

PhD Thesis

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**STEM CELL FATE IN CULTURED
HUMAN EPIDERMIS**

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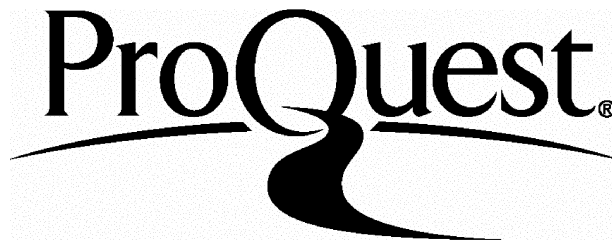
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ABSTRACT

The proliferating cells in the epidermis, stem cells and transit amplifying cells, can be separated to form enriched populations of each type by their ability to adhere to extra cellular matrix. Stem cells adhere rapidly to type IV collagen and give rise to large colonies in culture whereas slowly adhering transit amplifying cells form small colonies of terminally differentiated cells only.

I have developed and used a variety of lineage markers to follow individual cells and their progeny and study how different populations of cultured cells behave when at confluence, mimicking the epidermis in a steady state when the rate of cell renewal is balanced by that of differentiation.

Using various culture techniques I have looked at how the proliferating and differentiating cells of the epidermis may be organised *in vivo* and whether there is a relationship between the extracellular matrix of the dermis and the behaviour of the different types of epidermal cells in the steady state.

I have evidence from lineage analysis that clones derived from the putative stem cell population are large and long lived compared to clones founded by the transit amplifying population which are rapidly lost from reconstituted epidermis. I have also shown that the keratinocytes which contribute clones to a confluent cultured sheet, the stem cells, are likely to be a heterogenous population with variable fates.

By investigating the expression of adhesion molecules in the epidermis and of extracellular matrix in the basement membrane it is possible to suggest a model for the control of cell fate and stem cell longevity. I have evidence that variability of β_1 integrin expression in basal keratinocytes is accompanied by differences in cadherin expression and that the level of collagen IV varies in the basement membrane, potentially contributing to the definition of the stem cell niche.

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CHAPTER ONE

INTRODUCTION

The central theme of this thesis is to investigate stem cell fate in cultured human keratinocytes, the predominant cells in the epidermis, the outermost layer of the skin. The keratinocytes do not function in isolation and therefore the first part of the introduction is an overview of skin structure and function. The next section is a review of keratinocyte differentiation and includes more general reviews of some of the adhesion molecules on the cell surface which may be important in control of keratinocyte stem cell proliferation and differentiation. This is followed by sections on stem cells, including those in the epidermis, and on control of stem cell fate. The final part of the introduction looks at the methods and uses of lineage analysis in the investigation of cell fate.

1.1. The skin

The skin is not the largest organ in the body (Goldsmith 1990) as is often claimed. However it is indisputably the most apparent on casual glance, covering an area of 1.5 - 2.0 m² in an average adult, and most of its functions therefore involve interaction with the environment.

The skin acts as protection against physical, chemical and microbial assault, including ultra-violet irradiation, and as such is continuously exposed to injury. The major physiological functions include insulation, thermoregulation, fluid conservation, biosynthesis; for instance of vitamin D, excretion, immunological responses and, since the breast is a specialised adnexal organ, milk production. Also it is responsible for sensory perception of touch, pain, hot and cold. Last, but certainly not least, the whole cosmetics industry is based on the skin's aesthetic properties and sex appeal.

1.2. The structure of the skin

The structure and anatomy of the skin is well reviewed in a number of publications including (Goldsmith 1983; Rook *et al.* 1992; Weedon 1992). The skin consists of three distinct layers, the epidermis, dermis and subcutaneous fat, along with a number of adnexal

structures such as hair follicles, sweat glands and sebaceous glands plus a rich vascular and nerve supply, represented in Diagram 1.

1.2.i. Subcutaneous fat

Also called the *panniculus adiposus*, the deepest layer of the skin consists of lobules of fat cells and fibrous trabeculae containing vessels, nerves and lymphatics. It surrounds some of the deep adnexal structures, allows skin mobility, acts as a mechanical cushion, an insulator, and, like all adipose tissue, as a store of energy.

1.2.ii. The dermis

The supportive structure of the skin is provided by the dermis, composed of collagen, elastin and mucopolysaccharides within which are embedded cells, epidermal adnexae, vessels and nerve endings. The dermis is anatomically divided into two compartments, the papillary dermis and the reticular dermis.

The papillary dermis underlies the epidermis and surrounds the adnexae. It is composed of loosely arranged, fine collagen fibres, predominantly types III and I. The reticular dermis contains coarse, densely packed collagen, mostly type I, along with filamentous collagen, types V and VI.

The elastic network of the dermis consists of interconnecting layers of fibres including fibrillin, fibres of which run perpendicular to the basement membrane in the papillary dermis and connect the plexuses of elastin fibres in the papillary and reticular dermis.

The dermis also contains mucopolysaccharides, predominantly hyaluronic acid along with dermatan sulphate, chondroitin sulphate and heparan sulphate as well as water, salts and glycoproteins.

1.2.ii.a. Cells of the dermis

The cells of the dermis include fibroblasts, histiocytes, dermal dendrocytes, mast cells and lymphocytes.

Fibroblasts, probably along with keratinocytes (Compton *et al.* 1989; Regauer *et al.* 1990; Compton 1992) are responsible for the synthesis and repair of the dermis, producing collagen, elastin and laminin.

Histiocytes function as phagocytes, for antigen presentation and in the inflammatory response to insult or injury. Dendrocytes are also phagocytic and are probably bone marrow derived immune cells .

There are various subtypes of mast cell , all involved in release of chemical mediators of the inflammatory response, such as chemoattractants for other inflammatory cells and vasoactive mediators. The lymphocyte population consists mostly of T cells, which with the B cells , mediate the immune response.

1.2.ii.b. Dermal vessels

The blood vessels are arranged into two horizontal plexuses, at the junction of the dermis and subcutis and between the reticular and papillary dermis with arterioles and venules connecting the them. There is a capillary network in the papillary dermis which produces loops of capillaries in the dermal papillae. Both plexuses also contain lymphatic vessels.

The vessels supply nutrition and remove waste products to and from the skin and are also important in the inflammatory response and in thermoregulation.

1.2.ii.c. Nerves

The autonomic and sensory nerves of the skin also form a superficial and deep plexus, similar to the vessels. The autonomic nerves supply and control the smooth muscle of vessels and epidermal appendages and the sensory nerves transmit afferent impulses from receptors in the skin to the central nervous system.

The sensory receptors consist of free and encapsulated nerve endings. The free endings are mostly intraepidermal and sensitive to touch and pain. Some are associated with Merkel cells (see below) and thought to be mechanoreceptors. The encapsulated receptors are located in the dermis and are of at least 5 major types, the best known of which are Pacinian capsules (mechanoreceptors for vibration, pressure and tension) and Meissner's corpuscles (touch and tactile discrimination). Together the sensory nerves allow appreciation of touch, pressure, vibration, tension, spatial tactile discrimination, pain, temperature and itch as well as more complex sensations such as wetness.

1.2.iii. Basement membrane

The basement membrane is a thin sheet of specialised extracellular matrix at the dermal-epidermal junction, in direct contact with the basal cells of the epidermis. The membrane undulates over the dermal papillae and downward into the rete ridges, following the interlocking folds of the epidermis. Anchoring filaments, made of collagen VII (Burgeson 1993), extend from the membrane into the dermal collagen, along with dermal microfibrillary bundles. The main components (Timpl 1989) of the membrane itself include collagen IV, laminin (Tryggvasson 1993) (including epiligrin/kalinin/laminin 5 (Carter *et al.* 1991; Rousselle *et al.* 1991; Marinkovich *et al.* 1992; Marchisio *et al.* 1993), which is identical to nicein (Marinkovich *et al.* 1993)), heparan sulphate, proteoglycans and entactin/nidogen (Moscher *et al.* 1993). These components interact (Yurchenco and O'Rear 1994) and many are synthesised by, or synthesis is induced by, keratinocytes.

The basement membrane contains the extracellular matrix molecules in direct contact with the keratinocytes and functions to control epidermal cell growth, differentiation and polarity as well as to provide attachment for the epidermis (Adams and Watt 1993)

The membrane can be divided into three zones by electron microscopy - the lamina lucida (adjacent to the epidermis), the lamina densa and the lamina reticularis (adjacent to the papillary dermis).

1.2.iv. The epidermis

The epidermis is a cornified, stratified squamous epithelial sheet in which the predominant cell is the keratinocyte but contains other cell types, namely Merkel cells, Langerhan's cells and melanocytes. It is the epidermis that forms the barrier, and is the interactive layer, between the body and the environment.

1.2.iv.a. Keratinocytes

Keratinocytes form the major cell population in the epidermis and are arranged in layers above the basement membrane so that the rete ridges are perforated by upward, finger-like projections of the dermis, the dermal papillae (Diagram 1).

The layers of keratinocytes are divided morphologically into a basal layer, where most of the proliferating cells are situated, a spinous layer, a granular layer and an outermost cornified layer (*stratum corneum*) containing terminally differentiated, anucleate cells.

The basal layer comprises a heterogeneous population of proliferating and post-mitotic keratinocytes (Withers 1967; Potten and Hendry 1973; Barrandon and Green 1985; Jensen *et al.* 1985; Morris *et al.* 1985; Albers *et al.* 1986; Watt 1988) and supplies the cells for the other, more superficial layers. The keratinocytes of the basal layer are discussed further in the section on epidermal stem cells.

Cells migrate upwards from the basal layer during which time they undergo a process of terminal differentiation (Sun and Green 1976; Watt 1988) and are eventually shed from the surface of the epidermis. As keratinocytes migrate up through the epidermis they become flatter and enlarge. The spinous layer is so called because of the prominent intercellular connections, such as desmosomes, giving the appearance of spines around each cell. As cells move further up they produce cytoplasmic, basophilic keratohyaline granules, to form the granular layer. This layer is thought to be the main barrier to water soluble substances and contains large amounts of intercellular lipid. The cells leaving the granular layer undergo cell death and become flat anucleate corneocytes with no cytoplasmic organelles, possibly by an apoptotic process (Polokowska *et al.* 1994). The cells are filled with keratin filaments surrounded by an insoluble protein (cornified) envelope apposed to the inner plasma membrane. The corneocytes have a protective and barrier function for the underlying living layers.

The maintenance of the epidermis in a steady state requires a delicate balance between proliferation in the basal layer and cell loss from the stratum corneum (Watt 1988; Dover and Wright 1991). Cell loss is an active process and requires the enzymatic breakdown of lipids, such as cholesterol esters, in the intercellular space, and each cell needs replacement by cells reproduced by division in the basal layer.

1.2.iv.b. Merkel cells

Merkel cells contain loosely packed keratin and neurosecretory granules. They are associated with nerve endings (Pinkus corpuscles) and possibly act as slow adapting

mechanoreceptors. They have desmosomes and may be derived from the epidermis (Compton *et al.* 1990; Narisawa *et al.* 1993).

1.2.iv.c. Langerhan's cells.

Langerhan's cells are dendritic cells which interdigitate among keratinocytes and represent between 2 and 4 per cent of the total number of cells in the epidermis. The cell originates in the bone marrow, possesses receptors for IgG and C₃, is required for antigen processing and is possibly of the monocyte/macrophage lineage.

1.2.iv.d. Melanocytes

These are dendritic cells responsible for the production of pigment in the epidermis and hair. They are derived from the neural crest and migrate to the epidermis during development. Pigment-containing melanosomes (eumelanin which is brown/black and phaeomelanin which is yellow/red) are distributed to the keratinocytes via the dendritic processes where they are arranged perinuclearly and absorb ultra-violet light.

1.2.v. Epidermal adnexa

The adnexal structures of the skin are all derived from primitive epidermis and extend down into the reticular dermis.

1.2.v.a. Pilosebaceous unit

1.2.v.a.i Hair

The hair shaft grows from invaginations of epidermis, the hair follicles. The inferior part of the follicle is dilated into a bulb of epidermis and produces dead, anucleate, keratinised cells which form the hair shaft. The bulb encloses a connective tissue papilla, consisting of collagen, fibroblasts and a capillary loop, which is essential for hair growth. The shaft is surrounded by cylindrical structures, the inner and outer root sheaths. The outer root sheath is continuous with the overlying epidermis. The mid portion of the follicle contains an expansion, the bulge, which may contain the follicular stem cells (Cotsarelis *et al.* 1990). In the same region is the insertion of the *arrector pili* muscle which is anchored in the dermis.

Human hair grows in asynchronous cycles divided into phases of anagen (active phase, 2-6 years), catagen (regressing/involutory phase, 2-4 weeks) and telogen (resting phase, 2-4 months).

1.2.v.a.II Sebaceous glands

These glands develop from the mid-portion of the primitive follicle. The multilobular gland secretes lipid rich sebum into the follicle. The glands increase in size at puberty under androgenic stimulation but sebum function is unknown and is variously postulated to be emollient, bacteriostatic, pheromonal or for insulation.

1.2.v.b. Apocrine glands

Apocrine glands also develop from the primitive hair follicle, above the sebaceous gland, and are present in the axilla, groin, eyelids and external ear. The secretions are protein rich and release is controlled by the sympathetic nervous system. The function is unknown. The breast is a highly modified apocrine structure.

1.2.v.c. Eccrine glands

Eccrine glands are coiled, tubular structures in the deep dermis which produce a watery secretion important in thermoregulation, to dissipate body heat. They arise as separate appendages from the primitive epidermis and are also under control of the sympathetic nervous system.

1.2.v.d. Nails

The nail plate consists of hard keratin produced from differentiated cells of the nail matrix, the pale area at the proximal end of the nail. Proliferation of the matrix cells causes growth pressure which pushes the nail outward. The nail plate rests on the nail bed which produces differentiated epidermal cells that adhere tightly to the overlying plate.

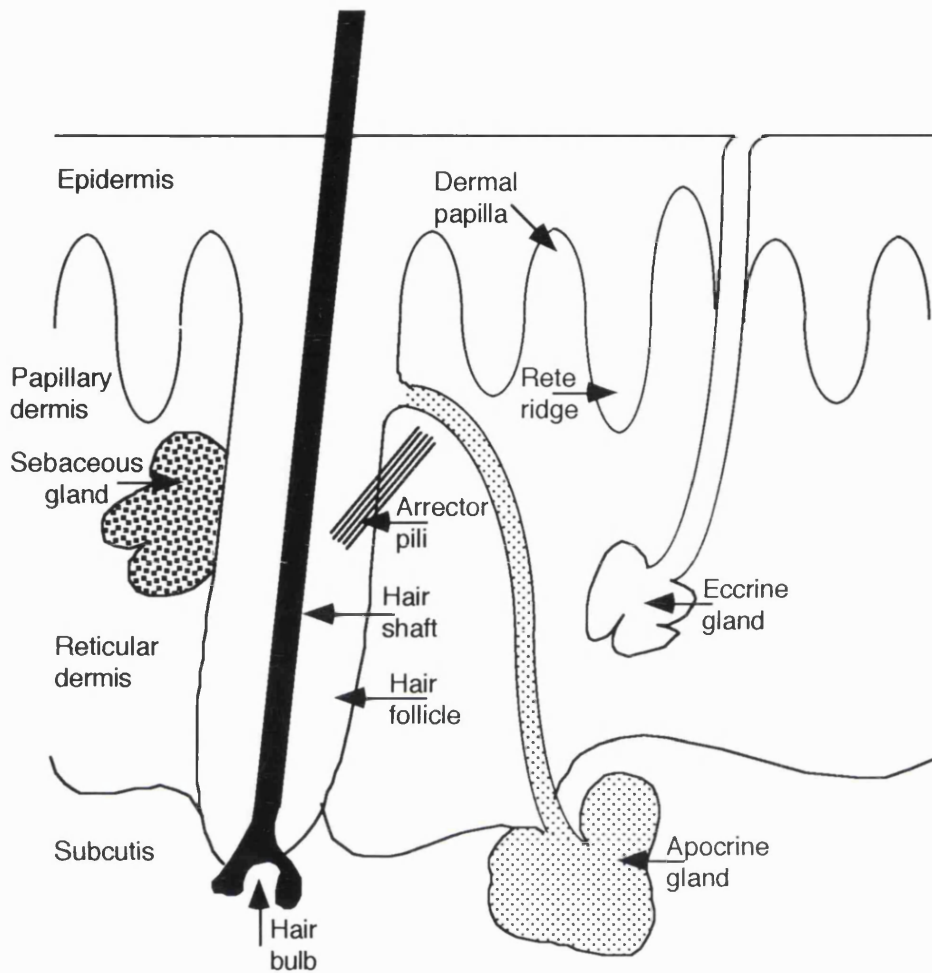


Diagram 1 : Representation of the anatomy of the skin.

1.3. The embryology of the skin

At 2 weeks gestation the embryo is covered by an ectodermally derived single layer of cuboidal cells which acquires a second layer by 4 weeks, the periderm. The periderm is transient and sloughs off when the underlying epidermis matures. An intermediate layer appears at 8 weeks, which increases in thickness and cell number until the periderm is lost at 24 weeks and the granular layer and stratum corneum form.

The pilosebaceous follicles appear as extensions of the epidermis into the dermis between the 3rd and 4th month and the sebaceous and apocrine primordia develop from the follicle in the 5th month. The sebaceous glands develop rapidly in the foetus, and are greatly enlarged compared to those of a child, but involute after the neonatorum to remain quiescent

until puberty. Apocrine development follows that of the sebaceous gland, around the 6th month, and the glands secrete during the last trimester but cease to function in the neonate.

Ecocrine glands develop around the 3rd month as downward buds from the epidermis and are differentiated by the 6th month.

The dermis arises from the somite mesoderm and the fibrous content of the mesenchymal dermis slowly increases, until a recognisable papillary and reticular division is apparent at 5 months, but does not fully mature until the 2nd postnatal year. The dermal papillae begin to develop during the 6th month following the formation of the secondary epidermal ridges in month 5.

1.4. Keratinocyte differentiation

The term "differentiation" of keratinocytes can have different meanings, for instance when referring to development, or morphology, such as the grading of malignant tumours, but when considering the individual cell the term refers to the process of terminal differentiation which the keratinocyte undergoes when a cell in the basal layer loses the capacity for unlimited self renewal and eventually becomes a cornified squame.

There are a number of markers of keratinocyte terminal differentiation and these are reviewed by both Fiona Watt and Elaine Fuchs (Watt 1989; Fuchs 1990).

1.4.i. Keratins

There are many different keratins, divided into two subfamilies, acidic and basic. They form intracytoplasmic filaments, for which one member of each subfamily is required. They have "preferred" pairings and all stratified epithelia express K5 and K14 (Purkis *et al.* 1990) and during terminal differentiation the epidermis expresses K1 and K10, seen in suprabasal keratinocytes. The epidermis also expresses K19 in small amounts, K9 in the palm and sole and the pair K6 and K16 in hyperproliferative epidermis, including in culture (Weiss *et al.* 1984; Kopan and Fuchs 1989).

1.4.ii. The cornified envelope and its precursors

Cells in the stratum corneum are surrounded by the insoluble cornified envelope (Sun and Green 1976) but protein precursors are present in the cytoplasm of cells in the spinous layer onwards. These proteins include involucrin, the most abundant and best characterised (Rice and Green 1979; Watt and Green 1981; 1983) and loricrin (Hohl *et al.* 1991). All of the genes encoding these proteins contain common tandem repeats. In human involucrin this represents around two thirds of the coding region and is the most modern part of the gene in terms of evolution (Watt 1983; Tseng and Green 1988). In involucrin, the amino acid repeats contain conserved glutamine residues and assembly of the envelope is by cross linking of involucrin catalysed by a transglutaminase (Thacher and Rice 1985).

Expression of involucrin is in the larger, differentiated cells (Watt and Green 1981) corresponding to all suprabasal cells in keratinocyte culture and the upper spinous layer and above *in vivo*.

1.4.iii. Filaggrin

Filaggrin (Dale *et al.* 1985) is a histidine rich basic protein, the inactive precursor of which (profilaggrin), along with loricrin, is a component of keratohyaline granules in the granular layer. Filaggrin is involved in the aggregation of keratin filaments in the stratum corneum.

1.4.iv. Peanut lectin binding glycoproteins, CD44

A lectin, peanut agglutinin (PNA), binds to suprabasal keratinocytes (Watt 1983) via the terminal galactose residue of CD44 (Hudson and Watt 1994) which is masked by sialic acid in basal cells (Keeble and Watt 1990; Watt and Jones 1992). This property allows the lectin to be used as a surface marker of differentiating keratinocytes.

1.4.v. Lipids

The lipid composition of the epidermis varies in the different layers. During terminal differentiation there is a decrease in phospholipids and an increase in sphingolipids, neutral lipids and cholesterol sulphate (Jetten *et al.* 1989). Intercellular lipid is secreted via structures

called lamellar bodies synthesised by the spinous cells and which fuse with the plasma membrane in the granular layer to release their lipid contents. These are important in the barrier function of the epidermis and are covalently bound to the cornified envelope (Wertz *et al.* 1989).

1.4.vi. Actin associated proteins

In common with other cells, keratinocytes change shape during differentiation (Watt 1986; Watt 1987), which is associated with alterations in the organisation of the actin cytoskeleton and associated proteins. Terminally differentiating keratinocytes have reduced levels of gelsolin, filamin, vinculin and talin while the level of actin remains unchanged (Kubler *et al.* 1991) with variation of the distribution of these proteins in suprabasal compared to basal keratinocytes.

1.4.vii. Integrins

It has recently become apparent that a family of cell surface adhesion molecules, the integrins (Albelda and Buck 1990), are important in regulating cell behaviour within many tissues, including skin (Watt 1986; Watt 1987; Adams and Watt 1989; Adams and Watt 1990; Marchisio *et al.* 1990; Nicholson and Watt 1991; Adams and Watt 1993; Hotchin *et al.* 1993; Jones *et al.* 1993; Jones and Watt 1993; Wang *et al.* 1993; Watt and Jones 1993; Watt *et al.* 1993; Hotchin *et al.* 1994) In cultured keratinocytes, integrins are localised to concentrations of molecules on the cell surface known as focal contacts, where they are involved in adhesion to ECM molecules and in cell spreading and migration.

The integrins are a superfamily of cell surface, heterodimeric, transmembrane glycoproteins which function as cellular receptors for ECM and molecules on other cells and reviewed by Hynes (1992). The heterodimer consists of one α subunit and one β subunit which are non-covalently linked. There are now more than 8 β subunits and 14 α subunits known and in general most α subunits form heterodimers with one or two β subunits, although some can form dimers with one of a number of β subunits. For instance α_v , α_1 , α_2 , α_3 and α_5 interact with β_1 only and form part of the β_1 family of integrins, also known as the VLA (Very Late Activation) proteins. Some integrins may have multiple forms, due to

alternative splicing, for instance there are 2 known variants of the β_1 subunit which have different cytoplasmic tails and one of which may be unable to reach the cell surface. Multiple forms of α_2 can be derived from one cDNA clone (Chan and Hemler 1993) and the α_6 subunit has two variants (Hogervorst *et al.* 1991; Hogervorst *et al.* 1993).

1.4.vii.a. Integrin-ligand binding

The ligand binding region involves the α and the β subunits. The association between the subunits is dependent on divalent cations and is probably mediated by the extracellular domains. The extracellular domain of the α subunit contains multiple cation binding sites, and of the β subunit contains four cysteine rich repeat sequences. Both subunits contain numerous disulphide bonds. Most subunits have a transmembrane domain and a short cytoplasmic tail, although the β_4 subunit cytoplasmic tail is 1000 amino acids, longer than the others.

Different integrins recognise peptide sequences in a wide variety of ligands. The sequence Arg-Gly-Asp (RGD) is recognised, for example, by $\alpha_5\beta_1$, $\alpha_v\beta_1$ and $\alpha_{IIb}\beta_3$ and is present in fibronectin and vitronectin (Felding-Habermann and Cherish 1993). Collagen type I contains Asp-Gly-Glu-Ala (DGEA) which is recognised by $\alpha_2\beta_1$. Some integrins recognise more than one sequence, for instance fibrinogen is also a ligand for $\alpha_{IIb}\beta_3$ (Hynes 1992) and this receptor recognises various fibronectin binding sites (Bowditch *et al.* 1991). Ligands such as laminin and fibronectin have various different binding sites (Timpl 1989; Tryggvasson 1993; Bowditch *et al.* 1994; Karecla *et al.* 1994) recognised by specific integrins. Some ligands must have more than one receptor since, for instance, α_5 knockout mice develop further than fibronectin knockouts, implying that $\alpha_5\beta_1$ is not the only receptor (Yang *et al.* 1993) and alternatively spliced fibronectin is recognised by $\alpha_4\beta_1$ (Wayner 1989; Guan and Hynes 1990).

The specificity of ligand - integrin interaction, reviewed by Humphries (1990) and Haas and Plow (1994), also depends on the type of cell expressing the integrin, as well as the subunits expressed.

Some integrins bind to other cell surface molecules rather than ECM. These ligands are members of the immunoglobulin superfamily and include ICAM 1, 2 and 3 and VCAM 1.

These interactions occur on the surface of cells of the immune system to allow activation, antigen presentation, proliferation and interaction with vascular endothelium. Integrins may also be involved in homotypic cell-cell adhesion, for instance $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in keratinocytes (Carter *et al.* 1990; Larjava *et al.* 1990; Sriramarao *et al.* 1993; Symington *et al.* 1993).

There may be competitive binding between ligands for a particular receptor, for example between fibronectin and V-CAM 1 for $\alpha_4\beta_1$ (Makarem *et al.* 1994).

1.4.vii.b. Integrin structure and function

The β cytoplasmic domains interact with the actin cytoskeleton via a number of cytoplasmic proteins with which there is co-localisation namely α -actinin, vinculin, talin, tensin, focal adhesion kinase and paxillin (Otey *et al.* 1990; Burridge *et al.* 1992; Zachary and Rozengurt 1992; Bockholt 1993; Otey *et al.* 1993; Johnson and Craig 1995) and are necessary for ligand binding (Marcantonio *et al.* 1990) and recruitment to focal contacts (Solowska *et al.* 1991; Ylanne *et al.* 1993). The α subunit cytoplasmic tail is permissive and regulatory of particular functions (O'Toole *et al.* 1991; O'Toole *et al.* 1994) and function of the cytoplasmic domains is important for signalling (Sastry and Horwitz 1993).

The cation binding sites are also important for function. Binding at this site results in conformational change and mutations in the cation binding site abolish ligand binding (Loftus *et al.* 1990).

Integrins exist in different affinity states and high affinity binding appears to require a particular conformational change (Neugebauer and Reichardt 1991; Faull *et al.* 1993; Faull *et al.* 1994)

1.4.vii.c. Integrins and signal transduction

It is now clear that integrins are important as signal transducers as well as adhesion molecules and are involved in "outside-in" (Burridge *et al.* 1992; Hynes 1992; Adams and Watt 1993; Bockholt 1993; Juliano and Haskill 1993; Sastry and Horwitz 1993; Schaller and Parsons 1994; Shattil *et al.* 1994) and "inside-out" (Sastry and Horwitz 1993; Faull *et al.* 1994; O'Toole *et al.* 1994) signalling events. Various intracellular events are associated with occupation of the integrin by ligand, including activation of the sodium proton antiporter with

rise of intracellular pH, raised intracellular calcium, tyrosine kinase phosphorylation and phosphorylation of focal adhesion kinase (Zachary and Rozengurt 1992; Schaller and Parsons 1994) Occupation of different receptors leads to different signalling events.

Events inside the cell can influence the binding affinity of the integrin and so influence cell behaviour ("inside-out signalling"). Mutation in the cytoplasmic tail can induce high binding affinity (O'Toole *et al.* 1994) and tyrosine or serine phosphorylation of the β_1 cytoplasmic tail can may inactivate the integrin. In keratinocytes the recruitment to focal contacts is blocked by antibodies, not only to the β_1 cytoplasmic tail but also by those to molecules involved in cell-cell adhesion such as cadherins and catenins (Hodivala and Watt 1994).

1.4.vii.d. Keratinocyte integrins

In keratinocytes, the integrins function to allow adhesion to the extracellular matrix of the basement membrane. The predominant integrins are receptors for collagen and laminin-1 ($\alpha_2\beta_1$), laminin-1 and laminin-5 (kalinin/epiligrin) ($\alpha_3\beta_1$), fibronectin ($\alpha_5\beta_1$) and vitronectin ($\alpha_v\beta_5$) (Carter *et al.* 1990; Adams and Watt 1991; Carter *et al.* 1991; Sonnenberg 1993; Watt and Hertle 1993; Watt and Jones 1993) plus a component of hemidesmosomes, $\alpha_6\beta_4$, which is a receptor for laminin-1 (Lee *et al.* 1992) and possibly laminin-5 (Nieson *et al.* 1994). $\alpha_3\beta_1$ may adhere only to laminin-5 and not to laminin-1 (Delwel *et al.* 1994).

The integrins are expressed only in the basal layer of the epidermis and play a role in stratification (Adams and Watt 1990; Hotchin and Watt 1992) and in the regulation of terminal differentiation. Terminally differentiating cells functionally down-regulate the integrins, so reduce ligand binding and as a result lose adhesiveness to the basement membrane (Watt and Green 1982; Watt 1984; Hotchin *et al.* 1993) enabling them to migrate upwards. The reduction of binding to fibronectin precedes the loss of integrin from the cell surface (Adams and Watt 1990; Nicholson and Watt 1991). The loss from the cell surface is due to decreased transcription and reduced maturation and transport of the protein (Hotchin and Watt 1992; Hotchin *et al.* 1994) regulation of which is via glycosylation. Expression of the differentiation marker, involucrin, by cells in suspension can be partially blocked by fibronectin (Adams and Watt 1989) and also a combination of fibronectin, collagen IV, laminin and antibodies against

integrins (Watt *et al.* 1993). Keratinocyte stem cells have higher levels of functional integrins than other basal keratinocytes (Jones and Watt 1993) and this is discussed in the section on stem cells.

Keratinocyte integrins are expressed at specific times during human embryogenesis and may be involved in regulation of skin development. Expression is also in particular sites and is related to sweat gland development and stratification (Hertle *et al.* 1991).

1.4.vii.e. Keratinocyte integrin expression in disease

Integrin expression is altered during wound healing (Grinnell 1992; Hertle *et al.* 1992; Gailit and Clark 1994), with increased expression of $\alpha_5\beta_1$, in response to exposed fibronectin, and with suprabasal expression of α_2 , α_3 , α_6 and β_1 (Hertle *et al.* 1992; Juhasz *et al.* 1993) in differentiating cells. The pattern of expression returns to normal two weeks after healing. Integrin expression alters during other hyperproliferative situations, including normal oral mucosa, psoriasis, eczema and lichen planus (Kellner *et al.* 1991; Ralfkiaer *et al.* 1991; Hertle *et al.* 1992; Pellegrini *et al.* 1992). In such situations the integrins are co-expressed with differentiation markers such as involucrin and such cells are also seen in Rheinwald Green keratinocyte cultures. The mechanism of suprabasal expression is not clear (Hertle *et al.* 1995), although protein synthesis is probably confined to the basal layer since *in situ* hybridisation shows mRNA at this site only. It is possible that the process of terminal differentiation is speeded up and integrin is left on the cell surface. It is not known if the suprabasal integrins are functional or not.

The expression can be radically altered in many tumours (Albelda 1993; Juliano and Varner 1993; Pignatelli and Vessey 1994), including those of keratinocytes. Reduced expression tends to be associated with altered phenotype, a poorly differentiated morphology (Jones *et al.* 1993; Sugiyama *et al.* 1993) and certain types of tumour show characteristic differences between them, for instance β_1 expression in basal cell carcinoma is high and in squamous cell carcinomas tends to be low (Peltonen *et al.* 1989). Loss of integrin may coincide with loss of ligand, for instance some oral squamous cell carcinomas lack $\alpha_6\beta_4$ focally and there is loss of collagen IV and laminin in the same areas (Downer *et al.* 1993). Expression may also be altered in potentially pre-malignant lesions such as human papilloma

virus infection of the uterine cervix and cervical intraepithelial neoplasia (CIN) (Hodivala *et al.* 1994).

1.4.viii. Cadherins

The cadherins are surface transmembrane proteins responsible for cell-cell adhesion (Takeichi 1990; Geiger and Ayalon 1992; Grunwald 1993), and are concentrated at cell junctions, including desmosomes and adherens junctions. Binding of cadherin molecules is homophilic, calcium dependent and there is indirect cytoplasmic association with intermediate filaments and the actin cytoskeleton.

The classical cadherins comprise E cadherin (uvomorulin), P cadherin and N cadherin and each has a unique pattern of expression. They each have a large extracellular domain with 4 internal repeats and 6 calcium binding sites plus a transmembrane domain and a short cytoplasmic tail. A single amino acid substitution in one calcium binding site can abolish E cadherin function (Ozawa *et al.* 1990).

1.4.viii.a. Cadherin structure and function

There is a histidine alanine valine (HAV) motif in the extracellular domain which is the recognition sequence for homophilic binding. The cytoplasmic tail binds to a group of proteins called catenins (Ozawa and Kemler 1992; Piepenhagen and Nelson 1993), which co-localise by immunoprecipitation with cadherins, and are able to regulate cadherin function (Hirano *et al.* 1992). They comprise α -catenin (a vinculin homologue (Herrenknecht *et al.* 1991)), β -catenin and γ -catenin (plakoglobin) (Knudson and Wheelock 1992). The complexes are linked to the cytoskeleton via α -catenin and different cadherin-catenin complexes exist, possibly at different sites on the cell (Hinck *et al.* 1994a; Nathke *et al.* 1994).

Cadherins are thought to be vital to the maintenance of the structural integrity of tissues and possibly to be involved in selective cell adhesion and cell sorting during morphogenesis (Nose *et al.* 1988; Takeichi 1991; Wheelock and Jensen 1992). Cells with a high density of cadherin will preferentially bind each other over cells with low density, forming spheres in suspension, a possible mechanism in organ formation (Steinberg and Takeichi 1994). Cadherins are also important for the polarisation of epithelial cells (Marrs 1993)

1.4.viii.b. Keratinocyte cadherins

Keratinocytes express P cadherin (basal layer) and E cadherin (all layers) (Nicholson *et al.* 1991). E cadherin is located in adherens junctions and is redistributed to cell-cell borders when cultured keratinocytes are switched from low to normal calcium concentrations, causing stratification. Addition of anti-E cadherin antibody prevents redistribution and stratification suggesting a role in epithelial morphogenesis (Wheelock and Jensen 1992; Hoidalva and Watt 1994).

There are a number of cysteine rich desmosomal cadherins (desmosomal glycoproteins, DG), reviewed by Arneman (1993) and Koch and Franke (1994), distributed in different patterns in the epidermis, some in the superficial layers (DG IV and V) and some more basal (DG II and III) or throughout (DG I), suggesting variable functions. Cadherin function is required for assembly of desmosomes and adherens junctions.

1.4.viii.c. Cadherins in epithelial neoplasia

Cadherins and catenins are thought to modulate tumour behaviour, in particular invasion and metastasis (Takeichi 1993). Reduction in E cadherin is associated with increased invasive potential and *vice versa* (Vleminckx *et al.* 1991) and a similar effect occurs with loss of α -catenin (Shiozaki *et al.* 1994). Such effects have been shown in carcinoma of the lung (squamous cell) (Bohm *et al.* 1994), breast (Oka *et al.* 1993), oesophagus (Doki *et al.* 1993), stomach (Mayer *et al.* 1993), bowel (Kinsella *et al.* 1993), bladder (Otto *et al.* 1994) and the head and neck (Schipper *et al.* 1991). *v-src* transformed cells show tyrosine phosphorylation of β -catenin, with loss of N cadherin function, correlated with loss of epithelial differentiation and increased invasiveness (Behrens *et al.* 1993).

1.5. Stem cells

Stem cells are most simply defined as those cells with both a high capacity for self renewal throughout life and the potential to produce differentiated progeny (Lajtha 1979) and therefore the capacity for asymmetric cell division. Stem cells may also be pluripotent, although this is probably not the case in adult epidermis, for example.

Stem cells in the developing embryo and those in the adult have different functions. Embryonic stem cells may give rise to new differentiated cell types, however these progenitor cell populations may not be self renewing, although they do have the capacity for asymmetrical cell division and for differentiation. Cell division in the adult differs in that it is usually required to maintain the number of differentiated cells at a constant level (in a steady state where cell loss is balanced by cell proliferation), replacing dead or lost cells, rather than to increase the number and type of cells as in the embryo. Adult tissues with a permanently renewing cell population (Leblond 1963), such as blood, epithelia and testis, contain cells with the ability for self renewal, asymmetric cell division and the production of differentiated daughters in which the process of differentiation is irreversible.

In the haematopoietic system and the intestinal epithelium the stem cells are pluripotent and in the epidermis there seems to be one pathway of differentiation (Diagram 2). In these systems the differentiated cells arise from stem cell daughters called transit amplifying cells or committed progenitors which have a limited capacity for self renewal. This amplifies the effect of a single stem cell division to produce relatively large numbers of terminally differentiated cells (Lajtha 1979).

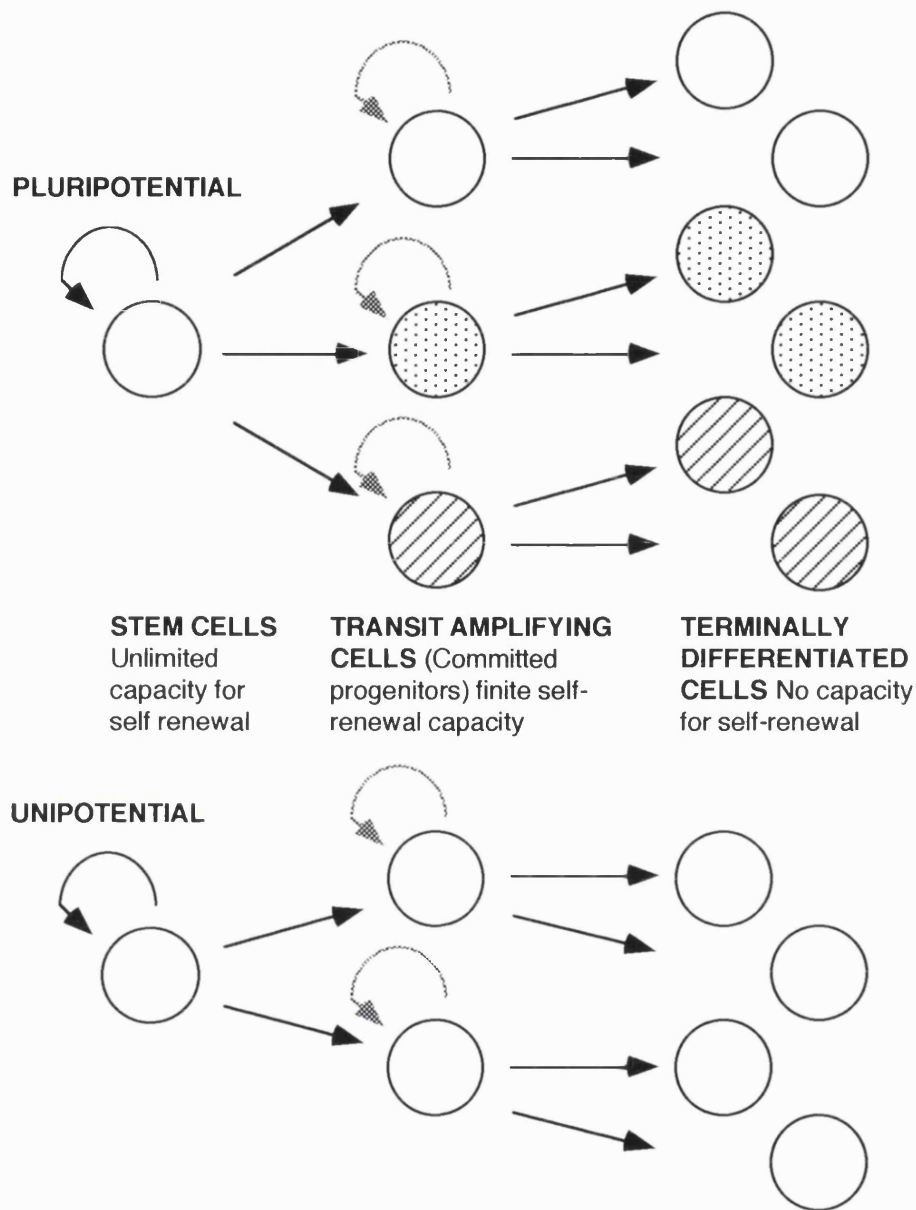


Diagram 2 : Models of stem cell division and amplification by committed progenitors. (Adapted from Hall and Watt 1989)

1.5.i. Epidermal stem cells

There is ample evidence that the proliferating population in the basal layer is heterogeneous. After radiation damage to mouse epidermis, only about 10% of the basal keratinocytes are able to form foci of regenerating epidermis (Withers 1967; Potten and Hendry 1973), the possible stem cells, whilst another 50% of the total number of basal cells can divide but cannot repopulate the epidermis (Potten 1974; Potten 1981), the transit amplifying cells, and 40% are post mitotic. Broadly similar results are seen in keratinocyte culture (Barrandon and Green 1987b).

Kinetic experiments looking at the incorporation and retention of tritiated thymidine in mouse epidermis, as well as work with keratinocytes *in vitro*, suggest that stem cells cycle more slowly than the transit amplifying population in situations other than hyperproliferative (culture, wound repair) since they retain tritiated thymidine for longer (Jensen *et al.* 1985; Albers *et al.* 1986). Cells whose tritiated thymidine labelled DNA retains the label for longer are those which have divided fewer times (Potten and Morris 1988) and these cells remain untritiated in pulse labelling experiments. They can also resist differentiation in culture due to TPA or suspension (Parkinson *et al.* 1983; Morris *et al.* 1985; Adams and Watt 1989).

Until recently there were no markers of stem cells or transit amplifying cells in the epidermis but there is evidence, some contradictory, as to where they are found *in vivo*. In some stratified epithelia evidence suggests that the stem cells occupy a specific location, for instance in the limbus of the cornea (Cotsarelis *et al.* 1989), or the deep invaginations of epithelium in the mouse tongue papillae (Hume and Potten 1976). The hair follicle is capable of reconstituting the epidermis and is therefore a source of stem cells (Lenoir *et al.* 1988) (also see below). In monkey palm epidermis the slowly cycling cells are in the troughs of the rete ridges (Lavker and Sun 1983).

In the mouse, in areas such as the dorsum, the epidermis is arranged in stacked columns (Mackenzie 1969; Christophers 1971) and it has been suggested that each represents an epidermal proliferative unit (EPU) comprising a stem cell surrounded by 5-6 transit amplifying cells and up to 4 post mitotic cells plus the suprabasal cells. The slowly cycling, tritiated thymidine retaining, cells are located toward the centre of each unit (Morris *et al.* 1985; Potten and Morris 1988). However in most instances the epidermis is not arranged in stacks and the pattern of mosaicism in chimeric mice may not be consistent with this model and suggests that the units may be polyclonal (Schmidt *et al.* 1987).

Patchy patterns of integrin expression (see below) suggest it is possible that the location of the stem cells may vary according to site in human epidermis, located at the base of the rete ridges in the palm and sole but at the top of the dermal papillae in the scalp and foreskin (see Jones and Watt (1995) and Chapter 5).

It has been suggested that Merkel cells may represent another differentiation pathway of a possible multipotent keratinocyte stem cell (Compton *et al.* 1990; Narisawa *et al.* 1993) in the same way as seems to occur with neuroepithelial cells in the intestinal epithelium (see below). Merkel cell tumours can be seen arising from areas of in-situ squamous cell carcinoma of the skin (Weedon 1992) which implies a close relationship in the pathways of differentiation.

1.5.i.a. Epidermal stem cells in culture

Human keratinocytes can be grown in culture under conditions in which they have many similarities to *in vivo* epidermis (Rheinwald and Green 1975). The cultured cells are able to form stratified sheets and express markers of terminal differentiation and therefore provide a model to investigate the growth and regulation of human epidermis. The conditions for culture are described in detail in Chapter 2. In summary a suspension of single cells is isolated from human epidermis, often neonatal foreskin, and cultured with a feeder layer of irradiated mouse 3T3 fibroblasts, which condition the culture medium also supplemented with hydrocortisone and cholera toxin, both of which stimulate proliferation (Rheinwald and Green 1975; Green 1977), epidermal growth factor which also increases the number of cell generations before senescence and stimulates outward migration of the proliferating cells at the edge of the colonies (Rheinwald and Green 1977) and insulin.

Stem cells must be retained in culture because cultured sheets of keratinocytes persist for many years when grafted onto burn patients (Compton *et al.* 1989; Compton 1992; 1993). Furthermore keratinocytes can be passaged at least 20 times before senescence and hair follicle stem cells are estimated to have the potential to produce up to 1.7×10^{38} progeny (Rochat *et al.* 1994). Barrandon (1987b) identified three types of colony forming cell in keratinocyte culture. Some form large colonies, with high growth potential, able to renew through many passages (holoclones) and others form small, abortive colonies containing only differentiated cells (paraclones). These are thought to correspond to stem cells and transit amplifying cells respectively. Some cells founded colonies intermediate between the two (meroclones). The founding cell type of meroclones is difficult to classify and may represent a stem cell which generates transit amplifying cells at a higher frequency than holoclone

founders. Subcloning from the parental colonies indicates that transitions from high to low growth potential occur but that the converse does not, as predicted by the stem cell model; holoclones are capable of producing more holoclones plus the other colony types but passaging of paraclones produces only terminally differentiated cells.

In contrast to haematopoietic tissue (see below), until recently there were no markers for the different sub populations of keratinocytes and it was only possible to identify stem cells in retrospect, according to their progeny. However Jones and Watt (1993) have shown that cultured keratinocytes expressing the highest levels of integrin, and with the ability to adhere rapidly to extracellular matrix molecules are most likely to contain the stem cell population. Cells sorted for high integrin levels by fluorescence activated cell sorting (FACS) have up to a four fold higher colony forming efficiency (CFE) than cells with low integrin levels on the cell surface. Cells adhering rapidly to collagen IV coated dishes had up to 5.5 times the CFE of unselected controls. Cells which stuck slowly to collagen IV (1-3 hours) formed mainly abortive colonies, and therefore represent the transit amplifying population, as predicted by Potten (Potten and Hendry 1973; Potten 1974; Potten 1981; Potten and Morris 1988). There is a linear relationship between log. integrin fluorescence and CFE, implying that rather than discrete cell subpopulations of the EPU model, there may be a continuum of proliferative and differentiative behaviour. In these experiments the sole criterion of colony formation was the ability to produce a clone containing more than 32 cells after 2 weeks in culture. There was a considerable variation in size and morphology of the clones and the high integrin expressing population probably included both holoclones and meroclones described by Barrandon and Green (1987b).

1.5.i.b. Location of epidermal stem cells

The same relationship between adherence and proliferative capacity applies to primary isolates of keratinocytes from foreskin. Using confocal microscopy it is possible to show that cells which express high integrin levels are located in patches, in specific positions of the epidermis, depending on body site, either in the depth of the rete ridges (palm) or the tips of the dermal papillae (foreskin, scalp) and that these sites correspond to the areas containing the fewest S-phase cells (Jones *et al.* 1995). The location of stem cells at the tips

of the dermal papillae is contrary to the usual dogma, Lavker (1983) showed that palm stem cells may lie in the troughs of the rete ridges and certainly observations such as that the highest concentration of melanin in keratinocytes is in the depths of the rete whatever the body site (personal observation) and of the shape and size of keratinocytes suggest the location may be in the rete ridges.

Patches of cells expressing high levels of integrin are also seen in confluent cultures and the size of patches is constant despite seeding density (Jones *et al.* 1995) This suggests that the patterning of proliferating cells in the basal layer is also an inherent property of the keratinocytes.

The location of stem cells in hair follicles has also been investigated. Microdissection of rat vibrissae (Kobayashi *et al.* 1993) suggests that the colony forming cells reside in the region containing the bulge and label retaining, slowly cycling, cells have been shown in the bulge of mouse follicles (Cotsarelis *et al.* 1990). However microdissection of human hair follicles followed by clonal analysis suggests that the colony forming cells are deep to the bulge, concentrated at the follicle midpoint (Rochat *et al.* 1994).

1.5.ii. Stem cells in other epithelia

The most extensively studied system is the gastrointestinal epithelium, in particular the small intestine. The lining of the small intestine is an epithelial monolayer arranged as crypts and villi. Cell proliferation is in the crypts from where the cells migrate and differentiate to be shed into the intestinal lumen from the tips of the villi (Potten and Loeffler 1990). As the cells migrate they cease dividing and differentiate into four main cell types: goblet, columnar and entero-endocrine cells on the villi plus Paneth cells which are in the base of the crypts and are probably lost by phagocytosis rather than shedding.

The four cell types seem to arise from a common progenitor stem cell. Radiation induced cell death results in phagocytosis of all cell debris by surviving cells and phagosomes are rapidly found in all the differentiated cell types (Cheng and Leblond 1974) and all cell types are found in adenomas and carcinomas, which are clonal in origin (Kirkland 1988; Ho *et al.* 1989). Each crypt contains a monoclonal population of cells derived from a single parent stem cell. Evidence for this comes mostly from chimeric mice or mice heterozygous for a

histochemical marker, along with experiments chemically inducing somatic mutations into the intestinal epithelium and transgenic mice, reviewed by Gordon (Gordon 1989; Gordon *et al.* 1992) (and see section on lineage analysis). Crypts always contain a single clonal population, either positive or negative for the marker, i.e. derived from a single parental type (Ponder *et al.* 1985; Griffiths *et al.* 1988; Schmidt *et al.* 1988; Winton *et al.* 1988; Thomson *et al.* 1990; Cohn *et al.* 1991). Gastric crypts are also monoclonal (Lorenz and Gordon 1993). Developmentally, adjacent crypts have a single progenitor so mutations introduced during embryogenesis occur in patches (Cohn *et al.* 1991).

The differentiation pathways are closely controlled, being sensitive to damage, such as radiation (Paulus *et al.* 1992), and to spatial information in respect to cell position within the crypt and the position of the crypt within the intestine (Roth and Gordon 1990). Roth and Gordon also suggest that entero-endocrine cells, of which there are multiple subtypes secreting different enteric hormones, may also have a sequential differentiation pathway. In the model proposed by Paulus (1993), differentiation into columnar or goblet cells occurs at a specific step in the pathway and is stochastic, and control is due to stem cell auto regulation, in particular following radiation induced apoptosis of villus cells (Potten *et al.* 1994).

The stem cells are located close to the base of the crypts (Potten and Loeffler 1990). Kinetic studies suggest that there are between 4 and 16 stem cells per crypt (Potten *et al.* 1987; Potten and Loeffler 1987; Gordon *et al.* 1992), each developmentally derived from a single clone as shown by the chimeric experiments described above, and further studies suggest that each crypt is maintained by one of these with a number of "transit stem cells" (Winton and Ponder 1990).

Work in other epithelia has indicated stem cell pluripotentiality in the prostate gland (Bonkoff *et al.* 1994), respiratory tract (Liu *et al.* 1994) and ovary (Boland and Gosden 1994). The probable site of stem cells in rat or rabbit cornea is thought to be the limbus, with the basal cells making up the transit amplifying population (Schermer *et al.* 1986; Zieske and Wasson 1993). Retroviral lineage studies in reconstituted epithelial grafts show pluripotentiality in rat tracheal epithelium (Engelhardt *et al.* 1991).

1.5.iii. Haematopoietic stem cells

The cells of the bone marrow, which produce the various cells of the peripheral blood and of the immune system, are the most extensively studied renewable stem cell system. The haematopoietic cells differentiate by the production of a number of progressively more restricted lineages (Brown *et al.* 1988; Hall and Watt 1989; Haig 1992; Ogawa 1993). The pluripotential haematopoietic stem cells (HSC) are located within the bone marrow and give rise to all the differentiated cell types present in the blood as well as the tissue components of the immune system. Experiments using lethally irradiated mice (in which the bone marrow is ablated) show that it is possible to reconstitute all blood cell types by bone marrow transplant and lineage marking of HSC's by chromosome markers or retrovirus has shown that all types of cell are derived from a single progenitor (Abramson *et al.* 1977; Spain and Mulligan 1992).

Identification of HSC's relies on expression of cell surface markers. In contrast to the other self renewing tissues, murine HSC's have a number of markers allowing them to be isolated in relatively pure form (Spangrude *et al.* 1988; Ploemaker and Brons 1989; Andrews *et al.* 1990; Jordan *et al.* 1990; Spangrude and Johnson 1990; Spangrude *et al.* 1991). However none of these markers is unique to stem cells and purification is dependent on screening for cells with high levels of the stem cell markers and low levels of the various markers of differentiated cells. Furthermore there is genetic variability in these markers between different strains of mice (Spangrude and Brooks 1993).

In humans the stem cell population, as determined by colony forming capacity and proliferative potential in culture, is included in a group of marrow cells positive for CD34 and can be further purified using an antigen Thy-1 (Baum *et al.* 1992; Mayani and Lansdorp 1994).

There is evidence that the population of cells capable of regenerating all haematopoietic lineages is functionally heterogeneous and that actively proliferating cells have a reduced stem cell activity in mice (Fleming *et al.* 1993; Rebel *et al.* 1994) and in humans (Mayani *et al.* 1993a; Mayani and Lansdorp 1994).

How environmental factors, such as cytokines, regulate cell fate in hand with pre-programmed cell commitment is discussed in section 1.6.

1.5.iv. Stem cells in other systems

Most other tissues are not continually turning over or self renewing during adult mammalian life (Leblond 1963) and consequently most studies concentrate on the analysis of developmental lineages, investigating the clonality of embryological stem cells. This has been done by using various markers of lineage, in particular retroviruses coding for histochemically detectable markers such as β -galactosidase. Studies, most extensively of the CNS and muscle, suggest that embryological stem cells follow similar rules as adult stem cells with pluripotential cells becoming progressively more restricted in their lineage potential, analogous to differentiation pathways in the adult tissues. Such pluripotential stem cells have been shown, using clonal markers, in rat retina (Turner and Cepko 1987) and cerebral cortex (Price *et al.* 1987; Price and Thurlow 1988; Raff and Lillien 1988; Price *et al.* 1991) mouse retina (Fields-Berry *et al.* 1992), mouse central nervous system (Cepko *et al.* 1990) and other tissues. Common clonality has been demonstrated between neurones and Schwann cells in rat neural crest (Stemple and Anderson 1992), *Xenopus* neural cell lineages (Wetts and Fraser 1991), neurones and glia in chicken neural crest (Bronner-Fraser and Fraser 1991; Frank and Sanes 1991) plus perineurium and fibroblasts (Bunge *et al.* 1989). Hughes and Blau (1992) found that lineage marked mouse muscle stem cells were multipotent and can differentiate into 8 fibre types when injected into mouse muscles, possibly depending on extrinsic factors.

1.6. Control of stem cell fate

1.6.i. Stem cell heterogeneity and division

All stem cells must be able to generate daughter cells with different fates (Wolpert 1988), some remaining as stem cells and some becoming transit amplifying cells. Theoretically this may be achieved by two possible mechanisms, either invariant, predetermined asymmetrical outcomes of cell division such that the daughters of a single division are always one stem cell and one transit amplifying cell, or by variation of outcome. If

stem cell division has a variable outcome, the possible results of the division could be two stem cells, two transit cells or one of each. If this is the case then stem cell fate would require a mechanism of regulation. There are three likely ways this could be achieved:

Stochastic

Environmental regulation

Heterogeneous stem cell population

Clearly these possible mechanisms need not be mutually exclusive and there is evidence for each occurring, reviewed in Hall and Watt (1989) and Horvitz and Herskowitz (1992). Evidence for stochastic division is provided by experiments which demonstrate that individual haematopoietic stem cells from mice can be cultured separately, but in exactly the same conditions, and give rise to pluripotential and committed progeny exhibiting differentiation along different lineages in culture (Johnson and Metcalf 1977; Mayani *et al.* 1993b).

Environmental regulation of outcome is seen in *Caenorhabditis elegans* vulval development for example where the fate of each precursor cell depends on signals from adjacent cells and mutations in the signalling pathway, e.g. Lin-12 alters cell fate and therefore vulval development (Greenwald and Rubin 1992; Horvitz and Herskowitz 1992).

In haematopoiesis, numerous growth factors are required for differentiation of stem cells (HSC's) into the various lineages (Allen *et al.* 1990; Dexter 1991; Haig 1992; Ogawa 1993), different lineages expressing different and progressively more restricted types of growth factor receptor. The role of these factors is unclear and they may be permissive of a particular pathway rather than directive (Mayani *et al.* 1993b).

Cell division can be driven by numerous growth factors or cytokines (Haig 1992; Verfaillie 1994), and different factors favour different pathways of differentiation (e.g. granulocyte/macrophage, erythroid or lymphoid). However these factors do not seem to determine the result of stem cell division as was first thought, but are permissive of a particular pathway, possibly by favouring expansion or survival of a subpopulation of lineage committed precursors (Mayani *et al.* 1993b). Stem cell division in HSC's may be symmetric or asymmetric but this is unaffected by combinations of cytokines/growth factors in culture.

Other factors may be important, such as contact with the marrow stroma or extracellular matrix, reviewed by Allen and Dexter (1990), although direct contact with the stromal cells is not essential in culture for long term maintenance of colony forming cells (Verfaillie 1994).

Similar control of cell fate is seen in other cell types. For example growth factors may restrict neural progenitor cells to a glial fate in the neural crest (Shah *et al.* 1994) and rat optic nerve (Raff and Lillien 1988).

The epidermis also proliferates under the control of growth factors (Watt 1988) such as epidermal growth factor (EGF) and there is considerable evidence that interactions with the extracellular matrix (ECM) can also regulate proliferation and terminal differentiation. During terminal differentiation, keratinocytes lose the ability to adhere to the basement membrane (Watt 1987) and fibronectin can inhibit terminal differentiation of keratinocytes in suspension (Adams and Watt 1989). As described above, the level and function of adhesion molecules (integrins) on the cell surface is related to the proliferative capacity in keratinocytes. Similar interactions have now been shown in haematopoietic tissue (Verfaillie *et al.* 1990; Gordon 1994; Rafii *et al.* 1994; Simmons *et al.* 1994) and in colonic carcinoma cell lines in which morphological differentiation is correlated with the ability to adhere to collagen (Richman and Bodmer 1988).

Evidence for stem cell heterogeneity supports the suggestion that there may be a continuum of stem cell renewal capacity rather than discrete populations of stem cells, with different proliferation potentials, and transit amplifiers. Some cells would have a high probability of self renewal and low likelihood of differentiation and, at the other end of the continuum, cells would be very likely to differentiate and have low self renewal capacity. It is possible that such a situation exists in the bone marrow (Lemischka *et al.* 1986) and in the epidermis where integrin levels are directly related to self renewal capacity ((Jones and Watt 1993) and Chapter 4).

Stem cell ageing, regulated by loss of telomeres for example, could be a cause of heterogeneity. For instance in the epidermis it may be that numbers of self renewing cells decrease with increasing donor age (Barrandon and Green 1987b). However this is disputed

by other workers and there is similar uncertainty about stem cell ageing in the bone marrow and it is possible that there is sequential activation of HSC's (Lemischka *et al.* 1986).

1.6.ii. The stem cell niche

It is proposed that stem cells reside in an optimal microenvironment, the stem cell niche (Schofield 1978). The niche would permit a high probability of self renewal but cell division only rarely. Of the daughter cells, only one would remain in the optimum niche and the other would be committed to terminal differentiation, or perhaps occupy another niche which permits a slightly lower probability of self renewal and higher chance of division and differentiation. This model suggests a hierarchy of "niceness" and that stem cell fate is probabilistic, which allows for stochastic events and a heterogeneous population of stem cells.

The niche itself would comprise other cells, such as bone marrow stroma, ECM and diffusible factors such as cytokines.

In keratinocytes the environmental requirements for self renewal and cell division include all three of these components. Culture of human keratinocytes requires the presence of feeder cells, derived from mouse 3T3 fibroblasts, and epidermal grafts require the appropriate cellular dermis or dermal fibroblasts. Hair follicles require specialised dermal cells from the hair papilla (Weinberg *et al.* 1993). It has been suggested that epidermal Langerhans cells are involved in defining the keratinocyte stem cell niche (Parkinson 1992). Culture, allowing self renewal and differentiation, also requires a variety of growth factors and the keratinocytes require attachment to substrate in the presence of extracellular matrix (Rheinwald and Green 1975; Rheinwald and Green 1977; Barrandon and Green 1987a)

As mentioned above (section on epidermal stem cells), stem cells are thought to reside in particular anatomical locations in some epithelia. It is not known what specific factors constitute the niche in these areas.

1.6.iii. Stem cells and adhesion

In both the bone marrow and the epidermis, cellular expression of specific adhesion molecules and high adhesive capacity to particular cells or substrates correlates with high proliferative potential. In the bone marrow this also correlates with pluripotentiality.

Progenitor stem cells require close association with stromal cells or basement membrane to allow self renewal, pluripotential differentiation and proliferation. Cultured keratinocytes terminally differentiate in the absence of attachment to extracellular matrix (Adams and Watt 1989), and the same is true for other epithelial cells. As already mentioned, keratinocyte proliferation *in vivo* and *in vitro* is related to high levels of integrin expression and function ($\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$) (Jones and Watt 1993).

In the bone marrow, haematopoietic progenitors have greater adhesiveness to certain substrates and possibly a specific profile of integrin expression and function. Progenitors adhere more efficiently than differentiating cells to plastic (Gordon 1994), bone marrow stromal cells (Verfaillie *et al.* 1990) and to bone marrow endothelial cells (Rafii *et al.* 1994). The progenitors may have either higher levels, or increased activation, of adhesion molecules such as $\alpha_4\beta_1$ integrin, CD44, PE-CAM 1 (CD31) (Simmons *et al.* 1994), and stem cell marker, CD34, is thought to be an adhesion molecule (Holyoake and Alcorn 1994; Majdic *et al.* 1994). Expression of integrins is regulated in part by cytokines in both haematopoietic cells (Simmons *et al.* 1994), possibly including the *c-kit* ligand (stem cell factor) (Kinashi and Springer 1994), and keratinocytes, for instance α_5 , α_v and β_5 (but not β_1) by TGF- β (Gallit *et al.* 1994) and α_2 by EGF (Chen *et al.* 1993). Cytokines, such as interferon- α , are able to restore adhesiveness to circulating chronic myelogenous leukaemia progenitor cells via restoration of function of β_1 integrins (Bhatia *et al.* 1994).

Differentiation in other cells is also regulated via integrin signalling pathways, such as β -casein production (Streuli *et al.* 1991) and morphogenesis in mammary epithelium via basement membrane proteins, collagen I and $\alpha_2\beta_1$ (Berdichevsky *et al.* 1992).

1.6.iii.a. Adhesion and patterning

Possible mechanisms for the association of adhesion molecules with tissue organisation and tumour progression are suggested by work in *Drosophila* and into mammalian oncogenes and tumour suppressor genes.

β and γ -catenin are members of the armadillo family of proteins which contain conserved repeat sequences and are involved in segment polarity and signal transduction in *drosophila*. In *Drosophila*, *armadillo* functions as a downstream target of a soluble factor, *wingless*, the mammalian homologue of which is Wnt-1, a proto-oncogene. The expression of these proteins and the functioning of the transduction pathway is required for segmental patterning during *drosophila* limb embryogenesis, possibly functioning in local morphogenesis or by maintaining the parasegmental borders allowing segmental separation via expression of proteins such as *engrailed* (Williams and Carroll 1993)

In mammalian cells, the armadillo type proteins have been shown to accumulate and stabilise their association with cadherins in response to Wnt-1 (as in *drosophila* (van Leeuwen *et al.* 1994)), perhaps allowing specification of cell boundaries by strengthening cell-cell adhesion (Hinck *et al.* 1994b).

β -catenin is also bound by the adenomatous polyposis coli (APC) gene product, a tumour suppressor gene, which competes with cadherin for binding. It is not clear whether APC has a negative or positive effect on cell adhesion or has an effect via the α -catenin link to the cytoskeleton (Su *et al.* 1993; Hulsken *et al.* 1994).

Integrin patterning is also seen in *drosophila*. The *drosophila* integrins PS1 and PS2 are restricted in their expression to dorsal and ventral aspects respectively in wing morphogenesis. They are targets for regulation by *apterous* and possibly *vestigial*, homeodomain and nuclear proteins respectively, which define the segmental boundary of the wing and control dorsal-ventral patterning (Williams and Carroll 1993; Gotwals *et al.* 1994).

In adult human epidermis, integrin expression is clearly restricted to a particular compartment, the basal layer. Within that layer there is a defined pattern of expression with patches of alternating high and low expressing cells, related to the rete ridges and suggesting

that levels of expression confer patterning to the epidermis (see section on location of epidermal stem cells above, Chapter 5 and Jones and Watt (1995)). Taken with other observations of proliferative potential mentioned above (section on epidermal stem cells in culture) and in Chapter 4, epidermal organisation may depend on adhesion molecule expression.

1.7. Lineage analysis and cell fate

Studies of cell lineage have been used to investigate cell genealogy in embryological development, for instance what cell types do particular precursors give rise to, and when, plus positional specifications during development (Price 1989). Lineage studies have also been used to understand stem cell clonality, pluripotentiality and progressive restriction of potential by investigating cell fate. The techniques have been of particular use to elucidate development of the nervous system and the neural crest as well as in investigation of haematopoietic stem cell fate.

The essential requirement for lineage analysis is an inheritable and detectable marker in the cell of interest. Several different types of marker have been used and these include retroviral vectors, label injection techniques, analysis of chimeras and *in vitro* cell cloning.

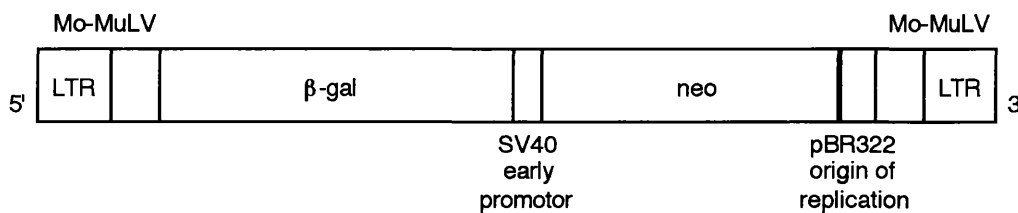
1.7.i. Retroviruses

Retroviruses are engineered to code for a histochemical marker, usually β -galactosidase (β -gal), which is introduced into the genome of the infected cell. A number of viruses have been used, in particular those based on the Moloney murine leukaemia virus (Sanes *et al.* 1986; Price 1987; Price *et al.* 1987; Price and Thurlow 1988; Cepko *et al.* 1990).

The histochemical marker is genetically encoded so it is inherited, undiluted, by all the progeny of the infected cell. These progeny are therefore able to produce the marker. The retroviruses are replication deficient and once incorporated into the host cell genome they are unable to produce viable, infective retroviral particles. They are therefore unable to infect any other cells and only the progeny of the infected cell will contain the viral DNA.

The BAG retrovirus (Price *et al.* 1987) is derived from the Moloney murine leukaemia virus and encodes for β -gal using the *Escherichia coli* gene, *lacZ*. It also contains a gene for neomycin phosphotransferase which confers G418 resistance on the infected cell, allowing selection of those cells. The infected cells express cytoplasmic β -galactosidase.

Diagram 3



The structure of the Bag vector, constructed by cloning the *E. coli* β -gal gene into the pDOL vector which is derived from the Moloney murine leukaemia virus (Price, Turner and Cepko, 1987).

Other vectors have been used, based on, for instance, the Rous sarcoma virus and some constructs cause expression of β -gal in the nucleus (Bonnerot *et al.* 1987) or use another detectable marker, such as placental alkaline phosphatase (Fields-Berry *et al.* 1992).

1.7.ii. Other genetic lineage markers

A number of inheritable genetic markers can be used for lineage analysis. Mice that are heterozygous for a gene on the X-chromosome show cellular mosaicism of gene expression due to X-inactivation. An example of this approach is to use the glucose-6-phosphate dehydrogenase (G6PD) gene (Griffiths *et al.* 1988), which shows either normal or low expression in the intestinal epithelium. Another gene used in this way is the phosphoglycerate kinase gene (Ponder *et al.* 1985). This approach has demonstrated the monoclonal origin of murine intestinal crypt epithelial cells.

Another way of marking lineage is to use chimeras of animals such that cells will show either positive or negative expression of the marker in question. The gene coding for the ability to bind the lectin *Dolichos biflorus* (DBA) has been used in this way in mice (Schmidt *et al.* 1988; Winton *et al.* 1988; Gordon *et al.* 1992). One problem with this method is that the resulting groups of marked cells tend to be very large because of the early stage in

embryogenesis that the chimeras are generated. Furthermore it is unclear how individual cells from different strains might co-operate and interact.

One way round these problems has been to use mice homozygous for G6PD or DBA and induce somatic mutations using a mutagen such as ethylnitrosourea. Mutation in the relevant marker gene results in loss of expression which acts as a marker of lineage for progeny derived from the mutated single cell (Griffiths *et al.* 1988; Winton *et al.* 1988).

Male/female chimeras in which cells either contain or lack the Y chromosome have also been used to mark lineage in the murine intestinal epithelium (Thomson *et al.* 1990). The Y chromosome can be visualised by using non isotopic *in situ* hybridisation using a DNA probe to a long repeated sequence on the chromosome, HY2.1 (Cooke *et al.* 1982; Burns *et al.* 1985). This method enabled demonstration of the clonal origin of intestinal endocrine cells with the other gut epithelial lineages and has also been used to study the survival of stem cells in skin grafts in humans (Brain *et al.* 1989; Burt *et al.* 1989).

Transgenic mice have also been used as a tool to study lineage in the intestine (Gordon 1989; Cohn *et al.* 1991; Gordon *et al.* 1992), the pulmonary epithelium (Hansbrough *et al.* 1992) and to investigate the multistep development of intestinal neoplasia (Kim *et al.* 1993). For example, to study the intestine, fusion transgenes comprising regulatory elements of the gene for a fatty acid binding protein (FABP) and a reporter or marker gene, human growth hormone were used. In normal mice, FABP is expressed in particular parts of the intestine at defined times. The transgenes were expressed inappropriately in other parts of the gut and this expression was switched off in patches at particular times during development. The findings confirmed crypt monoclonality and that villus epithelium was contributed to by multiple crypts. The patches of crypt expression suggest that stem cells in the patch give rise to subtly different progeny with slightly different differentiation programmes (spatial and temporal) compared to the adjacent patches, or that over a period of time the patch is resupplied by a single stem cell.

1.7.iii. Label injection lineage markers

A variety of labels can be injected into the cytoplasm of the cell of interest to trace the fate of the progeny. Examples include horse radish peroxidase and rhodamine dextran (Bronner-Fraser and Fraser 1991; Wetts and Fraser 1991). All these methods result in the progressive dilution of the label at each cell division but are useful for investigating the fate and lineage of cells which become widely dispersed during development.

1.7.iv. Lineage analysis in keratinocytes

Lineage has not been extensively studied in keratinocytes and a combination of approaches have been used. Chimeric mice have shed light on the organisation of proliferative units in the oral epithelium and elsewhere in the skin (Schmidt *et al.* 1987). Retroviral markers have been expressed in mouse skin and oral mucosa (Sanes *et al.* 1986; Carroll *et al.* 1993). In cultured keratinocytes a marker of lineage would be useful to study proliferative units when the culture has reached confluence and is in a steady state, where cell shedding and loss is balanced by the rate of proliferation (Watt 1988; Dover and Wright 1991).

The use of retroviral vectors, particularly those conferring neomycin resistance and selected using G418, in keratinocytes has been problematic. There is a reduction in proliferative potential and an increase in terminal differentiation (Stockschlaeder *et al.* 1991). Also some resistant, infected clones seem able to switch off expression of the marker enzyme (see Chapter 3). Jonathan Garlick (Garlick *et al.* 1991) showed stable infection of keratinocyte stem cells with minimal switching off of expression. Using the retrovirus as a marker in confluent oral keratinocyte cultures it was possible to find colonies with varying morphologies, providing strong evidence for the model of transit amplification in the proliferating population (Garlick and Taichman 1993). He also used this system to investigate clonality in an *in vitro* wound healing model (Garlick and Taichman 1994).

The use of an inheritable marker could also be used in cultured human epidermal keratinocytes to investigate stem cell fate, provided that there is no effect on proliferation or

differentiation. A candidate for such a marker is the steroid sulphatase gene which is deficient in recessive X-linked ichthyosis.

1.7.v. Steroid sulphatase (STS)

STS has a ubiquitous tissue distribution and is present in the Golgi, the endoplasmic reticulum, endosomes (Willemsen *et al.* 1988) and possibly lysosomes (Stein *et al.* 1989). It is a 63kD protein which has both N and C glycosylated terminal domains located extracellularly and connected by a hydrophobic domain which spans the membrane twice.

The enzyme hydrolyses sulphate esters of 3 β -hydroxysterols, including cholesterol sulphate and sulphated steroid hormone precursors such as 15 α -hydroxydehydroepiandrosterone sulphate. The protein is produced in the placenta where it is important for hydrolysing sulphated oestrogen precursor (Burns 1983; Yen *et al.* 1987). Cholesterol sulphate has a critical role maintaining intercellular lipid lamellae in the stratum corneum. Despite only representing 2.6% of the total lipid in the cornified layer, it is one of the few polar lipids and may be important for maintaining intercellular lipid bilayers and stabilisation of the plasma membrane.

In parallel with cholesterol sulphate, the highest STS activity is localised in the upper granular layer and stratum corneum with low activity in the basal layer. As such it is important in regulating desquamation by catalysing the hydrolysis of cholesterol sulphate and hence the breakdown of the lipid bilayers.

The STS gene is located on the distal portion of the short arm of the X-chromosome, close to the pseudoautosomal region, and there is no functional gene on the Y chromosome. The locus is also of interest because it escapes X-inactivation (Lyonisation) (Yen *et al.* 1987; Yen *et al.* 1988).

Recessive X-linked ichthyosis (RXLI) is an X-linked inherited disease caused by mutations in the STS gene and loss of STS activity. Male infants with this condition develop scaly skin at the age of around 3 months. The disease results from a disorder of desquamation and there is no hyperproliferation in the basal layer, as is seen in some other scaling disorders, such as other inherited ichthyoses and psoriasis. Despite loss of STS activity in all cells, ichthyosis is the only phenotypic abnormality except that intrauterine

production of placental oestrogen is very low (Williams 1983). The region of the X-chromosome where the STS gene is located is susceptible to deletion and therefore a number of contiguous gene defects may be present, resulting in associated disorders (Paige *et al.* 1994).

Patients with RXLI lack immunoreactivity with anti-STS antibodies (Epstein and Bonifas 1985; Conary *et al.* 1986) and the majority (80-90%) have a large deletion of the gene (Ballabio *et al.* 1987; Bonifas *et al.* 1987; Gillard *et al.* 1987), although there is genetic heterogeneity (Conary *et al.* 1987; Bonifas *et al.* 1990). Women who carry the gene have half the gene dosage, detectable by Southern blotting (Bonifas *et al.* 1990).

Keratinocytes from patients with RXLI can be cultured in standard conditions (Jensen *et al.* 1990). Mixing of normal and RXLI cells could provide a possible inheritable marker of lineage in cultured human keratinocytes.

1.8. Aims

The aims of my thesis were to investigate stem cell fate in cultured human epidermis in a steady state, when proliferation rate is balanced by terminal differentiation. To achieve this I tested various methods of lineage marking keratinocytes in culture and analysed the results obtained from the most successful of these methods. In addition I have looked at stem cell heterogeneity and the relationship of proliferative potential, adhesion molecule expression and the ability of cells to generate clones capable of regenerating an epidermal sheet. Finally I investigated the relationship of patterns of expression of adhesion and ECM molecules *in vivo*, *in vitro* and in pathological situations with particular interest in the location of the stem cells.

MATERIALS AND METHODS

All chemicals were supplied by Sigma Chemical Company (Poole, UK), BDH (Poole, UK) or Fisons (Loughborough, UK) unless stated otherwise.

2.1. Tissue Culture Media and Solutions

Media and solutions supplied by The Imperial Cancer Research Fund Central Cell Services are identified as (ICRF).

2.1.i. Phosphate Buffered Saline (PBS)

PBS (ICRF) comprised 8g NaCl, 0.25g KCl, 1.43g Na₂HPO₄ and 0.25g KH₂PO₄ in 1 litre of distilled water, titrated to pH 7.2 and autoclaved.

2.1.ii. PBSABC

PBS (ICRF) as above, supplemented with 1mM CaCl₂ and 1mM MgCl₂.

2.1.iii. Versene

Versene (ICRF) contains 8g NaCl, 0.2g KCl, 1.15g Na₂HPO₄, 0.2g Na₂EDTA and 1.5ml 10% phenol red per litre and was sterilised by autoclave.

2.1.iv. Trypsin

0.25% Trypsin in Tris saline (ICRF) was used with versene at 1 + 4 to detach and dissociate keratinocytes from culture dishes.

2.1.v. FAD Medium

FAD medium (Imperial Labs., UK, prepared by ICRF) consists of one part Ham's F12 medium, three parts Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/ml penicillin (Gibco BRL, Paisley, UK), 100 mg/ml streptomycin (Gibco BRL, Paisley, UK) and 1.8×10^{-4} M adenine. This was further supplemented with 10% batch tested fetal calf serum (FCS, Advanced Protein Products, Brierley Hill, UK), 0.5 µg/ml hydrocortisone (Calbiochem), 5µg/ml insulin (Sigma Chemical Co., Poole, UK), 10^{-10} M cholera toxin (ICN, High Wycombe, UK) and 10ng/ml epidermal growth factor (EGF) (Austral Biologicals, San

Ramon, CA, USA). This medium is known as FAD FCS HICE (Rheinwald and Green 1975; 1989).

2.1.vi. Fat free FAD

To remove retinoic acid from FCS the serum was made fat free using the method described by Rothblat (1976). Serum was added at 1:9 to precooled 1:1 ethanol:acetone at 4°C. This was left on ice for 4 hours with occasional stirring. The precipitated protein was collected on Whatman #1 paper by filtration, under suction, through a Buckner funnel. As the last of the solvent cleared, 25% volume of ethyl ether was added and suction continued until the protein was dry and flaky and no longer smelt of ether. The protein was resuspended in FAD at 80mg/ml, filtered and then added to FAD HICE at 10%.

2.1.vii. DMEM (E4 Medium)

E4 medium (ICRF) was supplemented with 10% batch tested donor calf serum (Gibco BRL, Paisley, UK), (E4 DCS) or 10% batch tested fetal calf serum (Advanced Protein Products, Brierley Hill, UK) (E4 FCS).

2.1.viii. Mitomycin C

Stocks of mitomycin C (Sigma) were made up at 0.4mg/ml in PBS, filter sterilised and stored at -20°C.

2.1.ix. Thermolysin

Working concentration of thermolysin (Sigma) was 0.5mg/ml in thermolysin buffer consisting of 10mM HEPES, 142mM NaCl, 6.7mM KCl, 0.43mM NaOH and 1.0mM CaCl.

2.1.x. G418

G418 (Gibco BRL, Paisley, UK) stocks were made up in sterile, distilled water at 100mg/ml and stored at -20°C.

2.1.xi. Chloramphenicol

Chloramphenicol (Sigma) was dissolved at 34mg/ml in 100% ethanol and stored at -20°C.

2.1.xii. Nystatin

Nystatin (Gibco BRL, Paisley, UK) was made as stock, 10,000 U/ml in PBS, and kept at -20°C.

2.1.xiii. Collagen IV

Collagen IV (Collagen VI, Sigma) stocks were made up in 0.01N acetic acid at a concentration of 2mg/ml, filter sterilised and stored at -20°C.

2.1.xiv. Bovine Serum Albumin (BSA)

10% stocks in PBS were stored at -20°C. For use to block coated plates, aliquots were heated to 80°C to denature and filter sterilised before dilution in PBSABC.

2.1.xv. Silastic sheeting

Silastic sheeting (Dow Corning, Michigan, USA) for handling epidermal sheets for transplant was washed in Lux soap flakes (Lever Bros. Corp., Kingston-Upon-Thames, UK), rinsed in distilled water, cut into 1cm squares and autoclaved before use.

2.2. Cell Culture

All cells were grown at 37°C in 5% CO₂ in a humidified incubator.

2.2.i. J2 3T3 Cells

A clone of 3T3 Swiss embryo fibroblasts, named J2, were selected by their ability to support keratinocyte growth and stocks were cultured in E4 DCS. When confluent, the cells were subcultured at 1:5 or 1:10 or used as a feeder layer to support keratinocyte cultures (Rheinwald and Green 1975; 1989). After mitotic inactivation by adding 4µg/ml mitomycin C for 2 hours at 37°C, the J2s were washed in versene and removed from the dish by incubation in versene for 5 minutes at 37°C. The cells were then spun down at 1000 rpm for 5 mins., resuspended in medium and seeded at 1:3. Keratinocytes could be added subsequently or in some experiments the J2s were placed onto recently subcultured keratinocytes.

2.2.ii. J2 neo cells

J2 neos, a generous gift from Barrandon and Green, are a strain of J2 3T3 cells selected for their ability to grow in G418 and used as feeders when selecting keratinocytes in G418. Culture conditions were as for J2s except that alternate feeds were supplemented with 200 µg/ml G418.

2.2.iii. PA 317 cells

PA 317 cells are amphotropic BAG-F retroviral producer cells capable of infecting keratinocytes (Price *et al.* 1987) kindly supplied by Dr. Jack Price, National Institute of Medical Research, London. Cells infected with the retrovirus are induced to produce the enzyme β -galactosidase which allows them to be stained with a substrate called X-gal. They were cultured in E4 FCS plus 600 µg/ml G418 and split at 1:10 when confluent. Mitomycin C treatment was as for J2s. All work using infective producer cells was carried out in a proscribed single cell culture hood and all waste was double bagged and autoclaved before incineration. Glassware was soaked in chlorox. The culture hood was washed in 70% industrial methylated spirit and uv irradiated for 30 minutes following use.

2.2.iv. Primary keratinocyte isolation

Fresh normal human neonatal foreskins were supplied by Dr. H. Barrie, Charing Cross Hospital, London. The origin of other strains is shown in the table listing sources of keratinocytes (section 2.10, page 77).

Under sterile conditions the keratinocytes were isolated by three methods :

2.2.iv.a. Explant culture

Each foreskin was scraped with a scalpel to remove as much of the dermis as possible while leaving the basal layer of the epidermis intact. The epidermal sheet was rinsed briefly in 200 U/ml nystatin, 200 IU/ml penicillin and 200 µg/ml streptomycin in PBS and then cut into pieces of 1-2 mm². These were attached to a culture dish by gentle scoring with a scalpel and a J2 feeder layer was added. The keratinocytes were allowed to grow out from the epidermal sheet and were gently trypsinised off at intervals without removing the

epidermal sheets. The feeder layer was then replaced and the keratinocytes allowed to grow out again.

2.2.iv.b. Trypsinisation

The foreskin was scraped and rinsed as above then cut into 5mm pieces and dissociated in a sterile Wheaton jar, with a magnetic stirrer suspended from the lid, containing 5ml trypsin and 5ml versene at 37°C for 30 minutes. The cells in suspension were added to 10mls of FAD FCS HICE and spun at 1000 rpm for 5 mins then plated at 10^5 cells per 25cm² (T25 flask) onto a feeder layer or frozen down. The remaining clumps of foreskin were retrypsinised in the Wheaton jar for a further 30 mins. This process was repeated until the number of cells yielded from each incubation began to decrease, usually after 4-5 incubations (Rheinwald 1989).

2.2.iv.c. Thermolysin/trypsin

The whole foreskin, including dermis, was rinsed in nystatin, penicillin and streptomycin as above and then chopped into pieces of 1cm². These were then placed in thermolysin at 37°C for 60 mins after which the epidermis could be peeled as a sheet away from the basement membrane and dermis. The epidermal sheet was incubated in 1:1 trypsin and versene for 10 mins at 37°C in a Wheaton jar. The single cell suspension was added to an equal volume of FAD FCS HICE, spun at 1000 rpm for 5 mins, resuspended in medium, counted and plated at 10^5 cells per 25cm² onto a feeder layer, or frozen down (Germain *et al.* 1993).

2.2.v. Keratinocyte subculture

The strains of keratinocytes used are shown in the table listing the source of keratinocytes (section 2.10, page 77). For all experiments cells used were between passage 2 and 7.

2.2.v.a. Normal keratinocyte culture

Keratinocytes were subcultured as described by Rheinwald and Green (1975). Cultures were grown on a feeder layer until just subconfluent then washed in versene

followed by brief incubation at 37°C in versene to remove any remaining J2s. The keratinocytes were then incubated for 5-10 mins at 37°C in 1:4 trypsin : versene, washed in FAD FCS HICE and centrifuged at 1000 rpm for 5 mins, resuspended, counted and seeded onto a mitomycin C treated J2 feeder layer at 10^5 per 25cm². The cultures were fed three times a week with FAD FCS HICE and reached confluence in 7-10 days. Cells were fed the day before further passage or experiment. All experiments using normal keratinocytes were on cells at passage 2-7.

2.2.v.b. Culture of RXLI keratinocytes

These strains of keratinocytes were isolated by the explant method described above and subcultured and passaged as for normal keratinocytes. The biopsies were kindly supplied by Dr. Mary Judge, Great Ormond Street Hospital, London.

2.2.v.c. Culture of XYY keratinocytes

Isolated by explant (XYA) or trypsinisation (XYB) these two cell lines were grown as for normal keratinocytes.

2.2.v.d. Culture of vp keratinocytes

Vp cells are a keratinocyte strain derived from normal foreskin (strain v) and transformed with HPV 16 (Pei *et al.* 1991). Vp were cultured in the absence of a feeder layer in FAD FCS HICE split at 1:10 or 1:20 when confluent. The cells used were between passage 60 and 70.

2.2.vi. Freezing cells

All cell types were frozen down in the same way. After removal from the flask and spinning down the cells were resuspended in FCS containing 10% DMSO and aliquoted into Nunc cryovials.(BRL, Paisley, UK) These were then placed in a container well insulated with cotton wool, which allows a slower rate of freezing, and put at -70°C for 24-48 hours. The vials were subsequently transferred to liquid nitrogen for storage.

2.2.vii. Thawing cells

Vials of frozen cells were thawed rapidly at 37°C, resuspended in the appropriate culture medium and spun down at 1000 rpm for 5 minutes. The supernatant was aspirated to remove the DMSO and the cells were resuspended in fresh medium, counted and plated out at 2×10^5 per 25cm^2 onto a feeder layer (keratinocytes) or at 1:1 (J2s or PA317).

2.2.viii. Growth Curves

The growth rates of RXLI cells were compared to that of normal keratinocytes by seeding 35mm plates, with a J2 feeder layer, with 2×10^4 keratinocytes per dish and counting the number of cells three times a week until confluence was reached. This was done by first gently removing the feeders from duplicate dishes with 2 washes of versene then trypsinising the keratinocytes and counting the cells using a haemocytometer.

2.2.ix. Cornified envelope formation

The number of cornified envelopes formed by RXLI cells was compared to normal by disaggregating dishes of cells when confluent with versene and trypsin and spinning down in medium with serum. The cell pellets were resuspended in 10mM Tris.Cl, pH 7.4, 1% β -mercaptoethanol (Sigma) and 1% SDS to lyse the living cells (Sun and Green 1976) and boiled for 10 mins. The mixture was then respun at 2000 rpm for 10 mins and the number of cornified envelopes counted using a haemocytometer.

2.2.x. Collagen coated membrane inserts

Keratinocytes were grown on 30mm, $0.4\mu\text{m}$ pore size cell culture chamber inserts either pre-coated with collagen (Transwell-COL inserts, Costar, MA, USA) or uncoated (Millicell-CM, Millipore, MA, USA), coated with type IV collagen (Sigma) at 3mg/ml in 0.01M acetic acid diluted 1:4 in 60% ethanol, applied to the insert membrane and allowed to dry in a cell culture hood. Keratinocytes were seeded at 5×10^5 to 10^6 cells per insert. A feeder layer of J2 cells were placed in the outer chamber formed by the culture plate as in diagram 1.

Keratinocytes were grown in the insert under medium until fully confluent when medium was removed from the inner chamber and the epidermal layer fed from underneath by replacing medium in the outer chamber three times a week for 7-10 days.

2.2.xi. Collagen gels

Collagen gels for supporting keratinocyte growth at the liquid/air interface were made from vitrogen 100 bovine dermal collagen (Collagen Corp., CA,

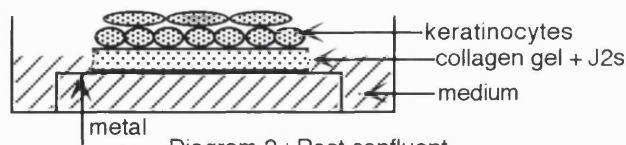


Diagram 2 : Post confluent keratinocytes grown on collagen gel after raising to the liquid/air interface and placing on a metal grid to allow feeding of the basal layer from below.

USA). The vitrogen was diluted in 0.012N HCl to a concentration of 2.34 mg/ml, to each 0.8mls of which was added, on ice and pre-cooled, 50 μ l 0.1M NaOH, 50 μ l 4.4% NaHCO₃, 0.1ml 10 x E4 (ICRF) and 5 x 10⁶ J2 cells in 0.1 ml E4. The final pH is 7.4. The gel was carefully mixed while on ice, avoiding air bubbles, and pipetted into 35mm culture dishes, 1ml per dish. The gel was allowed to set at 37°C for 1-2 hours then equilibrated with FAD HICE containing 20% FCS for 24 hours. Keratinocytes were seeded at 2 x 10⁵-5 x 10⁵ per dish and grown under medium until fully confluent. The gel was then gently removed from the dish, placed on a perforated metal plate and keratinocytes fed from underneath by replacing medium up to the level of the gel three times a week for 3 to 10 days, as illustrated in diagram 2.

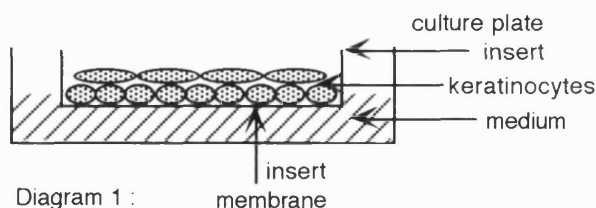


Diagram 1 : Post-confluent keratinocytes grown at the liquid/air interface on a membrane in a culture plate insert.

2.2.xii. Collagen IV coating of culture dishes

Culture dishes were coated with collagen IV (Sigma) at 100 μ g/ml in PBSABC for 1 hour at 37°C then blocked in 0.5mg/ml BSA in PBSABC before adhesion of keratinocytes in

serum free FAD HICE. Keratinocytes used in collagen adhesion assays were trypsinised as normal then resuspended in serum free FAD containing 5mg of soya bean trypsin inhibitor (Sigma) per 1ml of trypsin before counting and spinning down.

2.2.xiii. Keratinocytes selected on collagen IV

If the cells were to be selected on collagen IV then they were harvested in versene/trypsin as usual but, when the keratinocytes had disaggregated, the trypsin was blocked in serum free FAD containing soya bean trypsin inhibitor (Sigma), 5mg per 1ml of trypsin. The cells were spun at 1,000 rpm for 5 mins, resuspended in serum free FAD HICE, counted, serially diluted and plated onto a collagen IV coated plate or insert, without a J2 feeder layer, and incubated at 37°C for the appropriate length of time. The dishes were washed gently three times in serum free FAD to remove non adherent cells.

To select cells that adhered slowly to collagen IV the keratinocytes were incubated for one hour in serum free FAD HICE on a collagen coated plate and the non-adherent cells taken off, spun down, resuspended and counted. These were then plated onto collagen IV coated dishes and allowed to adhere in serum free FAD HICE for three hours.

All cells, fast and slow adhering, were switched to FAD FCS HICE after the same time, usually four hours. Control cultures were keratinocytes allowed to adhere for four hours to collagen IV without selection.

2.2.xiv. Deepidermidised dermis (DED)

Skin for the preparation of deepidermidised dermis was obtained from two different sources and these were treated differently.

2.2.xiv.a. Mastectomy specimens

These were kindly provided by Dr. Rosemary Millis, Hedley Atkins Breast Unit, Guy's Hospital. Pieces of skin uninvolved by tumour were cleaned in alcohol then cut out with a sterile scalpel, put into a sterile container and kept on ice until use or frozen to -80°C immediately. All the subcutaneous fat was removed. The epidermis was separated by immersion in PBS at 56°C for 2 minutes and then by carefully peeling the epidermis away as

one strip using sterile fine forceps. The dermis was then cut into 1cm^2 pieces and freeze/thawed between 2 and 5 times in liquid nitrogen.

2.2.xiv.b. Post mortem specimens

Skin from different body sites was washed in isopropanol before removal with a sterile scalpel, then washed in 70% ethanol and kept on ice in sterile containers before use. To prevent infection of the cultures it was necessary to immerse the skin in an antibiotic cocktail of 200 IU/ml penicillin, $200\mu\text{g/ml}$ streptomycin, 1mg/ml G418, $340\mu\text{g/ml}$ chloramphenicol and 200 U/ml nystatin for 24 hours at 4°C . The antibiotics were washed out by soaking in PBS for 24 hours at 4°C with three changes of PBS. The epidermis was then removed as above and the pieces of dermis stored at -80°C

2.2.xv. Keratinocyte culture on deepidermidised dermis

A piece of deepidermidised dermis (DED) of the required size, between 0.25cm^2 and 1.0cm^2 , was placed on a sterile metal perforated platform or into a millicell culture plate insert (Millipore) and the surface allowed to dry. Keratinocytes were applied to the surface of the dermis at various densities,

depending on the experiment, or as a sheet of cultured cells removed from the dish using Dispase. FAD FCS HICE medium was added such that the underside of the dermis was covered but the top surface, with

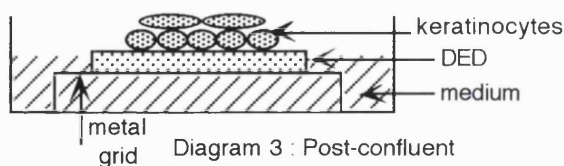


Diagram 3 : Post-confluent keratinocytes grown on deepidermidised dermis at the liquid/air interface on a metal grid to allow feeding of the basal layer from below. The dermis could also be placed in a culture plate insert.

covered but the top surface, with

basement membrane and keratinocytes, was at the liquid/air interface as illustrated in diagram 3. The cultures were grown for 2 to 4 weeks depending on the seeding density.

2.2.xvi. Dispase treatment of cultured keratinocytes

Sheets of post-confluent keratinocytes can be removed from the culture dish using an enzyme, Dispase (Boehringer Mannheim, Mannheim, Germany). 1 to 2 day post-confluent keratinocytes were washed in serum free E4 then incubated at 37°C in 2.5mg/ml dispase in

serum free E4 for approximately 30 minutes (Watt 1984). When the edges of the sheet had detached from the dish the rest of the sheet was peeled away with forceps. If the sheets were for transplant to deepidermidised dermis or nude mice they were washed 3 times in FAD FCS HICE and if for staining or cryosectioning then they were washed in E4 DCS followed by PBS.

2.2.xvii. Transplant of cultured epidermal sheets onto DED

1-2 day post-confluent cultures were removed with dispase then lifted using forceps and a piece of silastic to provide support. The basal layer of the sheet was placed onto the upper surface of the DED. The silastic sheeting was removed the following day. Cultures were grown for 4 weeks.

2.2.xviii. Transplant of cultured epidermal sheets into nude mice

Post-confluent keratinocyte cultures were removed from the dish (35mm diameter) using dispase and washed. The sheets were then transplanted, with basal layer most superficial, under skin flaps on the back of nude mice, with silastic as support, using the method first described by Barrandon (1988). Using this method the basal cells are apposed to the mouse dermis as shown in diagram 4. Transplants were harvested in 7-10 days.

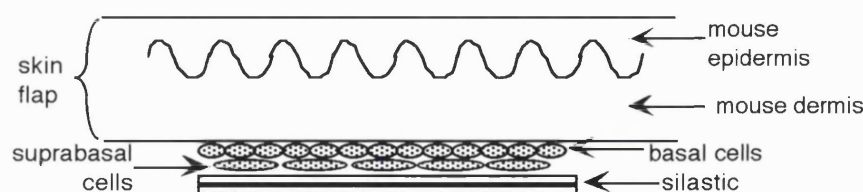


Diagram 4 : Transplant of cultured keratinocyte, dispase treated, sheets into nude mice.

2.2.xix. Infection of keratinocytes with BAG-F retrovirus

2.2.xix.a. Retroviral titres

The number of colony forming units per ml produced by the PA 317 packaging line was estimated using a method adapted from that of Morgenstern and Land (1991).

A confluent flask of PA 317 cells was incubated overnight in E4 DCS overnight. The medium was filtered through a 0.45µm filter to remove any remaining cells and put on to a T75 flask of J2 cells with the addition of 150µl of filter sterilised polybrene, 800µg/ml in PBS. The J2s were incubated for 2 hours at 37°C, 5% CO₂ in retroviral medium. The medium was replaced with fresh E4 DCS and the cells incubated at 37°C, 5% CO₂ for 24 hours. The J2s were then removed from the flask using versene and the cells split at dilutions between 1:1000 and 1:10⁶. The infected cells were grown in E4 DCS containing 400µg/ml G 418 until colonies were clearly visible. The colonies were stained with X-gal to confirm infection by retrovirus and then counted.

2.2.xix.b. Infection of keratinocytes

Mitomycin C treated PA 317 cells were split 1:3 into 75cm² (T75) culture flasks and allowed to adhere and spread for between 2 and 24 hours in FAD FCS HICE. Keratinocytes were added at 2 x 10⁵ per flask without changing the medium. The keratinocytes were allowed to grow for 4 days with the producer cells acting as feeders, supplemented by mitomycin C treated J2 neos if required, and with 2 changes of medium. After 4 days the producer cells were removed with versene and replaced by J2 neos. Selection of infected keratinocytes was by 150µg/ml G418, using non-infected keratinocytes to control for G418 effectiveness.

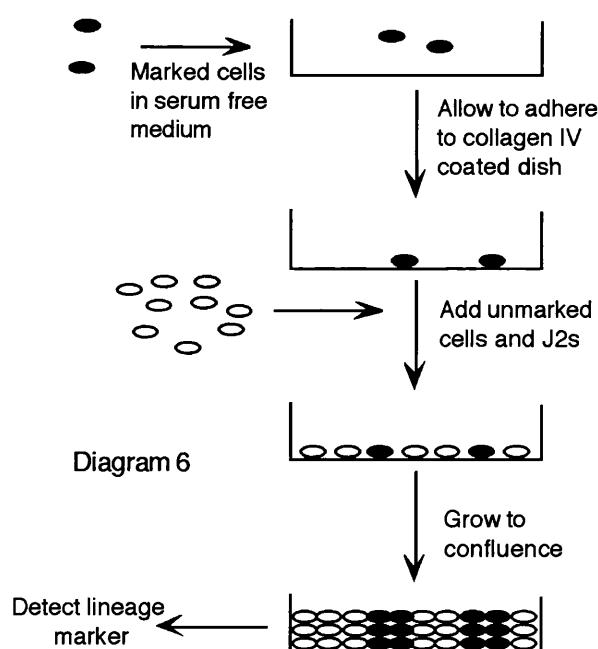
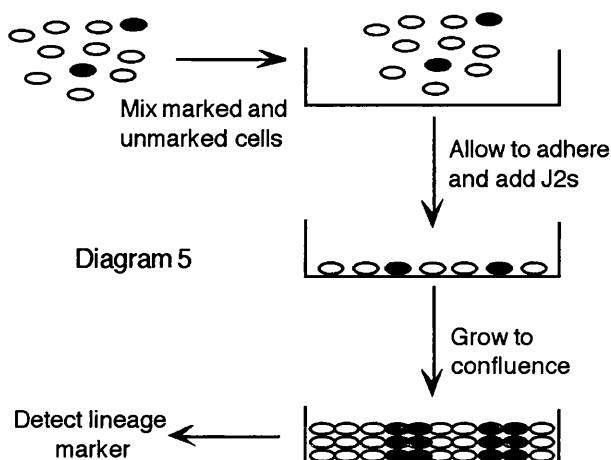
After around 10 days between 2 and 10 growing colonies remained in each flask. One flask was stained for β-galactosidase to ensure adequate expression and the rest of the keratinocytes were passaged by the standard method. Further passages of infected keratinocytes were not grown in G418.

2.2.xx. Keratinocyte lineage culture experiments

For lineage experiments, two lines of keratinocytes (one positive for the lineage marker and one negative) were mixed and co-cultured.

The keratinocytes were counted and each line serially diluted. Either the two lines were mixed in a tube at the appropriate ratio and the mixture of cells in suspension then plated in the usual way (diagram 5) or the cells positive for the marker were selected on collagen VI and the negative cells added subsequently (diagram 6).

Further details of the experiments investigating cell lineage are given in Chapters 3 and 4.



2.3. Cytochemistry and Immunofluorescence

All antibodies used are listed at the end of the chapter and were diluted for use in blocking solution, usually 10% FCS in PBS. Negative controls were using blocking solution only plus secondary antibody. Incubations and washes using fluoresceinated antibodies were performed in light tight containers.

2.3.i. Cytochemical stains

2.3.i.a. Haematoxylin

0.1% Mayer's haematoxylin (Sigma) was used to stain cells and sections by dipping for up to 5 mins then washing in running tap water.

2.3.i.b. Eosin

Eosin Y (Sigma) was used at 1% in water and sections washed in 50% ethanol.

2.3.i.c. Neutral red

Sections were dipped in a 1% solution of neutral red in water, carefully blotted dry on filter paper, then briefly immersed in 100% ethanol followed by xylene.

2.3.ii. DAB solution

One 10mg tablet of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) was dissolved in 10 ml of distilled water to which was added 10 μ l of 30% hydrogen peroxide (Fisons, UK). The solution was filtered through a 0.4 μ m filter before use and applied to sections for 3-4 mins according to the strength of the signal. Fresh solution was made up for each staining.

2.3.iii. Silane coating of 4 well slides

4 well slides obtained from C.A. Hendley Ltd, Loughton, UK were washed in hot tap water and washing up liquid then rinsed in distilled water. Slides were coated in silane by dipping into 2% solution of 3-aminopropylsilane in acetone, washed in acetone then twice in distilled water and allowed to dry.

2.3.iv. Gelatin coating of 4 well slides

4 well slides were washed as above then dipped in 2% gelatin, 1% chrome alum, allowed to dry and kept at 4°C.

2.3.v. Freezing of tissue for cryosection

Tissue was frozen by placing the specimen on a glass cover slip and submerging, either directly or covered in OCT (BDH, Poole, UK) at 4°C, into isopentane cooled by liquid

nitrogen. Frozen specimens were stored at -70°C. Sectioning was done by the ICRF Histopathology Unit, 6µm sections were cut and mounted on to silane coated 4 well slides and stored at -70°C.

2.3.vi. Gelvatol mounting solution

Gelvatol (Gelvatol, Monsanto Indian Orchard Plant, Springfield, MA, USA) was used for mounting all immunfluorescently stained sections. 2.4g of gelvatol were mixed with 6g of glycerol (BDH, Poole, UK) by vortexing. After the addition of 6mls of distilled water the mixture was left to stand at room temperature for 1.5 hours followed by adding 12.5mls 0.2M Tris pH 8.5. The solution was vortexed, heated to 50°C for 10 mins and left overnight on an end over end mixer. The solution was then spun down at 4000 rpm for 15 mins and the supernatant kept at 4°C or stored at -20°C.

2.3.vii. X-gal staining for β -galactosidase (β -gai)

Details of solutions used are listed in the appendix, stocks and solutions 1

2.3.vii.a. Whole cells

Colonies on culture dishes, dispase sheets or cells grown on cell culture inserts could be stained directly for β -gal. The cells were washed in PBS, fixed in 0.25% glutaraldehyde in PBS for 5 mins and washed briefly in PBS + 2mM MgCl₂. After 10 mins in detergent solution the cells were incubated overnight in X-gal solution at 37°C. Culture dishes and slides were washed in water then dehydrated through graded alcohols (50%, 70%, 95% and 100% ethanol) and dispase sheets post-fixed in 3.7% formaldehyde/PBS for paraffin embedding and sectioning (ICRF Histopathology Unit). Sections were counterstained with neutral red.

2.3.vii.b. Cryosections

Sheets of keratinocytes with multiple differentiated cell layers were better stained after sectioning to ensure adequate access of the stain to all layers. Sheets were fixed in 0.25% glutaraldehyde/PBS, incubated overnight in PBS + 2mM MgCl₂ + 30% sucrose at 4°C then frozen in OCT. 6µm sections were cut and mounted onto gelatin coated 4 well slides, post fixed in 0.25% glutaraldehyde for 10 mins on ice, washed for 5 mins x 2 in PBS + 2mM

MgCl₂ followed by 10 mins in detergent solution, all on ice. Slides were then incubated in X-gal solution at 37°C for 3 hours, washed in PBS, 2 x 5 mins, rinsed in water and dehydrated through graded alcohols, xylene and mounted in DPX (BDH).

2.3.viii. Staining of colonies using anti-keratin antibody

Small colonies of keratinocytes in dishes could be easily seen by staining with anti-keratin antibody LP34. Dishes of cells were fixed in 3.7% formaldehyde/PBS for 5 minutes then permeabilised on ice in pre-chilled methanol. Dishes were rinsed once in PBS then blocked for 20 mins in 10% FCS in PBS. LP34 supernatant, diluted 1:4 in 10% FCS/PBS, was applied for 15 mins at room temperature then washed 3 times in PBS. The second antibody was peroxidase conjugated rabbit anti mouse IgG applied for 15 mins. Finally the cells were washed and developed using DAB/H₂O₂.

2.3.ix. Immunofluorescent staining for steroid sulphatase (STS)

Whole cells or frozen sections were stained with the same method. Fixation was for 20 mins in 2% paraformaldehyde followed by washing in PBS + 0.1M glycine Tris, pH 7.2. Permeabilisation, if required, was in 0.1% triton-X-100/PBS for 10 mins, rinsed in PBS and blocked in 10% FCS/PBS. The primary antibody was kindly provided by Prof K. von Figura, Göttingen, Germany (Conary *et al.* 1986) and used at a 1:50 dilution for at least 1 hour at room temperature and slides were washed 3 times in PBS. This was followed by a 1:100 dilution of anti-rabbit secondary antibody, 1 hour at room temperature, conjugated either to fluorescein or biotin and three further washes in PBS. If a biotinylated secondary antibody was used slides were incubated in fluorescein conjugated streptavidin for 30 mins at room temperature and washed three times in PBS. All slides were rinsed in distilled water and mounted in gelvatol.

2.3.x. Immunofluorescent staining for integrins and cadherins

Cells were fixed in 3.7% formaldehyde/PBS for 10 mins at room temperature and washed in PBS three times. Frozen sections were air dried and dipped briefly in PBS. Results were unaffected if sections were fixed (3.7% formaldehyde/PBS) or unfixed. Blocking solution

was 10% FCS in PBS for 30 mins followed by primary antibody, generally at a dilution of 1:100 or as neat hybridoma supernatant, for 30 mins at room temperature. Slides were washed three times in PBS before incubation with secondary antibody at 1:100 dilution for 30 mins at room temperature followed by 3 further washes in PBS, rinsing in distilled water, and mounting in gelvatol. If the primary antibody was directly fluoresceinated, no secondary antibody was required and slides were washed and mounted immediately following incubation with the primary antibody.

2.3.xi. Immunofluorescent staining for involucrin

Whole cells were air dried onto a glass cover slip, fixed in 3.7% formaldehyde/PBS and permeabilised in methanol for 5 mins at room temperature. After washing in PBS the cells were stained using DH1 at 1:250 dilution with three washes in PBS and incubation with fluorescein conjugated anti rabbit-IgG diluted to 1:100. The cover slips were washed in PBS followed by distilled water and mounted in gelvatol. Photomicrographs of random fields were taken for counting the number of involucrin positive cells.

2.3.xii. Immunofluorescent staining for HLA subtypes

Whole cells were air dried onto a glass cover slip, fixed in 3.7% formaldehyde/PBS and blocked in rabbit serum for 15 mins at room temperature. Primary antibodies were used as neat supernatants, incubation was for 1 hour at room temperature followed by 3 washes in PBS. The secondary antibody used was a fluorescein conjugated anti-mouse IgG Fab fragment, incubated for 1 hour at room temperature. Cover slips were washed in PBS, rinsed in distilled water and mounted in gelvatol.

2.3.xiii. Immunofluorescent staining for collagen IV

Frozen sections were air dried, rinsed in PBS and blocked for 30 mins in 10% FCS/PBS. After 30 mins incubation with a fluorescein conjugated anti-collagen IV antibody slides were washed 3 times in PBS, rinsed in distilled water and mounted in gelvatol. 1µm slices of the sections were visualised using a BioRad MRC 600 confocal microscope and the intensity of fluorescence measured.

2.3.xiv. Confocal microscopy

Tissue sections, stained by fluorescein conjugated anti-collagen IV antibody, were examined with the 25x objective of the confocal microscope with an optical section thickness of 1 μ m. The fluorescence (pixel intensity) was measured from the confocal image along lines drawn across the basement membrane. 3-5 consecutive rete ridges or dermal papillae were measured in each experiment, with 3-8 readings per ridge/papilla.

2.3.xv. Fluorescein conjugation of primary antibody

The method was adapted from that of Harlow and Lane (1988). The antibody to be conjugated was dialysed at 4°C against distilled water overnight then dried in a speedvac (Savant A160) and resuspended in 0.1M sodium carbonate, pH9, at 2mg/ml. Fluorescein isothiocyanate (isomer 1, on celite, Sigma) was dissolved in DMSO at 1mg/ml and added to the antibody very slowly in 5 μ l aliquots, 50 μ l per 1ml of protein solution. The antibody was then left for 8 hours at 4°C and free fluorescein separated from antibody on a PD-10 Sephadex G-25 M column (Pharmacia, Uppsala, Sweden) equilibrated with PBS.

2.3.xvi. Staining of paraffin embedded tissue sections

Sections were deparaffinised by soaking 2 x 10 mins in xylene then rehydrated by dipping in graded alcohols, 3 mins each - 100%, 95%, 70%, 50% and PBS for 5 mins. Staining was then as for frozen sections, using the appropriate primary antibody active on paraffin sections.

2.3.xvii. Sections of healing wounds - suction blisters

Expression of adhesion molecules during wound healing was investigated using biopsies from suction blisters and supplied by Mark Hertle (Hertle *et al.* 1992). Briefly, blisters were induced on the thigh or upper arm of human volunteers by gentle suction, 600mmHg for 1-2 hours until a blood free bulla was formed. 6mm punch biopsies were taken at various time points, fixed in 4% paraformaldehyde, frozen in liquid nitrogen and sectioned.

2.3.xviii. Sections of oral squamous cell carcinomas

Frozen sections were generously supplied by Paul M. Speight, Department of Oral Pathology, Eastman Dental Hospital, London, UK. The carcinomas varied from well, through moderately, to poorly differentiated.

2.4. DNA preparation and purification

The recipes for the solutions used in the preparation and purification of DNA are listed in the appendix, stocks and solutions 2. Concentration of DNA was determined by absorbance at 260 and 280 nm on a spectrophotometer after each preparation. Restriction endonucleases were obtained from either Gibco BRL, Paisley, UK or New England Biolabs, Beverly, MA, USA and were used in the buffers supplied by the manufacturers.

2.4.i. Preparation of competent bacteria

Escherichia coli, either HB101 or DH5 α , were cultured in 10mls of L-broth, shaken at 37°C overnight. 1ml of the culture was added to 100 mls of L-broth and incubated, shaken at 37°C for 90-120 mins until the OD₆₀₀ was 0.3-0.5 then spun at 3,000 rpm for 5 mins. The pellet was resuspended in 50mls of 50mM CaCl₂, pH5.6, left on ice for 20 mins and spun at 3,000 rpm at 4°C for 5 mins. The pellet was resuspended in 10 mls 50mM CaCl₂, pH5.6, and left on ice until required, preferably for 24 hours, or frozen in 50% glycerol and stored at -70°C in 100 μ l aliquots.

2.4.ii. Transformation of competent bacteria

100 μ l of competent bacteria were thawed at room temperature until just liquid and put on ice for 10 mins. The DNA was added (1-100ng in less than 25 μ l), the cells left for 20 mins on ice and then heat shocked at 42°C for 3 mins followed by 2 mins on ice. 400 μ l of BHI at 37°C was added and the bacteria incubated for 45 mins at 37°C. The bacteria were then plated onto L-agar plates containing ampicillin (100 μ g/ml) (Sigma) and left overnight at 37°C.

2.4.iii. Extraction of genomic DNA

Cells from 3 x 75cm² (T75) flasks of confluent keratinocytes were trypsinised and spun down, in polypropylene tubes, in FAD FCS HICE at 1000 rpm for 5 mins. After removal of the supernatant (pellets could be stored at -70°C at this point) the pellet was resuspended in 5mls of TE8 plus 200µg/ml proteinase K (Sigma) and mixed well. SDS was added to a final concentration of 0.5% and this was incubated overnight at 37°C. An equal volume of Tris-equilibrated phenol (Amresco, Solon, Ohio, USA) was added and thoroughly mixed. After centrifugation for 10 mins at 1500 rpm at room temperature the aqueous layer was removed using a plastic pipette with the tip cut off. The phenol extraction was then repeated and an equal volume of chloroform/IAA added, well mixed and spun at 1500 rpm for 10 mins at room temperature, and the aqueous layer extracted as before. The solution of DNA was ethanol precipitated using 0.3M NaCl and 2 volumes of 100% ethanol pre-chilled to -20°C. The DNA precipitate was transferred to a microfuge tube, again using a "sawn off" pipette tip, washed in 70% ethanol and spun in a microfuge for 15 mins at room temperature. The supernatant was removed, the pellet allowed to air dry and was then resuspended in 100µl TE8.

2.4.iv. Extraction of plasmid DNA

This was by a standard alkaline lysis method adapted from the procedure published in Sambrook, Fritsh and Maniatis (1989). The pellet from a 400ml bacterial culture grown in L-broth plus appropriate antibiotic was resuspended in 18mls DNA lysis buffer at 4°C, on ice, to which was added 40 mls of alkaline SDS solution. After thorough mixing the mixture was left at room temperature for 10 mins. 20mls of ice cold potassium acetate solution was added, well mixed and left to stand on ice for 10 mins. The precipitate was removed by centrifugation at 4000 rpm for 15 mins at 4°C followed by filtration of the supernatant through multiple layers of gauze. 0.6 vols. of isopropanol was added to the filtered supernatant and left at room temperature for 10 mins. The DNA was then spun down at 5000 rpm for 15 mins at room temperature and the pellet washed in 70% ethanol, allowed to dry and resuspended in 3mls (for PEG precipitation) or 10mls (for caesium chloride gradient) of TE8.

2.4.v. Purification of plasmid DNA

Two methods were used, centrifugation in a caesium chloride gradient or precipitation with polyethylene glycol.

2.4.v.a. Caesium chloride gradient

To 10 mls of DNA in TE8 from alkaline lysis was added 10g of caesium chloride (Gibco BRL, Paisley, UK) and 1ml of ethidium bromide solution, 10mg/ml. When dissolved the solution was put into a sealable polyallomer tube (Beckman Instruments Inc., Palo Alto, CA, USA) and spun in an ultracentrifuge (Beckman L8-70M) at 60,000 rpm for 16 hours at 20°C using a 70.1.TI rotor. The plasmid band was usually visible in daylight but the tube was viewed in uv light if not. The band of plasmid DNA was removed from the tube using a needle and syringe, extracted three times in equal volumes of CsCl saturated isopropanol then precipitated in 0.3M sodium acetate and 2 volumes of 100% ethanol at 4°C, washed in 70% ethanol and resuspended in TE8.

2.4.v.b. Polyethylene glycol (PEG) precipitation

This method is from Sambrook, Fritsh and Maniatis (1989). To 3 mls of DNA in TE8 from alkaline lysis was added 3 mls of ice cold 5M lithium chloride (Gibco BRL, Paisley, UK), mixed and centrifuged at 10,000 rpm, 4°C for 10 mins. An equal volume of isopropanol was added to the supernatant, mixed and spun at 10,000 rpm for 10 mins at room temperature. The pellet was washed in 70% ethanol, dried and resuspended in 500µl TE8 plus 20µg/ml DNase free RNase and left at room temperature for 30 mins. 500µl PEG solution was added and the pellet recovered using a microfuge at 12,000g for 5 mins at 4°C. The pellet was resuspended in 400µl TE8 and extracted with phenol, phenol/chloroform and chloroform then precipitated in 2 volumes of ethanol with 100µl of 10M ammonium acetate, 10 mins at room temperature. The DNA was recovered by spinning at 12,000g at 4°C for 5 mins, washed in 70% ethanol at 4°C, dried and dissolved in 500µl TE8.

2.4.vi. Minipreps of plasmid DNA

Single bacterial colonies were picked and inoculated into 5 mls of L-broth plus ampicillin, 100µg/ml, and shaken at 37°C overnight. 1.5 mls of the culture was spun in a microfuge at 12,000g for 2 mins and the pellet resuspended in 100µl of miniprep lysis solution. To this was added 200µl of fresh alkaline SDS solution with rapid, gentle mixing and the mix left on ice for 5 mins. 150µl of ice cold 7.5M ammonium acetate was added, vortexed and microfuged at 12,000g for 5 mins. The supernatant was precipitated in 0.6 vols. of isopropanol, spun in a microfuge and the pellet washed in 70% ethanol, dried and resuspended in 50µl of TE8.

2.4.vii. Gel purification of DNA

DNA cut by restriction endonucleases was separated by horizontal electrophoresis in agarose (Gibco BRL, Paisley, UK) gel (0.8-2.0% according to DNA size) in TAE containing 1µg/ml ethidium bromide. The band of interest was cut out and purified by one of two methods, either by glassmilk in sodium iodide (GeneClean II kit, Bio 101 Inc, La Jolla, California, USA) as per the manufacturer's instructions or by spinning for 30 minutes in a microfuge through a 0.2µm filter (Costar, Cambridge, MA, USA). The filtered solution was extracted twice in phenol, phenol/chloroform and chloroform then precipitated in 0.3M sodium acetate, pH5.2 and 100% ethanol. Alternatively the band of interest was run into a gap cut in front of it in the gel, filled with TAE, by running the current at 100V and collecting and replacing the TAE every 30 sec., 5-10 times until the band had run out of the gel, viewed when transilluminated with uv light. DNA for the production of transgenic mice was further purified by using Elutip-d columns (Schleicher and Schuell, Dassel, Germany) as per the manufacturer's protocol.

2.5. Southern Blotting

All solutions are listed in the appendix, stocks and solutions 3.

2.5.i. Probe preparation

The probe used for Southern and Northern blots was prepared from pSVL-STs kindly supplied by Prof. K. von Figura, Göttingen, Germany (Stein *et al.* 1989). The plasmid was used to transform competent *E. coli* HB101 bacteria and purified as above. The plasmid was then cut with XbaI and BamHI giving a 2.4kb steroid sulphatase (STS) insert which was gel purified and labelled. Involucrin probe was cut from pl-2 (kindly provided by Dr Howard Green, Harvard Medical School and prepared by David Hudson, Keratinocyte lab, ICRF) using PstI then purified and labelled as below.

2.5.ii. Probe labelling

Probes were labelled by the random primer method with ^{32}P dCTP and Klenow (large fragment) polymerase (New England Biolabs, Beverly, MA, USA). 50µg of DNA was boiled for 5 minutes and mixed with 5µl OLB buffer, 1µl 10mg/ml nuclease free BSA (New England Biolabs, Beverly, MA, USA), 2.5µl of ^{32}P -α-dCTP at 10µCi/µl (Amersham International, Amersham, UK) and 2 units of Klenow made up to 25 µl with distilled water then incubated, at room temperature, overnight. Labelled probe was isolated using Sephadex G50 DNA grade Nick columns (Pharmacia, Uppsala, Sweden). Briefly the columns were equilibrated with TE7.5 and the labelled probe eluted with elution buffer, leaving unincorporated nucleotide in the column.

2.5.iii. Blotting

10µg of genomic DNA from each of the different keratinocyte lines was digested by EcoRI and the products mixed with loading buffer and separated on a 0.8% agarose/TAE gel in a horizontal electrophoresis tank (Gibco BRL, Paisley, UK) with TAE running buffer. The gel was depurinated using 0.25M HCl for 15 mins and denatured in 0.2M NaOH/0.6M NaCl, 30 mins, then washed twice in neutralising buffer.

The DNA was transferred to Hybond-N membrane (Amersham International, Amersham, UK) by blotting overnight at room temperature using 20 x SSC as buffer (Diagram 7). The membrane was then washed in 6 x SSC and baked for 2 hours at 80°C.

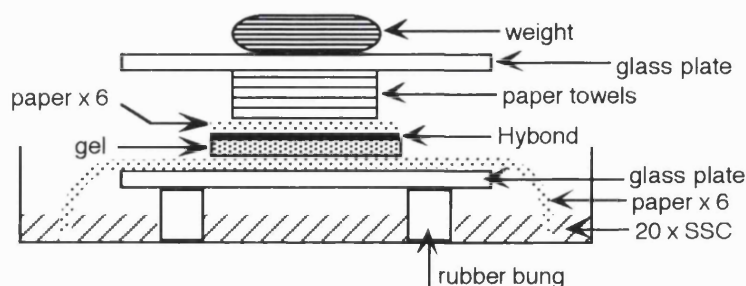


Diagram 7 : Method for transferring nucleic acids to a membrane by Southern or northern blotting.

2.5.iv. Prehybridisation

Blots were washed in 0.2 x SSC/0.5% SDS for 1 hour at 65°C to minimise background hybridisation then prehybridised in 12.5mls hybridisation buffer without probe for 4 hours at 42°C.

2.5.v. Hybridisation

50ng of labelled probe was boiled for 3 minutes then added with 12.5mls of a fresh batch of hybridisation solution to replace the prehybridisation buffer. Blots were incubated at 42°C overnight.

2.5.vi. Washes

The hybridisation solution was discarded and blots rinsed briefly in 2 x SSPE/0.1% SDS then washed for 15 minutes in 2 x SSPE/0.1% SDS at room temperature. Subsequent washes were 30 mins at 55°C in 2 x SSPE/0.1% SDS and 60 mins at 65°C in 0.1% SSPE/0.1% SDS. The blot was wrapped in Saran wrap and exposed to XAR-5 film (Kodak, Rochester NY, USA) for 2 to 6 days, depending on the probe, at -80°C.

2.5.vii. Stripping blots

To allow reprobing, the blots were stripped by incubating with 0.4M NaOH at 45°C for 30 mins followed by 0.1 x SSC/0.1%SDS/0.2MTris.HCl, pH7.5 for 30 mins at 45°C. The blots were checked to be free of probe by exposing to XAR-5 film overnight.

2.6. RNA preparation and Northern blotting

All solutions used are listed in the appendix, stocks and solutions 4 and were made up in RNase free distilled water, treated with DEPC if necessary. The probes used were as for Southern blotting.

2.6.i. RNA preparation

Total RNA was isolated from just preconfluent keratinocytes, fed the previous day, by removing the J2s in 3 x 75cm² flasks using versene then rinsing in PBS and lysing in 8 mls of guanidine thiocyanate (GIT) solution. The lysate was carefully added onto 4 mls of caesium chloride solution and spun in an ultracentrifuge (Beckman L8-70M) at 32,000 rpm, 20°C for 21 hours. The RNA pellet was washed in 70% ethanol and resuspended in distilled water plus 200µg/ml heparin, sodium salt (Sigma) and the OD₂₆₀ and 280 measured to determine the concentration. RNA was stored at -70°C.

2.6.ii. Preparation of poly A⁺ RNA

The method used was from Sambrook, Fritsh and Maniatis (1989). Columns were prepared using oligo-dT-cellulose (Sigma) suspended in 0.1m NaOH plugged with autoclaved glass wool. The column was washed with distilled water 3 times then with 1 x column loading buffer 5 times so the effluent reached pH 8. The RNA was heated to 65°C for 5 mins, cooled to room temperature then an equal volume of 2 x column loading buffer was added and the solution put down the column. The eluate was collected and 1 column volume of 1 x loading buffer applied to the column whilst still collecting eluate. The eluted solution was heated to 65°C for 5 mins, cooled and reapplied to the column. The column was washed with 5 volumes of 1 x column loading buffer. The poly A⁺ RNA was eluted using poly A⁺ elution buffer. The collected RNA was heated to 65°C for 3 mins, cooled and the NaCl concentration adjusted to

0.5M. The column was rinsed through with 0.1M NaOH followed by 1 x column loading buffer and the whole process repeated. Life is easier nowadays. The eluted poly A⁺ RNA was precipitated in 0.3M sodium acetate, pH 5.2, and 2 volumes of 100% ethanol at -20°C, microfuged, and the pellet air dried and resuspended in distilled water plus heparin, 200 µg/ml.

2.6.iii. Blotting

RNA was separated on a denaturing gel (Sambrook *et al.* 1989; Nicholson and Watt 1991) in a horizontal electrophoresis tank washed in 1M HCl and rinsed in distilled water. Gels were prepared by mixing 20mls of 37% formaldehyde, 10 mls 10 x HEPES buffer and 20 mls distilled water, heating to 56°C and adding 50 mls of 2% agarose (Gibco BRL, Paisley, UK) in water also at 56°C to form a 1% agarose gel. 3 µg of poly A⁺ RNA per sample in 4 µl of water was added to 16 µl sample buffer and heated to 70°C for 10 mins. 4 µl loading buffer was added and the gel run at 100V for 4 hours in northern running buffer. RNA markers (Gibco BRL, Paisley, UK) were run in one lane.

The RNA was transferred overnight in 20 x SSC to Hybond-N membrane (Amersham International, Amersham, UK) (Diagram 7), the membrane was rinsed in 6 x SSC, air dried, baked at 80°C for 1 hour, exposed to uv light for 4 mins, then wrapped in Saran wrap until hybridisation.

2.6.iv. Prehybridisation

In 12.5 mls hybridisation buffer without probe at 42°C for 4 hours.

2.6.v. Hybridisation

50ng of labelled probe was boiled for 3 minutes then added with 12.5mls of a fresh batch of hybridisation solution to replace the prehybridisation buffer. Blots were incubated at 42°C overnight.

2.6.vi. Washes

The hybridised blot was rinsed, then incubated twice at 42°C for 15 mins, in 2 x SSC/0.1% SDS followed by 30 mins in 1 x SSC/0.1% SDS at 42°C. The final wash was 0.1 x

SSC/0.1% SDS at 65°C for 15 mins. Blots were then wrapped in Saren wrap and exposed to XAR-5 film (Kodak, Rochester NY, USA) for between 1 and 7 days at -80°C.

2.6.vii. Stripping Blots

Before reprobing, the blots were stripped in 0.005M Tris.Cl, pH 8, 0.002M EDTA, pH 8 plus 0.1 x Denhardt's solution for 2 hours at 65°C. The blots were checked to be free of probe by exposing to XAR-5 film (Kodak, Rochester NY, USA) overnight.

2.7. In situ hybridisation (ISH)

All solutions used are listed in the appendix, stocks and solutions 5.

2.7.i. DNA-DNA hybridisation - Y chromosome

DNA-DNA hybridisation to detect the Y chromosome for lineage analysis was by non-isotopic ISH using a biotinylated probe.

2.7.i.a. Probe preparation

The probe for the Y chromosome was prepared from pHY2.1 (Cooke *et al.* 1982) (Amersham International, Amersham, UK). The plasmid was used to transform competent *E. coli* DH5 α bacteria and the plasmid DNA isolated as above. The plasmid was cut with HaeIII and the 2.1 kb insert isolated by gel purification, filtration of the cut band in a microfuge tube and phenol extraction. The probe hybridises to a long repeated sequence on the Y chromosome which is present in only small parts of the autosomal chromosomes.

2.7.i.b. Probe labelling

The insert HY2.1 was biotinylated using a nick translation kit (Gibco BRL, Paisley, UK) and biotin-7-dATP (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. The reaction conditions were 0.02mM each dGTP, dCTP, dTTP, 50mM Tris.HCl (pH 7.8), 5mM MgCl₂, 10mM β -mercaptoethanol, 10 μ g/ml nuclease free BSA, 1 μ g of HY2.1 DNA, 0.4mM biotin-7-dATP (in storage buffer), 0.04 U/ μ l DNA polymerase I / 4pg/ μ l DNase I (in storage buffer). The solution was well mixed, incubated at 15°C for 90 mins and stopped by

adding EDTA, pH8, to a concentration of 30mM and SDS to a concentration of 0.125%. The probe was recovered by precipitation in 0.3M sodium acetate and 2 volumes of ethanol.

2.7.i.c. Tissue preparation

2.7.i.c.I Whole cells

Cells grown on washed, autoclaved 4 well slides (C.A. Hendley Ltd, Loughton, UK) were fixed in 4% paraformaldehyde and rinsed in PBS.

2.7.i.c.II Dispace sheets

Confluent sheets were removed with dispace, washed in E4 DCS then PBS and fixed in 4% paraformaldehyde. The sheets were then processed and paraffin embedded by the ICRF Histopathology Unit and sectioned onto silane coated 4 well slides. Paraffin sections were dewaxed by soaking in xylene twice for 10 mins then rehydrated through graded alcohols (100%, 95%, 70%, 50% ethanol), 3mins each and soaked for 5 mins in PBS.

2.7.i.c.III Whole mounts

This technique allowed confluent keratinocytes to be used without shrinkage artefact seen with dispace sheets allowing accurate measurement of lineage marked (male) colony size. It also avoided the need to cut and hybridise large numbers of sections.

3-5 day post-confluent sheets of keratinocytes, grown in 35mm dishes, were carefully scraped from the dish using a small spatula and the epidermal sheet turned over and placed on a glass slide, 40mm x 80mm (Chance Propper Ltd, Smethwick, Warley, UK) using fine forceps. The basal keratinocytes were now upwards and the suprabasal cells in contact with the glass slides. The mount was carefully spread and allowed to air dry. When fully dry the cells were fixed in 4% pfa for 10 mins then rinsed in PBS.

2.7.i.d. Prehybridisation

This was the same for all types of tissue to be hybridised except for the proteinase K step. The length of incubation varied from batch to batch and had to be titrated for each type of experiment and each new batch of enzyme.

The slides or dishes were soaked in 0.02N HCl for 10 mins, washed in PBS 10 mins x 2 then dipped in 0.1% Triton X-100/PBS for 2 mins and washed twice for 5 mins in PBS. Digestion with pre-digested proteinase K (Sigma), 10µg/ml in TE7.4, was at 37°C for 5-10 mins (tissue sections), 10-15 mins (whole cells) or 15-45 mins (whole mounts). After the proteinase step, slides were washed twice in 2mg/ml glycine/PBS for 10 mins then post fixed in 4% paraformaldehyde for 5 mins. Slides were rinsed in distilled water and dehydrated through graded alcohols (50%, 70%, 95%, 100% ethanol) for 3 mins each and allowed to air dry.

2.7.i.e. Hybridisation

Labelled probe was added to hybridisation solution at 1ng/µl, applied to the dehydrated tissue and covered with a glass cover slip, ensuring no air bubbles were present. This required 6µl for 4 well slides and 50µl for the whole mounts. Cover slips for the whole mounts were 35mm diameter and specially made by Chance Propper Ltd, Smethwick, Warley, UK. The cover slips were sealed with vulcanising rubber bicycle tyre repair solution (Weldtite Products, South Humberside, UK) then placed into a pre-heated large biscuit tin and incubated at 98°C for 20 mins. This simultaneously denatures the probe and target DNA. The slides were then placed on ice for at least 10 sec. and hybridised, still sealed, at 42°C in a sealed humidified container overnight.

2.7.i.f. Washing

The cover slips were removed with fine forceps, very carefully under 2 x SSC, the slides placed in fresh 2 x SSC and washed at room temperature twice for 10 mins. Further washes were 30 mins at 62°C in 2 x SSC, twice for 10 mins at room temperature in 0.1 x SSC, once for 30 mins at 42°C in 0.1 x SSC and once in PBS for 5 mins at room temperature.

2.7.i.g. Immunohistochemistry

Slides were blocked in 1% H₂O₂ for 5 mins, rinsed in PBS and then blocked in 10% FCS/PBS for 20 mins. This was followed by incubation for 30 mins at room temperature in mouse anti-biotin antibody (Dako A/S, Glostrup, Denmark)(1:200 in 10% FCS/PBS) and three 5 min washes in PBS then a further incubation in biotinylated rabbit anti-mouse IgG antibody

(Dako)(1:100 in 10% FCS/PBS) for 30 mins at room temperature. After three washes in PBS the slides were incubated with streptABC/HRP (Dako) in 0.05M Tris.HCl, pH 7.6 for 30 mins and washed 3 x 5 mins in PBS.

2.7.i.h. Development

Slides were developed in DAB and counterstained in haematoxylin, as described in the section on cytochemistry, then dehydrated through graded alcohols as before followed by 1 min in acetone and 1 min in xylene. The slides were mounted in DPX (BDH) and coverslipped.

2.7.ii. RNA-RNA hybridisation

This was used to investigate the level of steroid sulphotase (STS) or β_1 integrin mRNA in cultured keratinocytes. Cells were grown on 8 chamber glass slides (Nunc, Gibco BRL, Paisley, UK). All solutions were made up in DEPC treated water or PBS. All prehybridisation and hybridisation steps were carried out using baked glassware, RNase free pipettes and tips, and any plastic was soaked in 10% H_2O_2 .

2.7.ii.a. Probe preparation

2.7.ii.a.I β_1 integrin probe

The probe was prepared from pFNR β_1 , kindly provided by E. Ruoslahti (Giancotti and Ruoslahti 1990), by Dr. Joseph Carroll, Keratinocyte lab, ICRF. The 678 bp PstI-HindIII fragment of pFNR β_1 was subcloned into pGEM3 (Promega, Madison, WI, USA) to produce p β aGEM3.

2.7.ii.a.II STS probe

The STS probe was prepared from pSVL-STS (see probe preparation for Southern blotting) by subcloning a 776 bp restriction fragment from a digest with PstI and XbaI (STS776) subcloned into Bluescript II SK+ (Stratagene, La Jolla, CA, USA). 5 μ g of pSVL-STS was digested with PstI and XbaI in the recommended buffers at 37°C for 1 hour and the digest gel purified to obtain STS776. The vector, Bluescript II SK+, was cut and purified in a similar way. After phenol extraction and ethanol precipitation with 10 μ g of carrier tRNA

(Sigma) the DNA was resuspended in 10 µl of TE8 containing 0.1 µg RNase A (Sigma) and incubated for 30 mins at 37°C. The two pieces of DNA were ligated using 1 µl of the Bluescript prep. and 3 µl of the STS776 prep., plus 200U T4 DNA ligase (New England Biolabs, Beverly, MA, USA) and the recommended buffer, in a total volume of 10 µl at 16°C overnight.

3 µl of the ligation solution was used to transform 100 µl of competent *E. coli* DH5α as described above and colonies picked, cultured and screened using the miniprep method described earlier. The presence of the insert in the vector was checked by restriction digest with PstI/XbaI and also a digest with BssHII. The correct orientation was ensured by digest with HindIII.

2.7.ii.b. Probe labelling

pβaGEM-3 was cut with EcoRI (SP6 promoter for antisense transcript) or BamHI (T7 promoter for sense transcript). pSTS776 was digested with BssHII which removed the T3 and T7 promoters plus the 776 bp insert. T3 transcribed for the sense and T7 for the antisense transcript. After digestion the DNA was extracted with phenol and chloroform then precipitated in 0.3M sodium acetate and ethanol. It was not necessary to gel purify the respective fragments before *in vitro* transcription.

Probes were labelled with ³⁵S-rUTP (Amersham International, Amersham, UK) using an RNA transcription kit (Stratagene, La Jolla, CA, USA). The reaction conditions were 40mM Tris.HCl, pH 8, 8mM MgCl₂, 2mM spermidine, 50mM NaCl, 0.4mM of each rATP, rGTP, rCTP, 0.04mM cold rUTP and 0.03M DTT. To this was added 1 µg of restricted DNA, 1U RNase-Block II, 3.5 µl of ³⁵S-rUTP (10mCi/ml, 800 Ci/mmol) and 10 U of T3 or T7 RNA polymerase or 20 U of SP6 RNA polymerase (Promega, Madison, WI, USA). The total reaction volume was 25 µl and incubation was 2 hours at 37°C. The transcript was precipitated in 2.5M ammonium acetate and 2 volumes of ethanol at -20°C, spun down in a microfuge and resuspended in 25 µl of DEPC water containing 10mM DTT and 1 in 100 RNase-Block II.

The number of counts per µl was determined by scintillation counting in Ecofluor.

2.7.ii.c. Checking the probe

0.5 x 10⁶ counts of labelled probe were run on a polyacrylamide gel to check the length of the probe. 25mls of Acryl-a-mix 6 (Promega, Madison, WI, USA) was mixed gently with 200µl of 10% ammonium persulphate and poured into a 0.5mm gel using a Mighty Small II (Hoefer) apparatus. The gel was allowed 1 hr to set and pre run for 15 mins at 15W, with TBE running buffer. The wells were flushed with TBE and loaded with 0.5 x 10⁶ labelled probe in loading buffer, boiled for 2 mins, and run at a constant 20W (~450V). The dye (xylene cyanol) runs at 150bp, the RNA markers were from Gibco BRL and labelled with ³⁵S. The gel was fixed in 10% methanol, 5% acetic acid and exposed for 2 hours with XAR-5 film (Kodak, Rochester, NY, USA).

2.7.ii.d. Prehybridisation

Slides were fixed in 4% paraformaldehyde/PBS for 10 mins at room temperature (at this point slides could be dehydrated in graded alcohols and stored at -80°C then rehydrated when required) then incubated with 20µg/ml predigested proteinase K (Sigma) in TE7.4 for 10 mins at 37°C. The slides were washed twice in 2mg/ml glycine/PBS for 5 mins and post fixed in 4% pfa/PBS for 5 mins. After rinsing in PBS the slides were acetylated using 1.25mls of acetic anhydride added, while stirring, to 500 mls of 0.1M triethanolamine, pH 8 for 10 mins, washed in PBS for 10 mins and dehydrated through graded alcohols, containing 0.1% DEPC.

2.7.ii.e. Hybridisation

1 x 10⁶ counts of labelled probe per slide were added to hybridisation buffer and heated to 80°C for 2 mins. 30 to 50µl of hybridisation solution was added to each slide and coverslipped using a 20 x 50mm coverslip rinsed in 70% ethanol and dried. Slides were placed in a humidified, RNase free chamber containing 1 x salts and 50% formamide to be hybridised overnight at 55°C.

2.7.ii.f. Washes

The slides were put in 50% formamide, 1 x salts at 55°C twice, for 30 mins followed by 4 x 15 min washes in TNE buffer. Slides were then incubated in TNE and 100µg/ml RNase

A at 37°C for 30 mins. Final washes were twice in 2 x SSC, 65°C for 30 mins and once in 0.5 x SSC, 65°C for 30 mins. The slides were dehydrated in graded alcohols, containing 0.3M ammonium acetate, as before and allowed to air dry.

2.7.ii.g. Autoradiography

Autoradiography was performed in a dark room (15W safe light, 902 filter). 12.5 mls of water at 45°C was made up to 25 mls with Ilford K5 emulsion (Ilford, Mobberley, UK) and left in a water bath at 45°C for 20 mins to melt. The emulsion was carefully stirred and left for another 20 mins so that there were no bubbles. Slides were dipped in emulsion, the reverse side wiped and then left to dry on an ice cold metal plate, in total darkness, or in a locked 'fridge (with no little man turning the light on!) for 3 hours or overnight. When dry, the slides were placed in a light tight plastic slide box containing silica gel which was sealed and left at 4°C for 3 to 14 days according to the probe and expected signal.

2.7.ii.h. Development

Slides were developed under the safety light in D-19 developer (Kodak, Rochester, NY, USA), 1:1 in distilled water, for 4 mins, stopped in 1% acetic acid for 1 min, washed in tap water for 1 min and fixed in Kodak film fixative, 1:5 in distilled water for 5 mins. The developed slides were rinsed in running tap water for 30 mins, counterstained if required (usually 10% Geimsa for 5 mins, washed in water for 3 mins) and air dried then mounted in DPX (BDH).

2.7.iii. Double labelling *in situ* hybridisation

Some experiments looking at the levels of integrin message in relation to individual clones in confluent whole mounts were attempted. In these experiments the prehybridisation steps were as for DNA-DNA hybridisation and the hybridisation buffer included 10mM DTT as well as the ³⁵S-β₁ integrin probe and the biotinylated HY2.1 probe for the Y chromosome. All solutions were made with DEPC treated distilled water or PBS. Posthybridisation washes were also as for DNA-DNA hybridisation with the addition of a 30 min incubation in 100μl/ml RNase A in PBS after the SSC washes. After the antibody and DAB steps the slides were

dehydrated in graded alcohols, containing 0.3M ammonium acetate, then coated in emulsion, exposed and developed as in RNA-RNA hybridisation.

2.8. Transgenic mice

The animal work was done by the ICRF animal unit and much of the microinjection of embryos by the ICRF transgenic unit. All solutions used are listed in the appendix, stocks and solutions 6.

2.8.i. Production of DNA constructs

Bovine keratin VI (equivalent to the human keratin 10 promoter) promoter was supplied as pKBVI-KSS, given by Dr. J. L. Jorcano, CIEMAT, Madrid (Blessing *et al.* 1989; Bailleul *et al.* 1990). The β_1 integrin cDNA was a generous gift from E. Rhouslati in pFNR β_1 (Giancotti and Ruoslahti 1990), derived from pECE with the β_1 DNA cloned into the EcoRI site. The α_2 construct was kindly given by Dr. J. Taylor-Papadimitriou and Dr. F. Berdichevsky (ICRF) cloned between the XbaI and KpnI sites of pJ7 Ω , which was originally derived from pSV2gpt and produced in Dr. H. Land's laboratory (ICRF).

2.8.i.a. pECEK10 β_1

A 3.4 kb proximal portion of the KVI promoter was cut from pKBVI-KSS using KpnI and BglII. This fragment (K10) was isolated by agarose gel purification and extracted using GeneClean. pFNR β_1 was cut at the KpnI site, which is upstream of the β_1 insert and the enzyme inactivated by heating at 65°C for 20 mins. To ligate the promoter into pFNR β_1 both linear pieces of DNA were blunt ended using T4 DNA polymerase (New England Biolabs, Beverly, MA, USA) in the supplied buffer for 30 mins at 37°C with 100 μ g/ml acetylated BSA and 0.2mM each of dATP, dGTP dTTP, dCTP and then purified using GeneClean. The two blunt ended pieces were ligated in equal proportions using T4 DNA ligase (New England Biolabs, Beverly, MA, USA) in the recommended buffer at 16°C overnight.

2.8.i.b. pJ7 Ω K10 α_2

The KpnI / BglII restriction fragment of the KVI promoter was ligated into the XbaI site of pJ7 Ω after blunt ending as for pFNR β_1 .

2.8.i.c. Transformation of bacteria

Competent HB101 bacteria were transformed using the method described in the section on DNA preparation, grown overnight on ampicillin L-agar plates and colonies selected for miniprep.

2.8.i.d. Selection of colonies

Miniprep. DNA was screened by cutting with SacI which also checked the orientation of the KVI insert. The positive clones with the correct orientation were selected and one of each construct grown up to produce the DNA for injection.

2.8.ii. Production of DNA for injection

2.8.ii.a. K10 β_1

The piece of DNA for injection was cut out from the construct pECEK10 β_1 by digestion with SphI followed by precipitation in ethanol then a further digest with Sall. The 7.1kb band containing the K10 promoter, the β_1 DNA and the SV40 poly A tail was separated on an agarose gel and purified using GeneClean resuspended in 100 μ l TE8 then further purified using an Elutip-d column (Schleicher and Schuell, Dassel, Germany) as per the manufacturer's protocol. The DNA was then precipitated in ethanol and the pellet washed twice with 70% ethanol and resuspended in injection buffer (sterile PBS). The concentration of the DNA was checked by running on an agarose gel against serial dilutions of DNA of a similar size and a known concentration. DNA for injection was diluted to 3ng/ μ l.

2.8.ii.b. K10 α_2

The DNA for injection was cut from the pJ7 Ω K10 α_2 construct using the restriction enzymes BspHI and Sall which gave a 9.7 kb fragment containing the K10 promoter, the integrin α_2 DNA and the SV 40 poly A tail. This was purified as for the K10 β_1 DNA described above.

2.8.iii. Injection of DNA into mouse embryos

Animals were kept and bred by the ICRF transgenic unit at Clare Hall who also performed much of the DNA injection and all of the surgery.

2.8.iii.a. Production of ova

Superovulated females (CBA/CS5 black F1 hybrids) were produced by giving intra-peritoneal follicle stimulating hormone (Folligon, pregnant mares serum, Intervet Labs Ltd), 5IU, on day 0 to a 4 week old mouse followed by intra-peritoneal human chorionic gonadotrophin / lutinising hormone (Chorulon), 5IU, on day 2 and adding a stud male mouse. Fertilisation was checked by looking for a copulation plug and ova were harvested the next day.

2.8.iii.b. Microinjection of fertilised ova

Ova were treated briefly with hyaluronidase (300µg/ml in M2) to remove cumulus (follicular) cells and washed in M2. The viable ova were selected (2 pronuclei, polar bodies, no adherent sperm) and incubated at 37°C, 5% CO₂ for 60 mins. Each ovum was injected with 1-2pl of DNA (3ng/µl) into one of the pronuclei using a glass micropipette and incubated for 60 mins at 37°C, 5% CO₂ in M2. 20-25 injected ova were implanted into 6-12 week old pseudopregnant (mated in oestrus with a vasectomised male) females through the fallopian ampulla (10-12 ova per tube).

2.8.iv. Isolation of genomic DNA from mice

Fresh ear tags from the mice were placed in ear buffer containing fresh proteinase K and digested for overnight at 55°C in microfuge tubes. The digest was heated to 100°C for 10 mins and cooled to room temperature. Any debris was spun out and the DNA stored at 4°C.

2.8.v. Screening of mouse DNA for transgene by PCR

The reaction mixture for PCR, containing dNTPs, primers and enzyme, was prepared as shown in the appendix, stocks and solutions section 6. Each reaction tube contained 11µl reaction mixture, 35µl water and 4µl of the ear snip DNA preparation. The final concentrations for the PCR were 0.4mM each, 20µM each primer and 0.6mM MgCl₂. The primers used were prepared by the ICRF oligonucleotide synthesis service and are shown in the PCR primer list. The conditions for the PCR were melting for 1 min at 95°C (extended to 4 mins for the first cycle), annealing for 1 min at 55°C and elongation for 1 min at 72°C. The cycle was repeated 30 times.

2.8.vi. Screening for transgenic mRNA by *in situ* hybridisation and nuclease protection assay

All this work was done in the keratinocyte lab., ICRF by Dr. Joseph Carroll. Paraffin and frozen sections of mice positive for transgenic DNA by PCR were hybridised with the appropriate ³⁵S labelled integrin riboprobe using the protocol for RNA-RNA *in situ* hybridisation to detect transgenic mRNA. The presence of message was also evaluated using a nuclease protection assay.

2.9. Microscopy and Image Analysis

The following microscopes were used :

Light, phase contrast, dark field, immunomicroscopy	Zeiss Axiophot
Drawing tube microscope	Lietz Diaplan
Drawing tube lens	12.5 x/18 periplan
Image scanner	LaCie □Silverscan
Image analysis	Apple Macintosh II
Image Programmes	ColorStudio 1.5 (Letraset, Paramus, NJ, USA) NIH Image 1.45 (Freeware)
Confocal microscope	BioRad MRC 600

2.10. Source of Keratinocytes

Name	Sex	Site of origin	Age of donor	Primary isolation	Given by
RXLI A	M	Thigh	10 y	Explant	Dr. M. Judge
RXLI B	M	Thigh	6 y	Explant	Dr. M. Judge
RXLI C	M	Thigh	Unknown	Explant	Dr. M. Judge
XYA	M	Buttock	47 y	Explant	
XYB	M	Forearm	20 y	Trypsin	
MEHA	F	Groin	5 y	Unknown	Dr. N. Kehinde
ka	F	Breast	22 y	Trypsin	
Other lines (A, Z, kb, kc, kj)	M	Foreskin	Infant	Various	

2.11. PCR primers

Primer target	Primer sequence 5'-3'
Keratin 10 promoter 1004-1034	GCAAGTAAGCAAACTCTAACACATGTGGG
β_1 integrin	GCTGGTGTTGTGCTAATGTAAGGCATCACA
α_2 integrin	ATAGGTCAACAGGACATTTATACACATCTC
Actin 5'	GTTTGAGACCTTCAACACCCC
Actin 3'	GTGGCCATCTCCTGCTCGAAGTC

2.12. Antibody List

2.12.i. Primary Antibodies

Specificity	Name	Species	Origin	Reference
Integrin α_2 subunit, extracellular domain	HAS6	Mouse	Keratinocyte lab. ICRF	(Tenchini <i>et al.</i> 1993)
Integrin β_1 subunit, extracellular domain	CD29	Mouse	Janssen Biochemica, Geel, Belgium	(Humphries 1990)
Integrin β_1 subunit, extracellular domain	4B7	Mouse	J. Marshall and I. Hart, ICRF, St. Thomas's Hosp., London.	
P-Cadherin, extracellular domain	NCC-CAD-299	Mouse	M. Takeichi	(Shimoyama <i>et al.</i> 1989)
E-Cadherin, extracellular domain	HECD-1	Mouse	M. Takeichi	(Shimoyama <i>et al.</i> 1989)
Steroid sulphatase	anti-STS	Rabbit	K. von Figura	(Conary <i>et al.</i> 1986)
Involucrin	DH1	Rabbit	D. Hudson, Keratinocyte lab, ICRF	(Dover and Watt 1987)
Keratinocyte keratins 10,18,4/14,6/16	LP34	Mouse	ICRF	(Lane <i>et al.</i> 1985)
Collagen IV		Mouse	ICRF	
HLA subtypes	W6/32, BB7.2, GAP A3, BB7.1	Mouse	Dr. J. Bodmer, ICRF	

2.12.ii. Secondary antibodies

Fluorescein conjugated anti-mouse and anti-rabbit antibodies were obtained from Sigma Chemical Co., Poole, UK or Jackson, Pennsylvania, USA. Biotin conjugated anti-rabbit or anti mouse antibodies and fluorescein conjugated streptavidin was bought from Amersham International, Amersham, UK or Dako A/S, Glostrup, Denmark.

CHAPTER THREE

LINEAGE ANALYSIS - TECHNIQUES

3.1. Introduction

To begin to understand stem cell fate in the epidermis it was necessary to assess the value of potential lineage markers. In the proposed experiments I intended to investigate colonies derived from a single cell within a confluent cultured epidermal sheet. This required the seeding of marked cells at a limiting dilution and detecting low numbers of colonies in a background of negative cells. A marker was needed that was stable, inherited through cell generations, expressed throughout differentiation, detectable in a large percentage of the marked cells and that had no effect on the behaviour of the keratinocytes. To analyse the results the marker needed to be detectable in the culture dish and in tissue sections.

The use of retroviruses to mark cell lineage has been widely used particularly in developmental studies of lineage (Price 1987; Price *et al.* 1991) and for studying haemopoietic stem cell fate (Spain and Mulligan 1992). The BAG-F retrovirus codes for the expression of β -galactosidase (Price *et al.* 1987) which can then be used as an inheritable marker of cell lineage. Advantages of this technique are that β -gal can be detected easily with a simple staining procedure and that mixes of marked and unmarked cells can be derived from the same source. However cells can switch off expression which may be a significant disadvantage if such an event was common enough.

The other strategy I used was to detect the Y chromosome, present only in male cells, as a marker to distinguish male colonies in a background of female keratinocytes. This technique, however, does not allow 100% detection in tissue sections (Brain *et al.* 1989) but the marker is stable.

Cells also display normal variations between different lines which I attempted to exploit as markers of lineage. One possibility was to use variations in the expression of HLA class I epitopes between keratinocytes derived from different individuals.

I also investigated the possibility of using cells derived from boys with the inherited skin disorder, recessive X-linked ichthyosis, who are deficient in steroid sulphatase (STS)

(Williams 1983). I have attempted to discriminate normal cells, expressing STS, from RXLI cells which do not express STS.

3.2. Results

3.2.i. Attempt to detect lineage using anti-HLA subtype antibodies

Three antibodies were tested, BB7.2 (HLA A2n69), BB7.1 (HLA B7) and GAPA3 (HLA A3) by staining a panel of cultured keratinocyte lines (lines a, s, t and z).

Although keratinocytes are known to express class I HLA molecules on the cell surface (Gielen *et al.* 1988; Symington and Santos 1990), and the positive control antibody, W6/32 (against an epitope common to HLA groups A, B and C), stained positive in all the cell lines investigated, the staining with HLA subtype antibodies was only weakly positive at best. For this reason it was not possible to distinguish between different cell lines and this method could not therefore be used for lineage analysis.

3.2.ii. Characterisation of recessive X-linked ichthyosis cell lines

Three strains of keratinocytes derived from boys with recessive X-linked ichthyosis (RXLI), and therefore deficient in STS, were used in these experiments. If it was possible to detect STS in cultured normal keratinocytes then RXLI cells could be used as negative background cells in lineage experiments using mixtures of normal and RXLI keratinocytes.

Although RXLI keratinocytes show no evidence of hyperproliferation *in vivo*, before any lineage analysis was attempted the RXLI cell strains were characterised to determine that growth and differentiation in culture were identical to normal keratinocytes and that the clinical diagnosis could be confirmed by Southern and northern blotting. Growth rates were determined by comparison with normal cell lines and differentiation in culture was measured by determining the number of involucrin positive cells (Dover and Watt 1987) and cornified cell envelopes (Sun and Green 1976) at confluence.

The genomic DNA, production of messenger RNA and expression of STS was investigated in cultured normal keratinocytes and RXLI cells.

3.2.ii.a. Morphology in culture

In standard Rheinwald and Green cultures the RXLI cells showed no obvious differences in morphology when compared to normal keratinocytes (Figure 1a,c,e).

3.2.ii.a.I Culture of RXLI cells in lipid free medium

Retinoids are known to suppress cornified envelope production in culture (Green and Watt 1982). To investigate if the RXLI cells would show any differences in morphology when compared to normal cells I looked at growth of keratinocytes in lipid free medium, which contains no retinoic acid (vitamin A). RXLI cells and normal cells showed no obvious changes in morphology.

3.2.ii.a.II Culture of RXLI cells on dermal equivalents

The morphology of RXLI and normal keratinocytes were compared when grown on collagen coated culture inserts and on deepidermidised dermis. There was no difference in morphology when grown on inserts, with no increase in the thickness of the cornified layer. When grown on deepidermidised dermis derived from breast the RXLI cells showed some thickening of the stratum corneum (Figure 2) but this was variable and sometimes also seen in normal cells.

3.2.ii.b. Growth rate of RXLI keratinocytes

The three lines of RXLI keratinocytes (A, B and C) were seeded at 2×10^4 , per 35mm dish. Duplicate dishes were harvested three times a week then the number of cells counted. Counting was continued until the keratinocytes were post-confluent and beginning to blister. Growth rates were compared with dishes of normal keratinocytes harvested and counted in the same way. All three of the RXLI lines had growth rates similar to normal keratinocytes (Figure 3a).

3.2.ii.c. Involucrin expression

The capacity of RXLI cells for terminal differentiation was compared to that of normal cells by counting the number of involucrin positive cells at confluence, after 20 days growth in

35mm dishes seeded at 2×10^4 per dish. The percentage of involucrin positive cells was around 30% in both the RXLI and normal keratinocytes (Table 1 and Figure 3b).

Keratinocyte strain	No. of exps.	Mean involucrin exp. (% cells) +/- SEM
RXLI A	4	30.97 +/- 0.80
RXLI B	5	31.99 +/- 1.29
RXLI C	3	31.33 +/- 3.48
Normal	5	29.92 +/- 1.45

Table 1: Involucrin expression in RXLI keratinocytes. There was no significant difference in expression between RXLI and normal cells (Unpaired *t*-test: df7, $p=0.47$; df8, $p=0.26$; df6, $p=0.59$ for RXLI A, B and C respectively).

3.2.ii.d. Envelope production

The number of cornified cell envelopes was determined at confluence after 20 days culture in 35mm dishes seeded at 2×10^4 . RXLI keratinocytes formed a similar number of envelopes in culture when compared to normal, approximately 5% (Table 2 and Figure 3b).

Keratinocyte strain	No. of exps.	Mean no. of envelopes (% cells) +/- SEM
RXLI A	5	5.50 +/- 0.43
RXLI B	7	4.68 +/- 0.42
RXLI C	3	5.09 +/- 0.72
Normal	5	5.85 +/- 0.94

Table 2: Cornified envelope formation in RXLI keratinocytes. There was no significant difference in the number of envelopes formed by RXLI and normal cells (Unpaired *t*-test: df8, $p=0.74$; df10, $p=0.23$; df6, $p=0.60$ for RXLI A, B and C respectively).

3.2.ii.e. Southern blots

The genomic DNA from cultured RXLI and normal cells was digested with EcoRI. Southern blots using a probe to STS showed 5 major bands in DNA from normal keratinocytes at 12, 9.5, 6.2, 5.0 and 4.3 kb. The two most prominent bands were 12 and 6.2

kb. The signal from female keratinocytes was approximately twice that from male. No bands were seen in blots of RXLI DNA (Figure 4a).

3.2.ii.f. Northern blots

Poly A⁺ RNA was isolated from the three RXLI lines and two normal lines and analysed by northern blotting and hybridised with the STS probe. Normal cells contained messenger RNA of two sizes, 7.0 and 5.2 kb, but there was no hybridisation to the RXLI RNA (Figure 4b). Loading and integrity of the RNA were checked by hybridisation to the involucrin probe, which gave a band of 2.1 kb in all lanes.

3.2.ii.g. Immunofluorescence

Cultured keratinocytes were immunofluorescently stained using antibody to STS. Staining of individual colonies showed mainly bright spots of cell surface staining in suprabasal cells and diffuse cytoplasmic staining in basal keratinocytes (Figure 1b,d,f). Staining of confluent cultures grown on 8 well slides showed granular staining of the most suprabasal cells at the cell-cell boundaries (Figure 1g,h).

Frozen sections of foreskin and confluent sheets of cultured normal keratinocytes showed a rather diffuse staining pattern with some cell surface staining. (Figure 1i) Expression of STS in foreskin was much higher than in cultured keratinocytes.

The background staining with the anti-STS antibody was high when applied to RXLI cells, especially in frozen sections. Expression of STS in culture was considerably lower than *in vivo*, and particularly low in basal keratinocytes. It is also possible that dispase digests the protein. These factors made identification of normal and RXLI cells in mixed culture unreliable, particularly in tissue sections of dispase sheets. Lengthy analysis was difficult with fluorescent antibodies because of fading and the signal in cultured cells was not high enough for reliable detection using peroxidase conjugated secondary antibody.

3.2.ii.h. In situ hybridisation

In situ hybridisation using the riboprobe STS 776 was performed on colonies of cultured cells and on paraffin embedded sections of dispase sheets. The signal in cultured cells was barely detectable (Figure 5) and no signal was seen above background in tissue

sections of dispase sheets. For this reason *in situ hybridisation* for STS was not of use in analysis of lineage.

3.2.iii. Marking of lineage using β -galactosidase (β -gal)

3.2.iii.a. Viral Titres

The retroviral titres were estimated by infecting J2 cells with supernatant from the packaging line. The infected J2s were split, selected in G418 and the colonies counted. The results show that the PA 317 cells produced 6.7×10^4 colony forming units per ml.

3.2.iii.b. Infection and culture of keratinocytes

Keratinocytes were infected with BAG-F retrovirus, coding for β -gal expression, using the producer line PA 317, and selected using G 418. The selected keratinocytes stained positive for β -galactosidase and were detectable when mixed with uninfected keratinocytes. Providing that the differentiated layers of the cultured epidermal sheets were not too thick then the stain penetrated to all layers of the culture. If there were significant layers of differentiated cells the cultures could be stained after paraffin section, allowing penetration of the stain to all levels of the culture. Retroviral infection seemed to reduce the capacity of the keratinocytes to survive through multiple passages: the maximum passage number achieved was 3 passages on from infection and after this the cultures became senescent.

Other disadvantages of infection of keratinocytes with BAG-F retrovirus were also seen and have been reported by others (Garlick *et al.* 1991 and Y. Barrandon, personal comm.). Some colonies showed no β -gal expression despite having the ability to grow in medium containing G 418 and other colonies had a mosaic pattern, suggesting that the expression of the enzyme was being switched off during cell division or differentiation. The number of colonies showing these features was variable and tended to increase with passaging. One infection showed over 50% mosaic colonies after selection in G 418. The features of these colonies are shown in Figure 6.

3.2.iv. Marking of lineage using the Y chromosome

Non isotopic *in situ* hybridisation using the probe to a repeated sequence on the Y chromosome, HY2.1, allowed colonies of male and female cultured cells grown on glass slides to be distinguished from one another, as shown in Figure 7a,b. After development with DAB the hybridised probe could be seen as a brown dot in the nucleus. The same technique could also be used to detect male cells in paraffin embedded sections of dispase treated sheets of keratinocytes. Characteristically the Y chromosome was seen close to the nuclear membrane. This meant that on sections, because cutting the sections bisects the nuclei, the Y chromosome sequence was no longer present in every partial male cell nucleus left in the section. Only approximately 60% were positive, making detection of small male colonies with HY2.1 in a background of female cells difficult. Hybridisation of some cell lines such as SCC4 and vp (HPV 16 transformed keratinocytes (Pei *et al.* 1991)) showed that these cells contained more than one copy of the repeated sequence (Figure 7c) and therefore it was possible to detect a higher proportion of male cells in sections, for instance vp showed more than 90% of cells positive as shown in Figure 8b.

By using cells grown from biopsies kindly given by men with an extra Y chromosome (XYY), I was able to increase the detection of male cells in tissue section by 25%-30% (Figures 8c and 9). This allowed a significant improvement in interpretation of the sections and colonies derived from male cells in a background of female cells were more easily seen. Attempts using thick sections did not improve the detection rate.

Experiments in which the probe was hybridised to whole mounts of cultured epidermal sheets gave close to 100% detection of male cells (Figure 9) and are described in more detail in Chapter 4.

Figure 1 : The morphology and immunofluorescent staining using anti-STS antibody in normal and RXLI keratinocytes in Rheinwald Green cultures and neonatal foreskin.

a-f : Morphology (phase contrast) (a,c,e) and anti-STS immunofluorescence (b,d,f) in RXLI (a,b) and normal (c-f) keratinocyte colonies in Rheinwald Green cultures. RXLI colonies have similar morphology but are negative with antibody staining. Normal colonies show diffuse cytoplasmic staining in the basal cells around the edge of the colony (short arrow) and focal membrane staining in the differentiated suprabasal cells (long arrow) (f).

g,h : Anti-STS immunofluorescence in a confluent Rheinwald Green culture of normal keratinocytes, showing phase contrast (g) and granular staining highlighting the superficial suprabasal cell membranes (h).

i : Anti-STS immunofluorescence in normal neonatal foreskin (frozen section) showing diffuse staining with some membrane staining in the *stratum spinosum*.

Scale bars : 50 μ m.

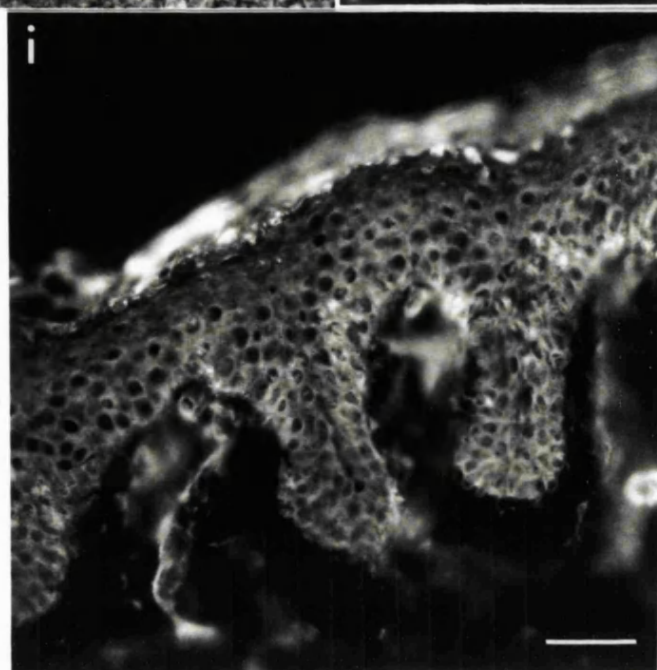
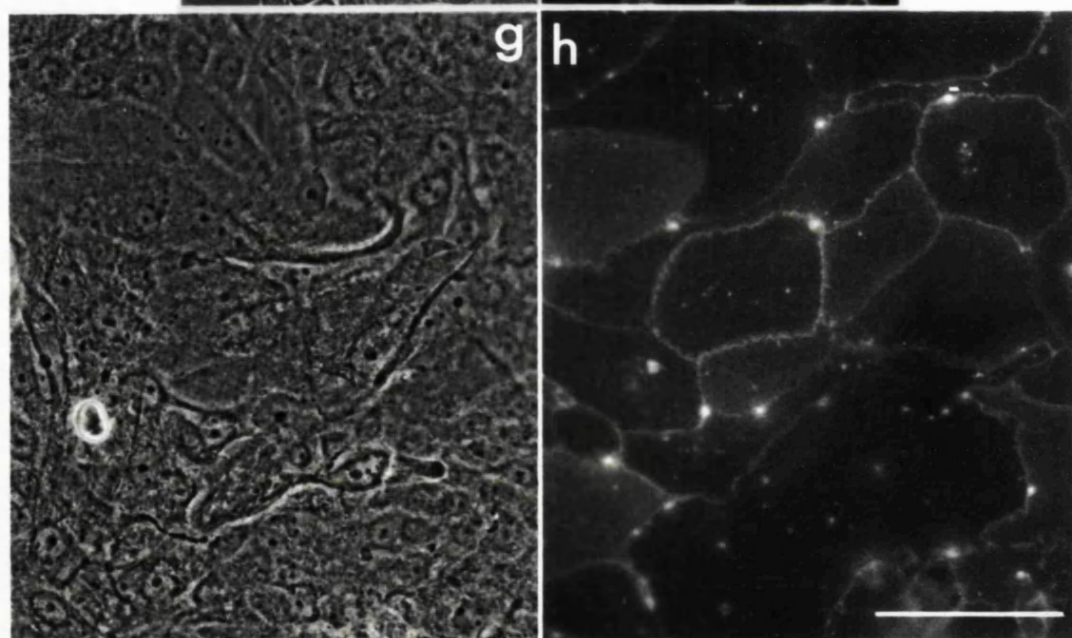
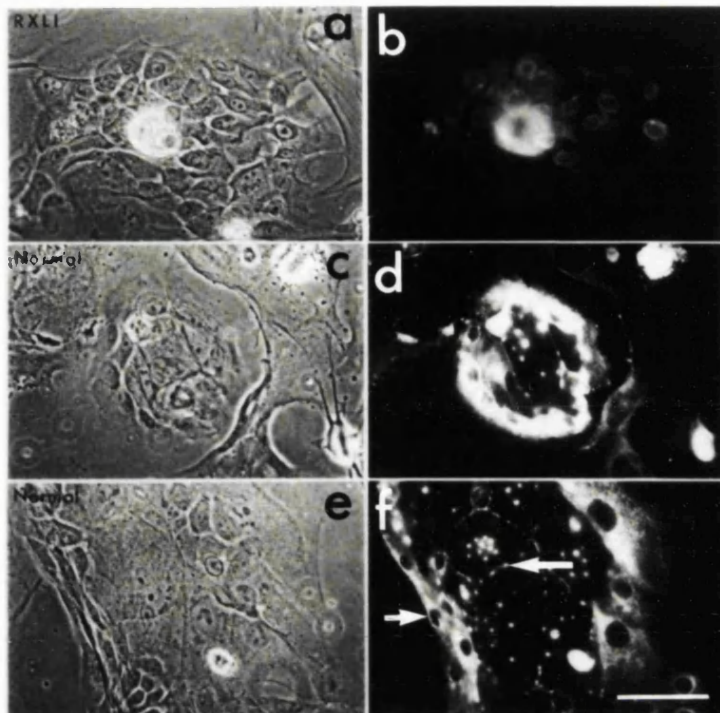


Figure 2 : Morphology of keratinocytes grown on de-epidermidised dermis from breast for two weeks (H+E).

a : Normal keratinocytes showing rete ridge pattern and normal differentiation with *stratum basalis*, *stratum spinosum* and *stratum corneum* but indistinct *stratum granulosum*.

b : RXLI keratinocytes showing focal increase in layers of the *stratum corneum*.

Scale bar : 50 μ m

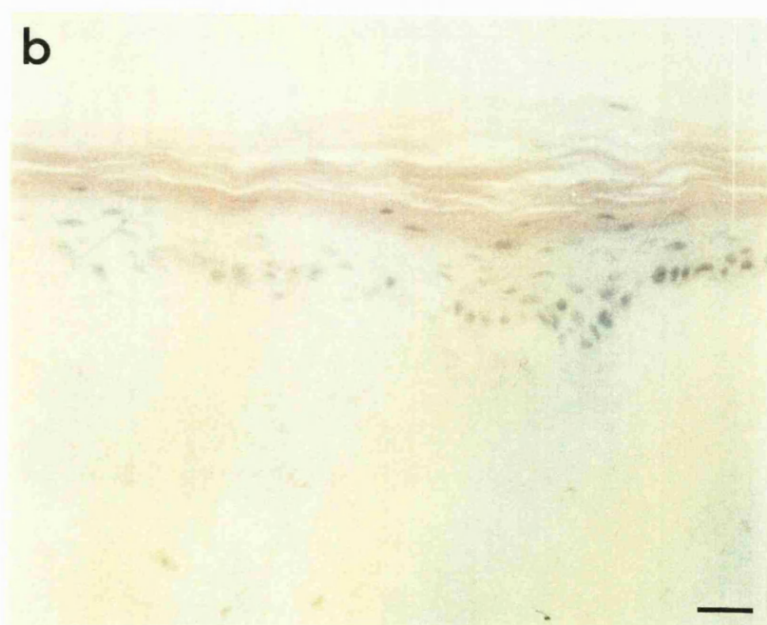
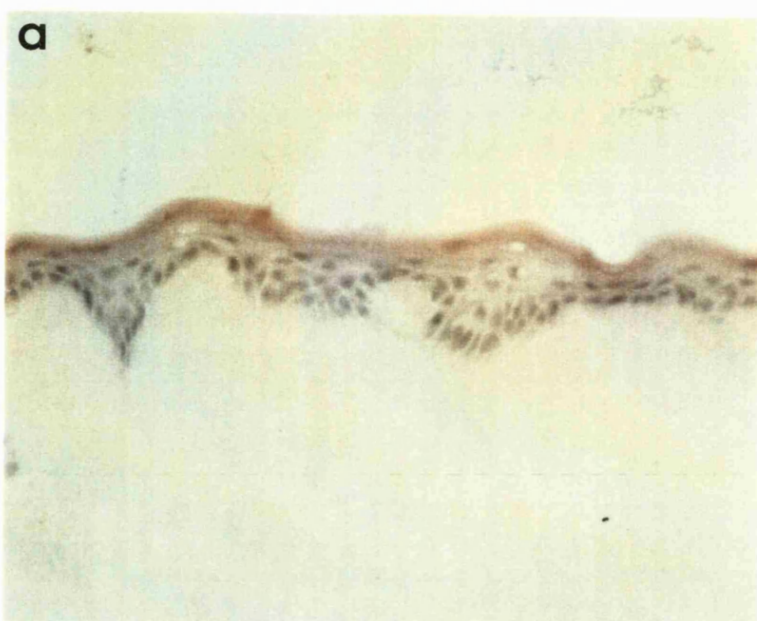


Figure 3 : Growth and differentiation of RXLI cells.

A : Growth rates of three lines of RXLI keratinocytes and a normal line. The growth curves are the mean values of between three and five experiments for each cell line, showing log number of cells per 35mm dish ($\times 10^{-4}$) versus days in culture, seeded at 2×10^4 per dish.

B : Comparison of involucrin expression and cornified envelope production (% of total cells) in confluent Rheinwald Green cultures of RXLI and normal keratinocytes. The values are the mean of between three and seven experiments for each cell line. Error bars show the standard error.

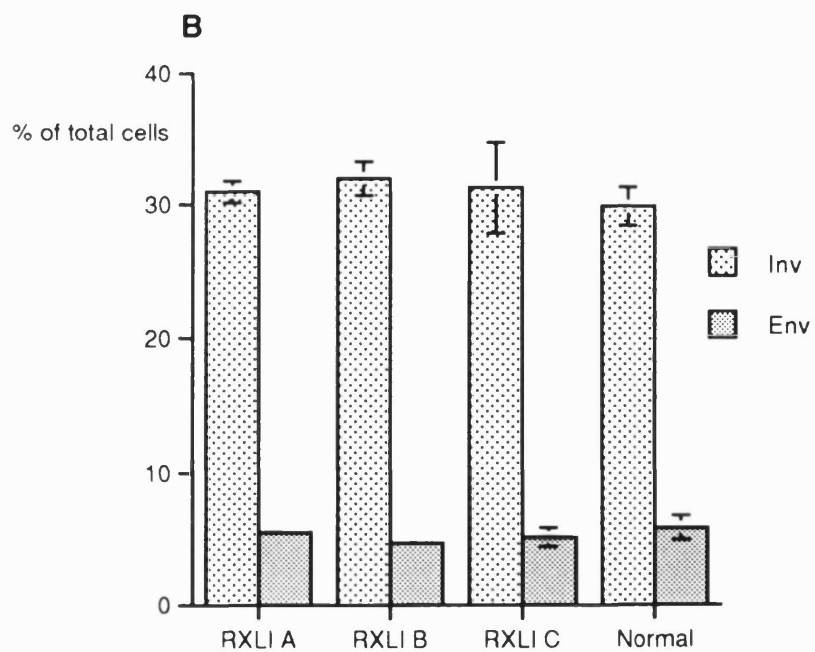
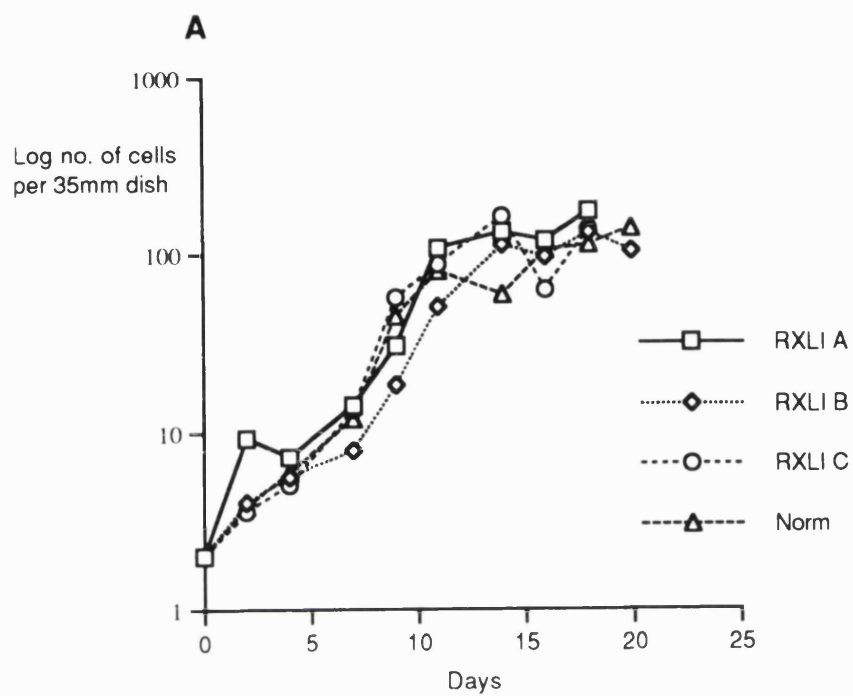
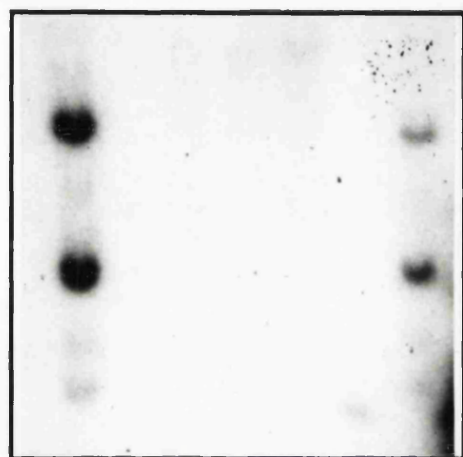


Figure 4 : Southern and Northern blots of normal and RXLI keratinocyte DNA and poly A+ RNA, extracted from subconfluent Rheinwald Green cultures and hybridised using a probe to STS.

A : Southern blot showing absent hybridisation to RXLI genomic DNA in three lines (RXLI A, B, C) and compared to normal keratinocyte DNA (N) following digestion by EcoR1. Normal DNA exhibits bands at 12, 9.5, 6.2, 5.0 and 4.3 kb and shows approximately twice the signal in female keratinocytes (f) compared to male (m). Loading was checked using a probe to involucrin (not shown).

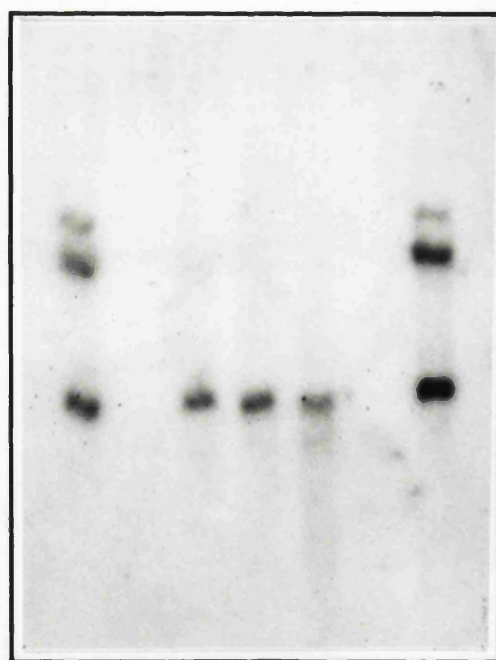
B : Northern blot showing lack of hybridisation to poly A + RNA in three lines of RXLI keratinocytes (RXLI A, B, C) and two bands in normal keratinocyte RNA (Norm) at 7.0 and 5.2 kb. Loading and integrity of the RNA is shown by hybridisation to the involucrin probe (2.1 kb).

A



N(f) RXLI A B & C N(m)

B



Norm RXLI A B & C Norm

STS

7.0 kb
5.2 kb

2.1 kb Invol.

Figure 5 : *In situ* hybridisation of RXLI and normal keratinocyte colonies in Rheinwald Green cultures on glass slides.

a,b : Normal keratinocytes hybridised with a sense STS probe showing background signal only (dark field) (b) with Giemsa counterstain (a).

c,d : RXLI keratinocyte colony (c) showing no hybridisation with antisense STS probe (d).

e,f : A normal keratinocyte colony (e) showing a small amount of hybridisation to the antisense STS probe in the basal cells at the edge of the colony (f) but note the high background.

Scale bar : 50 μ m.

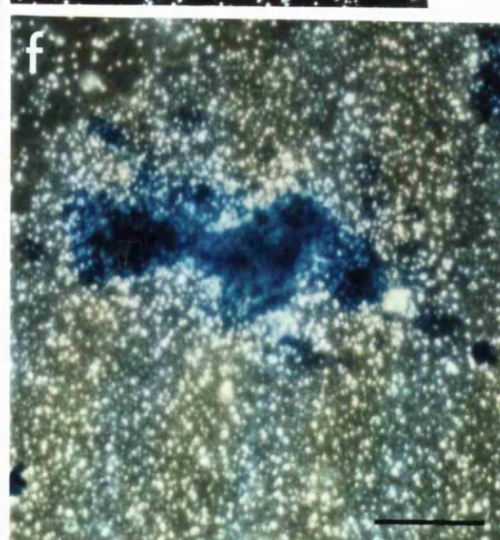
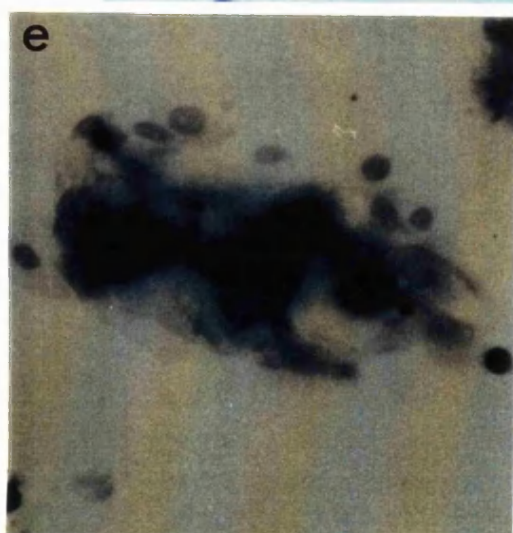
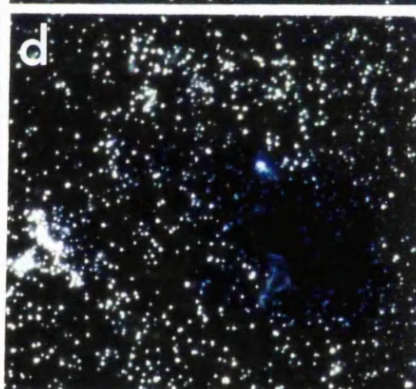
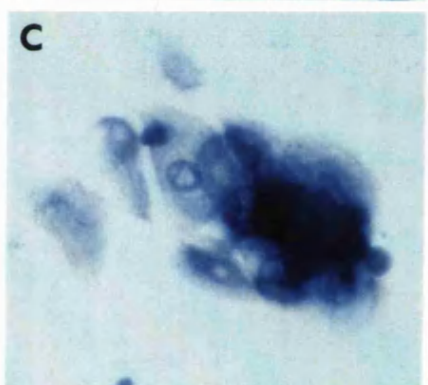
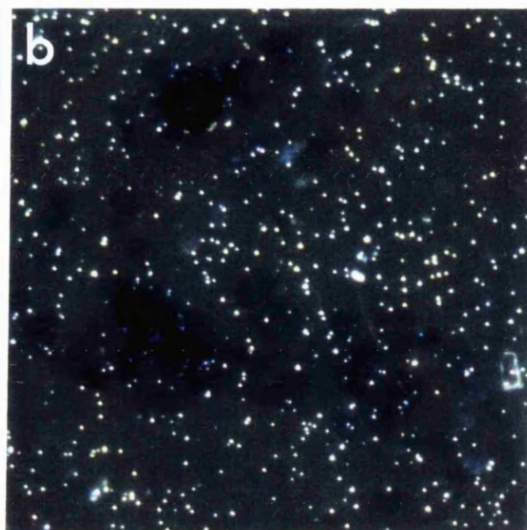
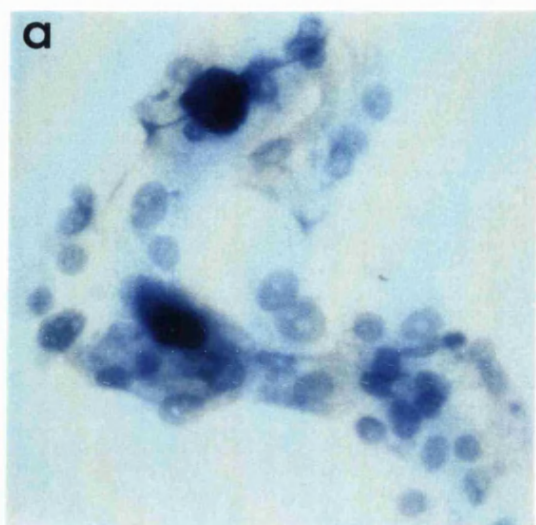


Figure 6 : Keratinocytes infected with the BAG-F retrovirus, selected in G 418, and stained for β -galactosidase using X-gal.

a,b : Colonies of Rheinwald Green cultured, BAG-F infected keratinocytes staining blue with X-gal. Note the heavily stained epithelioid spindle cells (b, right of picture). The unstained cells in the background are J2 feeder cells.

c : A single infected colony, grown in G 418, showing a mosaic pattern of staining in which some of the cells have switched off β -gal. expression.

Scale bar : 50 μ m.

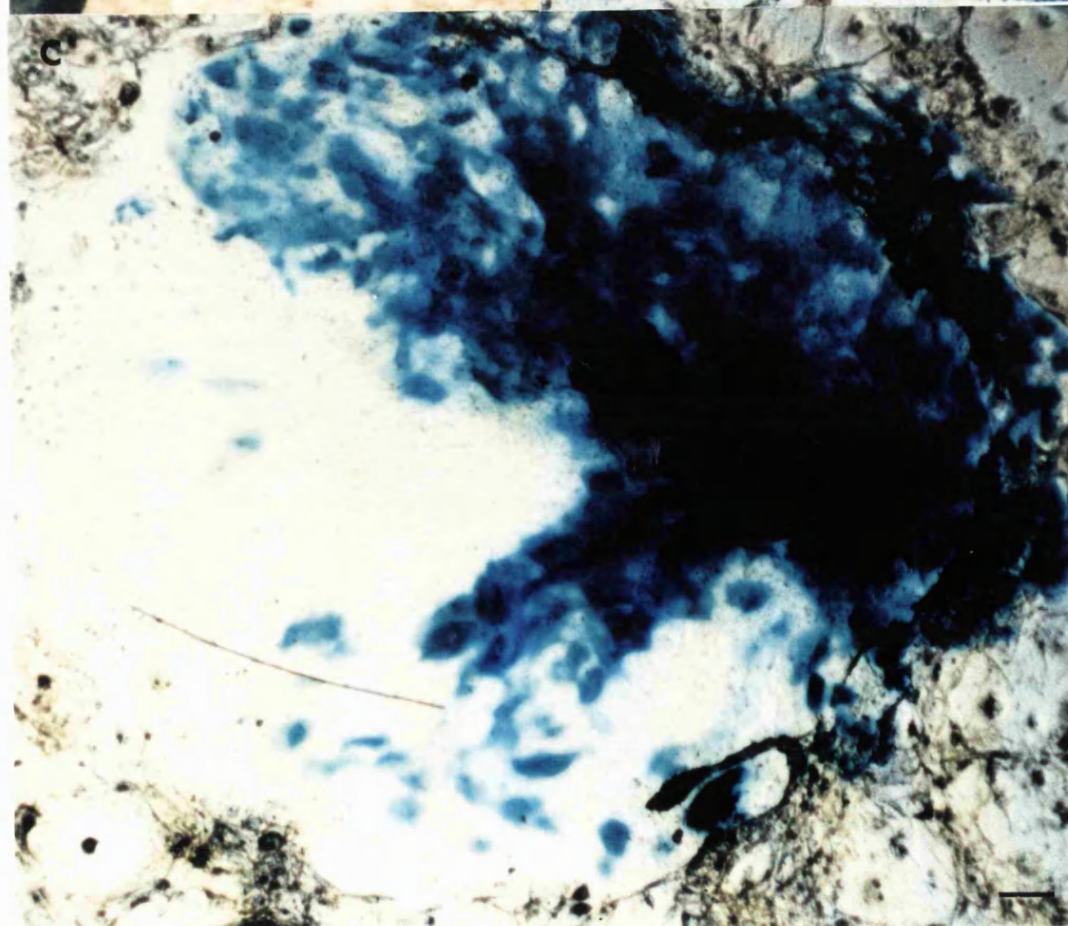
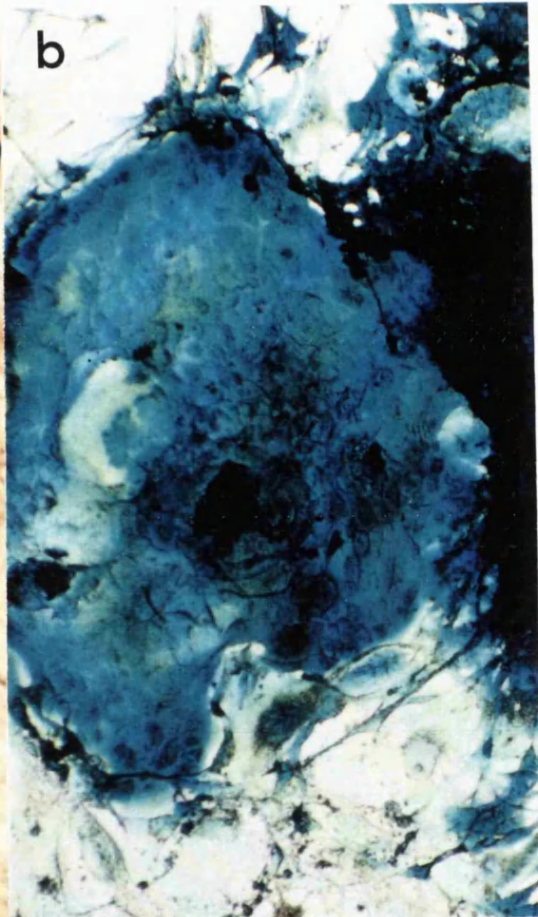
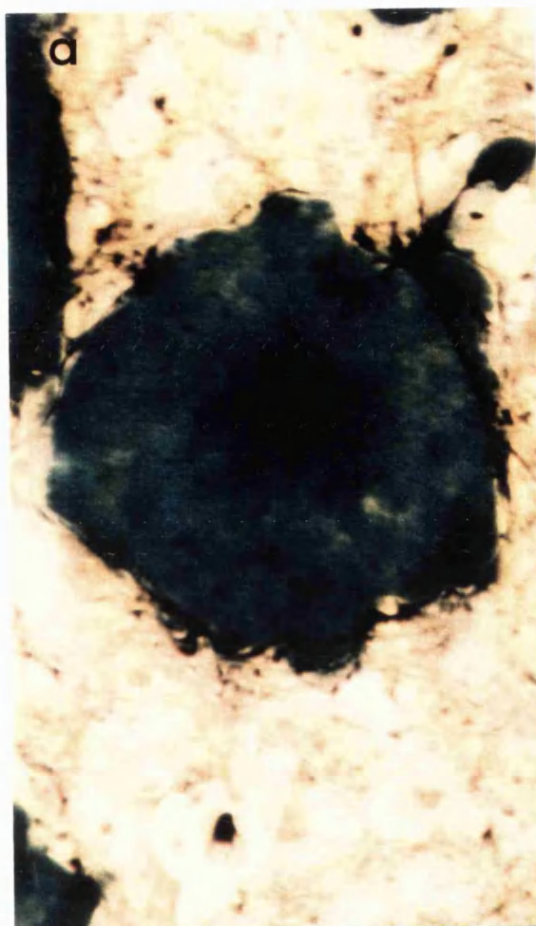


Figure 7 : Colonies of keratinocytes grown in Rheinwald Green cultures and hybridised *in situ* using biotinylated probe to the Y chromosome, HY2.1, and developed with horseradish peroxidase and DAB, then counterstained with haematoxylin.

a,b : Male keratinocytes show a single brown dot in the nucleus (b) whereas female cells are negative (a).

c : SCC4 keratinocytes, derived from a squamous cell carcinoma, contain multiple nuclear brown dots indicating multiple copies of the repeated sequence due to aneuploidy.

Scale bar : 50 μ m.

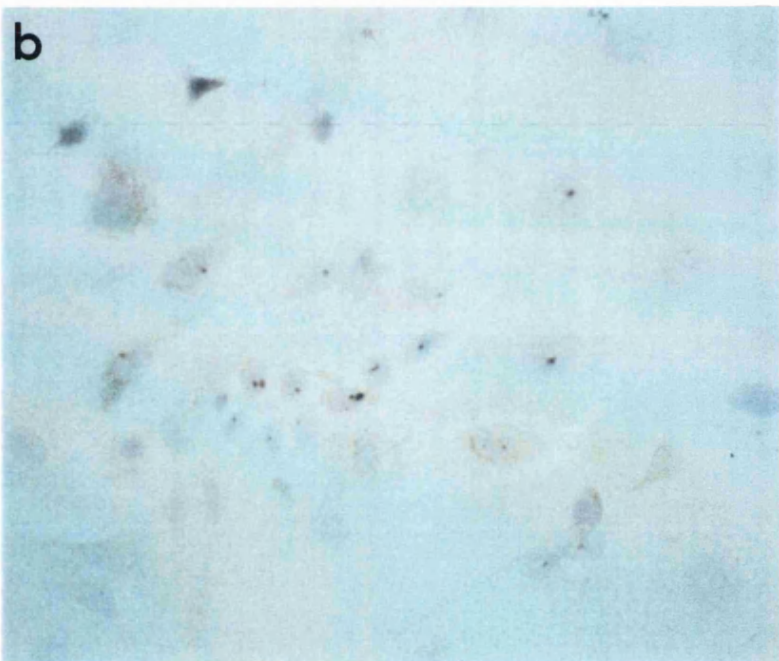
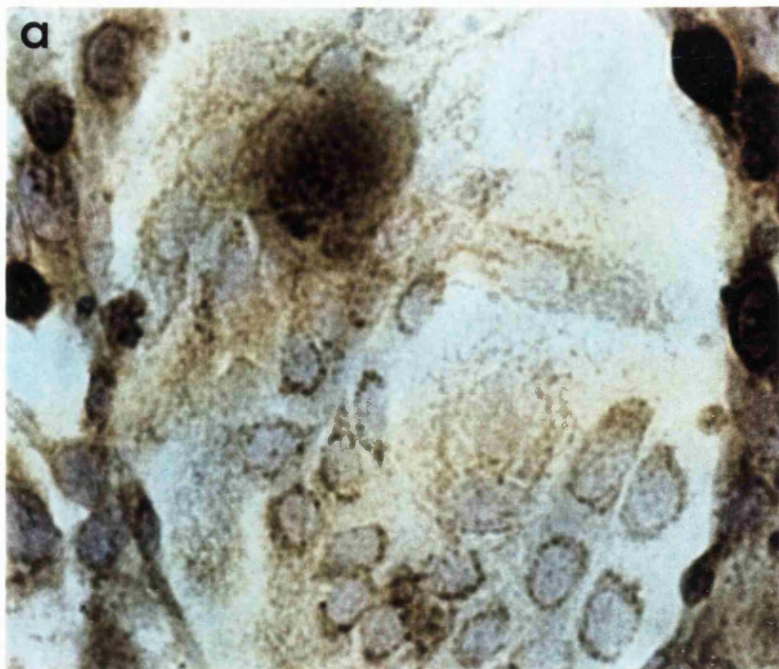


Figure 8 : Sections of paraffin embedded, dispase treated sheets of confluent Rheinwald Green keratinocyte cultures, *in situ* hybridisation to biotinylated HY2.1 probe for the Y chromosome.

a : Normal female keratinocytes, negative when probed with HY2.1, developed using horseradish peroxidase and DAB, counterstained with haematoxylin.

b : Polyploid human papilloma virus 16 transformed vp keratinocytes (Pei et al. 1991) showing multiple copies of the HY2.1 repeated sequence (brown nuclear dots), allowing detection of around 90% of the cells in sections.

c : XYY keratinocytes showing over 75% detection in sections, each nucleus contains two copies of the HY2.1 sequence.

Scale bar : 50 μ m.

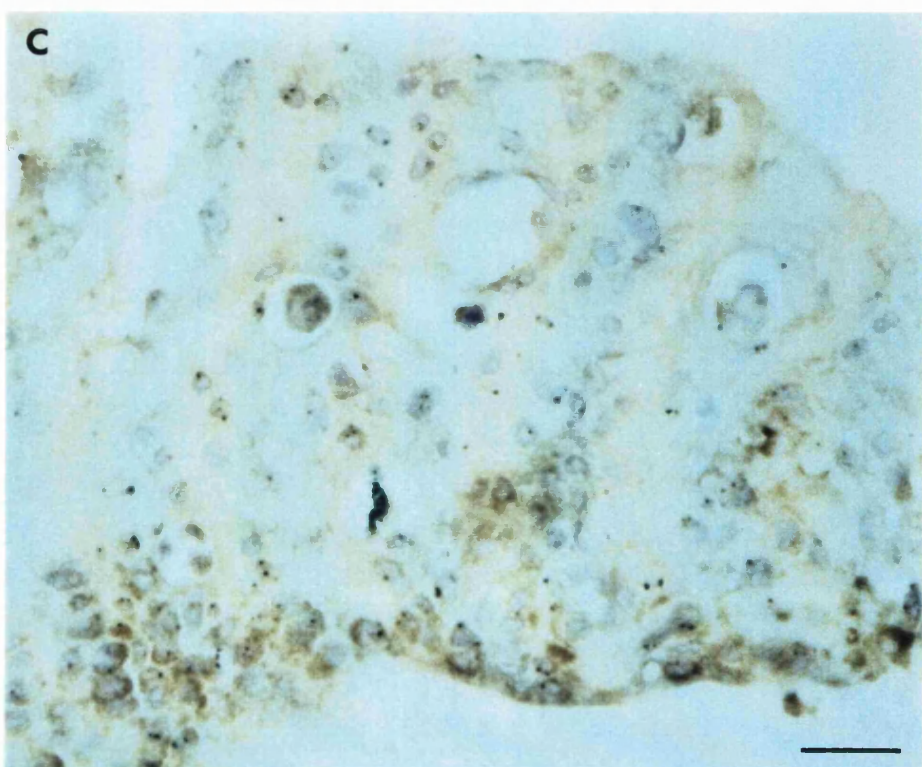
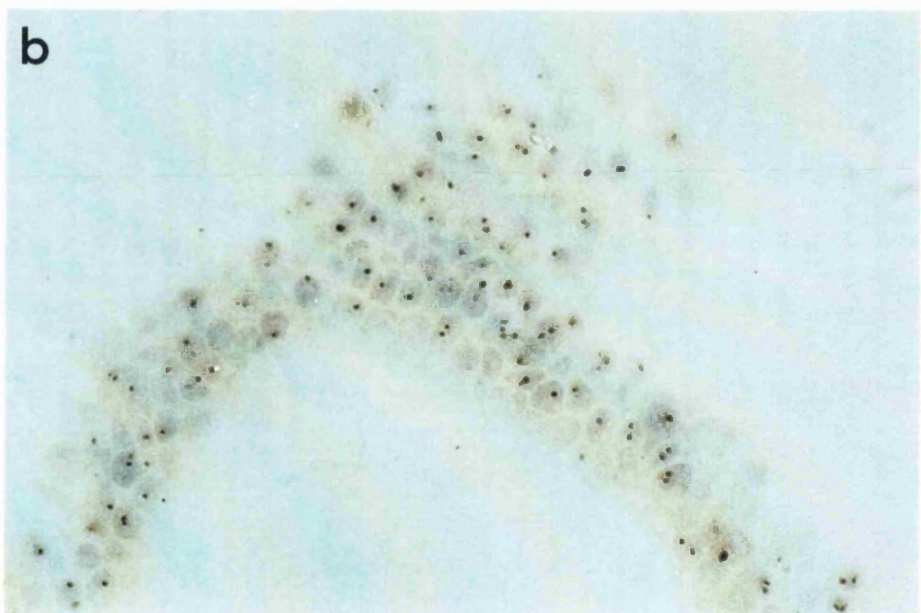
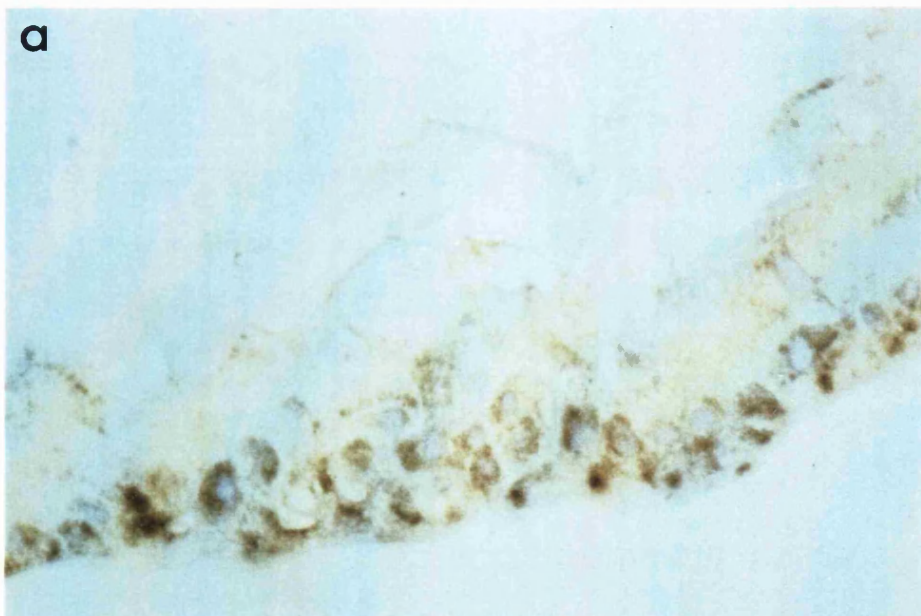
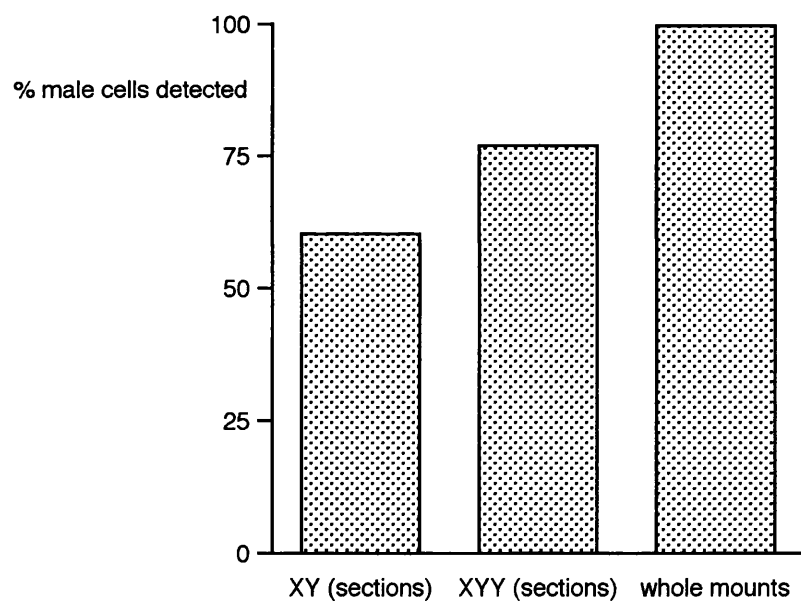


Figure 9 : Comparison of the percentage of male keratinocytes, grown to confluence in Rheinwald Green cultures, detected by *in situ* hybridisation using different methods; normal male cells (XY) (60.3%) or XYY cells (77.0%), dispase treated, paraffin embedded and sectioned, or normal male cells detected using the whole mount method (99.7%). The results are the mean of three readings for each method, counting 200 cells for each reading.



3.3. Discussion

Four different strategies were investigated for lineage marking in cultured human keratinocytes. All of the techniques produced some problems. The use of class I HLA antigens were not helpful as the levels of expression of individual antigens was not high enough in cultured keratinocytes, although all the lines tested showed positive staining with the antibody common to HLA A, B and C subgroups.

The morphology, growth and differentiation of RXLI cells in culture was the same as normal cells and this has been reported by other workers (Jensen *et al.* 1990). Clinically patients with RXLI have a thickened stratum corneum but this was not seen in standard Rhienwald Green cultures. This may be due to the abbreviated differentiation pathway in culture. The RXLI phenotype may be detectable in cells grown on deepidermidised dermis.

Retinoids are important in the differentiation pathway of keratinocytes, and high levels alter the terminal differentiation pathway, reducing cholesterol sulphate accumulation, cholesterol sulphotransferase and transglutaminase activity (Jetten *et al.* 1989), and also suppress envelope assembly (Green and Watt 1982), although they have no effect on involucrin expression. Retinoic acid is essential for epidermal development, and maturation is suppressed in transgenic mice expressing a dominant-negative retinoic acid receptor (Saitou *et al.* 1995) although deficiency in humans results in squamous metaplasia and keratinisation of mucous membranes. However removal of retinoic acid from the medium by extracting the fat soluble fraction did not alter the morphology of the RXLI or normal keratinocytes.

The sizes of the EcoR1 fragments of genomic DNA which hybridised to the STS probe were broadly similar to those found by other workers in human placenta (Bonifas *et al.* 1987; Conary *et al.* 1987; Yen *et al.* 1987). The sizes of the RNA bands on Northern blots were as seen by others in cultured fibroblasts (Conary *et al.* 1987; Yen *et al.* 1987; Yen *et al.* 1988)

My attempts to discriminate between cultured keratinocytes which do and do not express STS were unsatisfactory for the purposes of marking stem cell fate by lineage analysis. The levels of expression, as seen by immunocytochemistry, in cultured

keratinocytes were low, as indicated also by levels of messenger RNA, which required purification to poly-A⁺ RNA and a lengthy exposure before detection on northern blots and was very low when detected by *in situ* hybridisation.

It is possible that using culture methods which allow the full process of differentiation to occur, such as on deepidermidised dermis, would give higher expression, although differences in differentiation may occur between normal and RXLI keratinocytes.

The antibody used was also inconsistent when staining fibroblasts for STS (W. Rommerskirch, personal comm.) and it was not possible to use STS as a reliable marker of lineage.

In my experiments the capacity of retrovirally infected keratinocytes for repeated passage was severely limited. The cell lines used were usually capable of growth beyond passage 10 but, once infected by the BAG-F retrovirus at passage 4, were able to reach passage 7 at best. There are a number of possible reasons for this effect on the keratinocytes. It may be due to the virus itself, the high levels of β -gal transcribed and expressed in the cell under control of the viral LTR or possibly an effect of the lengthy exposure to G 418. The other possibility is that co-culture with the packaging line, PA 317, was detrimental to keratinocyte growth. On one occasion a culture of the PA 317 cells tested positive for mycoplasma although the keratinocytes themselves never tested positive, and nor were the packaging cells positive on any other occasion. A further problem was the overgrowth of cultures by retrovirally infected G 418 resistant epithelioid spindle cells, possibly ndk cells (Adams and Watt 1988).

Despite the difficulty in culturing the BAG-F infected cells a limited number of lineage experiments were possible. Staining with X-gal is a relatively simple technique in whole cells and tissue sections. The results of the experiments on dispase treated epidermal sheets and on keratinocytes grown on collagen coated culture inserts are described in Chapter 4.

The use of a biotin labelled DNA probe (HY2.1) to a sequence on the Y chromosome was the most successful method of detecting lineage to follow cell fate in cultured epidermis. The low rate of detection of male cells in sections has been previously reported (Brain *et al.* 1989) and I was able to partially overcome this by using XYY keratinocytes. The extra Y

chromosome increased the chances of at least one of the target sequences being in the section such that it was possible to detect male colonies with certainty. Individuals with the XYY chromosomal abnormality have no phenotypic abnormalities and the cells grew normally in culture. The results of experiments mixing XYY and female cells and detecting male colonies are described in Chapter 4.

The main drawback of the experiments with HY2.1 in sections was the long time needed to analyse results by microscopy. To find a single marked colony, after seeding at limiting dilution, involved hybridisation and examination of large numbers of sections. This problem was partially resolved by using whole mount preparations, described in Chapter 4.

Experiments with both sections and whole mounts of epidermal sheets involved many steps and were therefore more prone to fail than simpler protocols, the *in situ* hybridisation was the most fallible step, particularly the proteinase K digestion. Hybridisation to the keratinocyte DNA appeared less reliable than to other tissues used as controls, such as sections of carcinomas.

These studies provided me with two systems, β -gal and the Y chromosome, with the potential for use as markers of cell fate in analysis of lineage in confluent cultured keratinocytes. The results of these investigations are described in the following chapter.

CHAPTER FOUR

LINEAGE ANALYSIS - CELL FATE

4.1. Introduction

To investigate the fate of individual cells in a confluent cultured epidermal sheet, when the rate of proliferation in the basal layer is balanced by the rate of differentiation, I used two of the techniques described in Chapter 3, mixing keratinocytes which were positive for β -galactosidase (β -gal) with negative, or mixing male keratinocytes with female. By culturing the cells in different systems I have attempted to analyse the fate of different cells from the heterogeneous population of dividing keratinocytes, without prior selection.

In these experiments I wanted to establish;

1. A reliable assay, using the techniques discussed in Chapter 3, to detect colonies containing marked cells.
2. The efficiency of detection of colonies containing marked cells.
3. The limiting dilution of marked cells in an unmarked background which would ensure that all the marked colonies detected were clonal.
4. The morphology and heterogeneity of clones present in a confluent epidermal sheet.

I have also studied the fate of specific populations of proliferating keratinocytes, selecting the cells by their ability to adhere rapidly to collagen IV. Integrins are involved in the regulation of the onset of terminal differentiation (Adams and Watt 1989) and high proliferative capacity of keratinocytes is related to high surface expression of β_1 integrins and to a rapid rate of adhesion to extracellular matrix (ECM) proteins, such as collagen IV and fibronectin (Jones and Watt 1993). It is possible to separate cells with the characteristics of stem cells from those with characteristics of transit amplifying cells on this basis. I established assays to investigate whether cells adhering rapidly or slowly to collagen IV show any differences in fate when grown to confluence as part of an epidermal sheet, and to investigate if the morphology of clones derived from rapidly adherent cells show heterogeneity.

Finally, I attempted to look at the morphology of marked clones in keratinocytes grown in cultures for longer times than possible in Rheinwald/Green cultures.

4.2. Results

4.2.i. Experimental design of lineage experiments

4.2.i.a. Unselected keratinocytes

For lineage experiments, two strains of keratinocytes (one positive for the lineage marker and one negative), matched for similar growth rate, were counted and each line serially diluted. The two strains were mixed in a tube at the appropriate ratio and the mixture of cells in suspension was plated in the usual way, co-cultured on a feeder layer by the standard protocol (as shown in Chapter 2, diagram 5). All cultures were grown until 3-5 days post-confluent and harvested before the epidermal sheet became blistered.

In some experiments the keratinocytes were mixed then seeded and grown to confluence on collagen coated culture plate inserts. In all experiments, great care was taken to ensure even seeding of cells over the culture dish or insert.

4.2.i.b. Keratinocytes selected on collagen IV

Two types of experiment were designed to investigate the cells adhering rapidly and slowly to collagen IV. Cells, positive for the lineage marker, were harvested in trypsin, centrifuged in medium containing soya bean trypsin inhibitor and serially diluted in serum free medium.

The keratinocytes were plated onto collagen IV coated culture dishes and then treated in two different ways :

4.2.i.b.I Collagen IV selected cells on culture plate inserts

The cells positive for the lineage marker were allowed to adhere to the collagen coated culture plate in serum free medium, without feeder cells, for 20 minutes and the non adherent cells washed off. The adherent cells were removed with trypsin, centrifuged in medium containing serum, resuspended, counted, serially diluted in medium containing serum and seeded onto the culture plate inserts. Cells not adherent after 60 minutes on collagen were removed from the dish, serially diluted and seeded onto the inserts. Control keratinocytes were seeded, unselected onto the culture plate inserts.

4.2.i.b.II Collagen IV selected cells in Rheinwald and Green cultures

Keratinocytes positive for the lineage marker were allowed to adhere for 5 minutes, in serum free medium, to the collagen coated dish and then the non adherent cells were removed by repeated washing. In these experiments, panning of cells for 5 minutes on collagen IV resulted in more predictable numbers of cells adhering to the dish than panning for 20 minutes.

Unselected control cells were allowed to adhere to collagen for 4 hours in serum free medium and the non adherent cells removed. The controls therefore contained all the adherent cells. To select the slowly adhering cells the keratinocytes were allowed to adhere to collagen IV for one hour and the non adherent cells counted, diluted and transferred to further collagen IV coated dishes for 3 hours.

All the cells were switched to standard serum-containing medium at the same time point, 4 hours from the start of the experiment, and J2 feeder cells were added along with the background unmarked cells at the appropriate density (see Diagram 6, Chapter 2). Control dishes for rapidly adhering, slowly adhering and unselected cells were also set up with each experiment. The control dishes were used to count the number of adherent cells, or were cultured with J2s but no background unmarked cells for 10 days, the number of colonies counted and their areas measured after staining with anti-keratin antibody, LP34.

4.2.ii. Assays for detection of cells marked for lineage**4.2.ii.a. Rheinwald and Green cultures - β -galactosidase**

Epidermal sheets removed by treatment with dispase could be stained whole by immersion in X-gal and were then paraffin embedded and sectioned if required.

4.2.ii.b. Culture plate inserts - β -galactosidase

Cells grown on culture plate inserts at the liquid/air interface had a thicker cornified layer than standard submerged cultures and the X-gal stain was unable to penetrate the middle layers of the culture. The colonies positive for β -galactosidase were therefore detected by staining of frozen sections.

For the experiments using cells selected on collagen IV the cells were grown on culture plate inserts but kept submerged. These cultures did not produce as many cornified cells and all layers could be stained by immersing the insert in X-gal solution.

4.2.ii.c. Rheinwald and Green cultures - Y chromosome

Epidermal sheets containing mixes of male and female keratinocytes were removed from the culture dish using dispase, fixed in paraformaldehyde, embedded in paraffin and sectioned through the block to extinction. The sections were hybridised to the probe HY2.1, developed and detected by light microscopy. It was then possible to count the number of basal cells present in each marked colony, in each section.

A similar method was used for dispase treated epidermal sheets transplanted into nude mice.

4.2.ii.d. Whole mounts - Y chromosome

This technique allowed confluent keratinocytes to be used without the shrinkage seen with dispase treatment allowing accurate measurement of lineage marked (male) colony size. It also avoided the need to cut and hybridise large numbers of sections.

3-5 day post-confluent sheets of keratinocytes, either unselected or selected on collagen IV, grown in 35mm dishes, were carefully scraped from the dish using a small spatula and the epidermal sheet turned over and placed on a 40mm x 80mm glass slide, using fine forceps. The basal keratinocytes were now upwards and the suprabasal cells in contact with the glass slides (Diagram 1).

The mount was carefully spread and allowed to air dry. When fully dry the cells were fixed in 4% pfa for 10 mins then rinsed in PBS. These mounts could then be hybridised to the probe HY2.1 by the standard protocol and viewed by light microscopy.

Detection of the Y chromosome by this method was close to 100% (Chapter 3, Figure 9) and colonies could be found by careful low power light microscopy (Figure 1).

The area of the colonies of male cells was determined using a drawing arm on a light microscope to obtain an image of the colony on paper. The area was then measured using an image scanner and image analysis programmes on an Apple Macintosh II.

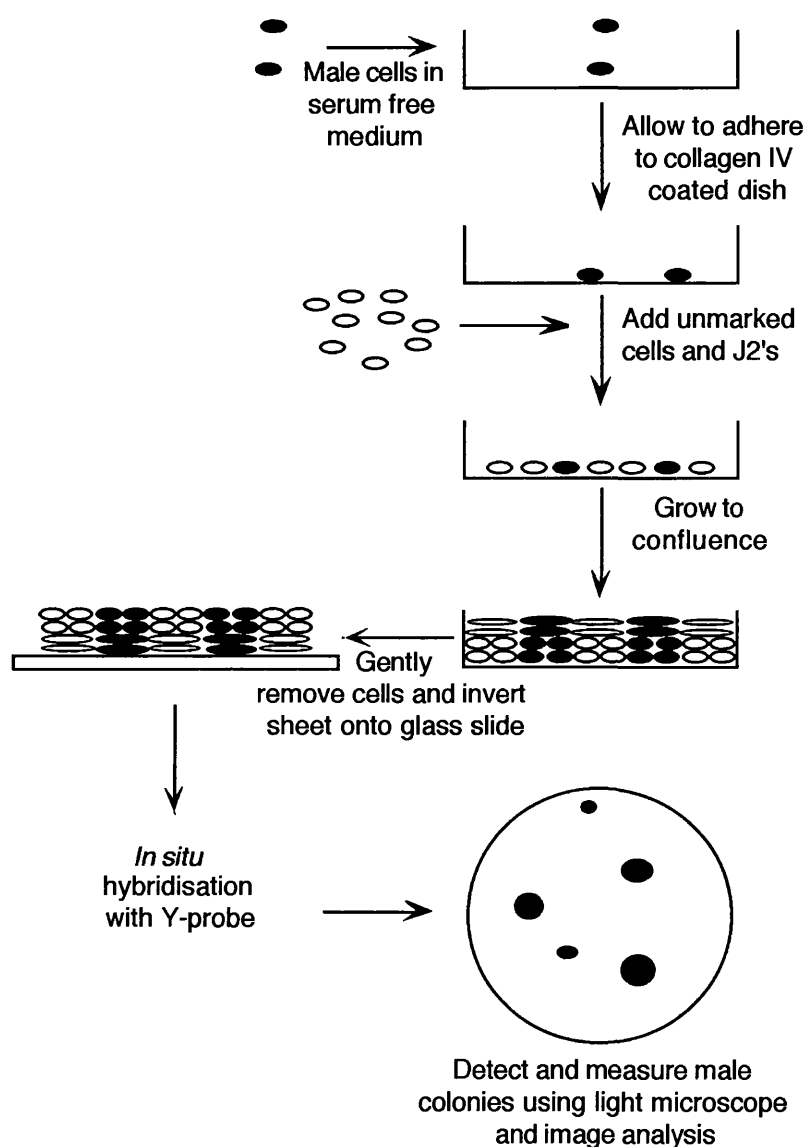


Diagram 1 : Method for detection of colonies founded by male cells selected on collagen IV.

4.2.iii. Efficiency of male colony detection by the whole mount method

The number of male colonies seen in the whole mounts (female seeding density of 5×10^4 per dish) was compared to the control plates containing male cells only, seeded at the same male cell density, without background female cells. The mean number of colonies per whole mount (6-9 whole mounts per experiment) was expressed as a percentage of the mean number of colonies per control dish (4-6 dishes per experiment) for each experiment (Table 1). Although variable from experiment to experiment, on average the number of colonies

detected by *in situ* hybridisation with the Y-probe in the whole mounts was 82.50 % (SEM +/- 22.21, range from 38.38% to 126.66% in 4 experiments) of the number of colonies seen on control dishes seeded with only the male cells, i.e. the predicted number of marked clones per epidermal sheet if all cells capable of producing colonies of over 32 cells were to contribute to the confluent epidermal sheet.

This result shows that most of the colonies on the control plates are seen by the detection method at seeding density of 5×10^4 female cells per 35mm dish. At lower and higher seeding densities fewer colonies were detected (see below).

Colonies per whole mount	Colonies per control dish	% colonies detected
3.17	6.25	50.67%
4.00	3.50	114.29%
9.50	5.00	126.66%
2.11	5.50	38.38%

Table 1: Efficiency of detection of colonies by the whole mount method, 4 experiments.

4.2.iv. Limiting dilution of marked cells to produce clonal colonies

XYYA keratinocytes (see Materials and Methods) were mixed with female keratinocytes at different ratios and seeded onto 2cm² culture dishes at various densities. The cultures were grown to confluence, treated with dispase, paraffin embedded and serial sectioned all the way through, at least 80 sections per epidermal sheet. These were then hybridised and developed. The number of colonies and the number of basal cells in each colony, in each consecutive section, were counted. This gave a maximum diameter for each colony expressed as number of cells. The results are shown in Table 2 and Figure 2.

		Cell ratio, Female:XYX					
		50:1		10:1		5:1	
		No. of colonies/ dish	Mean diameter of colonies (no. of cells)	No. of colonies/ dish	Mean diameter of colonies (no. of cells)	No. of colonies/ dish	Mean diameter of colonies (no. of cells)
Total no.	1250	1	60	5	61.00	10	111.30
of cells	5000	4	78.25	--		--	
per well	12500	18	112.5	--		--	

Table 2: Numbers and sizes of XYX colonies detected in sections by probe HY2.1.

The results show that a single marked colony per 2cm² dish was obtained at a seeding density of XYXA cells of 12.5 cells/cm² and a ratio of female to XYXA cells of 50:1. Because doublets of adherent keratinocytes after trypsinisation are rare (see below) this was assumed to represent a colony derived from a single cell and to be the limiting dilution. Increasing the number of positive cells in each dish resulted in more and larger colonies.

At higher seeding densities the XYX colonies were merged in such complex patterns that interpretation of the sections was not possible. At the lowest seeding densities of XYX cells the diameters (no. of cells) of the colonies show some variation but the averages were relatively constant (XX:XYX 5,000:100, 78.25 SEM +/- 9.64; 1,250:125, 61.0 SEM +/- 6.13). This possibly represents a variation in the proliferative capacity of individual colony forming cells. If the seeding density of the XYX cells was increased but the total seeding density remained low (1,250:250 per dish) then the average size of the colonies increased (111.3, SEM +/- 4.67), probably because some of the XYX colonies were merged. If the seeding density of the XYX keratinocytes was increased as well as the total seeding density (12.5 x 10³:250) a similar effect was observed on the average size of the colonies (112.5, SEM +/- 7.63), and more colonies were detected (Figure 2). This finding may be due to merging of

colonies combined with increased colony survival in the dishes seeded at higher density, which reached confluence considerably faster than those seeded with fewer keratinocytes.

The number of XYY cells seeded was important to ensure that all colonies were clonal and derived from a single cell, providing that the total seeding density was not too low. In all the subsequent experiments I used seeding densities based on the findings of this experiment. The limiting dilution of unselected, lineage marked cells was taken to be between 12.5 and 62.5 cells per cm^2 (100-500 per 35mm dish). These colonies are likely to be derived from a single cell, i.e. to be clonal, since they were not complex shapes as seen at higher seeding densities, were of similar size and unlikely to be derived from doublets. It was possible to vary the seeding density of background negative cells from between 2.5×10^3 to 2.5×10^4 per cm^2 (2×10^4 - 2×10^5 per 35mm dish).

4.2.v. Morphology of marked clones

4.2.v.a. Morphology of marked clones in cross section

In the above experiments, looking at serial sections of dispase sheets comprising mixes of XYY and female keratinocytes, of all the colonies, in all the sections examined none were seen containing only suprabasal cells, i.e. likely to have been derived from a transit amplifying cell. Typical colonies are shown in Figure 8 in Chapter 3. One colony was seen which contained only a few basal cells relative to the number of suprabasal cells, some of which overrode adjacent female basal cells, which I named the "top heavy colony."

Keratinocytes positive for β -gal were seeded onto culture dishes at a density of 200 cells per 35mm dish (25 cells/cm^2) and allowed to adhere. 5×10^4 negative cells were then added per dish. The cells were grown until 3 days post confluent then treated with dispase. The epidermal sheets were stained with X-gal, paraffin embedded and sectioned. The positive colonies all contained both basal and suprabasal cells (Figure 3a, b). The positive suprabasal cells were all directly above the positive basal cells, confirming the findings using XYY cells.

β -gal marked cells grown on culture plate inserts with negative cells seeded at high density, 5×10^5 or 10^6 in a 30mm insert, showed 2 types of colony. The most common were

those containing both basal and suprabasal cells but occasional colonies consisted of only suprabasal cells, suggesting that all of the cells in these colonies had undergone terminal differentiation and were founded by a transit amplifying cell. These colonies were underlain by negative cells (Figure 3c).

4.2.v.b. Morphology of clones in whole mounts

In whole mounts seeded at 5×10^4 cells per 35mm dish, the sizes of clones varied widely, in some experiments the area of the clones differed from smallest to largest by a factor of 70. Figure 4 shows the shape and size of some of the clones from one experiment.

4.2.v.b.i Effect of seeding density on the size and number of marked clones in an epidermal sheet

Whole mounts of a limiting dilution of male cells, seeded without selection on collagen, were plated with a varying number of female cells onto 35mm dishes and grown until post-confluent. The areas of the male clones were measured after *in situ* hybridisation of whole mounts.

As the female cell seeding density decreased, the area of the male clones increased as shown in Table 3. Two other observations were noticeable; the spread of clone size was widened, with some cells able to found very large colonies at the lower densities, whilst the number of male colonies was lower at the lower and higher seeding densities when compared to those seeded at 1×10^5 per 35mm dish ($2.5 \times 10^4/\text{cm}^2$) (χ^2 test, df 6, $p=0.05$). The results are shown in Figure 5.

Seeding density (total no. cells/dish)	Mean no. of clones	Mean area of clones (sq. mm.) +/- SEM	Variance
20,000	8.67	0.79 +/- 0.13	0.30
50,000	8.67	0.68 +/- 0.15	0.39
100,000	16.67	0.41 +/- 0.04	0.05
200,000	7.67	0.33 +/- 0.06	0.05

Table 3: Number and area of clones at different total seeding densities. Clone areas showed wide variation across experiments and were analysed by combining the percentile

rank of the clones from each experiment. These showed significant differences between clone areas as shown in Table 3a. The larger values of variance at the lower seeding densities illustrate the greater spread of clone areas in those dishes.

Seeding density (total no. cells/dish)	20,000	50,000	100,000
50,000	Not significant, $p>0.05$		
100,000	$p<0.005$	$p<0.05$	
200,000	$p<0.0005$	$p<0.005$	Not significant $p>0.05$

Table 3a: Differences between clone areas at various seeding densities. The percentile rank of clone areas in each experiment was combined and analysed (Mann-Whitney U test). The lower seeding densities contain significantly larger clones than the higher densities.

Plates seeded at very low density showed a trend towards a bimodal distribution of clone areas, not seen clearly in the control plates of male colonies only, although clone numbers were not large enough for this to be statistically proven.

4.2.vi. Relative proportions of different populations of cells adhering to collagen IV

The number of cells which adhered to collagen IV coated dishes was determined by allowing the cells in each population to attach, for up to 4 hours, then washing the dishes and staining the cells with 1% methylene blue. The number of cells attaching to the dishes showed some variability between experiments for all populations. The results are shown in Figure 6: approximately 5% of cells panned for 5 minutes were adherent, 40% of unselected (0-4 hours) and 25% of cells panned after 60 minutes (1-4 hours).

To show that any colonies derived from adherent cells were clonal, all adherent cells were counted and the percentage of cells adhering as couples (doublets), and therefore likely to produce mixed colonies, was established.

Adherence of cell doublets or involucrin positive cells was rare for all populations, cell doublets were under 1% and involucrin positive cells were under 5% of the total number of adherent cells. This confirms that all the experiments in which these cells were cultured produced colonies that were clonal, derived from single, basal cells.

4.2.vii. Colony forming efficiency of populations of cells with different properties of adhesion to collagen IV

On average, of 100 cells panned on collagen IV for 5 minutes, 2.2 colonies (SEM $\pm$ 0.303) were produced compared to 9.42 colonies (SEM $\pm$ 1.137) in the total population and 0.58 (SEM $\pm$ 0.068) of the keratinocytes which did not adhere within 60 minutes. The colony forming efficiency (CFE) was calculated as the percentage of adherent cells that formed colonies.

The percentage of cells in each population which were adherent and produced colonies of at least 32 cells was compared and the results shown in Table 4 and Figure 6. The keratinocytes adhering within 5 minutes to collagen had almost twice the CFE of unselected cells (paired *t* test: df 2, $p < 0.02$ (1 tailed) and around 17 times that of slowly adhering cells, (paired *t* test, df 2, $p < 0.005$) which had a CFE 10 times less than the unselected population (paired *t* test: df 2, $p < 0.0005$). These figures were constant between experiments.

Selection on collagen IV	Colony forming efficiency (No. of colonies as % of adherent cells) $\pm$ SEM
<5mins	43.05 $\pm$ 3.77
Unselected	23.89 $\pm$ 0.94
>60mins	2.42 $\pm$ 0.40

Table 4: Colony forming efficiency of cells selected on collagen IV.

4.2.viii. Effect of relative ability to adhere to collagen IV on the size of marked clones in an epidermal sheet

The areas of clones formed by male keratinocytes selected after 5 minutes adhesion to collagen (rapidly adherent), 1-4 hours adhesion to collagen (slowly adherent) or unselected, were measured. There was a constant background seeding density of 5×10^4 female cells per 35mm dish ($6.25 \times 10^3/\text{cm}^2$).

The areas varied between experiments and clones formed by slowly adhering cells showed no significant difference in area compared to those from unselected keratinocytes. The rapidly adhering cells produced clones with areas larger than those that adhered slowly and those of the unselected population. The results are more striking when the range of clone sizes in each population is considered, the rapidly adhering population formed large clones but the slowly adhering cells did not. The results of these experiments are shown in Table 5 and Figure 7. This effect on clone area was not seen consistently in the control dishes containing only male colonies, cultured for 10 days, stained with LP34 and the areas measured (Table 5a and Figure 8).

Selection on collagen IV	Mean no. of clones per dish	Mean area of clones (sq.mm) +/- SEM	Variance	Maximum clone area (sq.mm)	Minimum clone area (sq.mm)
<5mins	4.5	0.99 +/- 0.31	3.88	9.45	0.12
Unselected	3.7	0.62 +/- 0.86	0.86	4.45	0.07
>60mins	2.3	0.29 +/- 0.03	0.02	0.59	0.06

Table 5: The area of clones derived from populations of cells with different properties of adherence to collagen IV (3 experiments). The larger variance in the clones derived from rapidly adherent cells illustrates the greater range of clone area. The slowly adherent population shows a smaller range when compared to the unselected cells. There was variation in clone area between experiments and the results were analysed by combining the percentile rank of clone areas for each population of cells. Clones derived from rapidly adherent cells were significantly larger than those from unselected cells (Mann-Whitney U

test, $p < 0.005$) and those from the slowly adhering population (Mann-Whitney U test, $p < 0.005$).

Selection on collagen IV	Mean no. of clones per dish	Mean area of clones +/- SEM
<5mins	3.5	6.01 +/- 1.12
Unselected	7.8	6.63 +/- 0.75
>60mins	1.3	2.68 +/- 0.51

Table 5a: Areas of colonies on control plates. Control plates containing the same number of male cells as the whole mounts but without the background female cells. Analysis of the percentile rank of area did not reveal any significant differences between the clone derived from the various populations (Mann-Whitney U test, all p values greater than 0.1), despite the differences in mean area (see the discussion section of this chapter).

4.2.ix. Survival of clones selected by rapid adherence to collagen

To investigate if rapidly adhering cells gave rise to clones which survived for 10 days in a confluent epidermal sheet more frequently than those that adhere slowly to collagen, cells positive for β -gal expression were selected for 20 minutes on collagen IV, removed with trypsin and 200 cells seeded onto a culture plate insert. 200 unselected cells and 2000 cells which did not adhere to collagen IV in 60 mins were also seeded onto culture plate inserts. 5×10^5 negative cells were added to each insert and the cultures allowed to grow until 10 days post-confluent.

After 10 days the cultures were stained with X-gal (Figure 3d) and the numbers of positive clones in each dish were counted. The results of these experiments are shown in Table 6 and Figure 9.

Cells which adhered rapidly to collagen had around 6 times the colony forming capacity in this system when compared to the slowly adhering population.

Selection on collagen IV	Mean no. of colonies at 10 days (% of no. of seeded cells)
<20mins	30.38 +/- 5.73
Unselected	13.67 +/- 7.99
>60mins	5.23 +/- 2.91

Table 6: Mean number of β -gal marked colonies remaining 10 days post confluence.

There was no significant difference between the unselected population and either the rapidly adherent or the slowly adherent populations, however the rapidly and slowly adherent populations were significantly different (Student's unpaired *t* test, df 5, *p* = 0.025).

4.2.x. Attempts to determine cell fate by lineage analysis in longer term culture

I made some attempts to grow keratinocytes over longer periods than those possible with Rheinwald and Green cultures or culture plate inserts.

Transplants of dispase treated epidermal sheets were grown in nude mice using Barrandon's method (1988). These cultures survived for between 7 and 10 days and had a histological appearance close to that of normal epidermis. It was possible to section the grafts as a block including the mouse skin and detect male colonies by *in situ* hybridisation (Figure 10). However, large numbers of grafts would need to be performed to obtain data for only a short extension of time in culture so this method was not pursued.

Two methods of growing mixtures of male and female keratinocytes on deepidermidised dermis (DED) were tested. In some experiments the male and female cells were mixed before seeding onto the DED and others involved transplanting dispase treated epidermal sheets of male and female keratinocytes onto large pieces of DED. The former method gave sheets that were too delicate to be handled and the latter produced thick, tough sheets but in which most of the keratinocytes had differentiated by 2-4 weeks (Figure 10). I was unable to reliably stain these epidermal sheets, either as whole mounts or tissue sections, using the Y-probe because the sheets were of irregular thickness, difficult to handle or stick to glass slides. It is not clear why hybridisation to tissue sections was unreliable in

these experiments, but restraints of time meant that I was unable to pursue these investigations further.

Figure 1 : Keratinocytes grown to confluence in Rheinwald Green cultures and the sheets hybridised *in situ* using biotinylated probe HY2.1 to the Y chromosome by the whole mount method. Developed using horseradish peroxidase and DAB and counterstained with haematoxylin.

a,b : Low power photomicrographs of a sheet of HY2.1 positive male cells containing a single brown dot in the nucleus (b) and negative female cells (a).

c : Low power view of a single HY2.1 positive male clone (centre) in a background of negative female cells.

d : High power view of the edge of an HY2.1 positive male clone (right) and adjacent background negative female cells (left).

Scale bars : 50 μ m.

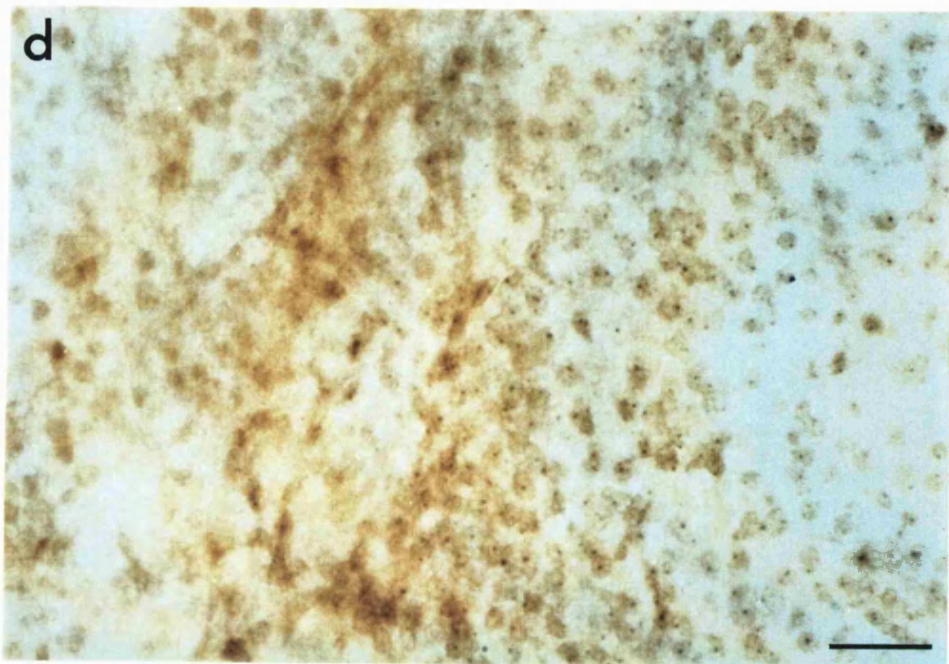
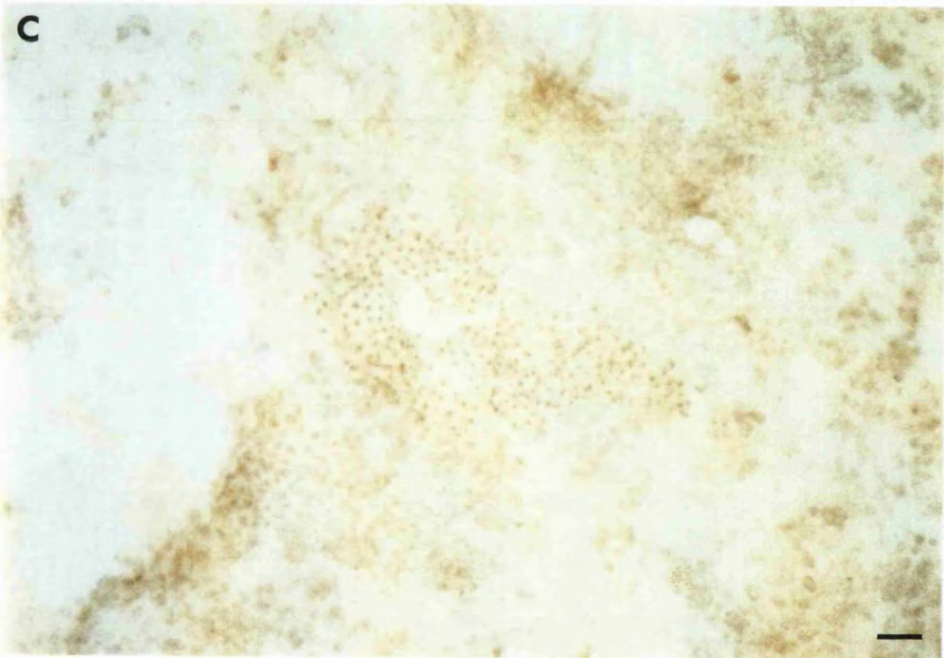
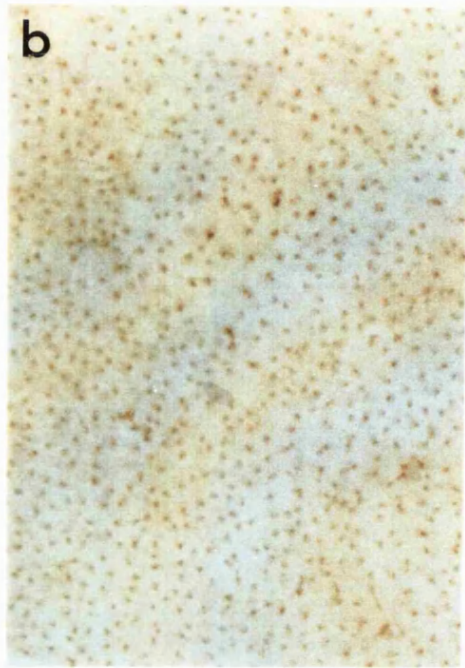
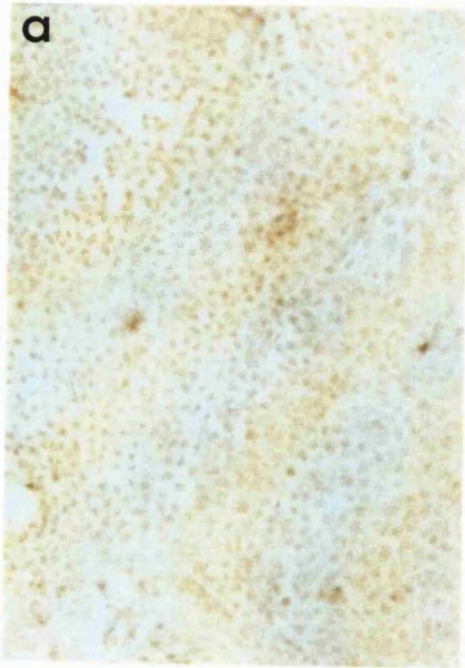


Figure 2 : Limiting dilution; effect of seeding density and female :
XYY ratio on XYA colonies. XYA keratinocytes grown in
Rheinwald Green cultures in duplicate 2cm² dishes with a
background of female cells to confluence, dispase treated,
paraffin embedded and serial sectioned. The dishes were
seeded at varying densities and ratios of female to XYY cells.
The serial sections were hybridised *in situ* using biotinylated
probe HY2.1 to the Y chromosome then the number of positive
basal cells in each colony in each section counted.

The higher ratios combined with the higher densities gave
complex patterns of merged positive colonies and the data is not
shown.

A : The mean number of XYY colonies per dish at various
female:XYY ratios and seeded at three different densities.

B : The mean size of XYY colonies (largest diameter, number of
cells) at various female:XYY ratios and seeding densities.

Increasing the seeding density with a constant ratio, or *vice
versa* results in an unpredicted increase in colony diameter,
indicating merging of colonies. Increasing the seeding density
causes an unpredicted increase in number of colonies, which
given colony merging, indicates survival of more colonies at
higher densities.

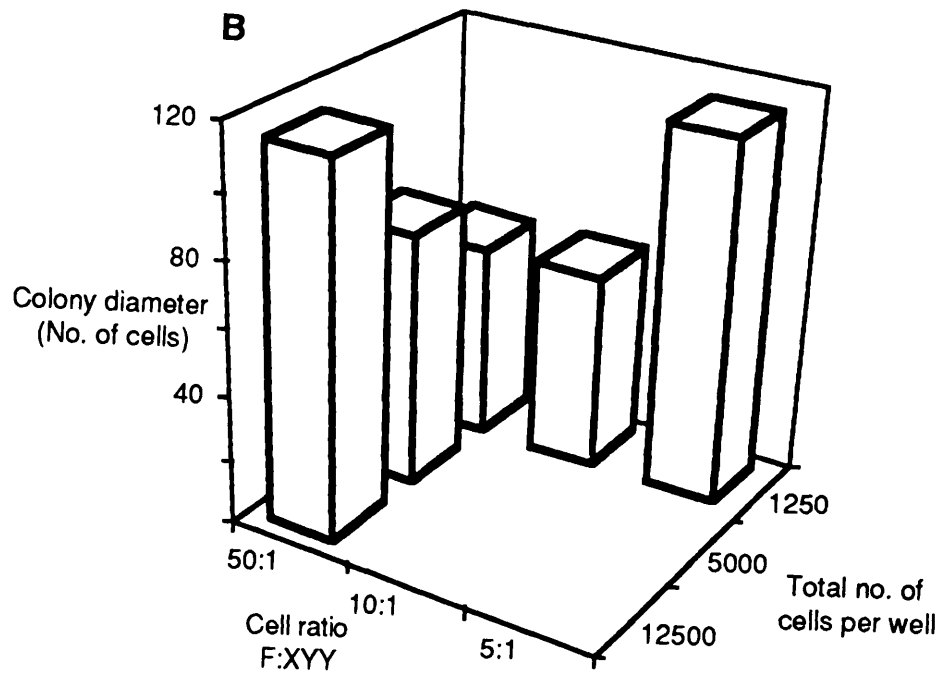
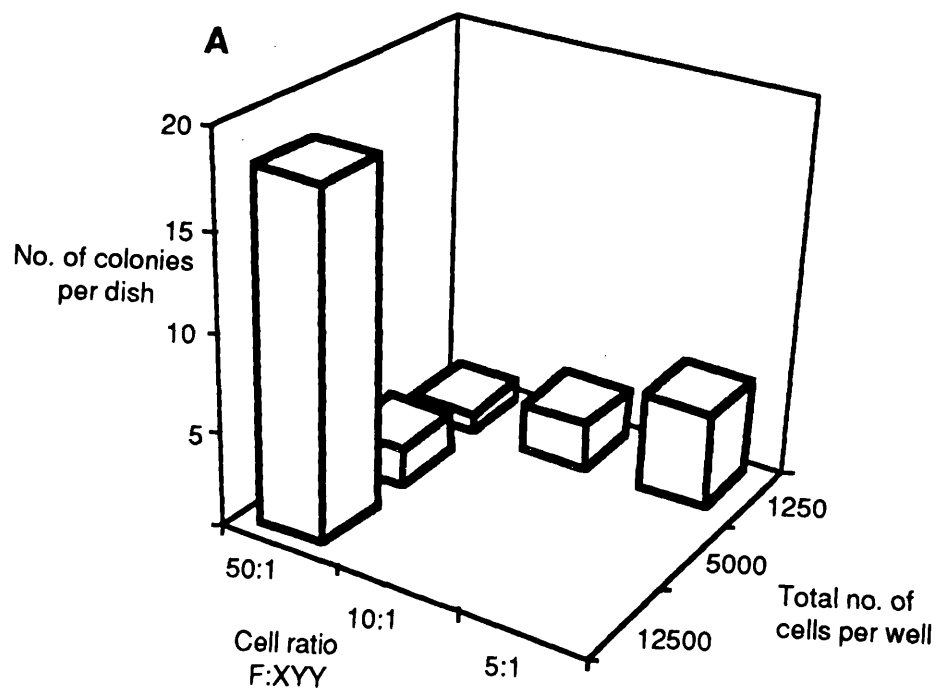


Figure 3 : X-gal staining of keratinocytes infected with the BAG-F retrovirus.

a : Mixed BAG-F infected and uninfected keratinocytes of the same line grown to confluence in Rheinwald Green cultures. The cultures were removed using dispase, stained with X-gal (blue), paraffin embedded, sectioned and counterstained with neutral red. The colony contains positive basal and suprabasal cells.

b,c : Mixed BAG-F infected and uninfected keratinocytes seeded at high density and grown on culture plate inserts for 10 days. The inserts were frozen and sectioned then stained with X-gal. A few colonies comprising suprabasal cells only were seen (b, phase contrast). All the cells in the control inserts containing infected cells only were stained blue (c).

d : A single β -gal positive colony grown on a collagen coated culture plate insert in a background of negative uninfected cells seeded at 5×10^5 per insert for 10 days, stained with X-gal and viewed directly.

Scale bar : 50 μ m.

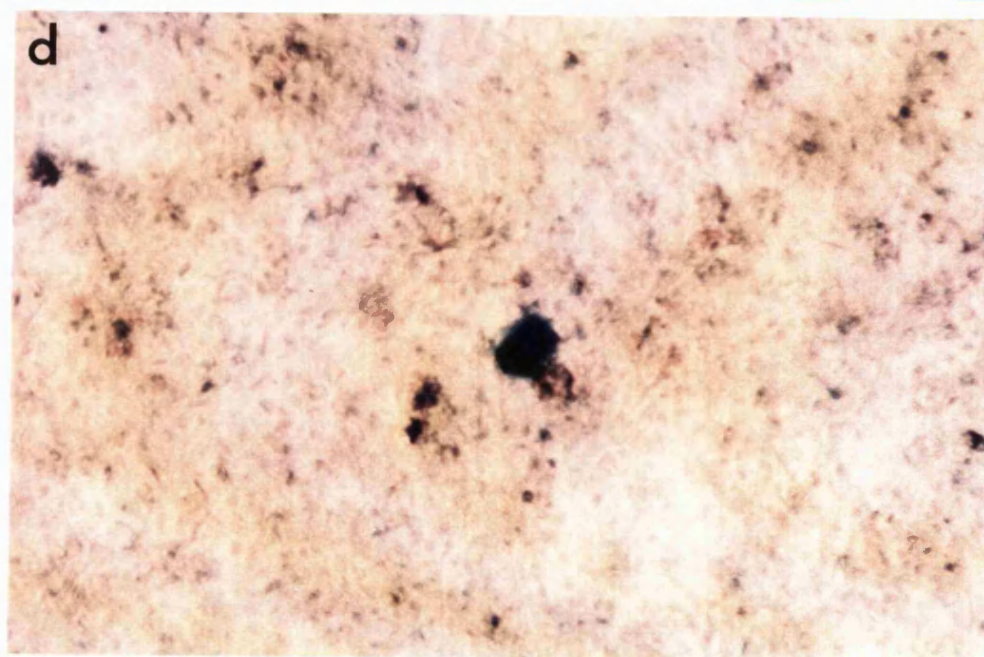
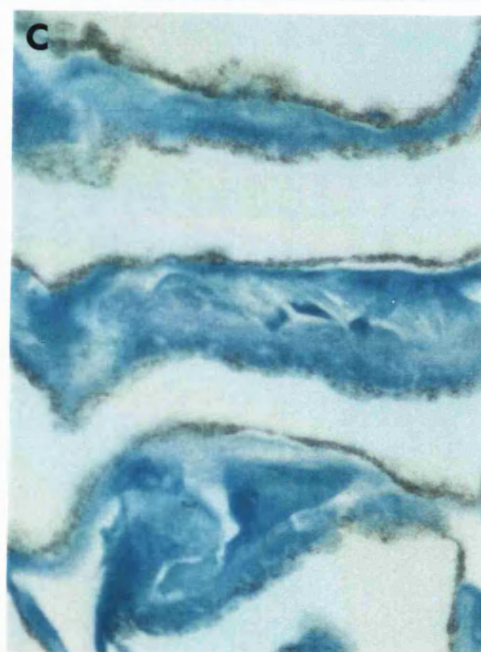
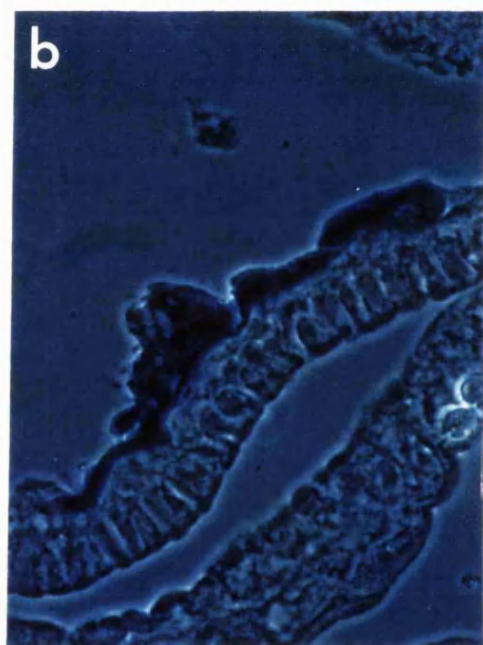
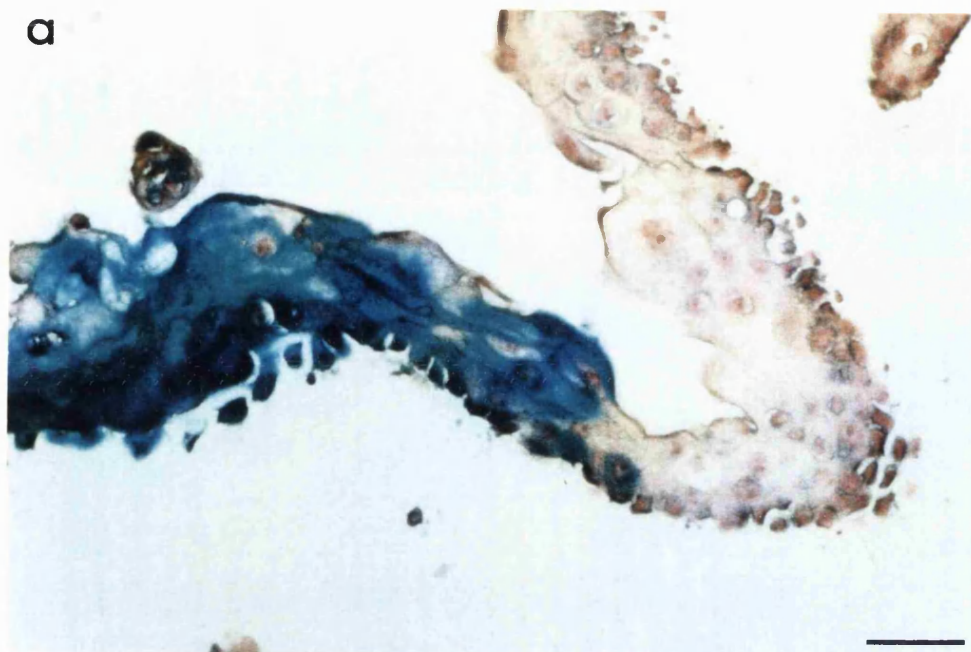
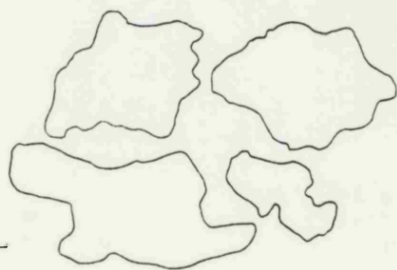
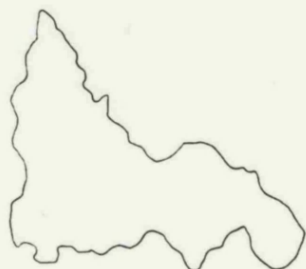
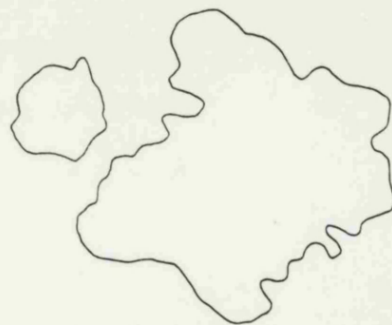
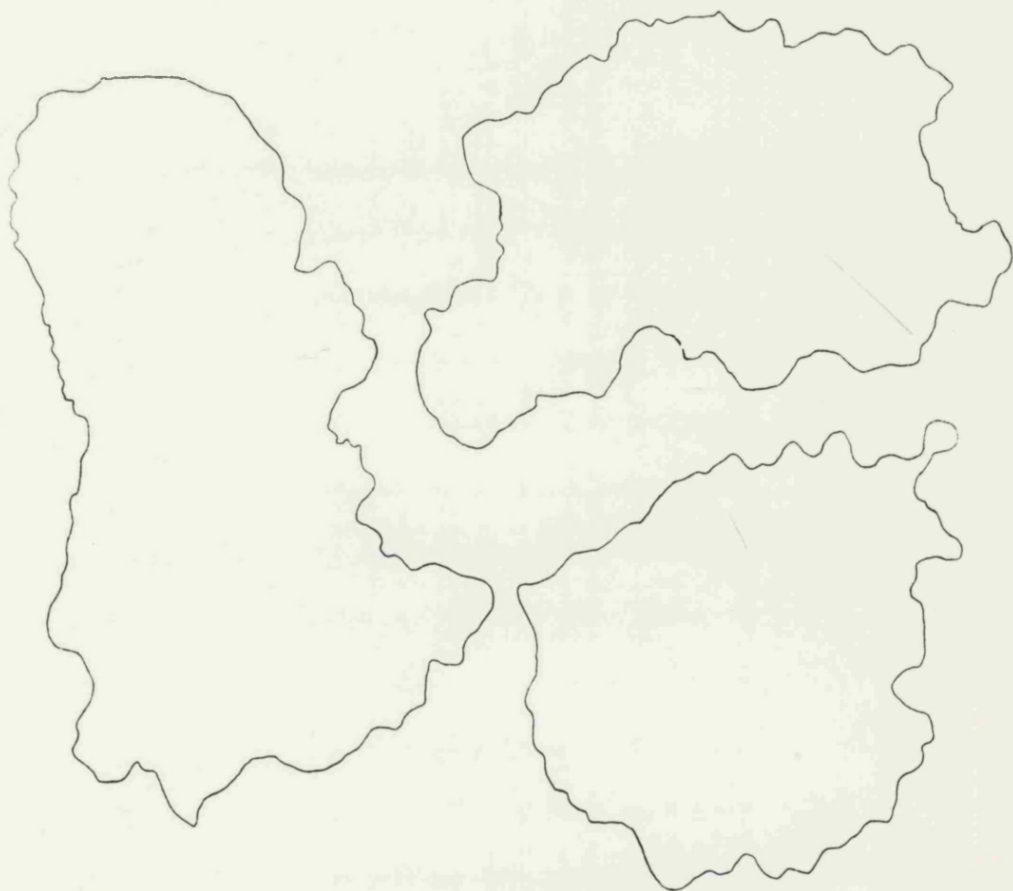


Figure 4 : The shape and size of clones of male keratinocytes, detected by *in situ* hybridisation using the biotinylated HY2.1 probe to the Y chromosome, in a background of female keratinocytes seeded at 5×10^4 cells per 35mm dish (Rheinwald Green culture, whole mounts). The clones were selected from one experiment and traced using a drawing arm on a Lietz Diaplan microscope.

Scale bar : 1mm.



1mm

Figure 5 : The effect of seeding density on the size and number of marked clones in an epidermal sheet. Detection of male clones was by *in situ* hybridisation with biotinylated HY2.1 probe to the Y chromosome, whole mount method. A and B are two typical experiments. A constant, limiting dilution of male cells was seeded, without selection on collagen, with a varying number of background female cells in excess and grown to confluence in Rheinwald Green cultures. Detected clones were traced using a drawing arm on a Lietz Diaplan microscope and the areas determined by image analysis.

The number of clones (shown at the top of each column) was higher when the total seeding density was 10^5 per dish compared to the other seeding densities. Areas decreased with increasing seeding density and the spread of areas tended to be larger at the lower densities. At the lowest densities the clones showed a trend toward a bimodal distribution of areas.

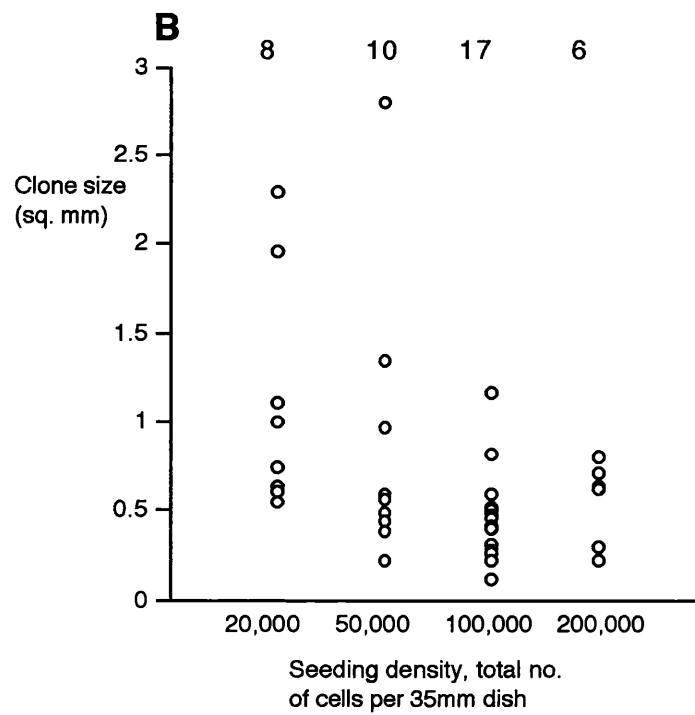
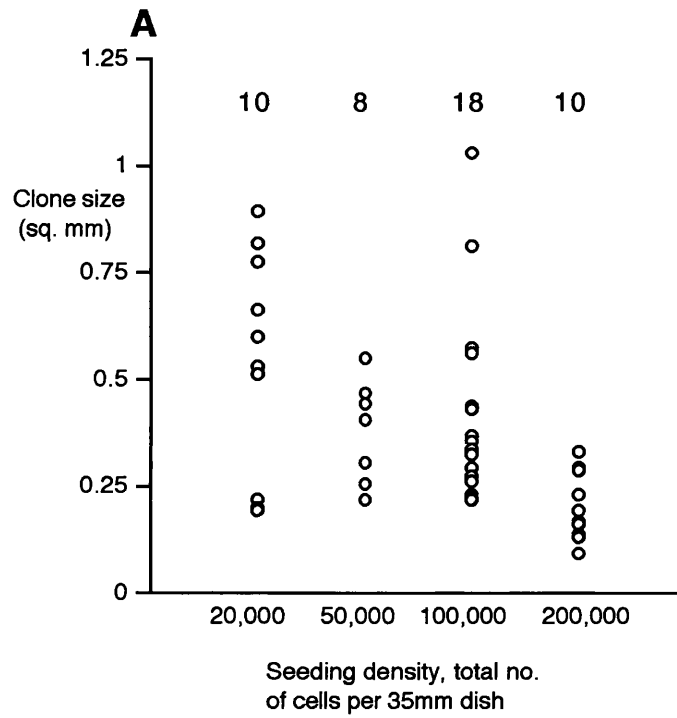


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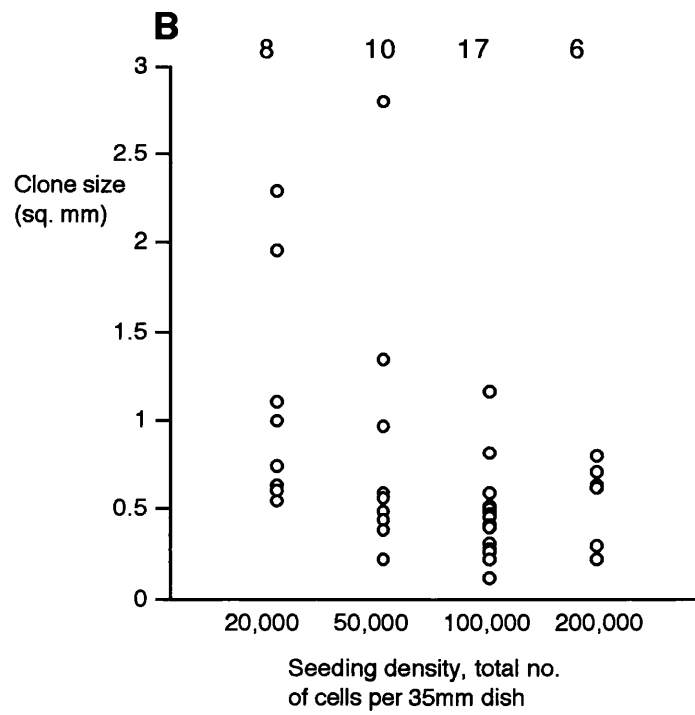
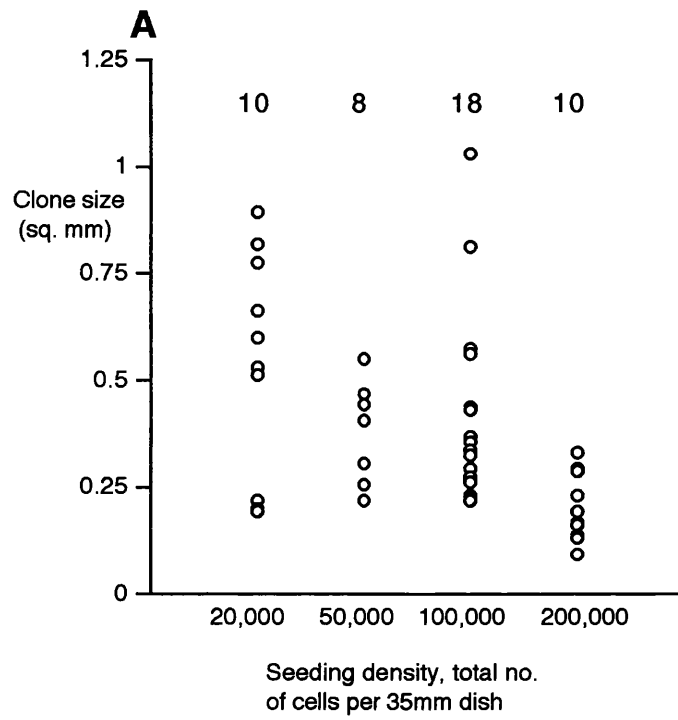


Figure 6 : Adhesion and colony forming efficiency (CFE) of keratinocytes selected on collagen IV.

A : The percentage of cells adherent to collagen IV selected for 5 minutes (<5m, 5.22% SEM $\pm$ 0.32), unselected (un), panned on collagen IV for 4 hours and representing the total adherent population, (37.58% SEM $\pm$ 6.06) and those adherent between 1 and 4 hours (>60m, 24.76% SEM $\pm$ 0.77). The results are the mean of 3 experiments, using duplicate dishes, for each population.

B : The CFE as the percentage of adherent cells forming colonies of over 32 cells in Rheinwald Green cultures when panned on collagen IV for 5 minutes (<5m, 43.05% SEM $\pm$ 3.77), unselected (un), panned on collagen IV for 4 hours (23.89% SEM $\pm$ 0.94) and those panned for between 1 and 4 hours (>60m, 2.42% SEM $\pm$ 0.40).

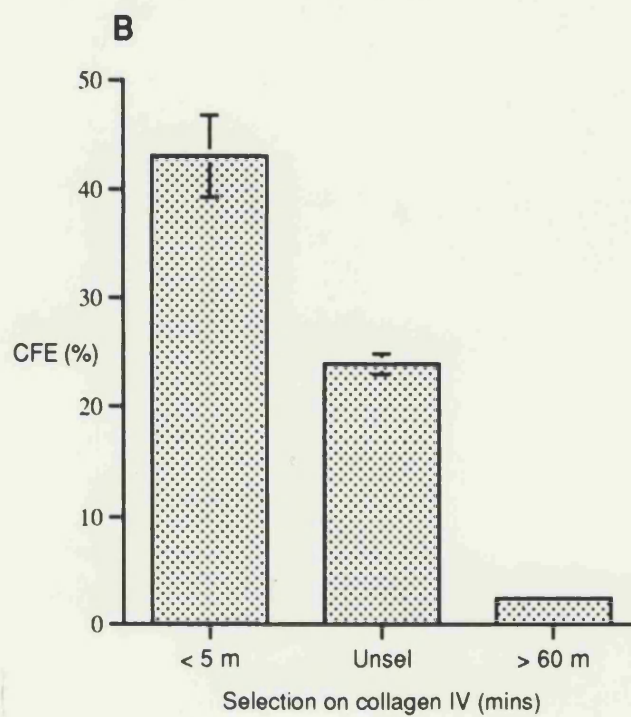
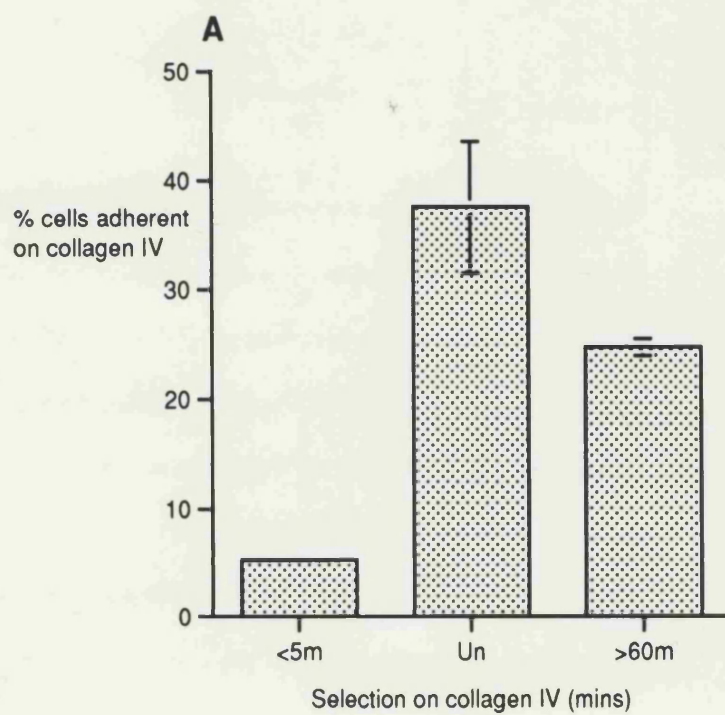


Figure 7 : The effect of selection on collagen IV on the area of clones of male keratinocytes in a confluent sheet of background female keratinocytes grown in Rheinwald Green cultures seeded at constant density (5×10^4). The clones were detected by *in situ* hybridisation to biotinylated HY2.1 probe for the Y chromosome by the whole mount method.

Cells were panned on collagen IV for 5 minutes (<5m), 4 hours, representing the whole adherent population (Unselected) and 1 to 4 hours (>60m).

A and B are two typical experiments. Rapidly adherent cells exhibit a greater spread of clone area, forming larger clones than unselected and slowly adherent keratinocytes.

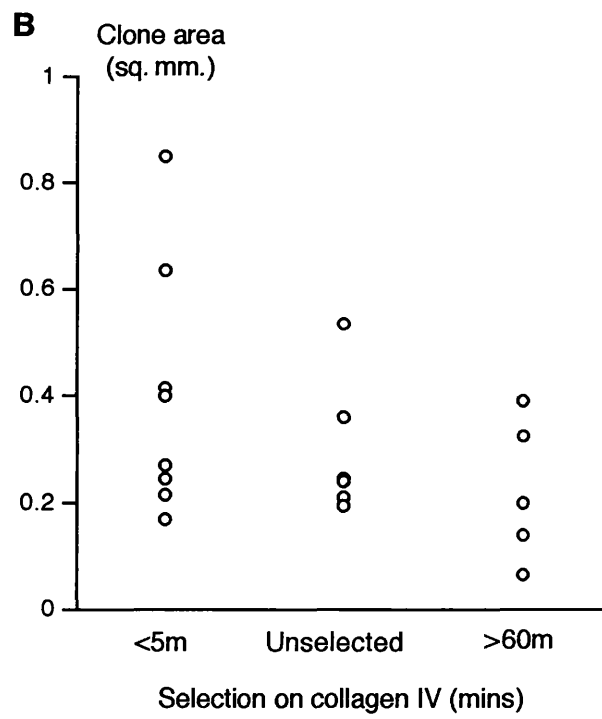
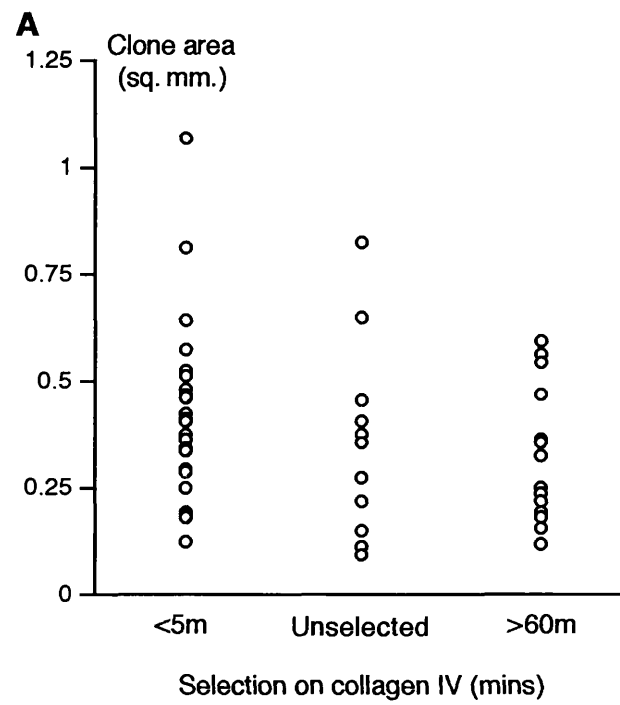
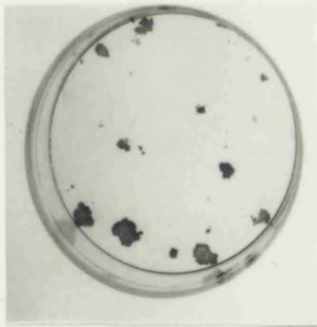
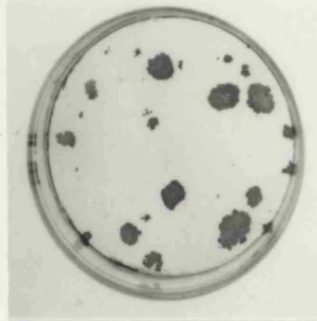


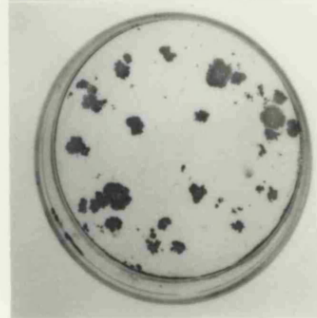
Figure 8 : Control plates for the selection of keratinocytes on collagen IV. Cells were panned on collagen IV for 5 minutes, 4 hours (Unselected) and 1 to 4 hours (Over 60 mins) then grown in Rheinwald Green cultures for 10 days. The colonies derived from each population did not show any consistent differences in area.



Under 5 mins, 200 cells



Unselected, 200 cells



Over 60 mins, 1000 cells

Figure 9 : The colony forming efficiency of clones surviving for 10 days in confluent cultures on culture plate inserts, of populations of cells selected on collagen IV. BAG-F infected keratinocytes were panned on collagen IV and those adherent in 20 minutes (<20m), those not adherent after 60 minutes (>60m) and the whole population of cells, unselected on collagen IV (Un) were seeded onto culture plate inserts in a background of uninfected cells seeded at high density (5×10^5). The β -gal positive clones were detected by direct staining of the culture with X-gal (see Figure 3). The values are the mean of three experiments.

Adherent cells (30.38% SEM $\pm$ 5.73) had six times the number of surviving clones compared to the non-adherent population (5.23 SEM $\pm$ 2.91) and unselected keratinocytes had an intermediate value (13.67% SEM $\pm$ 7.99).

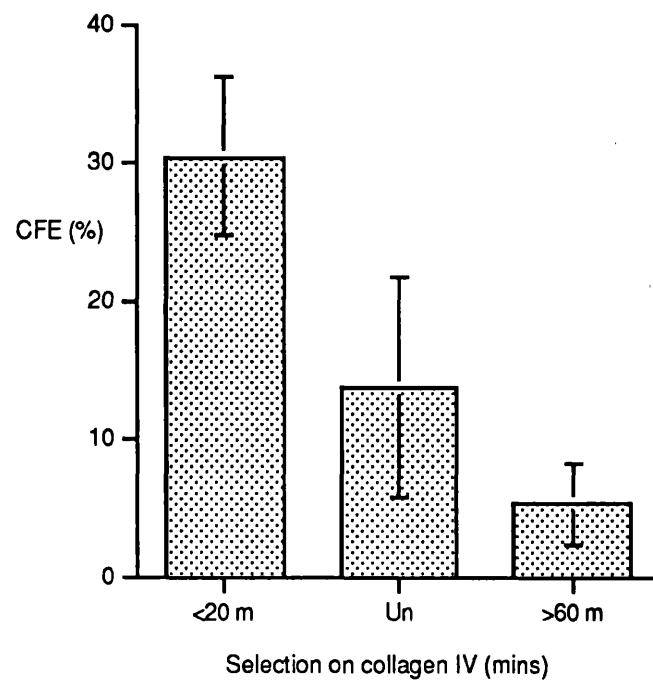
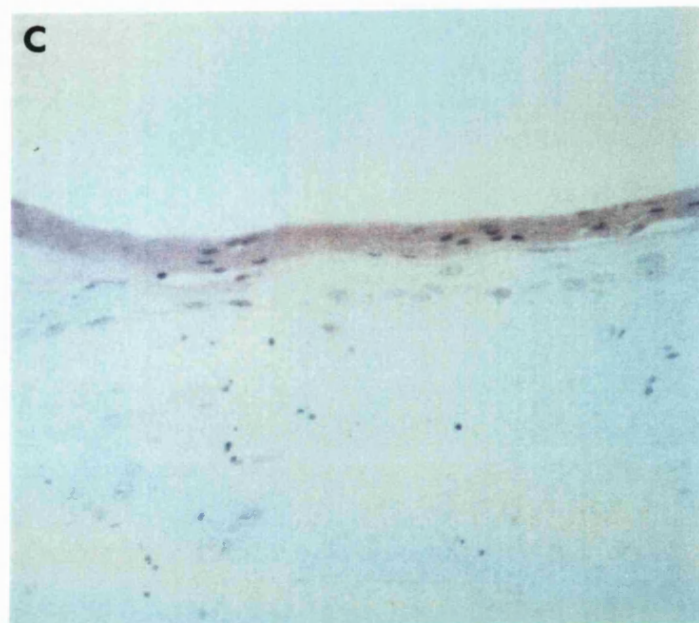
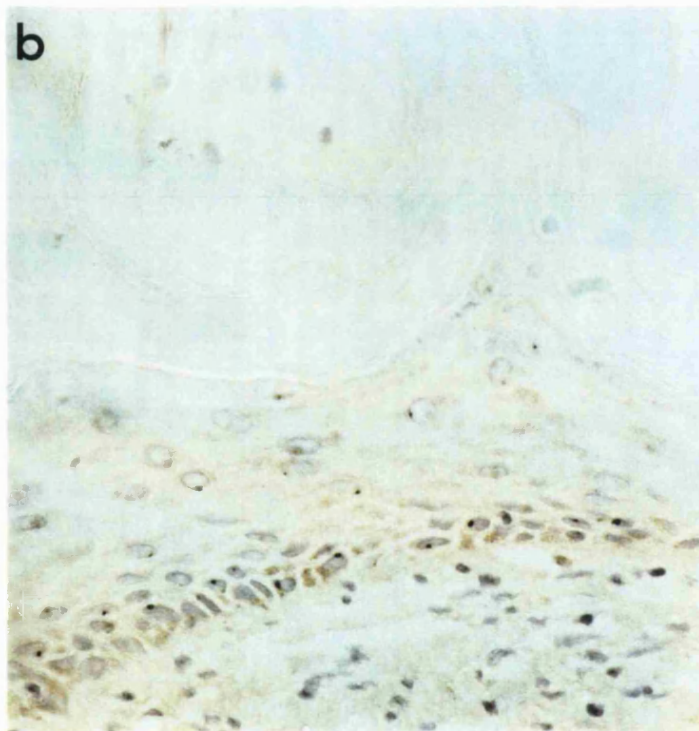
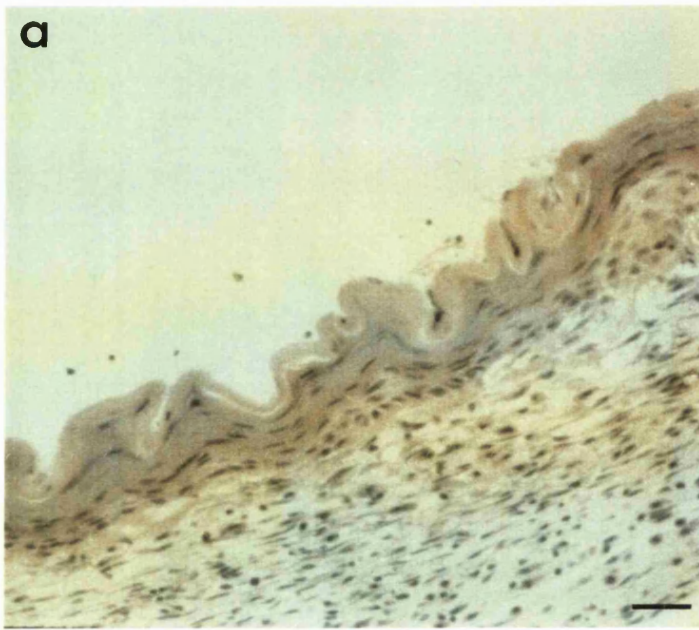


Figure 10 : Lineage analysis in long term keratinocyte cultures.

a : Keratinocytes grown to confluence in Rheinwald Green cultures, treated with dispase and grafted under a skin flap in nude mice (Barrandon et al. 1988) then harvested in 7 days (H+E). Scale bar : 50 μ m.

b : High power photomicrograph of a colony of male keratinocytes (brown nuclear dot) grown in Rheinwald Green cultures in a background of female cells and grafted into a nude mouse. The male cells were detected by *in situ* hybridisation of paraffin embedded sections using the biotinylated HY2.1 probe to the Y chromosome and developed using horseradish peroxidase and DAB.

c : Keratinocytes grown to confluence in Rheinwald Green cultures, dispase treated, grafted onto de-epidermised dermis then maintained for 4 weeks (H+E).



4.3. Discussion

Because of the difficulties in culturing the β -gal positive cells mentioned in Chapter 3 it was possible to obtain limited reliable information using this system. In experiments which were successfully concluded it was possible to stain all of the marked cells, for instance in cross-section, an advantage over the Y-probe method. The whole mount method using the Y-probe, however, also allowed detection of all of the marked cells but did not allow observation of cross-sectional morphology of clones.

Because of the nature of the lineage experiments, the number of colonies measured per experiment is low, however the results are reproducible between experiments.

The whole mount method was reliable both for high detection of positive cells in control sheets of only male cells, close to 100%, but also detected over 80% of the predicted number of marked clones in an epidermal sheet. This observation shows that most of the clones of over 32 cells in the low density control dishes without background unmarked female cells also contributed to the epidermal sheet at confluence. The reduction in the number of clones between the control dishes and the whole mount sheets could be for two reasons: either some clones were too small to detect or a proportion of clones, capable of producing over 32 cells in low density cultures, were lost from the confluent epidermal sheet. It is possible that the "top heavy colony" seen in the serial sections may represent such a clone.

I was able to establish the density at which marked cells could be seeded at limiting dilution and my initial experiments with lineage markers established that all cells which contribute colonies to the confluent epidermal sheet in Rheinwald and Green cultures give rise only to clones containing basal and suprabasal cells. There was no evidence of lateral movement of suprabasal cells between clones since only clones of this type are seen in cross-sections of dispase treated sheets.

These findings suggest that no colonies founded by transit amplifying cells survive to confluence. This agrees with Philip Jones' work where he shows that such cells form only small colonies in which all the cells are destined to differentiate and become involucrin positive (Jones and Watt 1993).

The whole mounts showed considerable differences in clone area between each experiment, depending on the behaviour of the background female cells and, in the control plates, the growth rate of the male keratinocytes. For this reason the mean area for each population could be skewed towards the experiment with larger clones in one population but low numbers of clones in another, meaning that differences in distribution within each experiment were not apparent. To counter this effect, all the statistical analysis in the whole mount experiments was done by giving each clone in each experiment a percentile rank, then combining these ranks from all the experiments. This has the effect of assigning the same score (100%), and therefore the same weighting, to the largest clone in each experiment and similarly to the smallest clone (0%), with the clones in-between having the appropriate intervening percentile rank. This data is not appropriate for a parametric test as it is of ordinal, rather than interval /cardinal status. For this reason the Mann-Whitney U test was used to obtain a probability (p) value.

In the whole mount experiments, at low total seeding density the range of areas of the clones forming the epidermal sheet was considerable and in one experiment the largest clone was 72 times larger than the smallest. Since the cells were seeded evenly this shows a considerable difference in the proliferative capacity of the cells in those clones. The effect of seeding density revealed that a few clones have the capacity to become very large. These are not seen at the high seeding densities, either because the sheet becomes confluent too quickly for them to grow or because the close proximity of other cells slows the proliferation rate.

The number of clones at low densities was less than at the higher densities. This implies that there were some clones incapable of surviving low density conditions which were still present at higher densities, since these reached confluence faster. It may be that some keratinocytes are able to form clones which have a limited capacity for proliferation, or at least in which the rate of differentiation is more rapid than proliferation under certain conditions. Such conditions would include low density culture when the rate of differentiation would "catch up" with the rate of proliferation and many of the cells would differentiate. These colonies are nevertheless able to reach a considerable size (between approximately 0.1 and

0.9mm² or 160-1620 cells (Barrandon and Green 1987b)) and can therefore contribute to the epidermal sheet in certain conditions, such as at higher seeding densities, when the high rate of differentiation and proliferation can be reversed before the clone reaches an endpoint, and the epidermal sheet reaches a steady state. The keratinocytes forming such clones may behave as a stem cell in a confluent epidermis, i.e. would proliferate slowly, give rise to differentiating progeny and also self renew as described by Lajtha (1979) and Hall and Watt (1989). However in some conditions, of which wound healing may be an example, the rapid rate of proliferation and even faster differentiation would lead to the loss of the clone from the epidermis. Such properties may be very useful in such conditions, allowing rapid healing, covering the wound rapidly with effective, differentiated cells but at the expense of the clone. Such clones possibly correspond to the meroclones described by Barrandon (1987b).

The number of positive clones at the highest density (2×10^5 per 35mm dish, $2.5 \times 10^4/\text{cm}^2$) was lower than expected. There are two possible reasons for this. The clones may have been too small to detect above background using the Y-probe method or the high seeding density may have caused the cells to differentiate or not proliferate at all. The first explanation is much the most likely. The size of clones detected at this density is fairly small (0.09-0.81 mm²) and even smaller clones would be impossible to distinguish reliably above the background female cells, since these also hybridise to the probe in low amounts making it difficult to pick out a group of only a few male cells. Observation of colonies seeded at very high density makes the other explanations less likely. Although cells seeded at very high density in the standard way may get overgrown by early proliferation of cells destined to differentiate, the larger growing colonies are still present and later catch up. Also if large numbers of keratinocytes are seeded onto collagen for up to 1 hour (even at very high densities of $3.5 \times 10^5/\text{cm}^2$) and the non-adherent (transit amplifying) population is then removed, the adherent cells form normal growing colonies unaffected by the close proximity of their neighbours.

In summary, the cells that give rise to confluent epidermal sheets in culture are heterogeneous, with widely varying capacities for proliferation relative to the rate of differentiation within the clone. Since stem cells are more likely to be rapidly adherent to

collagen IV than transit amplifying cells, I tried to discover if the stem cell population could be further divided by relative ability to adhere. The colony forming efficiency of rapidly adhering cells was twice that of the unselected controls and 17 times that of the slowly adhering cells.

When the rapid and slow adhering keratinocytes are compared in the whole mounts using male and female cells, with a constant total seeding density, the CFE was higher for the rapidly adhering cells, as expected. Also the clones in the epidermal sheet formed by the fast stickers were larger than those of the control total population and the slow stickers. This strongly suggests that the ability to adhere to collagen IV, and hence the level of functional integrin on the cell surface, is a measure of the "stemness" of a basal keratinocyte as postulated by Jones and Watt (1993).

Keratinocytes that did not adhere to collagen within 60 minutes did not form the very large clones formed by the rapidly adhering population. If ability to adhere is an indication of the extent of "stemness", then the cells which were "very stem", and therefore able to form the largest colonies, would be very adherent and have all adhered before 60 minutes, so completely removing them from the remaining population. The keratinocytes in the slowly adhering population that do form clones at confluence are not the transit amplifying cells identified by Potten (Potten and Hendry 1973; Potten 1974; Potten 1981; Potten and Morris 1988) since all such cells and their progeny have been lost before confluence and the smaller colonies forming these sheets contain a substantial number of cells, a clone of 1mm contains approximately 1600-1800 cells (Barrandon and Green 1987b) so the founding cell must have a considerable capacity for maintaining a proliferative unit in the epidermis, and for self renewal.

Heterogeneity in the proliferating compartment is related to the adhesive properties of the cell and the proliferative capacity of the cell, and therefore the type of clone it will form, is predictable according to its adhesive properties.

There are two main explanations for these findings. The first is that every founding cell has the same, predetermined number of cell divisions it can undergo during its life and that there is therefore a gradient of capacities seen at any one time, depending on how far along the pathway each cell has progressed. Progress along the pathway could be random or

the model would require the mechanism of an internal cellular clock, telling the cell how many divisions it had gone through. Such mechanisms have been postulated in other systems (Raff and Lillien 1988). In this case a long term clock - an internal cell calendar - would be required. Jones and Watt (1993) propose a continuum of cells with the ability to adhere to extracellular matrix being inversely related to the probability of a cell differentiating, the higher the adhesiveness, the higher the probability of self-renewal.

The other explanation is that the clone forming population of cells is divided into distinct groups. Some cells, capable of almost infinite self renewal, would be rapidly adhesive with high surface expression of functional integrins. These cells would fit the classical description of a stem cell (Lajtha 1979). Also in the clone forming population would be a discrete group, which in steady state conditions, such as in normal epidermis, behaved as a stem cell, proliferated slowly, were self renewing and gave rise to differentiated progeny, so fitting the definition of an epidermal stem cell. However under certain conditions the probability of differentiation of these cells and their progeny would be greater than the probability of self-renewal and the clone would eventually reach an endpoint when all the cells had terminally differentiated. The advantage of such a cell in stressful conditions, such as wounds, would be the ability to produce rapidly differentiating cells to protect the wound but at the relative expense of self-renewal. Such a system has been suggested in the haemopoietic system which may contain multipotent cells capable of rapidly repopulating the peripheral blood after insult but forming only relatively short lived clones. Other cells have the capability to repopulate the marrow in the long term (Lemischka *et al.* 1986). In the epidermis these cells are unipotent, which makes defining the sub-populations more difficult. It is possible that any discrete sub-populations of clone forming cells differed only in their progeny but not in the ability to self renew. Some cells may produce more daughter stem cells and others more daughter cells destined to differentiate. The difference between the populations would be due to the relative number of asymmetric divisions and divisions producing two stem cells.

The two possible mechanisms are not mutually exclusive, each cell may have a defined lifespan, measured by number of divisions, within each sub-population of clone

forming cells or there may be a continuum of probabilities of cell renewal ranging from infinitely high to low, independent of the age of the cell.

Given such heterogeneity of clone forming cells in the epidermis it is not clear whether the cells forming smaller clones represent true stem cells or a variety of "souped up" transit amplifying cell. This problem is largely a semantic one. In the haematopoietic system any multipotent cell, and therefore both the sub-populations, can be called stem cells. However no cells in the interfollicular epidermis have been shown to be multipotent as yet. Cultured epidermal sheets such as those used in my experiments are used for autografting onto patients with severe burns (Compton *et al.* 1989). The sheets are capable of forming a permanent, fully functional, normal epidermis in these patients which lasts for the lifetime of the patient (Compton 1993) and cells contributing clones to the sheet therefore deserve to be considered a stem cell. Stem cells do not necessarily have to last for ever, or even for the full life of the organism. Many cells defined "stem" during development give rise to multiple lineages of cells but do not survive themselves. It is not clear how long such clones would actually last in the epidermis, however results from Carolyn Compton's laboratory (personal comm.) suggest that keratinocytes from elderly patients have the same proliferative capacity as those from younger donors indicating that the stem cells have unlimited ability for self renewal.

In some low seeding density experiments there is a trend towards a bimodal distribution of colony area which suggests that there may be two different types of stem cell contributing to the epidermis. I have shown by these experiments that there is a hierarchy of stem cells that is predicted by their adhesiveness to extracellular matrix and suggest that this may be a continuum or consist of at least two discrete sub-populations of stem cell.

To observe clones formed by putative transit amplifying cells I used culture plate inserts, to increase the number of differentiated layers retained in the culture, and keratinocytes seeded at very high densities. In cross-section these colonies were small and consisted of only suprabasal cells with no underlying positive basal layer. This indicates that the founding cell divided a few times only and that all the progeny differentiated. Presumably

such clones are destined to leave the epidermal sheet eventually. These appearances have also been described by Jonathon Garlick (1993).

The results from the experiments on culture plate inserts indicate that cells with the ability to adhere rapidly to collagen also form clones which have enhanced ability to survive in confluent epidermal sheets for 10 days when compared to unselected cells and slowly adherent keratinocytes. Long term clonal survival is a further characteristic required of stem cells.

In conclusion, I have established reliable assays to detect lineage in marked cultured keratinocytes at a limiting dilution and used these to study the morphology of clones derived from those cells.

The experiments using lineage markers in cultured epidermal sheets have shown considerable heterogeneity in proliferative capacity of keratinocytes contributing clones to the sheet (stem cells) and that both proliferative capacity and ability of the clone to survive at confluence is related to the inherent adhesive properties of the cell.

INFLUENCES ON STEM CELL LOCATION AND PATTERNS OF KERATINOCYTE INTEGRIN AND CADHERIN EXPRESSION

5.1. Introduction

It has long been suggested that stem cells reside in specific locations where the microenvironment is optimal, the stem cell niche, and that the fate of their progeny depends on such microenvironmental factors (Schofield 1978; Hall and Watt 1989). Staining of epidermis from various sites by fluorescein labelled anti-integrin antibodies show patches of cells expressing higher levels of integrin. Their position suggests that the niche is in the depths of the rete ridges in the palm and sole but in all other body sites the stem cell niche may be at the tips of the dermal papillae where patches of basal keratinocytes with higher levels of surface integrin expression correlates with a lower number of cells in the cell cycle (Jones *et al.* 1995).

The results shown in Chapter 4 and those of others (Barrandon and Green 1987b; Jones and Watt 1993) demonstrate that the "stemness" of a keratinocyte is a property of that cell, and is related to integrin expression. However the cell would need to remain in the stem cell niche to retain its stem cell properties. How do the stem cells become located in a specific position in the epidermis? I have investigated the influence of the extracellular matrix of the dermis, including the basement membrane, on stem cell position, using the relative levels of integrin expression as a marker of stem cell patches.

Integrin expression in keratinocytes is regulated by cadherins (Hodivala and Watt 1994) and I was interested to investigate if cadherins exhibited similar heterogeneity of expression as the integrins in the proliferative compartment and in situations where integrin expression was altered, such as wound healing, keratinocytes cultured on collagen gels, (when expression becomes suprabasal) and pathological conditions such as psoriasis, oral squamous cell carcinoma and cervical intraepithelial neoplasia (Hertle *et al.* 1991; Grinnell 1992; Hertle *et al.* 1992; Hodivala *et al.* 1994). Finally, I attempted to discover the possible

effects of suprabasal integrin expression in the epidermis by generating transgenic mice expressing integrins under the control of a suprabasal promoter.

5.2. Results

5.2.i. Pattern of epidermal integrin expression *in vivo* and *in vitro*

Sections of non palmar-plantar epidermis stained with anti-integrin antibodies showed patches of bright and dull staining with the bright patches at the tips of the dermal papillae and the areas of low staining at the bottom of the rete ridges. In the palm and sole the pattern was variable, depending on the exact site of biopsy, but usually the reverse of that in other body sites (bright patches at the tips of the rete ridges). These patterns of staining are illustrated in Figure 1a,c.

The patterning of integrin expression into patches of high expression is also reflected in the messenger RNA levels of β_1 integrin in cultured keratinocytes, shown by the variation of the number of grains per cell after isotopic *in situ hybridisation* of keratinocyte colonies (Figure 2).

5.2.ii. Collagen IV levels in the basement membrane are related to patches of integrin expression

Frozen sections of skin from palm and abdomen were stained using a fluorescein conjugated anti-collagen antibody to establish whether there was variability in the amount of collagen IV along the basement membrane. The intensity of staining was measured using confocal microscopy.

The pattern of collagen IV staining in the basement membrane showed evidence of variability. This was most evident in the palm but also seen in the abdomen. The pattern was related to the integrin patches with the brightest areas at the tips of the rete in the palm and at the top of the dermal papillae in the abdomen. The intensity of staining was measured by confocal microscopy of the sections at 1 μm levels. This eliminated any effect of variation in thickness of the histological sections and of transverse sectioning of the basement membrane. The intensity of staining varied by a factor of approximately 2 between bright and

less bright areas of the basement membrane. The results and confocal images are shown in Table 1 and Figures 3 and 4.

Site	Total no. of readings	Mean fluorescence (arbitrary units) +/- SEM
Abdomen, deep rete ridges	43	70.23 +/- 7.69
Abdomen, top of papillae	42	139.05 +/- 7.77
Palm, deep rete ridges	71	79.86 +/- 4.81
Palm, top of papillae	83	169.04 +/- 5.88

Table 1: Relative fluorescence of the basement membrane in different sites labelled by fluoresceinated anti collagen IV antibody. The intensity of labelling at the top of the dermal papillae differs from that in the depths of the rete for both abdomen (Unpaired Student's *t* test, df 83, $p < 0.0005$) and palm (Unpaired Student's *t* test, df 152, $p < 0.0005$), which show the opposite pattern.

5.2.iii. Pattern of epidermal P-cadherin staining *in vivo* in relation to integrin expression

Staining of P-cadherin was low in all types of epidermis, and confined to the basal layer except in mucosa and hair follicles, where it was suprabasal. In palm, sole, abdomen, scalp and breast the pattern of P-cadherin was the same as the integrins, with bright patches in the same position, according to the origin of the skin (Figure 1b,d). In non palmar-plantar epidermis the cells expressing high levels of P-cadherin were at the tips of the dermal papillae and in the palm or sole the position could be at the papillae tips or the rete ridges, depending on the section, but always the same as the expression of integrin in that section.

5.2.iv. Pattern of epidermal E-cadherin staining *in vivo* in relation to integrin expression

E-cadherin was expressed in the basal and suprabasal layers of the epidermis in all the body sites investigated. The staining in the basal layer was relatively low compared with the suprabasal layers using the HECD-1 antibody but a patch pattern of staining in the basal layer could be seen in the palm and the foreskin (data not shown). In other body sites the

basal staining was too low to discern any variability. The cells in areas where expression of integrin and P-cadherin was high had the lowest staining with the anti E-cadherin antibody, with the intensity of the stain increasing in the integrin/P-cadherin dull patches. The intensity increased further still in the suprabasal cells.

5.2.v. Relationship between suprabasal expression of integrins and P-cadherin

5.2.v.a. Normal epithelia *in vivo*

Suprabasal integrin expression was seen in stratified epithelia in some areas, including the mucosal part of the foreskin, the oral mucosa and some hair follicles of the scalp. In all these cases the expression of P-cadherin was also suprabasal (Figure 5f-i).

5.2.v.b. Wound healing

Sections of wounds were stained for β_1 integrins and P-cadherin. The wounds were suction blisters on the inner thigh (Hertle *et al.* 1992). Wounds in three different subjects were stained at 168 hours after wounding (Figure 5a-e). Suprabasal staining was seen both in the healing wound itself, including the leading edge of the epidermis and in the epidermis immediately adjacent to the wound, but distinct from the leading healing edge. At all sites and all times, when integrins were suprabasally expressed, so was P-cadherin.

5.2.v.c. Cultured keratinocytes on collagen gels *in vitro*

Cultured keratinocytes were seeded onto collagen gels, grown to confluence and raised to the liquid/air interface. Frozen sections were stained at 3 and 7 days post-confluence with anti- β_1 integrin and anti-P-cadherin antibodies. There was marked suprabasal staining of both integrin and P-cadherin at 3 days which had become almost entirely basal by 7 days. Again P-cadherin suprabasal expression showed the same pattern of staining as β_1 integrins (Figure 6).

5.2.v.d. Oral squamous cell carcinoma *in vivo*

Frozen sections of 8 different oral squamous cell carcinomas were stained for $\alpha_2\beta_1$ and β_1 integrins plus P and E-cadherin. In general, tumours with high levels of integrin also

had a higher level of P-cadherin and lower E-cadherin staining (Table 2 and Figure 7).

The staining showed some correlation with the grade of tumour, although this was not invariable. Poorly differentiated tumours had a more basal type of staining throughout, with integrins and P-cadherin and less E-cadherin. Well differentiated tumours had a pattern of staining which reflected the expression in normal mucosa with predominantly basal integrins and P-cadherin. E-cadherin expression was in the differentiated areas.

SCC no.	α_2 integrin	β_1 integrins	P cadherin	E cadherin
1	+	+	+	-
2	n	n	n	n
4	n / -	n / -	n / -	n / -
5	n	n	n	n
7	n / +	n / +	n / +	n / -
8	+	+	+	-
9	- / -	+ / +	+ / -	- / -
10	-	-	-	-

Table 2: Adhesion molecules in squamous cell carcinoma. + = increased staining, n = staining pattern reflects normal distribution, - = reduced staining. A slash separates staining levels in well or moderately differentiated areas (before the slash) from poorly differentiated areas of the same tumour. Sections from SCC's 3 and 6 contained no tumour.

5.2.vi. Influence of the dermis on stem cell distribution

To discover if there are any features of the dermis which affect the distribution and pattern of cells which express high levels of integrins, which includes stem cells, I cultured keratinocytes derived from foreskin on deepidermidised dermis (DED) from different areas of the body. By using dermis from palm, sole, abdomen, breast and scalp I was able to investigate whether the origin of the dermis altered the expression pattern of integrins in the basal keratinocytes. Keratinocytes were cultured for two weeks on DED before sectioning and staining with fluorescein conjugated anti-integrin antibodies.

Culture of keratinocytes on DED produced an epidermis displaying a full programme of differentiation (Figure 8) and integrin staining was mostly confined to the basal layer.

Analysis of patterns of integrin expression was harder than *in vivo* because rete ridges were much less prominent and less frequent.

Multiple sections were examined from between 3 and 5 experiments with each type of DED. A pattern of integrin expression was taken to be two adjacent rete/papilla structures with alternating bright and dull staining.

When no rete ridges were present on the section it was still possible to see patches of high and low integrin expression. When there were rete on the section then in some areas the integrin pattern did coincide with the rete pattern. The distribution of integrin patches also corresponded with the type of dermis used, so cultures on palm and sole had patches of high integrin expression in the rete and on breast and interfollicular scalp patches of high expression were seen between the rete, so reflecting the *in vivo* pattern (Figures 9 and 10). Occasionally cultured keratinocytes colonised a follicular structure. The integrin staining was brighter around the infundibulum, the most superficial part of the follicle.

On sole and palm dermis the keratinocytes clearly organised such that the brighter patches were associated with the rete and the reverse was not seen. However the ridges were very shallow in the cultures. In the breast and scalp the association of bright cells with the top of the dermal papillae was less clear and less frequent, but examples could be found.

5.2.vii. Production of transgenic mice expressing suprabasal integrins

The transgenic DNA was prepared and injected into fertilised ova as described in Chapter 2. Using primers at the 3' end of the keratin 10 promoter and the 5' end of the integrin DNA, founder mice for 3 lines of mice containing the β_1 construct and 3 lines containing the α_2 construct were isolated as shown by screening ear snip DNA using PCR, an example of which is shown in Figure 11. The frequency of positive lines was below 5% for the α_2 construct but around 15% for the β_1 construct. Further investigation of these lines using *in situ hybridisation* and ribonuclease protection assays has shown that the mice containing the transgene do not contain mRNA for human integrin. The lines have been discontinued.

Figure 1 : Patterns of adhesion molecule expression *in vivo*. Immunofluorescence using anti- β_1 integrin and anti P-cadherin antibodies. The staining patterns were seen in multiple sections from at least three tissue specimens for each body site examined.

a,b : Palm (frozen section), showing high expression in the basal cells in the depths of the rete ridges (long arrows) of β_1 integrin (a) and P-cadherin (b) and low expression at the tips of the dermal papillae (short arrows).

c,d : Abdomen (frozen section), showing the reverse pattern of staining compared to the palm. There is high expression of β_1 integrin (c) and P-cadherin (d) at the tips of the dermal papillae (long arrows) and low expression in the depths of the rete ridges (short arrows).

Scale bar : 50 μm .

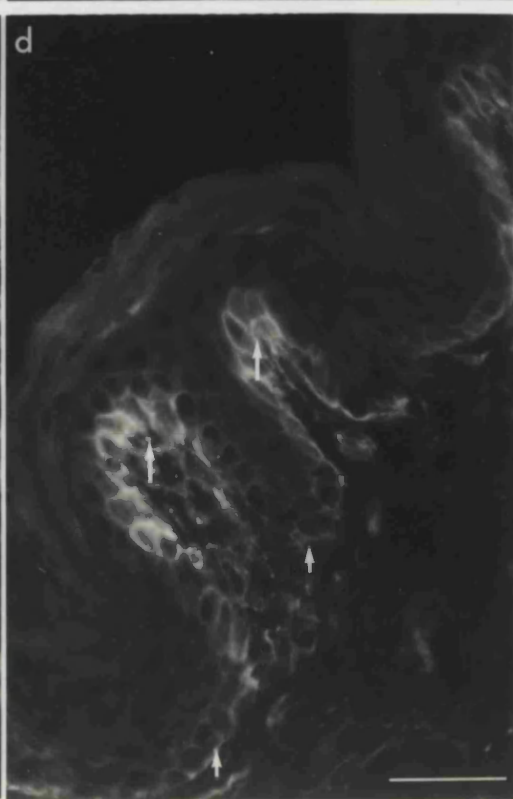
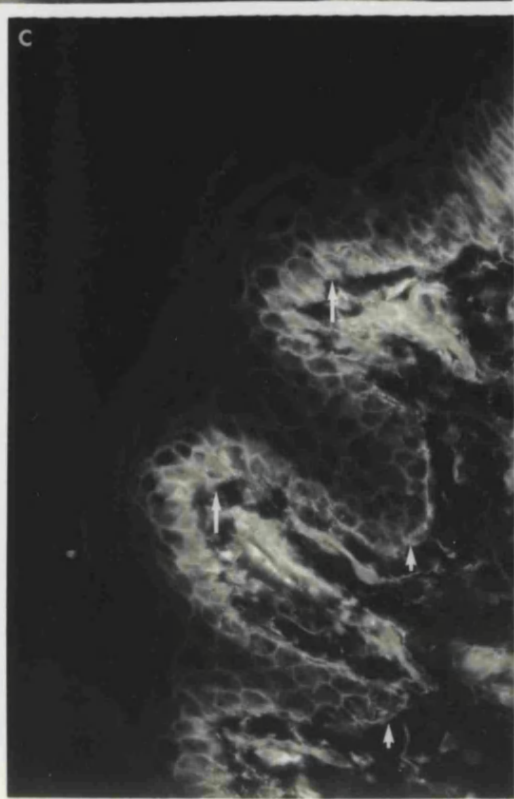
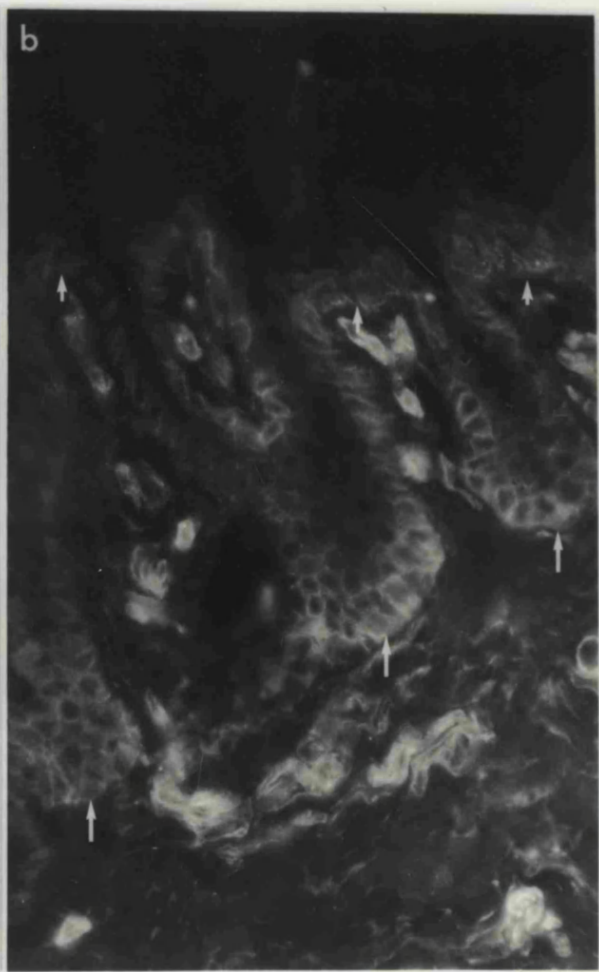


Figure 2 : *In situ* hybridisation of normal keratinocyte colonies grown in Rheinwald Green cultures using an antisense probe to β_1 integrin (b,d) and counterstained (Geimsa) (a,c). The basal cells at the edge of the colonies showed variation in the levels of RNA expression, some with high levels (long arrows) and others with low levels (short arrows), illustrating an inherent capacity of keratinocytes to form patches of high and low expression *in vitro*.

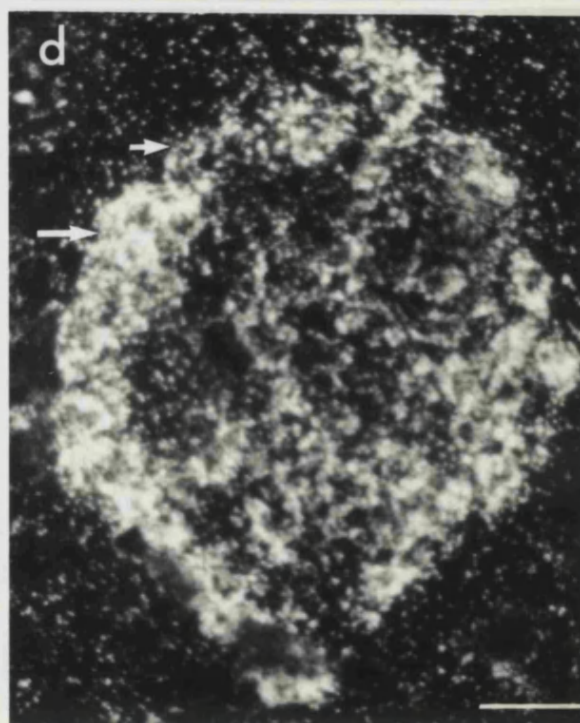
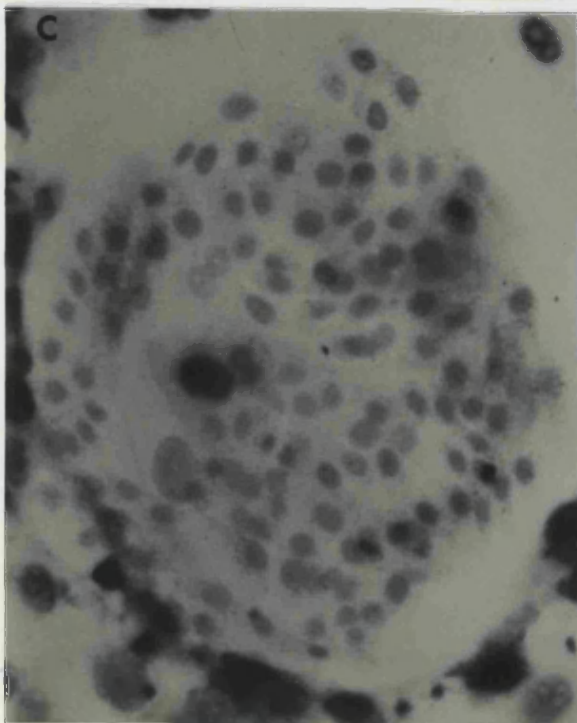
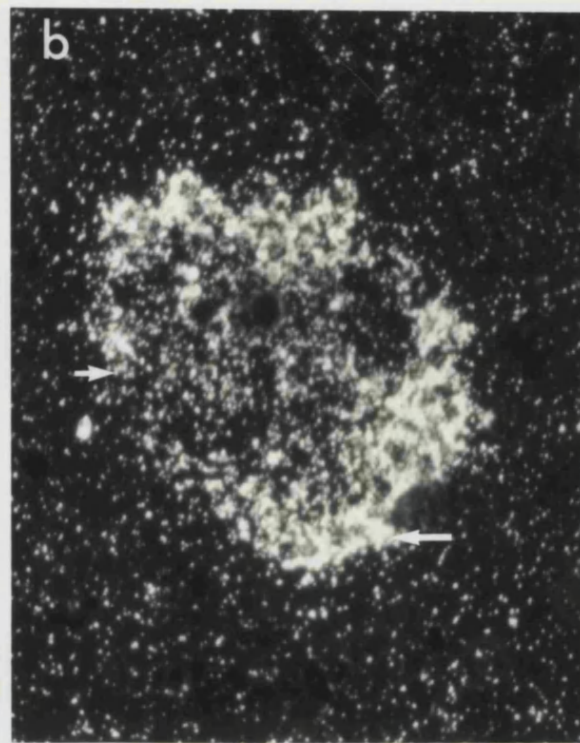
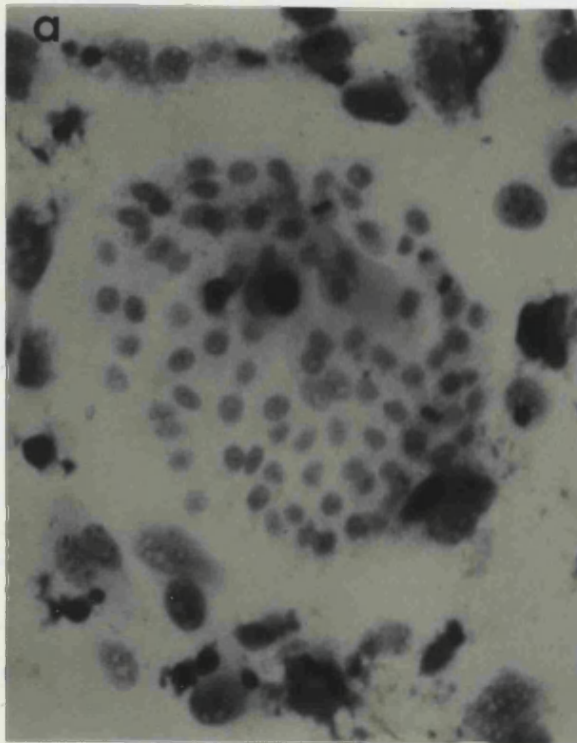


Figure 3 : Confocal microscopy images of palmar and abdominal skin stained with fluorescein labelled anti-collagen IV antibody. The palmar basement membrane (top figure) shows higher intensity of staining (red arrows) in the depths of the rete when compared to the tips of the dermal papillae (blue arrows). The abdominal basement membrane (bottom figure) exhibits the opposite staining pattern, with higher intensity at the tips of the dermal papillae (red arrows) and lower in the rete (blue arrows). The colour bar shows intensity of fluorescence, blue is low and red is high. The intensely staining structures in the dermis are blood vessels.

Scale bar : 50µm.

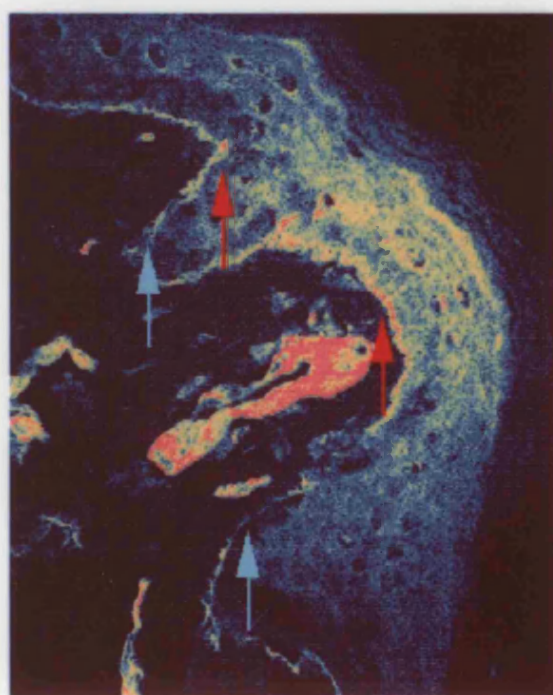
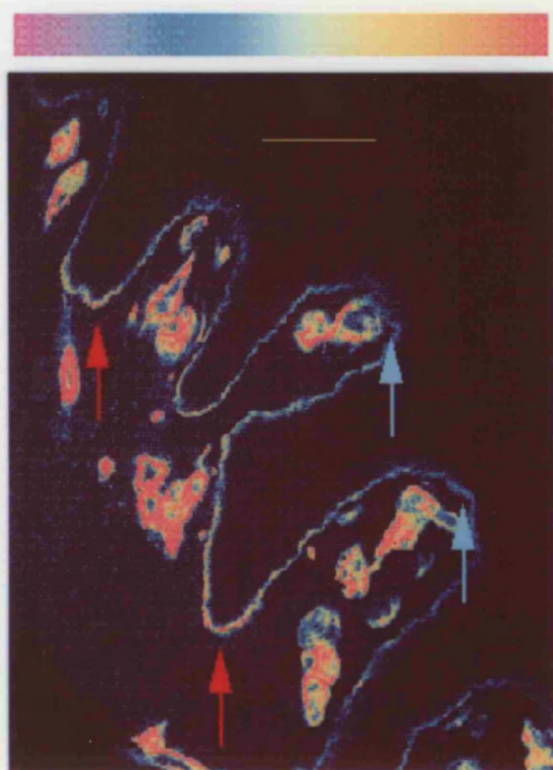


Figure 4 : Fluorescence of basement membrane using fluorescein labelled anti-collagen IV antibody. The intensity of fluorescence was measured in 1 μ m sections by confocal microscopy for the basement membrane in the palm and abdomen at dermal papillae tips and the depth of the rete ridges. The results are the mean of multiple readings from three experiments. Error bars are the standard error.

Fluorescence
(arbitrary units)

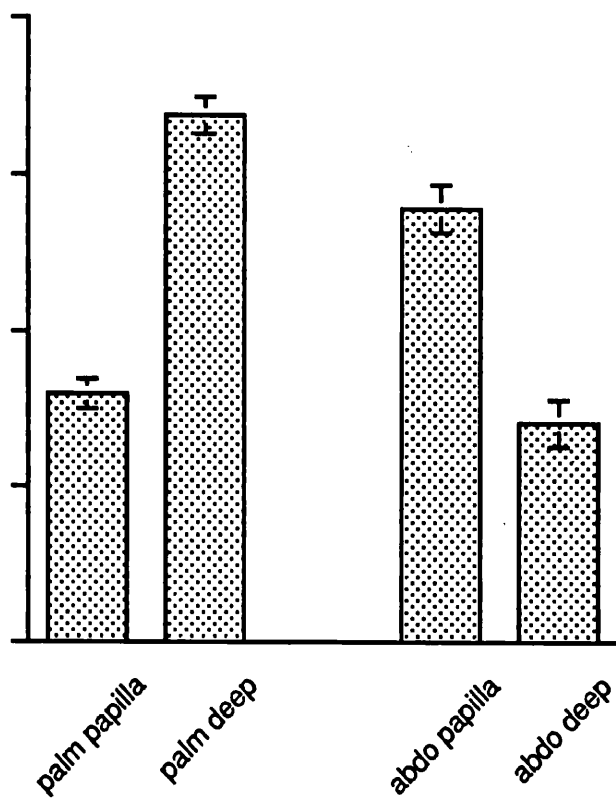


Figure 5 : Suprabasal expression of adhesion molecules in stratified squamous epithelium *in vivo*.

a : Low power photomicrograph of a wound after 168 hours (1 week) following suction blistering, showing re-epithelialisation of the wound bed (long arrow) and the undamaged epidermis adjacent to the wound (short arrow).

b,c : Immunofluorescence of the wound bed at 168 hours showing suprabasal expression of β_1 integrin (b) and P cadherin (c).

d,e : Immunofluorescence of the epidermis adjacent to the re-epithelialising wound at 168 hours showing suprabasal expression of β_1 integrin (d) and P-cadherin (e).

f,g : Immunofluorescence of normal oral epithelium showing suprabasal staining of β_1 integrin (f) and P-cadherin (g).

h,i : Immunofluorescence of normal scalp hair follicle (high power) showing suprabasal staining of β_1 integrin (h) and P-cadherin (i).

Scale bars : 50 μm .

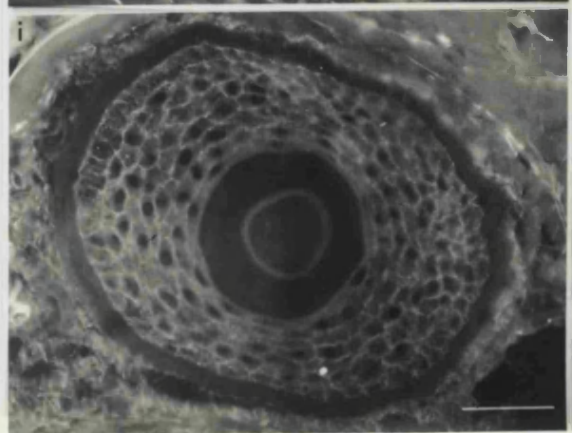
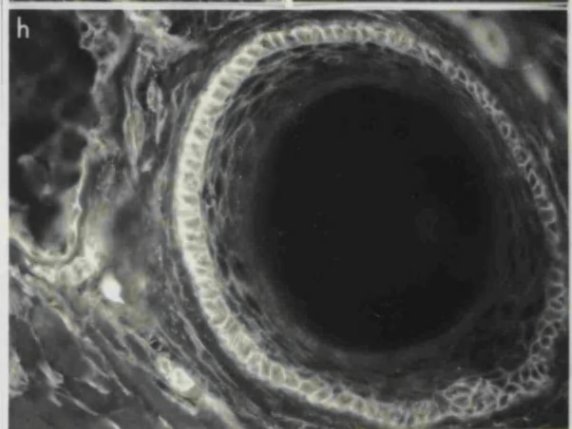
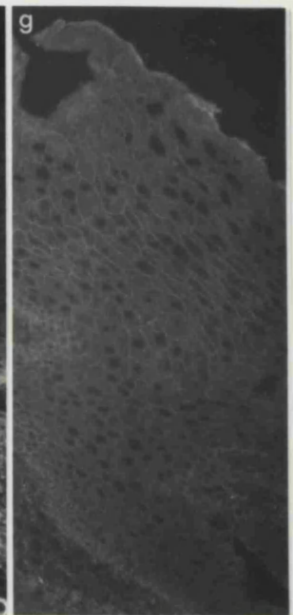
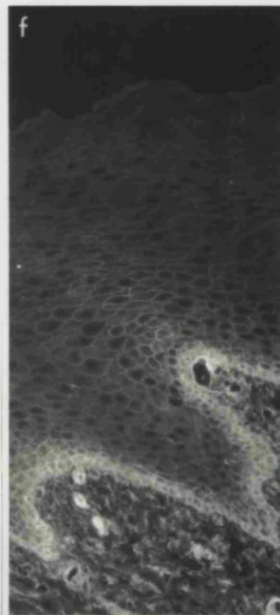
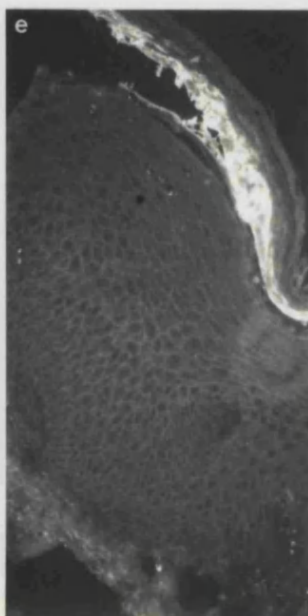
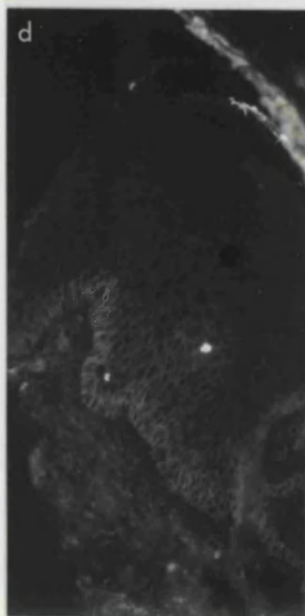
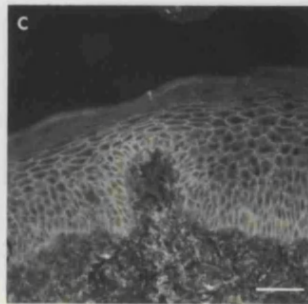
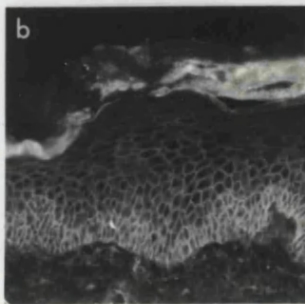


Figure 6 : Adhesion molecule expression in keratinocytes cultured on collagen gels.

a,c,e : 3 days post-confluence showing suprabasal staining of β_1 integrin (a) and P-cadherin (c) plus involucrin (e) which is expressed in all the suprabasal cells but not in the basal layer.

b,d,f : 7 days post-confluence when staining for β_1 integrin (b) and P-cadherin (d) is restricted to the basal layer as normal epidermis *in vivo*. Involcrin expression (f) in the suprabasal cells only is unchanged.

Basal layer = bl, suprabasal layers = s.

Scale bar : 50 μ m.

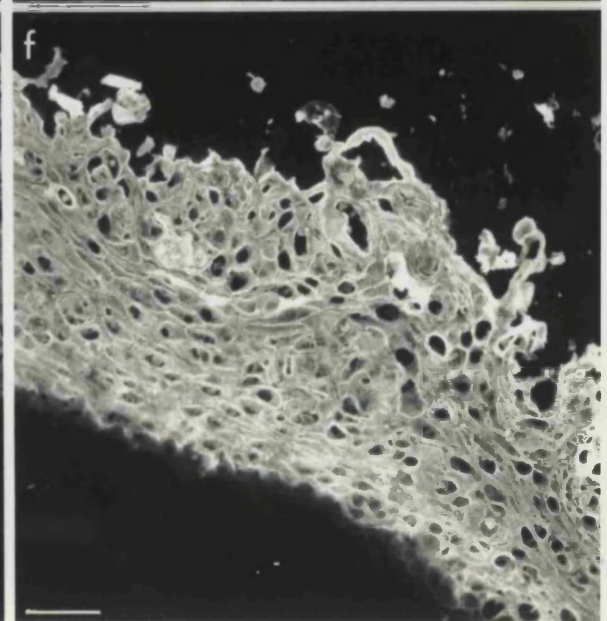
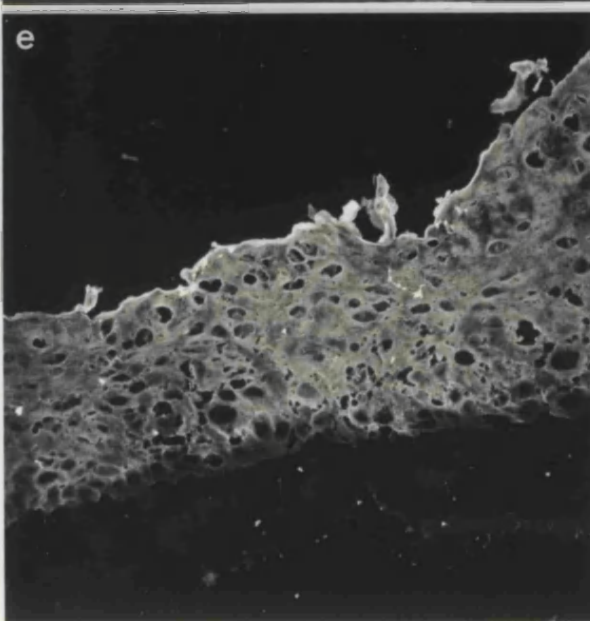
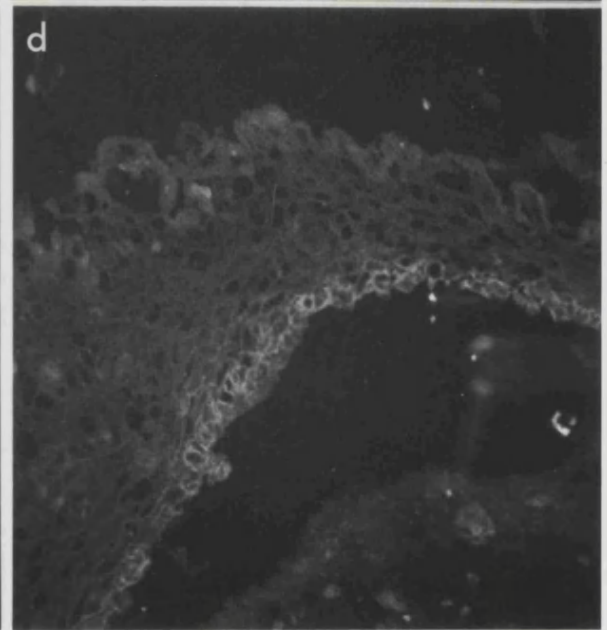
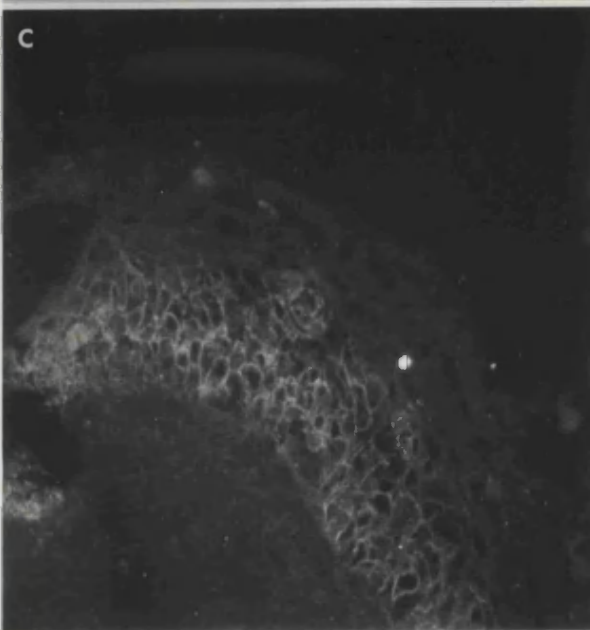
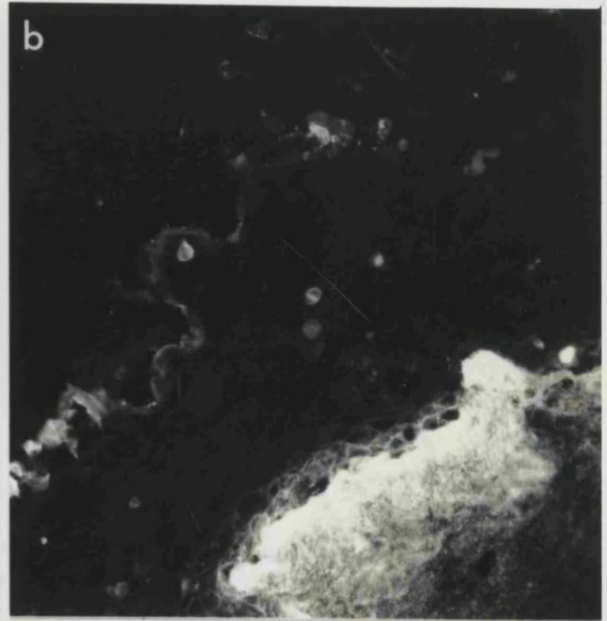
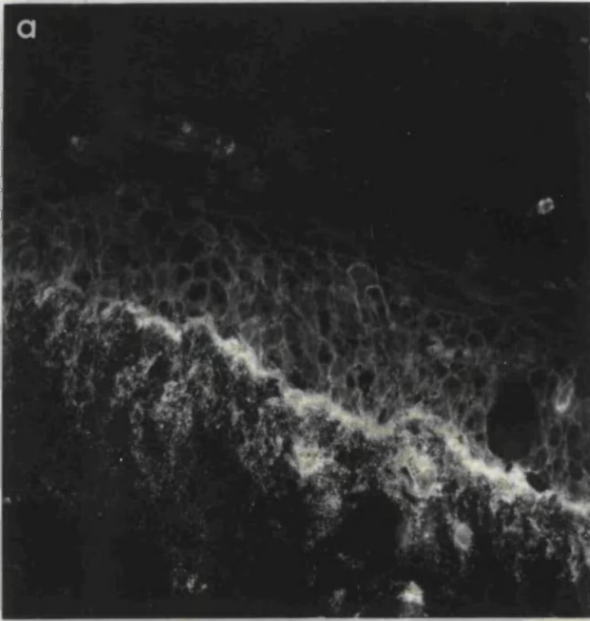


Figure 7 : Adhesion molecule expression in oral squamous cell carcinomas.

a,b : A moderately well differentiated carcinoma (frozen section) showing expression of β_1 integrin (a) and P-cadherin (b) throughout.

c,d : A moderately well differentiated carcinoma (frozen section) showing lack of expression of β_1 integrin (c) and P-cadherin (d) throughout.

Scale bar : 50 μ m.

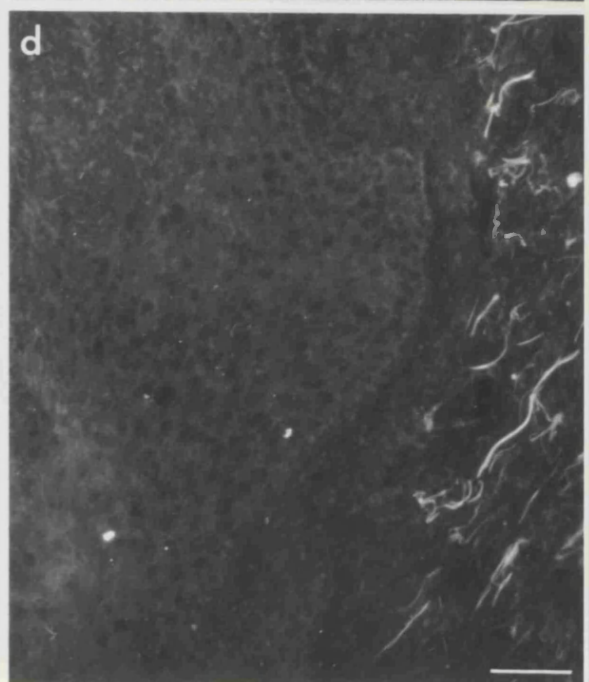
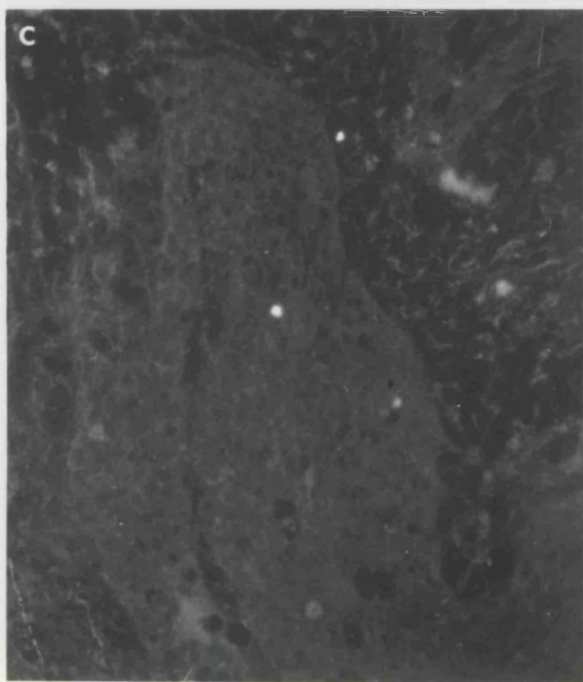
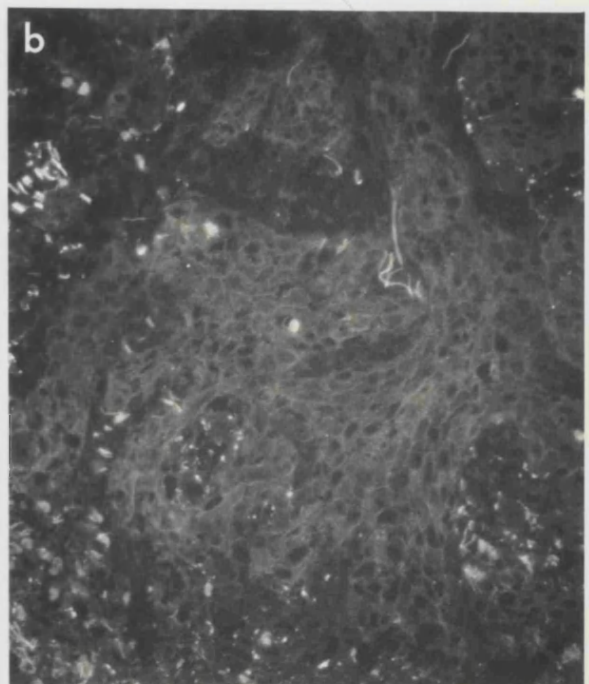
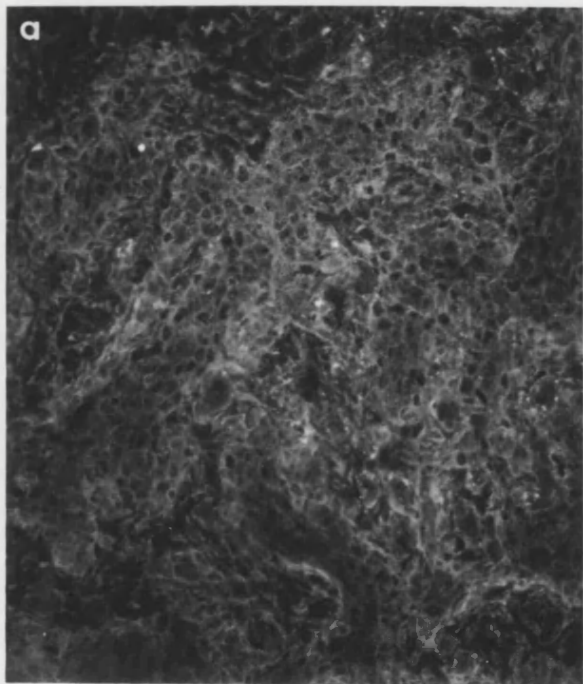


Figure 8 : Culture of keratinocytes on de-epidermidised dermis (DED).

a-d : Control dermis from foreskin (a), breast (b), sole (c) and scalp (d) without added keratinocytes showing no regeneration of the stripped epidermis (although occasional regeneration was seen within the hair follicles of the scalp).

e-h : Keratinocytes derived from foreskin cultured on DED from foreskin (e), breast (f), sole (g) and scalp (h) showing no obvious morphological differences (H+E).

Scale bar : 50 μ m.

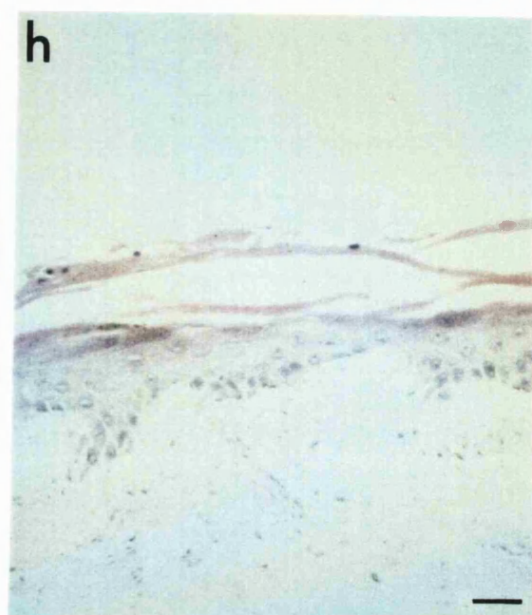
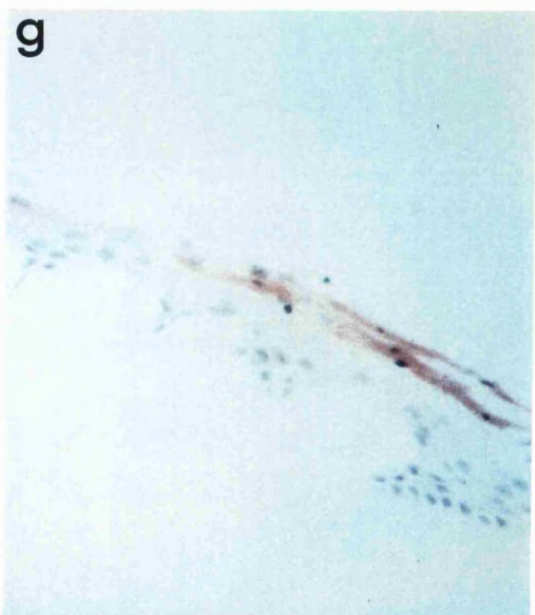
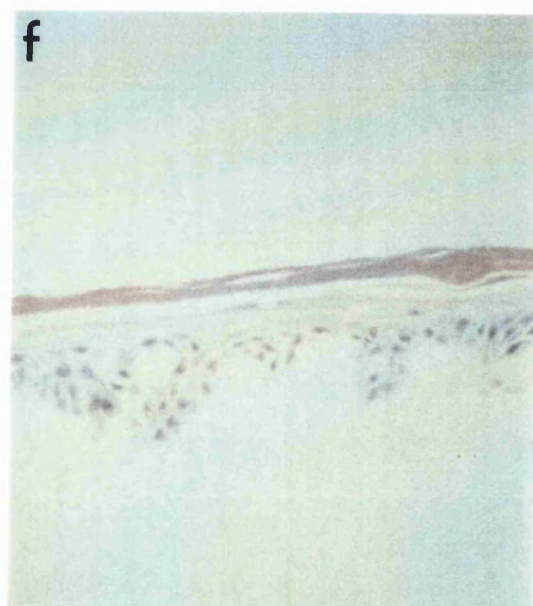
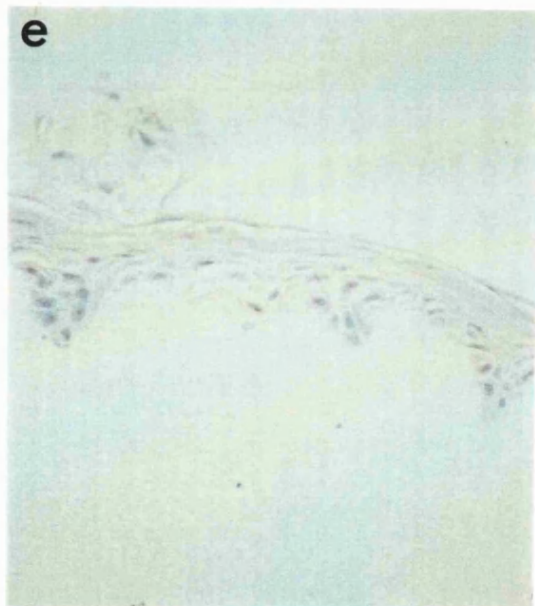


Figure 9 : Expression of β_1 integrin by immunfluorescence in basal keratinocytes cultured on de-epidermidised dermis (DED).

a,b,d : Low (a,b) and high power (d) photomicrographs of keratinocytes derived from foreskin, cultured on DED from the sole, showing possible high levels of β_1 integrin expression in the depths of the rete ridges (long arrows) compared to the tips of the dermal papillae (short arrows), and reflecting the normal pattern in the sole.

c,e : Low (c) and high power (e) photomicrographs of keratinocytes derived from foreskin, cultured on DED derived from breast, and suggesting a reverse of the pattern on sole DED. There is possible higher expression of β_1 integrin at the tips of the dermal papillae (long arrows) and lower levels in the depths of the rete ridges (short arrows) reflecting the normal pattern in the breast.

Scale bars : 50 μ m.

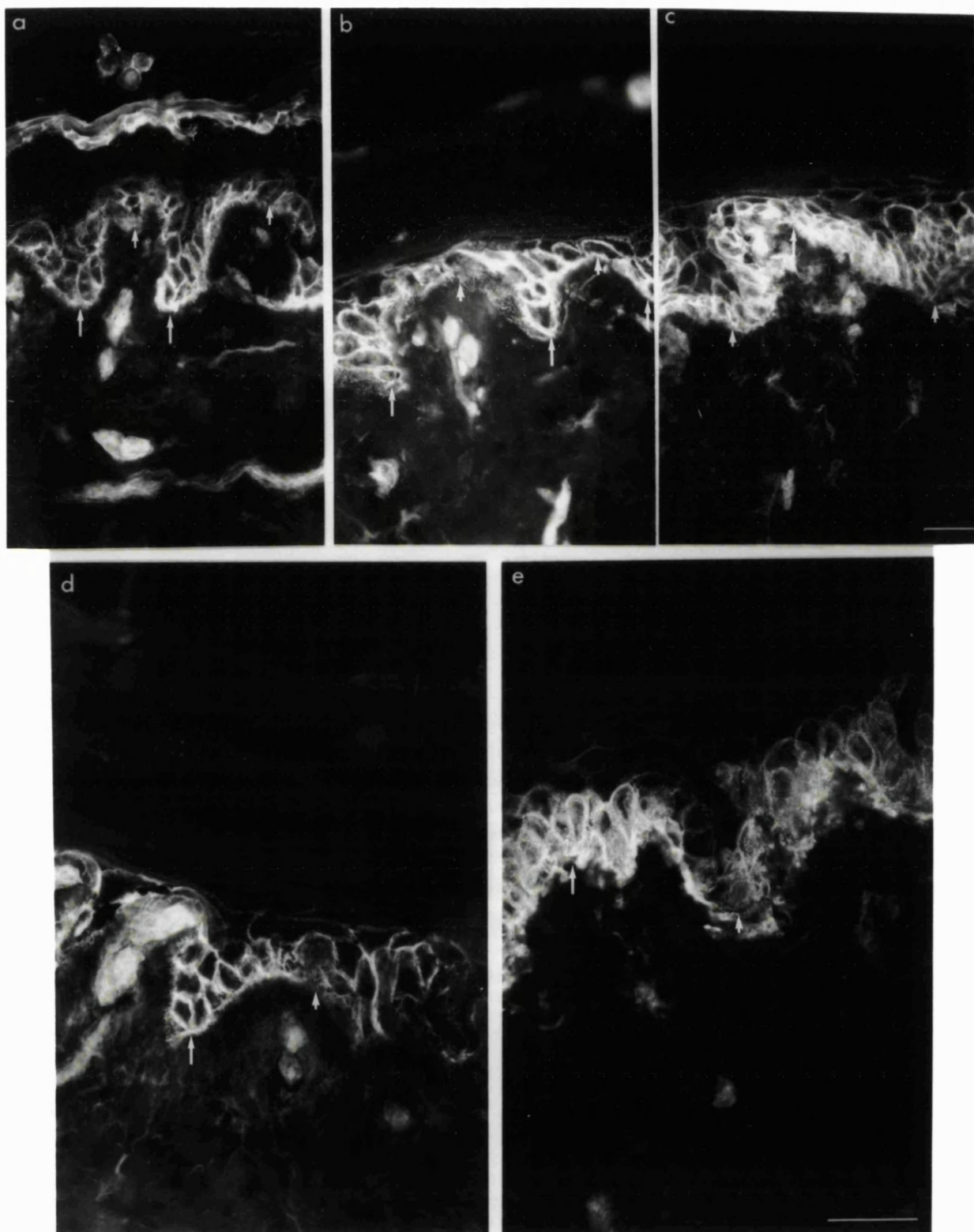


Figure 10 : Keratinocytes derived from foreskin and grown on de-epidermidised dermis (DED) from the sole (a) and scalp (b) immunofluorescently stained for β_1 integrin and showing opposite patterns of expression. There is higher expression in the rete on sole DED and above the dermal papillae on scalp DED.

Scale bar : 50 μm .

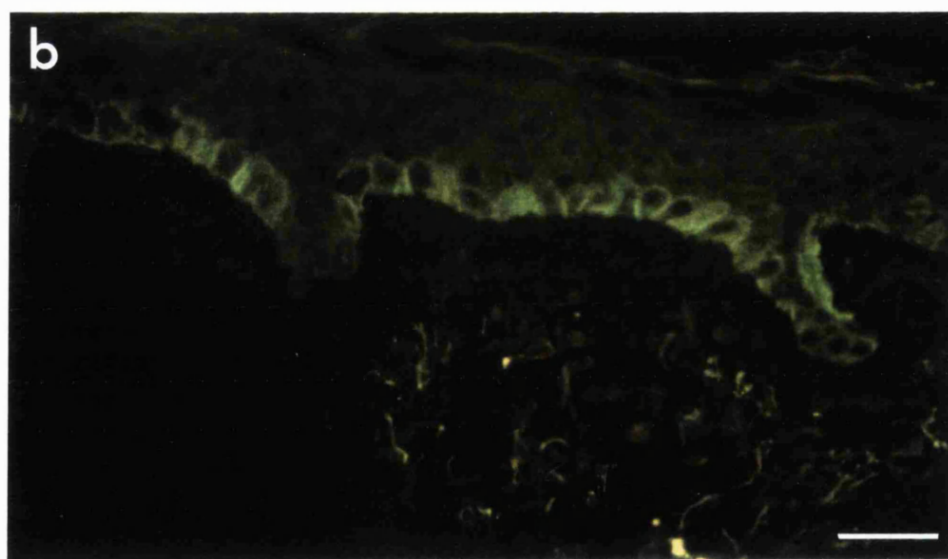
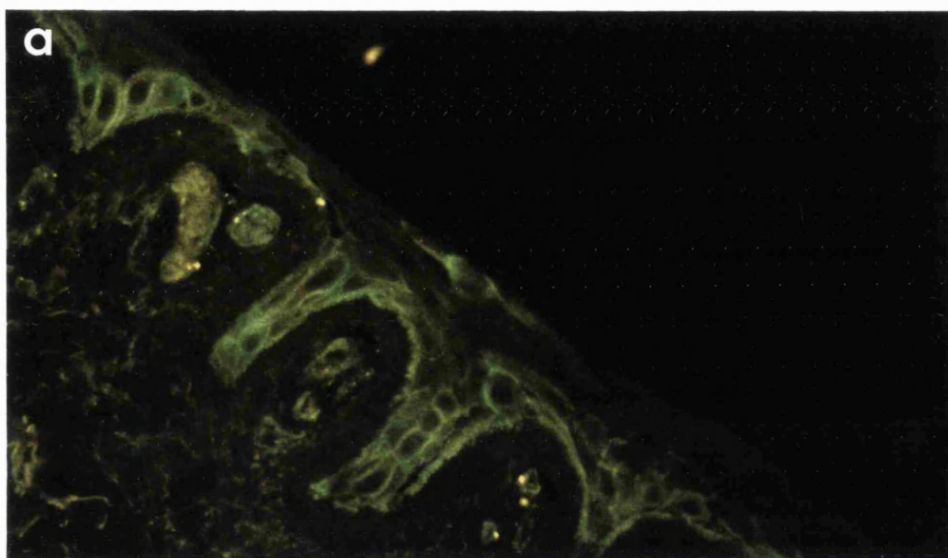
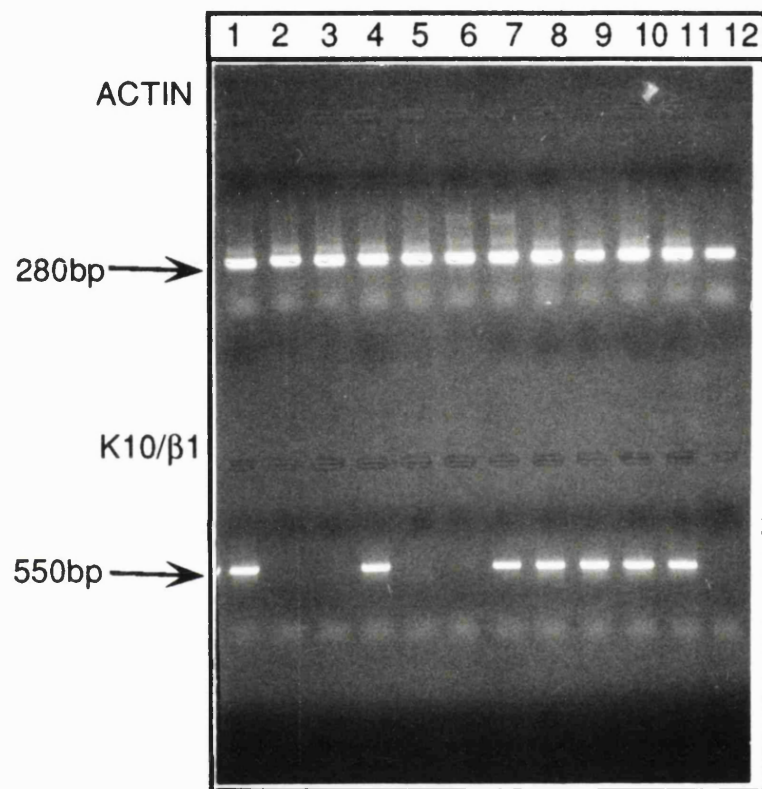


Figure 11 : The polymerase chain reaction products when screening DNA from mouse ear snips for the presence of transgenic β_1 integrin under control of the keratin 10 promoter. The actin gene was used as a control (280 bp). Lanes 1, 4 7, 8, 9 and 10 are positive for the transgene, giving a 550 bp product. Lane 11 is DNA from a known positive mouse and lane 12 is the negative control.



5.3. Discussion

The pattern of integrin expression in the basal layer of the epidermis suggests that the stem cells are situated at the tips of the rete ridges or dermal papillae depending on the body site (Jones *et al.* 1995). My results show that this type of pattern is reflected in the expression of the epidermal cadherins. P-cadherin is seen to show the same pattern of expression as the integrins, and E-cadherin, in the sites I tested and in foreskin (Jean-Pierre Molès, Keratinocyte lab, personal communication), has the exact opposite pattern of expression. Cells that are high in integrins are probably therefore high in P-cadherin and low in E-cadherin. In various particular circumstances, wound healing, oral mucosa, hair follicles and keratinocytes cultured on collagen gels, P-cadherin is seen in suprabasal keratinocytes and this is always when integrin expression is also suprabasal. The keratinocytes expressing suprabasal integrins also express markers of terminal differentiation, such as involucrin, keratin 10 and 16, and are not therefore stem cells (Hertle *et al.* 1992).

Further work by Jean-Pierre Molès in the Keratinocyte lab. has shown that integrins, γ -catenin and possibly P-cadherin have the opposite distribution pattern in basal cells compared to E-cadherin and β -catenin in foreskin *in vivo*. This pattern of distribution is exactly reproduced in epidermis cultured on deepidermidised dermis. In contrast, α -catenin does not show patchy variation of expression.

There seems to be a physiological link between expression of integrin and P-cadherin, which is seen even in pathological situations such as wound healing, tumours as well as *in vitro*. Blocking cadherins with antibodies prevents the redistribution of integrin on the cell surface usually seen when cultured keratinocytes are switched from low to high calcium medium (Hodivala and Watt 1994). This redistribution can also be blocked with anti-catenin antibodies injected into the cell (K. Hodivala, personal comm.).

It follows that epidermal stem cells should have high levels of surface integrin and P-cadherin and low levels of E-cadherin. This high level of expression of certain adhesion molecules may be important to strongly anchor the stem cell into the stem cell niche, the integrins to the extracellular matrix and P-cadherin to other cells in the basal layer. As the

stem cells divide, some daughter cells with a slightly lower level of expression and a lower potential for being a stem cell would therefore be forced from the niche and become "not so stem". Cells destined to be transit amplifying cells, with an even lower level of adhesion molecule expression, would produce daughters committed to terminal differentiation which functionally down regulate their surface integrins, then lose them altogether (Adams and Watt 1990; Hotchin and Watt 1992; Hotchin *et al.* 1993) and leave the basal layer. The cells which are committed to terminal differentiation, as well as having less adhesion to the basal layer, may even have increased expression of E-cadherin causing them to be "pulled up" into the suprabasal layer, ultimately to differentiate and be lost from the epidermis.

This process implies a continuous natural selection process in which the most appropriate cell, i.e. the cell with most proliferation potential is most likely to be kept in the stem cell niche by having more of the appropriate adhesion molecules than other cells. Cells with slightly fewer adhesion molecules would move to an environment which would be slightly less likely to maintain them in the basal layer and they would have a lower potential for proliferation.

The niche is partly defined by other, surrounding, cells, so cell-cell cohesiveness is also likely to be important. The relative cohesiveness of the cells in the basal and suprabasal layers, mediated by cadherins and other adhesion molecules, may act to stabilise cells in each particular layer and therefore maintain the integrity of epidermal organisation and dynamics. Cadherins are regulators of morphogenesis (Takeichi 1991) and able to mediate the organisation of cells into groups in suspension (Nose *et al.* 1988; Steinberg and Takeichi 1994) and stratification of cultured keratinocytes (Watt 1984; Watt *et al.* 1984; Hodivala and Watt 1994).

Since the pattern of integrin/P-cadherin staining is regular, and therefore so is the position of the stem cell niche, there must be something about the microenvironment in these patches of high expression which keeps the stem cells in that particular spot rather than anywhere along the basement membrane. Jones and Watt (1995) showed that this regular pattern of expression is probably imposed by the keratinocytes themselves, which form regular patches in culture, of similar size to those seen *in vivo*, and colonies in subconfluent

cultures also show variation of β_1 integrin RNA in the basal cells, suggesting that pattern formation is an inherent ability of the keratinocytes. Sheets of cultured keratinocytes grafted onto granulation tissue in burns patients are able to generate a fully formed dermis and basement membrane in 2 to 5 years unlike any other type of treatment (Compton *et al.* 1989; Compton 1992; Compton 1993). The process is faster if the cultured epidermis is accompanied by homograft dermis (Compton *et al.* 1993). The stem cells are possibly able to contribute to the formation of the niche and then remain within it. This has also been suggested for haematopoietic stem cells which exist in close association with bone marrow stromal cells, which also exhibit heterogeneity (Allen *et al.* 1990; Dexter 1991). There is considerable evidence that the epidermal basement membrane is synthesised by both keratinocytes and stromal cells with interaction between them being of importance. Fetal bovine keratinocytes, cultured on collagen gels, do not form basal laminae but addition of human fibroblasts results in formation of a basement membrane at the keratinocyte-fibroblast interface (Marinkovich, Keene *et al.* 1993). Both keratinocytes and fibroblasts (possibly a differentiated subset) are capable of synthesising the basement membrane components in culture but assembly requires interaction of both cell types.

The position of the integrin/P-cadherin patches is interesting. It has long been assumed that the "safest place" for the stem cell is deep in the rete ridges, however this does not seem to be where the stem cells are in non palmar plantar epidermis. There is no reason why the distance from the surface of the epidermis should be important to keep the stem cells protected - the difference in susceptibility from injury would be minimal - and far fewer cells are lost through injury during a lifetime than to the process of differentiation (approx. 1,000 billion). It may therefore be far more important for the stem cell to protect itself from ejection from the basal layer and this it is equipped to do by its stickiness to its niche. It is possible that increased frequency of injury to the palm and sole, especially the likelihood of burns, is a factor in requiring stem cells to be more deeply situated.

It is not clear why there was a variation in the position of the patches in palmar plantar epidermis. The possible explanation is that the pattern varies across the palm and

sole. The biopsies used were taken from different areas, some from the edge and some from the centre, although this was not recorded.

The existence of patches suggests that there must be some lateral movement of cells in the basal, or possibly the suprabasal, layers, as also implied by the EPU model in mice (Allen and Potten 1974; Potten 1974; Hume and Potten 1976; Potten 1981; Morris *et al.* 1985). My analysis of lineage marked cultured keratinocyte sheets shows that there is not likely to be much lateral movement of cells between proliferative units. However lateral movement within the EPU is possible as cells get displaced by division of adjacent cells. Movement may not be only lateral or only vertical but could be in a spiral fashion around the dermal papillae.

It was also possible to double hybridise for the Y chromosome and β_1 integrin, potentially to look at the relationship of clones of cells to the patches. This work is in the early stages but clearly some clones contain multiple patches of high integrin expressing cells.

It follows that the microenvironment along the basal layer is not uniform. The results from the experiments looking at the staining of the basement membrane with an anti-collagen IV suggest that this is the case. Furthermore the variation along the basement membrane corresponds to the variation in cell surface adhesion molecules and is of a similar order of magnitude. Keratinocytes are denied direct contact with stromal cells which the bone marrow stem cells require but do require attachment to ECM, and differentiate in suspension (Adams and Watt 1989). My results suggest that variation of the ECM molecules along the basement membrane does occur and therefore may potentially have some influence on the behaviour of cells in the basal layer. These results need careful interpretation since no control antibody against an ECM molecule which does not vary in intensity along the basement membrane was available.

Basement membrane structure varies between different anatomical sites and also shows variation in different, closely related microenvironments. The human corneal basement membrane shows topographical differences in expression of collagen IV and laminin isoforms, with variation of isoforms present in the central cornea, limbus and conjunctival basement membrane (Ljubimov *et al.* 1995) and the human ciliary body shows similar

variation of laminin isoforms (Wang *et al.* 1994). Heterogeneity of collagen IV and laminin isoforms is also detectable in the basal laminae of muscle fibres, which show variation between synaptic and extra synaptic portions. The composition of the basement membrane varies within some organs. For example, the monomeric elements from the globular domain of collagen IV show heterogenous distribution in renal basement membranes, with differences between the glomerulus, Bowman's capsule and proximal tubule (Desjardins *et al.* 1990) and this can also be seen for laminin, nidogen and heparan sulphate. The pulmonary airway shows variation in the distribution of sulphated glycosaminoglycans and glycoproteins between bronchioles and larger airways (Khosla *et al.* 1994). In human skin differences in the ultrastructure of the dermo-epidermal junction have been shown between the interfollicular areas and those in the hair follicle and hair bulb (Nutbrown and Randall, 1995). There are also focal gaps in the expression of collagen IV in the epidermal basement membrane, first noted in vitiligo but likely to be normal and associated with Merkel cells, melanocytes and infiltrating lymphocytes (Bose and Ortonne, 1994). In carcinomas, heterogeneity of basement membrane components is associated with the differentiation of the tumour, for example sulphated glycosaminoglycans in colorectal carcinoma (Malchiodi-Albedi *et al.* 1991) and collagen IV and laminin in oral squamous cell carcinomas (Downer *et al.* 1993). Temporal variations in basement membrane components may also be important, for instance during wound healing. In experimental models of wound healing, grafting epidermal sheets onto athymic mice, the components of the membrane appear at different times and this may influence keratinocyte migration and differentiation (Germain *et al.* 1995).

There are many examples of extracellular matrix molecules, including collagen, regulating epithelial cell behaviour, particularly differentiation, (Watt 1986; Adams and Watt 1989; Streuli *et al.* 1991; Berdichevsky *et al.* 1992; Adams and Watt 1993; Watt *et al.* 1993; Symington and Carter, 1995). If the level of occupied ECM receptor on the cell is important (Adams and Watt 1990; Watt *et al.* 1993) then the level of ECM molecules available to the cell will also be an important factor influencing the decision to differentiate.

It is notable that the microcapillary network in the non palmar-plantar dermis is directed up the dermal papillae, each papilla contains a capillary loop, and this would place

the stem cells in intimate contact with the circulation. The ECM at this site may also be specific, the oral mucosa of the mouse shows selective localisation of tenascin at the tips of the dermal papillae (Sloan *et al.* 1990).

The dermis may influence the position of the patches of integrin expression, and therefore of stem cells, in the basal layer. My experiments growing keratinocytes on DED, although preliminary, show that in some conditions in culture, the keratinocytes may take up the pattern associated with the dermis on which it is cultured. This may be due to an existing pattern of ECM on the basement membrane, such that the most adherent cells move to the stem cell areas as the seeded cells reorganise, or only remain stem cells if they happen to fall on one of those areas, or that only the stem cells adhere. In the absence of a pre-existing basement membrane the keratinocytes would be expected to impose their own pattern on the underlying tissue as in burns patients in whom grafted cultured keratinocytes maintain the patterns of growth and differentiation of their site of origin (Compton 1993).

The early results using cultures on DED need careful, guarded interpretation. All cultures showed patch formation, independent of the presence or absence of rete ridges. The ridge pattern was only maintained focally and occasionally not maintained at all. Controls of DED, without keratinocytes, left in culture medium for the same length of time as those with keratinocytes, showed loss of the rete pattern. These observations suggest that there is some degeneration of the DED matrix during the period of the culture. The culture is not in a steady state, and is more analogous to a healing wound when the basement membrane components are not fixed, so relationships between keratinocytes and ECM are not in equilibrium and may not directly relate to the situation in normal epidermis. Further, the separation of epidermis from dermis during preparation of the DED required heating to 56°C followed by repeated freeze-thaw steps. Although the basement membrane is still present after these steps, it is unknown whether alterations to the structure and function of the membrane components occurs. Interpretation of the sections, because of the small amount of DED available from certain sites, coupled with a high culture infection rate and the focal maintenance of the rete pattern, was necessarily subjective. Also in sections of DED cultures with shallow rete, a judgement as to the presence or absence of the rete structure had to be made and extension

of keratinocytes down adnexal structures, such as eccrine ducts, had to be excluded. It was particularly difficult to make such judgements on breast and abdominal dermis. However the pattern seen on the sole and palm, the reverse of the *in vivo* foreskin pattern, was more clear and did not seem to be simply due to chance since bright staining at the tips of the dermal papillae with adjacent dull staining in the rete depths was not seen. The presence of a pattern was taken to be alternating bright/dull staining over two adjacent rete/papillae. In the palm and sole the pattern occasionally extended further than this, to involve 3 or 4 rete structures. The observations provide tentative evidence that, if there is variation in the basement membrane microstructure as suggested by the experiments looking at collagen IV staining, ECM in the epidermal basement membrane contributes to the organisation of basal keratinocytes.

The patterning of keratinocytes and the association with adhesion molecules may also be important for cell signalling (Hynes 1992) as well as physically determining the position of the cell in the basal layer and the relative strength of its anchorage. Integrins are thought to act as mediators of "inside out" signalling events and regulate the onset of terminal differentiation in keratinocytes (Adams and Watt 1989; Watt *et al.* 1993). As mentioned in Chapter 1, adhesion molecules are involved in the transduction of patterning signals in *drosophila* limb development (Williams and Carroll 1993; Gotwals *et al.* 1994). Some of these molecules may be involved in the control of integrin expression and function (Hodivala and Watt 1994), in turn signalling alterations of cell behaviour. Inside out signalling may also be a result of the physical state of the cell, and therefore related to the number of adhesion molecules occupied, known as the tensegrity, in which the integrins act as mechanoreceptors and transmit mechanical signals to the cytoskeleton (Wang *et al.* 1993).

Suprabasal expression of integrins is associated with suprabasal expression of P-cadherin. In view of the expression of suprabasal differentiation markers rather than basal markers in these cells (Hertle *et al.* 1992), this is further evidence of the close relationship between the two adhesion molecules. Suprabasal expression occurred in stratified mucosa, hair follicles, healing wounds, cells adjacent to wounds and *in vivo*. In all these situations there is likely to be a high level of proliferation (Hertle *et al.* 1992) and it is possible that this is

the cause of some adhesion molecules remaining on the cell surface, and these molecules need not be functional. The integrins have a half life on the cell surface of 12 to 16 hours and may not be completely removed. Further information may be available from transgenic mice expressing suprabasal integrins to shed some light into the effects of this phenomenon in relation to wound healing and psoriasis and this work is continuing in the laboratory. Mice expressing suprabasal integrin under the control of the involucrin promoter (Carroll *et al.* 1993) have recently been produced (Joseph Carroll, personal communication), and some of these show abnormalities in the skin histologically very similar to psoriasis. The integrin in the suprabasal cells does not appear to be functional in adhesion assays but may still be involved in generating the inflammatory response, perhaps via direct interaction with inflammatory cells or via cell signalling and the production of soluble factors by the keratinocytes.

Expression of integrins and P-cadherin also seems to remain linked in pathological situations, such as oral squamous carcinoma, where the expression is linked to tumour grade.

In summary, I have shown that the expression of cadherins is related to the level of integrin expression and therefore probably to the "stemness" of keratinocytes in the basal layer. Cells with a high level of surface integrin also have higher levels of P-cadherin expression. The areas where these cells are positioned *in vivo* corresponds to areas of the basement membrane with more collagen IV.

CHAPTER SIX

GENERAL DISCUSSION

In the work presented in this thesis I have investigated four potential methods of marking lineage in cultured keratinocytes and used two of those techniques to investigate the fate of stem cells in the epidermis when in a steady state (Watt 1988; Dover and Wright 1991). Using β -gal and the Y chromosome as lineage markers I developed assays allowing me to investigate clonal fate of cells in cultured epidermis. I have demonstrated marked heterogeneity of stem cell fate in cultured epidermal sheets and shown that stem cell fate in cultured epidermis is related to adhesion ability, proliferating keratinocytes being either a continuum or distinct sub-populations with different capacities for reconstituting the epidermis. In addition I have demonstrated a link between the level of integrins and cadherins, most strongly with P-cadherin, in proliferating keratinocytes, and the possible existence of a physical stem cell niche comprising ECM molecules such as collagen IV.

I have also shown that RXLI cells have identical properties of growth and differentiation to normal keratinocytes in culture, and therefore have potential for use as a marker of lineage if detection of STS can be improved.

6.1. Stem cell fate and lineage analysis

The Y chromosome is the most reliable lineage marker for cultured keratinocytes, which had the major advantage of being a natural marker, so avoiding the problems of retroviruses in epithelial cells. However the complex methods required were less reliable, made the experiments very cumbersome and would not be suitable for long term cultures in which the epidermis would be thicker and less easy to manipulate.

Further improvement may be possible. As retroviral technology improves, viruses more suited to the epidermis may well be developed and better infection rates with more reliable growth and expression will be possible. Repeated passaging of oral keratinocytes using the BAG-F vector has been achieved by Garlick during the course of my studies and he described stem cell and transit amplifying colonies in oral keratinocytes grown on culture plate inserts (Garlick and Taichman 1993).

The EPU model in mouse skin suggested by Potten (Potten 1974; Potten 1981) suggests that terminally differentiated cells in the suprabasal layers are the progeny of the basal stem cell lying directly beneath. The patches of high integrin expressing cells seen in human epidermis show that the stem cells are clustered, suggesting a certain amount of lateral movement. My experiments into the lineage of stem cell progeny did not show any evidence of lateral movement in culture in the steady state. However the clone sizes investigated were larger than the size of the patches so movements within the patch could not be resolved and the cultures were confluent for a relatively short period of 3-5 days which may not be long enough for the organisation of the stem cell clusters and their progeny to mature. Further lineage experiments using cells seeded at very high density in conditions which allow longer culture, such as on de-epidermised dermis might resolve this problem. Experiments using fluorescent lineage markers to mark cells in very high density cultures may allow the cell lineage to be related to the pattern of integrin staining.

The heterogeneity of cells that give rise to clones capable of contributing to an epidermal sheet could be further investigated. Larger numbers of clones in each experiment would allow analysis of the relative numbers of the different types (areas) of clone and therefore of the founding cells. Further experiments specifically investigating the possibility of a bimodal distribution of clone area in low density cultures are possible but would require larger numbers of clones to achieve statistical significance. Such experiments may determine whether stem cells exist as a continuum with varying capacities for maintaining the epidermis or as discrete sub-populations.

In Chapter 4, I described some early attempts to culture keratinocytes over longer periods, up to 4 weeks, at confluence, which would allow tracing of lineage and stem cell fate over a long time course. Grafting onto nude mice is the most obvious way to achieve this, and has been achieved by workers using dermal/epidermal grafts and by workers in Lowell Goldsmith's laboratory (personal communication) using embryonic skin, but human fibroblasts are required in any of these systems. This would enable the design of experiments similar to those used in the study of haematopoietic lineage and cell fate.

It would be of interest to investigate other systems such as mouse keratinocytes with differences in expression of cytochemical markers, for instance keratinocytes from mice positive and negative for glucose-6-phosphate dehydrogenase may provide a useful rapidly detectable heritable marker in cultured cells.

6.2. Stem cell fate and adhesion molecules

The experiments carried out by Jones and Watt (1993) first showed that stem cells and transit amplifying cells could be separated by their relative adhesive properties and levels of β_1 integrins. The cells with high levels of integrin comprise about 40% of the basal cells, and are not all stem cells, which are predicted to be around 10% (Potten and Morris 1988) by kinetic studies. Further experiments using adhesion assays and cell sorting according to levels of integrins and cadherins would help isolate these populations to higher purity and to clarify the situation within the stem cell compartment and may help define the putative sub-populations of stem cells. The combination of two or more different stem cell markers has been highly successful in the study of haematopoietic stem cells (Baum *et al.* 1992; Holyoake and Alcorn 1994; Mayani and Lansdorp 1994). Cadherins are trypsin sensitive, so similar experiments would require an anti-cadherin antibody to the uncleaved part of the extracellular domain of the molecule.

One way to confirm the position of stem cells in the epidermis would be by microdissection of the rete, similar to that done by on the hair follicle (Rochat *et al.* 1994), and this may be possible where the papillary pattern is prominent such as the palm or abdomen.

The link between cadherins and integrins suggests a common pathway of regulation, possibly including gene products involved in patterning and polarity and the association with the *Wnt* family (Hinck *et al.* 1994b) mentioned in Chapters 1 and 5 is intriguing. Perhaps such products are involved in the control of expression of adhesion molecules and therefore their proliferative potential and fate. Further investigations into these genes may contribute to the understanding of stem cell fate.

The existence of a physical and chemical stem cell niche has been postulated. Positional information may be imposed by cells with inherent pattern forming ability or by graded environmental signals, or a mixture of both. The marking of the position of stem cells

in the epidermis makes associations with variations in the environment, particularly the extracellular matrix, much easier to determine. Further immunofluorescence and immunoelectronmicroscopy to investigate all the components of the basement membrane, such as laminin 1 and laminin 5 would allow a full picture of the distribution of the ECM molecules to be built up. A more precise picture would be obtained by investigating the distribution of basement membrane ECM subunits and isoforms, using directly conjugated antibodies and quantitative confocal microscopy. Further work in this area should reveal a molecule in the basement membrane zone which does not exhibit a variable distribution and could function as a control in such experiments. Possible candidate controls include $\alpha 4\beta 6$ integrin, bullous pemphigoid antigen (part of the collagen superfamily), collagen VII and entactin/nidogen.

Further work is also needed to study the distribution of E-cadherin in the basal layer and this is currently proceeding in the Keratinocyte laboratory. It may also be possible to monitor changes in the patterning and the niche over time during development and embryogenesis, wound healing and after grafting of cultured epidermis, in burns patients for example. This may give some insight into the balance between the inherent capacity of the stem cells to determine fate and the influences of the microenvironment. Patterning may be important in the embryological development of the epidermis, since patches of $\alpha 2\beta 1$ occur before 9.5 weeks gestation and the onset of stratification, perhaps via dermal-epidermal interactions.

Although the variation of basal cell adhesion molecule levels is clear by immunofluorescent light microscopy, the relative levels of cadherin staining in the basal layer could be measured by confocal microscopy.

Investigation of other systems would also be of interest. Of particular regard in this respect is the gut, where the position of stem cells is known. The relative levels of adhesion molecules and basement membrane components in the stem cell areas could be established. This would give insight into whether the patch distribution seen in the basal epidermis also applies in other epithelia.

After collection of more tissue, the preliminary findings using the deepidermidised dermis could be developed. The patterns of expression may be more apparent after shorter culture periods and staining of the cultures at various time points would be useful. At earlier time points it may be possible to investigate basement membrane ECM molecules and relate these to adhesion molecule patches, even in the absence of a rete pattern. Further experiments would also allow objective quantitation of adhesion molecule staining using confocal microscopy using directly conjugated primary antibodies. The use of frozen sections would allow investigation of more adhesion molecules.

In view of the findings that integrins are capable of transducing signals to the nucleus and regulate keratinocyte differentiation it seems likely that adhesion molecules play a role in controlling position and proliferation of keratinocytes rather than simply being markers of stem cells. Certainly it is reasonable to imagine that cells that are required to remain in the epidermis for the life of the individual should be the most adhesive to the basement membrane.

It will be of interest to use immunohistochemistry to investigate patterning in pathological epidermal processes when the anatomy of the skin is not disrupted, for instance *in situ* neoplasia (squamous carcinoma *in situ*, Bowen's disease, actinic keratosis, porokeratosis, intraepithelial neoplasia in the female genital tract), microinvasive tumours, benign hyperproliferative disorders such as viral warts, keratoacanthoma, psoriasis, chronic inflammatory conditions such as eczema and lichen planus. Similar patterning would be expected in other epithelial systems such as the intestinal epithelium, and may be of interest in conditions such as chronic ulcerative colitis, particularly in relation to the dysplasia which affected patients are predisposed.

Another likely area of dermato-pathological interest is invasive malignant melanoma. Early invasion is often as small dermal nests in which there is minimal proliferation and low metastatic potential (horizontal growth phase) whereas the vertical growth phase shows evidence of a higher proliferative rate and has an increased likelihood of distant metastasis. Comparison of the pattern of adhesion molecule distribution may be useful in the diagnosis of these two phases of malignant melanoma progression.

6.3. Conclusions

In conclusion, I have investigated various possible methods for marking clone forming human keratinocytes *in vitro* and have used some of these to provide direct evidence that the stem cell population in the epidermis is heterogeneous, in support of previous evidence of such variation. I have seen differences in the behaviour of proliferating cells destined to form an epidermal sheet, with their potential being related to their adhesive properties to extracellular matrix. I have shown that keratinocytes derived from individuals with recessive X-linked ichthyosis have the appropriate characteristics to be used in lineage experiments when grown in culture, if they can be effectively identified.

My investigations into expression and distribution of adhesion molecules, integrins and cadherins, and one of their ligands, the extracellular matrix molecule, collagen IV, suggested a close association between stem cell fate, adhesion molecules and a physical stem cell niche.

These observations imply that epidermal stem cells exist in a tight, carefully controlled niche, a sort of stem cell straightjacket, preventing them being lost to the skin. A possible model is shown in Diagram 1.

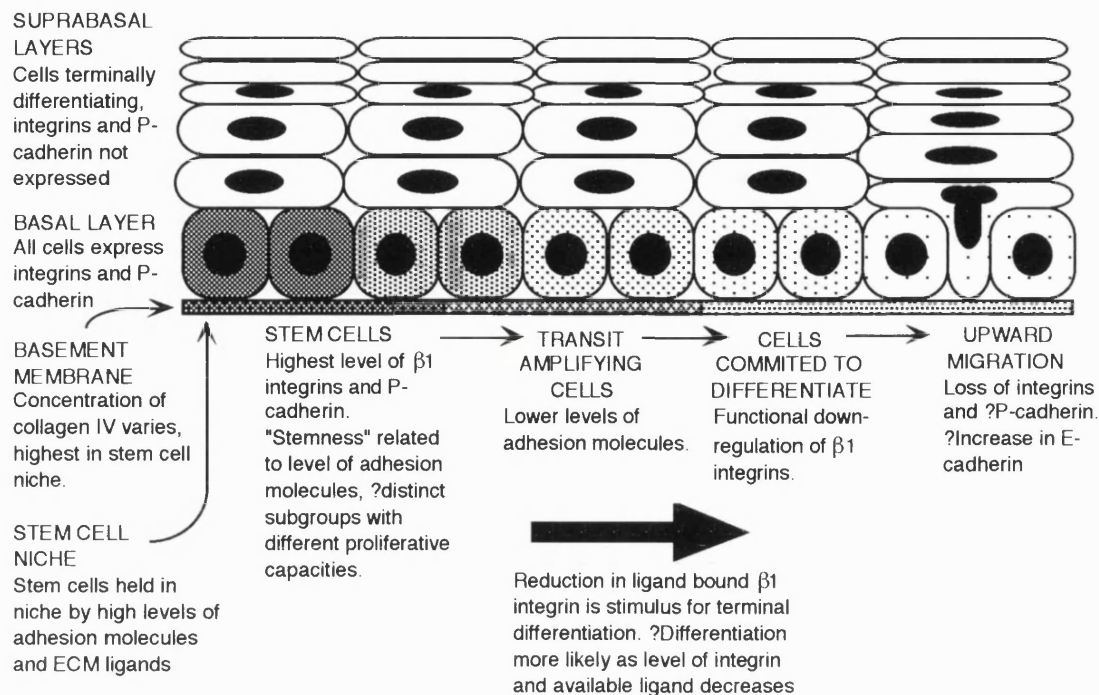


Diagram 1: Model of the relationship between keratinocyte "stemness," cell-ECM and cell-cell adhesiveness, proliferative capacity and terminal differentiation showing the possible sequence of events in the basal layer (adapted from Jones and Watt, 1993).

It is not clear how the delicate balance between the stem cell and its microenvironmental niche is controlled but it is clear that they cannot be considered in isolation and that adhesion molecules may be an important mechanism modulating that balance.

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Finally, I would like to thank my family, especially my wife Claire, for their loving support.

APPENDIX

Stocks and solutions 1

X-gal staining for β -galactosidase (β -gal)

X-gal detergent solution

1M MgCl ₂	2ml (2mM)
10% sodium deoxycholate	1ml (0.01%)
10% NP40	2ml (0.02%)
PBS	to 1L

X-gal stock

40mg/ml X-gal (Gibco BRL, Paisley, UK) in dimethylformamide (DMF), using glass pipettes, and stored at -20°C, in glass.

X-gal solution

K ₃ Fe(CN) ₆	1.64g (5mM)
K ₄ Fe(CN) ₆ ·3H ₂ O	2.1g (5mM)
1M MgCl ₂	2ml (2mM)
10% sodium deoxycholate	1ml (0.01%)
10% NP40	2ml (0.02%)
PBS	to 1L

Store in the dark at 4°C. Add X-gal to aliquots as required at a concentration of 1mg/ml. This can be reused for at least 1 month if stored dark at 4°C.

Stocks and solutions 2

DNA preparation and purification

Ampicillin Stock

100mg ampicillin / ml DW (1000 x). Filter sterilised and stored at -20°C.

L-broth

Provided by ICRF central stores

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
DW	to 1L

pH to 7.0 and autoclave.

BHI broth

Provided by ICRF central stores

BHI broth powder	37g
DW	to 1L

Autoclave.

L-agar

Provided by ICRF central stores

L-broth	1L
Bacto-agar	15g

TE8

1M Tris.HCl pH 8	10ml
0.5M EDTA pH 8	2ml
DW	to 1L

TE at other pH values was made by adjusting the pH of the Tris.HCl to the appropriate value.

10% SDS stock

Sodium dodecyl sulphate	10g
DW	100ml

Tris equilibrated phenol

Phenol (Amresco, Solon, Ohio, USA) was purchased pre-equilibrated with Tris.HCl, pH8 and stored at 4°C.

Chloroform

Chloroform : isoamyl alcohol at 25 :1.

Phenol/chloroform

Tris equilibrated phenol	50%
Chloroform/isoamyl alcohol	50%

DNA lysis buffer

1M glucose	5ml (50mM)
1M Tris.HCl	2.5ml (25mM)
0.5M EDTA	2ml (10mM)
DW	to 100ml

Alkaline SDS solution

5M NaOH	4ml (0.2M)
DW	86ml
10% SDS	10ml (1%)

Potassium acetate solution

5M potassium acetate	60ml
Glacial acetic acid	11.5ml
DW	28.5ml

Ethidium bromide

Ethidium bromide 10mg/ml in DW

CsCl saturated isopropanol

Isopropanol was stored above distilled water saturated with caesium chloride.

Sodium acetate stock

3M sodium acetate in DW, pH 5.2.

RNase A

RNase A (Sigma) stock solution was 10mg/ml. This was boiled for 10 mins to destroy DNases and stored at -20°C.

Polyethylene glycol (PEG) solution

1.6 NaCl containing 13% (w/v) polyethylene glycol (PEG 800).

Miniprep lysis buffer

1M Tris.HCl pH 8	5ml (50mM)
0.5M EDTA pH 8	2ml (10mM)

50 x TAE

Tris base	242g
Glacial acetic acid	57.1
0.5M EDTA pH 8	100ml

DNA loading buffers

1) 10 x OJ : 0.25% Orange G in 10 x TAE.

2) 6 x LB : 0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll 400 in water.

Agarose/TAE gels

DW 98ml

50 x TAE 2ml

Agarose (BRL, Ultrapure) 0.8-2%

Suspension boiled in a microwave oven until agarose dissolved then kept at 55°C.

Ethidium bromide (10mg/ml) 10µl

Stocks and solutions 3

Southern Blotting

OLB buffer

1M HEPES pH 6.6

0.25M Tris.HCl pH 8

0.1mM each dATP, dTTP, dGTP

0.36% β -mercaptoethanol

27 U/ml Hexadeoxynucleotides (Pharmacia, Uppsala, Sweden)

Stored at -20°C.

10% SDS stock

Sodium dodecyl sulphate	10g
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DW	100ml
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Nick column elution buffer

5M NaCl	0.4ml
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1M Tris.HCl pH 7.5	2ml
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0.5m EDTA pH 8	0.04ml
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10% SDS	3ml
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DW	to 100ml
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DNA loading buffer

6 x LB : 0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll 400 in water.

Neutralising buffer

Tris.HCl	68.65g
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Tris base	8g
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NaCl	87.65g
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DW	to 1L
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20 x SSC

NaCl	175.3g
Sodium citrate	88.2g
DW	to 1L (pH to 7.0 with HCl).

Hybridisation solution

100 x Denhardt's soln.	5ml (5 x)
Formamide	50ml (50%)
20 x SSPE	20ml (5 x)
10% SDS	10ml (1%)
10mg/ml denatured, sonicated salmon sperm DNA (Sigma)	1ml (100µg/ml)
DW	to 100mls

20 x SSPE

NaCl	175.3g
Sodium hydrogen phosphate.H ₂ O	27.6g
EDTA	7.4g
DW	840 ml
Autoclave (to dissolve), pH to 7.4.	
DW	to 1L

Denhardt's solution

Polyvinylpyrrolidone (PVP)	5g
Nuclease free bovine serum albumin (BSA)	5g
Ficoll 400	5g
DW	to 250ml

Filter sterilise and store at -20°C.

Stocks and solutions 4

RNA preparation and Northern blotting

Diethylpyrocarboate (DEPC) water or PBS

DEPC	400 µl
DW or PBS	400ml

Left overnight at room temperature then autoclaved.

Guanidine Thiocyanate (GIT) solution

GIT	50g
N-lauroylsarcosamine, sodium salt	0.5g
Tri-sodium citrate, pH 7.0	2.5ml
30% Antifoam A emulsion	0.33ml
DW	to 100ml (pH to 7.0 with NaOH)
β-mercaptoethanol	0.7ml

Filter (Whatman No. 1) and store at -20°C

Caesium chloride solution

CsCl	95.97g (5.7M)
3M sodium acetate pH 6.0	0.83ml (25mM)

10% SDS stock

Sodium dodecyl sulphate	10g
DW	100ml

Heat at 65°C for 30 mins.

2 x poly A⁺ column loading buffer

1M Tris.HCl pH 7.5	4ml (40mM)
5M NaCl	40ml (1M)
0.5M EDTA pH 8	0.8ml (2mM)
10% SDS	4ml (0.2%)
DW	to 100ml

Poly A⁺ elution buffer

1M Tris.HCl pH 7.5	1ml (10mM)
0.5M EDTA pH 8	0.2ml (1mM)
10% SDS	0.5ml (0.05%)
DW	to 100ml

Sodium acetate stock

3M sodium acetate in DW, pH 5.2.

10 x HEPES buffer

HEPES	119.15g (0.5M)
pH to 7.9	
0.5M EDTA pH 8	20ml
DW	to 1L

RNA sample buffer

Deionised formamide	10µl
37% formaldehyde	4µl
10 x HEPES	2µl

Add 4µl of RNA sample and 2 µl loading buffer.

RNA loading buffer

Glycerol	50%
10 x HEPES	50%
Bromophenol blue	0.25%

RNA running buffer

37% formaldehyde	200ml
10 x HEPES	100ml
DW	700ml

20 x SSC

NaCl	175.3g
Sodium citrate	88.2g
DW	to 1L (pH to 7.0 with HCl).

RNA hybridisation buffer

20 x SSPE	6.25ml
Deionised Formamide	12.5ml
100 x Denhardt's solution	1.25ml
10% SDS	1.25ml
10mg/ml Salmon sperm DNA	0.5ml
DW	3.25ml

20 x SSPE

NaCl	175.3g
Sodium hydrogen phosphate.H ₂ O	27.6g
EDTA	7.4g
DW	840 ml
Autoclave (to dissolve), pH to 7.4.	
DW	to 1L

Denhardt's solution

Polyvinylpyrrolidone (PVP)	5g
Nuclease free bovine serum albumin (BSA)	5g
Ficoll 400	5g
DW	to 250ml

Filter sterilise and store at -20°C.

Stocks and solutions 5

In situ hybridisation (ISH)

4% paraformaldehyde (20% stock)

Paraformaldehyde 200g (20%)

PBS 1L

Heat PBS to 70°C and add paraformaldehyde in a fume hood and stir. Use 5 M NaOH to dissolve then pH to 7.4. Store at -20°C and dilute in PBS for use.

Proteinase K stock solution

10mg/ml proteinase K (Sigma) stock, heated to 65°C for 30 mins, and stored at -20°C.

DNA-DNA in situ hybridisation buffer

20 x SSC 100 µl (2 x)

Deionised formamide 500 µl (50%)

50% dextran sulphate (preheated to 60°C) 100 µl (5%)

10mg/ml salmon sperm DNA 10 µl (100 µg/ml)

10% SDS 10 µl (0.1%)

50 x Denhardt's solution * 20 µl (1%)

Labelled probe 1 ng/µl

DW to 1ml

* Prepared using nuclease free, immunoglobulin free BSA (Sigma).

20 x SSC

NaCl 175.3g

Sodium citrate 88.2g

DW to 1L (pH to 7.0 with HCl).

Diethylpyrocarboate (DEPC) water or PBS

DEPC	400 µl
DW or PBS	400ml

Left overnight at room temperature then autoclaved.

20 x TBE buffer

Tris base	216g
Boric acid	110g
0.5M EDTA pH 8	80ml
DW	to 1L

Polyacrylamide gel RNA loading buffer

Formamide	85%
20 x TBE	5%
DW	5%
10% xylene cyanol	5%

4µl of buffer was added to 0.5×10^6 cpm labelled probe in 4µl of water.

Dithiothreitol (DTT)

Stock solutions were 0.75M or 1.0M, stored at -20°C.

10 x salts

Na ₂ HPO ₄	14.2g (100mM)
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Dissolve in a small amount DW and pH to 6.8.

NaCl	176.2g (3M)
1M Tris.HCl pH 7.6	100ml (0.1M)
0.5M EDTA pH 8	100ml (0.05M)
DW	to 1L

Ribosomal RNA

17.7 U/mg (Sigma). Dissolve in DEPC water to give a concentration of 10mg/ml, approx. 283µl per 50 U.

RNA-RNA in situ hybridisation buffer

10 x salts	100 µl (1 x)
Deionised formamide	500 µl (50%)
100 x Denhardt's solution	10 µl (1 x)
10mg/ml rRNA	30 µl (300 µl/ml)
50% dextran sulphate (preheated to 60°C)	200 µl (10%)
1M DTT	10 µl (0.01M)
Labelled probe	1 x 10 ⁶ cpm/section
DEPC water	to 1ml

TNE

NaCl	29.2g
1M Tris.HCl pH 7.6	10ml
0.5M EDTA pH 8	2ml
DW	to 1L

Stocks and solutions 6

Transgenic mice

M2 medium

Modified Krebs-Ringer solution containing HEPES, (Hogan *et al.* 1986).

PCR ear buffer

1M Tris.HCl pH 8	50µl
5M NaCl	4µl
10% SDS	10µl
Proteinase K 10mg/ml	100µl
DW	to 1ml

10 x PCR reaction buffer

Tris.HCl pH9.0	10mM
Ammonium Sulphate	75mM

PCR reaction mixture

For 14 tubes :

10 x reaction buffer	75µl
dNTP 20mM	1.5µl each
Primer oligonucleotide	18µl each
MgCl ₂ 25mM	45µl
Taq polymerase (1 unit/50µl)	1.5µl

Oligonucleotide primer stock solutions were diluted such that the final concentration

in the PCR tube was 20µM.

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