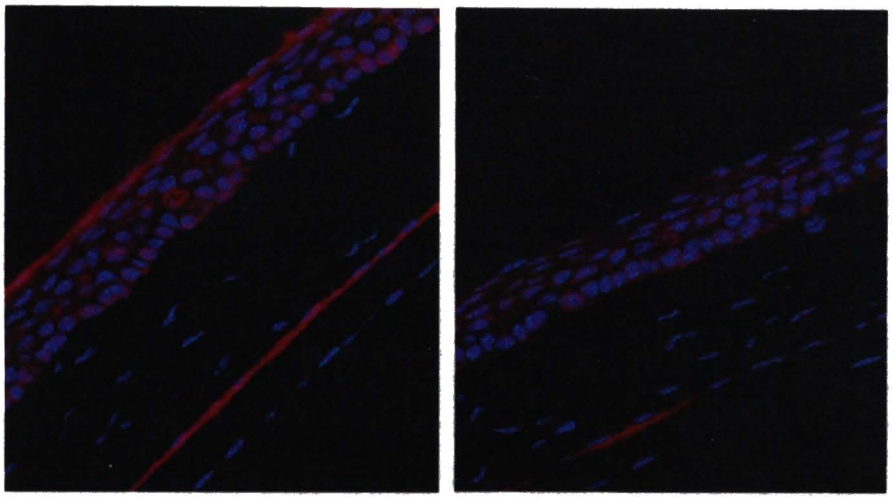


14-3-3σ

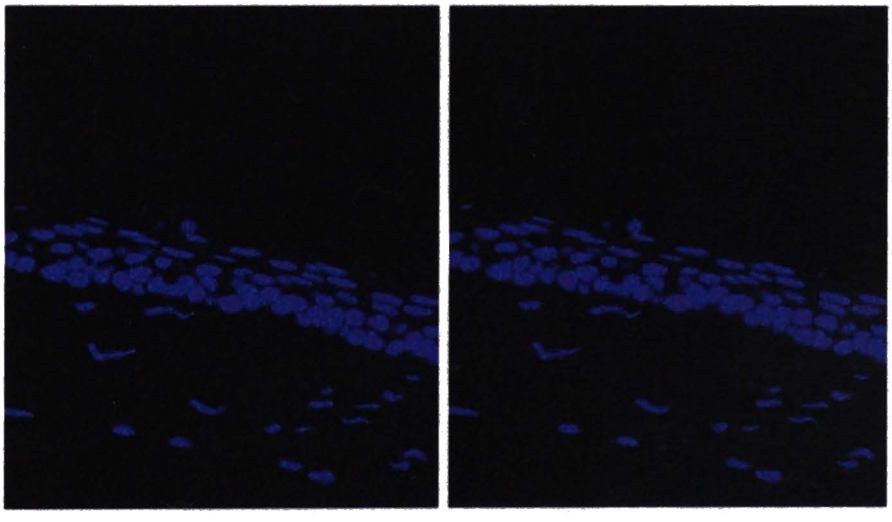


14-3-3θ

ER/+ WT(control)



14-3-3ζ



Control

Figure 6(5): Expression of 14-3-3σ in the corneal sections from 3 day, 1 month and 7 month old Er/+ mice and their age matched control as determined by Indirect Immunofluorescence.

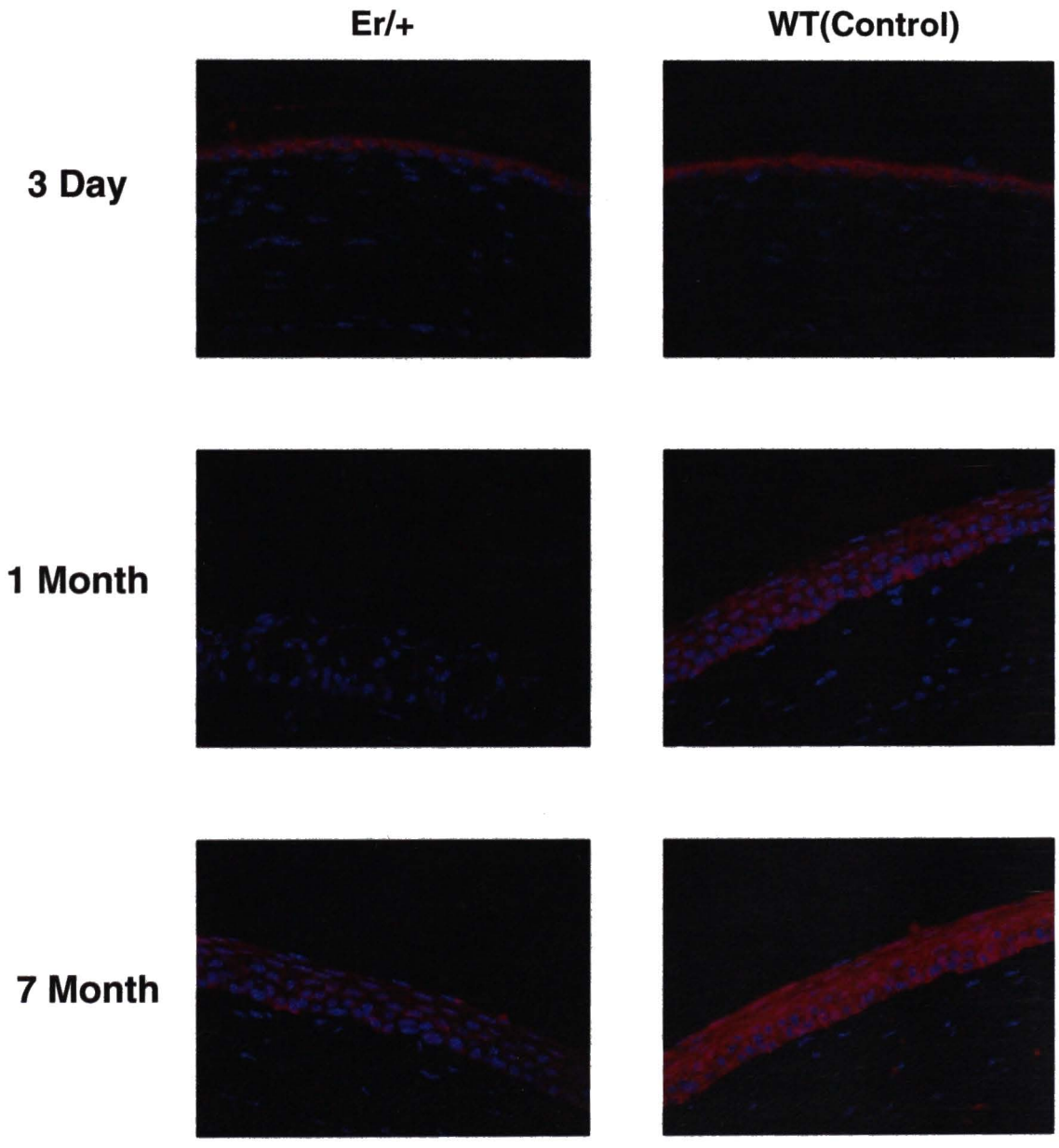
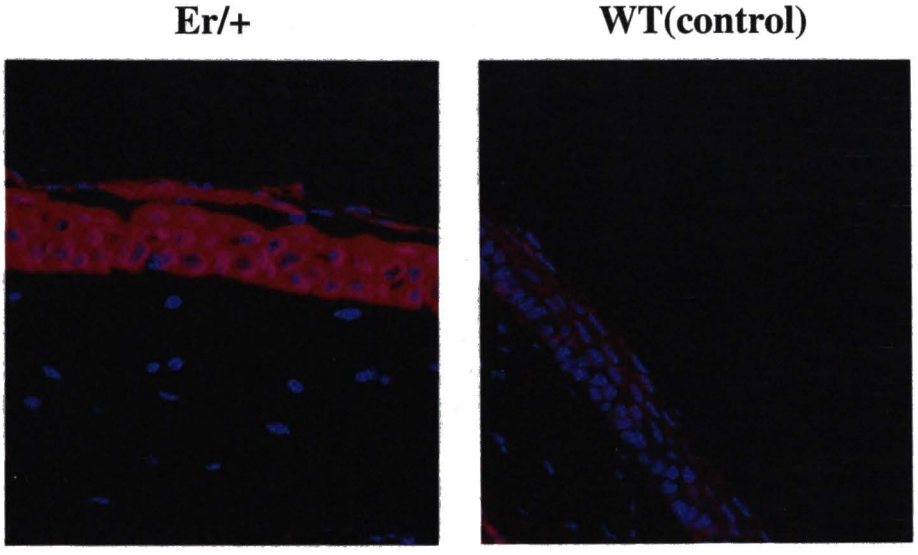
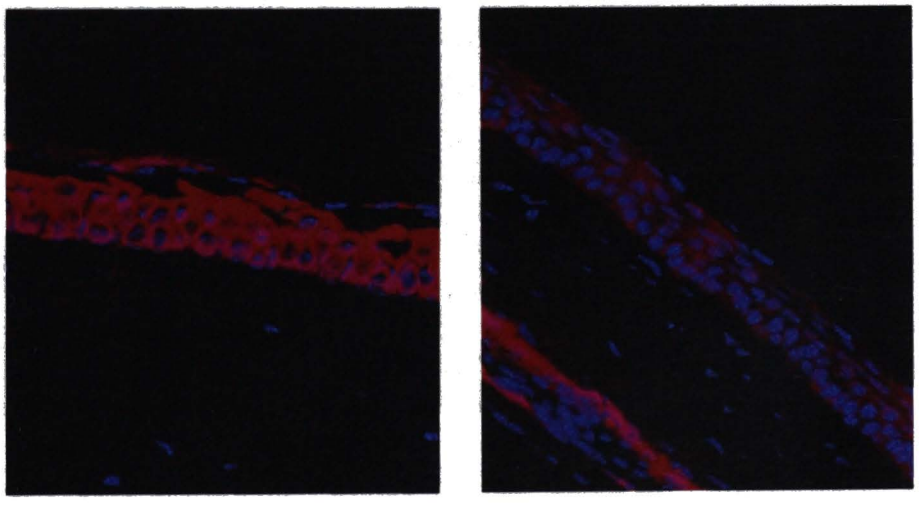


Figure 6 (6): Expression of keratins in the cornea sections from 7 month old Er/+ mice and their age matched control as determined by Indirect Immunofluorescence.

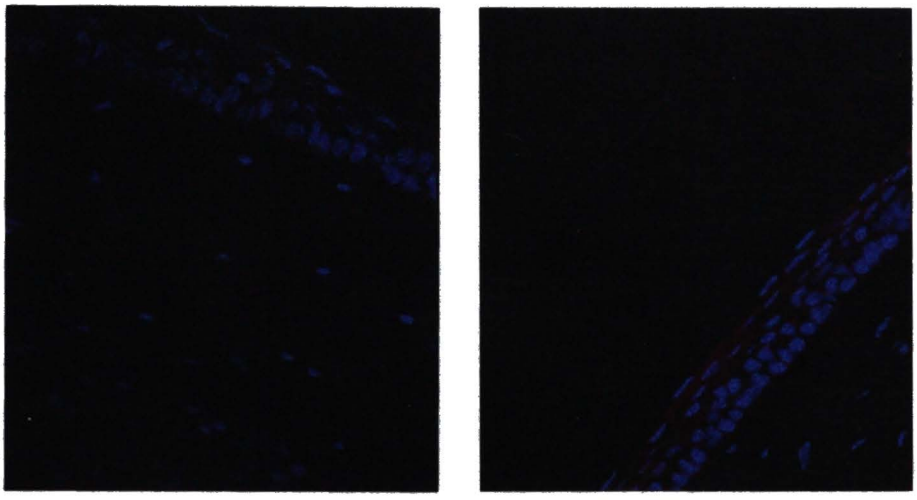


Cytokeratin 5

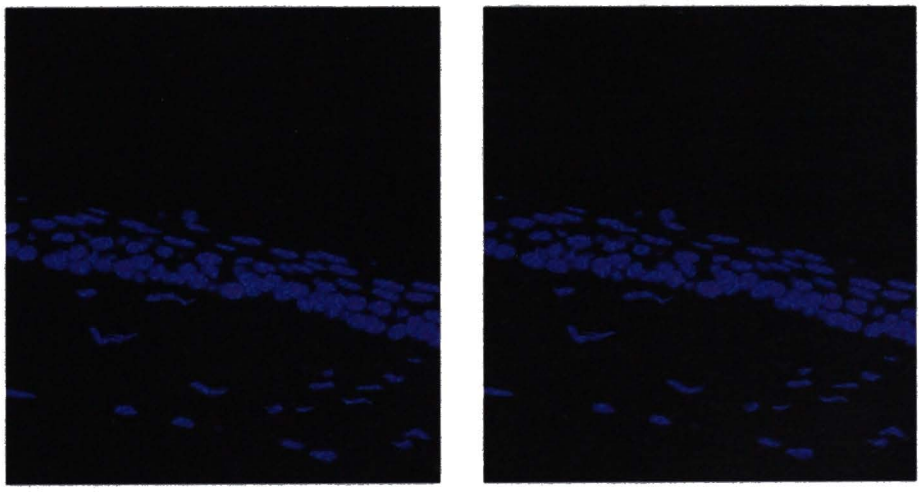


Cytokeratin 14

Er/+ WT(control)



Cytokeratin 12



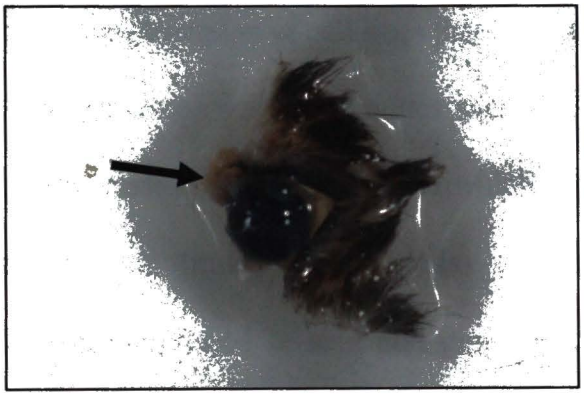
Control

Table 6(1): Comprehensive summary of the expression of 14-3-3 isoforms in the corneal sections from Er/+ mice and their age matched controls.

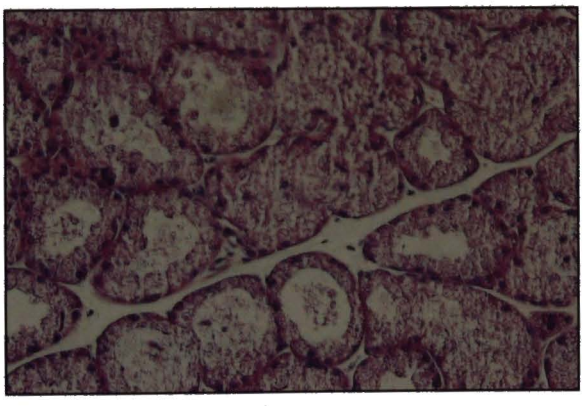
Age	WT	ER/+	Protein
3 Day	++	+	FTT σ
	+	+	FTT(β,ε,η,θ,ζ,γ)
	-	-	K12
1 Month	++	+	FTTσ
	+	+	FTT(β,ε,η,θ,ζ,γ)
	+	+++	K14
	+	-	K12
7 Month	++	+	FTTσ
	+	+	FTT(β,ε,η,θ,ζ,γ)
	+	+++	K14
	+	-	K12

Figure 6(7): (a) "Tumors" in the Eyelid margins of B6 strain Er/+ Mouse (arrow indicates the tumor in the eyelid margin) and (b) H&E stain of the section of the tumor.

(a)



(b)



CHAPTER VII

PRELIMINARY DATA FOR FUTURE STUDIES

Primary corneal epithelial cells have limited proliferative capacity and are hence difficult to work with. They are also subject to differences based on donor age, gender and method of tissue preservation. Difficulties in culturing primary corneal epithelial cells have lead to the generation of cell lines that have altered cell cycle control. These cell lines have been generated using techniques such as SV-40 T antigen, E6/E7 oncoproteins and ectopic expression of hTERT, all of which are global mechanisms of bypassing mortality checkpoints (immortalizing cells). Immortalization by 14-3-3σ down-regulation was first accomplished in skin and gingival keratinocytes using full-length 14-3-3σ antisense DNA. This method of *in vitro* life span extension is specific to epithelial cells which are the only cells that express 14-3-3σ.

We are the first to conduct a comprehensive study of the expression of the 14-3-3 family of proteins in the cornea and the conjunctiva. The 14-3-3σ isoform was shown to be specific to the corneal and conjunctival epithelium. 14-3-3σ expression increases with passage indicating that as the population of differentiating cells in the culture increases. Over-expression of 14-3-3σ in hTERT CEC induced the expression of involucrin, a marker of terminal differentiation. This data confirms the involvement of 14-3-3σ in corneal epithelial differentiation [Figure 7(1)]. Down-regulation of 14-3-3σ in corneal epithelial cells resulted in extension of life span in cells from several donors and also resulted in the generation of an immortal cell line. Characterization of these cells

confirms that these cells are highly proliferative like any cell line, but are similar to WT CEC cells in protein expression and differentiation. These FTT CEC cells however do not respond to adriamycin induced DNA damage like WT CEC cells but instead undergo apoptosis.

Our data also indicate that corneal epithelial cells and cell lines secrete 14-3-3 proteins (σ, γ and ζ, isoforms) and also that some of these isoforms (γ and ζ, isoforms) are also present in the tear fluid suggesting important extracellular functions for these proteins.

In vivo, the effect of down-regulation of 14-3-3σ was studied in the corneas of Er/+ mice, where a epithelial hyperproliferation due to impaired differentiation has been reported in the skin. Similar to the *in vitro* situation the corneal epithelial cells in these mice are highly proliferative as shown by the higher levels of cytokeratin 5 and 14 and lower levels of cytokeratin 12. However, there are no corneal tumors observed in these mice although skin tumors have been reported in these mice.

Future Directions

Future directions would involve determining the upstream and downstream regulators of 14-3-3 σ and elucidate the complete signaling pathway in corneal epithelial differentiation.

p63 as an upstream regulator of 14-3-3 σ

p63 was initially proposed as a putative marker of adult stem cells found in adult epithelial stem cell compartments (e.g. skin)[1] but p63 it was subsequently also found to be expressed in transient amplifying cells, so that it cannot be considered as a stem cell

marker.[2] p63 protein is a homolog of p53 that is crucial for maintaining epithelial homeostasis, and exists as six p63 transcripts that result from alternative splicing. TA and ΔN are the two main classes of the p63 protein and each of the classes consists of α, β and γ isoforms. TA and ΔN p63 have been shown to have contradicting functions with respect to epithelial cell proliferation and differentiation.

The ΔNp63 isoform suppresses both p53 and the TAp63. [2, 3] ΔNp63 regulates the stem cell fate by competing for the p53 – binding site on the **14-3-3 σ** promoter resulting in the repression of its function. As cells commit to differentiation, the ΔNp63 isoform expression is greatly reduced thereby lifting the suppression of and the suppression of **14-3-3 σ** transcription and expression. [4]**Δ**Np63α has also been shown to be a transcriptions enhancer of K14. Since with differentiation **Δ**Np63 levels decrease and there is also a corresponding decrease in K14 expression, **Δ**Np63α could be an important switch between differentiation and proliferation playing a dual role in keratin synthesis and 14-3-3σ signaling cascade. The role of p63 has been well studied in the skin where ΔN is the predominantly expressed isoform. ΔNp63α in keratinocytes has been shown to be a transcriptional repressor of **14-3-3 σ** and a transcriptional enhancer of K14. [5] This further emphasizes the role of **14-3-3 σ** in differentiation because K14 is a cytokeratin expressed in proliferating epithelial cells.

A Pan antibody against p63 that recognizes all p63 isoforms shows the presence of p63 in not confined to the basal layer of the corneal epithelium [Figure 7(2)]. This suggests that p63 proteins in general cannot be considered a corneal stem cell marker, as it is expressed outside the putative stem cell compartment/basal limbal epithelium.

In the corneal epithelia ΔNp63α has been shown by PCR to be the predominant isoform. [2] We have shown by indirect immunofluorescence that ΔNp63 localization is nuclear in the basal proliferating cells of the corneal epithelium, and cytosolic in the superficial/differentiated cells of both the central cornea and the limbus [Figure 7(3)]. We have also shown by indirect immunofluorescence that TAp63 expression is confined to the superficial layers of the central cornea and was absent in the limbus [Figure 7(3)].

Studies with corneal epithelial regeneration also suggest that ΔNp63α isoform is crucial for maintenance of proliferative potential of limbal stem cells following epithelial repair after an injury.[6] In yeast p53 has been shown to physically associate with ΔNp63α and targets it for degradation.[7], but the mechanism of degradation in not known. Hence studies of the intracellular translocation of these proteins might reveal additional information about the function of ΔNp63α and clarify its role in differentiation and hyper proliferation.

The expression levels and localization of the ΔNp63 isoforms in response to differentiation has not been studied in the corneal epithelium. Preliminary experiments indicate a decrease in ΔNp63 expression in response to differentiation in WT CEC, hTERT CEC and FTT CEC. This has been shown by western blot analysis [Figure 7(4)] and suggests that ΔNp63 is no longer transcribed and the existing protein is degraded. For the protein to be degraded it has to be transported to the cytoplasm. Preliminary experiments in FTT and hTERT CEC cells indicate the possible translocation into the cytoplasm when serum is present in the culture medium (i.e differentiation conditions) [Figure 7(6) & 7(7)]. Studies on WT CEC cells have been difficult due to the fact that

these cells appear to be committed to differentiation under normal culture conditions. However, these experiments have to be when repeated under normal and differentiation conditions over a period of time to confirm the translocation of the ΔNp63 protein to the cytosol.

p53 has the ability to target ΔNp63α for degradation[7]. Since the level of p53 has been shown to increase during differentiation ,[8-10] p53 may be one of the upstream regulators involved in the differentiation cascade. The cytosolic ΔNp63 could therefore be targeted for degradation. Since here is no known cytosolic function for ΔNp63, it would be interesting to elucidate a signaling pathway for the involvement of p63 in 14-3-3σ induced differentiation [Figure 7(10)].

14-3-3 σ induced cell cycle arrest

We have shown that 14-3-3 σ expression is directly involved in differentiation, so future experiments would intend to identify the downstream regulators. Cell proliferation and differentiation cannot occur simultaneously. In order for the epithelial cells to differentiate, the cells must arrest cell cycle progression, and this arrest can occur in the G1/S phase or the G2/M phase. 14-3-3σ expression is induced in response to gamma irradiation and other DNA damaging events and results in G2/M cell cycle arrest. In breast cancer cells over-expression of 14-3-3σ results in cell cycle arrest by sequestering cdc2 and cyclin B in the cytosol. Nuclear localization of these two proteins is crucial for entry into the M phase of the cell cycle. 14-3-3σ shares cyclin/cdc2 binding motifs with several cell cycle inhibitor proteins like p21, p27 and p57. Since 14-3-3 σ has been shown to bind and sequester cyclin:CDK complexes in the cytosol, it would be

important to identify if the cell cycle arrest during differentiation occurs in the G1/S or G2/M phase[Figure 7(11)].

Extracellular Role for 14-3-3 proteins

We have shown for the first time that 14-3-3 proteins (γ and ζ isoforms) are secreted by corneal epithelial cells, conjunctival epithelial cells and cells lines. We have also shown that these isoforms are present in the tear fluid which plays an important role in homeostasis of the ocular surface. The extracellular role of these proteins has not yet been studied.

We have shown that 14-3-3σ is also secreted by corneal epithelial cells, conjunctival epithelial cells and cell lines, and that the levels of secreted 14-3-3 σ is higher in differentiated (P5) WT CEC when compared to early passage (P2). This suggests a possible paracrine role for this protein in differentiation. We have shown using western blot analysis that in WT and FTT CEC cells this 14-3-3 σ is present as a dimer [Figure 7(8)]. Dimerization has been considered essential for its functionality. However, it is unlikely that 14-3-3 σ functions as a dimer outside the cell, but these novel aspects pf 14-3-3 σ biology need to be studied.

14-3-3 σ has been shown to be secreted by keratinocytes and has also been shown to increase with passage/differentiation in these cells. Most studies involving the extracellular 14-3-3 σ protein are concerned with the epithelial-mesenchymal communication and signaling in the skin. It has been shown that epidermis of the Er/Er mice when transplanted onto normal mice, was shown to "normalize" and undergo differentiation[11, 12] This suggests the involvement of environmental factors in

differentiation, hence it is possible that extracellular 14-3-3 σ proteins secreted by the surrounding normal differentiating epithelial cells may play a role in normalizing these cells. However, the mechanism of action of the extracellular protein has not been determined. Recent studies have shown that extracellular 14-3-3 σ abrogates the effects of IGF-1 and TGFβ-1 in dermal fibroblasts. This suggests that extracellular 14-3-3 σ could function by interacting (inhibiting or activating) with cell surface receptors. It is also interesting to speculate similar functions for the other two secreted 14-3-3 isoforms.

Figure 7(1): Proposed signaling cascade for 14-3-3σ protein in response to differentiation.

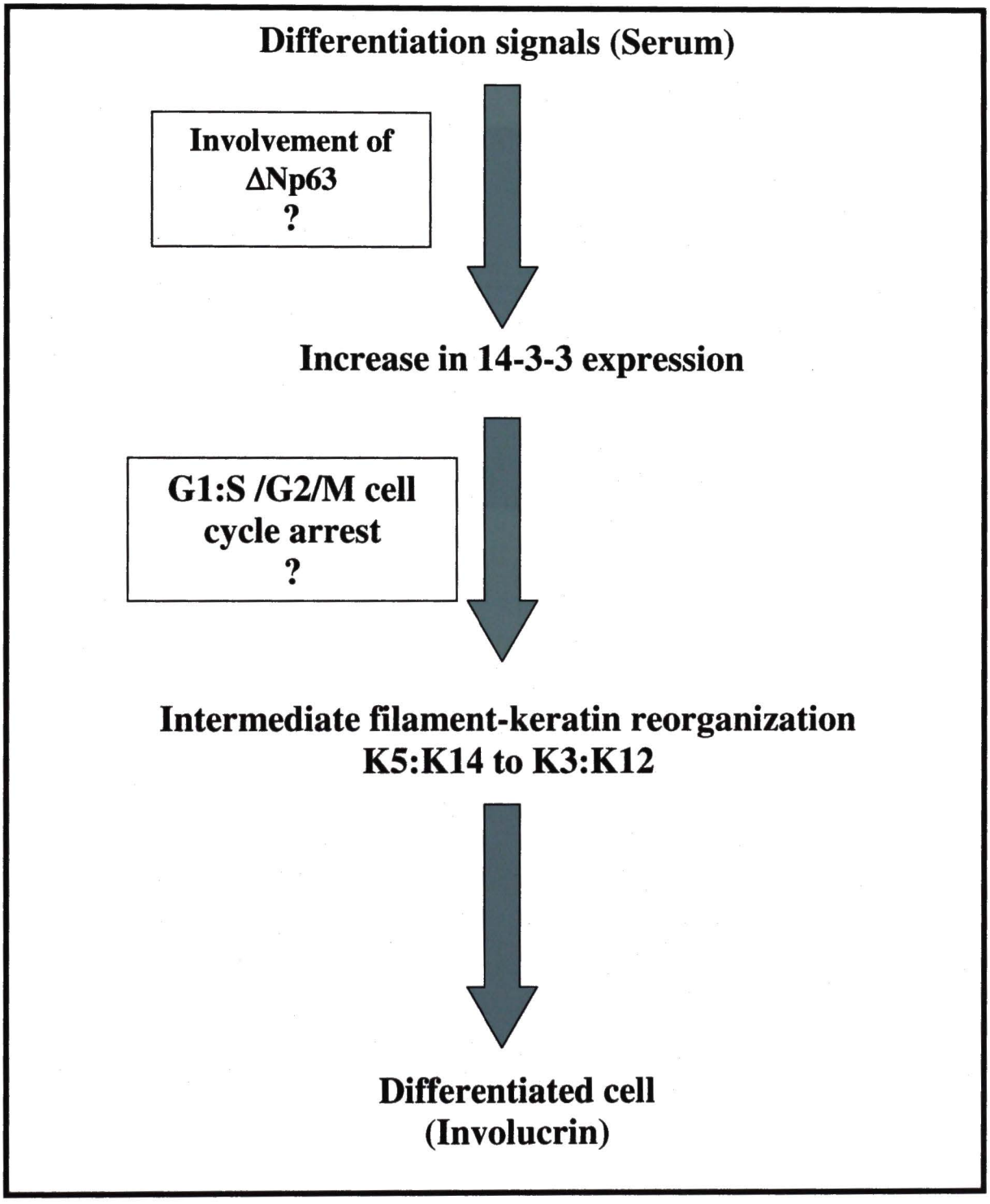
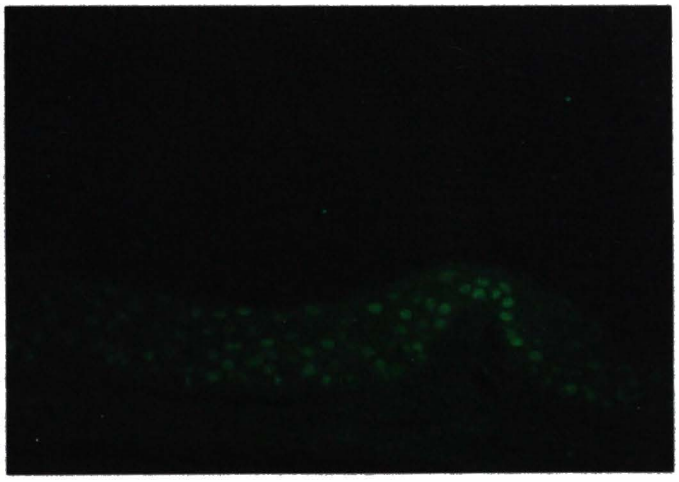


Figure 7(2): Expression of p63 in the human cornea as determined by Indirect Immunofluorescence. (a) Central Cornea and (b) Limbus.

(a)



(b)

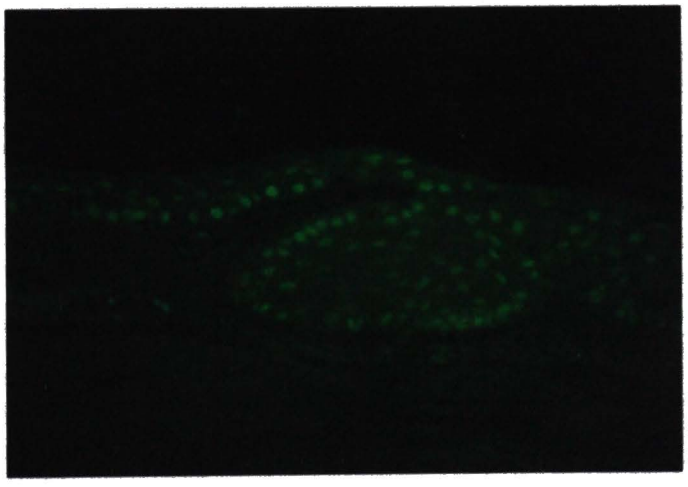


Figure 7(3): Expression of ΔNp63 and TAP63 in the human cornea as determined by Indirect Immunofluorescence.

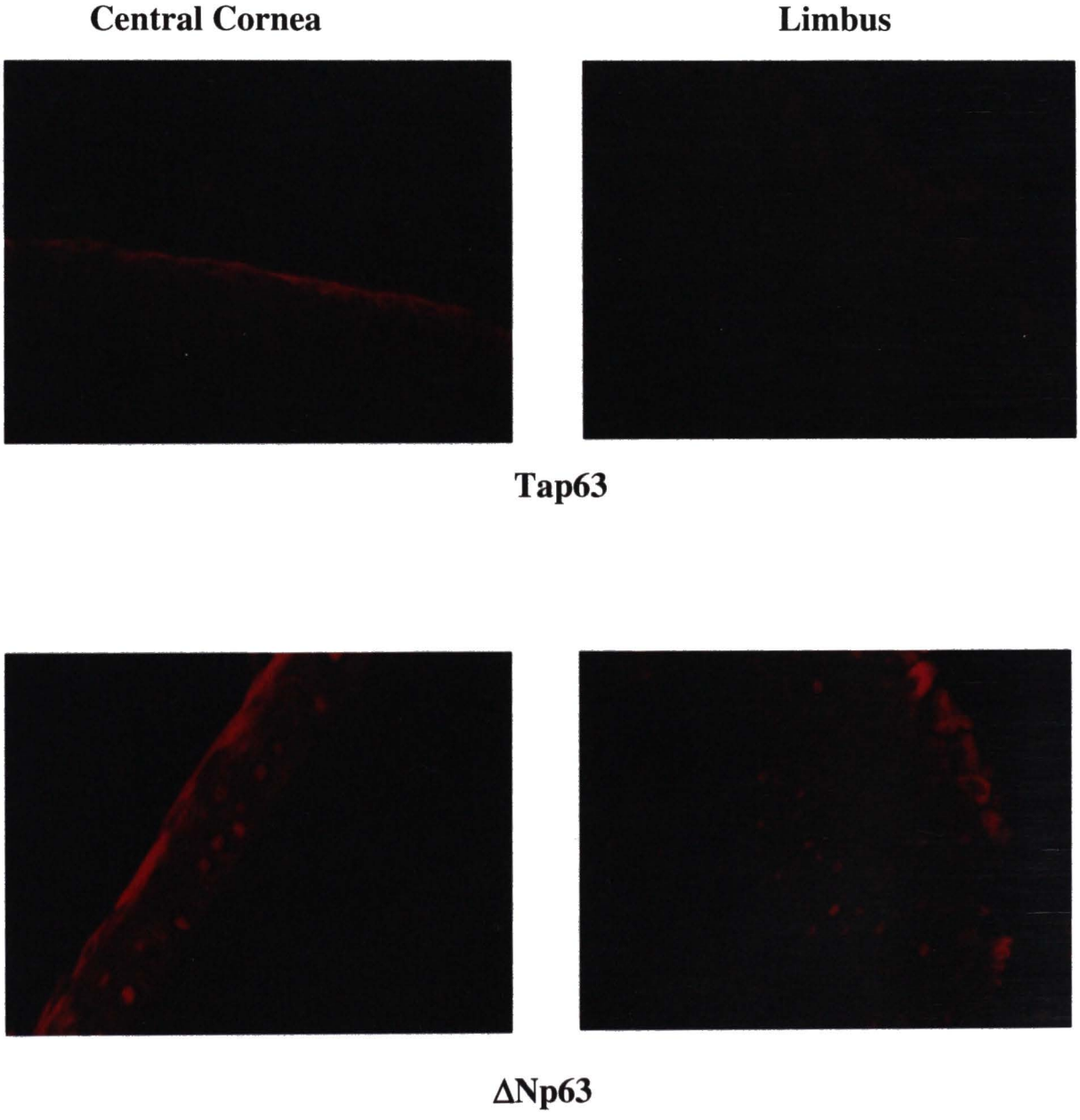
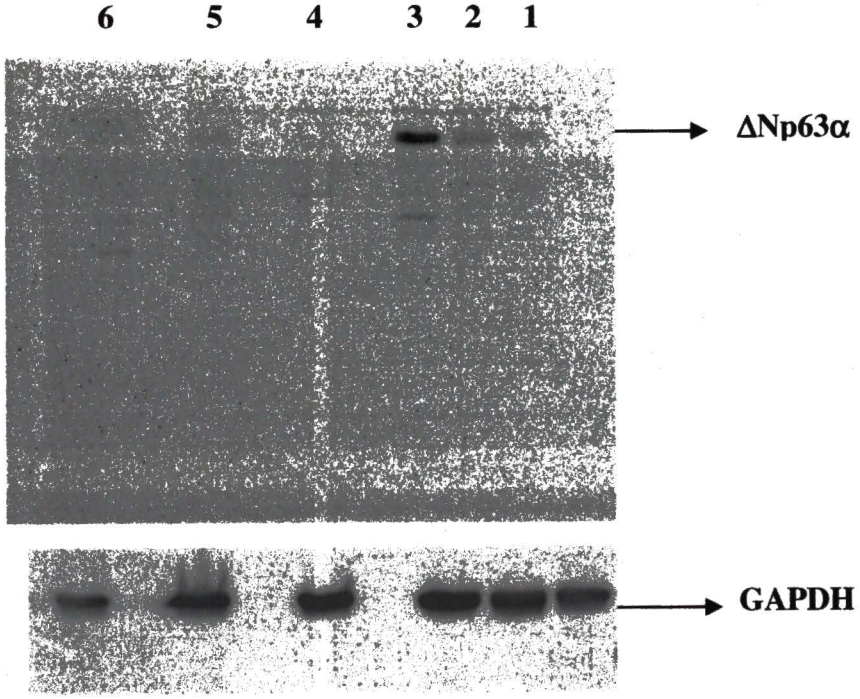


Figure 7(4): Western blot analysis to determine the expression of ΔNp63 expression in WT, hTERT and FTT CEC under normal and differentiation conditions.



1. WT CEC control (Epilife)
2. hTERT CEC Control (Epilife)
3. FTT CEC Control (Epilfe)
4. WT CEC differentiated (10% FBS)
5. hTERT CEC differentiated (10% FBS)
6. FTT CEC differentiated (10% FBS)

Figure 7(5): Indirect Immunofluorescence to determine localization of ΔNp63 expression in WT CEC cells under (a) Normal and (b) Differentiation conditions (24 hrs, 10% FBS). [Magnification -20X].

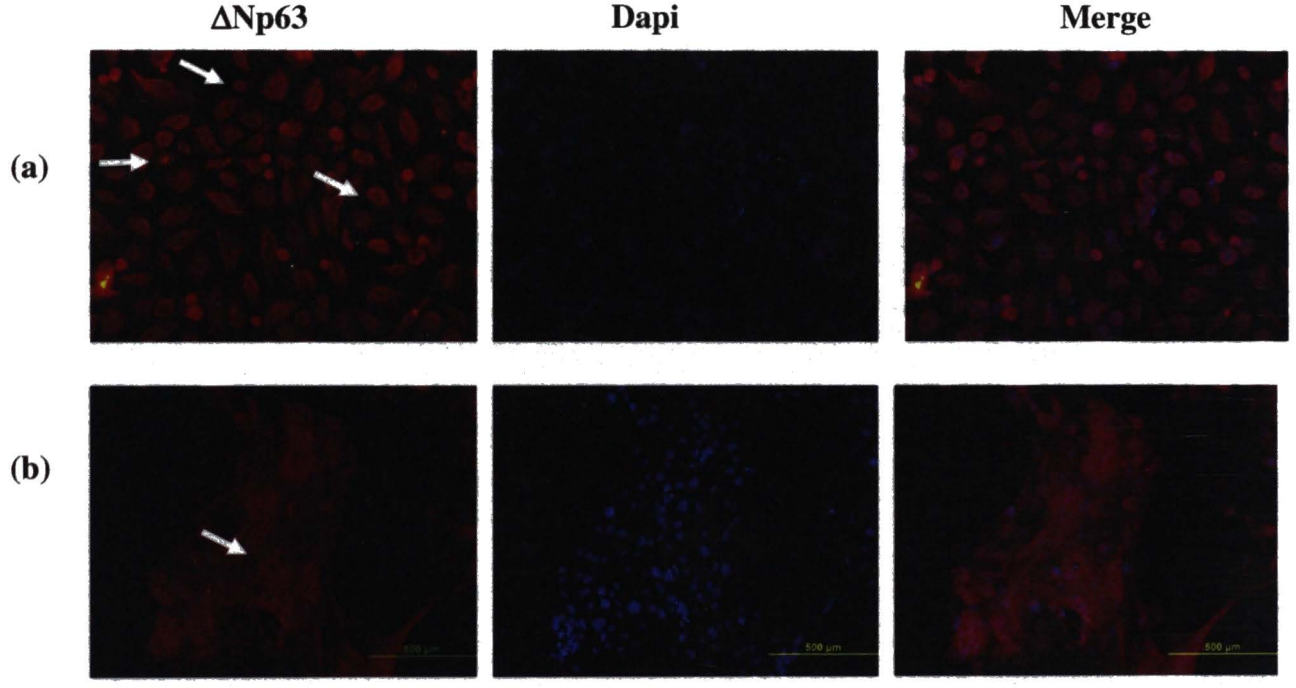


Figure 7(6): Indirect Immunofluorescence to determine localization of ΔNp63 expression in hTERT CEC cells (a) Normal and (b) Differentiation conditions (1 week, 10% FBS). [Magnification -20X]

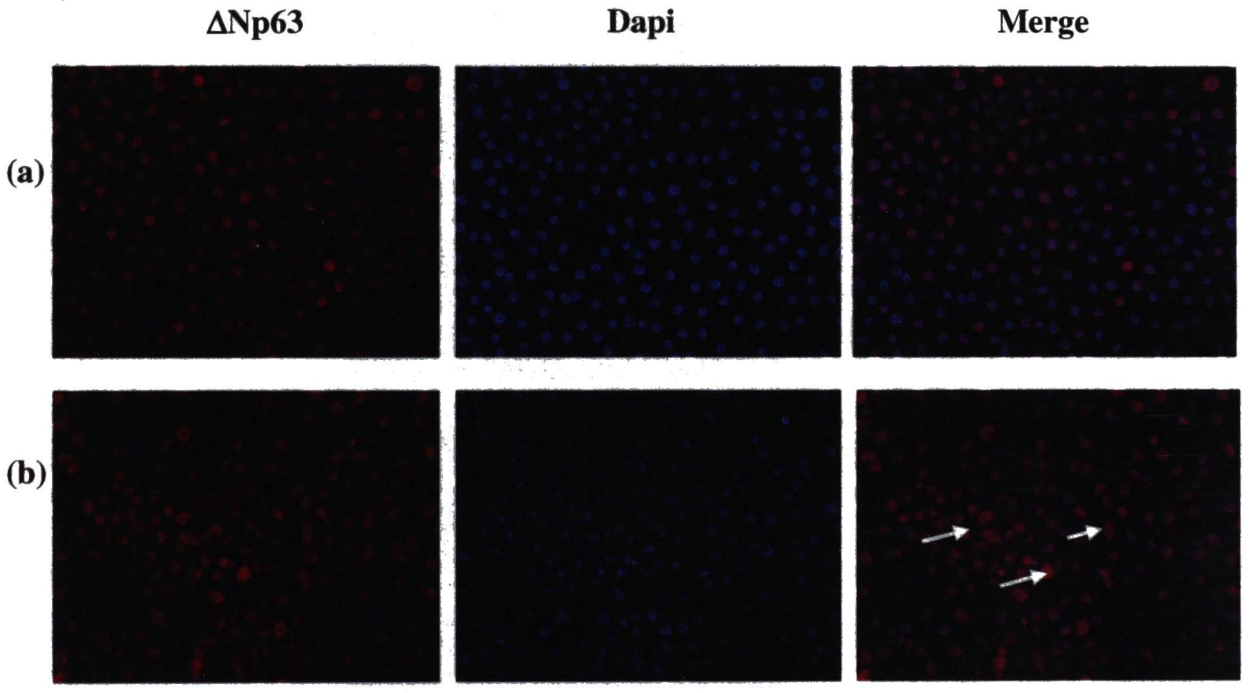
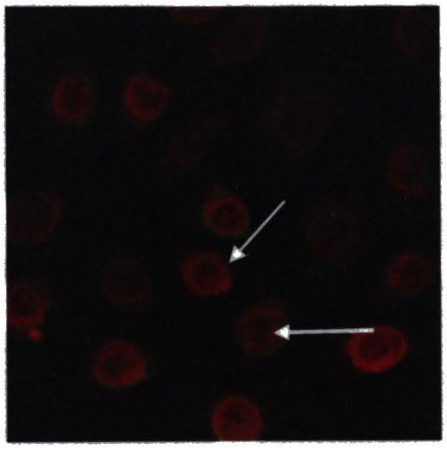


Figure 7(7): Indirect Immunofluorescence to determine localization of ΔNp63 expression (red) in FTT CEC cells (a) Normal and (b) Differentiation conditions (1 week, 10% FBS). [White arrows indicate localization of ΔNp63, Magnification-40X]

(a)



(b)

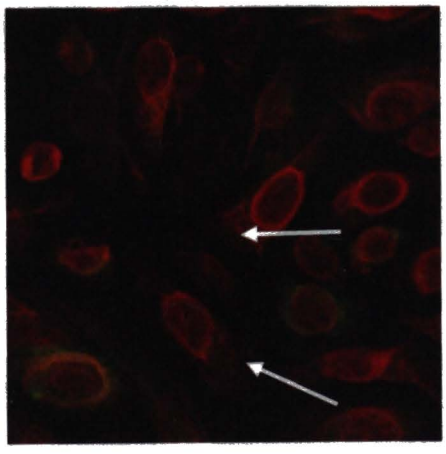
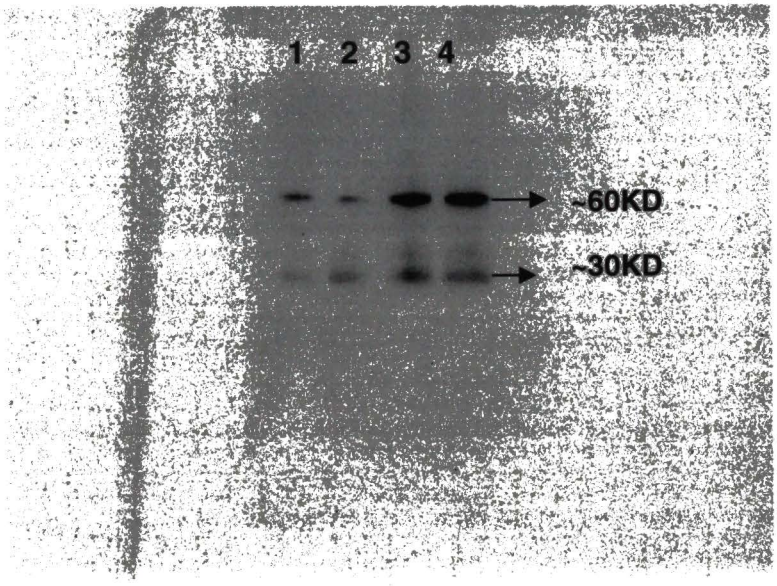


Figure 7(8): Dimerization of 14-3-3 σ proteins. WT CEC and FTT CEC cells were lysed and run on a native 12% SDS PAGE gel. Proteins were the transferred onto a nitrocellulose membrane and probed for 14-3-3σ expression by western blot analysis.



1 & 2 FTT CEC

3 & 4 WT CEC

Figure 7(9): Western blot analysis on 14-3-3σ secreted by WT CEC cells at passage 2 and passage 5.

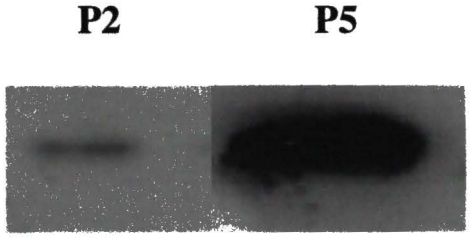


Figure 7(10): Proposed signaling cascade for p63 mediated 14-3-3σ expression.

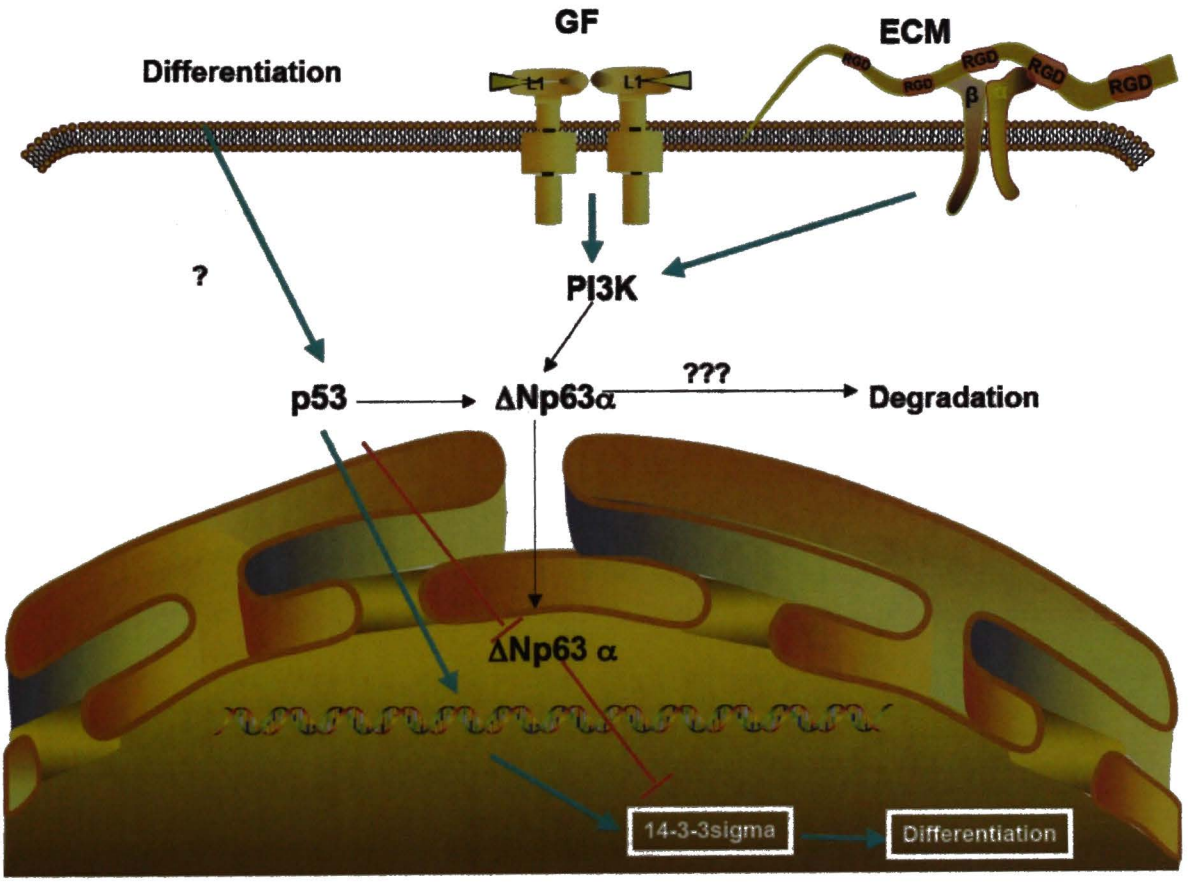
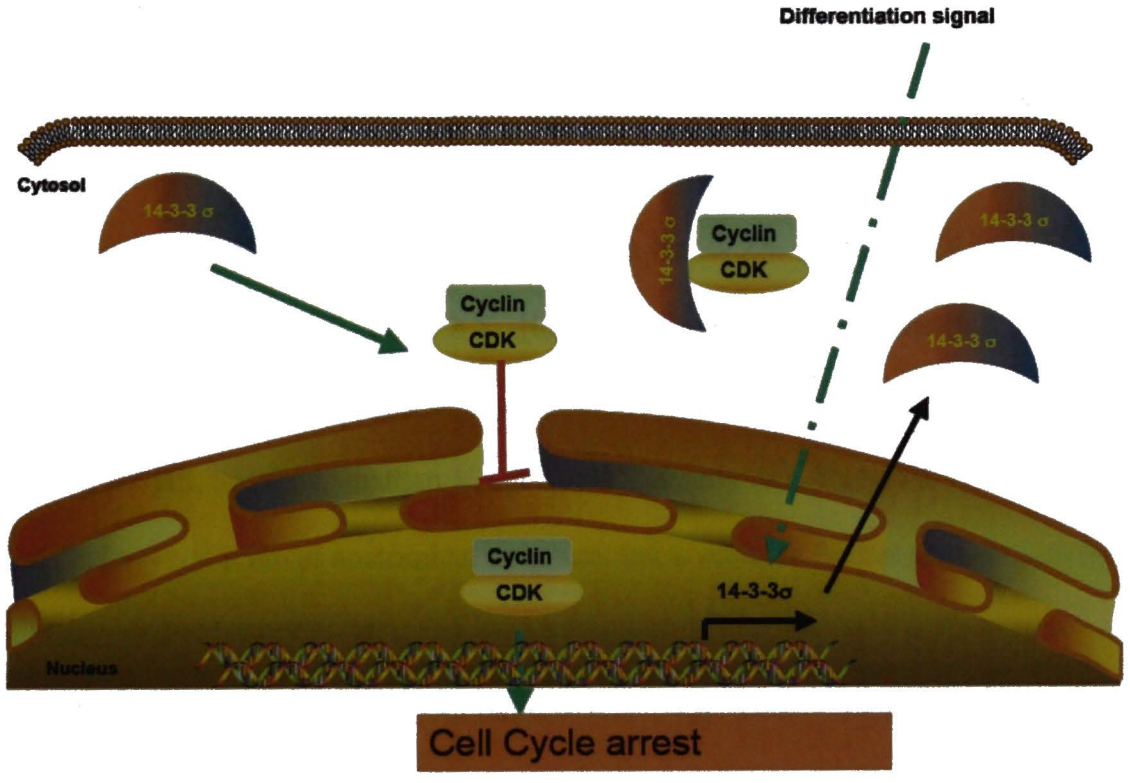


Figure 7(11): Proposed signaling cascade for downstream regulation of cell cycle arrest by 14-3-3σ.



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