ROLE OF β1 INTEGRIN IN EPIDERMAL DEVELOPMENT AND HOMEOSTASIS

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Abstract

Integrins are a ubiquitously expressed family of cell surface receptors that mediate cell-matrix and in some cases also cell-cell adhesion. In addition to just anchoring the cell to its surroundings, they play a crucial role in tissue morphogenesis and maintenance. In the epidermis β1 integrins have been implicated in regulating keratinocyte proliferation and differentiation.

The aim of the work presented in this thesis is to elucidate the role of β1 integrins in the epidermis by exploiting cells that lack the β1 subunit. Since deletion of the β1 integrin gene in mice causes embryonic lethality before the development of the epidermis, I have taken two experimental approaches: Differentiation of β1-null embryonic stem (ES) cells into keratinocytes in vitro and generation of β1-null keratinocytes via the Cre/Lox system.

ES cells that are homozygous null for the β1 integrin subunit fail to differentiate into keratinocytes in vitro but do differentiate in teratomas and wild-type/β1-null chimeric mice. I found that the impaired differentiation is due to a reduced sensitivity of β1-null ES cells to soluble growth factors in comparison to their wild-type counterparts. I showed that β1-null ES cells can be partially rescued by factors that are secreted by dermal fibroblasts. I could furthermore demonstrate, that TGFα, FGF10 and KGF had an inductive effect on keratinocyte differentiation in vitro.

By isolating keratinocytes from mice with a floxed β1 integrin gene that expressed Cre under the keratin 5 promoter and by expressing EGFP-Cre in fl/fl keratinocytes using retroviral infection, I could study the behaviour of β1-null keratinocytes in vitro. I showed that these cells have a reduced ability to adhere to various extracellular matrices and that they are not able to spread and to migrate due to a failure to organize the actin cytoskeleton and form focal adhesions. In addition, I demonstrated that β1-null keratinocytes have a lower proliferative index and start to express involucrin, a marker for terminal differentiation. Taken together, these data confirm the essential role of integrins in the epidermis and demonstrate that the loss of β1 integrins cannot be compensated for by upregulation of other integrins or signalling pathways.
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Abbreviations

AM12 gag pol + env AM12 packaging cells
bp base pairs
BrdU 5’ bromodeoxyuridine
BSA bovine serum albumin
CD cluster of differentiation antigen
DAB 3,3-diaminobenzene tetrahydrochloride
donor calf serum
ded de-epidermised dermis
DEPC diethylpyrocarbonate
DMEM Dulbecco’s modification of Eagle’s medium
DMSO dimethyl sulphoxide
DNase deoxyribonucleic acid endonuclease
dNTP deoxynucleotide triphosphate
ECM extracellular matrix
EDTA ethyldiaminotetraacetic acid, disodium salt
eGF epidermal growth factor
EGFP enhanced green fluorescent protein
FACS fluorescence activated cell sorter
FAD F12 + adenine + DMEM
FCS foetal calf serum
FITC fluorescein isothiocyanate
GPE gag pol + env86 packaging cells
H&E haematoxylin and eosin staining
HICE hydrocortisone, insulin, cholera enterotoxin and EOF.
HRP horseradish peroxidase
IF keratin intermediate filaments
K10 keratin10
kDa kilo Dalton
LDH lactate dehydrogenase
LRC label retaining cell
LTR long terminal repeats
MAPK mitogen activated protein kinase
MMTV Molony murine tumour virus
MOI multiplicity of infection
MoMuLV Molony murine leukemia virus
N-term amino terminus
O.D. optical density
PBS phosphate buffered saline
PBST PBS/Tween
PCR polymerase chain reaction
pen/strep penicillin/streptomycin
PFU plaque-forming units
PMSF phenylmethanesulphonyl fluoride
PVDF polyvinylidene fluoride
RNase ribonucleic acid endonuclease
rpm revolutions per minute
S.D. standard deviation
SDS sodium dodecyl sulphate
SSC salt sodium citrate buffer
TAE Tris-acetate-EDTA buffer
TBE Tris-borate-EDTA buffer
TCA Trichloroacetic Acid
TEMED N,N,N’,N’-tetramethylethylenediamine
TM transmembrane domain
WT wild type
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Integrins are a ubiquitously expressed family of cell surface receptors that mediate cell-matrix and in some cases also cell-cell adhesion. In addition to just anchoring the cell to its surroundings, they play a crucial role in tissue morphogenesis and maintenance. In the epidermis β1 integrins have been implicated in regulating keratinocyte proliferation and differentiation. This introduction will begin with a brief overview of the structure and function of the skin, followed by a short summary of ES cell culture. Finally, integrins and their role in the epidermis will be discussed.

1.1. STRUCTURE AND FUNCTION OF THE SKIN

The skin is the largest organ in the body. It consists of two layers which develop from different germ layers. The outer layer, the epidermis, is a stratified squamous epithelium derived from the ectoderm. The inner layer, the dermis, consists of connective tissue and is of mesodermal origin. Epidermis and dermis are separated by a basement membrane.

1.1.1. Epidermis and its appendages

*Interfollicular epidermis*

The epidermis is a squamous stratified epithelium. It is made almost entirely of keratinocytes (95%). Other cell types found in the epidermis include melanocytes, Langerhans cells (dendritic cells) and Merkel cells (sensory receptors).

During development the primitive epidermis is established when ectoderm and endoderm are defined in the inner cell mass of the blastocyst. It originates as a single cell layer that starts to form the periderm, a second, outer epidermal layer at the end of the first month in humans and by day 12 of embryonic development in mice (Weiss and Zelickson, 1975). The basal cells differ from the periderm cells in various aspects, but most importantly, some of them are probably cells with stem cell-like properties. (Bickenbach et al., 1986). At around 9-10
weeks estimated gestational age (EGA) in humans and between days 13 and 16 days in mice, after the embryonic – fetal transition, the epidermis becomes three layered and an intermediate layer is formed (Hertle et al., 1991; Fuchs, 1994; Weiss and Zelickson, 1975). During the early stages of development mitotic activity occurs in all layers (Fuchs, 1994), but as suprabasal cells begin to display morphological signs of differentiation, mitotic activity becomes restricted to the cells in the basal layer. On day 16 of mouse development, the intermedium layer is replaced by the strata spinosum and granulosum, and by day 17 the first cornified cells are observed. In human development, it takes about 24 weeks until all the epidermal layers are formed (Hertle et al., 1991).

The same pattern is observed in adult mammalian epidermis. Considering the skin's main function, to form an almost impenetrable barrier, and looking at a histological section might give the impression that the epidermis is a static tissue, but the opposite is the case. The epidermis is a very dynamic epithelium that is constantly being renewed, a process that involves much cell movement and trafficking from different compartments: there is horizontal movement of the undifferentiated cells in the basal layer and vertical movement up to the surface of the differentiating cells. In hairy skin, the movement is probably even more complex due to a contribution of cells from the hair follicle to the interfollicular epidermis (Jensen et al., 1999).

Morphologically, adult epidermis can be subdivided into four different layers that correspond to different stages of keratinocyte differentiation (Figure 1 D):

Stratum basale
The basal layer consists of a single layer of cuboidal cells that are all attached to the underlying basement membrane. It is the only epidermal cell layer that contains undifferentiated, proliferative keratinocytes. It is believed that there is a proliferative heterogeneity among basal cells, with epidermal stem cells that have unlimited proliferative potential generating a population of so called transit amplifying cells, defined as cells that are able to divide only a few times before they start to terminally differentiate (Potten, 1981).
Markers for the basal layer include the keratins K5 and K14, which form a heterodimer and are expressed in the basal cells of all stratified squamous epithelia (Fuchs, 1994).

**Stratum spinosum**

Keratinocytes that have committed themselves to terminal differentiation move out of the basal layer and enter the stratum spinosum, which consists of multiple cell layers. The name for this cell layer is derived from the intercellular bridges that are connected by desmosomes and look like spines under the light microscope. Cells in this layer (which have lost contact to the basement membrane) become more polygonal and start to flatten parallel to the skin surface. They synthesise differentiation specific keratin filaments and precursors of the cornified envelope: The keratins K1 and K10 are expressed instead of K5 and K14 directly above the basal layer and involucrin, a precursor of the cornified envelope is synthesised in the upper spinous layers of the epidermis. As the cornified envelope is formed, involucrin becomes crosslinked in the cornified envelope.

**Stratum granulosum**

Differentiation continues in the granular layer, as cells and synthesise profilaggrin, precursor of filaggrin, which is important for the aggregation of keratin filaments. Profilaggrin is found in keratohyalin granules that are abundant in these cells and give this layer its name.

**Stratum corneum**

The stratum corneum is the outermost layer of the epidermis. It consists of squames, dead keratinocytes whose nucleus and organelles were degraded by its own lysosomal enzymes but which are packed with keratin filaments embedded in a matrix derived from the keratohyalin granules. The cells are surrounded by an almost insoluble envelope that is formed by cross linking of involucrin, loricrin and other cornified envelope components.
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*Epidermal appendages*

The epidermal appendages are derived from epithelial germs that are formed during embryogenesis and lie (with the exception of nails) in the dermis. In mammals they include hair, sebaceous and sweat glands, and nails.

The formation of the appendages is dependent upon epithelial-mesenchymal interaction. The mesenchymal cells of the dermis have an inductive effect on the epidermal cells and determine the regional character of the skin (Billingham and Silvers, 1967; Hardy, 1992; Oshima et al., 2001).

The hair follicle contains dermal (dermal papilla and connective tissue sheath) and epidermal (matrix and inner and outer root sheaths) components. It has received much attention in the past years. Mutations and/or epidermal overexpression of developmentally regulated genes have primarily led to severe impairment of hair follicles (Gat et al., 1998; St-Jacques et al., 1998; Zhou et al., 1995; Huelsken et al., 2001). Furthermore, it has been proposed that the hair follicle is a major repository of keratinocyte stem cells in mouse skin (Taylor et al., 2000).

The development of the hair follicle can be divided into different stages. First the dermis initiates in the overlying epidermis the formation of an appendage (‘first dermal message’). The epidermis responds by beginning to form hair, feather buds or scale placodes - which appendage is actually formed depends on the origin of the epidermis, as dermal-epidermal recombination experiments between different classes have shown (Sengel and Mauger, 1976). The epithelial cells forming the hair follicle then induce mouse dermal cells to form the dermal papillae. This induction is followed by the second dermal message: ‘make hair follicle’. In contrast to the first dermal message, this second message is class specific. The resulting structure consists of a hair shaft surrounded by several concentric layers of epithelial cells that include the inner and outer root sheath, a mesenchymal sheath which surrounds this epithelial core, and a sebaceous gland, which is an outgrowth at the side of the hair germ (Figure 1 B). The hair and the sebaceous gland constitute the pilocebaceous unit.
Once the hair is formed, its lower part undergoes cyclic changes of growth and shedding. The three phases of follicular activity are known as growth (anagen; Figure 1.1 A), regression (catagen) and rest (telogen) (Paus et al., 1999). It is thought that the hair cycle in the adult reproduces some of the embryonic morphogenetic events (Oshima et al., 2001). At every start of the hair cycle, stem cells which reside in a specialised region of the outer root sheath known as the bulge come in contact with mesenchymal dermal papilla cells and are stimulated to exit this compartment and migrate down in the outer root sheath (ORS) to the hair bulb. While migrating, they contribute to the ORS differentiated layers and once they reach the tip of the hair follicle, they then commit themselves to become inner root sheath (IRS) and hair forming progenitors (Oshima et al., 2001). The catagen is initiated by FGF signalling (Hebert et al., 1994) and results in loss of proliferative activity in the matrix cells, leading to a cessation of hair elongation. During catagen, the lower (transient) portion of the hair follicle degenerates. In the third phase (telogen) the hair follicle is at rest and no morphological changes are detectable. In mice the first coat appears at day 4.5 after birth and results from growth of hair formed during embryogenesis. In contrast to humans, hair growth in mice is synchronised. Telogen is reached at day 16 after birth and the first postnatal cycle begins when mice are 4 weeks old.

1.1.2. The dermo-epidermal junction/ basement membrane

The basement membrane separates the epidermis from the underlying connective tissue. It is composed of highly organised extracellular matrix and plays a critical role in organising the skin which goes beyond the provision structural support. In the skin the basement membrane determines cell polarity and has a critical influence on the choice of cell fate. Assembly of a proper basement membrane is dependent on epidermal-mesenchymal interactions (Bohnert et al., 1986).

Depending on the tissue, the precise components of basement membranes vary, and there are also regional differences within the basement membrane of one tissue. The main components that are common to most basement membranes are collagen IV, perlecan, laminins and
nidogen. In skin, also type VII collagen is important. It forms the anchoring fibrils that link type IV collagen in the basement membrane to structures called anchoring plaques (Burgeson, 1993). Fibronectin is abundantly expressed in the dermis and the dermo-epidermal junction, but in normal adult skin there is little fibronectin expressed in the basement membrane itself (Couchman et al., 1990).

Nidogen is a single polypeptide chain that is folded into three globular domains connected by a rod or flexible link. It binds laminin, collagen IV, perlecan and calcium and is essential for connecting the networks of laminins and collagen IV in basement membranes (Dedhar et al., 1992).

Perlecan is the largest and most common proteoglycan in basement membranes. It serves many functions, including cell attachment, basement membrane assembly and binding of bFGF (Aviezer et al., 1994). Proteoglycans are a diverse family that is characterized by the covalent attachment of one or more glycosaminoglycans (GAG) side chains to a core protein. The highly acidic and hydrophilic GAG (repeating disaccharides) chains have a major influence on tissue hydration and elasticity, and can show high affinity binding to various ligands.

1.1.3. Dermis

The dermis lies between the epidermis and the subcutaneous fat. It supports the epidermis structurally and nutritionally. The main cells of the dermis are fibroblasts which are responsible for the synthesis of extracellular matrix (ECM). One of the most abundant components of the dermal ECM is collagen, which makes up 70-80% of the dry weight of the dermis (Hunter et al., 1995). Other components include elastin and proteoglycans. Although the skin consumes little oxygen, the dermis is highly vascularised which is important for thermoregulation. Even without the presence of foreign antigen, there are always small numbers of lymphocytes, mononuclear phagocytes, Langerhans cells and mast cells present. Other cells of the dermis include smooth and striated muscle, nerve cells and lymphatics, which form three extensive plexuses.
1.2. EMBRYONIC STEM CELLS

1.2.1. What are embryonic stem cells?

Embryonic stem (ES) cells are pluripotent, non-transformed stem cell lines that are derived from cells of the early epiblast, and can be propagated in culture (Brook and Gardner, 1997). Mouse embryonic stem cells were first isolated from inner cell masses by Evans and Kaufman (Evans and Kaufman, 1981) and Martin (Martin, 1981). A few years later it was shown that they are able to generate cells of all lineages when allowed to differentiate and that they are capable of contributing to many different tissues in chimeric mice, including the germline, when they are transferred back into mouse blastocysts (Bradley et al., 1984).

The concept of a pluripotent cell emerged from work on mouse teratocarcinomas, germ cell tumors that form various somatic tissues. Work in the 1960s and 1970s on cell lines established from these tumors, embryonal carcinoma (EC) cells, showed that they can serve as models for mammalian development, since they have the potential to differentiate in cells of all three embryonic germ layers \textit{in vivo} and \textit{in vitro} (Martin, 1980). EC cell lines that can differentiate derivatives from all three germ layers were also isolated from human teratocarcinomas (Andrews et al., 1984; Pera et al., 2000)

What defines an embryonic stem cell? One of the most important features of ES cells is their pluripotency. In mammals it was believed until recently that only the oocyte, zygote, early embryonic cells, primordial germ cells and tumours derived from them have the potential to differentiate into derivatives of all three embryonic germ layers (Desbaillets et al., 2000). A simple model would therefore be that a pluripotent cell from the ICM gives rise to committed daughter cells which are restricted to differentiate along specific lineages.

This unidirectional system has been challenged in the past years and a much more dynamic picture of the stem cell evolved. It was recently shown that adult-derived cells, which were already committed to a certain lineage, could under certain conditions (when mixed with ES
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Cells) give rise to all three germ layers (Kondo et al., 2000; Krause et al., 2001). These experiments suggest two possibilities. One is that tissue specific stem cells might be able to 'transdifferentiate' i.e. reprogramme their phenotype when challenged with a new environment. Another possibility is that there is no intrinsic difference between organ-specific and embryonic stem cells and that the concept of tissue-specific stem cells being restricted in their developmental potential is wrong (Anderson et al., 2001; Morrison et al., 2000; Lowell, 2000). One could carry this concept even further and say that being a stem cell is a function and not an entity and all cells have a decreasing but recruitable propensity to act as stem cells as they differentiate (Blau et al., 2001).

However, in contrast to the somatic cells isolated from adult tissue, both ES and embryonic germ (EG; derived from genital ridges of fetuses) (Matsui et al., 1992) can be cultured in an undifferentiated state for an unlimited period of time without losing their differentiation potential. This characteristic also distinguishes ES from EC cells, which often exhibit karyotype instabilities and variations in the ability to differentiate in multiple tissues (Thomson et al., 1998).

Taken together, the following criteria can be used to define an ES cell: (i) prolonged undifferentiated proliferation, (ii) clonally derived cultures that have the potential to form derivatives of all three germ layers, (iii) stable karyotype, (iv) derived from pre- or periimplantation embryo.

ES cells can be derived by isolating mouse blastocysts and placing them in culture where, after 5-6 days, the inner cell mass (ICM) has proliferated so much that it is easily recognised. The ICM is then removed from the trophoblast outgrowth and dissociated into smaller aggregates. Two days after the dispersal of the cells colonies of cells with different morphologies can be observed. The stem-cell-like cells, which can be identified by their appearance and the fact that they proliferate without changing their phenotype, are then selectively removed. The resulting ES cell lines can divide indefinitely without differentiating when placed in the appropriate culture medium. One of the factors that seem to be crucial for this suspension of developmental progress is LIF (leukemia inhibiting
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factor). LIF is a cytokine that acts through a receptor complex composed of a low affinity LIF receptor (LIFRβ) and gp130 (Nakashima and Taga, 1998). One of LIF's downstream effectors is STAT3, whose activity seems to be sufficient to keep ES cells in the undifferentiated state (Matsuda et al., 1999).

ES cells have revolutionised mammalian genetics because they have provided the basis for random mutagenesis and homologous recombination in mice. In addition to their use as a genetic tool, mouse ES cells are presently the only stem cell population that can be maintained indefinitely in vitro without differentiating. Thus, they provide a resource for investigating the parameters of stem cell growth and the factors that sustain proliferation at the expense of differentiation. Furthermore, with the isolation of human ES cells (Thomson et al., 1998; Reubinoff et al., 2000), translating stem cell research into clinical applications ('therapeutic cloning') has become a widely discussed prospect (Winston, 2001; Antoniou, 2001).

In vitro differentiation of ES cells

ES cells can differentiate into multiple lineages in culture. When the factors that keep them in an undifferentiated state are removed, ES cells differentiate spontaneously and - if cultured under appropriate conditions - generate cells of various lineages. The in vitro differentiation of ES cells provides a powerful system to analyse the events that take place during embryonic development: Several interesting and important insights into lineage commitment and maturation have been achieved by using this approach (Keller, 1995; Choi, 1998; Choi et al., 1998). The advantage of using this system over an in vivo approach is that to get access to normal embryo-derived precursors at this stage of development is often very difficult in animal models (Keller and Snodgrass, 1999). In addition, in vitro differentiation of ES cells allows the analysis of cells from knockout mice whose phenotype is early embryonic lethal. Differentiation of ES cells is easily induced and as outlined in the previous section, the difficulty of culturing ES cells lies more in stopping their developmental clock than in allowing them to differentiate.
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There exist various protocols for the \textit{in vitro} differentiation of ES cells. The general principle of all these methods is that the ES cells are removed from their feeder cells or the presence of LIF, and then cultured in a way that allows the formation of so called embryoid bodies, multicellular structures that contain elements of all three germ layers. This can be achieved by placing the cells in suspension either in methylcellulose containing media or by culturing the ES cells in 'hanging drops' (Figure 1.3). In some protocols the cells are also cultured directly on stromal cells rather than putting them in suspension first. Once embryoid bodies have formed, they can be transferred to standard culture conditions on tissue culture plastic for studies on adhesive cell development, in suspension cultures or be transplanted into recipient mice.

If embryoid bodies are kept \textit{in vitro}, a wide range of cell types start to evolve, among them hematopoietic, neuronal, epithelial, muscle and endothelial cells. Interestingly, the cells that differentiate \textit{in vitro} seem to undergo the same developmental program as their counterparts do to form the embryo \textit{in vivo} (Keller, 1995).

One major drawback of the differentiation that takes place in the embryoid bodies is that it is difficult to control and that until now it has not been possible to direct differentiation to only one cell type. However, in the last years immense progress has been made towards this end (Keller, 1995; Lumelsky et al.; 2001; Schuldiner et al., 2000). A range of growth factors has been identified that at least alter the relative proportions of a specific cell type: TGF-\(\beta\) for example has been shown to induce myogenesis (Rohwedel et al., 1994); retinoic acid directs cells towards neuronal differentiation (Bain et al., 1995), and accelerates cardiac differentiation (Wobus et al., 1997).

\textit{In vitro} differentiation of embryoid bodies also leads to the appearance of cells from stratified epithelia (Bagutti et al., 1996). The first epithelial cells appear 12 days after the induction of differentiation. After 3 additional days, the cells express keratins 8 and 18, which are markers of simple epithelium, and from the 21st day of differentiation onwards, markers of stratified epithelium appear. K8 and K18 expression is found at the 4- to 8-cell stage of preimplantation mouse development, in cells of the trophectodermal lineage.
(Oshima et al., 1983). K14 is first expressed in the single layered ectoderm (which will subsequently differentiate into epidermis) of mouse embryos, from E9.5 onwards in some cells, and from day 14.5 onwards K14 is expressed in all cells of the basal layer (Fuchs, 1994). The regional variation in sites of expression at day 9.5 is consistent with an inductive role of the underlying mesenchyme (Byrne et al., 1994).

K1 and K10 are first detected at E13.5, as the epidermis begins to differentiate into spinous and granular layers (Byrne et al., 1994). We have previously reported that there is a temporal sequence in the appearance of epithelial markers in wild-type ES cultures: K8 and K18 are expressed first, followed by K5/K14, then K1/K10 and another epidermal terminal differentiation marker, involucrin (Bagutti et al., 1996). It thus seems likely that the K14-positive cells that differentiate in ES cell cultures correspond to embryonic ectoderm.

1.3. INTEGRINS AND THEIR LIGANDS

Integrins regulate development and terminal differentiation in many cell types (Hynes, 1992; Hynes et al., 1992; Adams and Watt, 1993; Streuli et al., 1991). Integrins are large cell surface receptors found on almost all cells of multicellular organisms. They were discovered in the early 1980s (Tarone et al., 1982; Neff et al., 1982; Pytela et al., 1985), but not cloned until the mid 1990s (Hemler, 1999).

Integrins seem to be the main receptors by which cells adhere to extracellular matrix. In addition to ECM ligands many integrins have been shown to bind in other cell surface molecules. One distinctive feature of integrins is that cells can regulate their ligand binding activity.

1.1.1. Structure of integrins

Integrins are composed of two noncovalently linked type I transmembrane glycoprotein subunits called α and β. In mammals 19α and 8β subunit genes have been described so far which combine to give 25 heterodimers that differ in their ligand binding ability (Humphries, 2000b). In addition, splice variants have been found in the intra- and extracellular domains of
many integrin subunits, including α3, 6, 7 and β1, 3, 5 cytoplasmic domains and α6,7 and β3 extracellular domains (de Melker and Sonnenberg, 1999).

Structural knowledge of integrins is still limited. Until recently (with the exception of the I domain, a region in the α subunit which folds independently and can be expressed in recombinant form) the tertiary structure of integrins was not known. There are reports that the tertiary structure of αvβ3 has been solved (Science 293, 2001), but the original article was not yet available at the time of writing the thesis. Electron microscopy of integrins suggests that integrins have a globular head region that is connected to the membrane via two stalks (Nermut et al., 1988). The long extracellular domains of both the α and β subunits are responsible for ligand binding and subunit association, but the cytoplasmic tails also play a role in heterodimer association and the regulation of ligand binding (Takagi et al., 2001; Hughes and Pfaff, 1998). It was found that the membrane proximal association of both cytoplasmic tails via complementary negatively and positively charged residues constrains the integrin in an inactive state whereas lack of association in the membrane proximal regions activates the integrin.

The α subunit

The α subunits are composed of 1000-1150 amino acids and their mature size is 140-210 kDa. All integrin α subunits have seven amino terminal repeating sequences that fold into a β-propeller motif that is similar to that found in the β subunits of heterotrimeric G proteins (Springer, 1997).

The α1, α2, αL, αm, αx αD and αE subunits (Humphries et al., 2000; Humphries, 2000b; Hemler, 1999) contain an inserted domain (I domain) of about 200 amino acids between β sheets 2 and 3 of this β-propeller domain. Three dimensional structures of the I domain show that it has a domain with divalent cation coordination site (Michishita et al., 1993; Calderwood et al., 1997), which has been called 'MIDAS', for metal ion-dependent adhesion site. It has long been speculated that one of the coordination sites for this cation may be provided by peptide motifs found in most integrin ligands. Indeed, the crystal structure of the
complex of the α2 domain and a triple helical collagen peptide shows that the region of the I domain that coordinates the ion also engage the collagen (Emsley et al., 2000). There is also growing evidence that the I domains undergo conformational change upon activation and that this regulates cell adhesion (Leitinger and Hogg, 2000; Shimaoka et al., 2000). Those integrins that lack the I domain seem to use a site within the amino terminal half for ligand recognition, but the exact binding sites have not been determined (Kamata et al., 1995; Plow et al., 2000).

A region that is important for divalent-cation binding has been described in the last three or four C-terminal homologous repeats. The sequence has been described to be similar to the "EF hand" (although it recently has been proposed that these repeats more resemble a β hairpin loop than an EF hand (Springer et al., 2000), a motif that is found in many calcium binding proteins including calmodulin and myosin light chain).

Integrins that do not have an I domain often are proteolytically processed in a region close to the carboxyl end of the extracellular domain, yielding two peptides that are linked by disulfide bridges. The significance of this proteolytic processing is unclear, but it might play a role in receptor activation (Delwel et al., 1996).

Close to the transmembrane domain in the cytoplasmatic unit, all α subunits have a conserved 'GFFKR' region. Deletion of the membrane proximal GFKKR sequence constitutively activates LFA-1 (Hibbs et al., 1991), which suggests that this region is required to maintain the integrins in a low affinity state. Calreticulin, a calcium binding protein that seems to involved in transient elevation of calcium upon integrin ligation (Coppolino et al., 1997), directly interacts with the GFFKR motif. Calreticulin null cells have severely impaired integrin mediated cell adhesion (Coppolino et al., 1997). Another α subunit binding protein is caveolin-1, which is a transmembrane adaptor and has been reported to link Fyn (a tyroine kinase) and recruit Shc and subsequently Grb2 and Sos and thereby regulate Ras-ERK signalling (Wary et al., 1998).

With the exception of this membrane proximal region though, there is only a very low degree of sequence conservation between the different α subunits. However, within a given α...
subunit one can observe a very high degree of cross-species conservation of the cytoplasmic tails, indicating that cytoplasmic sequences are functionally important.

The β subunit

With the exception of β4, the β subunits have 730-800 amino acids and are 90-130 kDa in size (Hynes et al., 1992). They have an I domain-like motif in the amino-terminal half that contains residues essential for ligand and divalent cation binding (Huang et al., 2000). Mutations in this motif cause loss of adhesive function. Also in the extracellular domain, but nearer to the membrane, is a four repeating motif comprising the 'cysteine-rich', epidermal-growth factor (EGF)-like domains. This region appears to be important in the regulation of integrin function since activating and activation-dependent antibodies bind to this region (Humphries et al., 2000; Humphries, 2000a) and mutation in this region causes a constitutively active state (Nolan et al., 2000). Another domain that is found in the extracellular sequence is a module called PSI (plexins, semaphorins, integrins) (Bork et al., 1999).

The cytoplasmic tails of the β subunits have been studied extensively. The degree of sequence conservation between the cytoplasmic domains of the β subunit is higher than that of the α subunits (about 60%), and also the cross-species-homology is very high (96-100%) (Humphries, 2000b). An exception is the β4 cytoplasmic tail, which is much longer than the other cytoplasmic tails.

The β1 subunit

The β1 subunit cytoplasmic tail is sufficient to target proteins to focal adhesions, independent of the ability of the integrin to bind ligand (Takada et al., 1992). Three regions, cyto-1 to cyto-3, have been defined that are essential for the localisation of integrins to focal adhesions and cytoskeletal associations. Cyto-1 can bind α actinin (Otey et al., 1993), cyto-2 and cyto-3 contain 'NPXY' sequences that are important for integrin affinity. Other cytoplasmic subunit
sequences are also required for the modification of integrin adhesiveness (Hibbs et al., 1991; O'Toole et al., 1995).

To date, at least 12 proteins have been shown to bind to β1 integrin tails. They include talin (Horwitz et al., 1986), α-actinin (Otey and Burridge, 1990), filamin (Pavalko et al., 1989), ILK (Hannigan et al., 1996), FAK (Schaller et al., 1995), Paxillin (Schaller et al., 1995), Rack1 (Liliental and Chang, 1998), ICAP-1 (Chang et al., 1997) and CD98 (Zent et al., 2000). Two of these proteins are kinases: the focal adhesion kinase (FAK) and integrin linked kinase (ILK). It is not clear which, if any, role direct binding of FAK plays (Liu et al., 2000). FAK has been shown to bind isolates of cytoplasmic subunit fragments but it might also interact indirectly with the cytoplasmic domain via the binding of paxillin or talin (Borowsky and Hynes, 1998; Chen et al., 2000). ILK is a ubiquitously expressed serine-threonine kinase which takes part in focal adhesion formation (Hannigan et al., 1996) and is also involved in various other signalling pathways, including Wnt signalling (Delcommenne et al., 1998; Novak et al., 1998). Recently a new downstream target of ILK, affixin, has been found which is important for the initial formation of focal adhesions (Yamaji et al., 2001).

Talin has a molecular mass of approximately 230 kDa. It links the actin filament bundles to the cytoplasmatic subunit of integrins. The exact talin binding site has not been defined yet, but there exist point and deletion mutants that inhibit talin (Calderwood et al., 1999) and it has been shown that talin is essential for the localisation to focal adhesions (Reszka et al., 1992). α-actinin colocalizes with integrins in focal adhesions. The α-actinin binding site has been localised to the membrane – proximal half of the β1 tails (Otey and Burridge, 1990). Actin cross links and bundles actin filaments and can also bind various cytoplasmic and membrane bound proteins, including vinculin and α-catenin (Nieset et al., 1997). Filamin is an adaptor molecule (Ohta et al., 1999). It localises to the cortical cytoskeleton and along stress fibres, but also has been detected in focal adhesions (Pavalko et al., 1989). Its recruitment to focal adhesions is stimulated by mechanical stress and leads to actin recruitment (Glogauer and Ferrier, 1998). Rack-1 is a receptor for activated PKC. It contains 7 WD repeats. The importance of this interaction is not known yet (Zhang and Hemler, 1999; Liu 00). ICAP-1 (integrin cytoplasmic-domain-associated protein 1) binds specifically to
β1A. It is phosphorylated in response to cell adhesion to fibronectin. ICAP-1 might regulate cell spreading and migration in CamKII dependent manner (Bouvard and Block, 1998; Bouvard et al., 1998; Zhang and Hemler, 1999). CD98 is a type II transmembrane protein that might be able to regulate integrin activation (Zent et al., 2000).

Five splice variants of the cytoplasmic domains of the β1 subunit have been described (de Melker and Sonnenberg, 1999). The β1A variant is ubiquitously expressed, whereas the expression of the other splice variants is more restricted: β1B is found in the skin, liver, and in cardiac and skeletal muscle (Balzac et al., 1993). β1 C and β1 C-2 is found in haematopoietic cells, liver and kidney (Languino and Ruoslahti, 1992; Svineng et al., 1998), and β1 D is expressed in adult cardiac and skeletal muscle (Belkin et al., 1996; Zhidkova et al., 1995). β1B and C which both lack the cyto-2 and cyto-3 motif can not localize to focal contacts and induce tyrosine phosphorylation of cytoplasmic subunits (Meredith et al., 1995; Languino and Ruoslahti, 1992). The β1C splice variant has been shown to inhibit the cell cycle in late G1, and β1B can inhibit cell spreading and motility (Balzac et al., 1994; Meredith et al., 1995). In comparison to the β1A, β1B expression in keratinocytes is very low (Kee et al., 2000). β1A and D can both localise to focal contacts, but β1D lacks two consecutive threonine residues between cyto 2 and 3 which appear to be involved in the regulation of ligand binding (de Melker and Sonnenberg, 1999; Wennerberg et al., 1998) Mice with a null mutation of β1 D that express β1A instead develop normally but replacement of the β1A by the β1D isoforms results in embryonic lethal phenotype (Baudoin et al., 1998). β1B is not conserved in the mouse genome (de Melker and Sonnenberg, 1999).

1.3.2 Functional properties of integrins

Integrins have been extensively analysed by mouse genetics. The first report of a targeted mutation was the deletion of the α5 subunit by R. Hynes group in 1993 (Yang et al., 1993b), and since then most of the known integrin genes have been knocked out. These knockout studies showed that most integrins are indispensable for normal development. Deletion of the
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β1 integrin gene causes peri-implantation lethality, whereas mutations of the respective α subunits lead to different (mostly lethal) phenotypes at later stages of development (De Arcangelis and Georges-Labouesse, 2000).

The functions of individual integrins have been elucidated in vitro using various assays: ligand affinity columns, binding of soluble ligands to purified or cellular integrins, use of monoclonal antibodies that block or activate block ligand binding or the overexpression of individual subunits (and mutations therein) in cells.

Integrin binding activity is dependent upon divalent cations, which is explained by the MIDAS and β hairpin motifs in the extracellular subunits. The effects of cations on integrin binding is cell-type and integrin specific, but some cations, including Mn^{2+} promote a high affinity state under most conditions (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1991; Elices et al., 1991).

Integrins are capable of bidirectional signalling: they can transduce signals into the cells and activate signal transduction pathways (outside in signalling) and their functional activity (ligand binding affinity) can be modulated by intracellular events (inside out signalling) (Hynes, 1992). Communication between the inner and outer parts of the integrin takes place via conformational change and it recently has been proposed that during activation both subunits unclasp in the integrin C-terminal juxtamembrane region. This conformational change in the integrin is sufficient for inside out activation and association with other molecules; integrin clustering is not required (Takagi et al., 2001). Integrin function is also regulated by diffusion and clustering of integrins in the plasma membrane and lateral association with other membrane components such as TM4SF proteins or syndecans (Hemler, 1998; Woods and Couchman, 2000; Woods et al., 2000).

Upon engagement with ligand, integrins trigger intracellular signal transduction cascades. These signals are integrated with signals from other receptors, which is often facilitated by close proximity of key molecules in the cascade (Turner, 2000). Interactions can take place on several levels: Integrins have been shown to bind directly and indirectly to receptor
tyrosine kinases. For example they co-immunoprecipitate with the EGF receptor and PDGF receptor (Giancotti and Ruoslahti, 1999; Miyamoto et al., 1996). They also can bind the same intracellular kinases or adaptors as these growth factor receptors and activate signalling pathways similar to those of growth factor receptors, including tyrosine phosphorylation of intracellular proteins, elevation of intracellular calcium and activation of PKC (Clark and Brugge, 1995). A subgroup of β1 integrins for example can induce the recruitment of the adaptor molecules Shc and Grb2 and so activate MAPK (Wary et al., 1996). β1 integrins also have been shown to activate Abl (Lewis, 1996) and suppress ILK (Hannigan et al., 1996).

Ligation of all β1 integrins leads to FAK autophosphorylation, thereby creating a binding site for Src via its SH2 domain. Through the Src family kinases integrin then activates Ras, which leads to the activation of the classical MAPK cascade (Parsons and Parsons, 1997). Other signalling pathways that have been described are the activation of Rac and Rho via the GTPase activating protein Graf (Taylor et al., 1999) or via the recruitment of Nck (Schlaepfer et al., 1997).

Many of these signalling events take place in focal adhesions, which can form very large signalling complexes consisting of integrins and structural and signalling molecules. Focal adhesions are believed to form in a hierarchical manner. Experiments using fluorescent-protein tagged molecules showed that paxillin accumulates first, followed by α–actinin (Laukaitis et al., 2001). These complexes then grow in size and complexity, as various other components are recruited, including multiple actin binding –proteins and signalling molecules such as FAK, PKC isoforms, Src and Abl (Yamada et al., 1997). Focal adhesions contain also membrane proteins other than integrins. One example is syndecan-4, which localizes upon activation by PKC to focal adhesions (Baciuc and Goetinck, 1995).

### 1.3.3. Keratinocyte integrins

Keratinocytes express several integrins. The major integrins in keratinocytes are α2β1, α3β1 and α6β4. Additional integrins include ανβ5, ανβ6, α5β1 and α9β1. The latter three are induced in culture and on wounding (Watt and Hertle, 1994; Hakkinen et al., 2000; Stepp and Zhu, 1997). The majority of integrins have been identified by immunostaining and
further described by immunoprecipitations. Based on their function in the epidermis, they can be divided into two groups: The β1 integrins that associate with the actin cytoskeleton, and the α6β4 integrin that mainly associates with the intermediate filament network. The β1 integrins have a pericellular distribution, whereas α6β4 is enriched at the basement membrane (De Luca et al., 1992; De Luca et al., 1990; Hertle et al., 1991; Hertle et al., 1992).

α2β1 mediates cell adhesion to collagens I-IV. It also recognises laminin-1 (and possibly other laminins), but this activity is weaker than interactions with collagen and it is not clear whether it is important in the epidermis (Kirchhofer et al., 1990). The knockout of α2 has not been published yet.

α3β1 is primarily a laminin receptor. It interacts more strongly with laminin 5 and 10/11, but also mediates adhesion to other laminin isoforms and ligands such as collagen and fibronectin (Hemler, 1999). α3β1 associates with tetraspan molecules (Hemler et al., 1996). Insight into the importance of α3β1 in the skin came from the knockout of the α3 gene, which is lethal perinatally mainly due to defects in the kidneys and lungs. Neonatal skin of α3 knockout mice reveals regions of disorganised basement membrane from E15.5 onwards which is frequently accompanied by blistering at the dermal epidermal junction due to rupture of the basement membrane (DiPersio et al., 1997). Keratinocytes isolated from these mice can attach to laminin 5 through α6 integrins, but the lack of α3 causes a defect in cell spreading in comparison to wild-type cells. Hodivala-Dilke (Hodivala-Dilke et al., 1998) suggested transdominant inhibition of other integrins in mouse keratinocytes by α3β1 integrin: α3 – deficient keratinocytes showed an increase in fibronectin and collagen type IV receptor activities, and she also observed a higher concentration of actin-associated proteins such as vinculin, talin and alpha-actinin at focal adhesions in α3 deficient keratinocytes.

α5β1 recognizes various ligands that have a RGD motif such as fibronectin, vitronectin, tenascin and osteopontin. It is present at high levels during embryonic development in a wide range of tissues, in the adult its expression is more limited (Yang et al., 1993a). The knockout for α5 is embryonic lethal with mesodermal and vascular defects. α5 −/− cells can still
assemble a fibronectin matrix due to compensation by $\alpha v$ integrins (Hynes, 1996) $\alpha 5\beta 1$ is hardly detected in mature epidermis, but gets upregulated during wound healing and in cultured keratinocytes (Nazzaro et al., 1990; Hertle et al., 1991; Hertle et al., 1992). It has been proposed that exposure to dermal fibronectin is one of the signals that activates keratinocyte migration and proliferation during wound healing (Grinnell, 1992).

$\alpha 6\beta 4$ is primarily a laminin receptor. The distinctive feature of this integrin is the $\beta 4$ subunit, which has an unusually large cytoplasmic domain containing about 1000 amino acids instead of the about 50 amino acids found in the other $\beta$ cytoplasmic subunits. In contrast to the $\beta 1$ integrins, which are involved in the formation of focal contacts, $\alpha 6\beta 4$ forms hemidesmosomes, which are crucial for an intact dermo-epithelial junction. The knockouts of $\alpha 6$ and $\beta 4$ essentially have the same phenotype and result in perinatal lethality with severe blistering of the skin (Georges-Labouesse et al., 1996; van der Neut et al., 1996; Paus et al., 1999). Interestingly, in some $\alpha$ null mice $\beta 4$ is still expressed in keratinocytes, raising the possibility that $\beta 4$ is associated with another $\alpha$ subunit (Fassler et al., 1996).

Embryos that lack both $\alpha 3\beta 1$ and $\alpha 6\beta 5$ integrins develop epidermal blistering at stage E15.5 of development, but they also retain regions where the epidermis adheres to the basement membrane, suggesting alternative adhesion mechanisms (DiPersio et al., 2000). Interestingly, epidermal development and differentiation in these regions appears normal which shows that $\alpha 3\beta 1$ and $\alpha 6\beta 4$ are not essential for epidermal morphogenesis during skin development (DiPersio et al., 2000).

$\alpha 1\beta 1$ has been reported to be weakly expressed (Hertle et al., 1991; Buck et al., 1990) or absent (De Luca et al., 1990; Nazzaro et al., 1990; Zambruno et al., 1991). In culture, $\alpha 1$ is present only in trace amounts (Adams and Watt, 1991).

$\alpha V\beta 5$ binds vitronectin. It is readily detected in human keratinocyte cultures (Adams and Watt, 1991), but in vivo its expression is very low (Hertle et al., 1991) or undetectable (Pellegrini et al., 1992).

$\alpha v\beta 6$ recognises like $\alpha 5\beta 1$ the RGD sequence. It mediates cell attachment to fibronectin and tenascin and has been shown to exert a growth stimulatory effect on epithelial cells (Dixit et
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al., 1996). In addition it has been described to bind vitronectin (Huang, 1998). The knockout of the αv gene is either embryonic or perinatal lethal due to vascular defects (Bader et al., 1998), whereas the β6 knockout does not have an epidermal phenotype (Huang, 1998) suggesting, that αvβ6 is not essential in skin. However, it is upregulated in undifferentiated squamous cell carcinomas (Jones, 1997), and it recently has been shown that αvβ6 can regulate matrix metalloproteinase -9 and thereby promote invasion of squamous carcinoma cells (Thomas et al., 2001).

Other integrins reported to be expressed in keratinocytes αvβ8 and α9β1. αvβ8 has been described in the eyelid and in adult mouse epidermis (Stepp, 1999) recognises various RGD containing motifs. α9β1 binds tensacin and is upregulated during wound healing (Stepp and Zhu, 1997). It also has been described as a limbal marker, but is expressed throughout the basal layer of the epidermis (Stepp et al., 1995).

β1 knockout

The embryos die shortly after the initiation of implantation (Fassler et al., 1995; Stephens et al., 1995). The trophoblast differentiates without β1 and β1 is dispensable for the conversion of trophoectoderm to trophoblast. However the β1 null embryos cannot form extraembryonic endoderm and their ICM deteriorates. ES cells isolated from β1 null cells are viable, despite of ICM failure in vivo and in vitro. Scanning electron microscopy shows that the surface of β1 null ES cell colonies is irregular and that they seem to form fewer cell-cell junctions than wild type controls. β1 null ES cells do not adhere to fibronectin or laminin, but still exhibit a low level of vitronectin binding comparable to wild type cells. They also do not adhere well to a feeder layer of fibroblasts (Fassler and Meyer, 1995; Fassler et al., 1995; our own observation).

When injected into blastocysts of wt mice, β1–null ES cells contribute to all three germ layers, although β1– null endodermal cells are only found in the early embryos and not in
mature endoderm – derived lineages (Fassler and Meyer, 1995). Surprisingly, β1 null cells can participate in normal embryonic development, and chimeric mice are readily obtained if the overall contribution of the β1 null cells is lower than 25%. The percentage of β1 null cells in adult chimeric mice varies from tissue to tissue: Skin and skeletal muscle contain the highest levels of β1 integrin, whereas β1 null cells never contribute to hematopoietic organs such as bone marrow, thymus, spleen (Fassler and Meyer, 1995). The fact that the percentage of β1 null cells is relatively high in the skin of the chimeric mice was surprising, since in vitro data obtained from studies with human keratinocytes clearly indicated a crucial role for β1 integrins in controlling keratinocyte fate: Firstly, it has been shown that human epidermal stem cells can be distinguished from transit amplifying cells by the expression level of β1 integrins which is 2-3 higher in the stem cells. When human keratinocytes are analysed on the basis of whether they are rapidly (within 20 minutes) or slowly adhering to the β1 integrin ligand type IV collagen, the rapidly adhering cells have a high proliferative potential, whereas 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. This observation holds true whether cells are tested directly after isolating them from epidermis or have been in culture (Jones et al., 1995; Jones and Watt, 1993). Secondly, β1 integrin ligand binding is able to suppress suspension induced terminal differentiation of keratinocytes (Adams and Watt, 1989; Watt et al., 1993). The analysis of mutations in the β1 integrin subunit for the ability to regulate suspension-induced differentiation indicated that the signal transduced by β1 integrins is an instruction ‘do not differentiate’ (Levy et al., 2000). Interestingly, Levy et al. could also show that the regulation of terminal differentiation is independent of β1 mediated adhesion. In addition, the expression of a CD8/ β1 integrin chimera, which has earlier been shown to inhibit the function of endogenous integrins, leads to exit from the stem cell compartment and a lower colony forming efficiency in vitro via inhibiting MAPK (Zhu et al., 1999).
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All these findings argue for a role of β1 integrin in keeping keratinocytes in the stem cell compartment, but there are also arguments against this hypothesis. One is that not all cells expressing high levels of β1 integrin are stem cells. High β1 integrin marks 20-45% of the basal cells, which is at least double the proportion that are estimated to be stem cells in vivo (Jones et al., 1995; Jones and Watt, 1993).

Recently, mice with a targeted deletion of β1 integrin have been generated by using the cre-lox system. β1 integrin was deleted by crossing mice with a floxed β1 integrin to mice that carried Cre either under the K5 (Brakebusch et al., 2000) or under the K14 (Raghavan et al., 2000) promoter.

The mice die within the first six weeks after birth. Their basement membrane is severely disrupted and the skin shows extensive blisters at the dermal-epidermal junction. There is impaired proliferation of keratinocytes and hair matrix cells. Severe hair follicle abnormalities lead to dermal inflammation and subsequent fibrosis. In both mice a proliferation defect is observed, but they do not seem to have a higher rate of initiation of differentiation, arguing against a role of β1 integrin in keeping mouse keratinocytes in the stem cell compartment.
1.4. AIMS OF THESIS

The aim of the work presented in this thesis is to elucidate the role of β1 integrins in the epidermis by exploiting cells that lack the β1 subunit. Firstly, I have tried to investigate the role of β1 integrins in epidermal development using vitro differentiation of β1 null ES cells. Secondly, I have examined the role of β1 integrins in mouse keratinocyte in vitro using cells with a floxed β1 integrin.
A) H&E staining of mouse skin in anagen.

B) Stem cell model suggesting that transit amplifying cells have more restricted differentiation potential than stem cells. There might also be distinct transit cell populations for interfollicular epidermis, hair follicle and sebaceous gland.

C) Schematic representation of the structure of the hair follicle.

D) The layers of the epidermis and some of the markers of keratinocyte differentiation.
A STEM CELL

B STEM CELL

C STEM CELL

D STEM CELL

E STEM CELL

F STEM CELL

G STEM CELL

H STEM CELL

I STEM CELL

J STEM CELL

K STEM CELL

L STEM CELL

M STEM CELL

N STEM CELL

O STEM CELL

P STEM CELL

Q STEM CELL

R STEM CELL

S STEM CELL

T STEM CELL

U STEM CELL

V STEM CELL

W STEM CELL

X STEM CELL

Y STEM CELL

Z STEM CELL
Figure 1.2. Integrin mediated cell adhesion

A) Schematic representation of an integrin heterodimer

B) Schematic representation of focal adhesions and hemidesmosomes. Focal adhesions are formed by β1 integrins (indicated in red), hemidesmosomes by β4 integrins (indicated in blue).
A

Putative I-like domain

EF-Hand repeats

Cysteine rich region

Putative I-like domain

EF-Hand repeats

Cytoplasmic domain

Adapted from Newham & Humphries (1996)

B

ACTIN

ACTININ

VINCULIN

TENSIN

FAK

INTEGRIN

PAXILLIN

BPAG1

BPAG2

KERATIN IFs
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Figure 1.3. In vitro differentiation of ES cells

Schematic representation of the differentiation protocol used for the in vitro differentiation of ES cells.
In vitro differentiation of ES cells

formation of embryoid bodies  suspension  adherent

day 1  day 2  day 5/9  day 27

▲ Involucrin
K10, K1
K14, K5
K8, K18
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 (dH_{2}O) 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 Na_{2}HPO_{4} and 0.25g KH_{2}PO_{4} were dissolved in 1l dH_{2}O, the pH was adjusted to 7.2 and the solution was autoclaved. PBS_{ABC} was PBS supplemented with 1mM CaCl_{2} (B) and 1mM MgCl_{2} (C).

**Tris buffered saline (TS)**
10x stock solution was prepared by dissolving 24.2g Trizma base and 80g NaCl in 1l dH_{2}O. The pH was adjusted to 7.6 and the solution was autoclaved.

**EDTA solution (versene, ICRF)**
8g NaCl, 0.2g KCl, 1.15g Na_{2}HPO_{4}, 0.2g KH_{2}PO_{4} and 0.2g ethyldiaminotetraacetic acid (EDTA) and 1.5ml 1% (w/v) phenol red solution were dissolved in 1l dH_{2}O, the pH was adjusted to 7.2 and the solution was autoclaved.

**2x HBS for Calcium Phosphate Coprecipitation Transfection**
8.0 g NaCl, 6.5 g HEPES (sodium salt) and 10 ml of Na_{2}HPO_{4} stock solution (5.25 g Na_{2}HPO_{4} dissolved in 500 ml H_{2}O) ad 500ml; pH was adjusted to 7.0 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. It is used to metabolically inactivate J2-3T3 cells for the keratinocyte cultures. 4 mg 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 mouse embryonic fibroblasts, mitomycin C solution was added to the cell culture medium at a final concentration of 6 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

The wild type embryonic stem cell line D3 and the β1 deficient cell line D3/G201 were obtained from Reinhard Fassler. Mouse epidermal keratinocytes were isolated from newborn mice, grown and serially passaged as described below. Mouse embryo fibroblasts were used as feeder cells for supporting ES cell growth. Human dermal fibroblasts and human keratinocytes were isolated from neonatal foreskins and used for coculture with differentiating ES cells. Ecotropic retroviral packaging cells, GPE, and amphotropic packaging cells, AM12, and phoenix cell lines were used to generate high titer retroviruses for...
infecting mouse keratinocytes. 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 (human cells and ES cells) or at 32°C with 8% CO₂ (mouse keratinocytes). 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. Isolation and culture of primary mouse embryo fibroblasts

Mouse embryos were dissected at 13.5 days p.c. Head and liver were removed and the carcasses were transferred into a petri dish containing trypsin and minced with sterile razor blades. The minced embryos were then transferred in a 50ml screw cap tube containing trypsin/EDTA 1:3 and put on a shaker at room temperature for 25 mins. Cell suspension was passed through 70μm cell strainer and cells were plated on gelatin coated tissue culture dishes in DMEM (Gibco BRL) plus 10% FCS. When cells approached confluence they were harvested by rinsing them 2 times with trypsin and then washing the cells off by rinsing them in serum-containing culture medium. Mouse embryo fibroblasts (MEFs) cells were replated at a dilution of 1:5 to 1:10. MEFs were used as feeder cells for no more than 3 passages.

#### Preparation of MEFs as feeder cells

The culture of undifferentiated wild type ES cells is supported by co-cultivation with mitotically inactivated MEFs cells, which are referred to as feeder cells. Feeder cells were incubated with 0.6 μg/ml mitomycin C for 3h at 37°C in order to inhibit mitosis. The treated feeders were washed in PBS, harvested and plated at 1/2 confluent density. ES cells were added within 24 hours of preparing the feeder layer.

### 2.1.4. D3 and G201 ES cells

Undifferentiated ES cells were cultured in Dulbecco's modification of Eagles' medium (DMEM) with high glucose (Gibco, BRL) supplemented with 5000U/ml of leukemia inhibiting factor (LIF) (all reagents from Gibco, BRL). The wild type embryonic stem cell
line D3 was cultured on a feeder layer of mitomycin C treated primary mouse embryo fibroblasts. ES cells were passaged every 1-3 days.

Freezing and thawing of ES cells

Cells were harvested and centrifuged. 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 then 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 washed one time in PBSABC and then plated.

In vitro differentiation

A schematic representation of the in vitro differentiation protocol is shown in Figure 1.3. Embryoid body formation was initiated in 20μl hanging drops which contained 600 cells in DMEM supplemented with 20% (v/v) fetal calf serum (DCS), 2mM L-glutamine, nonessential amino acids, 50U/ml penicillin/streptomycin and 50μM β-mercaptoethanol (all reagents from Gibco, BRL). Thereafter, the embryoid bodies were cultured in suspension for another 3 (wild type) or 7 (β1 null) days. The cells were incubated for different times in suspension in order to achieve equivalent development (Bagutti et al., 1996). The 5 (wild type) and 9 (β1 null) day old embryoid bodies were allowed to attach to tissue culture dishes of dead, de-epidermised dermis (DED). Cells started to grow out from the embryoid bodies as soon as cells had attached. Outgrowths were examined at various stages of differentiation.

2.1.5. De-epidermised dermis (DED) culture

Preparation of DED

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 ES cells on DEDs

The vials were thawed and the pieces of DED were placed on tissue culture inserts (Becton-Dickinson) with the denuded epithelial surface uppermost and exposed to the air. ES cells were harvested and centrifuged at 1000rpm for 4 minutes. The cell pellet was resuspended in 50μl culture medium and then plated onto the denuded epithelial surface of the DEDs. Cultures were fed every 2-3 days for 2 weeks.

In some experiments DEDs were repopulated with dermal fibroblasts or keratinocytes before ES cells were added. To this end, DEDs were turned upside down and fibroblasts or keratinocytes were added. After 5 days, DEDs were orientated with the epidermal side up and ES cells were added.

2.1.6. Epidermal keratinocytes

Isolation and Culture of Primary Mouse Keratinocytes

Epidermal keratinocytes were prepared from newborn mice. 2-4 days old animals were killed by cervical dislocation, washed in 0.01 N iodine in PBS for 3 min, rinsed with PBS, washed in 70% ethanol for 3 min, and then rinsed in PBS. Tails were used for genotyping by PCR and prepared for frozen skin sections (see below). Skins were removed from the torso and then floated on 0.25% trypsin solution overnight at 4°C, with the epidermis facing upward. Skins were then transferred to a dry, sterile surface with the epidermis facing down, and the dermis was separated from the epidermis. The epidermis was minced, suspended in growth medium (see below), and then agitated to release keratinocytes. Suspensions were passed
through a sterile, 70-µm nylon filter (Becton Dickinson, Franklin Lakes, NJ) to remove cornified sheets. Keratinocytes were seeded onto tissue culture plates at a density of ~2-4 x10^5 cells/cm^2. A keratinocyte cell line was generated by repeated subcloning at high density as described (Romero et al., 1999).

*Keratinocyte culture medium (FAD+ FCS + HICE)*

Calcium free 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 and 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 µg/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 Laboratories) 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. Complete medium was stored at 4°C for up to 10 days. Mouse keratinocytes were cultured in medium with a reduced concentration of calcium ions in order to prevent differentiation. The FCS used to supplement low calcium FAD was pre-treated with Chelex to remove calcium ions.

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
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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. For keratinocytes infected with retrovirus, puromycin was added to the culture medium at 1μg/ml

Preparation of calcium-free serum

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. 24g swollen resin was added to 100ml FCS and the mixture was stirred at room temperature for 2 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 in aliquots at -20°C.

Serial culture of mouse keratinocytes

Mouse keratinocytes were grown on collagen coated dishes. Fresh medium was given to keratinocytes every 2 days. Keratinocytes were passaged just before they reached confluence. The cultures were rinsed once with versene and then 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. 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 and plated onto collagen coated dishes.

2.1.7. Retroviral producer cells and retoviral infection of keratinocytes

Retroviral producer cell culture

Helper free ecotropic packaging cells, GP + E-86 (abbreviated as GPE), and amphotropic packaging cells, GP + envAM12 (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 MoMuLVirus via homologous recombination events. The packaging cells were cultured in E4 medium
supplemented with 10% (v/v) FCS. For transfected or infected retroviral producer cells the culture medium was supplemented with 2.5 μg/ml puromycin. Phoenix ecotropic packaging cells were obtained from the American Tissue Culture Collection. Phoenix is a second-generation retrovirus producer lines derived from the human embryonic kidney 293 cell line for the generation of helper free ecotropic and amphotropic retroviruses. The lines had been created by placing into 293T cells constructs capable of producing gag-pol, and envelope protein for ecotropic and amphotropic viruses. For both the gag-pol and envelope constructs non-moloney promoters were used to minimize recombination potential. Different promoters for gag-pol and envelope were used to minimize their inter-recombination potential. Gag-pol was introduced with hygromycin as the co-selectable marker and the envelope proteins were introduced with diptheria resistance as the co-selectable marker.

Transfection of phoenix cells

Ecotropic and amphotropic virus producer cell lines were generated by transfecting the phoenix packaging cells with retroviral vector, and then using the virus containing supernatant to infect GPE or AM12. Using this ‘ping-pong’ protocol gives higher viral titers than from normally transfected GPE (Sally Lowell, personal communication). 8x10^6 phoenix cells were seeded onto a 100mm dish the day before transfection. The next day, about 5 minutes prior to transfection chloroquine was added to each plate to 25 μM final concentration.

To a 15 mL tube per 10 cm plate 10 μg DNA, 438 μl dd H2O, 61 μl 2M CaCl₂ was brought to a 500 μl total volume. 0.5ml 2xHBS was added, mixed and the HBS/DNA was added dropwise onto media.

24 hours post-transfection the medium was changed. Retroviral supernatants were collected at 65- to 90 hours after infection, filtered though a 0.45 μm filter to remove cells and added to GPE or AM12 cells.
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Infection of AM12 and GPE cells

AM12 and GPE cells were seeded on 100mm dishes at 1-2x 10^3 density the day before infection. 6 ml infection medium (virus-containing medium collected from the phoenix cells and supplemented with 8 µg/ml polybrene; Sigma) was added to the cultures. After 6h 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.

Pseudotype virus formation

Pseudotyped virus was prepared as described by Yee at al. (Yee et al., 1994). Briefly, phoenix-gp cells that expressed only gag-pol (no envelope protein) were plated the day before the transfection plated in E4 containing 10% FCS. The next day cells were cotransfected with a plasmid encoding vesicular stomatitis (VSV-G) envelope protein and the retroviral expression construct using superfect (Quiagen). 24hrs before the first collection of the viruses the medium was replaced by E4, 2% FCS. The viruses were collected 48hrs, 72hrs and 96hrs post-transfection. The supernatant was filtered through a 0.45µm filter (Nalgene filter 115ml), poured in a 50 ml falcon tube, snap frozen in liquid N2 and stored at -70 °C.

Concentration of the pseudotyped virus

Once the virus collection was finished, supernatant was thawed and transferred to a 30ml Beckman tube. It was centrifuged at 25 000rpm, for 1h30min using a SW 28 rotor. Supernatant was removed and the pellet resuspended in E4 containing 10% FCS. 100µl aliquots were snap frozen and stored at -70 °C.

Retroviral infection of keratinocytes

Keratinocytes were infected using AM12 or GPE supernatants or concentrated VSV G-pseudotyped virus. Virus was added to subconfluent mouse keratinocyte cultures in the presence of 8µg/ml polybrene. After 6 – 8 hours incubation cells were washed once with
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PBSABC to remove polybrene. Selection medium containing 1μg/ml puromycin was applied 48 hours later. The following retroviral vectors were used: pBabe chick β1 integrin (Levy et al., 1998), pBabe EGFPCre and LZRSpBMN EGFPCre (EGFPCre fusion protein and LZRSpBMN were kind gifts from J. Muller, ICRF)

2.1.8. Preparation of adenovirus and adenoviral infection of keratinocytes

Solutions: 0.1 M Tris-HCl, pH 8
5% Na-deoxycholate

Saturated CsCl solution: sold CsCl is mixed at room temperature in 120mM Tris-HCl, pH 8.0 and 1mM EDTA until saturated. CsCl was stored at 4°C, but used at room temperature.

DNAse I: 100mg of pancreatic DNAse was dissolved in 10ml of 20 mM Tris-HCl, pH 7.4, 50mM NaCl, 1 mM dithiothreitol, 0.1 mg/mL BSA, and 50% glycerol. Aliquots were stored at -20°C.

RNAse A: 100mg of RNAse A was dissolved in 10ml of 10mM Tris-HCl, pH7.4, 15 mM NaCl; aliquots were stored at -20°C.

50% confluent HEK293 were infected with a MOI of 5 pfu/cell in DMEM, 2% serum for 1 hour. After 2-3 days, when CPR was nearly complete (i.e. most cells were rounded but not all were detached) cells were harvested and resuspended in 0.1 M Tris-HCl, pH8.0. Cells were freeze-thawed 3 times to release virus. 0.1 volume of 5% Na-deoxycholate was added and cells were incubated on ice for 30 mins. After this incubation period 0.01 vol 2M MgCl, 0.005 vol DNAse I solution and 0.0005 vol RNase A solution was added. Utracentifugation was performed in an CsCl equilibrium gradient (2.3ml saturated CsCl + 2.4-2.8 ml virus suspension + 1.2 –1.6 ml 1 M Tris- HCl, ph8.0ml ) in a 6.3ml Beckman QuickSeal tube in a pre-cooled fixed angle rotor at 84 000g (70.1 Ti Beckman rotor, 35 000 rpm) for 16-20 hours.
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at 4°C. A single band with viral material was obtained and the fraction was collected by puncturing the tube with a needle. The virus was dialyzed in a ‘Slide – A – Lyzer Cassette’ (Pierce; 0.5 – 3ml sample volume) overnight at 4°C against 1000 volumes PBSABC containing 10% glycerol and stored in aliquots at –80°C.

Adenovirus titration

To determine the titre of the virus preparation, 50 000 293 cells/ well were plated on a 96 well plate. After the cells had adhered, medium was changed to 50 μl serum free DMEM containing adenovirus to each well. Serial dilutions of an initial 1:1000 dilution of the virus stock were performed. After 1 hour at 37°C 100ul normal DMEM containing 10% serum was added. Cultures were incubated for 4 days. If half the cells of a well were lysed, the concentration was scored as 0.5 pfu/150 μl.

Each new virus preparation was first tested on keratinocytes: In serial dilutions the amount of virus needed to get 90% lacZ positive cells was determined.

Infection of keratinocytes with adenovirus

Subconfluent keratinocytes were washed twice with PBS and the medium was changed to serum- free low Ca FAD. Virus was added in minimal volume. After 1-2 hours cells were washed and medium was changed to normal low- calcium FAD.

2.1.9. Time-lapse recording

Subconfluent keratinocytes were harvested and plated onto dishes coated in type I collagen or on a mixture of fibronectin, collagen I and serum at a density that allowed tracking of individual cells. Frames were taken every 5 minutes over a time of 22 hours using a Zeiss Axiovert 135 TV microscope fitted with a Hamamatsu CCD Orca –ER camera (Hamamatsu, Japan). Motility was measured using a cell tracking extension (ICRF) written for IPLab
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(Signal Analytics Inc., USA) and speed was calculated using a program written in Mathematica by Daniel Zicha (ICRF).

### 2.1.10. Adhesion assays using freshly isolated mouse keratinocytes

Keratinocytes from day 2 old mice were plated directly after isolation onto 96 well plates (5x10^4 cells/well) pre-coated with fibronectin, laminin, PDL, collagen I and collagen IV (BD Biocoat Cellware, Franklin Lakes, NJ). After overnight incubation, plates were washed twice with PBS and adhesion was quantified using a CytoTox 96™ colorimetric kit.

Vitronectin prepared from fresh frozen plasma according to the method of Yatohgo (Yatohgo et al., 1988) was a kind gift from Sam Janes. Coverslips and assay plates were coated overnight at 4° C with vitronectin in PBSABC at a concentration of 100 μg/ml.

Laminin 5 enriched keratinocyte extracellular matrix (KECM) was prepared by incubating a confluent culture of human keratinocytes overnight at 4°C in 20mM EDTA/PBS, 10μg/ml leupeptin, 1 mM PMSF and 10 μg/ml trypsin inhibitor. The next day cells were removed as a sheet. KECM matrix coated dishes were stored up to 5 days at 4°C.

### 2.2. IMMUNOLOGICAL METHODS

#### 2.2.1. General solutions

**Paraformaldehyde solution**

4% paraformaldehyde solution was prepared by adding paraformaldehyde powder (BDH) to PBS pre-warmed to 60°C. After 4 hours in a 60°C waterbath, paraformaldehyde was
completely dissolved. Aliquots were made and stored at -20°C. 4% paraformaldehyde solution was used to fix tissues and cells.

Gelvatol mounting solution
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.
2.2.2. Antibodies

All antibodies used are listed in Table 2.1 and 2.2.

Table 2.1. Primary antibodies

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Antigen specificity</th>
<th>Species</th>
<th>Dilution for IF or FACS</th>
<th>Originator/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL001</td>
<td>keratin 14</td>
<td>mouse monoclonal</td>
<td>1:2</td>
<td>Cell Structure Laboratory, ICRF</td>
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<tr>
<td></td>
<td>BrDU</td>
<td>mouse monoclonal</td>
<td>1:25</td>
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<tr>
<td>10D5</td>
<td>mouse αVβ6</td>
<td>mouse monoclonal</td>
<td>10 μg/ml IgG</td>
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</tr>
<tr>
<td>MB1.2</td>
<td>mouse β1 integrin</td>
<td>rat monoclonal</td>
<td></td>
<td>obtained from B Chan (Von Ballestrem et al., 1996)</td>
</tr>
<tr>
<td>H9.2B8</td>
<td>mouse integrin αV chain</td>
<td>armenian hamster</td>
<td>10 μg/ml IgG</td>
<td>purchased from RDI;</td>
</tr>
<tr>
<td>GoH3</td>
<td>human α6 integrin</td>
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<tr>
<td></td>
<td>mouse keratin 1</td>
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<td>2.5μg/ml</td>
<td>purchased from BabCO</td>
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</table>
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<table>
<thead>
<tr>
<th>Antibody/Marker</th>
<th>Source</th>
<th>Dilution/Concentration</th>
<th>Vendor</th>
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<td>Heparan sulfate (10E4 epitope)</td>
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<tr>
<td>Ki67</td>
<td>rabbit polyclonal</td>
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<td>20 μg/ml IgG</td>
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<tr>
<td>ERLI-3 mouse involucrin</td>
<td>rabbit polyclonal</td>
<td>1:800</td>
<td>(Li et al., 2000)</td>
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<tr>
<td>LP34 pan-keratin</td>
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<tr>
<td>mouse keratin 10</td>
<td>rabbit polyclonal</td>
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<td>BabCO</td>
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<td>collagen type IV</td>
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<td>SQ37C cornifin</td>
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<td>1:2500</td>
<td>kind gift of A. Jetten</td>
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<tr>
<td>paxillin</td>
<td>mouse monoclonal</td>
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Filamentous actin was detected using Texas-red-conjugated phalloidin (Sigma).
### Table 2.2 Secondary antibodies

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<th>Antigen specificity</th>
<th>Conjugate</th>
<th>Species</th>
<th>Dilution</th>
<th>Reference</th>
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<td>mouse IgG, whole molecule</td>
<td>Alexa 488</td>
<td>Rabbit</td>
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<td>purchased from Molecular Probes</td>
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<td>mouse IgG, whole molecule</td>
<td>Alexa 594</td>
<td>Rabbit</td>
<td>1:400</td>
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<td>Goat</td>
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<td>purchased from Molecular Probes</td>
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<tr>
<td>rabbit IgG, whole molecule</td>
<td>Alexa 594</td>
<td>Goat</td>
<td>1:400</td>
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<tr>
<td>rat IgG, whole molecule</td>
<td>Alexa 488</td>
<td>Goat</td>
<td>1:400</td>
<td>purchased from Molecular Probes</td>
</tr>
<tr>
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<td>Alexa 594</td>
<td>Goat</td>
<td>1:400</td>
<td>purchased from Molecular Probes</td>
</tr>
<tr>
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<td>1:100</td>
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<tr>
<td>rabbit IgG</td>
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<td>Swine</td>
<td>1:100</td>
<td>purchased from DAKO</td>
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<td>1:100</td>
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<td>rat IgG, whole molecule</td>
<td>Texas Red</td>
<td>Donkey</td>
<td>1:100</td>
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</tbody>
</table>
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2.2.3. Preparation of cells for immunofluorescence staining

Cells were cultured on tissue culture plastic microscope slides (Nunc) or on glass coverslips (Chance Propper 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 they were fixed in 4% paraformaldehyde solution for 20 minutes at room temperature. Cells were rinsed three times in PBS and (if necessary) 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 PBSABC containing 2% FCS to reduce binding to non-specific proteins for at least 30 minutes at room temperature. 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 three times in PBS and then incubated in blocking solution.

To stain keratinocyte cultures for focal adhesion components, cells were fixed for 5 minutes with 4% paraformaldehyde in PBS containing 0.1% Triton X-100. For staining of keratin filaments, ES cultures were fixed with ice cold methanol/aceton 2:3 for 6 minutes.

2.2.4. Preparation of cryosections of DED and mouse epidermis

Small pieces of DED or unfixed neonatal mouse skin were embedded in OCT and snap-frozen in chilled isopentane in liquid nitrogen. The tissue embedded in OCT compound was stored at -70°C. Using a cryomicrotome 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.
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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 acetone for 5 minutes.

2.2.5. 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.

Involucrin staining

To determine the proportion of involucrin-positive cells in cultures of primary keratinocytes, single cell suspensions were air-dried at 37°C dried on microscope slides and fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilised with 0.1% Triton-X 100 for 7 min, blocked in 2% FCS in PBS for 60 min at RT and then incubated in an anti-involucrin antiserum (ERLI-3 (Li et al., 2000), dil 1:900); After
incubation coverslips were washed twice and incubated for 15 min in secondary antibodies conjugated with AlexaFluor 488.

Nuclei were stained with Hoechst 33258 and the percentage of differentiated cells was determined by counting involucrin positive cells versus nuclear staining.

BrdU staining for immunofluorescence
Preparation of cells for immunofluorescence staining
10 μM BrdU (Sigma) (from 2 mM stock in dH2O stored at -20°C in the dark) was added to the keratinocyte cultures for 1 hour.

Fixation and staining for incorporated BrdU
Culture medium was discarded from cells and they were rinsed twice in PBS before fixing in 4% paraformaldehyde solution for 10 min at room temperature. The specimens were rinsed twice in PBS, incubated for 10 min with 2 M HCl and 0.5% (v/v) Triton X-100 in PBS, washed twice in PBS, and then blocked for 30 min to reduce binding to non-specific proteins in blocking solution (3% (w/v) BSA, 10% (v/v) serum (foetal bovine or goat (Gibco-BRL), 5% (w/v) fat-free dried milk powder, 0.01% (v/v) Tween-20 in PBSA). Cells were then washed twice in PBSA and incubated with 60 μl of undiluted anti-BrdU antibody (Becton-Dickinson) for 45 min in the dark. Cells were washed 5-6 times with 0.01% (v/v) Tween-20 in PBSA. Alexa Fluor 488 anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) was diluted 1:400 in blocking solution with 0.1 μg/ml Hoechst 33258 and incubated on the cells for 30 min. Cells were washed 5-6 times and mounted in mounting medium under a coverslip.
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2.2.6. Flow cytometry

Cell Surface Epitopes

Single cell suspensions of keratinocytes isolated directly from mouse epidermis or of keratinocyte cultures harvested with trypsin/EDTA solution were incubated in primary antibodies diluted in 2% FCS PBSABC on ice for 20 min, washed, and incubated on ice for 15 min with an Alexa Fluor 488 conjugated secondary antibodies or biotinylated antibody followed by 10 min incubation on ice with RPE-Cy5 conjugated streptavidine. Cells were washed 4 times and then resuspened in PBSABC. Immediately before analysis on a Becton-Dickinson FACScan, TO-PRO-3 (Molecular Probes, Leiden) was added to the sample for viability gating. In some experiments the differentiating cells were gated out on the basis of forward and side scatter as previously described (Jones and Watt, 1993). Cells were stained and washed in v-bottom 96 well plates.

Intracellular Epitopes

Keratinocytes were fixed in 4% paraformaldehyde for 10 min at room temperature and then 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 0.22μm filter and stored at 4°C. FSP was used instead of PBS and cells were kept at room temperature at all times.

FACS sorting

Cells were harvested and resuspended in ice-cold medium. 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.
CHAPTER 2 Materials and Methods

Sorting for chick β1 integrin

The method was as described for staining cell surface epitopes except cells were kept in ice-cold culture medium instead of PBSABC.

2.3. PROTEINS

2.3.1. Extraction of total proteins

SDS extraction buffer was used (1% SDS, 2 mM CaCl₂ and 15 mM Tris-HCl, pH 7.5). Cells were washed twice in PBS and lysed for 10 minutes on ice. The cells were scraped from the petri dishes, boiled for 5 minutes and centrifuged at 14000 rpm for 10 minutes at 4°C. The protein lysates were assayed for protein content. 1 mM PMSF and 1 μg/ml leupeptin were then added and the lysates were aliquoted and stored at -70°C until use.

2.3.2. Measuring protein concentration

Protein concentration was determined using the Bradford assay. Reagents were obtained from Bio-Rad. A standard curve was prepared by making triplicate dilutions of a standard protein solution (BSA, 2 mg/ml). Lysis buffer was used as the "blank" and to dilute samples. Samples were added into triplicate wells in a 96-well plate (Immunlon Dynatech). The assay was carried out according to manufacturer's instructions. The O.D.₆₉₀ was measured for the contents of each well on a Titretek Multiskan MCC/340 MKII spectrophotometer. The protein concentration of sample was determined against the standard curve.

2.3.3. SDS PAGE and immunoblotting

4 x sample buffer (reducing)

<table>
<thead>
<tr>
<th>60 mM</th>
<th>0.5M Tris pH 6.8</th>
<th>1.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>glycerol 50%</td>
<td>5 ml</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% SDS</td>
<td>2 ml</td>
</tr>
<tr>
<td>14 mM Mercaptoethanol</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>0.1% Bromphenol blue 1 %</td>
<td>1 ml</td>
</tr>
<tr>
<td>A.d.</td>
<td>2.8 ml</td>
</tr>
</tbody>
</table>

Aliquots of the sample buffer were stored at -20°C

0.5 M Tris-HCL, pH 6.8:
60.57 g Tris was made up to 1000 ml of dH₂O and adjusted to pH 6.8.

1.5M Tris-HCL, pH 8.8:
180.71 g Tris was made up to 1000 ml of dH₂O and adjusted to pH 8.8.

1x SDS-PAGE running buffer:
20 mM Tris base, 190 mM glycine (Sigma), 0.1% SDS, pH 8.3

Blocking buffer:
5% (w/v) fat-free milk powder and 0.01% (v/v) Tween-20 in PBSA

Semi-dry transfer buffer

10x stock solution comprised 0.2 M Tris-HCI, pH 7.5 and 1.5 M glycine. The semi-dry transfer buffer was made by diluting the stock solution 10 fold in 0.1 % SDS and 20 % (v/v) absolute methanol.

10% SDS resolving gel

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M TRIS PH 8.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>
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A.d. 3.96 ml  
30%-0.8% Acryl-Bisacryl 3.33 ml  
10% APS 0.1 ml  
TEMED 0.01 ml

5% SDS stacking gel

5 M tris pH 6.8 1.25 ml  
10% SDS 0.05 ml  
A.d. 2.81 ml  
30% -0.8 % Acryl-Bisacryl 0.84 ml  
10% APS 0.05 ml  
TEMED 0.005

**SDS-PAGE**

Vertical gel electrophoresis apparatus systems (Model SE400, Hoefer Scientific Instruments) were used. 1.5mm thick gels were prepared between glass plates. After pouring resolving gel solution, gels were allowed to polymerise at room temperature for a minimum of 1h. A 1.5mm thick comb was inserted to create wells and then the stacking gel was poured and left to polymerise. Samples were applied to the wells using capillary pipette tips. 10µl pre-stained rainbow molecular weight markers (Amersham) were added to 10µl Laemmli sample buffer, which were boiled for 5 minutes and then loaded into one of the wells. Samples were electrophoresed at 60V until the dye front had just entered the resolving gel. The voltage was increased to 120V. Alternatively, samples were electrophoresed overnight at 40-50V.

**Semi-dry transfer protocol**

Up to 25µg protein lysate was loaded per track on an SDS-PAGE gel and electrophoresed. After electrophoresis, proteins were transferred onto a Millipore Immobilon PVDF
(polyvinylidifluoride) membrane (pre-wetted in absolute methanol) using a semi-dry transfer unit (Millipore MilliBlot graphite electroblotter I system, Hoefer Scientific Instruments) at 200mA for up to 1h. To verify transfer, the membrane was stained with Ponceau S for 5 minutes at room temperature, washed twice with water and position of transfer was marked. The membrane was then rinsed briefly in PBS containing 0.05% Tween –20 (PBST; Sigma) and then was blocked in 5% milk powder solution in PBST 0.05% for 1h at room temperature.

**Blotting with antibodies and ECL**

Membranes were incubated with antibody diluted in 2.5% milk powder solution in PBS containing 0.05% Tween-20. Incubation with primary antibody was for 1h at room temperature or overnight at 4°C with agitation. This was followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham). After each antibody incubation, membranes were washed three times with PBST. Detection of HRP-bound antibody was by chemiluminescence kit (ECL, Amersham) which was used according to manufacturer's instructions. If required, the membrane was then stained for total protein with Ponceau S solution (Sigma) to look at efficiency of transfer and then washed 5-6 times with PBSA.

**Coomassie blue staining**

After electrophoresis, gel was transferred to a plastic container and 5 gel volumes of 0.25% Coomassie brilliant blue R-250, 50% methanol and 10% acetic acid were added. Gel was incubated for 2 hours on a shaker at room temperature. Stain was then removed and gel was destained by successive incubations in destain (5% methanol, 7.5% acetic acid) at room temperature on a shaker.
2.3.4. Staining for lacZ activity

LacZ activity was stained according to Hogan et al. (Hogan et al., 1994).

The following solutions were freshly prepared:

Fixative
1 M phosphate buffer (pH 7.3)
0.2 % Glutaraldehyde
5 mM EGTA (made from a stock of 0.1 M at pH 8.0)
2 mM MgCl₂

Wash solution
0.1 M phosphate buffer (pH 7.3)
2 mM MgCl₂
0.01% sodium deoxycholate
0.02% Nonidet P-40

Staining solution
0.1 M phosphate buffer (pH 7.3)
2 mM MgCl₂
0.01% sodium deoxycholate
0.02% Nonidet P-40
5 mM potassium ferricyanide
5 mM potassium ferrocyanide
1 mg/ml f.c. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (made from a 25 g/ml stock in dimethylformamide).
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Staining:
Cells were fixed for 5 minutes, rinsed in the wash solution three times at room temperature for 7 minutes and then incubated in the staining solution at 37 °C for 3 hours in the dark.

2.4. MOLECULAR BIOLOGY

2.4.1. Stocks and general solutions for DNA techniques

All reagents used were of molecular biology grade. When possible, solutions were autoclaved after preparation to destroy DNAses.

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. 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.

Tris/EDTA buffer (TE)
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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 1000ml.

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 1000ml.

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

Agarose gel
0.8-2% (w/v) ultra pure agarose (Gibco BRL) was melted in a microwave oven in 0.5x TBE or 1xTAE buffer. Ethidium bromide was added at 0.05 µg/ml to agarose solution before casting in gel mould. DNA was electrophoresed at constant voltage of 80-100V in 1x TAE or 0.5 x TBE 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.
2.4.2 General DNA techniques

Isolation of genomic DNA

Tail snips were placed in 0.7 ml buffer A (50mM Tris, pH 8.0; 100mM EDTA, 100mM NaCl, 1% SDS, 500μg/ml proteinase K) and incubated overnight at 55°C. Proteins were extracted with 1 volume phenol/chloroform at room temperature for 15 mins. Samples were centrifuged at 13000rpm for 10 min in a benchtop centrifuge (Eppendorf). DNA was precipitated with 0.8 volumes isopropanol and pelleted at 13000rpm for 15 min. Pellets were washed with 70% ethanol and resuspended in 100 μl TE buffer or water.

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 using a Qiagen plasmid mini kit (Qiagen Ltd.). 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.
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**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, pH 8.0 or dH2O.

**Restriction digestion**

DNA (0.2-2μg/μl) was incubated in the appropriate buffer with 1U/μg restriction endonuclease in a volume of 30μl at the appropriate temperature for 1-2 hr.

**Quantitation of DNA**

DNA was placed in a quartz cuvette with path length of 1cm and the absorbance at wavelengths 260nm and 280nm was read in a spectrophotometer. An OD$_{260}$ of 1 corresponds to 50μg/ml double stranded DNA, 40 μg/ml RNA and 33 μg/ml single stranded DNA. The ratio OD$_{260/280}$ provides an estimate of the purity of DNA solutions and usually was ~1.7.

**Cohesive-end ligation**

Vector and insert were digested. The vector was dephosphorylated to prevent its re-circularisation by incubating the DNA with 0.5U/μg alkaline phosphatase (CIP, New England Biolabs) at 37°C for 1hr. Vector and insert were gel-extracted and ligations were performed using 200-300ng total DNA and 400U T4 DNA ligase (New England Biolabs or Gibco BRL) in a total volume of 20μl for ≥12hr at 16°C or for 2 hours at room temperature. The vector to insert molar ratio usually was 1:2, 1:5 and 1:10. 1μl of the ligation reaction was transformed into competent bacteria.

Retroviral vector pBabe puro
pBabe puro was a gift from Dr H. Land (Morgenstern and Land, 1990). 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.

![Diagram of pBabe puro](attachment:diagram.png)

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

**Figure 2.1. Retroviral vector pBabe puro**

Modified from (Morgenstern and Land, 1990)

**Subcloning of cDNA constructs into pBabe puro**

The cDNAs for EGFPCre were provided by Joyce Muller (ECBL, ICRF) cDNA was cut out from the vector by EcoRI and SalI digestion, purified, and subcloned into the SalI I and EcoRI cut pBabe puro retroviral vector.

The final recombinant retroviral vectors were prepared using a Qiagen maxi-prep column and subjected to transfection into ecotropic GPE packaging cells.
Transformation of bacteria

25µl of chemically competent bacteria (One Shot Top 10, Invitrogen) were transformed according to manufacturer’s instruction. Briefly, bacteria were thawed and left on ice. DNA (0.1µg for ligations or 1 ng for plasmids) was added, mixed and left on ice for 30 minutes. The bacteria were then heat-shocked at 42°C for 90 seconds and transferred onto ice for 2 minutes. 4 volumes of SOC medium (ICRF central services) were added and the tube was agitated gently in a 37°C water bath for 60 minutes. 20-100 µl from each transformation culture was spread out onto agar plates containing ampicillin and incubated overnight at 37°C. Single colonies were picked with a sterile loop to inoculate LB media.

PCR

The following primers were used

for lox genotyping

T56 5'-AGGTGCCCTCCCTCTCTAGA-3'
L1 5'-GTGAAGTAGGTGAAAGGTAAC-3'

for Cre genotyping

CRE5' 5'-AACATGCTTCTCAGTCGG-3'
CRE3' 5'-TTCGGATCATCAGCTACACC-3'

The annealing temperature was calculated using the following formula:

\[ T = 3\sum G/C \text{-basepairs} + 2\sum A/T \text{-basepairs} \]

For amplifying genomic DNA, reactions were carried out with 100-200ng DNA and 15pmol primer 200µM dNTP (Ultrapure dNTP, Pharmacia) and 5U Taq DNA polymerase (ICRF) in
a total volume of 100μl using a thermal cycler (PTC-225, MJ-Research). 10% DMSO was added to some reactions to reduce secondary DNA structures.

The following PCR conditions were used:

1 cycle: denaturation at 94°C for 5min and “hot start”

35 cycles:
- denaturation at 94°C for 40sec
- annealing at 55°C for 40sec
- extension at 68°C for 35 sec

1 cycle: extension at 68°C for 5min
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2.5. 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.
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., Aryshire, 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.
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.
CHAPTER 2 Materials and Methods

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, Carlifornia, USA.
Pharmenus, 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, Carlifornia, 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: SOLUBLE GROWTH FACTORS RESTORE THE ABILITY OF β1 INTEGRIN DEFICIENT ES CELLS TO DIFFERENTIATE INTO KERATINOCYTES

3.1. INTRODUCTION

β1 integrins have been implicated in the regulation of proliferation and differentiation in adult epidermis (Watt, 2001). Since targeted deletion of the β1 gene in mice is lethal before development of the epidermis (Fassler and Meyer, 1995; Fassler et al., 1995; Stephens et al., 1995). Claudia Bagutti, while she was postdoc in the lab, tried to differentiate β1 integrin knockout ES cells into epithelial cells in order to investigate the role of β1 integrin subunit in keratinocyte development. She found that in contrast to wild type cells β1-null ES cells failed to undergo differentiation into keratinocytes in vitro, but that they did form cysts expressing markers of keratinocyte differentiation when allowed to form teratomas by subcutaneous injection in mice. In addition, the epidermis of chimeric mice containing wild type and β1-null cells was normal, even in patches of β1-null tissue (Bagutti et al., 1996).

These results were interesting, since they suggested that factors provided by the surrounding wild type cells could enable the β1 null cells to differentiate. The data presented in this chapter were published in a paper of which I am joint first author (Bagutti et al., 2001). Claudia initiated the search for these factors and when she left I completed the project.
3.2. RESULTS

3.2.1. In vitro differentiation of $\beta_1$-null cells

To induce differentiation, ES cells were aggregated in hanging drops for two days to form embryoid bodies and then maintained in suspension in bacterial dishes for 3 (wild type cells) or 7 ($\beta_1$ null cells) days (Figure 1.3). $\beta_1$ null ES cells grow more slowly than wild type cells (data not shown; Aumailley et al., 2000), so to ensure that cells were plated at the same stage of development, $\beta_1$ null cells were kept in suspension for 5 days longer than wild type cells. Embryoid bodies were then plated on gelatin coated plates. After 16-26 days in culture they were analysed for keratinocyte differentiation markers by using western blots and immunofluorescence (Figure 3.1).

I could reproduce the finding that the $\beta_1$ integrin null ES cells have an impaired ability to differentiate (Bagutti et al., 1996). Wild-type cells expressed keratin 14 which is a marker for stratifying squamous epithelium from day 21 onwards, but $\beta_1$ null cells were not or only to a very low percentage keratin 14 positive (Figure 3.1.).

3.2.2 Basement membrane does not stimulate ES cell differentiation into keratinocytes

Although $\beta_1$ null cells fail to undergo differentiation into keratinocytes in vitro, they start to express markers of stratifying epithelium in teratomas and wild-type/$\beta_1$ null chimeric mice (Bagutti et al., 1996). One explanation for this difference may lie in the observation that $\beta_1$ null cells assemble a basement membrane in teratomas and chimeric mice, but not in vitro. Defective ECM production has been described in $\beta_1$ null cells (Aumailley et al., 2000; Wennerberg et al., 1996). The failure to differentiate might therefore be caused by the inability of the $\beta_1$ null cells to assemble an ECM, rather than lack of $\beta_1$ integrin per se.

I therefore investigated whether providing $\beta_1$ null ES cells with an intact basement membrane could overcome the failure of $\beta_1$ null cells to differentiate. The basement membrane was obtained by using a de-epidermised dermis (DED). DEDs are prepared by
peeling off the epidermis from a skin sample and then killing dermal cells by repeated cycles of freezing and thawing. At the epidermal side of the dermis remains an essentially intact basement membrane (Bagutti et al., 2000) and references cited therein; Figure 3.4A).

ES cells were differentiated into embryoid bodies and on day 5 (wild type cells) and 9 (β1 null cells) after induction of differentiation plated on the DED and grown for another 16 to 26 days. Both wild type and β1 null cells formed multiple cell layers (Figure 3.2.). On DEDs populated with wild type ES cells, structures resembling cysts of stratified squamous epithelia were observed. These structures were absent in the cell layers formed by β1 null cells (Figure 3.2.). Staining of these cysts with keratinocyte markers shows that they are indeed similar to the epidermis: the periphery of the cysts is formed by keratin 14 positive cells that express α6β4 integrin in a polarised manner, whereas cells in the centre are keratin 14 negative, but keratin 10 positive (Figure 3.3.). The cysts are surrounded by collagen IV but a discrete basement membrane was not observed (Figure 3.4B). No cells expressing keratins 14 or 1 were observed in cells differentiated from β1 null embryoid bodies.

The results from these experiments show that (i) sustained interaction with a basement membrane does not stimulate keratinocyte differentiation (since the cysts were formed at all levels in the layers) and (ii) that the provision of a basement membrane does not restore the ability of β1 null embryoid bodies to differentiate into keratinocytes.

3.2.3. Fibroblasts stimulate ES cells to differentiate into keratinocytes

Another reason for the ability of β1 null cells to differentiate in vivo but not in vitro, might be that the lack of β1 integrins reduces the sensitivity to soluble differentiation factors and that these soluble factors are abundant in vivo but not in vitro. Because of the strong inductive effect of the mesenchyme on the epidermis during embryonic development we investigated whether factors secreted by dermal fibroblasts could overcome the failure of β1 null embryoid bodies to give rise to keratinocytes. DEDs were repopulated with dermal
fibroblasts by seeding the cells on the surface opposite the basement membrane and incubating the DEDs upside down. After an incubation period of 10 days, the DEDs were turned around and embryoid bodies were seeded onto the basement membrane. I also tested whether factors secreted by keratinocytes had an effect on ES cell differentiation by seeding on some DEDs keratinocytes instead of dermal fibroblasts. The cultures were grown for 16 to 26 days and then analysed for keratinocyte differentiation markers.

As shown in Figure 3.5., incorporation of fibroblasts into DED stimulated differentiation of both wild type and β1 null embryoid bodies into cells of stratified epithelium. The number of wild type K14-positive cysts increased from 4.6 ± 2 (per section of DED without fibroblasts) to 15.6 ± 6 (per section of DED containing fibroblasts) (Figure 3.5 A, B). β1 null ES cells did not form cysts when cultured on DED containing fibroblasts. However, small clumps of K14 positive, K1 negative β1 null cells were formed in the presence of fibroblasts (Figure 3.5 C). In contrast, factors secreted from human keratinocytes did not have an inductive effect on ES cell differentiation. The number of K14 positive cysts formed by wild type ES cells (2.8 ± 1) was not significantly different from the number in standard DED cultures (4.6. ± 2) and K14 positive β1 null cells were not observed (Figure 3.5).

To establish whether there was direct contact between the differentiating ES cells and the dermal fibroblasts, I stained a DED with anti-vimentin to detect both ES cells and fibroblasts. As shown in Figure 3.6., the fibroblasts did not grow far into the DED and did therefore not make direct contact with ES cells. It was thus likely that secreted factors were responsible for the differentiation-promoting effects of fibroblasts.
3.2.4. ES cell differentiation into keratinocytes is stimulated by secreted fibroblast factors

The above experiments show that a factor secreted by mesenchymal cells increases the frequency by which wild type ES cells differentiate into keratinocytes and induce differentiation of K14 positive β1 null cells for the first time in vitro. We next wanted to test whether soluble factors alone (without incubation on DED) could account for the induction of differentiation by fibroblasts.

Wild type and β1 null embryoid bodies were plated onto tissue culture plastic and cocultured with dermal fibroblasts which were seeded in an insert with a pore size of 0.4 μm that would allow the diffusion of soluble growth factors but prevent direct contact between fibroblasts and ES cells. Cells were cultivated until day 27 after induction of differentiation and then stained for keratinocyte differentiation markers.

In the scoring, three different types of keratin 14 positive groups of ES cells were discerned: patch, cluster and colony (Figure 3.7.). In both patches and clusters there is no extensive contact between the keratin 14 positive cells, and they only differ in their size: a patch consists of less than 20, a cluster of 20-100 keratin 14 positive cells. Colonies in contrast consist of large, densely packed groups of keratin 14 positive cells resembling keratinocyte colonies.

When wild type keratinocytes were differentiated, most keratin 14 positive cells were found in either patches or clusters. Upon coculture with fibroblasts, the formation of colonies was increased (Figure 3.8). The keratin 14 positive colonies were multilayered and suprabasal cells expressed the differentiation marker keratin 10 (Figure 3.9.). The formation of clusters or patches in wild type cells was slightly reduced in the presence of dermal fibroblasts (Figure 3.8.).
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The presence of fibroblasts had an even more significant effect on differentiation of β1 null cells. There was an induction of keratin 14 positive patches, clusters and colonies: the mean number of patches per dish was 8.8 ± 3.9 versus 1.3 ± 0.2 (patches), 4.3 ± 0.4 versus 0.7 ± 0.1 (clusters), and 1.3 ± 0.4 versus 0 (colonies) (Figure 3.8.). These experiments show that differentiation of both wild type and β1 null ES cells into keratinocytes is induced by growth factors and that contact with a basement membrane is not necessary to induce differentiation.

3.2.5. Effects of specific growth factors on ES cell differentiation

To identify which growth factors were responsible for the inductive effect of dermal fibroblasts on ES cell differentiation, we decided to add candidate growth factors to the differentiating cells and investigate whether they could substitute for dermal fibroblasts.

I have used bFGF, TGFα, TGFβ, KGF, and aFGF and FGF10 to stimulate the cells. These factors were chosen because they are known to affect keratinocyte proliferation or differentiation. Keratinocyte growth factor (KGF/FGF7) is secreted by dermal fibroblasts and is known to be a major factor in promoting epidermal cell proliferation and differentiation (Guo et al., 1993). Both FGF7 and FGF10 bind the IIb variant of FGFR2 and are synthesised predominantly in tissue mesenchyme (De Moerlooze et al., 2000). aFGF and bFGF stimulate keratinocyte growth (O'Keefe et al., 1988; Shipley et al., 1989). Transforming growth factor α (TGFα) also has been described as key regulator of epithelial growth and differentiation and overexpression of TGFα in the basal layer leads to epidermal hyperplasia and hypertrophy (Vassar and Fuchs, 1991). Transforming growth factor β (TGFβ) has been described to have an influence on keratinocyte differentiation (Cui et al., 1995). Recombinant growth factor concentrations were chosen according to manufacturer's recommendation.

ES cells were allowed to differentiate into embryoid bodies for 5 and 9 days respectively and then transferred into tissue culture plastic dishes. They were grown for another 16-26 days in
the presence or absence of growth factors (Figure 1.3). The culture medium was changed every second day and fresh growth factor was added each time.

Figure 3.10 shows the result of an individual experiment. Analysis of pooled data from all the growth factor experiments is presented in Table 3.1. The amount of patches, colonies and clusters in the control cultures was subtracted from the values obtained with the growth factor treated cells and Student's $t$ test was performed. In wild type cultures KGF and FGF10 increased the number of K14-positive colonies and TGF β increased cluster formation ($p < 0.05$); none of the other factors stimulated differentiation significantly (Table 3.1).

In β1 null cultures aFGF and bFGF had no effect and the positive effects of TGFβ were not statistically significant (Table 3.1). KGF, FGF10 and TGFα all had positive effects on differentiation of K14 positive cells. No colonies formed in KGF treated cultures (Table 3.1), but cluster formation was stimulated. TGFα primarily stimulated formation of clusters and patches (Table 3.1) although colony formation was also increased in some experiments (Figure 3.10). Overall, FGF10 was the most potent growth factor for stimulation of differentiation by β1-null cells, as evidenced by significant induction of colonies, clusters and patches (see Table 3.1).

To test whether FGF10, TGFα and KHF could account for the activity of HDF to induce differentiation, conditioned medium was assayed for growth factor production. We were unable to detect TGFα production either by using western blot or an ELISA assay, however, Claudia could show that HDF did produce KGF and FGF10 in the concentration range that we used for stimulating the cells (Bagutti et al., 2001).

### 3.3. DISCUSSION

The presence of β1 integrins is a prerequisite for ES cells to differentiate into keratinocytes *in vitro*, but not in teratomas and the skin of wild type/β1 null chimeric mice (Bagutti et al., 1996). There are at least two possible explanations for this observation: (i) β1 null cells have
reduced sensitivity to a differentiation factor in comparison to wild type cells. (ii) β1 null ES cells can (in contrast to their wild-type counterparts) not produce a differentiation factor, but might be able to recognise it.

One reason for why β1 null embryoid bodies can differentiate into keratinocytes in vivo but not in vitro might be their defect in extracellular matrix assembly (Aumailley et al., 2000; Bloch et al., 1997; Sasaki et al., 1997; Stephens et al., 1993). There are a number of observations that would argue that this is the case: differentiation in many tissues is dependent upon the assembly of a basement membrane (Ashkenas et al., 1996; Murray and Edgar, 2001; Smyth et al., 1999; Williamson et al., 1997). In β1 null cells, the assembly of a basement membrane correlates with the ability of these cells to differentiate into keratinocytes. In vitro no deposition of basement membrane components such as laminin and collagen IV is observed in β1 null embryoid bodies and their outgrowths (Bagutti et al., 1996; Aumailley et al., 2000). However, under conditions in which β1 null cells are able to differentiate into keratinocytes, in β1 null teratomas and in wild-type/β1 null chimeric mouse skin, a basement membrane is assembled as judged by immunofluorescence microscopy (Bagutti et al., 1996). Although the basement membrane of β1 integrin-deficient teratomas shows a disorganised deposition of several basement membrane proteins on an ultrastructural level (Sasaki et al., 1998), it seems conceivable that the failure of β1 null cells to differentiate into keratinocytes in vitro may reflect the lack of a basement membrane.

When we tested this hypothesis by providing β1 null ES cells with an epidermal basement membrane by seeding them on DED, we found that it is not sufficient to restore the capacity of β1 null ES cells to differentiate into keratinocytes. However, when we repopulated DED with dermal fibroblasts, we could show that factors secreted by dermal fibroblasts do have inductive activity. Furthermore, the fact that HDF supernatants also can induce differentiation in plastic culture dishes suggests that the basement membrane is not necessary for the induction of keratinocyte differentiation.
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How can these findings explain the differences between β1 null and wild type cells in vitro and why β1 null cells can differentiate in vivo but not in vitro? The HDF coculture experiments provide evidence that β1 null cells have reduced sensitivity to soluble factors that direct the differentiation of ES cells towards becoming keratinocytes. Our hypothesis is that for wild type cells the levels of growth factors in the serum and produced by other differentiating cell types are sufficient to induce keratinocyte differentiation. However, β1 null ES cells are less sensitive than wild type cells to these factors and therefore need higher concentrations than present in serum for differentiation. In vivo the amounts of these differentiation factors are higher and this is why β1 null cells are able to differentiate.

Although the degree of differentiation achieved with conditioned medium or growth factors was always lower in β1 null than in wild type cultures, the relative increase in K14-positive cells in response to the factors was greater in β1 null cultures, and in the case of TGFα only the β1-null cells showed a significant response (Table 3.1). This would be consistent with the idea that concentrations of growth factors present in the standard cultures are not limiting for wild-type cells, but are limiting for β1-null cells. A synergy of integrins with growth factors is well established (Sastry et al., 1996) and activation of signalling pathways by growth factors can be dependent on integrin-mediated adhesion (Renshaw et al., 1997). In agreement with this hypothesis β1 null ES cells show a defect in MAPK activation upon serum in preliminary experiments (data not shown). It would be very interesting to further investigate MAPK signalling in β1 null ES cells, but there are technical problems that would need to be solved first.

The positive effects of fibroblasts on keratinocyte differentiation are consistent with the inductive role of the dermis during embryonic development (Sengel and Mauger, 1976). One of the prototypes of epithelial mesenchymal interaction is the FGF signalling unit, consisting of FGF, FGFR and HSPG (Plotnikov et al., 1999). Epithelial FGFRs interact with mesenchymal ligands across the basement membrane. KGF and FGF10, which are secreted by dermal fibroblasts, were potent stimulators of ES cell differentiation. Furthermore, both
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growth factors bind the same receptor, the IIIb isoform of FGFR2. Mice lacking the FGFR2 gene die at the same stage of development as the β1 null embryos (Arman et al., 1998) and mice with a targeted deletion of the IIIb splice variant have impaired epidermal development (De Moerlooze et al., 2000).

In contrast to KGF and FGF10, which was readily detected in HDF conditioned supernatants, TGFα was not found. This was to be expected since in the skin TGFα is produced by keratinocytes and not by the fibroblasts. However, KGF can induce TGFα expression and secretion in cultured keratinocytes (Dlugosz et al., 1994) and coculture of HDFs could have an inductive effect on keratinocyte differentiation via this pathway.

The β1 null phenotype is not completely rescued by the coculture with HDFs or the addition of growth factors. β1 null cells cannot form cysts and they have a lower frequency of colony formation. While wild-type ES cells were capable of further epidermal differentiation in vitro, as evidenced by formation of K1/K10-positive cysts in the DED cultures and suprabasal K10-positive layers in colonies on plastic, I could never observe K10 positive cells. This may be the result of their decreased sensitivity to additional inducing factors. It would be very interesting to examine other factors. For example Wnts and BMPs are known to affect commitment of embryonic cells to epidermal versus neuronal lineages (Sasai and De Robertis, 1997; Wilson et al., 2001). In chick epiblast high level of Wnt signalling permits the expression and signalling of BMP-4, which then directs the cells to an epidermal fate (Wilson et al., 2001). BMP-4 is reduced in β1 null cells (Rohwedel et al., 1998) and this might be a reason for their impaired ability to differentiate into epidermis.

Our data would predict that embryoid bodies that lack the receptors for some of the growth factors we have tested show impaired ectodermal differentiation but can be rescued by increased ECM adhesiveness. Indeed, in a recently published paper it was shown that the failure in epithelial differentiation of embryoid bodies with a dominant negative mutation of FGFR2 can be partially rescued by extracellular matrix components (Li et al., 2001).
lack of FGFR signalling suppresses collagen and laminin synthesis in embryoid bodies (Li et al., 2001). Together with the here presented finding that β1 null cells have an impaired sensitivity to FGFR mediated signalling, this might also explain the earlier mentioned observation that β1 null embryoid bodies can assemble an extracellular matrix in vivo but not in vitro.
Figure 3.1. Wild type, but not β1 null embryoid bodies differentiate into keratinocytes in vitro.

A) Immunoblot detection of keratin 14.
Equal amounts of wild type (D3) and β1 null (G201) cells were loaded. As loading control the gel was stained with coomassie blue.

B) Wild type embryoid bodies were photographed under phase contrast microscopy at 26 days of differentiation and stained for nuclei (blue) and keratin 14 (green).
Scale bar: 100 μm
Figure 3.2. Differentiation of embryoid bodies on DED.

H&E staining and schematic representation of differentiated wild type and β1 null embryoid bodies.
Scale bar: 160 μm
A: wild type

B: β1 null

Dermis

K1

K14

bm
Figure 3.3. Immunofluorescence staining of cysts formed by wild type ES cells seeded onto DED.

Cysts were labelled with antibodies to keratin 14, keratin 10 and the α6 integrin subunit. Scale bar: 40 μm (K14 and K10), 80 μm (α6).
keratin 14  keratin 10  integrin α6 subunit
Figure 3.4. Collagen type IV distribution in DED.

A) Immunofluorescent labelling of DED with anti human type IV collagen antibodies before the addition of embryoid bodies.

B) Cysts formed by wild type ES cells were labelled with antibodies to mouse type IV collagen.
Figure 3.5. The presence of dermal fibroblasts stimulates ES cells to differentiate into keratinocytes.

A) Graph showing the number of keratin 14 positive groups of cells per section DED cultures of wild type (wt) and β1 null (ko) embryoid bodies incorporating human dermal fibroblasts (+ Fibros) or human epidermal keratinocytes (+ Kerats), or without added fibroblasts or keratinocytes (control).

B) Keratin 14 positive cyst of wild type cells.

C) Keratin 14 positive patch of β1 null ES cells.

Scale bar: 130 μm (B), 60 μm (C)
Number of K14-positive cysts

![Image of cysts and bar chart showing the number of K14-positive cysts for different conditions: control, wt, ko, +Fibros, +Kerats.]
Figure 3.6. Immunofluorescence staining with anti vimentin of a β1 null ES culture on DED containing fibroblasts.

ES cells (above the broken line which denotes position of the DED basement membrane) and fibroblasts are vimentin positive. Solid line denotes lower surface of DED.

Scale bar: 130 μm
Figure 3.7. Types of keratin positive ES cell groups.

Immunofluorescence staining of ES cell groups formed by wild-type embryoid bodies for keratin 14 and corresponding phase images. Three different types of keratin 14 positive groups were discerned:

A) patch (less than 20 keratin 14 positive cells)
B) cluster (20-100 keratin 14 positive cells)
C) colony (large, densely packed group of keratin 14 positive cells)

Scale bar: 50 μm (A-C)
Figure 3.8. Effects of coculture with HDFs on differentiation of ES cells into keratin positive cells.

Graphs showing the absolute numbers of keratin 14 positive groups of cells (colonies, clusters, patches) in the presence (+F) or absence (co) of HDF conditioned medium.

A) wild type cells.
B) β1 null cells.

Data represents means +/- standard deviation of two (A) or three (B) independent experiments.
Wild-type

a

b

K14-positive groups of cells

+ F, co colonies + F, co clusters + F, co patches

K14-positive groups of cells

+ F, co colonies + F, co clusters + F, co patches

β₁-null
Figure 3.9. Keratin positive ES cell groups formed by wild type embryoid bodies cocultured with HDF.

A) Immunofluorescence staining of keratin 14 and corresponding phase image.

B) Immunofluorescence staining of keratin 10 and corresponding phase image.

Scale bar: 75 μm (A and B)
Figure 3.10. Effects of specific growth factors on ES differentiation

Differentiation of wild type (A) and β1 null (B) ES cells in presence or absence (co) of various growth factors: acidic and basic FGF (at final concentrations of 50 and 10 ng/ml respectively), KGF (100 ng/ml), FGF10 (2 ng/ml), and TGFα (25 ng/ml).
Wild-type

Colonies □ Clusters □ Patches

β1-null

Colonies □ Clusters □ Patches
### Table 3.1. Statistical analysis (p values) of the results of all experiments with specific growth factors.

p values < 0.05 indicate significant difference between growth factor treated and control.

*Number of colonies in control and KGF-treated cultures was zero. Number of independent experiments and concentration of each growth factor: aFGF (3; 50 ng/ml), bFGF (3; 10 ng/ml), KGF (3; 100 ng/ml), FGF10 (6; 20 ng/ml), TGFα (4; 25 ng/ml), TGFβ (3; 5 ng/ml).
CHAPTER 4: DELETION OF β1 INTEGRIN IN MOUSE KERATINOCYTES

4.1. INTRODUCTION

β1 integrin mediated adhesion has been implicated in regulating terminal differentiation and it had been proposed that epidermal stem cells expressed higher levels of β1 integrin (Jones et al., 1995; Jones and Watt, 1993; Zhu et al., 1999). This finding had been challenged when mice with a skin-specific deletion of β1 integrin were generated. *In vivo* β1 null keratinocytes had a reduced proliferation rate, but the regulation of terminal differentiation did not seem perturbed (Brakebusch et al., 2000; Raghavan et al., 2000). However, *in vivo* the deletion of β1 integrin might be compensated by various mechanisms. We were therefore interested to analyse β1 null keratinocytes *in vitro*.

I isolated keratinocytes from mice with a keratinocyte restricted deletion of the β1 integrin gene and, in addition, deleted β1 integrin *in vitro* by expressing Cre recombinase in β1 integrin fl/fl keratinocytes. β1 null keratinocytes exhibited defects in adhesion, spreading, and migration. Furthermore, the differentiation marker involucrin became upregulated in primary β1 null cells, indicating that the removal of β1 integrin in mouse keratinocytes induced cell differentiation *in vitro*.
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4.2. RESULTS

4.2.1. Isolation of keratinocytes from the K5Cre fl/fl β1 fl/fl mouse and genotyping

To delete β1 integrin in keratinocytes, mice whose complete coding and 3’ non-coding region of the β1 gene was flanked by loxP sites were crossed with mice that expressed Cre recombinase under the control of the keratin 5 promoter (Brakebusch et al., 2000). A promoterless lacZ gene had been introduced downstream of the stop codon, resulting in lacZ expression driven by the endogenous β1 integrin promoter after Cre mediated recombination and hence allowing monitoring of deletion of the β1 integrin gene (Figure 4.1 A and 4.1B).

For the isolation of keratinocytes, mice were killed between days one and five after birth. Epidermal keratinocytes isolated from individual mice were cultured separately. To determine the β1 status of the individual littermates, they were genotyped by PCR. Genomic DNA was isolated from tail snips and analysed for the presence of floxed or wild type alleles of β1 integrin and for the presence of Cre recombinase (Figure 4.1C).

4.2.2. FACS analysis of integrin levels

The presence of Cre does not necessarily mean that efficient recombination has taken place. Therefore, to examine whether β1 was still present in cells isolated from a mouse that expressed K5 Cre and was heterozygous for the floxed allele, β1 integrin surface levels were investigated in freshly isolated keratinocytes via FACS analysis. The analysis shows that in two day old mice most of β1 integrin has been removed (Figure 4.2B). To test whether other integrins were affected by the absence of β1 integrin, I also analysed the surface levels of β4 and αv integrins. In agreement with earlier reports (Bagutti et al., 1996; Raghavan et al., 2000; Brakebusch et al., 2000), β4 surface integrin expression was reduced to about 50% of wild type control levels (Figure 4.2A). αv integrins were only expressed at very low levels in freshly isolated mouse keratinocytes, and the surface level of αv did not differ between
wild type and Δ/Δ keratinocytes (Figure 4.2C). β1 integrin levels of keratinocytes with only one copy of the β1 integrin gene had the same β1 integrin levels as their wild type controls (Figure 4.2E).

4.2.3. Morphology of β1 null keratinocytes in culture: Impaired adhesion and spreading of β1 null keratinocytes

Keratinocytes isolated from individual mice were plated onto tissue culture plastic, vitronectin, fibronectin and collagen I. Within one litter, keratinocytes with different genotypes were compared: homozygous for β1 deletion, heterozygous for the β1 deletion, and no deletion (mice without the presence of Cre).

Adhesion and spreading on vitronectin was poor in cells regardless of their β1 integrin expression (Figure 4.3A). On fibronectin and collagen a major difference between cells that had a homozygous deletion for β1 integrin and their littermates was observed. Most of the β1 null keratinocytes did not adhere and the spread cells in Figure 3a are melanocytes, as determined by their pigmentation. In contrast, all wild type and heterozygote keratinocytes adhered and spread on collagen and fibronectin.

To further analyse the morphology of β1 null keratinocytes, cells were fixed after 24 or 48 hours in culture and then stained with an antibody against paxillin to test if cells were able to form focal adhesions and with TRITC- conjugated phalloidin to visualise microfilaments. Figure 4.4. shows that β1 null cells were not able to organise their actin cytoskeleton or to form focal adhesions: most cells remained rounded and even those which were able to spread to a small extent never displayed stress fibres like wild type or heterozygote keratinocytes did.

To quantitate the extent of adhesion of β1 null keratinocytes they were plated onto 96 well plates coated with various substrates. Since freshly isolated mouse keratinocytes adhered
very slowly, cells were allowed to adhere overnight before they were fixed and the percentage of adherent cells was scored. On poly-d-lysine in both wild type and β1 null keratinocytes, 18% of the plated cells adhered (+/- 2 and 1.5 respectively). In agreement with reports that the α5β1 integrin is not expressed in steady state skin, the adhesion on fibronectin was barely above adhesion on poly-d-lysine (23 (+/- 2.8) in wild type vs 17 (+/- 2) in β1 null keratinocytes). On the other substrates tested, there was a significant difference between β1 deficient and wild type keratinocytes: In case of collagen I 54 (+/- 9) vs 13 % (+/- 4), Laminin 36 % (+/- 5.8) vs 0.8% (+/- 15) and Collagen IV 49% (+/- 9.5) vs 17% (+/- 3.4).

I also tried to plate keratinocytes onto DEDs or laminin 5 enriched keratinocyte extracellular matrix, but the β1 null keratinocytes did also not adhere to these matrices (data not shown).

4.2.4. β1 null keratinocytes exhibit reduced proliferation and increased differentiation in vitro

It has been reported for various cell types, that lack of integrin- mediated adhesion induces apoptosis (Frisch and Ruoslahti, 1997). To test whether removal of β1 integrin in primary mouse keratinocyte induced apoptosis, cells were plated after isolation onto different substrates and allowed to adhere. After five days in culture they were fixed and nuclear fragmentation was scored. The lack of β1 integrin does not induce apoptosis in keratinocytes, regardless of the substrate the keratinocytes were plated on: The percentage of positive cells was 0.4% in β1 null and 0.5% in wild type keratinocytes (Figure 4.6A).

I next asked whether the β1 null keratinocytes were able to undergo cell division and compared the BrdU incorporation in β1 null and wild type keratinocytes. Four days after isolation, keratinocytes were incubated for 1 hour with BrdU and then fixed. Incorporated BrdU was detected using a monoclonal anti-BrdU antibody. Figure 4.6B shows that some β1
null cells did incorporate BrdU, although to a lower extent than wild type keratinocytes (+/− 1.4) vs 5.5 (+/− 0.7).

In human keratinocytes β1 integrin has been implicated in the regulation of terminal differentiation (Adams and Watt, 1989). Involucrin is a marker of keratinocyte differentiation both in vivo and in vitro. I therefore investigated whether β1 null cells had a different percentage of involucrin expression in comparison to wild type cells. Mouse keratinocytes were isolated from a litter that consisted of β1 null and wild type mice and plated overnight onto collagen I coated dishes. After 22 hours in culture cells were trypsinised, dried onto glass coverslips, fixed and permeabilised and then stained with antiserum against mouse involucrin. β1 null keratinocytes had 35 times more involucrin expressing cells than their littermate controls (35% (+/− 15) positive cells versus 1 % (+/− 1) in wild type keratinocytes (Figure 4.7).

To test if involucrin upregulation could also be observed on other substrates, keratinocytes were plated directly after isolation onto fibronectin, laminin, collagen I and vitronectin coated coverslips, fixed after 48 hours in culture, and then scored for their involucrin expression. β1 null cells always expressed higher levels of involucrin than the wild type control keratinocytes, regardless of the matrix they were plated onto (Figure 4.8A).

One explanation for why the β1 null cells induced the differentiation marker involucrin might be that the impaired ability to spread induces differentiation (Watt, 1987). I therefore plated cells on PDL and stained them after 48 hours in culture for involucrin. Even on PDL, where wild type cells were not able to spread, the level of involucrin was about five times higher in β1 deficient cells than in wild type cells (Figure 4.8A and B). Another reason for the higher levels of involucrin in the β1 null cells might be that the involucrin levels were already higher in the β1 null cells at the time of isolation. I therefore analysed levels and distribution of involucrin before keratinocytes were placed in culture. In FACS, involucrin staining of β1 null and wild type cells was similar and the percentage of involucrin positive cells was not higher in β1 null keratinocytes (Figure 4.9B). In agreement with published data obtained from the K5Cre fl/fl and the K14 Cre fl/fl mice (Brakebusch et al., 2000; Raghavan...
et al., 2000), there was also no difference in distribution of involucrin and a second early differentiation marker, keratin 10, in skin sections (Figure 4.9).

4.2.5. Infection of mouse keratinocytes with an adenovirus vector expressing Cre recombinase

In addition to analysing the β1 null keratinocytes that were isolated form the K5 Cre fl/fl mice, I tried to investigate the effect of the deletion of the β1 integrin gene in keratinocytes in vitro. I generated a spontaneously immortalised fl/fl keratinocyte cell line by serial passage of neonatal keratinocytes at high density for 10 passages, after which the proliferation rate of the cells started to increase.

I set out to try to delete β1 integrin in the fl/fl keratinocytes by expressing Cre recombinase using an adenoviral vector. Adenovirus can be grown to high titers and can infect dividing as well as quiescent cells (Anton and Graham, 1995). AdCre1, a replication-defective human adenovirus (Ad) type 5 vector containing the gene for Cre recombinase under the control of the human cytomegalovirus immediate early promoter (Anton and Graham, 1995), was provided by Cord Brakebusch and Reinhard Fassler. I prepared adenovirus stocks by infecting 293 cells, which can be used to propagate the virus, and then purified it by equilibrium gradient ultracentrifugation. Keratinocytes were infected by incubating them for 1-2 hours in serum-free medium containing virus. To test whether keratinocytes were successfully infected and whether the Cre recombinase was active, I monitored cells for lacZ expression two days after infection. At a concentration of 100 pfu/cell, recombination had taken place in all cells, whereas infection with a concentration of 1 pfu/cell resulted in excision of β1 in only a minority of cells (Figure 4.10).

**Adenovirus is toxic for keratinocytes at the levels needed for downregulation of β1 integrin.**

When fl/fl keratinocytes were infected with an amount of virus that resulted in a downregulation of β1 integrin to about 50% of its control surface levels (Figure 4.11), I could observe a detachment of almost all keratinocytes. To test for any toxic effect of the
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virus, I infected a wild type mouse keratinocyte cell line established in our laboratory (Romero et al., 1999) in parallel with the fl/fl keratinocytes (Figure 4.11). It turned out that also in this line cells detached after infection with the adenovirus, although not to the same extent as the fl/fl keratinocytes. This suggested that cells detached at least partly because of an unspecific side effect of the virus. An ELISA for histone-associated DNA fragments, which are indicative of apoptosis confirmed that the levels of adenovirus needed to induce Cre mediated recombination and downregulation of \( \beta_1 \) integrin were toxic for mouse keratinocytes (Figure 11).

4.2.6. Expression of Cre recombinase using retrovirus.

Since the adenovirus showed considerable toxicity, I set out to take another approach using retroviral instead of adenoviral vectors.

Construction of retroviral vectors
cDNA of a fusion of EGFP (enhanced GFP) and Cre recombinase was obtained from Joyce Muller (ECBL, ICRF) and I subcloned the cDNA into the retroviral vector, pBabe puro. Both ecotropic retroviral producer cell lines, GPE, and amphotropic producer lines, AM12 were generated by first transfecting the packaging cell line Phoenix with the retroviral vector, and then using the Phoenix supernatant to infect GPE or AM12. For the EGFP Cre ecotropic producer cells, the 20% of the population of the highest EGFP expression was selected by FACS. Polyclonal lines were used for all infections.

Transduction of mouse epidermal keratinocytes with EGFP-Cre retrovirus:
The standard protocol used to transduce primary keratinocytes with retrovirus is to cocultivate the keratinocytes with producer cells (Levy et al., 1998). However, in the low calcium conditions used for mouse keratinocytes, the packaging cell lines detached easily and I therefore used supernatants for the infection. There was no significant difference between the infectivity of GPE and AM12, and for safety reasons I decided to use GPE for
the infections. I tested whether Cre was functionally active by analysing lacZ expression and
downregulation of β1 integrin.
The percentage of EGFP positive cells correlated with the percentage of lacZ positive cells
(data not shown). However, in many infections I did not see a downregulation of the β1
integrin cells in all EGFP positive cells between 72 and 120 hours after infection (Figure
4.12). Since I saw the first lacZ positive cells as early as 48 hours after infection with EGFP,
and because the half life of β1 integrin on the cell surface is between four and eight hours
(Hotchinson and Watt, 1992), the finding that I still had EGFP-positive, β1 integrin positive
cells suggested that Cre recombinase was not efficient: It removed only one copy of the gene
(hence the lacZ positive cells), but since the cells still had one copy of the gene, they retained
surface β1 integrin expression. Immunofluorescence staining of EGFP positive cells in
comparison to EGFP negative cells was therefore difficult to interpret (Figure 13). Some
EGFP positive cells did round up (Figure 4.13D), but others looked similar to the uninfected
keratinocytes and I could not see a difference in paxillin and α6 integrin staining in some of
the cells (Figure 4.13B and C). I tried another approach to circumvent this problem,
reasoning that a higher titer might allow more copies of cre recombinase to be incorporated
per cell and therefore lead to a better ratio of GFP positive/β1 integrin negative cells. One
way to increase the titer is to concentrate the virus by physical methods such as
ultracentrifugation. The drawback of this method is that the virus gets damaged, which
partially can be attributed to the instability of the virus-encoded envelope protein (Yee et al.,
1994). However, it has been suggested that the stability of retroviruses can be increased by
the generation of vesicular stomatitis virus (VSV) G pseudotyped retroviral vectors (Burns et
al., 1993). I obtained 293 gp cells, a cell line that stably expressed gag and pol proteins of
MoMLV, and cotransfected them with VSV-G and pBabe puro EGFPCre or with
LZRSpBMN EGFPCre (Joyce Muller, ECBL, ICRF). The concentrated pseudotype virus
yielded a better EGFP positive/β1 negative ratio. β1 integrin started to disappear from the
cell surface from day three after infection onwards and by day four after infection most cells
had lost the β1 integrin. Interestingly, the β1 null keratinocytes disappeared in the following
two days and the fraction of β1 positive cells increased (Figure 4.14).
In next tested whether fl/fl keratinocytes underwent terminal differentiation upon retroviral expression of EGFPCre. When passage I fl/fl keratinocytes were infected with LZRSpBMN EGFPCre and analysed for the induction of differentiation markers, higher levels of involucrin (data not shown) and cornifin (Figure 4.15 A) were observed in the EGFPCre positive cells in comparison to the uninfected, EGFPCre negative cells on day 5 after infection. 23.2 (+/− 17) % of the EGFPCre postive cells expressed cornifin, whereas 3.2 (+/−1)5 of the EGFPCre negative cells stained positive for cornifin. Since not all EGFPCre positive cells did downregulate β1 integrin (Figure 4.14), the actual percentage of cornifin positive cells in β1 null cell is probably even higher.

Next I analysed the fl/fl keratinocytes in order to test whether removal of β1 integrin had the same effects on spreading in high passage keratinocytes (which might have upregulated other integrins) as in K5 Cre keratinocytes. fl/fl keratinocytes were infected with LZRSpBMN EGFPCre and on day 6 after infection two populations were sorted: EGFPCre negative, β1 integrin positive, and EFGFPCre positive and β1 integrin negative. Equal amounts of cells were plated on vitronectin, fibronectin and collagen 1 coated coverslips and allowed to adhere overnight. The next day cells were fixed and stained with TRITC-conjugated phalloidin to visualize actin microfilaments. In agreement with the results obtained using the K5 Cre fl/fl keratinocytes, only a few of the β1 null cells adhered, and those that did were not able to organise their actin cytoskeleton and could not spread.

In order to find out what was happening to these cells, I decided to film them over a period of 24 hours on days four and five after infection. Frames were taken under fluorescence illumination to detect GFP positive cells and under phase contrast to visualise the uninfected cells and keratinocyte morphology. As a control some of the cells that were plated for the movie were stained for β1 integrin and a FACS analysis was performed to see how many of the GFP positive cells had downregulated β1 integrin (Figure 4.16). The β1 null keratinocytes behaved very differently from the uninfected cells. Similar to the cells that were isolated from the K5 Cre fl/fl mice, the keratinocytes were not able to spread and the cells were not motile. Interestingly though, they seemed to stick firmly to their surface, presumably via other integrins such as αv or by non-integrin dependent mechanisms. They
exhibited many extensions, which do not seem to be retraction fibres, but filopodia (see Figure 4.17). In contrast to the uninfected keratinocytes, the β1null cells were not motile. Wild type keratinocytes moved at a speed of 21.2 (+/− 0.506) μm/hr, whereas β1 integrin deficient keratinocytes moved at 2.56 (+/− 0.085) μm/hr. Surprisingly, although the cells do not seem to be able to organise their cytoskeleton, they were still able to undergo cell division (see film and Figure 4.18). However, the cell cycle time of β1 null cells seemed to be longer: when the amount of cell divisions were quantified, the β1 null keratinocytes underwent 1 cell division/ cell/ 22 hours whereas the wild type keratinocytes in the same dish underwent 2/cell divisions/ cell/ 22 hours.

As a control I infected the fl/fl keratinocytes with a retroviral vector encoding chick β1 integrin ((Levy et al., 2000) and Figure 4.19). When filmed, the GFP positive, mouse β1 integrin negative cells behaved like wild type keratinocytes in their extent of spreading and motility (Figure 4.20 and Figure 4.21), indicating that the chick integrin can rescue the mouse keratinocytes.

4.3. DISCUSSION

In this chapter I presented evidence that β1 integrin is indispensable for keratinocyte adhesion, spreading and migration in vitro, and that lack of β1 integrin induces differentiation in vitro.

β1 integrin has been studied extensively in human keratinocytes. High surface levels of β1 integrins are a marker for stem cells in human interfollicular epidermis (Jones and Watt, 1993; Jones et al., 1995). Furthermore, a dominant negative β1 integrin construct, when expressed in human keratinocytes, stimulated exit from the proliferative compartment (Zhu et al., 1999). The massive induction of involucrin in the K5 Cre mice would support this hypothesis. An important control would be to look at other differentiation markers. I tried to stain freshly isolated keratinocytes from fl/fl K5 Cre mice with keratin 10 and cornifin in one experiment, but did not get conclusive results. Unfortunately, I did not have enough K5 Cre...
mice to repeat this experiment. However, the upregulation of cornifin and involucrin in fl/fl keratinocytes expressing EGFPCre indicates that the β1 null cells indeed induce differentiation.

It has been shown that cell shape can influence the proliferation and terminal differentiation of keratinocytes and furthermore, that human keratinocytes start to differentiate within 3 days not allowed to spread (Watt et al. 1989). One could therefore argue that the inability of β1 null keratinocytes to spread causes them to undergo terminal differentiation. However, when wild type keratinocytes were prevented from spreading by plating them on poly-d-lysine, they did not start to induce their involucrin expression (Figure 4.8), indicating that terminal differentiation is induced by a different mechanism. Furthermore, since β1 null keratinocytes induced differentiation independently of the substrate they were plated onto, one could speculate that involucrin is induced by a mechanism that is independent of cell adhesion. It has already been proposed that β1 integrins regulate keratinocyte adhesion and differentiation by different mechanisms (Levy et al., 2000).

I had a number of technical difficulties to overcome before I could study the effects of the removal of β1 integrin on keratinocytes in culture. The adenovirus turned out to have toxic side effects and was therefore not feasible. Using a retroviral vector solved this problem, but this approach had the disadvantage that often only one copy of the β1 integrin gene appeared to be removed and hence the cells remained in effect in a wild type state. It would therefore be better to use keratinocytes with a fl/- rather than a fl/fl genotype.

αv integrins mediate cell adhesion to various matrix proteins including fibronectin, vitronectin and tenascin. Keratinocytes express a number of αv integrins: αvβ5 and αvβ6 for example. The β1 null cells expressed low levels of αv integrins, which did not seem to become upregulated in keratinocytes to compensate for the loss of β1 integrins. αvβ6 has been shown to be critical for keratinocyte migration on fibronectin and vitronectin (Huang, 1998). However, the films of the EGFPCre infected cells show that keratinocytes require β1
integrins for spreading and migration and that other integrins cannot compensate for the loss of β1 integrins. One striking observation was that the β1 null keratinocytes stuck well to the surface and that they were still able to undergo cell division. A number of questions arise from these observations. How do these cells stick? How can these cells undergo cell division despite the fact that their actin cytoskeleton seems to be highly disorganised?

For the films the cells were plated on glass coverslips that had been coated with collagen I (film I and II; Figures 4.17, 4.18, 4.21). However, since the keratinocytes were plated on these dishes for 24 hours before the filming was started, additional extracellular matrix might have been produced by the keratinocytes themselves. In addition, cells were plated in the presence of serum, which contains extracellular matrix components like fibronectin. Fibronectin has a collagen binding site (Hemler, 1999) and might therefore adhere well to collagen coated dishes and thus provide the β1 null keratinocytes a matrix they can adhere to. This might be an explanation for the paradoxical observation that β1 null keratinocytes always adhered better to collagens than to laminin1 (paradoxical, because α6β4 can bind laminin1 (Sasaki and Timpl, 1999). One possibility is, therefore, that the cells still adhered via integrins other than β1 integrins. Possible candidates are α6β4 integrin which in high passage keratinocytes did not become downregulated upon deletion of the β1 integrin gene (data not shown) and αv integrin.

Integrin receptor function is required in most cell adhesion assays, perhaps because integrins can interact with and reorganize the cytoskeleton, strengthening weak primary interactions (Reichhardt, 1999). At the same time there exist additional cell surface molecules that interact with the extracellular matrix and β1 integrin cells might adhere via them. For example, cells could adhere via syndecan 1 or syndecan 4. However, keratinocytes have to be trypsinised for both the isolation from mouse skin and for passaging cultured keratinocytes. Trypsin treatment removes syndecans from the cell surface (Jalkanen et al., 1987; my own observation), making adhesion assays to test whether syndecans can account for residual adhesion in β1 null cells difficult.
CHAPTER 4 \( \beta_1 \text{ null Keratinocytes} \)

Figure 4.1. Conditional \( \beta_1 \) integrin knockout

A) Schematic representation of the wild type \( \beta_1 \) integrin gene, the targeting construct and the knockin (Brakenbusch and Fassler)

B) \( \text{lac Z} \) staining of mouse keratinocytes

After recombination, the \( \beta_1 \) integrin promoter transcribes the \( \text{lacZ} \) cDNA

Keratinocytes isolated from \( \Delta/\Delta \) (M1), \( +/- \) (M2) and \( \text{fl}/+ \) (M3) mice. Note the lack of spreading in \( \Delta/\Delta \) keratinocytes.

C) PCR analysis

DNAs were isolated from littermates and PCR was performed to test for the presence of K5 Cre and floxed and wild type \( \beta_1 \) integrin alleles. PCR fragments were resolved by agarose gel electrophoresis and visualised by ethidium bromide staining. When compared to the wild type \( \beta_1 \) integrin PCR product, a larger PCR product was present if the gene has been replaced by the knockin. The heterozygote animals (M2 and M3) showed two PCR products.
Brakebusch and Fassler
Figure 4.2. Surface expression of integrins before and after deletion of β1 integrin.

A) FACS profiles of freshly isolated keratinocytes from two day old littermates that were homozygous for the floxed β1 integrin allele in the presence (Δ/Δ) or the absence (fl/fl) of K5 Cre. Keratinocytes were stained with antibodies against β1, β4, αv and αvβ6 integrin.

B) β1 integrin surface level of keratinocytes homocysogyous and heterocysogyous for the floxed β1 integrin.

C) and D) αv β6 and αv integrin surface level of keratinocytes homocysogyous and heterocysogyous for the floxed β1 integrin.

E) β1 integrin surface levels in β1 fl/fl and β1 fl/Δ animals
Figure 4.3. β1 null keratinocytes exhibit impaired adhesion and spreading.

Keratinocytes were isolated from β1 null and wild type littermates and plated onto collagen, fibronectin and vitronectin coated dishes. Phase photographs of cultures were taken after four days.

Scale bar: 20 μm
Collagen I

Fibronectin

Vitronectin

β1 null

wild type
Figure 4.4. Loss of focal adhesions and stress fiber formation in β1 null keratinocytes.

Wild type (A,B,C) and β1−deficient (A’, B’, C’) keratinocytes were grown for two days and examined for F-actin (B, B’) and focal adhesions (C, C’) by immunofluorescence staining for paxillin and staining of F-actin with phalloidin. A, A’ is an overlay of the two stainings. Scale bar: 35 μm
Figure 4.5. Adhesion of wild type vs β1 integrin deficient keratinocytes.

Keratinocytes isolated from two day old mice were plated onto 96 well plates (5x10^4 cells /well) pre-coated with fibronectin, laminin, PDL, collagen I and collagen IV. Adhesion was quantified using a CytoTox 96™ colorimetric kit. Error bars represent standard deviation of the mean of triplicate samples within one experiment.
Figure 4.6. β1 integrin deficient keratinocytes incorporate less BrdU, but do not apoptose.

A) Keratinocytes from wild-type and β1 deficient cells were fixed after 4 days in culture and nuclear fragmentation was detected using the ‘Apoptosis Detection System’ from Promega.

B) Keratinocytes were isolated from wild type and β1 deficient mice and kept in culture four days. BrdU was then added for 1hr. Cells were fixed and incorporation was detected using immunofluorescence staining using an antibody against BrdU.

Error bars in A and B represent standard deviation of the means of replicate samples.
A

% of BrdU positive cells

wild type

β1 null

B

% of apoptotic cells

β1 null

wild type
CHAPTER 4  \( \beta_1 \) null Keratinocytes

Figure 4.7. Lack of \( \beta_1 \) integrin induces involucrin expression \textit{in vitro}.

A) Immunofluorescence staining of the differentiation marker involucrin. Wild type and \( \beta_1 \) null keratinocytes were fixed after 24 hrs in culture.

B) Percentage of involucrin expressing cells. Error bars represent standard deviation of the mean of the results obtained from two K5Cre fl/fl and two wild type littermates. The experiment was repeated four times with similar results.
% of involucrin positive cells

β1 null

wild type

A

B

wild type

β1 null
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β1 null Keratinocytes

Figure 4.8. Involucrin is induced independently of the substrate the cells are plated onto and is not linked to the extent of cell spreading.

A) Percentage of involucrin expressing cells. Keratinocytes were stained after 3 days in culture.

B) Overlay of an immunofluorescence staining for involucrin (green) and F-actin (red).

Scale bar: 50 μm
Figure 4.9. Involucrin and keratin 10 expression in fl/fl K5Cre mice

Frozen sections of knockout and wild type newborn mouse skin were processed for immunofluorescence and visualised by confocal microscopy. 2 A) and B): K10 (green), integrin α6 subunit (red), nuclei (blue). 2 C) and D): involucrin; dotted lines denote location of basement membrane. Note that in day two knockout and wild type skin the differentiation markers involucrin and keratin 10 show a similar staining intensity and spatial distribution. Scale bar: A-D: 50 μm

E) FACS analysis of involucrin in wild type and knockout mice: Keratinocytes were isolated from day two old animals and stained for involucrin. Gate M1 indicated keratinocytes with high expression of involucrin.
β1 null

wild type

K10

INV

E

<table>
<thead>
<tr>
<th>β1 null</th>
<th>wild type</th>
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![Histograms](E)
Figure 4.10. Cre recombinase is efficiently introduced into keratinocytes by adenoviral infection.

fl/fl keratinocytes were infected with 0 – 100 pfu/cell, fixed 48 hrs after infection and stained for lacZ expression. A: 0 pfu/cell; B: 1 pfu/cell; C: 10 pfu/cell; D: 100 pfu/cell.
FIGURE 4.11. Adenovirus has toxic side effects at the MOI needed for efficient downregulation of β1 integrin.

This is the result of a single experiment. The same results were obtained when the experiment was repeated three times. fl/fl keratinocytes and +/+ keratinocytes were infected with adenovirus. 120 hrs after infection cultures were fixed and stained with bromphenol blue (A) and for β1 integrin (B) to quantify its downregulation. Some of these cells were also used in a ‘Cell Death ELISA’ to test whether adenovirus induced apoptosis (C).
A

Co +Vi

MK

fl/fl

B

Counts

0 30 60 90 120 150

0 10^1 10^2 10^3 10^4

FL1-H

β1 integrin

-Ci

+Vi

2nd ab co

C

absorbance

1400 1200 1000 800 600 400 200

0

+Vi

-Vi
Figure 4.12. FACS analysis of β1 integrin.

fl/fl keratinocytes were retrovirally infected with pBabe EGFPcre. Note that the β1 integrin levels in the GFP positive cells was almost the same as in the GFP negative cells.
A, B: FACS profiles showing expression of EGFP (A) and EGFP and β1 integrin (B) in populations of fl/fl keratinocytes that have been infected with pBabe EGFPcre.
C) β1 integrin expression of GFP negative cells (region 5 (R5) in A).
D) β1 integrin expression, when gates are set for GFP positive (region 6 (R6) in A) cells.
DATA.002

DATA.002

DATA.002

Iox18
Figure 4.13. Immunofluorescence staining of fl/fl keratinocytes infected with EGFP Cre.

Immunofluorescence staining for β1 integrin (A), α6 integrin (B), paxillin (C), and E-cadherin (D).

Scale bar: 20 μm (A-D)
**β1 integrin**

**α6 integrin**

**paxillin**

**E-cadherin**
Figure 4.14. Downregulation of $\beta_1$ integrin after infection with EGFP-Cre.

FACSs profiles showing the timecourse of the downregulation of $\beta_1$ integrin in fl/fl keratinocytes after infection with LZRSpBMN EGFP-Cre. Note that the $\beta_1$ integrin negative cells disappear after a few days.
Figure 4.15. Effects of retroviral infection with EGFP-Cre on mouse keratinocytes.

A) Percentage of cornifin positive cells in cultures of passage 1 fl/fl keratinocytes that have been infected with LZRSpBMN EGFP-Cre. Cells were trypsinised on day five after infection, dried onto coverslips and stained for cornifin. The percentage of cornifin positive cells in EGFP positive and EGFP negative cells was determined. Error bars represent the standard deviation of the mean of four samples within one experiment.

B) Two colour FACS sort of fl/fl keratinocytes (cellline): GFP negative, β1 integrin positive (R1) and GFP positive, β1 integrin negative (R2) keratinocytes were sorted.

C) Equal amounts of sorted cells (the same as in B) have been plated on vitronectin (C, C'), fibronectin (D, D') and collagen I (E, E'). C, D, E, were β1 integrin positive (G1 in B), C', D', E' were β1 integrin negative (G2 in B). Cells were allowed to adhere overnight and then 'fixed and F-actin was stained using TRITC-phalloidin. Nuclei were visualised with DAPI. Scale bar: 20 μm (C-E, C'-E')
β1 integrin

% of cornifin positive cells

A

B
CHAPTER 4

Figure 4.16. FACS for β1 integrin.

On day five after infection cells that were infected in parallel to the keratinocytes used for the movie were stained with an antibody against β1 to monitor how many of the GFP positive keratinocytes were β1 integrin negative.

A) Forward and sideward scatter of mouse keratinocytes. Only cells within the gate were analysed.

B) Negative control for the β1 integrin antibody

C) GFP FACS profile of infected keratinocytes.

D) Dot plot showing a two colour analysis of the infected keratinocytes

E) β1 integrin expression in the uninfected keratinocytes (cells within region 2 (R2) in C were gated)

F) β1 integrin expression in the GFP positive keratinocytes (cells within region 3 (R3) in C were gated).
Figure 4.17. Phase photograph of fl/fl keratinocytes infected with EGFPCre.

Phase photograph of fl/fl keratinocytes infected with EGFPCre at t = 0 hours of the movie. In colour are the tracks of two EGFPCre infected (dark and light blue tracks) and uninfected (red and green tracks) keratinocytes. Cells were infected in 1 cm dishes with virus in the presence of polybrene for six hours. On day three after infection cells were plated onto 35 mm dishes. The next day (day four after infection) filming was started. Frames were taken in phase and fluorescence at five minutes intervals over a period of 22 hours.
Figure 4.18. Phase and fluorescence photographs of different frames.

Same experiment as Figure 4.17.

A) Phase photograph of fl/fl keratinocytes infected with EGFPCre. Phase pictures of frames at different timepoints. Arrows point at two EGFPCre infected (dark and light blue arrows) and uninfected (red and green arrows) keratinocytes.

B) Fluorescence photographs which where taken in parallel to the phase pictures.
Figure 4.19. Chick β1 integrin expression in fl/fl keratinocytes.

FACS profile of fl/fl keratinocytes expressing chick wild type β1 integrin (blue).
The second antibody control is shown in red.
Figure 4.20. Downregulation of mouse $\beta_1$ integrin after infection with EGFPcre in fl/fl and fl/fl + chick $\beta_1$ integrin keratinocytes

Flow cytometry demonstrates that the $\beta_1$ integrin subunit is missing in 50% of EGFP positive keratinocytes. Some of these cells were used in the movie in Figure 4.21.

A) GFP and second antibody negative control.
B) B1 integrin positive/ GFP negative control.
C), D) Two colour FACS analysis of EGFPcre infected fl/fl keratinocytes (C) and of fl/fl keratinocytes that express chick $\beta_1$ integrin (D).
Figure 4.21. Chick β1 integrin transduced keratinocytes remain spread after infection with EGFP Cre retrovirus.

fl/fl keratinocytes were infected with an EGFP Cre expressing retrovirus and examined five days later (see also Figure 4.20). Overlay of phase and fluorescence pictures of fl/fl (A) and fl/fl + chick β1 integrin (B) at t = 0 hours of a movie that was taken over a period of 24 hours.
CHAPTER 5: DISCUSSION

In this thesis I have described my findings on the role of β1 integrins in epidermal development in vitro using the differentiation of ES cells as a model system. I found that the failure of β1 null ES cells to differentiate into keratinocytes can be overcome by dermal fibroblast derived growth factors. I also described the effects of the removal of β1 integrins on keratinocytes in vitro in an attempt to investigate the role of β1 integrins in epidermal homeostasis. In this chapter I will suggest some experiments to address the questions that have remained unanswered.

5.1. DERMAL FIBROBLAST - DERIVED GROWTH FACTORS RESTORE THE ABILITY OF β1 INTEGRIN DEFICIENT ES CELLS TO DIFFERENTIATE INTO KERATINOCYTES

Evidence was presented that factors produced by mesenchymal cells could induce β1 null ES cells to differentiate into keratinocytes in vitro. Normally, β1 null ES cells do not differentiate into complex epithelial cells (Bagutti et al., 1996). I have shown that the failure of β1 null keratinocytes is at least to a certain extent due to a reduced sensitivity to soluble growth factors and that specific factors, particularly FGF7 and FGF10, enhance differentiation as monitored by keratin 14 expression.

An important question that needs to be answered next is by which mechanism the mesenchyme-derived growth factors enhance differentiation of ES cells. Is the expression of the growth factor receptors decreased on the β1 null cells? Do β1 and growth factor receptor signalling pathways converge? If yes, at which point(s) of the signalling cascade?
It would be interesting to examine whether ES cells that lack the receptors for some of the growth factors we have tested show impaired epidermal differentiation. As already mentioned in Chapter 3, ES cells with a dominant negative FGFR have a defect in epithelial differentiation, which can be partially rescued by extracellular matrix components (Li et al., 2001). The dominant negative FGFR mutation has a broader effect than mutations of individual FGFR isotypes (Li et al., 2001) and to further investigate which signalling pathway is involved in epidermal differentiation, it would be useful to analyse ES cells from knockout mice. Another possibility would be to introduce activated molecules that are downstream of either β1 or the FGF receptor (for example activated p42/p44 MAPK; (Zhu et al., 1999) into the β1 null ES cells and then to test whether they can influence the fate of β1 null ES cells when they are induced to differentiate. However, since these signalling cascades are activated by a series of other receptors and their activation is spatially and temporally tightly regulated, the results obtained by expressing a constitutive activated protein need to be interpreted with caution.

It would be very difficult to address growth factor responsiveness using a biochemical approach in the in vitro differentiation system. Using the cells that grow out from the embryoid bodies is not feasible since these populations are heterogeneous and cells from different lineages may respond differently to any particular growth factor.

An approach to investigate the role of β1 integrin in epidermal development in vivo could be undertaken by analysing mice with a floxed β1 integrin gene. Crossing fl/fl β1 mice with the K5Cre and K14 Cre transgenics (Brakebusch et al., 2000; Raghavan et al., 2000) did not address this question. Both keratins are detected in a few cells from E9.5, but only from day E14.4 onwards they can be found in all cells of the basal layer (Fuchs and Byrne, 1994). At this stage a multilayered epidermis has already formed. In addition, it seems that Cre has to accumulate until it efficiently recombines the floxed sites of the β1 integrins and for this reason, some of the K5Cre and K14 Cre mice that have been described to be still mosaic for
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General Discussion

the β1 integrin deletion at time of birth (Brakebusch et al., 2000; Raghavan et al., 2000). To analyse the role of β1 integrin in epidermal development, one would therefore have to cross mice with a floxed β1 integrin gene with mice that have Cre recombinase under the control of a promoter which is expressed in simple epithelial cells before the development of the multilayered epidermis. One candidate promoter would be the keratin 18 promoter. Keratin 18 expression is found very early, at the 4- to 8- stage of development (Oshima et al., 1983). However, since this keratin is expressed in many simple epithelial cell types (Kulesh and Oshima, 1988), these mice might die due to defects in other organs before a multilayered epidermis has developed.

5.2. ADHESION, SPREADING, AND MOTILITY OF β1 NULL KERATINOCYTES IS SEVERELY COMPROMISED

β1 null keratinocytes were not able to spread, could not organise their actin stress fibres and displayed a defect in focal adhesion formation. Furthermore, β1 null keratinocytes could not migrate. The small GTPases Rho, Rac and Cdc42 are involved in many of these processes and can be activated by integrin mediated adhesion (Takai et al., 2001). It therefore seems likely that the β1 null keratinocytes have a defect in the activation of these small GTPases. If their activity is indeed lower in the β1 deficient keratinocytes one could try to rescue their phenotype by retrovirally expressing activated Rho and Rac in fl/fl keratinocytes before deleting the β1 integrin by retroviral infection with Cre. However, these experiments are technically difficult since mouse keratinocytes are not easy to infect. In addition, similar experiments have already been performed in cells that expressed a dominant negative β1 integrin (Berrier et al., 2000). In these cells activated R-Ras, Rac1, PI 3-kinase and PKCɛ could rescue cell spreading, but these signalling proteins required intact β1 cytoplasmic domains on the integrins mediating adhesion to restore cell spreading. A question that remains to be answered is how the β1 null cells were able to stick at all. Because of technical problems I was not able to perform short term (1 –2 hours) adhesion
assays in the presence of cycloheximide. It is therefore possible that an altered production of extracellular matrix proteins by β1 null keratinocytes and hence different ECM surface between wild type and β1 null cells accounts for some of the differences in cell adhesion.

Another factor that needs to be taken into consideration is that I did all my adhesion assays in the presence of serum. It would be important to try to perform an adhesion assay under serum free conditions or to remove the fibronectin by affinity chromatography on gelatin sepharose.

In a study that determined the effects of the loss of β1 integrins in vitro and during wound healing (A crucial role of β1 integrins for keratinocyte migration in vitro and during cutaneous wound repair. Grose, R., Hutter, C., Bloch W., Thorey, I., Watt, F., Fassler R., Brakebusch C., and Werner S.; submitted for publication) it was shown that in vivo the loss of β1 integrins caused a severe defect in wound healing. In support of the in vitro findings described in this thesis, epithelial migration during wound repair in fl/fl K5Cre mice was delayed dramatically. Interestingly, although in fl/fl K5Cre mice the onset of keratinocyte migration was severely retarded in comparison to wild type mice, the keratinocytes could finally migrate over the wound bed, suggesting that compensatory mechanisms take place. Such mechanisms are probably based on the expression of non-β1 integrins by keratinocytes and on the presence of the corresponding ligands.

It would be interesting to investigate whether adding of inflammatory cytokines could induce migration in the β1 null keratinocytes also in vitro.

5.3. β1 NULL KERATINOCYTES HAVE A LOWER PROLIFERATION RATE THAN WILD TYPE KERATINOCYTES AND UNDERGO TERMINAL DIFFERENTIATION IN VITRO

I could show that in vitro mouse keratinocytes undergo terminal differentiation upon removal of β1 integrin. These findings agree with earlier work on human keratinocytes isolated from interfollicular epidermis that suggest that β1 integrins are important to keep these cells in the stem cell compartment (Adams and Watt, 1989; Jones et al., 1995; Jones and Watt, 1993).
CHAPTER 5 ___________________________ General Discussion

There exist no molecular markers for mouse epidermal stem cells, therefore the criterion for identifying stem cells in the skin is to analyse the replicative behaviour of a cell: \textit{in vivo} the label retaining cells are believed to identify stem cells and the corresponding cells \textit{in vitro} are those with the clonogenic potential (Oshima et al., 2001). Clonogenicity assays are not possible with mouse keratinocytes (Oshima et al., 2001), but since \( \beta_1 \) null keratinocytes differentiate upon placing them in culture, it seems likely that also in mouse keratinocytes the level of integrin correlates with proliferative potential \textit{in vitro}. Indeed, the BrdU incorporation of \( \beta_1 \) null cells is both \textit{in vitro} and \textit{in vivo} lower than in wild type cells (Brakebusch et al., 2000; Raghavan et al. 2000; Chapter 4), the proliferation rate of the cultured \( \beta_1 \) deficient keratinocytes being almost halved. However, the expression of the differentiation markers involucrin, cornifin and keratin 10 did not seem to change (Brakebusch et al., 2000; Raghavan et al. 2000; my own observation). This raises the question how relevant the \textit{in vitro} observations are. The fact that wild type keratinocytes do not induce involucrin within 24 hours when they are plated in conditions where they are not able to spread is an indication that the induction of differentiation in \( \beta_1 \) null keratinocytes is due to the lack of \( \beta_1 \) integrin and is not an unspecific response because the cells cannot spread. The difference between the \textit{in vitro} and \textit{in vivo} results could reflect the lack of certain growth factors and a proper basement membrane and intercellular adhesion in culture.


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