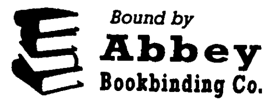

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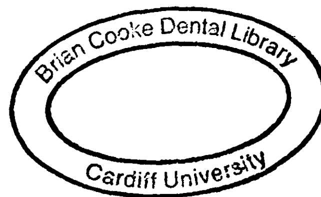

STEM CELLS IN HUMAN ORAL EPITHELIUM

Thesis submitted for the fulfilment of the requirements of the degree of Doctor of Philosophy of the University of Wales College of Medicine.

November 2004

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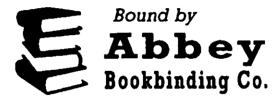

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This thesis is dedicated to Mum and Dad who have been a constant source of support and inspiration.

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ABSTRACT

The stratifying epithelia of skin and mucosa continuously renew their structure by cell division, but only a small fraction of proliferative cells, the stem cells, are capable of continuous self-renewal. The staining distribution of various stem cell and differentiation markers were investigated in normal human mucosal epithelium. To determine the extent of regional variation in patterns of stem cell behaviour in human epithelia, human mucosal keratinocytes were amplified *in vitro*. Using retroviral vectors, a sub-population of these cells were transduced with genes for histochemically detectable markers to permit lineage analyses. Organotypic culture methods were used to generate epithelia with normal patterns of cell kinetics and differentiation and these were examined after maintenance *in vitro* or after transplantation back to *in vivo* sites in immune deficient mice. The clonal lineages resulting from stem cell patterns were compared and the number of functional stem cells were assessed from the size of stable clonal units regenerated. The cell cycles of keratinocytes grown on plastic compared to organotypic culture were also investigated by flow cytometry to see if the organotypic cultures provided a suitable stem cell model. Results included the following. Antibody staining in palate revealed that K15 and K19 identified zones of positively stained basal cells at the epithelial rete tips. These K15 and K19 positive regions were negative for differentiation markers K6 and K16, indicating that cells in these regions were undifferentiated and could include stem cells; Organotypic cultures gave similar staining profiles to palate, and using retrovirally stained subpopulations of cells and transplantation to SCID mice, clonal units were identified which did not correspond to the primitive rete that had reformed. Flow cytometry work showed that the cell cycles of cells grown *in vitro* on plastic were faster than the cell cycles of organotypic cultures.

ABBREVIATIONS

AP	alkaline phosphatase
BM	basement membrane
BGA	blood group antigens
BSA	bovine serum albumin
CT	connective tissue
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EGF	epidermal growth factor
EPU	epidermal proliferative unit
EtBr	ethidium bromide
EtOH	ethanol
FAD	F12 + adenine + DMEM
FACS	fluorescence activated cell sorter
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
H&E	haematoxylin and eosin staining
IBL	interpapillatory zone
IF	keratin intermediate filaments
IHC	immunohistochemistry
IL-1	interleukin-1
K	keratin
KDa	kilo Dalton
KGF	keratinocyte growth factor
LRC	label retaining cell
NCS	newborn calf serum
PBL	papillae of the supporting connective tissue

PBS	phosphate buffered saline
PE	phycoerythrin
pfa	paraformaldehyde
PI	propidium iodide
RNase	ribonucleic acid endonuclease
rpm	revolutions per minute
SC	stem cells
SCC	side scatter
S.D.	standard deviation
TA	transit amplifying cell
TD	terminally differentiated cell

CHAPTER ONE

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Stratified squamous epithelia, including oral epithelia, continuously renew their structure by division of cells in the basal strata to produce differentiating cells that ascend through the epithelium eventually to be shed from the epithelial surface (Squier, 1976). These processes of cell proliferation and differentiation maintain the steady state that determines normal epithelial structure. Stratified epithelium exists normally in a steady state and for every cell division in the basal layer, one cell enters the maturation pathway and as this cell begins to mature, cells lying above it mature and desquamate. Thus, the cell moves up through the epithelium towards the surface, and the orderliness of this sequence of events, together with cell synthesis associated with maturation, leads to the appearance of the various strata. The thickness of the different strata and the overall thickness of the epithelium are determined by a steady balance between cell formation, the rate at which cells pass through each of the strata and the rate at which they are desquamated. Changes in rates of cell formation and maturation induced using friction, tape or chemical agents, lead to marked hyperplastic changes in skin and oral mucosa (Mackenzie and Miles, 1973). Thus the rate of cell proliferation and cell maturation seem to be linked. The patterns of cellular renewal in mucosal epithelia significantly influence the mechanisms that control tissue homeostasis and many aspects of mucosal disease (Hume and Potten, 1979). Epithelia are able to respond to the altered physiological requirements, for example, by increased proliferation for tissue regeneration after wounding (Mackenzie, 1987), but altered patterns of proliferation also underlie pathological changes such as those occurring during carcinogenesis (Meyer and Schroeder, 1975). It is now generally accepted that epithelial renewal is associated with a stem and amplifying pattern of cell proliferation but, as yet, there is little information about either the number or distribution of stem cells in human oral mucosa.

1.2 Structure of keratinising epithelia

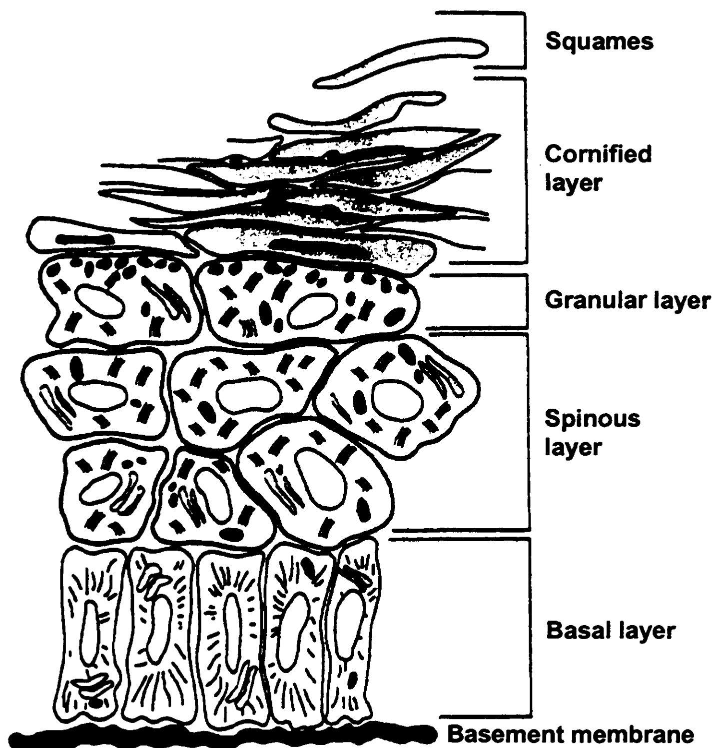
Human skin is made up of two tissue layers, the epidermis and the dermis. The epidermis is the outer layer of skin and provides the organism with a physical barrier which protects it from the environment. On most sites of the body the epidermis varies in thickness from 75 to 150μm, the exception to this being the palms and soles where the thickness is 0.4 to 0.6mm or greater.

The epidermis has an outer layer of anucleated cornified cells (the stratum corneum) and a living inner layer from which the surface cells arise by differentiation. The principal cell type here, the epidermal cell, is commonly called a keratinocyte because of the fibrous proteins, called keratins, which make up the vast majority of the cell cytoplasm. The epidermis also contains populations of other cell types such as melanocytes, Langerhans' cells and Merkel cells. Keratinocytes originate from the superficial ectoderm of the embryo and assemble into layers and in some tissues, into columns. All keratinocytes contain rough endoplasmic reticulum, Golgi, ribosomes and mitochondria. The density volume of these organelles varies according to the cell strata. The keratinocyte plasma membrane is a lipid bilayer that becomes modified by submembranous components as it undergoes terminal differentiation giving it a thickened appearance.

The dermis, which underlies the epidermis, is a dense fibroelastic connective tissue and constitutes the bulk of the skin. The thickness of the dermis varies depending with the body site. Vascular and nerve networks are situated in the dermis, as are specialised excretory and secretory glands and keratinised appendages. Beneath the skin is a layer of subcutaneous tissue (or hypodermis) which is composed of loose areolar connective tissue or fatty tissue.

Keratinised oral mucosa is very similar in structure to epidermis, as they are both stratified squamous epithelia. Hard palate is an example of keratinised oral mucosa and is typically subdivided into the following four layers, as shown in figure 1.1.

Figure 1.1 Structure of stratified squamous epithelium. The figure illustrates the four layers, basal, spinous, granular and cornified. All cells above the basal cells are considered to be suprabasal.



Basal layer (stratum basale)

The basal layer of keratinocytes is the germinative layer. Basal keratinocytes are cuboidal in shape and rest on the basement membrane (BM). Basal cells are bordered laterally by other basal cells and superiorly by suprabasal spinous cells. The cells contain a nucleus which is oval and elongated, and an array of cytoplasmic intermediate filaments (IF), organelles such as Golgi complexes, mitochondria, endoplasmic reticulum and ribosomes, and markers of cell replication such as centrioles and nucleoli. The keratins are precursors of the IF, which are organised in bundles surrounding the nucleus. The structure, composition, organisation and stability of the keratin filament cytoskeleton gives the basal cell mechanical strength, and makes up 30% of the basal cell protein. The suprabasal cells contain more than twice this quantity of keratin. The keratinocytes attach

to each other by highly developed cellular attachments called desmosomes which are the points of mechanical adherence between the cells (Chambers and Rényi, 1925).

Spinous layer (stratum spinosum)

The spinous layer comprises several layers of polyhedral cells lying above the germinal layer. As cells leave the basal layer they lose contact with the extracellular matrix proteins, and begin to express markers of terminal differentiation such as involucrin (Adams and Watt, 1989). The 3 to 4 layers of cells in this layer show a gradient in size, orientation, shape and cytoplasmic structure. The spinous cells start oval and become flattened as they near the granular layer. The spinous cells contain large, dense bundles of keratin filaments. The keratin is organised concentrically around the nucleus and small bundles of filaments extend up to the periphery of the cell into the desmosomes. Cells in the spinous layer begin to make precursors of the cornified envelope of the keratinocyte cells, such as involucrin (Rice and Green, 1977).

Granular layer (stratum granulosum)

The granular layer is a layer of flattened nucleated cells which contain distinctive cytoplasmic inclusions, the keratohyalin granules. This granular layer is typically 3 to 4 cells thick but is thicker in palm and sole. Keratohyalin granules are composed of the IF-associated protein, profilaggrin that is deposited at the points of intersection of keratin filament bundles. Profilaggrin is a precursor for filaggrin, which is involved in the aggregation of keratin filaments (Rothnagel and Steinert, 1990). The keratohyalin granules become larger as the cells move to the outermost granular layer. In some cells, the keratohyalin granules form interconnecting masses that appear to involve the majority of the keratin filaments in the cell. Loricrin is another structural protein that later becomes incorporated into the cornified envelope (Mehrel *et al.*, 1990). Granular cells synthesise and cross-link a number of structural proteins that form the cornified cell envelope of the stratum corneum cell and the keratin filaments become organised into larger bundles called macrofibrils (Kopan *et al.*, 1987). As the cells in the granular layer transit to the first cornified layer, their cytoplasmic organelles and nuclei are lost. The permeability of the cell increases which activates the enzyme transglutaminase which

catalyses cross-linking of nonkeratin proteins into an envelope located beneath the plasma membrane.

Cornified layer (stratum corneum)

The stratum corneum consists of multiple layers of terminally differentiated keratinocytes suspended in an extracellular lipid matrix. It is typically made up of 15-20 layers of flat anucleate cells, termed squames. The number of cell layers in the stratum corneum varies between the thick skin of the palms and soles, and the thin skin of the body. These cornified cells are about 0.5μm thick and have diameters of between 30-40μm compared to basal cells which have a diameter of 6-8μm (Plewig and Marples, 1970). These flat, plate-like cornified cells become modified so that they adhere to one another but they also need mechanisms to come apart. The superior and inferior surfaces of the cells have ridges, undulations and villi that allow them to become interlocked. The borders of adjacent cells overlap, interdigitate and insert into each other. These squames are dead and provide mechanical protection. In some keratinising tissues, these squames are arranged as ordered stacks of hexagonal squames (Mackenzie, 1970; Potten, 1974). By staining sheets of epidermis, it is possible to visualise a group of 10-12 basal cells underneath each squame. The desmosomes become structurally modified as basal keratinocytes differentiate through the stratum granulosum and stratum corneum. Before desquamation, the desmosomes at the outer layer of the stratum corneum are degraded.

1.3 Dermis

The dermis is made up of collagen fibres, elastic fibres and an interfibrillar gel of glycoseaminoglycans, salts and water to make a dense fibroelastic connective tissue. The main cell of the dermis is the dermal fibroblast, which synthesises and monitors the structural elements. Collagen, which makes up a large part of the skin, provides the tensile strength of the dermis. Collagen type I is the major collagen of the dermis, making up about 62%, with collagen type III being less abundant, making up 15%. Between the bundles of collagen is a network of fibres that gives skin its elastic properties. Perivascular mast cells are found in the connective tissue along with tissue macrophages.

The connective tissue supports the fibroblasts and encloses a nerve network, epithelial glands, keratinising appendages and a microcirculatory vascular and lymphatic system. The fibroblasts are an important cell type within the connective tissue as they provide the necessary requirements for the growth of the keratinocytes.

1.4 Basement membrane

Cells in the basal layer are in contact with an underlying basement membrane (BM) composed mainly of type IV collagen (Yaoita *et al.*, 1978) and laminin. Also present in the BM are proteoglycans and nidogen (Burgeson and Christiano, 1997). Dermal fibroblasts, together with keratinocytes, secrete these extracellular matrix proteins (Marinkovich *et al.*, 1993). The BM separates the epithelium from the connective tissue and provides a scaffold which allows the basal keratinocytes to adhere via $\alpha6\beta4$ integrin within the hemidesmosomal complex to its ligand kalinin/laminin-5 in the extracellular matrix (Tomakidi *et al.*, 1997).

Below the plasma membrane of the basal cells is an electron-lucent zone termed the lamina lucida. Below this is an electron dense amorphous layer, the lamina densa, which has loops of collagen type VII anchoring fibrils inserted into it. Collagen fibrils interlock with the anchoring fibrils forming a lower layer. At the sites of hemidesmosomes, the anchoring fibrils in the lamina densa break into smaller filaments, traverse the lamina lucida and approach the plasma membrane of the basal cells (Susi *et al.*, 1967). Microfilaments in the lamina densa are composed of type IV collagen and are associated with non-collagenous glycoproteins. The lamina lucida consists of fine filaments, the glycoprotein laminin (Terranova *et al.*, 1980) and the proteoglycan heparin sulphate (Kanwar and Farquhar, 1979).

1.5 Adhesive cell junctions of keratinocytes

Desmosomes and hemidesmosomes

Cells are linked to each other and to the underlying matrix by specialisations of the plasma membrane called desmosomes and hemidesmosomes. Desmosomes are focal junctions between adjoining keratinocytes that are primarily involved in adhesion, and are present in all layers except the stratum corneum. Desmosomes are made up of intra- and extra-cellular components that function in cell-cell adhesion and attachment of filaments within the cell. The desmosome spans two adjacent cells, forming plaques. Keratin filaments insert into the plaque via plaque proteins that bind keratin polypeptides (Stappenbeck and Green, 1992). Some of the desmosomal transmembrane glycoproteins belong to the cadherin family (Collins *et al.*, 1991). Keratin filaments formed by K5 (K=keratin) and K14 *in vivo* associate both with hemidesmosomes and with desmosomes in the basal layer (Hutton *et al.*, 1998). *In vitro* studies suggest that the interaction between desmosomes and filaments occurs through binding of the desmoplakin tail segment to the head domain of type II epidermal keratins (Jakic-Razumovic *et al.*, 1998).

Unlike desmosomes, hemidesmosomes are asymmetrical structures that connect cells to the basal lamina. They also have bundles of IF anchored to a dense plaque but the extracellular region is associated with anchoring filaments that span the basal lamina and connect the plasma membrane to thick anchoring fibrils in the stroma (Kowalczyk *et al.*, 1999). The basal keratinocytes adhere to the BM proteins via integrins. Integrins are heterodimeric glycoproteins made up of an α and a β subunit (Hynes *et al.*, 1999). A variety of integrins are expressed in keratinocytes such as $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta4$. Integrin $\alpha2\beta1$ binds collagen type I, collagen type IV and laminin 1. Integrin $\alpha3\beta1$ binds laminins 1 and 5 (Adams and Watt, 1991) and integrin $\alpha6\beta4$ binds laminin 1 (Carter *et al.*, 1990). Cultured keratinocytes express $\alpha5\beta1$ and $\alpha v\beta5$ integrins which bind fibronectin and vitronectin respectively. Keratinocytes are linked to the underlying membrane via two types of junction, hemidesmosomes and focal contacts. Hemidesmosomes link keratin filaments to BM proteins via the $\alpha6\beta4$ integrin. Expression of the integrins is predominantly confined to the basal cell layer *in vivo* and *in*

vitro, however $\alpha 6$ and $\beta 4$ show a stronger concentration at the BM zone. *In vivo* and *in vitro,* $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins are expressed at the lateral and apical borders of basal cells, but it is not known whether they are involved in cell-cell adhesion (De Luca *et al.*, 1990; Hertle *et al.*, 1991).

Desmosomes and hemidesmosomes occupy over 50% of the cell membrane of epidermal keratinocytes. Cultured keratinocytes cohere by forming intercellular adherens junctions in addition to desmosomes and attach to the substratum primarily by means of focal contacts. The adhesive components of focal contacts and hemidesmosomes are members of the integrin family.

Adherens and gap junctions

Cultured keratinocytes also form focal contacts with their substrate and form adherens and gap junctions with each other in both culture and tissue (Salomon *et al.*, 1988). Adherens junctions are sites of cell-cell and cell-substrate contact, and like desmosomes have a cytoplasmic plaque and membranous domain. Gap junctions which serve as intercellular routes of chemical communication are seen frequently in cultured keratinocytes grown in low calcium medium but few are seen within the epidermis.

Homophilic interactions between transmembrane proteins called cadherins mediate cell-cell adhesion between adjacent cells at adherens junctions (Yap *et al.*, 1997). The actin cytoskeleton inside the cell is linked to these junctions. Keratinocytes express two cadherins, E- and P-cadherin. The cytoplasmic tail of cadherin binds to the cytoplasmic proteins α catenin, β catenin and plakoglobin. Catenins can regulate cadherin function. Desmogleins and desmocollins, which are specialised members of the cadherin superfamily, mediate cell-cell adhesion at desmosomes. The formation of desmosomes, as well as adherens junctions, depends on the presence of extracellular calcium.

1.6 ORAL MUCOSA

1.6.1 Introduction

Oral mucosa has either a keratinised, non-keratinised or specialised epithelium. Non-keratinised epithelium forms the surface of the soft palate, ventral surface of the tongue, floor of the mouth, alveolar mucosa, vestibule and cheek. Keratinised epithelium makes up the lips and is found in less flexible masticatory mucosa, such as hard palate and in parts of the dorsum of the tongue and gingiva. The palatal epithelium is a predominately orthokeratinised tissue with finger-like projections called rete separated by connective tissue papillae. The average thickness of the palatal epithelium is 0.25mm (Jani and Bhargava, 1976), but is thinner over the connective tissue papillae. As with epidermis, the cells in oral mucosa change their shape and size during maturation. In non-keratinised epithelium basal cells are cuboidal or oval whereas those in keratinised epithelium are more columnar. In keratinised epithelium cells assume a polygonal shape in the lower spinous layer and become flattened in the upper stratum spinosum. Maturation in palatal epithelium is very similar to that of epidermis. Suprabasal cells in the deeper regions of the palatal rete retain a similar shape and pattern of histological staining as the basal cells which has been suggested to indicate a slower rate of maturation in these regions (Nair and Schroeder, 1981). As these cells move towards the surface, they increase in size. The nuclei of basal cells are large in relation to the size of the cell, and account for up to a third of the tissue volume in the basal layer (Meyer and Schroeder, 1975).

1.7 Cell renewal in palatal epithelium

The epithelial lining of the oral cavity maintains its natural structural integrity and protective function by a continuous process of cell renewal. Stratified squamous epithelia can be classified as having three phases of activity. A progenitor compartment, which is made up of stem and amplifying cells; a maturing compartment, which is made up of transit amplifying cells, and an outer functional protective compartment which is made up of post mitotic and terminally differentiated cells.

The new cells are produced by mitosis in the progenitor compartment and migrate through the maturing and outer compartments to the surface from where they are shed. Under steady state conditions, the rate of loss of cells from the surface is balanced by the production of new cells, and cells in the basal layer represent those in the progenitor compartment. Cell division is not randomly distributed along the BM, instead it is organised with clusters of DNA synthesising and dividing cells, suggesting that the basal cells are heterogeneous.

1.8 Non-keratinocytes in oral mucosa

There are four kinds of cells which make up oral mucosal epithelium and epidermis. The major cell is the keratinocyte and the other non-keratinocytes are Langerhans' cells, melanocytes and Merkel cells. The non-keratinocytes constitute up to 10% of the epithelial population in mouse ear epidermis but this value is less in human oral mucosa, and appear as clear cells in histologic sections as their nuclei are surrounded by a clear halo or space. Melanocytes and Merkel cells are usually present in the basal layer, and Langerhans' cells in the spinous, and sometimes granular layers. These non-keratinocytes do not persist *in vitro.*

Langerhans' cells are formed in the bone marrow and have an immune function in stratified squamous epithelia. They are distributed as a suprabasal network (Schroeder and Theilade, 1966; Waterhouse and Squier, 1967). Langerhans' cells possess specific immunological receptors and cell surface antigens, structurally they have an elongated cell body and long dendritic processes. They can be seen from the third suprabasal cell row up to the granular cell layer. Langerhans' cells processes radiate laterally between adjacent spinous cells and the dendrites of one cell are very close to the tips of another.

Melanocytes originate in the neural crest, they appear as dendritic cells in the basal layer and contain characteristic melanin-containing organelles called melanosomes. They do not form desmosomal attachments with keratinocytes. Melanocytes confer a protective

function in skin against ultraviolet rays but it is not known what function they perform in oral mucosa.

Merkel cells appear as clear cells near the base of the epithelial ridges of oral epithelium and epidermis (Squier and Hill, 1994). They either occur singly or in clusters in the basal layer (Hashimoto, 1972) and are slightly larger than the surrounding keratinocytes with which they form a few desmosomal attachments. This cell is sometimes in contact with the basal lamina with which it forms hemidesmosomes. Merkel cells are neurosecretory cells of the skin and have epithelial features such as expression of K8, K18, K19 and K20. The nucleus and cytoplasm of a Merkel cell is much less opaque than in keratinocytes and the nucleus is deeply invaginated (Garant *et al.*, 1980). Over the surface of the Merkel cell small cytoplasmic processes are evenly placed at 1-2μm intervals and project into adjacent keratinocytes. Merkel cells are scarcely distributed in adult human skin but are present in hair follicles and have been localised to the basal layer of the bottom of the rete ridges in palm and sole (Fradette *et al.*, 1995).

1.9 The culture of human keratinocytes

Much of the current understanding of human keratinocytes comes from the development of techniques for growing keratinocytes in the laboratory. It is possible to disaggregate epithelium with proteolytic enzymes and to seed individual keratinocytes in culture but unless the cells are seeded at a very high density subculturing is unsuccessful. Rheinwald and Green (Rheinwald and Green, 1975) showed that it was possible to grow and subculture single cell suspensions of keratinocytes on an irradiated feeder layer of 3T3 mouse embryo cells which secrete growth factors and extracellular matrix proteins (Alitalo *et al.*, 1982) that encourage keratinocyte attachment and proliferation. Irradiating the 3T3 cells stops them from growing but not from secreting the necessary factors, and this is also achieved by treatment with Mitomycin C (Lechner *et al.*, 1981).

The culture medium developed by Rheinwald and Green for keratinocyte growth consists of one part Hams' F12 medium and three parts Dulbecco's Modification of Eagle's

Medium (DMEM) which is supplemented with foetal bovine serum (FBS), adenine, insulin, hydrocortisone, cholera toxin and epidermal growth factor (EGF) (Rheinwald, 1989). Cholera toxin has a potent effect in increasing the overall rate of cell proliferation and increases the proportion of small cells in the colonies (Rheinwald and Green, 1975; Green, 1978). EGF prolongs the number of cell generations before senescence without affecting the growth rate (Rheinwald and Green, 1977) and also stimulates proliferation by stimulating outward migration of rapidly dividing keratinocytes at the edges of the colonies (Barrandon and Green, 1987a). Keratinocytes grown this way can expand rapidly in culture and keratinocyte sheets grown in this way can be used as skin grafts in burns patients (Nanchahal *et al.*, 2002).

Keratinocyte cell culture allows the production of large numbers of keratinocytes from small biopsies. Keratinocytes from neonatal foreskin can undergo 80-100 population doublings before senescence (Rheinwald, 1989). Cultures from older donors have a reduced population doubling capacity compared to younger donors (Gilchrest, 1983). In culture, keratinocytes can become confluent as stratified sheets some 6 to 8 layers thick. Proliferation is confined to the basal layer which show some limited expression of markers, and the suprabasal cells express markers of the spinous, granular and cornified layers. In culture, the cell layers are flattened and distinct granular and cornified layers do not form, but once these cultures are grafted onto human recipients or mice, the histological appearance of the cell layers becomes normal suggesting deficiencies in the cell culture environment. Keratinocytes from other stratified squamous epithelia can also be grown using the Rheinwald and Green method (Rheinwald and Green, 1975). It is possible to culture keratinocytes on plastic, with natural or artificial substrates in the presence of other cells, in a variety of media, and to graft them onto animals.

1.10 Epithelial and fibroblast proliferation

Epithelial cells and fibroblasts grown *in vitro* gradually lose their ability to proliferate. When normal embryonic fibroblasts from skin are cultured, the cells are able to go through about 50 population doublings before senescing. When fibroblasts are derived

from adult human tissue, they are only able to undergo about 20 population doublings before dying. This suggests that the age of the donor affects the population doubling potential. This phenomenon is known as the Hayflick effect (Hayflick and Moorhead, 1961). Also, cells derived from long-lived species can undergo more population doublings than cells derived from short-lived species.

In senescent cells, many changes occur including the loss of proliferative potential and reduced protein synthesis, but little is known about the primary cause of cell ageing and cell death. The Hayflick effect might only be a tissue culture artefact as, *in vivo,* the small number of stem cells present that are capable of indefinite multiplication continually produce a population of cells that have less proliferative potential (Hayflick, 1965). Culture conditions could eliminate the small stem cell fraction leaving the rest of the cells to undergo limited numbers of divisions.

1.11 Epithelial-mesenchymal interactions regulate growth and differentiation *in vitro*

In conventional cell culture, when keratinocytes are grown on plastic and submerged in medium, their differentiation program is only rudimentarily achieved with deficiencies in both the structural arrangement of the tissue and in the expression of differentiation markers. This is due to the lack of proper epithelial-mesenchymal interactions which control epithelial homeostasis *in vivo* (Fusenig, 1994). Connective tissue influences are essential for regular epithelial growth and differentiation. Work in developmental biology has indicated that during embryogenesis, the normal development of epithelia of many organs depend on epithelial-mesenchymal interactions (Sawyer and Fallon, 1983). It has been demonstrated that the underlying mesenchyme has both instructive and permissive effects on morphogenesis and *in situ* differentiation of adult epithelia when epithelial and connective tissue components of different organs are used (Mackenzie and Dabelsteen, 1987). Transplantation studies with isolated and cultured keratinocytes showed that the deficient display of epithelial growth and differentiation characteristics *in vitro* was due to the absence of a mesenchymal influence operating. These studies demonstrate that

epidermal homeostasis and differentiation programs are regulated by diffusible factors provided by the mesenchyme, and the isolated and cultured epithelial cells do not lose their intrinsic potential for tissue specific differentiation, but do still respond to appropriate extrinsic stimuli (Mackenzie and Dabelsteen, 1987). These findings were important for the development of culture model systems to study epithelial-mesenchymal interactions *in vitro*. Cocultured keratinocytes induce mesenchymal feeder cells to express growth factors known to exert stimulatory effects on keratinocytes in a paracrine fashion, such as keratinocyte growth factor (KGF) and interleukin 6 (Aaronson *et al.*, 1990). Epithelial-mesenchymal interactions are difficult to study under *in vivo* conditions as there are so many variables involved and experimental conditions cannot be properly controlled and modified. Some understanding of how the mesenchyme controls epithelial differentiation has been investigated by the development of *in vitro* models such as the organotypic culture system.

There is evidence that epidermal tissue homeostasis is regulated by a cytokine network between keratinocytes and dermal fibroblasts and there is also a strong dependence of keratinocyte growth *in vitro* on mesenchymal interactions as was demonstrated in two-dimensional feeder layer cocultures of keratinocytes and 3T3 cells (Rheinwald and Green, 1975). This mesenchymal support is based on matrix components and diffusible factors produced by fibroblasts (Smola *et al.*, 1993). The molecular basis of dermal-epidermal interactions has been further elaborated in a more physiologic context *in vitro,* using organotypic culture models. In these organotypic culture models a novel double paracrine mechanism by which keratinocytes control their proliferation has been documented (Maas-Szabowski *et al.*, 2001). Through the release of interleukin-1 (IL-1), they induce growth factors such as KGF in dermal cells, which in turn stimulate keratinocyte proliferation (Smola *et al.*, 1993). KGF is a product of the fibroblasts and is produced by mesenchymal cells only and the receptor is exclusively found in epithelial cells. Immortal cell lines of epithelial and mesenchymal cells can be used in culture but some cell lines may have lost their differentiation capacity or aberrantly express differentiation markers without the mesenchymal support. This was demonstrated with the spontaneously immortalised HaCaT keratinocyte cell line (Ryle *et al.*, 1989).

1.12 STEM CELLS

1.12.1 Introduction

Human epidermis is continuously renewed and the outermost cell layer is shed from the body approximately every 24 hours (Baker and Kligman, 1967; Jansen *et al.*, 1974; Roberts and Marks, 1980). This is the main route by which cells are lost from both epidermis and oral mucosa and there is no evidence that apoptosis occurs in normal healthy epithelium (Gandarillas *et al.*, 1999). The mechanism of balancing the rate of cell division with the rate of cell loss is imperative for epithelial homeostasis and must be maintained for the lifetime of the organism. This balance between proliferation and terminal differentiation ensures that the epithelium maintains a constant thickness. In addition, epithelium is able to regenerate and re-establish homeostasis following injury (Potten, 1981). These epithelial properties are reliant on a population of cells, known as stem cells.

Many different types of stem cells maintain the mammalian skin and its associated structures such as hair follicles, sebaceous glands and sweat glands. In interfollicular epidermis, a steady flow of differentiating keratinocytes are supplied by epidermal stem cells located in the basal layer (Watt and Hogan, 2000) and in the deep rete ridges (Lavker and Sun, 1982).

In most mammalian tissues a proliferative hierarchy of stem, amplifying and terminally differentiating cells is responsible for tissue renewal. In rapidly proliferating tissues such as cutaneous epithelium a stem cell can be defined as an undifferentiated, generally quiescent cell that has a high proliferative potential that gives rise both to other stem cells and to proliferative transit amplifying cells. These transit amplifying cells are responsible for the immediate replenishment of cells that are lost to the environment after terminal differentiation (Cotsarelis *et al.*, 1999). One property of stem cells is that their division is asymmetric giving rise both to cells that are identical to themselves and to cells that are different. The mechanism for this may be intrinsic or extrinsic. An interesting issue is

how such asymmetry is programmed into stem cells that are sometimes both identical to the parent cell and other times both different. If epithelial homeostasis is to be maintained, the rate of production of new cells in the basal layer must be balanced by the rate of loss of terminally differentiated cells from the tissue surface. Thus on average for each stem cell division, one daughter remains a stem cell and the other will eventually terminally differentiate.

A minimal definition of a stem cell is a cell with an extensive self-renewal capacity that extends throughout the life span of the organism (Lajtha, 1979), but particular stem cell systems exhibit additional tissue specific properties. In stratifying epithelia, three points are of importance. First, only a fraction of the proliferative basal cells is capable of indefinite self-renewal, i.e., are stem cells. Second, each stem cell division in the steady state results in one daughter cell that remains a stem cell and another cell that is committed to differentiate. Third, the committed daughter cells undergo a limited number of amplification divisions in order to increase cell production before they terminally differentiate.

The stem cells occupy precise positions within the epithelium but it is not clear yet how they are different intrinsically from other proliferative cells. If the function of a stem cell is determined by its position within the tissue, it is still unclear what characterises these positions, niches or microenvironments (Hall and Watt, 1989), and how these intrinsic properties change when these cells are placed in culture.

1.13 Types of stem cells

1.13.1 Pluripotent stem cells

Pluripotent stem cells can give rise to every cell type in the animal body and are not derived from adult but from embryonic tissues. There are three types of mammalian pluripotent cell lines that have been isolated: embryonal carcinoma (EC) cells which come from the stem cells of testicular tumours; embryonic stem (ES) cells which are derived from pre-implantation embryos; and embryonic germ (EG) cells which are

derived from primordial germ cells of the post-implantation embryo (Donovan and Gearhart, 2001). Pluripotent stem cells are derived from germ cells and blastocysts of discarded human embryos. All other stem cells are derived from cells of the animal body, the somatic cells. In adult animals, only the germ cells retain the ability to make a new organism, a feature known as developmental totipotency.

1.13.2 Adult stem cells

Adult stem cells were initially thought to have a limited potential and be restricted to the production of differentiated cells, but a range of adult somatic stem cells can be induced by appropriate developmental signals to contribute to the formation of a much wider range of tissues than had been previously thought. Neural stem cells can form blood-forming and muscle tissue (Bjornson *et al.*, 1999); mesenchymal stem cells can produce differentiated cell types in the brain (Mezey *et al.*, 2000); human dermal stem cells can make neurons, glia, smooth muscle and adipocytes *in vitro* (Toma *et al.*, 2001); and murine epidermal stem cells retain the capacity to produce cells of all three germ layers (ectoderm, mesenchyme and neural-crest) *in vivo* (Liang and Bickenbach, 2002). Nuclear cloning experiments have demonstrated that the nuclei of somatic cells can be reprogrammed to allow them to become totipotent and recapitulate development (Wakayama *et al.*, 1998), but in the normal life cycle of animal species they do not do so.

1.13.3 Haematopoietic stem cells

Haematopoietic stem cells are multipotent and produce erythrocytes and all the types of white blood cells. Haematopoietic stem cells have been isolated from mice and humans and have been shown to be responsible for the generation and regeneration of the blood-forming and immune systems. Stem cells from a variety of organs might have the potential to be used for therapy in the future, but haematopoietic stem cells which are the vital elements in bone marrow transplantation, have been used extensively for the treatment of leukaemias.

1.13.4 Intestinal stem cells

Epithelial cells in the small and large intestine originate from stem cells that are located towards the base of the crypts of Lieberkühn (Potten *et al.*, 1997). These crypts are small inpocketings of the gut surface lined by a single layer of columnar cells that lie against a BM. Lineage-tracing experiments have identified 4 to 5 stem cells that reside near the bottom of each crypt. The stem cell progeny differentiate into four main cell types - Paneth, enteroendocrine, goblet and columnar, these cells differentiate as they migrate up and down the walls of the crypt. Lineage labelling has still not pinpointed the exact number and location of stem cells in the basal region of the crypt (Spradling *et al.*, 2001).

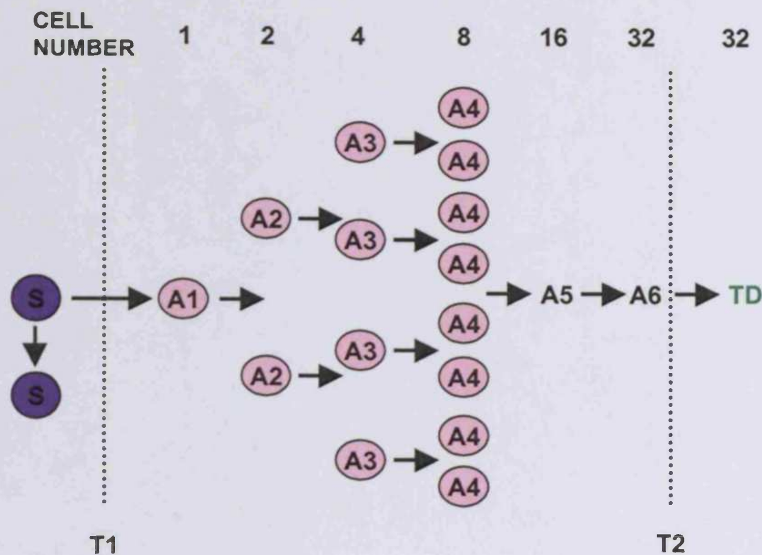
1.14 Stem cell location

Stem cells in human tissues cannot be localised by *in vivo* labelling methods and evidence for stem and amplifying cells in human epithelia has been gained mainly from a range of *in vitro* studies (Cotsarelis *et al.*, 1999). Individual human epidermal keratinocytes are capable of clonal growth *in vitro* (Rheinwald and Green, 1975) and although many cells have only limited growth potential, some cells form colonies that show expansive growth and can be repeatedly passaged (Barrandon and Green, 1987b). The "clonogenic" cells that give rise to such colonies are smaller than other cells and are assumed to correspond to the stem cells present *in vivo* (Barrandon and Green, 1985). Assays of the clonogenicity of cells isolated from micro-dissected regions of human epithelia have confirmed that clonogenic cells (Rochat *et al.*, 1994; Cotsarelis *et al.*, 1999) are localised to sites such as the limbal region of the cornea (Cotsarelis *et al.*, 1989) and the bulge region of hair follicles (Cotsarelis *et al.*, 1990), the anatomical sites shown to contain label retaining cells in murine epithelia (Cotsarelis *et al.*, 1999). It is thought that stable microenvironments might control the stem cells and these regions have been called 'niches'. A niche is considered to be a subset of tissue cells and extracellular substrates that can indefinitely house one or more stem cells and control their self-renewal and progeny production *in vivo.*

1.15 Regulation of stem cells

Figure 1.2 illustrates a scheme for stem cell renewal that is hypothesised to function in normal epithelia. Each stem cell division normally produces one stem cell (S) and one amplifying cell (A1). The amplifying cell undergoes a series of divisions (A2-A6) to produce terminally differentiating cells (TD). In figure 1.2, one stem cell division is followed by six amplifying divisions to produce 32 differentiating cells, which is enough to maintain a murine epidermal unit for a month. The three cell compartments (S, A and TD) are separated by two transitions, T1 and T2. Lack of transition from S to A leads to the accumulation of stem cells and the increased transition of S to A leads to stem cell loss and atrophy. The T2 transition controls the number of differentiated cells produced per stem cell division, if the tier is shifted to the left, the cell number halves, and if the tier is shifted to the right, the cell number doubles. This cascade system theoretically leads to epithelial homeostasis and can also be used to describe epithelial pathoses. For example, if the division rate of the stem cells increases this could lead to a type of hyperplasia associated with the promotion of carcinogenesis, whereas if the division rate of the amplifying cells increases, this could lead to benign epithelial hyperplasia (Mackenzie and Bickenbach, 1982). During cell division, cells are at particular risk to genetic damage or change, and the infrequent division of epithelial stem cells may be a protective mechanism against tumour development.

Figure 1.2 Stem cell proliferation system. This figure illustrates a scheme for stem cell renewal in epithelia. Each stem cell division normally produces one stem cell (S) and one amplifying cell (A1). This amplifying cell undergoes a series of divisions (A2-A6) to produce terminally differentiating cells (TD). The cell number after each cell division appears at the top of the figure.



Terminal differentiation both removes cells from the proliferative pool and leads to the formation of functional cells, and is considered to be the primary homeostatic control for cell production (Hall and Watt, 1989). Alternative pathways of programmed cell death exist, and apoptosis is a widespread mechanism that deletes developmental cells, eliminates damaged or altered cells and occurs in a range of epithelial pathologies including UV damage, but it is uncertain whether apoptosis plays a physiological role in the homeostasis of stratified squamous epithelia (Haake and Polakowska, 1993). Terminal differentiation has been considered to be a modified form of the apoptotic pathway (Polakowska *et al.*, 1994). An apoptotic event deletes only one cell, and apoptosis is rarely seen in normal epidermis. Within the stem cell proliferative hierarchy a single apoptotic event could have an influence on cell production. If an apoptotic event occurred at the stem cell level, it would delete an entire clone. An apoptotic event at the stem cell daughter level would delete all cell production for one stem cell cycle, and an

apoptotic event at the first amplification level, half of all cell production would be deleted.

There is now evidence that stem cell fate is responsive to extrinsic signals (Watt, 1998). Several molecules have been implicated in regulating the decision of a stem cell to become a transit amplifying cell. High levels of β1 integrin activate MAPK (mitogen-activated protein kinase) mediated signals that are required for keratinocytes to remain as stem cells (Zhu *et al.*, 1999). Stem cells have higher levels of non-cadherin-associated β-catenin than transit amplifying cells *in vitro,* and experiments using dominant negative and active β-catenin mutants have provided evidence that β-catenin signalling can maintain a stem cell fate (Zhu and Watt, 1999). Similar approaches have shown that c-Myc acts selectively on stem cells, driving them into the transit amplifying compartment (Gandarillas and Watt, 1997).

1.16 Spatial arrangement of stem cells and transit amplifying cells

1.16.1 Murine stem cells

Early radio-labelling studies of mucosal epithelial basal cells suggested that a particular basal cell divides or differentiates as a chance event (Leblond, 1964), but non-random patterns of cell division in murine epidermis (Mackenzie, 1970), together with other cell kinetic properties (Potten, 1981), led to a concept of self-renewing stem cells that divide both to maintain themselves and to produce other daughter cells that amplify the cell population by undergoing several more divisions before differentiating

1.16.2 Label retaining cells

Tritiated thymidine labelling studies have identified slowly cycling cells in mouse epidermis that are positioned towards the centre of a cluster of basal cells associated within each EPU (Potten, 1974). This was also demonstrated by labelling basal cells and waiting for the label to dilute to background levels in the faster cycling cells (Bickenbach, 1981; Morris *et al.*, 1985). Labelling the cells of young adult or neonatal mice continuously was most effective and gave high labelling indices. The cells that

retain the label for longer than the average were termed label retaining cells (LRCs) and were assumed to have divided less frequently and be more slowly cycling. There is a subpopulation of slowly cycling LRCs in rodent oral epithelium and epidermis that are located in the deep tips of epithelial rete, that retain a DNA label for at least 72 days whereas most of the labelled basal cells have left the basal layer within 2 to 3 days (Bickenbach, 1981). Other cell kinetic analyses and modelling studies have shown that the stem cells are more slowly cycling and have a cell cycle time of 200 hours (Potten *et al.*, 2000). The LRC work also shows that most of these labelled cells are located towards the centre of the EPU, which is the site suggested for the stem cells (Mackenzie and Bickenbach, 1985). The loss of label in the faster cycling cells could be attributed to the duration of the cell cycle, or the cells may be dividing transit amplifying cells from the basal layer that migrate to the upper layers. LRC studies in murine epithelia with more complex structures have localised the stem cells at the base of the tongue papillae and in the bulge region of hair follicles and it is thought that stem cells are distributed in relation to tissue architecture. Further evidence that LRCs could be stem cells comes from the evidence that LRCs are more clonogenic than other cells (Morris and Potten, 1994; Potten, 1974).

1.16.3 Epidermal proliferative unit

The cells in the basal layer on mouse back skin are arranged in a specific pattern that relates to the columnar organisation of the cells in the suprabasal layers. These columns of cells can be seen from the appearance of the epidermal surface after silver staining the epidermal sheets. These columns represent the proliferation of a group of about 10 to 11 basal cells that are located immediately below the column. These columns of basal and proliferating cells represent proliferative units and have been termed epidermal proliferative units (EPU) and within the EPU each basal cell has a characteristic position (Potten, 1974) and studies have shown that the mitotic cells are located at the boundaries of the hexagonal cell columns (EPU) (Mackenzie, 1970). Other studies have suggested that the basal layer has considerable cellular heterogeneity (Allen and Potten, 1974; Bickenbach, 1981; Lavker and Sun, 1982; Barrandon and Green, 1985; Staiano-Coico *et al.*, 1986). Radiobiological data suggest that each EPU possesses a single central

clonogenic cell that shows less frequent labelling than the peripheral cells (Potten, 1974), and that this cell may function as a stem cell or clonogenic cell and comprises about 10% of the basal cells. The cell cycle of the stem cells have been estimated to be twice that of the transit amplifying cells, and this slow cell cycle has been used as a means of identifying the stem cells.

In simple flat stratified epithelia such as the skin on the back of the mouse, the stem cells must be in the basal layer as cell proliferation occurs only in the basal layer. In more complex undulating stratified epithelia that contain rete ridges or pegs the stem cells have been identified in the basal layer but may be at various positions within it. In the dorsal surface of the tongue and filoform papillae in mouse, the stem cells have been identified as being in the deepest part of the basal layer that projects into the connective tissue (Hume and Potten, 1976).

1.16.4 Human epidermis

Human epidermis is markedly different from mouse epidermis in that dorsal mouse skin has only two living layers and is relatively flat whereas human epidermis has many cell layers and although the epidermal surface is flat, the basal layer undulates. The regions where the epidermis projects down into the dermis are called rete ridges, and the areas where the dermis reaches highest into the epidermis are called the dermal papillae (Odland, 1991).

An interesting question is whether proliferative units in human epidermis are arranged in the same way as the EPUs in mouse dorsal skin. Human epidermis does not have such a clear alignment between the basal cells as the columns of squames in mouse epidermis (Mackenzie and Zimmerman, 1981). It is not feasible to pulse label human skin with radioactive markers, but this has been carried out on palm skin of monkeys where tritiated thymidine was taken up less frequently by keratinocytes at the rete bases than in the rest of the basal layer. These studies have led to the proposal that the stem cells reside in the rete, and that the arrangement of the stem cells is related to the pattern of the rete rather than to the organisation of squames on the epidermal surface as in mouse.

It has been predicted that each columnar unit of structure in epidermis is the clonal lineage of a single stem cell (Mackenzie and Bickenbach, 1985). This has been confirmed by marking murine epithelial cell lineages with a replication-deficient retroviral vector (Mackenzie, 1997) and human oral keratinocytes have a clonal pattern of growth *in vitro* (Lindberg and Rheinwald, 1990). 25-40% of basal cells have been defined as β1 integrin bright (Jensen *et al.*, 1999) which would suggest that within a β1 integrin bright patch the stem cells would be interspersed with transit amplifying cells. From whole-mount labelling of human epidermis it appears as though the stem cells are in clusters which are surrounded by transit amplifying cells.

1.16.5 Lineage marking

Keratinocyte lineage markers are useful tools as they can be used to test models of epithelial organisation. There are two approaches to label cells experimentally and identify their progeny. The first approach is dyes that stain cell membranes or cytoplasm but these become diluted as the cell divides, and progeny can only be tracked up to a certain point. The second is to use genetic markers which are stably inherited. This allows the progeny to be tracked over the long term. These markers can be delivered to the target cell and integrated into its genome by using replication deficient retroviral vectors (Price, 1987). Retroviral technology has been optimised and is used for transduction of human keratinocytes. LacZ and alkaline phosphatase DNA have been put into these retroviruses, and have been used as lineage markers in mouse epidermis (Ghazizadeh *et al.*, 1999; Ghazizadeh and Taichman, 2001) and in human keratinocytes (Kolodka *et al.*, 1998). In murine epidermis, these lineage marking studies have indicated that the units of structure are clonal with each being renewed by a single stem cell (Mackenzie, 1997; Ghazizadeh and Taichman, 2001).

1.16.6 Transfection

Transfection of primary cell lines and stem cells can be a problem. Most methods work efficiently for cell lines, but in culture fail to transfect primary cell lines (Price *et al.*, 2001). Types of transfection include transient and stable transformants, and to detect gene expression reporter genes are assayed for and/or the analysis of RNA is carried out. The

development of reporter gene systems and selection methods for stable gene expression of transferred DNA have greatly expanded the applications for gene transfer technology.

There are two general approaches for delivering or introducing nucleic acids into mammalian cells (Unger, 1997). Transfection is the process whereby the nucleic acid sequences (as well as proteins and oligonucleotides) are introduced by either biochemical of physical processes. Biochemical methods include DEAE-dextran, calcium phosphate co-precipitation and liposome-mediated transfection processes. Physical transfection methods include the direct microinjection of materials, biolistic particle delivery (both methods deliver the materials by mechanically perforating the cell membrane), and electroporation. Electroporation exposes target cells to brief, defined electrical pulses to create transient pores that allow nucleic acids and proteins to cross the cell membrane. The following have been carried out on human epidermal keratinocytes - electroporation, lipofection, calcium phosphate co-precipitation, DEAE-dextran and polybrene-mediated transfection (viral infection) (Jiang *et al.,* 1991). Calcium phosphate co-precipitation is very cheap and is used on many cell types but it is generally not suitable for keratinocyte transfections, especially primary cultures as it is very inefficient. Lipofection reagents do vary in quality, but newer generations of reagents have vastly improved transfection efficiencies, but the lipids used can lead to cell cycle arrest or apoptosis.

The second general approach for delivering nucleic acids into cells is transduction. Transduction is a viral mediated process whereby target cells are transduced with a virus whose genome carries an inserted cloned sequence. While this can be an efficient method for some cell lines and tissues, the limitation of this technique is that it requires host cells to posses a viral receptor in order to achieve high efficiency transfer. This requirement limits the broad utility of this method of transfection to only select systems. While biochemical methods do not obtain the efficiencies of newer technologies, they do offer the following advantages: published protocols are available for numerous cell lines; these methodologies do not require removal of adherent cells for transfection to occur; and laboratories incur minimal expenditure for equipment.

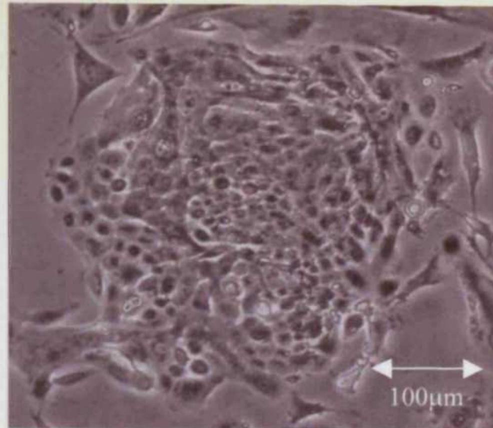
1.17 Tissue culture and stem cells

Epithelial stem cells differentiate when they are held in suspension (Adams and Watt, 1990) and *in vivo* epithelia degrade in the absence of connective tissue influences (Mackenzie, 1984; Hill and Mackenzie, 1989). When human epidermal keratinocytes are cultured they retain some of the characteristics of the tissue from which they are derived and this in theory makes keratinocytes a useful experimental model for studying stem cell properties.

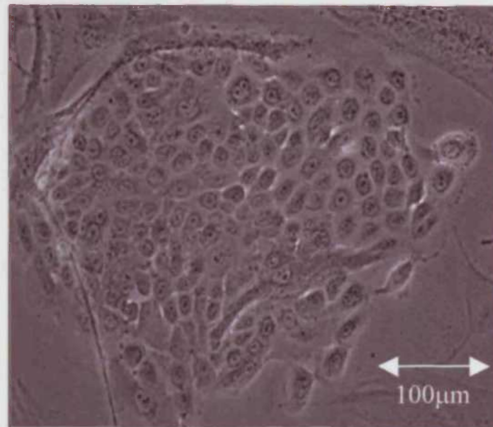
Keratinocyte cultures eventually senesce after several passages suggesting that stem cells do not persist in culture. However, when cultures are grafted onto suitable recipients, normal epidermis is reformed and survives for years, indicating that stem cells must persist at least in early passage cultures (Gallico, III *et al.*, 1984). There is evidence for proliferative heterogeneity in culture and subpopulations of keratinocytes have been identified that differ in cell cycle time and DNA synthesis, and withdrawal from the cell cycle has been shown to occur in a specific subset of dividing cells (Dover and Potten, 1983; Albers *et al.*, 1986; Albers *et al.*, 1987). The proliferative potential of individual keratinocytes has been shown to be inversely correlated to their size.

In culture, three types of colonies are formed *in vitro* by human keratinocytes, these are defined by appearance on the basis of the type of colony they found (Barrandon and Green, 1987b). One, the holoclone, is distinguished by a high reproductive capacity and the absence of terminal differentiation. The cells in the holoclone are small and compact. A second, the paraclone, consists of cells that stop dividing after a about 15 rounds of mitosis and undergo terminal differentiation. The cells in the paraclone are large with distinct nuclei. The third, the meroclone, is a mixture of cells of growth potential and represent a transitional stage between the holoclone and paraclone. The cells in the meroclone appear as a mixture of small and large cells. The transitions from holoclone to meroclone to paraclone are unidirectional and result in a progressive restriction of growth potential (Barrandon and Green, 1987b). It is believed that the founder cells of the holoclones are stem cells and those of the paraclones are transit amplifying cells. Cells in

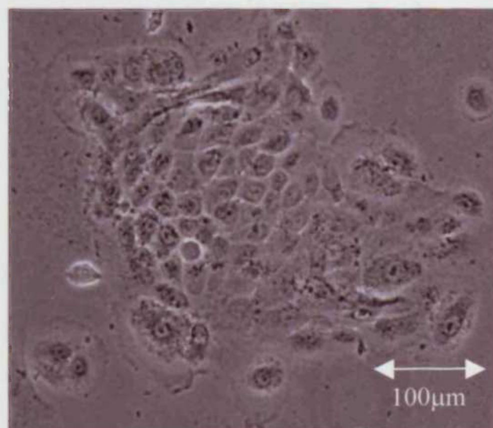
the paraclones have been shown to be able to undergo 15 population doublings producing approximately 1000 cells prior to terminal differentiation (Barrandon and Green, 1987b). The holo-, mero- and paraclone assay is labour intensive and time consuming so a simpler approach has been investigated by scoring terminal or abortive colonies (less than 32 cells per clone) (Watt, 2000). The keratinocyte colonies were classified as stem cells if there were more than 32 cells per colony. These examples show the ambiguity of the classification of stem cells *in vitro*. Examples of a holoclone, meroclone and paraclone are shown in Figure 1.3. Figure 1.3A shows small cobblestone-like cells which make up the compact holoclone. Figure 1.3B shows a meroclone which is made up of a mixture of small and larger cells. Figure 1.3C shows a paraclone made up of larger differentiating cells.



A



B



C

Figure 1.3 Palatal epithelial cells grown on plastic showing a holoclone (A), a meroclone (B) and a paraclone (C) (magnification x10)

1.18 Stem cell markers in stratified squamous epithelia

Many markers have been proposed to identify stem cells in various epithelial tissues. So far, no single stem cell property can reliably identify stem cells in any epithelial tissue. In most tissues, stem cells are few in number and as a result stem cells will need to be identified prospectively and purified carefully in order to study their properties. Some of the potential stem cell markers that have been described are outlined below.

β1 integrin

When basal cells were fractionated by flow cytometry there is a log linear relationship between the level of β1 integrin expression on basal cells and their proliferative capacity *in vitro* (Jones and Watt, 1993). Keratinocytes with characteristics of stem cells were isolated from cultured human epidermis on the basis of high β1 integrin expression and rapid adhesion to extracellular matrix proteins such as collagen type IV. Proliferating cells that adhered more slowly had characteristics of transit amplifying cells, and after one to five rounds of division all their daughters underwent terminal differentiation. The rapidly adhering cells resembled stem cells and over 80% of the cells that adhere within 20 minutes formed large colonies containing over 5000 cells. Stem cells have also been shown to express 2- to 3-fold higher levels of β1 integrin than transit amplifying cells (Jones *et al.*, 1995) using clonogenicity assays.

High levels of β1 integrin have also been used as a stem cell marker (Jensen *et al.*, 1999) within human epidermis of foreskin, breast and scalp with clusters of high level β1 integrin expression at the tops of the dermal papillae surrounded by β1 integrin dull cells which extend down into the rete. In contrast, in thickened epidermis such as palm and sole the β1 integrin bright cells were at the rete tips.

α6 integrin

Keratinocyte integrins play a role in controlling epidermal differentiation and morphogenesis, this suggests that differences in integrin expression or function may provide a marker to distinguish stem cells from transit amplifying cells (Jones and Watt,

1993; Watt and Jones, 1993; Watt *et al.*, 1993). High expression of $\alpha6$ integrin combined with the low expression of the surface antigen recognised by the monoclonal antibody 10G7, a transferrin receptor, have been proposed to identify stem cells (Li *et al.*, 1998). The $\alpha6^{bri}10G7^{dim}$ cells exhibit the greatest regenerative capacity of the basal cells, and also represent a minor subpopulation of immature epidermal cells which are quiescent at the time of isolation as determined by cell cycle analysis. The $\alpha6^{bri}10G7^{dim}$ population has also been shown to contain a purer fraction of keratinocyte stem cells than a $\beta1^{bri}10G7^{dim}$ population (Kaur and Li, 2000).

K19

K19 has a wide tissue distribution in that it is present in both stratified squamous and simple epithelia, unlike the other keratins that are usually present in either stratified or simple epithelia. Variations in K19 expression in various tissues have been observed, this could be due to the synthesis of it to compensate for reduced production of type I keratin, or overproduction of type II keratin (Stasiak *et al.*, 1989). It has been reported that whenever two different epithelial cell phenotypes coexist in close proximity to one another, and that they may have arisen from the same progenitor cell pool, K19 is abundant (Stasiak *et al.*, 1989). Also, where the tissue location of the progenitor compartment is known, K19 is often present.

K15

K15 is a type I keratin that does not have a defined type II partner. K15 is expressed by a subset of keratinocytes in the outer root sheath of the hair follicle (Waseem *et al.*, 1999). In hyperproliferative epidermis such as psoriasis and hypertrophic scars, K15 expression is downregulated and K15 is also absent from keratinocytes growing as skin equivalent cultures.

The hair follicle, like the epidermis contains a population of stem cells that is able to generate large numbers of epidermal keratinocytes (Rochat *et al.*, 1994). The localisation of stem cells in the hair follicle has relied upon three complementary approaches, the detection of slowly cycling cells (Lavker *et al.*, 1993), the detection of high colony

forming cells (clonogenicity assays), and immunohistochemical staining. Hair follicle stem cells have been localised to the bulge area within the hair follicle, with K15 and K19 being preferentially expressed in these hair follicle stem cell zones (Michel *et al.*, 1996; Lyle *et al.*, 1999). There still remains some controversy as to the contribution of hair follicle stem cells to the steady state of interfollicular epidermis.

bcl-2

The expression of this anti-apoptotic gene has been suggested as an epithelial stem cell marker (Polakowska *et al.*, 1994). It has been hypothesised that cell death is intrinsically programmed into the cell genome and proceeds unless suppressed by appropriate signals (Raff *et al.*, 1994) but would be held at bay in the stem cells by the expression of bcl-2.

p63

p63 is a member of the p53 tumour suppressor family of proteins, and plays a critical role in maintenance of progenitor-cell populations which are required for epithelial development and morphogenesis. In human epidermis, hair follicles and stratified epidermal cultures, p63 is expressed in the nuclei of cells that are either proliferating or possess the ability to multiply (Parsa *et al.*, 1999). p63 has been used to distinguish stem cells from their transit amplifying progeny in the basal cells of the limbal epithelium in cornea (Pellegrini *et al.*, 2001). In genetic studies using p53 transgenic knockout mice, the mice had no epidermis present showing that p53 plays a role in epidermal formation, and it is likely that p63 does too (Greenhalgh *et al.,* 1996).

Delta 1

In many developing tissues, signalling mediated by the transmembrane protein Delta 1 and its receptor Notch 1 inhibits differentiation. Delta 1 expression is confined to the basal layer of epidermis, with highest expression in those regions where stem cells reside (Lowell *et al.*, 2000). High Delta 1 expression by stem cells may have three effects: a protective effect on stem cell proliferative potential by blocking Notch signalling; enhanced cohesiveness of stem cell clusters; and signalling to cells at the edges of clusters to become transit amplifying cells (Lowell *et al.*, 2000).

1.19 Uncertainty of stem cell positions

As described above, localisation of stem cells in human epithelia has been hindered by the lack of markers that are expressed solely by stem cells (Cotsarelis *et al.*, 1999). However, the early stages of differentiation of epithelial basal cells are characterised by the loss of expression of some molecules and the gain of expression of others. The negative or positive staining of basal cells for such molecules can be thus used to identify sub-populations of basal cells that are enriched for stem cells, as shown by their clonogenicity or label retention (Mackenzie *et al.*, 1989). Various members of the keratin family of IF molecules have been reported to be differentially expressed during epithelial differentiation. Typically, all of the basal cells of stratifying epithelia express K5 and K14 and, as they differentiate, new regionally-dependent keratins are expressed suprabasally. In some tissues, certain keratins have been reported to show greater expression in putative stem cell zones. For example, both K15 and K19 stain the bulge region of hair follicles, where stem cells are localised, and K19 is also reported to stain stem cell zones in other tissues (Michel *et al.*, 1996; Lyle *et al.*, 1999). Human epidermal stem cells are also reported to express higher levels of β1 and α6 integrins than amplifying cells (Jones and Watt, 1993; Li *et al.*, 1998). Staining sections and sheets of human epidermis for β1 integrin indicates that the most strongly expressing cells lie over the tips of the connective tissue papillae (Jensen *et al.*, 1999), but there appears to be a marked variation in stem cell distribution, as demonstrated by the reverse pattern of staining of sections of oesophageal mucosa, where the strongest expression of β1 integrin is found in the basal cells at the deep tips of the epithelial rete (Seery and Watt, 2000).

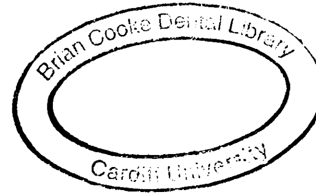
1.20 Skin equivalents

De-epidermalised dermis (DED) is an alternative method to organotypic cultures and is made by growing primary human keratinocytes on a sheet of dead dermis (Prunieras *et al.,* 1983). Pieces of DED can be obtained from cadaveric human skin stored in 80% glycerol from the European Skin Bank in Holland (Waseem *et al.,* 1999). Primary dermal fibroblasts are allowed to attach overnight on the underside of the DED. The dermis is

turned over and primary keratinocytes are seeded into a stainless steel ring placed over the dermis. After the cells have attached, the steel ring is removed and the keratinocytes allowed to grow in FAD for 4 days to form a monolayer following which the dermis is raised to a metal grid to form an air-liquid interface. The cultures stratify into multilayered epithelia and display features of differentiation including tonofilaments, desmosomes and membrane coating granules (Selvaratnam *et al.,* 2001). The cultures also show positive expression of proliferation and ECM proteins (Ojeh *et al.,* 2001).

Cultured epidermis has been extensively used as autografts for the permanent coverage of full-thickness burn wounds, excised burn scars and tattoos. Cultured epidermis is available as allografts to aid the healing of chronic skin ulcers of deep dermal burns (Oshima *et al.,* 2002).

Skin equivalents or substitutes are mostly used in the treatment of burn injuries and wounds such as diabetic skin ulcers. The role of skin substitutes is important in burn surgery. Epidermal injuries are healed by regeneration of the epidermis by keratinocytes from the periphery of the wound and their proliferation, which leads to healing without scars. However if the dermis is injured epidermal recovery is harder since fibroblast proliferation, which eventually leads to collagen formation, is very slow, and can even result in an unepithelialised, open wound. In order to shorten the healing process, skin substitutes are used either temporarily or permanently. DED with cultured keratinocytes layered directly onto it prior to implantation onto dermal wounds is one form of skin equivalent. Other skin substitutes can be divided into two categories, skin substitutes for wound closure and skin substitutes for wound cover. Examples of skin substitutes for wound cover are Biobrane, Transcyte, Apligraf (Graftskin), Dermagraft and cultured allogeneic keratinocytes. Examples of skin substitutes for wound closure are Alloderm, Integra, cultures autologous keratinocytes and composite epidermal-dermal skin substitutes.



1.20.1 Organotypic cultures

Organotypic cultures are cultures in which keratinocytes are grown on a substrate that resembles the dermis and is sometimes known as a 'dermal equivalent'. When cultured keratinocytes are grown as organotypic cultures, with dermal fibroblasts or 3T3s incorporated into a collagen matrix, the degree of morphological differentiation achieved is much greater than when grown on plastic with a feeder layer of 3T3 cells (Bell *et al.*, 1981; Asselineau and Prunieras, 1984). Early approaches to forming a dermal equivalent included culturing the keratinocytes on collagen type I films or gels (Karasek and Charlton, 1971). Organotypic cultures mimic the natural tissue architecture of epithelium, although in a simplified form, and such conditions enable keratinocytes to regenerate their tissue-specific differentiation program *in vitro* (Bell *et al.*, 1981). It has been possible to study many aspects of epithelial biology using the organotypic culture system. These include re-epithelialisation during wound closure (Garlick and Taichman, 1994) where a combination of migration, proliferation and differentiation of keratinocytes occurs.

Organotypic cultures can regenerate patterns of epithelial organisation that are closely similar to those of the normal tissue of origin. For experimental purposes, organotypic cultures can theoretically provide essentially unlimited amounts of experimental material. In addition, the epithelium can be removed in minutes which prevents cell changes that may occur during lengthy enzyme treatment needed for skin and mucosal separation. Retroviral labelling to examine clonal structure is also possible. Organotypic cultures represent a three-dimensional, high density culture, regenerated from isolated lineages of cells that are then combined, their interaction studied, and their response to exogenous stimuli can be characterised.

The usual process of differentiation as cells move from the basal to suprabasal layer is accompanied by altered cellular expression of molecules such as the keratins and blood group antigens (BGAs). When organotypic cultures are made using epidermal cells plated on collagen containing fibroblasts, the cultures stratify and show *in vivo*-like expression of differentiation markers and more normal cell kinetic properties (Parenteau *et al.*,

1992). Organotypic cultures made of human skin or oral mucosa have also been used as grafts to aid the healing of wounds (Nanchahal *et al.*, 2002). Using autologous organotypic cultures in the healing of wounds or burns overcomes the problems associated with tissue rejection due to immunological responses.

The original method of organotypic culture construction by Bell and subsequent modifications are still used (Bell *et al.*, 1981; Parenteau *et al.*, 1991). During the construction of organotypic cultures, a large number of variables are involved, such as the number, quality and tissue of origin of the cells used, the construction and contraction of the fibroblast gel, the timing involved during the process of adding the keratinocytes and what growth medium is used. These variables make the organotypic culture model quite difficult to standardise and results are not always reproducible.

1.21 MARKERS OF EPITHELIAL DIFFERENTIATION

1.21.1 Introduction

Keratin proteins have diverged to perform specific functions in higher eukaryotic epithelia (Hutton *et al.*, 1998). Keratin networks are a universal feature of epithelia, but their composition, organisation and density vary greatly, and appear to be tailored to the different shapes and structural requirements of individual epithelial cells. Each keratin polypeptide is the product of a distinct keratin gene and there is conservation of gene sequence and protein structure across species (Leigh *et al.*, 1993). Control of keratin gene expression is operated at transcriptional and post-transcriptional levels, with some post-translational modifications. Monoclonal antibodies are used to examine precise tissue distribution of keratin epitopes (Leigh *et al.*, 1993).

1.22 Keratin intermediate filaments

The large multigene family of closely related IF proteins are made up in part, of low-sulphur α-type keratin filament proteins which are found in various epithelial cells of the skin and its appendages. These IF proteins are major components of the cytoskeleton and provide mechanical integrity to cells. If this is absent the cells become fragile and easily rupture upon mild physical trauma. To date, about 40 IF proteins displaying structural and functional heterogeneity have been identified and characterised using biochemical analysis from two-dimensional gel electrophoresis on the basis of charge plus molecular weight. Specific monoclonal antibodies have been used as an alternative method of identifying the IF proteins by either immunoblotting or *in situ* immunohistochemistry techniques. Cytoplasmic IF proteins converge on the nuclear pore to provide structural integrity throughout the cell.

Keratins are classified by number. K (keratin)1 is the largest and most basic and K19 the smallest and one of the most acidic (Moll *et al.*, 1982). There are 16 human acidic (type I) keratin IF proteins. These range in size from 40-65 kDa and cover the acidic pH range (pI 4.5-6). There are 13 human neutral basic (type II) keratin IF proteins. These are larger and are in the size range of 50-70 kDa and are more basic (pI 6.5-8.5). All other types of IF proteins, types III to VI, such as vimentin, desmin, glial fibrillary acidic protein, perpherin, neurofilament triplets, α-internexin, laminins A and C, and nestin, are classified on the basis of amino acid sequence data. The type I and type II keratins are co-expressed and self-associate into coiled-coil heterodimers. The size of the keratin IFs have been determined by SDS-polyacrylamide gel electrophoresis and are shown in table 1.1 (Chu and Weiss, 2002).

Table 1.1 Sizes of keratins Type I and II

Keratin Type II (basic)	Size (kDa)	Keratin Type I (acidic)	Size (kDa)
1	67	9	64
2	65	10	56.5
3	64	11	56
4	59	12	55
5	58	13	51
6	56	14	50
7	54	15	50
8	52	16	48
		17	46
		18	45
		19	40
		20	46

1.23 Structure of intermediate filaments

Keratin filaments together with actin microfilaments and microtubules make up the cytoskeleton of vertebrate epithelial cells. The keratins belong to a family of IF proteins, these associate laterally and form 10nm diameter filaments from end-to-end and about 20,000 heterodimers assemble into an IF. The major structural characteristic of all IF proteins is the central α-helical rod domain. This domain is highly conserved in size and structure.

The individual polypeptide chains or subunits have a central rod domain consisting of 4 α-helical regions separated by non-helical linkers. The central domain is flanked by an amino terminal domain (head) and carboxy terminal domain (tail). The central rod domain is about 47nm long and accounts for about 38kDa in molecular weight terms. As proteins they exist only as type I-type II pairs and not singly. The smallest keratin, K19 is only 40kDa and possesses a rod domain with only a minimal of amino (N) and carboxy (C) terminal sequences. These N- and C-terminal sequences are often unique and have highly specialised primary and secondary structures which differ between the types of IF proteins and also between the different keratins.

The sequence of the rod domains for IF proteins of the same type is highly conserved (60-75% homology), but less conservation (30-40% homology) is observed between types, including type I and type II keratins. Levels of keratin IF reach up to 80% of the total cell protein in terminally differentiated keratinocytes. Keratins are cytoplasmic and at the cell periphery they link to the desmosome junctions which form cell-cell and cell-substrate attachment plaques. The keratin-desmosome joining creates a three-dimensional network through the epithelium.

Tissue distribution of expression of individual or groups of IF proteins is specific and predictable indicating that these stable proteins which make up the cytoskeleton play an important role in tissue differentiation. Newly synthesised, differentiation-specific, keratin polypeptides are added to the keratins that are already present when the cells move out of the basal layer into the spinous layer, thus increasing the quantity and diversity of molecular species of keratins (Tseng *et al.*, 1982). This role may determine cell behaviour in the early stages of commitment to differentiation, or in the later stages of differentiation. The tissue specific expression of the IF is useful for characterising differentiated cells. If a certain keratin is a marker of differentiation it could be said that it is a negative stem cell marker.

Type I keratins can polymerise with many type II keratins *in vitro*, but *in situ* the keratins are predominantly co-expressed in defined pairs. Each pair is characteristic of a particular epithelial phenotype (Eichner *et al.*, 1984). A characteristic tissue distribution is shown by the major keratins with even sub-population of cells within a tissue showing specific phenotypes. This is thought to reflect a physiological difference between the cell populations.

1.24 Keratin genes

The keratin genes produce the major structural components of the skin or external barrier layer. The keratin IF genes are localised to different chromosomes. Generally type I keratins are located in two clusters on chromosome 17, one cluster on the short arm and

the other on the long arm. The type II keratins are localised to a single cluster on chromosome 12 (Bowden, 1993). The cellular arrangement within the epidermis and the building up of extensive keratin cytoskeletal network results in the skin having a protective function. The keratins are very stable as intermolecular protein interactions take place in the early stages of their assembly (Fuchs, 1993).

1.25 Simple and stratifying epithelium

There are two distinct categories of epithelia, simple or stratifying, which are characterised by specific keratin distribution (Purkis *et al.*, 1990). Simple epithelial cells retain contact with the basal lamina and have a free apical surface and express the primary simple epithelial keratins, type I K18 and type II K8. The simple epithelial keratins K8 and K18 are the first keratins to be expressed in the embryo. K8 and K18 also persist into adult tissues and in simple epithelial cells, and also as the sole keratins in hepatocytes. Keratins type I K14 and type II K5 are stratification-specific keratins and are expressed by all stratifying squamous epithelia (Purkis *et al.*, 1990).

1.26 Keratin expression in various epithelia

Epidermis: K1 and K2, the type II basic keratins, and K9 and K10, the type I acidic keratins, are the largest keratins and are expressed in the differentiating cells of interfollicular epidermis (Fuchs, 1995) but K2 and K9 are only minor components in epidermis.

K6 and K16 are expressed in epidermis but only in response to wounding or distress of the epidermis and in hyperproliferative states such as psoriasis (Leigh *et al.*, 1995). K15 is also a minor component of the epidermis, as is K19, and both are localised to the germinative compartment.

Palmar/plantar: The epidermis of human palmar and plantar skin (ridged skin) express a more complex pattern of keratins than trunk epidermis, which could be due to the greater

stress that they have to withstand (Swensson *et al.*, 1998). K9 is abundant in palmar and plantar epidermis, with K1 and K10 expressed in the upper part of the pilosebaceous duct that is continuous with the epidermis. K5 and K14 are also expressed in basal and early differentiated cells. Specific expression patterns of keratins K6, K16 and K17 have been observed indicating regional epidermal adaptations to a high cell turnover rate. Nests of K17 positive cells are observed at the bottom of the deep primary ridges indicating heterogeneity of basal cells. This suggests that these K17 positive nests may include stem cells (Swensson *et al.,* 1998).

Cornea: K3 and K12 are expressed in the stratified epithelium of the cornea and are specific for the differentiation of this tissue (Liu *et al.*, 1994). K5 and K14 are also expressed in basal and early differentiated cells.

Oesophagus: K4 and K13 are expressed in the differentiated cells of the oesophagus, tongue, epiglottis, various internal organs and the apocrine glands of the axilla. A smaller type I keratin, K20, is also found in these epithelia.

Hair follicle: K5 and K14 are expressed in the outer root sheath cells and in cells of the sebaceous gland duct. K6 and K16 are constitutively expressed and K15 and K17 are expressed in the outer root sheath cells with K19 being a minor keratin localising to the stem cell compartment.

Sweat and sebaceous glands, trachea and mammary gland ducts: K7 and K19 are expressed in sweat glands, mammary gland ducts and in the trachea wall. K17 is expressed in mammary gland duct and tracheal epithelium.

Intestine, liver, colon and kidney: These simple epithelia express K8, K18 and sometimes K19 expression is observed.

1.27 Altered keratin expression

The following physiological and pathological factors are known to affect the expression of keratins.

Psoriasis

Psoriasis is a chronic inflammatory and proliferative skin disorder in which K6 and K16 are expressed (Rao *et al.*, 1996). Changes in keratin expression suggest that there is abnormal keratinocyte differentiation and abnormal keratinocyte proliferation in patients with psoriasis.

Wound healing

Re-epithelialisation is a central event in wound healing. It involves the migration and cornification of perilesional basal keratinocytes across a wound bed to cover it (Rao *et al.*, 1996). During wound healing the basal keratinocytes express keratins K6 and K16 unlike normal skin which does not expresses K6 and K16. If there is extreme damage to the skin, abnormal wound healing takes place resulting in hypertrophic scars or keloids. In keloid skin there is an increase in the expression of K14 suggesting that altered keratin expression might influence abnormal wound healing. Wound healing or re-epithelialisation has also been investigated using organotypic cultures (Garlick and Taichman, 1994). The expression of K16 has been investigated in response to wounding in oral mucosa (Dabelsteen *et al.,* 1998). In normal oral mucosa K16 has a patchy suprabasal distribution but after wounding K16 was uniformly present in all cell layers.

Retinoids

Retinoids are synthetic vitamin A analogues that are be used as treatment for a variety of dermatological disorders. They are found to alter keratin gene expression and modulate filament formation by regulating transcriptional or post-translational modifications of keratin polypeptides. Retinoids can modulate proliferation and differentiation of epidermal keratinocytes *in vivo* and *in vitro* (Kopan *et al.*, 1987). Organotypic cultures need retinoic acid added to the medium for enhanced expression of markers of

differentiation (Kautsky *et al.,* 1995). In organotypic culture, the optimal concentration of retinoic acid for improved stratification and differentiation is 10^{-9}M. At a lower concentration there is evidence of hyperkeratinisation, and at a higher concentration the expression of both K1 and K10 and filaggrin is reduced and the cultures become hyperplastic (Dale *et al.,* 1990).

1.28 Keratins in oral epithelia

Stratified squamous epithelial lining or oral mucosa covers and protects the oral cavity and can be keratinised or non-keratinised. Keratinised epithelia can be either parakeratinised (nucleated stratum corneum) or orthokeratinised (stratum corneum is free from nuclei) (Squier, 1976). The non-keratinising oral mucosa forms the soft palate, cheeks, lips, floor of mouth and the alveolar mucosa. The masticatory mucosa, which is keratinised, forms the hard palate, which is orthokeratinised, and the gingiva, which is parakeratinised.

The pattern of keratin synthesis in human oral orthokeratinised epithelium from hard palate is basically similar to that produced in epidermis apart from the expression of K6 and K16 which is present in hard palate but not in normal epidermis (Clausen *et al.*, 1986). K8 and K7 are not expressed by normal oral keratinocytes (Morgan *et al.*, 1987) but are expressed in oral mucosa of oral cancer patients (Ogden *et al.*, 1993a). These simple epithelial keratins are expressed in poorly differentiated tumours (Morgan *et al.*, 1987) and also in well-differentiated tumours (Ogden *et al.*, 1993b).

1.29 Orthokeratinised epithelia

Stratified orthokeratinised epithelia make up the palate, maxillary tuberosity, edentulous ridges and the tips of the filoform papillae. K1, K2 and K10, which are specific to epidermal type differentiation, are found in these epithelia. These keratins are absent from the basal cells and the deep cells of the stratum spinosum, but are expressed in the

upper stratum spinosum and stratum granulosum becoming weaker in the stratum corneum.

Keratin expression in orthokeratinised oral epithelia: K4 and K13 are mostly absent from epidermis but they are specific for non-keratinised mucosal epithelia, but a few cells in the suprabasal layer of keratinised oral mucosa are positively stained when antibodies for K4 and K13 are used (Morgan *et al.*, 1987). K6 and K16 are also expressed by the superficial cells and are usually expressed by epithelia in hyperproliferative states undergoing high cell turnover, such a psoriasis, but as the stratified epithelia of the oral mucosa is in a high turnover state this keratin pair is expressed.

Antibodies against K7, K8, K18 and K19 react negatively to all stratified keratinised epithelia except for a few isolated basal or parabasal cells. These isolated cells are found in the tips of the epithelial rete and are thought to be neuroendocrine Merkel cells (Morgan, 1986).

1.30 Parakeratinised epithelia

This type of epithelia is characterised by a stratum corneum with pycnotic nuclei and poorly defined granular cell layer. It also covers the attached and marginal gingivae and the fungiform papillae. More cells stain for K4 and K13 in parakeratinised than orthokeratinised epithelia. Some basal cells in the parakeratinised epithelia, such as gingiva, express K19 as opposed to the K19 positive Merkel cells in orthokeratinised epithelia (Bosch *et al.*, 1989).

1.31 Stratified non-keratinised epithelia

The stratified nonkeratinised epithelia cover the alveolar mucosa, the gingival sulcus and the internal surfaces of the cheeks and lips, the floor of the mouth, the lateral and ventral surfaces of the tongue, the lateral aspects of the filoform and fungiform papillae, the interpapillary mucosa of the dorsal surface of the tongue and the soft palate.

Nonkeratinised squamous epithelia are made up of three layers the basal, suprabasal and superficial layers. These layers have been termed the stratum basale, which contains small cells, stratum filamentosum that is made up of large, polyhedric cells and the stratum distendum where the cells are flattened. These epithelia lack a stratum granulosum and stratum corneum and are characterised as such by the presence of a stratum distendum (Schroeder, 1981,)

Oesophageal type K4 and K13 are expressed in suprabasal cells in non-keratinised epithelia, but no epidermal-type K1 or K10 is expressed (Moll *et al.*, 1982). The basal layer contains the stratification-specific keratins K5 and K14. Most basal cells also express K19 but the other simple epithelial-type keratins are not expressed (Morgan *et al.*, 1987). K15 and K17 have a varied pattern of expression. Alveolar mucosa contains traces of K17 (Ouhayoun *et al.*, 1985) but no K15, and the ventral surface of the tongue contains K15 but no K17. Cells in the lateral surface of the tongue however, contain traces of K15 and K17 (Sawaf *et al.*, 1990).

1.32 <u>Keratin expression in hard palate</u>

Previous staining of keratins in hard palate have reported staining of K1 in the more superficial layers above the level of the rete, K13 staining in a few suprabasal cells, K14 expression in the whole of the epithelium and K19 was not expressed at all. Organotypic cultures made of palatal keratinocytes plated on de-epidermised dermis showed no staining with K1, K13 was expressed in the parakeratotic horny layers, K14 was expressed in the whole of the epithelium, and K19 was expressed in the uppermost part of the epithelium (Cho *et al.*, 2000).

1.33 <u>Keratin expression in organotypic cultures</u>

Organotypic cultures made using cells derived from hard palate, gingiva and alveolar mucosa have been made using homotypic fibroblasts and their keratin profiles examined

(Shabana *et al.*, 1991). Oral epithelia stratify and differentiate when plated on collagen gels containing fibroblasts but a difference occurs in the epithelia formed from keratinised and non-keratinised sites (Shabana *et al.*, 1991). The epithelium of palatal origin had elongated non-nucleated superficial cells, polyhedral spinous cells and cuboidal basal cells. The superficial cells of the gingival and alveolar mucosal organotypic culture epithelium retained their nuclei and had polyhedral spinous cells and cuboidal basal cells. The basal cell keratins, K5 and K14 and the hyperproliferative keratins K6 and K16 were also found in all the epithelia. The terminal differentiation keratins K1, K2, K10 and K11 were found in the suprabasal cells of the palatal epithelia. These keratins were not found in the gingival and alveolar epithelia except for weak labelling of a few individual superficial gingival cells. Anti-K13 and K14 antibodies labelled suprabasal cells in all organotypic cultures with K13 labelling stronger and more homogeneous in the alveolar cells. K4 weakly labelled suprabasal cells in all organotypic cultures. The whole of the epithelium of all organotypic cultures stained with anti-K18 antibody. Anti-K19 antibody stained strongly in the alveolar mucosal cells with labelling occurring in the superficial cells while individual cells were stained weakly in the palatal cells.

1.34 <u>Markers of early differentiation - blood group antigens</u>

The blood group antigens (BGAs) can be used as markers of differentiation. The BGAs of the A, B, H and MN systems of erythrocytes are also found on oral keratinocytes (Dabelsteen *et al.*, 1982). A, B and H BGAs are glycolipids and glycoproteins which arise from three independent but related gene systems. The H antigen is the chemical substance that is converted into the A or B antigen under the influence of the A or B genes by the addition of a simple sugar. The BGAs are components of larger molecules. Two types of carbohydrate chains, type 1 and type 2 carry the A, B and H antigens and these chains are distributed differently in various organs.

The BGAs, A, B or H may be expressed on the cell membranes of oral keratinocytes and also on other stratified epithelia such as oesophagus, vagina and uterine cervix. The

BGAs are expressed in most human epithelial tissues in such a way that the expression of distinct carbohydrates is restricted to specific tissues and cell types. In stratified squamous epithelium, cells express a series of BGA precursors as they differentiate and move from basal to superficial layers. Epidermal keratinocytes only express the H antigen.

The presence of A, B and H antigens on human oral epithelial cells is related to the maturity of the cells, the differentiation pattern and the type of carbohydrate chain which carries the antigen. BGAs A and B are synthesised by the addition of terminal carbohydrates to a precursor substance with BGA H activity (Watkins, 1966). The basal layer, which lacks the BGAs A, B and type 2 chain H antigen, has an H-antigen precursor molecule called N-acetyl-lactosomine. Type 2 chain H-antigen is also found at a lower level than the A and B antigens. Type 2 chain H antigen is expressed on basal cells, parabasal cells and cells in the spinous layer, whereas the A and B antigens have a limited distribution in the spinous layer.

The oral epithelium of blood group O persons, which lack the A and B antigens on epithelial cells, can function without the final sugar in the carbohydrate chain of the BGA. This makes it unlikely that A and B antigens function as receptors for molecules such as hormones or vitamins but may have a role in intercellular adhesion and communication. The different levels of expression of antigens A, B and H (type 2 chain) in epithelium suggests a stepwise building up of carbohydrates during differentiation of epithelial cells. It may also indicate a chemical change taking place in the cell membrane which occurs at the same time as changes in cell size, shape and maturity. In human oral mucosa, basal cells express short and unbranched carbohydrates, whereas the layers above express carbohydrates with longer sugar structures (Dabelsteen *et al.*, 1991). Parabasal cells express shorter carbohydrate structures than spinous cells, which in turn express structures shorter than those in the superficial part of the epithelium.

Keratinised oral mucosa such as palate mainly express carbohydrates that are precursors of the BGAs but only type 2 chains like H1 and Le^y, and mucin-like type 3 chains have

been detected (Ravn and Dabelsteen, 2000). The BGA H1 has been previously reported to be expressed in the basal and suprabasal layers with the BGA Le^y expressed suprabasally (Dabelsteen *et al.*, 1991). The BGAs are expressed in most human epithelial tissues in such a way that the expression of distinct carbohydrates is restricted to specific tissues and cell types.

1.35 Cell cycle of keratinocytes

The cells within the progenitor compartment perform two functions, to maintain the proliferative potential of the epithelium, and also to produce cells which mature and maintain tissue function. During mitotic cell cycle the stem cell divides to produce two daughter cells, one of which remains in the proliferative cell cycle (stem cell), and one which divides and enters the differentiation pathway and eventually terminally differentiates and is then lost from the surface.

The cell cycle can be divided into 4 phases. Figure 1.4 shows the various phases of the cell cycle, which are,

G1 the post mitotic, pre-synthetic gap.

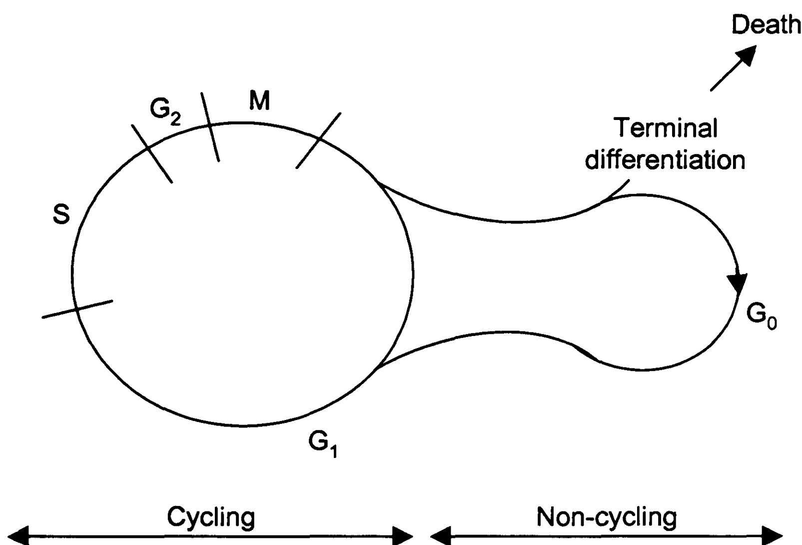
S synthesis, where the DNA is replicated

G2 the post-synthetic, pre mitotic gap

M mitosis, where cell division occurs as a process of prophase, metaphase, anaphase and telophase.

An additional phase, G0, has been postulated as there is a wide variation in the duration of G1 (Lajtha, 1963). G0 cells are those that are not actively undergoing cell cycle (but the cells are capable of participating in the cell cycle) and therefore remain in a stationary G1 phase. This population of G0 cells could explain why some basal keratinocytes do not take up a label (Potten *et al.*, 1982) and as such they could be a subpopulation of quiescent cells, which are slowly cycling stem cells.

Figure 1.4 A diagram of the cell cycle. The figure includes one possible configuration for a resting phase and a path for exit from the cycle for differentiation or cell death. The cycling component consists of cells in the G1 phase, which is the most variable part of the cycle. Cells then move into the S phase during which the cell doubles its DNA complement. Cells then enter a second gap phase, G2 which then leads to mitosis, M, which concludes with cell division to produce two daughter cells. These may both proceed through new cycles by entering a new G1, or enter a differentiation path, or a path leading to cell death.



The duration of each of the phases in the cell cycle in oral epithelium of rat and mouse are not well established but are thought to be approximately 8 hours in S phase, 2 hours in G2 phase, 1½ hours in M phase, and with a variable time of between 14-150 hours in G1 phase.

Flow cytometry is a technique used to assess the cell cycle. Cells are treated with a dye that binds the DNA stoichiometrically. The cells are passed through the flow cytometer as a single cell suspension where a laser is used to excite the intercalated dye. The resulting fluorescence emission collected by an array of detectors is used to quantitate the amount of DNA in the cell.

1.36 Aims and objectives of this thesis

Hair and cornea are large epithelial structures which are maintained by their own reservoir of stem cells, various murine epithelia are simpler in structure and have small units of structure and are maintained by one stem cell per clonal unit. A transitional epithelium with an intermediate structure is hard palate which is made up of well-defined rete in an ordered arrangement. To date, stem cells have been identified as being situated in different positions within epithelia, in epidermis it is suggested that the stem cells are located over the tips of the connective tissue papillae tips (Jensen *et al.*, 1999) and in mucosal epithelia the stem cells lie at the deep tips of the epithelial rete (Seery and Watt, 2000). As no single stem cell marker has been identified to date, the aim of this thesis was to combine and compare two different methods, staining for differentiation markers and putative stem cell markers, and lineage analysis. It was investigated if stem cell patterns could be re-established in organotypic culture by comparing the palate staining profiles with those obtained with the organotypic cultures. A characteristic feature of stem cells is their slow cell cycle and therefore the cell cycles of cells grown on plastic and organotypic cultures were compared. Organotypic cultures were also constructed using a proportion of virally transduced keratinocytes and the histochemically detectable patterns produced could be identified.

To summarise, the aims of this thesis were to undertake the following,

1. To investigate the distribution of stem cells in human hard palate by the epithelial expression of putative markers.
2. To investigate whether organotypic cultures can provide a suitable stem cell model system.
3. To determine if the cell cycles of epithelial cells in organotypic cultures differ from those of cells grown on plastic.
4. To compare the positions of stem cells as determined by two different mechanisms, staining, and lineage analysis.

CHAPTER TWO

CHAPTER 2

MATERIALS AND METHODS

2.1 General cell culture

All double distilled water was autoclaved and was obtained from the Department of Matrix Biology and Tissue Repair Research Unit (UWCM, Cardiff).

Phosphate buffered saline (PBS) was made by adding 1 tablet of PBS (Sigma-Aldrich Company, Fancy Road, Poole, Dorset, UK) to 100ml of double distilled water and was autoclaved.

All cells were grown on tissue culture standard plastic T75 flasks in a humidified incubator at 37°C with 5% CO_2. All media or solutions that were added to cells were warmed to 37°C in a water bath.

Human oral mucosal keratinocytes and fibroblasts were isolated from palatal mucosa as tissue removed from healthy patients, with consent, during surgical exposures of unerupted canines.

3T3 cells were obtained as frozen stocks from Swiss mouse embryo fibroblasts and for retroviral work a neomycin resistant strain was used.

2.2 Human epithelial cells

The following medium was made for the culture of all keratinocytes.

Solution A

375ml of Dulbecco's minimal essential medium (DMEM, Invitrogen, 3 Fountain Drive, Inchinnan Business Park, Renfrew, UK) with high glucose was added to 125ml Ham's F-12 medium (Invitrogen). 9.1mg adenine (Sigma) was dissolved in 4ml of DMEM/Ham's F-12. This was then added back to the 500ml DMEM/Ham's F-12 mixture.

FAD medium

50ml foetal bovine serum (FBS, Invitrogen), 0.5ml hydrocortisone stock solution, 0.5ml cholera toxin stock solution, 0.5ml epidermal growth factor (EGF, Sigma), 0.25ml insulin stock solution and 5ml antibiotic antimycotic (Invitrogen) were dissolved in 450ml solution A. This was filter sterilised and stored at 4°C.

Hydrocortisone 1000 x stock solution: 4mg hydrocortisone (Sigma) was dissolved in 10ml of solution A. This was stored in aliquots at 4°C.

Cholera toxin 1000 x stock solution: 1mg cholera toxin (Sigma) was dissolved in 119ml solution A. This was stored in aliquots at -20°C.

Epidermal Growth Factor (EGF)1000 x stock solution: 100µg EGF (Sigma) was dissolved in 10ml solution A. This was stored in aliquots at -20°C.

Insulin preparation: A 10mg/ml stock solution was prepared by adding 100mg of insulin (Sigma) to 10ml distilled water and 100µl of glacial acetic acid (Sigma).

Final concentrations of components of FAD medium

DMEM	67.5%	Ham's F-12	22.5%
FBS	10.0%	Hydrocortisone	400ng/ml
Cholera toxin	10^{-10}M	EGF	10ng/ml
Adenine	0.089mM	Insulin	5µg/ml
Antibiotic antimycotic	500units/ml		

Keratinocyte isolation from human palatal tissue

The tissue was collected in FAD + 3X antibiotic antimycotic, and excess connective tissue and fat was removed. If the sample was large, it was cut into strips approximately 3mm wide. The sample was placed in 3 to 4 changes of FAD + 3X antibiotic antimycotic over the course of a day and stored at 4°C overnight.

The medium was removed and the tissue was washed once in PBS, 5ml of cold 0.25% trypsin was added and the sample stored at 4°C for at least six hours. The contents were poured into a petri dish and the epithelial sheet was removed using forceps. The remaining connective tissue was scraped using the forceps to remove any loosely adherent epithelial cells. The epithelial sheet, dissociated cells and remaining trypsin were collected and transferred to a 15ml tube and 5ml of warm trypsin was added to disassociate the epithelial sheet into a single cell suspension, FAD was added to neutralise the trypsin and the sample was centrifuged at 1000rpm for 10 minutes. The pellet was resuspended in 2 ml of FAD and transferred to a flask of mitomycin C treated 3T3 cells.

As previously described (Gao and Mackenzie, 1992) keratinocytes were plated in FAD and, after colony formation and expansion towards confluence, passaged at a 1:10 dilution ratio for amplification.

Human epithelial cell culture from frozen stocks

Human epithelial cells were grown using Mitomycin C (Sigma) treated 3T3 cells as a feeder layer. Mitomycin C treated 3T3 cells were plated at 6 x 10^5cells/T75 flask. The 3T3 medium was removed from the flask and replaced with 10ml of FAD medium and a cryovial of epithelial cells was defrosted and plated onto the 3T3 cells. The flask was then placed in 5% CO_2 incubator at 37°C.

To passage epithelial cells

The FAD medium was removed and cells were washed twice with PBS. 2.5ml of warmed trypsin/EDTA (Sigma) was added and the flask was placed in CO_2 incubator until cells

were detached from flask, this can be aided by gentle tapping. When the cells had lifted 5ml of FAD medium was added to the flask, the cell solution was placed in a tube and spun for 10 minutes at 1000rpm. The supernatant was removed and cells were plated at $1.3x10^4$cells/cm^2 onto Mitomycin C 3T3 cells.

Cryopreservation

The cells were lifted as described previously and the pellet was resuspended in 0.1ml DMSO (Sigma) and 0.9ml FBS per cryovial. The cryovial was placed in a Nalgene Mr. Frosty freezing container (Sigma) and was left at -70°C overnight. The frozen cryovials were then stored in liquid nitrogen.

2.3 Human fibroblasts

Fibroblast medium

50ml FBS (final concentration 10%) and 5ml antibiotic antimycotic (final concentration 500units/ml) was added to 450ml DMEM, this was sterile filtered and stored at 4°C.

Fibroblast isolation from human palatal tissue

The connective tissue was minced very thoroughly with a scalpel and placed in a tube with 5-10ml of fibroblast medium. The sample was centrifuged at 800rpm for 5 minutes and was resuspended in 0.5ml of fibroblast medium. Meanwhile the collagen gel was prepared by adding 4ml collagen to 0.5ml 10X DMEM and 0.5ml FBS with the addition of 2 to 3 drops of 1N NaOH until the mixture becomes a light pink in colour. The fibroblasts in 0.5ml medium were added to this mixture and were plated into 2 wells of a 6 well plate. This was allowed to gel in the incubator for 5-10 minutes and then 2ml of fibroblast medium was added per well. These gels were maintained in culture to allow cell outgrowth (Igarashi *et al.*, 2003) until enough fibroblasts had expanded from the minced tissue and were then treated with warm collagenase (Sigma), centrifuged and plated at $1.3x10^4$cells/cm^2 for amplification into flasks.

Human fibroblast culture from frozen stocks

The cryovial was removed from the liquid nitrogen and was thawed quickly at 37°C in the water bath. The thawed cells were then added to a T75 flask containing 10ml of fibroblast medium which was then placed in $C0_2$ incubator.

To passage fibroblasts

The fibroblast medium was removed and cells were washed twice with PBS. 2.5ml of warmed trypsin/EDTA was added and the flask was placed in $C0_2$ incubator until cells were detached from flask, this can be aided by gentle tapping. When the cells had lifted 5ml of fibroblast medium was added to the flask, the cell solution was placed in a tube and spun for 5 minutes at 800rpm. The supernatant was removed and cells were plated as required in fibroblast medium.

To cryopreserve the cells they were lifted as described previously and the pellet was resuspended in 0.1ml DMSO and 0.9ml FBS per cryovial. The cryovial was placed in a Mr. Frosty freezing container and was left at -70°C overnight. The frozen cryovials were the stored in liquid nitrogen.

2.4 3T3 cells (feeder cells)

3T3 medium

50ml newborn calf serum (final concentration 10%, NCS, Invitrogen) and 5ml antibiotic antimycotic (final concentration 500units/ml) was added to 450ml of DMEM medium. This was sterile filtered and stored at 4°C.

To passage 3T3 cells

These cells were subcultured before becoming confluent to retain their original growth characteristics. The cells were passaged as for fibroblasts at a 1:10 dilution ratio for amplification and plated in 3T3 medium.

Mitomycin C treatment of 3T3 cells

To a 2mg vial of Mitomycin C (Sigma) 4ml of sterile DMEM was added and was filtered to produce a working solution of 0.5mg/ml. The solution must not be exposed to light so all tissue culture flasks that were to be treated were covered in blue roll and the tubes of mitomycin C were covered in foil. 0.1ml of mitomycin C was added to 5ml of 3T3 medium in each flask and incubated for 3 hours (final concentration 0.01mg/ml). The mitomycin C treated 3T3 cells were washed with 3 changes of PBS, then 3T3 medium was added and the cells were placed back in the incubator overnight.

To plate mitomycin C treated 3T3 cells

The cells were lifted as for fibroblasts (section 2.3) and plated at a density of 6×10^5 per T75 flask (8×10^3cells/cm^2). If cells were not to be used immediately, they were refrigerated for future use for up to 5 days. The treated 3T3 cells were left for 4 hours or overnight before plating the epithelial cells on top.

3T3 cell culture from frozen stocks

The cryovial was removed from the liquid nitrogen and was thawed quickly at 37°C in the water bath. The thawed cells were then added to a T75 flask containing 10ml of 3T3 medium which was then placed in $C0_2$ incubator.

To cryopreserve the cells they were lifted as described previously and the pellet was resuspended in 0.1ml DMSO and 0.9ml NCS per cryovial. The cryovial was placed in a Mr. Frosty freezing container and was left at -70°C overnight. The frozen cryovials were the stored in liquid nitrogen.

2.5 Freezing sections of fresh tissue

Fresh tissue specimens were frozen on cork disks (RA Lamb, Eastbourne, UK) using Cryomatrix (Shandon, 93-96 Chadwick Road, Astmoor Industrial Estate, Runcorn, Cheshire, UK) in an isopentane/liquid nitrogen bath and stored at -80°C until used. The specimens were sectioned perpendicular to the epithelial surface in a cryostat and were

cut at a thickness of 5μm, collected on Polysine microscope slides (Shandon), air dried for 1 hour and frozen at -20°C in foil until used.

2.6 Haematoxylin and eosin (H&E) staining

This was carried out in the Dental School Pathology Laboratory (UWCM, Cardiff) by Mrs. K. Allsop. Samples were fixed in 4% paraformaldehyde, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (H&E).

2.7 Immunofluorescent staining of palate sections, tissue culture flask bases and organotypic cultures

For all staining, samples were fixed in an ice-cold acetone:methanol mixture for 10 minutes. The excess fixative was tapped off and samples were blocked in a blocking buffer (PBS containing 0.2% Triton-X and 2% bovine serum albumin) for 1 hour. Multiple samples on the same surface or slide were separated by drawing around with a Pap pen (Sigma). 20-50μl of the appropriate primary antibody (table 2.1) was added to each sample and the slides were placed in a humid container and left at 4°C overnight.

The slides were rinsed in 3 changes of PBS over 30 minutes. The secondary fluorescein isothiocyanate (FITC) labelled antibody (DakoCytomation, Denmark House, Angel Drove, Ely, Cambridgeshire, UK) (table 2.2) was diluted 1:80 in blocking buffer, filtered and was added to each section and left for 2 hours at room temperature. The antibody was shaken off and rinsed in PBS as before. Samples were counterstained with Hoechst 33258 (1μg/ml) (Dako) for 5 minutes, rinsed in water, dried and mounted in antifade medium (Johnson, 1981). The samples were viewed and photographed at standard magnification on an Olympus Provis fluorescent microscope at wavelengths 420-490nm and 330-385nm for FITC and Hoechst respectively. Separate images for FITC and Hoechst were obtained and, where necessary to illustrate tissue morphology were combined in Photoshop 5 (Adobe).

The controls included omission of the primary antibody, secondary antibodies alone, comparison of staining patterns between the different antibodies used, and the conformity of staining to known patterns of antigen distributions.

Table 2.1 Primary antibodies

Antibody name	Antigen specificity	Species	Source	Reference
BTAN20	Human Notch 1	rat monoclonal IgG	1	(Blaumueller and Artavanis-Tsakonas, 1997)
C17.9C6	Notch, intracellular domain	mouse monoclonal IgG	1	(Fehon *et al.*, 1990)
C458.2H	Notch, extracellular domain, EGF repeats #12-20	mouse monoclonal IgG	1	(Diederich *et al.*, 1994)
C594.9B	Delta, extracellular domain	mouse monoclonal IgG	1	(Ye *et al.*, 1999)
C651.6DbHN	Human Notch 2	rat monoclonal IgG	1	(Blaumueller and Artavanis-Tsakonas, 1997)
F461.3B	Notch, extracellular domain, EGF repeats #5-7	mouse monoclonal IgG	1	(Rand *et al.*, 2000)
TS1.15H	Human Jagged 1	rat monoclonal IgG	1	(Gray *et al.*, 1999)
I12 anti-Hoxb4	Hoxb4 (mouse)	rat monoclonal IgG2a	1	(Gould *et al.*, 1998)
A12	Cyclin A	mouse monoclonal IgG1	1	(Knoblich and Lehner, 1993)
2E8 anti-laminin	Laminin	mouse monoclonal IgG1, kappa	1	(Engvall *et al.*, 1986)
AMF-17b	Vimentin	mouse monoclonal IgG1, kappa	1	(Isaacs *et al.*, 1989)
SY5	Involucrin	mouse monoclonal IgG	1	(Hudson *et al.*, 1992)
M3F7	Type IV collagen	mouse monoclonal IgG	1	(Foellmer *et al.*, 1983)
VM-1	Keratinocyte, basal, cell attachment antigen	mouse monoclonal IgG	1	(Oseroff *et al.*, 1985)
5E1	Sonic hedgehog	mouse monoclonal IgG1	1	(Ericson *et al.*, 1996)
P4G11	Beta1 integrin	mouse monoclonal IgG1	1	(Wayner *et al.*, 1993)
P5D2	Beta1 integrin	mouse monoclonal IgG1	1	(Wayner *et al.*, 1993)
A11B2	Beta1 integrin	rat monoclonal IgG1	1	(Damsky *et al.*, 1992)
MPIIIBIO	Osteopontin	mouse monoclonal IgG1	1	(Gorski *et al.*, 1990)
AON-1	Osteonectin	mouse monoclonal IgG3	1	(Bolander *et al.*, 1989)
N-262	c-Myc	rabbit polyclonal IgG	11	(Alitalo *et al.*, 1983)
	LHM2	mouse supernatant	12	(Kupsch *et al.*, 1995)
	NG2	rabbit polyclonal	12	(Belachew *et al.*, 2002)
Gap 28H	Connexin 26 (Cx26)	rabbit polyclonal		(Kuraoka *et al.*, 1993)
DE9	Beta1 integrin (human)	mouse monoclonal IgG1	2	(Bergelson *et al.*, 1992)
CD49f, VLA-6	Alpha6 integrin (human)	mouse monoclonal IgG	2	(Li *et al.*, 1998)
A4A	p63	mouse monoclonal IgG2a	3	(Yang *et al.*, 1998)
3S3	Beta1 integrin-FITC (CD29)	mouse IgG1	4	(Gao *et al.*, 1995)
GoH3	Alpha6 integrin-FITC (CD49f)	rat IgG2a,kappa	5	(Aumailley *et al.*, 1990)
MIB-1	Ki-67	mouse monoclonal IgG1	3	(Key *et al.*, 1993)
111.6	EGFR-10	mouse monoclonal IgG1	6	(Kawamoto *et al.*, 1983)
AF 138	Keratin 5	rabbit polyclonal	7	(Roop *et al.*, 1984)
6B10	Keratin 4 Ab-1	mouse monoclonal IgG1	6	(van Muijen *et al.*, 1986)
1C7 + 2D7	Keratin 13 Ab-3	mouse monoclonal IgG2a + IgG2b	6	(van Muijen *et al.*, 1986)
E3	Keratin 17 Ab-1	mouse monoclonal IgG2b/kappa	6	(Guelstein *et al.*, 1988)
Ks20.8	Keratin 20	mouse monoclonal IgG2a, kappa	8	(Moll *et al.*, 1982)
3B9	N(euronal)-cadherin	mouse monoclonal IgG1-kappa	9	(Takeichi, 1991)
4A2C7	E(pithelial)-cadherin	mouse monoclonal IgG1-kappa	9	(Takeichi, 1991)
LHK1	Keratin 1	mouse monoclonal (supernatant)	10	(Fuchs and Green, 1980)
LHK6	Keratin 6	mouse monoclonal (supernatant)	10	(Lane *et al.*, 1985)
LP5K	Keratin 7	mouse monoclonal (supernatant)	10	(Lane *et al.*, 1985)
LE41	Keratin 8	mouse monoclonal (supernatant)	10	(Makin *et al.*, 1984)
LHP2	Keratin 10	mouse monoclonal (supernatant)	10	(Leigh *et al.*, 1993)

LL001	Keratin 14	mouse monoclonal (supernatant)	10	(Purkis *et al.*, 1990)
LHK15	Keratin 15	mouse monoclonal (supernatant)	10	(Waseem *et al.*, 1999)
LH025	Keratin 16	mouse monoclonal (supernatant)	10	(Wetzels *et al.*, 1991)
LE61	Keratin 18	mouse monoclonal (supernatant)	10	(Lane *et al.*, 1985)
LP2K	Keratin 19	mouse monoclonal (supernatant)	10	(Stasiak *et al.*, 1989)

Sources

1 Developmental Studies Hybridoma Bank, Iowa, USA

2 TCS Biologicals, Buckinghamshire, UK

3 Dako, Glostrup, Denmark

4 Serotec Ltd, 22 Bankside, Kidlington, Oxford, UK

5 Pharmingen, San Diego, California, USA

6 NeoMarkers, Fremont, California, USA

7 Covance, Richmond, California, USA

8 Dako, California, USA

9 Zymed Laboratories Inc., South San Francisco, California, USA

10 St. Bartholomew's and the Royal London School of Dentistry Queen Mary and Westfield College, London, UK

11 Santa Cruz Biotechnology Inc, 2145 Delaware Avenue, Santa Cruz, California, USA

12 Chemicon International, Chandlers Ford, Hampshire, UK

Primary antibody dilutions

All the mouse supernatants were used neat as were all the antibodies from the Developmental Studies Hybridoma Bank (except for involucrin). All others were used at 1:80 apart from the following.

c-Myc 1:20

Cx26 1:250

E-cadherin 1:100

N-cadherin 1:100

Involucrin 1:500

Table 2.2 Secondary antibodies

Secondary antibodies	Dilution	Source
Rabbit anti-mouse immunoglobulins-Fluorescein isothiocyanate (FITC)	1:80	Purchased from Dako, UK
Swine anti-rabbit immunoglobulins-Fluorescein isothiocyanate (FITC)	1:80	Purchased from Dako, UK
Rabbit anti-rat immunoglobulins-Fluorescein isothiocyanate (FITC)	1:80	Purchased from Dako, UK

2.8 Organotypic cultures

Organotypic culture set up (grid method)

Human palatal fibroblasts were lifted using trypsin, made into a suspension with fibroblast medium, centrifuged, resuspended in fibroblast medium and counted using a haemocytometer. To make 5 organotypic gels the following was carried out. The fibroblasts were suspended to achieve the required final concentration of 0.5×10^6 per well in 0.5ml of fibroblast medium. This was kept on ice until needed. To make the organotypic gel, 0.5ml of 10X DMEM and 0.5ml of FBS were added to 4ml of collagen (extracted from rat-tail tendon, Rowling *et al.,* 1990) and was mixed gently in a tube on ice. 1N NaOH was added slowly to the mixture until it was neutralised as indicated by a change from a yellow colour to an orange/pink colour. The fibroblast mixture was added to the gel mixture being careful not to introduce bubbles and 1ml was placed into each of 5 wells of a 24-well plate. The plate was placed in the incubator for 5 minutes to set, fibroblast medium was then added and the plate was returned to the incubator overnight.

The epithelial cells were removed from flask bottoms using trypsin, neutralised with FAD, counted and plated in FAD at 1×10^6 per well on the organotypic gels (containing fibroblasts). These were left overnight in a CO_2 incubator to begin the process of re-epithelialisation.

The next day, collagen coated nylon disks were placed on wire grids on which the organotypic gels were placed. FAD medium wad added until it touched the disks and the

cultures were maintained in this manner for 1 to 3 weeks, replacing the medium every 1 to 2 days. FAD medium was used to feed these cultures with the addition of ascorbic acid (final concentration 50μg/ml) and all trans retinoic acid (final concentration 10^{-9}M) (both from Sigma).

Organotypic culture set up (insert method)

This method was carried out using the same procedure described above with the fibroblast gel mixture being added into special inserts (Falcon, 69 Union Terrace, York, UK) instead of into the 6-well plate. These inserts slot into a special 6 well companion plate (Falcon). The insert has a membrane at its base which is transparent and made of a low pore density polyethylene terephthalate and the pore size was 3μm. The epithelial cells were then added after a day and the cultures were maintained in FAD at the air-liquid interface for up to 3 weeks.

Epidermal organotypic cultures

The same method as above was used except for using dermal fibroblasts in the collagen gel and epidermal keratinocytes were plated above.

Examination of organotypic culture

Once the organotypic culture had been maintained for 14 days, half was fixed in buffered formalin for wax embedding, and the other half was frozen for sectioning on a cryostat. The organotypic cultures were histologically studied to see if stratification and differentiation had occurred by staining with H&E and various antibodies. The H&E staining gave an indication of the general appearance of the organotypic culture while immunofluorescent techniques were used to examine patterns of differentiation.

Freezing organotypic cultures

The cultures were removed from the nylon meshes and were soaked in a 2M solution of sucrose for up to half an hour until the cultures were crisp, this was done to facilitate sectioning on a cryostat. The culture was then folded in half and frozen on to a cork disk and processed as for fresh tissue (section 2.5).

2.9 HaCaT cell line

The HaCaT cell line (from Petra Boukamp, German Cancer Centre, Heidelberg, Germany) was grown using a 3T3 feeder cell layer in a Class II safety cabinet before being made up into the organotypic cultures (section 2.8), frozen (section 2.5) and sectioned on a cryostat.

2.10 OKF6/TERT-1, -2, and N-TERT cell lines

The OKF6/TERT-1, -2, and N-TERT were all grown in a Class II safety cabinet using a 3T3 feeder layer in culture flasks until the flasks were about 60% confluent before being made up into organotypic cultures as described in section 2.8.

2.11 Retroviral transduction

Culture and use of PA317/LAPSN virus producing cells

All the following work was carried out in a Class II safety cabinet following the correct handling procedures for genetically modified organisms.

PA317/LAPSN viral producing cells were grown in DMEM + 10% FBS and the medium was changed 3 times a week and the cells were subcultured before becoming confluent. If the cells become confluent the titre of the virus would be affected. To collect the supernatant the virus-containing medium was transferred to a tube and was centrifuged at 800rpm for 5 minutes to remove cells and debris. The virus-containing supernatant was transferred to a tube and was used immediately or was frozen at -70°C for future use as it

stays stable for up to a year. To select the PA317/LASPN cells, the cells were incubated in 0.75mg/ml of G418 for 7-10 days. For cell maintenance, the cells were then grown with the concentration of G418 at 0.5mg/ml.

An enhanced green fluorescent protein (EGFP) vector called MGIN which has a neomycin (*neo*) insert that allows G418 selection of transduced cells was initially used to transduce palatal keratinocytes. This EGFP transduction was carried out in the laboratory of Dr. R. Darley (Haematology, UWCM) .A β-galactosidase (β-gal) vector was used for the transduction of the epidermal and palatal keratinocytes, it was similar to the above vector.

To transduce epithelial cells

Polybrene (Sigma) is known to enhance the transduction of the virus and 10μl of 4mg/ml of polybrene was added to the cells for 2 hours before transduction. To transduce the cells, 10ml of viral supernatant, 5ml of FAD and 15μl of polybrene was added to each T75 flask. The flask was left overnight and the transduction was repeated using the same procedure as above.

The supernatant was removed and FAD was added and left overnight in the incubator. To select for transduced cells, the antibiotic G418 (Sigma, stock 100mg/ml) was added at various concentrations to different flasks. The concentrations that were used were 0.5mg/ml, 0.75mg/ml and 1.0mg/ml and these doses were added with medium daily until the cells began to die off, this could take up to 7 days.

Alkaline phosphatase (AP) staining with TR/Napthol or BCIP/NBT

The staining solutions were made up following the manufacturer's instructions. For the BCIP/NBT stain, 1 tablet of BCIP/NBT (Sigma) was added to 10ml of water. For the TR/Napthol (Fast Red) stain, 1 tablet of the buffer was added to 1ml of water and when this was dissolved, the TR/Napthol tablet (Sigma) was added. Both solutions should be used within 1 hour.

For smears and cryosections: Frozen sections and smears were fixed in 4% paraformaldehyde for 10 minutes.

For epithelial sheets from organotypic cultures: The cultures were removed from the collagen-impregnated mesh and were incubated in 5ml of 3mM EDTA to dissociate the epithelial sheet from the fibroblast layer. These were placed in the water bath and were periodically shaken to assist in the removal of the epithelial sheets. Once the sheet was separated it was placed in a 6-well plate and 4% paraformaldehyde was added for 10 minutes.

For both procedures, the paraformaldehyde was removed and the slides or sheets were washed 3 times over 20 minutes with 1X TRIS buffer. The substrate was added to the sections, smears or cultures and was left at room temperature from 10 minutes to 2 hours for a colour change to be detected under the microscope. When the colour had developed the sample was washed in water to stop the reaction, dried and mounted in Crystal Mount (Sigma).

β-galactosidase (β-gal) staining with X-gal solution

A ferri/ferro cyanide solution was made by adding 0.422g of 5mM potassium ferrocyanide, 0.329g of 5mM potassium ferricyanide and 0.081g of 2mM magnesium chloride to 200ml of PBS, this was filtered and stored in the dark. A 50mg tablet of X-Gal (Sigma) was added to 1.25ml of DMSO and allowed to dissolve. 50μl of the X-Gal solution was added to 2ml of the ferri/ferro cyanide solution, this was filtered and stored frozen.

Enhanced Green Fluorescent Protein

Enhanced green fluorescent protein (EGFP) is a convenient way of visualising transduced cells. EGFP is encoded by a number of plasmids, the plasmid used was MGIN. When visualised under UV light, the cells that have taken up the EGFP DNA and express the EGFP protein appear bright green. EGFP expression is visible about 8 hours post transduction and is maintained for several days.

Cell cloning

This procedure was carried out as the PA317/LAPSN viral producer cells appeared initially not to be producing a high enough virus titre as the cells were not transfecting at a high efficiency. 3T3 cells were used to determine the titres of the retroviral producer cells by the addition of viral supernatant, then staining the 3T3s with Fast Red or BCIP.

The cells were grown in culture for a few days before being lifted using 2ml trypsin to produce a single cell suspension. 4ml of medium was added to stop the action of the trypsin and the cells were counted and diluted to produce a range of seeding densities. The ranges chosen were 10, 50 and 100 cells/ml and 10μl of medium containing cells was added to each well of a 96-well plate. The well was placed in the incubator overnight and when the cells had attached, each well that had only one cell in it was marked. When a colony had formed from a single cell, the colony was isolated using trypsin and was plated into a T25 flask with FAD medium and the growth was monitored. When the flask was nearly confluent it was split for expansion in culture, and a cell smear was made and stained for AP.

2.12 Flow cytometry

The following was carried out to assess the best method to fix and/or permeabilise the cells and when to add the antibody before running the cells on the flow cytometer. This was investigated using a combination of the following variables.

Fix (F)

2% paraformaldehyde (pfa) or 70% ethanol (EtOH) was used, or cells were not fixed at all.

Stain (S)

Antibodies against α6 or β1 integrins, which were labelled with FITC, were added to the cells.

Permeabilise (P)

0.1% Triton X-100 was added to the cells.

Combinations of variables tested for flow cytometry work

The following combinations for fixing, staining and permeabilising the cells were examined to see which gave the better result.

F (pfa)	+	S	
F (EtOH)	+	S	
No fix	+	S	
S	+	F (pfa)	
S	+	F (EtOH)	
S	+	no fix	
S	+	P (Triton-X)	
S	+	F (EtOH)+	P (Triton-X)

Preparation of cells for flow cytometry from organotypic cultures

After the required time in culture the organotypic culture was removed from the nylon mesh using forceps and divided into equal pieces, depending on what processing methods were to be carried out. Part of the culture was placed in 4% paraformaldehyde overnight for histology, staining with H&E. Another part of the culture was processed for cryosectioning (section 2.5 and 2.8). The remaining culture was placed in 3ml of warm EDTA and left for up to 1 hour in a 37°C water bath, shaking the tube periodically.

When the epithelial sheet was separated from the underlying connective tissue it was placed in a tube containing 2ml of warm 0.25% trypsin. This was placed in the water bath for up to 10 minutes until the epithelial sheet had disassociated into a single cell suspension. FAD medium was added to the suspension to stop the action of the trypsin. The suspension was filtered using a cell strainer (Falcon, pore size 0.4μm) into as many fresh tubes (Falcon) as necessary and centrifuged at 1000rpm for 10 minutes.

The supernatant was discarded, PBSA (1% FBS was added to PBS without Mg^{2+} and Ca^{2+}, Invitrogen) was added and the tubes were centrifuged. The supernatant was discarded and the pellet was mixed in the residual liquid. The pellet was resuspended in 10µl of the appropriate FITC labelled antibody and left in the dark at room temperature for 1 hour.

PBSA was added to the sample and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cell pellet was fixed by resuspending the cell pellet in ice-cold 70% ethanol for 5 minutes on ice. The sample was centrifuged then washed twice with PBSA. The tubes containing the cell pellets were then taken to the flow cytometry facility. 400µl of facsflow (made of PBS and antibiotic antimycotic, Becton Dickinson, BD Biosciences, Cowley, Oxford, UK), 40µl of 0.5mg/ml of propidium iodide (PI, Sigma) and 50µl 1mg/ml of RNaseA (Sigma) was added to each tube and the samples were placed in a water bath for 30 minutes. RNaseA was added to destroy any RNA present as PI stains RNA as well as DNA.

The cells were run on a FACScalibur (Becton Dickinson) where data was collected for 10000 events which were later analysed using WinMDI 2.8 and Cylchred programs.

Flow cytometry on primary and immortal cell lines

A variety of different primary and immortal cell lines were grown in tissue culture flasks with feeder layers, and as organotypic cultures, and were subjected to the refined protocol and run on the flow cytometer. Palatal keratinocytes (PE), OKF6/TERT-1 keratinocytes, OKF6/TERT-2 keratinocytes and N-TERT keratinocytes were used from culture flasks and as cells derived from the organotypic cultures. The anti integrin antibodies α6 and β1 were chosen as they were previously proposed to be possible stem cell markers.

Addition of anti-α6 and -β1 integrins

Initially the experiment was carried out using both the anti-α6 and -β1 integrin antibodies in the same tube, as the α6 integrin was labelled with FITC and the β1 integrin was labelled with phycoerythrin (PE). The rationale for this was that α6 integrin stained a subset of the β1 integrin population which would give a purer stem cell population (Kaur and Li, 2000).

CHAPTER THREE

CHAPTER 3

STAINING PATTERNS OF PALATAL EPITHELIUM

3.1 Introduction

The aim of this study was to clarify the pattern of stem cell distribution in human oral mucosal epithelium. The tissue examined was the mucosa of anterior hard palate as this has a marked, and typically regular, pattern of epithelial rete to which cell differentiation can be related. Studies in chapter 5 use the method of lineage marking and transplantation of organotypic cultures to SCID mice to see if clonal units could be visualised. This has previously been used to identify clonal units in murine epidermis (Mackenzie, 1997). As these studies produced results in conflict with a range of previous observations, the pattern of differentiation in palatal rete was examined in detail by staining with a range of antibodies to known epithelial differentiation markers and to putative stem cell zones. The results localise stem cells to the apical epithelial tip indicate and suggest that this distribution results from an intrinsic epithelial pattern modulated by connective tissue influences.

Choice of antibodies

Antibodies against α6 and β1 integrins; K15; K19; Delta and its receptor Notch (Lowell *et al.*, 2000); and p63 (Pellegrini *et al.*, 2001) were chosen as it had been proposed that they localise stem cells. Antibodies against other keratins were also used as they are markers of differentiation and give an indication of the tissue maturity. Ki-67 was used as it shows the position of the actively cycling proliferative cells, but not cells in the resting phase such as stem cells. BGAs H1 and Le^y were used as they have also been used as markers of early differentiation. Involucrin was used as it is a marker of terminal differentiation and therefore should not stain undifferentiated cells. The oncoprotein c-Myc was used as its expression promotes differentiation of stem cells and it therefore might act as a marker of differentiation (Gandarillas and Watt, 1997; Waikel *et al.*, 2001).

NG2, an antibody against mouse chondroitin sulphate and LHM2, against its human homologue have both also been suggested as stem cell markers. Levels of E-cadherin have been reported as being heterogeneous with low levels over the CPT in foreskin and high levels at the ERT in palm, a pattern related to stem cell position (Moles and Watt, 1997). Vimentin was used to identify the connective tissue components. The other antibodies were investigated to see if any stem cell related patterns were observed.

3.2 Materials and methods

3.2.1 Tissue Samples

Specimens of palatal mucosa were obtained, with informed consent, as tissue removed from 20 healthy patients during surgical exposures of unerupted canines. Specimens were transported to the laboratory at 4°C in a tissue culture medium termed FAD (Wu *et al.*, 1982). All specimens were processed for frozen sectioning and, when of sufficient size, part was removed to generate cell cultures. Specimens were either (a) fixed in 4% formalin, processed for wax embedding and sectioning, and stained with H&E or for histo-blood group antigens (BGAs) or (b) frozen in Cryomatrix (Shandon) in an isopentane/liquid nitrogen bath, sectioned perpendicular to the epithelial surface in a cryostat set at a thickness of 5μm and collected for immunofluorescent staining on Polysine microscope slides (Shandon).

3.2.2 Immunofluorescent methods

Methods for staining with all antibodies except those against the BGAs are detailed in Chapter 2.

Staining for BGAs was carried out in the laboratory of Prof. Erik Dabelsteen, (School of Dentistry, Department of Oral Diagnostics, Copenhagen, Denmark). The antibody against BGA H was BE2 and the antibody against BGA Lewis y (Le^y) was AH6. Sections cut at 5μm were dewaxed and brought to water before applying the appropriate antibody for 20 hours at 4°C. Slides were rinsed in 3 changes of PBS and incubated for 40 minutes with rabbit anti-mouse antibody using the Dako EnVision System, and then labelled with

horseradish peroxidase (HRP). Additional samples were also stained after treatment with neuraminidase (Mandel *et al.*, 1991) which detects sialated structures.

3.3 Results

3.3.1 Haematoxylin and eosin (figure 3.1)

The H&E stained sections of palate showed a thick orthokeratinised stratified epithelium with a smooth surface and deep finger-like projections into the connective tissue. The underlying lamina propria had the appearance of a dense collagenous tissue that interdigitated with the epithelial rete making up the lower two-thirds of the epithelium. There was some variability in the lengths of the rete from specimen to specimen but all samples had a similar overall tissue morphology.

3.3.2 Tissue architecture

The connective tissue papillae interdigitate with the epithelium. The uppermost part of the papillae, the CPT, appear to be of a uniform height where they project, and come to a point in the epithelium. The uppermost points of the CPT are homogeneously rounded in contrast to the ERT. The base of each connective tissue papillae that adjoins the lowermost part of the epithelium appears to be less uniform in shape. The ERT are not necessarily rounded and fit into the irregular connective tissue papillae bases.

3.3.3 Immunofluorescent staining

Immunofluorescent staining gave consistent and well defined staining patterns for each of the antibodies used and, except where indicated, similar staining patterns were obtained for all specimens. Antibody staining was consistently found to be distributed in relation to tissue architecture and cell maturity. To simplify the explanation of the staining patterns, the following abbreviations have been used, connective tissue papillae tip the most superficial part of the connective tissue (CPT), and epithelial rete tip the deepest part of the epithelium (ERT).

3.3.4 Patterns of staining with markers of basal cells

α6 integrin (figure 3.2A)

Binding of antibodies against α6 integrin was localised to the region of epithelial/connective tissue interface with the appearance of a continuous band. Staining appeared stronger at the interface in the region of the CPT and was typically weaker, and sometimes absent, at the ERT. Typically the lateral surfaces of the basal cells were unstained but over the tips of CPT there was weak staining of the whole periphery of basal cells. Staining for α6 integrin was also seen on some connective tissue elements, apparently capillaries.

β1 integrin (figure 3.2B)

β1 integrin was localised to basal cells and typically, a single layer of basal cells showed staining of the entire cell periphery. As observed with α6 integrin, β1 integrin staining appeared somewhat stronger for cells overlying the CPT. A few layers of suprabasal cells in the region of the ERT were weakly stained as were some suprabasal cells over the connective tissue papillae tips. Staining for collagen IV was also undertaken to define clearly the region of the basal lamina and, unlike staining for α6 and β1, staining for collagen IV was found to be uniform along the whole of the epithelial/connective tissue interface (figure 3.6A) suggesting that this particular uniform staining pattern is not a sectioning artefact.

K19 (figure 3.2C)

Staining for K19 was found to be rather variable but two distinct patterns of staining could be identified. Typically, staining was localised to basal cells with basal cells at the ERT being stained more strongly. Staining of basal cells over the CPT was variable but usually weak or absent. Additionally, K19 strongly stained individual cells lying singly or in clusters at the ERT. This staining, as discussed below, appeared to correspond to Merkel cells (figure 3.10). In some samples large stretches of epithelium showed no basal cell staining at all and only Merkel cell staining was present.

K15 (figure 3.2D)

The pattern of staining for K15 was similar to that for K19 but the bright staining of Merkel cell clusters seen with K19 was absent. Staining was largely restricted to basal cells with stronger staining of cells at the deep tips of the ERT. In some specimens suprabasal cells were also stained in this region Although the distribution of staining for K15 was similar to that for K19, it was more consistent and less patchy in that most rete were stained.

K5 and K14 (figures 3.3A and B respectively)

Staining for both K5 and K14 was cytoplasmic, excluded the nuclei and was present in all epithelial strata from the basal cells to the stratum corneum.

3.3.5 Staining with differentiation markers

K6 and K16 (figures 3.3C and D respectively)

K6 was present in most of the suprabasal cells of the epithelium above the level of the CPT. Staining of suprabasal cells in the core of the epithelial rete was also present but the basal and some suprabasal cells at the ERT were unstained. In most specimens, areas of the sections could be found where K6 also stained basal cells at the tips of the CPT (figure 3.3C). K16 had a staining pattern essentially similar to K6 in that the suprabasal cells stained above the level of the CPT and down into the core of the rete. K16 appeared to be expressed earlier than K6 with staining of basal cells over the CPT extending down to include basal cells approaching the ERT.

K1 and K10 (figures 3.4A and B respectively)

K1 and K10 stained most of the suprabasal cells above the level of the CPT with K1 expressed lower in the epithelium than K10 and extending down into the rete cores. In some regions the staining for K1 reached almost to the basal layer over the CPT.

K4 and K13 (figures 3.4C and D respectively)

The staining for K4 and K13 was not uniform and was present only in some regions of epithelium. When present it was found high suprabasally with a chevron pattern reaching down into the epithelial rete. K13 staining was similar to K4 but reached deeper into the rete.

Blood group antigens H1 and Le^y (figures 3.5C and D respectively)

Staining with the antibody against BGA H1 was localised to the cell periphery. Staining extended into the spinous layer with no basal cell staining. Stained cells reached into the rete cores but staining became weaker and sparser towards the ERT. The highest concentration of stained cells was over the CPT but not including the basal cells, and at the rete base. BGA Le^y was localised to the cell surfaces of patches of spinous cells, either in clusters or as individual cells. Some cells were very strongly stained whereas others were much weaker.

3.3.6 Staining with other antibodies

K7, K8, K17, K18, K19 and K20 (figures 3.10A-F respectively)

Antibodies against keratins K7, K17, K18 and K20 each stained cells near the ERT that were found singly or in clusters. Antibodies against K17 and K18 also showed patchy and apparently artefactual staining in the high suprabasal region. This staining corresponded to Merkel cells.

Ki-67 (figure 3.5A)

Ki-67 staining was seen in the nuclei of cells in the lower half of the epithelium below the CPT. Labelled cells were rarely seen higher than the tops of the CPT and the greatest number of stained cells were in the upper part of the epithelial rete where about 25% of the cells were stained. Fewer stained cells were found in the lower half of the rete and the zone of basal cells showing positive staining for K19 and K15 contained fewer cells stained for Ki-67.

p63 (figure 3.5B)
p63 staining was observed in all basal and suprabasal cells within the rete up to the level roughly corresponding to the top of the CPT. There was some clustering of stained cells over the CPT and the suprabasal cells appeared to be weaker stained than the basal cells.

Collagen IV (figure 3.6A)
The interface between the epithelium and CT stained as continuous line. Connective tissue elements, such as blood vessels were also strongly stained.

Laminin (figure 3.6B)
Staining for laminin was similar to that observed with collagen IV, the interface between epithelium and CT stained as a continuous band with staining of CT elements.

Vimentin (figure 3.6C)
This antibody stained elements within the CT and also a few elements within the lower part of the epithelium.

EGFR-10 (epithelial growth factor receptor-10) (figure 3.8D)
This antibody stained the cell surface of all cells from the ERT to the stratum corneum. The cytoplasm in the cells of the rete seem to be weakly stained compared to cells closer towards the cornified layer from the CPT level upwards. At high power it appears that the cells at the ERT in the basal and suprabasal level are more strongly stained than the cells further up the rete.

LHM2 (figure 3.7A)
This antibody stained the basal cells strongly over the CPT and became weaker down towards to ERTs. It was possible to see the cells of the basal layer becoming weaker stained from the CPT down the rete to the basal cells at the ERT which were unstained.

NG2 (figure 3.7B)

The staining for this antibody was similar to LHM2 with strong basal staining at the CPT but with the basal cells staining all the way down the ERTs. All cells in the basal layer in the rete were stained with some weaker than others.

Sonic hedgehog (figure 3.7C)

The cell membrane of all cells in the epithelium and also the cytoplasm of suprabasal cells stained with this antibody.

Keratinocyte basal (figure 3.7D)

This antibody strongly stained the cell surface of all cells in the whole of the epithelium, and the cytoplasm of these cells were also weakly stained.

E-cadherin (figure 3.8A)

The cell surface outlines stained very strongly with this antibody appearing as a single cell band over the CPT and as a thicker band of stained cells into the suprabasal cells about half-way up the rete. Cells at the ERT were weakly stained under the strongly stained band.

N-cadherin (figure 3.8B)

The cytoplasm of a few individual cells near the ERT were stained with this antibody.

Involucrin (figure 3.8C)

This antibody against involucrin stained cells in the stratum corneum. The antibody stained the cell surface and cytoplasm but not the nuclei of the cells.

Delta (extracellular domain) (figure 3.9A)

With this antibody staining identified individual cell outlines that were visible around the ERT.

Notch 2 (figure 3.9B)

This antibody stained the cell membrane and cytoplasm of every cell in epithelium.

Osteopontin (figure 3.9C) **and osteonectin**

Antibodies against both of these gave similar results. The whole epithelium including nuclei and cytoplasm stained with staining slightly weaker at the ERT.

c-Myc (figure 3.9D)

Individual cells stained at the ERT, apparently in the suprabasal layer. There were varying numbers of stained cells per ERT.

No results were observed with the antibodies against Jagged, Hoxb4, Cyclin A, Notch 1, Notch intracellular domain, Notch extracellular domain EGF repeats #5-7 and Notch extracellular domain EGF repeats #12-20.

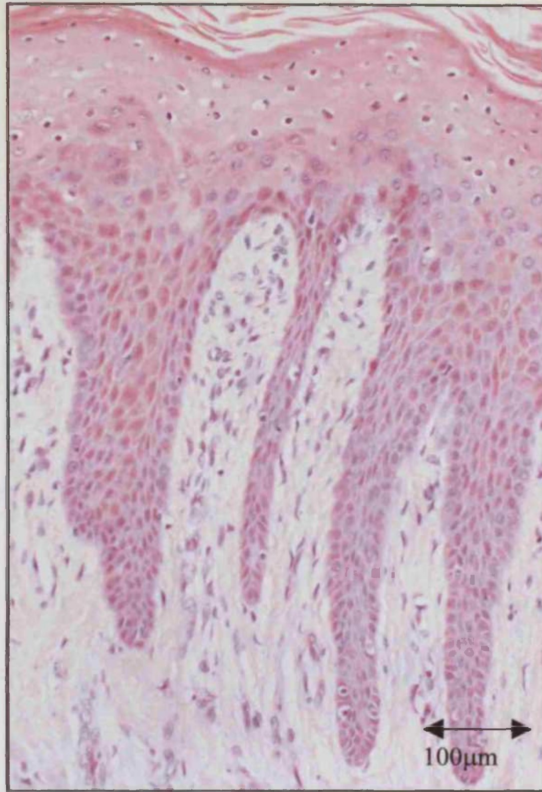
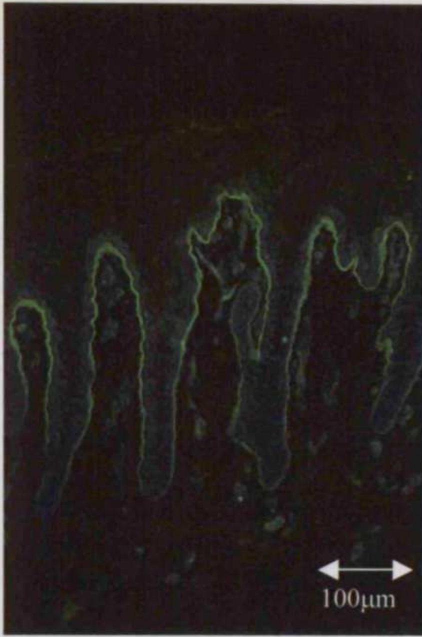


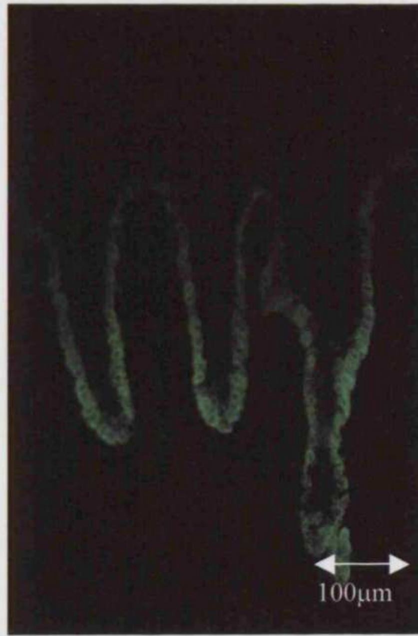
Figure 3.1 Palate section stained with H&E (magnification x20)



A

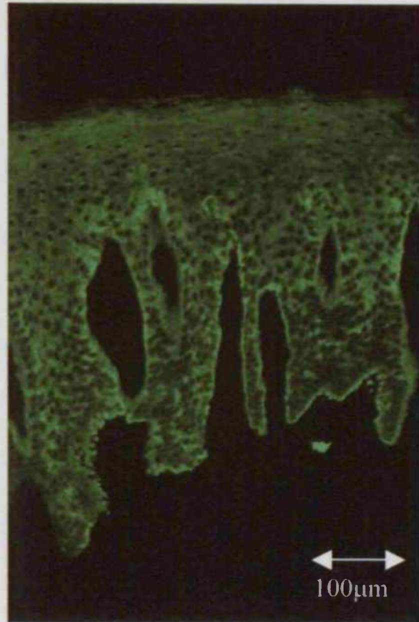
B

C



D

Figure 3.2 Palate stained with α6 integrin (A), β1 integrin (B), K19 (C) and K15 (D) (magnification x20)



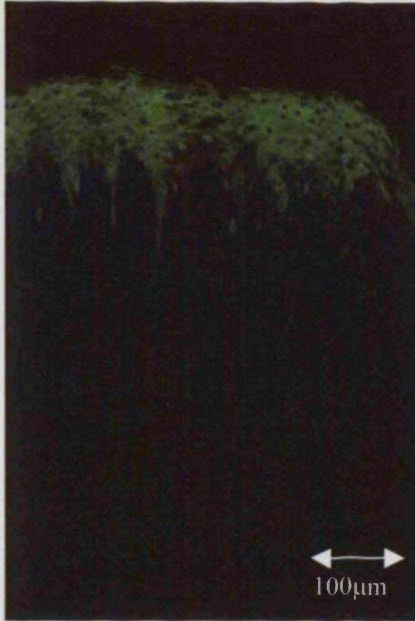
A

B

C

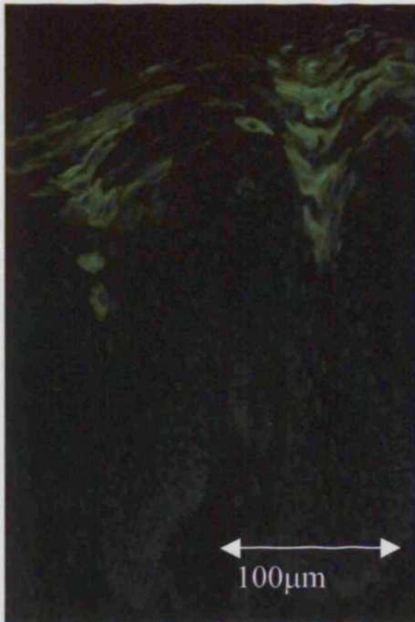
D

Figure 3.3 Palate stained with K5 (A), K14 (B), K6 (C) and K16 (D) (magnification A&B x20, C&D x40)



A

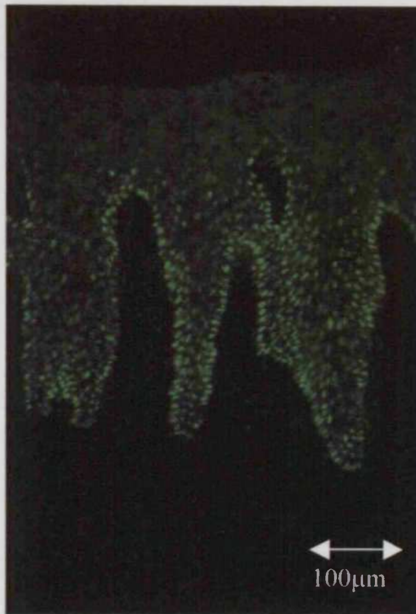
B



C

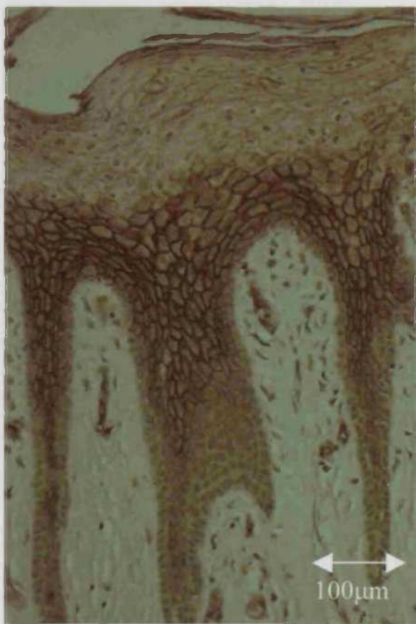
D

Figure 3.4 Palate stained with K1 (A), K10 (B), K4 (C) and K13 (D) (magnification A&B x20, C&D x40)

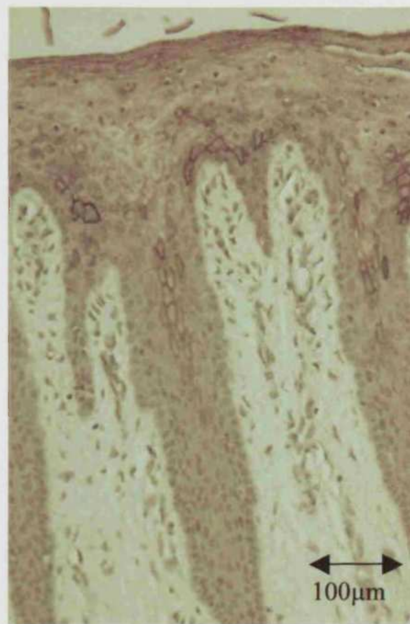


A

B

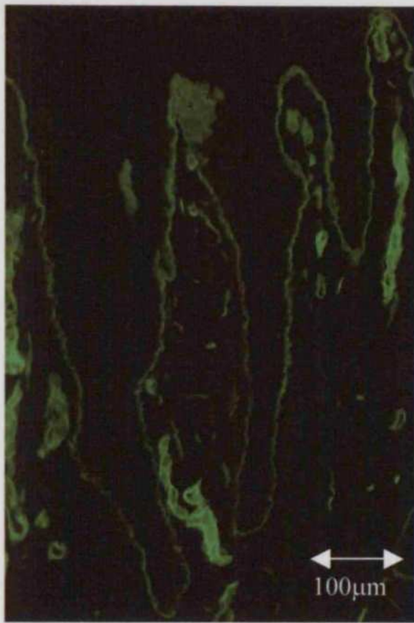


C

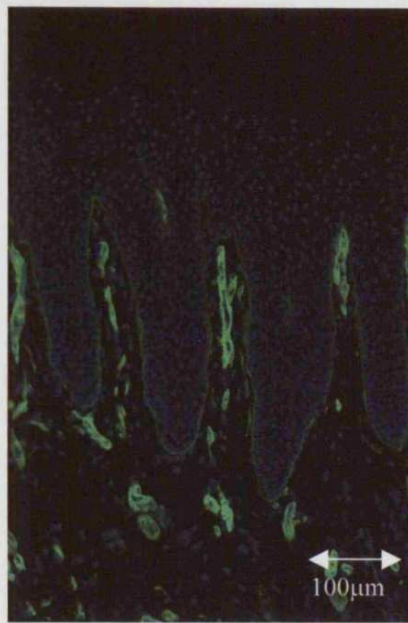


D

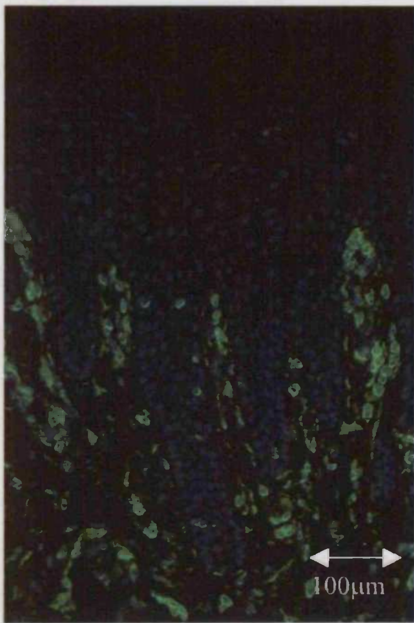
Figure 3.5 Palate stained with Ki-67 (A), p63 (B), BGA H1 (C) and BGA Le^y (D) (magnification x20)



A



B

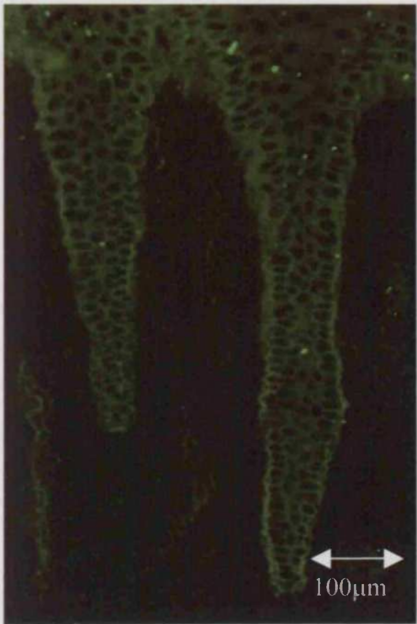


C

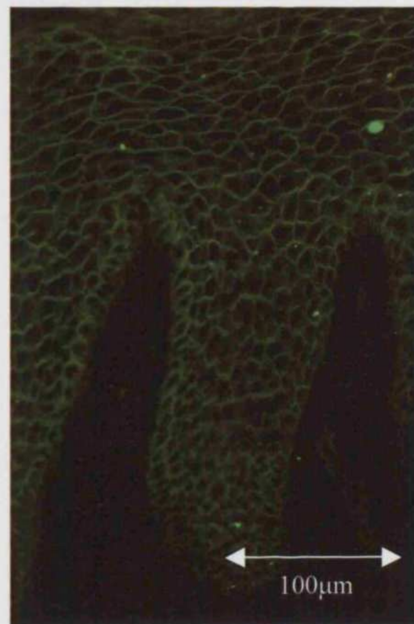
Figure 3.6 Palate stained with collagen IV (A), laminin (B), vimentin (C) (magnification x20)

A

B



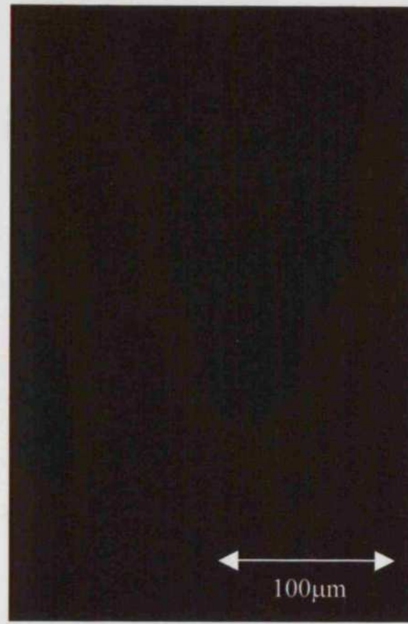
C



D

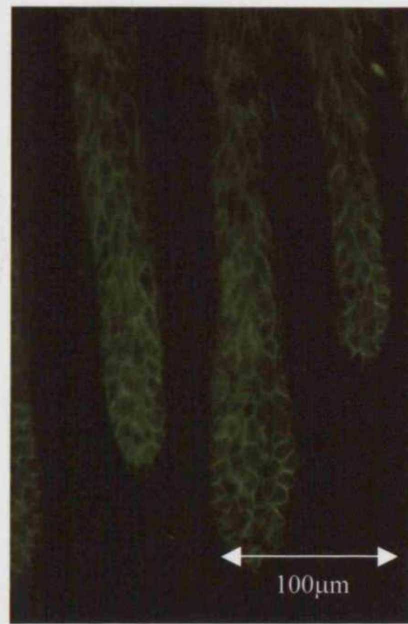
Figure 3.7 Palate stained with LHM2 (A), NG2 (B), sonic hedgehog (C) and keratinocyte basal (D) (magnification A&C x20, B x10, D x40)

A



B

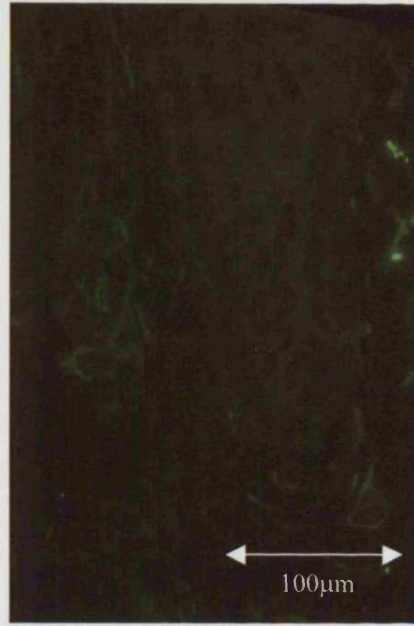
C



D

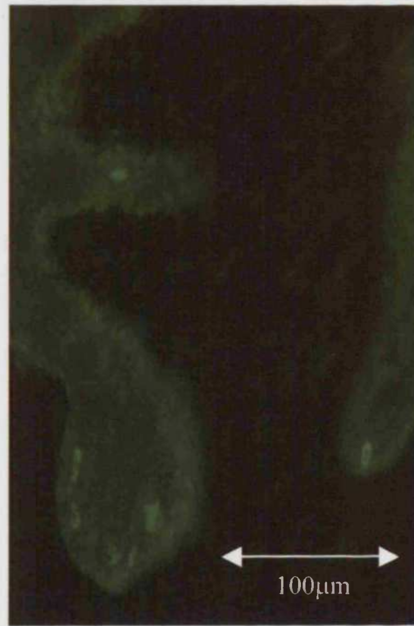
Figure 3.8 Palate stained with E-cadherin (A), N-cadherin (B), involucrin (C) and EGFR-10 (D) (magnification A&C x20, B&D x40)

A



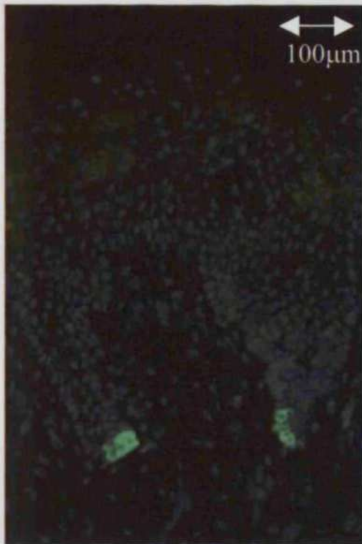
B

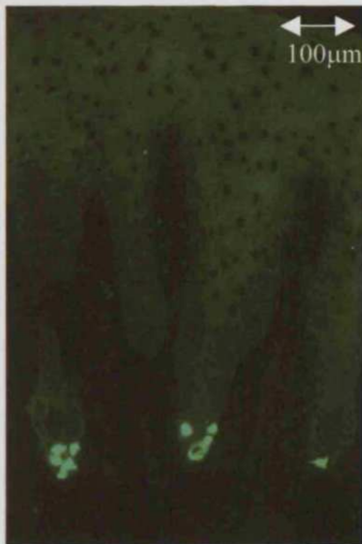
C



D

Figure 3.9 Palate stained with Delta (A), Notch 2 (B), osteopontin (C) and c-Myc (D) (magnification x40)





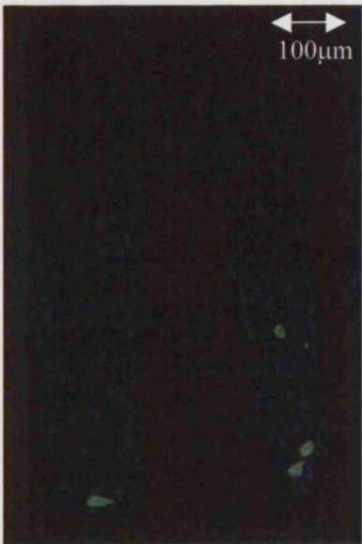


Figure 3.10 Palate stained with K7 (A), K8 (B), K17 (C), K18 (D), K19 (E) and K20 (F) (magnification x20)

3.4 Discussion

Many studies have described the patterns of expression of keratins and other antibodies in oral mucosa (Morgan *et al.*, 1986; Morgan *et al.*, 1987). However, relatively little work has been undertaken on normal human palate using immunofluorescent methods, but work has been carried out in human gingiva. The present study was undertaken with the aim of detecting differences in the patterns of basal cell staining that might be used to localise stem zones.

3.4.1 Stem cells

Stratified squamous epithelia are renewed by a hierarchy of dividing cells that is ultimately maintained by a small subpopulation of stem cells. Epithelial stem cells are considered to be undifferentiated cells that are relatively slowly cycling but have a high self-renewal potential. At anatomical sites such as hair and eye, large epithelial structures exist which are maintained by their own reservoir of stem cells. In hair the stem cells are located within the bulge region of the hair follicle (Cotsarelis *et al.*, 1990), and in the eye the stem cells are located in the limbal region of the cornea (Cotsarelis *et al.*, 1989). Epithelia with smaller units of structure, such as epidermis and ear epithelium have one stem cell per unit as explained by the EPU (Potten, 1974). Palate falls between these two models and has an intermediate type of structure, it has large rete suitable for study and it is thought that the stem cells within it correspond to these rete. There is conflicting evidence as to where the stem cells are, they may lie over the CPT, lie in the ERT, or be anywhere in between.

3.4.2 Tissue architecture

From the cryosections of palate, which shows the ERT as rounded structures, it is unlikely that the cryosection cuts through the very tip of every rete. It is possible that the rete have been cut close to the tip giving a rounded appearance in cross-section, but not including the actual tip. If a positive stem cell marker or zone is identified, it is possible that it will not be present in every rete and will therefore only be seen in some rete.

3.4.3 Differentiation markers

As stem cells are the least differentiated cells, differentiation markers, such as some of the keratins were used to see where cell differentiation occurred in the epithelium. K5 and K14 were the first pair of keratins to be expressed in the palatal epithelium with the whole of the epithelium stained from the basal cells upwards continuing into all strata. K6 and K16 were the second pair of keratins to be expressed with K16 expressed slightly earlier than K6. There was some K16 staining in the basal cell layer over the CPT reaching into the suprabasal layers where K6 also stained. K1 and K10 were the next pair of keratins to be expressed with staining starting above the level for K6 and K16, with keratins K4 and K13 the last pair to be expressed very high up in the epithelium.

When these keratins are expressed they correspond to states of cell differentiation with the least differentiated cells corresponding to cells in the basal layer that includes the stem cells, and the most differentiated cells residing in the uppermost layers the upper granular and cornified layers. K19 and K15 have both been associated with stem cells in hair follicles and from the staining observed, correspond to the least differentiated cells which in palate, were located in the ERT. Even though the $\alpha6$ and $\beta1$ integrins have been identified as putative stem cell markers, in palate this does not hold true as the position of the $\alpha6$ and $\beta1$ integrin staining appears at the CPT which from the keratin staining, does not include the least differentiated cells. In human epidermis, cells that stain strongest with the putative stem cell marker $\beta1$ integrin are identified as lying at the tips of the CPT (Jensen *et al.*, 1999), whereas in the human oesophageal epithelium the putative stem cells were situated in the ERT (Seery and Watt, 2000). Given the observations obtained in this study it could be suggested that the least differentiated cells which correspond to the stem cells, reside somewhere in the ERT.

Keratin distribution is specific for each epithelial tissue and varies according to the differentiation status of the cells. In epidermis, the basal cells express K5 and K14 and suprabasal cells, which are more differentiated, express K1 and K10. Skin also contains K19 which is only present in a small proportion of cells. In adult human hairy skin sites K19 is restricted to the bulge area of the hair follicle. In human glabrous skin K19

expressing cells were found in the basal layer of palm and sole (Michel *et al.*, 1996), localised in the deep part of the rete ridges. Simple epithelia generally express the simple keratins K7, K18 and K20, and K17 is typically expressed in hyperproliferative squamous epithelia and also in basal cells of complex epithelia (Chu and Weiss, 2002). In palate however, these keratins, K7, K8, K17, K18 and sometimes K19, stain Merkel cells which are non-epithelial in origin.

3.4.4 Other antibodies

The other antibodies gave a range of staining patterns with laminin and collagen IV giving similar results staining the interface between the epithelium and the CT, and also some CT elements. Vimentin, which also stains CT elements, stained Langerhans' cells and elements within the lower part of the epithelium. Keratinocyte basal and sonic hedgehog both gave similar staining profiles with all cell surfaces throughout the epithelium stained. Involucrin is a marker of terminal differentiation in keratinocytes and it is expressed in the stratum corneum which can be observed clearly in figure 3.8C. After this, it is cross-linked in the cornified layer during the construction of the cornified envelope, of which it is a major component (Hoeller *et al.*, 2001). EGFR-10 gave an interesting result with cell surfaces in the lower part of the ERT strongly stained compared to the rest of the epithelium. Delta, c-Myc and N-cadherin stained individual cells within the rete but these cells appeared to be randomly situated and not in any regular position within the rete. Staining with osteopontin became weaker towards the ERT as did staining for Notch 2. E-cadherin gave an unusual profile with a single line of cells stained over the CPT which then reach into the rete cores as a thicker band of stained cells. NG2 recognises chondroitin sulphate and LHM2 is the human form of NG2. Both of these antibodies stained the basal cells strongly over the CPT with basal cell staining reaching down the rete with varying degrees of staining at the ERT.

In palate, which has a well developed rete structure, mitotic activity has been postulated to occur at the base of the ridges (Loe *et al.*, 1972), which is analogous to the clusters of dividing cells at the base of the crypts in the rat small intestine (Uddin *et al.*, 1984).

However, staining for the cell cycle protein Ki-67 occurred at an intermediate position between the base and tip of the epithelial rete.

3.4.5 Previously reported antibody staining

In foreskin, breast and eyelid skin, β1 integrin-bright patches overlie the dermal papillae (Jones *et al.*, 1995). Using whole mount labelling of sheets of normal foreskin, the β1 integrin-bright cells were also seen in the epithelium at the dermal papillae tips (Jensen *et al.*, 1999) with the patches of bright cells being separated from one another by areas of integrin-dull cells. Interestingly, α6 integrin positive staining cells, another putative stem cell marker, did not correlate to β1 integrin staining at the dermal papillae tips. Suprabasal marker K10-stained cells, were always found in the integrin-dull regions of the basal layer and none in the integrin-bright stem cell clusters.

In oesophagus, β1 integrin expression was confined to the basal layer in the IBL but in the papillae of the supporting PBL there was suprabasal expression at the tips of the papillae. β1 integrin staining was weaker in the IBL than the PBL (Seery and Watt, 2000). By combining fluorescence activated cell sorting (FACS) with *in vitro* clonal analysis evidence was obtained that the IBL is enriched for stem cells. The proportion of clonogenic cells in the IBL (integrin-dull) was two fold higher than in the PBL (integrin-bright). This evidence is in contrast to epidermis where clonogenic cells are enriched in the β1 bright population (Jones and Watt, 1993).

Basal cells also express other integrins. In normal human epidermis expression of the integrins is restricted to the basal layer with α2β1, α3β1 and α6β4 being the predominant integrins. α6β4 is exclusively expressed in the hemidesmosomes (Sonnenberg *et al.*, 1991) and binds to epiligrin in the basement membrane (Carter *et al.*, 1991). Basal cells express high levels of the adhesion molecule α6 and low levels of the transferrin receptor 10G7 (Li *et al.*, 1998) and it was shown that the cells with the phenotype $\alpha6^{bri}10G7^{dim}$ represented the epidermal stem cell population as the cells exhibited the greatest regenerative capacity *in vitro* and were quiescent at the time of isolation from the

epidermis as determined by cell cycle analysis. Murine *in vivo* studies suggest that epidermal stem cells constitute between 1 and 10% of the basal layer and the $\alpha6^{bri}10G7^{dim}$ suggested keratinocyte stem cell population made up 4% of the basal cells (Li *et al.*, 1998).

Significant amounts of K15 are present in the basal layer of several stratified epithelial tissues and may be a marker for stem cells in hair follicles (Lyle *et al.*, 1998). In frozen unfixed sections, expression of K15 was completely restricted to the basal layer of epidermis (Porter *et al.*, 2000) and was basally restricted in buccal and palate mucosa. K15 was expressed at lower levels in plantar epidermis where groups or single cells were observed usually deep in the epidermal ridges of the foot sole. K15 is expressed in the basal cells of the outer root sheath of hair follicles and in the luminal cells of the secretory sweat glands.

3.4.6 Blood group antigens

In stratified squamous epithelium cells express a series of histo-BGA precursors as they differentiate and move from basal to superficial layers (Dabelsteen *et al.*, 1991). Keratinised oral mucosa, like palate, mainly express carbohydrates that are precursors of the histo-BGAs but only type 2 chains like H1 and Le^y, and mucin-like type 3 chains have been detected (Ravn and Dabelsteen, 2000). The BGAs are expressed in most human epithelial tissues in such a way that the expression of distinct carbohydrates is restricted to specific tissues and cell types. From the staining seen for the BGAs it is apparent that the expression of H1 is seen earlier in the differentiation of the keratinocytes than Le^y.

3.4.7 General conclusions

From the staining patterns seen in the palate sections, if the integrins were thought to be the stem cell markers it could be postulated that the stem cells reside at the CPT, but if K19 and K15 were used as stem cell markers, the stem cell zone would appear to be at the ERT. $\alpha6$ and $\beta1$ integrins are being expressed in the wrong place if they were to be used as stem cell markers as they are being expressed in the differentiated cells as stained

by K6 and K16. It is apparent therefore that $\alpha6$ and $\beta1$ integrins are not acting as stem cell markers as is the case in some of the reported epithelial tissues. They appear instead to co-localise in the basal layer with stronger expression at the more differentiated position overlying the CPT which conflicts some previous studies.

K15 and K19 stained cells that are situated at the ERT and correspond to regions of cells that lack differentiation. K19 staining (excluding the Merkel cell staining) localises to the highest points towards the ERT as does the K15 staining. K6 and K16 stained cells at the CPT and correspond to differentiated cells. From these results, staining of K15 and K19 which corresponds to undifferentiated cells at the ERT, while the differentiated cells stained by the other keratins localise further down the rete towards the CPT. As the stem cells are undifferentiated, it appears that the stem cells localise to a stem cell zone in the ERT. So far it is not known how many stem cells there are per stem cell zone. These results confirm that stem cell localisation may depend on the anatomic site.

It is apparent that there is regional variability in the position of stem cells in different tissues. It is possible that stem cells reside in a niche or microenvironment and that their position is dependent on environmental factors.

CHAPTER FOUR

CHAPTER 4

STAINING PATTERNS OF PALATAL KERATINOCYTE COLONIES

4.1 Introduction

The aim of this study was to see if keratinocytes grown *in vitro* on plastic would grow and start to differentiate in culture to form an epithelial-like structure, and if these colonies function in a stem cell-like manner. Also investigated, using a panel of antibodies, was whether the colonies gave staining profiles that could be compared to palate *in vivo*. The rationale for this was to see if a subpopulation within a colony could be identified as stem cells. It is much easier to grow keratinocytes *in vitro* on plastic than in more complex cultures such as organotypic cultures which is why this method was investigated before embarking on the more complex experimental set up of organotypic cultures.

Analyses of clonal growth in human keratinocytes was carried out by Barrandon and Green (Barrandon and Green, 1987b). Barrandon and Green defined three types of proliferating keratinocyte on the appearance of the type of clone they formed *in vitro* before and after passaging the cells. Individual clones were disaggregated and replated and the behaviour of the secondary clones were evaluated. If no secondary clones formed or consisted wholly of terminally differentiated cells the founder clone was classified as a paraclone. If 0 to 5% of the clones were terminal the clone was classified as a holoclone. Meroclones were intermediate in their behaviour with between 5 and 100% of the clones being terminal. The holoclones have the greatest self-renewal potential and are likely to be founded by stem cells.

When grown on plastic in culture, human keratinocytes eventually give rise to the different types of colonies mentioned above. If these colonies are founded by stem cells, all the cells appear small, are positioned close together and have the appearance of

cobblestones. These colonies are classified as holoclones. When these cells begin to differentiate they begin to enlarge and the colonies lose some of their cobblestone-like appearance, these colonies are the meroclones. When all the cells in the colony have enlarged, they are classified as a paraclone. Previous work has shown that keratinocytes in primary cultures proliferate and after having reached confluence, stratify to form multilayered sheets that resemble squamous epithelium, and present some aspects of normal epithelial differentiation *in vivo* (Watt, 1989; Fuchs, 1993).

4.2 Materials and methods

Palatal keratinocytes were seeded at a relatively low density at about 1×10^5 in tissue culture flasks on a feeder layer of mitomycin C treated 3T3s and were fed with FAD. When the colonies were about 20-30 cells in size the FAD medium was removed from the flask, the cells were rinsed in 10ml PBS, and fixed in 5ml 4% paraformaldehyde for 10 minutes. The top and sides of the flask were removed using a small drill to release the flask base (where the cell colonies were situated). The base of the flask was divided into sections using a Pap pen (Sigma) and the standard immunohistochemistry methods described in section 2.7 were carried out.

4.3 Results

α6 integrin (figure 4.1A)

It was possible to see the hexagonal outline of the cell surfaces stained strongly with this antibody and weaker staining could be seen on the rest of the surface. The cells overlap and where this happens staining appears stronger. A few localised patches of very strongly stained cells were visible but these appeared to be random and not associated with cell size or being suprabasal cells. Some cells were larger than others.

β1 integrin (figures 4.1B and C)

The hexagonal cell peripheries appeared to be very specifically stained where cells touched each other forming a very clear line of staining. It was possible to see the nuclei

of the cells and these appeared to have a halo of staining surrounding them. Some of the smaller cells seemed to be more strongly stained than the other larger cells giving the appearance of strongly stained patches.

K15 (figure 4.2A)

Cytoplasmic staining was seen mainly with this antibody but weak nuclear staining was also observed. Smaller cells appeared to be more strongly stained than the larger cells and these smaller cells seem to be centrally placed within the colony.

K19 (figure 4.2B)

This antibody stained the cytoplasm of the cells. It stained localised patches of individual cells within each colony. The marked staining corresponded to a particular sized cell and stained only the small cells.

K4 (figure 4.3A)

Hoechst staining was included in order to see the colony outline. This antibody stained only a few cells in the whole colony, and from the Hoechst staining it was possible that the K4 stained cells were suprabasal as their nuclei do not correspond to any of the Hoechst stained nuclei. The stained cells were large and flat in appearance.

Connexin 26 (Cx26) (figures 4.3B and C)

This antibody stained the cytoplasm and nucleus of every cell and also stained projections joining the cells to one another. The cell membrane was clearly visible and staining was stronger here. All cells were roughly the same size (figure 4.3B).

The other image shows a colony of different sized cells (figure 4.3C). The largest cells were assumed to be the most differentiated cells and these stained cells appeared to be more opaque than in the smaller, less differentiated cells.

K14 (figure 4.4A)

With this antibody only the cytoplasm had stained and none of the nuclei. The staining observed was uniform in all cells of the colony and the hexagonal outline of the cells was visible. In colonies that had begun to differentiate, the differentiated cells became much larger and the nuclei have stained weakly.

K6 (figures 4.4B and C)

The staining observed with this antibody was cytoplasmic and the larger, more differentiated cells were more strongly stained than the smaller less differentiated cells. Staining levels for this antibody varied in intensity and corresponded to cell size with the larger cells more strongly stained than the smaller cells. The overlying suprabasal cells appear to be uniformly stained with no visible nuclei. The stronger staining cells appear to be localised in groups and not randomly placed.

K10 (figure 4.5A)

This antibody stained only the few large suprabasal differentiated cells and dead cells in the colony. The cytoplasm was stained with the strongest staining seen around the nucleus.

K8 (figure 4.5B)

Hoechst staining shows that the cells stained here were a subset of the whole colony. The stained cells appear to be in clusters but vary in their staining intensities with strong and weak stained cells within the same cluster. All the stained cells were small in size.

K18 (figure 4.5C)

Staining with this antibody was cytoplasmic in appearance. Various cells within the colony show a much stronger staining profile, but these strongly stained cells corresponded to cell size with scattered small cells more strongly stained.

Ki-67 (figure 4.6A)

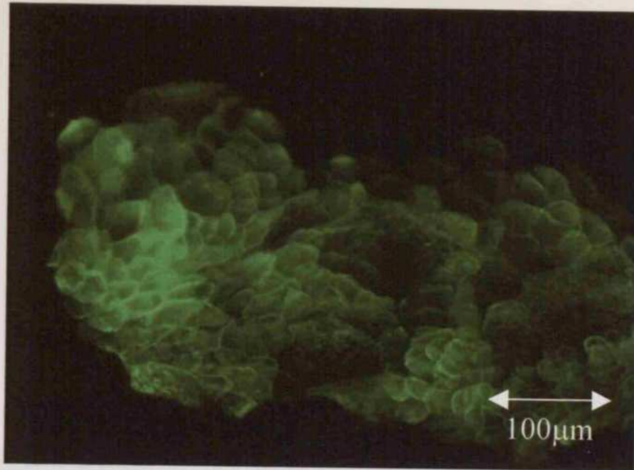
This antibody stained elements within the nuclei which probably correspond to segments on the chromosomes. All cells were stained and at least two grains could be seen per nucleus. Some of the nuclei appeared larger in size with more stained elements within them. There were a range of staining intensities but this could be due to the staining procedure or suprabasal cells masking the antibody staining.

p63 (figure 4.6B)

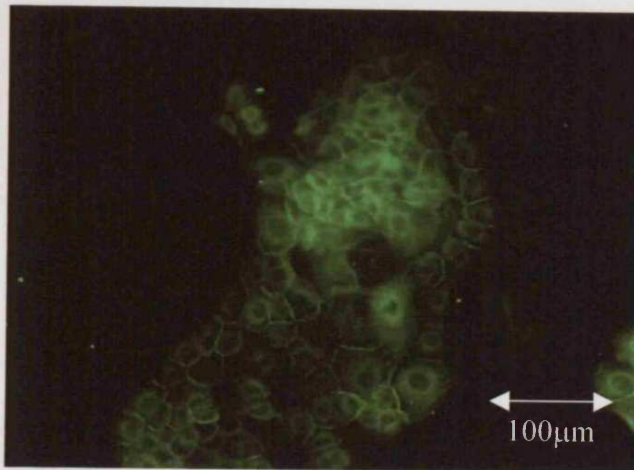
This antibody stained the nuclei of all the cells in the colony. Neither the cytoplasm nor the cell membrane were stained.

Vimentin (figure 4.6C)

This antibody stained all cells and the staining observed was mainly cytoplasmic. The vimentin antibody was against human vimentin and is a marker of human fibroblasts. The murine origin of the 3T3s cause them not to be stained with this anti-human antibody and their Hoechst stained nuclei can be visualised.



A



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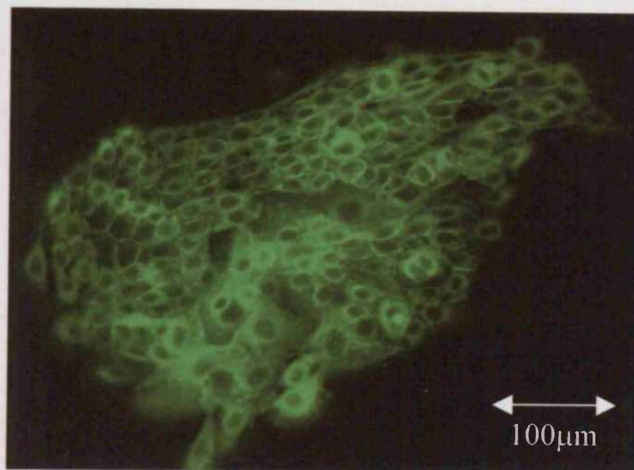
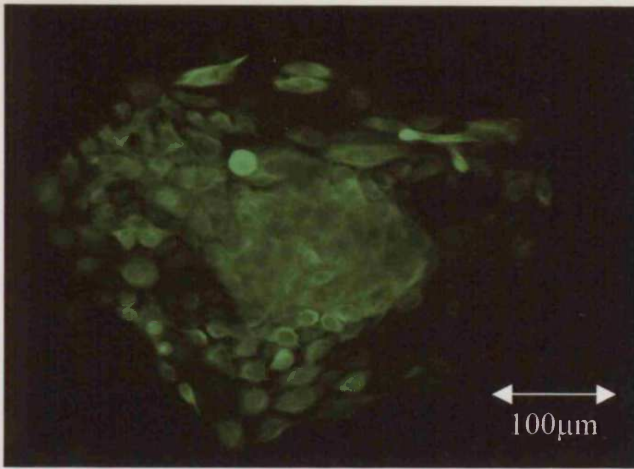
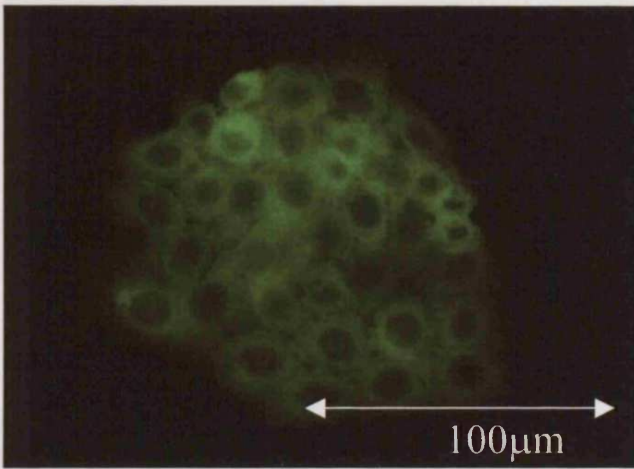


C

Figure 4.1 Palatal epithelial cell colonies stained with $\alpha6$ integrin (A) and $\beta1$ integrin (B and C) (magnification x 10)

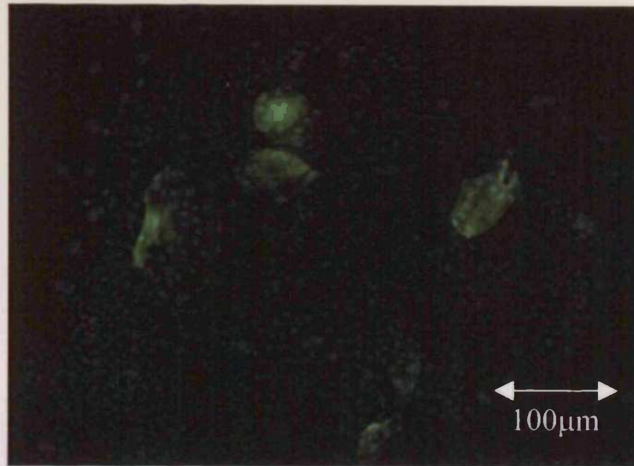


A

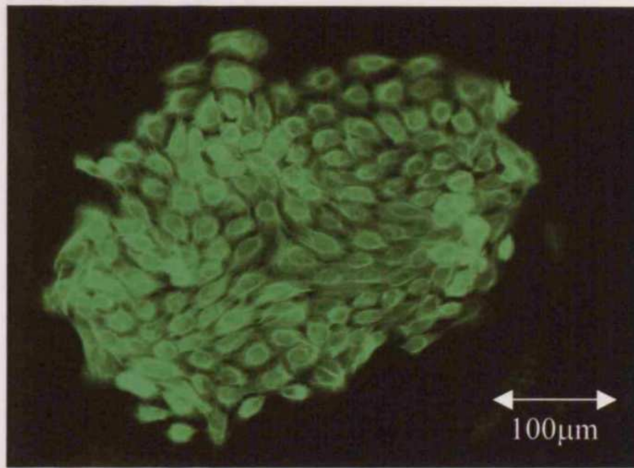


B

Figure 4.2 Palatal epithelial cell colonies stained with K15 (A) and K19 (B) (magnification A x 10, B x 40)



A



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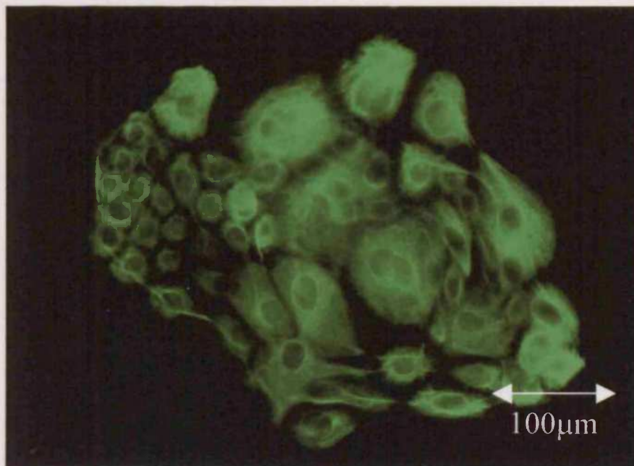
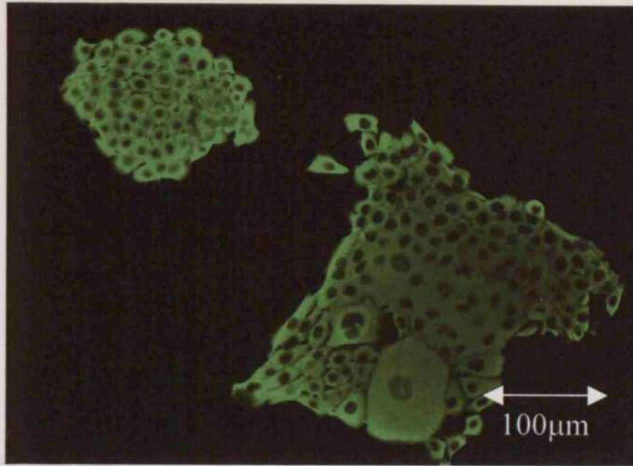
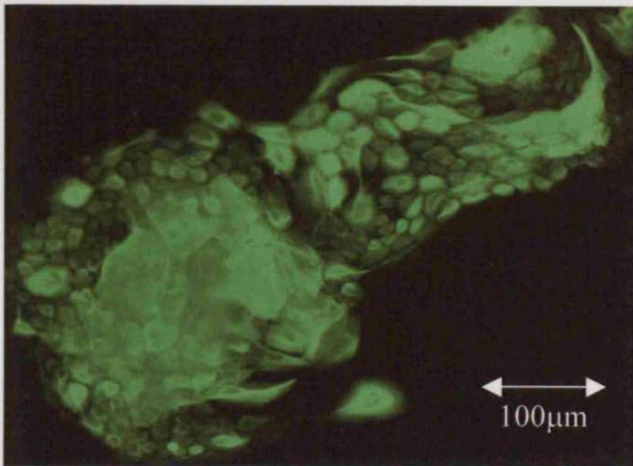


C

Figure 4.3 Palatal epithelial cell colonies stained with K4 (A) and Cx26 (B and C) (magnification x 10)



A



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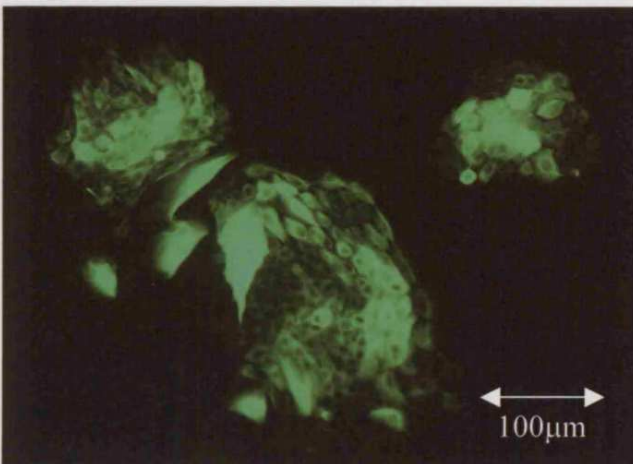
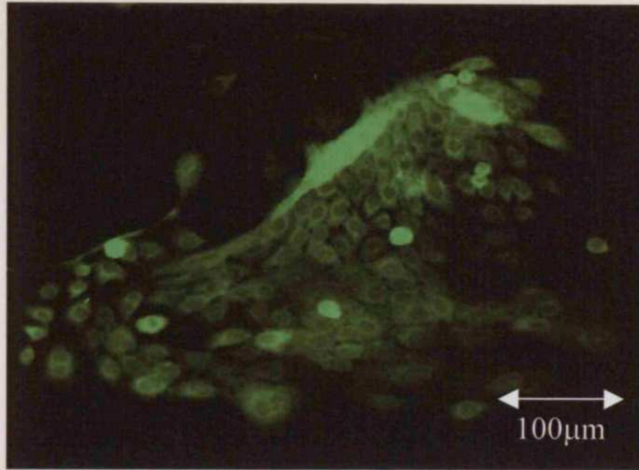


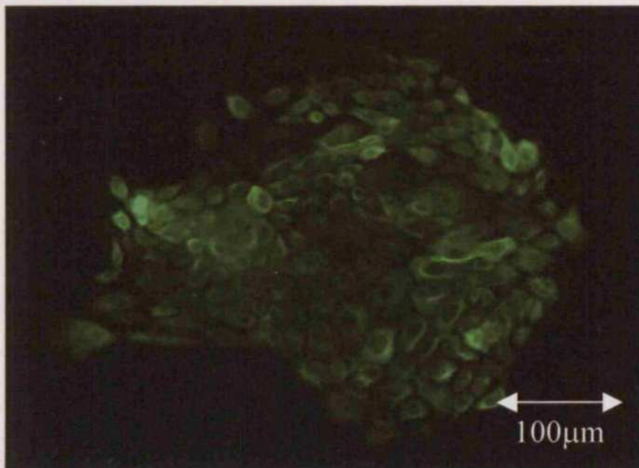
C

Figure 4.4 Palatal epithelial cell colonies stained with K14 (A) and K6 (B and C) (magnification x 10)



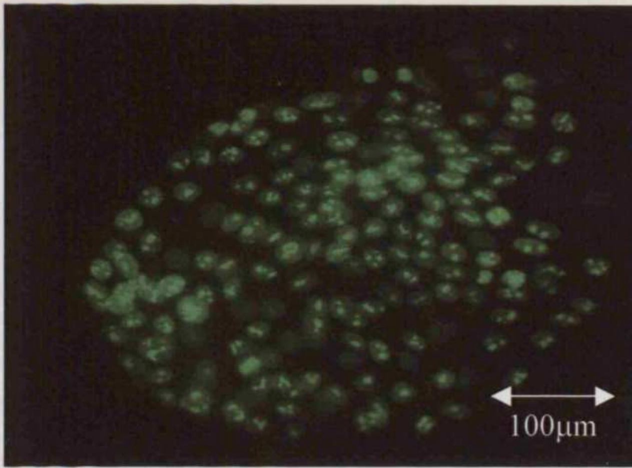
A

B



C

Figure 4.5 Palatal epithelial cell colonies stained with K10 (A), K8 (B) and K18 (C) (magnification x 10)



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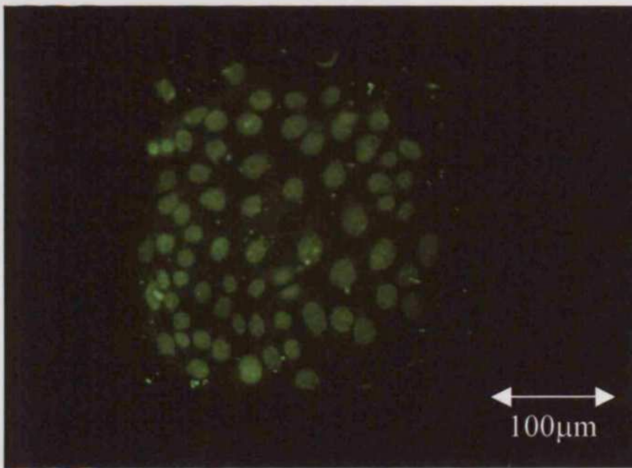
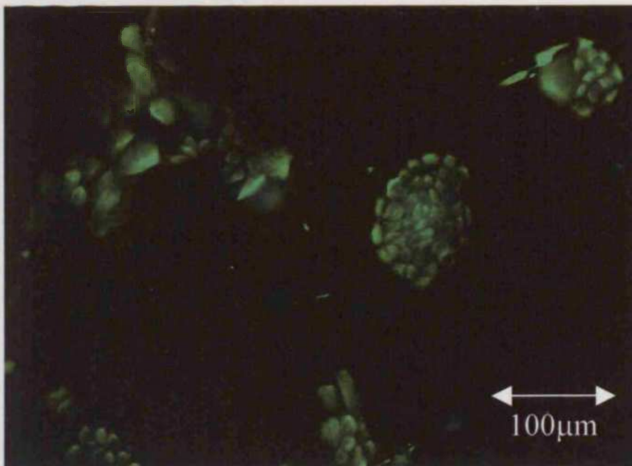


B



C

Figure 4.6 Palatal epithelial cell colonies stained with Ki-67 (A), p63 (B) and vimentin (C) (magnification x 10)

4.4 Discussion

From the staining patterns observed it can be concluded that the staining profiles obtained were different to those observed with the palate staining in chapter 3. There could be a number of reasons for these differences. Cells in culture cannot be directly compared to *in vivo* situations as the image that was observed here was a 2-dimensional overview mainly of the culture surface and not a cross-section as with the palate samples in chapter 3.

In the colonies stained with Cx26, K14, Ki-67, vimentin and p63, the staining observed was relatively uniform in appearance. α6 integrin, β1 integrin, K15, K19, K4, K8 and K18 appear to have very distinct differences in their staining intensities with strong and weak stained cells within each colony. Only K6 produced a range of staining intensities within the individual colony. The antibody against K10 stained the large differentiated suprabasal cells. From the staining observed with Ki-67, which is a protein that is expressed during the cell cycle but not during G0, it is apparent that none of the cells in the colony are in G0. Cx26 is a gap junction protein and staining with this antibody showed attachments between the cells. Staining in some of the colonies, including β1 integrin, K6 and K15, corresponded to cell size with smaller cells more strongly stained with β1 integrin and K15, and the larger cells stained with K6.

As reported, paraclones can undergo 15 population doublings prior to terminal differentiation producing approximately 1000 cells that are attributable to a single transit amplifying cell (Barrandon and Green, 1987b). The existence of meroclones could be indicative of heterogeneity within the stem cell compartment or could indicate that stem and amplifying cells represent opposite extremes of a spectrum of behaviour (Jones and Watt, 1993). A functioning stem cell population needs to be present and would equate to the holoclone type of colony. Stem cell markers would not be found in the paraclone type of colony and as analyses of the proliferative behaviour of the colonies was not possible it must be assumed that a mixture of colony types were present in this study giving a range of staining profiles.

From the shape, size and arrangement of the cells within the colonies, identifying the colonies as holo, mero or paraclones was not always possible, and classifying the colonies as such was not a feasible option. The holo, mero and paraclones are not being formed as expected, and therefore the staining is not consistent to what would be expected from previous staining observed using murine keratinocytes (Tudor *et al,* 2004). Murine keratinocytes grown *in vitro* showed three types of colonies. The first contained large flattened cells, showed little growth, and was thought to correspond to paraclone-like type of colony. The second colony was made up of smaller cells surrounded by a zone of larger flattened cells, this was thought to correspond to the meroclone-like type of colony. The third type of colony had an additional central zone made up of small closely packed cells, this was thought to correspond to the holoclone-like type of colony. Staining of these murine colonies showed that the inner cells in the holoclone-like colony did not stain for K6, an early differentiation marker, but did stain for K15, the putative stem cell marker. This suggests that these inner cells are the stem cells or contain a high proportion of stem cells. The palatal epithelial cells grown *in vitro* did not show these types of colonies and the staining profiles did not correspond to those seen in the murine colonies. Groups of smaller cells that could correspond to undifferentiated cells were present but they were not at the centre of the colonies. From the staining profiles obtained, it is clear that there was heterogeneity between the cells in each colony and also between the colonies. In this study the three different types of colonies were not identified, but some interesting staining profiles were observed.

To conclude, while the staining patterns observed do give some information about the cells, not enough can be deduced about their states of differentiation and whether the cells are stratifying *in vitro* to produce an *in vivo* stratified epithelial-like structure. It is also worth noting that the keratinocyte cells may be undifferentiated but not stem cells, and that these keratinocyte colonies are not a good stem cell model.

CHAPTER FIVE

CHAPTER 5

ORGANOTYPIC CULTURES

5.1 Introduction

Keratinocytes grown on plastic in culture do not grow and differentiate without the aid of matrix and mesenchymal interactions and only form thin multilayered sheets when confluent (Fuchs and Green, 1980).

To study molecular mechanisms of the epithelium-mesenchymal regulations *in vitro*, while also maintaining the main principles of skin biology, organotypic cultures were designed. Organotypic cultures were developed as a culture system or model as it would be advantageous to have an *in vitro* system that mimicked the *in vivo* situation. To improve, cultures supplemented with physiological substrates, such as collagen, were developed to create more *in vivo*-like conditions for keratinocytes. The matrix is lifted to the air-liquid interface so that the upper cell layers are air-exposed and the lower basal layer is close to the matrix that diffuses the nutrients from the growth media (Stark *et al.*, 1999). The collagen gels contract depending on the number of cells and the concentration of collagen they are made out of. To prevent the contraction of the gels the number of fibroblasts can be decreased or the concentration of collagen increased. As the collagen gel contracts and produces a densely organised lattice, the fibroblasts reduce their proliferative activity and protein synthesis (Coulomb *et al.*, 1984). Collagen type I is isolated from rat tail tendons or it can be bought commercially. Commercially available culture devices have also been developed and consist of a filter membrane holder that fits into a multi-well dish.

Organotypic cultures were used in this study as it is possible to choose what is put into the system, and to know that there are not any unknown elements present in the cultures. The fibroblasts and epithelial cells of choice can be added to the system forming an

adequate basement membrane zone. This indicated that there is confidence in using the organotypic culture system and not having to rely on DED which could be very variable in quality. Advances in tissue culture techniques have allowed the development of *in vitro* organotypic cultures that regenerate the patterns of cell behaviour and differentiation similar to those found *in vivo* (Parenteau *et al.,* 1992; Kautsky *et al.,* 1995).

It was hypothesised that organotypic cultures could resemble human palate *in vivo* and provide a good human stem cell model system that mimics the *in vivo* situation. H&E sections and the staining patterns of a panel of antibodies were examined to see if the distribution of the various differentiation markers in the organotypic cultures matched or closely resembled those of palate *in vivo* (Chapter 3). Organotypic cultures would also form an experimental model as they can be produced without the need for invasive surgery to obtain the experimental material.

5.1 1 Organotypic cultures made from immortal cell lines

The aim of the organotypic culture system was to produce an *in vitro* stem cell model. The organotypic cultures produced above were not always uniform in appearance which is important if a stem cell model is to be reliable. When making organotypic cultures from keratinocytes, it is desirable to use keratinocytes at very early passages, ideally from passage 1 to passage 4. As this was not always possible and as the organotypic cultures did not appear to be at their optimal appearance it was decided to use immortal cell lines to see if the organotypic cultures produced had a better morphological appearance.

5.1.2 HaCaT cell line

The first cell line to be examined was the HaCaT cell line. By using cell lines the variety of donors is excluded and the cells are available in unlimited quantity. The immortal skin epidermal cell line HaCaT, although cytogenetically abnormal, it does exhibit a high differentiation potential under *in vivo* and *in vitro* conditions (Boukamp *et al.*, 1988). For

this reason, this cell line has been used extensively as a substitute for normal human keratinocytes. HaCaT cells made into organotypic cultures form a more normal epidermis when transplanted onto nude mice (Breitkreutz *et al.*, 1998), but do show delayed epidermal reorganisation compared to skin keratinocytes. This indicated that the HaCaT cells had not lost their capacity for normal differentiation and epidermal tissue organisation.

Previous work has shown that under organotypic conditions, HaCaT cells can reconstitute a more normal structured epithelium (Schoop *et al.*, 1999). This was achieved using an increased number of fibroblasts and the epithelium that formed expressed most differentiation markers and closely resembled an epidermis. Some deficiencies in the formation of the stratum corneum occurred indicating that further improvements needed to be made.

5.1.3 OKF6/TERT-1, -2, and N-TERT cell lines

Other cell lines were used which were OKF6/TERT-1 and OKF6/TERT-2, which were derived from normal human oral mucosal epithelium, and N/TERT-1, which was derived from normal human epidermis. All three cell lines were cultured from clinically and genetically normal tissues. They were generated in the laboratory of J.G. Rheinwald (Brigham and Women's Hospital and Harvard Institutes of Medicine, Boston, USA) and were a kind gift from him.

Normal human somatic cells have a limited capacity to replicate in culture and progressive shortening of telomeres eventually leads the cells to cell senescence. Mid-life-span cultures of epidermal and oral mucosal keratinocytes were infected with amphotropic retroviral vectors encoding hTERT (Dickson *et al.*, 2000). By introducing hTERT, the catalytic subunit of the telomerase holoenzyme, into those epithelial cells, after periods of between 3 weeks up to 3 months, rapidly dividing immortalised cells eventually arose. The OKF6/TERT-1 and -2 cell lines were derived and cultured from floor-of-mouth mucosa which is why the organotypic cultures showed a nonkeratinising

type of differentiation and not a keratinising type of differentiation as would be expected if the cell lines were derived from palate.

As the organotypic cultures formed with the OKF6 cell lines showed good patterns of differentiation and as this thesis was aimed at identifying cell lineages and stem cells, the next part of the work was to see if individual cells could be transduced with virus in plastic culture, before being mixed with untransduced cells and made into organotypic cultures. It was hoped that some of the cells transduced would be stem cells and could be used to see if stem cell patterns or zones were recreated when the cells were made up into organotypic culture. If successful, when the organotypic cultures were sectioned, clones or zones of labelled cells would then be visible.

5.1.4 Retroviral transduction

Genes can be harnessed as *in situ* cell markers for clonal analysis of lineage. Recombinant retroviruses are used to mediate the transfer of foreign genes into cells. Viruses that are unable to replicate are used so that when they are integrated, their genome should be transmitted only to the clonal descendants of the transduced cell. The ability to transduce cells with foreign genes has made several strategies possible for experimental studies and for gene therapy. By using suitable vectors for gene transfer, the stable *in vitro* expression of a wide range of foreign genes has been possible but the loss of long term gene expression when the transduced cells are returned to the *in vivo* environment can be a problem (Fenjves, 1994).

In general, it is best to transduce primary keratinocyte cell lines at no later than passage 2. As this was not always possible, cell lines were also used. Retroviruses were used for the transduction of keratinocytes, and one advantage of using them is that the transduction efficiencies can be much higher than transfection techniques as the gene transfer is very efficient. Lineage studies of human epithelia were undertaken by the transduction of keratinocytes to produce populations of marked and unmarked cells. An immortal cell line and normal palatal and epidermal keratinocytes were transduced with retroviral

supernatant, made into organotypic culture using the method in chapter 2, and some of the organotypic cultures were transplanted into SCID (severely combined immunodeficient) mice. These were examined by UV for the EGFP transduction, or for histochemically detectable markers, either AP or β-gal. This was expected to allow the visualisation of stable clonal units that were regenerated in the experimental epithelia.

5.2 Results

Preliminary experiments were carried out to investigate the optimal number of epithelial cells and fibroblasts that were needed to produce a good thickness epithelium in the organotypic cultures. It was also important that the cultures could be maintained for up to 2 weeks and that the epithelium was still organised after this time. A variety of different cell combinations were also investigated including using mitomycin C treated 3T3 cells and fibroblasts instead of fibroblasts that have not been treated with mitomycin C. Two different types of organotypic culture set up were also investigated with the methods described in section 2.8. The inserts were an easier method as there was no need to coat the nylon disks with collagen and place the gels onto the wire grids, but this method usually resulted in thin, disorganised and patchy epithelia which was not suitable for further examination. The grid method was far more reliable and reproducible, and the epithelia formed appeared more organised with stratification and differentiation

Types of cells used in collagen gel

The collagen gels were made using mitomycin C treated 3T3 cells and mitomycin C treated fibroblasts as well as untreated fibroblasts. The epithelia formed using the 3T3 cells and fibroblasts both treated with mitomycin C were very poor in appearance, with virtually no epithelial growth. Using the untreated fibroblasts, the reformed epithelia showed some degree of stratification and differentiation.

Numbers of cells used in organotypic culture

The total numbers of epithelial cells and fibroblasts used per well were investigated to see which gave the more *in vivo*-like looking epithelium with epithelial stratification and

differentiation. The number of epithelial cells per well that were used included, 0.5 x 10^6, 1 x 10^6 and 2 x 10^6. The number of fibroblasts that were used included, 0.5 x 10^6, 1 x 10^6 and 2 x 10^6 fibroblasts per well.

Epithelial cell numbers

With 2 x 10^6 epithelial cells per well, there were far too many cells present in the culture and the epithelium formed was very disorganised in appearance indicating that there was no need for so many cells. Cultures made with 0.5 x 10^6 epithelial cells generated very thin epithelia with numerous holes within them.

Fibroblast cell numbers

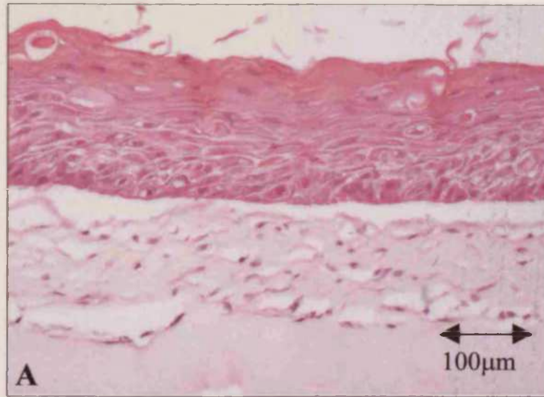
With 2 x 10^6 and 1 x 10^6 fibroblasts per well the collagen gel appeared crowded. Since the cultures made with 0.5 x 10^6 fibroblasts seemed to support adequate epithelial growth it was decided that 2 x 10^6 and 1 x 10^6 fibroblast cells per well were too many and 0.5 x 10^6 fibroblasts provided sufficient support. All organotypic cultures contracted to a small degree initially. Gels with a high number of fibroblasts (2 x 10^6) showed the greatest degree of contraction.

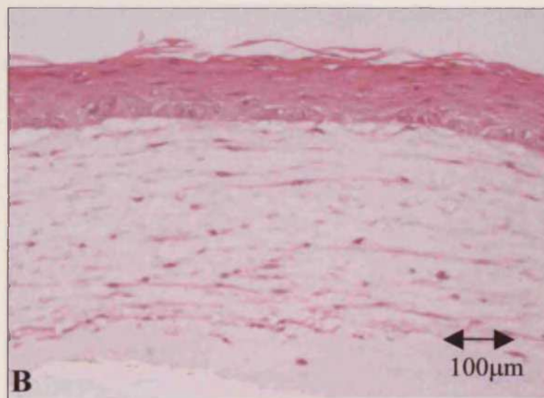
5.2.1 General appearance of palatal epithelial and fibroblast organotypic cultures

Organotypic cultures showing the greatest degree of organisation were made using palatal fibroblasts without any mitomycin C treatment using 0.5 x 10^6 fibroblasts and 1 x 10^6 palatal epithelial cells and these are shown in figures 5.1A-C. The epithelial surface is orientated towards the top of each image with the fibroblast-containing collagen gel at the bottom. The fibroblasts always appeared healthy and were evenly scattered throughout the collagen gel.

After 14 days in culture, the H&E stained sections showed that the covering epithelium appeared to be stratified and differentiated but often in a disorganised manner, and was anywhere between 2-10 cell layers thick. Cultures maintained for more than 14 days often started to become disorganised and began to disintegrate. The organotypic cultures

made with normal palatal epithelial cells and fibroblasts reformed an epithelium showing some degree of change towards restoration of epithelial structure. The basal cells appeared cuboidal in shape. The middle cells appear to correspond to the granular and spinous layers, and the superficial cells became progressively flattened with no nuclei as they entered the differentiation pathway and ascending into the stratum corneum. The junction between the collagen and the epithelium appeared flattened and may be weak, resulting in artificial separation between the two tissues. The thickness of the epithelium formed varied greatly between the different experiments, but also between organotypic cultures within the same experiment. The uppermost cornified cells eventually detached and sloughed off, these squames could sometimes be seen above the epithelial sections.





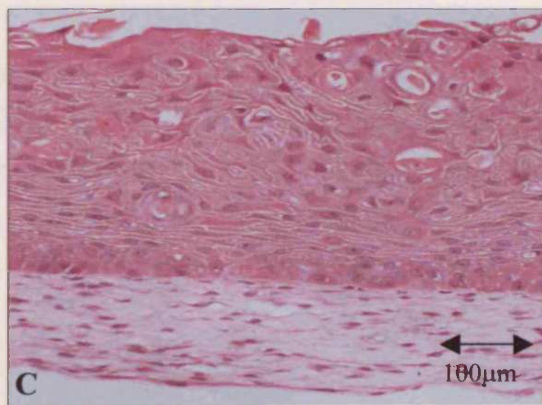


Figure 5.1 Organotypic cultures made from palatal epithelial cells stained with H&E (A to C) (magnification A,C x 20, B x 10)

5.2.2 Antibody staining of organotypic cultures

The following panel of antibodies was chosen as they were used in the previous chapters and gave an indication of the states of epithelial differentiation and of tissue maturity and architecture.

α6 integrin (figure 5.2A)
The whole of the epithelium stained weakly, with the basal cells are more strongly stained than the rest of the epithelium.

β1 integrin (figure 5.2B)
The cells in the basal layer stained very strongly with a visible band of staining at the connective tissue (collagen and fibroblasts) and epithelial interface.

K15 (figure 5.2C)
K15 was not expressed throughout the whole of the epithelium.

K19 (figure 5.2D)
Individual cells in the basal layer stained, these appeared to be randomly placed within this layer. Cells in the upper stratum corneum also stained.

K5 (figure 5.3E) **and K14**
The basal cells were strongly stained with background staining in the rest of the epithelium, the staining was cytoplasmic in appearance.

K6 (figure 5.2F)
It was possible to see the cells that made up the epithelium very clearly. The staining was cytoplasmic and not nuclear, and was present in the whole of the epithelium. It was possible to see that the basal cells were rounded, and that as the cells differentiated towards the epithelial surface, their nuclei were lost as the cells began to flatten.

K16 (figure 5.3A)

The whole of the epithelium was stained with the staining cytoplasmic in appearance.

K10 (figure 5.3B) **and K1**

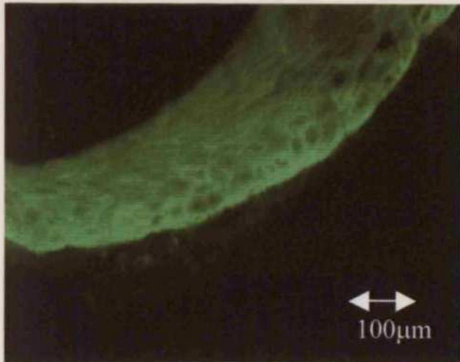
All suprabasal cells stained with this antibody.

K13 (figure 5.3C) **and K4**

The cells in the upper layers of the epithelium stained with no basal cell staining.

E-cadherin (figure 5.3D)

This antibody stained the cell peripheries clearly with all cells of the epithelium stained, but with weaker staining towards the upper epithelial surface.



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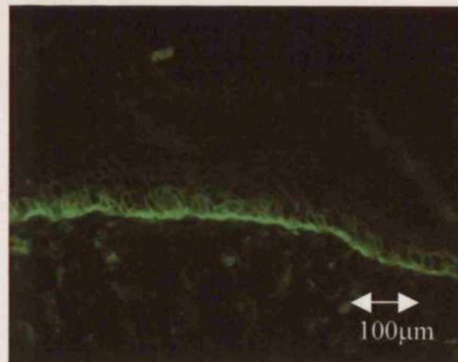

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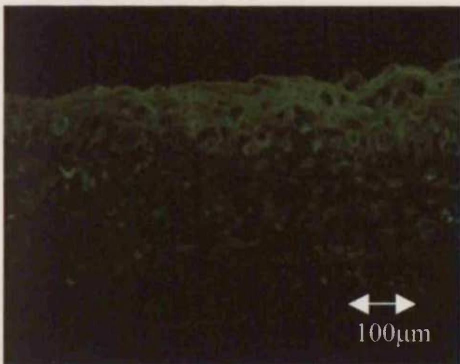

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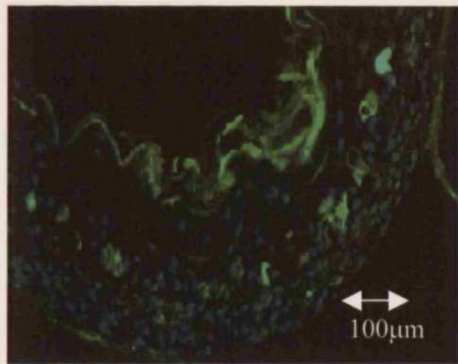

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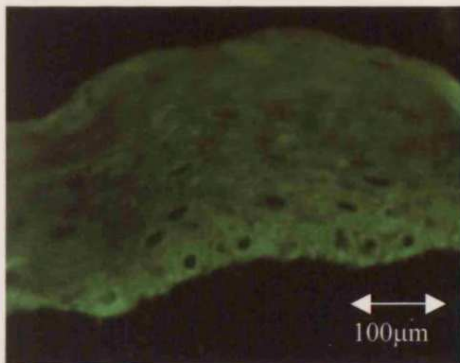

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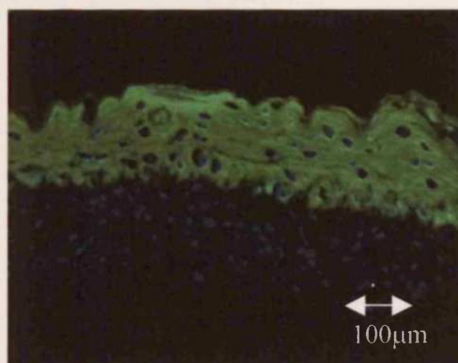
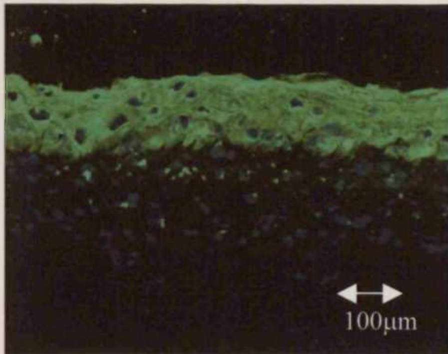


F

Figure 5.2 Palatal epithelial cells made up into organotypic culture then stained with various antibodies. α6 integrin (A), β1 integrin (B), K15 (C), K19 (D), K5 (E) and K6 (F) (magnification A-D,F x10, E x20)



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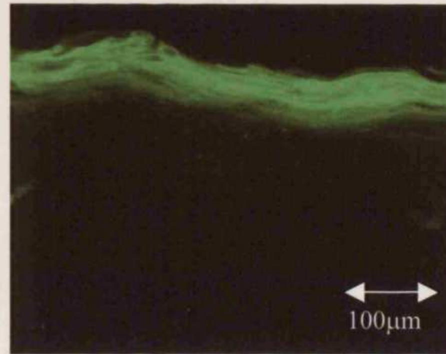
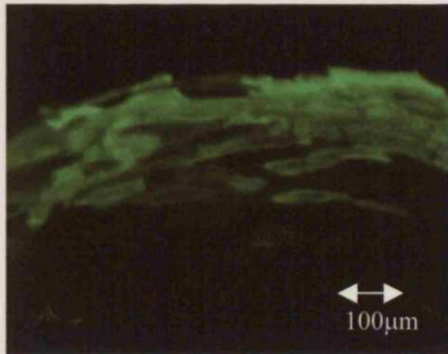


B



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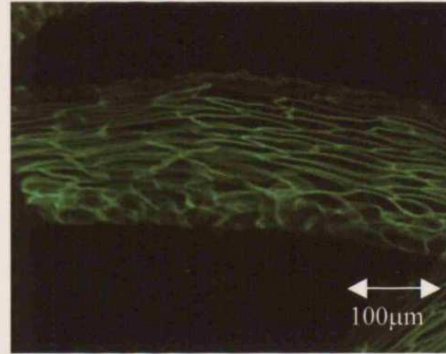


D

Figure 5.3 Palatal epithelial cells made up into organotypic culture then stained with various antibodies. K16 (A), K10 (B), K13 (C), and E-cadherin (D) (magnification A,C, x10, B,D x20)

Table 5.1 Organotypic culture v palate antibody staining

Antibody	Epitope	Organotypic culture	Palate *(in vivo)*
LHK1	Keratin 1	Suprabasal	Suprabasal
6B10	Keratin 4 Ab-1	High suprabasal	High suprabasal
AF 138	Keratin 5	Basal	All strata
LHK6	Keratin 6	All strata	Suprabasal
LP5K	Keratin 7	NR	Merkel cells
LE41	Keratin 8	NR	Merkel cells
LHP2	Keratin 10	Suprabasal	Suprabasal
1C7 + 2D7	Keratin 13 Ab-3	High suprabasal	High suprabasal
LL001	Keratin 14	Basal	All strata
LHK15	Keratin 15	NR	Basal at rete tips
LH025	Keratin 16	All strata	Suprabasal
E3	Keratin 17 Ab-1	NR	Merkel cells
LE61	Keratin 18	NR	Merkel cells
LP2K	Keratin 19	Random basal cells	Basal at rete tips
DE9	Beta1 integrin (human)	Basal	Basal
CD49f, VLA-6	Alpha6 integrin (human)	All strata	Basal lamina
4A2C7	E-cadherin	All strata	Over CPT

NR = no result

5.2.3 EGFP retroviral transduction of palatal epithelial cells

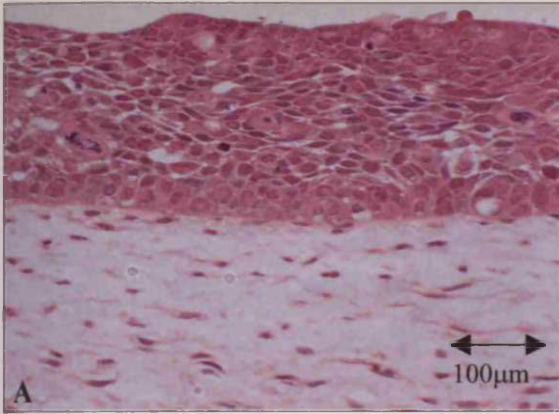
The palatal epithelial cells were transduced using the MGIN plasmid which contained EGFP and a neomycin (*neo*) insert by Dr. R. Darley. The cells used were at the lowest possible passage number, but as the number of transduced cells was quite low it was necessary to expand the cells in culture. 3% of EGFP transduced cells were added to untransduced cells and made into organotypic cultures. The organotypic cultures were very messy in appearance and were hard to maintain successfully in culture for the usual 14 days. When they were viewed histologically, the cultures were disorganised. The usual cuboidal basal cell layer was absent, and the suprabasal cells were arranged in a manner where the cells were disordered and there were no distinct layers of cells, there were also spaces between the cells within the epithelia. When the fresh frozen tissue was cryosectioned and viewed under UV light, only a few random cells fluoresced green. None of these cells were in the basal layer and none were in any clonal zones.

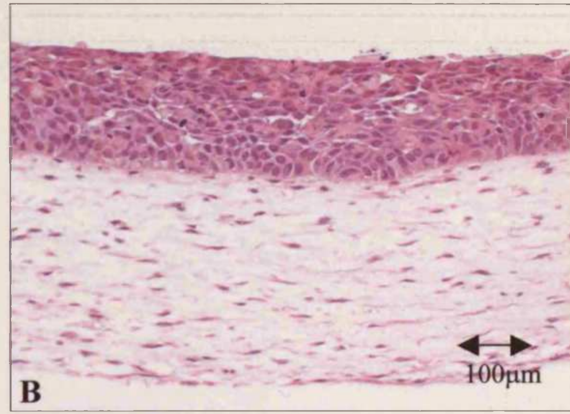
5.2.4 HaCaT organotypic cultures

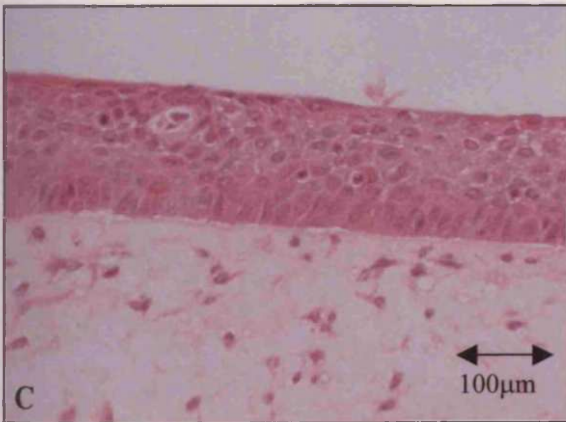
The organotypic cultures formed stratified in a non-keratinised manner and had no morphological pattern of differentiation (figures 5.4A and B) and did not appear to be stratifying and differentiating in the manner previously described (Schoop *et al.*, 1999). The fibroblasts were evenly spaced and appeared healthy and all the epithelial cells in the organotypic culture appeared to be the same in size and appeared as if no degree of differentiation had occurred at all. The basal cells did appear slightly cuboidal in shape but the rest of the cells remained small and rounded throughout the epithelium, and the whole epithelium was very compact. The epithelium formed was thicker than the organotypic cultures made with normal palatal keratinocytes. As the organotypic cultures formed using the HaCaT cells did not differentiate as anticipated, other cell lines were investigated.

5.2.5 OKF6/TERT-1, -2 and N-TERT organotypic cultures

The organotypic cultures made with the OKF6/TERT-1 and -2, and the N-TERT keratinocytes were examined for histology by H&E (figures 5.4C and D show OKF6/TERT-1 and N-TERT respectively, figures 5.5A-C show OKF6/TERT-2). The sections stained with H&E showed good morphology with the cultures showing some signs of stratification and differentiation. The basal cells were not as cuboidal in appearance compared to the normal palate and HaCaT organotypic cultures. The epithelium formed was thicker than the palatal and HaCaT organotypic cultures, with between 12 to 20 cell layers in thickness. The OKF6/TERT-2 organotypic cultures were closer in appearance to the *in vivo* structure of palate than the palatal and HaCaT organotypic cultures and this model was considered a satisfactory one. The fibroblasts appeared healthy and were evenly scattered throughout the collagen gel.







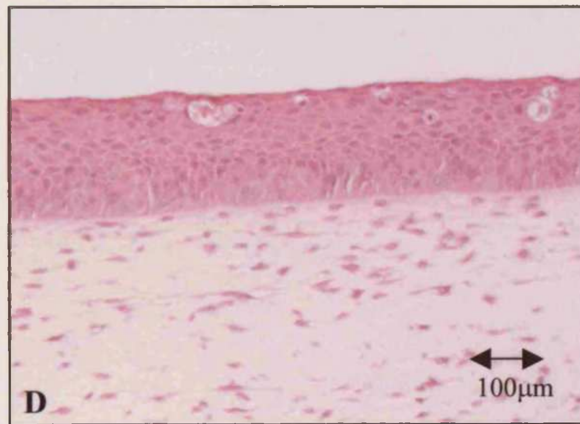
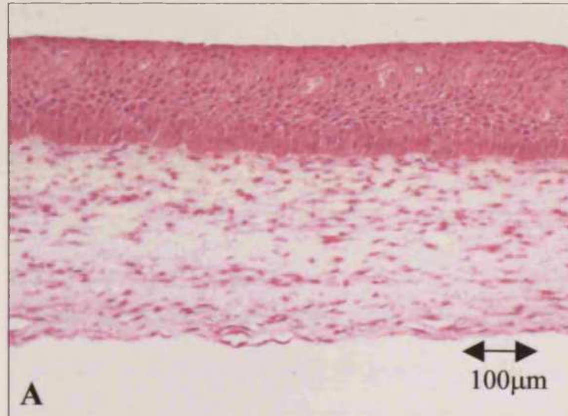
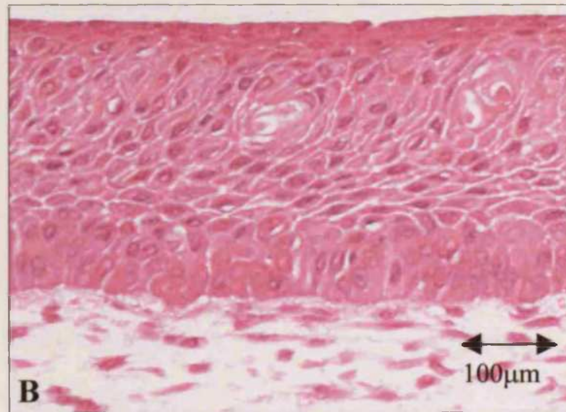


Figure 5.4 Organotypic cultures made from HaCaT (A and B), OKF6/TERT-2 (C) and N-TERT (D) cells stained with H&E (magnification A,C x 20, B,D x 10)





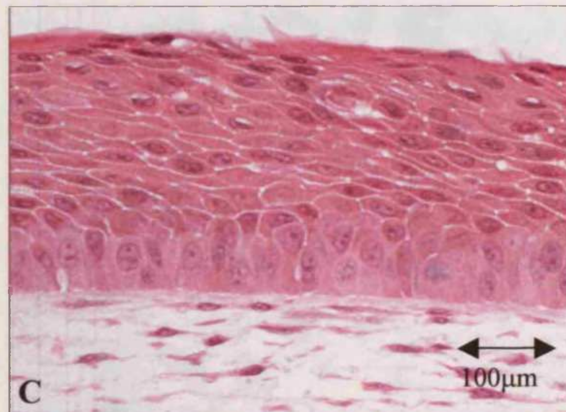


Figure 5.5 Organotypic cultures made from OKF6/TERT-2 cells stained with H&E (A to C) (magnification A x 10, B,C x 20)

5.2.6 Retroviral transduction

OKF6/TERT-2

As the OKF6/TERT-2 cell line appeared to show a slightly better degree of stratification, this was the cell line chosen to be made into organotypic culture after the cells had been transduced using an alkaline phosphatase (AP) vector that produces retrovirus. The PA317/LAPSN vector codes for AP and has a neomycin (*neo*) insert that allows G418 selection of transduced cells. The transduced cells would be identifiable by staining for AP using either fast red or BICP detection kits.

The medium containing retrovirus did not transduce the OKF6/TERT-2 cells. It was thought that the concentration of virus was too low and did not have a high transduction rate so it was deemed necessary to re-clone the producer cells to establish a 100% AP positive producer cell line (section 2.11). This cell line was then used to successfully transduce the OKF6/TERT-2 cell line and organotypic cultures were constructed using varying percentages of transduced cells mixed with normal cells.

Figure 5.6 shows examples of cryosections stained with BCIP. The BCIP stain sometimes over-stained certain parts of the cryosections and also cells at the upper surface of the organotypic cultures.

The various percentages of transduced cells investigated included 1%, 3%, 5%, 8% and 10%. The 1% value produced no visibly detectable transduced cells. The 10% level produced too many cells to be able to identify columnar units. The results produced appear to show that the most adequate percentage of transduced cells to use was 3% (figures 5.6A-C).

The whole epithelial sheets formed from the organotypic cultures were removed from the collagen gel and were also stained for AP with Fast Red. These were viewed from above to give an indication of the success of the estimation of the numbers of transduced and

non-transduced cells used (figures 5.6D and E). As can be seen the clonal units of AP positive stained cells were well dispersed throughout the epithelial sheet.

Epidermal and palatal keratinocytes

Figure 5.7D shows the range of sizes of cells that were initially transduced with AP. The larger cells are thought to correspond to terminally differentiated cells, the medium sized cells differentiated cells, and the smaller cells corresponding to stem and early amplifying cells. Normal epidermal and palatal keratinocytes were transduced with retroviral supernatant and were made into organotypic cultures using between 10 and 20% transduced cells mixed with untransduced cells. These were maintained in culture for 16 days before being transplanted into SCID mice for up to 12 weeks. It was not possible to maintain the organotypic cultures for longer than 16 days before transplantation.

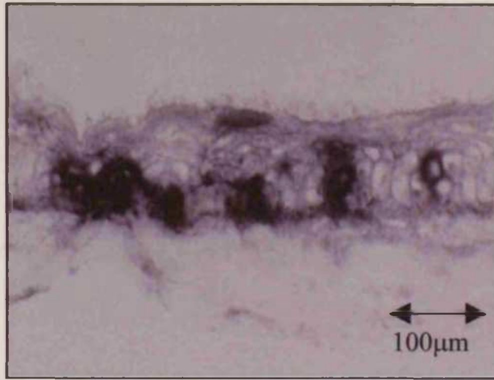
Epidermal keratinocytes

Two weeks after transplantation the reformed epidermis was hyperplastic and contained large irregular clusters of labelled cells that appeared to be differentiating. A cryosection of the organotypic cultures made out of epidermal keratinocytes that were transplanted into SCID mice for 12 weeks can be seen in the lower panel of figure 5.7B with normal human epidermis shown in the upper panel of figure 5.7B. The cells stained for β-gal appear blue in colour. By 12 weeks, hyperplasia had diminished and the number and size of the labelled cell clusters was reduced. The histochemically detectable units produced were spaced at intervals and not in clusters.

Palatal keratinocytes

A cryosection of the organotypic cultures made out of palatal keratinocytes that were maintained in culture for 5, 10 and 21 days, and stained for β-gal can be seen in figure 5.7A. After 21 days in culture, it was possible to see that a rete pattern was nearly regenerated. As the organotypic cultures could not be maintained for longer than 21 days in culture, it was not possible to see if the rete patterns of normal palate would completely regenerate *in vitro*.

16 day organotypic cultures made of palatal cells were also transplanted into SCID mice for up to 12 weeks. After this time, it was possible to see units of AP positive cells stained red, as shown in figure 5.7C. After transplantation into SCID mice the palatal keratinocytes reformed quite thick epithelia with an undulating epithelial-connective tissue interface and rete-like structures. The clonal cell lineages developed took the form of columns of cells running through the full thickness of the epithelium. The columns were narrower than the rete structures and bore no fixed relationship to them.



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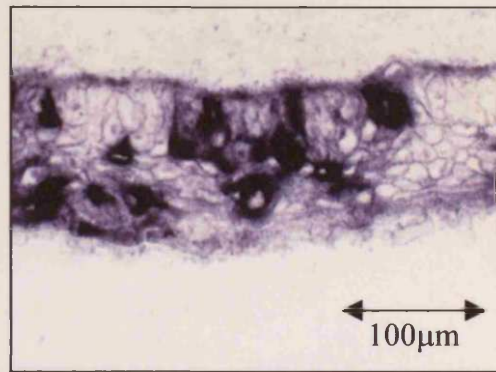

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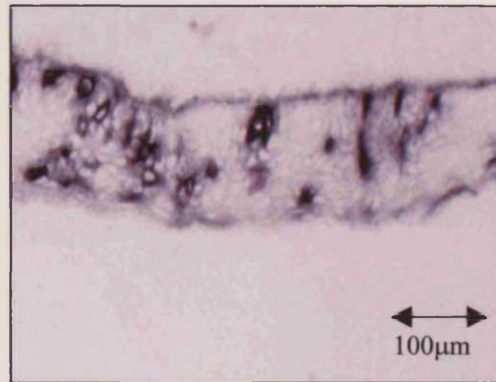

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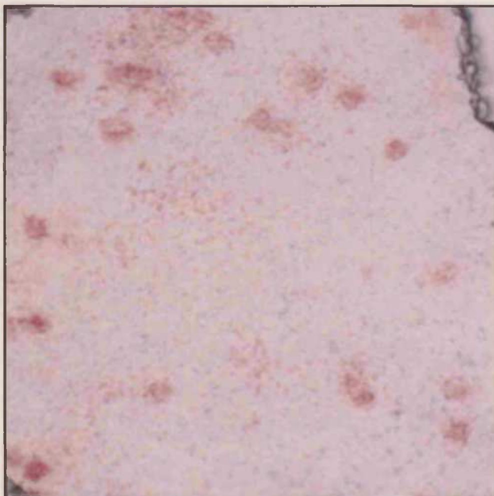

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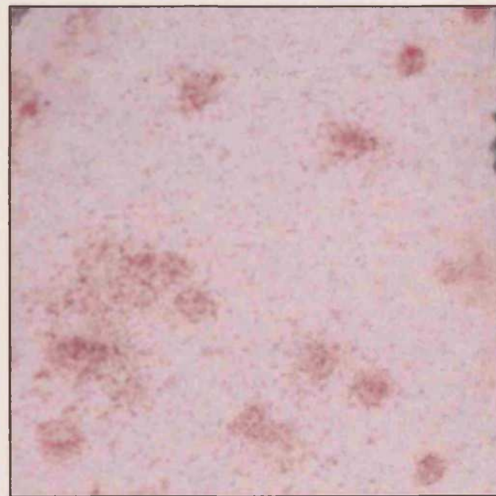

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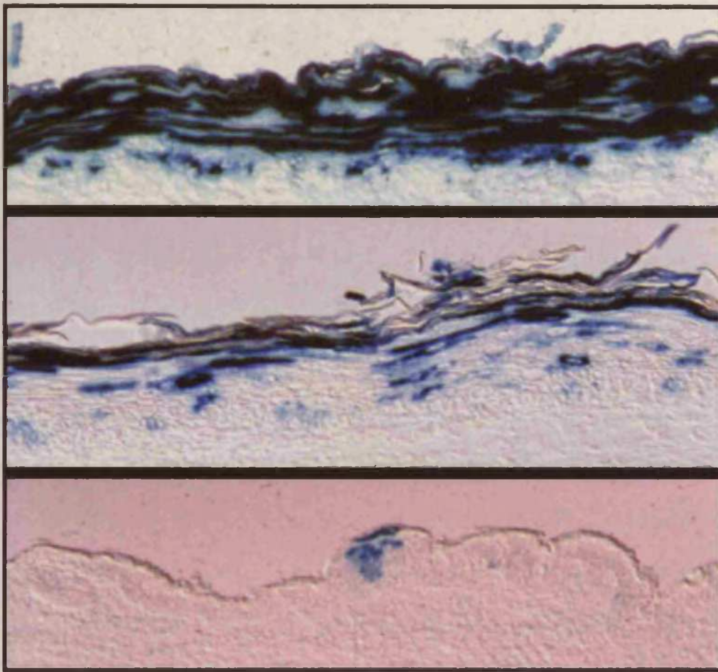
Figure 5.6 BCIP staining of cryosections of OKF6/TERT organotypic cultures (A-C). Fast Red staining of OKF6/TERT organotypic culture epithelial sheet (D,E)



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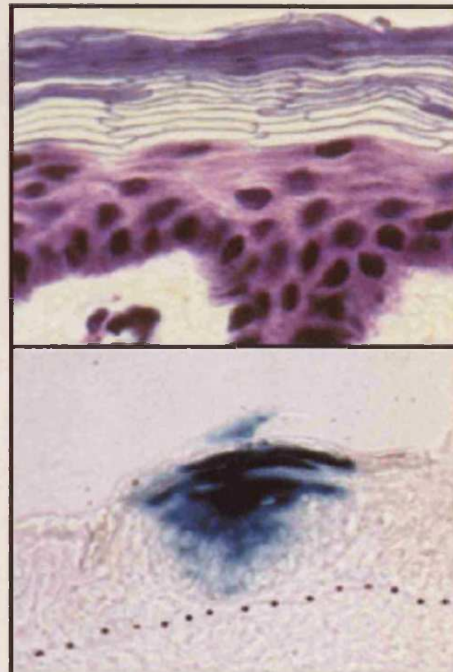

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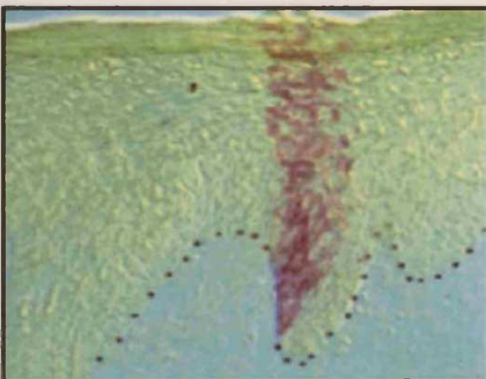

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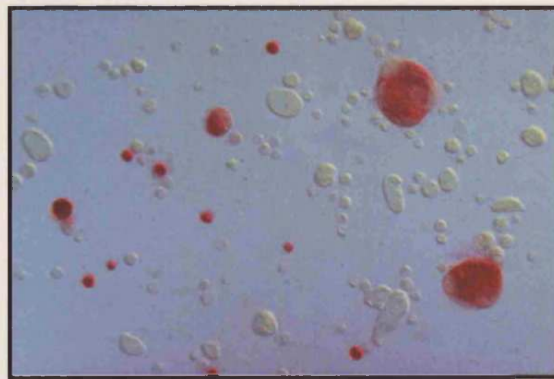


D

Figure 5.7 A shows a panel of palatal organotypic cultures after 5 days, 10 days and 21 days, respectively, in culture. The upper panel in B shows the cells in the upper layers overlapping and forming units in epidermis, the lower panel shows an epidermal organotypic culture that has been transplanted for 12 weeks, a single column of transduced cells can be seen. C shows a palatal organotypic culture that has been transplanted for 12 weeks showing a column of transduced cells. D shows a range of sizes of AP positive cells.

5.3 Discussion

The organotypic cultures stratified and showed moderate patters of histological differentiation. The basal cells appeared cuboidal in shape and the cells of the uppermost layer corresponding to the stratum corneum appeared flattened. It was possible to see in some H&E sections that the flattened cells were being sloughed off as squames (figure 5.1). The cells between the basal and cornified layers lost their roundness and began to flatten as they progressed through the layers towards the upper surface. The staining distributions obtained with the various antibodies on the organotypic cultures did not correspond to those of palate *in vivo* and differences were observed. If the organotypic cultures were kept for longer than 14 days, the epithelium started to disintegrate and was very difficult to freeze and cryosection. Maintenance of organotypic cultures *in vitro* provides information about the success of the techniques employed in the setting up of the cultures and about the basic pattern of tissue clonality. A more *in vivo*-like pattern of epithelial stratification and differentiation may have been achieved if the cultures had been maintained for longer but unfortunately, it was not possible to maintain the organotypic cultures in a very good condition in culture for much more than two weeks.

The images shown (Figures 5.1A-C) looked the most *in-vivo*-like of a large series of similar experiments, indicating that the organotypic cultures were not always reproducible, and the thickness of the epithelial layer varied greatly.

5.3.1 Antibody staining of organotypic cultures

As can be seen in table 5.1 there are a few discrepancies in the antibody staining profiles between the organotypic cultures and palate *in vivo*. The most obvious of these is that α6 integrin stained the whole of the organotypic culture epithelium, and not the basal cells and basal lamina as in palate. β1 integrin stained the basal cells in both tissues. K5 stained the whole of the epithelium in palate but stained the basal cells stronger in the organotypic culture. K6 and K16 stained the whole of the epithelium in the organotypic cultures whereas in palate antibodies against these keratins stained suprabasally and the basal cells were left unstained. K4 and K13 stained cells in the upper suprabasal layers of

the organotypic cultures, with K13 staining these cells at various intensities. K10 and K1 stained suprabasal cells as in palate. This is almost equivalent to the staining observed with these antibodies in palate, but as no rete are present in the organotypic cultures the chevron pattern that corresponded to the ERT in palate, was not observed. K15 was not expressed in the epithelium in the organotypic cultures. This did not correspond to the staining in palate where zones of stained cells appeared at the ERT, but did correspond to K15 which was not expressed in primary keratinocytes grown on DED at the air-liquid interface (Waseem *et al.,* 1999). No individual groups or clusters of cells in the basal layer could be observed in the organotypic cultures. K19 gave an interesting staining profile with individual cells stained in the basal layer of the organotypic cultures. E-cadherin stained the whole of the epithelium in the organotypic culture. This did not correspond to the staining seen in palate which was over the CPT and into the rete core.

5.3.2 EGFP retroviral transduction of palatal epithelial cells

The organotypic cultures that were made from the EGFP transduced cells were very inadequate in appearance producing disorganised epithelia and with only a few transduced cells visible after maintenance in culture. Because of the appearance of the cultures, it was thought that the palatal epithelial cells had been passaged too many times before being made into organotypic culture. This would deplete the supply of any stem cells that had been transduced and the quality of the cells does decline with increased passage number.

5.3.3 OKF6/TERT-1, -2 and N-TERT organotypic cultures

From the organotypic culture work using the immortal cell lines OKF6/TERT-1 and -2, and N-TERT it was possible to see that the epithelia formed were much better than the epithelium formed from the HaCaT cell line. OKF6/TERT-2 demonstrated the best degree of stratification which was why this cell line was chosen to be investigated further. The organotypic cultures appeared to have retained the capacity for normal keratinocyte differentiation as they showed the keratinocytes had stratified to a degree and had begun to undergo a non-keratinising form of suprabasal differentiation.

5.3.4 Retroviral transduction - OKF6/TERT-2 organotypic cultures

In the cryosections of most of the transduced organotypic cultures, except for the 1% level, is was possible to see small individual units of AP positively stained cells. Some of these appeared in the upper strata of the cultures which indicated that these cells were not stem cells and were probably transit amplifying cells at the time of transduction. The AP positive cells in the basal layer could correspond to stem cells.

5.3.5 Retroviral transduction - epidermal organotypic cultures

The labelled cells produced could be seen to be spaced in clusters, suggesting that each unit was the product of one transduced cell and not more than one cell clumped together in a unit. The labelled cell units that remained roughly corresponded morphologically to the columnar units present in human epidermis (Mackenzie *et al.*, 1981) indicating that thin human epidermis, like murine epidermis, consists of small clonal units of structure.

5.3.6 Retroviral transduction - palatal organotypic cultures

The units formed in the palatal derived transplanted organotypic cultures did not correspond to the rete structures which was unanticipated. It was expected that the palatal stem cells would lie at the deep tips of the rete as this is their position in murine palate and human oesophageal mucosa, and it was anticipated that the whole rete would be clonal.

Having used both primary cell lines and immortal cell lines to construct organotypic cultures, there are advantages and disadvantages to their use. The extensive use in culture of immortal cell lines indicates that there is little risk from routine cell culture. As HaCaT, OKF6/TERT-1, OKF6/TERT-2 and N-TERT are not fully characterised it is wise to regard all such materials as potentially infectious and all work was carried out in a class II safety cabinet. Cell lines offer the following advantages over primary cell lines: reproducibility; cell that are readily available; cells can be banked and harvested when needed; cells can be checked for pathogens. Immortal cell lines do have disadvantages as they are often cytogenetically abnormal and as such, maybe not always such a good model cell line compared to primary cell lines. Primary cell lines are invaluable to culture

work but as sources are often scarce and limited if possible to low passage number, immortal cell lines are reverted to. Both primary and immortal cell lines are receptive to gene transfer.

CHAPTER SIX

CHAPTER 6

FLOW CYTOMETRY

6.1 Introduction

For organotypic cultures to form a suitable *in vitro* stem cell model, the cells within the cultures need to be behaving in an *in vivo*-like manner. It was hypothesised that the cell cycles of the organotypic cultures would differ from the cell cycles of cells grown on plastic. There were two aims to this part of the study, the first was to see if there was a difference in the cell cycles of cells grown on plastic compared to cells made up into the organotypic cultures, by using flow cytometry. The second aim was to see if there was a difference in the levels of expression of $\alpha 6$ or $\beta 1$ integrins in culture and as the cells pass through the cell cycle. Preliminary experiments were first carried out to see what the most suitable method to prepare the cells for the flow cytometer.

6.1.1 Data collection and analysis

The experimental cells were run through the flow cytometer and the data for 10,000 events was collected for each sample. These results were saved and later interpreted using the WinMDI computer program. The data analysed in WinMDI is usually displayed as a histogram (figure 6.1E) and dual parameter dot plots (figures 6.1A-D). The histogram plots the magnitude of the FL2-area (PI) measurement against the number of events exhibiting a given magnitude.

Having manipulated the data in WinMDI the profile for FL2-area (PI) was saved and imported into a cell cycle analysis computer programme called Cylchred. This programme gives a percentage value for the number of cells in G0/1, S and G2/M phases. The programme marks provisional G0/1 and G2/M markers as determined by an algorithm. If the markers were not appropriate, they could be moved but they only need

to be placed approximately as the algorithm is iterative. G1, G2 and S phase are the percentage distribution of cells within the fitted region.

It is important that the cells are in a single cell suspension and should contain as little debris and as few dead cells and clumps as possible. Experiments were carried out to assess the best method to fix and/or permeabilise the cells and when to add the antibody before running the cells on the flow cytometer. Cells have to be fixed before running on the flow cytometer and permeabilised to allow the PI to enter the cell.

6.1.2 Histograms

The DNA flow histogram provides a snapshot of the proportion of different kinds of nuclei present at a particular moment. A band pass filter of 515-535mm (green, FITC) and a long pass filter of 630mm (red, PI) were used to measure light. The area/peak ratio from red fluorescence was used to exclude doublets of diploid cells and gate the real single tetraploid cells for further analysis

6.2 Results

The cells fixed in the 70% ethanol were better than those fixed in 2% paraformaldehyde. Staining the cells with the appropriate antibody first gave a better result than staining at a later stage. Even though the cells should need to be permeabilised to allow the PI to pass through the membrane, permeabilisation was unnecessary as it has no effect on the cells at all. Therefore the order used for all the following experiments was staining with the appropriate antibody (S) then fixing (F) the cell sample with ethanol.

6.2.1 Dot plots

Figures 6.1 to 6.19 show typical dot plots and DNA histograms for each of the six sub-populations. Figure 6.1A is a dot plot and shows forward scatter (FSC) against side scatter (SCC) which gives an indication of the size and granularity of the cells. The FSC signal is sometimes called a size or volume signal. It is related to the size and volume of the cells, but is also related to other factors such as the refractive index of the cell. Some

measurements are amplified logarithmically and not linearly. The linear measurements are arbitrary. The rougher, more irregular or granular a particular cell is, the more it scatters the illuminating beam to the side. The intensity of this SCC light is related to the cell's surface texture and internal structure as well as to its size and shape.

In a typical flow cytometer, two of the photo-detectors measure forward-angle scatter light and SSC light in order to provide some information about the physical characteristics of the cell. The other three or more photo-detectors are equipped with coloured filters to provide information about the fluorescent light being emitted by the cells. The characteristics registered on the photo-detectors are known as measured parameters in the flow cytometer system.

Figure 6.1B shows FL1-height (FITC is always FL1) against FL2-area (which in this case is PI). The FL1-height gives an indication of the level of fluorescence. Figure 6.1C shows FSC-height against plus FL1-height, which also gives an indication of the level of fluorescence of the fluorochrome. Figure 6.1D shows FL2-width against FL2-area, which gives a good view of cells in the various phases of the cell cycle by their PI staining. The blue histogram in figure 6.1E (measurement of PI staining against the number of cells) is the gated region from figure 6.1D. The blue histogram is the sum total of the red histogram (which corresponds to cells in G0/G1 and G2/M phases), and the green histogram (which corresponds to cells in S phase). This is the DNA histogram.

Figure 6.1 A-E Example dot plots and DNA histogram

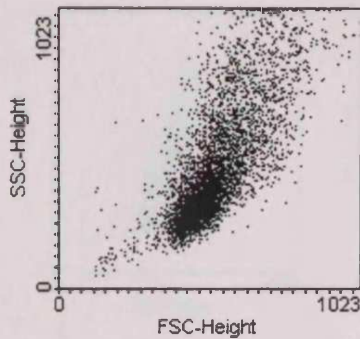


Figure A Dot plot showing forward scatter (FSC) against side scatter (SCC)

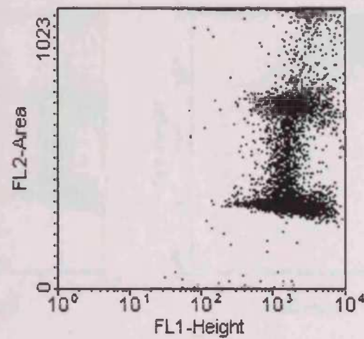


Figure B Dot plot showing FL1-height (FITC) against FL2-area (PI)

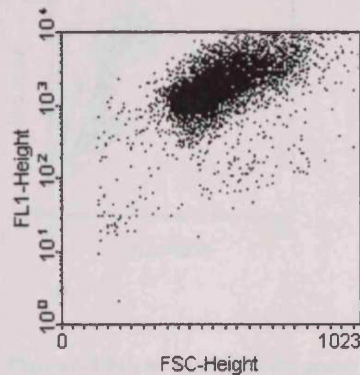


Figure C Dot plot showing FSC-height against FL1-height

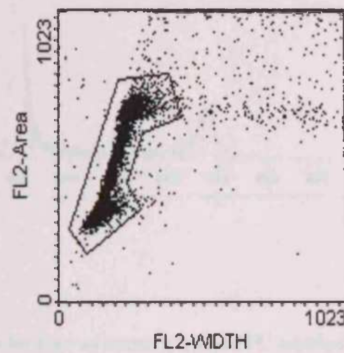


Figure D Dot plot showing FL2-width against FL2-area. The gated region is used for the DNA histogram in figure E

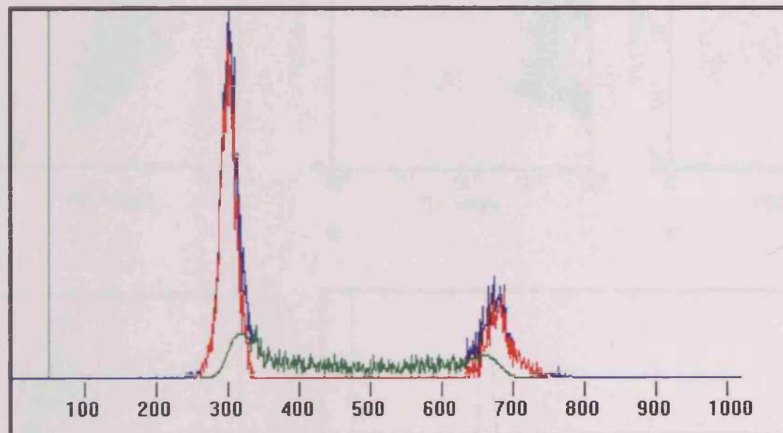


Figure E DNA histogram showing cells in G0/G1 phase corresponding to the first peak, S phase cells as the green part, and cells in G2/S phase corresponding to the second smaller but wider peak. Using the Cylchred program on this histogram gives values for the percentage of cells in each phase of the cell cycle.

Figure 6.2 Palatal epithelial cells grown on plastic stained for flow cytometry with FITC labelled α6 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

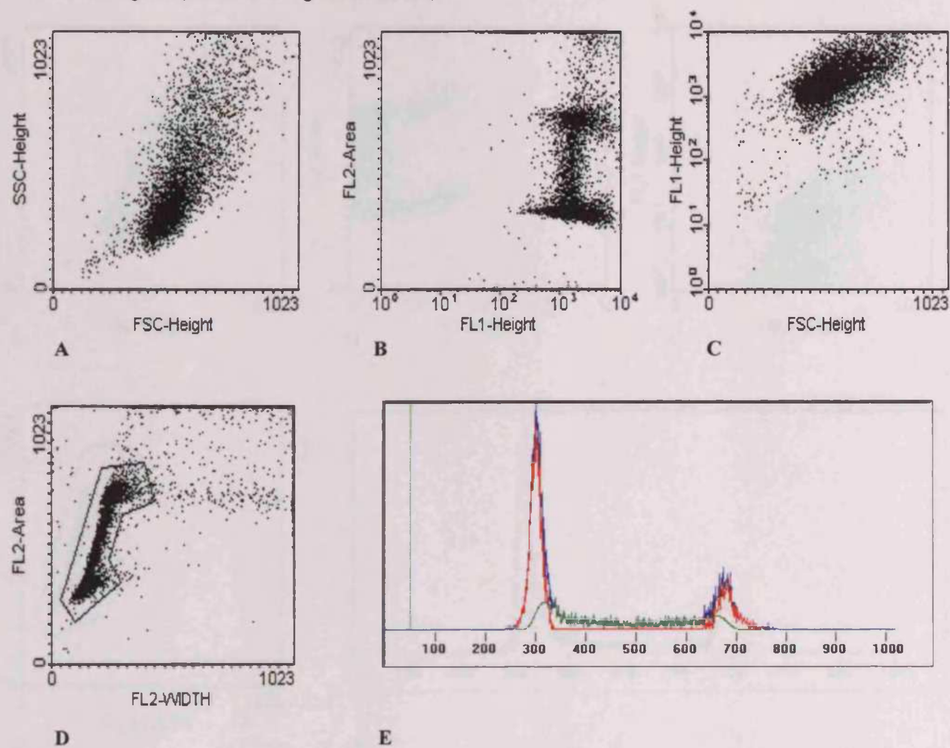


Figure 6.3 Palatal epithelial cells grown on plastic stained for flow cytometry with FITC labelled β1 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

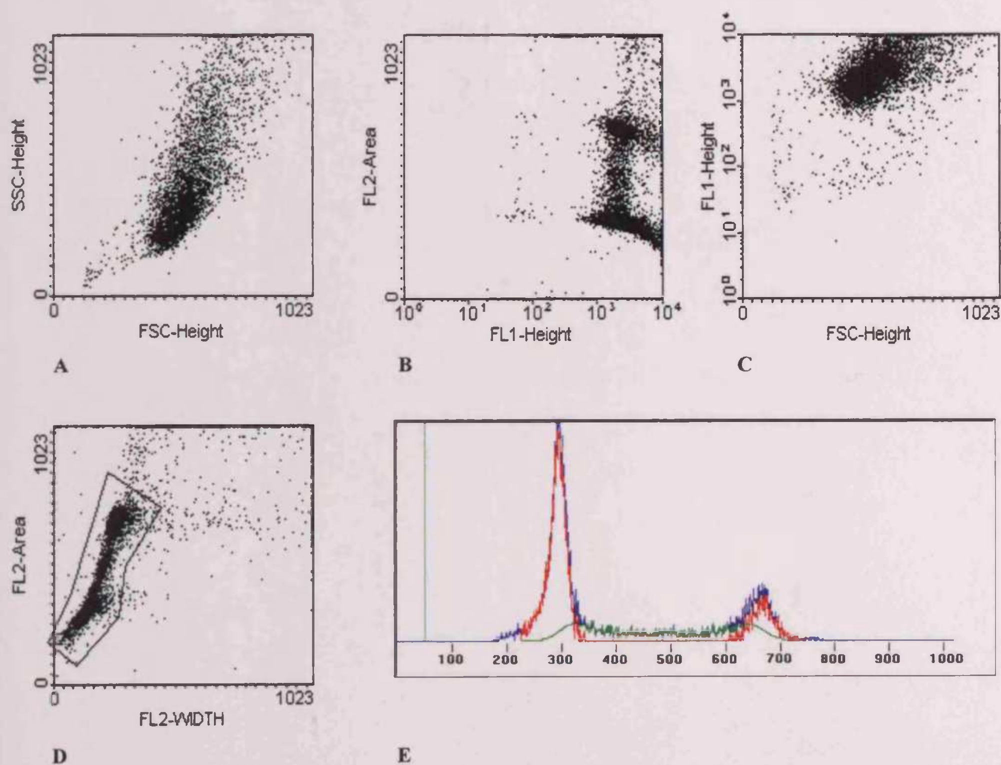


Figure 6.4 Palatal epithelial cells grown on plastic for negative control. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

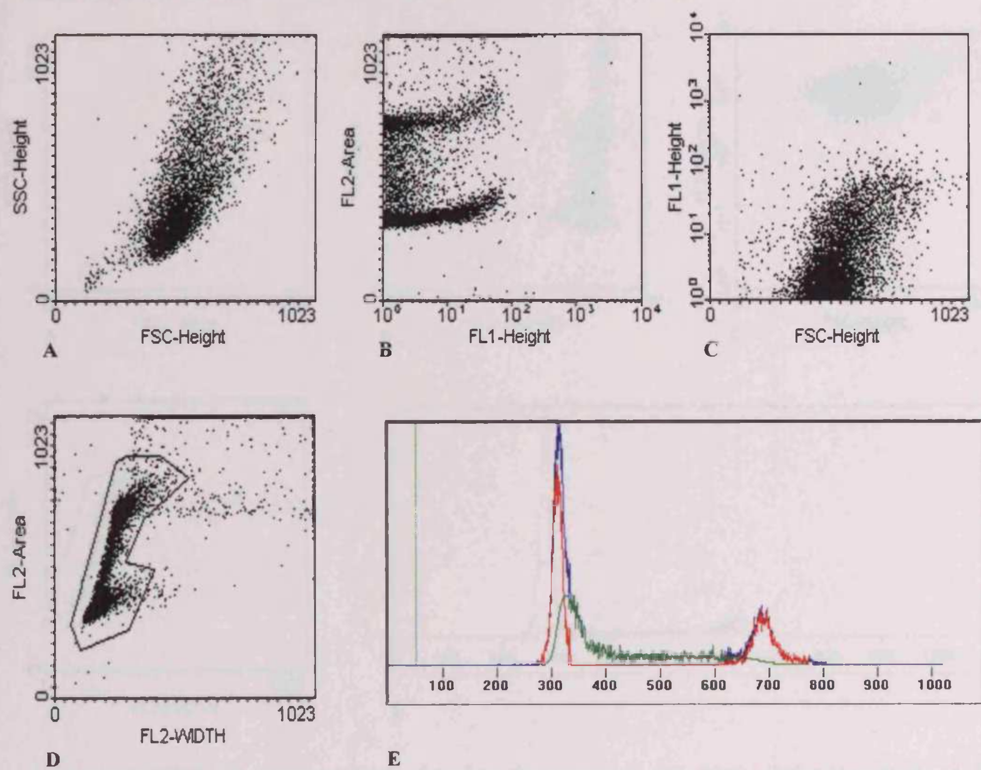


Figure 6.5 N-TERT cells grown on plastic stained for flow cytometry with FITC labelled $\alpha6$ antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

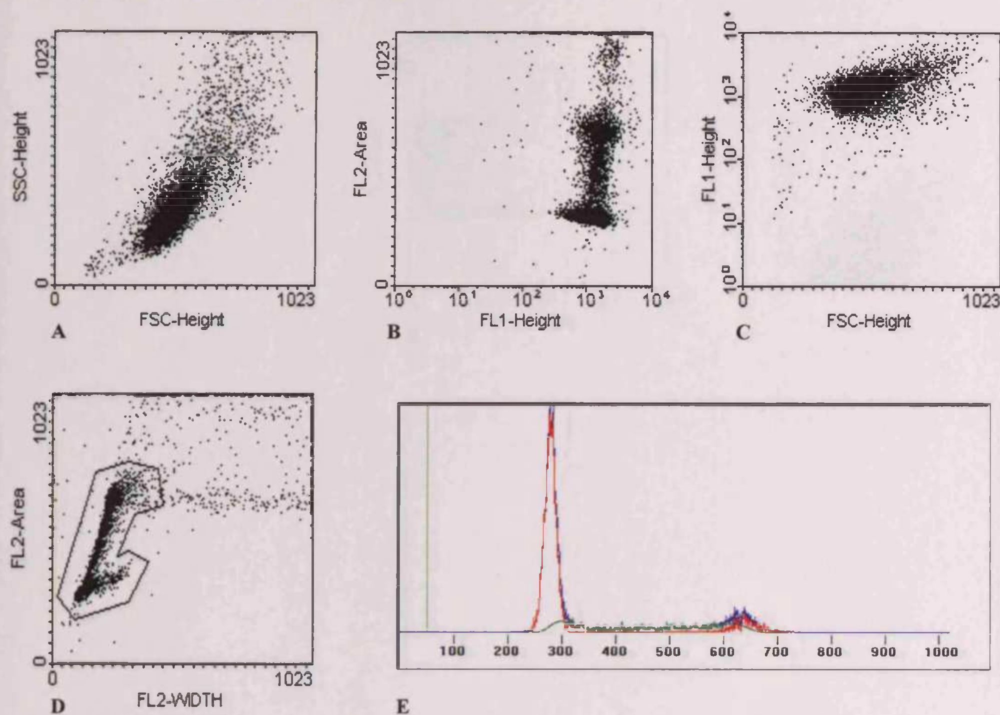


Figure 6.7 N-TERT cells grown on plastic stained for flow cytometry with FITC labelled $\beta1$ antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

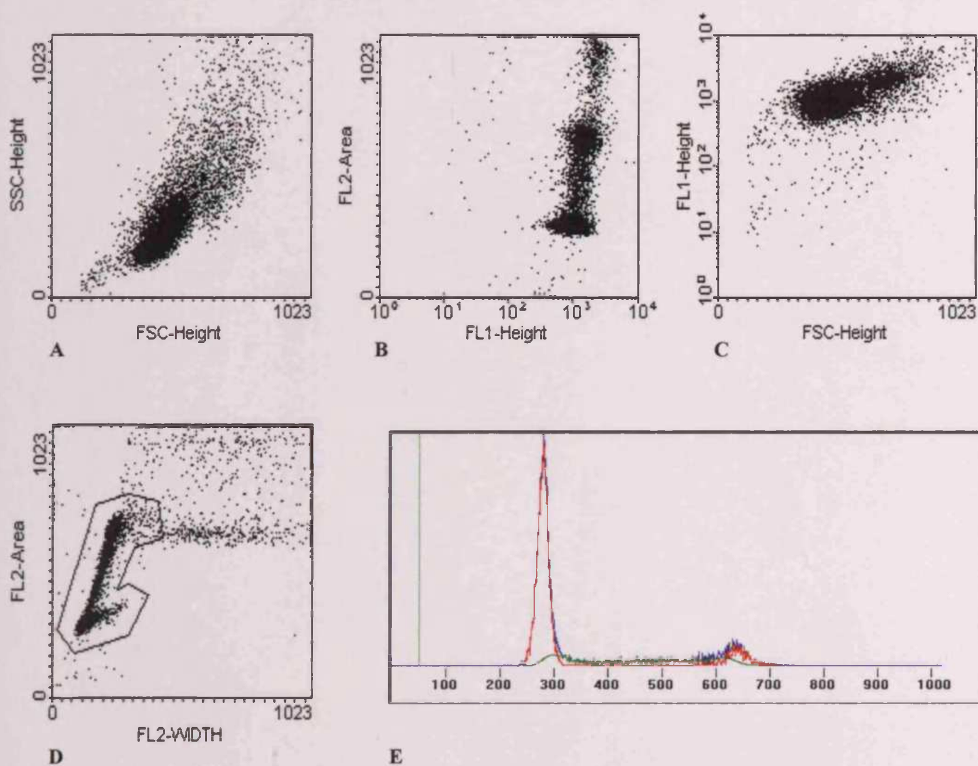


Figure 6.7 N-TERT cells grown on plastic for negative control. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

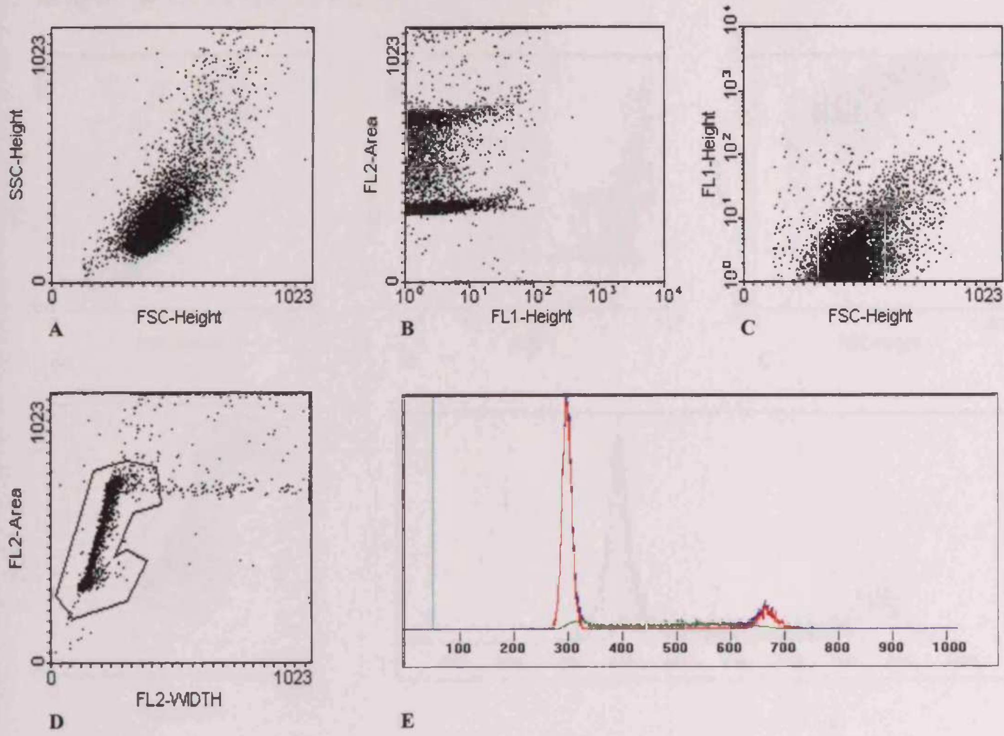


Figure 6.8 OKF6/TERT cells grown on plastic stained for flow cytometry with FITC labelled α6 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

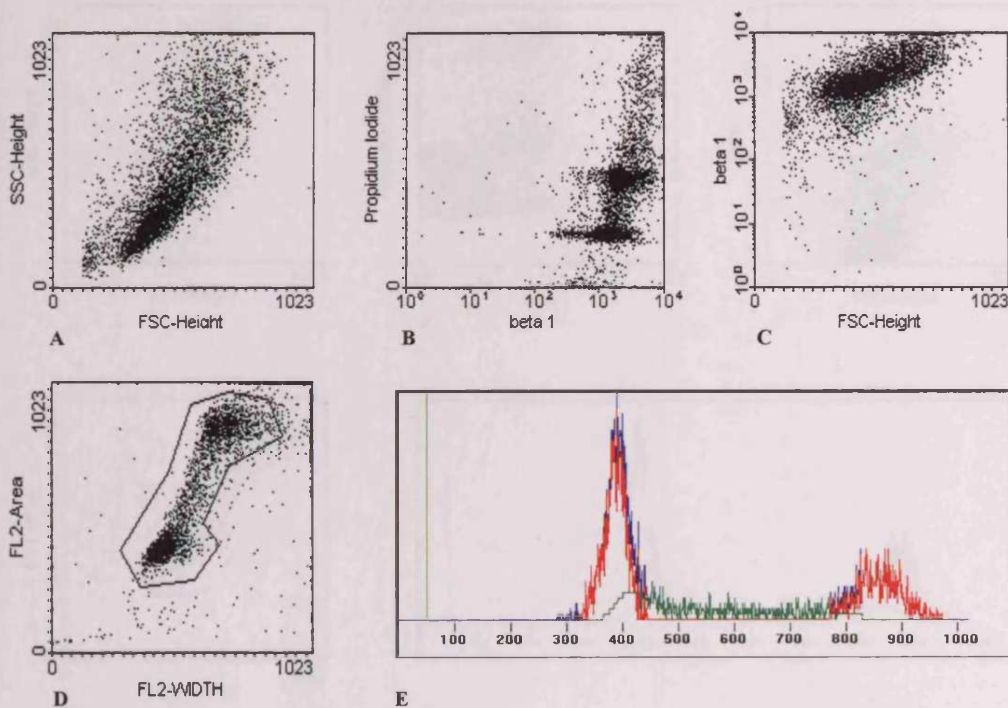


Figure 6.9 OKF6/TERT cells grown on plastic stained for flow cytometry with FITC labelled β1 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

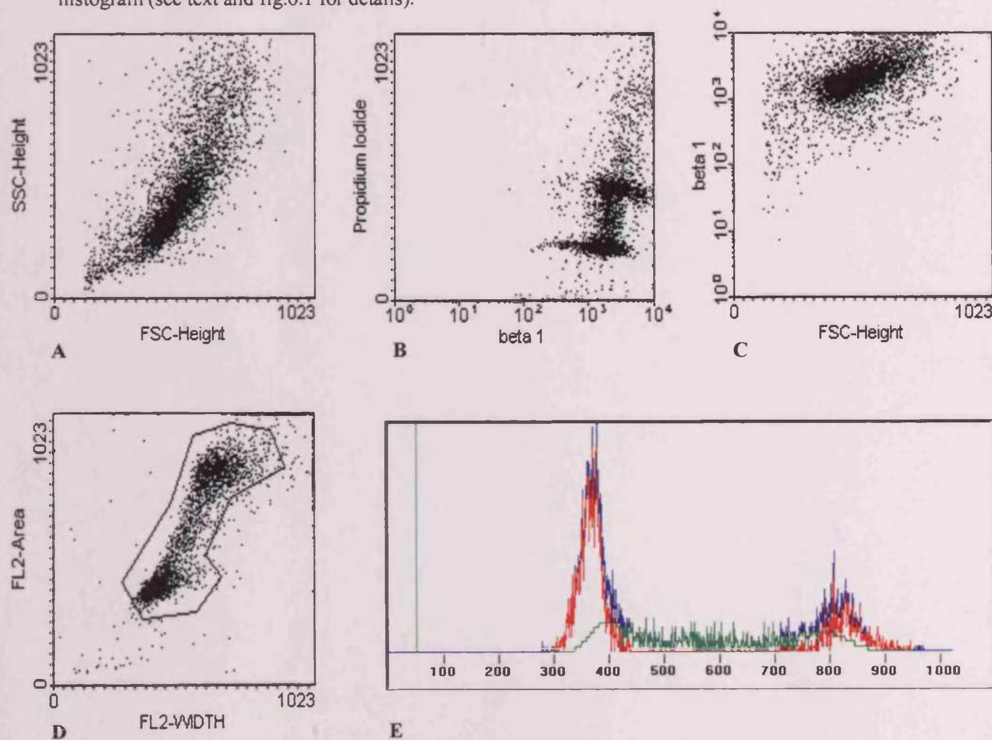


Figure 6.10 OKF6/TERT cells grown on plastic for negative control. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

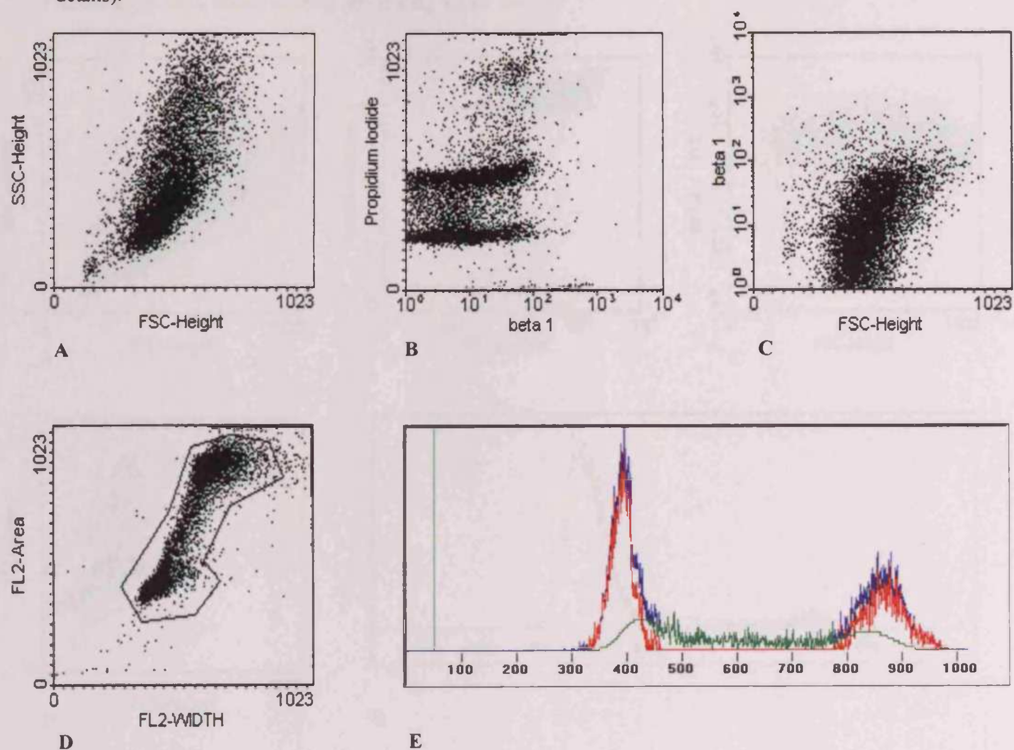


Figure 6.11 Palatal epithelial cells grown in organotypic culture stained for flow cytometry with FITC labelled α6 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

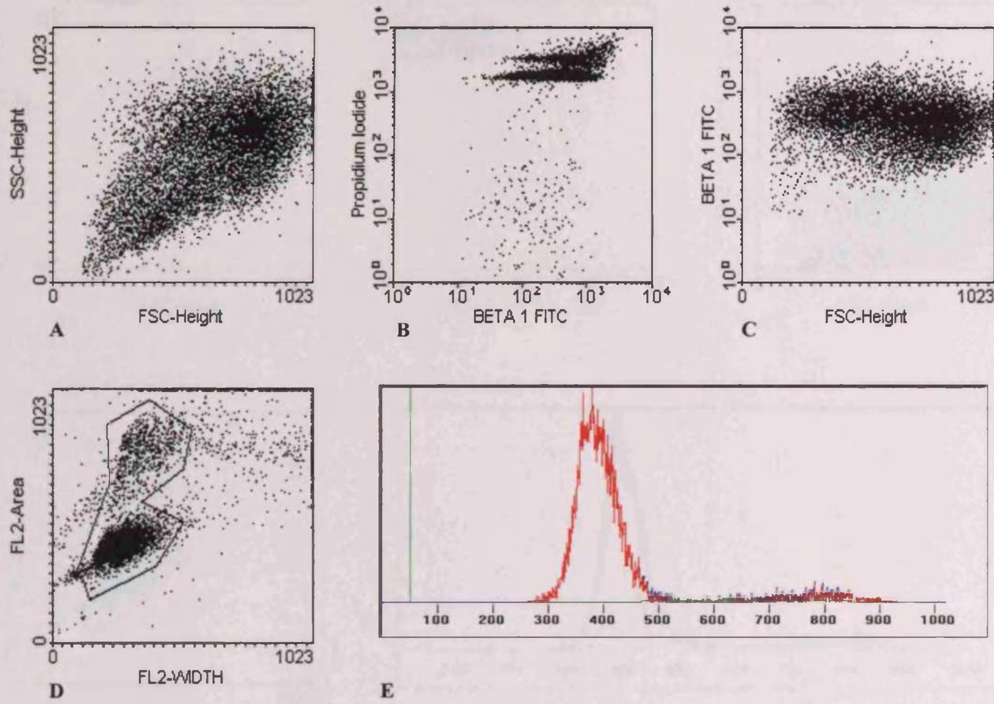


Figure 6.12 Palatal epithelial cells grown in organotypic culture stained for flow cytometry with FITC labelled β1 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

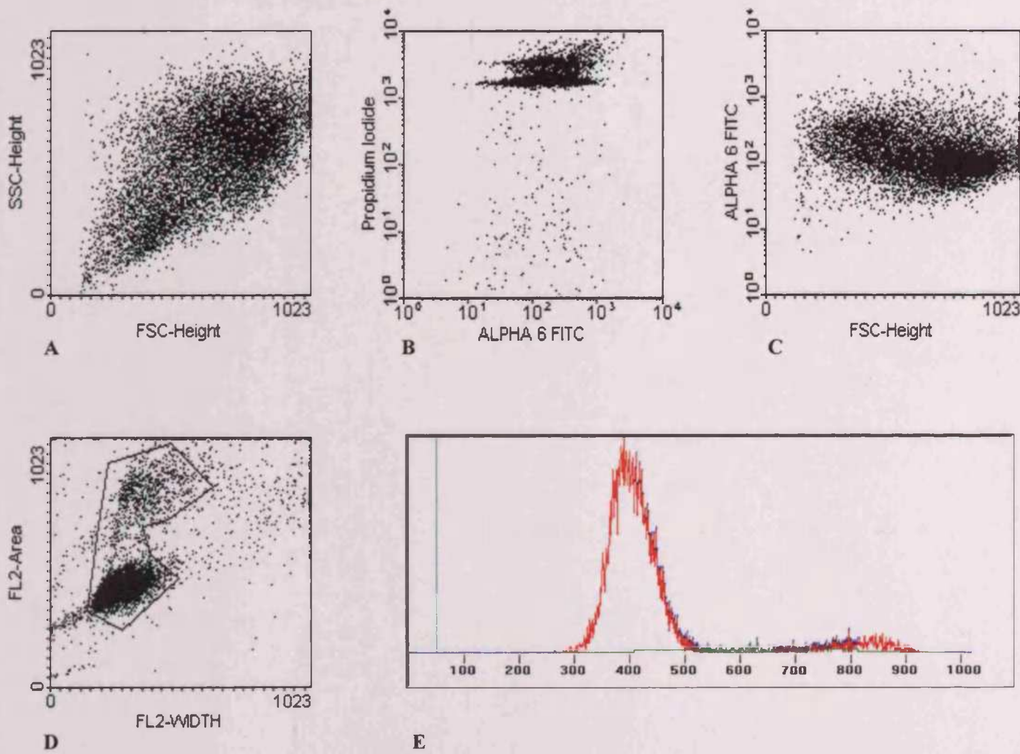


Figure 6.13 Palatal epithelial cells grown in organotypic culture for negative control. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

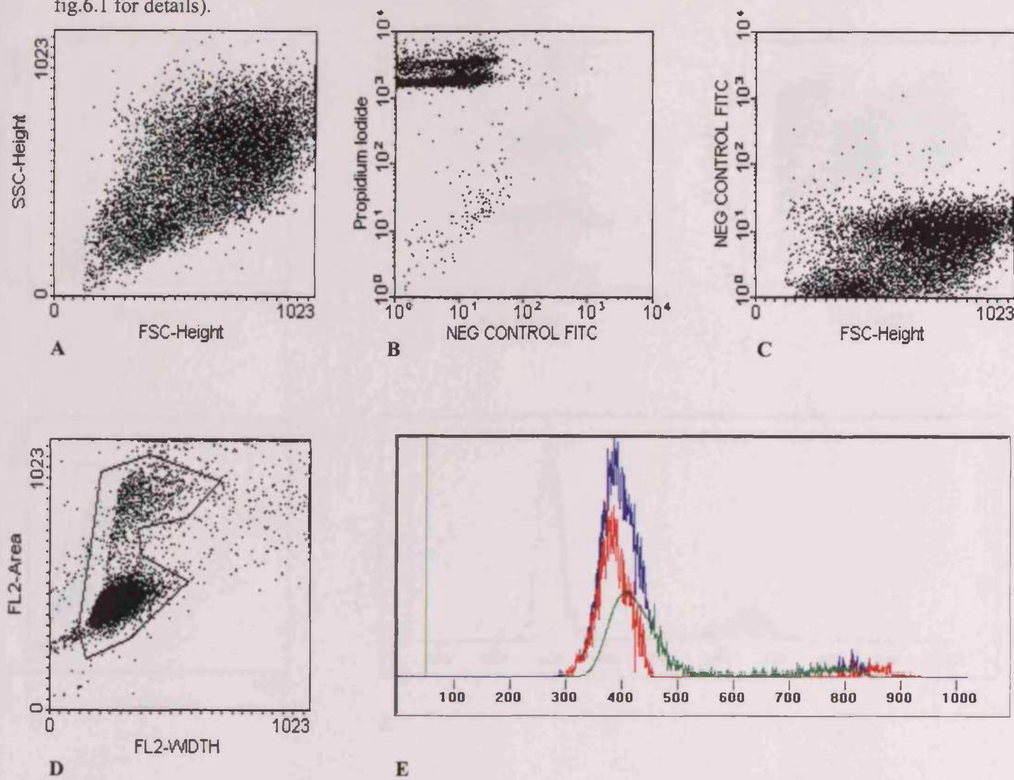


Figure 6.14 N-TERTcells grown in organotypic culture stained for flow cytometry with FITC labelled $\alpha6$ antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

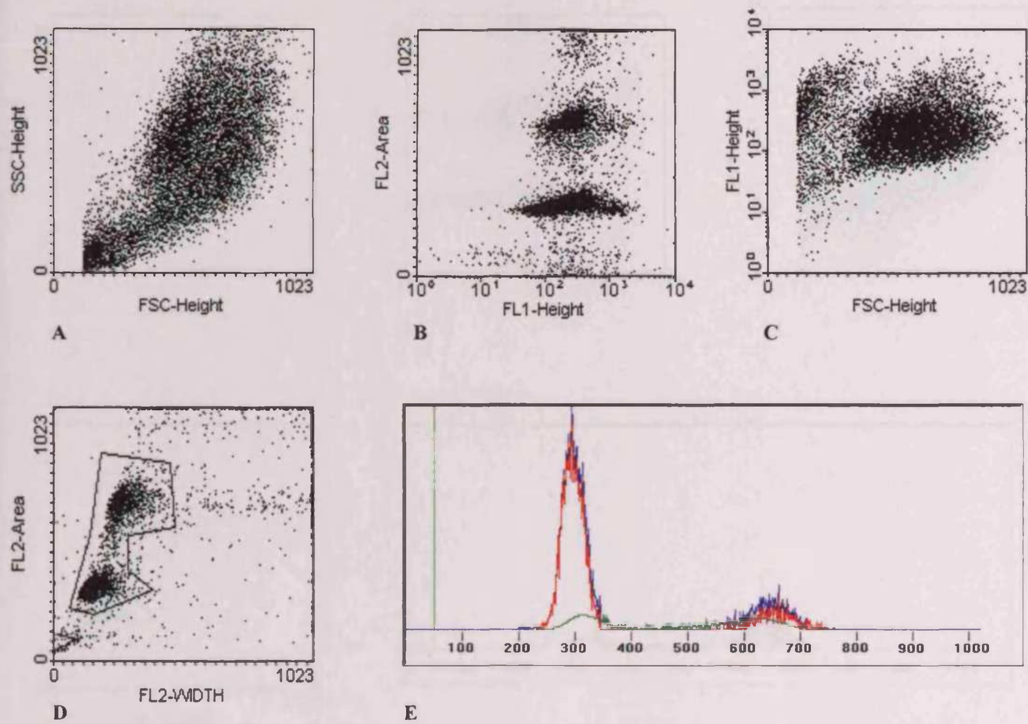


Figure 6.15 N-TERT cells grown in organotypic culture stained for flow cytometry with FITC labelled $\beta1$ antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

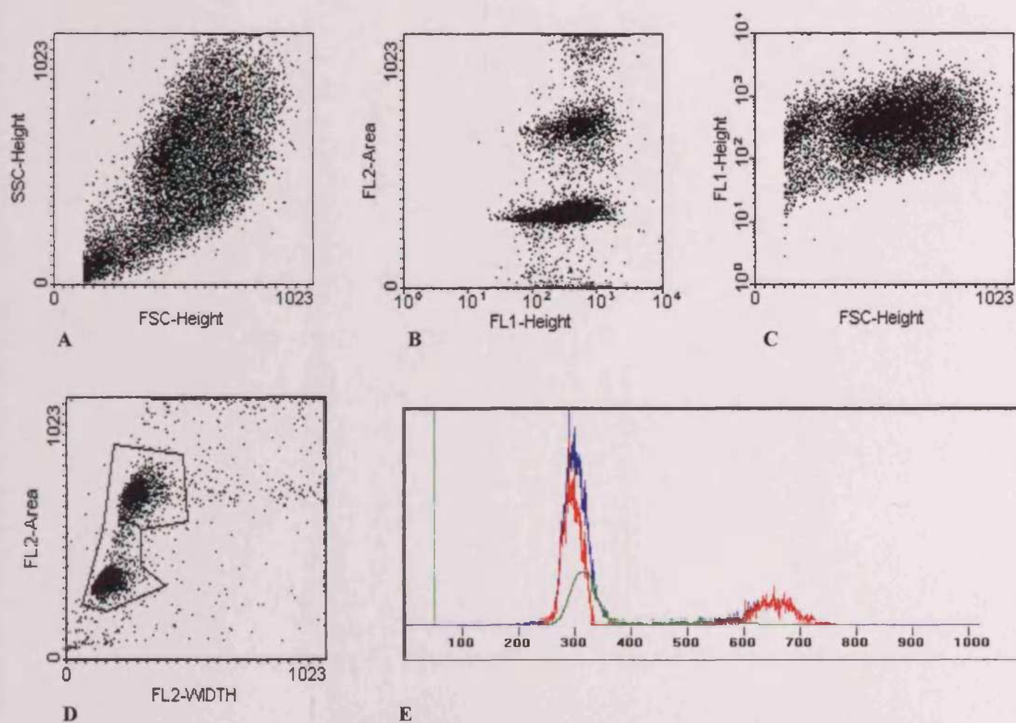


Figure 6.16 N/TERT cells grown in organotypic culture for negative control. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

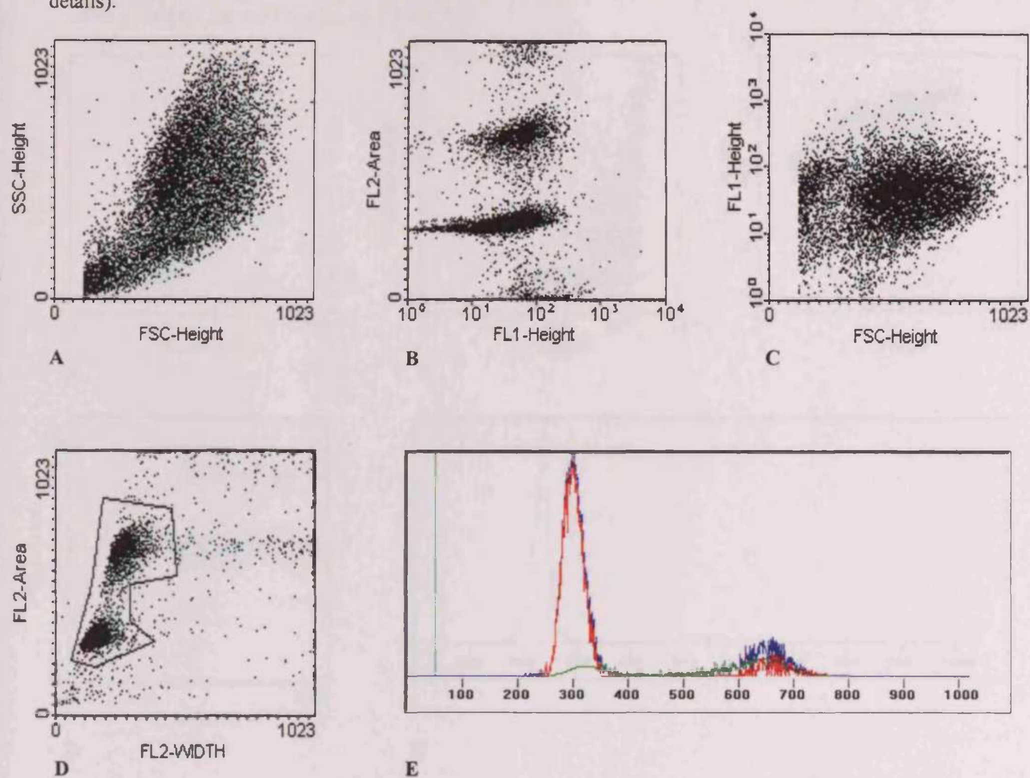


Figure 6.17 OKF6/TERT cells grown in organotypic culture stained for flow cytometry with FITC labelled α6 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

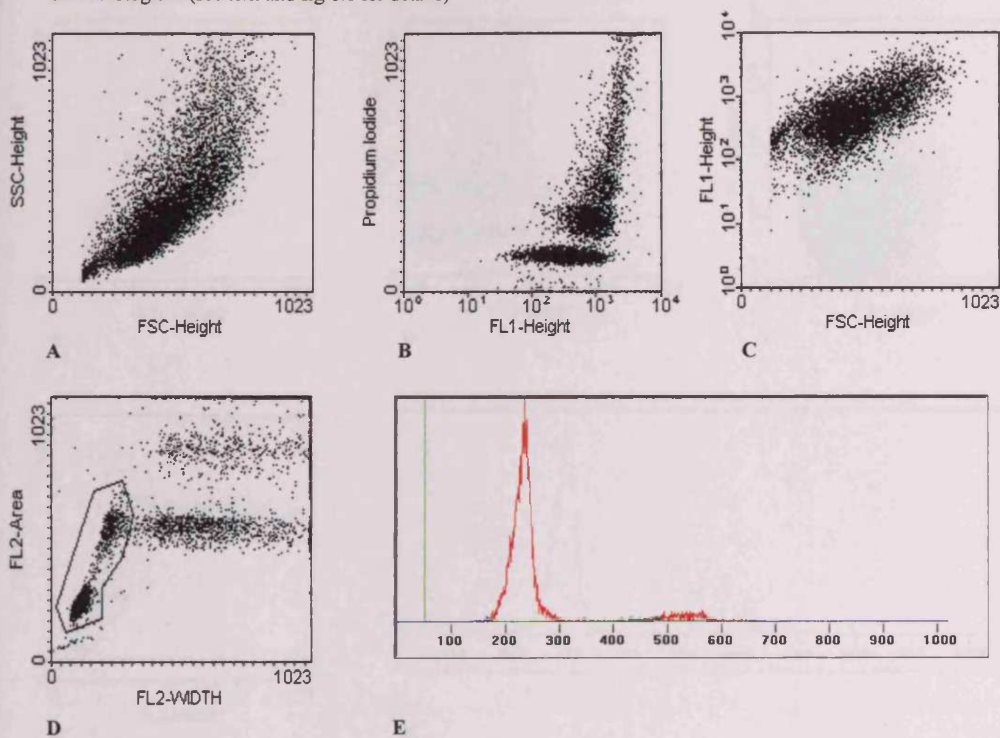


Figure 6.18 OKF6/TERT cells grown in organotypic culture stained for flow cytometry with FITC labelled β1 antibody. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).

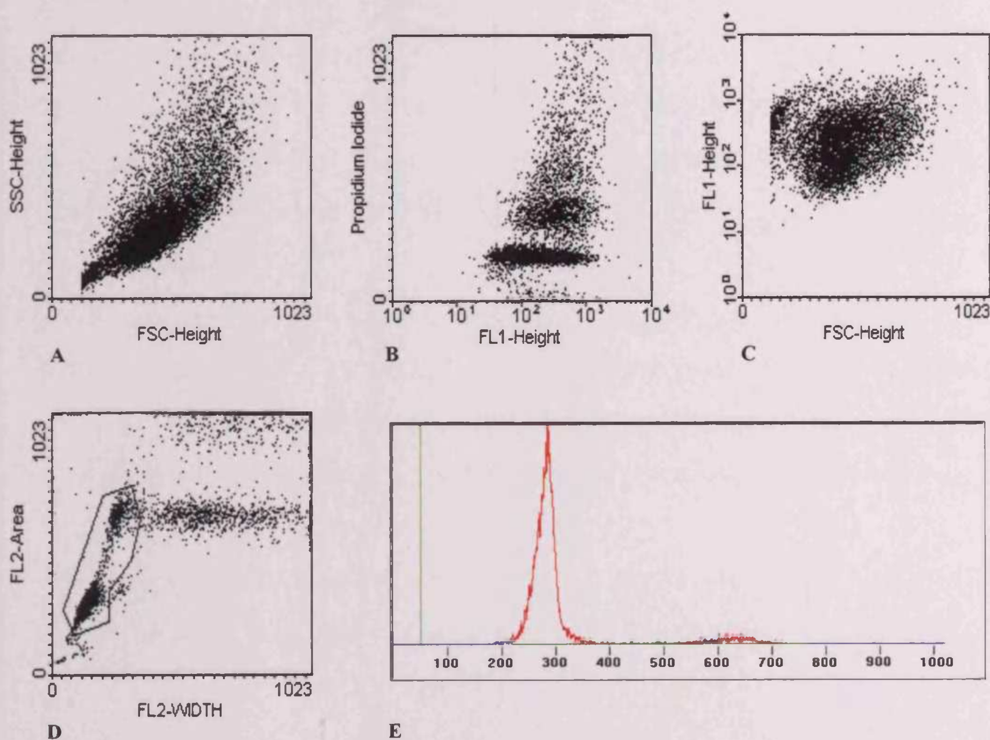
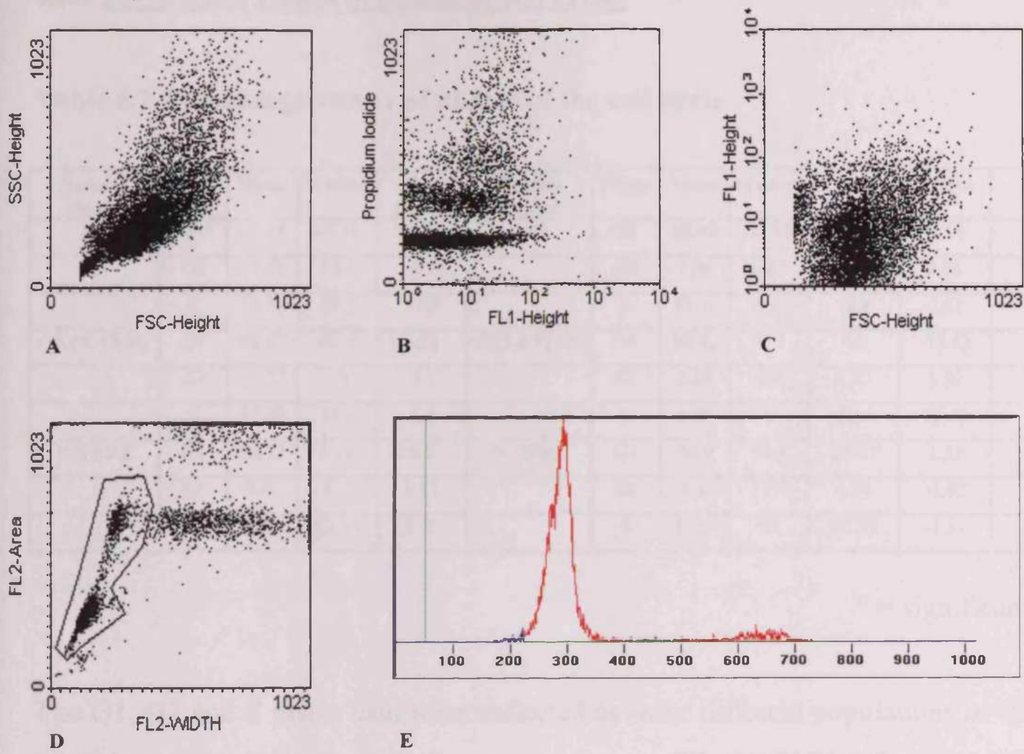


Figure 6.19 OKF6/TERT cells grown in organotypic culture for negative control. (A) FSC against SSC, (B) FL1 against FL2, (C) FSC against FL1, (D) FL2-width against FL2-area, (E) DNA histogram (see text and fig.6.1 for details).



6.2.2 Percentage values of phases of cell cycles

Table 6.1 Percentage values of phases of the cell cycle

Sample plastic	Phase	Mean	Median	S.D. plastic	Sample organo	Phase	Mean	Median	S.D. organo	t-value	p value
PE	G1	47.16	47.85	8.81	**PE**	G1	60.42	57.35	18.63	-2.34	0.024*
	G2	17.48	18.4	3.3		G2	7.09	6.35	3.4	8.93	<0.001*
	S	35.36	37.5	7.29		S	32.48	35.05	18.6	0.52	0.609
OKF6/TERT	G1	48.67	49.75	6.21	**OKF6/TERT**	G1	89.42	92.1	4.8	-15.63	<0.001*
	G2	14.27	13	7.5		G2	5.88	6.2	1.87	3.89	0.0012*
	S	37.08	36.6	4.1		S	4.69	3	5.14	13.47	<0.001*
N-TERT	G1	66.17	66.6	3.8	**N-TERT**	G1	50.9	54.8	24.17	1.52	0.153
	G2	9.47	9.7	1.83		G2	11.41	11.4	4.36	-1.02	0.324
	S	24.38	23.85	3.31		S	37.67	33	24.25	-1.31	0.211

* = significant

The G1, G2 and S phase data were collected as three different populations so that it was possible to compare them. The populations were PE, OKF6/TERT and N-TERT, and each of these populations was split into two sub-populations; cells grown on plastic (plastic), and cells made up into organotypic cultures (organo) for analysis. The values in table 6.1 show what percentages of the cells in each sample are in the different phases of the cell cycle. Table 6.1 also shows the mean, median and standard deviation (S.D.) for each phase (G1, G2 and S) within each population, and the t-value and p-value for each phase (G1, G2 and S) within each sub-population.

To begin, data within each of the six sub-populations was analysed to see if it was normally distributed. This is carried out by assessing if the mean and median of G1, G2 and S for each of the six sub-populations are similar. As they were, it was assumed that the data was normally distributed and parametric tests could be carried out.

The t-test is the parametric test that is carried out if samples are normally distributed, but are not identical populations. t-tests were carried out between the plastic and organotypic

cultures as these subpopulations are not identical populations. t-tests give a p value which is considered significant at less than a value of 0.05 (<0.05).

6.2.3 Anti-α6 and -β1 integrin antibody staining of keratinocytes and analysis by flow cytometry

After the process of producing a single cell suspension of the cells, either anti-α6 or β1 integrin antibody was added to the cells. Previous work (Jones *et al.*, 1995) has shown that all cells in the basal layer express β1 integrin but that stem cells express three times this amount. As stem cells are thought to be small and also express three times more β1 integrin than other basal cells, and be in a G0 resting phase, it was investigated to see if it was possible to view a population of cells that were small, that expressed high levels of β1 and were in the G1 phase of the cell cycle. In the same way, α6 integrin (Li *et al.*, 1998) is thought to be a stem cell marker and the experiment was also carried out using an anti-α6 integrin antibody.

Figures 6.2 to 6.19 show the same set up of plots as 6.1A to 6.1E. Some of the FL1-height axes have been labelled as beta1 (β1), alpha6 (α6) or negative control (-ve), and the FL2-area axes as propidium iodide.

Fluorescence values for α6 and β1 integrins

Fluorescence values for α6 and β1 were obtained by plotting a histogram of FL1-height against the number of events (data not shown). By applying a statistics window to this histogram, a value is given for the median fluorescence. The median value for the negative control samples was subtracted from the α6 and β1 values and these values were then averaged for α6 and β1 integrins in each subgroup. The levels were compared for each sample and are shown in table 6.2.

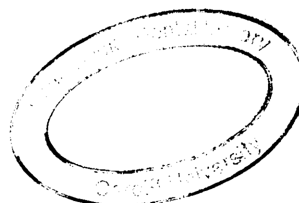


Table 6.2 Fluorescence values

Sample	Median Beta 1	Median alpha 6	Negative Control	Mean beta 1 minus Negative control	Mean alpha 6 minus Negative control
Plastic					
PE9	1433.01	881.68	1.07		
PE9	827.88	1345.57	1.55	1129.14	1112.32
PE11	1654.82	2308.24	2.48	1652.34	2305.76
PE12	1684.85	1730.94	1.88	1682.97	1728.12
OKF6/TERT	1746.58	1826.92	5.47		
OKF6/TERT	1843.42	1980.96	8.82	1787.86	1896.8
N-TERT	1074.61	1286.41	2.82		
N-TERT	1134.19	1263.46	2.02	1101.98	1272.52
Organotypic cultures					
PE9	378.55	121.88	7.77		
PE9	445.08	143.3	5.83		
PE9	441.09	86.6	7.84		
PE9	421.7	152.61	9.31	413.92	118.41
PE11	410.47	170.01	14.72		
PE11	425.51	243.62	13.95		
PE11	461.38	223.7	15.54	417.72	197.71
PE10	938.98	552.32	42.55		
PE10	973.38	537.61	44.51		
PE10	938.98	685.39	42.55	907.24	548.57
OKF6/TERT	655.25	142.02	7.1		
OKF6/TERT	523.3	528.03	5.08		
OKF6/TERT	572.55	240.6	6.21		
OKF6/TERT	523.3	237.14	6.32	562.42	280.77
N-TERT	325.27	237.14	39.6		
N-TERT	291.64	235.01	40.68		
N-TERT	342.89	250.29	39.95	279.86	200.74

The results for each individual experiment were kept separate for analysis, but within each experiment, cells of the same type were grouped together.

6.3 Discussion

6.3.1 Percentage values of cell cycle phases

Percentage values of cell cycle phases of palatal epithelial cells (PE)

From the results in table 6.1, in the PE cultures it can be seen that the difference in the percentage of cells in G1 is significant as it is higher in the organotypic culture with a p value of 0.024 (which is <0.05). The difference in the percentage of cells in G2/M is significant, as it is higher in the plastic group with a p value of <0.001, which is significant if <0.05. This is consistent with the hypothesis that the cells grown on plastic cycle faster than the cells in organotypic culture. There is no significant difference in the percentage of cells in S phase.

Percentage values of cell cycle phases of OKF6/TERT cells

In the OKF6/TERT cultures, the difference in the percentage of cells in G1/0 is significant, as it is higher in the organotypic culture with a p value of <0.001. The difference in the percentage of cells in G2/M is significant, as it is higher in the plastic group with a p value of 0.001185. The difference in the percentage of S cells is also significant, as it is higher in the plastic group with a p value of <0.001.

Percentage values of cell cycle phases of N-TERT cells

In these N-TERT cultures there were not any significant differences between the plastic and organotypic culture sub-populations. This may be due to the fact that the organotypic cultures formed using the N-TERT cells did not stratify and differentiate in the same manner as the OKF6/TERT organotypic when sections were examined after H&E staining. As the cells were less organised in organotypic culture, it is possible that the cells in the cultures were more like the cells grown on plastic thus forming more of a sheet of keratinocytes as opposed to an epithelium.

All three populations

From the significant values in the results (PE and OKF6/TERT), it appears that the plastic groups were cycling faster than the organotypic culture, as there was a higher

percentage of cells in the G2/M fraction in the plastic group compared to the organotypic group. The percentage of cells in the G1 fraction was higher in the organotypic group which suggests that a greater proportion of the cells were either resting and not cycling or have not yet begun to make more DNA in preparation for a new cycle. These results relate to a slow cell cycle in the organotypic cultures, which may be indicative of a population of slowly cycling stem cells within these cultures.

6.3.2 α6 and β1 integrin staining by flow cytometry

When the control samples of each group were set up without any FITC labelled antibody (figures.6.4, 6.7, 6.10, 6.13, 6.16 and 6.19), it can be seen from figures 6.4B, 6.7B, 6.10B, 6.13B, 6.16B and 6.19B that there are not many cells stained along the FL1-height x-axis above the level of about 10^2. The few cells that are present above this level on the x-axis are the cause of autofluorescence. In figures 6.4C, 6.7C, 6.10C, 6.13C, 6.16C and 6.19C the FL1-height y-axis also shows that the level of fluorescence is low and is below 10^2 with any fluorescence above this level due to autofluorescence. The plots for α6 and β1 integrin are quite similar within each group and only when a statistical window was applied were any differences observed.

Figures 6.2, 6.3 and 6.4 show palatal epithelial cells grown on plastic that have been analysed on the flow cytometer. Figures 6.11, 6.12 and 6.13 show palatal epithelial cells from organotypic culture which have very different profiles from the cells grown on plastic (figures. 6.2, 6.3 and 6.4). Both integrin plots in each group (figures 6.2 and 6.3; 6.11 and 6.12) appear very similar to each other which is why the fluorescence values from the histogram need to be calculated.

The same pattern can be seen with the N-TERT keratinocytes with the integrin profiles within the group similar (figures 6.5 and 6.6; 6.14 and 6.15) but with different profiles between the cells grown on plastic and those from the organotypic cultures.

This pattern was also observed with the OKF6/TERT keratinocytes with the integrin profiles within the group appearing similar (figures 6.8 and 6.9; 6.17 and 6.18), but the

profiles between the cells grown on plastic and those from the organotypic cultures different.

6.3.3 Fluorescence values of α6 and β1 integrins

From the results in table 6.2 it can be seen that in the organotypic cultures the level of α6 integrin was always lower than for β1 integrin, except in one sample where the values were very close. In the cells grown on plastic the level of β1 integrin was lower than α6 integrin, except in one sample. The median fluorescence values for the cells grown on plastic were much higher than for organotypic culture. The same number of cells were collected for each sample so this discrepancy is not as a result of different cell numbers. As the flow cytometer method requires cells to be as single cells, these median fluorescence values indicate that the cells grown on plastic must be larger or have a greater number of integrin receptors per cell, as the fluorescence value is much higher, and that the cells in organotypic culture are smaller or have a smaller number of receptors.

A subset of cells was gated on high and low fluorescence values as stem cells are thought to have higher β1 integrin expression, and the cell cycle for these were calculated to see if there was a difference in the distribution of the different phases of the cell cycle. There was no visible difference between proportion of cells in the various phases in these two subpopulations (data not shown).

Another subset of cells was also gated for low FSC and SSC as this represents a population of small cells. The percentage of cells in the different phases of the cell cycle was calculated for this subset and compared with an ungated sample (data not shown). There was no visible difference between the proportion of cells in the various phases of the cell cycles between these two subsets either.

CHAPTER SEVEN

CHAPTER 7

GENERAL DISCUSSION

Stem cell patterns in palate

It is apparent from the staining profiles obtained with the palate sections that none of the antibodies used in the experiment was able to identify individual stem cells, although it is possible that two of the antibodies examined did identify reliably small stem cell zones making palate an interesting tissue to study stem cells. When staining with the early differentiation markers, K6 and K16, the basal cells at the ERTs did not stain. These areas are thought therefore to correspond to the least differentiated cells. From these observations, it could be deduced that the least differentiated cells, which are thought to correspond to a stem cell population, form the basal layer of the ERT region. The basal layer of cells that did not stain positively for K6 and K16, did stain for keratins K15 and K19. K15 and K19 only stained cells in the basal layer, and localised cells to the ERTs. Although it appears that these zones of K15 or K19 positive cells could contain stem cells, is not known how many stem cells there might be per stem cell zone. As K6 and K16 stained cells in the same position as the integrins did, it is apparent that the staining for $\alpha6$ and $\beta1$ integrins in the tissue corresponded to areas of early differentiation and not to stem cell zones.

Possible relationships between rete and stem cells give conflicting results. LRCs in murine tissue suggest that one or more stem cell appear to lie at the ERT; clusters of stem cells stained with $\beta1$ integrin lie over the CPTs are described in some human studies such as oesophagus (Seery and Watt, 2000); and work carried out using organotypic cultures transplanted onto SCID mice show that the stem cells maintain narrow columnar zones that do not correspond to rete at all. These differences might be due to regional variations but more information is needed to clarify this. Stem cells form only a small subpopulation of the proliferative basal cells, and to date a pure population of stem cells has not been isolated without ambiguity. Little is known about the mechanisms that

regulate stem cell self-renewal or the differentiation of stem cell progeny, but with progress, the answers might become clearer. The behaviour and distribution of these stem cells differ considerably from one species to another and even between different regions of squamous epithelia within the same animal.

The staining profiles obtained with the *in vitro* colonies of palatal epithelial cells (chapter 4) did not clarify any of the staining profiles obtained with the palate sections but some interesting observations were made. Staining in some of the colonies, including β1 integrin, K15 and K6 did correspond to cell size with the smaller cells strongly stained for antibodies against β1 integrin and K15, and the larger cells to K6.

Organotypic cultures and stem cells

The three-dimensional nature of organotypic cultures, taken with their more normal patterns of differentiation, provides an environment that more closely mimics the *in vivo* environment. The organotypic cultures formed were very variable in quality and the staining profiles of the various antibodies were not always satisfactory. The staining profiles obtained with the various antibodies did not always correspond to what would be predicted from the palate staining. K5, K13 and β1 integrin had the same staining profiles but the other antibodies did generate different staining profiles. Discrepancies between the *in vivo* and *in vitro* antibody staining are well documented and were not entirely unexpected, as it is difficult to get normal patterns of differentiation in organotypic culture.

The organotypic cultures made from the HaCaT cells lacked differentiation so other cell lines were investigated. The OKF6/TERT cell lines produced satisfactory organotypic cultures with an epithelia of good thickness. These cell lines were investigated further to see if it was possible to retrovirally transduce a small population of the cells, which were made into organotypic culture, in order to view any clonal lineages that would descend from any transduced stem cells. The ideal percentage of transduced cells to use in the OKF6/TERT organotypic cultures was 3%, as clones were beginning to be visible. Transplantations of the epidermal and palatal organotypic cultures were carried out in

SCID mice in order to view the long term persistence of the clones formed in these cultures. The results show that the marked cell clusters formed in the epidermal organotypic cultures corresponded to the columnar units in human epidermis, and it could be proposed that these labelled units correspond to stem cell clones. This is in contrast to the palatal organotypic cultures where the columns of labelled cells formed did not correspond to the bigger morphological units of rete structures. Instead, the columns of cells were narrower than the rete structures and did not arise from the putative stem cell zones situated in the rete tips.

Cell cycles of different epithelial populations

The flow cytometry work showed significant statistical values between the cell cycles of some of the groups. The groups of PE and OKF6/TERT cells grown on plastic were cycling faster than the organotypic culture groups, as there was a higher percentage of cells in the G2/M fraction. The percentage of cells in the G1 fraction was higher in the organotypic group, which suggests that a greater proportion of the cells were either resting and/or not cycling. This indicates that they were cycling slower than the plastic group and that the cells in the organotypic cultures are under better proliferative control.

Palatal epithelial cells from organotypic culture have a very different flow cytometry profile compared to the cells grown on plastic. Both the integrin plots in each group appeared very similar to each other, this was why the fluorescence values from the histogram were calculated. This pattern was also observed with the OKF6/TERT keratinocytes with the integrin profiles within the group appearing similar, but the profiles between the cells grown on plastic and those from the organotypic cultures appeared different.

In the organotypic cultures the level of $\alpha6$ integrin was always lower than for $\beta1$ integrin, and in the cells grown on plastic the level of $\beta1$ integrin was lower than $\alpha6$ integrin, except in one sample. The median fluorescence values for the cells grown on plastic were much higher than for organotypic culture. As the same number of cells were collected for each sample and as the flow cytometer method requires cells to be as single cells, these

median fluorescence values could indicate that the cells grown on plastic are larger as the fluorescence value is much higher. Since the antibody levels reverse, these values can be considered to be a genuine cell effect. No differences were observed when the cell cycles of cells with extremes of fluorescence or extremes of cell size were analysed.

A good stem cell model?

The staining profiles of the various antibodies in palate showed the location of the undifferentiated cells and identified a stem cell zone in the ERT within which the stem cells reside. When human palatal keratinocytes were grown *in vitro* on plastic the colonies formed corresponded to the holo-, mero- and paraclone types of colonies which were not consistent to a good stem cell model because differentiation markers were not expressed in a step-wise manner as would be expected. Keratinocytes grown on plastic did not form a stem cell culture model however this could be improved by the use of organotypic culture models. The staining profiles of the various antibodies in palate were compared to those in the organotypic cultures to see if they were similar and the organotypic cultures seemed to be a satisfactory stem cell model. This was tested in more detail with the use of retroviral vectors to transduce keratinocytes that formed clonal units founded by stem cells in organotypic culture. After these were transplanted to SCID mice it could be seen that a number of stem cell clonal units populated each rete. This was in contrast to the staining profiles obtained with the putative stem cell zone markers K19 and K15, which indicate that there was only one stem cell clonal unit per rete in human palatal epithelium.

Future prospects

A number of recent advances have been made in stem cell research. These include the use of confocal and laser capture microscopy for identifying and studying stem cell regions, the use of β-catenin as a potential stem cell marker, and evidence that oesophageal epithelium has a similar stem and amplifying pattern to palate. The methods and results for these techniques are outlined below.

Confocal microscopy

The method for preparing a sample for confocal microscopy involves the blocking and permeabilisation of epithelial sheets before the addition of primary antibody (Braun *et al.*, 2003). The epithelial wholemounts were then washed, secondary antibody added and then the sheet was washed and mounted. Images were acquired using a confocal microscope, with a number of optical sections of each epithelial sheet captured with a typical increment of 1-3μm. Samples were scanned from the dermal to the epidermal surface to a total thickness of 40-80μm.

In limbal-corneal epithelium the stem cells were exclusively localised to the basal epithelial layer of the limbal domain (Romano *et al.*, 2003). Studies have been conducted to determine whether this spatial stem cell arrangement is reflected in differences in the cell size between limbal and corneal cells. *In vivo* confocal microscopy was used to scan and measure the size of the cells of the central corneal and superior limbus from the superficial to the basal cell layer. Limbal and corneal sheets were isolated by dispase digestion and dissociated into single cells by trypsin digestion. The FSC (forward scatter - cell size) and SCC (side scatter - granularity/cytoplasmic complexity) light scattering properties of the cells were determined by flow cytometry. The basal cells of the limbal and corneal zones were shown to have diameters of about half the values of the corresponding superficial cell layers. The limbus contained a substantial proportion of very low FSC and SCC cells for which there was no corneal counterpart indicating that the smallest cells were located in the limbal basal epithelium, this feature may help isolate corneal stem cells located in the limbus.

Laser capture microdissection

It is possible to section a papillae where the even numbers of sequentially numbered sections were subjected to confocal microscopy (a camera system was used to capture overlapping digital images from all regions of a specific papillae), and the odd numbered sections were prepared then the overlapping images were pieced together for laser capture microdissection (LCM). LCM represents a way to recover epithelial cells from selected papillae and to profile these cells (Wong *et al.*, 2000). Cells in fresh-frozen

tissue sections can be marked for LCM by direct histochemical or immunohistochemical staining. An alternative was to stain an adjacent section and use it as a guide for the microdissection, this is termed navigated-LCM. The section preceding the one targeted for LCM was stained with antibody to identify the desired region. Electronic images were captured from the entire stained section and used to assemble a panoramic view. The composite image was projected on a computer screen. The electronic image template was used as a guide to navigate the microdissection and the process can be repeated through a group of serial sections.

Navigated-LCM can also be used to recover DNA and intact RNA from specific cell populations. Real-time quantitative RT-PCR and/or DNA microarray profiling of gene expression would allow biology and pathobiology to be defined at the interface between normal and abnormal cellular cohorts.

β-catenin

Advances now indicate that there are a number of stem cell repositories within the epidermis, two of which, the interfollicular epidermis and the bulge region of the hair follicle, may supply each other when damaged (Janes *et al.,* 2002). The protein β-catenin has recently been proposed to control the behaviour of stem cells (Alonso and Fuchs, 2003). β-catenin, a multifunctional protein is stabilised when cells receive a Wnt signal. The Wnt signalling pathway has been linked to the ability of skin epithelial cells to acquire and/or maintain features of multipotent stem cells. β-catenin is required to activate members of a DNA-binding protein family called the Lef/1Tcf family. In skin, Wnt signals are received by multipotent embryonic skin epithelial cells before their commitment to form a hair follicle. When specialised skin mesenchymal cells inhibit a second signalling pathway, the bone morphogenetic protein (BMP) pathway, the multipotent epithelial cells express Lef1 and become committed to forming a hair follicle. Both the Wnt and BMP signalling pathways appear to be functionally important to making a hair follicle as mice with disrupted function of Lef1 or β-catenin are all severely impaired in their ability to form hair follicles. In postnatal skin, only bulge stem cells are thought to retain multipotency. When β-catenin is constitutively stabilised in

transgenic mouse skin, the adult interfollicular epidermis behaves like embryonic skin, seemingly able to choose between an epidermal and hair follicle fate. When the specialised mesenchymal cells of the hair follicle were exposed to Wnt signalling, they also appear to retain the hair follicle-inducing power. It appears that Wnt signals may be able to act on both the epithelium to induce stem cell-like properties and on the mesenchyme to maintain its stem cell recruiting properties. The degree of Lef1/Tcf activity may also be critical in determining the outcome of stem cell lineage determination. When Lef1 is overexpressed in the skin and oral epithelium, occasional hairs and teeth were seen in inappropriate places. Recently a more global role has been proposed for β-catenin and its partners in stem cells and fate specification.

Oesophageal epithelium

Oesophageal epithelium has a similar tissue architecture to palatal epithelium. Oesophageal epithelium has a basal zone made up of a single layer of cells that adhere to the basement membrane, and a variable number of cells above this (epibasal layers). The lamina propria invaginates the epithelium at regular intervals, producing tall papillary structures, which are analogous to the CPT in palate. This divides the basal layer into two components, one flat (interpapillary basal layer, IBL) and one covering the papillae (papillary basal layer, PBL). Cellular proliferation was limited to the basal layer and the cells were thought to progress towards the oesophageal lumen (Seery, 2002). Detailed analysis of mitotic figures in the oesophageal epithelium combined with immunohistochemical staining for proliferating cells demonstrated a highly complex pattern of cell proliferation in the basal zone. The proliferating cells were more common in the epibasal layer and within the basal layer, the mitotic cells were four times more common in the PBL than in the IBL. Also the orientation of the mitoses was heterogeneous in the basal layer, with cells in the PBL dividing symmetrically relative to the underlying stroma, yielding two daughter cells in contact with the basement membrane. Cell division in the IBL was asymmetrical and occurred at right angles to the underlying basement membrane, yielding one daughter cell that remained in the basal layer and one that entered the epibasal layer - the putative transit compartment. β1 integrin expression was heterogeneous in the oesophageal basal layer with cells in the

IBL expressing lower levels than cells in the PBL. Stem cells of the epidermis express higher levels of β1 integrin compared to the putative stem cells of the IBL. When these cells were grown at clonal density, the IBL cells were two-fold enriched of cells capable of forming large actively growing colonies (Seery and Watt, 2000), indicating that these cells might be candidates for oesophageal epithelial stem cells.

Keratin expression was investigated, K14 and K15 levels were high in the PBL and epibasal layers and patchy in the IBL. K4 expression in the papillary region was detectable from the second epibasal layer onwards but did not appear until the third epibasal layer in the interpapillary region. Therefore in terms of differentiation markers, the cells in the IBL were the least differentiated.

Differences between epidermal and oesophageal keratinocytes may reflect differences in the role of epithelial-mesenchymal interactions in the control of stem cell function in the two cell types. Epidermal keratinocytes can regulate stem cell proliferative activity and number independently of interactions with the dermis. In contrast, the basement membrane of the oesophageal epithelium plays a role in controlling oesophageal stem cell behaviour. Interactions between oesophageal stem cells and the oesophageal basement membrane determine the asymmetric orientation of cell division and dictate tissue architecture (Seery and Watt, 2000).

Every cell in the IBL was unlikely to be a stem cell and the status of the cells in the PBL was difficult to define in terms of stem or amplifying-cell model. It is thought that these cells were an intermediate between the stem cells of the IBL and the putative transit amplifying cells of the epibasal layers. The lineage relationship between the cells of the IBL and the PBL were unknown, but the papillae may form by the sideways expansion of cells in the IBL. The progeny of the rare cell divisions parallel to the basement membrane in the IBL could contribute to the PBL.

This data is consistent with the results obtained in this thesis with the palate investigation in chapter 3, with the IBL corresponding to the ERT in palate, and the PBL

corresponding to the CTP. This would strengthen the idea that the oesophagus and palate have similar stem and amplifying cell positions within the tissue, which are different to epidermis. The least differentiated cells reside in the ERT/IBL, which would correspond to the stem cells, the stem/early amplifying cells reside in the CPT/PBL, with the late transit amplifying cells in the suprabasal layers.

Stem cells

Adult stem cells are much rarer than embryonic stem cells making adult stem cell lines difficult to establish, however adult stem cells could in the future be used to regenerate specific cells for the person they came from, making new organs for transplant. As these stem cells creating the new organ would come form that particular person's body, it would not be rejected. Human stem cells could be used for beneficial research as they can be used to test and study the affects of new drugs. This would allow for non-human testing, as well as more rapid and efficient testing. Stem cells could also be used for studying diseases and conditions caused by abnormal cell behaviour such as excess division of cells of the wrong differentiation of cells. These studies could lead to a cure for cancer, birth defects and a long list of genetic diseases. Human stem cells are also being studied for the regeneration of damaged tissues, and cells could be differentiated into the types of cells needed to repair damage to the body from diseases such as Parkinson's, diabetes, Alzheimer's, heart disease and arthritis. Stem cells could also be differentiated to repair damage from injuries such as spinal cord injuries, burns and strokes. Stem cell research can only lead to a better and brighter medical future for everyone. Drugs could be developed to specifically target diseases efficiently and quickly, cell damage will be able to be reversed restoring an injured body to its original state, and transplants could be made without concerns about availability and rejection.

Future prospects for the study of epithelial stem cells are exciting, the skin, like bone marrow, could be a readily accessible source of stem cells for therapeutic intervention and there is evidence of skin stem cell plasticity (Janes *et al.,* 2002). Initial work has implicated the integrins as cell surface markers that are not specific but can be used to enrich populations of cells for stem cells. Delta1 and p63 have also been suggested to

play a role in stem cell patterning. β1 integrins, Notch, c-Myc and β-catenin all regulate the size of the stem cell compartment *in vitro* and evidence from transgenic mice suggests that they are also important *in vivo* (Watt, 2002). Recently, Wnt signalling, β-catenin and Lef1/Tcf transcriptional regulation have been implicated in stem cell maintenance and/or lineage determination. Despite these advances, scientists have not yet been able to reliably isolate stem cells from skin and oral epithelia to exhaustively study their functional and transcriptional characteristics.

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