

DELTA AND THE FATE OF STEM CELLS IN HUMAN EPIDERMIS

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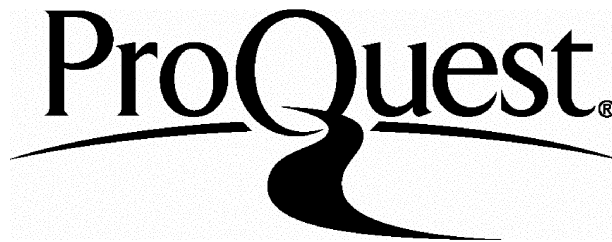
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ABSTRACT

Human epidermis is renewed throughout life from a population of stem cells, which are located in evenly spaced patches within the basal cell layer. Those daughters of stem cells that are destined to differentiate are called transit amplifying cells, and these are located in the remaining regions of the basal layer that surround the stem cell patches. Cell movement and cell differentiation must be tightly coordinated in order to maintain the organisation of the tissue.

In this thesis I describe a stably heritable lineage marking technique based on Green Fluorescent Protein (GFP) which I have used to follow the clonal progeny of single cells within a cell culture model of human epidermis. Using this technique, I found that stem cell progeny tend to remain in cohesive clusters whilst transit cell progeny move more freely amongst their unlabelled neighbours.

Signals from surrounding keratinocytes influence the differentiation of epidermal stem cells, but the nature of the signals is unknown. I found that the transmembrane signalling protein Delta is expressed in stem cells. I overexpressed Delta in primary human keratinocytes by retroviral infection and used GFP as a lineage marker to follow the effect of Delta on the fate of neighbouring cells within a reconstituted epidermis. The results of these experiments suggest that Delta signalling promotes differentiation at the boundary of stem cell clusters. I also obtained evidence that Delta promotes cohesiveness between groups of keratinocytes. This function of Delta requires the intact cytoplasmic domain of the protein and appears to be independent of effects on stem cell differentiation. Thus, Delta may have a dual role in the epidermis: regulating stem cell differentiation and location.

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A B B R E V I A T I O N S

AH	azaserine hypoxanthine
AJ	adherens junction
AP	ammonium persulphate
AM12	gag pol + envAM12 packaging cells
BHI	brain heart infusion
bp	base pairs
BrdU	5' bromodeoxyuridine
BSA	bovine serum albumin
CD	cluster of differentiation antigen
CFE	colony forming efficiency
cfu	colony forming units
CNS	central nervous system
COL	collogen
C-term	carboxy terminus
DAB	3,3-diaminobenzedene tetrahydrochloride
DCS	donor calf serum
DED	de-epidermised dermis
DEPC	diethylpyrocarbonate
DIMP	dimethyl pimelimidate
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
DNase	deoxyribonucleic acid endonuclease
dNTP	deoxynucleotide triphosphate
DSL	Delta/ Serrate/ Lag2
DTT	dithiolthreitol
ECM	extracellular matrix
EDTA	ethyldiaminotetraacetic acid, disodium salt
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EPU	epidermal proliferative unit
EBP	ezrin binding protein
FACS	fluorescence activated cell sorter
FAD	F12 + adenine + DMEM
FCS	foetal calf serum
FITC	fluorescein isothiocyanate

FN	fibronectin
FSG	fish skin gelatin
GPE	gag pol + env86 packaging cells
HA	influenza hemagglutinin
H&E	haematoxylin and eosin staining
HLH	helix loop helix
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HES	Hairy and Enhancer of Split
HGF	hepatocyte growth factor
HICE	hydrocortisone, insulin, cholera enterotoxin and EGF.
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
IC	intracellular domain
ICRF	Imperial Cancer Research Fund
IF	keratin intermediated filaments
IRES	internal ribosomal entry site
K10	keratin10
K	keratinocyte
kDa	kilo Dalton
LDH	lactate dehydrogenase
LNR	notch/ lin12 repeat
LRC	label retaining cell
LTR	long terminal repeats
MAPK	mitogen activated protein kinase
MF	actin microfilaments
MMTV	Molony murine tumour virus
Mo MuLV	Moloney murine leukemia virus
MOPS	3-[N-Morpholino]propane-sulfonic acid
N-term	amino terminus
O.D.	optical density
PBS	Phosphate buffered saline
PBST	PBS/Tween
PCR	polymerase chain reaction
pen/strep	penicillin/streptomycin
PI-3 kinase	phosphatidylinositol 3-kinase
PMSF	phenylmethanesulphonyl fluoride
polyHEMA	poly(2-hydroxyethyl methacrylate)
PVDF	polyvinylidene fluoride
RBPJk	recombination signal sequence binding protein for Jk genes

RNase	ribonucleic acid endonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
S	stem cell
SC	stem cell
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SSC	salt sodium citrate buffer
SuH	Suppressor of Hairless
SuH ^{DBM}	Suppressor of Hairless DNA binding mutant
TA	transit amplifying cell
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TCA	Trichloroacetic Acid
TCP	Tissue Culture Plastic
TD	terminally differentiated cell
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TM	transmembrane domain
TS	Tris buffered saline
Wg	Wingless (Drosophila)
Wnt	mammalian homologue of Wingless
WT	wild type

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PUBLICATIONS

Some of the data described in this thesis has been presented in the following publications:

Jensen, U. B., **Lowell, S.** and Watt, F. M. (1999). The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. *Development* **126**, 2409-2418.

Lowell, S., Jones, P., Le Roux, I., Dunne, J. and Watt, F. M. (2000). Stimulation of human epidermal differentiation by Delta-Notch signalling at the boundaries of stem-cell clusters. *Curr Biol* **10**, 491-500.

CHAPTER ONE

INTRODUCTION

1.1 Background

Human epidermis is renewed throughout life from a population of stem cells, which are located in evenly spaced patches within the basal cell layer. Those daughters of stem cells that are destined to differentiate are called transit amplifying cells, and these are located in the remaining regions of the basal layer that surround the stem cell patches. The decision of a stem cell daughter to either self-renew or to become a transit amplifying cell is influenced by its neighbours. The stability of stem cell patterning in human epidermis may also depend on co-ordination between a cell's differentiation decisions and its movement within the basal layer. Delta, a transmembrane ligand for the Notch receptor, is widely used to mediate local intercellular signals between stem cells of several other tissues. The role of Delta in epidermal stem cell fate and patterning is the subject of this thesis.

1.2 Structural and functional organisation of human epidermis

The epidermis is the outer layer of the skin. It is made up of multiple layers of keratinocytes, which are named for their characteristic intracellular network of keratin intermediate filaments. Other cell types found within the epidermis include melanocytes, which synthesise melanin pigment, Merkel cells, which are sensory receptors, and Langerhans cells, which play a role in antigen presentation to the immune system. The epidermis is separated from the underlying dermis by a basement membrane. Beneath the dermis lies a layer of subcutaneous fat, which forms the deepest layer of the skin (Nasemann *et al.*, 1983).

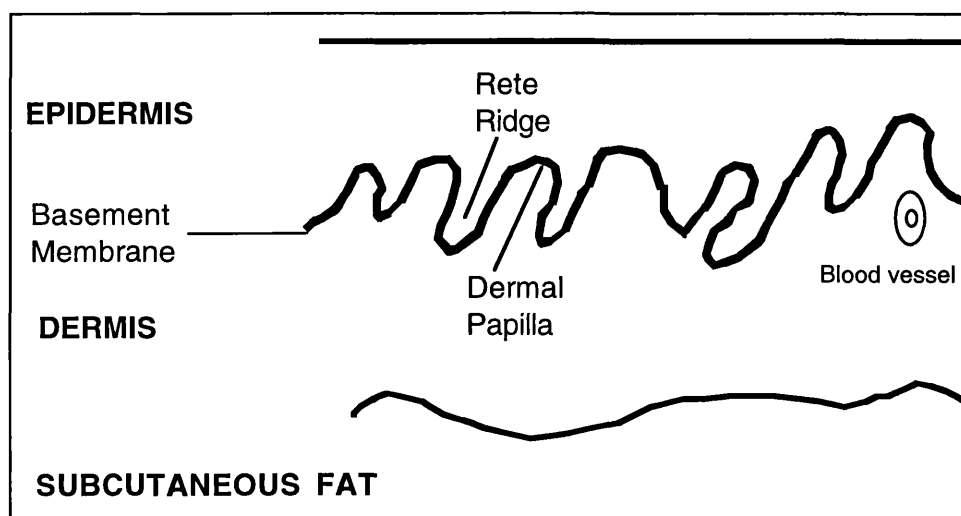


Figure 1.1 The structure of skin

1.2.1 Functional organisation of epidermis

The cells in the outermost layer are the tough squames, which are responsible for the barrier function of the skin, whilst the cells in the basal layer are undifferentiated proliferative cells which are responsible for regeneration of the epidermis. Dead squames are continually lost from the surface of the skin, and are replaced by cells moving upwards from the basal layer. As the keratinocytes lose contact with basement membrane, the terminal differentiation programme is activated and the keratinocytes become progressively larger and flatter. The epidermis can be subdivided into four

layers, each with distinctive histological features that reflect the successive stages of keratinocyte differentiation (Watt, 1989; Fuchs, 1990).

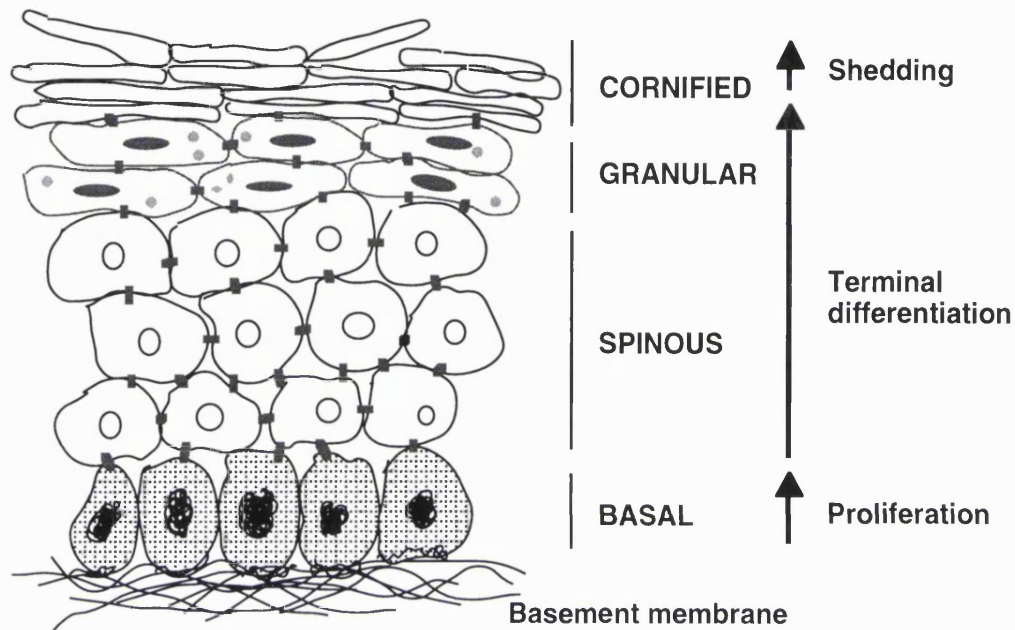


Figure 1.2 The Layers of the Epidermis

Modified from (Nasemann *et al.*, 1983) by A. Zhu: reproduced with permission.

1.2.2 Basal layer

The cells in the basal layer are in contact with an underlying basement membrane. The most abundant protein in the basement membrane is type IV collagen (Timpl, 1989). Other components include laminins, proteoglycans and nidogen (Burgeson and Christiano, 1997). Keratinocytes and dermal fibroblasts co-operate to secrete these extracellular matrix (ECM) proteins (Marinkovich *et al.*, 1993). Basal keratinocytes adhere to the basement membrane proteins via integrins, which are heterodimeric glycoproteins comprised of an α and a β subunit (Hynes, 1999). The most abundantly expressed integrins in keratinocytes are: $\alpha 2\beta 1$, which binds type 1 collagen, type IV collagen, and laminin 1; $\alpha 3\beta 1$, which binds laminin 1 and laminin 5 (Adams and Watt,

1991), and $\alpha 6\beta 4$ which binds laminin-1 (Carter *et al.*, 1990). Cultured keratinocytes also express $\alpha 5\beta 1$, which binds fibronectin and, $\alpha v\beta 5$, which binds vitronectin.

Keratinocytes adhere to the underlying matrix via two different types of adhesive junction, focal contacts and hemidesmosomes. Focal contacts link actin microfilaments to basement membrane proteins via integrins together with an intracellular complex of intermediate proteins such as talin, vinculin and α actinin. The type of integrin found in a focal contact depends on the nature of the ECM that it is attached to.

Hemidesmosomes link keratin filaments to basement membrane proteins via the $\alpha 6\beta 4$ integrin. High levels of the integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ are also expressed at the lateral and apical borders of basal cells, *in vivo* and *in vitro*, although it is not clear whether they are involved in cell-cell adhesion (De Strooper *et al.*, 1988; De Luca *et al.*, 1990; Hertle *et al.*, 1991).

1.2.3. Spinous layer

As soon as cells leave the basal layer they lose contact with the extracellular matrix proteins and begin the terminal differentiation programme (Adams and Watt, 1989). The 4-5 layers above the basal layer contain cells in the earlier stages of differentiation. These cells become progressively larger as they move further away from the basal layer. The name “spinous” comes from the abundant spiny-looking desmosomal junctions that connect adjacent cells (Holbrook, 1994). Cells in the spinous layer begin to make precursors of the cornified envelope, such as involucrin (Rice and Green, 1979). Involucrin *in vivo* starts to appear several layers above the basal layer, but in cultured epidermis it appears from the first suprabasal layer upwards: keratinocytes in culture express involucrin within 24 hours of losing contact with the basement membrane (Watt, 1988).

1.2.4. Granular layer

Keratinocytes in this layer are characterised by electron dense granules that contain loricrin and profilaggrin. Profilaggrin is a precursor for filaggrin, a protein thought to be involved in the aggregation of keratin filaments that toughens the differentiated keratinocyte (Rothnagel and Steinert, 1990). Loricrin is a structural protein that will later become incorporated into the cornified envelope (see below) (Mehrel *et al.*, 1990; Holbrook, 1994). The granular layer is 3-4 cells thick in thin skin and thicker in palm and sole.

1.2.5. Cornified layer

This is the outer layer of epidermis. It forms the tough impermeable barrier that protects the body from the environment. It contains wide flattened keratinocytes called squames, 40um in diameter and only 0.5 um in height (Odland, 1991). These are dead cells whose only function is mechanical protection. The organelles and nucleus are degraded, the cytoplasm is filled with keratin filaments, and a tough meshwork of crosslinked proteins called the cornified envelope lies beneath the plasma membrane (Nemes and Steinert, 1999).

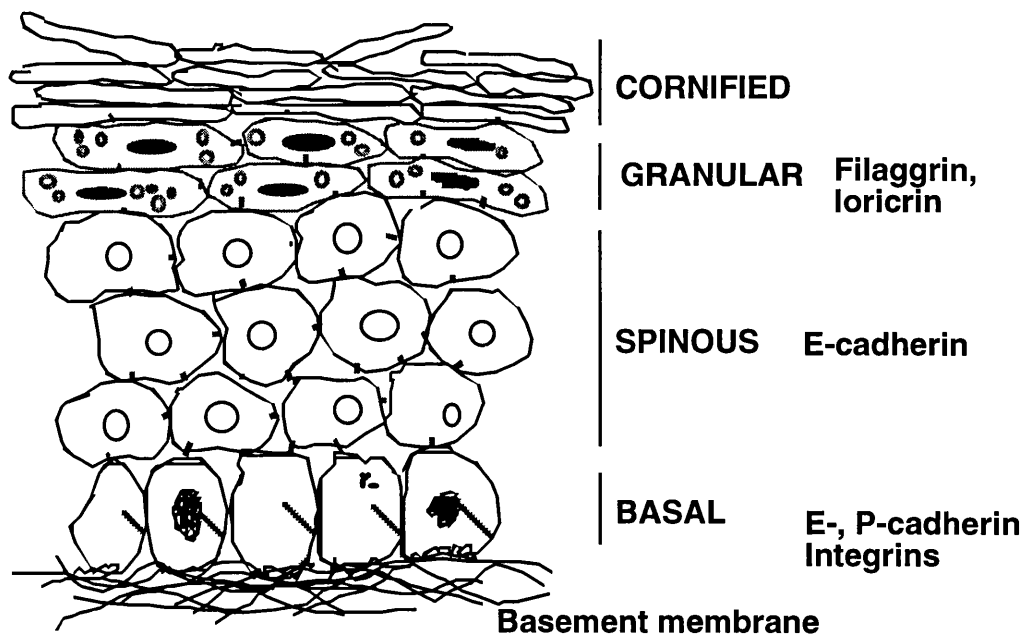
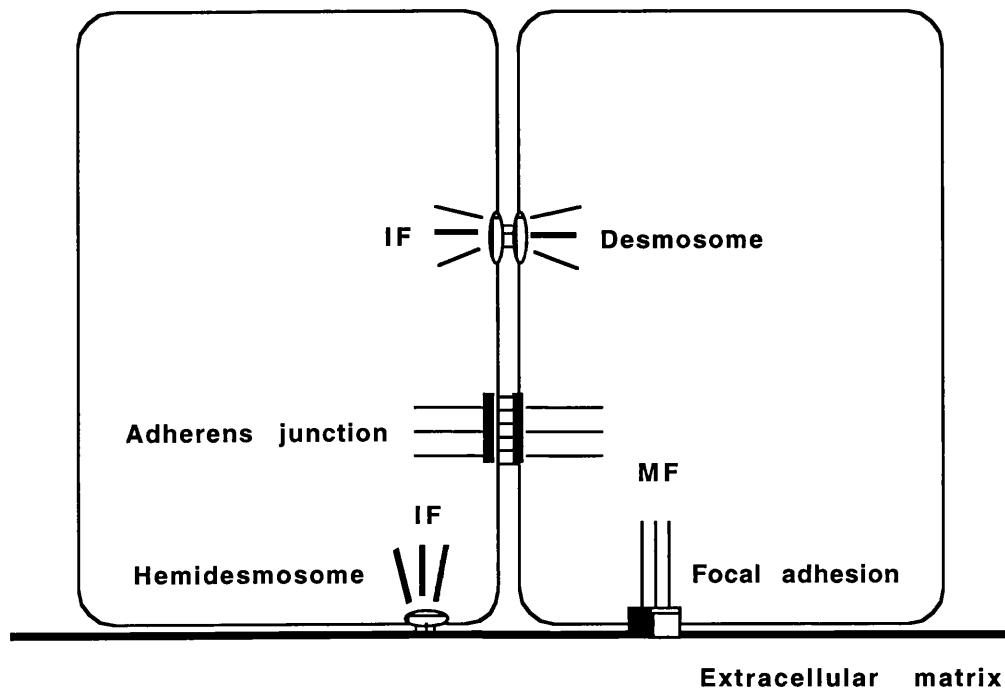


Figure 1.3 Markers of keratinocyte differentiation.



IF: Intermediate filaments MF: microfilaments.

Figure 1.4 Adhesion structures in keratinocytes

1.2.6. Cell-cell adhesion

Homophilic interactions between transmembrane proteins called cadherins mediate cell-cell adhesion between adjacent cells at adherens junctions (Yap *et al.*, 1997). These are linked inside the cell to the actin cytoskeleton. Formation of adherens junctions depends on the presence of extracellular calcium (Takeichi, 1977). Keratinocytes express two of the classical cadherins, E and P cadherin. The cytoplasmic tail of cadherins binds to cytoplasmic proteins called α catenin, β catenin and plakoglobin. Catenins can regulate cadherin function and have an independent signalling function in the Wnt signalling pathway (Gumbiner, 1995). Specialised members of the cadherin superfamily, the desmogleins and desmocollins, mediate homophilic cell-cell adhesion at desmosomes. These are anchored to the network of intermediate filaments within the cell (Kowalczyk *et al.*, 1999). Like adherens junctions, the formation of desmosomes depends on the presence of extracellular calcium. Other adhesion molecules expressed by keratinocytes include CD44 (Hudson *et al.*, 1995) and syndecans (Sanderson *et al.*, 1992), both of which mediate calcium independent adhesion. The contribution of these molecules to adhesion in the epidermis is not clear.

1.2.7 Hair

Hair follicles are found all over the body, apart from the palms, soles and foreskin. The follicle is enveloped in a sheath of connective tissue made up of type I and type III collagen. This is continuous with the papilla, a protrusion of the dermis into the base of each follicle. The epithelial components of the follicle are the outer root sheath, the inner root sheath, and the cells surrounding the dermal papilla, which are called the matrix. The major cell type of the hair follicle is the keratinocyte (Chapman, 1986). A basement membrane, containing type IV collagen, laminin, nidogen, and proteoglycans, separates the epithelial cells from the papilla and the connective tissue sheath. Hair follicles also have an associated sebaceous gland, which secretes lipid-rich sebum into the upper hair follicle in order to lubricate the skin and hair.

Proliferation is confined to cells in the matrix and the outer root sheath below the insertion of the arrector pili muscle (Chapman 1986). The central matrix cells give rise to the cortex cell and ultimately to the keratinous fibres that form the hair, whilst the outer matrix cells generate the hair cuticle. Human hair grows through asynchronous cycles, which are divided into three phases. During anagen phase (2-6 years), the matrix cells proliferate and the hair grows. Mitotic activity regresses during the catagen phase (2-4 weeks) and during telogen phase (2-4 months), the follicle lies dormant (Chapman, 1986).

1.3 Epidermal stem cells

1.3.1. Epidermal turnover

The human epidermis is continually renewing itself. All of the squames that make up the outermost cell layer are shed from the body approximately every 24 hours (Baker and Kligman, 1967; Jansen *et al.*, 1974; Roberts and Marks, 1980). This is the major route by which cells are lost from the epidermis: there is no evidence that apoptosis, as conventionally defined (Kerr *et al.*, 1972) occurs in normal healthy epidermis (Gandarillas *et al.*, 1999). Cells are replenished by the proliferation of undifferentiated keratinocytes in the basal layer. Each squame on the skin surface occupies

approximately the same area as 25 basal cells: this means that 4% of basal cells would in theory need to divide each day to maintain a steady state. This number is in agreement with the proportion of proliferative cells that have been measured by pulse labelling of human subjects with tritiated thymidine (Dover, 1994), or using markers of proliferation such as proliferating nuclear antigen or Ki67 (Pierard-Franchimont and Pierard, 1989; Jakic-Razumovic *et al.*, 1992; Hall *et al.*, 1993; Jensen *et al.* 1999). The fact that the epidermis is able to regenerate the outermost cornified layer every day, and the basal layer approximately once a month, makes the epidermis highly dependent on a population of stem cells.

1.3.2. What is a Stem Cell?

Writing in 1901, Adami noted that in normal epithelia the “actively-functioning and fully-developed cell, as such, does not undergo mitosis or show evidence of multiplication”. He proposed that epithelial tissues such as the epidermis must contain “proliferous or ‘mother’ cells, cells which themselves throughout life do not attain full differentiation but which give off daughter cells and the daughter cells it is which develop into the fully differentiated functional cells”. The proliferation of the ‘mother cells’ would be regulated by signals from the surrounding daughter cells (Adami, 1901). Adami’s definition of a “mother cell” is effectively the same as the current definition of an epidermal stem cell: a cell with a high capacity of self-renewal and the ability to generate daughter cells that undergo terminal differentiation (Potten and Morris, 1988; Morrison *et al.*, 1997; Watt, 1998). However, is difficult to assess whether any particular cell possesses these defining features, and the identification of stem cells in the epidermis remains a controversial area.

Stem cells in other tissues are often capable of generating more than one type of differentiated cell (Morrison *et al.*, 1997; Watt and Hogan, 2000). There is some evidence that interfollicular keratinocyte stem cells are also pluripotent: when grafted into an empty hair follicle they can differentiate to produce normal hair (Reynolds and Jahoda, 1992). For the most part, however, the only fate decision faced by stem cells in interfollicular epidermis is whether to self-renew or to differentiate into a squame.

1.3.3. Proliferative heterogeneity

The first direct evidence for the existence of stem cells in self-renewing adult tissues came, not from the epidermis, but from the haemopoietic system. Individual bone marrow cells, when transplanted into the spleens of recipient mice, were capable of generating large numbers of blood cell precursors of several different lineages. Serial

transplantation of these cells showed that they had a high capacity to self-renew (Till and McCulloch, 1961; Becker *et al.*, 1963; Curry and Trentin, 1967). By analysing haematopoietic stem cells (HSC) in vitro, it was shown that they do not give rise to differentiated cells directly, but via populations of proliferative committed progenitor cells (Morrison and Weissman, 1994). Many differentiated progeny can be generated from a single stem cell division, leading to the proposal that stem cells spend most of their time withdrawn from the cell cycle in a resting state called Go (Becker *et al.*, 1965; Lajtha, 1979).

By analogy with HSC, it was proposed by Potten that epidermal stem cells generate differentiated progeny via a population of “transit amplifying cells”, defined as cells that are able to divide only a few times before all of their progeny terminally differentiate (Potten, 1974; Potten, 1976; Potten, 1981). Most of the cells in the basal layer of mouse or human epidermis have the capacity to proliferate, even though most of them will remain quiescent in the steady state (Withers, 1967; Potten and Hendry, 1973; Potten and Morris, 1988). The transit-amplifying hypothesis would predict that not all of the proliferative cells are stem cells. The first evidence for this came from in vivo studies in mouse. When mouse epidermis is regenerated after skin irradiation, only about 10% of basal keratinocytes are able to form detectable foci of new epidermis (Potten and Hendry, 1973). This was taken as evidence that the majority of basal cells are not stem cells, based on the assumption that most stem cells will be in Go and thus remain unaffected by the dose of radiation. Although the validity of this approach has been questioned, (Withers, 1967; Wright and Alison, 1984; Archambeau, 1987), the existence of a transit amplifying compartment has since been supported by further evidence (see below) and is now widely accepted.

The advantage of having a transit-amplifying population within the epidermis may be that it allows the stem cells to remain relatively quiescent. Each cell division carries the risk of acquiring a mutation, and this risk is particularly high in the epidermis, which is exposed to a mutagenic onslaught from the outside world (Cairns, 1975). If a transit-amplifying cell is able to divide 5 times, then each stem cell division need only divide once on average to generate 32 cells. Mutations acquired during transit-amplifying cell divisions pose little danger since these cells are all destined to be shed from the surface of the skin within a month.

1.3.4. Tissue culture models for the study of epidermal stem cells.

The study of human epidermal stem cells has been greatly facilitated by the ability to grow human keratinocytes in culture supported by a feeder layer of mitotically inactive

3T3 cells (Rheinwald and Green, 1975). Human keratinocytes can be passaged several times before they undergo senescence and do not usually undergo spontaneous transformation. There is good evidence that keratinocyte cultures contain stem cells: cultures are able to generate normal epidermis for many years when they are grafted onto a suitable recipient. This has proved very useful for treating patients who have lost large areas of their epidermis through burns (Gallico *et al.*, 1984; Compton *et al.*, 1989; Compton *et al.*, 1998).

Keratinocyte cultures provide strong evidence for proliferative heterogeneity, confirming Potten's transit-amplifying hypothesis. Keratinocytes display their full proliferative capacity when they are plated at clonal density (Barrandon and Green, 1987a). Some generate thousands of progeny, giving rise to large actively growing colonies, whilst others are only able to divide a few times before all of their progeny undergo terminal differentiation, resulting in an abortive colony. Barrandon and Green (1987a) defined three categories of proliferative keratinocyte, based on a detailed clonal analysis. Keratinocytes with the highest proliferative capacity generate clones termed holoclones, in which over 95% of the cells can generate actively growing colonies after subcloning. Clones in which all of the cells lack proliferative capacity, or generate only abortive colonies after subcloning, are termed paraclones. An intermediate type of clone, called a meroclone, contains a mixture of cells of different growth potential and represents a transitional stage between a holoclone and a paraclone. Subcloning experiments have demonstrated that the transitions from holo- to mero- to paraclone are irreversible (Barrandon and Green, 1987a).

Thus, cultures of human keratinocytes contain stem cells, and these are likely to be contained within the populations that display a high proliferative capacity when plated at clonal density. It is likely that both holoclones and meroclones contain stem cells, because keratinocytes isolated from the epidermis of elderly people appears to yield few or no holoclones (Barrandon and Green, 1987a).

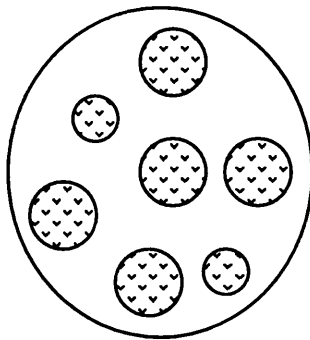
A simple form of clonal analysis, in which keratinocytes are seeded at clonal density and cultured for only two weeks, has been used as a convenient way to assess the proportion of stem cells and transit amplifying cells within a proliferative population (Jones and Watt, 1993; Gandarillas and Watt, 1997; Zhu and Watt, 1999). Cells that give rise to abortive colonies in this assay are assumed to arise from transit amplifying cells. Cells that give rise to large actively growing colonies after two weeks are assumed to include the stem cells, although it should be noted that not all of the cells within this population may be capable of regeneration over the long term. This type of assay will therefore tend to overestimate the proportion of stem cells (Cotsarelis *et al.*,

1999). Typically, only about 50% of the proliferative keratinocytes isolated from normal epidermis generate abortive colonies in this type of assay. A more rigorous way to identify stem cells would be to measure their capacity to self-renew over a much longer time period (Mathor *et al.*, 1996). Unfortunately, because stem cell fate can only be reliably determined on a clonal basis, long-term analysis requires subcloning experiments, which are very labour-intensive and not feasible for use as a routine assay. Another important limitation of clonegenicity assays, whether short term or long term, is that they cannot be used to identify stem cells prospectively.

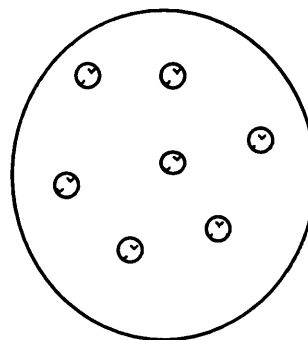
1.3.5. Stem cell markers

In the haematopoietic system, stem cells can be prospectively distinguished from committed progenitors on the basis of differences in the expression of cell surface markers (Spangrude *et al.*, 1988). Analysis of epidermal stem cells was hampered for many years by the lack of molecular markers that can distinguish stem cells from transit-amplifying cells. Some progress has been made in recent years towards this end.

The fact that keratinocyte integrins play a role in controlling epidermal differentiation and morphogenesis (Watt *et al.*, 1993) led to the suggestion a few years ago that differences in integrin function or expression may provide a marker to distinguish stem cells from transit-amplifying cells (Jones and Watt, 1993). Sorting basal keratinocytes by flow cytometry provided evidence to support this hypothesis: there is a log linear relationship between the level of surface $\beta 1$ integrin expression on basal cells and their proliferative capacity in vitro. When basal cells are fractionated on the basis of how long it takes for them to adhere to dishes coated with the $\beta 1$ integrin ligand type IV collagen, over 80% of the cells that adhere within 20 minutes go on after two weeks to form large circular colonies containing over 5000 cells. The cells that adhere slowly divide only a few times before all of their progeny undergo terminal differentiation. Thus, rapidly adherent cells resemble stem cells and the slowly adherent cells behave like transit-amplifying cells (Figure 1.5). This holds true whether cells are tested directly after isolating them from epidermis, or have spent some intervening time in culture (Jones and Watt, 1993; Jones *et al.* 1995).

**STEM CELLS**

Form big colonies in vitro
High $\beta 1$ integrin expression
Cells adhere quickly

**TRANSIT AMPLIFYING CELLS**

Form abortive colonies in vitro
Low $\beta 1$ integrin expression
Cells adhere slowly

Figure 1.5 Stem cells and Transit amplifying cells in vitro

Other molecules that have been proposed as stem cell markers include the combination of high $\alpha 6$ integrin expression and low expression of the surface antigen recognised by monoclonal antibody 10G7 (Li *et al.*, 1998). The $\alpha 6$ -bright-10G7-dull cells are relatively quiescent in vivo and populations of these cells have very high long-term proliferative capacity. It would be interesting to know the regenerative capacity of the individual cells within these populations in order to assess to what extent they might be enriched in stem cells.

It is not yet possible to unambiguously identify a stem cell prospectively. High $\beta 1$ integrin marks 20-45% of the basal cells, which is at least double the proportion that are estimated to be stem cells in vivo. Furthermore, at least 20% of $\beta 1$ integrin-bright cells form abortive colonies after two weeks in clonal culture. Markers are currently being sought that might identify purer populations of stem cells. It is, however, possible that the regenerative behaviour of a cell depends to some extent on an invariant probability of undergoing differentiation. This would mean that some of the cells that do not behave as stem cells, in vivo or in vitro, are not intrinsically different from those cells that do behave as stem cells, making the isolation of “pure” stem cell populations an unattainable goal.

1.3.6. Regulation of stem cell fate

Whenever a stem cell divides, each of the two daughters faces the decision of whether to remain as a stem cell, or to become a transit-amplifying cell and ultimately to terminally differentiate. How is this decision regulated *in vivo*? In theory, the number of stem cells could be kept constant by ensuring that every stem cell division is strictly asymmetric, giving one stem and one transit-amplifying daughter. However, this mechanism alone could not explain how the stem cell pool in the epidermis can expand in response to tissue damage. Indeed, there is now abundant evidence that stem cell fate is responsive to extrinsic signals (Watt, 1998, and see below). An alternative strategy to limit stem cell numbers is to ensure that stem cells can only exist in a restricted environment which provides factors that maintain them and excludes factors that induce differentiation: i.e. a “stem cell niche”. The cell culture models of epidermis described in section 1.3.4 have made it possible to investigate the regulation of human epidermal stem cell fate. In recent years, optimised retroviral transduction protocols have greatly improved our ability to genetically manipulate primary human keratinocytes (Levy *et al.*, 1998). Thanks to this technological advance, several molecules have now been implicated in regulating the decision of a stem cell to become a transit-amplifying cell (Figure 1.6).

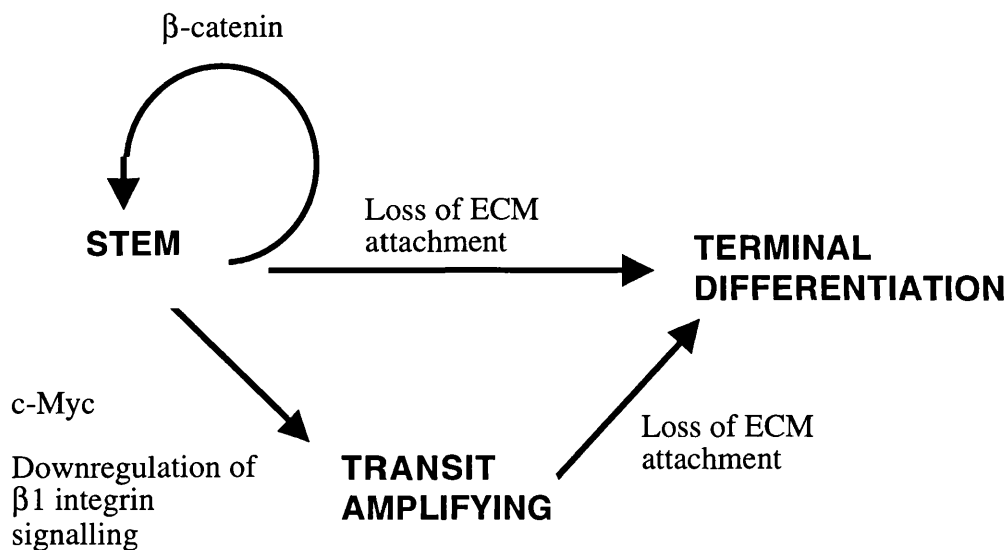


Figure 1.6 Regulation of stem cell fate.

Integrins in focal contacts are able to transduce signals from the ECM into the cell in addition to their mechanical adhesive function (Yamada and Miyamoto, 1995): high levels of $\beta 1$ integrins activate MAPK mediated signals that are required for keratinocytes to remain as stem cells (Zhu *et al.*, 1999). Stem cells have higher levels of non-cadherin-associated β catenin than transit-amplifying cells in vitro; experiments using dominant negative and active β -catenin mutants have provided evidence that β catenin signalling can maintain a stem cell fate (Zhu and Watt, 1999). Similar approaches have shown that cMyc promotes transition of keratinocytes from stem cell to transit-amplifying cell (Gandarillas and Watt, 1997).

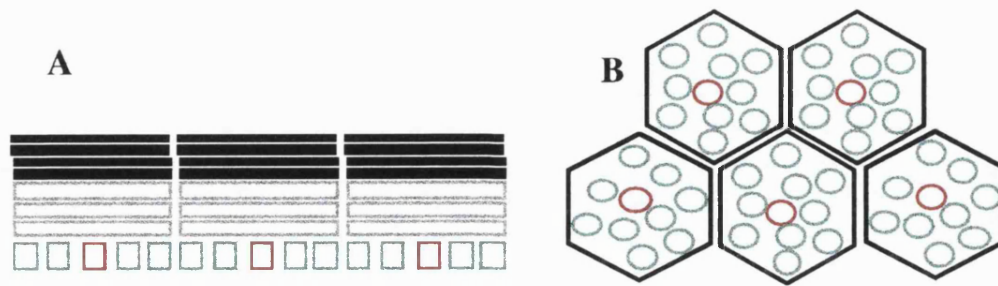
1.3.7. The spatial arrangement of stem cells and transit-amplifying cells

How are stem cells and transit-amplifying cells organised within the basal layer? For many years there were no molecular markers that could be used to distinguish stem cells from transit-amplifying cells in vivo. Early workers therefore sought to identify stem cells based on the assumption they would be the most quiescent cells in the tissues.

Mouse epidermis

When mice are given a series of tritiated thymidine injections, almost 100% of the cells in the epidermis become labelled, and after three months some cells still retain label (label retaining cells: LRC) (Bickenbach, 1981). Since the label would become diluted as cells divide, it was argued that the LRC must be relatively quiescent and therefore might be stem cells (Potten, 1981). By extracting cells from labelled mouse epidermis and placing them in culture, it has been demonstrated that LRC are still capable of proliferation (Morris and Potten, 1994).

In mouse dorsal epidermis, the squames in the upper layer of the dorsal epidermis are arranged in ordered columns that are aligned with cells in the basal layer. These columns can be made clearly apparent by swelling the epidermis with an alkaline solution (Mackenzie, 1970). The basal cells that lie under the centre of each cornified cell are less likely to be in mitosis than the cells under the edge of the cornified cells (Mackenzie, 1970; Christophers, 1972) and are more likely to be LRC (Potten and Morris, 1988). These observations led to the Epidermal Proliferative Unit model, according to which the central basal cell is the stem cell that maintains all the differentiated cells in the column that is aligned above it (Figure 1.7).



Cells in the basal layer of mouse dorsal epidermis are aligned with columns of squames in the cornified layer (A: shown in cross section B: shown from above). The central cell (shown in red) represents the stem cell and the peripheral cells (shown in green) represent the transit amplifying cells and the committed cells.

Figure 1.7 The Epidermal Proliferative Unit

Adapted from Potten and Morris (1988)

Stem cells in hair follicles

The hair follicle contains a population of stem cells that can generate large numbers of epidermal keratinocytes. In partial thickness burns, islands of regenerating epidermis can be seen to grow outwards from the remnants of hair follicles (Bereiter-Hahn, 1986). LRC are found in a region of the outer root sheath that lies adjacent to the insertion of the arrector pili muscle, known as the bulge region. This region has been microdissected and shown to contain cells that found keratinocyte colonies in vitro (Yang *et al.*, 1993; Rochat *et al.*, 1994). It has recently been shown that, even in the absence of extensive epidermal ablation, cells from mouse hair follicles migrate into interfollicular epidermis in vivo. This was demonstrated within the highly proliferative epidermis of the newborn mouse and after induction of proliferation by wounding in adult mice (Taylor *et al.*, 2000). The relative contribution of interfollicular and hair - follicle stem cells to the steady state interfollicular epidermis remains a controversial issue.

Human epidermis

The morphology of human epidermis is considerably different from that of mouse epidermis. Dorsal mouse skin has only 2 living cell layers and is relatively flat, whereas human epidermis has many cell layers, and although the surface of the epidermis is flat, the basal layer undulates. Regions where the epidermis projects down into the dermis are called rete ridges, and areas where the epidermis reaches closest to the surface of the skin are called dermal papillae (Odland, 1991) (Figure 1.1). Furthermore, most regions of human skin contain very few hair follicles.

Are proliferative units in human epidermis arranged in the same way as described above for mouse dorsal epidermis? Human epidermis does not have such a clear alignment between particular basal cells and columns of squames as mouse epidermis (Mackenzie *et al.*, 1981). It is not feasible to pulse label the skin of humans with radioactive markers, but these types of experiments have been carried out in the palm (non hair-bearing) skin of monkeys. Tritiated thymidine is taken up less frequently by keratinocytes at the base of the rete ridges than in the rest of the basal layer. These non-proliferating cells were interpreted as being stem cells, although, unlike in mouse (Morris and Potten, 1994), it has not been shown that they retain proliferative capacity. These studies led to the proposal that the stem cells reside in the rete ridges, and that the arrangement of stem cells is related to the pattern of rete ridges rather than to the organisation of squames on the surface of the epidermis.

Using high-level $\beta 1$ integrin expression as a marker, it has been possible to localise stem cells in human epidermis (Jones and Watt, 1993; Jensen *et al.*, 1999). The basal cells with highest integrin expression are localised in evenly spaced clusters containing approximately 40 cells. With the exception of palm and sole epidermis, these clusters are always on the tops of the dermal papillae. They are surrounded by an interconnected region of integrin-dull cells, extending down into the rete ridges. Thus the integrin-bright clusters form discrete islands surrounded by a “sea” of integrin-dull cells (Figure 1.8).

It is unlikely that all of the integrin-bright cells are stem cells. Kinetic considerations predict that only 10% of the basal population are stem cells whereas 25- 40% are defined as integrin-bright. It is therefore possible that within an integrin-bright patch, stem cells are interspersed with transit-amplifying cells. However, there is some evidence that this is not the case (Jensen *et al.*, 1999). Transit-amplifying cells can be identified on the basis that they have a high probability of active proliferation, commitment to differentiation and exit from the basal layer. Attempts have been made

in the past to identify the proliferating and differentiating cells in serial histological sections of human epidermis; however these cells are relatively rare, and it has been difficult to get a clear impression of their distribution within the basal layer. Jensen developed a whole mount approach that makes it possible to visualise proliferation and differentiation in the basal layer of an intact epidermis. This has made it clear that the integrin-dull regions of human epidermis contain the vast majority of actively proliferating cells as assessed by BrdU incorporation or expression of the proliferation marker, Ki67 (Figure 1.9 A). Cells expressing the early differentiation marker K10 are found exclusively in the integrin-dull regions as are cells that appear to be moving upwards out of the basal layer (Figure 1.9 B).

The above model predicts that at least 25% of basal cells (the integrin bright population) have a low probability of active proliferation or terminal differentiation. One interpretation would be that some of the integrin bright cells constitute an “intermediate population” between stem and transit cells. The characteristics of this putative intermediate population cannot be investigated until we have stem cell markers that are more precise.

1.3.8 Autoregulation of stem cell fate

The patterning of stem cells has shed some light on the regulation of stem cell fate. Sheets of keratinocytes cultured in vitro contain evenly spaced patches of integrin-bright cells, with similar number and spacing to the stem cell patches in vivo, showing that epidermal stem cells can become arranged in a spatially patterned array in the absence of dermis (Jones *et al.*, 1995). The size and spacing of these integrin-bright patches is independent of the number or proportion of integrin-bright cells that are included in the seeding population. A single keratinocyte can generate a sheet of epidermis covering an average area of 17mm² in which there are over 150 separate patches of integrin-bright basal cells (Figure 1.10). Thus, stem cell number and patterning can be autoregulated in the absence of cues from underlying dermis. This implies that stem cell fate is not intrinsically predetermined. It also implies that if a stem cell niche can be said to exist, then it is not simply a specialised region of the dermis but must instead be defined in terms of the environment provided by neighbouring keratinocytes. This “niche” would also have the ability to become established as a consequence of autoregulatory feedback signals.

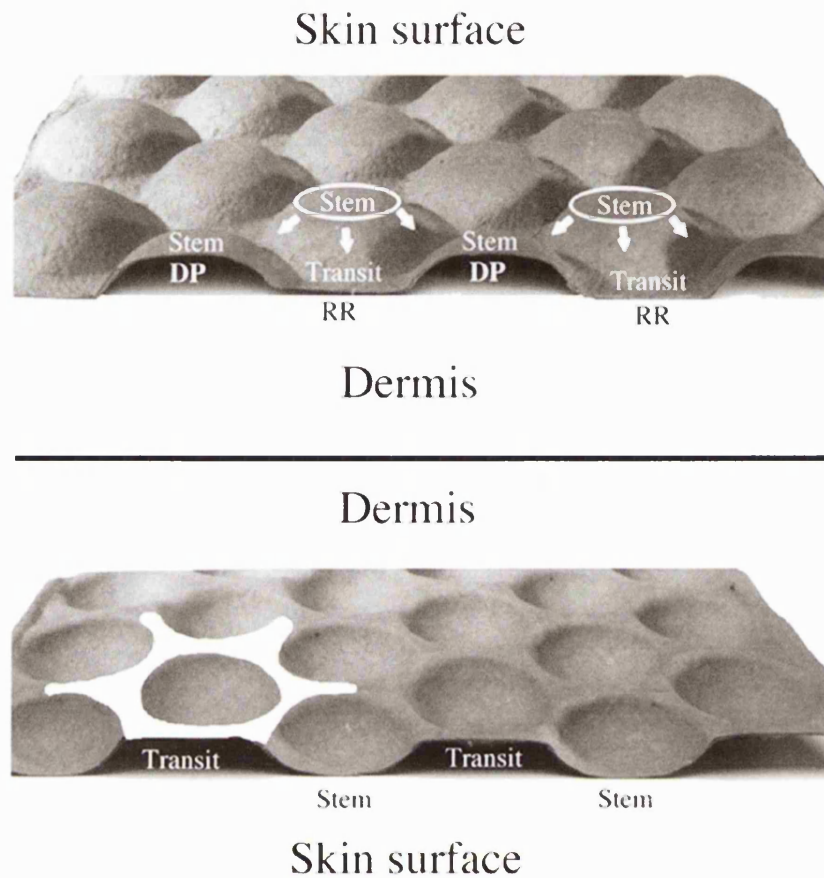


Figure 1.8 Model showing the arrangement of stem cells and transit amplifying cells in basal layer of human epidermis

Stem cell patches (Stem) are at the tips of the dermal papillae (DP) and the transit amplifying cells (Transit) are at the tips of the rete ridges (RR). The arrows in the top panel represent movement of transit amplifying cells away from the stem cell patches. The transit amplifying compartment extends from the periphery of each stem cell patch to the tips of the rete ridges and is continuous throughout the tissue (white area bottom panel). Note that the epidermis is shown in opposite orientations in the two panels.

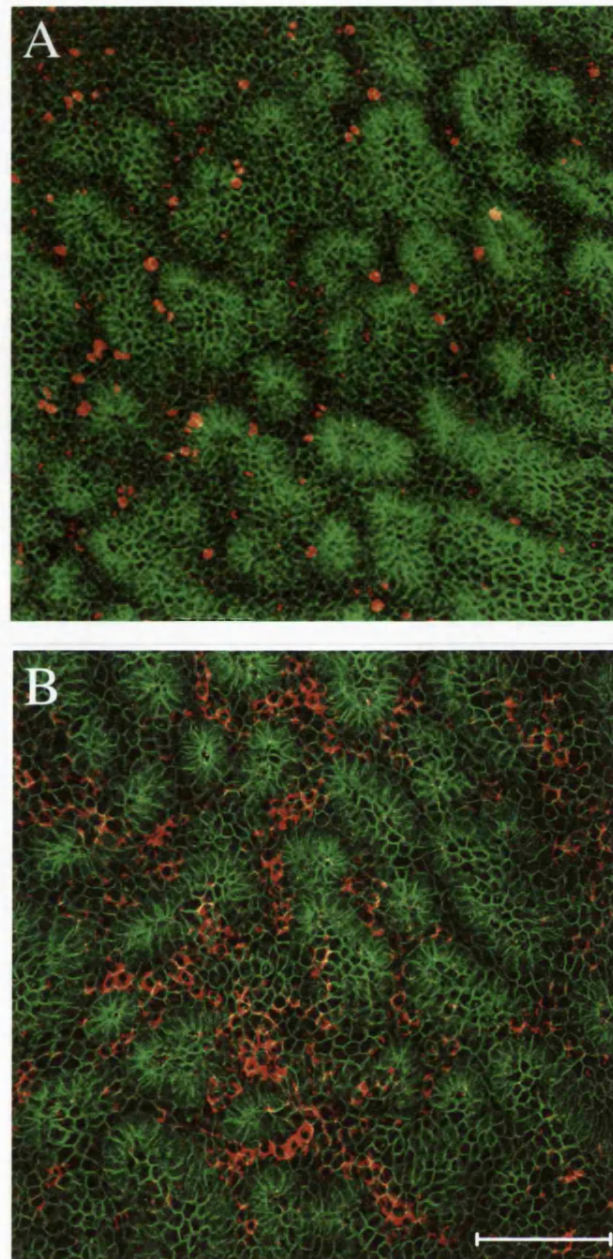


Figure 1.9 The spatial arrangement of stem cells and transit amplifying cells in the basal layer of human epidermis.

A: Confocal micrograph of a whole-mount breast skin epidermis stained for beta-1 integrins as a marker of stem cells (green) Ki67 as a marker of actively proliferating cells (red).

B: Confocal micrograph of whole-mount foreskin epidermis stained for beta-1 integrins as a marker of stem cells (green) and keratin 10 as an early marker of terminal differentiation (red). Only those keratin-10 positive cells that are still attached to the basement membrane are visualised.

Scale bars: 100µm (figure reproduced from Jensen et al 2000))

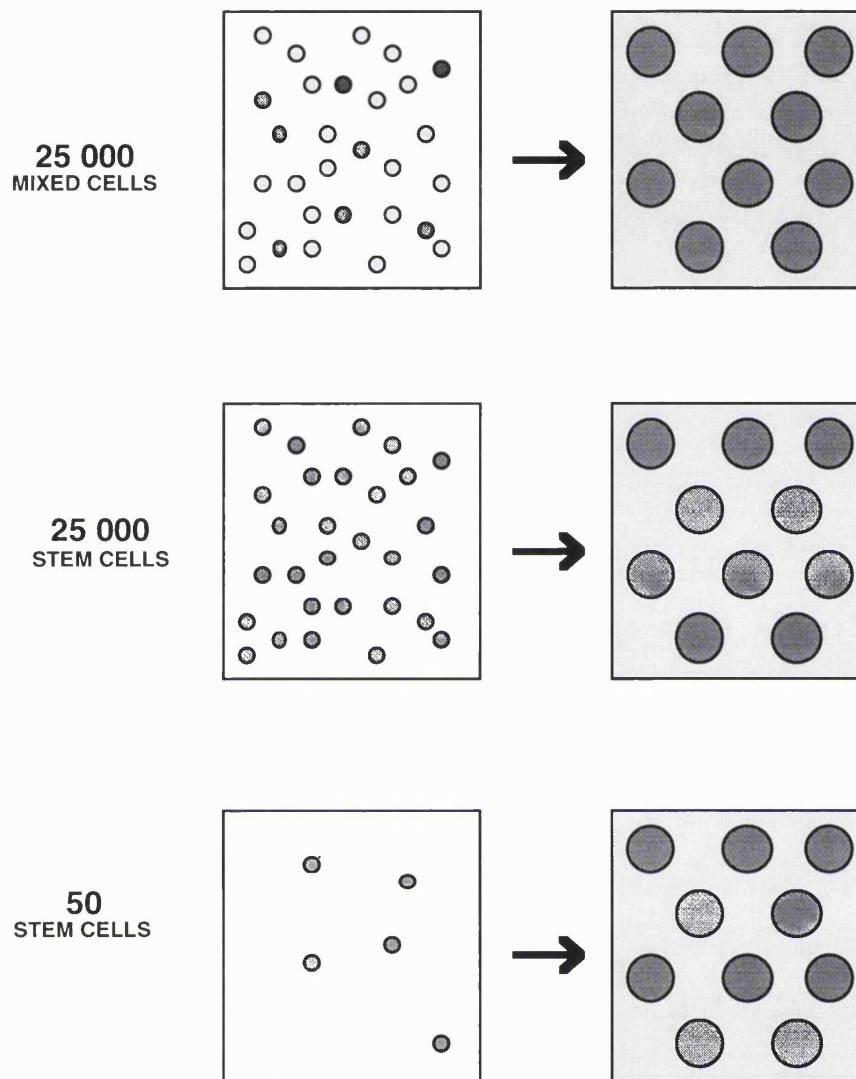


Figure 1.10 Autoregulation of stem cell fate in vitro

Seeding-populations of keratinocytes are represented in the left-hand panels and the final confluent cultures are represented in the right hand panels. Regions containing integrin-bright cells are shaded dark grey and regions containing integrin-dull cells are shaded pale grey. The integrin-bright cells in the final confluent sheet are arranged in evenly spaced clusters, whose size and spacing is independent of the number of stem cells in the seeding population. Figure reproduced from (Jones, 1997)

All the molecules that have been so far demonstrated to regulate epidermal stem cell fate act cell autonomously. Two commonly used ways of assessing stem cell fate are to carry out cloneogenicity assays and to quantitate surface integrin expression by flow cytometry, both of which involve analysis of isolated cells. This makes it difficult to measure how a cell's fate decisions might be influenced by its neighbours. This problem could be overcome by using lineage markers to mark a particular cell amongst unmarked neighbours. It would then be possible to either follow the fate of the marked cell and its progeny within the intact culture, or else to distinguish and analyse marked cells after the culture has been disaggregated.

1.3.9. Lineage marking

Keratinocyte lineage markers are useful tools for several purposes. They can be used to test models of epidermal organisation (see section 1.3.10), and they make it possible to investigate how a keratinocyte's fate can be influenced by its neighbours (see section 1.3.8). Lineage marking could also be used to study certain intrinsic properties of keratinocytes that would not be apparent in isolated cells, for example a cell's mobility relative to its clonal siblings.

There are two general approaches to experimentally label cells and identify their progeny. Some markers, for example fluorescent lipophilic dyes that stain the cell membrane, or cytoplasmic dyes, will become diluted as the cell divides. These dyes are useful for revealing the proliferative history of a cell because the intensity of the dye will be related to the number of divisions that relate it to the original labelled cell. However, the progeny can only be tracked until the marker becomes diluted to undetectable levels and so this approach has only limited usefulness for lineage marking.

A second approach is to use genetic markers which will be stably inherited, allowing all the cells progeny to be tracked over the long term. There are several naturally occurring genetic variations that might be exploited for lineage marking studies. Y-chromosomes distinguish male (XY or XYY) keratinocytes from female keratinocytes. A lack of steroid sulphatase expression distinguishes keratinocytes derived from patients with recessive X linked ichthyosis from keratinocytes derived from unaffected donors. Similarly, different individuals express different HLA class 1 epitopes. However, none of these potential markers are easy to detect and the marked populations cannot be reliably distinguished from the unmarked cells (Rytina, 1996).

Alternatively, markers can be delivered to the target cell and integrated into its genome using replication incompetent retroviral vectors (Price, 1987). Retroviruses were first successfully used to lineage mark cultured keratinocytes with lacZ as long ago as 1988 (Watt, 1988) and it was proposed at that time that this could provide a way to follow the fate of clonal progeny of individual cells without having to work with isolated clones. Since that time, retroviral technology has been optimised and is now widely used for transduction of human keratinocytes. LacZ has been successfully used as a lineage marker in mouse epidermis (for example, Ghazizadeh *et al.*, 1999) and in human keratinocytes (for example, Kolodka *et al.*, 1998).

1.3.10. Lineage marking can be used to test models of epidermal organisation.

Different models of epidermal organisation can be tested using lineage marking. According to the Epidermal Proliferative Unit model (Potten 1974), a single stem cell lies at the base of a column of suprabasal cells and is surrounded by transit-amplifying and committed cells (section 1.3.7: Figure 1.7). This model does not require any lateral movement of the keratinocytes, the suprabasal terminally differentiating cells being the progeny of the stem cells directly beneath them. According to the model described for human epidermis by Jensen *et al.* (1999), stem cells are clustered in discrete patches of around 40 cells, with the transit-amplifying compartment forming a continuous network around these patches (section 1.3.7: Figures 1.8 and 1.9). This differs from the EPU model in several ways. It suggests that each proliferative unit is maintained by more than one stem cell, and so clonal progeny might not be arranged in discrete units. It predicts lateral migration of cells within the basal layer, since cells must move from the stem cell clusters down into the transit-amplifying compartment. Clonal progeny would therefore occupy a much wider area than predicted by the EPU model. Some lateral migration would also be predicted within the suprabasal layers: cells appear to exit the basal layer exclusively from the integrin-dull compartment, and so some of them must move laterally to lie over the integrin-bright compartment.

Thus, the EPU model predicts that a lineage marked stem cell will give rise discrete narrow column of labelled progeny, whilst the model of Jensen *et al.* predicts that clonal progeny will be arranged over a wider area, in which marked cells would be interspersed with unmarked cells within both the basal layer the suprabasal layers. The predictions arising from the EPU model are supported by lineage experiments in mice either within epidermis that has been reconstituted from grafts of cultured mouse keratinocytes (Mackenzie, 1997) or by homologous recombination, with the lacZ gene (Byrne and Fuchs, 1993). As discussed above (section 1.3.7), human epidermis may not be organised in the same way as mouse epidermis. It is not feasible to lineage mark

human epidermis *in vivo*. However, sun exposed skin acquires frequent 53 mutations, which are harmless and easily detectable, and so can be exploited as an *in vivo* lineage marker. Clones of p53 mutant cells have provided evidence that basal cells migrate laterally along the basement membrane before entering the suprabasal layer (Jonason *et al.*, 1996; Ren *et al.*, 1997; Jensen *et al.*, 1999). Whether such patterns of migration occur in the absence of p53 mutation is not known. Lineage marking experiments based on grafted human keratinocytes (Kolodka *et al.*, 1998) appear to indicate that proliferative units are wider and less well defined than those described above for mouse keratinocytes (Mackenzie, 1997). However, it is not clear to what extent these grafts accurately represent a steady state epidermis *in vivo*.

1.4 NOTCH SIGNALLING

1.4.1. Notch signalling regulates the patterning of cell fates.

In many developing tissues, stocks of stem cells need to be retained at least until they have generated enough differentiated cells to complete the tissue. This depends on signalling between cells so that each cell knows what its neighbour is doing. Notch signalling is a mechanism for controlling cell fate through such local intercellular interactions.

Notch was first described in *Drosophila* as a mutation resulting in notches at the wing margin (Moohr, 1919). Stronger lethal loss-of-function mutations were discovered by Poulson (Poulson, 1937). The most obvious defect in Notch mutant flies is that they have a huge excess of neural cells, but abnormalities can also be found in many other tissues, including examples of tissues derived from all three germ layers (Hartenstein *et al.*, 1992). This multitude of defects led Poulson to describe the Notch mutant as “a kind of hopeless monster”. As well as being used many different tissues of the *Drosophila* embryo, Notch signalling has also been conserved through evolution, being found in organisms ranging from sea urchins to humans. It is now clear that Notch is widely used as a tool for regulating differentiation decisions through local cell interactions.

1.4.2. Notch pathway

The Notch gene encodes a single pass transmembrane receptor that becomes activated by transmembrane ligands Delta and Serrate (also called Jagged in vertebrates) on neighbouring cells (Artavanis-Tsakonas *et al.*, 1999). Upon activation, Notch undergoes a set of proteolytic cleavages, ultimately releasing an intracellular fragment called NotchIC (Schroeter *et al.*, 1998). The Notch signalling pathway is unusual in that it involves no intermediate steps or cascades of secondary messengers: Notch IC moves directly to the nucleus where, by interaction with the DNA binding protein, Suppressor of Hairless (SuH: also called RBPJk in vertebrates) it activates transcription of target genes (Figure 1.11). (Tamura *et al.*, 1995; Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). The target genes vary according to cell type and context. Common vertebrate targets are the bHLH transcription factors of the Hairy and Enhancer of Split (HES) family (Fisher and Caudy, 1998). There is some evidence that Notch can signal independently of SuH in certain situations (Shawber *et al.*, 1996b; Matsuno *et al.*, 1997; Wang *et al.*, 1997; Ordentlich *et al.*, 1998). However in most cases it is clear that SuH is the major activator of Notch signalling.

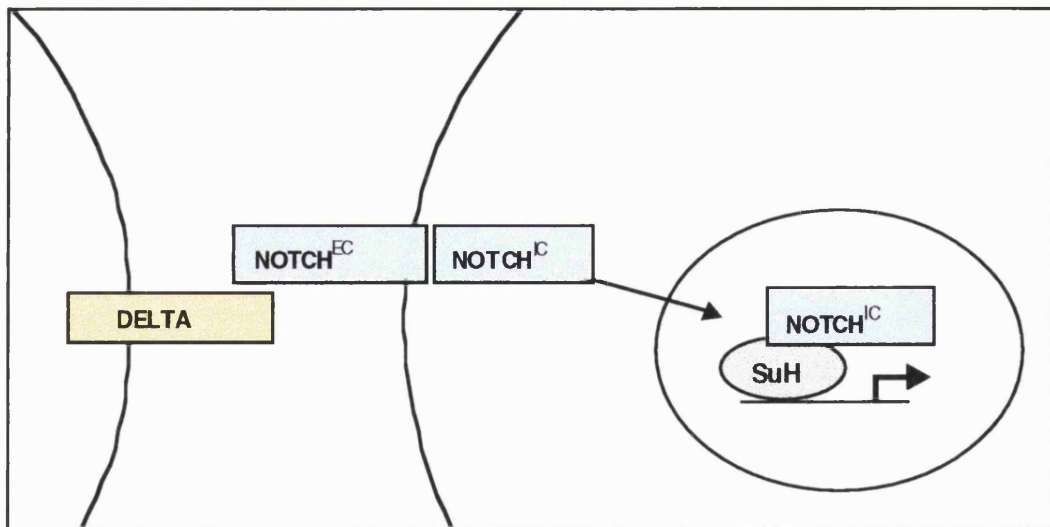
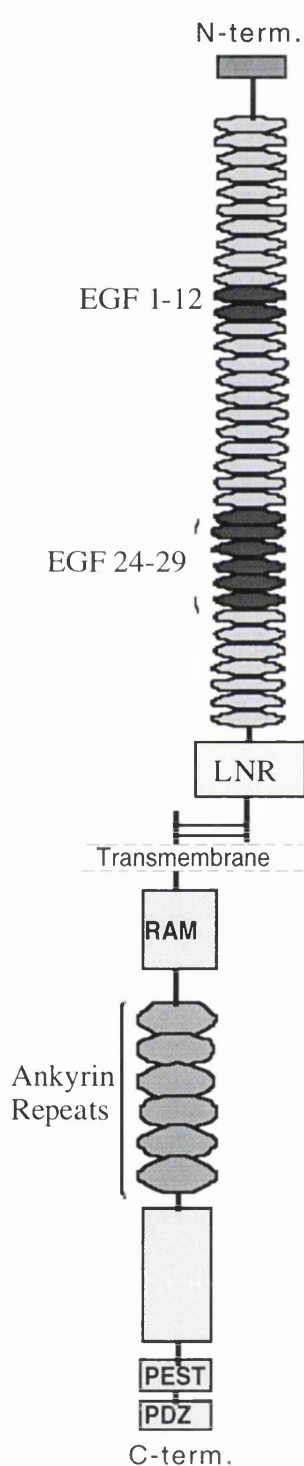


Figure 1.11: The Notch signalling pathway

1.4.3. Structure of Notch and its ligands

Structure-function analysis has mainly focused on *Drosophila* Notch and its ligands. However, all of the structural elements described below can be recognised in their homologues in other species, including humans.

Figure 1.12 Notch



Notch

Notch is a 300kd single pass transmembrane receptor (fig 1.12). Notch is cleaved in the trans-Golgi network by a furin-like convertase and appears on the cell surface as a heterodimer (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). The extracellular domain contains 36 tandem EGF like repeats. EGF repeats 11 and 12 are necessary and sufficient to physically bind ligand in trans (Rebay *et al.*, 1991; Lieber *et al.*, 1992) whilst a region at around EGF repeats 24-29 mediates inhibitory interactions with ligand in cis, although it is not yet clear whether this region directly binds ligand (section 1.4.5) (De Celis and Bray, 2000). Close to the transmembrane domain lie three cysteine-containing repeats of unknown function termed the LNR (Lin12 Notch Repeats: Lin12 is worm Notch homologue).

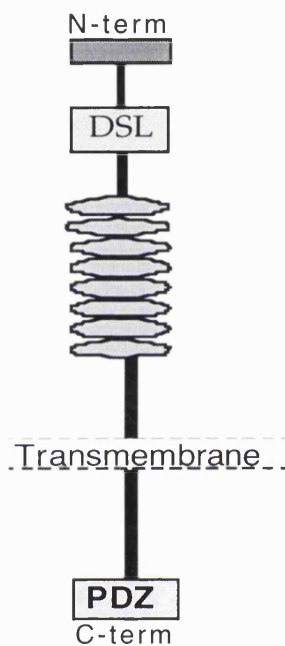
The intracellular domain contains a region called the RAM domain that binds SuH. Binding of SuH is facilitated by an adjacent region containing six tandem ankyrin repeats (motifs associated with protein-protein interactions). Intracellular modulators of Notch activity such as Numb and Dishevelled interact with a region lying just C-terminal to the ankyrin repeats. The intracellular terminus is the least conserved region amongst the Notch family members, although they do all share a PEST sequence (proline-glutamate-serine-threonine rich region), a PDZ protein interaction domain, and a nuclear localisation signal.

Four mammalian Notch receptors have been identified which share an overall identity of about 50% between themselves and the single *Drosophila* Notch protein:

Notch1/TAN-1 (Ellisen *et al.*, 1991; del Amo *et al.*, 1993), Notch2 (Weinmaster *et al.*, 1992), Notch3 (Lardelli *et al.*, 1994), and Notch4/int-3 (Uyttendaele *et al.*, 1996).

Delta

Delta is a 90 kDa transmembrane ligand for the Notch receptor. (Figure 1.13)



The extracellular domain of Delta bears 8 EGF-like repeats and a 45 amino acid motif termed the DSL domain from Delta Serrate Lag2 (Lag2 is a worm Notch ligand). The DSL domain can be described as a modified EGF-like repeat, lacking one of the six characteristic cysteine residues: this domain is required for function in invertebrates (Muskavitch, 1994). The intracellular domain contains a hydrophobic motif at the C-terminus, whose sequence suggests that it is recognised by PDZ domain proteins

Figure 1.13 Delta

The short cytoplasmic domain is the most mysterious region of the Notch ligands. It is known to be required for proper Notch activation, and mutants that lack most of the intracellular domain take on a dominant negative role (Henrique *et al.*, 1997).

Surprisingly, these mutants have been shown to block Notch signalling when they are expressed within the signal-receiving cell, but the molecular basis for this is unknown.

It has been proposed that the intracellular domain of Delta might mediate heterodimerisation or protein trafficking (Fleming, 1998), or that it might even mediate signalling into the cell, by analogy with the bidirectional signalling mediated by the ephrin receptor/ligand family (Bruckner and Klein, 1998). Three Delta mammalian Delta homologues have been identified: Delta1 (Bettenhausen *et al.*, 1995), Delta3, (Dunwoodie *et al.*, 1997) and Delta4 (Shutter *et al.*, 2000).

Serrate

Serrate has the same structural features as Delta, with the exception that Serrate contains an additional extracellular cysteine-rich domain of unknown function, and insertions that interrupt some EGF-like repeats. It is these structural differences that categorise a Notch ligand as a Delta or Serrate family member. Two vertebrate Serrate homologues have been identified (Lindsell *et al.*, 1995; Shawber *et al.*, 1996a).

1.4.4. Functional differences between Delta and Serrate

Both Delta and Serrate bind EGF repeats 11 and 12 on Notch, and the two ligands can perform each others function if they are interchanged in *Drosophila* (Gu *et al.*, 1995). However, they have different expression patterns, and loss of function analysis indicates that they have different functions during development, both in flies and in mammals (section 1.4.6). Deletion analysis of Notch has shown that EGF repeats 24-25 are required in different ways by the two ligands (Lawrence *et al.*, 2000). This is a region known to interact with modifiers of Notch.

1.4.5. Notch regulators

Fringe

Several factors can act cell autonomously to adjust the ability of Notch to respond to ligand. Fringe is a glycosyltransferase that can modify the extracellular domain of Notch and modulate its response to ligand (Bruckner *et al.*, 2000; Moloney *et al.*, 2000). For example in the fly wing disc, Fringe prevents Notch from being activated by Serrate without blocking activation by Delta (Fleming *et al.*, 1997; Panin *et al.*, 1997). Fringe has three vertebrate homologues: Radical, Lunatic and Maniac (Johnston *et al.*, 1997).

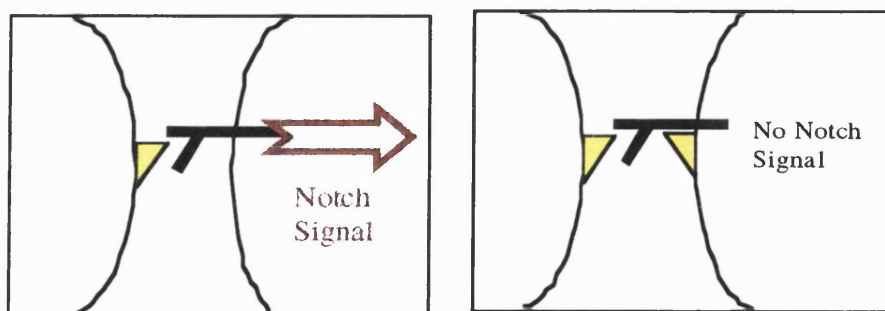
Numb

Numb is a cytoplasmic membrane-associated protein that binds to the cytoplasmic domain of Notch and antagonises its function (Guo *et al.*, 1996). Numb was first described in *Drosophila* neurogenesis, where it localises asymmetrically in mitotic precursor cells such that it only becomes inherited by one of the daughter cells. The daughter that inherits Numb will be insensitive to Notch signalling and consequently follows a different fate from its sibling (Spana and Doe, 1996). Asymmetrical cell division is also known to occur in vertebrate CNS development, and vertebrate Numb

homologues have been found to be asymmetrically localised in some mitotic neural precursors (Wakamatsu *et al.*, 1999).

Cell-autonomous action of Notch ligands

Delta and Serrate, in addition to activating Notch in neighbouring cells, can act cell autonomously to inhibit Notch activation (Micchelli *et al.*, 1997). Activation of Notch by ligand depends on the EGF repeats 11 and 12, whilst the inhibitory effects of ligand depend on a region at around EGF repeats 24-29 (de Celis and Bray, 2000). It is not yet known if this region directly binds ligand in cis, but Delta and Notch have been seen to colocalise in cis on the surface of cultured cells (Fehon *et al.*, 1990). This has led to the suggestion that Notch and Delta expressed on the surface of the same cell can interact, and that this interaction somehow renders Notch unavailable for signalling. We have, at present, no clue as to the molecular basis of this Delta-mediated inhibition of Notch signalling, and there are some exceptions, such as the retina, in which Delta can be overexpressed at high levels without inhibiting Notch (Henrique *et al.*, 1997). It is worth noting that these exceptions are always tissues in which the Notch ligands would never normally be found on two adjacent cells (section 1.3.7). The negative effect of the ligands will affect the polarity of Notch signalling: signals will pass from a cell expressing higher levels of ligand to a cell expressing lower levels but not in the opposite direction (Figure 1.14).



Delta (yellow triangle) activates
Notch in a neighbouring cell

Delta can act cell autonomously
to block Notch activation

Figure 1.14: Non-autonomous and autonomous action of Delta

1.4. 6. Functions of vertebrate Notch and its ligands

Targeted mutations and/or naturally occurring human mutations have revealed that Notch performs many functions, including neurogenesis, somitogenesis, angiogenesis, lymphoid development, and skeletal patterning.

Notch

Mice lacking Notch1 die early in development with defects in the nervous system and the somites (Swiatek *et al.*, 1994; Conlon *et al.*, 1995). Chromosomal translocations that result in a constitutively active truncated form of Notch1 have been found in certain T cell acute lymphoblastic leukemias (Ellisen *et al.*, 1991). Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is an inherited syndrome associated with point mutations in the extracellular domain of the human Notch3 receptor (Joutel *et al.*, 1996), although it is not known whether these mutations activate or inactive Notch signalling. MMTV insertional mutations that activate Notch 4 are associated with mammary tumours in mice (Gallahan and Callahan, 1987).

Delta

Mice lacking Delta1 die early in development, with defects that are similar to those seen in the Notch1 mutants (Hrabe de Angelis *et al.*, 1997). Deletion of murine Delta3 (Kusumi *et al.*, 1998), or natural mutations in its human homologue (Bulman *et al.*, 2000), cause axial skeletal defects. A new member of the Delta family, Delta 4, has recently been cloned from mouse and humans (Shutter *et al.*, 2000). Delta4 expression is restricted to the developing vasculature, and is able to signal through Notch1 and Notch4: deletion of Notch4 in the absence of Notch1 results in severe defects in angiogenic vascular remodelling early in development (Krebs *et al.*, 2000).

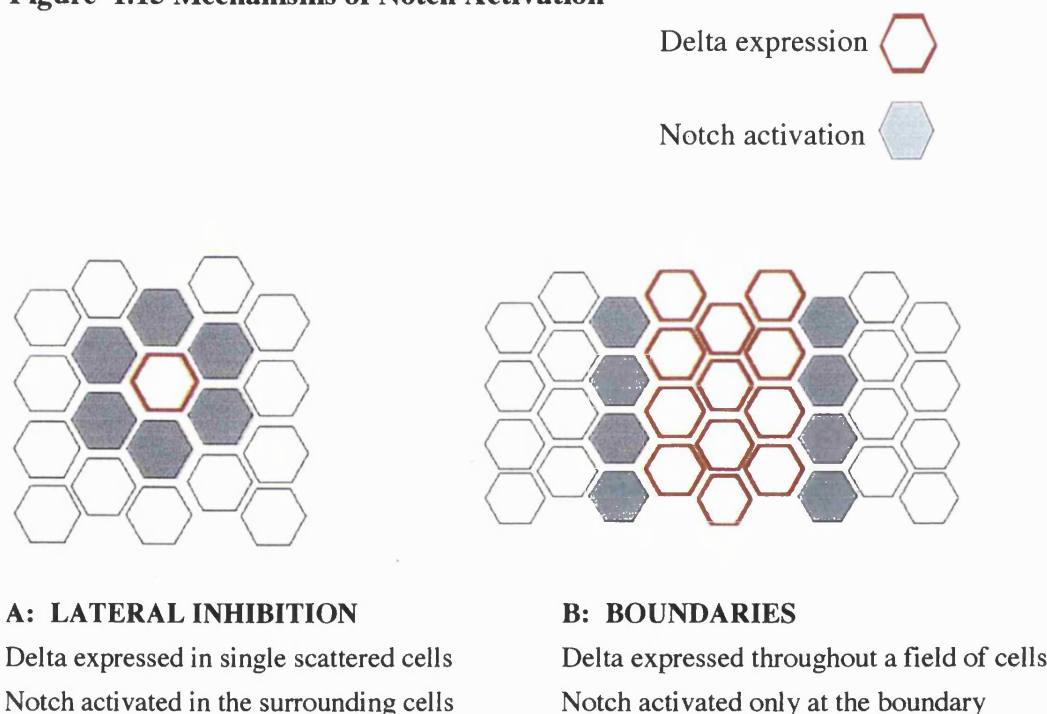
Serrate

Serrate1 null mice die from haemorrhage early during embryogenesis, exhibiting defects in remodelling of the embryonic and yolk sac vasculature (Xue *et al.*, 1999). Alagille syndrome, an autosomal dominant disorder characterised by developmental abnormalities of the heart, skeleton, muscle, liver, and eyes, is associated with mutations that are predicted to result in a truncated extracellular fragment of Serrate1. (Li *et al.*, 1997; Oda *et al.*, 1997): it is not clear whether this is a loss or function or gain of function mutation. Disruption of murine Serrate2 has revealed an essential role for this gene in limb, craniofacial, and thymic development (Jiang *et al.*, 1998).

1.4.7. How does Notch regulate cell fates?

Notch signalling is used by cells to monitor the differentiation status of their neighbours, so that they can take this information into account when making their own differentiation decisions. Various tissues have adapted this signalling pathway to their particular needs, and so the mechanistic details differ between tissues. In most cases, Notch signalling has been found to act either in binary lineage decisions mediated by lateral inhibition, or in boundary formation (Figure 1.15).

Figure 1.15 Mechanisms of Notch Activation



Lateral Inhibition

The lateral inhibition model explains how a single cell becomes selected for a particular fate from amongst a group of equivalent precursors (Figure 1.15A). Two classic examples are the selection of sensory bristles in *Drosophila*, or retinal neurons in vertebrates (Lewis, 1996). In both cases, cells that start to embark on the neural differentiation pathway express Notch ligands on their surface. These ligands activate Notch in neighbouring cells, inhibiting neurogenesis and thereby preventing them from adopting the same fate. In addition to regulating differentiation Notch downregulates expression of its own ligands. Initial stochastic differences in ligand expression between neighbouring cells will therefore become amplified and stabilised by a

negative-feedback loop. This results in the characteristic “salt and pepper” expression pattern of Delta in single scattered cells that is seen in several neurogenic tissues.

Boundary formation

During boundary formation, for example in *Drosophila* imaginal discs or the vertebrate somites, Notch ligands are expressed uniformly throughout fields, or clusters, of cells (Figure 1.15B). In this case ligand expression no longer responds to negative feedback; rather positive feedback may operate to stabilise the uniform expression (Hrabe de Angelis *et al.*, 1997). Activation of the Notch pathway occurs only at the outer borders of the Delta-expressing clusters: this drives the border cells to follow a specific differentiation program. It is not clear in all cases why Notch does not become activated within the cluster of ligand-expressing cells, but one explanation is that Serrate and Delta themselves can act autonomously to protect cells from Notch activation (see section 1.4. 5). Coexpression of Fringe can also prevent Notch from being activated by Serrate, but, at least in the *Drosophila* wing disc, it does not protect against activation of Notch by Delta (Panin *et al.*, 1997).

1.4.8. Regulation of differentiation, proliferation and apoptosis.

Notch signalling is widely used for regulating differentiation decisions. In many cases it acts to maintain stem cell populations by inhibiting differentiation, but in other cases Notch actively promotes differentiation along particular lineages (Morrison *et al.*, 2000). Notch activation can also inhibit apoptosis in murine thymomas (Deftos *et al.*, 1998) and erythomas (Shelly *et al.*, 1999) and this may be the basis of the oncogenic effect of the activating Notch mutations that are associated with lymphomas (Pear *et al.*, 1996). Notch mutations affect proliferation in the fly wing and leg disc, but this is most likely an indirect effect because it is not restricted to mutant clones (Go *et al.*, 1998; de Celis *et al.*, 1998).

1.4.9. Regulation of adhesion and cytoskeletal reorganisation

There is some indirect evidence that Delta can influence cell-cell adhesion. Delta function is required to prevent intermingling between the dorsal and ventral compartments of the fly wing disc: clones of cells lacking Delta will transgress the boundary. (Micchelli and Blair, 1999). Similarly, Fringe affects the ability of cells to violate the dorso-ventral cell boundary. This is independent of effects on Notch activation: Fringe has the same influence even within fields of ubiquitous activated Notch (Rauskolb and Irvine, 1999). In vertebrates, the Delta/Notch pathway is required for maintaining boundaries between somites. It is not required for establishing the

primary metameric pattern or for differentiation but rather it is required for precise positioning of segment boundaries: in mice with targeted deletion of Delta, the boundaries lose definition (Hrabe de Angelis *et al.*, 1997).

Delta can also influence cytoskeletal remodelling. Delta and Notch regulate the outgrowth of neurites and axon migration, and these effects are independent of the influence of Notch signalling on neural differentiation (Giniger, 1998; Franklin *et al.*, 1999; Hassan *et al.*, 2000). The morphogenic movements that drive dorsal closure (changes in cell shape and motility) are also dependent on Notch but independent of the downstream components of the conventional Notch signalling pathway (Zecchini *et al.*, 1999).

1.4.10. Notch and skin

Notch, its ligands and components of the Notch pathway are all expressed in developing vertebrate epidermis, in patterns that vary with the terminal differentiation state of the keratinocytes (see table 1.1). Notch and its ligands become upregulated in squamous cervical tumours, suggesting that Notch might regulate proliferation of human keratinocytes (Gray *et al.*, 1999).

	Location	Reference
Delta1	Basal layer	(Lindsell <i>et al.</i> , 1996; Beckers <i>et al.</i> , 1999)
Delta3	not detected	(Dunwoodie <i>et al.</i> , 1997)
Delta4	not detected	(Shutter <i>et al.</i> , 2000)
Serrate1	Suprabasal layers	(Lindsell <i>et al.</i> , 1995)
Serrate2	Basal layer	(Shawber <i>et al.</i> , 1996a; Luo <i>et al.</i> , 1997)
Notch1	All layers	(Weinmaster <i>et al.</i> , 1992)
Notch 2	not detected	(Weinmaster <i>et al.</i> , 1992)
Notch 3	no published data	
Notch 4	no published data	

Table 1.1 Expression of Notch and its ligands in epidermis



CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1. General Cell Culture Solutions

The Central Cell Services of Imperial Cancer Research Fund provided sterile distilled deionised water (dH₂O) and solutions that are indicated by 'ICRF'. All reagents used were of tissue culture grade and kept sterile.

Phosphate buffered saline (PBS, ICRF)

8g NaCl, 0.25g KCl, 1.43g Na₂HPO₄ and 0.25g KH₂PO₄ were dissolved in 1l dH₂O, the pH was adjusted to 7.2 and the solution was autoclaved. PBSABC was PBS supplemented with 1mM CaCl₂ (B) and 1mM MgCl₂ (C).

Tris buffered saline (TS)

10x stock solution was prepared by dissolving 24.2g Trizma base and 80g NaCl in 1l dH₂O. The pH was adjusted to 7.6 and the solution was autoclaved.

EDTA solution (versene, ICRF)

8g NaCl, 0.2g KCl, 1.15g Na₂HPO₄, 0.2g KH₂PO₄ and 0.2g ethyldiaminetetraacetic acid, disodium salt (EDTA) and 1.5ml 1% (w/v) phenol red solution were dissolved in 1l dH₂O, the pH was adjusted to 7.2 and the solution was autoclaved.

Trypsin solution (ICRF)

8g NaCl, 0.1g Na₂HPO₄, 1g D-glucose, 3g Trizma Base, 2ml 19% (w/v) KCl solution and 1.5ml of 1% phenol red solution were dissolved in 200ml dH₂O, the pH was adjusted to 7.7 and 0.06g penicillin and 0.1g streptomycin (Gibco BRL) were added. 2.5g pig trypsin (Difco, 1:250) was dissolved in 200ml dH₂O; air was bubbled through the solution until the trypsin dissolved. The trypsin solution was added to the Tris-buffered saline, made up to 1l with dH₂O, sterilised by filtration through 0.22µm filter (Millipore) and stored at -20°C.

Mitomycin C stock solution

Mitomycin C is an inhibitor of DNA synthesis and nuclear division (Tomasz *et al.*, 1987). It is used to metabolically inactivate J2-3T3 cells for the keratinocyte cultures. 4mg mitomycin C powder (Sigma) was dissolved in 10ml PBS. The stock solution (0.4 mg/ml) was sterilised by filtration through a 0.22µm filter, aliquoted and stored at -20°C. In the treatment of J2-3T3 cells, mitomycin C solution was added to the cell culture medium at a final concentration of 4 µg/ml.

Puromycin stock solution

100mg puromycin powder (Sigma) was dissolved in 50ml PBS. The stock solution (2 mg/ml) was sterilised by filtration through a 0.22µm filter, aliquoted and stored at -20°C.

2.1.2. Cultured Cell Types

Human epidermal keratinocytes were isolated from neonatal foreskins, grown and serially passaged as described below. J2-3T3 cells were used as feeder cells for supporting keratinocyte growth. Puromycin resistant J2-3T3 cells (J2-puro) were used as feeder cells for retrovirally infected human keratinocytes. Ecotropic retroviral packaging cells, GP + E, and amphotropic packaging cells, AM12, were used to generate high titre retroviruses for infecting human keratinocytes. HeLa cervical carcinoma cells were used to determine the titres of retroviral producer cells. All cell lines were cultured on plastic dishes or flasks of tissue culture grade (Becton-Dickinson or Nunc) in a humidified incubator at 37°C with 5% CO₂. Media or any solutions added to cells were first warmed to 37°C. All cell lines were confirmed by the ICRF Cell Production Unit as being negative for mycoplasma infection.

2.1.3. J2-3T3 Cells and J2-puro Cells*J2-3T3 and J2-puro culture medium (E4 + DCS)*

J2-3T3 cells were cultured in Dulbecco's modification of Eagles' medium (DMEM) (E4, ICRF) supplemented with 10% (v/v) donor calf serum (DCS, Gibco BRL). J2-3T3 cells transfected with the puromycin resistance gene (J2-puro) were cultured in medium containing 2.5 µg/ml puromycin.

J2-3T3 and J2-puro cell cultures

Clone J2 of 3T3 Swiss mouse embryo fibroblasts is a clone selected for its ability to support keratinocyte growth (Rheinwald and Green, 1975; Watt, 1998). When J2 cells approached confluence they were harvested by rinsing with versene and incubating at

37°C in trypsin diluted 1:5 in versene for 5 minutes. The trypsin was then inactivated by dilution in serum-containing culture medium and the cells were replated at a dilution of 1:10 or 1:20. J2-3T3 cells were maintained in culture for no more than 2 months before discarding and replacing with a freshly thawed vial of low-passage cells, because when J2-3T3 are maintained for many months in culture they tend to become transformed.

Freezing and thawing of J2-3T3 cells

Cells were harvested as described above. The cell pellet was resuspended gently in 3ml DCS containing 10% (v/v) sterile dimethyl sulphoxide (Gibco BRL). 1ml cell suspension was frozen in each cryotube (Nunc) in an insulated box at -70°C overnight and the transferred to liquid nitrogen for long term storage. Cells were thawed by transferring the cryotube of cells from liquid nitrogen directly to a water bath at 37°C. As soon as the cell suspension was thawed, it was added to 10ml medium and centrifuged at 1000rpm for 4 minutes. The recovered cells were plated onto a 75cm² flask.

2.1.4. Epidermal Keratinocytes

Keratinocyte culture medium (FAD+ FCS + HICE)

FAD powder (F12 + adenine + DMEM: Imperial Labs) was supplemented with 3.07 g/l NaHCO₃, 100 IU/l penicillin and 100 µg/l streptomycin. FAD medium (ICRF) was bubbled with CO₂ until the pH dropped below 7.0, then sterilised by filtration through a 0.22µm filter. Medium was stored at 4°C until use.

Stock solutions of additives were kindly prepared by Simon Broad (Keratinocyte Lab, ICRF). 10⁻⁵ M cholera enterotoxin (ICN) was stored at 4°C. Hydrocortisone (Calbiochem) was dissolved in 95% ethanol at 5 mg/ml and stored at -20°C. 100 mg/ml recombinant human epidermal growth factor (Austral Biologicals) was prepared by first dissolving in 1/100 volume 0.1M acetic acid (BDH) before adding to FAD medium containing 10% (v/v) batch-tested foetal calf serum (FCS, Imperial Labs.) and stored at -20°C. The additives were combined into a 1000x 'cocktail' (HCE): 1ml hydrocortisone, 100µl cholera enterotoxin and 1ml epidermal growth factor stock solutions were added to 7.9ml FAD medium with 10% FCS and stored at -20°C. The final concentrations in the medium were 10⁻¹⁰ M cholera enterotoxin, 0.5 µg/ml hydrocortisone and 10 µg/ml epidermal growth factor. 1000x insulin stock solution (5 mg/ml in 5mM HCl, Sigma) was stored at -20°C. The final concentration in the medium was 5µg/ml insulin. Complete keratinocyte medium (FAD + FCS + HICE) was prepared by adding 10% (v/v) FCS, 'cocktail' and insulin solutions to the FAD

medium prior to use (Watt, 1998). For keratinocytes infected with retrovirus, puromycin was added to the culture medium at 1µg/ml (Zhu and Watt, 1996). Complete medium was stored at 4°C for up to 10 days.

In some experiments keratinocytes were cultured in medium with a reduced concentration of calcium ions in order to prevent assembly of adherens and desmosomal junctions (Hodivala and Watt, 1994). Low calcium FAD medium was prepared in the same way as normal FAD except that calcium salts were omitted from the DMEM and F12 formulations. The FCS used to supplement low calcium FAD was pre-treated with Chelex to remove calcium ions, as follows: Chelex 100 resin (100-200 mesh, sodium form; BioRad) was swollen at 40g/l in distilled water, titrated to pH 7.4 with NaOH and filtered through Whatman No.1 paper. 20g swollen resin was added to 50ml FCS and the mixture was stirred at room temperature for 3 hours. The Chelex was then removed by filtration through Whatman No.1 paper and the FCS was sterilised through a 0.22µm filter. Sterile chelated FCS was stored at -20°C. Complete low-calcium keratinocyte medium was prepared by adding 10% (v/v) chelated FCS to low calcium FAD, supplementing with HICE cocktail in the same concentration as described for standard FAD medium, and adding 1% of complete standard keratinocyte medium. The calcium concentration of complete low calcium FAD medium is 0.1mM free calcium ions.

Preparation of J2-3T3 cells as feeder cells

The culture of human keratinocytes is supported by co-cultivation with mitotically inactivated J2-3T3 cells, which are referred to as feeder cells (Rheinwald and Green, 1975). Feeder cells were incubated with 0.4 µg/ml mitomycin C for 2-3h at 37°C in order to inhibit mitosis. The treated feeders were then harvested and plated at 1/3 confluent density. Keratinocytes were added within 24 hours of preparing the feeder layer. J2-puro cells were used as feeders for keratinocytes infected with retroviruses.

Isolation of primary human keratinocytes

Neonatal foreskins were kindly provided by Dr Cohen of the Fitzroy Clinic, London. Isolation of primary keratinocytes was carried out as soon as possible after circumcision (Watt, 1998). Under sterile conditions, using a pair of forceps and curved scissors, a piece of foreskin was trimmed of dermal and fatty tissues. The foreskin was cut into pieces of about 5mm² and transferred into a Wheaton Cellstir (Jencons) containing 5ml trypsin and 5ml versene and stirred over a magnetic stirrer at 37°C. Dissociated cells were collected every 30 minutes and added to 5ml keratinocyte culture medium. The number of cells obtained was estimated using a haemocytometer. Dissociation of cells from the tissue was continued with addition of fresh versene and trypsin solution. This procedure was repeated 2 to 3 times before the number of cells obtained started to

decrease. The yield from a neonatal foreskin was usually between $1-5 \times 10^7$ cells. Feeder cells had been plated onto 25cm^2 flasks in readiness. Isolated cells were pooled, pelleted and plated at a density of 10^5 cells per 25cm^2 flask. Cells were cultured until just confluent. One flask of cells was tested for mycoplasma infection by the ICRF Cell Production Unit, while the remaining cells were harvested and frozen at 10^6 cells per ml as for J2-3T3 cells.

Serial culture of human keratinocytes

Frozen keratinocytes (passage 1-4) were thawed as described for J2-3T3 cells. The strains used were named z, n, km, kq, kp. Each strain corresponds to keratinocytes isolated from a single individual. The usual number of cells seeded in $1 \times 75\text{cm}^2$ was 2×10^5 actively growing cells; 5×10^5 cells from a frozen cryotube were plated in a 75cm^2 flask to allow for loss of viability resulting from freezing and thawing. Fresh medium was given to keratinocytes every 2 days. A day prior to any experimental manipulation, keratinocytes were fed with fresh medium.

Keratinocytes were passaged just before they reached confluence. The cultures were rinsed once with versene and then incubated with versene for 5-10 minutes at 37°C . This treatment caused any remaining feeder cells to detach. Keratinocytes would round up but would not detach from the flask. The versene solution was discarded and the remaining keratinocytes were incubated in 5ml trypsin/versene solution (1 part trypsin and 4 parts versene) at 37°C for about 10 minutes, until all keratinocytes had detached from the flask. 5ml medium was added to the suspension and the number of cells was counted using a haemocytometer. The cells were pelleted and resuspended in medium as described and plated onto flasks with feeder cells; 10^5 cells were added to a 25cm^2 flask or 2×10^5 cells were added to a 75cm^2 flask. Keratinocytes were discarded after 6 passages.

In some experiments, confluent keratinocyte cultures were removed as intact sheets. The cultures were incubated in 2.5 mg/ml Dispase II (Boehringer Mannheim) in DMEM buffered with 20mM Hepes at 37°C for 30 minutes. Using forceps, the sheet of cultured cells was gently peeled away from the tissue culture dish and fixed immediately in 4% paraformaldehyde.

2.1.5. Retroviral Producer Cells

Retroviral producer cell culture

Helper free ecotropic packaging cells, GP + E-86 (abbreviated as GP + E), and amphotropic packaging cells, GP + *env*AM12 (abbreviated as AM12), were obtained

from Dr P. Patel of the Institute of Cancer Research, London. These cells were designed in conjunction with the pBabe puro vector to reduce the risk of generation of wild type Mo MuLVirus via homologous recombination events (Markowitz *et al.*, 1988b; Markowitz *et al.*, 1988a). The packaging cells were cultured in E4 medium supplemented with 10% (v/v) FCS (Sigma). For transfected or infected retroviral producer cells the culture medium was supplemented with 2.5 $\mu\text{g/ml}$ puromycin. Harvesting, freezing and thawing retroviral producer cells was carried out in the same way as described for J2-3T3 cells.

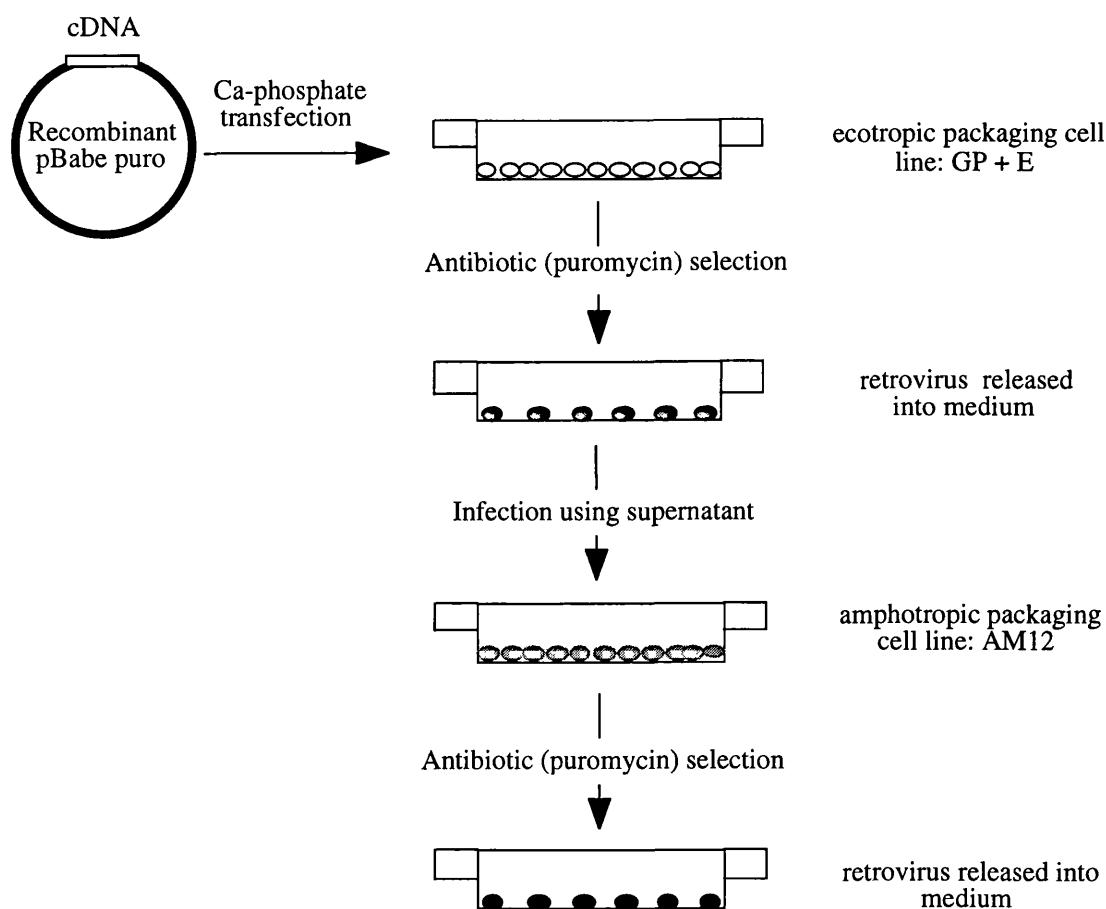


Figure 2.1. Procedures for generating retroviral producer cells

Adapted from a figure drawn by A. Zhu

Transfection of GP + E cells.

Ecotropic virus producer cells lines were generated by transfecting the packaging cells with retroviral vector (Figure 2.1). The ecotropic GP + E cells were seeded onto a 100mm dish at $2-5 \times 10^5$ the day before transfection. In a round bottom plastic tube (6ml, 12 x 75mm), 20µg DNA was made up to 160µl with 1mM Tris-HCl, pH7.6, 0.1mM EDTA, mixed thoroughly but gently on a whirring mixer and incubated for 5 minutes at 37°C. 160µl of 0.5M CaCl₂ and 0.1M HEPES pH6.7 solution pre-warmed to 37°C was added, mixed thoroughly and incubated for 10 minutes at 37°C. 320µl of 0.28M NaCl, 0.75mM Na₂HPO₄, 0.75mM NaH₂PO₄, 0.05M HEPES, pH6.7 solution was pre-warmed to 38°C and added drop-wise while gently vortexing the contents of the tube. This was incubated for 15 minutes at 37°C and then added to the cells whilst gently swirling the medium. The cells were left in the incubator at 37°C for about 16h and then inspected under a microscope for presence of a suspension of fine, black precipitate in the medium. The medium was discarded and a sterile solution of 25mM Tris-HCl, pH7.4, 137mM NaCl, 5mM KCl, 0.7mM CaCl₂ and 0.6mM Na₂HPO₄ was added to the cells and incubated for 10 minutes at 37°C. This was repeated twice and then the medium was replaced. Puromycin (2.5 µg/ml) was added to the medium 48h after transfection. The selection medium was changed every 2 days until cells reached confluence. At this point, the selection medium was replaced with half the volume of normal culture medium (E4 + FCS), and active retroviral particles were collected in the supernatant for 24 hours. Floating cells were removed from the virus-containing medium by filtration through a 0.22µm filter. The filtered supernatant was used directly to infect amphotropic AM12 cells.

Infection of AM12 cells

Producer lines that are generated by retroviral infection have higher viral titres than those generated by transfection (Morgenstern and Land, 1990), hence virus released into the culture medium by confluent GP + E cells was used to infect the amphotropic packaging line AM12 (Markowitz *et al.*, 1988a) (Figure 2.1). AM12 cells were seeded on 100mm dishes at $1-2 \times 10^5$ density the day before infection. 2.5ml infection medium (virus-containing medium collected from the GP + E cells and supplemented with 8 µg/ml polybrene; Sigma) was added to the AM12 cells. After 3h infection at 37°C the infection medium was replaced with fresh culture medium (E4 + FCS). The selection medium containing 2.5 µg/ml puromycin was applied 48h later and changed every 2 days until cells reached confluence.

The viral titres of the polyclonal AM12 populations were assessed by infection of HeLa cells as described below. In cases where the titre was above 10^5 cfu/ml and the encoded protein was expressed at high levels in the majority of the cells, then the polyclonal

AM12 population was used for infection of keratinocytes. Otherwise, the population of AM12 was improved by selecting those cells with the highest levels of expression. The level of expression of the virally encoded gene correlates with the titre of the virus produced by AM12 cells (Levy *et al.*, 1998). For virus encoding the intrinsically fluorescent marker protein EGFP (Enhanced Green Fluorescent Protein), EGFP-negative or EGFP-dull cells were eliminated by FACS. Some viral constructs contained genes followed by an IRES (Internal Ribosomal Entry Site)-EGFP tag. These were also selected by FACS on the basis of EGFP expression. In cases when the encoded genes might be harmful (e.g. potential oncogenes), the FACS selection step was carried out on the ecotropic GPE producer cells to avoid having to remove live amphotropic virus from the virus containment facilities (see Figure 2.2). In each case, the virus produced by highly expressing GPE populations produced highly expressing populations of AM12 that did not need any further selection.

Ring cloning of AM12 cells

Packaging cells that did not contain EGFP were selected by ring cloning. The polyclonal population of infected AM12 cells were seeded on 100mm dishes at densities ranging from 100 to 2000 cells /dish and fed every third day until drug resistant colonies were visible without the aid of a microscope (normally 5-6 days after plating). At least 30 widely separated colonies, each containing at least 100 cells, were chosen and marked on the bottom of the dishes. The culture medium was then removed and glass cloning rings (5mm diameter, 8mm height) dipped into autoclaved silicon grease were placed over marked colonies. 50µl trypsin/ versene solution was added into each ring to release cells from the dish. Cloned cells were transferred to 24 well plates and eventually expanded onto 100mm dishes. Expression of exogenous genes in cloned AM12 cells were examined by indirect immunofluorescence staining. The conditioned medium from 2-day post-confluent, positive AM12 clones was collected, filtered and subjected to retroviral titration and wild-type virus detection.

Cultivation of HeLa cells for titration

The titre of retroviral stock depends might be expected to depend upon the target cell type. HeLa cells are human epithelial carcinoma cells and were chosen for the titration because of their similarity to human keratinocytes. HeLa cells were obtained from the ICRF Cell Production Unit. The culture medium comprised DMEM (E4, ICRF) supplemented with 10% (v/v) FCS (Imperial Labs.). For HeLa cells that had been exposed to retrovirus cell culture medium was supplemented with 0.4 µg/ml of puromycin. HeLa cells were harvested by rinsing in trypsin/versene solution and

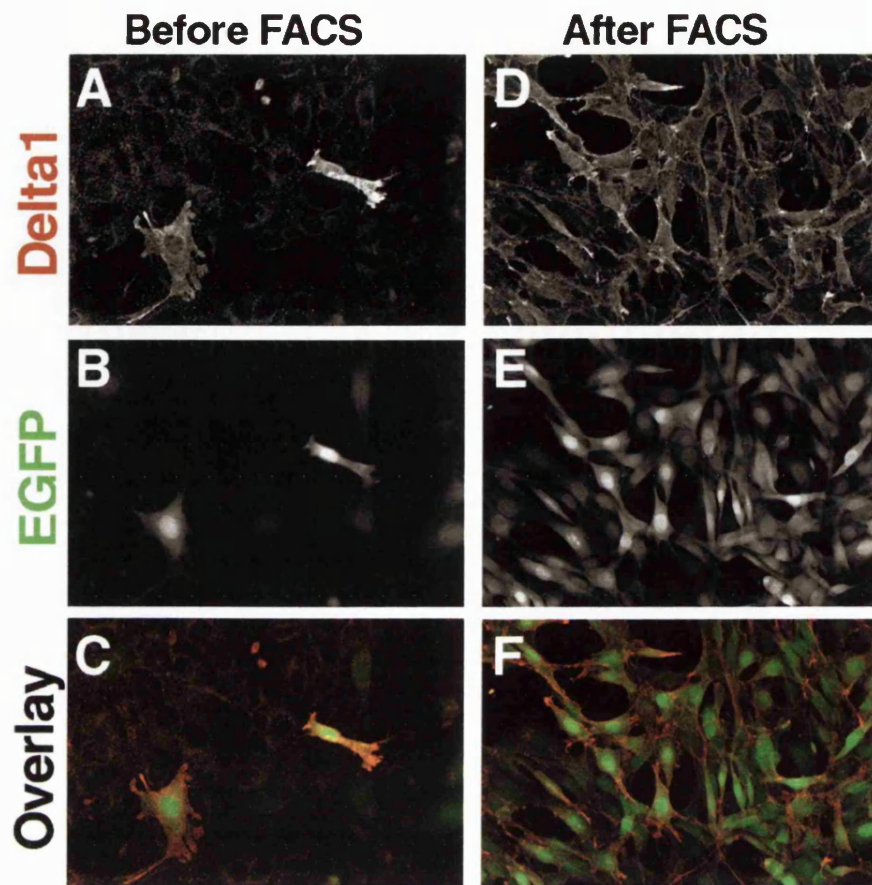


Figure 2.2: FACS selection of packaging cells containing EGFP-tagged constructs

GPE packaging cells were transfected with pBabepuro-Delta1-EGFP and subjected to puromycin selection for 7 days. Many cells were puromycin-resistant but did not express high levels of Delta. (A) High levels of EGFP expression correlated with high levels of Delta1 expression (B,C) The EGFP-negative population were eliminated by FACS (D-F)

incubating in trypsin for 5 minutes at 37°C. For routine passaging, cells were usually split at a dilution of 1:20.

Titration of retroviral producer cells

HeLa cells were seeded on 100mm dishes at 4×10^5 density the day before titration. 50µl filtered supernatant of AM12 producer cells was added to 9.95ml fresh culture medium containing 8 µg/ml polybrene. The resulting infection solution was used to infect HeLa cells at 37°C. The infection medium was discarded 4h later and fresh culture medium was added to the cells and incubated overnight at 37°C. Infected HeLa cells were then split with a series of dilutions (1:5, 1:10, 1:50, 1:100 and 1:500) and grown in the selection medium containing 0.4 µg/ml puromycin for a week. Surviving colonies were fixed in 3.7% formaldehyde (BDH) and stained with 1% methylene blue (Sigma). Blue colonies were counted and the number of counted colonies was converted to the retroviral titre using the following formula: retroviral titre (colony forming unit/ml) = 200 x number of the colonies x dilution factor.

Expanding retroviral producer clones

Amphotropic producer clones were selected based on their expression levels, as examined by indirect immunofluorescence or flow cytometry, and on their retroviral titres. In every clone examined high expression levels correlated with high retroviral titres. Clones with the highest titres and expression levels were selected, expanded and used to infect primary human epidermal keratinocytes.

Infection of human keratinocytes

In some cases retroviral infection was carried out by plating human keratinocytes (10^5 per 100mm dish) onto 70% confluent AM12 producer cells that had been pre-treated with 4 µg/ml mitomycin C. After 2 days, 1 µg/ml puromycin was added to the medium. The producer cells were removed by versene treatment after 3-4 days and replaced with puromycin resistant J2-3T3 cells, J2-puro.

Retroviral infection of keratinocytes or J2-3T3 cells with some constructs was carried out using virus-containing supernatant: the procedure was the same as for infection of AM12 (described above) except that virus was collected from AM12 producer cells rather than GPE producer cells. When keratinocytes were to be doubly infected with a Delta retrovirus and pBabe puro-EGFP, they were grown for at least 7 days in puromycin after infection with Delta before harvesting and replating on EGFP retroviral producer cells. Where necessary, any EGFP-negative keratinocytes were eliminated by FACS. Infected keratinocytes were used for experiments immediately or following 1-4 passages on J2-puro in medium supplemented with 1 µg/ml puromycin.

2.2. Analysis of Keratinocytes in Culture

2.2.1. Counting Viable Cells Using Trypan Blue

Trypan blue solution was made by dissolving 20mg trypan blue powder (Gibco BRL) in 50ml PBS. Crystals were removed using a 0.22µm filter. Cells were harvested with versene/trypsin solution. 100µl cell suspension was mixed with 100µl trypan blue solution. Cells were scored in a haemocytometer; only nucleated, small golden cells were scored as viable cells; giant golden cells and blue or dark brown cells were regarded as nonviable.

2.2.2. Setting up Growth Curves

Equal numbers of viable cells were seeded onto 35mm dishes in the presence of mitomycin C-treated feeder cells. Growth curves were obtained by harvesting triplicate dishes at 2-3 day intervals for 24 days. When the cells (in 1ml versene/trypsin solution) detached from the dishes they were transferred to counting vials with 7ml Isoton (Coulter Electronics Ltd.). The dishes were then rinsed with 2ml Isoton, which was added to the cell/Isoton, suspension in the counting vials (total volume 10ml). The cells were counted on a Coulter Counter (Coulter Electronics Ltd.).

2.2.3. Clonogenicity Assays

Equal numbers (100-1000) of viable keratinocytes were plated per 35mm dish in triplicates on top of mitomycin C-treated J2-3T3 feeder cells. After 2 weeks the cultures were washed with PBS and the cells were fixed in 3.7% formaldehyde for 5 minutes at room temperature. After further washing in PBS, the cultures were stained for 30 minutes at room temperature with rhodanile blue (Rheinwald and Green, 1975; Jones and Watt, 1993). All the visible colonies (i.e. >2 cells) were scored on each dish and colony forming efficiency was calculated as % of plated cells that formed colonies. Abortive colonies were defined as colonies that were less than 0.4mm in diameter and contained fewer than 40 cells, the majority of which were large and terminally differentiated.

2.2.4. De-epidermised Dermis (DED) Culture

Preparation of DED

The method for growing keratinocytes on DED was based on the Pruniéras model (Pruniéras *et al.*, 1983) to achieve histological differentiation as close as possible to the epidermis *in vivo*. Human breast skin was cut into 10cm² pieces and the subcutaneous fat was scraped off. The skin was heated in PBS to 56°C for 20 minutes and the epidermis was peeled off. The remaining dermis was cut into 1cm² pieces, placed into cryovials and snap frozen in liquid nitrogen. The vials were allowed to thaw at room temperature for 20 minutes and then frozen again in liquid nitrogen. This cycle of freezing and thawing was repeated 10 times in order to kill all of the cells in the dermis. After the last cycle the vials were stored at -70°C until use.

Growing keratinocytes on DEDs

The vials were thawed and the pieces of DED were placed on sterile metal grids or tissue culture inserts (Becton-Dickinson) with the denuded epithelial surface uppermost and exposed to the air. 10⁵ keratinocytes were harvested using the versene/trypsin treatment, washed twice in the culture medium and centrifuged at 1000rpm for 4 minutes. The cell pellet was resuspended in 20µl culture medium and then plated onto the denuded epithelial surface of the DEDs (Figure 2.3). Cultures were fed every 2-3 days for 1-3 weeks.

DED cultures containing EGFP cells were rinsed in PBS and analysed on a BioRad confocal microscope to detect EGFP positive cells. In some experiments the reconstituted epidermis was removed from the DED as an intact sheet using 2.5 mg/ml Dispase. Other DED cultures were kept intact and either fixed in normal buffered formalin (for paraffin sections) or snap-frozen in isopentane pre-cooled in liquid nitrogen and stored at -70°C until use (for frozen sections).

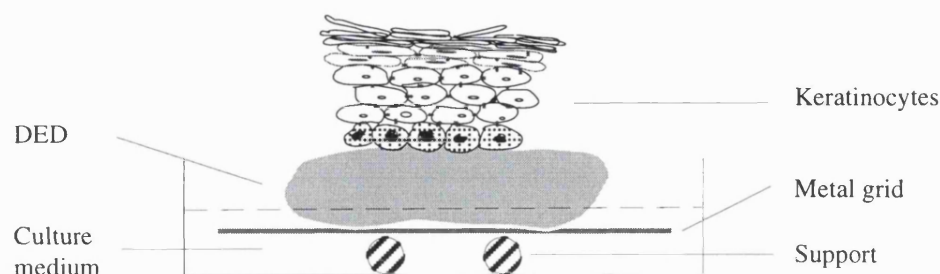


Figure 2.3. Schematic drawing of keratinocytes grown on DED

Grafting

Grafts of DED cultures were performed using 6-week-old athymic Balb/C nude mice who were anaesthetised by inhalation of Fluothane (Zeneca). A 1cm linear incision was made into the full thickness of the mouse skin. Underlying connective tissue was separated from the skin in a small area anterior to the incision using a blunt seeker and the DED was carefully placed into the sub-dermal space, keeping the keratinocytes uppermost. The incision was closed using 9mm Clay Adams clips (Becton Dickinson) and the mice were allowed to recover. The grafts were removed after killing the mice by CO₂ asphyxiation (Levy *et al.*, 1998). Qualified staff in the ICRF animal unit carried out all procedures on live animals.

2.2.5. Induction of Terminal Differentiation*Medium for keratinocyte suspension culture*

Keratinocytes were induced to undergo terminal differentiation by placing them as a single cell suspension in methylcellulose supplemented medium. 3.5g methylcellulose (Mecel, viscosity 4000 centipoises, Aldrich Chemical Co.) was autoclaved in a 400ml centrifuge tube together with a magnetic stirrer bar. 180ml FAD was heated to 60°C and added to the tube. The contents were stirred at room temperature for 30 minutes and then at 4°C overnight (Green, 1977; Adams and Watt, 1989). After addition of 20ml FCS the medium was centrifuged at 9,500rpm in a Beckman J2-21 at 4°C for 30 minutes. Methyl cellulose (1.75%, w/v) medium was aliquoted and stored at -20°C until use. Immediately before use, HICE solutions were added as for keratinocyte medium (section 2.x) and mixed thoroughly.

Preparation of polyHEMA-coated dishes

To minimise attachment of suspended keratinocytes to the tissue culture dish, bacteriological grade plastic petri dishes were used. Petri dishes were coated with polyHEMA (type NCC, cell culture grade, Hydro Medical). A 10% polyHEMA (w/v) stock solution in 95% ethanol was prepared by mixing end over end overnight at room temperature. To coat dishes, stock solution was diluted to 0.4% in ethanol/acetone (50%/50%, v/v). 5ml diluted solution was added to a 100mm dish and swirled quickly to coat the base of the dish and then removed immediately. This was repeated once. Coated dishes were left under sterile conditions to dry completely in a tissue culture hood before use.

Measuring the kinetics of induction of terminal differentiation.

Newly confluent cultures of keratinocytes were incubated briefly with versene to remove any remaining feeder cells, harvested using versene/trypsin and resuspended at

10^6 cells per ml in complete FAD medium. The cells were then added slowly to the methyl cellulose medium whilst swirling the medium, to ensure that keratinocytes were homogeneously distributed in the suspension. Keratinocytes were at a final cell density of 10^5 cells per ml. 50ml cell suspension was poured into 100mm polyHEMA-treated dishes and cultured for 4, 8, 12, 16 or 24 hours in a humidified 37°C incubator. The starting population of cells (i.e. before suspension) was recovered from the methyl cellulose medium immediately. Keratinocytes were recovered from suspension by diluting methyl cellulose medium 10 fold with ice cold versene and centrifuging in 250ml conical bottom Beckman bottles at 2000rpm for 10 minutes at 4°C. The cell pellet was resuspended gently in 15ml ice-cold PBS. To assess the extent of induction of terminal differentiation, 5×10^5 cells were fixed in 4% paraformaldehyde and permeabilised with 0.3% saponin (Sigma), then stained for involucrin, a marker of keratinocyte terminal differentiation, and analysed by flow cytometry.

2.2.6 Adhesion selection of populations of keratinocytes enriched for stem or transit cells

Populations enriched with stem cells or transit amplifying cells can be selected on the basis that keratinocyte stem cells adhere more rapidly than transit amplifying cells to type IV collagen (Jones and Watt 1993). Tissue culture dishes (Becton-Dickinson) were coated overnight at 4 °C with 10µg/ml type IV collagen (Sigma) and blocked with 0.5mg/ml heat denatured BSA (Sigma) at 37 °C for one hour. Keratinocytes were plated at low density and allowed to attach to the dishes for 20 minutes at 37 °C. Cells that adhere within this time constitute the stem cell- enriched population. Non adherent cells were removed and plated on a separate collagen coated dish. Cells within this population that had attached after 60 minutes constituted the population enriched in transit-amplifying-cells. Cells that had not attached included the terminally differentiated cells.

2.2.7 Cell motility

Subconfluent keratinocytes were harvested and plated onto dishes coated in type IV collagen at a density that allowed tracking of individual cells. Any cells that had not attached after 20 minutes were removed. The remaining stem cell-enriched population was kept humidified at 37°C with 5% CO₂ and videotaped for 22 hours. Frames were taken every 2 minutes using Olympus IMT1 or IMT2 inverted microscopes driven by Broadcast Animation Controllers (BAC 900) and fitted with monochrome CCD cameras and video recorders (Sony M370 CE and PVW-2800P, respectively). Recordings were digitised and the sequence of all frames was run on a PC. Motility was measured using a cell tracking extension (ICRF) written for IPLab (Signal

Analytics Inc., USA) and speed was calculated using a program written in Mathematica by Daniel Zhica (ICRF).

2.2.8 Assessment of cell spreading

Keratinocytes were harvested and plated onto bacteriological dishes that had been coated in 50 µg/ml type IV collagen or 25 µg/ml fibronectin or onto uncoated tissue culture plastic. The procedure for coating dishes with extracellular matrix proteins is the same as described above for collagen (section 2.2.6). The dishes were incubated at 37°C to allow the cells to attach. At various time points, cells were fixed in 4% formal saline and stained with methylene blue.

Speed of spreading

About 100 (range 95- 105) randomly selected cells from each dish were examined at high resolution by videomicroscopy and scored (blinded) according to whether they were still completely rounded or had started to spread.

Size of spread cells

A digital image was captured of each of 100 randomly selected cells from each of the dishes that had been incubated for 120 minutes. The area of each of these cells was measured using Kinetic image analysis software.

2.3 Lineage Marking Experiments

2.3.1. PKH labelling

2.5×10^6 keratinocytes were labelled in 1ml 2µM PKH26 solution (Sigma) according to the Manufacturer's instructions. The viability of labelled cells was checked by trypan blue exclusion and found to be in excess of 90%. PKH26 has 2 excitation maxima at 515nm and 551nm and an emission maximum at 576nm (Horan *et al.*, 1990), and was visualised using a Zeiss Axiophot fluorescence microscope (Carl Zeiss).

2.3.2 Culture of EGFP-labelled stem cells or transit amplifying cells within confluent sheets of uninfected keratinocytes.

EGFP labelled keratinocyte populations ($2 \times 5 \times 10^3/\text{cm}^2$) enriched in stem cells or transit amplifying cells were selected on the basis that keratinocyte stem cells adhere more rapidly than transit amplifying cells to type IV collagen (section 2.2.6). After the selection procedure, each dish contained fewer than 10^2 EGFP stem- enriched or transit-

enriched keratinocytes/cm². 10⁵ /cm² uninfected keratinocytes were added to the dishes of EGFP keratinocytes to achieve a confluent layer of cells within 24 hours.

Cells were either fixed on the dish in 4% formaldehyde after 5 days, or removed as a sheet using 2.5 µg/ml Dispase 7 days after plating. EGFP positive colonies of keratinocytes were scored using a Zeiss Axiophot fluorescence microscope (Carl Zeiss). Dispase sheet of keratinocytes were also stained for β1 integrin using Alexa 594-conjugated P5D2 and the distribution of EGFP-positive clones relative to integrin-bright patches was analysed on a Zeiss 510 upright confocal microscope.

Quantification of integrin staining was performed as follows. Using the Lookup Tables provided as part of the computer software, false colour was applied to the images by designating the pixel intensities (range: 0-255 units) different codes: blue for the top 1/3 (i.e. brightest fluorescence), red for the bottom 1/3 (i.e. dullest) and green for the remaining 1/3 (i.e. medium).

2.3.3. Delta lineage marking experiments

Second passage keratinocytes were infected with pBabe puro-Delta1 (K-DELTA), pBabe puro-DeltaT (K-DeltaT) pBabe puro-SuH^{DBM} (K-SuH^{DBM}) or empty vector (wild type keratinocytes; K-WT). Cells that underwent a second infection with EGFP are designated K-DELTA-EGFP, K-DeltaT-EGFP, K-SuH^{DBM}-EGFP or K-WT-EGFP. All Delta1 or DeltaT infected cells were immunostained with SER20 to confirm that close to 100% of infected cells were expressing high levels of Delta1. K-SuH^{DBM} infected cells were immunostained with an antibody against the HA tag to confirm that close to 100% of infected cells were expressing the tagged SuH^{DBM} construct.

Terminal differentiation of lineage marked cells

EGFP labelled cells were cocultured with either unlabelled K-WT or K-DELTA. In experiments to measure terminal differentiation, 10⁴ EGFP labelled cells were mixed with 10⁵ unlabelled cells of the same strain and passage number and seeded on 35 mm diameter tissue culture dishes. After 24 hours cultures were examined under a UV inverted microscope to check that EGFP cells were in contact with unlabelled cells. Cultures were grown for 5, 10 or 15 days. At each of these time points EGFP-positive colonies were photographed and triplicate dishes were then trypsinised, counted and fixed in 4% paraformaldehyde for 10 minutes at room temperature. In some experiments 10⁶ cells were permeabilised and stained for involucrin using SY5. For each sample, 10⁴ EGFP positive cells were analysed by FACS. Forward and side scatter gates were set to separate basal cells from terminally differentiated suprabasal cells. (Jones and Watt, 1993)

Size and morphology of lineage marked clones

Some experiments were designed to score the size and morphology of individual EGFP clones. These experiments were carried out in the same way as described in the previous section, with the following modifications. Only 200 EGFP cells were mixed with 10^5 unlabelled cells, so that marked colonies would be widely spaced. Cultures were seeded on tissue culture plastic (Becton-Dickinson) for 5, 10 and 15 days. At each time point, intact (i.e. non-trypsinised) sheets of cells were fixed with 4% paraformaldehyde at room temperature. Some sheets of cells were stained for $\beta 1$ integrins using P5D2 (Jensen *et al.*, 1999)

2.3.4. Confrontation assay

The confrontation assay was designed to test whether two populations of cells tend to freely intermingle or whether they remain in different compartments separated by a clear boundary (the design of the assay is illustrated in Figure 5.5, which shows a control assay carried out on two populations of wild type keratinocytes). One of the two populations of cells was labelled by retroviral infection with EGFP. Both the labelled and the unlabelled populations were harvested using trypsin/ versene and concentrated to 4×10^5 cells/ml by centrifugation at 1000 rpm. For each of the two cell populations, a 50 μ l droplet containing 2×10^4 cells was placed onto an area of uncoated tissue culture plastic measuring 0.21cm^2 . The two droplets were placed with a distance of 2mm between them at their closest edges. A template beneath the tissue culture plastic was used to guide the area and the position of each droplet. The same template was used for all experiments. Cells were allowed to attach for 4 hours within the separate droplets. The dishes were then flooded with culture medium, unattached cells were removed and the cells were cultured from then on under a shared pool of cultured medium to allow the two populations to migrate towards each other. In some cases the cells were cultured in low-calcium medium after the first four hours. All cultures were fed daily for 1-7 days and then fixed with 4% paraformaldehyde. In some cases intact cultures were removed from the tissue culture dish using Dispase before fixing.

2.3.5. Sorting assay

The sorting assay was designed to test whether two intermingled populations will sort out from each other (see section 5.2.4). One of the two populations of cells was labelled by retroviral infection with EGFP. Both the labelled and the unlabelled populations were harvested using trypsin/ versene and concentrated to 5×10^5 cells/ml by centrifugation at 1000 rpm. 10^5 EGFP labelled cells were mixed thoroughly with

5×10^5 unlabelled cells and plated at a total density of 1.1×10^5 cell/cm² on tissue culture plastic. The cultures were maintained in the standard way for 24 hours then fixed with 4% paraformaldehyde.

2.4 Immunological Methods

2.4.1. General Solutions

Paraformaldehyde solution

The stock solution of 10% paraformaldehyde solution was prepared by adding paraformaldehyde powder (BDH) to PBS pre-warmed to 60°C and stirring in a fume hood until the solution appeared completely clear. Aliquots were stored at -20°C. 4% paraformaldehyde solution was used to fix tissues and cells. The frozen solution was thawed completely at 37°C and then filtered through a 0.22µm filter before diluting to 4% with PBSABC.

Blocking solution

Blocking solution for the anti-Delta SER20 antibody contained 10% FCS and 3% BSA in PBSABC. For other antibodies, blocking solution contained 0.2% cod fish skin gelatin (FSG, Sigma) solution in PBS. The stock solution of 1% (w/v) was prepared, filtered through 0.22µm filter and stored sterile at room temperature.

Gelvatol mounting solution

The Gelvatol mounting solution was prepared as described by Harlow and Lane (Harlow and Lane, 1988). 2.4g Gelvatol (Monsanto Chemicals) was mixed with 6g glycerol (Sigma) and vortexed. 6ml dH₂O was added, mixed and left to stand for 90 minutes at room temperature. 12.5ml of 200mM Tris-HCl, pH8.5 was added and the solution was vortexed, heated to 50°C and vortexed again. Heating and vortexing were repeated three times and the solution placed on an end over end mixer overnight at room temperature. The solution was then centrifuged at 2000rpm for 10 minutes at room temperature and stored in aliquots at 4°C.

2.4.2 Antibodies

All antibodies used are listed in Table 2.1 and 2.2.

Table 2.1. Primary antibodies

Antibody name	Antigen specificity	Species	Dilution	Reference
SER 20	Chick Delta1	rabbit polyclonal	1:80	(Henrique <i>et al.</i> , 1997)
TAN 20	Human Notch1	rat monoclonal	10 µg/ml IgG	(Blaumueller <i>et al.</i> , 1997)
bhN6	Human Notch2	rat monoclonal	10 µg/ml IgG	(Blaumueller <i>et al.</i> , 1997)
P5D2	human β1 integrin	mouse monoclonal	10 µg/ml IgG	obtained from DSHB; (Dittel <i>et al.</i> , 1993)
CD29 conjugated to FITC	human β1 integrin	mouse monoclonal	10 µg/ml IgG	purchased from DAKO; (Koenigsmann <i>et al.</i> , 1992)
GoH3	human α6 integrin	rat monoclonal	10 µg/ml IgG	purchased from Serotec; (Sonnenberg <i>et al.</i> , 1991)
HECD-1	human E-cadherin	mouse monoclonal	1:200	Kind gift of Drs M. Takeichi and S. Hirohashi; (Shimoyama <i>et al.</i> , 1989)
clone 14	mouse β-catenin	mouse monoclonal	1:200	purchased from Transduction Laboratories
DPI/II	desmoplakin I and II	mouse monoclonal	1:200	purchased from ICN
E1/28	Human CD44	mouse monoclonal	10 µg/ml IgG	(Isacke <i>et al.</i> , 1986)
anti- vinculin	Human Vinculin	mouse monoclonal	1:100	Purchased from Serotec

LP34	pan-keratin	mouse monoclonal	10 µg/ml IgG	Cell Structure Laboratory, ICRF; (Lane <i>et al.</i> , 1985)
SY5	human involucrin	mouse monoclonal	10 µg/ml IgG	(Hudson <i>et al.</i> , 1992)
Anti-Filaggrin	human filaggrin	rabbit polyclonal	1:100	Purchased from Babco (Lynley and Dale, 1983)
SQ37C	human cornifin	rabbit polyclonal	1:100	Kind gift of A. Jetten (Fujimoto <i>et al.</i> , 1997)
IB5	CD63 (late endosomes and lysosomes)	mouse monoclonal	1:100	Kind gift of M. Marsh, University College London
Y-11	HA epitope tag	rabbit polyclonal	1:200	purchased from Santa Cruz

Filamentous actin was detected using Texas-red-conjugated phalloidin (Sigma) diluted at 1: 500 (Wulf *et al.*, 1979).

Table 2.2 Secondary antibodies

Antigen specificity	Conjugate	Species	Dilution	Reference
mouse IgG, whole molecule	Alexa 494	Rabbit	1:400	purchased from Molecular Probes
mouse IgG, whole molecule	Alexa 594	Rabbit	1:400	purchased from Molecular Probes
rabbit IgG, whole molecule	Alexa 494	Goat	1:400	purchased from Molecular Probes
rabbit IgG, whole molecule	Alexa 594	Goat	1:400	purchased from Molecular Probes
rat IgG, whole molecule	Alexa 494	Goat	1:400	purchased from Molecular Probes

rat IgG, whole molecule	Alexa 594	Goat	1:400	purchased from Molecular Probes
mouse IgG, whole molecule	FITC	Goat	1:100	purchased from Jackson ImmunoResearch
mouse IgG, whole molecule	Texas Red	Donkey	1:100	purchased from Jackson ImmunoResearch
rabbit IgG, whole molecule	FITC	Goat	1:100	purchased from Jackson ImmunoResearch
rabbit IgG, whole molecule	Texas Red	Donkey	1:100	purchased from Jackson ImmunoResearch
rat IgG, whole molecule	FITC	Donkey	1:100	purchased from Jackson ImmunoResearch
rat IgG, whole molecule	Texas Red	Donkey	1:100	purchased from Jackson ImmunoResearch

2. 4.3 Preparation of Cells for Immunofluorescence Staining

Cells were cultured on tissue culture plastic microscope slides (Nunc) or on glass coverslips (Chance Proper Ltd.). Coverslips were first boiled in 7x detergent (ICN) for 30 minutes to remove silicone coating. They were rinsed thoroughly first in tap water and then in dH₂O. Washed coverslips were rinsed briefly in absolute ethanol and spread out on filter paper to dry completely before autoclaving.

Fixation of cells

Culture medium was discarded from cells and they were rinsed in PBS before fixing in 4% paraformaldehyde solution for 20 minutes at room temperature. The specimens were rinsed three times in PBS and then quenched in 50mM NH₄Cl (prepared just before use) for 10 minutes at room temperature. The specimens were rinsed three times in PBS, and then incubated in blocking solution, to reduce binding to non-specific proteins, for at least 30 minutes at room temperature.

Permeabilisation of cells

If the epitope of the primary antibody was intracellular, cells were paraformaldehyde-fixed and then permeabilised using 0.1% Triton X-100 in PBS for 4 minutes at room temperature before rinsing thrice in PBS and then incubated in blocking solution.

2.4.4. Preparation of Cryosections of DED and human foreskin

Small pieces of DED or human neonatal foreskin were placed in a plastic mould containing O.C.T. compound and frozen for 1-2 minutes on isopentane pre-cooled in liquid nitrogen. The tissue embedded in O.C.T. compound was stored at -70°C. Using a cryomicrotome (Reichert-Jung), 6µm sections were cut from the tissue block in an orientation perpendicular to the surface of the tissue. The sections were mounted onto slides (Superfrost Plus, BDH) and stored at -70°C. All sectioning was performed by the ICRF Histopathology Unit. To examine the morphology of the tissues frozen sections were stained with haematoxylin and eosin (H&E). For immunofluorescence staining, frozen sections were thawed at room temperature for 30 minutes. Sections were either stained unfixed, or fixed in 3% paraformaldehyde for 20 minutes at room temperature, or fixed in ice cold methanol for 5 minutes.

2.4.5. Immunofluorescence Staining Protocol

Tissue sections or cultured cells were incubated in blocking solution for 30 minutes at room temperature. Primary antibodies were diluted in the blocking solution (dilutions shown in table 2.1) and applied to cells for 1h at room temperature. The cells were rinsed three times in blocking solution and then incubated with the secondary antibody diluted in blocking solution (dilutions shown in table 2.2) for 1h at room temperature. The cells were then rinsed three times in blocking solution, once in PBS, and once in dH₂O, before mounting in Gelvatol solution.

In co-localisation experiments using a double immunofluorescence method, cells were stained with two primary antibodies. Both primary antibodies were of distinct species and these were subsequently probed with species-specific secondary antibodies conjugated to different fluorophore. The antibodies were applied in the following sequence: first primary; first secondary; second primary; second secondary, with thorough washes in blocking solution between each antibody application. Stained samples were viewed with a Zeiss Axiophot microscope (Carl Zeiss) or with a Nikon Diaphot 200 inverted microscope linked to an MRC-1000 laser scanning confocal microscope attachment (Bio-Rad).

2.4.6 Flow Cytometry

Cell Surface Epitopes

Keratinocyte were harvested with versene/trypsin solution. At least 5×10^5 cells were required per antibody incubation. The cells were resuspended in ice cold complete medium and transferred to a 1.6ml Eppendorf tube. The cells were pelleted by pulse spinning in a bench top centrifuge (14000rpm for approximately 8 seconds), resuspended in 100 μ l primary antibody diluted 1:100 in medium and incubated for 20 minutes at 4°C. The cells were agitated occasionally. 1ml medium was added and cells were pelleted again. Cells were washed twice in medium and then incubated with secondary antibody diluted 1:100 in medium for 20 minutes at 4°C. Cells were washed twice in medium and once in PBS. Finally cells were resuspended in 200 μ l PBS and transferred to round bottom plastic FACS tubes (6ml, 12 x 75mm) (Boehringer Mannheim). Immediately before analysis on the Becton-Dickinson FACScan, a drop of propidium iodide (5 mg/ml) was added to the sample for viability gating. 10000 events were acquired in list mode for each sample. In some experiments the differentiating cells were gated out on the basis of forward and side scatter as previously described (Jones and Watt, 1993).

Intracellular Epitopes

Harvested keratinocytes were permeabilised by resuspending in 10% FCS and 0.3% saponin in PBS (FSP) for 20 minutes at room temperature. The stock saponin solution was 3% (w/v) solution in PBS, filtered through a 0.22 μ m filter and stored at 4°C. 5×10^5 cells were stained for each sample. The method was as described for staining cell surface epitopes except cells were resuspended in FSP instead of medium and after the final antibody incubation, keratinocytes were fixed in 1% paraformaldehyde for 5 minutes at room temperature. Keratinocytes were resuspended in 200 μ l PBS and analysed on a Becton-Dickinson FACScan machine (Gandarillas and Watt, 1997)

FACS Sorting on the basis of EGFP fluorescence.

Cells were harvested and resuspended in ice-cold culture medium at a density of 5×10^5 cells/ml. EGFP positive cells were sorted within 30 minutes after trypsinisation. No processing is required to detect EGFP fluorescence. The FACS machine was chilled to approximately 4°C and cells were collected directly into complete ice-cold culture medium. After collection, cells were spun down at 1000rpm and replated in pre-warmed culture medium.

2.5 Molecular Biology

2.5.1. Bacterial Media and Selection Antibiotics Stocks

Brain Heart Infusion (BHI) broth (ICRF)

This comprised 3.7% Brain Heart Infusion (BHI) broth powder (Difco) in dH₂O. The solution was autoclaved.

L-broth (ICRF)

L-broth comprised 1% Bacto-Tryptone (Difco), 0.5% yeast extract (Difco) and 170mM NaCl and was sterilised by autoclaving.

L-agar (ICRF)

L-agar comprised 1.5% bacto-agar (Difco, w/v) in L-broth. The agar was dissolved by heating in a microwave oven and allowed to cool to 50°C before adding the selection antibiotic. The solution was then poured into 100mm bacteriological petri dishes and left to set on a level platform. Agar dishes were stored at 4°C, agar side up.

Ampicillin stock solution

Ampicillin (Sigma, stock 100 mg/ml in dH₂O) was used as a selection antibiotic and was added to BHI or L-agar to a final concentration of 100 µg/ml.

2.5.2. Bacterial Transformation

Buffers I and II for preparing competent bacteria

Buffer I comprised 30mM sodium acetate, pH6.0, 50mM MnCl₂, 100mM RbCl, 10mM CaCl₂ and 15% (v/v) glycerol. The solution was adjusted to pH5.8, filtered through a 0.22µm filter and stored sterile at 4°C. Buffer II comprised 10mM 3-[N-Morpholino]propane-sulfonic acid (MOPS, Sigma), pH7.0, 10mM RbCl, 75mM CaCl₂ and 15% (v/v) glycerol.

Preparation of competent bacteria

A single colony of bacteria strain Escherichia coli DH5α was inoculated overnight in BHI medium and grown in a 37°C agitator (250rpm). 2ml overnight culture was used to inoculate 200ml BHI and cultured in a 37°C agitator until O.D.₆₀₀ was about 0.6 (for 3-4h). The bacteria were pelleted by centrifugation at 3000rpm for 10 minutes at room temperature. Bacteria were resuspended in ice cold buffer I and incubated on ice for 10

minutes. Bacteria were pelleted again by centrifugation at 3000rpm for 10 minutes at 4°C. The pellet was resuspended in 4ml ice cold buffer II and aliquoted in Eppendorf tubes which were pre-cooled in dry ice. Aliquots were stored at -70°C. Competence was tested by transformation with a known plasmid. Typically, 10µl of competent bacteria were able to produce approximately 10⁶ colonies per µg DNA.

Transformation of bacteria

50µl of competent bacteria were thawed at room temperature and then incubated on ice for 10 minutes. DNA (0.1µg for ligations or 1 ng for plasmids) was added, mixed and left on ice for 20 minutes. The bacteria were then heat-shocked at 42°C for 90 seconds and transferred onto ice for 2 minutes. 4 volumes of BHI medium were added and the tube was agitated gently in a 37°C water bath for 40 minutes. 100µl culture was spread out onto plates containing L-agar and ampicillin and incubated overnight at 37°C. Single colonies were picked with a sterile loop to inoculate LB media.

2.5.3 General Solutions for DNA Techniques

All reagents used were of molecular biology grade. Methods and solutions prepared were according to Sambrook *et al.* (1989). When possible, solutions were autoclaved after preparation to destroy DNases.

Tris/EDTA buffer (TE)

TE was used as a general storage buffer for DNA and comprised 10mM Tris-HCl and 1mM EDTA, pH8.0.

Tris-acetate-EDTA buffer (TAE)

A 50x stock solution was prepared by dissolving 242g Trizma base and 57.1ml glacial acetic acid (BDH) in dH₂O. 100ml 0.5M EDTA, pH8.0 were added and the final volume was made up to 1l.

Tris-borate-EDTA buffer (TBE)

A 10x stock solution was prepared by dissolving 54g Trizma base and 27.5g orthoboric acid (BDH) in dH₂O. 20ml 0.5M EDTA, pH8.0 was added and the final volume was made up to 1l.

SSC (ICRF)

A 20x stock solution was prepared by dissolving 175g NaCl and 882g sodium citrate in 1l dH₂O.

Plasmid preparation solutions I, II, and III

Solution I comprised 50mM glucose, 25mM Tris-HCl, pH8.0 and 10mM EDTA pH8.0 and 5 mg/ml lysozyme (Sigma). Solution II comprised 0.2M NaOH and 1% (w/v) SDS and was prepared freshly. Solution III comprised 3M potassium acetate and 11.5% (v/v) glacial acetic acid to give pH4.8.

Agarose/TAE gel

This was used in the electrophoresis of DNA. 0.8-2% (w/v) ultra pure agarose (Gibco BRL) was melted in a microwave oven in 1x TAE buffer. Ethidium bromide was added at 0.05 µg/ml to agarose solution before casting in gel mould. Typically, DNA was electrophoresed at constant voltage of 80-100V in 1x TAE buffer.

DNA loading buffer

6x DNA gel loading buffer comprised 0.25% bromophenol blue (Sigma), 0.25% xylene cyano (Sigma) and 30% (v/v) glycerol in dH₂O. The loading buffer was stored at 4°C.

2.5.4. General DNA Techniques*Phenol/chloroform extraction*

This was used to remove protein from preparations of DNA. 1 volume of phenol (pH7.8-8)/chloroform/isoamyl alcohol (25:24:1 by volume, Amresco) was mixed with 1 volume of DNA solution, vortexed thoroughly and centrifuged at 14000rpm for 5 minutes at room temperature. As much as possible of the upper aqueous phase (DNA) was removed without disturbing the interface. Recovered DNA solution was then further mixed with an equal volume of chloroform and extracted as for phenol/chloroform/isoamyl alcohol.

Ethanol precipitation of nucleic acids

Sodium acetate (pH5.2) was added to DNA solution to a final concentration of 0.3M. The solution was mixed thoroughly before 2 volumes of ice cold ethanol were added, mixed thoroughly and left at -20°C to precipitate for at least 30 minutes. DNA was pelleted by centrifugation at 14000rpm for 10 minutes at room temperature, washed with 70% ethanol, air-dried and dissolved in TE, pH8.0 or dH₂O.

Quantitation of nucleic acids

Nucleic acids in solution were diluted and placed in a clean quartz cuvette with a path length of 1cm. The absorbance at wavelengths 260nm and 280nm was measured using a spectrophotometer (Pharmacia Biotech, Ultrospec 2000). An O.D.₂₆₀ reading of 1

corresponded to 50 µg/ml double stranded DNA, 40 µg/ml RNA and 33 µg/ml oligonucleotides.

Preparation of plasmid DNA

To screen colonies after ligation and transformation, single colonies were picked and inoculated in LB-media containing a suitable antibiotic, then grown overnight in a 37°C agitator. Plasmid DNA was isolated from the pelleted bacteria by resuspending in 100µl ice cold solution I, mixing with 200µl solution II followed by 150µl solution III, centrifuging at 14000rpm for 10 minutes at room temperature, and finally precipitating with 0.7 volumes of isopropanol. Precipitated DNA was washed in 70% ethanol, air dried and finally dissolved in 50µl TE pH 8.0 containing 100 µg/ml RNase A (Sigma). In some experiments plasmid DNA mini preparations were performed by an AutoGen 740™ robot (Integrated Separation Systems).

For maxi preparations, 0.5ml of the 10ml overnight culture was added to 100ml LB medium containing a suitable antibiotic and grown overnight in a 37°C agitator. Bacteria were pelleted by centrifugation at 6000rpm for 15 minutes at 4°C in a Beckman J2-21 centrifuge. The plasmid DNA was purified using a Qiagen plasmid maxi kit (Qiagen Ltd.) according to the manufacturer's instructions.

Purification of DNA fragment from Agarose gels

DNA fragments were purified from agarose gels using a GeneClean II kit (Bio 101 Inc.). Briefly, the gel slice containing the DNA fragments was cut using a clean scalpel, weighed and dissolved in 3 volumes of NaI stock solution at 50°C. Glassmilk suspension was added and the solution was mixed end over end for 30 minutes at room temperature to allow the DNA fragments to bind to the Glassmilk. The glassmilk/DNA complex was pelleted by pulse spinning and washed three times in New Wash. Bound DNA was then eluted twice in TE, pH8.0 or dH₂O.

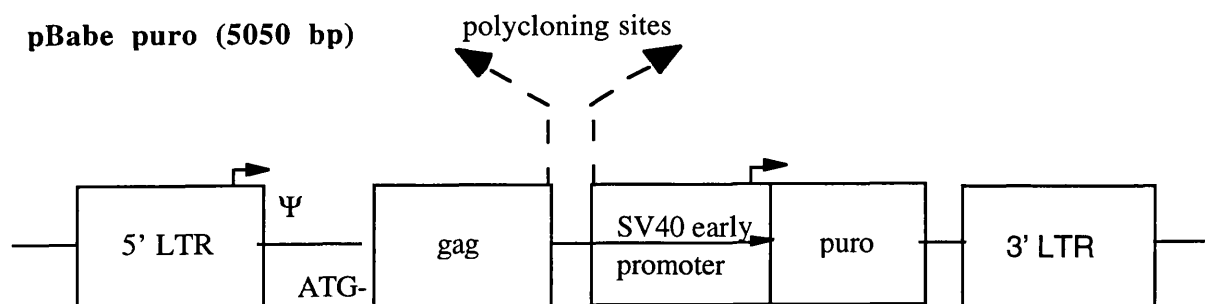
Enzymatic manipulation of DNA fragments

Restriction enzymes and T4 DNA ligase were purchased from NEB and used for restriction digestions and DNA ligations, respectively. DNA polymerase Klenow fragment (NEB) and dNTPs (Gibco BRL) was used to blunt end DNA fragments. Calf intestinal alkaline phosphatase or shrimp alkaline phosphatase (Boehringer Mannheim) was used to remove 5' phosphate groups from DNA fragments.

2.5.5. Manipulation of cDNA Constructs

Retroviral vector pBabe puro

pBabe puro was a gift from Dr H. Land (Morgenstern and Land, 1990; Morgenstern and Land, 1991). It is a high titre, direct orientation, replication deficient retroviral vector which expresses the inserted gene from the promoter within the Mo MuLV long terminal repeats (LTR) and an RNA encoding puromycin resistance marker from the SV40 internal early promoter (Figure 2.4). Homologous *env* coding sequences are deleted in the vector (ATG⁻ *gag*) without compromising recombinant vector titre. In combination with the complementary helper-free packaging cell lines AM12 and GP + E (which lack the packaging signal, Ψ), pBabe puro provides an extremely efficient retroviral infection system to transduce cultured primary human epidermal keratinocytes without helper retrovirus contamination (Zhu and Watt, 1996; Levy *et al.*, 1998). Competent bacterial strain DH5 α was used as the host for transformations, so as to avoid high recombination frequencies resulting from the presence of two LTRs in the retroviral DNA.



Polycloning sites: BamH1, BstX1, SnaB1, EcoR1, BstX1 (reverse orientation), SalI

Figure 2.4. Retroviral vector pBabe puro

Modified from (Morgenstern and Land, 1990)

List of cDNA constructs

All the cDNA constructs used for generation of recombinant retroviral vectors are listed in Table 2.3.

Table 2.3. List of cDNAs used for constructing retroviral vectors

Encoded protein	cDNA size	Reference
Full length mouse Delta1	~2200bp	(Henrique <i>et al.</i> , 1997)
Mouse DeltaT mutant	~1760bp	(Henrique <i>et al.</i> , 1997)
Full length human Serrate1	~3700bp	I. Le Roux, ICRF.
Human SerrateT mutant	~ 3300bp	I. Le Roux, ICRF.
Xenopus Suppressor of Hairless DNA binding mutant + HA tag	~ 1500bp	(Wettstein <i>et al.</i> , 1997) HA tag added by I. Le Roux ICRF
Enhanced GFP mutant (EGFP)	~ 700bp	purchased from Clontech
Internal Ribosomal Entry Site (IRES) followed by EGFP	~ 1500 bp	(Pear <i>et al.</i> , 1998)

Subcloning of cDNA constructs into pBabe puro

The cDNAs for full length mouse Delta1, mouse DeltaT, full length human Serrate1, and human SerrateT were provided by Isabelle Le Roux (Developmental Genetics Lab, ICRF) and Jenny Dunne (Lymphocyte Activation Lab ICRF). Each of these cDNAs was cut out from the vector pks by *EcoRI* and *BamH I* digestion, purified, and subcloned into the *BamH I EcoRI* cut pBabe puro retroviral vector.

A DNA binding mutant of Xenopus Suppressor of Hairless was provided by C. Kintner (Salk Institute, La Jolla). An HA (influenza hemagglutinin) epitope tag was added by

Isabelle Le Roux using PCR. The tagged cDNA was subcloned into the EcoR1 and Sal1 sites of pBabe puro.

EGFP (Clontech) was subcloned into the BamH1 and Sal1 sites of pBabe puro. The IRES-EGFP was obtained from Julie Miller (Lymphocyte Activation Lab ICRF) and was cloned into the EcoR1 and Sal1 sites of pBabe puro

For subcloning, before each ligation, the 5' phosphate groups of the restriction enzyme-cut pBabe puro were removed using alkaline phosphatase in order to prevent vector self-ligation. T4 DNA ligase was used in all the ligation experiments. The molar ratio between insert and vector was 10:1. Orientation of the resulting ligation products was checked by specific restriction enzyme digestions. The final recombinant retroviral vectors with the correct orientation of the inserts were prepared using a Qiagen maxi-prep column and subjected to transfection into ecotropic GP + E packaging cells.

2.5.6 General Solutions for RNA Techniques

Methods and solutions prepared were according to (Sambrook *et al.*, 1989), unless otherwise stated. All reagents used were of molecular biology grade.

DEPC dH₂O

In order to inhibit any contaminating RNases, Diethyl pyrocarbonate (DEPC, Sigma) was added to dH₂O to a final concentration of 0.1% and left overnight in a 37°C water bath. The treated water was then autoclaved.

Guanidinium isothiocyanate (GIT) homogenisation buffer

This comprised 4M GIT, 0.1M Tris-HCl, pH7.5 and 1% β-mercaptoethanol. 50g GIT was dissolved in 10ml of 1M Tris-HCl, pH7.5 and 89ml dH₂O. The solution was filtered through Whatman 1MM paper and stored at room temperature. 1ml β-mercaptoethanol was added prior to use.

Sodium phosphate buffer

The stock solutions of this buffer were 1M Na₂HPO₄ and 1M NaH₂PO₄. For sodium phosphate buffer at pH7.2, 36ml Na₂HPO₄ and 14ml NaH₂PO₄ were mixed together.

2.5.7. Northern Blotting

Preparation of RNA in Formaldehyde gel sample buffer

Total RNA was kindly provided by Phil Jones and Alberto Gandarillas. Up to 12µg RNA was resuspended in 4µl DEPC dH₂O and added to 14.5µl cocktail A which consisted of a mixture of 1ml deionised formamide, 100µl 10x formaldehyde gel running buffer and 350µl 12.3M formaldehyde. The sample was heated at 65°C for 15 minutes and then chilled on ice, and centrifuged briefly. 2µl of cocktail B was added: cocktail B comprised 50% glycerol, 1mM EDTA and 0.25% EDTA, and 1µg ethidium bromide. The sample was then loaded on a formaldehyde gel.

Formaldehyde RNA gel

1.8g agarose was melted in 108ml dH₂O and cooled to 60°C and was then added to 15ml of 10x formaldehyde gel running buffer (0.2M MOPS, 10mM EDTA and 80mM sodium acetate) and 27ml stock formaldehyde solution (37%, BDH). The solutions were mixed and then poured into a gel casting tray. The gel casting tray and apparatus were treated with 3% hydrogen peroxide solution and DEPC-dH₂O prior to use. The final composition of the gel was 1.2% agarose gel in 2.2M formaldehyde and 1x formaldehyde gel running buffer.

RNA gel electrophoresis

The 1.2% agarose formaldehyde gel was electrophoresed in 1x formaldehyde gel running buffer for 4-6h at 10V/cm at room temperature in a fume hood. After electrophoresis, the gel was photographed.

Northern blotting

After electrophoresis, RNA was partially hydrolysed by soaking the gel for 20 minutes in 50mM NaOH and then neutralising the gel for 30 minutes in several batches of 20x SSC. The RNA was transferred from the gel to Hybond-N (Amersham) membrane by capillary action using 20x SSC as the transfer buffer. The transfer was completed overnight at room temperature. The membrane was then rinsed in PBS, baked for 2h at 80°C and stored in Saran wrap at room temperature until required for hybridisation.

Radiolabelling probes for Northern hybridisation

25-200ng gel-purified DNA fragments were denatured at 95°C for 5 minutes and radiolabelled by random priming using the Rediprime DNA labelling system (Amersham) and 50µCi [³²P]-α-dCTP (ICN). The kit was essentially based on the method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983) with the exception that the heat-denatured DNA fragments were labelled for only 15 minutes at 37°C,

using nonamers as primers instead of hexamers. Un-incorporated [^{32}P]- α -dCTP was removed using a Nick column (Pharmacia). The cDNAs used as probes for Northern hybridisation are listed in table 2.4.

Table 2.4 List of cDNAs used as probes for Northern hybridisation

cDNA	Expected band size	Reference
Human Notch1 (Tan 1)	~1000bp	(Austin <i>et al.</i> , 1995)
Human Delta1	~ 4400bp	(Lowell <i>et al.</i> , 2000)
Human involucrin	~2100 bp	(Gandarillas and Watt, 1995)
Human 18S RNA	~ 2000 bp	(Gandarillas and Watt, 1995)

Northern hybridisation and washing

Hybridisation was carried out following the method of Church and Gilbert (Church and Gilbert, 1984). To prevent the non-specific binding of probe to RNA the blot was incubated in 7.5ml of Northern blot hybridisation solution (0.2M sodium phosphate buffer pH7.2, 1mM EDTA, 1% BSA, 7% SDS, 45% formamide) in a Techne Hybridiser HB-1 for 4h at 42°C. The labelled probe (2×10^6 cpm/ml) was heat denatured for 5 minutes at 95°C, rapidly cooled on ice, then added to the membrane in 12.5ml of Northern blot hybridisation solution. The hybridisation was completed overnight at 42°C. The hybridisation solution was discarded and the membrane was washed once in Northern blot wash buffer (40mM sodium phosphate buffer pH7.2, 1mM EDTA and 1% SDS) for 30 minutes at 42°C. This was followed by 2 washes for 15 minutes at 65°C. The membrane was monitored with a Geiger counter to check background. Membranes were wrapped in Saran wrap and exposed to Kodak XAR-5 film for autoradiography.

2.6 List of Suppliers and Distributors

Aldrich Chemical Company Ltd. Dorset, UK.
Amresco, Solon, Ohio, USA.
Amersham International, Amersham, Buckinghamshire, UK.
BDH Laboratory Supplies Inc., Hemel Hempstead, Hertfordshire, UK.
Beckman Instruments, Palo Alto, California, USA.
Becton-Dickinson, Lincoln Park, New Jersey, USA.
Bio 101 Inc. La Jolla, California, USA.
Bio-Rad Laboratories Inc. Hemel Hempstead, Hertfordshire, UK.
Boehringer Mannheim UK Ltd. Lewes, East Sussex, UK.
Calbiochem -Novabiochem (UK) Ltd. Nottingham, UK.
Carl Zeiss Ltd. Welwyn Garden City, Hertfordshire, UK.
Chance Propper Ltd., Swethwick, Warley, UK.
Clontech, Palo Alto, UK.
Coulter Electronics Ltd. Harpenden, Herts, UK.
DAKO A/S, Denmark.
Developmental Studies Hybridoma Bank (DSHB), University of Iowa, Iowa, USA.
Difco Laboratories, Manston, Wisconsin, USA.
Eastman Kodak Co. is distributed by Sigma Chemical Co.
EOS Electronic, South Glamorgan, Wales, UK.
European Collection of Animal Cell Cultures (ECACC), Salisbury, UK.
Flow Laboratories Ltd., Ayrshire, Scotland, UK.
Genetics Research Instrumentation Ltd. Dunmow, Essex, UK.
Gibco BRL/Life Technologies Ltd. Paisley, Renfrewshire, UK
Hoefer Scientific Instruments is distributed by Biotech Instruments Ltd., Beds, UK.
Hydro Medical Sciences Division, Brunswick, New Jersey, USA.
ICN Pharmaceuticals Ltd. Thame, Oxon, UK.
Imperial Laboratories (Europe) Ltd. Andover, Hampshire, UK.
Integrated Separation Systems, Natick, Maryland, USA.
Jackson Immunoresearch Laboratories, Luton, Bedfordshire, UK
Jencons, Leighton Buzzard, Beds, UK.
Monsanto Chemicals. Springfield, Massachusetts, USA.
Millipore, Harrow, Middlesex, UK
Molecular Probes, Leiden, Netherlands.
New England Biolabs (NEB). New York, USA.
Nunc A/S, Roskilde, Denmark.
Perkin-Elmer Co. Foster City, California, USA.

Pharmacia Biotech. Uppsala, Sweden.
Pharmingen, San Diego, California, USA.
Pierce, Rockford, Illinois, USA.
Premier Brands UK Ltd. Knighton, Stafford, UK.
Promega UK Ltd. Southampton, UK.
Qiagen Ltd. Crawley, UK.
RND Systems, Abingdon, Oxford, UK.
Santa Cruz Biotech. Inc. Santa Cruz, California, USA.
Seikagaku Corp. Tokyo, Japan.
Serotec Ltd. Kidlington, Oxford, UK.
Scotlab Ltd. Coatbridge, Strathclyde, Scotland, UK.
Sigma Chemical Co. Poole, Dorset, UK.
Transduction Laboratories, Lexington, Kentucky, USA.
US Biochemical Corp. Cleveland, Ohio, USA.
Vector Laboratories, Burlingame, California, USA.
Whatman International Ltd. Maidstone, Kent, UK
Zeneca Pharmaceuticals, Macclesfield, UK

CHAPTER 3

LINEAGE MARKING

3.1. Introduction

I set out to find a suitable lineage marker for primary human keratinocytes, primarily so that I would be able to track individual cells and their clonal progeny within confluent cultures and investigate how the fate of keratinocytes is influenced by surrounding cells.

There are two general types of markers that can be used to experimentally label cells and identify their progeny. Some markers, for example fluorescent lipophilic membrane dyes, will become diluted as the cell divides. These types of markers are useful for revealing the proliferative history of a cell but can only be used over the short term for lineage tracing. I tested the suitability of one such dye, PKH 26, as a short-term keratinocyte lineage marker.

A second approach is to use genetic markers which are stably inherited, allowing all the cells progeny to be tracked over the long term. One of the most reliable ways to do this is to use replication incompetent retroviral vectors to integrate genes encoding marker proteins into the genome of target cells (Watt, 1988; Garlick and Taichman, 1992). Retroviruses encoding the marker protein β galactosidase have been successfully used for lineage studies, both in mouse epidermis *in vivo* and in epidermis that has been reconstituted from cultured cells (Mackenzie, 1997; Kolodka *et al.*, 1998; Ghazizadeh *et al.*, 1999). One disadvantage of β galactosidase is that it cannot be detected in living cells. EGFP (Enhanced Green Fluorescent Protein) is an intrinsically fluorescent marker protein that has been used successfully as a lineage marker both in cell lines and in developing organisms (Gubin *et al.*, 1997; Brand, 1999). I constructed a retrovirus encoding EGFP and tested its suitability as a lineage marker for keratinocytes. I went on to use EGFP as a lineage marker in experiments to compare the motility of stem cells with transit amplifying cells within confluent sheets of keratinocytes.

3.2. Results

3.2.1 *Is PKH a useful lineage marker for keratinocytes?*

PKH26 (Sigma) is an aliphatic red fluorescent reporter molecule that becomes incorporated into the cell membrane (section 2.3.1). It becomes progressively diluted as cells grow and divide. I tested how many times a labelled cell could divide before the label becomes so dilute that it can no longer be detected. When keratinocytes were labelled with 2 μ M of PKH, the fluorescence became undetectable after 6-7 cell divisions (Figure 3.1). Those cells that are still fluorescent in Figure 3.1C are most likely differentiated cells that are not dividing. At this concentration the dye had no detectable effects on keratinocyte proliferation: the colonies were identical in size and appearance to those founded by keratinocytes that had been incubated in the dye-diluent only or in PBS. It is likely that keratinocytes would remain fluorescent over a greater number of cell divisions if they were stained with higher concentrations of dye. However, higher concentrations of dye are not recommended by the manufacturer because they can interfere with membrane viability.

This marker is suitable for use in short term experiments to follow the division of individual cells and has the advantage that labelling is a quick and simple procedure. However it is not useful for longer- term experiments.

3.2.2 *Constructing a EGFP retrovirus*

One way to stably lineage mark a cell is to engineer a replication incompetent retrovirus to deliver a gene that encodes a suitable marker protein. This gene will become integrated into the genome of any cell that is infected by the virus, and the marker-protein will consequently be stably expressed in all of the progeny of that cell.

The green fluorescent protein (GFP) is an intrinsically fluorescent protein, originally isolated from the bioluminescent jellyfish *Aequorea victoria*. It has been widely used to study cell lineage in various different organisms during development (Brand, 1999). Mutant forms of GFP have been made that display modified fluorescence spectra and an increased extinction coefficient, for example 'enhanced' GFP (EGFP) (Clontech). EGFP seemed likely to be a suitable lineage marker for keratinocytes.

Infection of primary keratinocytes with retroviruses is now a well-established procedure (Levy *et al.*, 1998), largely because primary human keratinocytes are difficult to transfect stably with plasmid DNA constructs (Hengge *et al.*, 1995). Close to 100% infection can be achieved with retroviral vectors and during repeated passaging there is a stable expression of the transduced gene (Garlick *et al.*, 1991; Mathor *et al.*, 1996; Levy *et al.*, 1998). I made replication incompetent retrovirus that encoded EGFP, as follows: EGFP was subcloned into the retroviral vector pBabe puro. The pBabe puro-EGFP construct was then transfected into the ecotropic packaging line, GPE. Stably transduced puro-selected GPE were sorted by FACS to eliminate any cells with low or no EGFP expression (cells outside gate M1-A in Figure 3.2A). Supernatant from the EGFP positive GPE cells was used to infect the amphotropic packaging line AM12. After puromycin selection, close to 100% of the AM12 were EGFP positive (Figure 3.2B). The cells with the lowest EGFP expression were discarded by FACS in order to ensure that all of the virus made by the sorted AM12 will efficiently encode EGFP (cells outside gate M1-B in Figure 3.2B). This will also ensure that the sorted AM12 generate virus of high titre, since the titre of the virus has been shown to correlate with the levels of expression of the virally encoded genes in AM12 cells (Levy *et al.*, 1998). Keratinocytes were infected with viral supernatant from the sorted AM12. Close to 100% of infected keratinocytes were EGFP positive after puromycin selection (Figure 3.2C: 96% of cells lie within gate M1-C).

3.2.3 Is EGFP a useful lineage marker for keratinocytes?

A useful lineage marker should have the following properties:

- It should have no effect on keratinocyte viability, proliferation or differentiation.
- It must be heritable as cells divide over the course of the experiment. For longer term experiments it would have to be stably heritable.
- It should be easily detectable so that marked cells can be reliably distinguished from unmarked cells.

An ideal lineage marker would also be detectable in living cells.

3.2.4. EGFP does not influence keratinocyte behaviour

EGFP is widely used as a lineage marker or a reporter, because it does not influence the behaviour or affect the health of most cell types (Gubin *et al.*, 1997). It has even been expressed in every cell of transgenic mice (Okabe *et al.*, 1997) or *Drosophila* (Plautz *et al.*, 1996) with no deleterious effects. Keratinocytes that had been stably transduced with EGFP looked healthy and grew normally, reaching confluence at about the same

time as cells that had been transduced with the empty vector. In order to determine whether there were any subtle effects on stem cell differentiation, I carried out a cloneogenicity assay to measure the proportion of clonogenic cells and stem cells in the transduced population.

Keratinocytes (kn3) were stably infected with the EGFP retrovirus (K-EGFP) or empty retrovirus (K-WT). Close to 100% of the EGFP infected cells were positive for EGFP. Cloneogenicity assays were carried out as described in section 2.2.3. EGFP had no significant effect on the number of proliferative cells (CFE) or on the proportion of stem cells (table 3.1)

3.2.5. EGFP is stably heritable.

The ideal lineage marker should be stably inherited by all of the progeny of the labelled cell. Figure 3.3 shows that fluorescence is not lost from any cell with time in culture. Keratinocytes stably transduced with EGFP continue to fluoresce for at least 5 passages (5 weeks or 35 cell divisions). Cells also retain EGFP expression for at least 8 weeks when grown as a reconstituted epidermis on DED (see Figure 3.8). After initial puromycin selection, keratinocytes can be grown in the absence of further drug selection without loss of EGFP expression, as has been demonstrated for other cell types (Gubin *et al.*, 1997).

Although EGFP expression does not decrease over time within the population as a whole, there is some heterogeneity of expression. Terminally differentiated cells tend to be brighter than small actively proliferating cells. This may reflect the fact that EGFP is a very stable protein. Once a cell has terminally differentiated, the protein will continue to be transcribed but will no longer be diluted by cell division, so protein levels will build up in the cytoplasm. Heterogeneity of expression is not a problem for lineage studies because levels never fall to below detectable levels in any cell.

3.2.6. EGFP is readily detectable

Labelled cells can be identified by fluorescence microscopy.

A second requirement for a good lineage marker is that labelled cells should be easily distinguishable from unlabelled cells. Figure 3.4 A shows a confluent sheet of keratinocytes 3 days after seeding a single EGFP-positive cell amongst unlabelled cells. The culture was seeded at a high density (10^4 cells/cm²) so that the culture became confluent after 1 day. All of the clonal progeny of the original EGFP labelled cell can

be clearly distinguished from unlabelled cells using fluorescence microscopy (see also Figures 3.5 and 3.6).

Labelled cells can be identified by FACS.

EGFP labelled cells can also be readily distinguished from unlabelled cells by FACS analysis. Figure 3.4 B shows the EGFP fluorescence profile of a culture that had been seeded with 1 EGFP cell to every 4 unlabelled keratinocytes and grown at confluence for 5 days before disaggregating the cells using trypsin/EDTA and subjecting them to FACS analysis. The EGFP-positive population lie within gate M1 (18.1% of the total population). This technique makes it possible to quantify the proportion of EGFP cells within the culture and to follow the expression of differentiation markers in the labelled population independently from the unlabelled population.

3.2.7 EGFP lineage marking can be used to distinguish self-renewing colonies from abortive colonies within confluent cultures.

Figure 3.5 A shows a confluent sheet of keratinocytes 24 hours after a single EGFP positive cell has been seeded amongst unlabelled cells. The culture was seeded at a high density (10^4 cells/cm²) so that the culture became confluent after 1 day. Figure 3.5 B-D shows different examples of clones that arose from single EGFP labelled cells after 5 days (B) or 15 days (C, D).

When keratinocytes are grown at confluence, they stratify into several cell layers which are organised in a similar way to the epidermis *in vivo*; The basal layer contains undifferentiated proliferative cells whilst the upper layers contain terminally differentiated cells (Watt, 1988). In the five-day old culture shown in Figure 3.5 B all of the cells in the labelled colony are in the basal layer (basal cells distinguished by $\beta 1$ integrin immunofluorescence: red in Figure 3.5). The cultures in Figure 3.5 C and D were allowed to grow for 15 days. After this time the morphology of the EGFP labelled clones fell into one of two categories: the type of colony shown in Figure 3.5 C was large, containing many cells that populated both the basal and suprabasal layers. This is the type of colony that would be predicted to arise from a stem cell. The type of colony shown in Figure 3.5 D contained few cells, all of which were in the suprabasal layer. Figure 3.5 D shows seven large, flat, green cells lying over the top of the smaller integrin-positive basal cells. This is the type of colony that would be predicted to arise from a transit-amplifying cell. EGFP lineage marking can therefore be used to distinguish self-renewing colonies from abortive colonies within confluent cultures.

3.2.8 EGFP can be used to follow the behaviour of living cells.

Time lapse microscopy can simultaneously follow EGFP labelled cells (using UV fluorescence microscopy) and surrounding unlabelled cells (using conventional microscopy). Figure 3.6 B shows a near-confluent sheet of keratinocytes 24 hours after a single EGFP positive cell had been seeded amongst unlabelled keratinocytes at a density of 10^4 cells/cm². In this culture the EGFP labelled cell had already divided once during the first 24 hours (Figure 3.6 A, B). The culture was filmed starting from 24 hours after plating the culture and finishing 3 days later, with exposures at 10-minute intervals. At each time point a photo was taken under fluorescence excitation to visualise the EGFP and then immediately afterwards a phase photograph was taken. A selection of still frames from the movie is shown in Figure 3.6.

The time lapse phase microscopy movie of the confluent sheet of cells revealed that there was a significant amount of “jostling” and displacement of cells relative to each other during the course of film. The clonal progeny of the EGFP labelled cell remain together for the first 2 days, despite being vigorously buffeted by the movements of surrounding cells, but by the third day the EGFP labelled clonal progeny began to break away from each other (Figure 3.6 G, H).

It would be interesting to use the time-lapse approach to compare the behaviour of stem cells and transit amplifying cells. However, it has the disadvantage that only a limited number of EGFP labelled cells can be followed in the field of view of the microscope, and so a large number of time lapse sessions might be required to gather statistically significant information. For this reason I did not pursue the time-lapse approach for comparing the behaviour of stem cells and transit amplifying cells. I instead analysed lineage marked cells in cultures after they had been fixed (section 3.2.9): this limits the analysis to a single time point per culture, but has the advantage that all of the labelled colonies in the culture can be analysed.

3.2.9 Using EGFP to compare the behaviour of stem and transit amplifying cells

Stem cell progeny are more cohesive than transit amplifying cell progeny

A population of keratinocytes was stably transduced with EGFP. EGFP labelled stem and transit amplifying cells were selected by adhesion to type IV collagen as described in section 2.2.6. They were then mixed with unlabelled cells that had not been selected on collagen at a ratio of 50-70 unlabelled cells per EGFP labelled cell, and plated at sufficient density that a confluent stratified sheet was formed within 24 hours (10^4 cells per cm²). Five days later the size and distribution of EGFP-labelled cells in clones founded by stem or transit amplifying cells were determined. At this time the average

number of cells per colony was not significantly different between the two populations (21 cells per colony in the stem cell-enriched population; 22 cells per colony in the transit amplifying cell-enriched population). However there was a striking difference in morphology, with stem clones being compact (>50% of cells per clone in contact with one another; Figure 3.7A) and transit amplifying clones being more dispersed (<30% of cells in contact; Figure 3.7 B) ($p < 0.01$, χ^2 test). The proportions of compact colonies were 74/103 (stem) vs. 25/78 (transit) in one experiment and 135/170 (stem) vs. 20/56 (transit) in a second experiment.

Clonal progeny are more cohesive within integrin-bright regions than within integrin-dull regions.

High $\beta 1$ integrin expression marks keratinocyte stem cells. In confluent cultured keratinocytes, integrin bright cells are found in a patterned distribution of evenly spaced patches (Jones *et al.*, 1995). I used EGFP to compare the behaviour of cells that lay within the integrin bright stem cell patches with cells that lay within the surrounding integrin dull, transit amplifying, regions.

Cultures were set up as described in the previous section, but were continued for a further 2 days (i.e. for a total of 7 days after plating) and EGFP visualisation was combined with $\beta 1$ integrin labelling in sheets that had been detached from the culture dish using Dispase. Clones were detected that encompassed both integrin-bright and -dull areas (Figure 3.7 C, D). Twenty colonies were examined. EGFP-positive cells in the integrin-dull areas were scattered while those in the integrin-bright areas were compact; both compact and scattered cells could be found within a single stem cell clone (Figure 3.7 C, D). Large, suprabasal, integrin negative cells were also found in the clones and they sometimes overlay integrin-bright areas that did not contain any EGFP-positive cells (Figure 3.7 C, D).

In the clone illustrated in Figure 3.7 D, a false colour scale has been applied to the integrin fluorescence. The brightest cells are visualised in blue/green and duller cells are visualised in red. The EGFP-positive cells in the area with the highest level of $\beta 1$ integrins (labelled s) are compact whereas the cells within the adjacent region (labelled TA) are scattered. Suprabasal, terminally differentiating (i.e. integrin negative) cells are also shown (labelled td).

3.2.10. Following the behaviour of stem cells over several weeks in reconstituted epidermis

Keratinocytes that are seeded on decellularised deepidermalised dermis (DED cultures) can grow to reconstitute a stratified epidermis which is histologically identical to epidermis *in vivo* (section 2.2.4). These cultures remain stable for up to four weeks when cultured at an air-liquid interface in a tissue culture dish (Rikimaru *et al.*, 1997) or for at least 4 months when grafted under the skin of a nude mouse (Levy *et al.*, 1998). DED cultures serve as an experimental model of intact human epidermis. I set out to see if EGFP can be used to follow the behaviour of stem cell progeny over long periods of time within reconstituted epidermis in DED cultures.

Establishing the optimum proportion of cells that should carry the label

I grew DED cultures seeded with mixtures of EGFP labelled and unlabelled keratinocytes. The aim was to follow the clonal progeny of individual cells. This required that the EGFP were seeded far apart from each other within the cultures in order to ensure that groups of labelled cells were clonally derived. In practice this means that there should be fewer than 10 colonies per DED so that they are far enough apart from each other to be sure that neighbouring clones do not merge. Since the vast majority of labelled cells are lost from the culture within the first few weeks through terminal differentiation, it was necessary to carry out pilot experiments to establish how many EGFP cells should be seeded. I found that 100 EGFP cells give rise to an average of four colonies after 3 weeks. In longer-term experiments, as many as 10^4 EGFP cells were needed in the seeding population in order to ensure that widely spaced EGFP colonies would persist for 9 weeks: it was not possible to count the exact number of colonies because of the poor quality of the cultures at 9 weeks.

Following lineage marked cells in DED cultures.

100 or 10^4 EGFP labelled cells were mixed with 10^5 unlabelled cells and seeded onto DEDs. Some DEDs were instead seeded with 10^5 cells that had all been labelled with EGFP: this acts as a positive control to check that EGFP expression is not lost from any progeny of the labelled cells during the course of the experiments. The cultures were all grown at an air: liquid interface: some of them were cultured in this way for 3 weeks whilst others were removed after 10 days and grafted onto nude mice for a further 8 weeks.

After three weeks the morphology of the EGFP labelled clones on DEDs fell into one of two categories: the type of colony shown in Figure 3.8 A contained several hundred cells. This is the type of colony that would be predicted to arise from a stem cell. The

type of colony shown in Figure 3.8 B contained few cells, many of which were large, which is a characteristic feature of a differentiated cell. This is the type of colony that would be predicted to arise from a transit amplifying cell. The small colonies tended to be scattered, whilst the large colonies tended to be cohesive. None of the cells in the control DED had lost expression of EGFP (Figure 3.8 C)

After a further eight weeks EGFP cells were still visible within the reconstituted epidermis (Figure 3.8 D, E), and none of the cells had lost EGFP expression in the control DED (3.8 F). A few regions contained vertical columns of EGFP labelled cells that might represent a proliferative unit (Figure 3.8 D). Elsewhere in the DED there was evidence of extensive lateral movement in the suprabasal layers (Figure 3.8 E).

The reconstituted epidermis that had formed after 8 weeks was in every case disappointingly thin and fragile with little histological evidence of a healthy proliferative basal layer. It was easily damaged during sectioning, and disintegrated completely during attempts to remove it as an intact sheet using Dispase. It is therefore questionable whether the epidermis had remained healthy for long enough to reach a steady state.

3.3 DISCUSSION

PKH as a lineage marker

The first lineage marker that I tested was the lipophilic dye, PKH. I found that PKH could not be used to reliably mark cells over more than a few cell divisions, and I did not use it in any of the experiments described in this thesis. However, PKH could be useful for certain types of experiment. The fact that the dye becomes diluted as cells divide means that it can reveal the proliferative history of each individual cell, the intensity of staining being directly related to the number of divisions that relate it to the original labelled cell. The brightness of each cell can be quantified using flow cytometry (Jones, 1995). One potential use for PKH is to distinguish asymmetric from symmetric cell divisions. For example, it could be used to test the hypothesis that every transit amplifying cell divides strictly symmetrically, such that all of its progeny will differentiate after the same number of cell divisions. When labelled keratinocytes are plated at clonal density, this hypothesis predicts that all the differentiated progeny within any abortive colony will contain an equal amount of dye. Although I did not carry out a detailed clonal analysis using PKH, I did notice that single cells could stop

dividing and differentiate within a clonally derived colony. This suggests that transit amplifying cells are capable of undergoing asymmetric cell divisions. Whether or not they do so in vivo is another matter.

It should in principle be possible to use PKH for testing hypotheses that are more complex than the one outlined above. Any hypothetical pattern of symmetric and/or asymmetric cell divisions arising from an individual cell should give rise to a predictable distribution of dye amongst the cells progeny. Laser scanning microscopy could be used to quantitate the brightness of each cell within a clone. This technique measures fluorescence according to the same principle as flow cytometry, except that rather than analysing cells suspended in a moving stream of liquid, adherent cells on a microscope slide or culture dish are analysed one by one on a moving microscope stage.

EGFP as a lineage marker

Green Fluorescent Protein has been widely used as a stable marker protein in other organisms and cell types (Gubin *et al.*, 1997; Brand, 1999). In this chapter I confirmed that it is also a good lineage marker for primary human keratinocytes.

Live cell studies

One useful feature of EGFP is that it can be easily detected in living cells, allowing lineage-marked cells to be followed by time-lapse microscopy. EGFP could be used to identify a particular experimentally modified cell within an otherwise wild type culture. For example, the label could identify a cell transduced with a molecules involved in cell-cell adhesion or cell migration. Time-lapse analysis could then be used to follow the speed with which the cell moves through the culture, and whether or not it stably associates with particular neighbours.

Very little is known at present about how stem cells remain quiescent in vivo. EGFP could be used to test whether keratinocyte proliferation can be reversibly suppressed by neighbouring wild type, or genetically modified, keratinocytes. For example, if it is observed that EGFP labelled cells fail to proliferate amongst particular neighbours within a mixed culture, a live-cell FACS sort could be used to extract the labelled cells, thus releasing them from the influence of those neighbours. The labelled cells would then be plated at clonal density in order to assess their full proliferative capacity. The advantage of using lineage markers in this type of experiment is that they allow wild type cells to be examined amongst modified neighbours,

Multi-colour labelling

Several variants of GFP have been engineered that fluoresce at different wavelengths, giving Blue fluorescent protein, Yellow fluorescent protein, and Cyan fluorescent protein (Clontech). A protein containing a red fluorophore has also recently been isolated from a species of Indo-Pacific sea anemone (Clontech). Thus, different populations can be simultaneously labelled with different colours within the same culture. Some potential uses of multi-colour labelling are discussed below.

Controlling for loss of expression of the lineage marker

One problem commonly associated with retroviral transduction of keratinocytes is that the target cells are able to “switch off” expression of the integrated foreign gene (Watt, 1988; Rytina, 1996). This might be due to inactivation of the viral promoter by methylation (Hoeben *et al.*, 1991; Challita and Kohn, 1994). The fact that gene silencing can occur makes it difficult to be sure that any given unlabelled cell is not in fact a descendent of a labelled cell. A second coloured label could be used to control for the possibility of gene silencing. For example the fate of green fluorescent cells could be followed amongst a population of red fluorescent cells instead of amongst a population of unlabelled cells. This type of control might be particularly important in long term grafting experiments, but is less important for the experiments described in this thesis, which never extended beyond a few weeks: I did not see any evidence that keratinocytes lose expression of EGFP within 8 weeks of labelling.

Using EGFP to follow cell fate decisions

Using lineage marking, it becomes feasible to follow the fate of clonal progeny without having to use isolated cells. This makes it possible to study the influence of surrounding cells on cell fate. The fate of the labelled cell can be assessed in two ways. One way is to directly measure proliferation and differentiation in the labelled population by flow cytometry after disaggregation of the mixed culture. I used this approach in experiments described in Chapter four. The other approach is to analyse, within an intact culture, the clones that arise from labelled cells cultured amongst unlabelled neighbours. Examples of these types of experiment are described in Chapters four and five, and are discussed further below.

Clonal analysis

In the experiments described in section 3.2.7, I demonstrated that it is possible to test the self-renewal capacity of clones over the course of 2 weeks within a confluent culture. In principle it should be possible to extend such experiments over many months in cultures that have been stabilised by grafting onto nude mice (Barrandon *et al.*, 1988; Mackenzie, 1997; Kolodka *et al.*, 1998; Levy *et al.*, 1998). This would

provide a more rigorous assay for stem cell fate, since it would measure the regenerative capacity of individual clones over the long term. However, it is not feasible to use such an assay on a routine basis unless a method can be found to stabilise epidermal cultures over the long term without using mice.

Clonal analysis of labelled cells within confluent cultures relies on the assumption that adjacent clonal units will remain distinct from each other. However, in my experiments I saw a surprising lack of cohesiveness between clonal progeny. Labelled cells would frequently migrate away from the parent colony and intermingle with surrounding unlabelled cells. This means that, in order to carry out a reliable clonal analysis, lineage labelled cells need to be seeded far apart from each other within a confluent culture so that adjacent colonies remain clearly separate. In practice this means that large numbers of cultures may be needed in order to analyse significant numbers of labelled clones. This situation could be improved somewhat by using several different coloured markers to distinguish the different clones.

Organisation of the proliferative unit

It is still a matter of speculation whether human keratinocytes migrate away from their clonal siblings within the basal layer of the steady state epidermis in vivo (see sections 1.3.7 and 1.3.10). One viewpoint would be that, by analogy with the mouse Epidermal Proliferative Unit (EPU) model, clonal progeny remain as a discrete coherent unit underlying a single stack of squames, generated by a single central stem cell (Potten and Morris, 1988). This model implies that keratinocytes do not move sideways. An alternative point of view arises from the proposed arrangement of stem cells in clusters, surrounded by an interconnected network of transit amplifying cells, the two cell types therefore residing in adjacent but distinct regions of the epidermal basal layer (Jensen *et al.*, 1999). This model implies that there is lateral movement of transit amplifying cells away from the stem cell clusters.

Although I observed that labelled clones do not remain as cohesive units within a reconstituted epidermis, my experiments never extended for more than 9 weeks and therefore cannot be taken as evidence for the behaviour of clonal progeny in steady state epidermis. Even when epidermis is reconstituted on DED and grafted under the skin of nude mice, it can remain in a hyperproliferative state for at least 8 weeks (Levy *et al.*, 1998). Epidermal cultures reconstituted from mixtures of unlabelled and beta galactosidase- labelled human keratinocytes have been maintained for up to 40 weeks as grafts on mice (Kolodka *et al.*, 1998). During the hyperproliferative phase in the first few weeks the clones did not remain cohesive. However, as hyperplasia gave way to a steady state, the clones resolved into vertical columns that, in most cases, contained no

unlabelled cells within the central core of the column (Kolodka *et al.*, 1998). This might imply that these proliferative units are maintained by a single stem cell. It is notable that the clonal units appear to span at least 10 cell diameters and that labelled cells intermingle with unlabelled cells at the edges of the colonies. These observations are compatible with the idea that transit-amplifying cells may migrate away from the parent stem cell towards the edges of the clonal unit. Interestingly, in similar experiments, labelled mouse keratinocytes give rise to columns of cells that are only 1 cell wide in the upper layers and fewer than 5 cells wide in the basal layer, as would be predicted by the EPU model (Mackenzie, 1997). It would be very interesting to investigate whether this reflects a difference between mouse and human keratinocytes. Although my experiments did not provide any direct evidence regarding the organisation of the proliferative unit in the steady state, they did demonstrate that EGFP will be a useful tool for gathering such evidence in the future.

Intrinsic regulation of cell motility

As discussed above, it seems possible that transit cells move away from the stem cell clusters within steady state human epidermis. This might suggest that transit amplifying cells are intrinsically more motile than their stem cell parents. Alternatively, all basal cells might be equally motile, movements either occurring randomly or being influenced only by extrinsic factors such as the local composition of the basement membrane. It is worth noting that keratinocytes also need to migrate along the basement membrane during wound healing (Martin, 1997). It may be advantageous to ensure that the first cells to repopulate a wounded area are transit-amplifying cells, stem cells only being recruited later in case of need. This is another reason in favour of the prediction that stem cells will be intrinsically more restricted than transit amplifying cells in their ability to move across the basement membrane.

I found that there are intrinsic differences between stem cells and transit amplifying cells in the way that they move amongst their neighbours when they are placed amongst identical surrounding keratinocytes in confluent cultures. Specifically, the colonies founded by stem cells are more cohesive than the colonies founded by transit amplifying cells. This can be accounted for at least in part by differences in motility: stem cells move more slowly than transit amplifying cells when plated as isolated cells on collagen (Jensen *et al.*, 1999). Cell motility depends on the level of integrin expression, and so the higher motility of transit amplifying cells is likely to reflect their lower levels of $\beta 1$ integrin expression (Huttenlocher *et al.*, 1985). However, it is possible that other factors contribute to the difference in motility between stem and transit amplifying cells (Nobes and Hall, 1995; Klemke *et al.*, 1997). Furthermore, clonal cohesiveness will depend not only on cell motility but also on cell-cell adhesion.

Lineage marking can be used to investigate the factors that regulate cell movements and clonal cohesiveness. Experiments of this type are described in Chapter five.

In summary, I have successfully developed EGFP lineage marking in keratinocytes. Using this lineage marker, I have revealed a previously unrecognised lack of cohesiveness amongst a subpopulation of basal keratinocytes (the transit amplifying cells) that is consistent with a model in which stem cells are clustered in discrete islands amongst a sea of intermingled transit amplifying cells.

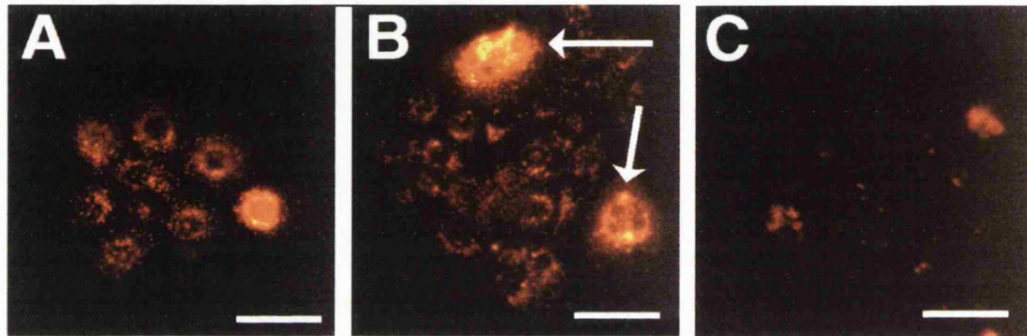


Figure 3.1 PKH as a short-term lineage marker

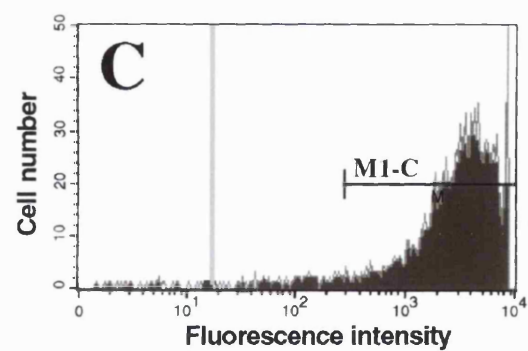
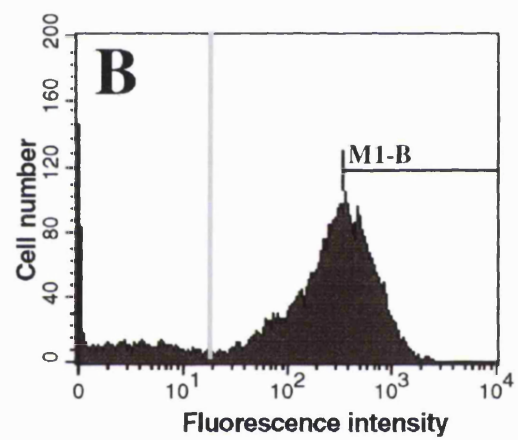
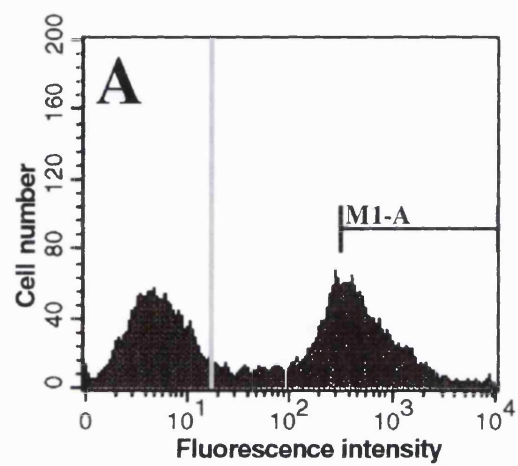
Keratinocytes were stained with 2 μ M PKH and allowed to grow for 3 days (A) 5 days (B) or 7 days (C). The dye becomes diluted as cells divide. Dye is still reliably detectable after 8 cell divisions (A) but becomes barely detectable after 5 days and is undetectable after further cell divisions. Note that the dye remains detectable in the larger differentiated cells that are not actively dividing (arrows in B).

Scale bar: 20 μ m

Figure 3.2 Expression of EGFP packaging cell lines and in keratinocytes

FACS profiles showing EGFP fluorescence. The vertical grey line represents the upper limit of the negative control (cells not transduced with EGFP).

- A:** GPE transfected with pBabe puro-EGFP. The EGFP positive GPE cells were selected by FACS (gate M1-A: top 32%)
- B:** AM12 infected with ecotropic EGFP virus. The EGFP positive AM12 cells were selected by FACS (Gate M1-B: top 33%)
- C:** Keratinocytes infected with amphotropic EGFP virus. Close to 100% of infected keratinocytes were EGFP positive (Gate M1-C: 96.2%).



	% COLONY FORMING EFFICIENCY	% STEM CELL CLONES
K-EGFP	15.1 ± 1.9	50.4 ± 2.9
K-WT	16.3 ± 2.6	48.2 ± 1.6

TABLE 3.1. Effect of EGFP on colony forming efficiency and % stem cell clones.

Keratinocytes transduced with EGFP (K-EGFP) or empty retroviral vector (K-WT) were cultured on a feeder layer of J2-3T3 cells. Data are means ± s.d. from triplicate dishes in a single, representative, experiment. $p > 0.1$ for both %CFE and % stem cell clones: Student's t test.

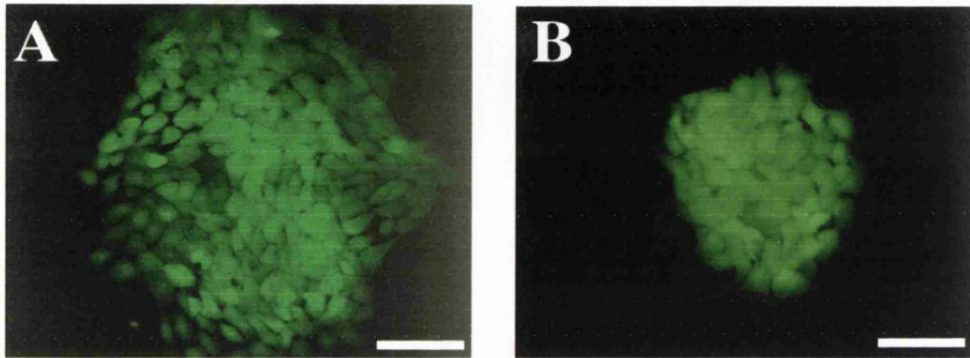


Figure 3.3 EGFP is stably inherited as keratinocytes divide

Keratinocytes infected with EGFP retrovirus .

A: 5 days post infection (Passage 1)

B: 14 days post infection (Passage 2)

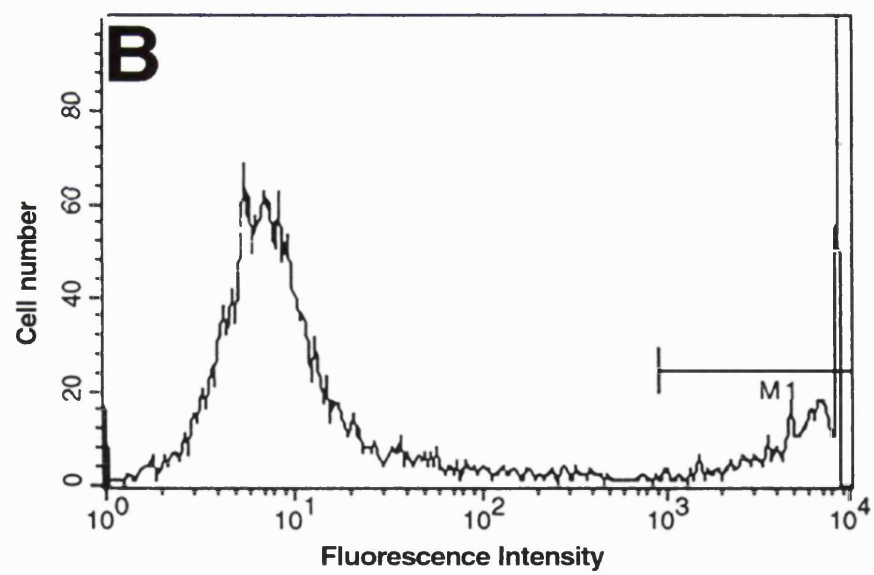
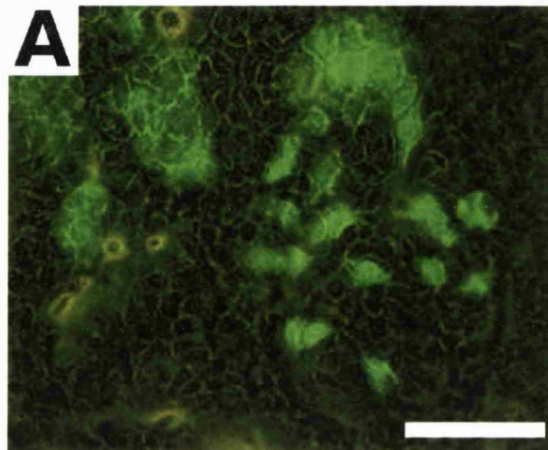
These pictures show individual clones from sparsely seeded cultures. Every cell in the clone is EGFP positive, which indicates that the fluorescent label is stably inherited.

Scale bar 50 μ m.

Figure 3.4 EGFP labelled keratinocytes can be clearly distinguished from unlabelled neighbours within mixed cultures:

A: A single EGFP labelled cell was seeded amongst unlabelled cells at confluence and allowed to grow for 3 days. In this photo, a fluorescence image is shown superimposed on a phase image. All of the EGFP labelled progeny can be easily distinguished from the surrounding unlabelled cells. Scale bar 50µm.

B: EGFP labelled keratinocytes were seeded amongst a fourfold excess of unlabelled cells at a total density of 10^4 cells/cm² and cultured for 5 days. The culture was disaggregated and analysed for GFP fluorescence by flow cytometry. The EGFP positive cells (18.1% of the total population) lie within the region marked M1.



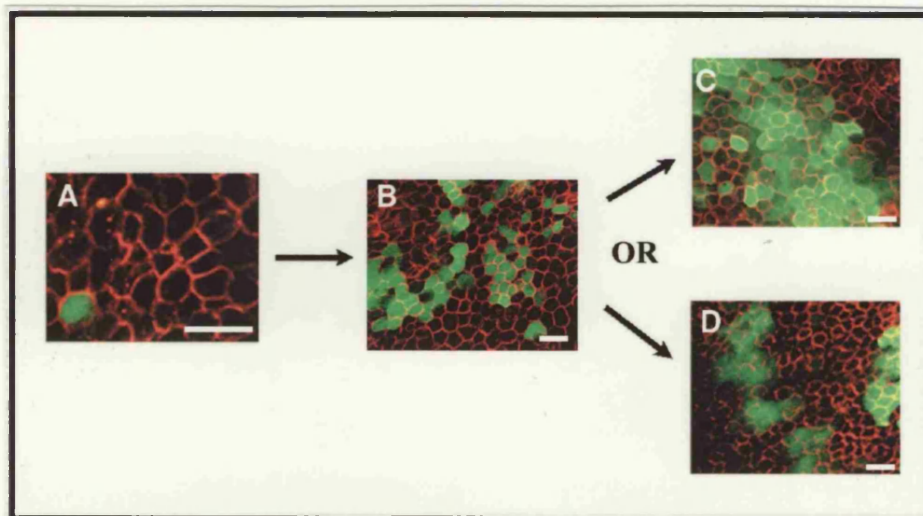


Figure 3.5. EGFP can be used to distinguish self-renewing colonies from abortive colonies within confluent cultures of unlabelled keratinocytes.

Panel A shows a confluent sheet of keratinocytes 24 hours after a single EGFP positive cell had been seeded amongst unlabelled cells. The culture was seeded at a high density (10^4 cells/cm²) so that the culture became confluent after 1 day. Figure 3.5 B-D show different examples of the clones that arose from single EGFP labelled cells after 5 days (B) or 15 days (C, D). These cultures have been immunostained for $\beta 1$ integrin as a marker of the basal cells (Red). Scale bar 50 μ m.

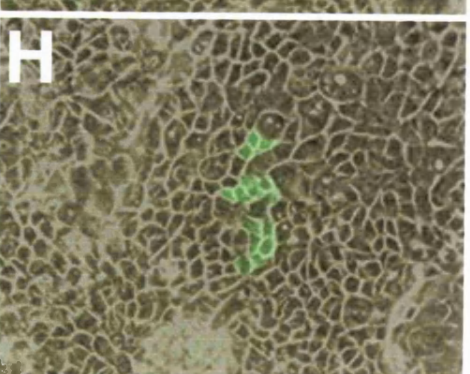
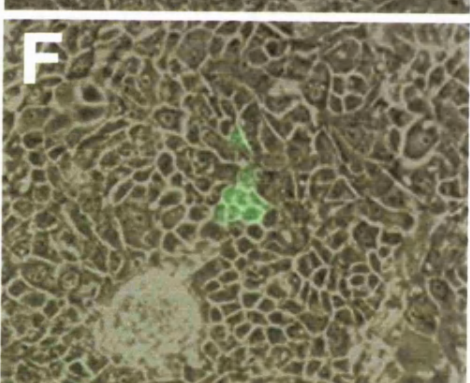
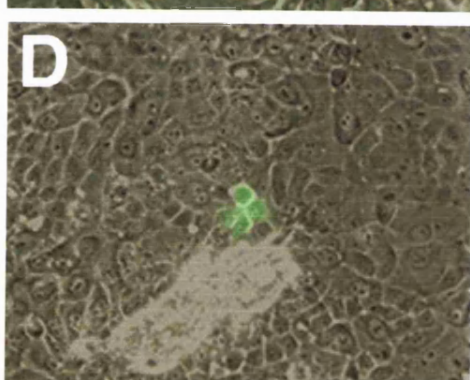
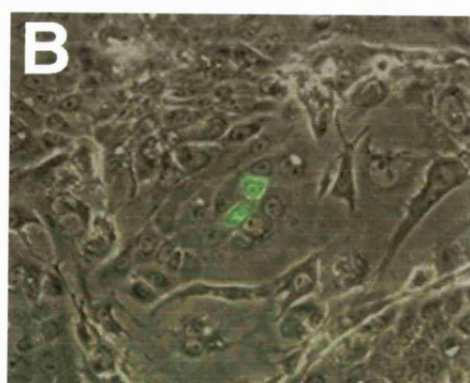
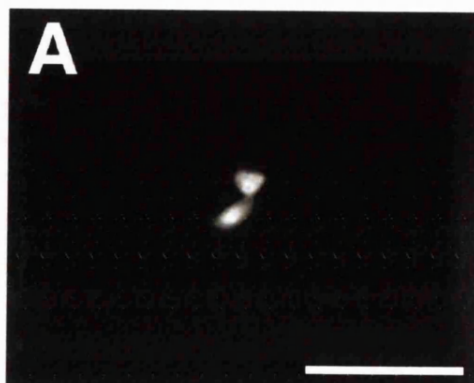
Figure 3.6. EGFP can be used to follow the behaviour over time of living cells within confluent sheets of unlabelled keratinocytes.

This figure shows a selection of still frames taken from a movie of a single EGFP cell dividing amongst unlabelled cells. The culture was filmed by time lapse microscopy starting 24 hours after seeding the culture and continuing for 3 days with exposures at 10-minute intervals.

The pictures shown here were taken at 24 hours (A,B), 48 hours (C,D), 72 hours (E,F) or 96 hours (G,H) after seeding the culture. At each time point a photo was taken under fluorescence excitation to visualise the EGFP (white in A ,C, E, G: green in B, D, F, H) and then immediately afterwards a phase photograph was taken. Panels B, D, and H show the fluorescence image superimposed on the phase image.

All the cells in the culture are in motion throughout the movie. The clonal progeny of the EGFP labelled cell remain cohesive for the first 2 days.

Scale bar 100 μ m.



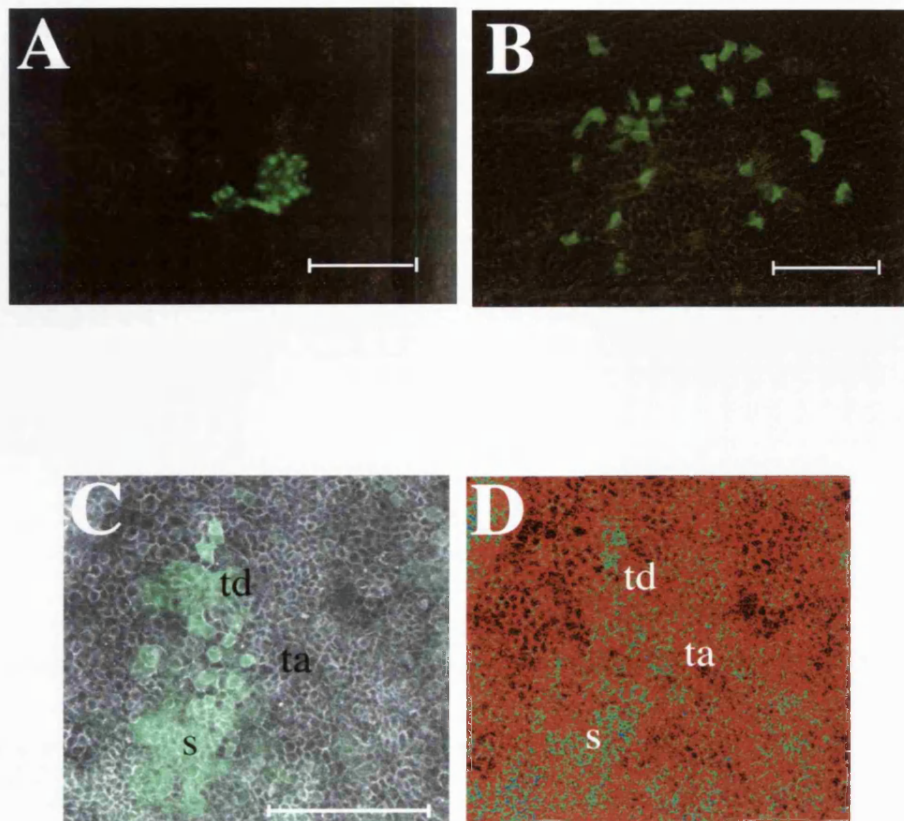


Figure 3.7. Comparing the behaviour of stem cells and transit amplifying cells

This figure shows the clonal progeny of EGFP labelled stem cells (A, C, D) and transit amplifying cells (B) within a confluent sheet 5 days (A,B) or 7 days (C,D) after plating.. The culture in C has been stained for $\beta 1$ integrin (shown in black and white).

In D the $\beta 1$ integrin fluorescence is presented using false colour imaging to quantify the fluorescence intensity. The strongest signals are represented in blue/green and the weakest signals in red. Cells within the clone are interpreted as being stem cells (s), transit amplifying cells (ta) or suprabasal terminally differentiating cells (td).

Scale bars: 10 μ m (A,B), 100 μ m (C,D).

Figure 3.8 Long-term lineage analysis

EGFP labelling can be used to follow the behaviour of stem cell progeny over long periods of time within reconstituted epidermis.

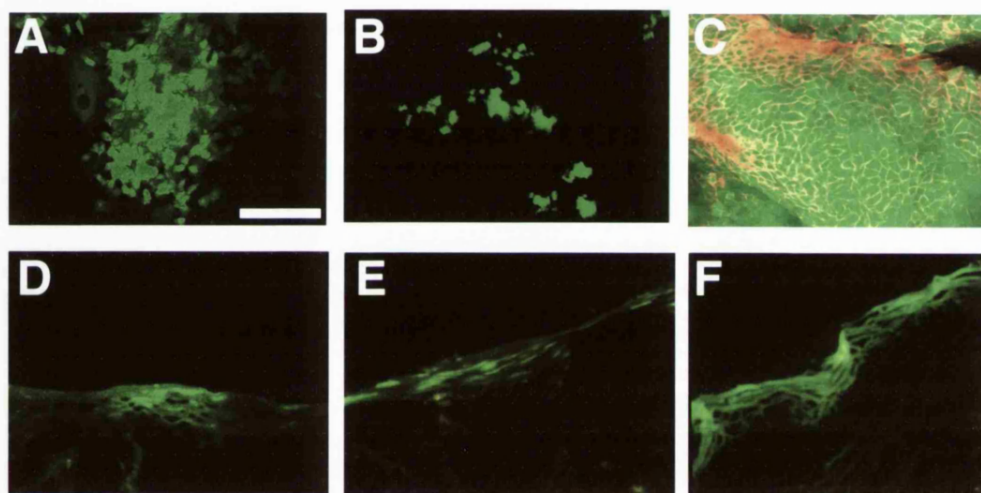
A,B: Examples of the two typical types of EGFP labelled colony that arise from a EGFP labelled cell grown amongst unlabelled neighbours on a de-epidermalised dermis for 3 weeks. Photograph taken from above of an intact epidermal sheet.

C: Control: to show that EGFP continues to be expressed in all cells over the course of the experiment. The experiment is the same as described in A,B except 100% of the seeding population were labelled with EGFP. The final culture has been counterstained in red for $\beta 1$ integrin (appears yellow), to allow each basal cell to be distinguished from its neighbours. None of the cells have lost the EGFP label. Photograph taken from above of an intact epidermal sheet

D,E: Examples of the two typical types of EGFP labelled colony that arise from a EGFP labelled cell grown amongst unlabelled neighbours on a de-epidermalised dermis for 9 weeks. Photograph taken from a lateral histological section of the epidermis.

F: Control: to show that EGFP continues to be expressed in all cells over the time of the experiment. The experiment is the same as described in D,E except 100% of the seeding population were labelled with EGFP. Photograph taken from a lateral histological section of the epidermis.

The DEDs in D-F were grafted onto nude mice 10 days after seeding, in order to stabilise the reconstituted epidermis over the time of the experiment.



CHAPTER 4

THE INFLUENCE OF NOTCH/DELTA SIGNALLING ON KERATINOCYTE DIFFERENTIATION.

4.1. Introduction

Keratinocytes must communicate with each other to ensure that number of stem cell daughters that differentiate is balanced by the number that remain as stem cells (Jones *et al.*, 1995). However, we do not know the nature of the signals that mediate this intercellular communication. In Chapter 3 I showed that EGFP can be used as a lineage marker to distinguish stem and transit amplifying colonies within a confluent culture. This makes it possible for the first time to examine signals between cells that might regulate cell fate.

As discussed in Chapter 1 Notch signalling is a good candidate to be such a signal. I therefore set out to investigate whether Notch signalling might play a role in controlling the number or spatial distribution of stem cells in interfollicular human epidermis. My first objective was to find out whether Notch and its ligands are expressed in the relevant cell compartments in human epidermis. I then went on to construct retroviral vectors, which I used to manipulate Notch activity in primary human keratinocytes. Notch has two classes of ligands: the Delta family and the Serrate family. The first part of this chapter deals with Delta. I found that Delta/Notch signalling promotes differentiation of keratinocytes at the boundaries between regions of high and low Delta1 expression. In the second part of this chapter I briefly describe my attempts to detect Serrate1 and to manipulate its function in keratinocytes. I was not able to reach any conclusions about the function of Serrate/Notch signalling in epidermal keratinocytes.

4.2. Results

4.2.1. Expression of Delta1 and Notch1 by human keratinocytes

Degenerate primer PCR was carried out by Phil Jones in the lab to identify Delta homologues expressed by cultured human keratinocytes. Six out of six clones sequenced corresponded to human Delta1 (Gray *et al.*, 1999) (Figure 4.1). Neither of the two other members of the vertebrate Delta family, Dll3 or Dll4, were detected.

In situ hybridisation of sections of human foetal and adult skin, carried out by the ICRF in situ hybridisation service, showed that Delta1 mRNA was confined to the basal layer of the epidermis and absent from the underlying dermis (Figure 4.2 A-D). Expression within the basal layer was not uniform; rather, there were groups of cells that had higher levels of mRNA than their neighbours and these groups tended to lie at the tips of the dermal papillae, where the underlying dermis comes closest to the surface of the skin. In situ hybridisations for Notch1 were not performed because good monoclonal antibodies were already available: The Delta1 in situ hybridisations were carried out at a time when the Delta1 antibody had not yet been made.

Immunofluorescence labelling of neonatal and adult epidermis with a polyclonal antibody to chick Delta1 also showed expression confined to the basal layer. The staining intensity varied within the basal layer in the same way as found by in situ hybridisation (Figure 4.2E)

In contrast to Delta1, Notch1 protein was detected in all the living layers of the neonatal and adult epidermis, the staining being weak in the basal layer and most intense in the suprabasal layers (Figure 4.2F). Two different monoclonal antibodies against Notch1 (Tan20 and Tan18) gave identical staining patterns. Notch 2 was barely detectable in human epidermis by immunostaining (not shown). Notch 3 and 4 were not examined.

This expression pattern is consistent with Northern blots of Notch1 and Delta1 expression in cultured human epidermal keratinocytes, carried out by Phil Jones (Figure 4.3). Populations were enriched for stem cells (S), transit amplifying cells (TA) or suprabasal, terminally differentiating cells (TD) by differential adhesiveness to type IV collagen as described in section 2.2.6. Blots were probed for involucrin, a marker of terminal differentiation that is selectively expressed in suprabasal keratinocytes (Jones and Watt, 1993) and, as a loading control, for 18S RNA. Two transcripts

(approximately 4.0 and 4.6 kb) were detected with a probe to human Delta1, potentially reflecting differences in polyadenylation (Gray *et al.*, 1999). Delta1 mRNA was detected in stem and transit amplifying cells, but was virtually undetectable in terminally differentiating cells. When the Delta1 signal was quantitated relative to the 18S RNA loading control, the mRNA was approximately two fold more abundant in stem cells than transit amplifying cells (1.7 fold in Figure 4.3A; average of two fold in three independent batches of keratinocytes), consistent with the in situ hybridisation results in Figure 4.2 A-D. Notch1 mRNA (transcript size approximately 10kb) was detectable in stem cells, but was upregulated in terminally differentiated keratinocytes (Figure 4.3B),

4.2.2. Retroviral transduction of keratinocytes

There are two widely used approaches to manipulating Notch function. One is to misexpress constitutively active or dominant negative forms of Notch (Rebay *et al.*, 1993), another is to misexpress normal or dominant negative forms of the ligands that activate Notch (Henrique *et al.*, 1997). I took the second approach, with the view that activation of Notch by the ligand might be more physiologically relevant than using constitutively active Notch mutants, and that Notch might respond differently to different ligands.

Construction of retroviral vectors

cDNAs were obtained from Isabelle Le Roux (Developmental Genetics Lab ICRF) and Jenny Dunne (Lymphocyte Activation Lab, ICRF) that encode full length mouse Delta1 (bp 14 to 2190) and a truncated mutant, DeltaT, lacking all but 13 of the amino acids in the intracellular domain (bp 14 to 1756). This mutants act as a dominant negative inhibitor of Notch activation, rendering cells unable to respond to Delta signals from their neighbours (Henrique *et al.*, 1997). Mouse Delta1 shows close homology to human Delta1 (Figure 4.1)

A DNA binding mutant of *Xenopus* Suppressor of Hairless, SuH^{DBM}, was obtained from Chris Kintner (The Salk Institute, La Jolla). This mutant acts as a dominant negative inhibitor of the Notch signalling pathway since it binds to activated Notch but is unable to bind DNA and so cannot activate transcription of target genes (Wettstein *et al.*, 1997). This mutant was modified by Isabelle Le Roux (ICRF) using PCR to include an HA tag at the 3' end.

I subcloned these cDNAs into a high titre, direct orientation retroviral vector, pBabe puro (see section 2.5.5.). For the Delta1 vector I constructed both untagged and IRES-

EGFP tagged versions. The IRES encodes an internal ribosomal entry site: the Delta1-IRES-EGFP is therefore transcribed as one continuous strand of mRNA but is translated as two separate proteins (Pear *et al.*, 1998). As a control I constructed a vector containing only the IRES-EGFP.

The IRES-EGFP tagged version of the construct was used for infection of 3T3-J2 cells. The tag was needed because 3T3-J2 cells tended to lose expression of Delta1 over time, even though they did not lose puromycin resistance. Cells that lose Delta1 expression will also lose EGFP expression, and so the tag allows the Delta1-negative population to be eliminated by FACS sorting on the basis of EGFP fluorescence. It is not clear why J2 fibroblasts tend to lose expression of Delta1, but this seems to be a problem specific to the Delta1 protein since other proteins, for example EGFP, can be stably expressed by J2 cells without any need of further selection. Keratinocytes maintained good expression of Delta1 over several passages, as monitored by immunostaining, without the need for further selection, and so the EGFP tag was not necessary and untagged constructs were used in all keratinocyte infections.

Construction of retroviral producer cells

Both Ecotropic retroviral producer cell lines, GPE, and amphotropic producer lines, AM12, were generated (see section 2.1.5). Although clonal cell lines were made for the Delta AM12 producer cells, none of the 24 screened by immunostaining had expression levels that were as high as the polyclonal population, and so polyclonal lines were used for all infections (Figure 4.4 A-B). For the IRES-EGFP tagged ecotropic producer cells, the population with the top 20% EGFP expression was selected by FACS. Immunostaining with the anti-Delta1 antibody confirmed that every EGFP positive cell was positive for Delta1 (Figure 4.4 D). This virus was only used for infecting J2-3T3 cells, which are mouse cells, and so it was not necessary to make amphotropic virus producer cells. Clones of SuH^{DBM} AM12 virus producer cells were screened by immunostaining for the HA tag. One of two selected clones is shown in Figure 4.4 F. Only early passages of retroviral producer cells were used for infection of primary human keratinocytes and J2-3T3 cells.

Transduction of human epidermal keratinocytes with Delta retroviruses

The standard protocol used to transduce primary human epidermal keratinocytes with retrovirus is to co-cultivate the keratinocytes with amphotropic producer cells which have been treated with mitomycin C (Rheinwald and Green, 1975). I found that Delta producer cells tended to promote differentiation of co-cultivated keratinocytes, making it very difficult to obtain actively growing infected populations. A likely explanation for this, in the light of the findings described later in this chapter is that Delta1 protein

on the surface of the producer cells activates the Notch pathway in adjacent keratinocytes. I overcame this problem by using retroviral supernatant to infect keratinocytes. The proportion of cells transduced in this way was close to 100%, both on first infection and in subsequent passages. Immunofluorescence was used to compare the level and subcellular localisation of transduced and endogenous Delta1 (Figure 4.5)

When overexpressed, Delta1 and DeltaT accumulated at cell-cell borders (Figure 4.5 A,B). The antibody that we used was raised against a peptide region of Delta1 that is highly conserved between mouse and human (this region is highlighted on the Delta1 sequence alignment in Figure 4.1) and could also therefore be used to detect the endogenous protein (Figure 4.1E). In empty vector infected keratinocytes, endogenous Delta1 was weakly expressed and was localised in a punctate distribution at cell-cell borders (Figure 4.5 C). The overexpressed proteins therefore accumulated at the same cellular location as the endogenous protein.

4.2.3. Effects of uniformly overexpressed Delta1 or DeltaT on keratinocyte growth and terminal differentiation.

Clonegenicity assays

In order to find out how keratinocytes respond to uniform overexpression of Delta1, I compared keratinocytes overexpressing Delta1, or the dominant negative mutant DeltaT, or empty retroviral vector. Keratinocytes stably transduced with each construct were seeded at clonal density on a wild type J2-3T3 feeder layer. 14 days later the dishes were fixed and stained with rhodanile blue (Figure 4.6). For each construct two parameters were measured: colony forming efficiency (CFE) and % colonies attributable to stem cell founders (section 2.2.3) (Zhu and Watt, 1999). Delta1 expression did not significantly increase CFE ($p > 0.1$) or the proportion of stem cell clones ($p > 0.1$) in three separate experiments (Figure 4.6 A,B; compare K-WT and K-DELTA in Table 4.1). DeltaT caused an increase in CFE ($p < 0.05$) but did not increase the percentage of stem cell clones ($p > 0.1$) (Figure 4.6 C; K-DeltaT in Table 4.1).

FACS analysis of $\beta 1$ integrin expression

Another way to distinguish stem cells from transit amplifying cells is that stem cells express twofold higher levels of $\beta 1$ integrin on their cell surface (Jones and Watt, 1993). I therefore examined surface levels of $\beta 1$ integrins. Subconfluent populations of keratinocytes expressing empty vector, Delta1 or DeltaT were immunolabelled with an

antibody specific for the extracellular domain of human $\beta 1$ integrin (CD29 FITC) and the basal populations of cells, as judged by forward and side scatter, were analysed by flow cytometry. Uniformly expressed DeltaT had no effect on surface $\beta 1$ integrin levels in subconfluent cultures (Figure 4.7 A,B). Uniformly expressed Delta1 also had no effect on surface $\beta 1$ integrin levels in cultures up to three weeks post infection (Figure 4.7 A). However, after being in culture for longer than this, K-DELTA often had lower $\beta 1$ integrin levels than K-WT (Figure 4.7 B).

FACS analysis of terminal differentiation

The degree of terminal differentiation in a population of keratinocytes can be evaluated by comparing the expression of a terminal differentiation marker, involucrin, by FACS analysis. There was an increase in terminal differentiation in K-DELTA but not in K-DeltaT (Figure 4.8).

Growth curves

When the total number of cells per dish was compared at intervals for up to 25 days after plating, there was little difference between the growth rates of K-WT, K-DELTA and K-DeltaT and all three populations reached the same confluent density (Figure 4.9).

Epidermis reconstituted on DEDs

Epidermis reconstituted on DEDs from K-DELTA had morphology very similar to the epidermis that was reconstituted from K-WT, both resembling normal human epidermis in vivo (Figure 4.10 A,B). In contrast, epidermis reconstituted on DEDs from K-DeltaT had an abnormal morphology. The cells in the upper layers failed to flatten, the cornified cells were nucleated and there were many gaps between the cells (Figure. 4.10 C). One explanation for this defect could be that DeltaT delays the onset of the terminal differentiation program or that it blocks some stage of keratinocyte maturation. However, I found that K-DeltaT undergo suspension induced terminal differentiation at the same rate as K-WT (section 2.2.5: data not shown) and that cornifin and filagrin, markers of the later stages of terminal differentiation, were expressed at comparable levels in postconfluent cultures of K-DeltaT and K-WT according to FACS analysis (data not shown).

The abnormal morphology of the K-DeltaT DED cultures might represent a defect in homeostasis that is not apparent within subconfluent cultures. It might also be that the defect seen in the DEDs represents a defect in cell morphology or adhesion rather than a delay or a block in the differentiation programme. The effect of Delta1 on cell morphology and adhesion is discussed in Chapter 5.

Uniform Delta expression versus boundaries of Delta expression

The results I have described so far indicate that when Delta1 or DeltaT are uniformly overexpressed, they have little effect on keratinocytes growth or differentiation in the short term. However, K-DeltaT appear to have a defect in differentiation that manifests itself when epidermis is reconstituted on DEDs from K-DeltaT. Also, K-DELTA cultures did show some tendency to contain more differentiating (Figure 4.7) or differentiated (Figure 4.8) cells than controls, particularly after longer time in culture. Delta1 expression in these later cultures tends to become more heterogeneous and can no longer be considered to be uniform.

It has been observed in other tissues that uniform expression of Delta1 fails to activate Notch signalling, but that Notch can become activated in a cell when the amount of Delta1 on its surface falls beneath a certain threshold whilst levels of expression of Delta1 on its neighbours remain high: Notch signalling is active at cell boundaries in several developmental contexts (Bray, 1998) (see 1.4.7). Therefore while uniform overexpression of Delta1 did not affect the proportion of stem cells it seemed possible that a wild type keratinocyte would respond to high Delta1 expression on a neighbouring cell. Since human keratinocytes are routinely cultured on a feeder layer of J2-3T3 cells, then a simple way to create a boundary between cells expressing different levels of Delta1 was to culture wild type keratinocytes (K-WT) with feeder cells that had been transduced with Delta1 (J2-DELTA).

4.2.4. Engineering feeder cells to express Delta1

J2-3T3 cells were transduced with Delta1-IRES-EGFP virus or with control IRES-EGFP virus, and EGFP-positive cells were then selected by FACS (Figure 4.11A,B,C,D). Delta1 expressed on the surface of J2-DELTA was frequently concentrated at sites of contact with neighbouring keratinocytes (Figure 4.11E: note that in Figure 4.11E, J2 cells were used that contain the untagged version of Delta1 (i.e. no EGFP) whilst keratinocytes were labelled with the EGFP retrovirus described in chapter 3, so they would be visible in the picture). Delta1 was undetectable by immunofluorescence in untransduced J2-3T3 (Figure 4.11 C).

J2-DELTA markedly decreased both CFE and % stem cell clones ($p < 0.05$) (K-WT + J2-DELTA compared with K-WT + J2-WT; Figure 4.11 F,G; Table 4.2). When keratinocytes were removed from J2-DELTA after 7 days and replated on normal J2-3T3 (J2-WT) they did not regain proliferative capacity (CFE for keratinocytes removed from J2-DELTA: $4 \pm 1\%$; for keratinocytes removed from J2-WT: $20 \pm 1\%$). As a negative control I used DeltaT to render the keratinocytes unresponsive to Delta signals

from neighbouring cells. When keratinocytes expressing DeltaT were cocultured with J2-DELTA, CFE was similar to the control combination of K-WT + J2-WT (Table 2) and % stem cell clones was increased ($p < 0.05$).

The experiments described in this chapter so far show that when Delta1 was uniformly expressed in a population of keratinocytes there was little effect on the proportion of stem cells: the experiments in the present section showed that when keratinocytes were exposed to neighbouring cells with high levels of Delta1 proliferative potential was strongly and irreversibly reduced.

4.2.5. Creating boundaries between keratinocytes expressing high and low levels of Delta1

The patchy Delta1 expression pattern in the epidermal basal layer (Figure 4.2, 4.3) and the results of the experiments described above might indicate that Notch/Delta signalling would tend to decrease proliferative potential and stimulate terminal differentiation at the interface between keratinocytes expressing high and low levels of Delta1. In order to examine this, I transduced keratinocytes with a retroviral vector encoding EGFP and used EGFP as a lineage marker in confluent cultured sheets of keratinocytes (see Chapter 3). Epidermis reconstituted in this way has the same organisation as epidermis in situ, with stem and transit amplifying cells in the basal layer and terminal differentiation occurring in the suprabasal layers (Watt, 1988). The fate of individual EGFP expressing cells was monitored in the four types of mixing experiment shown in Figure 4.12 A-D.

In (A) an EGFP labelled wild type keratinocyte (K-WT) is exposed to wild type neighbours.

In (B) an EGFP labelled wild type keratinocyte is exposed to keratinocytes transduced with Delta1 (K-DELTA).

In (C) an EGFP labelled keratinocyte expressing DeltaT (K-DeltaT) is exposed to keratinocytes transduced with Delta1; this acts as a negative control because expression of DeltaT renders cells unresponsive to Delta signals from neighbouring cells. In some experiments a DNA binding mutant of Suppressor of Hairless (SuH^{DBM}) was also included as an alternative method of blocking the Delta response (Wettstein *et al.*, 1997)

In (D) an EGFP labelled keratinocyte expressing Delta1 (K-DELTA) is exposed to neighbouring cells that also overexpress Delta1 (K-DELTA) and thus there is uniform high Delta1 expression throughout the culture. Figure 4.13E shows an example of an EGFP labelled wild type keratinocyte surrounded by unlabelled K-DELTA, shown 24 hours after plating.

4.2.6. Localisation of Delta1 in the mixing experiments

Delta1 localised preferentially at cell borders that were in contact with other cells that overexpressed Delta1, rather than at borders between K-WT and K-DELTA (Figure 4.13 A). EGFP marked wild type cells in contact with K-DELTA frequently showed punctate cytoplasmic staining for Delta1 (Figure 4.13 B). Double labelling for Delta1 and a lysosomal marker indicated that cytoplasmic Delta1 was primarily in lysosomes (Figure 4.13 C). The majority of Delta1 in the lysosomes must have come from the neighbouring cell because very little lysosomal Delta1 was detected when two wild type cells were in contact. These observations fit well with the finding that in *Drosophila* Delta is rapidly cleared from the cell surface by endocytosis (Kooh *et al.*, 1993) and uptake of Delta from neighbouring cells by transendocytosis is part of the Notch signalling process during cell fate determination (Seugnet *et al.*, 1997; Klueg and Muskavitch, 1998).

4.2.7. Delta1 promotes terminal differentiation of neighbouring keratinocytes

Number of EGFP labelled clones persisting in the cultures after 2 weeks

The fate of EGFP marked keratinocytes in the mixing experiments was monitored for up to 15 days after plating. Once all the progeny of a transit-amplifying cell have undergone terminal differentiation, they are shed from the outermost layer of the epidermis. In contrast, stem cell progeny can persist in the basal layer for long periods of time (see chapter 3: (Potten and Morris, 1988; Watt, 1998). Thus if Delta1 induces neighbouring keratinocytes to leave the stem cell compartment there should be a progressive loss of EGFP marked clones. This was quantified by seeding cells at confluent density with a ratio of 1 EGFP positive cell to 500 unmarked cells, thereby ensuring that groups of EGFP positive cells were clonally derived.

After 5 days there was no significant difference between the total number or size of colonies arising from wild type cells surrounded by wild type neighbours (CFE 47.5%; mean number of cells per colony 7.2) or wild type cells surrounded by K-DELTA neighbours (CFE 57.1%; mean number of cells per colony 7.6). However, very few (none in the experiment shown in Table 4.3) clones of wild type cells persisted for 15 days when they were surrounded by K-DELTA neighbours ($p < 0.05$). The number of clones remaining at day 15 was equal when wild type cells were surrounded by wild type neighbours, when K-DELTA cells had K-DELTA neighbours or when K-DeltaT had K-DELTA neighbours (Table 4.3).

Analysis of EGFP cells by flow cytometry

It is not feasible to analyse the fate of large numbers of EGFP marked cells within confluent sheets using the type of clonal analysis described in the previous section. However, large numbers of cells can instead be analysed as a population using flow cytometry. I increased the ratio of marked to unmarked cells to 1:10 and used flow cytometry of disaggregated cultures to determine the proportion of EGFP expressing cells that were in the basal layer or were undergoing terminal differentiation. Basal and suprabasal (differentiating) keratinocytes can be distinguished on the basis of forward and side scatter (Jones and Watt, 1993). In postconfluent sheets of wild type keratinocytes the proportion of terminally differentiated cells is approximately 50-60% (57% in the experiment described in table 4.5). For these experiments I included the DNA binding mutant of Suppressor of Hairless (see section 4.2.5) as a second negative control in addition to the DeltaT mutant.

At day 5 there was no significant difference between the number of EGFP-positive basal K-WT in combination with unmarked K-WT ($7.8 \pm 0.5 \times 10^4$) or in combination with unmarked K-DELTA ($10.2 \pm 0.3 \times 10^4$) and no difference in the proportion of terminally differentiated cells ($47.1 \pm 2.4\%$ with K-WT; $54.5 \pm 1.8\%$ with K-DELTA). However, at day 15 the number of EGFP-marked basal cells was significantly reduced when EGFP marked K-WT were in contact with K-DELTA, declining to 0.5×10^4 from a seeding density of 10^4 . In all other cell combinations the number of EGFP-marked basal cells increased during the same period (Table 4.4)

Corresponding to the decrease in basal cells at day 15 there was an increase in the proportion of terminally differentiating cells in the K-WT-EGFP + K-DELTA combination ($p < 0.05$). I confirmed that the increased number of cells with high forward and side scatter correlated with induction of involucrin expression: $80 \pm 5\%$ of wild type cells exposed to K-DELTA were involucrin positive, compared with $62 \pm 4\%$ of wild type cells exposed to wild type neighbours. In contrast there was no significant difference between the percentage of terminally differentiated cells in the other combinations ($p > 0.1$ in table 4.5). The results suggest that Delta1-expressing cells did not suppress proliferation or induce wild type cells to undergo terminal differentiation directly (day 5 results), but rather promoted entry into the transit amplifying compartment, the cells dividing a small number of times prior to terminal differentiation and eventual loss from the cultures (day 15 results).

Morphology of EGFP clones in the mixing experiments

The morphological appearance of the EGFP marked clones is shown in Figure 4.14. This illustrates that on day five the wild type clones with K-DELTA neighbours were of a similar size to the clones in the other mixing experiments (Figure 4.14 A,C,E,G,I). However by day 15 the wild type clones with K-DELTA neighbours were small, with all the cell having a terminally differentiated (i.e. large, suprabasal) morphology as would be predicted for clones that are being deleted from the cultures through terminal differentiation (Figure 4.14 D). There was no evidence of cells undergoing nuclear fragmentation, which would have been indicative of apoptosis, in any of the mixing experiments.

There were differences in the morphology of DeltaT and DeltaI expressing clones. Clones of K-DeltaT tended to be more scattered at day 15, intermingling more freely with their unlabelled neighbours, than clones of K-DELTA, K-SuH^{DBM}, or wild type cells (Figure 4.14 F: compare with B, H, J). Note that this is why the colony shown in Figure 4.14 F appears to contain relatively few cells: these colonies scatter over a large distance and so many cells are outside the field of the photo (see Table 4.4. for quantification of the total number of basal EGFP cells). Figure 4.15 shows the difference between wild type and DeltaT clones when they are cultured amongst K-DELTA. Cultures in Figure 4.15 A,C,E have been stained for $\beta 1$ integrin as a marker of the basal cells, and cultures in Figure. 4.15 B,D,F are shown at low magnification. Figure 4.15 C (equivalent to the culture shown in 4.15 F) illustrates that the K-DeltaT-EGFP colonies contain basal cells that freely intermingle with unlabelled cells, and so they scatter over some distance. In contrast Figure 4.15 C,D (equivalent to the culture shown in 4.14 D) shows that the EGFP cells are not in the basal layer but are large, flat, terminally differentiated cells lying in the suprabasal layers of the culture.

Number of EGFP labelled clones persisting in the cultures after 3 weeks

Confluent cultures are more stable over the long term when they are grown on decellularised deepidermalised dermis (DED cultures) rather than on tissue culture plastic (Rikimaru *et al.*, 1997). Some mixing experiments were therefore extended to three weeks on DED cultures (Figure 4.16). This confirmed the results shown in Table 4.3 and Figure 4.14: EGFP labelled wild type cells exposed to K-DELTA neighbours never formed colonies that were retained in the cultures for three weeks. (0 colonies on 6 independent DED cultures): only remnants of EGFP colonies were seen on the surface of the DED (Figure 4.17 B). In contrast, large EGFP colonies persisted in the culture for at least three weeks when K-WT-EGFP were combined with K-WT (Figure. 4.16A: average of 4.1 colonies on each of 6 independent DED cultures) or K-DELTA-EGFP were combined with K-DELTA (Figure 4.16C: average of 5.2 colonies on each

of 3 independent DED cultures). Unfortunately the numbers of EGFP colonies are small in these experiments because the EGFP cells need to be seeded very sparsely to ensure that any EGFP colonies are clonally derived. Also, it is not possible to completely disaggregate and extract all of the keratinocytes from these cultures, which precludes FACS analysis and cell counting. This means that the data gathered from these experiments do not approach statistical significance.

4.2.8. Expression of Numb in human epidermis

From the experiments described so far in this chapter, I concluded that Delta/Notch signalling promotes differentiation in neighbouring cells. I also concluded that cells that express high levels of Delta1 themselves are protected from Notch signals: this might explain how stem cells *in vivo* are protected from Notch-induced differentiation. However, there are other proteins that can act autonomously to inhibit Notch activity, and it is possible that they may protect keratinocyte stem cells from differentiation *in vivo* (section 1.4.5). One of these proteins is Numb.

Numb is an intracellular protein that acts cell autonomously to inhibit Notch activation. It is used as a localised fate determinant during the stereotyped asymmetrical cell divisions of *Drosophila* neurogenesis (Jan and Jan, 1998). Staining for Numb in dividing neuroblasts shows a characteristic “crescent” and one end of the cell. It is not known whether Numb is always used as a localised fate determinant, or if also acts as a Notch inhibitor in other contexts, for example in tissues where stem cell division is not stereotypically asymmetric, such as in the epidermis (Watt and Hogan, 2000). One hypothesis would be that Numb is expressed in the stem cell clusters to protect them from Notch-mediated differentiation signals.

I stained sections of human epidermis with a polyclonal antibody raised against chick Numb (Figure. 4.17 A,B) (Wakamatsu *et al.*, 1999). According to this staining, Numb is localised to cell-cell contacts in a punctate distribution, reminiscent of the distribution of Notch1 and Delta1. Numb seems to be expressed in all cells of the epidermis and is distributed evenly around the membrane of each cell, with the exception of the basal layer where it is only on the apical and lateral membranes. There is no evidence for a localised Numb “crescent” in any cell, nor of any variation in Numb expression in different regions of the basal layer. In sections from some individuals there were periodic basal cells that stained very brightly with the Numb antibody (Figure. 4.17A: arrow). These cells always had a characteristic morphology, being attached to the basal layer by a thin stalk of cytoplasm with the main body of the cell reaching into the first

suprabasal layer. Cultured keratinocytes also express Numb at cell-cell contacts: again there was no evidence that Numb was preferentially inherited by any particular cells within dividing colonies (Figure 4.17 C, D).

4.2.9. Expression of Serrate1 by human keratinocytes

Serrate1 is another Notch ligand that is known to be expressed in epidermis (see section 1.3.10). Northern blots, carried out by Phil Jones, suggest that Serrate1 is expressed in primary keratinocytes and that it becomes transiently upregulated in transit amplifying cells then downregulated upon terminal differentiation (not shown). This suggests that Serrate1 might have a complementary expression pattern to Delta1: Delta1 in the stem cell compartment and Serrate1 in the transit amplifying compartment. This would be reminiscent of the expression patterns of Delta and Serrate in other tissues where Notch signalling establishes boundaries. For example, in the *Drosophila* imaginal discs Delta is expressed in an adjacent compartment to Serrate, and Notch is activated at the boundary between the two compartments (de Celis *et al.*, 1996).

Unfortunately further studies of Serrate1 in human epidermis have been hampered by a lack of antibodies that recognise human Serrate1. None of the available Serrate antibodies stain human epidermis: nor do they stain positive-control sections of retrovirally infected chick embryos that miss-express human Serrate1, as confirmed by in situ hybridisation. My attempts at in situ hybridisation for Serrate1 in human epidermis were not successful.

4.2.10. Retroviral transduction of keratinocytes with Serrate

In the hope that this problem would be overcome during the course of the project, I set out to manipulate Serrate1 function in human keratinocytes using the same approach that I described for Delta1. I constructed retroviral vectors containing cDNAs for full length human Serrate1 (bp361 to 4027) and a truncated mutant, SerrateT (bp261 to 3667). This mutant is predicted to act as a dominant negative in a similar way to the Delta mutant described in section 4.2.2, rendering cells unable to respond to Serrate signals from their neighbours (Rebay *et al.*, 1993). Both of these cDNAs were obtained from Isabelle Le Roux, Developmental Genetics lab, ICRF. I added an IRES-EGFP tag to both of these constructs to allow transduced cells to be identified. As a control I constructed a vector containing only the IRES-EGFP. I subcloned these cDNAs into a high titre, direct orientation retroviral vector, pBabe puro (section 2.5.5).

Virus packaging cell lines were constructed as described in 2.1.5. The ecotropic GPE packaging cells were selected by FACS to eliminate EGFP negative cells before collecting virus to infect AM12 (Figure. 4.18 A-C). It was not possible to select AM12 populations using FACS, because amphotropic Serrate1 virus cannot be removed from the virus isolation lab for safety reasons.

4.2.11. Serrate1 and SerrateT inhibit keratinocyte growth.

Initially I followed the standard protocol of keratinocyte infection by coculture with mitomycin treated virus producer cells followed by puromycin selection (section 2.1.5). It proved very difficult to obtain actively growing cultures from keratinocytes that had been infected with either Serrate1 or SerrateT. In contrast, keratinocytes that had been infected with empty pBabe-IRES-EGFP vector founded healthy actively growing EGFP positive populations (not shown). It was possible either that the Serrate1 producer cells themselves might be inhibiting keratinocyte growth, or else that keratinocytes were not successfully infected and so were killed by puromycin. I excluded both these possibilities in subsequent experiments (Figure. 4.18 D-F): I seeded 10^4 normal keratinocytes (kq4) per 10cm dish on a normal mitomycin treated J2 feeder layer and infected the cells with viral supernatant rather than by coculture with AM12 cells. The cells were cultured for 2 weeks in the absence of puromycin selection before fixing the dishes and staining with Rhodanile blue. There is a clear reduction in actively growing colonies in cells infected with Serrate1 (Figure 4.18 D) or SerrateT (Figure 4.18 E) in comparison with cells infected with EGFP only (Figure 4.18 F).

Quantitative comparisons between Serrate1 and SerrateT cannot be made from this experiment; Since no puromycin was added to these cultures, those cells that did grow normally may represent uninfected cells and the difference between the two constructs may represent differences in infection efficiency. This experiment was repeated but the cultures were fixed after 7 and 10 days and analysed by fluorescence microscopy for EGFP expression. Any actively growing colonies in either Serrate1 or SerrateT infected dishes did not express detectable EGFP: EGFP expression was only found in small abortive colonies. After 10-15 days EGFP positive cells appeared to be necrotic (not shown). It does not appear that Serrate1 or SerrateT induce terminal differentiation. Cells that remained intact after 16 days after infection did not upregulate involucrin as assessed by FACS analysis (Figure. 4.18 G). The expression of Serrate1 and SerrateT at these levels therefore appears to be toxic to keratinocytes and further analysis was not possible.

4.3 Discussion

I found that keratinocytes exposed to Delta signals from their neighbours were driven to enter the differentiation pathway, unless they overexpressed high levels of Delta1 themselves. This is the first evidence that Notch signalling influences stem-cell fate in interfollicular human epidermis.

Notch signalling has been most extensively studied during embryonic development, but there is now growing evidence that this pathway continues to regulate cell fate in self-renewing or regenerating adult tissues. The best-studied examples come from the haematopoietic system. The Notch ligand Serrate1 continues to be expressed postnatally in the bone marrow stroma (Jones, 1998). Maturation of cultured haematopoietic precursor cells can be inhibited using constitutively active forms of Notch or by overexpression of Serrate1 in neighbouring stromal cells (Milner *et al.*, 1996; Jones *et al.*, 1998; Varnum-Finney *et al.*, 1998; Carlesso *et al.*, 1999), although a recent study has suggested that the Notch pathway can accelerate rather than inhibit differentiation (Schroeder and Just, 2000). Notch and its ligands are also expressed in the thymus, where they have been shown to influence the CD4/CD8 lineage decision of differentiating T-cells during, and possibly beyond, development. (Robey *et al.*, 1996; Washburn *et al.*, 1997). Notch signalling also appears to be important beyond development in the inner ear of the chicken: unlike mammals, birds are able to regenerate sensory hair cells after injury, and regeneration is accompanied by increased expression levels of Delta1 (Stone and Rubel, 1999). It has been shown previously that Notch and its ligands are also expressed in adult human epithelia (Gray *et al.*, 1999) but there have been no previous reports addressing the functional importance of Notch signalling in adult epidermis.

As most of my specimens came from non-hair bearing skin, I did not examine the distribution of Delta1 and Notch1 in hair follicles. Others (Kopan and Weintraub, 1993) have reported that Notch is expressed in the proliferative matrix cells of mouse hair follicles, and is downregulated as cells begin terminal differentiation. Transgenic mice have been generated in which Notch is constitutively activated within the cortex cells (one type of hair follicle precursor cell), at the time that they would normally begin to differentiate. Activated Notch does not appear to affect precursor cell proliferation, but does result in abnormal differentiation of neighbouring medulla and cuticle cells of the hair (Lin *et al.*, 2000). Since the affected cells are not descended from the cortex cells and do not express Notch target genes, then the defect must be an indirect consequence of Notch activation. It is still not clear whether Notch signalling

has any direct influence on stem cell fate in the hair follicle. Delta1 is also expressed in developing chick epidermis (Chen *et al.*, 1997; Crowe *et al.*, 1998; Viallet *et al.*, 1998): The expression of Delta1, in single scattered cells, together with the results of retroviral misexpression of Delta1, suggest that Delta1 acts by a lateral inhibition-type mechanism to inhibit the initiation of feather buds (Crowe *et al.*, 1998). There have been no reports that address the function of Delta1 on the regions of post-natal epidermis that lie between the hairs (in mammals) or the feathers (in birds).

There are two general types of mechanism by which Notch signalling regulates the patterning of cell fates: lateral inhibition and boundary formation (Bray, 1998) (section 1.4.7). The expression of Delta1 in contiguous groups of cells in the epidermal basal layer, and the distribution of epidermal stem cells as clusters rather than as single cells, are not consistent with a lateral inhibition mechanism. Furthermore, Notch signalling during lateral inhibition generally maintains a stem cell fate (Lewis, 1996), whereas in keratinocytes it promotes stem cell differentiation. My results are more consistent with a boundary mechanism, in which Notch becomes activated only at the interface between high and low regions of Delta expression. I would predict that Notch activation in human epidermis would occur within the basal layer at the interface between groups of cells at the tops of the dermal papilla and the cells further down towards the rete ridges (Figure 4.19). This model is based on my finding that Delta1 is expressed more highly in groups of cells at the tops of the dermal papillae, and that Notch signalling promotes the transition from stem cell to transit amplifying cell.

An important test of my model would be to directly identify the cells *in vivo* in which Notch signalling is active, for example by *in situ* hybridisation for target genes of the Notch pathway. The model shown in Figure 4.19 predicts that the target genes would be expressed in a ring of cells encircling the tips of the dermal papillae. However, at present we do not know what the target genes of the Notch pathway are in keratinocytes. Target genes in *Drosophila* include members of the Enhancer of Split family of bHLH transcription factors (Bailey and Posakony, 1995; de Celis *et al.*, 1996). Hes-1, a vertebrate homologues of Enhancer of split, has been shown to be a direct target of the Notch pathway in rat myogenic precursor cells and in human epithelial cell lines (Jarriault *et al.*, 1995; Fisher and Caudy, 1998; Kuroda *et al.*, 1999; Ohtsuka *et al.*, 1999). I was able to detect Hes1 in human keratinocytes by Northern hybridisation, and interestingly it appears to be upregulated as keratinocytes differentiate (not shown). It should be possible to test whether Hes1 is a target of the Notch pathway in keratinocytes. One way to do this might be to compare Hes1 expression in wild type keratinocytes, keratinocytes containing dominant negative Delta or SuH mutants, and keratinocytes cocultured with cells that are overexpressing Notch

ligands. A direct transcriptional response would not be distinguishable from an indirect response by monitoring the expression of endogenous Hes1 in such experiments, but Hes1 reporter constructs could be used to test for a direct transcriptional response (Jarriault *et al.*, 1998).

It is not clear how the expression pattern of Delta1 in epidermis is established and maintained. In other tissues, Delta can regulate its own expression by feedback signalling (Bray, 1998). In tissues where Notch signalling operates by lateral inhibition, the expression of Delta1 in a pattern of single scattered cells becomes established and maintained through negative feedback (Lewis, 1996) (section 1.4.7). In tissues where Delta is expressed in contiguous groups of cells, for example in the vertebrate somites, negative feedback does not operate: instead positive feedback appears to come into play in order to maintain uniform Delta1 expression (Hrabe de Angelis *et al.*, 1997). It would be very interesting to investigate whether Delta1 regulates its own expression in keratinocytes. It might be possible to use human-specific probes in Northern hybridisation to assess whether levels of endogenous Delta1 are affected by overexpression of mouse Delta1, or whether they are affected by blocking the Notch signalling pathway. It is not clear whether positive feedback in somites is mediated by the conventional Notch signalling pathway (Hrabe de Angelis *et al.*, 1997). A more attractive hypothesis for keratinocytes would be that positive feedback is a cell autonomous response to Delta1, independent of the signalling pathway that drives differentiation at the boundaries of Delta1 expressing cell clusters.

Positive feedback might help to stabilise Delta1 expression within stem cell clusters, but it cannot explain how the pattern of evenly spaced Delta1-expressing clusters becomes established in the first place. Signalling through the wingless pathway can stimulate Delta expression in *Drosophila* (de Celis and Bray, 1997). Wnts, which are the vertebrate homologues of wingless, signal via β -catenin. This might explain why there is elevated Delta1 expression in the epidermal stem cell patches, as there is increased β -catenin activity in epidermal stem cells, at least in vitro (Zhu and Watt, 1999). Constitutively active or dominant negative components of the Wnt signalling pathway could be tested to see if they can directly affect expression of Notch or its ligands (Gat *et al.*, 1998; Zhu and Watt, 1999).

My results suggest that Delta/Notch signalling promotes keratinocyte differentiation. I also found that stem cells express the highest levels of Delta1, both in vivo and in culture. This raises the question of how the stem cells are protected from Notch-induced differentiation. One possible explanation comes from another of my findings: that keratinocytes with high Delta1 were not themselves stimulated to differentiate.

Further evidence in favour of this explanation comes from the *Drosophila* wing disc, where misexpression of physiological levels of Delta can render cells unresponsive to Delta signals (Micchelli *et al.*, 1997). However, the mechanistic basis for this is not known.

Delta1 may not be the only molecule that helps to protect stem cells from Notch-induced differentiation *in vivo*. Fringe is a glycosyltransferase that can modulate Notch's response to ligand (section 1.4.5). Vertebrate Fringe homologues are expressed in developing mouse epidermis (Thelu *et al.*, 1999). They drop to barely detectable levels in postnatal mouse epidermis but become upregulated during wound healing (Thelu *et al.*, 1999). This raises the interesting possibility that the Fringe family of proteins acts to prevent Notch-induced differentiation during epidermal regeneration, thus allowing expansion of the stem cell pool to repopulate the wounded region.

I investigated the expression pattern of another modifier of Notch signalling, Numb, but I found no evidence that Numb is expressed at higher levels in stem cells than in transit amplifying cells, either *in vivo* or in culture. However, I noticed that occasional single cells stained very brightly for Numb, and that these cells always had a characteristic morphology, being attached to the basal layer by a thin stalk of cytoplasm with the main body of the cell reaching into the first suprabasal layer. This morphology strikingly resembles that of mitotic cells in the basal layer of the oesophagus (Seery and Watt, 2000) and in the ventricular zone of the developing neuroepithelium (Matsuzaki, 2000). In both these tissues, the nucleus migrates up into the suprabasal layers during S phase whilst the cell body remains attached to the basement membrane by a thin cytoplasmic stalk. It would be interesting to see if the Numb-bright cells in human epidermis are also in S phase. Alternatively, they might represent cells in the process of exiting the basal layer and beginning terminal differentiation (Jensen *et al.*, 1999). However, the Numb-bright cells are relatively rare (fewer than 1 in 100 cells) which would not be consistent with bright numb staining indicating every differentiating or dividing cells. Bright expression might either be limited to a subset of differentiating/dividing cells or else must be very transient. These questions could be answered using markers for proliferation and early differentiation. Unfortunately I was unable to obtain further supplies of the anti-Numb antibody so these questions remain open. The apparently ubiquitous expression of Numb in both steady state and actively growing keratinocytes raises the questions of whether Numb does block Notch activation in keratinocytes, and if so, what are the threshold levels of Numb required to inhibit Notch activity. It should be possible to answer this by overexpressing Numb in keratinocytes.

Serrate can functionally substitute for Delta in *Drosophila* (Gu *et al.*, 1995), although their different expression patterns, both in flies and vertebrates suggest that they perform different functions in vivo (Lindsell *et al.*, 1996). Misexpression of Serrate1 clearly does not affect keratinocytes in the same way as misexpression of Delta1. This could be a consequence of experimental differences: for example, Serrate1 might have been overexpressed at much higher levels than Delta1. Alternatively it may be that keratinocytes respond differently to physiological levels of Delta1 and Serrate1 in vivo. I attempted to provide the Serrate signal to normal keratinocytes in trans by expressing Serrate1 in feeder cells. Unfortunately, J2 cells did not support Serrate1 or SerrateT expression: the small proportion of cells that were EGFP positive after infection failed to grow after FACS sorting (not shown). Other fibroblast lines can support Serrate1 expression (Jones *et al.*, 1998). It may be possible to find a cell line that can support Serrate1 expression and act as a suitable feeder layer for keratinocytes. Another possibility would be to expose keratinocytes to purified Serrate1 protein that has been immobilised on the tissue culture surface or on beads (VarnumFinney *et al.*, 1999)

The pattern of expression of endogenous Delta1 (Figures 4.1 and 4.2) suggests that populations of cells expressing the highest levels of Delta1 may be enriched for stem cells. One way to test this directly would be to FACS sort populations with high or low Delta1 expression and follow their proliferative capacity in clonal culture. In principle this seems possible: Delta1 is a transmembrane protein, and the Delta1 antibody recognises the extracellular domain. However, Delta1 does not remain on the cell surface when the cells are in suspension. This is consistent with the observation that in adherent keratinocytes Delta1 only localises to those regions of the membrane that are in contact with neighbouring cells (see Figure 4.14A) and that Delta can be rapidly cleared from the cell surface by endocytosis (Kooh *et al.*, 1993): Figure 4.14 C). Delta1 therefore cannot be tested as a stem cell marker by FACS, and will not be useful as a marker for prospective isolation of stem cells from population of keratinocytes.

Figure 4.1 Amino acid sequences for Delta1

Amino acid sequence for Human Delta1 (Gray *et al.*, 1999) shown in alignment with mouse Dll-1 (Bettenhausen *et al.*, 1995) and Chicken Delta-1 (Henrique *et al.*, 1995). Residues shaded dark grey are identical; residues shaded pale grey are similar.

The SER 20 antibody (Henrique *et al.*, 1997) was raised against the chick peptide sequence highlighted in yellow. Regions in green correspond to the primers used in the PCR to identify Delta homologues expressed by cultured human keratinocytes. The DeltaT mutant lacks all the amino acids C-terminal to the residue marked with the red arrow.

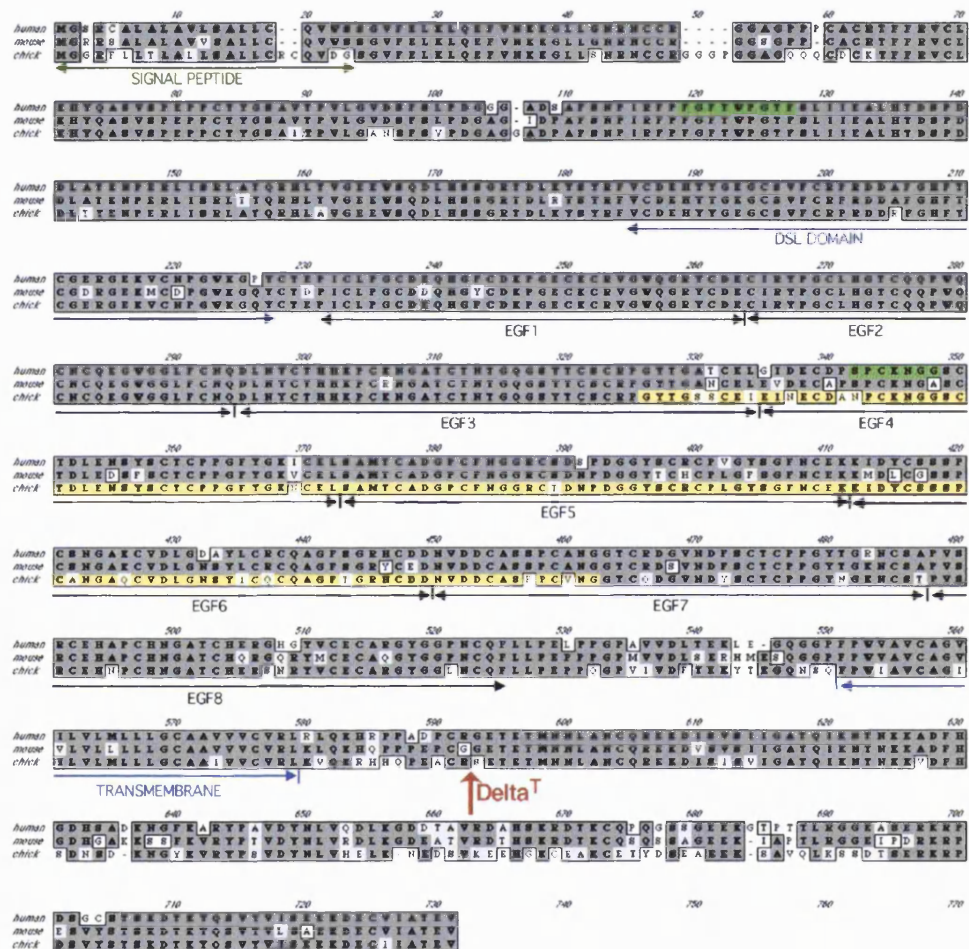


Figure 4.2: Expression of Delta1 and Notch1 in human skin.

A-D: In situ hybridisation of mid gestational foetal skin (A,B) or adult foreskin (C,D) with a probe against Delta1 mRNA. A,C: dark field illumination of B,D respectively.

E, F: Immunofluorescence staining of neonatal foreskin with antibodies to Delta1 (E) or Notch1 (F). Arrows in A,C,E indicate tips of dermal papillae. Scale bars: 50 μm .

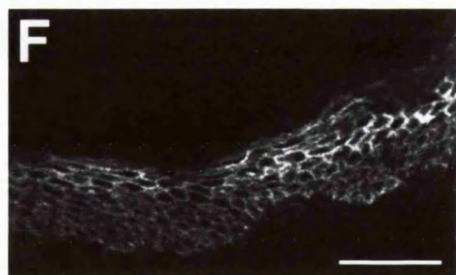
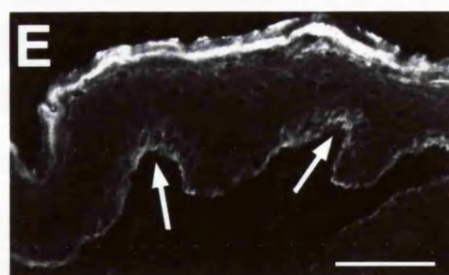
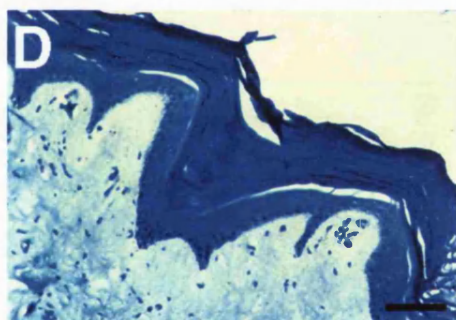
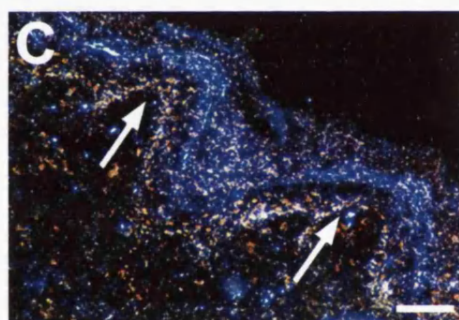
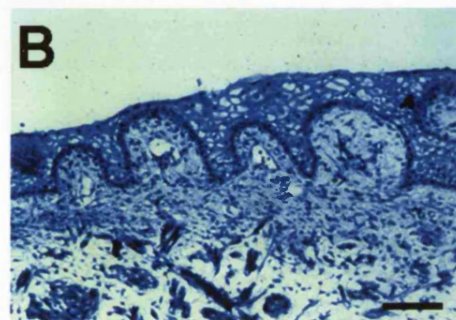


Figure 4.3 Northern blots of cultured human keratinocytes.

Populations enriched for stem (S) transit amplifying (TA) and terminally differentiated cells (TD) were compared. Blots were probed for Delta1 (A) and Notch1 (B). Blots were reprobed for involucrin, as a marker of terminal differentiation, and 18S RNA, as a loading control.

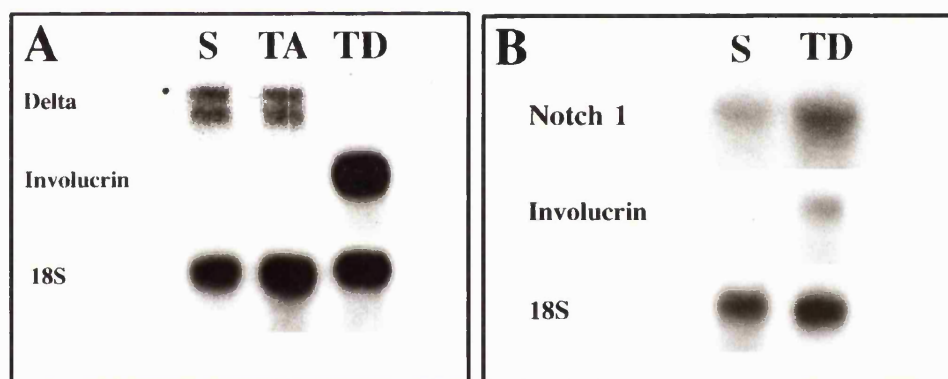


Figure 4.4 Virus packaging cell lines.

A-E: Immunostaining for Delta1 using the polyclonal antibody SER 20 (Red in D,E) of AM12 stably transduced with pBabe puro-Delta1 (A), pBabe puro-DeltaT (B), or empty pBabe puro vector (C) or GPE stably transfected with pBabe puro Delta1-IRES-EGFP (D) or empty pBabe puro IRES-EGFP vector (E) after FACS selection for EGFP positive cells (green).

F,G: immunostaining for the HA tag of AM12 stably transduced with pBabe puro-SuH^{DBM} (F) or empty pBabe puro vector (G).

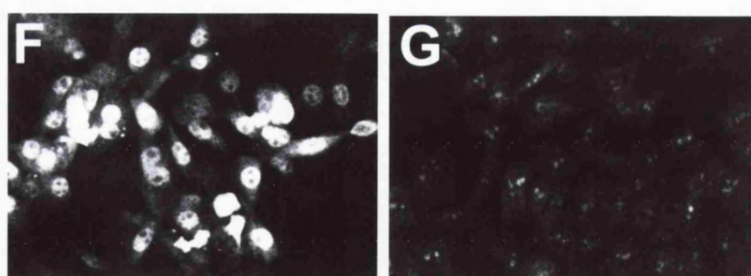
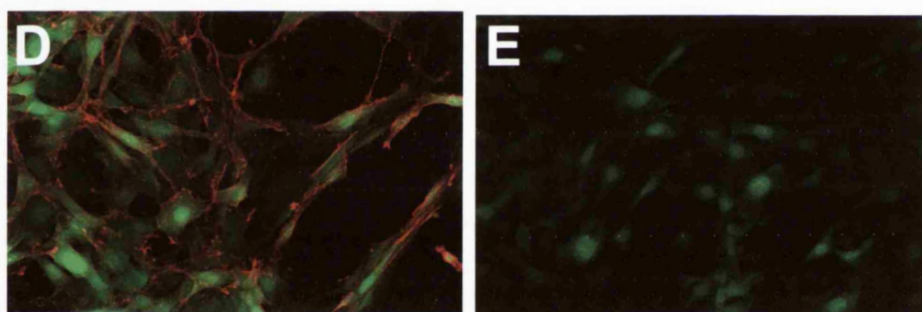
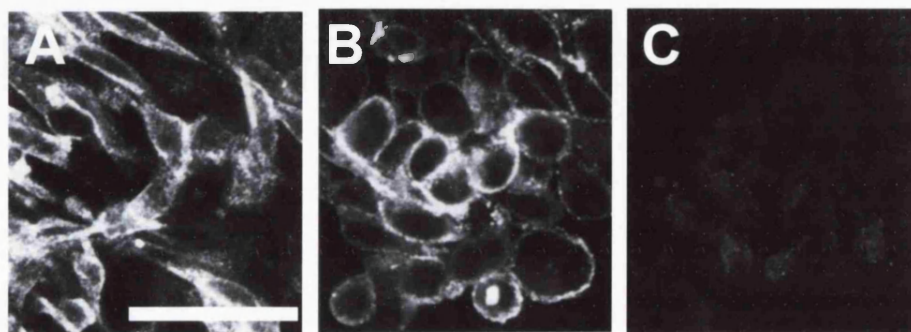


Figure 4.5 Expression of Delta1 in retrovirally transduced keratinocytes.

Primary keratinocytes stably transduced with pBabe puro-Delta1 (A), pBabe puro-DeltaT (B), or empty pBabe puro vector (C). All panels show immunostaining for Delta1 using the polyclonal antibody SER 20.

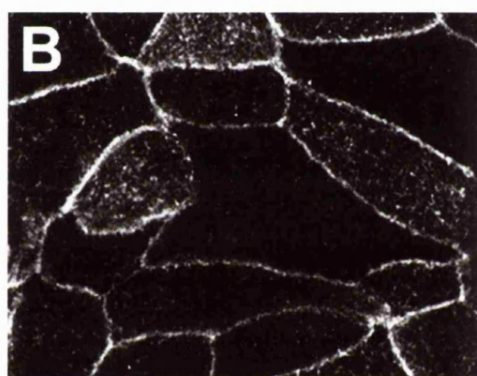
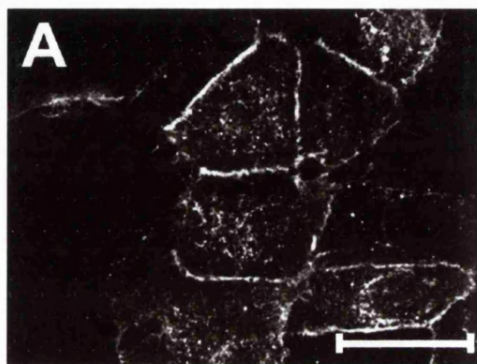


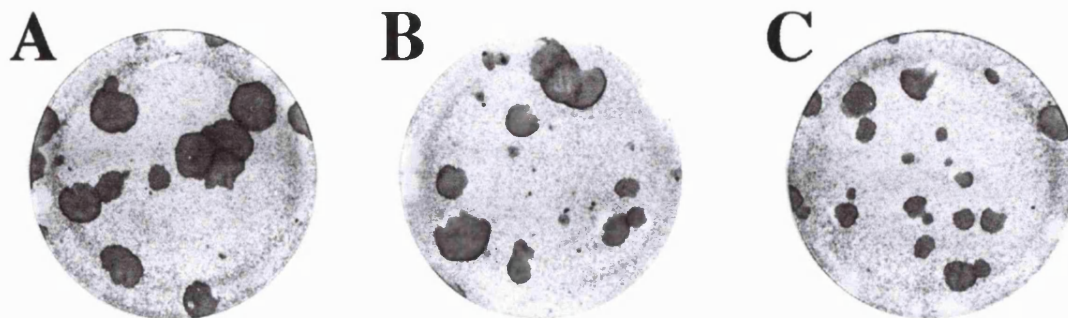
Figure 4.6 Clonogenicity assays.

K-WT (A), K-DELTA (B) or K-DeltaT (C) were seeded at low density on a J2-3T3 feeder layer in 35 mm diameter dishes and fixed with Rhodanile blue after 14 days.

TABLE 4.1. Effect of uniform Delta1 expression on colony forming efficiency and % stem cell clones.

Keratinocytes transduced with the following retroviral vectors were cultured on a feeder layer of J2-3T3 cells: empty vector (K-WT); full length Delta1 (K-DELTA); truncated Delta1 (K- DeltaT). Data are means \pm s.d. from triplicate dishes in a single, representative, experiment. Values that differ significantly from control are marked * .

(Students t-test: $p < 0.05$; all other significance values > 0.1).



	% COLONY FORMING EFFICIENCY	% STEM CELL CLONES
K-WT	15.0 ± 2.6	56.7 ± 7.1
K-DELTA	27.3 ± 3.7	56.0 ± 4.3
K-DeltaT	35.0 ± 2.0 *	66.2 ± 0.4

Figure 4.7 Surface expression of $\beta 1$ integrin.

FACS profiles showing K-WT (red), K-DELTA (green) or K-DeltaT (blue) either one week (A) or four weeks (B) post infection, stained with directly conjugated CD29 antibody against $\beta 1$ integrin, a marker of early keratinocyte differentiation. Keratinocytes stained with directly conjugated irrelevant antibody (anti-CD8) are shown in black.

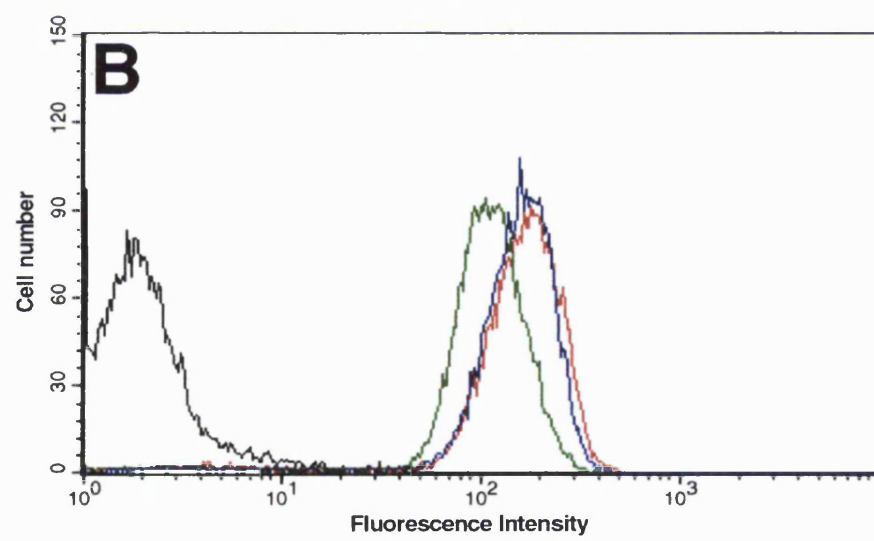
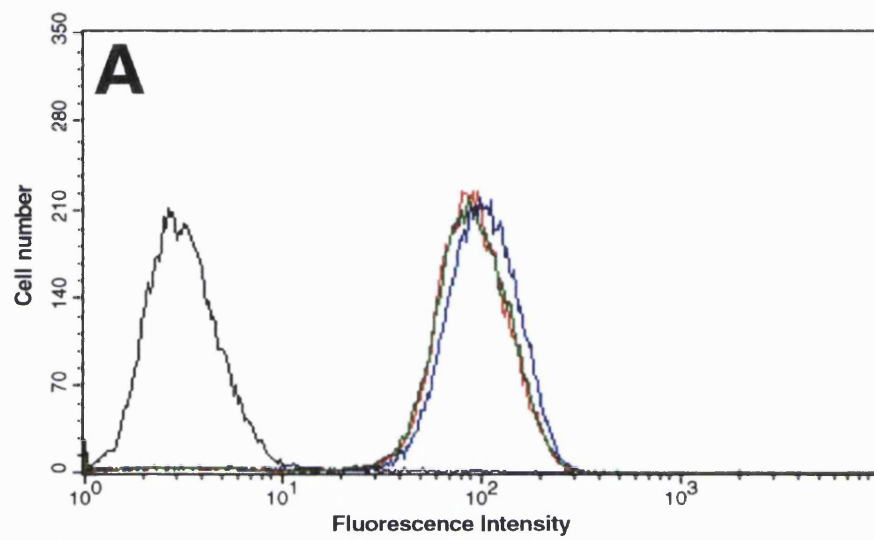


Figure 4.8 Involucrin expression

FACS profiles showing K-WT (red), K-DELTA (green) or K-DeltaT (blue) stained with SY5 antibody against involucrin, a marker of early keratinocyte differentiation. Gate M1 indicates keratinocytes with high expression of involucrin. The proportion of keratinocytes that fall within gate M1 are: 50.1% (K-WT); 62.4% (K-DELTA) and 32.6% (K-DeltaT). Keratinocytes stained with secondary antibody only are shown in black. (0.1% fall within gate M1).

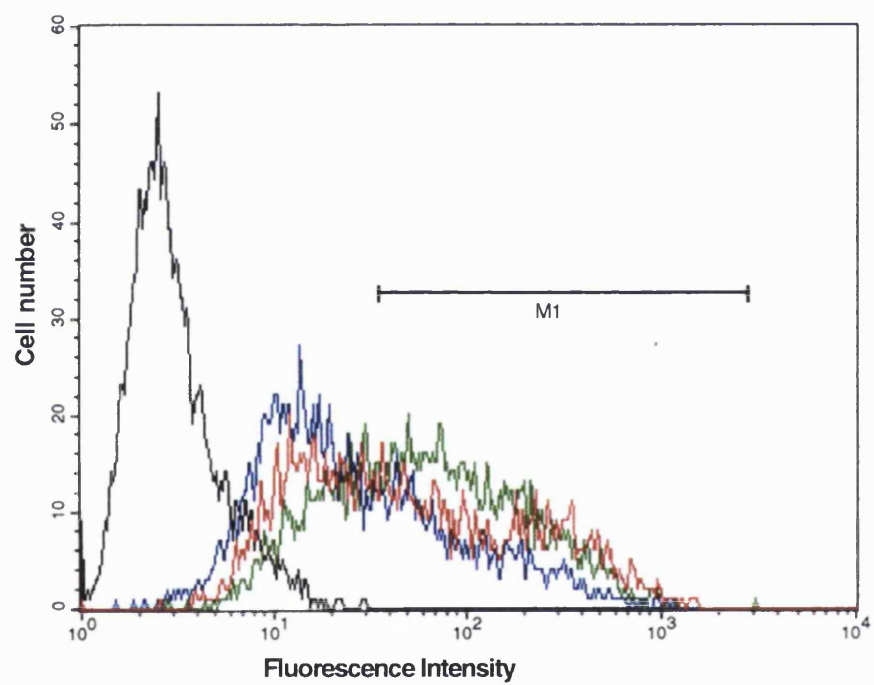


Figure 4.9 Growth curves

Growth curves of K-WT (Red), K-DELTA (Green) and K- DeltaT (Blue).

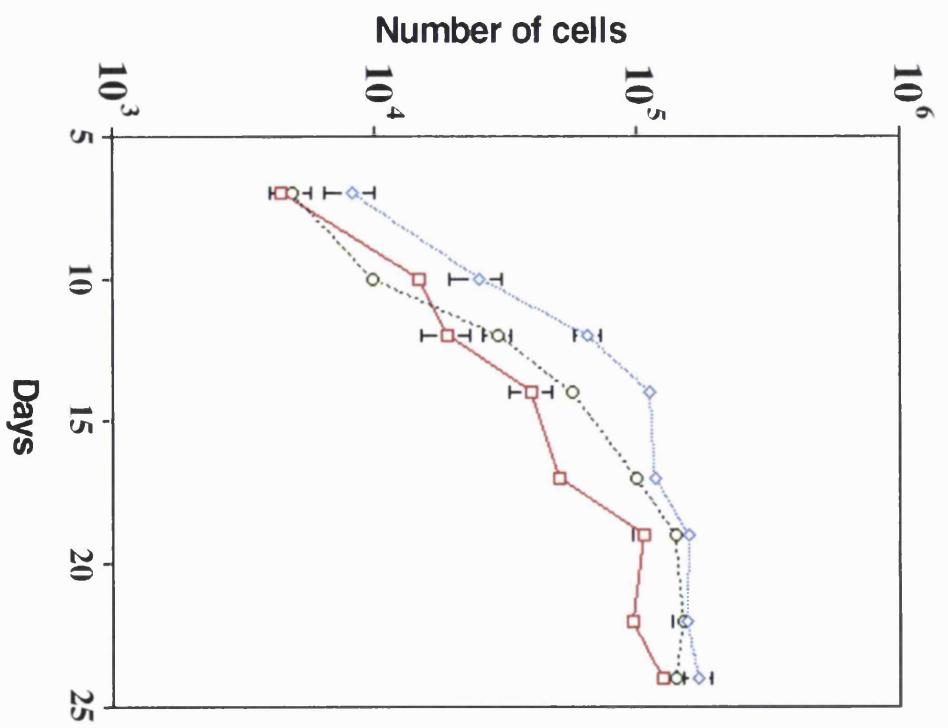
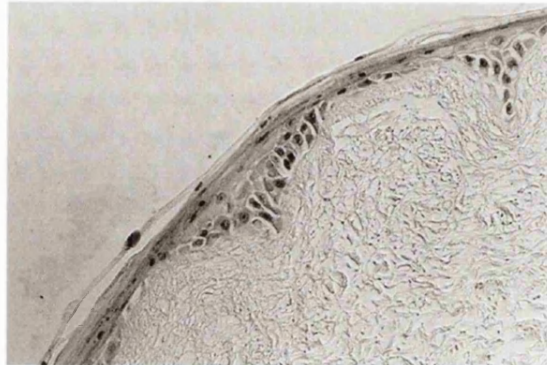


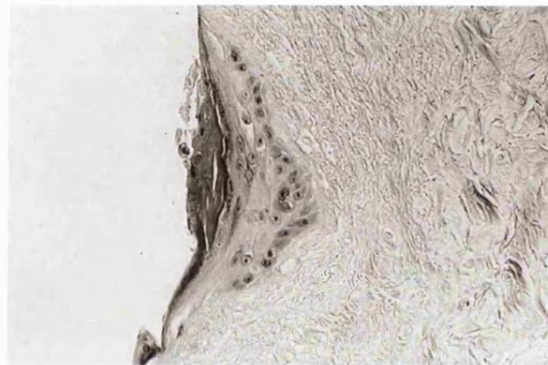
Figure 4.10 DED cultures.

Reconstituted epidermis grown on DED from K- WT (A), K-DELTA (B) or K-DeltaT (C).

A **K-WT**



B **K-DELTA**



C **K-DeltaT**

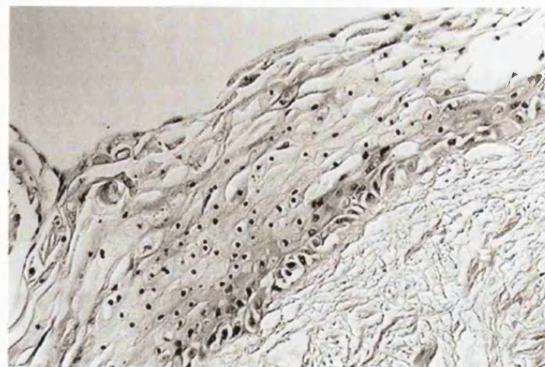
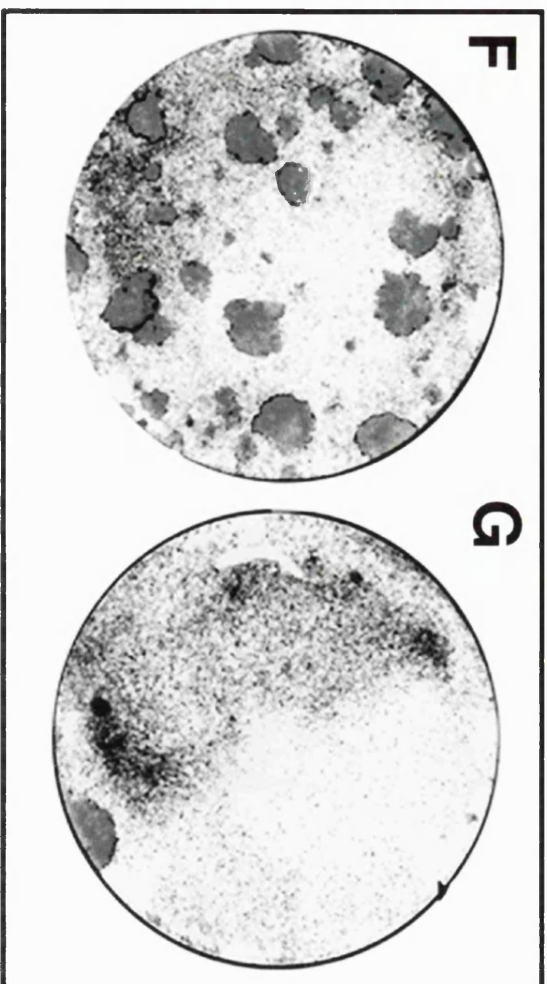
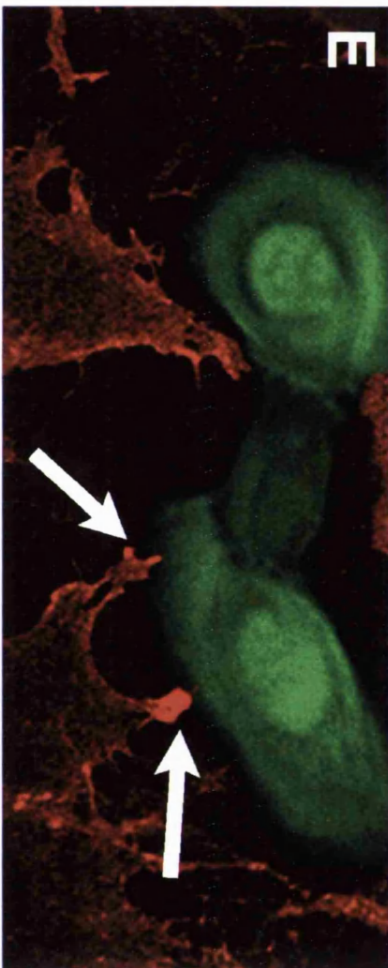
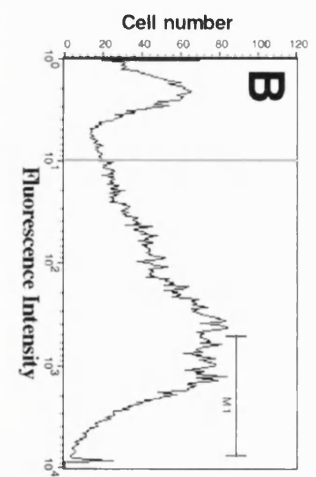
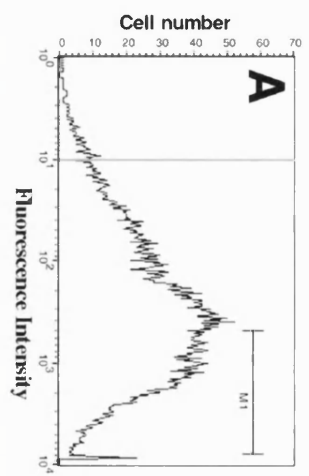


Figure 4.11 Clonegenicity assays of keratinocytes cultured on J2-DELTA feeders

A, B: FACS profiles showing expression of EGFP in populations of J2 cells that have been infected with pBabe puro-IRES-EGFP (A) or pBabepuro-DELTA-IRES-EGFP (B) viral supernatant. The vertical grey line indicates the upper limit of the negative control (uninfected J2 cells). Cells that lay outside region with high EGFP expression (gate M1) were discarded.

C-E: Immunofluorescence staining for Delta1 (Red) of J2-WT (C), J2-DELTA (D) or K-WT-EGFP on a feeder layer of J2-DELTA cells (E) . Arrows indicate Delta1 staining concentrated at points of contact between the J2 cells and the keratinocytes. The keratinocytes in this picture are labelled with EGFP so that they can be easily seen.

F, G: Clonegenicity assays. Wild type keratinocytes were seeded at low density on a feeder layer of J2-WT (F) or J2-DELTA (G) in 35 mm diameter dishes and fixed with Rhodanile blue after 14 days.



	% COLONY FORMING EFFICIENCY	% STEM CELL CLONES
K-WT + J2-WT	13.1 \pm 1.0	48.5 \pm 1.4
K-WT + J2-DELTA	4.3 \pm 0.9*	10.8 \pm 7.2*
K- DeltaT + J2-DELTA	12.33 \pm 0.27	58.86 \pm 1.13 [†]

TABLE 4.2. Cloneogenicity assays of keratinocytes cultured on J2-DELTA feeders

Keratinocytes transduced with empty vector (K-WT) or truncated Delta1 (K- DeltaT) were cultured on a feeder layer of wild type J2-3T3 cells (J2-WT) or J2-3T3 cells transduced with full length Delta-1 (J2-DELTA). Data are means \pm s.d. from triplicate dishes in a single, representative, experiment. Values that are significantly less than controls are marked * and values that are significantly greater than controls are marked † (Student t-test: $p < 0.05$; All other significance levels are greater than 0.1)

Figure 4.12. Design of keratinocyte mixing experiments.

The following cartoons illustrate the combinations of cells tested in the mixing experiments. Green represents EGFP labelling, and the thicker red line indicates Delta1 overexpression. The **X** represents overexpression of dominant negative constructs.

A: K-WT-EGFP surrounded by unlabelled K-WT;

B: K-WT-EGFP surrounded by unlabelled K-DELTA;

C: K-DeltaT-EGFP or K-SuH^{DBM}-EGFP surrounded by unlabelled K-DELTA;

D: K-DELTA-EGFP surrounded by K-DELTA.

E shows a photograph of a mixed culture of K-DELTA and K-WT-EGFP (the combination illustrated in B) stained for Delta1 (red).

Scale bar: 10 μ m.

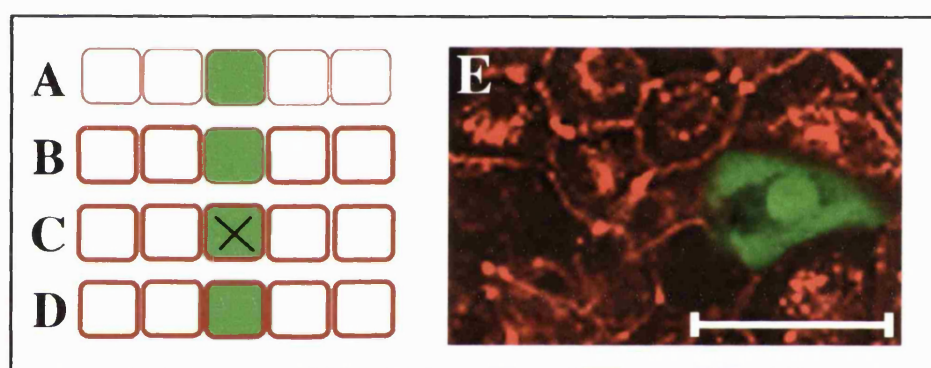
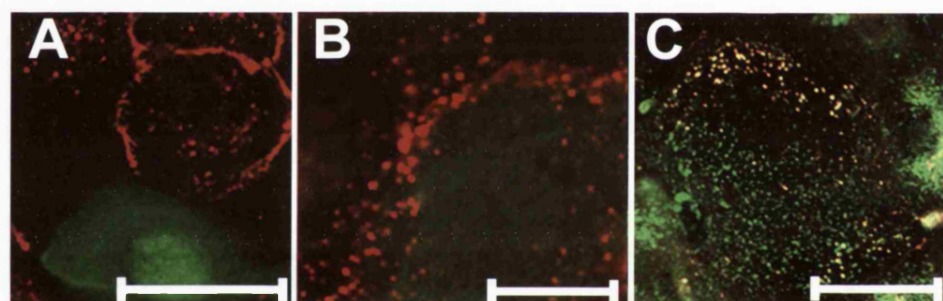


Figure 4.13 Localisation of overexpressed Delta1 in the mixing experiments

A, B: Mixed culture of K-DELTA and K-WT-EGFP stained for Delta1 (red). In A, note strong intercellular Delta1 staining between two K-DELTA but weak staining between K-DELTA and K-WT-EGFP.

C: K-DELTA cell double labelled for Delta1 (red) and a lysosomal marker (green).

Scale bars: 10 μm (A), 2.5 μm (B), 5 μm (C).







EGFP colonies persisting for 15 days	
	13.7 ± 0.8
	0.0 ± 0.0*
	15.8 ± 1.9
 DeltaT	13 ± 0.36

TABLE 4.3. Persistence of EGFP-marked clones in keratinocyte mixing experiments.

See Figure 4.12 legend for explanation of the different cell combinations. 100 EGFP cells were seeded per cm². The ratio of marked to unmarked cells was 1:500. The number of EGFP colonies present on the dish 15 days later is shown. Data are means ± s.d. of data from triplicate dishes in a single, representative, experiment. Values that differ significantly from controls are marked * (Student t-test: p<0.05: All other significance levels are greater than 0.1)






Total number of basal EGFP cells after 15 days	
	$2.1 \pm 0.8 \times 10^4$
	$0.5 \pm 0.1 \times 10^4 \text{ †}$
 DeltaT	$5.5 \pm 0.6 \times 10^4 *$
 SuH ^{DBM}	$20.5 \pm 1.3 \times 10^4 *$
	$3.6 \pm 0.5 \times 10^4$

TABLE 4.4. Total number of basal EGFP cells remaining after 15 days in keratinocyte mixing experiments.

See Figure 4.12 legend for explanation of the different cell combinations. 10^4 EGFP cells were seeded per cm^2 . The ratio of marked to unmarked cells was 1:10. The number of EGFP marked basal cells after 15 days are shown for each cell combination. Data are means \pm s.d. of data from triplicate dishes in a single, representative, experiment. Values that are significantly less than controls are marked * and values that are significantly greater than controls are marked † (Student t-test: $p < 0.05$; All other significance levels are greater than 0.1)

Proportion of EGFP labelled cells that are terminally differentiated (%)

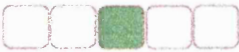




	DAY 5	DAY 15
	47.1 ± 2.3	56.6 ± 4.9
	54.5 ± 1.8	87.6 ± 1.4 *
 DeltaT	51.4 ± 4.4	64.0 ± 0.4
 SuH ^{DBM}	46.1 ± 9.1	47.3 ± 6.4
	46.1 ± 9.1	69.0 ± 1.3

TABLE 4.5. Terminal differentiation in EGFP cells in keratinocyte mixing experiments.

See Figure 4.12 legend for explanation of the different cell combinations.

10⁴ EGFP cells were seeded per cm². The ratio of marked to unmarked cells was 1:10.

Data are means ± s.d. of data from triplicate dishes in a single, representative, experiment. Values that differ significantly from controls are marked * (Student t-test: p<0.05; All other significance levels are greater than 0.1)

Figure 4.14. Fate of keratinocytes in mixing experiments (1) .

Size and morphology of EGFP labelled clones surrounded by unmarked cells in confluent cultures 5 days (A,C,E,G,I) or 15 days (B,D,F,H,J) after seeding.

A, B: K-WT-EGFP surrounded by unlabelled K-WT;

C, D: K-WT-EGFP surrounded by unlabelled K-DELTA;

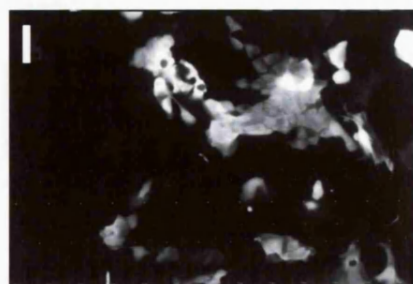
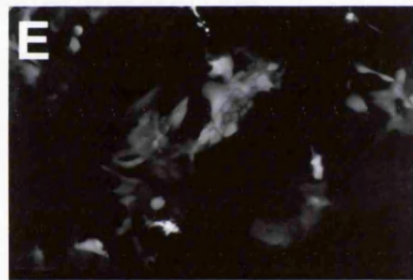
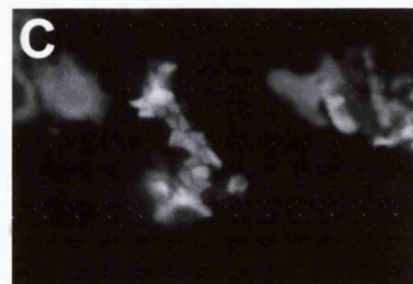
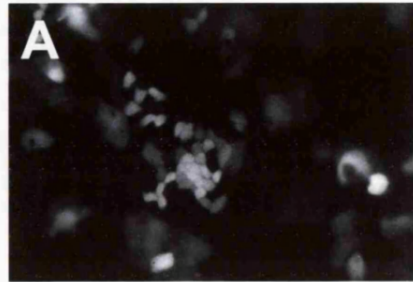
E, F: K-DeltaT-EGFP surrounded by unlabelled K-DELTA;

G, E: K-SuH^{DBM}-EGFP surrounded by unlabelled K-DELTA;

F, G: K-DELTA-EGFP surrounded by unlabelled K-DELTA.

Cells were seeded at a ratio of 1 EGFP positive cell per 10 unmarked cells and a total of 1.1×10^5 cells (i.e. 10^4 EGFP-positive + 10^5 unmarked cells) were plated per dish. Scale bars: 100 μm .

DAY 5



DAY 15

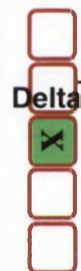
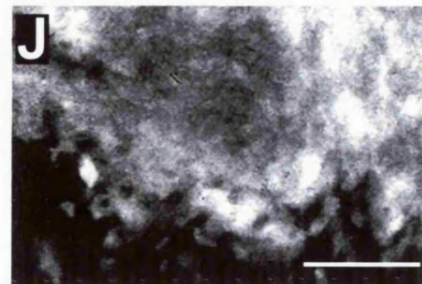
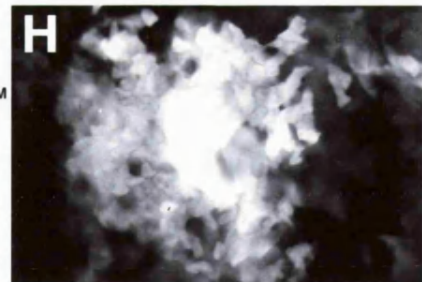
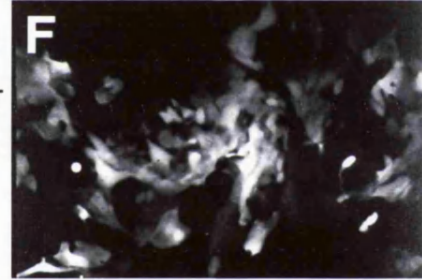
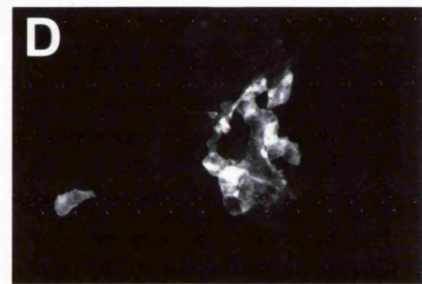
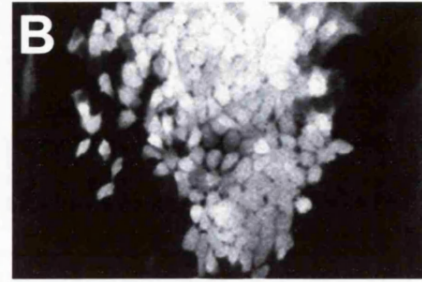


Figure 4.15 Fate of keratinocytes in mixing experiments (2) .

This figure illustrates more clearly the difference between the colonies shown in Figure 4.15 B (shown in panels A,B of this figure), D (shown panels C,D of this figure) and F (panels E,F, of this figure).

A, B: K-WT-EGFP surrounded by unlabelled K-WT;

C, D: K-WT-EGFP surrounded by unlabelled K-DELTA;

E, F: K-DeltaT-EGFP surrounded by unlabelled K-DELTA;

The cultures in A,C and E have been stained with P5D2 antibody against $\beta 1$ integrin as a marker for basal cells (red). The colonies in A and C contain EGFP labelled cells in the basal layer whilst the cell shown in B is lying above the basal cells, in the suprabasal layers. The cultures in B,D and F are shown at low magnification to illustrate that the DeltaT cells found colonies that scatter over a wide area (F).

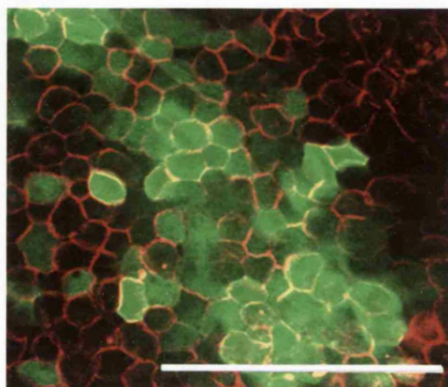
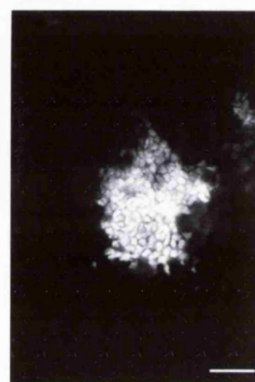
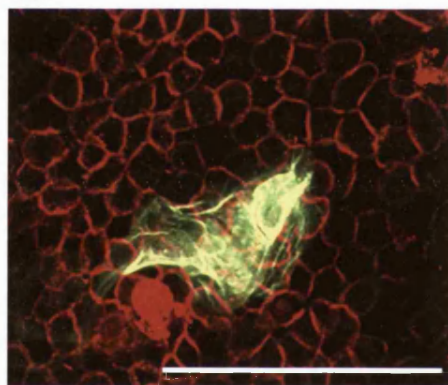
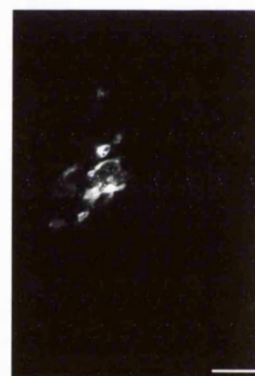
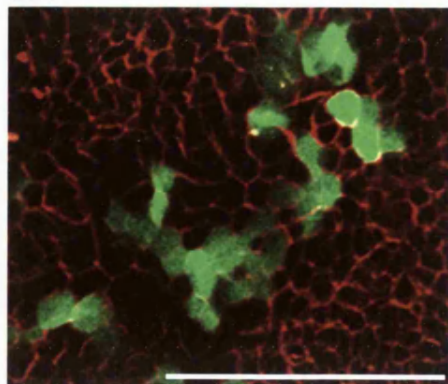
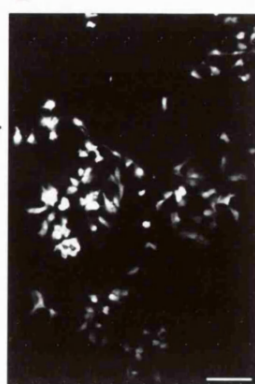
A**B****C****D****E****F**

Figure 4.16 Mixing experiments on DEDs

EGFP labelled clones surrounded by unmarked cells in DED cultures 3 weeks after seeding.

A: K-WT-EGFP surrounded by K-WT

B: K-WT-EGFP surrounded by K-DELTA

C: K-DELTA-EGFP surrounded by K-DELTA

Cells were seeded at a ratio of 1 EGFP positive cell per 10^4 unmarked cells and a total of 10^5 cells were plated on each DED.

Scale bar: 50 μ m

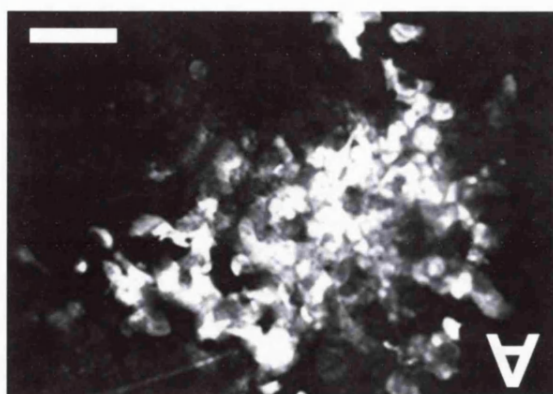
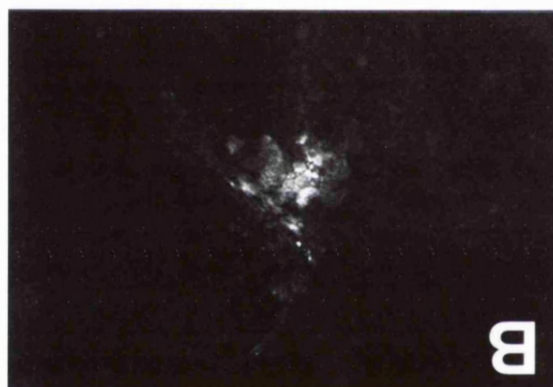
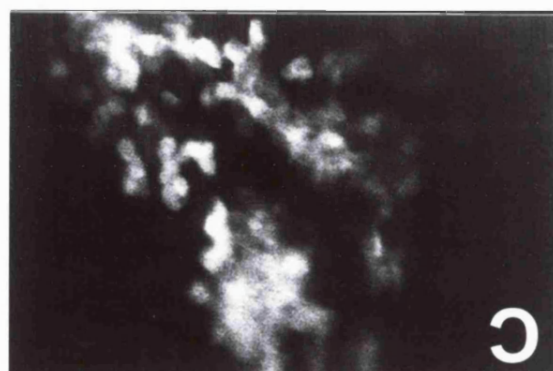


Figure 4.17 Expression of Numb in human keratinocytes

Sections of human foreskin epidermis (A,B) or colonies of normal human keratinocytes (C,D) stained with a polyclonal antibody against chick Numb. Arrow in A indicates the typical appearance of occasional cells that stain very brightly for Numb.

Scale bars: 50 μm in A and B, 10 μm in C, 40 μm in D.

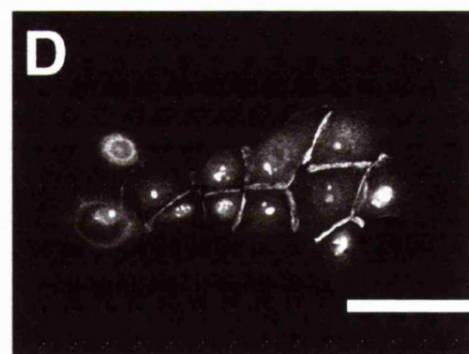
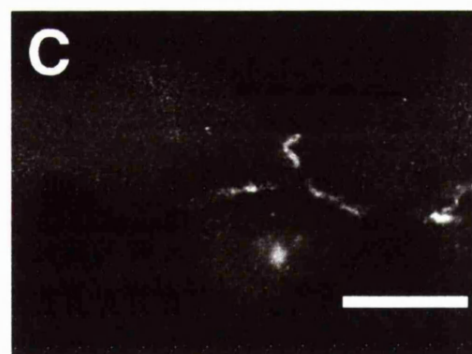
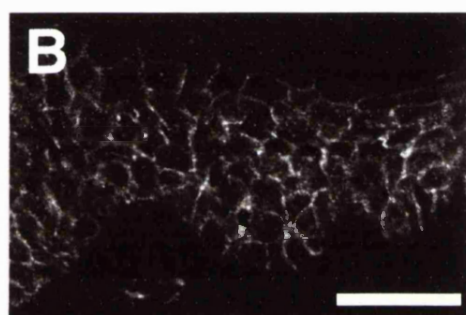
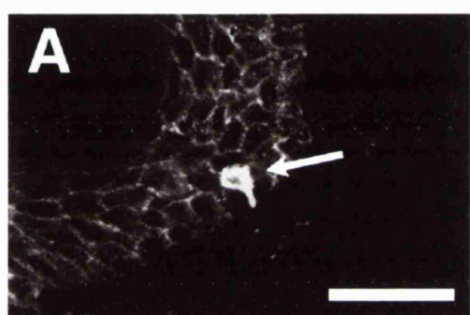


Figure 4.18 Misexpression of Serrate1 and SerrateT in keratinocytes

A-C FACS profiles showing EGFP expression in GPE packaging cells transfected with pBabe puro-Serrate1-IRES-EGFP (A) pBabe puro-SerrateT-IRES-EGFP (B) or pBabe puro-IRES-EGFP (C). Cells that lay outside the EGFP positive region (gate M1) were discarded

D-F 10⁴ normal keratinocytes (KQ4) were infected with pBabe puro-Serrate1-IRES-EGFP (D), pBabe puro-SerrateT-IRES-EGFP (E) or pBabe puro-IRES-EGFP (F) viral supernatant. The cells were cultured for 2 weeks in the absence of puromycin selection before fixing the dishes and staining with Rhodanile blue.

G FACS profiles showing keratinocytes stably transduced with pBabe puro-Serrate1-IRES-EGFP (Red) pBabe puro-SerrateT-IRES-EGFP (Green) or with pBabe puro-IRES-EGFP (Black) stained with SY5 antibody against involucrin, a marker of early keratinocyte differentiation. The negative control profile (keratinocytes stained with secondary antibody only) is indicated with a dotted line. Keratinocytes had been infected with viral supernatant, subjected to puromycin selection for 4 days to ensure that all the cells in the subsequent analysis were successfully infected, and then those cells that survived were grown for a further 10 days before harvesting and analysing by FACS for involucrin expression.

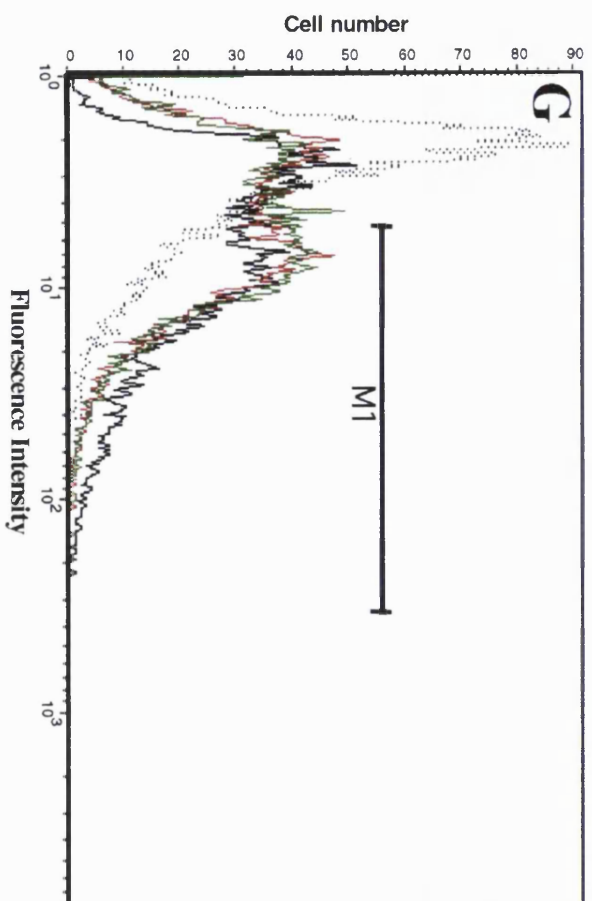
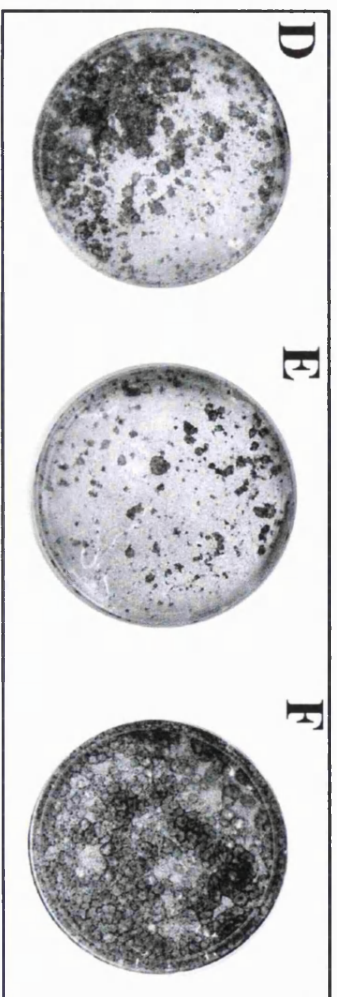
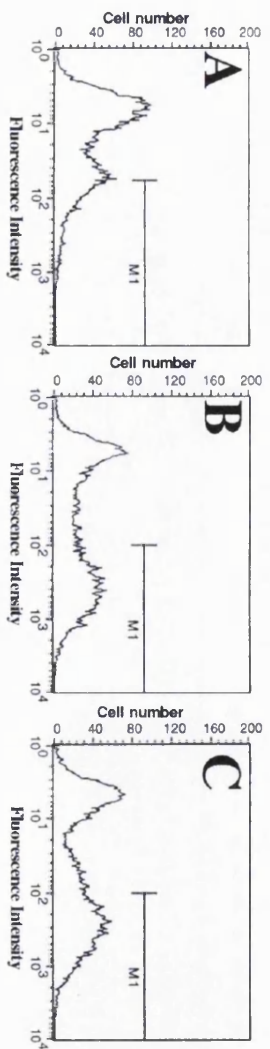
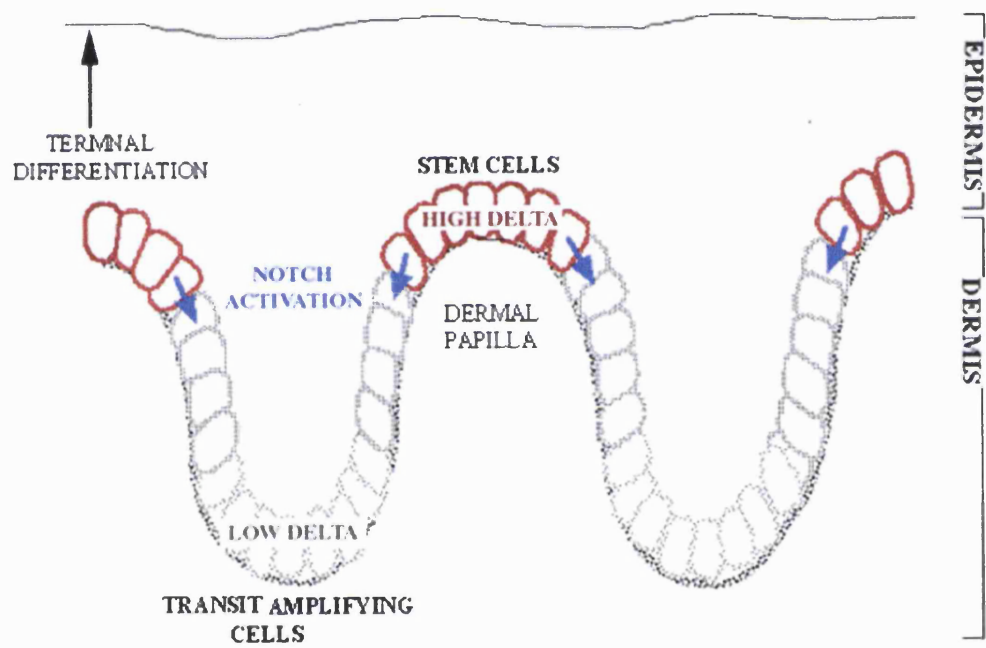


Figure 4.19 Model of Notch signalling in human epidermis.

Clusters of stem cells above the dermal papillae are shown expressing high levels of Delta1. The remaining basal cells, with low levels of Delta1, are transit amplifying cells or cells that are committed to undergo terminal differentiation. Notch is activated at the boundaries between the stem cell clusters and neighbouring transit amplifying cells. Cells that have left the basal layer and are undergoing terminal differentiation do not express Delta1.



CHAPTER 5

THE INFLUENCE OF DELTA ON CELL LOCATION

5.1. Introduction

The arrangement of stem cells and transit amplifying cells in distinct regions of the epidermal basal layer (Jensen *et al.*, 1999) implies that there is lateral movement of transit amplifying cells away from the stem cell clusters. The stability of this arrangement might depend on stem cells being restricted from moving outside the stem cell clusters, and their transit-amplifying daughters acquiring the ability to move relatively freely amongst their neighbours. Keratinocytes also need to migrate along the basement membrane during wound healing (Martin, 1997). It may be advantageous to ensure that the first cells to repopulate a wounded area are transit-amplifying cells, stem cells only being recruited later in case of need. This is another reason to predict that stem cells will be more restricted than transit amplifying cells in their ability to move across the basement membrane.

In chapter three I described intrinsic differences between stem cells and transit amplifying cells in the way that they move amongst their neighbours when they are placed amongst identical surrounding keratinocytes in confluent cultures. Specifically, the colonies founded by stem cells are more cohesive than the colonies founded by transit amplifying cells. This can be accounted for at least in part by differences in motility: stem cells move more slowly than transit amplifying cells when plated as isolated cells on collagen (Jensen *et al.*, 1999). In principle it could also be accounted for by differences in cell-cell adhesion. While we have no direct evidence at present that stem and transit cells differ (quantitatively or qualitatively) in their affinity for neighbouring cells, there is evidence that they differ in their expression levels of E-cadherin *in vivo* (Molès and Watt, 1997).

In the course of the mixing experiments described in Chapter 4 I noticed an influence of Delta1 on the cohesiveness of keratinocyte colonies within a confluent sheet. Delta1 had this effect in situations where it did not influence cell fate. This led to the idea that Delta1 may have a dual role, independently regulating both the differentiation and location of keratinocytes. Although Delta is best known as a regulator of differentiation, there is already a growing body of evidence that it can also influence cell cohesiveness and cytoskeletal remodelling in other systems (section 1.4.9). I

therefore set out to confirm the observation that Delta1 can influence a cell's movements relative to its neighbours within a confluent sheet of cells. I then addressed the question of how Delta1 might influence keratinocyte cohesiveness.

5.2. Results

5.2.1 *The effect of Delta1 on the cohesiveness of clonal progeny*

Delta1 promotes cohesiveness of clonal progeny within confluent cultures

As a normal keratinocyte divides within a confluent sheet, it can either remain in contact with its progeny, giving rise to a cohesive clone, or its progeny can move apart, generating a non-cohesive clone in which the majority of sibling cells are not in contact with one another (see Chapter three). The property of “cohesiveness” will depend upon a combination of cell-cell adhesion and relative cell motility.

Keratinocytes containing either empty retroviral vector (K-WT), Delta1 (K-DELTA), the truncated Delta mutant (K-DeltaT) or the dominant negative Suppressor of Hairless mutant (K-SuH^{DBM}) were labelled with EGFP and seeded sparsely amongst unlabelled cells (1 labelled cell to 100 unlabelled cells) at confluent density. After 5 days I scored each EGFP labelled colony according to whether it was cohesive (more than 50% of the cells were in contact within a single group) or non-cohesive (fewer than 30% of cells were in contact). Intermediate colonies were excluded from the analysis. Thirty clones were scored from each of three separate experiments (Table 5.1). I found that full length Delta1 promoted cohesiveness: keratinocytes that overexpressed Delta1 tended to give rise to clones that were more compact than wild type clones when they were within a confluent sheet of wild type cells (Figure. 5.1: compare B with A). DeltaT acted in the opposite way: DeltaT clones were more scattered than wild type clones (Figure. 5.1: compare C with A). The dominant negative Suppressor of Hairless mutant, which blocks the Notch signalling pathway (Chapter 4), had no significant effect on keratinocyte cohesiveness (Figure 5.1: compare D with A). The data suggests that Delta1 depends upon its intracellular domain to promote cohesiveness, and that DeltaT acts in a dominant negative way to prevent endogenous Delta1 from performing this function. The effect of Delta1 and DeltaT appear to be independent of the conventional SuH-dependent Notch signalling pathway.

I had previously used this same assay to compare the cohesiveness of stem cells and transit amplifying cells, and found that stem cells tend to give rise to clones that are

more cohesive than those that arise from transit cells (section 3.2.9). However, it is unlikely that the effect of Delta1 on cohesiveness is secondary to an effect on cell fate: K-WT, K-Delta1 and K-DeltaT populations all contain similar proportions of stem cells, as assessed by their proliferative capacity at clonal density (section 4.2.3: Table 5.1). I attempted to test the fate of the cells in the cohesiveness assay more directly by counting the number of “stem cell colonies” that arose from the EGFP labelled cells within confluent cultures after 15 days (table 5.1). However it was difficult to score the number of individual colonies that arise from K-DeltaT because clonal progeny do not tend to remain as cohesive colonies (see above and also Figure. 4.15). This means that the figure of 10.5% stem cell colonies (Table 5.1) is likely to be an underestimate.

5.2.2 Delta1 has no obvious influence on cohesiveness of isolated clones

I next asked whether Delta1 has similar effects on the cohesiveness of cells when they are growing as isolated clones, rather than as clones within confluent cultures. For example, if DeltaT reduces cell-cell adhesion then it might cause cells to visibly “scatter” from isolated clones, as happens when keratinocytes express a dominant negative E-cadherin mutant (Zhu and Watt, 1996). I compared isolated colonies of cells expressing Delta1, DeltaT or empty vector and did not see any obvious effects on colony cohesiveness (Figure. 5.2 A-C: compare with Figure. 5.1: note that the cells in the present experiment must completely detach from other keratinocytes when they move away from these colonies: this is unlike the colonies in Figure. 5.1 which are lineage labelled clonal progeny within a confluent sheet of unlabelled cells.) It seemed possible an effect of Delta1 on cell-cell adhesion might be difficult to detect in the presence of the strong adhesion mediated by the adherens junctions and desmosomes. I therefore carried out the same experiment in low calcium cultures, in which adherens junctions and desmosomes do not assemble (Hodivala and Watt, 1994). I could not detect any difference between the three cell types (Figure. 5.9 D-F). I concluded that Delta1 does not have any obvious effect on cell-cell adhesiveness. However, it is still possible that the effects seen in confluent cultures (Figure. 5.1 B, C) are a consequence of smaller differences in cell-cell adhesion that might not be detectable in this assay.

5.2.3 Assays to test keratinocyte cohesiveness

During development, for example in *Drosophila* imaginal discs, Delta can help maintain boundaries by preventing intermingling between adjacent populations (Micchelli and Blair, 1999). The observations described above (Figure. 5.1) suggest that this might also be the case for keratinocytes: for example Delta1 might act to prevent stem cells from intermingling with transit amplifying cells. I set out to design an assay to test

whether Delta1 can influence intermingling between two different keratinocyte populations. I tried two approaches:

- 1) I designed an assay to test whether two populations that are initially intermingled will tend to separate out over time. I call this the sorting assay.
- 2) I designed an assay to test whether 2 populations that are initially separate will tend to intermingle over time. I call this the confrontation assay.

5.2.4 Sorting assay

EGFP labelled cells were mixed with a tenfold excess of unlabelled cells and plated at confluence, such that the EGFP cells were evenly dispersed amongst the unlabelled cells (see section 2.3.5 for full experimental details). Each of the four possible combinations of labelled or unlabelled K-WT with labelled or unlabelled K-DELTA were compared. The same combinations were tested using K-DeltaT in place of K-DELTA.

After 24 hours in culture most of the EGFP cells were still evenly dispersed as single cells or doublets amongst the unlabelled cells (Figures. 5.3 and 5.4). In all of the experimental conditions there were only a very few clusters that contained more than three EGFP cells in contact with each other (range 17-31 clusters/cm²). Two strains of keratinocytes (kq and kn) gave similar results. This assay therefore did not provide any evidence that K-DELTA or K-DeltaT can sort out selectively from K-WT cells. Keratinocytes start to divide 24 hours after plating; this means it is not possible to prolong the assay period so as to test whether cells selectively sort out from each other over longer time periods.

5.2.5 Confrontation assay

The confrontation assay was designed to test whether two populations of cells tend to intermingle freely or whether they remain in different compartments separated by a clear boundary. The design of the assay is illustrated in Figure 5.5, which shows a control assay carried out on two populations of wild type keratinocytes (see section 2.3.4 for full experimental details). Two populations of cells, one of which is EGFP labelled, are seeded at high density in adjacent droplets that are close but not in contact: the edges of each drop are 2mm apart (Figure 5.5 A). After 24 hours the two populations of cells have migrated towards each other to form a continuous confluent sheet. At this stage there is a well-defined boundary between the two populations (Figure 5.5 B). Over the next few days, the two populations intermingle and the

boundary loses definition. I established from pilot experiments there is considerable intermingling between wild type populations three days after seeding (Figure 5.5 D). In subsequent experiments I therefore examined the cultures at only two time points: at 24 hours, to check that the two populations were in contact with a defined boundary, and at 72 hours, to assess whether the two populations had intermingled. I photographed the basal layer at three randomly chosen 2mm boundary regions by confocal microscopy and counted the number of EGFP positive cells that had moved into the unlabelled territory.

Testing Delta1 and DeltaT in the confrontation assay

I found that K-DELTA tended not to intermingle with wild type cells after three days (Figure. 5.6 C). However, the effect was not absolute, and a few cells did transgress the boundary: I checked to see if these cells were in the basal layer or the suprabasal layer by counterstaining the cultures for $\beta 1$ integrin as a marker of basal cells. The majority of cells that had moved into the unlabelled compartment were suprabasal (Figure. 5.6 D: Table 5.2). This suggests that K-DELTA do not intermingle with KWT in the basal layer. In contrast, K-DeltaT behaved like wild type controls, intermingling freely with wild type cells within the basal layer (Figure. 5.6 E, F). These observations were consistent between three independent experiments each including either duplicate or triplicate dishes for each experimental condition. In two additional experiments the experiments were carried in low calcium medium for the final 48 hours. Under these conditions adherens junctions and desmosomes do not form. The results were similar to those shown in Figure 5.6 for experiments carried out in normal (high calcium) medium (Figure. 5.7: Table 5.2). Delta1 therefore appears to promote cohesiveness independently of adherens junctions or desmosomes.

The results presented so far in this chapter suggest that Delta1 can promote cohesiveness between keratinocytes. Cohesiveness will depend on a combination of cell-cell adhesion and cell motility. The remainder of this chapter describes my attempts to understand how Delta1 promotes cohesiveness.

5.2.6. Determination of stem cell motility

K-WT, K-DELTA, K-DeltaT or K-SuH^{DBM} were harvested from subconfluent cultures. Populations enriched in stem cells were selected on the basis of adhesion to collagen IV as described in section 2.2.6: briefly, cells were plated onto collagen-coated dishes and incubated at 37°C for 20 minutes in normal medium. Any cells that had not attached after this time were considered not to be stem cells and were removed. The remaining stem cell-enriched populations were then cultured in either low calcium medium or

normal medium and each dish was photographed over 24 hours by time lapse microscopy. Thirty randomly chosen individual cells per sample were tracked manually. Cell speed was calculated using Mathematica (Table 5.3).

Two experiments were carried out for each cell type in normal (high calcium) medium. Under these conditions cell motility might be influenced by the adhesive contacts made with other cells during the course of the experiments. Three additional experiments were carried out in low calcium for each cell type, using two different strains of keratinocytes (kq and kn). Under these conditions, cells do not form stable intercellular adhesive contacts and so these experiments measure their motility as single cells. Under both conditions K-DeltaT were significantly more motile than controls. The motility of K-DELTA and K-SuH^{DBM} did not differ significantly from controls.

5.2.7 Assessment of spreading

K-WT, K-DELTA, K-DeltaT or K-SuH^{DBM} were harvested from subconfluent cultures. Some cells from each dish were analysed by flow cytometry for expression of surface $\beta 1$ and $\alpha 6$ integrins. 10^4 keratinocytes per cm^2 were plated on type IV collagen (50ug/ml), fibronectin (25ug/ml) or uncoated tissue culture plastic (TCP) and incubated at 37°C for 10, 20, 40, 80 or 120 minutes. At each time point, one dish for each experimental condition was quickly washed and fixed in 4% formaldehyde. The cells were stained with Nile blue.

Speed of spreading

100 randomly selected cells from each dish were examined at high resolution by videomicroscopy and scored according to whether they were still completely rounded or had started to spread. Scoring was performed in a blinded fashion. The cells had been seeded sparsely so they would not be able to influence each other's spreading behaviour. Those occasional cells that were close to or in contact with another cell were excluded from the analysis.

K-DeltaT spread more rapidly than K-WT or K-DELTA or K SuH^{DBM} on type IV collagen and on tissue culture plastic. There was no difference in the speed with which cells spread on fibronectin, all four cell-types spreading very rapidly (Figure. 5.8).

K-DeltaT and K-SuH^{DBM} expressed similar levels of surface $\beta 1$ integrin compared with wild type control cells, and K-DELTA had lower levels of surface beta1 integrin than controls (Figure 5.9 A). All four cell types expressed similar surface levels of $\alpha 6$ integrin (Figure. 5.9 B).

Size of spread cells

In order to assess the final spread size of the cells on type IV collagen and on tissue culture plastic, a digital image was captured of 50 randomly selected cells from each of the dishes that had been allowed to attach for 2 hours. The area of each of these cells was measured using Kinetic image analysis software (IP labs). There was a wide variation in cell size after spreading, and the differences between cell-types were not significant (Table 5.4),

After 2 hours, K-WT, K-DELTA cells plated on tissue culture plastic had smooth round edges and had not started to spread although they had extended fine filopodia (Figure 5.9 C, D). K-DeltaT in contrast showed a striking difference in morphology: almost half of the cells had started to extend wide veil-like lamellipodia and smaller cytoplasmic protrusions, which gave an uneven edge to the cell (Figure 5.9 E). K-SuH^{DBM} were slightly larger than K-WT but did not exhibit lamellipodia.

I used an image analysis programme to measure dispersion, which is a property that indicates how smooth the cells perimeter is. Circles or ellipses have dispersion value 0. The computer programme detected the edge of the cell according to the difference in pixel intensity between the dark Nile-blue-stained cell and the pale background. This threshold level was adjusted for each cell to ensure that the computer-generated silhouette accurately represented the outline of the cell. The fine filopodia are not included in the computer-generated outline. The results indicate that the perimeter of the K-DeltaT cells are not as smooth as the perimeters of the other three cell types (Table 5.4), although again there was considerable variation within each experimental group and the differences were not significant.

5.2.8 Cell-cell adhesion molecules

The localisation of E-Cadherin appears normal in K-DELTA and K-DeltaT, although it was notable that Delta1 and E-Cadherin staining was often complementary, with E-Cadherin staining being weakest at those intercellular contacts where Delta1 staining was most intense and vice versa (Figure 5.10 A, B, D, E, arrows). No such relationship was observed when Delta1 and desmoplakin staining was compared (Figure 5.10 G, H). Adherens junctions and desmosomes localise normally to the interface between K-DELTA and K-WT in mixed cultures (Figure 5.10 J, K).

5.2.9 Delta1 interaction with the actin cytoskeleton

Delta1 and DeltaT both colocalise with actin at the cell membrane (Figure. 5.11 A, B). Latrunculin (Sigma) is a drug that disrupts the organisation of the actin cytoskeleton. Latrunculin was diluted to 400ng/ml in culture medium and added to K-WT, K-DELTA or K-DeltaT for 3.5 hours at 37°C. The cultures were then washed in PBS, fixed immediately in 4% paraformaldehyde, and stained for actin and Delta1. When the actin cytoskeleton is rearranged using this drug treatment Delta1 and DeltaT remain on the cell surface, but change from a fine evenly distributed punctate distribution to a coarser distribution of large actin-containing points (Figure 5.11 C, D, G, H). Similar changes in Delta1 localisation can be seen when the actin cytoskeleton is rearranged by placing keratinocytes in low calcium (Figure. 5.11 I, J). Therefore, Delta1 localisation appears to depend to some extent upon the actin cytoskeleton. The redistribution of Delta1 with latrunculin does not reflect a general disruption of the cell membrane or its contact with neighbouring cells because desmoplakin, a transmembrane protein that is anchored to the intermediate filament network, does not change its redistribution upon latrunculin treatment (Dicolandrea *et al.*, 2000). Intermediate filaments are not affected by latrunculin treatment.

The Delta mutant that lacks most of the intracellular domain has an identical distribution to full length Delta1 in all cases. It therefore seems unlikely that Delta1 binds directly to the actin cytoskeleton inside the cell. It may be indirectly linked via another actin-linked transmembrane protein. The actin cytoskeleton associates with integrins, cadherins and CD44. I found that Delta1 and DeltaT colocalise with CD44 both at cell-cell contacts and in microvilli on the surface of keratinocytes (Figure. 5.12). Delta1 showed partial localisation with adherens junctions, although Delta1 remains on the surface in low calcium in the absence of adherens junctions (Figure 5.10 C, F; Figure 5.11 G,H). Delta1 does not localise to regions where keratinocytes are attached to the basement membrane by either of the two types of integrin-mediated adhesion structures, hemidesmosomes (Figure 5.12 A-C) or focal contacts (Figure. 5.12 D-H).

5.3 Discussion

In this chapter I presented evidence that stem cells expressing a dominant negative version of Delta1 can acquire the motile behaviour that is characteristic of transit cells, whilst retaining their stem cell identity. They have increased motility on collagen and can move more freely away from their clonal siblings within confluent cultured sheets. Full length Delta1, in contrast, appears to promote cohesiveness between clonal progeny. In vivo, Delta1 is expressed at highest levels within the stem cell clusters. My results might suggest that Delta1 acts through its intracellular domain to restrict the mobility of stem cells within the basal layer,

It is an attractive idea that both differentiation and mobility of keratinocytes might be regulated by the same molecule in a tissue like the epidermis where cell location is tightly co-ordinated with cell differentiation (Jensen *et al.*, 1999). Delta1 would not be the only molecule to have such a dual role. Integrins not only mediate adhesion to the underlying matrix, but can also independently activate signalling pathways that regulate the transition from stem cell to transit amplifying cell (Levy *et al.*, 2000; Zhu *et al.*, 1999).

Assays to test keratinocyte cohesiveness

The initial indication that Delta1 might influence keratinocyte cohesiveness came from the mixing experiments described in Chapter four. K-DeltaT, when cultured within confluent sheets of K-DELTA, generated clonal progeny that tended not to remain in contact with each other, but rather would disperse over some distance (Figure 4.15). In this chapter I showed that Delta1 and DeltaT influence keratinocyte cohesiveness within confluent sheets of wild type cells. Cohesiveness was not affected by the dominant negative SuH^{DBM} mutant, which indicates that Delta1 does not affect clonal cohesiveness via the conventional Notch pathway. It is safe to assume that the SuH^{DBM} mutant does act in a dominant negative manner to block Notch signalling in keratinocytes, since these same populations of K-SuH^{DBM} were not able to respond to Delta1 in the mixing experiments described in Chapter 4.

One important feature of my observations on the cohesiveness of clonal progeny is that they appear to be independent of effects on cell differentiation. The most direct way to

demonstrate this would have been to score the colonies for cohesiveness on day 5 after plating, and then allow those same colonies to grow for a further 10 days in order to assess whether they form abortive or actively growing colonies. This would require very wide spacing between the EGFP cells in order to identify each individual colony, because the DeltaT colonies tend to disperse over a wide area. In my experiments, I did not seed the EGFP cells sufficiently sparsely to obtain reliable clonal data after 15 days. However, the cloneogenicity assays that I carried out on the same cell populations, plated as isolated cells at clonal density, provide reasonable evidence that the proportion of transit amplifying cells in populations of K-DELTA and K-DeltaT do not correlate with the proportion of non-cohesive colonies.

Using my confrontation assay, I was able to show that populations of K-DELTA tend not to intermingle with populations of K-WT within the basal layer. One criticism of this assay is that Notch signalling is likely to become activated in the wild type keratinocytes at the interface with K-DELTA keratinocytes. Indeed, when I attempted to extend the intermingling assay for 7 or 10 days, I saw a striking increase in large flat differentiated wild-type cells at the interface with K-DELTA, but not at the interface with K-WT or K-DeltaT. A lack of intermingling between K-DELTA and K-WT within the basal layer after more than 7 days might therefore be a consequence of the fact that the boundary cells have been induced to differentiate and move into the suprabasal layers. The assay seems reasonably valid over the course of my 5-day experiments since I have never detected any increase in suprabasal cells within the first 5 days of exposure to K-DELTA (Chapter 4).

Does Delta1 influence cell-cell adhesion?

My observations might suggest that K-DELTA are more adhesive to each other than to K-WT. It is not possible to use conventional cell aggregation assays to measure differences in keratinocyte cell-cell adhesiveness. Instead, I designed an assay based on selective sorting of two keratinocyte populations. I was not able to detect selective sorting-out of any of the different keratinocyte populations that I tested using this assay. However, it is difficult to draw any conclusions in the absence of a positive control to check that this assay is a valid way to detect differences in cell affinity. E-Cadherin has been shown to mediate selective sorting in other systems (Godt and Tepass, 1998), and keratinocytes have been generated that overexpress a dominant negative E-Cadherin mutant (Zhu and Watt, 1996), so these might provide a useful positive control. If the assay can be validated in this way, then it would be interesting to test whether those

keratinocyte that express the highest levels of $\beta 1$ integrin can selectively sort out from those keratinocytes that express lower levels of $\beta 1$ integrin.

One hypothesis would be that Delta1 influences the function of cell-cell adhesive junctions. Delta1 partially colocalises with adherens junctions and with desmosomes (Figure. 5.10 C.F), raising the possibility that it could directly influence either their adhesive function or the speed with which they can be released and reattached. On the other hand, the observation that K-Delta1 are still more cohesive in low calcium medium (Figure. 5.7) argues against the idea that Delta's effect on cohesiveness depends totally on a cadherin-mediated or desmosome-mediated mechanism.

Another hypothesis would be that Delta acts as a cell adhesion molecule itself. It was originally proposed 10 years ago that Delta and/or Notch might act directly as cell-cell adhesion molecules (Fehon *et al.*, 1990). They contain EGF repeats, a feature common to several adhesive proteins (see for example Litvinov 1994). I found that both Delta1 and DeltaT localise to cell-cell contacts in subconfluent keratinocyte cultures. Endogenous Delta1 can only be weakly detected but was also localised to cell-cell contacts (Figure. 4.5). Therefore, in subconfluent cultures, Delta1 is in the right location to act as an adhesion molecule. Furthermore, the localisation of Delta to cell-cell contacts is insensitive to calcium levels (see Figure 5.11): this would be consistent with the observation that K-Delta1 are more cohesive in low calcium as well as in high calcium medium (Figure. 5.7; Table 5.2). However, there are reasons to think that Delta1 is unlikely to be an adhesion molecule. I observed that once keratinocytes become confluent, Delta1 is mainly found in vesicles inside the cell, with only a little remaining on the cell surface. This is consistent with the observation that endogenous Delta1 is predominantly vesicular in developing *Drosophila* and vertebrate tissues (there is some evidence that endocytosis of Delta plays an important integrative role in Notch signalling (Parks *et al.*, 2000)). These data suggest that Delta is a dynamic molecule that does not sit stably on the cell surface, which makes it hard to see how Delta1 itself could act as an adhesion molecule. Furthermore, cell aggregation experiments have shown that, although Delta can bind Delta on neighbouring cell, the interaction is weak and seems unlikely to contribute much to cell-cell adhesiveness *in vivo* (Fehon *et al.*, 1990).

The influence of Delta1 on spreading and motility

DeltaT increased the motility and speed of spreading of keratinocytes on type IV collagen. This could not be attributed to differences in cell fate or integrin expression,

since populations of DeltaT contained similar proportions of transit amplifying cells, and expressed similar levels of surface beta1 and alpha6 integrins compared with wild type controls. However, it was not so clear whether the effects of DeltaT on motility and spreading were independent of the conventional Notch signalling pathway. The SuH^{DBM} mutant did slightly increase both motility and speed of spreading in some cases, and although these effects never reached statistical significance, it seems unsafe to draw any definite conclusions about the role of the conventional Notch signalling pathway.

I did not detect any effect on motility when I overexpressed full length Delta1 in keratinocytes. It is possible that Delta1 might influence stem cell motility under different experimental conditions. My experiments were carried out, in the presence of EGF which strongly stimulates cell motility (Barrandon and Green, 1987b). It would be interesting to see if K-DELTA is able to reduce stem cell motility under submaximal stimulation. It is also possible that the effects of Delta1 are already maximal in untransduced stem cells. It would be useful to check whether Delta1 can reduce the motility of transit amplifying populations.

Cells initiate migration by reorganising the actin cytoskeleton to extend sheet like cytoplasmic protrusions called lamellipodia (Small *et al.*, 1999). These structures become stabilised by forming integrin-mediated anchoring contacts with underlying basement membrane (Nobes and Hall, 1995; Small *et al.*, 1996). However, lamellipodia can form initially without anchorage of the cell to a substrate (Small *et al.*, 1999), I found that almost half of the DeltaT cells extended unusually broad lamellepodia in the absence of any exogenous extracellular matrix substrate. It would be interesting to investigate whether the protrusions seen on tissue culture plastic are anchorage-independent by staining the cells for the two different types of stable anchoring contacts used by keratinocytes: focal complexes and hemidesmosomes.

How might Delta1 influence spreading and motility?

When the actin cytoskeleton is rearranged by latrunculin treatment or by lowering the concentration of calcium, both full length and truncated forms of Delta1 change from a fine to a coarse punctate distribution along the cell membrane. Delta1 and DeltaT show almost complete colocalisation with CD44, a transmembrane protein that is linked to the actin cytoskeleton inside the cell.

CD44 is an integral membrane glycoprotein that is a receptor for the basement membrane protein hyaluronan (Underhill, 1992; Hudson *et al.*, 1995). CD44 can also influence cell motility, independently of its ability to mediate adhesion (Peck and

Isacke, 1996; Peck and Isacke, 1998). This is in keeping with the finding that CD44 induces cytoplasmic protrusions, even when the extracellular domain has been replaced with an irrelevant protein domain that cannot bind to extracellular matrix (Yonemura and Tsukita, 1999). Thus, the intracellular domain of CD44 is implicated in the same events that are affected by the DeltaT mutant.

Regulation of intracellular cytoskeletal events by CD44 is dependent on intracellular binding to ezrin (Legg and Isacke, 1998; Yonemura and Tsukita, 1999). Ezrin acts as a reversible link between CD44 and the actin cytoskeleton (Bretscher, 1999), becoming activated in response to various signals from the Rho family of small GTPases (Hall, 1998; Shaw *et al.*, 1998). There is also evidence that ezrin can in turn activate Rho, leading to a positive feedback cycle (Bretscher, 1999). Ezrin binds to a protein called EBP50, which contains two PDZ interaction domains: this protein has been proposed to act as a scaffold for the attachment of specific membrane proteins to Ezrin (Reczek *et al.*, 1997; Reczek and Bretscher, 1998). It is worth noting that the cytoplasmic tail of Delta1 contains a conserved domain that is predicted to interact with PDZ-containing proteins. Given the colocalisation of Delta1 with CD44, it is possible that Delta1 forms part of the cluster of regulatory proteins, including EBP50, Ezrin and Rho, which associate with the cytoplasmic tail of CD44. It would be very interesting to carry out biochemical studies to directly test whether Delta1 can interact directly with EBP50, Ezrin or CD44. Unfortunately I was not able to determine whether endogenous Delta1 colocalises with CD44 in cultured keratinocytes, because the Delta1 antibody staining was too weak. It is notable, however, that the location of Delta1 *in vivo* is very reminiscent of the staining pattern for CD44 (Hudson *et al.*, 1995), both proteins localising strongly to regions where basal keratinocytes contact the underlying basement membrane, and weakly to cell-cell contacts.

One, very speculative, hypothesis is that Delta1 inactivates CD44 by preventing it from binding to ezrin. In the absence of Delta1 (or, experimentally, when endogenous Delta1 is displaced by DeltaT), ezrin would be free to link CD44 to actin, facilitating the extension of lamellipodia and initiation of migration in response to the appropriate stimuli.

What is the function of the Delta1 IC domain

The intracellular domain of Delta1 is relatively well conserved within vertebrates, but nothing is known about its function. Biochemical studies are currently underway in other labs to identify proteins that interact with the intracellular domain of Delta1, but no candidates have emerged as yet. The quantifiable effects of DeltaT (and potentially

of full length Delta1) on cell motility provide an assay to assess the effects of smaller deletions of the intracellular domain of Delta1. For example, would Delta1 increase cell motility when only the putative PDZ-interacting domain is deleted? Given the ambiguous results obtained with the SuH mutant, it would also be interesting to test whether the ability of Delta1 mutants to block Notch is separable from the ability to increase motility.

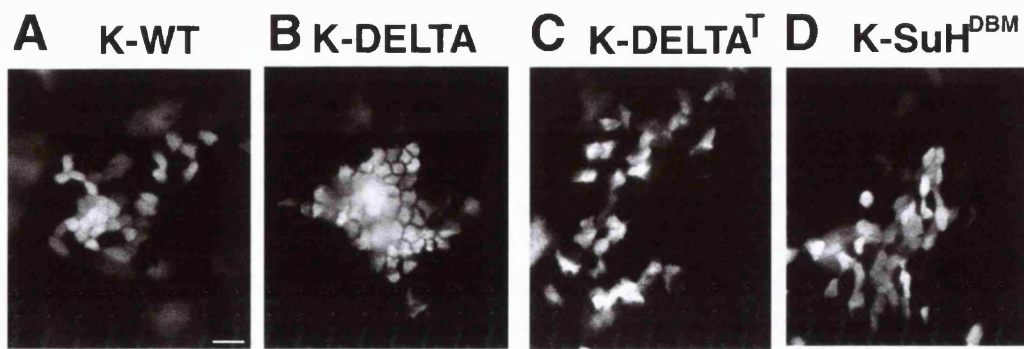
Figure 5.1 Cohesiveness of colonies within confluent cultures

A-D Morphology of colonies arising from K-WT-EGFP (A), K-DELTA (B), K-DeltaT (C) or K-SuH^{DBM} (D) 5 days after seeding within a confluent sheet of unlabelled wild type cells. The colonies are photographed on a fluorescence microscope to visualise EGFP labelled cells: unlabelled cells are not visible. Scale bar: 20µm

Table 5.1 Cohesiveness of colonies within confluent cultures

Colonies were scored as cohesive or non-cohesive after 5 days (see text). For comparison, the proportion of stem cells is given for each cell population. Stem cells are defined as cells that give rise to non-abortive colonies, either when seeded at clonal density or within a confluent culture.

Data are means \pm s.d. from 3 experiments. Values that are significantly different from controls are marked * (Students t-test: $p < 0.05$; all other significance levels are greater than 0.1)



	K-WT	K-DELTA	K-DELTA ^T	K-SuH ^{DBM}
% Cohesive colonies	53.6 ± 4.6	70.2 ± 6.0*	35.6 ± 5.2*	50.0 ± 7.1
% Stem cells (clonal density)	56.7 ± 7.1	56.0 ± 4.3	66.2 ± 0.4	n/d
% Stem cells (confluence)	13.7 ± 0.8	14.7 ± 1.9	10.5 ± 0.6*	n/d

Figure 5.2 Cohesiveness of isolated colonies

Morphology of K-WT (A,D), K-DELTA (B,E) or K-DeltaT (C,F) colonies 5 days after seeding on a feeder layer of J2 cells. Cells are grown in normal medium (A-C) or in low calcium medium (D-F) The cultures are stained with FITC linked to an anti- pan keratin antibody, and photographed under fluorescence excitation so that all of the keratinocytes but none of the J2 cells can be seen.

Scale bar: 50 μ m

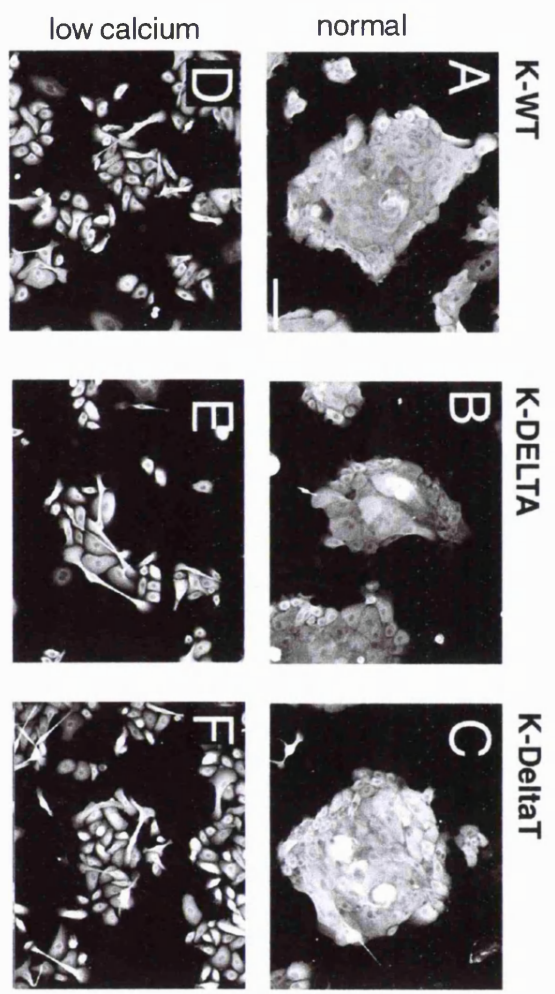


Figure 5.3 Testing K-DELTA in the sorting assay

EGFP labelled cells were thoroughly mixed with unlabelled cells and plated at confluence for 24 hours. The following mixtures were tested:

A,B K-WT EGFP with K-WT **C,D** K-DELTA- EGFP with K-WT
E,F K-WT-EGFP with K-DELTA **G, H** K-DELTA-EGFP with K-DELTA

The cultures shown in A,C,E,G were counterstained with an antibody against Delta1 (RED). Cultures shown in B,D,F,H show the EGFP cells only: surrounding unlabelled cells are not visible. A,C,E show examples of “clustered” colonies; G shows unclustered cells (see text).

Scale bars: 20µm (A,C,E,G) 100µm (B,D,F,H).

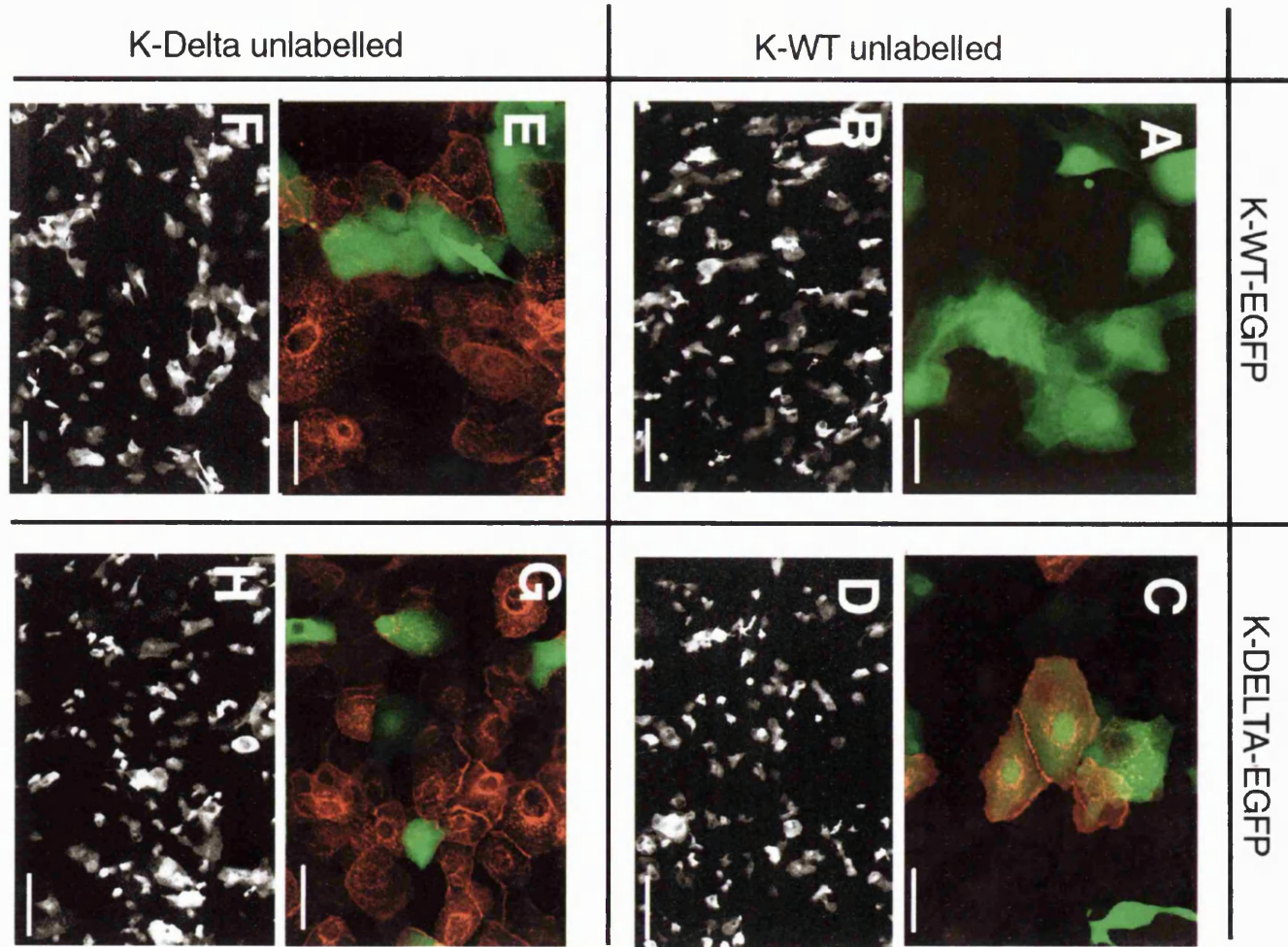


Figure 5.4 Testing K-DeltaT in the sorting assay.

EGFP labelled cells were thoroughly mixed with unlabelled cells and plated at confluence for 24 hours. The following mixtures were tested:

A: K-WT-EGFP with K-WT

B: K-DeltaT-EGFP with K-WT

C: K-WT-EGFP with K-DeltaT

D: K-DeltaT-EGFP with K-DeltaT

These pictures show the EGFP cells only: surrounding unlabelled cells are not visible.

Scale bar: 100 μ m

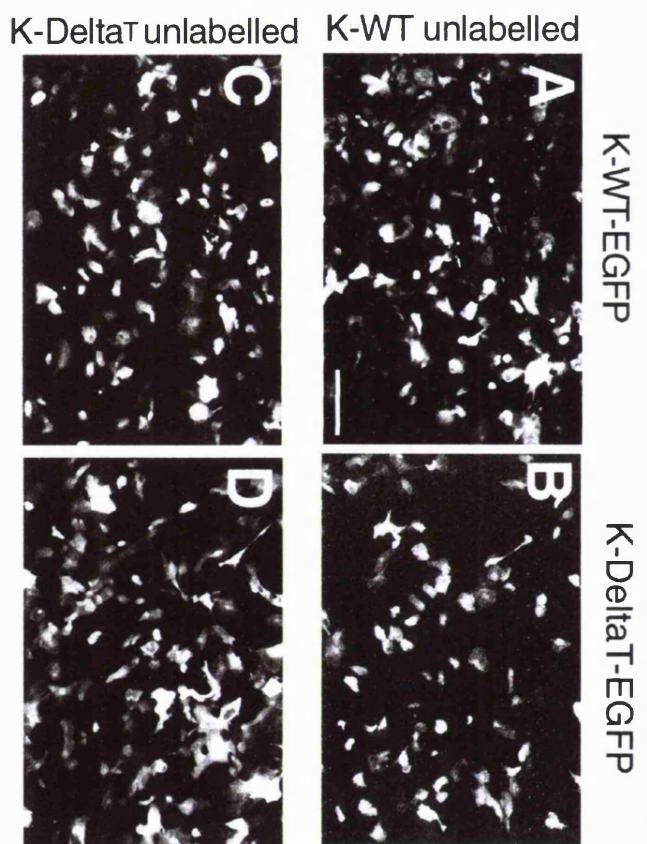


Figure 5.5 Design of the confrontation assay

A: EGFP labelled wild type cells and unlabelled wild type cells, photographed under fluorescence illumination 4 hours after seeding in separate 50ul droplets with a 2mm gap between them. The grey area represents the location of the unlabelled population: the black region in this picture is unpopulated tissue culture plastic.

B-D: After 24 hours (B) the two populations have moved into the gap and joined together to form a continuous confluent layer of cells. The black regions in these pictures are now populated with unlabelled cells.

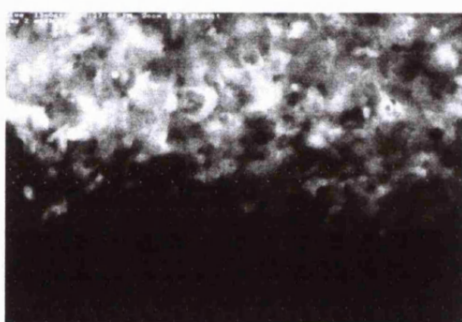
Typical boundary regions were photographed after 24 hours (B) 48 hours (C) or 72 hours (D).

Scale bar 100µm

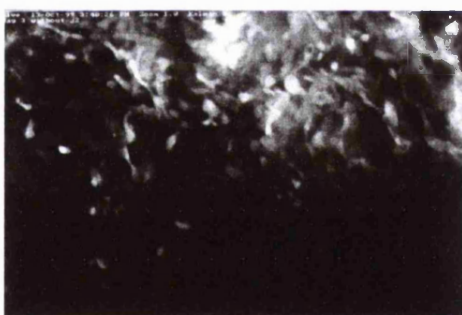
A
4h



B
24h



C
48h



D
72h



Figure 5.6 Confrontation assays: normal medium

Confrontation cultures were set up as described in Figure 5.4 and photographed after 72 hours. The following mixtures of cells were tested:

- A,B:** K-WT-EGFP and K-WT
C,D K-WT-EGFP and K-DELTA
E,F K-WT- EGFP and K-DeltaT

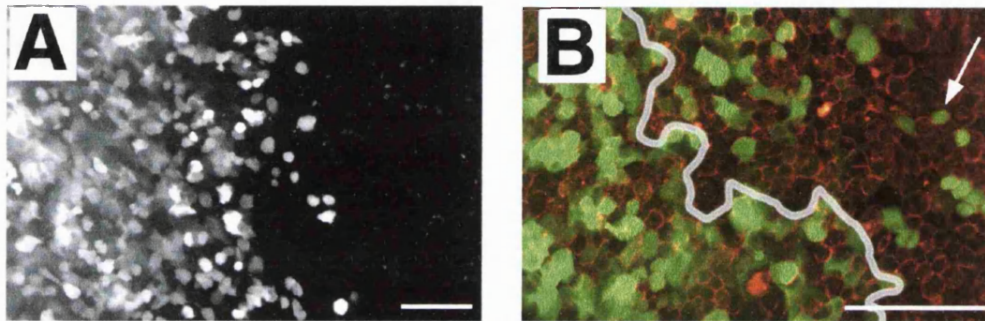
The cultures in B,D,F have been counterstained for $\beta 1$ integrin (red) to distinguish basal cells from suprabasal cells: basal EGFP cells express integrins and so they are outlined in red (an example is marked with an arrow) whilst suprabasal EGFP cells do not express integrins so they are not outlined and appear more diffuse (an example is marked *)

Note that EGFP expression is not uniform in the labelled population.

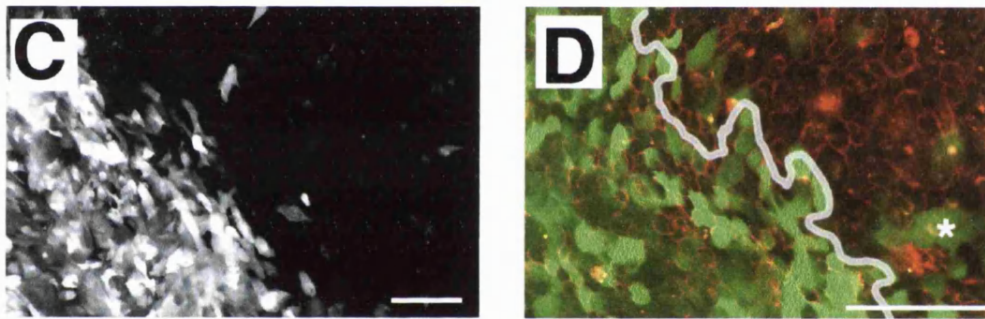
A grey line marks the boundary between the EGFP labelled population and the unlabelled populations in B,D,F. Note that it is much more difficult to define a clear boundary in B and F than it is in D.

scale bars: 100 μ m

K-WT



K-DELTA



K-DeltaT

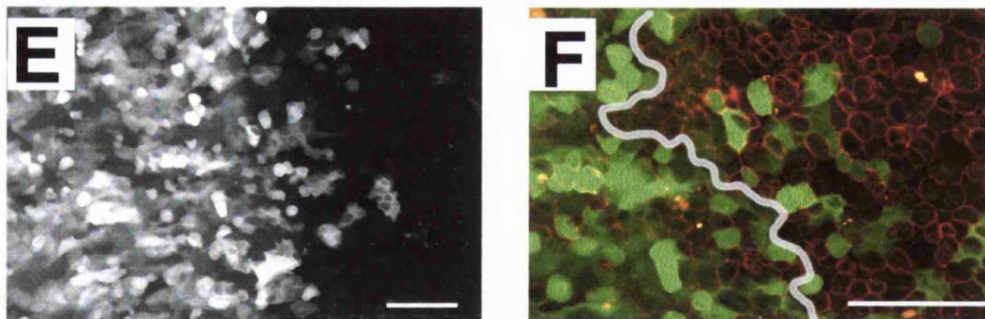


Figure 5.7 Confrontation assays: low calcium medium

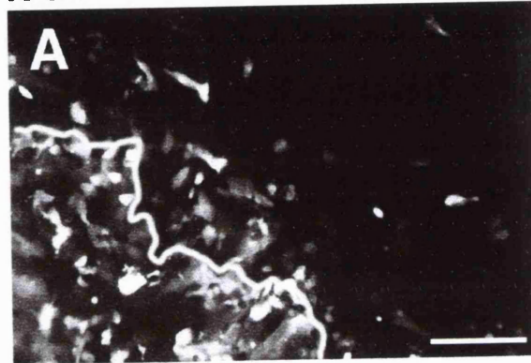
Confrontation cultures were set up as in Figure 5.6 except the cultures were incubated in low calcium medium for the final 48 hours. Cultures were photographed 72 hours after seeding. The following mixtures of cells were tested:

- A:** K-WT-EGFP and K-WT
- B:** K-WT-EGFP and K-DELTA
- C:** K-WT-EGFP and K-DeltaT

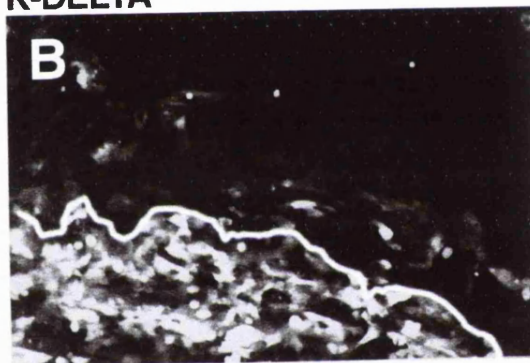
A white line marks the boundary between the EGFP labelled population and the unlabelled populations.

Scale bars: 100µm

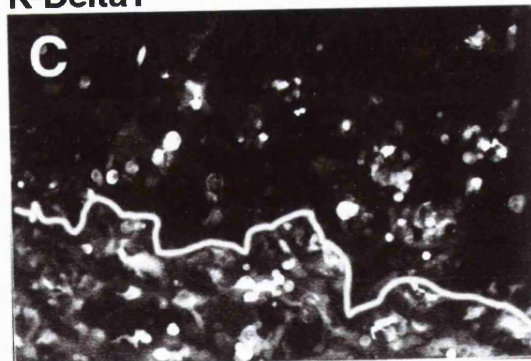
K-WT



K-DELTA



K-DeltaT



	Number of cells that transgressed boundary		% of transgressing cells that were in the basal layer	
	High calcium	Low calcium	High calcium	Low calcium
K-WT	38.2 ± 22.1	78.0 ± 14.4	75.9 ± 8.9	66.8 ± 4.2
K-DELTA	16.2 ± 7.5*	36.3 ± 7.5*	46.5 ± 14.9*	33.0 ± 4.7*
K-DeltaT	27.3 ± 12.9	71.0 ± 11.4	69.6 ± 6.0	68.3 ± 4.4

Table 5.2 Confrontation assay

Confrontation assays were set up as described in Figures 5.6 and 5.7. Three boundary regions were chosen at random each measuring 2mm. The number of EGFP positive cells that had moved into the labelled territory was measured both within the basal and the suprabasal layers using a confocal microscope

Data are means ± s.d. from 3 experiments. Values that are significantly different from controls are marked * (Students t-test: $p < 0.05$; all other significance levels are greater than 0.1)

Speed $\mu\text{m}/\text{min}$	K-WT	K-DELTA	K-DeltaT	K-SuH ^{DBM}
High calcium:	0.50 ± 0.19	0.52 ± 0.15	$0.65 \pm 0.2^*$	0.63 ± 0.27
Low calcium:	0.52 ± 0.24	0.47 ± 0.22	$0.66 \pm 0.31^*$	0.51 ± 0.24

Table 5.3 Speed of cell migration on collagen IV

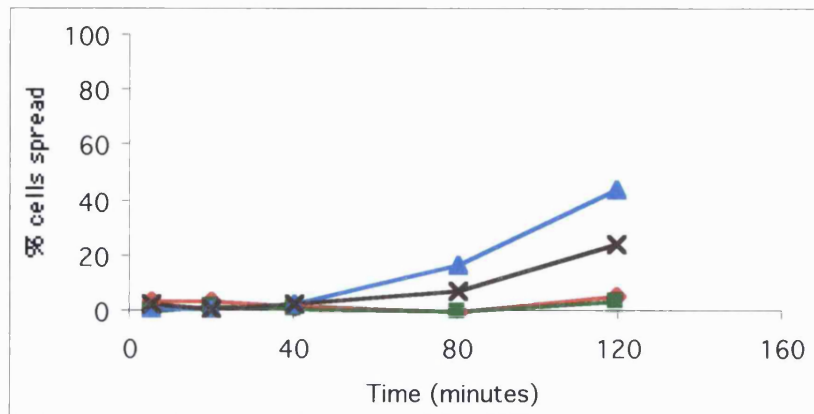
The motility of cells plated on type IV collagen was measured over 24 hours. Cells were either in normal medium (High calcium) or in medium that had a reduced concentration of calcium ions in order to prevent the formation of stable cell-cell contacts (Low calcium).

Data are means \pm s.d. from 3 experiments. Values that are significantly different from controls are marked * (Students t-test: $p < 0.05$; all other significance levels are greater than 0.1)

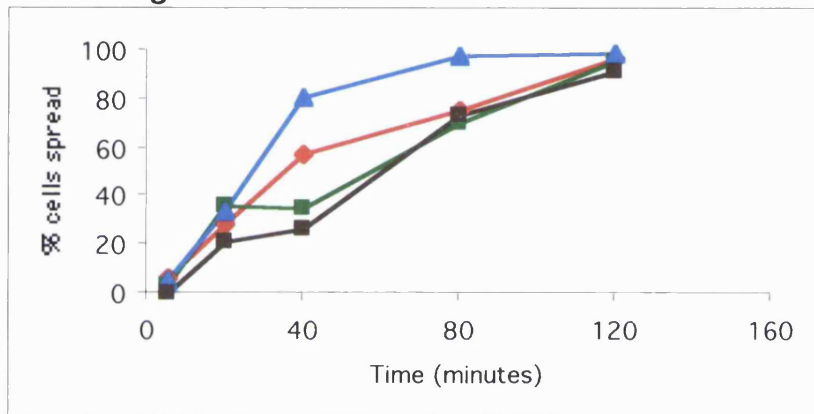
Figure 5.8 Kinetics of cell spreading

K-WT (red) , K-DELTA (green) , K-DeltaT (blue) or K-SuH^{DBM} (grey) were plated on uncoated tissue culture plastic (A) type IV collagen (B) or fibronectin (C) and incubated at 37°C for 10, 20, 40, 80 or 120 minutes. 100 randomly selected cells from each dish were examined at high resolution by videomicroscopy and scored according to whether they were still completely rounded or had started to spread.

A Tissue Culture Plastic



B Collagen IV



C Fibronectin

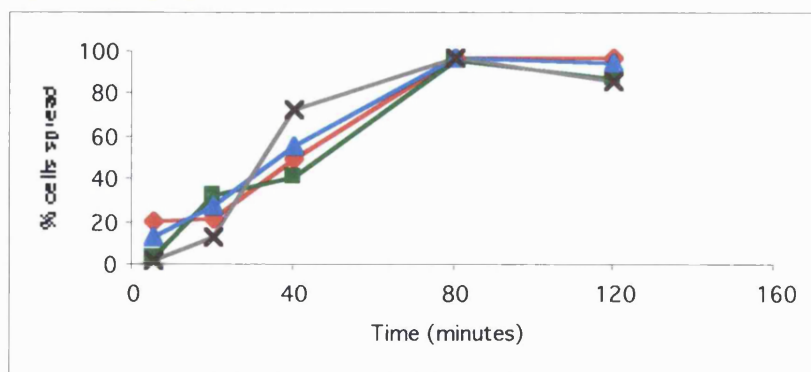


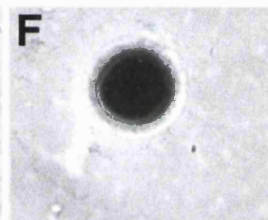
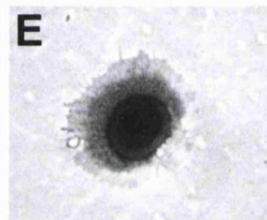
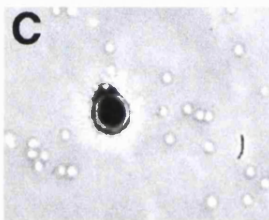
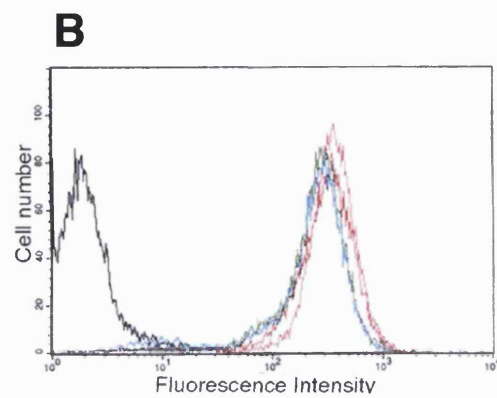
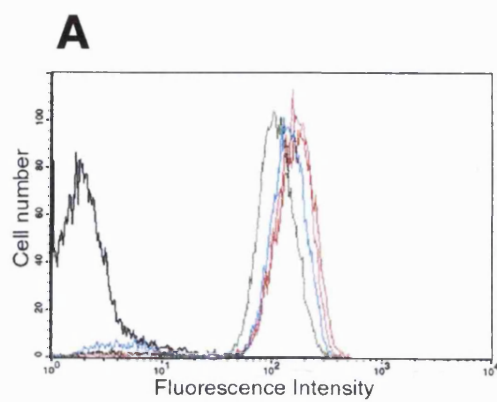
Figure 5.9 Cell spreading

A-B FACS profiles showing surface expression of $\beta 1$ integrin (A) or $\alpha 6$ integrin (B) of K-WT (red) , K-DELTA (green) , K-DeltaT (blue) or K-SuH^{DBM} (pink). The black peak represents K-WT cells stained with an irrelevant antibody.

C-F: Phase contrast photographs showing K-WT (C) , K-DELTA (D) , K-DeltaT (E) or K-SuH^{DBM} (F) 2 hours after plating on uncoated tissue culture plastic

Table 5.4: Cell spreading

Cells were plated for 2 hours on type IV collagen or on tissue culture plastic. Kinetic image analysis software was used to measure total cell area and dispersion (see text). Data are means \pm s.d. from a total of 50 cells. No values that are significantly different from controls (Students t-test: $p > 0.1$)



Spread area	K-WT	K-DELTA	K-DeltaT	K-SuH ^{DBM}
Area Collagen IV	8.3 ± 6.31	7.7 ± 7.3	9.3 ± 5.7	9.9 ± 6.7
Area TCP	2.2 ± 2.1	2.1 ± 3.1	2.7 ± 2.8	2.5 ± 2.7
Dispersion TCP	0.04 ± 0.04	0.03 ± 0.04	0.07 ± 0.09	0.03 ± 0.10

Figure 5.10: Distribution of cell adhesion molecules

A-I: Double label immunofluorescence of K-DELTA (A-C, G-I) or K-DeltaT (D-F) stained with antibodies against Delta1 1 (A,D,G,; red in C,F,I) and E-cadherin (B,E; green in C,F) or desmoplakin (H,K; green in I). C,F,I are higher magnification views the boxed areas in A,B and D,E and G,H, respectively.

Arrows in A,B,D,E show complementary staining intensity for Delta1 and E-cadherin at cell-cell borders (open arrows: high Delta1, low E-cadherin; closed arrows: high E-cadherin, low Delta1).

J-K: Mixed cultures of K-DELTA with KWT-EGFP were stained for E Cadherin (J: Red) or desmoplakin (K: Red). Green is EGFP.

Scale bar: 10 μm (A,B,D,E,G,H) or 5 μm (C,F,I,J,K)

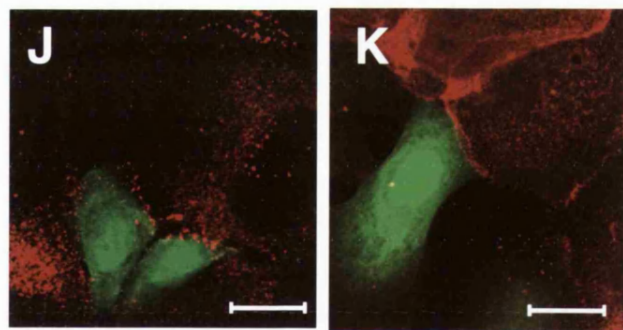
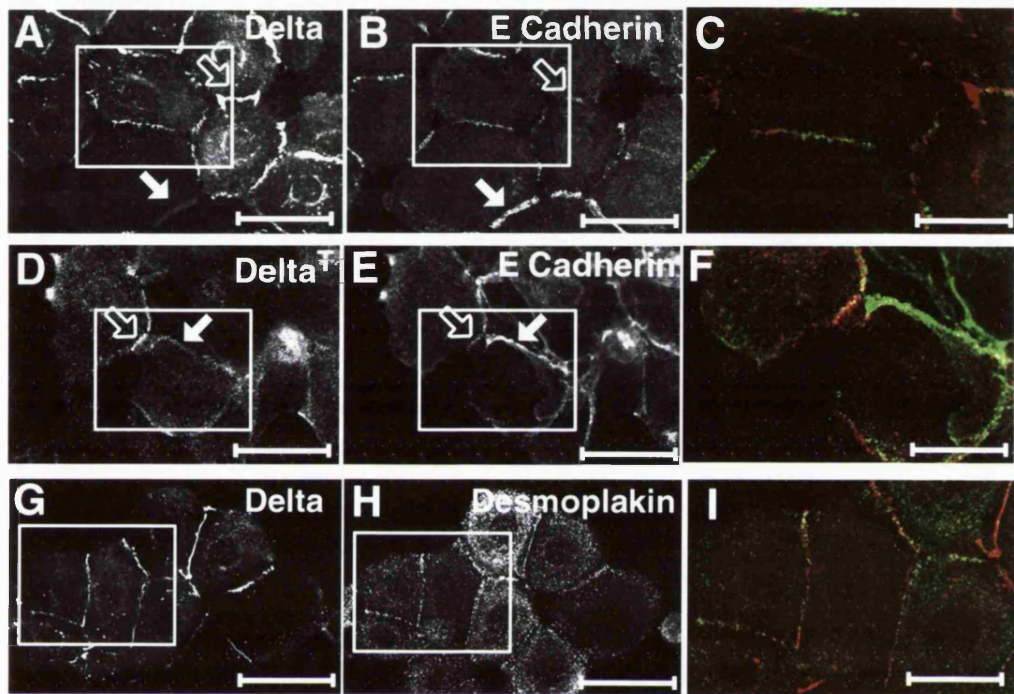


Figure 5.11: Colocalisation of Delta1 and DeltaT with actin.

A-D: Subconfluent keratinocyte cultures overexpressing Delta1 (A,C) or DeltaT (B,D) cultured in normal medium (A,B) or in the presence of Latrunculin (C,D) were stained for Delta1 (green) and actin (Red). Scale bars: 20µm.

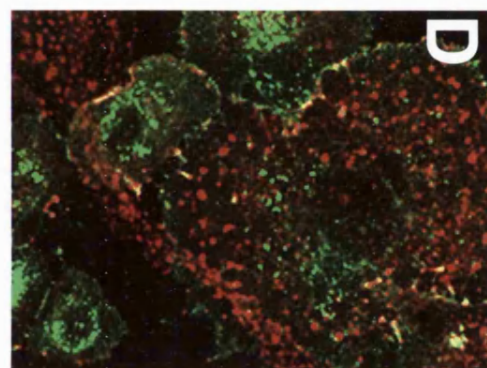
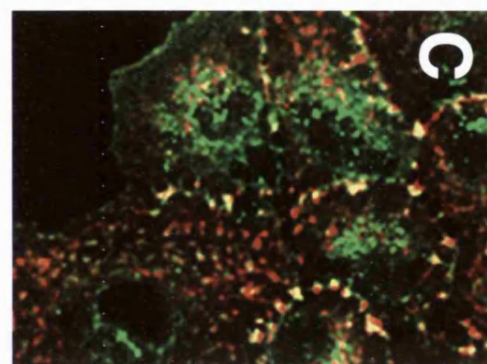
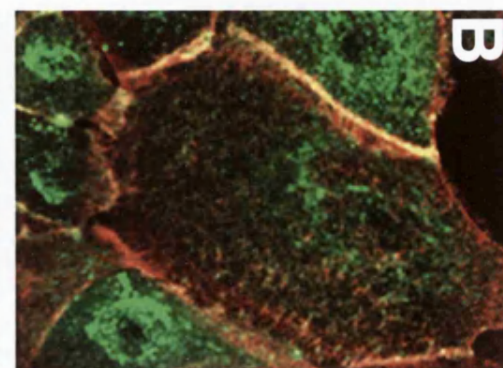
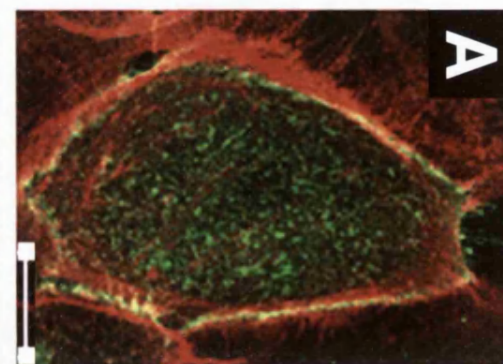
E-J: Subconfluent keratinocyte cultures overexpressing Delta1 (E,G,I) or DeltaT (F,H,J) cultured in normal medium (E,F) or in the presence of Latrunculin (G,H) or in low calcium medium (I,J) and stained for Delta1. Scale bar: 10µm

K-DELTA

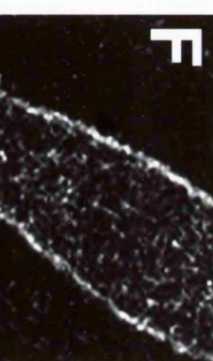
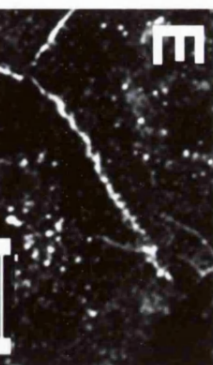
K-DeltaT

Untreated

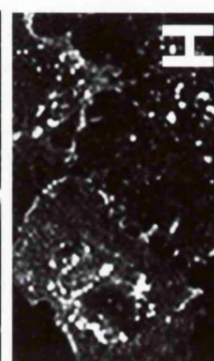
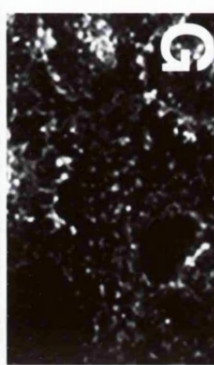
Latrunculin



normal



latrunculin



low calcium

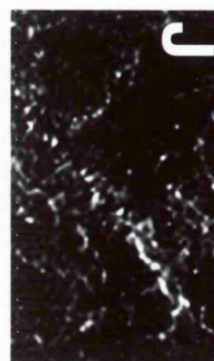
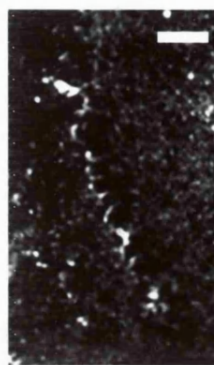


Figure 5.12 : Colocalisation of Delta1 and DeltaT with CD44

Subconfluent keratinocyte cultures overexpressing Delta1 (A,C) or DeltaT (B, D) cultured in normal medium (A,B) or in low calcium medium (C, D) were stained for Delta1 (green) and CD44 (Red)

Scale bars: 10µm.

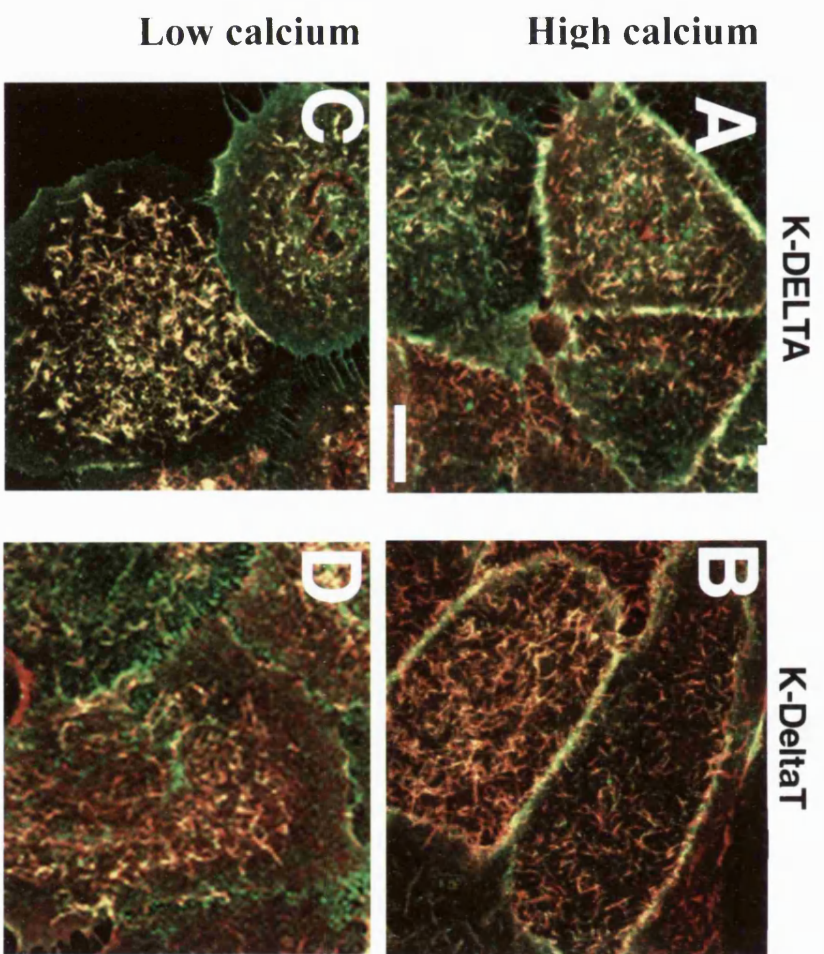
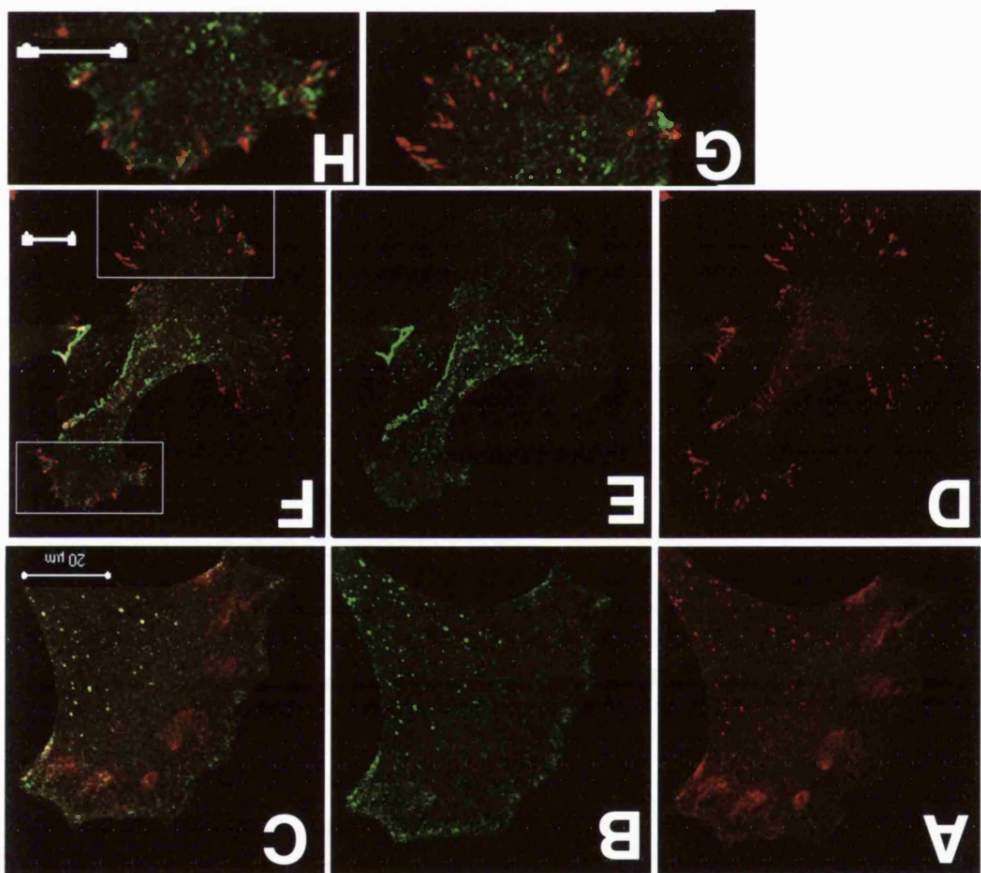


Figure 5.13: Delta1 does not localise to integrin-mediated contacts with the basement membrane.

Subconfluent keratinocyte cultures overexpressing Delta1 cultured in normal medium on type IV collagen were stained for Delta1 (green: B,C,E,F,G,H) and $\alpha 6$ integrin, a component of hemidesmosomes (Red: A,C) or vinculin, a component of focal contacts (Red: D,R,G,H). G and H show magnified views of the boxed areas in F. Scale bars: 10 μ m.



CHAPTER 6

GENERAL DISCUSSION

In this thesis I have presented evidence that the transmembrane protein Delta influences stem cell fate in human epidermis. I have found that Delta1 is expressed in human epidermis, and that it can be found at highest levels in the putative stem cell population. I have also obtained evidence for three different functions of Delta1 in cultured human keratinocytes:

- signalling to neighbouring stem cells to instruct them to become transit amplifying cells.
- acting cell-autonomously to render cells deaf to Delta signals from their neighbours
- promotion of cohesiveness within groups of Delta1-expressing cells

On the basis of these conclusions, I present a model of how Delta1 may function in human epidermis *in vivo*. This model is based on the assumption that stem cells are located in clusters, as proposed by Jones et al (1995) and Jensen et al (1999).

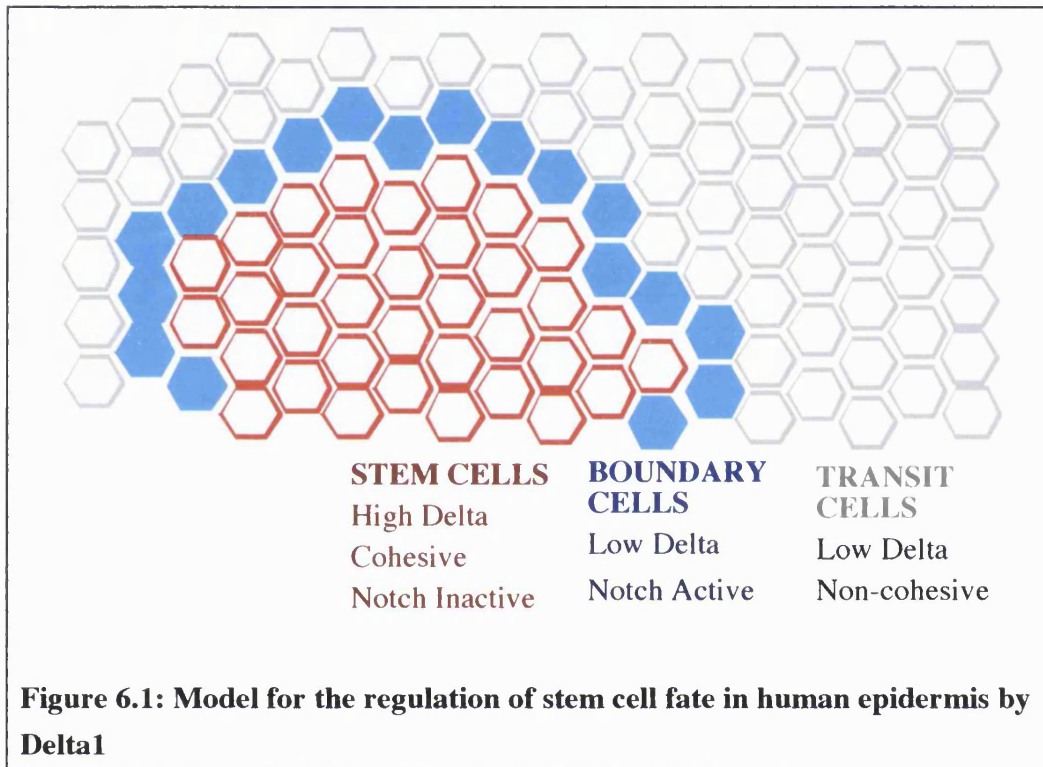
Model for the regulation of stem cell fate in human epidermis by Delta1

I propose that Delta/Notch signalling promotes differentiation at the boundary between the stem-cell clusters and the surrounding transit amplifying cells, thus ensuring that stem cell fate is restricted to the cells within the cluster (Figure 6.1). In this context, differentiation means the transition from being a stem cell to being a transit amplifying cell. Stem cells are protected from Notch activation by virtue of expressing high levels of Delta1, making it important that cells expressing high levels of Delta1 do not migrate out of the cluster. This is achieved by Delta1 promoting cohesiveness within groups of Delta1-expressing cells. This function of Delta1 is independent of the Notch pathway: Notch is only activated at the boundary of high-Delta1 expression, whereas cohesiveness is promoted in all cells expressing high levels of Delta1.

Endogenous Delta1 expression

An important feature of my model is that Delta1 is expressed at higher levels in stem cells than in transit amplifying cells. This difference was apparent both in immunostaining and in situ hybridisation of skin sections. Northern blotting confirmed that a difference is also seen between cultured stem and transit amplifying cells. However, both the immunostaining and the in situ hybridisation can only detect endogenous Delta1 weakly, which makes it difficult to assess whether or not there is a

clear boundary, or a sharp gradient of expression, between high and low expressing cells. Good anti-Delta1 antibodies need to be developed in order to clarify this. In the absence of such antibodies it is also difficult to analyse more clearly the subcellular localisation of endogenous Delta1 and to test its association with proteins such as CD44 and actin that might mediate Delta's influence on cell cohesiveness.



Cross talk with Wingless signalling

Delta1 is likely to act in combination with other signalling pathways in the epidermis, one of the prime candidates being the Wingless (Wg) pathway. *Drosophila* Wg, and its vertebrate homologues the Wnts, are secreted signalling proteins that are important during the development of several tissues, including many which also depend on Notch signalling (Cadigan and Nusse, 1997; Uyttendaele *et al.*, 1998). There is plenty of genetic evidence for cross talk between the Notch and Wg pathways, but the evidence is often confusing and contradictory, probably because the two pathways intersect both directly and indirectly at multiple levels (Panin and Irvine, 1998). At the molecular level, Dishevelled (dsh), which is a component of the Wg signalling pathway, can directly bind to the carboxy terminus of Notch and antagonise its function (Axelrod *et al.*, 1986).

Some members of the Wnt family are expressed in human keratinocytes (Alan Zhu, pers. comm.), but at present we do not know their expression pattern within human epidermis. There is, however, evidence that the Wnt signalling pathway is more active in stem cells than in transit amplifying cells, at least in culture, and that this pathway can maintain a stem cell fate (Gat *et al.*, 1998; Zhu *et al.*, 1999). It is interesting that Wg signalling in *Drosophila* can upregulate expression of Delta whilst antagonising Notch function (de Celis and Bray, 1997). This could explain two features of my model: stem cells have high levels of Delta1 expression but low levels of Notch activation.

Stem cell patterning

In this thesis I have proposed that Delta1 helps to maintain the patterned distribution of stem cells, but my model cannot explain how the size of the stem cell patches becomes established in the first place. Notch signalling can regulate the patterning of cell fates only at a single cell resolution: the arrangement of stem cells in clusters might instead suggest a mechanism based on a longer-range signal. Wingless is one of the few signalling molecules that has been directly shown to act as a long range morphogen (Turing, 1952; Neumann and Cohen, 1997a), and interestingly its range of action has been estimated at 5-10 cell diameters (Zecca *et al.*, 1996; Neumann and Cohen, 1997b). The integrin bright patches in epidermis have a radius of around 5 cell diameters (Jones *et al.*, 1995). At present we do not know which cells in the epidermis are the source of Wnt protein, but the fact that the Wnt pathway appears to be more active in stem cells than in transit amplifying cells might imply that Wnts are secreted by stem cells (Zhu and Watt, 1999).

How might a gradient of Wnt protein become translated into a binary fate decision at the boundary between stem and transit cells? Cooper and Bray have shown that a gradient of Wg in the *Drosophila* eye creates a difference in Notch signalling capacity between adjacent cells along the gradient by promoting Delta1 expression in the cells exposed to the higher levels of Wg. Subsequent feedback in the Notch pathway amplifies this difference, resulting in a boundary between cells with high-Delta expression/low-Notch activity, and cells with low-Delta expression/high-Notch activity (Cooper and Bray, 1999). Thus, one model for the epidermis would be that the patterning of stem cells becomes established by the interplay of Wg and Notch.

Notch signalling during terminal differentiation

In this thesis I have focussed on how Notch signalling regulates the balance of cell fates within the proliferative population of epidermis. However Notch1 is expressed in all the epidermal cell layers and becomes upregulated as terminal differentiation proceeds. It is therefore possible that Notch influences the terminal differentiation programme of keratinocytes. It is unlikely that Delta would act as the Notch ligand in terminally differentiated keratinocytes because it can only be detected in the proliferative basal layer. This role might instead be filled by the Serrate family of Notch ligands: Serrate1 has been found in the suprabasal layers of developing rat epidermis (Lindsell *et al.*, 1995). What role might Notch play in the suprabasal layer? Keratinocytes at each successive stage of differentiation are arranged in discrete layers: this might indicate that keratinocytes communicate with each other in order to coordinate the precise timing of each stage of the differentiation programme. One hypothesis would be that this communication is mediated via the Notch receptor.

Specificity of response to Notch

Notch signalling can mediate a wide range of different events, and can respond differently to its various ligands in many developmental contexts (Bray, 1998), and possibly also in the epidermis (discussed above). Understanding how different target genes are regulated may help explain how specificity of response is achieved. The events downstream of Notch signalling are poorly characterised in vertebrates, and in most cases the direct target genes are not known. In *Drosophila*, Notch signalling drives expression of a family of bHLH transcription factors called the enhancer of split [E(spl)] family. Individual family members have distinct expression patterns. These distinct expression patterns are driven by a small evolutionarily conserved region on the enhancer of each family member. Notch activation alone can only recapitulate expression from this enhancer in a subset of locations, indicating that Notch activity must be integrated with other transcriptional regulators, (Cooper *et al.*, 2000). The vertebrate E(spl) homologue, Hes1, has been shown to respond to Notch activation in epithelial cell lines (Jarriault *et al.*, 1995) and I have found that Hes1 is also expressed in primary human keratinocytes, including in differentiated keratinocytes.

A novel member of the Hes family has recently been found to be expressed in human epidermis (P. Jones, pers comm). It would be interesting to compare the expression patterns of these genes and investigate if they are targets of Delta and/or Serrate mediated Notch signals.

Future prospects

The future prospects for the study of epidermal stem cells are exciting. The ability to follow individual cells using lineage marking has opened up the possibility of studying new aspects of stem cell fate. The work presented in this thesis supports the idea that the same mechanisms used in patterning the developing embryo also regulate the ongoing self-renewal of adult tissues such as the epidermis. The investigation of these patterning mechanisms will no doubt continue to be a fruitful direction for future research.

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