An Investigation of Keratin 15 Function by Small Interfering Ribonucleic Acid Technology

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An Investigation of Keratin 15 Function by Small Interfering Ribonucleic Acid Technology

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Declarations

I declare that the experiments described in this thesis were carried out by myself (Miss Cheryl Lancaster) in the laboratory of my supervisor (Dr Arto Määttä) in the School of Biological and Biomedical Sciences, Durham University. This thesis has been composed by myself and is a record of work that has not been previously submitted for a higher degree.

Miss Cheryl Lancaster

I certify that the work reported in this thesis has been carried out by Miss Cheryl Lancaster, who, during the period of study, has fulfilled the conditions of the Ordinance and Regulations governing the Degree of Doctor of Philosophy.

Dr Arto Määttä

Abstract

The research presented in this thesis endeavours to further understanding of the cytoskeletal protein keratin 15 (K15), and the role this protein has on maintaining the stem cell niche. This study has focused on studying the role of K15 by using small interfering ribonucleic acid (siRNA) to knock-down K15 in different cell lines (HaCaT cells, MET 1, 2 and 4 cells and normal human epidermal keratinocytes). To observe the effect of K15 on stem cells, adhesion, migration, differentiation and proliferation were assessed following K15 knock-down by siRNA.

Although cell adhesion was not affected by K15 knock-down, cell spreading and morphology was affected. K15 knock-down cells spread more quickly than their control cells, and cells were larger following K15 ablation. Cell migration was studied using the scratch wound assay. Cells without K15 were less motile than K15-positive cells. Involucrin expression was observed as an indicator of differentiation. Following K15 knock-down, involucrin expression increased, indicating differentiation. Differentiation was assessed using the calcium switch assay, where higher levels of involucrin were observed in K15 siRNA transfected cells after only 6 hours in high calcium media. Cell proliferation was measured using the MTT assay, and K15 knock-down cells were shown to proliferate to a greater extent than control cells.

Tissue sections were also probed for K15 and CD34. K15 was observed in the basal layers of the epidermis and around the hair follicle in rat, mouse and human adult skin. This study also observed a CD34-positive/K15-positive cell population and a CD34-negative/K15-positive population in adult human interfollicular epidermis. For comparison between 2D cell culture and tissue sections, 3D (organotypic) cultures were utilised. Variable K15 expression was observed in the squamous cell carcnoma lines MET 1, MET 2 and MET 4. In HaCaT cells, K15 was observed to a greater extent at the base of the culture, similar to that observed in the epidermis.

K15 ablation has been shown to affect cell spreading and morphology during adhesion, cell migration, differentiation and proliferation. These results suggest that K15 does impact on the stem cell nature of keratinocytes, although the mechanisms require further investigation.

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CHAPTER 1:
INTRODUCTION

1: Introduction

1.1: The Structure and Development of the Epidermis

The epidermis consists of several layers of stratified epithelium, and basement membrane. From the innermost layer to the outermost layer, the epidermis comprises the basal layer, spinous layer, granular layer and the stratum corneum. Under normal conditions, cells divide by mitosis at the basal layer and move upwards towards the skin surface (for further details, see section 1.1.1). As the cells move towards the surface, they change in size and protein expression profile, eventually becoming keratinised and forming the hydrophobic outermost layer of the skin; this process takes around thirty days.

There are several populations of epidermal stem cell, important in maintaining the skin and hair follicle. Populations of epidermal stem cells have been identified in the bulge region of the hair follicle, the interfollicular epidermis (IFE) and the sebaceous glands (Braun *et al*., 2003; Tiede *et al*., 2007; Kaur, 2006; Bieniek *et al*., 2007, Abbas and Mahalingam, 2009). These niches are thought to be conserved through specific ectopic protein expression, although most of these proteins have yet to be definitely identified. Two keratins have been investigated as potential stem cell markers: K15 and K19. K15 and K19 are observed in the human and mouse bulge (Michel *et al*., 1996; Lyle *et al*., 1998; Braun *et al*., 2003; Kloepper *et al*., 2008; Hoang *et al*., 2009) and the basal layer of the epidermis in human skin (Michel *et al*., 1996; Kloepper *et al*., 2008). K15 is considered important as it may be an early marker of differentiation, expressed prior to the fate of the keratinocyte being decided (i.e. either hair-like or epidermal) (Porter *et al*., 2000; Abbas and Mahalingam, 2009). K19 may be expressed following commitment to an epidermal fate (Morris, 2004; Kloepper *et al*., 2008; Abbas and Mahalingam 2009).

Ghazizadeh and Taichman (2001) used label-retaining studies to show that stem cells resided in the basal layer, the sebaceaous gland and the hair, an observation suggesting that distinct stem cell populations exist, each able to produce daughter cells capable of differentiating along each of the epidermal lineages. Differentiation of these cells however usually occurs down a restricted lineage, in response to signals from the local environment (Niemann and Watt, 2002). It is also possible for these cells to differentiate down a different lineage, in response to wounding, for example. Wherever these stem cells are, it is likely that they are related (Ghazizadeh and Taichman, 2001).

1.1.1: Skin Development

The epidermis stratifies during embryogenesis, forming as a result of a complex 'stratification program' (Koster and Roop, 2007). The epidermis forms from the ectoderm; in mice this occurs during E8.5-18.5, requiring (as yet unknown) signals from the underlying dermis, although some studies suggest that β-catenin expression is involved in this process (for example, Pearton *et al*., 2005), as are bone morphogenic protein (BMP) and Wnt, members of the developmental signaling pathways (Lefort and Dotto, 2004). A change in the keratin expression pattern also occurs as cells move towards the outermost layers of the skin; this increases the strength of the cell at a cost to motility (Fuchs and Cleveland, 1998). The ectoderm expresses the cytoskeletal proteins keratin (K)8 and K18, whereas cells with an epidermal fate express K5 and K14 (see section 1.2.4). Once terminally differentiated, the cells express K1 and K10; the transcription factor p63 is specific to this lineage (Green *et al*., 2003), and is necessary for K5 and K14 expression, particularly ΔNp63α (an isoform lacking the 'p53-like N-terminal transactivation [TA] domain') (Parsa *et al*., 1999; Yang *et al*., 1998).

The mature epidermis can be considered to consist of four stages of differentiation (Fuchs, 1990). The first layer to form is the basal layer, which, via asymmetric cell division, gives rise to the second layer: the periderm (this layer is shed before birth, and so can only be seen in embryos). Basal cells have contact with the mature basement membrane, and express K5 and K14. Unlike other keratinocytes, the specialised epithelial cells are responsible for cohesion, barrier function and renewal (Nelson and Sun, 1983). An intermediate layer (expressing K1) can then form between the first two layers (Lechler and Fuchs, 2005); the only known transcription factor required for the production of the intermediate layer is ΔNp63α (Nguyen *et al*., 2006). It is this intermediate layer that develops spinous cells (the cells that eventually become granular and cornified cells), again via asymmetric division of basal cells detached from the basement membrane. The intermediate layer is eventually replaced by these cells (Smart, 1970). Spinous cells also express K1 and K10, considered to be two of the earliest markers of terminal differentiation (Fuchs and Green, 1980). Granular cells express filaggrin, and the cornified layer precursors involucrin and loricrin, and transglutaminase, the enzyme responsible for crosslinking the cornified envelope components (Dale *et al*., 1985). In human skin, the intermediate layer undergoes apoptosis, required for the development of the mature basal layer and further stratification (Haake and Cooklis, 1997). In mice the cells of the intermediate layer become the spinous cells, and no cells are lost through apoptosis (Weiss and Zelickson, 1975); this development begins at the dorsal surface, and spreads towards the ventral surface, and takes around 24 hours *in utero* (Hardman *et al*., 1998). The last stage of differentiation is the dissolution of organelles and nucleus, to form the cornified envelope (the cells are then metabolically inert) (Tomic-Canic *et al*., 1998).

It is important for stratification that premature terminal differentiation of cells from the basal layer is precluded. This is achieved by repressing expression of genes that would allow or cause differentiation (Koster and Roop, 2007).

As previously mentioned, in the mature epidermis, no intermediate layer exists; instead, the keratinocytes of the basal layer differentiate directly into the (post-mitotic) spinous cells. This appears to be controlled by the Notch pathway (Nickeloff *et al*., 2002). A further trigger of differentiation (*in utero* and beyond) is a calcium ion (Ca^{2+}) gradient (which increases with maturity). An increase in extracellular Ca^{2+} concentration is also involved in forming the epidermal barrier, spinous layer and granular layer (Yuspa *et al* 1989; Koster and Roop, 2007).

The last step in the production of mature skin is barrier formation. Several transcription factors have been implicated in this phase of development; one of the most studied is Klf4, which is expressed in the upper spinous and granular layers. Genetically altered mice which lack this transcription factor ($Klf4^{-/-}$) display normal spinous development, but poor cornifed envelope formation (causing barrier defects). Application of ectopic Klf4 expression is shown to accelerate barrier formation. Further work has shown that the transcription factors Grhl3 and Get1 are also involved, but mostly via cell adhesion and lipid metabolism routes, and Grhl3-deficient mice display altered filaggrin, loricrin and involucrin expression. The skin barrier requires the formation of tight cell-cell junctions to help prevent water loss (as observed in claudin-1-deficient mice). The cornified envelope cells also produce lamellar granules filled with lipids. These lipids are extruded into the cornified envelope protein scaffold, producing a hydrophobic (and therefore waterproof) layer, preventing unregulated fluid escape or permeation (Kalinin *et al*., 2002).

After this stage, cells stop metabolic and transcriptional activity and undergo a process similar to a type of programmed cell death (Gandarillas, 2000). These cell

remnants are eventually sloughed from the surface of the skin, being replaced by differentiating cells moving upwards from the basal layer: a process that takes between 10 and 14 days (Potten, 1974).

1.1.1.1: Calcium Gradient-Driven Differentiation

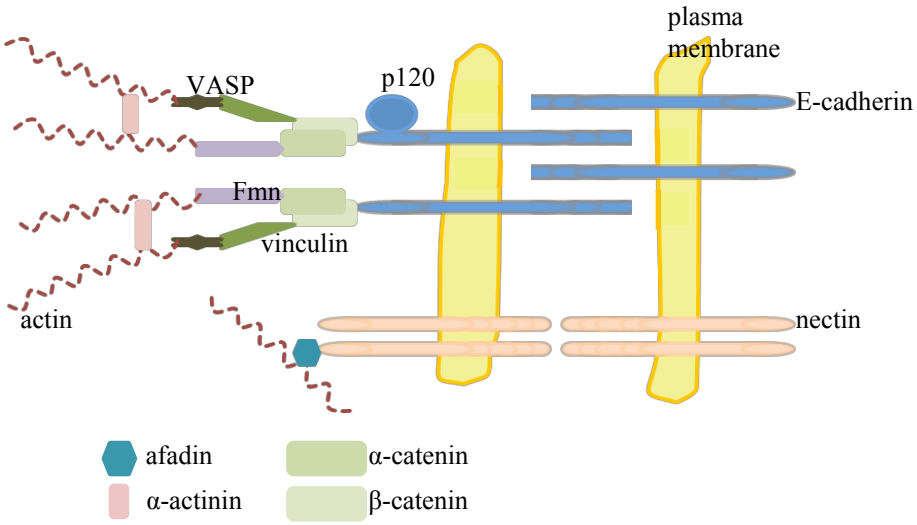
A change in calcium concentration has been observed between the basal layer (low calcium – up to 0.5mM) and the granular layer (high calcium – from 1mM) *in vivo*, influencing a change in keratin expression from K14 and K5 in the basal layers to K1 and K10 in the granular layers (Yuspa *et al*., 1989). Any increase in extracellular calcium concentration is followed by increases in both diacylglycerol and phosphatidylinositol metabolism via protein kinase C (PKC) (Lee and Yuspa, 1991). PKCα is specific to keratinocytes, and is responsive to changes in Ca^{2+} concentration (Eckert *et al*., 2004). PKCη appears in spinous cells during their transition to granular cells (coinciding with a decrease in both K1 and K10, and increases in loricrin, filaggrin and transglutaminase) (Eckert *et al*., 2004). Ca^{2+}-independent PKCη can also induce differentiation in granular cells; these cells however do have Ca^{2+}-sensing receptors, part of the Ca^{2+} binding protein family (Bikle *et al*., 1996). Interestingly, mice lack this receptor, and reduced amounts of both filaggrin and loricrin have been found in this animal compared to humans. Increased extracellular calcium has been shown to activate the phospholipase C pathway (via a calcium receptor), which ultimately results in a rise in intracellular inositol triphosphate (IP_3) and a rise in intracellular calcium. This intracellular calcium activates chloride channels in the cell membrane, resulting in hyperpolarisation; voltage-independent cation channels also become permeable (also mediated by a calcium receptor), allowing calcium ion movement. These actions combined produce a swift rise in intracellular calcium, followed by a prolonged increase (Bickle *et al*., 1996). Bickle *et al*. (1996) have also demonstrated that undifferentiated cells are more sensitive to extracellular calcium than their more differentiated counterparts. Extracellular calcium also affects other mechanisms important in differentiation. Epidermal cadherins, for example, are calcium dependent, their extracellular domain containing calcium ion binding sites, where Ca^{2+}-binding is necessary for cell-cell adhesion (important in keratinocyte differentiation) (Lefort and Dotto, 2004).

In vitro, monolayers of cells cultured in low levels of extracellular calcium (<0.1mM) can be induced to differentiate with the addition of media containing 1-2mM Ca^{2+}. This differentiation can be seen in changes to protein markers of differentiation; for example, changes in the keratin expression pattern and other markers, such as involucrin (Ponec *et al*., 1988). (Further examples of the use of calcium to induce differentiation *in vitro* are given in section 3.1.4.)

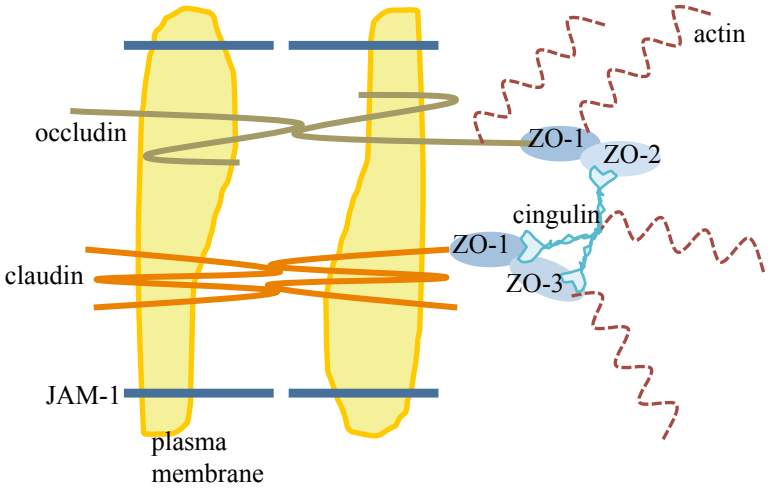
1.1.1.2: Selected Markers of Differentiation

Cell-Cell Junctional Proteins

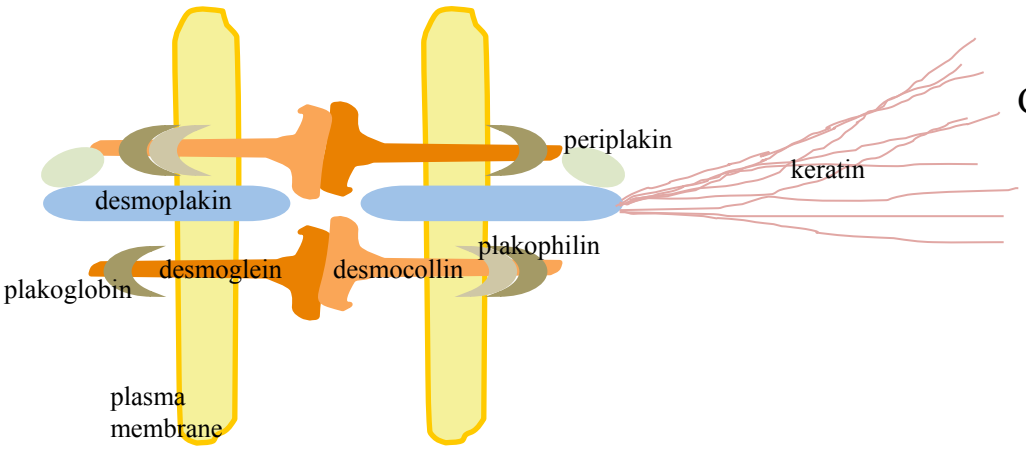
There are several types of cell-cell junctions which form at various stages of differentiation (*Figure 1.1*). The specific proteins associated with each type of junction can therefore be used to identify differentiation states. For example, desmosomes have two roles in keratinocytes: to participate in growth, differentiation and motility, and as an anchor for scaffold structures, such as the keratin network



A Adherens Junction



B Tight Junction



C Desmosome

Figure 1.1: Schematic diagrams of cell-cell junctions in epithelial cells

Adherens junctions (A) join adjacent cells via actin filaments, through transmembrane cadherins. E-cadherin is the direct binding partner of β-catenin, which binds to several other proteins, including α-catenin. This complex then binds to the actin cytoskeleton.

Tight junctions tightly join cells between the apical and basolateral membrane regions (B). Zonular occludens proteins 1, 2 and 3 (ZO-1, ZO-2 and ZO-3) form complexes with actin, whilst claudins and occludins mediate binding of the membranes.

Desmosomes (C) contain several specialised proteins, including the plakins (such as plectin, periplakin, envoplakin), the desmogleins and desmocollins and armadillo proteins (plakophilin and plakoglobin), which anchor intermediate filaments between cells.

(Allen, Yu and Fuchs, 1996; Hatzfeld, 2007). Hemidesmosomes are also linked to the keratin network, and form between epithelial cells and the underlying basement membrane, in a region known as the basement membrane zone' (BMZ) (Hashmi and Marinkovich, 2011). Interactions between the extracellular proteins and adjacent keratinocytes in this region help regulate apoptosis, proliferation, adhesion, migration and differentiation (Miner and Yurchenco, 2004). Hemidesmosomes consist of four transmembrane proteins (pemphigoid antigen, CD151 and the two subunits of α6β4 integrin) (Sterk *et al.*, 2000). The α6β4 integrin interacts with laminin-332 in the BMZ (Tsuruta *et al*., 2008) and the keratin cytoskeleton (Jones, Hopkinson and Goldfinger, 1998). The latter is mediated by plectin, important in stabilising the hemidesmosome (Andrä *et al*., 1997). Desmoplakin is the largest of the desmosomal proteins, which form oligomers with keratins which translocate to the cell borders (Godsel *et al*., 2005; Al-Amoudi *et al*., 2011). Schoop *et al*. (1999) and Laprise *et al*. (2004) have demonstrated that desmosomes (and their constitutive protein expression levels) may directly correlate with keratinocyte differentiation and proliferation.
Adherens junctions are important in maintaining the structural integrity of tissues (Lewis *et al*., 1994), and E-cadherin is the major cadherin in the human epidermis (Hung *et al*., 2006). Like desmoplakin, E-cadherin layers, has been shown to link to the keratin network (Koch and Franke, 1994; Szegedi *et al*., 2008) and actin via α- and β–catenin (Kippenberger *et al*., 2005a).
Zonula occludens protein-1 (ZO-1) is a protein found in tight junctions, located in the granular layer of the epithlium. Other tight junction proteins include occludin, ZO-2, ZO-3, cingulin, 7H6, symplekin, claudin-1 and claudin-4 (Stevenson and Keon, 1998; Brandner *et al*., 2002; Peltonen *et al*., 2007). Tight junctions are important in forming a permeable barrier regulating movement of solutes, water and immune cells in simple epithelia (Stevenson and Keon, 1998), and connecting to the actin cytoskeleton (Tsukita, Furuse and Itoh 1999). ZO-1 is a member of the membrane-associated guanylate kinase homologue (MAGUK) protein family, and is thought to contribute to correct protein organisation of the tight junction plaque (Stevenson and Keon, 1998; González-Mariscal, Beranzos and Avila-Flores 2000).

Involucrin
Involucrin was first described by Rice and Green in 1979, who identified an 83kDa protein which was incorporated into the cornified envelope. Involucrin is expressed in differentiating keratinocytes and, as a precursor of the cross-linked envelope, is a marker of early differentiation (Watt, 1983; Crish *et al*., 2002). This envelope consists of several proteins, covalently cross-linked into a submembranous structure (Rice and Green, 1979). Involucrin is expressed after the cell has left the basal layer and before envelope cross-linking in the spinous and granular layers; this is at the same time as transglutaminase expression occurs, which is required for cornified envelope formation (Eckert *et al*., 1993; Tong *et al*., 2006). Involucrin (alongside filaggrin and loricrin) serves as a calcium-dependent substrate for transglutaminase 1, also important in catalysing cornified envelope formation (Schroder *et al*., 1992; LaCelle *et al*., 1998; reviewed by Hitomi, 2005). When synthesised, involucrin is localised next to the interior face of the plasma membrane, then cross-linked to other proteins by transglutaminase 1 (Thacher and Rice, 1985).
In vivo, as keratinocytes differentiate, the cells of the granular and spinous layers become increasingly permeable to calcium (Rice and Green, 1979). Increased levels of intracellular calcium affect PKC, in turn increasing involucrin and transglutaminase 1 expression. As described later (section 3.1.4), PKC has a role in

differentiation, and has been shown to affect involucrin gene expression by inducing its promoter activity (Papp *et al*., 2003; Eckert *et al*., 2004). Other protein kinases have also been shown to increase involucrin expression, including cAMP-dependent protein kinase A (PKA) and cGMP-dependent protein kinase G (PKG) (Paramio and Jorcano, 1997). Increased cAMP levels were also shown to increase involucrin levels in HaCaT cells by Mammone *et al*. (1998) and Kawabata *et al*. (2002), along with other differentiation markers including K1, K10 and transglutaminase.

Filaggrin

Filaggrin was first purified in 1977, referred to as the stratum corneum basic protein (Dale, 1977), and is named as a shortened version of 'filament aggregating protein'.

In mouse keratinocytes cultured in high calcium media (1.2mM), filaggrin synthesis occurred (Dale *et al*., 1983). Filaggrin is synthesised as a histidine-rich, phosphorylated polyprotein precursor, which is processed into 35kDa filaggrin subunits by the activity of phosphatase and proteolysis during terminal differentiation (Steven *et al*., 1990; McGrath, 2008). The human precursor is comprised of 10-12 filaggrin subunits, separated by linker peptides; this is known as profilaggrin (Presland *et al*., 1992; McGrath, 2008). Filaggrin does not appear *in vivo* any lower than where keratohyalin granules occur (i.e. in the granular layer). More filaggrin is observed throughout the stratum corneum, where it is incorporated into the matrix (Dale and Ling 1979; Stevens *et al*., 1990). As previously mentioned, transglutaminase is required to cross-link filaggrin with loricrin and involucrin (Thacher and Rice, 1985). Eventually, in the upper stratum corneum, filaggrin is degraded into free amino acids, which helps to maintain epidermal hydration (Scott, Harding and Barrett, 1982). The influence of filaggrin as a natural moisturiser is evident, as individuals with 10 filaggrin repeats in profilaggrin have dryer skin than those with 12 repeats (McGrath, 2008).

Recently, siRNA transfection methods have been used to knock-down filaggrin expression in organotypic skin models (Mildner *et al*., 2010). K1, K2 and K10 expression and organisation was normal, and a generally normal stratum corneum morphology was observed. However, keratohyalin granule numbers were reduced and lamellar body formation was disrupted. Lack of filaggrin also reduced the urocanic acid concentration, making the organotypic skin more susceptible to UVB-induced apoptosis. Mildner *et al*. (2010) suggest that their results also question the role of filaggrin as an IF aggregator whist establishing a previously unknown link between filaggrin and UVB protection.

1.1.2: Hair Follicle Structure and Development

Hair follicle formation takes place during skin development in the foetus. Mesoderm-derived cells develop into the connective tissue hair follicle sheath and dermal papilla whilst ectodermal hair follicle stem cells produce all the epithelial components of the hair follicle (Schneider, Schmidt-Ulrich and Paus, 2009). The hair shaft is a product of the hair follicle, a complex 'miniorgan' of the skin, made up of the sebaceous gland, the arrector pili muscle, the apocrine gland and the pliosebaceous unit (with associated structures) (Schneider, Schmidt-Ulrich and Paus, 2009). The hair shaft consists of compacted, fibrous, terminally differentiated keratinocytes (trichocytes). The hair itself grows from a group of cells at the base of the hair follicle, called the bulb, and is replaced with each cycle of growth. The follicle cycles, as hair growth occurs and ceases. At this resting stage (telogen), the lower follicle involutes and shortens, before regenerating to form a new hair (Morris *et al*., 2004). During normal cycling in the hair follicle, the different regions of the follicle change in both form

and function. In catagen, the upper third of the follicle remains whole (along with the mesenchymal cells of the dermal papilla), as the keratinocytes of the lower follicle are destroyed. It is the upper portion that contains the stem cells.

Stem cells of the hair follicle reside in a niche known as the 'bulge' (Cotsarelis *et al*., 1990), which have been identified by activity of the promoter of the keratin 15 gene (Liu *et al*., 2003). The bulge can be identified as the point of insertion of the arrector pili muscle (Cotsarelis *et al*., 1990). Oshima *et al*. (2001) generated chimeric follicles in mice by replacing the wild-type hair follicles with those from Rosa 26 transgenic mice (which express a *lacZ* reporter gene under control of the SV40 promoter). The Rosa 26 cells were found to contribute to all the lineages of the hair follicle: the outer root sheath, the inner root sheath and the hair shaft. The progeny of the transplanted cells also contributed to the interfollicular epidermis and the sebaceous gland. The bulge cells migrated to the base of the follicle during hair growth.

It is not yet known conclusively whether the bulge cells contribute directly to hair regeneration or not (Panteleyev, Jahoda and Christiano, 2001; Lavker *et al*., 2003; Myung *et al*., 2009); Morris *et al*. attempted to address this in their 2004 study by producing a murine mutant, which showed as *lacZ*-positive if the *KRT15* promoter was active. The *lacZ*-positive cells were found in the bulge, in the lower region of the hair follicle and the hair shaft, demonstrating that bulge cells are at least partly responsible for regeneration of the follicles. Label-retaining studies in mice also showed that cells from the bulge migrated towards the bulb of the follicle to a far greater extent than those which migrate upwards towards the surface (Braun *et al*., 2003) (see section 1.4.2.4). The same study allowed murine follicles to be observed over several time-points up to 42 days after birth, showing differences in different stages of hair follicle growth. For example, anagen follicles at PN35 appeared longer and had more obvious bulges than those at PN28 (early anagen). At PN42, the follicles were in telogen and so were very short; the label-retaining cells were observed extending from the follicle base to just below the sebaceous gland. During this time however, no significant migration occurred during anagen, and no significant depletion of cell number was observed. In another study, to determine whether the cells of the bulge region were necessary for epidermal renewal, Ito *et al*. (2005) targeted the *KRT15* promoter with a suicide gene encoding the herpes simplex virus thymidine kinase (HSV-*TK*). This demonstrated that ablation of the bulge cells led to survival of the epidermis, but not the hair follicle, which was completely lost. This study then demonstrated that the cells of the bulge region were required for maintenance of the hair follicle, but not the epidermis. However, bulge cells were recruited into the epidermis following epidermal injury. After several weeks, most of the bulge cells were again eliminated from the epidermis.

More recently, Romano *et al*. (2010) have demonstrated that in transgenic mice expressing ΔNp63α, the hair follicle stem cell niche becomes depleted. In normal mouse skin, ΔNp63α is expressed in the bulge (Rendl, Lewis and Fuchs, 2005), where it is thought it maintains the niche by balancing proliferation and differentiation of cells (Romano *et al*., 2010). In mice overexpressing ΔNp63α, K15, Sox9 and S100-A6 expression are all lost, suggesting a depletion of the stem cell niche. At PN16 in transgenic mice, hypertrophy of the follicles was observed and the hair shafts had been replaced by keratinised tissue. This suggests that the keratinocytes from the hair follicle had switched to an interfollicular epidermal fate under the influence of ΔNp63α, affecting hair follicle development and later, cycling (Romano *et al*., 2010). Examination of the hair follicles in the transgenic mice revealed a defect in differentiation, confirmed by lack of Gata3 expression

(previously described by Kaufman *et al*., 2003). Levels of β-catenin, also demonstrated to be necessary for hair follicle formation (Andl *et al*., 2002), were reduced in the transgenic mice, further suggesting impeded differentiation of hair follicle cells (Romano *et al*., 2010). Ki67 immunofluorescence also suggested loss of proliferation potential. ΔNp63α has previously been shown to induce K1 expression (Ogawa *et al*., 2008), and Romano *et al*. (2010) demonstrated that overexpression of ΔNp63α led to K5 and K14 expression throughout the epidermis and increased K1/10 expression. Loricrin expression was reduced, suggesting a decrease in the number of terminally differentiated cells. Filaggrin and involucrin levels however increased, which Romano *et al*. suggest could be due to compensatory mechanisms and/or direct transcriptional effects of the overexpressed ΔNp63α. Western blotting results suggested that the basal and spinous layers of the epidermis expanded in mice overexpressing ΔNp63α.

1.2: The Cytoskeleton

The cytoskeleton has played an important role in the evolution of multicellular organisms, allowing cell compartmentalisation, specialisation, attachment, movement and division (Fuchs and Karakesisoglou, 2001; Fletcher and Mullins, 2010). The cytoskeleton is maintained through regulated self-assembly, depending on physical constraints, such as size of the cell. The architecture of the cytoskeleton is also capable of controlling some physical properties of the cell (such as size, rigidity, adhesions and shape), as well as maintaining links with the cell's external environment (for example, Jamora and Fuchs, 2002). The ability to attach to a surface, such as to extracellular matrix, allows cells to spread, which increases the opportunity for cells to attach to each other. Cytoskeletal networks are dynamic – whilst resisting deformation, they are also capable of reorganisation (e.g. during cell division and in cells subjected to external stress). This is important in maintaining the integrity of the cell and intracellular compartments and location of organelles (for example, Hershberg, 2002). Cytoskeletal proteins also provide a scaffold for receptor proteins, protein kinases, motor proteins and signal transduction (Gniadecki, Olszewska and Gajkowska, 2001; Lund *et al*., 2010).

The cytoskeleton is made up of three major filament types: microtubules, actin filaments (or 'microfilaments') and intermediate filaments (IFs) (*Figure 1.2*). What differs between them is their polarities, the associated proteins, their mechanical stiffness and their assembly dynamics (for example, Heidemann *et al*., 1999; Kasas *et al*., 2005; Parsons, Horwitz and Schwartz, 2010). Actin filaments and microtubules, for example, can polymerise and depolymerise to produce forces capable of changing cell shape and guiding the formation of intercellular compartments. Several other classes of protein also aid in producing the complex cytoskeletal networks, including depolymerising and severing factors (which disassemble filaments), polymerases (to aid in filament growth), stablilising proteins and crosslinkers (to reinforce network structures once they are organised) and capping proteins (to terminate filament growth) (see section 1.2.3).

1.2.1: Microfilaments

Microfilaments comprise actin polymers (F-actin), actin-binding proteins and associated proteins (Stossel, 1993; Winsor and Schiebel, 1997), which together work for cell motility, polarity, contractility, cytokinesis, intracellular transport and phagocytosis (Schmidt and Hall, 1998; Iwatsuki and Suda, 2010). Actin is a 43kDa globular protein consisting of 375 amino acids, containing two domains separated by

a cleft. The monomer is highly conserved (Kron *et al*., 1992), highlighting the importance of this protein. Globular actin (G-actin), the monomer, when bound with ATP can polymerise to form F-actin-ATP. F-actin-ADP can also exist following hydrolysis. Actin filaments are polarised, with growth occurring preferentially at one end, designated '+' or barbed end, and dissociation of monomers facilitated by hydrolysis of ATP from the opposite end, designated '-' or pointed end. Thus, F-actin is highly dynamic, and filaments containing actin proteins are capable of spontaneous formation (via non-covalent bonds). Actin associates with other proteins in the cell; to date more than 150 proteins have been identified with actin-binding domains.

Actin filaments are less rigid than microtubules, although when in large numbers, crosslinker proteins create highly organised, stiff structures. Highly branched actin filaments support the leading edge of motile cells whilst bundles of filaments aid in cell-cell communications and chemotaxis (Zani, Indolfi and Edelman, 2010). Rho, Rac and Cdc42 aid in coupling the actin cytoskeleton at sites where membrane trafficking and exocytosis occur (Gasman *et al*., 1999). These actin structures are also dynamic. Assembly and disassembly occurs in response to local signaling systems; for example, branching networks are formed at the leading edge of motile cells in response to chemotaxis (as in crawling leukocytes). The growth of filaments stop when a capping protein attaches, preventing further actin monomers from attaching to the filament (Cooper and Sept, 2008). Filopodium-like structures containing actin may protrude some length from the cell in order to establish cell-cell contacts; the extreme lengths of some of these structures demonstrate that actin needs to interact with the plasma membrane to stabilise the protrusion (Liu *et al*., 2008).

Myosin proteins attach to actin filaments, and play a role in the organisation of the actin cytoskeleton. Not all actin structures require myosin (such as the structure at the

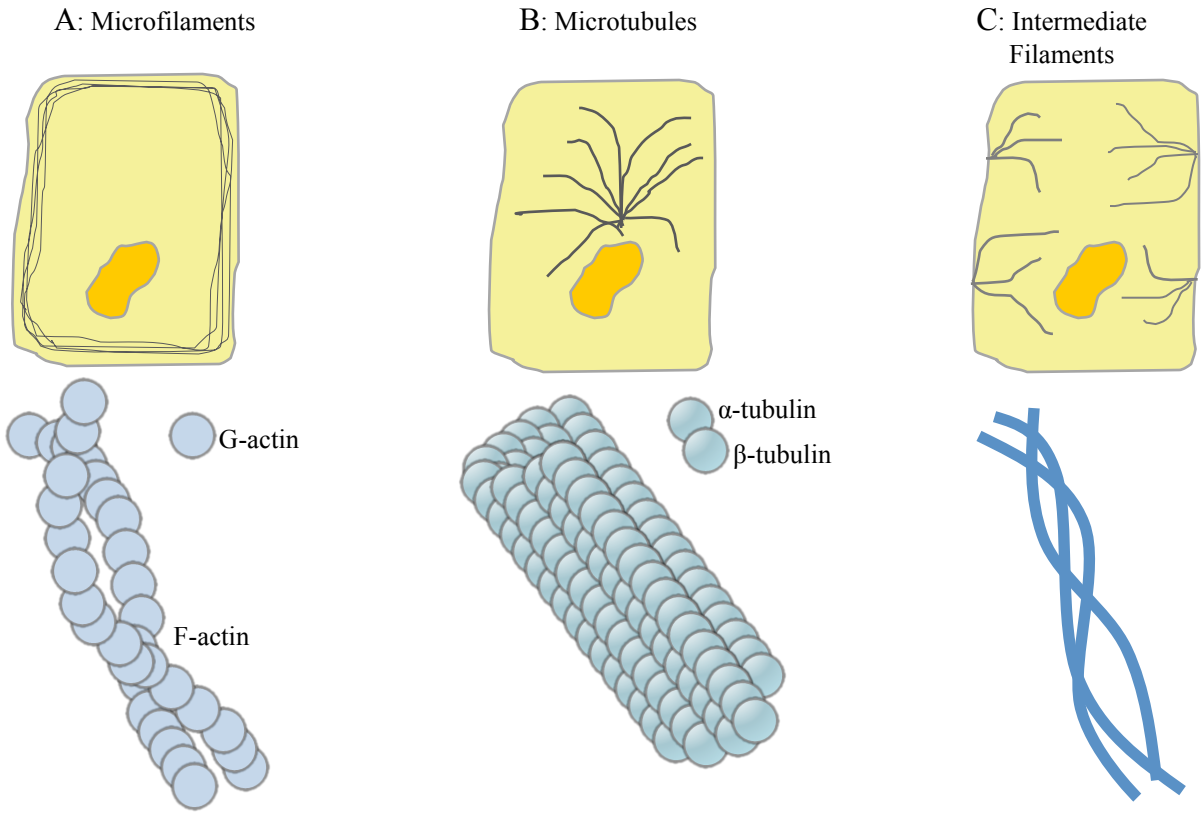


Figure 1.2: Schematic diagrams of the cytoskeletal filament networks
Microfilaments, or actin filaments (A) are two-stranded helical polymers of actin. This polymerisation of G-actin produces F-actin filaments. Although found throughout the cell, actin filaments are generally observed beneath the plasma membrane.
Similarly, microtubules (B) are also polymers, of α- and β-tubulin. These heterodimers arrange to form protofilaments, which then arrange into hollow cylinders. One end of the micrtubule is usually attached to a microtubule organising centre (MTOC).
Intermediate filaments (C) are long, rope-like filaments, which extend across the cell. This provides mechanical strength, which varies depending on the composition of the filament (to allow for migration, for example).

leading edge of a motile cell); the contractile structure at the rear of a motile cell does however. Myosin proteins also act upon actin filaments in stress fibres, enabling cell contraction in response to their external environment (Koenderink *et al*., 2009; Fletcher and Mullins, 2010). Crosslinker proteins (such as α–actinin) allow some flexibility, as the actin bindng sites rotate. The architecture of the actin network is therefore determined by the kinetics of this interaction. Also, actin filaments are produced depending upon the dissociation rate of these crosslinkers; where this level is low, more branching occurs (apparently randomly). At higher dissociation rates, filaments become bundles (Wachsstock *et al*., 1994). Other actin associated proteins bind to other cytoskeletal structures. For example, α-catenin, β-catenin and E-cadherin assemble into 'puncta' (Yonemura *et al*., 1995; Adams *et al*., 1996) and become the site at which vinculin and VASP can reorganize and polymerise actin (Vasioukhin *et al*., 2000; Vaezi *et al*., 2002). α-catenin is also required for the binding of the cytoskeleton to adherens junctions (Vasioukhin *et al*., 2001). In addition, the actin nucleation-promoting factor WHAMM binds to actin, microtubules and membranes, whilst the GTPase Rac1 stimulates actin polymerization (creating lamellipodial protrusions) (Waterman-Storer *et al*., 1999; Campellone *et al*., 2008).
As keratinocytes differentiate, they produce tighter cell-cell junctions (see section 1.1.1.2). This is to produce the barrier skin is required to be, preventing, for example, water loss and infection. Actin has been shown to have an important role in this, contributing to cell reshaping, cadherin oligomerisation (Mége, Gavard and Lambert, 2006), relocation of soluble desmoplakin and plakophillin (Jones and Goldman, 1985; Godsel *et al*., 2005) and finally production of tight junctions (Tsukita *et al*., 1999). Actin rearrangement during differentiation is affected by increases in extracellular calcium (for example, Vasioukhin *et al*., 2000). Cell shape has been shown to be an important factor in epithelial cell differentiation; reduced cell surface area promotes differentiation (Connelly *et al*., 2010). Cells cultured on larger surfaces have been shown to contain more filamenous actin (F-actin) than globular actin (G-actin). Preventing actin polymerisation by culturing keratinocytes on small surfaces has also been shown to inhibit differentiation (Connelly *et al*., 2010). It is proposed that G-actin affects differentiation by binding to MAL (megakaryotic acute leukaemia) protein in the cytoplasm. This prevents MAL protein binding to serum response factor (SRF), inhibiting transcription (mediated by FOS and JUNB, both SRF targets). This suggests that without G-actin, MAL protein-mediated SRF activation promotes differentiation in epidermal cells, and leads Connelly *et al*. (2010) to suggest that actin is a primary sensor for epidermal stem cell differentiation.

1.2.2: Microtubules

Microtubules are the most complex cytoskeletal polymers with regards to their assembly and disassembly dynamics (Brangwynne *et al*., 2006). Actin and myosin stress fibres attach to the rigid microtubule network during membrane rearrangement and cell motility (Hawkins *et al*., 2010). Their stiff nature also allows radiating of the polymers facilitating intracellular trafficking (Fletcher and Mullins, 2010).
In comparison with microtubules, which rely on the cellular concentration of subunits and regulatory proteins, microtubule polymers are capable of much quicker polymerisation and depolymerisation (Holy and Leibler, 1994); this process is known as 'dynamic instability' (Hawkins *et al*., 2010). This allows cell motility as well as cellular organisation (such as vesicle and organelle separation) (Mitchison and Kirschner, 1984; Gross, Vershinin and Shubeita, 2007) and essential spindle

formation during mitosis (Jordan and Wilson, 2004). Again the strength of microtubules is essential here, as they need to be able to withstand moving large structures (such as the chromosomes).

Microtubules interact with proteins named microtubule associated proteins (MAPs). MAPs are considered to be important in linking microtubules to other cytoskeletal components, supporting the notion that all cytoskeletal proteins work together in the cell (for example Mandelkow and Mandelkow, 1995; Vanier *et al*., 2003; Faller and Brown, 2009). These associated proteins (of which more than 600 have been identified) can manipulate the stability and rigidity of microtubules, as well as crosslinking them into bundles (Hawkins *et al*., 2010). For example, much work has been carried out neuronal MAPs, and different proteins are observed in the axon (increasing stability and rigidity) and dendrites (stiffening the microtubule structure to a lesser extent than those in the axon) (reviewed by Hawkins *et al*., 2010). Other MAPs (EB1 and CLIP-170) are observed at the barbed end of the filament, where the fastest growth and shrinkage occurs; it is possible that these proteins correct defects occurring during polymerisation. E-MAP-115 has been shown to have a role in maintaining microtubule stability in keratinocytes, and is upregulated during terminal differentiation (Lee *et al*., 2005). MAPs also exist to destabilise the microtubule; depolymerising kinesins (such as MCAK) act at the ends of the microtubule to remove dimers, whereas microtubule severing proteins (such as katanin) act all along the microtubule to remove dimers from the lattice, weakening the structure (Hawkins *et al*., 2010). Where MAPs are defective or mutated, disease can occur. Parkinson's disease, Huntington's disease and Alzheimer's disease have all been linked with abnormal MAPs, although exactly how these are affecting microtubules to lead to disease states are currently unknown. This does demonstrate however that the flexibility and stability of microtubules is essential for function, and where these properties are not altered appropriately, disorders can occur (Hawkins *et al*., 2010).

Like the microfilament actin, microtubules are polar. Because of this structural polarity, both actin and microtubules are useful as tracks for other molecules which prefer to move in a single direction (Fletcher and Mullins, 2010); microtubules are also relatively straight, which aids efficient long-distance transport (Hawkins *et al*., 2010). Microtubules are large filaments, each composed of 13 protofilaments, stacked side-to-side. Each protofilament is 25nm in diameter and is comprised of a cylindrical ring of 13 hetrodimers of α and β tubulin; these form a sheet that is then rolled into a tube. There are several isoforms of α and β tubulin, encoded on different genes, which can also be altered post-translationally, producing microtubules with different biochemical compositions (Linhartová *et* al., 1992; Lee *et al*., 2005). The heterodimers can associate or dissociate from either end of the microtubule after the tube nucleates. The – end of the microtubule associates with the MTOC (microtubule-organising centre), whilst assembly occurs at the free, + end. Disassembly can occur at both ends upon microtubule disassociation with the MTOC (Kitanishi-Yumura and Fukui, 1987; Lee *et al*., 2005). This lattice can adapt depending on cell type; for example, cilia microtubules are made up of an A-lattice (which is staggered) and a B-lattice (which has α-α and β-β lateral interactions except at the seam with the A-lattice) (Brinkley, 1997 and Hawkins *et al*., 2010). The individual microtubule polymers are important as, unlike the other cytoskeletal proteins, microtubules do not function as large branched networks (Hawkins *et al*., 2010).

Lechler and Fuchs (2007) describe microtubule arrangement during epithelial differentiation; in the basal layer, microtubules are apically organised. In stratified

cells however, microtubule networks are concentrated at cell-cell borders; there is also no obvious MTOC in these cells. In contrast, in simple epithelia, despite the lack of MTOC, microtubules exhibit an apical-basal orientation. There is an abundance of the class II β-tubulin isoform in the granular layer of the epidermis, although this does not extend into the cornified envelope; as keratinocytes differentiate, microtubules are degraded (Lee *et al*., 2005). In less differentiated NHEK cells, class II β-tubulin is present in the nucleus (Lee *et al*., 2005), as has been described in other cell types (Ranganathan *et al*., 1997; Walss, Kreisberg and Ludueňa; 1999, Walss-Bass Kreisberg and Ludueňa, 2001; Xu and Ludueňa, 2002). As the cells differentiate, there is acetylation of microtubules which become increasingly dense; both acetylated tubulin and class II tubulin stabilise the microtubules in differentiating NHEK cells (Lee *et al*., 2005). Increased levels of class II β-tubulin are also observed in squamous cell carcinomas; low levels however are observed in hyperproliferative keratinocytes (such as in psoriasis). Lee *et al*. (2005) suggest that this indicates the class II β-tubulin expression is correlated with differentiation.

It has also been shown that desmoplakin affects organisation of the microtubule cytoskeleton during epithelial differentiation (Lechler and Fuchs, 2007). Without desmoplakin, epithelial cells cultured in high calcium media do not lose their MTOC nor develop a cortical microtubule network. It was also suggested that desmoplakin-dependent re-localisation of the microtubule-anchoring protein ninein to desmosomes could affect microtubule reorganisation to the cell cortex.

1.2.3: Intermediate Filaments

Intermediate filaments (IFs) are the least stiff of the three cytoskeletal polymers; because of this, they are more resistant to tensile forces than compressive forces. IFs are thought to have a major role in structurally supporting the cell; this can improve cell and tissue integrity (for example, through desmosomal cell junctions) and allow organisation of cells into tissues, such as muscle and the skin. The IF cytoskeleton spans between the nucleus and cell membrane, anchored through desmosomal and hemidesmosomal linker proteins. This organisation allows flexibility as well as structural stability, which is important in maintaining the integrity of epithelial tissues and coping with mechanical stress. For example, in the epithelial cells of the oesophagus, keratin networks help prevent damage to the cell through shear stress (Lane *et al*., 1982; Fuchs and Cleveland 1998; Uitto, Richard and McGrath, 2007; Flitney *et al*., 2009). This said, IFs are not polar so do not facilitate directional movement of molecular motors (unlike actin and microtubules). Associated crosslinker proteins, such as plectin, link IFs together as well as to actin and microtubules. Another function of IFs is to connect the plasma membrane to the nuclear compartments (the nucleus and cytoplasm contain different IF populations). Lamins are Type V IFs which contribute to the mechanical integrity of the nucleus. Phosphorylation of these lamins by cyclin-dependent kinases help the mitosis process by triggering nuclear envelope breakdown (Tsai *et al*., 2006). The nucleus itself is surrounded by a 'cage' of cytoplasmic IFs (Gniadecki et al., 2001).

IFs belong to a large multigene family, comprising over 70 members – one of the largest in the human genome (Hesse *et al*., 2001; Uitto, Richard and McGrath, 2007; Szeverenyi *et al*., 2008). Genetic mutations resulting in abnormal or absent IFs have been linked with many human diseases, including progeria, epidermolysis bullosa simplex, motor neuron disease (also known as amyotrophic lateral sclerosis) and a predisposition to liver disease (for a review of diseases caused by keratin mutations, see Uitto, Richard and McGrath, 2007).

1.2.3.1: Intermediate Filament Nomeculature

IFs can be divided to give six subfamilies (see *Table 1.1*). These were created based on their dynamic properties, role in signalling, mechanical stabilisation, participation in cytoskeletal crosstalk and motility; grouping them by cell type was not an option as different IFs were expressed throughout differentiation in many cell types (for example, Franke *et al*., 1978; reviewed by Eriksson *et al*., 2009). Similarities in structure eventually lead to classification based on the rod domain amino acid sequences, secondary structure and net acidic charge (Steinert and Roop, 1988; Eriksson *et al*., 2009). The cytoplasmic IFs are grouped according to their sequence homology (Type I-IV), and the nuclear lamins form Type V. More recently, a separate class (Type VI) was created for nestin (Herrman and Aebi, 2000), although now eye lens IFs are also included (Szeverenyi *et al*., 2008).

(Type I and II [the cytokeratins] will be discussed in detail in section 1.2.4.1.)

Type III IFs include glial fibrially acidic protein (GFAP) (found in astrocytes and glia), peripherin (in neurons), desmin (a major constituent of smooth muscle) and vimentin (often observed in cells of mesenchymal origin). Vimentin has also been described in epithelial carcinoma cell lines, such as HeLa cells (for example, Moskalewski and Thyberg, 1984; Fortier *et al*., 2010). In quiescent endothelial cells, the vimentin network shrinks toward the perinuclear region in response to platelet-derived growth factor, and is continually rearranged in epithelial cells in response to chemical or mechanical stimulation (Tang, 2008). Using siRNA to downregulate vimentin expression in endothelial cells, Lund *et al*. (2010) demonstrated that both migration and proliferation levels reduced. Since it was demonstrated that vimentin colocalises with VASP, suppression of vimentin resulted in translocation of VASP from focal adhesions to the perinuclear region of cells. The colocalisation of VASP with vimentin allows the phosphorylation of VASP; where vimentin levels are suppressed, VASP phosphorylation does not occur (Lund *et al*., 2010). Taken together, Lund *et al*., 2010 suggest a role for vimentin in controlling endothelial cell morphogenesis. There is also evidence that during mitosis, the type III IFs (and keratins) become spot-like structures during the transition of prophase to metaphase despite the observation of extensive networks in interphase (Tang, 2008). During mitosis, reorganisation of vimentin IFs has been shown to be mediated by phosphorylation of the head domain by p34cdc2 kinase (Chou, Ngai and Goldman, 1991), and more recently Bub1, a serine/threonine kinase protein (Ando *et al*., 2008).

The type IV IFs are usually observed in nerve cells and muscle. In neurons, type IV IFs are implicated in the growth of the axon, and it has been suggested that neurofilaments may facilitate elongation of axons inhibiting their retraction and stabilisation of the cytoskeleton (Lin and Szaro, 1995; Walker et al., 2001). Type IV IFs observed in neurons include neurofilaments (heavy, medium and light) and nestin (sometimes classified separately) (Szeverenyi *et al*., 2008). Another type IV IF is synemin, which is found in striated and smooth muscle, co-localising with vimentin filaments and interacting with desmin (both type III IF proteins) (Tang, 2008).

Type V IFs are the nuclear lamins – major components of the nuclear lamina, closely associated with the inner nuclear membrane in non-mitotic cells. Further investigation revealed three distinct proteins, named A, B and C; A and C are derived from alternate splicing of the same gene (*LMNA*). Later, further proteins were identified (such as lamin B1 and lamin B2) (Höger *et al*., 1990; reviewed by Genschel and Schmidt, 2000). Lamin A is, so far, the IF with the most number of identified mutations (currently around 230); these cause a complex set of diseases, including lipodystrophies, progeria syndromes and muscular dystrophies (Worman *et al*., 2009).

Due to the role of the lamins in the nuclear lamina, depolymerisation occurs between prophase and metaphase of dividing cells – vital for DNA distribution. Repolymerisation occurs following mitosis (Bridger *et al*., 2007).
Type VI IFs are the eye lens IFs. There are currently two proteins identified: phakinin (or CP49) and filensin (or CP115) (Hess *et al*., 1993; Merdes, Gounari and Georgatos, 1993). These proteins are dissimilar to other IFs because of differences in their structure; the 5nm filament is dotted with beads periodically, making the ocular lens cytoskeleton appear as a beaded filament (Maisel and Perry, 1972; Hess *et al*., 1996). This said, genomic analysis has suggested that they are highly conserved amongst species (Hess *et al*., 1996).

Table 1.1: Six Classes of IF

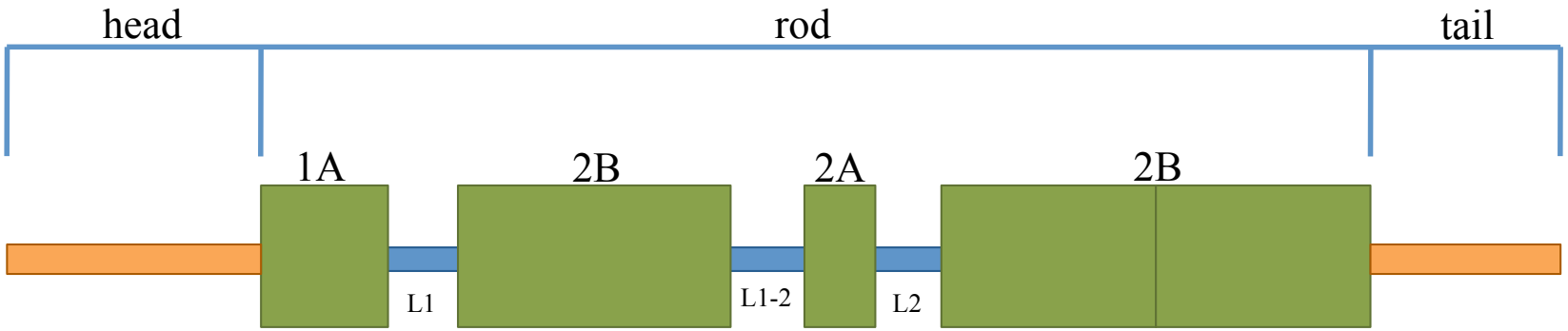
Type	Protein Components	Tissue Specificity
Type I	Acidic keratins	Epithelia
Type II	Basic keratins	Epithelia
Type III	Vimentin, desmin, GFAP, peripherin	Mesenchyme, muscle, glia, astrocytes, peripheral (and some central) nervous system
Type IV	NF-L, -M, -H, Internexin, Nestin	Central nervous system, neuroepithelial stem cells
Type V	Lamins	All cell types
Type VI	CP49/phakinin, filensin/CP115	Eye

1.2.3.2: Intermediate Filament Structure
Intermediate filaments contain an α-helical rod domain, usually 310 residues long and flanked with non-helical carboxy- and amino-terminals. These terminals vary depending on IF type (Coulombe *et al*., 2001; Parry *et al*., 2007; Goldman *et al*., 2008). The rod domain contains four subunits (1A, 1B, 2A and 2B), also known as heptad repeats (Coulombe *et al*., 2001; Qin, Buehler and Kreplak, 2010). These regions facilitate dimerisation between rod domains of separate IFs. The carboxy- (C) terminal domain of IFs contributes to filament assembly; the amino- (N) terminal domain is essential in formation of the tetramer, an early assembly intermediate (*Figure 1.3*).
The rod domain of the protein consists of two α-helices; the α-helical structure aids in dimerisation. Helix I (closer to the N-terminal) contains the LNDR motif, which is highly conserved and critical to IF assembly. Mutations in this region have been shown to cause skin blistering diseases (for example, Hess *et al*., 1996). Helix II (closer to the C-terminal) contains the TYRKLLEGE motif, which is also highly conserved. In K14 a RELLEGEDAL deletion in the C-terminal caused filament collapse (Albers and Fuchs 1987; Hatzfeld and Weber 1991).
IFs are capable of spontaneous assembly, forming non-polar tetramer filaments in the absence of ATP and GTP (Strelkov, Herrmann and Aebi, 2003). These tetramers have a diameter of 8-9nm. Free tetramers are rarely observed in cells, which are capable of regulating assembly of into rope-like filaments by phosphorylation of specific serine residues in the amino head domain (Izawa *et al*., 2006). It is only the α–helical rod domain that is required for this dimerisation, and subsequent tetramer formation, to occur (Parry and Steinert 1992; Qin, Buehler and Kreplak, 2010).

In particular, type III IFs have shown that at neutral pH, dimers associate rapidly forming half-staggered, anti-parallel tetramers; these associate to form unit-length filaments (ULF) (Goldman *et al*., 2008). Free tetramers are rarely observed in the cell, as serine-specific residues in the N-terminal are phosphorylated as a method of regulating IF assembly (Hatzfeld and Weber, 1990; Izawa et al., 2006). These IF tetramers therefore assemble into rope-like polymers, which measure 7-10nm in diameter (Gniadecki et al., 2001; Qin, Buehler and Kreplak, 2010). They may also form ULFs, which are comprised of four octamers (Sokolova *et al*., 2006). Using electron microscopy and scanning force microscopy, Kirmse *et al*. (2007) demonstrated that IFs (in this study, vimentin) grow from the addition of ULFs (not dimers or tetramers); this has also been demonstrated in peripherin (Chang *et al*., 2006). Nonfilamentous peripherin particles comprise dimers, tetramers and ULFs (Chang and Goldman, 2004).

Recently, there has been more focus on the atomic level. The crystal structures of some IF protein domains, such as α–helices and coiled coils, have been elucidated by crystallography. Papapostolou *et al*. (2007) built on this work by developing peptides with sticky ends, which would form coiled coils; this was important as coiled coil proteins do not crystallise in filament form (Smith and Parry, 2008). This is due to the imperfect 3D structure produced as IFs polymerise and become filaments, although sophisticated computer modeling software has overcome this issue. Bertaud, Qin and Buehler (2009) identified that 'biological protein materials' feature, for example, sliding and folding; this requires the disruption of hydrogen bonds and covalent crosslinks. This is particularly the case in the cytoskeleton, which are required to have strength, elasticity and robustness. They therefore suggest that closer examination is needed alongside the macroscale studies (such as those described above). Bertaud, Qin and Buehler (2009) and Herrmann *et al*. (2009) suggest that this 'multiscale' understanding (using computational techniques) will provide further advances in the field.

A



B

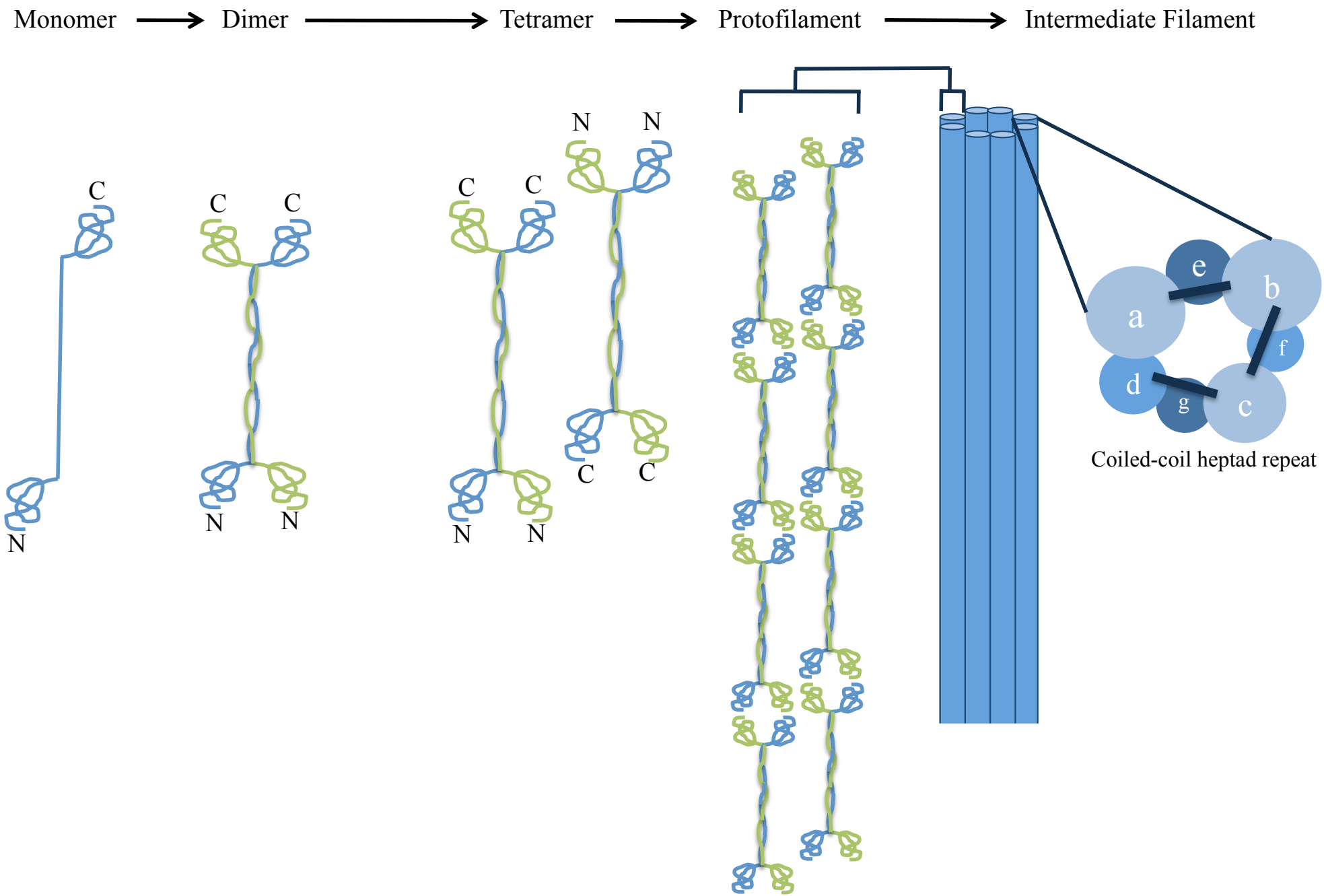

Monomer
Dimer
Tetramer
Protofilament
Intermediate Filament
C
N
a
b
c
d
e
f
g
Coiled-coil heptad repeat

Figure 1.3: **The Structure of Intermediate Filaments**

Schematic diagram of the structural domains in intermediate filaments (A). The head, tail, rod and linker domains are shown. The head domain is thought to adopt a globular structure. The rod domains, which form the coiled-coil heptad repeat (see B), are interrupted by linker regions (L1, L1-2, L2).

Intermediate filaments are made up of fibrous proteins (fibrils) which together form a rope-like structure. This structure allows intermediate filaments to withstand large forces, important in anchoring organelles and maintaining cell structure (B). The monomer contains a head domain, a tail domain and a central α-helical rod domain of approximately 310 amino acids (350 in the nuclear lamins). The central rod domains of two monomers wind around each other to form the dimer. The dimers than associate in antiparallel to form tetramers. Protofilaments are formed from end-to-end associations of tetramers, which then laterally form filaments. Each filament contains eight protofilaments, which wind around each other to form the rope-like structure.

Phosphorylation of Intermediate Filaments

Cytoskeletal proteins require reorganisation during mitosis, which is, at least in part, controlled by phosphorylation, particularly during S and G2/M phases of the cycle (Celis *et al*., 1983; Chou and Omary, 1994); the first evidence of this was observed in vimentin. Phosphorylation state-specific antibodies, which recognise phosphorylated serine/threonine residues (Inagaki *et al*., 1997), can be used to detect phosphorylation of IFs at the cleavage furrow (Nishizawa *et al*., 1991). *In vivo* analysis has demonstrated the importance of Rho-kinase, Aurora-B and Polo-like kinase 1 in phosphorylation (Kosako *et al*., 1997; Goto *et al*., 2003; Yamaguchi *et al*., 2005; Izawa and Inagaki 2006). IF organisation is regulated by protein kinases causing phosphorylation as this induces disassembly; conversely, protein phosphatases dephosphorylate IFs, allowing assembly. This ensures a constant turnover of filaments and the soluble pool of IF fragments. Most phosphorylation sites are in the N-terminal domain. During mitosis, phosphorylation of IFs is observed throughout the cell. In a study specifically observing GFAP phosphorylation, Ser8 phosphorylation began in prometaphase and continued through metaphase before declining; Thr7, Ser13 and Ser38 phosphorylation occurred during anaphase, continuing through telophase and only decreasing as the cell was exiting mitosis. This suggested that at least two different kinases are acting on GFAP during mitosis (Matsuoka *et al*., 1993; Izawa and Inagaki 2006). Cdk1 was shown to be one of these kinases (Tsujimura *et al*., 1994a), which was also shown to act on Ser55 of vimentin (Tsujimura *et al*., 1994b). The second was Rho-kinase (Kosako *et al*., 1997). In cells transfected with a GFAP mutant (in which Thr7, Ser13 and Ser38 had been changed to alanine residues), a formation referred to as an 'IF bridge' was created between unseparated post-mitotic cells, demonstrating that no IF disassembly had occurred (Yasui *et al*., 1998). In a similar experiment using vimentin, IF bridges were also observed between daughter cells (Yasui *et al*., 2001). Kops, Weaver and Cleveland (2005) have described how defects in mitosis (such as those described here) promote aneuploidy and tumourigenesis. Izawa and Inagaki (2006) speculate that impaired phosphorylation of IFs may contribute to the formation of tetraploid cells (with two nuclei); in normal cells this would cause apoptosis. Without a tetraploid 'checkpoint', it is possible that tumourigenic aneuploid cells may develop. A similar phenomenon has been observed in K8/K18 knockout mice, which are approximately 100 times more sensitive to TNF-induced apoptosis than control littermates (Caulin *et al*., 2000). Epithelial cells are more resistant to TNF-induced apoptosis; an association between TNFR1-associated death domain protein (TRADD) and K18 may attenuate the activated TNFR1, reducing the effects of TNF (Inada *et al*., 2001). A similar mechanism of K14-TRADD interaction has been suggested to have a role in EBS (Yoneda *et al*., 2004). Inhibition of K5 T150 phosphorylation in K5 P152L mutants may also contribute to EBS (Toivola *et al*., 2002).

An example of the role of IF phosphorylation in the stress response can be seen in liver cirrhosis. Mouse models expressing the K8 G61C mutation, which has been shown as a predisposing factor in human cirrhosis, demonstrate reduced keratin phosphorylation, as the mutation inhibits Ser73 phosphorylation which normally occurs through stress-activated proteins, such as JUNK and MAPK (Ku and Omary, 2006). In an injury model of lung alveolar epithelial cells, the keratin cytoskeleton has been shown to disassemble when exposed to shear stress; this co-insides with the PKCδ-mediated phosphorylation of K8 at Ser73 (Ridge *et al*., 2005). Flitney *et al.* (2009) also demonstrated that K18 was phosphorylated at Ser33 during shear stress. During shorter periods of stress, this Ser33 phosphorylation occurred closer to the

nucleus (where forces were greatest) (Barbee *et al*., 1995); increases in duration or shear force caused further phosphorylation, gradually advancing outward from the nucleus. It was shown through electon microscopy that shear stress promotes the bundling of pre-existing keratin filaments, creating tonofibrils. The role of phosphorylation in response to shear stress then is to reinforce the keratin cytoskeleton (demonstrating that phosphorylation is not always associated with filament disassembly) (Flitney *et al*., 2009). Osmotic stress has also been shown to induce K20 phosphorylation in the small intestine; in colon-carcinoma derived cell lines Caco and HT29 cells, K8 was also altered under osmotic stress conditions (Tao *et al*., 2006a). In hyposmotic conditions, K8 Ser431 becomes dephosphorylated (and hyperphosphorylated in hypertonic conditions); K8 Ser73 (phosphorylated during apoptosis and mitosis – Liao *et al*., 1997) becomes hyperphosphorylated after exposure to both hyposmotic and hypertonic conditions (Tao *et al*., 2006a). In contrast, K18 phosphorylation sites were unaffected in these conditions.

Phosphorylation has also been shown to have an effect on cell-cell junctions and cell motility, affecting wound healing. For K17, this is an indirect effect, demonstrating the importance of keratins as scaffolds for other proteins, in addition to their role in maintaining mechanical integrity of cells. Small epithelial cells and delayed wound closure has been reported in K17-null embryos (Mazzalupo *et al*., 2003); cultured K17-null keratinocytes are also small and demonstrate a ~20% reduction in protein synthesis (Kim, Wong and Coulombe, 2006). It is possible that the epithelium-specific mTOR kinase isoform 14-3-3 K17 associated protein regulates K17 distribution (Hermeking and Benzinger, 2006). Hypophosphorylation of K17 allows 14-3-3 to relocate to the nucleus; this prevents activation of mTOR (mammalian target of rapamycin), a member of the phosphatidylinositol 3-kinase-related kinase family, which affects cell growth and the rate of cell replication (Kim *et al*., 2006; Cao and Wan 2009). K8 and K18 are also required at 14-3-3 binding sites during mitosis (Ku *et al*., 2002). Cdc2/CyclinB phosphorylation and dephosphorylation help to regulate mitosis through multiple signal cascades. Action of the phosphatase Cdc25 (which dephosphorylates Cdc2/CyclinB during G2/M phase transition of the cell cycle) is regulated by binding to 14-3-3; again hyperphosphorylation of K8/K18 increases 14-3-3-keratin interaction (Margolis *et al*., 2006). K8 Ser73 has also been shown to be phosphorylated by the stress-activated kinase p38, a function important in causing reorganisation of the filament *in vitro* (Ku *et al*., 2002). Toivola *et al*. (2002) have further demonstrated that p38 is also capable of phosphorylating K6b and K5 in a similar manner to K8, although K6b and K5 are phosphorylated on serine and threonine, as opposed to K8, which is only phosphorylated on serine. Enhanced activity of p38 and mitogen-activated protein kinases (MAPK) has also been shown to induce differentiation in keratinocytes. The activity of p38 increased in NHEK cells exposed to sulphur mustard, causing K1 and loricrin levels to rise (Popp *et al*., 2011). Hyperphosphorylation of 65kDa, 60kDa and 55kDa keratins were observed following treatment with okadaic acid in murine keratinocytes (Kasahara *et al*., 1993). This phosphorylation, occurring at several sites in each keratin, caused a change in solubility of the keratins and reorganisation of the keratin network (which eventually collapsed into perinuclear aggregates) (Kasahara *et al*., 1993); this has also been demonstrated in SCC-13 cells (Toivola *et al*., 2002). It has since been demonstrated that in primary keratinocytes, K4, K5 and K6 are phosphorylated and become solublised following treatment with okadaic acid (Toivola *et al*., 2002).

Intermediate Filament Dynamics

As previously mentioned, intermediate filaments are dynamic proteins, forming dimers and filaments, assembling and disassembling as cells change shape, size, order, and differentiate. Immunofluorescence has been used extensively to study cytoskeletal networks in cultured cells. In a process called 'dynamic subunit exchange', it was noted that ectopically expressed keratin and vimentin could become incorporated into endogenous intermediate filaments (Albers and Fuchs, 1989; Ngai *et al*., 1989); similar results were observed when intermediate filaments were mincroinjected into cultured cells (Vikstrom, Borisy and Goldman, 1989; Miller, Vikstrom and Goldman, 1991). Later, fluorescence recovery after photo-bleaching (FRAP) was used to confirm that subunit exchange could occur along the length of vimentin and keratins (Vikstrom *et al*., 1992; Yoon *et al*., 1998; Yoon *et al*., 2001), and that phosphorylation sites are important in this exchange. It has also been possible to measure the rate of subunit turnover using FRAP (for example, Yoon *et al*., 2001). Using GFP reporter proteins, Windoffer and Leube also demonstrated that individual IFs could change shape (Windoffer and Leube, 1999). Since the observation that vimentin translocation is dependent on dynein and kinesin (Gyoeva and Gelfand 1991; Prahlad *et al*., 1998; Helfand *et al*., 2002), it has been noted that IFs are not only structural proteins, but organisers of different cellular processes (Eriksson *et al*., 2009). Also assisting in this view were increasing numbers of diseases attributed to intermediate filaments, without being easily related to structural function (for example, see *Table 1.2*).

Table 1.2: Keratin Diseases

Keratin	Disease	
K1/K10	Ichthyosis hystrix of Curth-Macklin	A disorder characterized by the appearance of ridges on the skin surface. The hyperkeratosis observed may be caused by hyperproliferation, a response to the cell fragility caused by K1/10 mutations.
	Epidermolytic hyperkeratosis	This condition is characterized by hypertrophy, blistering and redness. Electron microscopy has shown that affected suprabasal cells often demonstrate clumping of the keratin cytoskeleton.
	Striate keratoderma	A type of palmoplantar keratoderma characterized by hyperkeratosis on the palms of the hand.
K2e	Ichthyosis bullosa of Siemens	A milder form of epidermolytic hyperkeratosis, localised to flextures. Aggregation of keratin filaments can be observed in the spinous and granular layers.
K3/K12	Meesman epithelial corneal dystrophy	Characterised by the development of multiple opaque spots on the epithelium of the cornea.
K4/K13	White sponge nevus of Cannon	This condition affects the non-cornifying stratified squamous epithelial tissues, such as in the mouth. The white plaques and loose skin in the mouth are caused by fragility of the suprabasal cells of buccal epithelium.

Keratin	Disease	
K5/K14	Epidermolysis bullosa simplex	A skin blistering condition caused by fragility of the basal layer. This is a result of mutations in K5 or K14 which disrupt the formation of K5/K14 filaments. Different mutations result in different phenotypes.
K6a/K16	Pachyonychia congenita type 1	Also known as Jadassohn-Lewandowsky syndrome. Characterised by hyperkeratosis of the nail bed and distortion and thickening of the nail plate. Oral leukokeratosis is also seen.
K6b/K17	Pachyonychia congenita type 2	Also known as Jackson-Lawler syndrome. Symptoms as for type 1, without the oral leukokeratosis. Those with type 2 are prone to pilosebaceous cysts, hair abnormalities, vellus hair cysts and natal teeth.
	Steatocystoma multiplex	The sebaceous cysts which occur in steatocystoma multiplex are known as steatocysts, and can occur over the entire body. In some patients, mild keratoderma and nail changes are observed.
K6hf	Pseudofolliculitis barbae	A condition increasing the risk of ingrowing hair occurrence and follicular infections.
	Loose anagen syndrome	Hair in anagen is easily removed and demonstrates no inner or outer root sheath and a ruffled cuticle. Causes thinning hair in children.
K8/K18	Cryptogenic cirrhosis	Liver tissue is replaced by fibrotic scar tissue in liver disease. This results in loss of liver function.
K9	Epidermolytic palmoplantar keratoderma	This condition occurs as mutant K9 weakens the cytoskeleton in suprabasal cells of the palms and soles of the feet. This results in thickening of the palms, which is characteristically yellow and has an erythematous border.

More recently, keratins have been observed by incorporating a fluorescently-tagged keratin with an EGFP (enhanced green fluorescent protein) construct. This demonstrated that keratins not only formed mature IFs, but also in two types of particles. These were denoted S (for static, or slow) particles, which are less motile, and F (fast) particles, which are more dynamic. The swift movement of these F particles is aligned with microtubules (Liovic *et al*., 2003). Wöll, Windoffer and Leube (2005) observed similar results, identifying that keratin motility relied on microtubules and intact actin filaments. Furthermore, Kölsch, Windoffer and Leube (2009) demonstrated that focal adhesion-dependent polymerization of keratin occurs in developing lamellipodia. In addition, the transportation of newly-formed keratin particles is initially mediated by actin; this mechanism then would allow extension of the keratin IF network to the leading edge of cells during migration, important in wound healing.

1.2.4: The Keratins

The keratin network occupies the cytoplasm between the nucleus and the plasma membrane, where they act as protein scaffolds. Once at the cell membrane, keratins

aid in the attachment of individual cells to each other through desmosomes. As epithelial cells detach from the basal layer and move towards the skin surface, the IF network changes to increase the tensile strength of the cell. This appears as a change in the keratin expression pattern (Fuchs and Cleveland, 1998; Gu *et al*., 2007).
The important role of keratins is highlighted by the symptoms of many genetic diseases caused by keratin mutations (Uitto *et al*., 2007; Moll, Divo and Langbein, 2008; Omary *et al*., 2009); for example, epidermolysis bullosa simplex, where mutations in K14 or K5 cause different severities of blistering, depending on the mutation, to the extent that such blistering can be fatal (Coulombe, Kerns and Fuchs, 2009). Keratins also have regulatory and highly specialized functions in the cell (Eichner *et al*., 1986; Magin, Vijayraj and Leube, 2007), and different keratins in different cell types may have different regulatory functions (discussed in more detail in section 1.2.4.2).

1.2.4.1: Type I and Type II Keratins
The keratin genes are part of a large family, comprising 54 members, divided into two groups based on sequence homology (Gu *et al*., 2007; Moll, Divo and Langbein, 2008). In 2006, Schweizer *et al*. instigated a new consensus nomenclature for mammalian keratins, which has since been widely adopted (for example, Karantza, 2010).
Acidic Type I keratins generally have a lower Mr than the basic Type II keratins (see Moll, Divo and Langbein, 2008). Equimolar association of both types are required for polymer assembly (Eichner *et al*., 1986).
The Type I keratins are named K9-10, K12-20, K23-28 and K31-40 (hair keratins); basic keratins are named K1-8, K71-80 and K81-86 (hair keratins). There are 28 type I keratin genes (11 hair keratins and 17 epithelial keratins) and 26 type II genes (6 hair keratins, 20 epithelial keratins). These genes are designated as *KRT1*, *KRT2*, *KRT3* etc. and clustered at two chromosomal sites: 12q13.13 (type II keratins and K18) and 17q21.2 (type I keratins excluding K18) (Moll, Divo and Langbein, 2008). Although most keratin pairs are consistently found together (for example, K10 and K1), this is not always the case (for example, K5 dimerises with both K14 and K15).

1.2.4.2: Keratin Expression Patterns in Skin
Not all keratin proteins are expressed in all epithelial cells, and cell type can often be identified by their specific keratin expression pattern. For example, more stratified epithelia express keratins 5/6, 10, 15 and 14, whereas simple epithelia express simple epithelial keratins: 7, 18, 19 and 20 (see *Table 1.3*).
As cells of the epidermis differentiate, the keratin expression pattern changes (Gu et al., 2007). In the basal layers, K5/K15 and K5/K14 are most prominent. K1 and K10 (which replace K5 and K14) are therefore considered early-stage differentiation markers.

Table 1.3: Human Keratin Nomenclature and Gene Designation

Keratin Type	Protein Designation	Gene Designation
Type I Human Epithelial Keratins	K9	*KRT9*
	K10	*KRT10*
	K12	*KRT12*
	K13	*KRT13*
	K14	*KRT14*
	K15	*KRT15*
	K16	*KRT16*
	K17	*KRT17*
	K18	*KRT18*
	K19	*KRT19*
	K20	*KRT20*
Type I Human Epithelial Keratins	K23	*KRT23*
	K24	*KRT24*
	K25	*KRT25*
	K26	*KRT26*
	K27	*KRT27*
	K28	*KRT28*
Type I Human Hair Keratins	K31	*KRT31*
	K32	*KRT32*
	K33a	*KRT33A*
	K33b	*KRT33B*
	K34	*KRT34*
	K35	*KRT35*
	K36	*KRT36*
	K37	*KRT37*
	K38	*KRT38*
	K39	*KRT39*
	K40	*KRT40*
Type II Human Epithelial Keratins	K1	*KRT1*
	K2	*KRT2*
	K3	*KRT3*
	K4	*KRT4*
	K5	*KRT5*
	K6a	*KRT6A*
	K6b	*KRT6B*
	K6c	*KRT6C*
	K7	*KRT7*
	K8	*KRT8*
	K71	*KRT71*
	K72	*KRT72*
	K73	*KRT73*

Keratin Type	Protein Designation	Gene Designation
	K74	*KRT74*
	K75	*KRT75*
	K76	*KRT76*
	K77	*KRT77*
	K78	*KRT78*
	K79	*KRT79*
	K80	*KRT80*
Type II Human Hair Keratins	K81	*KRT81*
	K82	*KRT82*
	K83	*KRT83*
	K84	*KRT84*
	K85	*KRT85*
	K86	*KRT86*

1.2.4.3: The Epithelial Keratins

This section will describe the keratins in epithelial tissue. This will begin with K8/K18, a marker of embryonic cells committed to an epidermal fate. K19, a possible marker of epithelial stem cells, is then discussed. This is followed by basal keratinocytes K5 and K14, then K1/K10, observed in more differentiated keratinocytes. The discussion of K6, K16 and K17 follows, since these keratins are not normally present in the epidermis.

The epithelial keratin not discussed in this section is K15; since this protein requires a more detailed discussion, it will be reviewed in section 1.3.

K8/18

K8 is considered to be one of the earliest markers of embryonic cells committed to an epidermal fate. In a study assessing *in vitro* differentiation of embryonic stem cells, Troy and Turksen (2005) describe 'epithelial progenitor cells' expressing K8 (as well as K19 and K17); these cells expand and differentiate into K14-positive cells (and continue towards terminal differentiation. K8/18 is not present in human adult skin, but is present in some cell lines, such as HaCaT cells (Kazerounian, Uitto and Aho, 2002).

In the embryo, murine K8 can be detected at the 8-cell stage before becoming restricted to the trophectoderm and extraembryonic endoderm at the blastocyst stage (Jackson *et al*., 1980; Brûlet *et al*., 1980; Oshima *et al*., 1983; Thorey *et al*., 1993).

Studies utilising K8 deletion have demonstrated the role of K8 in inflammation and epithelial barrier integrity (Baribault *et al*., 1994; Habtezion *et al*., 2005). Different *KRT8*$^{-/-}$ animals however demonstrate conflicting results; for example, *KRT8*$^{-/-}$ *Xenopus* embryos show defective wound healing whereas *KRT8*$^{-/-}$ mice demonstrate no difference in wound closure compared to the wild type (these mice also develop colonic hyperplasia); these different results are possibly due to subtle cell-type specific or species differences in keratin requirements for wound healing (Long *et al*., 2006). In a study downregulating K8 in simple epithelia, Long *et al*. (2006) demonstrated that lack of K8 reduces epithelial integrity and affects migration and wound healing *in vitro*. Long *et al*. (2006) showed that K8 was essential for the integrity of migrating MCF-7 cells, where the keratin network is re-distributed to the edge of the epithelial sheet.

Both K8 and K18 expression increases following IL-6 increase (which can occur pathologically in diseases such as inflammatory bowel disease), and phosphorylation of K8 was induced (Wang *et al*., 2007). K18 phosphorylation allows an interaction between K8 and K18 and the 14-3-3 protein family, promoting depolymerisation and the intracellular distribution of K8 and K18 *in vitro*. K8 and K18 are also phosphorylated in response to cell stress (Ridge *et al*., 2005). Phosphorylation at Ser-431 increases during mitosis following filament reorganisation and epidermal growth factor exposure (Ridge *et al*., 2005), whereas K8 Ser-73 phosphorylation is mediated by PKCδ. It is this inhibition of PKCδ which prevents keratin disassembly in shear-stressed alveolar epithelial cells (Ridge *et al*., 2005). As well as affecting simple epithelial wound healing and migration, RNAi depletion of K8 has also been shown to result in a breakdown of cell-cell adhesions (and redistribution of cytolinker proteins to the cytosol from the cell border) (Long *et al*., 2006).

K19

KRT19 is part of chromosome 17q21 and comprises 6 exons. As yet, no disease or condition has been associated with *KRT19* mutation(s) (Whittock, Eady and McGrath, 2000), and K19 knockout mice do not display early embryonic defects. However, when bred with *KRT18*$^{-/-}$ mice, these K18/K19-null mice suffer from early embryonic lethality (E10.5), since both embryonic type I keratins have been removed (Hesse *et al*., 2000). It has been suggested that this is due to cytolysis and necrosis of trophoblast giant cells. E9.5 *KRT18*$^{-/-}$*/KRT19*$^{-/-}$ embryos were associated with trophoblast cells which had begun to separate from the maternal tissue due to necrosis. Hesse *et al*. (2000) demonstrated that these trophoblast giant cells did not have a keratin cytoskeleton through immunofluorescence; only K8 aggregates were observed.

The K19 protein is not restricted to stratified or simple epithelia, but has been described in the hair follicle bulge (Whittock, Eady and McGrath, 2000), nipple epidermis (Stasiak *et al*., 1989), epidermal basal cells (Whittock, Eady and McGrath, 2000), superficial layers of the conjunctiva (Krenzer and Freddo, 1997) and corneal epithelial cells (Offord *et al*., 1999). K19-positive cells have also been identified in tumours; K19 is often (but not always) observed in basal cell carcinomas, and occasionally observed in sebaceous tumours (Heyl and Mehregan, 2008). Low levels of the soluble fragments of K19 in the blood stream are observed in patients with differentiated thyroid carcinomas, although this increases in more poorly differentiated carcinomas (Giovanella *et al*., 2008). When blood levels of the soluble faction were investigated in breast cancer patients, no correlation was observed (Marrakchi *et al*., 2008). An investigation of full length circulating K19 showed that the protein was expressed and released by breast cancer cell lines and colorectal cancer cell lines; this was an active process, not a consequence of cell death (Alix-Panabières *et al*., 2009).

K19 is also suggested to be a marker of epithelial stem cells in different tissues. For example, Brembeck *et al*. (2001) suggest that the *KRT19* promoter may be useful in studying transdifferentiation of epithelial cells in the stomach and pancreas. K19 immunofluorescence has also been used alongside 3H-thymidine incorporation (producing label retaining cells) to label epidermal stem cells both *in vivo* and *in vitro* (Larouche *et al*., 2005). Low levels of K19 have been observed in murine embryonic stem cells, whereas human embryonic stem cells express moderate levels of K19 mRNA. Significant levels of K19 mRNA are expressed in human embryonal carcinoma cells (Maurer *et al*., 2008). In the hair follicle, K19-positive cells have been observed in the ORS (alongside K15) (Raposio *et al*., 2007). K19 is also

observed in the bulge of human hair follicles (Kloepper *et al*., 2008). Extensive investigation by Kloepper *et al*. (2008) has demonstrated in human hair follicles, K19 was present in the outermost layer of the ORS around the bulge region; heterogenous immunoreactivity was described around the bulge and distal to it.

K5/K14

K5 and K14 are expressed in mitotically active basal cells of the epithelium in the genito-urinary tract, digestive system, mammary glands and the skin. Mutations in *KRT5* and *KRT14* are known to cause forms of the epidermal disorder epidermolysis bullosa simplex (Casatorres *et al*., 1994; Romano *et al*., 2009). EBS patients display a large range of mutations in *KRT5* or *KRT14*; patients with severe EBS frequently tend to have mutations near to the end of the α–helical rod region, necessary for filament elongation. Milder forms of the condition display mutations outside of the α–helical domains (Chan *et al*., 1994). For example, the highly conserved regions at the end of the α-helical 1A rod domain is connected to the 2B end of the next dimer by a 10-11 residue head-to-tail overlap. Point mutations in these regions may affect filament formation (Steinert *et al*., 1993). It is in these regions where mutations may occur in *KRT5* or *KRT14*, causing EBS-DM (Dowling-Meara type) (Liovic *et* al., 2001; Müller *et al*., 2006). As mutations in these regions affect keratin filament assembly at an early stage, a more severe phenotype is expected (Irvine and McLean, 1999; Porter and Lane, 2003). This had previously been demonstrated by Letai *et al*. (1993): by engineering several transition mutations *in vitro*, the group showed that different keratin mutations had more or less severe affects on assembly, disease phenotype and susceptibility to mutagenesis. This said, more recently, Arin *et al*. (2010a) have highlighted that established genotype-phenotype correlations do not always accurately predict the effects of the disease on individuals – this is possibly due to the high number of *KRT5* and *KRT14* mutations; more than 150 have been reported so far on the Interfil.org website (http://www.interfil.org - Szeverenyi *et al*., 2008). Furthermore, there are two rare subtypes of EBS where the phenotype includes pigmentation: EBS-MP (EBS mottled pigmentation) (Fine *et al*., 2008) and EBS-MCE (EBS migratory circinate erythema) (Gu *et al*., 2003), which are not fully understood.

K8 and K18 (so-called 'simple epithelial keratins') are expressed in the embryo first, before K5 and K14 (the 'stratified epithelial keratins'). Romano *et al*. (2009) suggest that this is a critical event in development of the commitment to stratification in the epidermis. Inactivation of *KRT14* results in basal keratinocytes which lack a significant keratin network, leading to cytolysis (due to the inability to cope with the mechanical stresses of the skin). This EBS-like phenotype results in death of *KRT14*-null mice ~2 days after birth (Lloyd *et al*., 1995). This also indicates that K5/K15 keratin filaments are not capable of providing enough mechanical stability to support the integrity of the basal cells without K5/K14 fibres. Paladini and Coulombe (1999) suggest that this raises the question as to whether basal cells require K14 to achieve a functional keratin filament network. Lloyd *et al*. (1995) observe that there is no mechanism for upregulating K15 or any other type I keratin in the absence of K14, although Paladini and Coulombe (1999) claim that there is an increase in K15 levels in the absence of K14. In addition, K5 levels were not as reduced in the *KRT14*-null mice as expected in this study, given that K5 has been shown to degrade where K14 is not present (Chan *et al*., 1994). Where targeted deletion of K5 is used, research has suggested that K5 may also be a partner of K17 (as well as K15 and K14), and may induce K6 expression (this is different to the reaction of cells to K14 deletion) (Peters *et al*., 2001). Studies utilitising cells from patients with EBS can aid in research on

the effects of K5 and K14 mutations or ablation. For example, Morley *et al*. (2003) demonstrated that EBS keratinocytes migrated faster (following scratch wounding) than control keratinocytes (suggesting that this may be due to upregulation of stress-activated kinase pathways in these cells). Liovic *et al*. (2009) demonstrated that cell junction components are downregulated in EBS cells, caused by mutations in the helix boundary motifs of either K14 or K5 (the underlying mechanisms however are unclear). This finding was supported by creating the same mutation from wild type keratinocytes. In addition, real-time PCR was used to assess which junction proteins were affected. These included plakophilin 1, periplakin, laminin, α6-integrin, desmoglein, desmocollin, connexion, claudin and E-cadherin. Liovic *et al*. suggest that this may explain some EBS pathologies, such as acantholysis, and faster wound migration. The usual stresses coupled with the weakened cell junctions may contribute to a possible increased risk of non-melanoma skin cancers in EBS patients. Other studies have remained concerned with the mechanical stability of cells with K5 or K14 mutations; for example, Russell, Ross and Lane (2010) demonstrate that extracellular signal-regulated kinase (ERK) is expressed in mechanically stressed cells, inducing apoptosis.

It is also possible that mutations may also affect the role of keratins beyond mechanical stability. For example, Liovic *et* al. (2001) suggest that the location of the protein defect may be secondary in importance to the nature of the amino acid change when referring to genotype-phenotype correlations; no specific references to any function other than mechanic stabilisation were made however. Later work did demonstrate the effect of other proteins associated with keratins in EBS patients (Liovic *et al*., 2009; as described previously).

Bruen *et al*. (2004) studied burn re-epithelilisation in mice by creating transgenic mice expressing green fluorescent protein driven by the K5 promoter. The enhanced green fluorescent protein (EGFP) was observed in basal cells throughout the mouse, and the expression was strongest in the epidermis. It was noted that K5 promoter activity increased up to 75% during wound closure (up to approximately day 15 post-wounding). After this, K5 promoter activity gradually decreased, although was still greater than normal levels 21 days after wounding (Bruen *et al*., 2004). Further work has been carried out to investigate the initiation of K5 (and K14) expression. Several cis-regulatory regions in *KRT5* and *KRT14* have been identified, mostly toward the 5' end (which are highly conserved between these particular keratins) (Sinha *et al*., 2001; Kaufman *et al*., 2002); several expected transcription factors bind to the DNA regions studied, including AP-1 and AP-2, Sp1 and Ets (Romano *et al*., 2009). None of these transcription factors are restricted to the basal layer however. More recently, p63 and in particular the ΔNp63 isoform (which is restricted to the basal layer), has been identified as affecting K14 expression and K5 expression (Romano *et al*., 2007; Romano *et al*., 2009).

K1/K10

K1/K10 filaments are considered to be less dynamic than many other keratin pairs, suggesting that these fibres are important in forming a more rigid cell structure (Coulombe and Omary, 2002). K10 has been shown to aid cells resist mechanical trauma (Santos *et al*., 2002a), and K10 mutations have been shown to lead to severe skin fragility syndromes, such as epidermolytic hyperkeratosis (Syder *et al*., 1994; Reichelt and Magin, 2002). K10 has also been suggested to play a role in influencing cell cycling: ectopic expression of K10 has been shown to induce cell cycle arrest in proliferating cells (both *in vivo* and *in vitro*) (Santos *et al*., 2002b). Ectopic expression of K10 in the basal layer (in this case, under control of the *KRT5*

promoter) impaired tumour development and inhibited cell proliferation (Santos *et al*., 2002b). K10 levels are reduced during hyperproliferation, whether normal (such as in wound healing) or pathological (as in epidermal tumours, for example) (Santos *et al*., 2002b). Previously, Fuchs and Green (1980) had suggested that this was due to K10 replacing K14 as keratinocytes lose contact with the basement membrane and entered terminal differentiation, becoming post-mitotic in the process (i.e. K10 is important in maintaining and/or inducing the post-mitotic state of suprabasal cells). This is thought to be through the induction of the oncoprotein c-Myc, important in growth control and cell cycle progression; c-Myc expression is minimal under normal conditions, but increased levels were found in the basal layer in *KRT10*$^{-/-}$ mice (Reichelt and Magin, 2002). In this study, the basal cells were also found to increase in size (also thought to be as a result of increased c-Myc levels). c-Myc plays a role in cell proliferation and differentiation: induction drives quienscent cells into the cell cycle (Yu *et al*., 2009). Overexpression of c-Myc has been identified in several tumours, for example, breast cancer (Aulmann *et al*., 2006). Arnold and Watt (2001) have also demonstrated that activation of c-Myc can drive keratinocyte stem cells to the transit amplifying compartment, stimulating proliferation and differentiation along the sebaceous and epidermal lineages. Furthermore, it has been shown that c-Myc affects transcription of proteins important in maintenance of the cytoskeleton and cell adhesion. This resulted in inhibited cell motility (and wound healing). Cell spreading was also shown to be impaired (Frye *et al*., 2003). *KRT10*$^{-/-}$ mice have a faster keratinocyte turnover than wild-type mice; Reichelt *et al*. (2004) suggest that this accelerated turnover increases the rate of elimination of keratinocytes at the early stage of tumour development. This increase in turnover is likely to be mediated by the activation of MAPK pathways in the epidermis (Reichelt *et al*., 2004). Recently, AP-2α has been shown to block some of the detrimental effects of c-Myc overexpression in HaCaT cells, such as an increase in reactive oxygen species (ROS) and apoptois (Yu *et al*., 2009). AP-2 has also been shown to affect K10 expression directly through interaction with C/EBPα and C/EBPβ - C/EBPα and C/EBPβ have three binding sites on the *KRT10* promoter region. This mechanism is threefold: 1) AP-2 and C/EBPβ are most common in the lower epidermis and C/EBPα in the upper epidermis; 2) both C/EBPα and C/EBPβ have binding sites in the AP-2α promoter region; 3) there are AP-2 binding sites in the C/EBPα promoter region (which suppresses C/EBPα transcription) (Maytin *et al*., 1999). Zhu *et al*. (1999) also demonstrated that K1 and K10 expression was directly controlled (at least partially) by C/EBPβ in BALB/MK2 keratinocytes, and C/EBPβ-deficient mouse skin demonstrated decreased K1 and K10 expression in a hyperplastic epidermis. A K10 knockout mouse has been developed which has provided a murine model of the skin disorder epidermolytic hyperkeratosis. Reichelt *et al*. (1997) suggest that the skin fragility observed in K10 knockout mice is as a consequence of the decrease in K1/K10 filaments and the compensatory increase in K6/K16 filaments, which are not as robust (Porter *et al*., 1996). Where no K10 is present in *KRT10*$^{-/-}$ mice, hyperproliferation was demonstrated, alongside induction of K6 and K16 (normally present in wound healing - and therefore hyperproliferative - situations) (Reichelt and Magin, 2002); McGowan and Coulombe (1998) previously suggested that hyperproliferation could occur because the presence of K10 is not compatible with cell migration or proliferation. Reichelt and Magin's study also found that there was no spontaneous tumour formation in *KRT10*$^{-/-}$ mice. Following *in vivo* ablation of K10 in suprabasal keratinocytes in mice, Reichelt and Magin (2002) demonstrated that proliferation was not induced in these cells, but in cells residing in the basal layer,

suggesting that hyperproliferation is an indirect consequence of K10 ablation, and that signaling must occur between these cell compartments. Furthermore, Reichelt and Magin suggest that this could be due to susceptibility to mechanical stress and the signaling events which are involved, or through cytokines such as TGF-β. Further work using targeted deletion of K10 has demonstrated an increase in the proliferation and differentiation of sebocytes; sebaceous glands of *KRT10*$^{-/-}$ mice demonstrate increased cell turnover as well as an increase in secretions, such as wax esters, cholesterol esters and triglycerides (Reichelt *et al*., 2004). This suggests that lack of K10 pushes cells towards sebocyte differentiation in this region.

K6/16/17

There are three isoforms of K6, as listed in *Table 1.2*. K6a is more abundantly expressed than K6b; this is most obvious in mRNA studies of skin explant cultures (Tyner, Eichman and Fuchs, 1985). K6 is expressed in glandular epithelia, the outer root sheath of the hair follicle, the stratified epithelia of the oesophasgus and oral mucosa and in the tongue (Moll *et al*., 1982; Ouhayoun *et al*., 1985; Stark *et al*., 1987). K6 is not usually present in epidermis (with the exception of some specific sites such as soles of the feet and palm), and K16/K6 induction is associated with a hyperproliferative state, such as during wound healing, disease, or cancer. K6 expression is also induced when primary epithelial cells are seeded in culture. Epidermal injury triggers induction of K6, K17 and K16 in activated keratinocytes at the wound edge (where K1 and K10 expression decreases). Following wound closure, this pattern of keratin expression is reversed (Wong and Coulombe, 2003). Transgenic mice null for isoforms of K6 (K6a and K6b) die shortly after birth, due to the fragility of the epithelia in the oral mucosa; in their 2003 study, Wong and Coulombe use an *ex vivo* skin explant culture and *in vivo* tissue grafting to study K6 following injury. K6a/K6b-null keratinocytes demonstrated a greater ability to migrate compared to the wild-type, and K16 levels were also decreased. The cells at a wound edge were also observed to be more fragile than controls. In explants from transgenic mice where K16 is upregulated, a delay in outgrowth is observed, and wound healing is slower than normal (Wawersik *et al*., 2001).

In terms of primary structure, K6 is closely related to K5 and K16 and K17 are related to K14. Due to their similarity in structure to K5 and K14, and the downregulation of K1 and K10 following induction of K6 and K16 and K17, Wong and Coulombe (2003) suggest that K6, K16 and K17 are intermediates between these other keratin pairings, particularly in terms of mechanical properties. Reduced levels of K6/K16 leave keratinocytes in a state observed *in vivo* in the basal layer (i.e. K5/K14-positive cells), which are more pliable and better equipped for migration. K1/K10 however equip cells with greater mechanical strength, but without the same pliability. Furthermore, Wong and Coulombe (2003) suggest that K6/K16/K17 expression at the wound edge is a compromise between these two states – the pliability (for migration, for example) and the mechanical strength to withstand the wound environment.

K6 has also been shown to be a marker of early mammary gland development (without being normally present in the adult) (Grimm *et al*., 2006). In C/EBPβ null mice however, K6 can be observed in the mature mammary gland (Grimm *et al*., 2002).

K17 is observed in both adult and foetal skin, although is expressed more so in foetal skin. A study by Coolen *et al*. (2010) assessed K17 expression throughout development. K17 was observed in the basal layer and periderm, and intermittently in the intermediate layer at 13-14 weeks post-conception. Expression in the basal layer reduces between 16 and 20 weeks, whilst remaining constant in the periderm up to 22

weeks. K17-positive cells in the hair follicles can be seen as they begin to develop at around 16 weeks (Coolen *et al*., 2010). It is likely that the early K17-positive cells in the developing epidermis have a non-epidermal fate, such as contributing to the sebaceous gland, periderm or hair follicle. K17 remains in the hair follicle of human adults in the ORS and medulla, and appears to be weakly expressed in cells lining the dermal papilla (Langbein *et al*., 2010). Mutations in *KRT17* give rise to steatocystoma multiplex (typified by sebaceous gland cysts) and type 2 pachyonychia congenital (which affects epithelial appendages, particularly nails) (Munro *et al*., 1994; also reviewed by Irvine and McLean, 1999). K17-null mice develop alopecia in the week after birth; this correlates with hair shaft fragility and apoptosis of the hair bulb (McGowan *et al*., 2002). Tong and Coulombe (2006) have demonstrated that this apoptosis is due to the premature onset of catagen. Primary keratinocytes of K17-null mice grown in culture were also more sensitive to TNFα via TRADD and, as discussed previously, K17 is involved in 14-3-3 mediated regulation of mTOR. Ablation of TNFα, required for timely anagen-catagen transition in mice, partially rescues the hair-cycling defect observed in K17-null mice (Tong and Coulombe, 2006). K17 induction has also been associated with inflammation, and markers of immune cells in *KRT17*$^{-/-}$ mice were reduced compared to controls (DePianto *et al*., 2010).
K17 is also expressed in some squamous cell carcinomas and cervical intraepithelial neoplasias; this correlated with increasing lesion grade (Ikeda *et al*., 2008).

1.3: Keratin 15
K15 is a Type I keratin of 50kDa, which forms heterodimers with the Type II keratin K5 (Chu and Weiss, 2002). The K15 protein had been described in several papers as an acidic keratin as a minor component in epithelia (Whitbread and Powell, 1998), sebaceous glands, hair follicle ORS and the human adult epidermis (Moll *et al*., 1982). The majority of publications describing K15 study have focused on K15 as a stem cell marker because of its high expression in the hair follicle bulge.

1.3.1: Keratin 15 Gene
Nozaki *et al*. (1994) first isolated the K15 gene (*KRT15*) in mice and determined its nucleotide sequence, following isolation of K19, which was shown to have a high sequence homology to human *KRT15*; for example, there are homologous sequences around the TATA boxes in the 5' upstream regions of both *KRT15* and *KRT19* genes. The first antibody created against K15 was a guinea pig polyclonal antibody (clone gp15.1); the antigen is the C-terminal 'tail' region (and part of the rod domain) of human K15. As with other type I keratins (apart from *KRT18* and *KRT19*), *KRT15* has seven introns and eight exons. In another of the early papers discussing K15 in relation to K14 ablation, Jonkman *et al*. (1996) suggest that following K14 ablation, increased levels of K15 are observed; three years later, Paladine and Coulombe (1999) published similar findings (discussed above). Jonkman *et al*. (1996) suggest that K15 did not aggregate to higher-order bundles, and this reduced the stability of the cell (as previously observed by Lloyd *et al*., 1995).

1.3.2: Keratin 15 Expression
K15 is specifically expressed in the basal cells of the sebaceous gland, epidermis and tongue, stomach, bronchial and cervical epithelia. In addition, K15 expression has been described throughout the oesophageal epithelium and the hair follicle (Leube *et al*., 1988; Smedts *et al*., 1993; Lloyd *et al*., 1995; Waseem *et al*., 1999; Liu *et al*., 2003) – in particular, the stem cells of the hair follicle. This latter is currently still

under debate, with different K15 antibodies staining different cell populations (for example, Poblet *et al*., 2006; Kloepper *et al*., 2008; Tiede *et al*., 2010). There is also debate over which cell population K15 is present in, depending on differentiation of cells occurring in or around the bulge region of the hair follicle (see section 1.1.2).
Whitbread and Powell (1998) described K15-positive cells throughout the hair follicle and the outer root sheath (ORS) of sheep. Cells in the bulge region of the hair follicle however were described as negative for K15, and the protein was observed at variable levels in the basal layer. In contrast to K15 expression in human tissue, K15-positive cells were only noted in the basal layer of the oesophagus in sheep. Whitbread and Powell (1998) also demonstrated that there were no consequences of overexpression of K15 in transgenic mice (with a cDNA sheep *KRT15* construct). From their results, Whitbread and Powell suggested that K15 expression marks an early stage of keratinocyte differentiation, preceding the decision to become epidermal or hair-like. Likewise, Waseem *et al*. (1999) demonstrated that K15 was present in the basal keratinocytes of stratified epithelia, and that the ORS was K15-positive. In addition to the work by Jonkman *et al*. (1996) (which also utilised sections from patients who did not express K14) and Paladini and Coulombe (1999) (who used K14-null mice), Waseem *et al*. demonstrated that in sections from an individual in which both K14 alleles were naturally ablated, K14 ablation in the ORS resulted in increased K15 protein and mRNA expression throughout the follicle. Conversely, K15 levels were seen to be reduced in activated (hyperproliferative) keratinocytes, which can be observed in conditions such as psoriasis – i.e. *KRT15* expression is downregulated in order to maintain an activated phenotype in keratinocytes. This is also observed *in vitro* in 3D cultures, were hyperproliferative cells are K15-negative. Once grafted onto mice however, cells began to express K15 (Smiley *et al*., 2006). Studies into psoriasis suggested that there are three key processes in psoriasis: proliferation, differentiation and inflammation (for example, Das *et al*., 1992). A suggested marker of successful treatment is an increase in K15 levels (van der Velden *et al*., 2010).

1.3.2.1: Keratin 15 Expression in Carcinomas
Research studying K15 in cancers was also being carried out during the late 1990s. Jih *et al*. (1999) studied trichoepitheliomas and basal cell carcinomas from different patients for K15, using the C8/144B antibody (this DAKO monoclonal antibody was originally raised against CD8, although Jih *et al*. established that it also cross-reacted with K15). All trichoepitheliomas and some tumours of follicular origin were found to be K15-positive; conversely, all squamous cell carcinomas studied were K15-negative. From their results, Jih *et al*. concluded that trichoepitheliomas and basal cell carcimomas originate from the (K15-positive) bulge region of the hair follicle. Utilising the same antibody to observe K15, Kanitakis *et al*. (1999) suggested that basal cell carcinomas were K15-positive, and were therefore not differentiating towards hair bulge cells. Furthermore, Kanitakis *et al*. suggested that based on their results, K15 immunostaining was useful in differential diagnosis between trichoepitheliomas and basal cell carcinomas. It has further been suggested that there is peripheral localisation of K15 in trichoepithelioma compared to basal cell carcinomas (Choi *et al*., 2008). In cutaneous mixed tumours (i.e. those which contain both mesenchymal and epithelial cells, and can be apocrine or eccrine in nature), some regions were shown as K15-positive (using the C8/144B antibody); these were noted to be in 'less differentiated' regions of the tumours (Minami *et al*., 2004). A small percentage (5%) of breast carcinomas were also found to be K15-positive (Celis *et al*., 2007). However, use of the C8/144B antibody in identifying the K15 protein was recently questioned by Pontiggia *et al*. (2009), who demonstrated that the human

stratum basale was C8/144B-negative, but K15-positive. Bieniek *et al*. (2007) have also published finding several other tumours which contained K15-positive cells, including sebaceous carcinomas, adenomas and hyperplasias. The K15-positive cells were observed in the basal, less differentiated regions of the tumour. More differentiated cells were negative for K15. Such findings could be considered as evidence for a (K15-positive) cancer stem cell population. More recently, the presence of 'tumour-initiating cells' has been discussed, which are also positive for some stem cell markers, such as CD44. These cells may not respond effectively to common therapeutic procedures (Costea *et al*., 2008). Harper *et al*. (2010) have recently shown that populations of malignant human epithelial cells which have stem cell-like properties are more resistant to apoptosis. This is likely to be associated with an extended G2 cell cycle phase. In order to overcome this therapeutically, Harper *et al*. (2010) suggest targeting G2 checkpoint proteins, since those cells released from G2 are more prone to apoptosis.

1.3.2.2: Regulation of K15 Expression

Transcription Factors

Some initiation of keratin expression occurs at the transcription factor level (Roop *et al*., 1987). Most of the influential transcription factor binding sites are observed upstream of the gene, but some remain in introns and downstream regions. Cell type-specific enhancer regions have been observed in proliferating stratifying epithelial cells, yet not in their simple epithelial counterparts (Blessing *et al*., 1987).

The only common transcription factor binding site found in all keratin gene promoter regions is the TATA box (or a variant) in the minimal core promoter region, showing that the TFIID transcription factor is essential in initiating all keratin gene transcription. This is flanked by an Sp1 binding site, which is essential for the increased activity of the promoter (Delouis *et al*., 2005).

Groups of keratins then have other transcription factors in common, for example, the CCAAT box. There are several CCAAT-enhancer binding proteins (C/EBP) including (at least) C/EBPα, C/EBPβ (also known as LAP, CRP2, NF-IL6, IL6DBP or NF-M), C/EBPδ (or CRP3 or NF-IL6β), C/EBPγ (Ig/EBP-1), CRP1, CHOP10 (or GADD153) and d/CEBP, all members of the DNA binding protein family bZIP. Heterodimerisation allows interaction between C/EBPs and other transcription factors, such as NF-κB (Stein *et al*., 1993) and AP-1 (Hsu *et al*., 1994); this adds a further level to the functional properties of these transcription factors. Oh and Smart (1998) have demonstrated that C/EBPα, C/EBPβ and C/EBPδ mRNA are expressed at relatively high levels in murine epidermis and in primary keratinocytes (although C/EBP mRNA levels do not consistently coincide with protein levels i.e. protein expression is controlled at the translational level or post-transcriptionally). The expression of C/EBPα and C/EBPβ is upregulated in suprabasal keratinocytes *in vivo* and during calcium-induced differentiation of primary keratinocytes *in vitro*. Most C/EBPα present in the epidermis is restricted to the nuclei and cytoplasm of suprabasal cells, although some is observed in a few basal cells. However, as C/EBPα is observed in keratinocytes cultured in 0.05mM Ca^{2+}, Oh and Smart (1998) suggest that there is a function for C/EBPα in basal keratinocytes *in vivo*. During calcium-induced differentiation *in vitro*, Oh and Smart also observed increased levels of C/EBPα and C/EBPβ, further suggesting that these isoforms are important in regulating genes affecting differentiation (or genes expressed during differentiation). Without C/EBPα and C/EBPβ in the epidermis, there is increased keratinocyte proliferation and impaired differentiation.

Sp1 is a zinc-finger-containing DNA binding protein, distributed throughout several cell types. There are four domains (A, B, C and D) required for optimal transcription, which is always enhancing (as opposed to suppressive). However, in the epidermis, Sp1 activates transcription of genes for cornified envelope precursors and keratins throughout differentiation (Eckert, Crish and Robinson, 1997).
Additional Sp family proteins have also been identified, named Sp2-Sp8; unlike Sp1, these can be both activators and suppressors of genes (Erkert, Crish and Robinson, 1997; Bouwman and Philipsen, 2002). Masson-Gadais *et al*. (2006) demonstrated that both Sp1 and Sp3 are expressed by both cultured adult keratinocytes and newborn foreskin keratinocytes. Terminal differentiation also correlated with loss of Sp1 and Sp3. Culturing cells in different levels of calcium also affected Sp1 and Sp3 levels in primary keratinocytes. Furthermore, it was demonstrated that post-translational phosphorylation affected the DNA binding ability of Sp1 and Sp3 (Masson-Gadais *et al*., 2006).
Activator protein 1 (AP-1) is important in upregulating several differentiation specific proteins involved in the conversion of basal keratinocytes to spinous and granular cells (Rossi *et al*., 1998). AP-1 consists of heterodimers between fos (c-fos, fosB, fra1 and fra2) and jun (junB, junD and c-jun) transcription factors as well as homodimers of the latter (Angel and Karin, 1991). c-jun and junD have been shown to positively regulate epidermal specific genes, whereas junB acts as a suppressor (Rossi *et al*., 1998).

The KRT15 *Promoter*
Following a paper by Whittock *et al*. (2000) describing the genomic organisation of K15, further research was carried out to study the promoter region of *KRT15* and the transcription factors associated with it. As well as the work by Whittock *et al*. (2000), Talbot, Loring and Schorle (1999) also identified a potentially important transcription factor in *KRT15* expression by examining the skin of AP-2α–deficient mice. AP-2 levels have been reported to decrease during keratinocyte differentation (Wanner *et al*., 1996). There are five known murine AP-2 proteins expressed in the skin, and AP-2α has been shown to be the most highly expressed (Byrne *et al*., 1994). In contrast, low AP-2 mRNA levels were observed in postconfluent HaCaT cells; it is possible that this is either due to loss of proliferative capacity and/or initiation of differentiation. It has also been observed that *in vivo*, AP-2α is expressed to a greater extent in basal cells (i.e. those not actively cycling). From these results, Wanner *et al*. (1996) suggest that AP-2 has an important role at the onset of differentiation in activated keratinocytes. AP-2 has also been observed in embryonic mice (E15.5), localised to the (proliferating) basal keratinocytes (and not observed in the differentiating suprabasal cells) (Byrne *et al*., 1994). AP-2α is normally present in the nucleus of some basal and suprabasal cells of the epidermis (Wang *et al*., 2006). In an AP-2α knock-out targeted to the epithelium (using 'Cre-lox' methods), there were increased levels of epidermal growth factor receptor (EGFR, also known as ErbB1) in the epithelium compared to controls, leading to hyperproliferation (Wang *et al*., 2006). It was also noted that other AP-2 proteins did not compensate for the loss of AP-2α. AP-2α apparently represses K5 expression in cultured keratinocytes (Byrne *et al*., 1994), although *in vivo* it must be considered that K5 expression is restricted to the basal layer whereas AP-2 is not restricted to this region. It has been demonstrated *in vitro* however that Akt, phosphoinositol-3-kinase and MAPK fail to function properly; this suggests that downregulation of AP-2 expression can contribute to tumourigenesis (Wang *et al*., 2006).

Growth Factors

Growth factors have also been demonstrated to affect *KRT15* expression. TGFβ was shown to suppress *KRT15* when expressed during wound healing (where K14 levels increased) (Werner and Munz, 2000). As in Waseem *et al*. (1999), activated keratinocytes were K15-negative. K15 expression was also noted to be suppressed by TNFα, EGF and KGF. In HaCaT cells, increased TGFβ1 was shown decrease K15 levels (Werner and Munz, 2000). Transforming growth factor (TGF)α is one of the major factors controlling growth of epidermal cells. In normal skin, TGFα has a role in controlling epidermal thickness during development and differentiation (Vassar and Fuchs, 1991). Using the K14 promoter to target expression of TGFα to the squamous epithelia, the regions observed to be most responsive to this overexpression of TGFα were those areas of skin which were normally thick and where hair follicle density was low. In these areas, these qualities were emphasised. The increased epidermal thickening was found to be due to an increase in the number of basal, spinous, granular and stratum corneum cells coupled with cell hypertrophy. It was also observed that EGFR expression was not affected by TGFα (Vassar and Fuchs, 1991). When calcium is used to initiate differentiation *in vitro*, TGFα prevents the expression of K1 and K10 whilst K8 and K18 are expressed.

Due to the role of TGFα in epidermal thickening in normal skin, it is also thought to be involved in psoriasis (Vassar and Fuchs, 1991); presumably this is through the same route as the role KGF has in psoriasis (see Das *et al*., 2009).

TGFβ also regulates keratinocyte function. Unlike TGFα however, TGFβ suppresses cell proliferation and induces synthesis of extracellular matrix proteins. TGFβ1 is also a tumor suppressor in normal keratinocytes, acting via Smads (and other pathways) (Bae *et al*., 2009). Alternately, TGFβ1 may also enhance the malignant properties of tumor cells by affecting epithelial-mesenchymal transition (EMT), invasion, metastasis or anti-tumor immunity; this alteration may depend on other pathways, such as *ras* which are activated in tumors (Bae *et al*., 2009) or Wnt/β-catenin activity (Roarty *et al*., 2009).

Although the TGFβ isoforms (TGFβ1-3 in mammals) are very similar (conserved across species with 70-80% homology), they are differentially expressed *in vivo* in embryogenesis, carcinogenesis and tissue repair (for a review, see Cho *et al*., 2004). This suggests distinctive roles for these isoforms. TGFβ1 is localised to the upper, more differentiated layers of the epidermis. TGFβ2 and TGFβ3 immunostaining suggests that these isoforms are present in the suprabasal layers. A role for TGFβ has also been described in psoriasis; in transgenic mice where TGFβ expression was controlled by the K5 promoter, severe inflammation of the skin was observed (Han *et al*., 2010).

K15 expression has also been shown to be affected by several other factors. Decreases in insulin and insulin-like growth factor also led to decreases in K15 levels *in vivo* (and further study demonstrated a loss in label retaining cells); Rac was shown to aid recovery (Stachelscheld *et al*., 2008). Previously, retinoic acid, glucocorticoid receptors and NF-κB were shown to suppress the *KRT15* promoter, whilst C/EBPβ and AP-1 induced it (Radoja *et al*., 2004). As thyroid hormone and IFN-γ were also shown to activate the *KRT15* promoter, Radoja *et al*. (2004) also suggested that these may be potential treatments for EBS (i.e. to aid upregulating K15 to compensate for inadequate K14). As well as in HaCaT cells, K15 was also studied in a human breast epithelial cell line by Badock *et al*. (2001). Badock *et al*. suggest that K15 is

responsible for the mechanical integrity of these cells, and that apoptosis-induced cleavage (by caspase) results in three fragments of the K15 protein.
Binding of epidermal growth factor (EGF) to its receptor (EGFR) has been shown to increase motility, affect proliferation and cause degradation of the extracellular matrix in several cell types (Jiang *et al*., 1993). Binding causes dimerisation of the EGFR activating the intracellular protein tyrosine kinase. This begins a chain of phosphorylation events conveying the signal from the cell membrane to the nucleus, where nuclear proteins regulate gene expression and cell division (Jiang *et al*., 1993; Tomic-Canic *et al*., 1998). In keratinocytes, the activation of the EGFR causes proliferation and degradation of the extracellular matrix which allows migration and cell spreading (for example, Barrandon and Green, 1987b; Jiang *et al*., 1993; Hernández-Quintero *et al*., 2006). An increase in β-catenin is also observed in keratinocytes following treatment with EGF (Hernández-Quintero *et al*., 2006). This response occurs in both pathological situations and under normal circumstances, such as in embryonic development (where EGFR are observed throughout all layers). (EGFR are also observed in the adult, although primarily in the basal layer and occasionally in the suprabasal layers.)
Keratinocyte growth factor (KGF), part of the fibroblast growth factor (FGF) family, is synthesised by cells in the dermis and active in the epidermis (Guo *et al*., 1993). Guo *et al*. (1993) suggest that KGF interferes with the signaling of some mesenchymal-epithelial interactions, affecting development, differentiation and growth of skin. In generated skin equivalents *in vitro*, added KGF induced thickening, flattening of the rete ridges, some disorganization including the granular layer and stratum corneum and morphological changes in basal cells. Immunostaining for Ki67 also demonstrated that proliferation extended above the basal layer and into the suprabasal layer, whilst K10 and transglutaminase expression was delayed (Andreadis *et al*., 2001). It is also possible that the hyperproliferation of keratinocytes observed in psoriasis is (at least partially) due to excess KGF, possibly secreted by activated T-cells (Das *et al*., 2009).

1.3.2.3: Keratin 15 Expression in the Foetus
Islam and Zhou (2007) identified epidermal stem cells from goat foetus by their rapid adherence onto collagen type IV *in vitro*; these cells were also K15-positive. Islam and Zhou also noted that these cells had a high nuclear to cytoplasmic ratio, typical of stem cells (see also Liu *et al*., 2008). Talbot *et al*. (1999) observed that no K15-positive ectodermal cells were observed where lens induction occurs in the eye in AP-2α–deficient mice. In human eye development, limbal cells were identified as K15-positive, and remained so throughout embryonic development (from week 6 onwards) (Lyngholm *et al*., 2008).

1.3.2.4: Keratin 15 in the Hair Follicle Bulge
There is an ongoing debate about whether there are stem cell populations, progenitor cell populations or TAC populations in and around the hair follicle, despite the isolation and culture of several cell types from the region (for example, see Gutiérrez-Rivera *et al*., 2010). Hair follicle transplants of both upper and lower portions of the dissected follicle result in survival following transplantation. The use of keratinocyte cultures and skin patches (for covering burns for example) have established through lineage tracing that ‘gene-corrected’ stem cells remain stable throughout the life of the patient (Mavillo *et al*., 2006; Gutiérrez-Rivera *et al*., 2010). These studies and results suggest that several populations are available in and around the hair follicle

which can maintain the structure, which contributes to the ongoing controversy regarding the cell population with the greatest *in vitro* proliferative potential.
In order to establish whether bulge cells were stem cells (and whether a suggested sub-population of K15-positive cells were bulge stem cells), the presence of other, more established stem cell markers have been examined in the hair follicle. Trempus *et al*. (2003) observed the staining pattern of CD34 (a blood system stem cell marker) in mice, claiming co-localisation with K15 in label retaining cells. CD34-positive cells were observed in the membrane of the bulge region in mice, and these cells were in G0 or G1 phase, indicating a higher proliferation potential that CD34-negative cells (which were found to be in G2/M or S phase). A later study in human skin described a different CD34-positive population residing in the ORS below the bulge during anagen (no CD34-postive cells were identified during catagen or telogen). There was also no K15/CD34 co-localisation (Poblet *et al*., 2006). Raposio *et al*. (2007) suggest that there are some CD34-positive cells in the bulge region in human hair follicles, but these constitute a very low percentage (1-2%).
Kloepper *et al*. (2008) suggest that the best *in situ* markers of human bulge cells are K15, K19 and CD200 (these cells were also CD34- and nestin-negative). These cells were shown to be unaffected by aging. Gutiérrez-Rivera *et al*., (2010) suggest that in human skin CD34-negative cells are stem cells or early progenitor cells, whilst CD34-positive cells may be 'transit-amplifying precursors' for hair follicle sheath cells. The CD34-postive cells of the ORS however apparently had the same properties (particularly with regards to migration and their ability to create an epidermis in short-term organotypic cultures) as CD34-negative cells residing in the bulge region and IFE. Hedgehog (Hh) affects these K15-positive, CD200-positive, K19-positive cells; Hh levels are raised in the bulge region, and is reduced in differentiated keratinocytes. *In vitro*, Hh was shown to maintain the bulge phenotype by sustaining K15 and K19 levels (Rittié *et al*., 2009).
Similarly to Trempus *et al*. (2003), Han *et al*. (2003) observed K15 expression in foetal murine epidermal stem cells (~95% of which were in G1 phase, indicating slow cycling cells). The use of label retaining studies to establish stem cell populations was also utilised by Braun *et al*. (2003), who studied LRCs in the hair follicle. Despite changes in hair follicle morphology throughout hair cycling, the permanent region of the follicle contained K15-positive LRCs (see also Nijhof *et al*., 2006).
Another group have however suggested that stem cells in the bulge region are K15-negative. Amoh *et al*. (2005a) used nestin (an established neural stem cell marker) to separate stem cells from other cells in the bulge. Nestin-positive, CD34-positive, K15-negative cells isolated from the bulge were shown to be capable of differentiating into keratinocytes, melanocytes, smooth muscle cells and neurons *in vitro* (Amoh *et al*., 2005a) as well as Schwann cells and blood vessels (Amoh *et al*., 2005b; also Hoffman, 2007). From this study, Amoh *et al*. (2005b) suggest that K15-negative cells in the bulge are multipotent stem cells, whereas K15-positive cells are more differentiated. Later, Amoh *et al*. (2008) also demonstrated that hair follicle stem cells were capable of promoting repair of severed nerves in mice. Kanoh *et al*. (2008) also considered hair follicle stem cells to be nestin-positive, and these cells gave rise to the ORS. It is likely that these are mesenchymal cells.
Despite the studies by Amoh *et al*. however, K15 continues to be used to mark epidermal bulge cells, which were frequently referred to as stem cells. For example, K15 has been used as a bulge stem cell marker in a study investigating the effects of laser hair removal (where the bulge region was seen to remain unchanged) (Orringer *et al*., 2006). In order to more closely study bulge cells, an *in vitro* cell line was established from human hair follicles (Tel-E6E7 cells); these cells were all K15-

positive (Roh *et al*., 2008). The study of the bulge region in alopecia has also utilised K15 as a marker. For example, in primary scarring alopecia, K15-positive cells were observed throughout the stages of the scarring alopecia, suggesting that this condition is not caused by the destruction of the bulge stem cells (Pozdnyakova and Mahalingam, 2008, Hoang *et al*., 2009). Only around 50% of non-scarring alopecia bulge regions were positive for K15, although K15 was observed in the ORS of both scarring and non-scarring alopecia samples (Hoang *et al*. 2009). Similarly, K15-positive bulge cells have been shown to be involved in the condition cutaneous lupus erythematosus. Observed changes in the K15 expression of the bulge suggests a stem cell involvement; this may explain the scarring and irreversible alopecia associated with the condition. Expression of K15 reduces as stem cells then become damaged (Al-Refu *et al*., 2009). The role of bulge cells in primary cicatricial alopecia has also been investigated; this type of alopecia is caused by destruction of the bulge, described as an 'immune privileged' area. Increases in MHC I and II in the bulge region leads to a decrease in K15 levels and ultimately eradication of the bulge (Harries *et al*., 2010).
This said, Wu *et al*. (2005) warn that study of the bulge region using immunofluorescence needs careful controls, as autofluorescence is common in this area.

Studies of Keratin 15 Promoter Activity in the Hair Follicle
As well as elucidating the presence of the K15, some studies have examined the promotor region of *KRT15* to look for activity. For example, Liu *et al*. (2003) created *KRT15/lacZ* transgenic mice to measure K15 promoter activity throughout the life of the mouse. Bulge cells remained K15-positive, despite *KRT15* promoter activity changing with age. *KRT15* promoter activity also correlated with levels of differentiation throughout the epithelium (Liu *et al*., 2003). In a study assessing the contribution of bulge stem cells to wound healing, Ito *et al*. (2005) used *KRT15* promoter activity as a marker of bulge stem cells and to elucidate their role following wounding in mice. Ito *et al*. established that bulge cells were recruited to the epidermis following injury, producing TACs for acute wound repair; these were found to be eliminated from the healed wound site within weeks following healing. By inserting a suicide gene under the *KRT15* promoter (herpes simples virus thymidine kinase – HSV-*TK*), in the same paper Ito *et al*. (2005) report that the hair follicle is lost, but the epidermis survives. Driven by the *KRT15* promoter, the HSV-*TK* converts the nucleoside analog ganciclovir into a toxic anolog in mice; administration of ganciclovir caused bulge cell death and gastro-intestinal injuries. When skin containing this same insertion was grafted onto immunodeficient mice and ganciclovir administered, hair loss began 4 days post-treatment. By 8 days post-treatment, hair follicles had been lost from the graft. This demonstrated that bulge cells were not required for epidermal survival.

1.4: Aims of the Present Study
K15 expression is generally recognised as a signal of early stage keratinocyte differentiation, and a marker of bulge cells, considered stem cells of the hair follicle. However, *KRT15* expression has also been demonstrated in interfollicular epidermal cells. More recently, publications have suggested that K15-positive cells may not be stem cells, but cells which are more differentiated.
The aim of this study then is to use siRNA to knock-down K15 in keratinocytes and assess the effect of K15 on proliferation, differentiation, adhesion, spreading and

migration in both keratinocytes and squamous cell carcinoma cells. This may give further indication of the differentiation state of cells which express K15.

Chapter 2:
Materials and Methods

2: Materials and Methods

All chemicals and reagents used in this study were of analytical or tissue culture grade.

2.1: Cell Lines and Culture Methods

Five cell lines have been used in this study. The most extensively used were HaCaT cells, a spontaneously transformed human immortalised cell line that has been used extensively in the study of differentiation. HaCaT cells, originally described by Boukamp *et al*. (1988), were established from a section of full thickness skin from a 62 year old male. The section had fat and dermis removed before being placed in trypsin solution, allowing the epidermis to be separated from the dermis and cultured. HaCaT cells became so widely used as they could be easily maintained in culture, were nontumourigenic and did not require, for example, infection with SV40 or similar to achieve immortality. Boukamp *et al*. (1988) cultured the HaCaT cells to at least passage 140 and noted their continued capacity for normal differentiation and few (if any) chromosomal abnormalities.

The MET cell lines, originally described by Proby *et al*. (2000) are spontaneously immortalised cells taken from a patient with squamous cell carcinoma. MET 1 cells were isolated from a primary cutaneous tumour, MET 2 and 3 from local reoccurances and MET 4 from a distant metastases. Since MET 2 and 3 cells are similar, only MET 2 cells were used in this study. Further details about this cell line are described in Chapter 5.

The fifth cell type used in this study is the normal human epidermal keratinocyte (NHEK) (PromoCell, UK). These cells are primary cells isolated from juvenile foreskin. These cells will only proliferate up to passage 3 or 4 before terminally differentiating. This meant that large numbers of these cells could not be cultured, and not all experiments could be carried out using these cells. This said however, they were useful as a 'normal' comparison to HaCaT cells.

2.1.1: Cell Maintenance

HaCaT and MET cells used in this study were cultured at 5% CO_2 in Dulbecco's modified Eagle's media (DMEM) (Sigma-Aldrich, UK) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, UK) and 1% antibiotics/antimycotics (Invitrogen, UK). Stock cells were cultured in T75 flasks (Greiner Bio-one, UK), and not grown above 80% confluency (preventing differentiation). Cells were passaged using 0.25% trypsin (Sigma-Aldrich, UK) in tissue culture grade 1x PBS (Sigma-Aldrich, UK); all washes were also carried out using 1x PBS.

To ensure that similar passages of cells were used throughout, stocks of cells were frozen for later use. Cells were centrifuged at 800rpm for 5 minutes before any remaining liquid was discarded, leaving the cell pellet. The cell pellet was then re-suspended in appropriate media and serum containing 10% dimethyl sulphoxide (DMSO) (Sigma-Aldrich, UK) and frozen in cyrovials (Fisher Scientific, UK) to -80°C. For longer term storage, cells were stored in a liquid nitrogen cell bank.

2.1.2: siRNA Transfection For Protein Depletion

siRNA ('short interfering RNA') is a well defined method used to knock-down a protein of interest (in this study, K15) (see section 3.1.2 for details of the mechanisms involved).

In this study, the same K15 siRNA was capable of knocking-down K15 in all cell lines. The siRNA was part of a pre-designed selection from Ambion (see *Table 2.1*).

Table 2.1: Details of pre-designed siRNA for K15 (Ambion).

	Sense	Antisense
Sequence (5' – 3')	GCUCAUUUGUACAGUGUCUtt	AGACACUGUACAAAUGAGCtt
Length	21	21
Percent G/C	38.00%	38.00%
Molecular Weight	6585.2	6700.2
Molar Extinction Coefficient	201800	214600

To transfect each cell line, oligofectamine (Invitrogen, UK) was used at 100nM siRNA 24 h prior to transfection, cells were washed and antibiotic/antimycotic-free media added to the flask(s) or plate(s). This was replaced by fresh media containing 1% antibiotic/antimycotics 6 h post-transfection. This prevented prolonged cellular exposure to oligofectamine and reduced the risk of infection.
After immunofluorescence analysis, the optimum knock-down was achieved in each cell type. In HaCaT, MET 1, MET 2 and MET 4 cells, the optimum knock-down of K15 occurred at 48 h, lasting until 72 h. By 96 h, the K15 levels appeared normal. In NHEKs, the optimum was later, at 72 h, with some recovery (not 100%) by 96 h (see section 3.3.3.3). These optimums were considered for all experiments.
For details of the siRNA mechanism, please refer to section 3.1.2.

2.1.3: Calcium Switch Assay

For immunofluorescence staining, cells were seeded onto 13mm glass coverslips in 24-well plates (Greiner Bio-one, UK); for protein extraction, cells were seeded into 90mm petri dishes (Greiner Bio-one, UK). Cells were then cultured in low calcium (0.07mM) media (defined keratinocyte serum-free media [Gibco, UK]). At various time-points, media was switched to high calcium media (defined keratinocyte serum-free media with additional calcium (1.5mM). Cells were cultured for 96 hours; control cells were grown in low calcium media for 96 hours. Experimental cells had their growth media changed from low to high calcium media for 72 h and 6 h, to assess the short-term and longer term effects of high levels of extracellular calcium.
Cells grown on coverslips for immunofluorescence were washed and fixed in 4% paraformaldehyde (Agar Scientific, UK). Cells in petri dishes for protein extraction were processed as detailed in sections 2.4.1 and 2.4.2.
Additional calcium was added from tissue-culture quality 1M stock of $CaCl_2$. To produce 1.5mM calcium media, 75µl was added to 50ml defined keratinocyte serum-free media (defined keratinocyte serum-free media contains 0.07mM calcium).

2.1.4: Adhesion Assay

Cells cultured in T75 flasks were removed using trypsin, then centrifuged (800rpm, 5 minutes) to produce a cell pellet. Cells were resuspended in growth media (with serum) and counted, using a haemocytometer. $5x10^5$ cells were then seeded onto glass coverslips in 24-well plates (Greiner Bio-one, UK). At pre-determined time-points, cells were gently washed in PBS (to remove any non-adhering cells) then fixed in 4% paraformaldehyde (Agar Scientific, UK) for immunofluorescence

staining. For live cell imaging, cells were and maintained in culture conditions (37°C and 5% CO_2) for the duration of the experiment. Phase contrast images could then be taken at pre-determined intervals, at 10x objective.

2.1.5: Scratch Wound Assay

Cells were grown to 100% confluent monolayers on 13mm glass coverslips in 24-well plates (Greiner Bio-one, UK). A 200µl pipette tip (Star Lab, UK) was then used to scratch through the monolayer. At varying time points following the scratch, cells were fixed using 4% paraformaldehyde, and followed by immunofluorescence probing to observe any changes at the edge of the wound. For live cell imaging scratch wound assays, cells were seeded onto 6-well plates (Greiner Bio-one, UK). Once confluent, the monolayer was scratched and the plate mounted to the microscope (see section 2.3.3), and kept at 37°C and 5% CO_2 for the duration of the experiment. Phase contrast images could then be taken at pre-determined intervals, at 10x objective.

2.1.6: MTT Assay

The 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay has been used as a colorimetric approach to studying cell proliferation *in vitro* (for example, by Ahmadian *et al*., 2009), by assessing the activity of mitochondrial dehydrogenase (where soluble yellow MTT becomes insoluble purple formazan). As this assay is light-sensitive, all steps were carried out in the dark as far as possible.

An MTT stock solution of 5mg/ml (0.25g of MTT in 50ml PBS) was made and stored at -20ºC until required. A working solution of 0.5mg/ml was then made from the stock.

Cells were counted using a haemocytometer, seeded into 6-well plates (Greiner Bio-one, UK) and allowed to adhere for at least 4 hours. Culture media was then removed and 1ml MTT working solution added to each well. The plate was then incubated at 37ºC and 5% CO_2 for 2 hours. As cells metabolise the yellow MTT, purple formazan is produced. The cells were then washed twice in PBS and 300µl of 10% DMSO and 90% isopropanol was added; the formazan is soluble in this solution. The resulting solution was centrifuged (13000rpm, 5 minutes) and 200µl added to wells of a 96-well plate (Greiner Bio-one, UK). The plates are then read at OD 540nm.

To correctly ascertain cell numbers in experimental procedures, a standard curve was previously created using the following numbers of cells: 0, $5x10^4$, $1x10^5$, $2x10^5$, $4x10^5$, $6x10^5$, $8x10^5$, $1x10^6$, $2x10^6$.

For the adhesion assay, cells were exposed to the MTT prior to being trypisinised, the cell number counted using a haemocytometer, and re-seeded for 40, 90 or 120 minutes. For cells to be seeded for 40 minutes, MTT was added 80 minutes prior to re-plating. For 90 minutes, MTT was added 30 minutes prior to re-plating. For the 120 minute timepoint, cells were trypsinised and counted, and the MTT solution added once the cells were re-plated. Once re-plated, all cells continued to be exposed to the MTT solution, making a total of 2 hours exposure. The cells were then treated as above.

2.1.7: Organotypic Cell Culture

Previously, cells have been seeded onto collagen gels which could be raised to the air-liquid interface (which in keratinocytes, promotes stratification) (for example, Schoop *et al*., 1999). Collagen gels are made using 1 volume of rat tail collagen (First Link, UK), 7 volumes of 10xMEM medium (Sigma-Aldrich, UK) and 1 volume of 1M NaOH (some methods describe the addition of 1 volume of 0.5-$5x10^6$/ml fibroblasts

in FBS, although this was not used in this study). All liquids were cold (4ºC) at the time of mixing to facilitate solidification. Once mixed, 3ml of the collagen liquid was added to each well of a 6 well plate, and incubated at 37°C and 5% CO_2 for at least 15 minutes to solidify. Once solidified, cells can be seeded directly onto the surface of the gel, facilitated by a sterile metal ring (to keep the cells on top of the gel following addition of media). Media is added to the well to cover the gel. The gel is incubated and submerged for 48h, after which the media can be changed and the collagen gel lifted. The gel is raised onto a sterile metal grid and the metal ring surrounding the seeded cells removed. Media is added to the well to reach the surface of the gel, without submerging the cells. This creates the air-liquid interface that facilitates stratification. The culture is then incubated for at least 7 days (with adequate media changes).

More recently, an adaptation to the method has been suggested. As opposed to seeding cells directly onto the collagen gel, a thin scaffold is placed on top of the collagen gel and cells seeded on top of this scaffold. The scaffold is porous, and cells can penetrate the surface of the scaffold before adhering, producing a 3D environment from which stratification can begin (Bokhari *et al*., 2007; Knight *et al*., 2011). The scaffold has been utilised in this study for seeding HaCaT and MET cells. Some 3D cultures used in this study were donated by Dr. R Carnachan (University of Durham/ReInnervate Ltd.).

The whole organotypic culture (i.e., cells, collagen and scaffold) is then fixed and stained using the same techniques used in tissue fixation and staining (see section 2.2.1).

2.2: Histology

2.2.1: Preparation of Tissue Sections

2.2.1.1: Frozen Sections

Murine and rat skin is thin enough to be sectioned whole. Back skin was used for sectioning. For ease, hair from the samples to be taken was removed; to minimise damage to the skin section, depilatory cream is used. The sample(s) of skin can then be removed with a scalpel.

The sample is immediately embedded in a mold in Cryo-M-Bed (Bright, UK), carefully to avoid air bubbles. To freeze the sample quickly, the mold can be held over liquid nitrogen. Once frozen, the mold can be placed into the liquid nitrogen until all samples have been collected. The molds can then be stored until sectioned and mounted onto slides.

Sections were taken at 7µm; this was found to be suitable for appropriate immunofluorescence and for integrity of the tissue. Slides could then be stored at -20°C.

Slides of adult human skin sections (donated by those undergoing cosmetic surgery procedures) were donated by Dr N Ojeh (University of Durham).

2.3: Immunofluorescence

2.3.1: Immunofluorescence of Cell Monolayers

Cells were grown in 24-well plates on uncoated 13mm glass coverslips. Cells were fixed with 4% paraformaldehyde in PBS (from 16% paraformaldehyde stock solution [Agar Scientific, UK]) for 10 minutes, then permeabilised in 0.05% Triton X-100 (Sigma Aldrich UK) (1:1 methanol:acetone fixation was also tested, although paraformaldehyde fixation was observed to produce better results). Following permeabilisation, cells were blocked with 0.2% fish skin gelatin (Sigma Aldrich UK). After washing, primary antibody was added and cells incubated for 1 h at room

temperature. Primary antibodies were detected using Alexa 488 or 594-labelled secondary antibodies (Invitrogen, UK); cells were incubated with secondary antibody for 45 minutes. Filamentous actin was stained with TRITC labeled phalloidin (Sigma Aldrich, UK) for 45 minuites.
Between steps, cells were washed three times with wash buffer (1x PBS, 0.02% BSA). All steps following addition of the secondary antibody were carried out in the dark (including storage).
In addition to the last wash step, some samples were also stained using the DNA marker DAPI (4',6-diamidino-2-phenylindole) (Invitorgen, UK) at 100ng/ml. This stains nuclei blue.
Coverslips were mounted sample side-down onto glass slides using Immunomount (Thermo, UK) and dried overnight. Slides could then be stored at 4ºC without further exposure to light.

2.3.2: Immunofluorescence of Tissue Sections
The slides of frozen sections were removed from the freezer and allowed to air-dry (30 minutes). The slides are then placed in an ice-cold methanol/acetone bath for 10 minutes. The slides are again allowed to air-dry (5 minutes). Slides are then washed in a 1xPBS bath for 5 minutes. At this stage it is useful to use a hydrophobic pen to draw around the section(s) to be probed. A 1:5 dilution of gelatine from fish skin (Sigma Aldrich, UK) in 1x PBS was prepared, and added directly onto the section, until completely covered. The slides were incubated in a humidified chamber for 20 minutes. The serum block was then removed and the primary antibody added, again directly to the section. The slides were placed in the humidified chamber for 90 minutes at 37°C. The excess antibody was removed and the slides washed in a cold (4-8°C) PBS/BSA bath for 5 minutes. The secondary antibody was then added directly to the section, and the slides again placed in the humidified chamber for 60 minutes. The slides were then washed twice in PBS. At this point DAPI staining can be carried out if required. Coverslips were mounted onto the sections after staining, and the slides air-dried overnight. The slides were stored at 4-8°C.

2.3.3: Microscopy
Samples were viewed using the Biorad Radiance 2000 confocal microscope with LaserSharp software (BioRad) or using a Zeiss Axiovert microscope with Axiovision software. 40x and 63x/1.40 oil immersion and 10x and 20x lenses were used. The power of the laser, the iris and gain were adjusted to achieve the best signal-to-noise ratio. Images were captured at a resolution of 1024x1024.
Live cell imaging was carried out using the Live Cell Imaging Zeiss Axiovert microscope with AxioCam and Axiovision software. 10x and 20x lenses were used.
Images were generated and linear adjustments made (brightness and contrast only) using Image J software (NIH).

2.4: Protein Analysis
2.4.1: Whole Cell Protein Extract
The cell media was aspirated from the adherent cells, which were then washed twice in PBS. The cells were lysed on ice using 1x Laemmli's Buffer (1% sodium dodecyl sulphate, 20%v/v glycerol, 1mM ethylenedinitrilotetraacetic acid, 50mM trishydroxymethylaminomethane-hydrochloric acid [pH 6.8]) supplemented with protease inhibitor cocktail; 1 tablet/10ml buffer (Roche, UK). Circular movements were made on the bottom of the dish with a cell scraper to dislodge the cells and release the intercellular proteins. Protein degradation is avoided by use of protease

inhibitor cocktails and low temperatures. The suspension is then placed in an eppendorf tube and boiled for 5 minutes, then homogenised using a 25G syringe needle; this shears DNA, which can make the product sticky and difficult to use in later applications. At this stage the extract can be frozen (-20ºC short term, -80ºC long term).

2.4.2: Cytoskeletal Protein Extract

The cells were lysed on ice with a small amount of 50mM trishydroxymethylaminomethane-hydrochloric acid ('Tris buffer'); circular movements were made on the bottom of the dish with a cell scraper to dislodge the cells and help release the intercellular proteins. Following lysis and homogenisation, cell extracts were mixed with solution 1 (20mM trishydroxymethylaminomethane-hydrochloric acid [pH 7.4], 0.6M KCl, 1% Triton X-100 [Fisher Scientific, UK] plus protease inhibitor cocktail), and centrifuged at 14000g for 15 minutes at 4ºC. The supernatant is discarded, and the previous step repeated twice. The pellet is then re-suspended in solution 2 (20mM trishydroxymethylaminomethane-hydrochloric acid [pH 7.5], 9M urea, 10% β-mercaptoethanol plus protease inhibitor cocktail). The cytoskeletal extract is then ready for running on a 1D gel (section 2.4.4).

2.4.3: Colorimetric Protein Concentration Quantification

The concentration of the protein extract is calculated via the bicinchonic acid, or BCA method. The kit used to carry out this assay is the BCA Protein Kit (Pierce, UK). The standards were made up as indicated from the stock protein solution in the kit. Standard protein concentrations were: 0, 0.25, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0mg/ml. The protein extract is also diluted to 1:10. The BCA solution is made (also from two solutions provided in the kit) and added to the standards and the diluted protein extract. All samples are then incubated at 37ºC for 10 minutes.

The samples and standards are read on a Beckman DU-600 spectrophotometer. The standards produce a standard curve when read at OD 562nm. The concentration of the protein extract can then be inferred from the standard curve.

2.4.4: Gel Electrophoresis

1D gels were run using the Thermo Electron Mini Gel System, using SDS (sodium dodecyl sulphate); a detergent which, due to its anionic nature, denatures proteins. Protein samples to be tested were mixed with equal volumes of Laemmli's buffer (with an additional 0.2% bromophenol blue), and run on pre-made 4-12% Bis-Tris gels (Invitrogen, UK). The gels were run at 100V through the stacking gel then 200V through the resolving gel in MOPS running buffer (0.2% 20xMOPS [Invitrogen, UK]).

For 2D gel electrophoresis, samples were prepared as previously described. Samples were run on pre-made Bis-Tris gels (Invitrogen, UK) with an IPG strip (Invitrogen, UK). The gels were run at 200V.

2.4.4.1: Coomassie Blue staining of 1D Gels

Coomassie Blue solution (0.1% Coomassie Blue G-250 [Sigma Aldrich UK], 10% acetic acid, 40% methanol) was utilised to visualise the proteins separated in the gel. The gels were stained with Coomassie Blue overnight at room temperature. Destain (10% acetic acid, 10% methanol in dH_2O) was then used to remove excess Coomassie Blue over several hours at room temperature; the destain was changed frequently.

2.4.4.2: Immunoblotting ('Western Blotting')

Proteins separated in the gel were transferred to Hybond nitrocellulose membranes (Amersham Pharmicia Biotech, UK) in NuPAGE transfer buffer (Invitrogen, UK). The method used for the transfer is known as wet blotting, as the transfer occurs within a cassette enveloped in transfer buffer. A current is passed through the container for 1h at 30V. Following the transfer, the gel may be discarded, and the nitrocellulose membrane was rinsed in 1x TTBS (10mM trishydroxymethylaminomethane-hydrochloric acid, 137mM NaCl, 2.68mM KCl and 0.2% Tween 20 [Fisher Scientific, UK]). After rinsing, the membrane was stained with Ponceau-S red (Sigma-Aldrich, UK), a stain which allows evaluation of transfer efficiency (including equal loading). Membranes were then blocked for 2 hours at room temperature (or at 4ºC overnight) in 5% non-fat milk powder in 1x TTBS. The membranes were probed with the primary antibody diluted in 3% non-fat milk powder in 1x TTBS for 1 hour at room temperature followed by a 1 hour room temperature incubation with the horseradish-peroxidase (HRP) conjugated secondary antibody. Between incubations, membranes were washed with 1x TTBS for 3x 5 minutes.

Antibodies bound to the membrane were visualised using Enhanced Chemiluminescence (Amersham Pharmacia Biotech, UK), and FujiFilm Intelligent Dark Box II. Image J software could then be used to calculate changes in intensity.

Table 2.2: Primary antibodies utilised in this study

Primary Antibody	Species	Name	Source	IF (Working Solution)	WB (Working Solution)
Desmoplakin	Mouse	DP1/2	ICN	1:100	
E-cadherin	Mouse		BD Biosciences Pharmingen	1:500	
Filaggrin	Rabbit		Covance	1:200	
Involucrin	Mouse	SY5	Sigma	1:200	
Keratin 6	Mouse		Abcam	1:100	
Keratin 8	Mouse	AE3	Abcam	1:100	
Keratin 14	Rabbit	AF64	Covance	1:100	
Keratin 15	Mouse	LHK15	Thermo Scientific	1:1000	
Keratin 15	Chicken	HK15	Covance		1:1000
Keratin 15	Mouse	C8/144B	Abcam		
Keratin 17	Mouse	E3	Millipore	1:200	
Alexa-Fluor Phalloidin 594 (Actin)			Invitrogen	1:600	
Vinculin	Mouse	V-11-5	Sigma	1:500	
ZO-1	Rabbit		Abcam	1:50	

(IF = immunofluorescence; WB = western blotting)

Table 2.3: Secondary antibodies utilized in this study

Secondary Antibody	Species	Company	IF	WB
Donkey	Anti-chicken immunoglobulins HRP	DakoCytomation		1:1000
Goat	Alexa-Fluor Anti-mouse IgG 594	Invitrogen	1:800	
Goat	Alexa-Fluor Anti-mouse IgG 488	Invitrogen	1:800	
Goat	Alexa-Fluor Anti-rabbit IgG 594	Invitrogen	1:800	
Goat	Alexa-Fluor Anti-rabbit IgG 488	Invitrogen	1:800	
Donkey	Alexa-Fluor Anti-mouse IgG 594	Invitrogen	1:800	
Donkey	Alexa-Fluor Anti-mouse IgG 488	Invitrogen	1:800	
Donkey	Alexa-Fluor Anti-rabbit IgG 594	Invitrogen	1:800	
Donkey	Alexa-Fluor Anti-rabbit IgG 488	Invitrogen	1:800	
DAPI		Invitrogen	1:1000	

(IF = immunofluorescence; WB = western blotting)

Chapter 3: K15 Downregulation in Epithelial Cells, and the Effects on Adhesion and Migration

3.1: Introduction

3.1.1: Aims

This chapter aims to achieve an overview of the effect of K15 expression on keratinocytes. It has previously been established that K15 is a marker of undifferentiated cells (see section 1.4). In order to elucidate a function for K15 in these undifferentiated cells and the effects its ablation has, K15 siRNA transfection was used to knock-down K15 expression in HaCaT cells, which normally express K15. The effect of K15 ablation on cell proliferation and differentiation will be discussed.

3.1.2: The siRNA Transfection Method

RNA interference (RNAi) is a method of post-transcriptional gene-silencing (see *Figure 3.1*). It is thought that this mechanism originally evolved to protect the host genome against viruses in organisms such as the fungus *Neurospora crassa* and the nematode *Caenorhabditis elegans*; some protozoa and plants also utilise this defence mechanism (Tenllado *et al*., 2004). In mammalian cell studies, RNAi is mediated by small interfering RNAs (siRNAs) that are usually 20-25 nucletides long. The small size of these siRNAs does not trigger the usual antiviral response to double-stranded RNA (dsRNA) in mammalian cells (i.e. RNA degradation and inhibition of protein synthesis), and so can successfully be used in experimentation (Bass, 2001). Successful protein knock-down using siRNA techniques in mammalian cell culture was first achieved by Elbashir *et al*., 2001b. This technique has also been used in keratinocytes *in vitro* (for example, Boczonadi *et al*., 2007; Chang *et al*., 2011).

3.1.2.1: The RNA-induced Silencing Complex

The RNA-induced silencing complex (RISC) is part of the defence mechanism developed by higher cells in defence against viral infection. The RISC uses siRNA or micro RNA (miRNA) as a template; when RNA complementary to this template is recognised, an RNase is activated and the RNA is cleaved. This rids the cell of the infectious viral vector. The RISC is utilised in laboratory siRNA methods to ultimately prevent translation by stopping ribosome translation of the RNA or cleaving the native complimentary RNA (for a review of the RISC, see Filipowicz, 2001; Sontheimer, 2005; Rawlings, Krishnan and Walter, 2011).

The siRNA, transfected into cells, assembles into endoribonuclease-containing complexes and unwinds, creating the RISC. The RISC includes a RISC loading complex (RLC), containing a Dcr-2/R2D2 heterodimer, which binds the siRNA. The argonaute protein (part of the core RISC) then displaces the Dcr-2/R2D2, and cleaves the sense strand of the dsRNA, activating the RISC (Rivas *et al*., 2005). Hydrolysis of ATP at this point is thought to accelerate this process, although it appears not to be an absolute requirement. The antisense strand of RNA then guides the RISC to the homologous mRNA, resulting in cleavage of the mRNA. Release of the two mRNA fragments from the cleavage process requires ATP, unlike the previous dsRNA cleavage. In the cytoplasm, the 5' end fragment of cleaved mRNA is degraded by an exonuclease complex (the exosome). It is possible that the 3' end fragment is not degraded in human cells (Holen *et al*., 2002; Holmes *et al*., 2010). The RISC also dissociates from the cleaved mRNA to repeat cleavage with other targets.

This whole process prevents protein translation, resulting in protein knock-down, and allowing protein function to be inferred from its absence. A key experimental parameter to be considered here is the half-life of the protein to be studied.

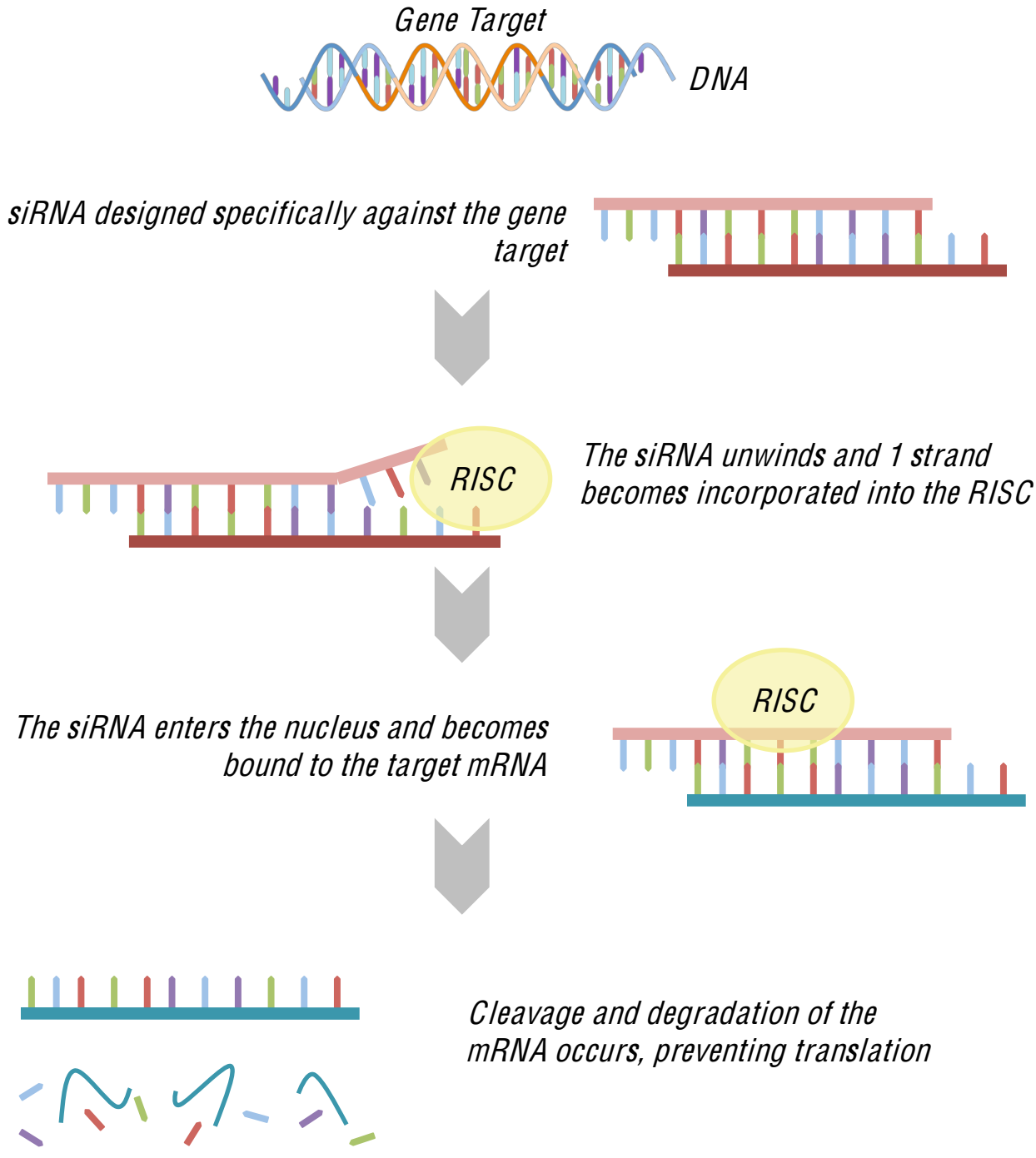


Figure 3.1: Gene silencing by siRNA
Expression of selected genes can be inhibited by transfecting cells with double-stranded siRNA. Inside the cell, the siRNA unwinds and becomes incorprated into the RISC. This then binds to the endogenous mRNA. If pairing occurs, then the native mRNA is severed and undergoes degradation.

More recently, some adjustments to the method have been suggested to increase efficiency. For example, incorporation of poly(γ-glutamic acid) has been shown to increase cellular uptake of the siRNAs (Liao *et al*., 2010), and polymers have been developed with similar effect (Takemoto *et al*., 2010).

3.1.3: Keratin Expression in HaCaT Cells

HaCaT cells are a spontaneously transformed human epithelial cell line from adult skin. This cell line has become a useful tool in research as these cells maintain their capacity for epidermal differentiation and are nontumourigenic. DNA fingerprint testing and chromosomal analysis demonstrated that the genetic composition of these cells was unaffected in long-term culture (>140 passages) (Boukamp *et al*., 1988).
HaCaT cells express a wide variety of keratins, and are capable of associating into stratified, epidermal organisation, including the changes in keratin expression throughout the stratified layers. *In vitro*, HaCaT cells proliferate with a low degree of differentiation, expressing keratins associated with the basal layer. Once confluent, layers begin to form; HaCaT cells stratify spontaneously and the keratin expression pattern changes in a similar way to that observed in normal skin (Merne and Syrjänen, 2003; Sun *et al*., 2007; Prado *et al*., 2011). As these cells are, at present, the only widely available clonal cell line which have the capacity to differentiate in this way, this cell line was considered the most appropriate for use in this study.

3.1.3.1: Previous Studies of HaCaT Cells

When HaCaT cells were cultured on collagen and allowed to stratify, involucrin and filaggrin expression has been observed as in normal skin sections (i.e. localized to the stratum corneum and stratum granulosum respectively) (as described in, for example, Kim *et al*., 2010b). Basal and suprabasal cells were identified using the Pab421 antibody, specific for epidermal basal cells, and by a K10/11 antibody (as previously described by Moll *et al*., 1982). These findings correlated with keratin analysis by gel electrophoresis. When compared with normal cells from adult foreskin and adult thigh skin, HaCaT keratin expression was similar. This included strong expression of K1, K10, K5 and K14. K6 and K16, observed in hyperproliferative epidermis, were also observed in HaCaT cells and normal cells, following transplantation. Some K4 and K13 expression was also observed. HaCaT cells also express K17, which is only present in the epidermis in a hyperplastic state (for example, as observed by Müller *et al*., 2006). Boukamp *et al*. (1988) highlight that the keratin expression pattern observed in HaCaT cells is comparable to the (hyperplastic) morphology of transplants and transplanted mouse keratinocytes.
Boukamp *et al*.'s initial study (1988) was followed by a comparison between HaCaT cells and tumourigenic HaCaT-ras clones (Ryle *et al*., 1989). HaCaT cells, as well as expressing K5, K6, K14, K16 and K17, were also shown to express K7, K8, K18 and K19. These keratins are generally associated with simple epithelia. Ryle *et al*. (1989) noted that these keratins were expressed whilst the HaCaT cells were cultured at low densities. Confluent HaCaT cells expressed K4, K13 (as previously described) and K15; Ryle *et al*. contribute the expression of these keratins to the onset of stratification.

Further study was carried out on the differentiation capability of HaCaT cells. Breitkreutz *et al*. (1998) carried out HaCaT surface transplants on the backs of nude mice, comparing appearance and protein expression to normal human keratinocytes grafted in the same way. At day 1-4, the initial regeneration phase, poorly organised stratified epithelia formed, similar in appearance to normal keratinocytes. By day 7,

proliferation had become restricted to basal layers, which were K14 and β1-integrin positive. There was also strong suprabasal staining of K1 and K10. By day 14, the late markers K2e, loricrin and filaggrin had appeared. Differentiation continued with keratinisation by 3 weeks and a switch from parakeratotic to orthokeratotic by 6 weeks. This is comparable to keratinisation observed in normal keratinocytes grafted in the same way. Furthermore, Breitkreutz *et al*. studied ultrastructural features, such as matrix and basement membrane components. Again this was similar to the production of proteins in normal keratinocyte grafts, with laminin-5, collagen IV, BM-laminin and collagen VII deposition respectively. To further examine the differentiation capacity of HaCaT cells, Schoop *et al*. (1999) compared stratification of HaCaT cells on athymic mice and on collagen gels. Although orthokeratotic keratinisation was not reached, differentiation up to this point was comparable. K10, K16, involucrin and transglutaminase I were identified in suprabasal layers after one week in culture, followed by K2e, loricrin and filaggrin after 2-3 weeks. This coincided with a restriction in proliferation to basal cells.
Differentiation in HaCaT cells has also been studied in monolayers, using changes in external calcium concentration to induce differentiation. This will be discussed in section 3.1.4.
The results described above indicate that HaCaT cells demonstrate differentiation typical of normal keratinocytes; as the study of K15 would need to note any changes in differentiation, HaCaT cells are a useful model to study differentiation in keratinocytes, in both 2D and 3D culture.

3.1.4: The 'Calcium Switch' Assay

In vitro, monolayers of keratinocytes cultured in low levels of extracellular calcium (<0.1mM) can be induced to differentiate with the addition of media containing 1-2mM Ca^{2+}.
This differentiation can be seen in changes to protein markers of differentiation; for example, changes in the keratin expression pattern and other markers, such as involucrin (Ponec *et al*., 1988; see Tu *et al*., 2008 and Tu, Chang and Bikle, 2011 for reviews of the role of the calcium receptor in differentiation).
A change in calcium concentration has been observed between the basal layer (low calcium – up to 0.5mM) and the granular layer (high calcium – from 1mM) *in vivo*, influencing a change in keratin expression from K14 and K5 in the basal layers to K1 and K10 in the granular layers (Yuspa *et al*., 1989). Any increase in extracellular Ca^{2+} concentration is followed by increases in both diacylglycerol and phosphatidylinositol metabolism via protein kinase C (PKC) (Lee and Yuspa, 1991). PKCα is specific to keratinocytes, and is responsive to changes in Ca^{2+} concentration (Eckert *et al*., 2004). Ca^{2+}-independent PKCη appears in spinous cells during their transition to granular cells (coinciding with a decrease in both K1 and K10, and increases in loricrin, filaggrin and transglutaminase) (Eckert *et al*., 2004). PKCη can also induce differentiation in granular cells; these cells however do have Ca^{2+}-sensing receptors, part of the Ca^{2+} binding protein family (Bikle *et al*., 1996). Further research has shown that overexpression of PKCη induces differentiation and G1 arrest (Kashiwagi *et al*., 2002). Interestingly, mice lack this receptor, and reduced amounts of both filaggrin and loricrin have been found in this animal compared to humans (for example, Komuves *et al*., 2002). Stanley and Yuspa (1983) and Yuspa *et al*. (1989) describe the actions of murine keratinocytes in low and high calcium media: in low calcium cells proliferate without stratifying, whereas those cells in high calcium media develop cell-cell contacts, stratify and cornify. DNA synthesis is

inhibited, and the cell eventually dies. The same occurs in both HaCaT cells and normal human keratinocytes (Micallef *et al*., 2009). This is comparable to cells *in vivo* (Stanley and Yuspa, 1983). Bikle *et al*. (1996) showed that this rise in extracellular calcium shifted the intracellular calcium concentration, and it was this 'double' action which initiated the differentation response.

Increased extracellular calcium has been shown to activate the phospholipase C pathway (via a calcium receptor), which ultimately results in a rise in intracellular inositol triphosphate (IP_3) and a rise in intracellular calcium. This intracellular calcium activates chloride channels in the cell membrane, resulting in hyperpolarisation; voltage-independent cation channels also become permeable (also mediated by a calcium receptor), allowing calcium ion movement. These actions combined produce a swift rise in intracellular calcium, followed by a prolonged increase (Bikle *et al*., 1996). Bikle *et al*. (1996) have also demonstrated that less differentiated cells are more sensitive to extracellular calcium than their more differentiated counterparts (the molecular biology of calcium signaling has been reviewed more recently by Breitwieser, 2008). Extracellular calcium also affects other mechanisms important in differentiation. Epidermal cadherins, for example, are calcium dependent, their extracellular domain containing calcium ion binding sites, where Ca^{2+}-binding is necessary for cell-cell adhesion (important in keratinocyte differentiation) (Lefort and Dotto, 2004; Tu *et al*., 2008). Intracellular and extracellular calcium concentrations have been shown to affect cell-cell junctions in several ways. For example, cell-cell junction formation in keratinocytes depends on actin fibre rearrangement, which occurs during the differentiation process, initiated by an increase in extracellular calcium (for example, Vasioukhin *et al*., 2000; Umapathy *et al*., 2010).

In low calcium media, both mouse and human keratinocytes fail to form desmosomes. Once elevated above 0.1mM calcium, adherens junctions form within 5 minutes and desmosomes form over the following 2 hours (Hennings *et al*., 1980; Hennings, Holbrook and Yuspa, 1983; Mochizuki *et al*., 2002). In DJM-1 keratinocytes (a squamous cell carcinoma line, where desmosome formation is similar to that observed in normal keratinocytes), desmocollin 3 interacts with plakoglobin after 10 minutes, and adherens junctions form. Desmocollin 3 is then phosphorylated and interacts with desmoglein 3, initiating desmosome formation (Kitajima, Inoue and Yaoita, 1987; Aoyama *et al*., 2009). Desmosomes have also been shown to be resistant to disruption by chelexed extracellular calcium (Watt, Mattey and Garrod, 1984); it has been hypothesised that this 'hyper-adhesiveness' is to maintain epidermal integrity. When keratinocytes are cultured at lower densities however, desmosomes become calcium-dependent (Kimura, Merritt and Garrod, 2007). Hyper-adhesiveness is also lost during wound healing, when desmosomes become calcium-dependent (Wallis *et al*., 2000; Kimura, Merritt and Garrod, 2007). There is some evidence to suggest that this calcium-dependence is triggered by PKC, which alters the configuration of cadherins in the desmosome, reducing their binding affinity (Wallis *et al*., 2000; Garrod *et al*., 2005). Kimura, Merritt and Garrod (2007) have demonstrated that desmosomes in both simple and stratified epithelia have similar adhesive properties, capable of hyper-adhesion.

S100 proteins are similar to calmodulin proteins, although are able to bind to calcium in the millimolar range (as opposed to the micromolar range) (Heizmann and Cox, 1998). They have dual roles as chemoattractants and signal transductors, which are thought to be influential in keratinocyte differentiation and psoriasis (Jinquan *et al*., 1996; Broome, Ryan and Erkert, 2003).

3.1.5: Organotypic Cell Culture

Organotypic (also known as 'raft') cultures have been utilized in the study of differentiation and stratification (for example, Asselineau and Prunieras, 1984). These cultures are generally a collagen gel (often impregnated with fibroblasts) onto which keratinocytes can be directly seeded. The gels are then raised so that the seeded cells are at the air-liquid interface, encouraging stratification and differentiation. This 3D culture has been shown to be similar to *in vivo* conditions, making it an appropriate model to study skin, particularly human skin, where *in vivo* possibilities for study are limited. For example, Slavik *et al*. (2007) used the model to study Wnt signaling, which was shown to induce differentiation in the organotypic keratinocyte culture. Keratins have also been studied using this technique. Chamcheu *et al*. (2009) have used organotypic cultures seeded with cells from EBS patients, demonstrating that cells with mutant K5 or K14 were capable of stratifying *in vitro* in an identical fashion to normal controls (differences were observed in keratin filaments following heat shock compared to controls). Okugawa and Hirai (2008) have also used organotypic cultures seeded with HaCaT cells to observe how K1 and K14 expression were affected by syntaxin2 (a stromal signaling factor) signaling.

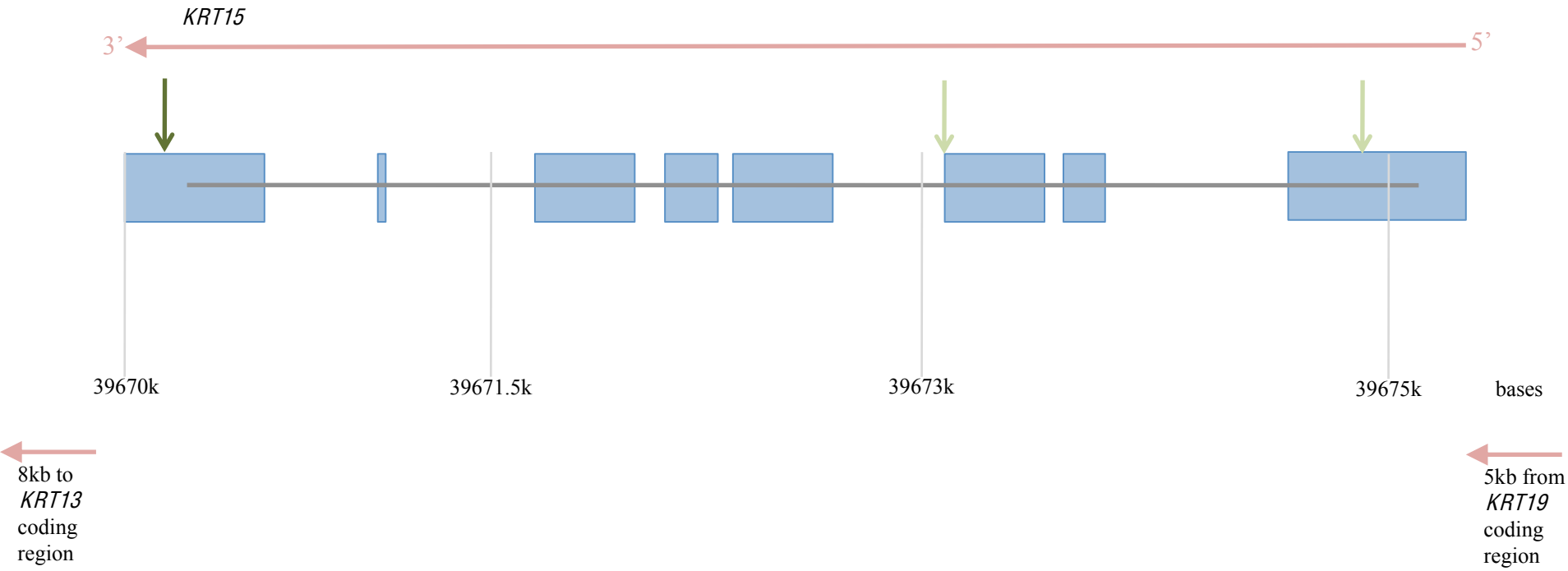
3.2: Materials and Methods

The materials and methods used in investigation of the effect of K15 on differentiation and proliferation are described in Chapter 2. No changes or variations were made to the methods described.

The schematic diagram in *Figure 3.2* shows the site of the pre-designed siRNA used in this study (see also section 2.2). Although the other siRNAs shown were tested in HaCaT cells, similar knock-down effects were noted in each (as demonstrated by immunofluorescence). The siRNA chosen also worked in NHEK cells and MET cell line cells.

Figure 3.2: Schematic Diagram of *KRT15*.

Schematic diagram of the K15 gene highlighting exons and siRNA sites. Also included is the normal K15 amino acid sequence. The pre-designed siRNA utilised in this study is highlighted, although the two other siRNAs shown were also tested.

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  1 MTTTFLQTSS STFGGGSTRG GSLLAGGGGF GGGSLSGGGG SRSISASSAR  50
 51 FVSSGSGGGY GGGMRVCGFG GGAGSVFGGG FGGGVGGGFG GGFGGGDGGL 100
101 LSGNEKITMQ NLNDRLASYL DKVRALEEAN ADLEVKIHDW YQKQTPTSPE 150
151 CDYSQYFKTI EELRDKIMAT TIDNSRVILE IDNARLAADD FRLKYENELA 200
201 LRQGVEADIN GLRRVLDELT LARTDLEMQI EGLNEELAYL KKNHEEEMKE 250
251 FSSQLAGQVN VEMDAAPGVD LTRVLAEMRE QYEAMAEKNR RDVEAWFFSK 300
301 TEELNKEVAS NTEMIQTSKT EITDLRRTMQ ELEIELQSQL SMKAGLENSL 350
351 AETECRYATQ LQQIQGLIGG LEAQLSELRC EMEAQNQEYK MLLDIKTRLE 400
401 QEIATYRSLL EGQDAKMAGI GIREASSGGG GSSSNFHINV EESVDGQVVS 450
451 SHKREI
```

KRT15 amino acid sequence

Key

- coding region
- exons
- gene
- Site of siRNA used in this study
- Other Ambion pre-designed siRNA sites

3.3: Results

3.3.1: Experimental Approach

The aim of the work described in this chapter was to establish whether K15 siRNA transfection would be a useful tool in studying the effect of K15 ablation in human skin. Initially, immunofluorescence was used to investigate where the K15 protein is present in mammalian skin. Previous studies had shown contradictory results of K15 expression, so it was considered useful to establish where the K15 protein exists in mammalian skin.

In order to explore the importance of K15 expression in skin, K15 siRNA transfections were carried out in monolayer HaCaT cells. Immunofluorescence, immunoblotting, cell viability tests and gel electrophoresis were used to demonstrate the effects of K15 knock-down on these cells in monolayer cultures. Similarly, immunofluorescence was carried out in NHEK cells following K15 siRNA transfection.

To investigate the effects of K15 ablation on differentiation, K15 siRNA transfections were carried out in cells which were cultured in either high or low calcium media.

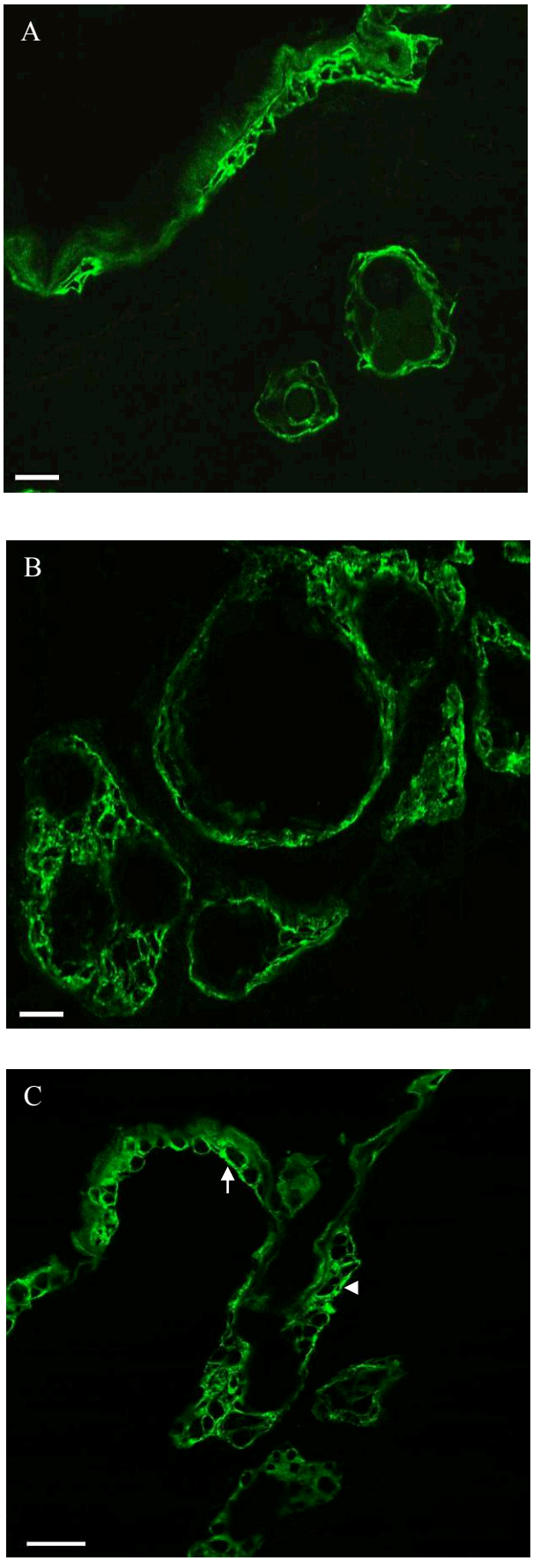


Figure 3.3: Immunofluorescence of K15 in adult rodent skin
Shows K15 in the basal layers of the IFE (A) and hair follicles (B) of rat skin and K15 immunofluorescence in the basal layers of the IFE (arrow) and hair follicles (arrowhead) of mouse skin (C) (scale bar = 10μm). K15 immunofluorescence can be observed around the ORS (B, C arrowhead). K15 can be seen in the basal layer of the IFE, with residual K15 above this layer, and very low levels/no K15 observed any closer to the skin surface (A, C arrow).

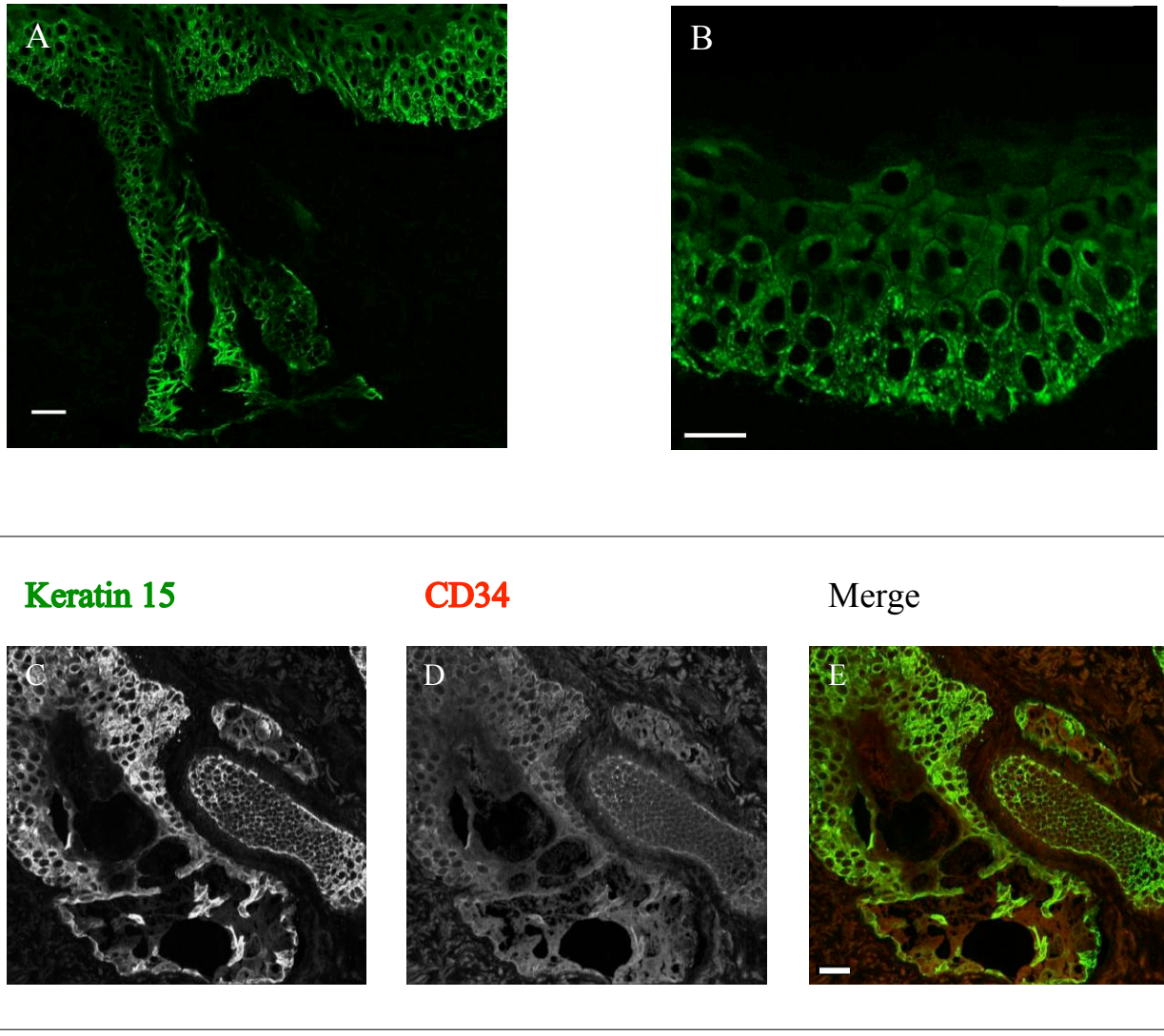


Figure 3.4: Human whole skin sections probed for K15 (green) and CD34 (red).
K15 immunofluorescence staining of a hair follicle and the IFE (A) (scale bar= 10µm). Although there are some regions around the hair follicle which appear brighter, in this plane the ORS appears surrounded by K15-positive cells.
The IFE (B) (scale bar = 10µm) shows that the K15 is expressed to a greater extent in the basal layers, and the amount of K15 reduces as the cells differentiate and move upwards towards the skin surface.
Merge of a section through a hair follicle (E), with K15 (green, C) and CD34 (red, D) immunofluorescence. CD34 is present throughout at low levels, whilst K15 can be observed specifically in the cells surrounding the hair follicle. (scale bar = 10um).
It is likely that the low levels of CD34 observed here is a non-specific cross reaction.

3.3.2: K15 Expression in Mammalian Skin

Indirect immunofluorescence was used to detect K15 expression in rat, mouse and human skin. K15 is present in the same regions in both mouse and rat (*Figure 3.3*). This includes a K15-positive outer root sheath and interfollicular epidermis (IFE). In the IFE, K15 is expressed to a greater extent at the base (for example, 'arrow' *Figure 3.3* C). Here K15 is more prominent than in cells closer to the surface of the skin. K15 is expressed in the basal layer, and as the cell differentiates and moves towards the surface of the skin, it is possible that expression desists and only residual K15 remains. Due to the angle of the murine skin section, the cells between the hair follicle and the IFE can be seen. The cells in this region are also K15-positive (as also suggested by Liu *et al*., 2003). This region can not be observed in the rat sections (*Figure 3.3* A, B), however K15-positive cells can clearly be observed around the hair follicle and in the basal layers of the epidermis.

K15 is present in similar regions in human skin as in rodent skin i.e. around the hair follicle and in the basal layers of the epidermis. In *Figure 3.4* A, the hair follicle and outer root sheath can be seen to contain K15. The cells of the outer root sheath just below the sebaceous gland however appear to contain more K15 than surrounding cells. This region has previously been described as the bulge (i.e. the stem cell niche) (Ohyama *et al*., 2006), and cells here are actively expressing K15 (Kloepper *et al*., 2008).

The basal layers of the epidermis are K15 positive (*Figure 3.4* B); however, K15 can also be observed above the basal layers (probably residual). As the cells begin to stratify closer to the surface of the epidermis, the K15 protein remains, but is present to a lesser extent than in the basal layers. This residual K15 however does not last throughout all layers of the epidermis, and as the cells flatten most cells have metabolised any remaining K15. K15 then can not be observed in the flat cells at the surface of the skin.

CD34 has previously been suggested as a marker of stem cells in the bulge region of the hair follicle; Trempus *et al*. (2003) identified CD34 in the bulge region of murine hair follicles (co-localised with label-retaining cells), for example. Cotsarelis (2006) however suggests that CD34 is not expressed in the (K15-positive) human bulge region (in the telogen hair follicle); this finding has recently been supported by Inoue *et al*., (2009) utilising flow cytometry and immunocytochemistry techniques.

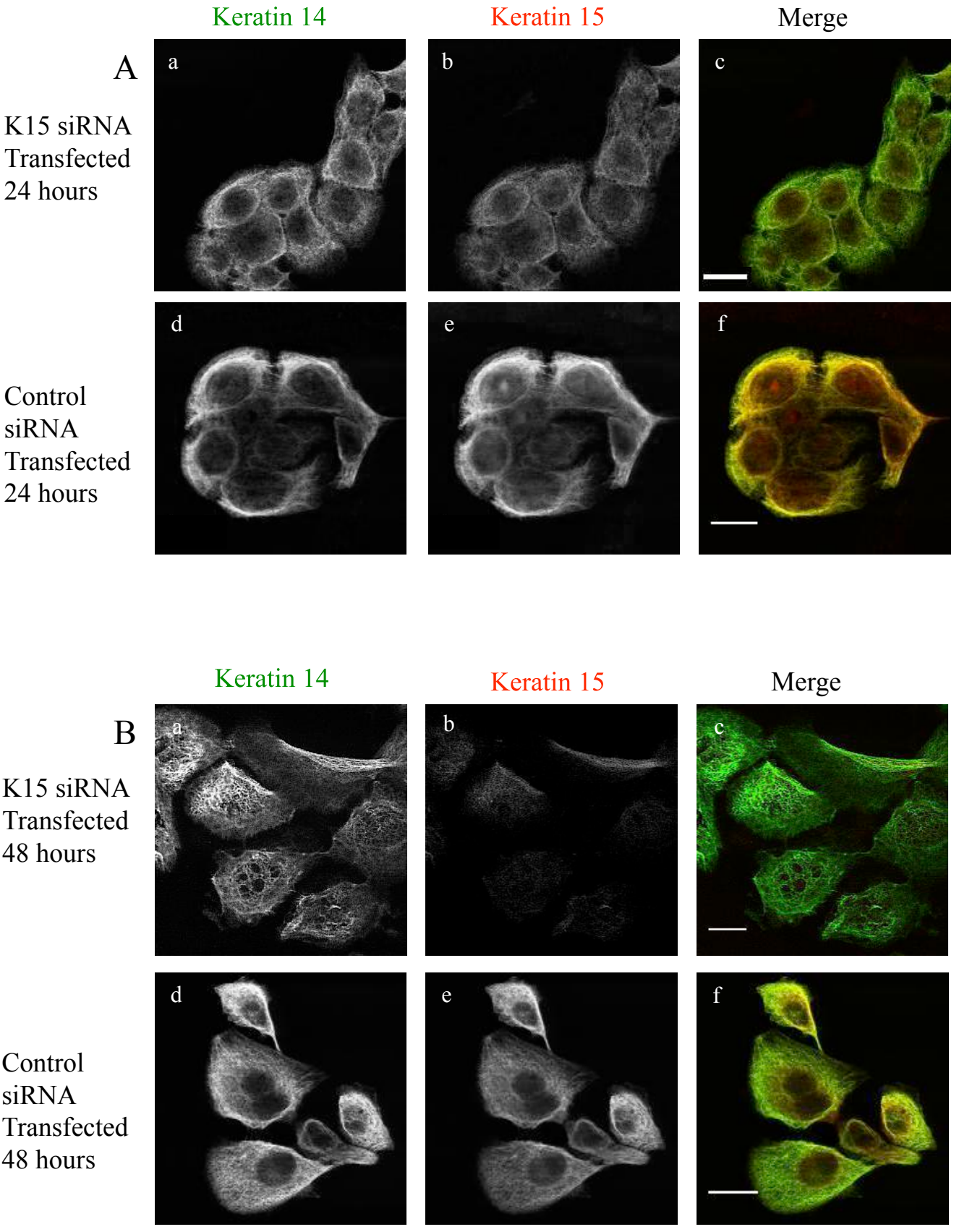
For these reasons it was considered useful to compare K15 and CD34 expression around the hair follicle in this study. As expected, there is no CD34 in the epidermis, but K15 can clearly be observed (*Figure 3.4* C, D, E). Very low levels of CD34 are observed in the human IFE here (D and E), and it is likely that this is background immunofluorescence. The section appears to stain uniformly for CD34, again indicating that no specific CD34 staining is occurring. This would correspond with those observations made by Cotsarelis *et al*. (2006) and Inoue *et al*. (2009).

3.3.3: K15 siRNA Transfection in HaCaT and NHEK Cells

3.3.3.1: K15 siRNA Transfection in HaCaT Cells

In the first instance, the efficiency of siRNA knock-down of K15 was evaluated by double immunofluorescence of K15 and K14 proteins. Time points of 24, 48, 72 and 96 hours after the transfection were chosen to identify the optimal period of K15 expression reduction for future studies. In order to establish that no autofluorescence was occurring, fixed cells were 'stained' with only secondary antibody and examined. This demonstrated that the fixed cells did not autofluoresce and that the secondary antibody did not bind to IF proteins nonspecifically, confirming that any immunofluorescence observed was as a result of the presence of primary antibody on

a sample. In addition, use of double immunofluorescence with an anti-K14 antibody was designed to introduce an internal control for successful staining.


Keratin 14
Keratin 15
Merge
A
K15 siRNA Transfected 24 hours
Control siRNA Transfected 24 hours
a
b
c
d
e
f
B
K15 siRNA Transfected 48 hours
Control siRNA Transfected 48 hours

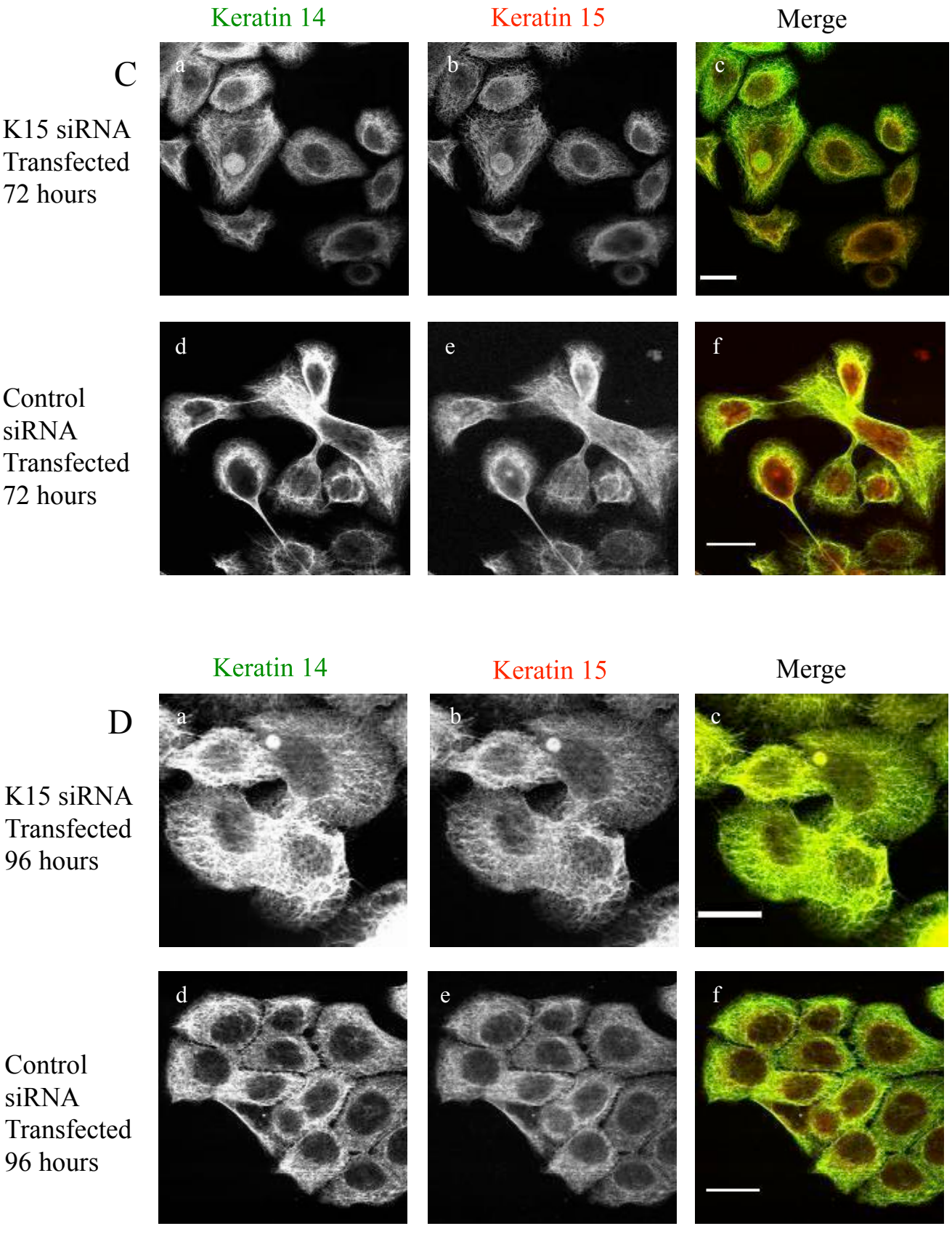

Keratin 14
Keratin 15
Merge
C
K15 siRNA
Transfected
72 hours
a
b
c
Control
siRNA
Transfected
72 hours
d
e
f
Keratin 14
Keratin 15
Merge
D
K15 siRNA
Transfected
96 hours
a
b
c
Control
siRNA
Transfected
96 hours
d
e
f

Figure 3.5: Expression of K14 and K15 in K15 siRNA knockdown HaCaT cells.
Single plane confocal images of immunofluorescence showing both K14 and K15 in HaCaT cells following K15 siRNA transfection and control siRNA transfection (scale bar = 10um).
24 h post-transfection, K14 expression appears normal in both K15 siRNA transfected (A, a) and control siRNA transfected (A, d) HaCaT cells. Normal levels of K15 can be observed in control siRNA transfected HaCaT cells (A, e) with slightly lower levels observed in K15 siRNA transfected HaCaT cells (A, b).
At 48 h post-transfection, K14 levels are similar in both K15 siRNA transfected cells (B, a) and control siRNA transfected cells (B, d). Levels of K15 appear similar to control levels in control siRNA transfected cells (B, e), however are greatly reduced in K15 siRNA transfected HaCaT cells (B, b). This can also be observed in the merge image (B, c).
By 72 h post-transfection, K15 levels in K15 siRNA transfected HaCaT cells have recovered slightly (C, b) compared with 48 h post-transfection (B, b). K14 expression in K15 siRNA transfected cells remains normal (C, a), as does K14 (C, d) and K15 (C, e) following control siRNA transfection.
Levels of K15 at 96 h post-transfection with K15 siRNA (D, b) are similar to levels at 96 h post-transfection with control siRNA (D, e). This suggests that the effect of K15 siRNA transfection is reversible in HaCaT cells. K14 levels remain the same in both K15 siRNA transfected (D, a) and control siRNA transfected HaCaT cells (D, d).
Based on data from this experiment, the most effective knockdown of K15 following siRNA transfection of HaCaT cells is 48 h post-transfection. This was considered for all further experiments.

In *Figure 3.5* A, the organisation of K15 and K14 arrangement appears similar, with the keratin network observed throughout the cytoplasm up to the edges of the cell; this can be particularly seen in *Figure 3.5* A, f, as the merge image suggests K14 and K15 co-localisation (yellow in the merged image). Far less K15 can be observed at 48 h post-transfection in K15 siRNA transfected cells compared to control siRNA transfected cells (*Figure 3.5* B, b), although K14 staining intensity (*Figure 3.5* B, a) appears similar to that observed in control siRNA transfected cells (*Figure 3.5* B, d). This suggests that the reduced K15 staining is not an artifact of the staining method. K14 network may appear over the nucleus to a greater extent in K15 siRNA transfected cells than in control siRNA transfected cells, although to accurately determine whether this is the case, several different optical slices would be required. At the 72 h time point, K15 expression begins to return (*Figure 3.5* C, b). Arrangement of K15 in these cells appears similar to control transfected cells, suggesting that no permanent changes have occurred preventing K15 from being expressed normally or localising normally. K14 expression remains similar at 72 and 96 h post-transfection, suggesting that any compensatory mechanism which may be occurring is removed when K15 expression returns. Thus, it was established that the most efficient time point for K15 knock-down by siRNA transfection was 48 h post-transfection (*Figure 3.5* B, b) after similar decreases in K15 expression were observed in repeat experiments. This was taken into consideration when planning subsequent experiments. A similar knock-down effect was also noted using another prepared K15 siRNA from Ambion (data not shown), suggesting that further testing of other siRNAs was not required.

In order to confirm the immunofluorescence findings, immunoblotting was carried out on whole protein extracts taken at 48 h post-transfection. Samples were run on a 1D and 2D gel, which were transferred to nitrocellulose via wet blotting. These results confirmed successful knock-down of K15 compared to the control siRNA transfected sample (*Figure 3.6*; *Figure 3.7*). The pI of K15 is 4.9 (MW 50kDa) (Kanitakis *et al*., 1999), however appears higher in this 2D blot (*Figure 3.6*). The same is true of K14, with a pI of 5.09 (MW 51.6kDa) (Fang, Zeng and Guo, 2008). It is possible that this may be due to an experimental error or possibly due to glycosylation/phosphorylation. In the 2D electrophoresis, two spots observed on the control sample blot suggest some phosphorylation, a common post-translastional modification of keratins regulating the filament assembly (for example, as described by Liao *et al*., 1996) (*Figure 3.6*). There is only a weak indication of one of the two spots in the K15 siRNA transfected sample, also suggesting the reduction of protein expressed in the K15 siRNA transfected cells. This also confirms the specificity of the antibody used, as no cross-reaction with any other keratin was observed. *Figure 3.6* also shows the K14 immunoblot in control siRNA transfected and K15 siRNA transfected samples. This was carried out as a control. The two spot patterns look identical, suggesting that K15 siRNA transfection has no effect on K14 expression or post-translational modification of K14 (as the patterns were similar). Staining for K14 also indicates equal loading (since imunofluorescence had previously demonstrated that transfection did not affect K14 expression). The faint line upper of the spots observed on the K14 blot may be an unreduced keratin dimer, or cross-reaction of the antibody with another keratin.
Western blotting (*Figure 3.7*) further demonstrates the reduced K15 48 h post-transfection and the return of expression at 72 h. This has been quantified using Image J, where the graph produced show the changes in staining intensity. After transfer to the nitrocellulose, Ponceau staining was carried out to establish equal loading of the protein extracts used.

3.3.3.2: Keratin Expression in K15 siRNA Transfected HaCaT Cells

It was considered important to assess the effect of K15 ablation on the expression of other keratins, as it has previously been suggested that keratin expression can change in response to expression of other keratins. For example, keratins related to K17 (such as K16) have been shown to be upregulated in *KRT17*-null mice (McGowan *et al*., 2002). It has also been demonstrated using RNAi that severe suppression of dominant keratins in non-small cell lung cancer results in suppression of all other keratins, whilst moderate suppression results in downregulation of complimentary keratins and upregulation of other keratins (Kanaji *et al*., 2006).

Although normally only expressed in hyperproliferative keratinocytes, K6 is constitutitively expressed in HaCaT cells (Boukamp *et al*., 1988; Mommers *et al*., 2000; Ojeh *et al*., 2008a). Examination of K6 expression and cellular arrangement may indicate if any other keratins that are normally expressed in HaCaT cells were affected by K15 ablation. Expression levels appear similar with a concentration of filament bundles around the nucleus, although K6 can be observed throughout the cytoplasm (*Figure 3.8* A, B). It has been suggested that K6 and K17 expression in normal keratinocytes are an 'intermediate' between basal K5/K14 expression and differentiated cell K1/K10 expression, allowing pliability (for migration) yet providing a certain amount of mechanical stability (Wong and Coulombe, 2003).

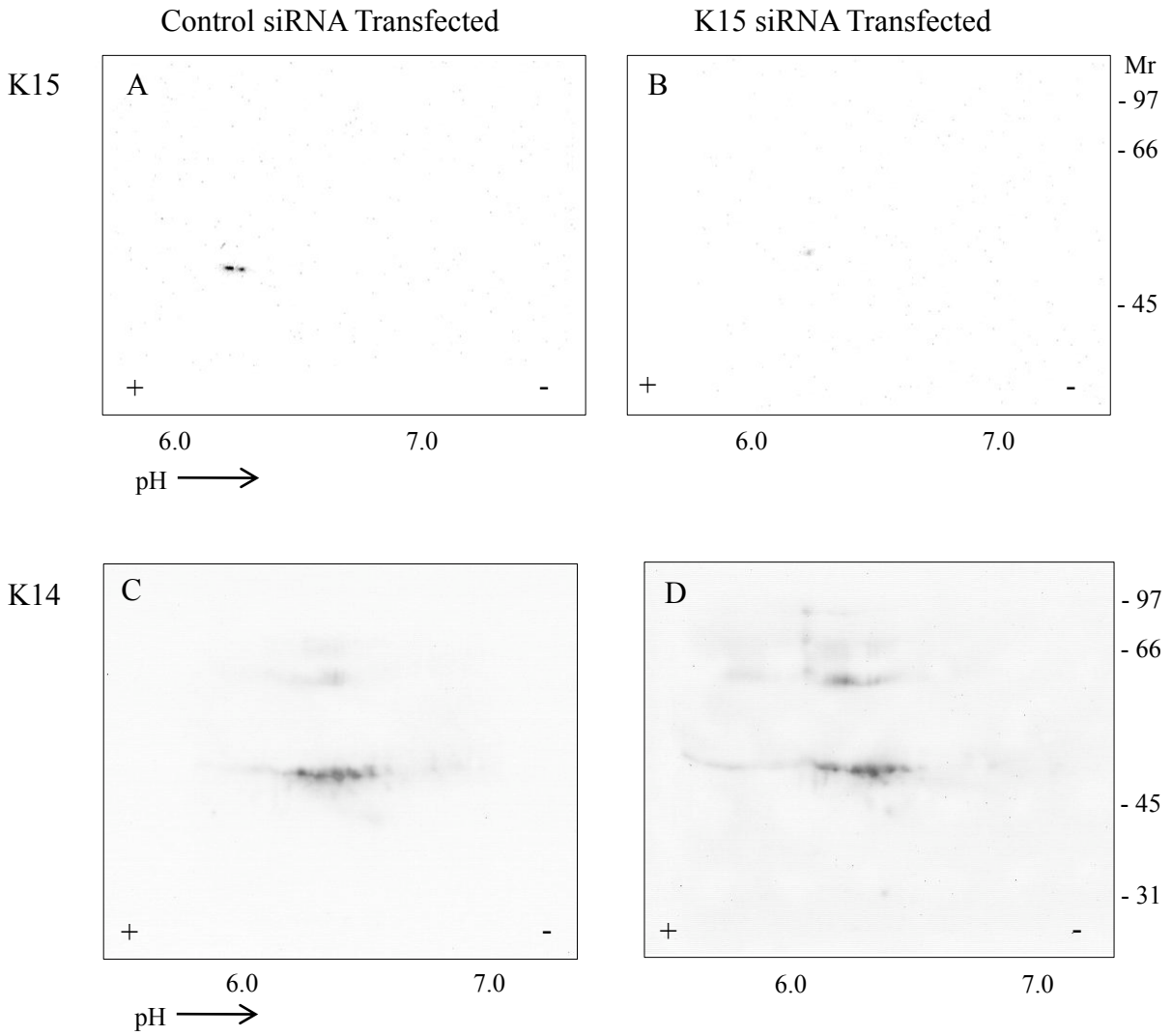


Figure 3.6: Immunoblotting following K15 siRNA transfection in HaCaT cells
2D immunoblotting of K15 and K14 from whole cell protein extract of K15 siRNA transfected and control siRNA transfected HaCaT cells (obtained 48 h post-transfection).
The 2D immunoblotting demonstrates a reduction in K15 in K15 siRNA transfected cell protein extract (B) compared to the control siRNA transfected protein extract (A).
The spotting pattern observed for K14 suggests that K14 expression is similar in both control (C) and K15 siRNA transfected (D) cell protein extracts.

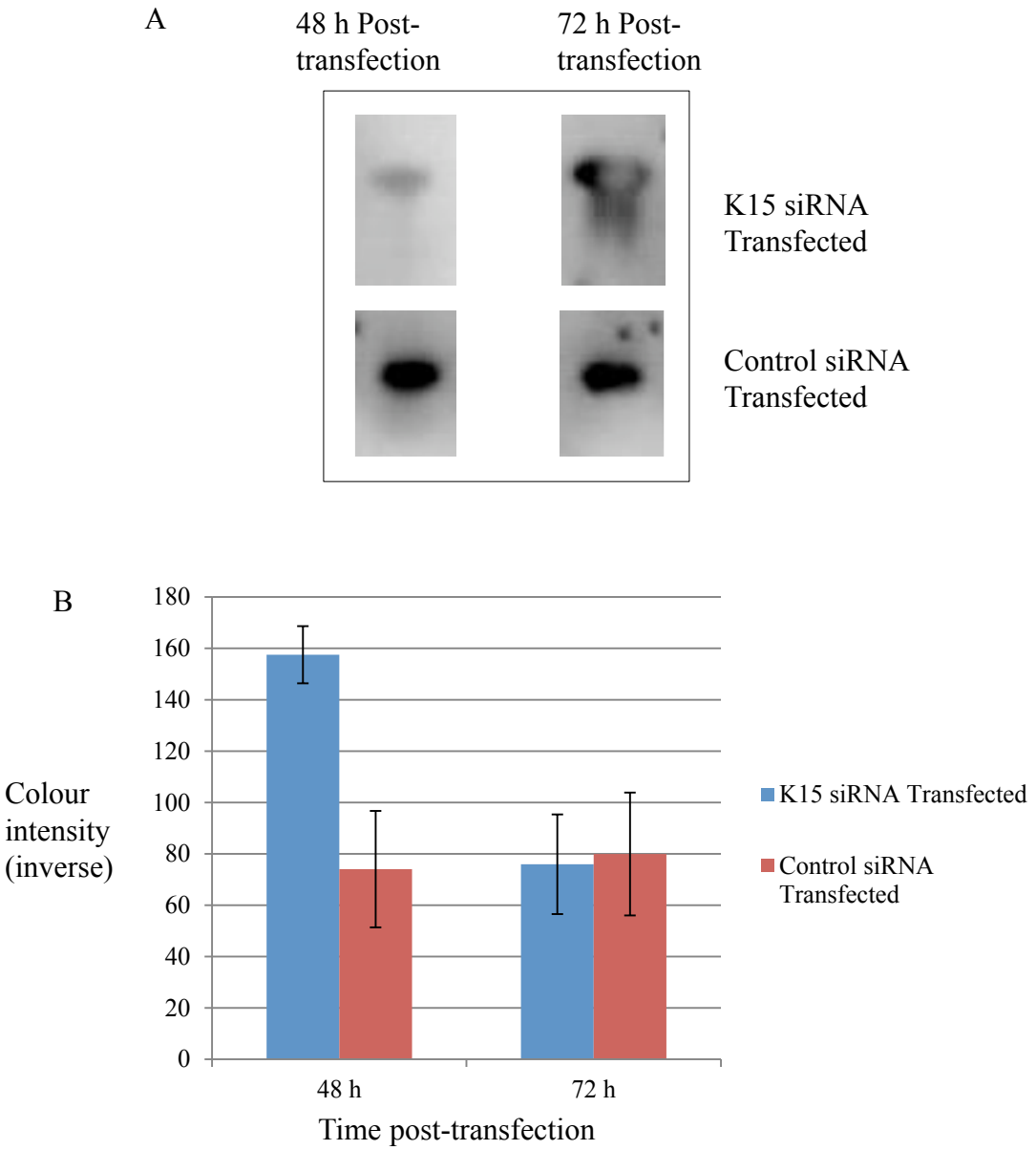


Figure 3.7: K15 Immunoblotting following K15 siRNA transfection in HaCaT cells.
At 48 h post-transfection, K15 levels can be seen to be lower than control siRNA transfected HaCaT cells (A). Recovery of K15 in K15 siRNA transfected cells can be observed by 72 h post-transfection. No change in the amount of K15 can be observed in the control siRNA transfected cells. Ponceau staining (not shown) was used to demonstrate equal loading.
Using Image J, the intensity of the staining of the immunoblots in A were measured (B) (error bars = standard deviation). The control siRNA transfected blots at both 48 and 72 h post-transfection are similar, suggesting that K15 expression in these cells is unchanged between these timepoints. The intensity of K15 staining at 72 h post-transfection in K15 siRNA transfected cells is comparable to the control siRNA transfected cells, suggesting similar amounts of K15 in each protein extract. At 48 h post-transfection in K15 siRNA transfected cells, the inverse intensity suggests that there is less K15 available for staining 48 h post-transfection in K15 siRNA transfected HaCaT cells. This result is comparable to those observed using immunofluorescence staining in transfected HaCaT cells (*Figure 3.5*).

Like K6, K17 is normally expressed in HaCaT cells, so it would not be a useful indicator of differentiation, but would establish further whether K15 ablation affected arrangement of other keratins. K17 expression and arrangement appears similar in both K15 siRNA transfected and control siRNA transfected cells, suggesting that K15 ablation does not affect K17 expression in HaCaT cells. K8 is usually associated with simple epithelia, although is also expressed in HaCaT cells (Ryle *et al*., 1989; Kazerounian, Uitto and Aho, 2002). Like K17, K8 is also expressed in epithelial progenitor cells, as shown in a study describing differentiation of embryonic stem cells *in vitro* (Maurer *et al*., 2008). K8 has been shown to have a role in cell integrity, for example, during epithelial wound healing (Baribault *et al*., 1994; Habtezion *et al*., 2005; Long *et al*., 2006). Depletion of K8 can affect wound healing, migration and cell-cell adhesions; it was deemed necessary to determine whether K8 levels were reduced following K15 siRNA transfection to ensure that if migration or cell-cell adhesions were affected, a role for K8 could be investigated further. K8 expression in HaCaT cells has previously been shown to be variable and heterogenous expression was also observed here (*Figure 3.8*, *Figure 3.9*). This makes determining the effect of K15 ablation on K8 expression and appearance more difficult to establish, however there appear to be no large differences between K8 in K15 siRNA transfected and control siRNA transfected cells. Although assessment of K5 expression and arrangement would have been useful, immunofluorescence staining did not produce results of a high enough quality to determine arrangement of K5 within the HaCaT cells.

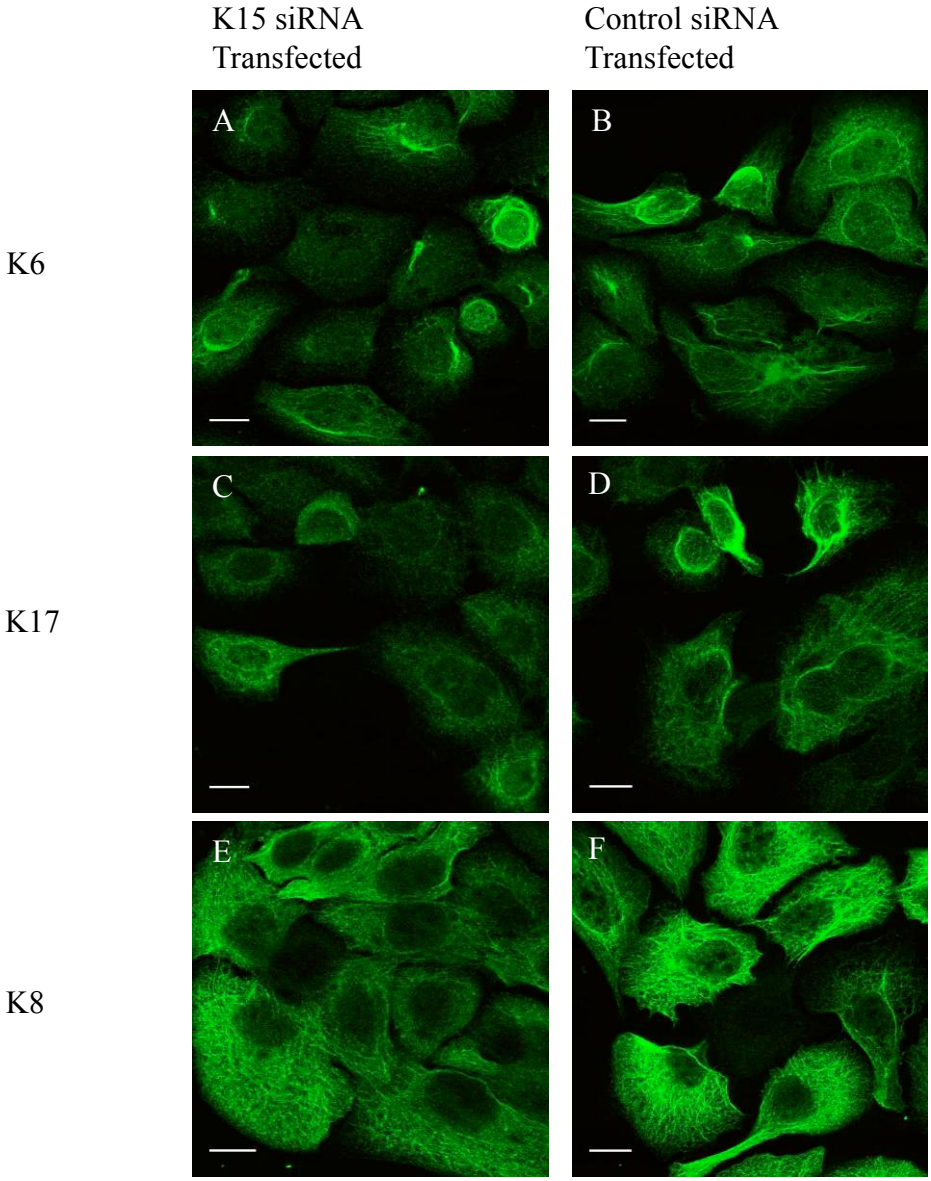
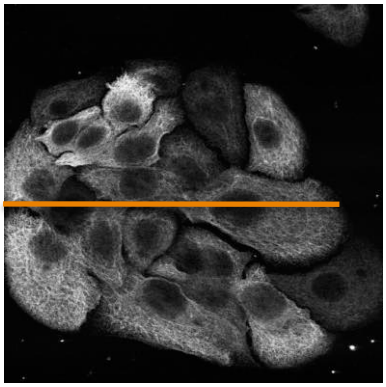
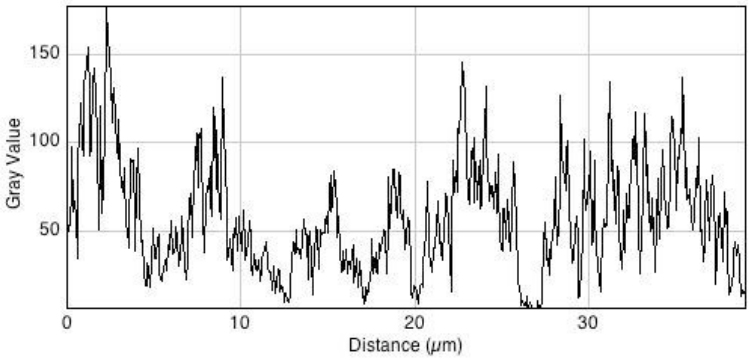


Figure 3.8: Keratin expression in control siRNA transfected and K15 siRNA transfected HaCaT cells.
Similar expression patterns can be seen in both K15 siRNA transfected and control siRNA transfected HaCaTs for K6 (A, B) and K17 (C, D). K8 expression in K15 knock-down cells (E) appears slightly reduced as compared to control siRNA transfected cells (F) (scale bar = 10μm).

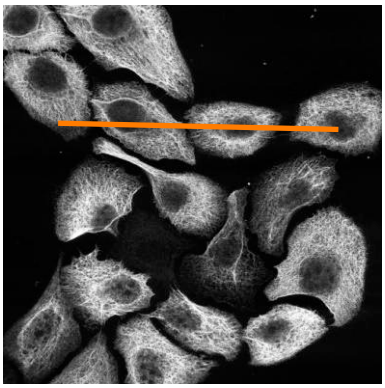
A

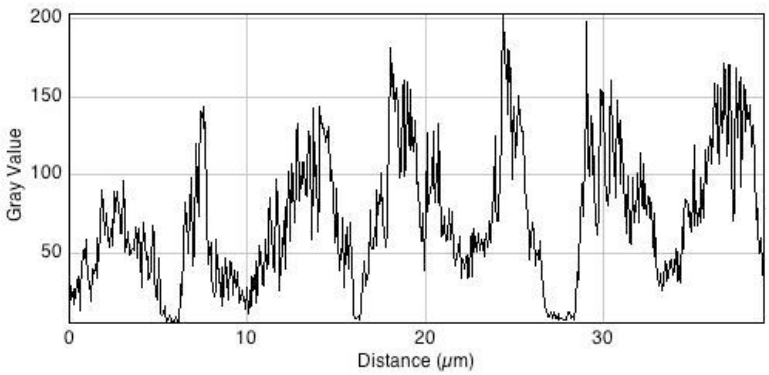
K15 siRNA Transfected





Control siRNA Transfected





B

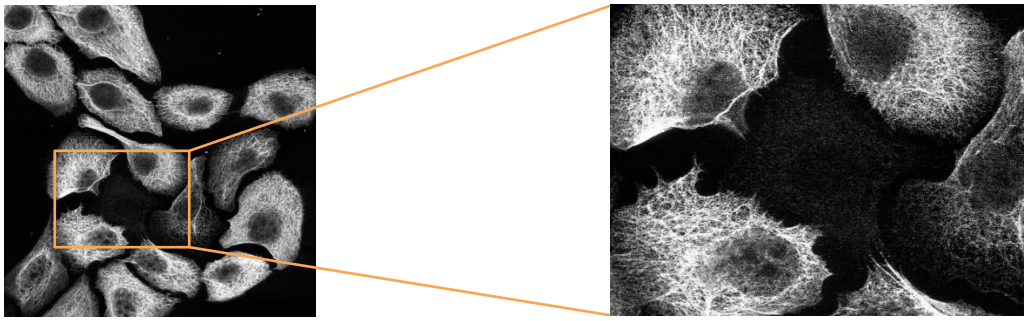


Figure 3.9: Variable K8 expression in HaCaT cells.

A: Measurement of average intensity of K8 staining in HaCaT cells. In order to examine the reduction in K8 expression, Image J software was used to analyse the intensity of the 'grey value'. The graphs indicate that the K8 staining is not as intense in the control cells compared to the K15 knock-down cells. The line indicates the cross-section where the staining intensity was measured.

B: This control siRNA transfected image of K8 shows where a cell is present which demonstrates a low level of K8 expression compared to nearby cells. Similar variation can be observed in K15 knock-down cells (see A).

3.3.3.3: K15 siRNA Transfection in NHEK Cells

In order to assess whether similar K15 ablation could be produced in normal keratinocytes, K15 siRNA transfection and control siRNA transfections were carried out using NHEK cells. The same procedure was used as with HaCaT cells, although the optimum knock-down for K15 was 72 h post-transfection (*Figure 3.10*). K15, K14 and actin arrangement were observed using immunofluorescence post-transfection. There is little difference between K14 expression in K15 siRNA transfected cells compared to control siRNA transfected cells (*Figure 3.10* B, E). Similarly, actin expression and localisation remains unchanged following K15 ablation (*Figure 3.10* C, F). However, due to limited numbers of NHEK cells available (for example, these cells can only be cultured to approximately passage 4 before differentiation), few other experiments were carried out using these cells. As similar results were obtained with HaCaT cells, these were considered a suitable immortalised cell-line compromise, with almost unlimited cell numbers available for experimentation.

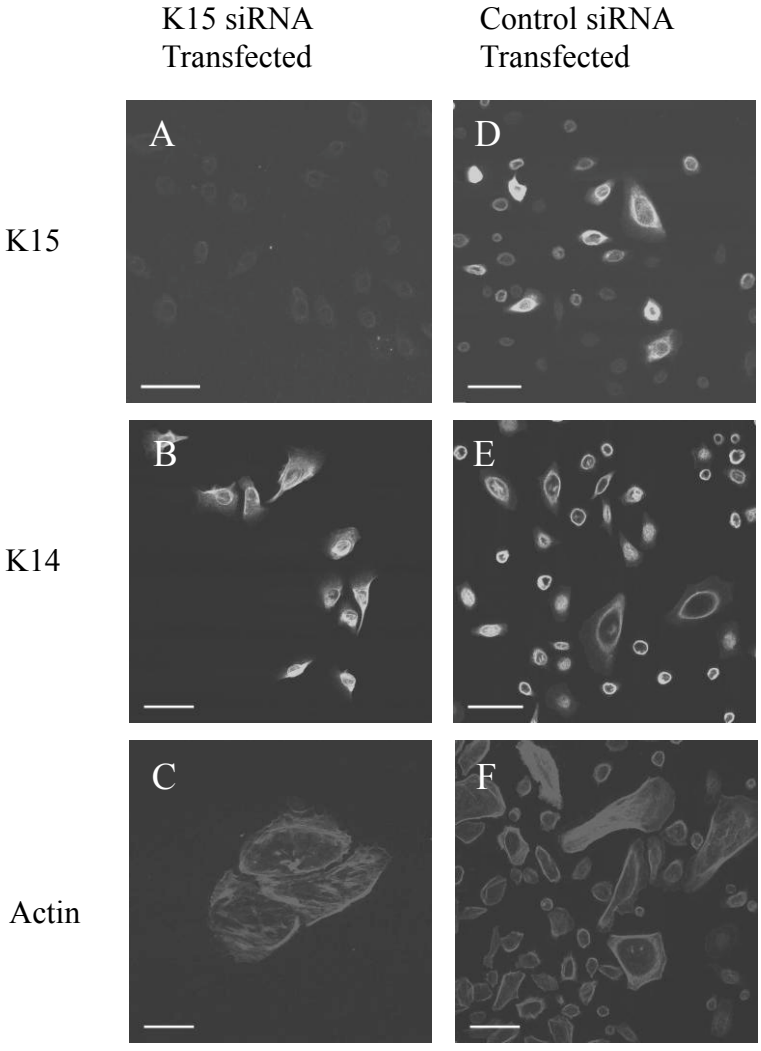


Figure 3.10: Expression of K14 and K15 in K15 siRNA knockdown NHEK cells. Single plane confocal images of immunofluorescence showing K15, K14 and actin in NHEK cells following K15 siRNA transfection (scale bar = 20μm). The levels of K15 in the K15 siRNA transfected cells can be seen to be lower than in control cells, demonstrating successful K15 knock-down. Neither K14 or actin organisation have been changed by K15 knock-down.

3.3.4: Cell Viability following K15 siRNA Transfection

An MTT assay was used in order to assess how HaCaT cell viability was affected by K15 ablation (*Figure 3.11*). $1x10^5$ cells were seeded and allowed to settle (0 h timepoint), then transfected using either K15 siRNA or control siRNA. 48 h post-transfection, an MTT assay was carried out. Whilst there had been some increase in cell number in control siRNA transfected cells, this increase was greater in K15 siRNA transfected cells. The relatively low numbers of cells used here were due to the restrictions on the cell numbers that could be successfully and efficiently transfected. However, the difference between K15 siRNA transfected and control siRNA transfected cell numbers at 48 h post-transfection remains significant ($p=0.001$).

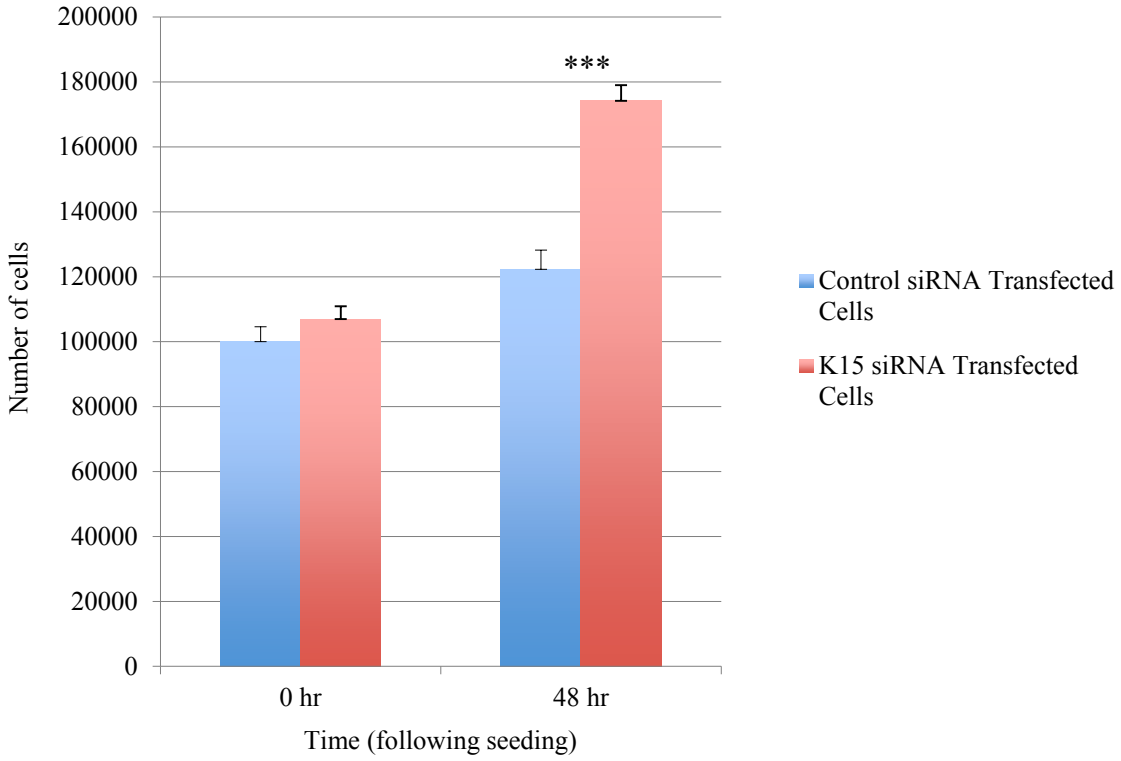


Figure 3.11: Proliferation of K15 siRNA transfected and control transfected HaCaT cells.

Cell numbers were calculated using the MTT assay and a standard curve (error bars = standard deviation). An increase can be observed in the number of viable cells observed post-seeding in both control siRNA and K15 siRNA transfected cells, however a greater increase is observed in K15 siRNA transfected cells. This difference is significant (***) (p=0.001).

3.3.5: Differentiation of K15 siRNA Transfected HaCaT Cells

3.3.5.1: Effect of K15 Knock-down on Involucrin and Filaggrin

It has been established that K15 is expressed in basal keratinocytes, and that expression is lost as cells differentiate and move upwards towards the surface of the skin. It was considered an important part of this study to establish whether loss of K15 was a factor in inducing differentiation. To investigate whether spontaneous differentiation (without calcium switch) was occurring, immunofluorescence was used to identify differentiation markers in HaCaT cells following K15 siRNA transfection.

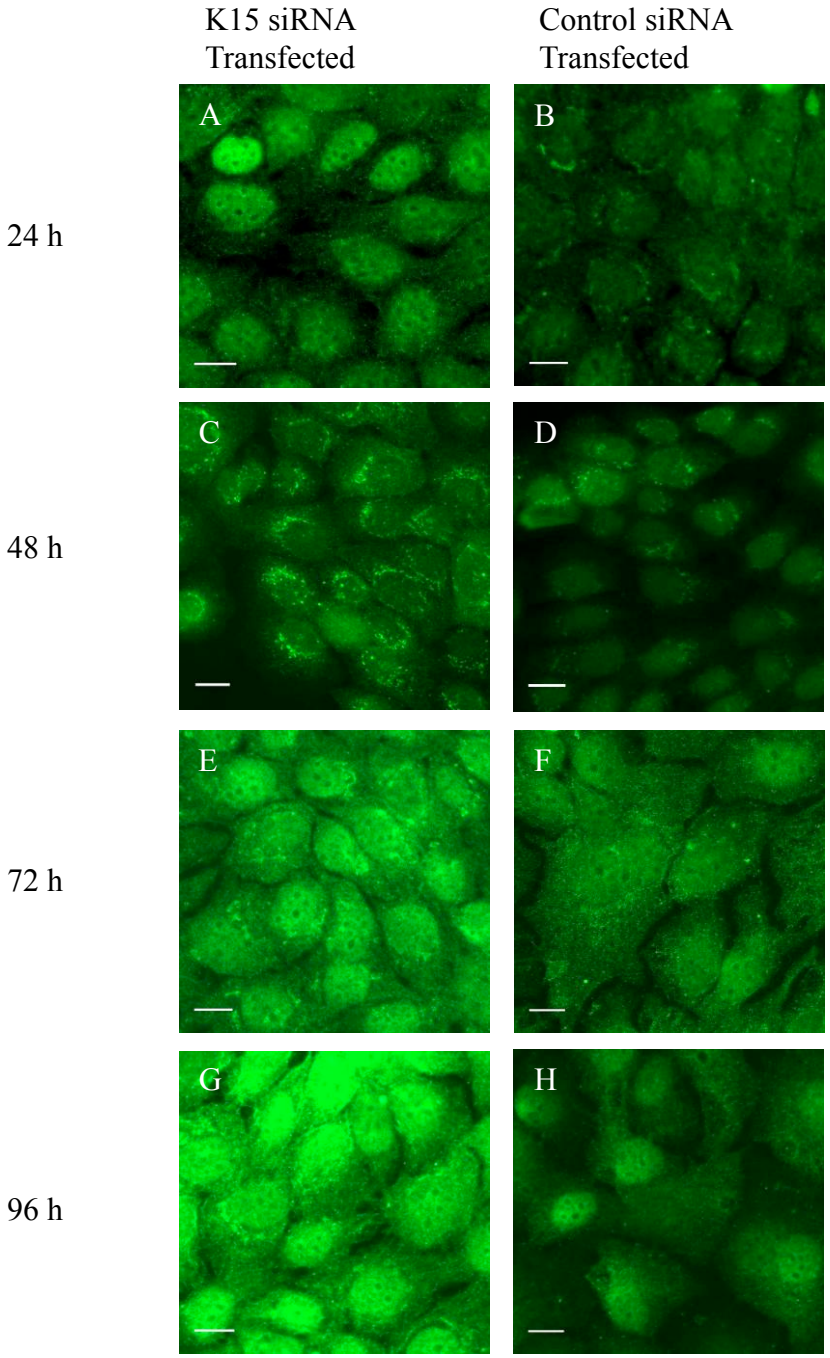


Figure 3.12: Expression of involucrin following siRNA transfection in HaCaT cells.
Single plane confocal images of immunofluorescence showing involucrin in HaCaT cells following K15 siRNA transfection (scale bar = 10μm).
As K15 expression is reduced using K15 siRNA transfection, involucrin levels increase (A, C, E, G). The increase continues after K15 expression has been shown to return to normal 96 h post-transfection (G).
There appears to be less involucrin in the control siRNA transfected cells (B, D, F, H) compared to the K15 siRNA transfected cells, although the expression pattern is similar (i.e. concentrated around the nucleus).
Where levels of involucrin are high, some non-specific staining can be observed in the nucleus (for example, G). This is like to be as a result of cross-reaction of the rabbit antibody.

Figure 3.12 shows involucrin immunofluorescence between 24 and 96 h post-transfection. There is some variation in the involucrin expression between these time points in the control siRNA transfected HaCaT cells. At 24, 72 and 96 h however (*Figure 3.12* B, F and H), involucrin appears to be more highly concentrated around the nucleus (some non-specific staining of the nucleus is also observed, possibly due to the use of a rabbit antibody). This also occurs in the K15 siRNA transfected cells. Throughout the experiment, involucrin staining becomes more intense in the K15 ablated cells. The perinuclear staining pattern however is comparable with control cells (for example, at 48 h post-transfection [*Figure 3.12* C and D]).
Involucrin, as a precursor of cross-linked envelope assembly in skin, is considered an early marker of keratinocyte differentiation (Watt, 1983; Crish *et al*., 2002). As increased levels of involucrin are associated with differentiation, this result suggests that K15 siRNA transfected cells may be becoming more differentiated than control siRNA transfected counterparts. Involucrin has a role as a substrate for transglutaminase 1, an important step in catalyzing the formation of the cornified envelope (Schroder *et al*., 1992; LaCelle *et al*., 1998; Hasegawa *et al*., 2011). Following synthesis then, involucrin is localised to the interior face of the plasma membrane, before being cross-linked by transglutaminase 1 (Thacher and Rice, 1985; Steinert and Marekov, 1997; Candi *et al*., 2001). This suggests that involucrin should localise at the cell membrane prior to cross-linking in cornified envelope formation. It appears that this could be happening in *Figure 3.12* G, where bright immunoflourescence staining throughout the cell may indicate involucrin localisation at the cell membrane. This is in contrast to earlier time points where involucrin appears as perinuclear granules (*Figure 3.12* C). Possible non-specific staining can also be observed in the nucleus of some cells. As K15 has previously been shown to be expressed in undifferentiated cells in the skin, it may be that K15 expression (either directly or indirectly) affects cell differentiation. This theory required further investigation.

We next investigated filaggrin immunofluorescence following transfection and in untransfected HaCaT cells. Very low levels of non-specific staining were observed (data not shown), suggesting that filaggrin is not a protein expressed in HaCaT cells without calcium switch. As filaggrin is usually expressed in the granular layer and above, it suggests that HaCaT cells are not differentiated enough to express this protein *in vitro*. Similar, low level non-specific filaggrin staining was observed in K15 siRNA transfected cells, indicating that even without K15, HaCaT cells are not differentiated up to a granular level 'stage'. Papp *et al*. (2003) demonstrated that low levels of filaggrin were expressed in cultured HaCaT cells, increasing upon confluency (approximately 6 days post-seeding). However, in order to increase efficiency of transfection, in this experiment cells were only cultured to approximately 60-70% confluency (at 48 h post-transfection). This may also explain the reduced levels of filaggrin observed.

3.3.5.2: Effects of K15 Knock-down on Cell Junctional Proteins
With the differences observed in involucrin expression, it was considered useful to assess the effect of K15 ablation on cell junctional proteins. Cell junctions are known to be affected during differentiation; for example, undifferentiated cells have fewer desmosomes than suprabasal cells. As cells differentiate they migrate upwards forming more desmosomes and assembling tight junctions (and expressing tight junction proteins) to contribute to the skin barrier function. To establish the type of

cell-cell interactions present in control transfected and K15 siRNA transfected HaCaT cells, E-cadherin, desmoplakin and ZO-1 expression and localisation were observed.

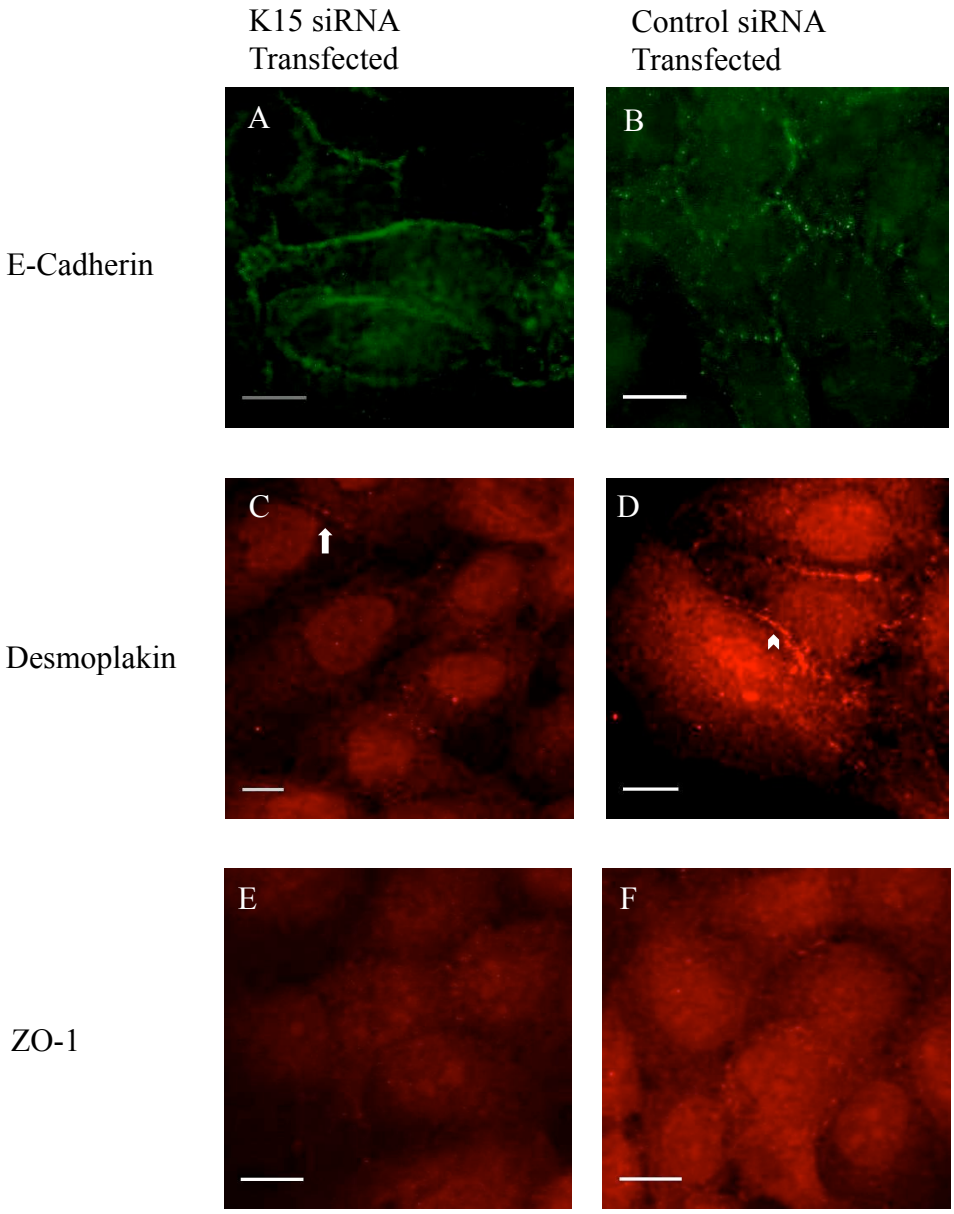


Figure 3.13: Expression of desmoplakin, E-cadherin and ZO-1 following K15 siRNA transfection in HaCaT cells.

Single plane confocal images of immunofluorescence showing desmoplakin, E-cadherin and ZO-1 in HaCaT cells following K15 siRNA transfection (scale bar = 10μm).

E-cadherin expression can be observed at cell-cell junctions in both control siRNA transfected (B) and K15 knock-down (A) HaCaT cells.

Some desmoplakin at cell junctions can be observed in control siRNA transfected cells (D, arrowhead) and K15 siRNA transfected cells (C, arrow).

No ZO-1 can be seen at cell-cell junctions in control siRNA transfected (F) or K15 siRNA transfected (E) HaCaT cells.

E-cadherin expression was assessed using immunofluorescence; cells were fixed and stained 48 h post-transfection (*Figure 3.13* A and B). Cell-cell junctions in both control and K15 knock-down cells can be seen to stain for E-cadherin, and intensity of staining appears similar in both K15 knock-down cells and control siRNA transfected cells. No increase or decrease was observed in E-cadherin following K15 ablation. This suggests that ablation of K15 does not affect adherens junction formation.

Desmoplakin is associated with desmosomes, which are formed after the initial cell-cell interactions involving E-cadherin. The role of desmoplakin is to anchor the keratin network to the desmosomal cadherins (Godsel *et al*., 2005); it was therefore considered useful to assess the effect of K15 ablation on desmoplakin expression and localisation. Wan *et al*. (2007b) demonstrated that increased cell proliferation was observed in desmoplakin knock-down HaCaT cells. Here desmoplakin expression was observed by immunofluorescence in HaCaT cells after siRNA transfection. In control siRNA transfected cells, desmoplakin can be observed clearly at cell borders (*Figure 3.13* C, arrowhead). Without calcium switch not all cells formed desmosomes, however desmoplakin was detected at cell borders. This suggests that K15 ablation does not affect desmoplakin expression or desmosome formation. This is as expected, since no changes in E-cadherin expression are observed.

The expression and subcellular localisation of the tight-junction protein ZO-1 was also assessed using immunofluorescence. At 48 h post-transfection, HaCaT cells were fixed in paraformaldehyde and stained. Low levels of cytoplasmic staining can be seen in both control siRNA transfected and K15 siRNA HaCaT cells; this suggests that neither control siRNA transfected nor K15 siRNA transfected HaCaT cells produce tight junctions (*Figure 3.13* E and F). Tight junctions are associated with well differentiated keratinocytes, so formation of these junctions in HaCaT cells cultured without high levels of calcium is unlikely. Although some differentiation of HaCaT cells may be occurring in K15 ablated cells, differentiation does not occur to an extent where tight junctions begin to form. If cells could be cultured to a more confluent stage, it may be possible to obtain evidence of tight junction formation. However, the 30-50% confluency required for effective transfection coupled with the transient nature of the K15 siRNA transfection prevents the possibility of culturing HaCaT cells for the length of time required to establish these higher levels of confluency.

3.3.5.3: Effects of High Extracellular Calcium on K15 siRNA Transfected HaCaT Cells

Since it is possible that K15 ablation may be promoting differentiation in HaCaT cells (as indicated by involucrin immunofluorescence), a calcium switch experiment was carried out to determine whether any similarities could be observed. Increases in extracellular calcium concentration have been shown to increase differentiation in keratinocytes (for example, as described by Yuspa *et al*., 1989; Kolly *et al*., 2005). There are several mechanisms which can affect many proteins in keratinocytes dependent on extracellular and intracellular calcium, such as the PKC pathway (see section 3.1.4).

HaCaT cells were transfected and culture media was switched to either low calcium or high calcium media, for 6 h (at 42 h post-transfection) or 72 h (where transfection was carried out 24 h after the initial change of media to high or low calcium media). Cells were then fixed and immunofluorescence used to observe K15, K14 and

involucrin. This was to ensure that the cells were all harvested at the point of maximum K15 ablation.

Table 3.1: Timeline of Calcium Switch Assay

6 hour time point	**72 hour time point**
HaCaT cells seeded ↓ Cells transfected ↓ *42 h later* Media switched to either high or low calcium media ↓ *6 h later* Cells fixed and immunofluorescence carried out	HaCaT cells seeded ↓ Media switched to either high or low calcium media ↓ *24 h later* Cells transfected ↓ *48 h later* Cells fixed and immunofluorescence carried out

Firstly, the effect of increased extracellular calcium on K15 levels in HaCaT cells was assessed (*Figure 3.14*). K15 siRNA transfected cells consistently show a lack of K15. Thus, siRNA ablation could be carried out in high calcium conditions. Control siRNA transfected cells all show that HaCaT cells express K15, regardless of extracellular calcium concentration. This also demonstrated that transfection of cells in high calcium media was equally efficient. Similar levels of K15 are expressed throughout, with K15 being seen throughout the cytoplasm up to the edges of the cells, and around the nucleus. As K15 is considered a marker of less differentiated cells, it was not expected for keratinocytes to express K15 when cultured in high calcium media for 72 h. This may be because of the unusual keratin expression pattern observed in HaCaT cells, or due to relatively long half-life of intermediate filament proteins.

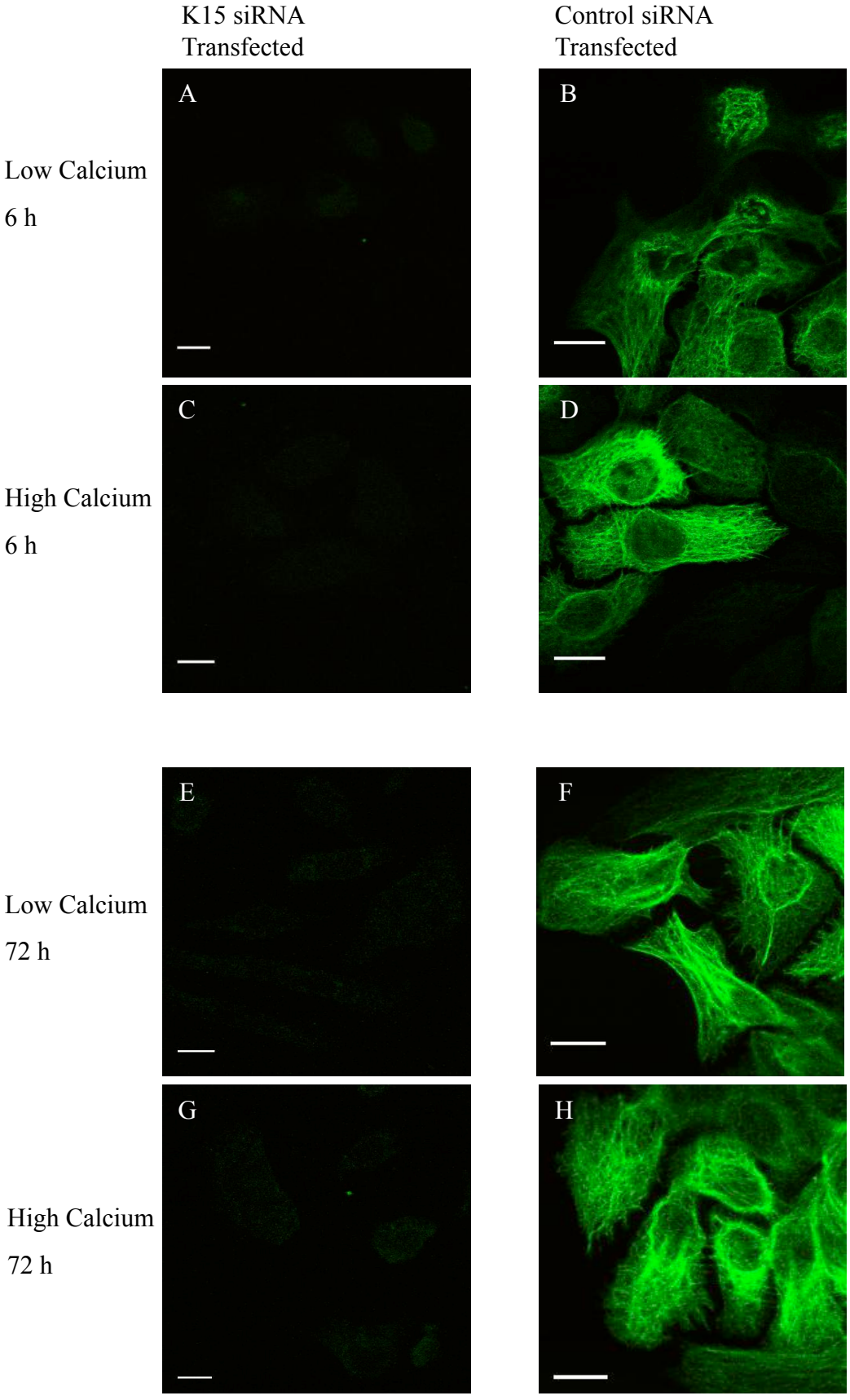

K15 siRNA Transfected
Control siRNA Transfected
A
B
Low Calcium 6 h
C
D
High Calcium 6 h
E
F
Low Calcium 72 h
G
H
High Calcium 72 h

Figure 3.14: Expression of K15 in HaCaT cells following siRNA transfection after growth in high or low calcium media.

Single plane confocal images of immunofluorescence showing K15 in HaCaT cells following K15 siRNA transfection (scale bar = 10μm).

Low levels of K15 can be observed in K15 siRNA transfected cells after 6 h in low calcium media (A). K15 expression in control siRNA transfected cells (B) appears normal, with K15 throughout the cytoplasm to the edge of the cell. After culture in high calcium media for 6 h, low level K15 expression can be seen in K15 siRNA transfected cells (C). In control siRNA transfected cells (D), some heterogeneic K15 expression can be observed, where some cells express more K15 than others.

Low levels of K15 can be observed in K15 siRNA transfected cells (E) after culture in low calcium media for 72 h. Expression of K15 in control siRNA transfected HaCaT cells (F) appears similar to K15 as observed at 6 h in low calcium media (B); expression is observed throughout the cytosplasm, to the edge of the cell. Despite some heterogeneity after 6 h in high calcium media, no similar heterogeneity is observed after 72 h in control siRNA transfected HaCaT cells (H). Although high calcium media induces differentiation in HaCaT cells, K15 expression remains. K15 can be seen throughout the cytoplasm through to the edges of the cell. Thicker K15 ‘cables’ can be observed around the nucleus. Low levels of K15 are observed in K15 siRNA transfected cells (G).

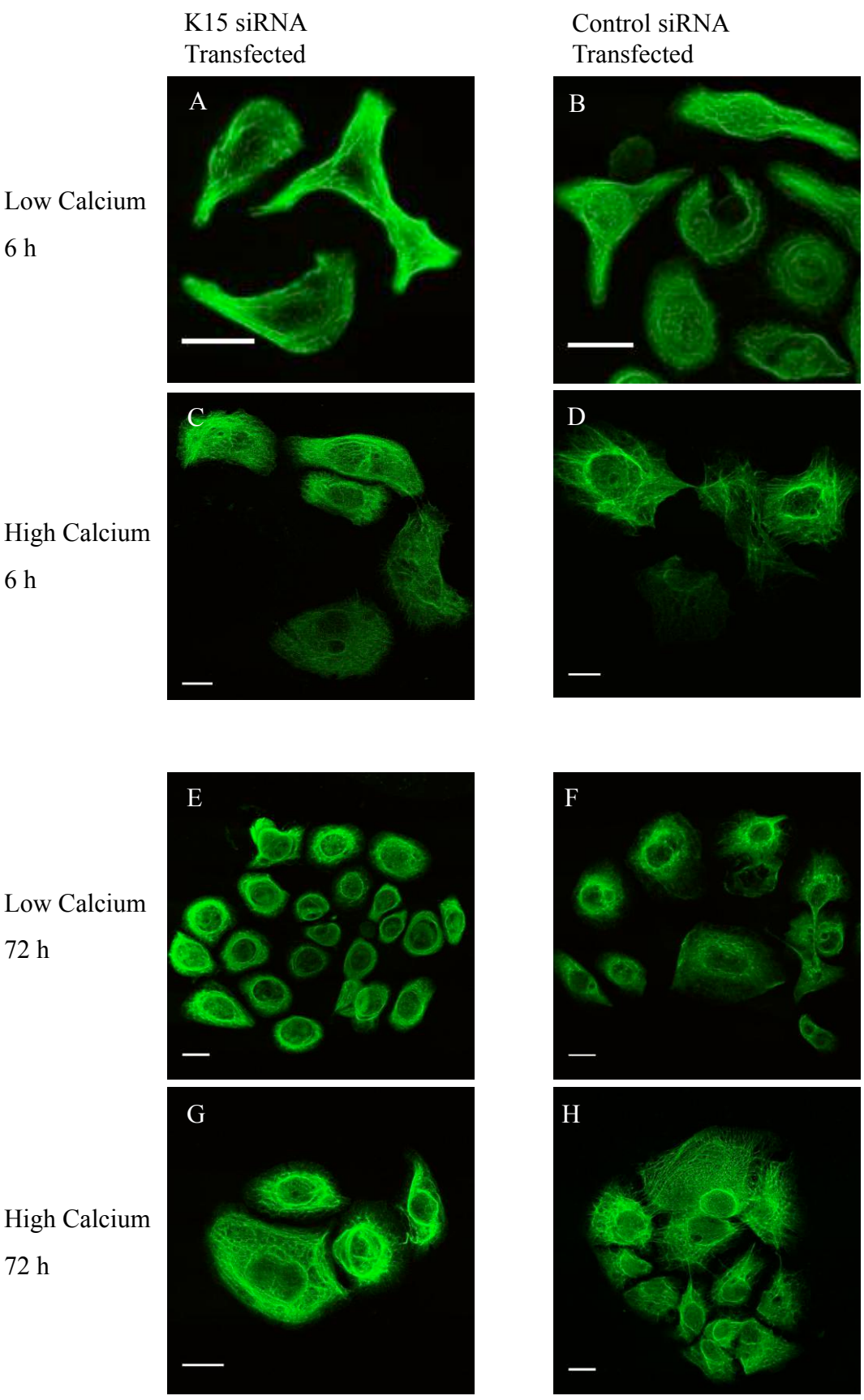

K15 siRNA
Transfected
Control siRNA
Transfected
A
B
Low Calcium
6 h
C
D
High Calcium
6 h
E
F
Low Calcium
72 h
G
H
High Calcium
72 h

Figure 3.15: Expression of K14 in HaCaT cells following siRNA transfection after growth in high or low calcium media.

Single plane confocal images of immunofluorescence showing K14 in HaCaT cells following K15 siRNA transfection (scale bar = 10μm).

Previous results have suggested that following K15 siRNA transfection, K14 is not observed to the edge of the cell. This was not observed here after 6 h in low calcium media (A), as K14 was observed throughout the cytoplasm, around the nucleus and up to the edges of the cell. This was also observed in control siRNA transfected cells (B). In both K15 siRNA transfected cells (C) and control siRNA transfected cells (D), K14 fibres can be observed throughout the cytoplasm and around the nucleus after 6 h in high calcium media; this was comparable to K14 expression in HaCaT cells grown in low calcium media.

Similar expression is observed in both control siRNA transfected (F) and siRNA transfected cells (E) after culture in low calcium media for 72 h, as K14 is expressed around the nucleus and throughout the cytoplasm. In control siRNA transfected cells, K14 can be observed up to the edge of the cell (F); however staining intensity appears reduced at the cell edge in K15 siRNA transfected cells (E), as observed previously following K15 siRNA transfection. After 72 h in high calcium media, K14 is expressed throughout the cytoplasm and around the nucleus in both K15 siRNA transfected (G) and control siRNA transfected cells (H). K14 staining intensity also decreased at the cell edge in K15 siRNA transfected cells (G). This is not observed to the same extent in control siRNA transfected cells (H). K14 expression appears increased in K15 siRNA transfected cells after 72 h in high calcium media (G) compared to cells cultured for 6 h in high calcium media (C).

For comparison, K14 immunofluorescence was also carried out on transfected cells cultured in high or low calcium media (*Figure 3.15*). In K15 siRNA transfected cells, K14 can be observed throughout the cytoplasm and around the nucleus; after 6 h culture in high or low calcium media, no differences could be observed in K14 expression or localisation. K14 staining could not be observed up to the cell edges in K15 siRNA transfected cells after 72 h in either high or low calcium media (*Figure 3.15* E and G). This is comparable to results described in Chapter 4 (*Figure 4.6*, *Figure 4.7*) and support the observation that K14 organisation is dependent on K15 expression. Other than this slight change, K14 expression appears similar to control siRNA transfected cells; K14 expression level in high and low calcium-treated cells does not appear to change.

To establish whether this was occurring, Image J software was used to quantify any changes in K14 arrangement during cell spreading (*Figure 3.16*); this is calculated as intensity of 'grey value'. The graphs produced show that K14 staining intensity peaks in the cytoplasm, before a drop is observed in the nucleus (this is due to the plane of the images taken). In the untransfected HaCaT cells, the intensity of K14 staining is relatively uniform throughout the cytoplasm, and can be observed to the edge of the cell. Similarly, in control siRNA transfected cells, the staining intensity peaks in the cytoplasm, although the staining is not as intense at the edges of the cell, the peak is reached in a relatively short distance. In K15 siRNA transfected cells however, the staining intensity at the edge of the cell is far lower than in control cells; a peak of similar intensity is achieved in the cytoplasm closer to the nucleus. This again shows that in K15 ablated cells there may be altered cytoskeletal protein arrangements at the cell edges during spreading (for further details on this see section 4.3.2.4).

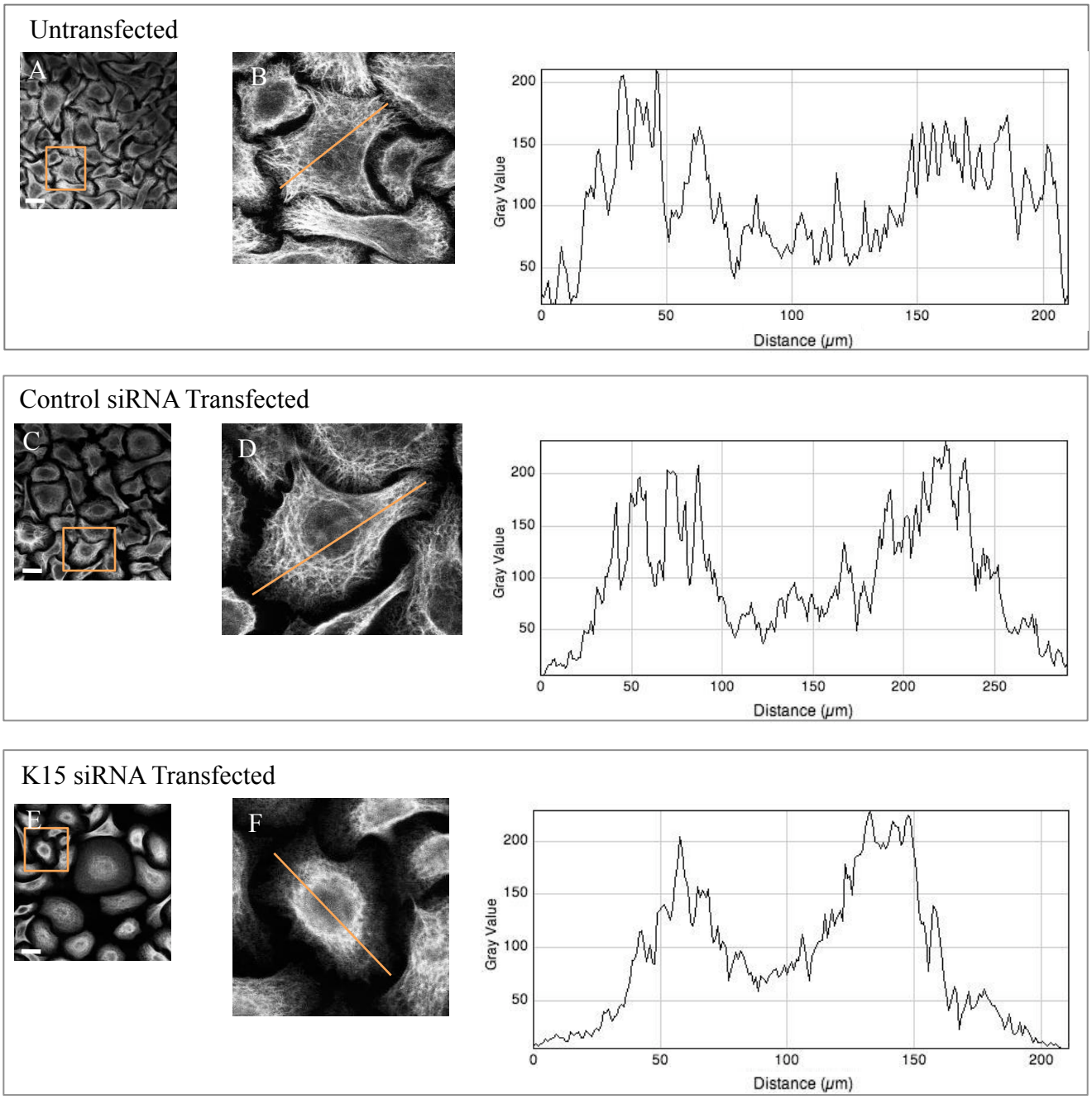


Figure 3.16: Staining intensity measurement of K14 in untransfected, control siRNA transfected and K15 siRNA transfected HaCaT cells.

Single plane confocal images of untransfected (A, B), control siRNA transfected (C, D) and K15 siRNA transfected (E, F) cells stained for K14 were analysed using Image J in order to examine any change in K14 expression (scale bar = 10μm). The line (B, D, F) indicates the cross-section of the cell where the staining intensity was measured. Peaks can be observed around the nucleus. The plots for the control cells appear similar, although the cell size of the control siRNA transfected cell is slightly larger than the untransfected cell. A comparison of the K15 siRNA transfected cell and the similarly sized untransfected cell suggest that although the peaks are similar, there is a reduction in the amount of K14 at the edge of the cell in the K15 siRNA transfected cell compared to the untransfected cell.

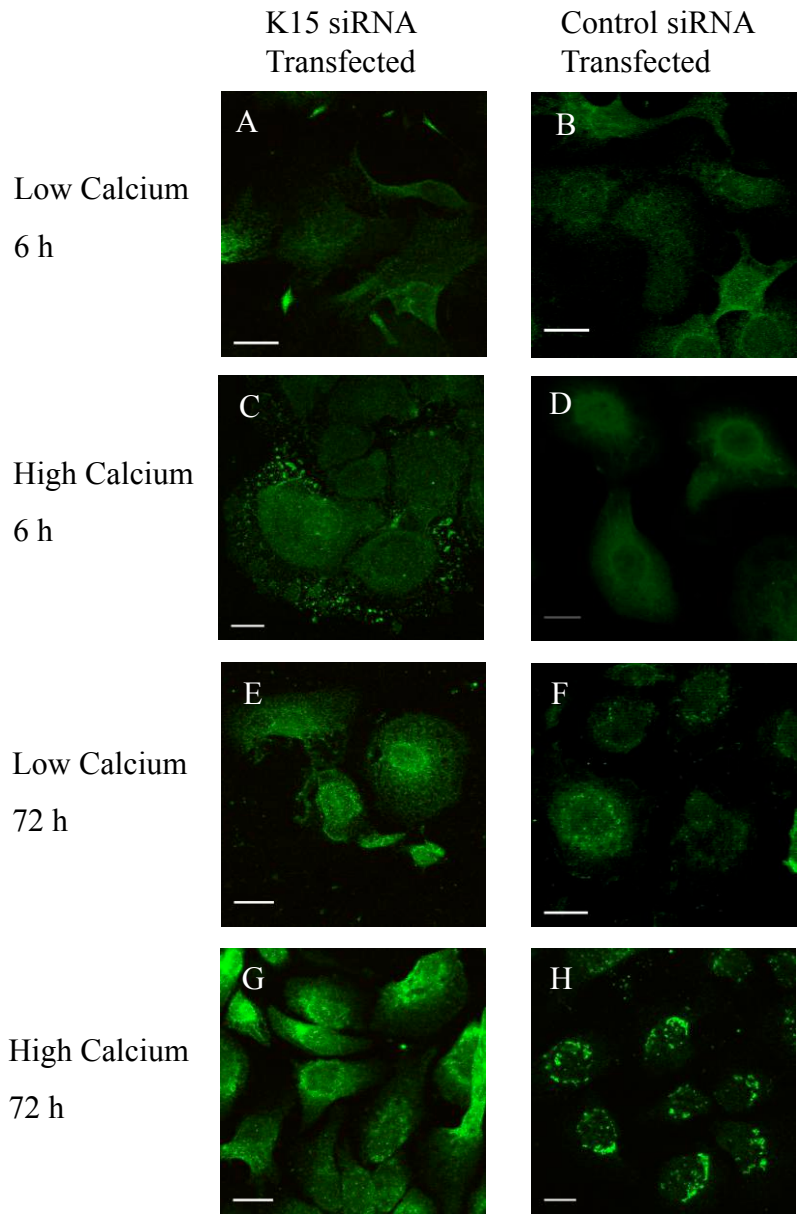


Figure 3.17: Expression of involucrin in HaCaT cells following siRNA transfection after growth in high or low calcium media.

Single plane confocal images of immunofluorescence showing involucrin in HaCaT cells following K15 siRNA transfection (scale bar = 10μm).

Low levels of involucrin are observed in both control siRNA transfected (B) and K15 siRNA transfected (A) cells after 6 h culture in low calcium media. Both appear similar, with involucrin expressed throughout the cytoplasm without a granaular appearance. Similar levels of involucrin can be observed in both K15 ablated cells (C) and control cells (D) after 6 h culture in high calcium media; involucrin can be observed throughout the cytoplasm. In control siRNA transfected cells, this concentrates around the nucleus to an extent, and in K15 siRNA transfected cells the involucrin appears more granular.

Following 72 h culture in low calcium media, expression of involucrin in both control (F) and K15 ablated cells (E) appears similar to that observed after 6 h in low calcium media, with involucrin expressed at relatively low levels throughout the cytoplasm. The staining intensity of involucrin in K15 siRNA transfected cells is slightly greater than in control siRNA transfected cells. After 72 h culture in high calcium media, involucrin staining varies between K15 ablated and control HaCaT cells. In K15 siRNA transfected cells (G), staining is observed throughout the cytoplam, with some stronger staining around the nucleus; in some cells this appears granular. Regions around the nucleus are also strongly stained in control siRNA transfected cells (H), in a definite granular appearance, with little other staining appearing throughout the remainder of the cytoplasm.

Following 6 h exposure to low calcium media, no change in involucrin can be observed between K15 siRNA transfected cells and control siRNA transfected cells (*Figure 3.17* A and B). Low calcium conditions do not induce differentiation, which may contribute to the generally low levels of involucrin observed. An increase in involucrin can be observed following 6 h exposure to high calcium media however (*Figure 3.17* C and D). Involucrin in both control siRNA transfected and K15 siRNA transfected cells is uniformly present throughout the cell, although to a greater extent in K15 knock-down cells. It was previously demonstrated that involucrin expression possibly increases in K15 ablated cells (*Figure 3.12*); the increase in extracellular calcium could also be enhancing an effect. Longer exposure to low levels of extracellular calcium appear to have little effect on involucrin expression in control siRNA transfected cells (*Figure 3.17* F). Slightly more involucrin can be observed in the K15 siRNA transfected HaCaT cells (*Figure 3.17* E), suggesting that even at low levels of extracellular calcium, K15 ablation does have an effect on involucrin expression (and therefore differentiation). This suggests that K15 ablation alone can affect involucrin levels (as previously observed in cells cultured without exposure to higher concentrations of extracellular calcium, in *Figure 3.12*). There is some increased involucrin expression in control siRNA transfected cells following 72 h exposure to high calcium (*Figure 3.17* H), which indicates that these cells have commenced differentiation. A further increase can be observed in K15 siRNA transfected cells (*Figure 3.17* G). This said, the immunofluorescence data presented is not entirely conclusive; further examination of involucrin would be required to assess expression more conclusively.

To assess the effect of the calcium switch assay on other keratins in HaCaT cells, cytoskeletal extracts were produced from cells cultured in high and low calcium media following transfection with either control or K15 siRNA. Since this method had already demonstrated that no other keratins were affected by K15 ablation or the transfection procedure, any changes observed would be as a result of changes in extracellular calcium concentration after 72 h.

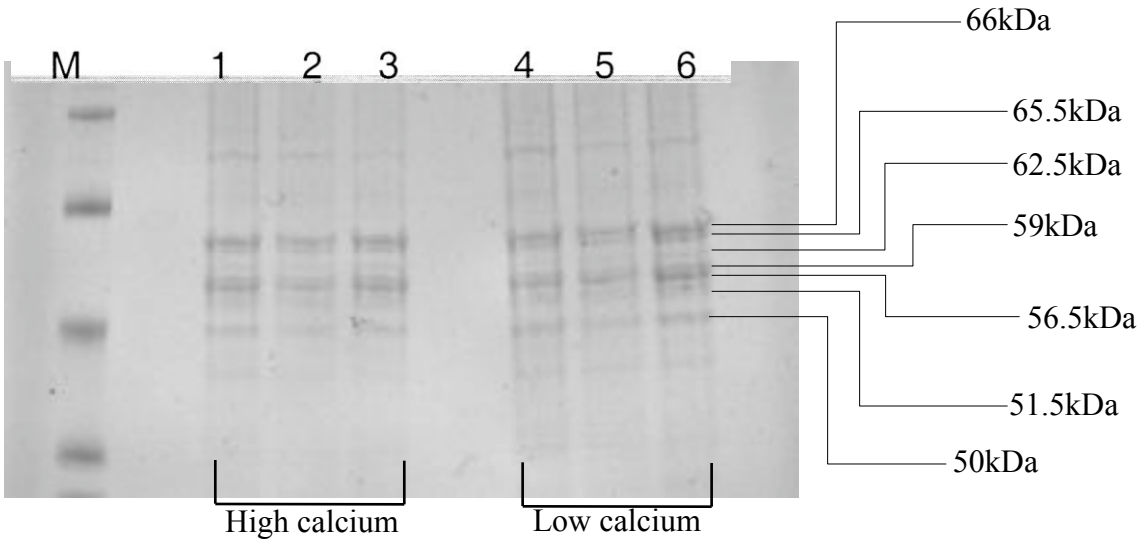


Figure 3.18: HaCaT cytoskeletal protein extracts from a calcium switch assay. Samples 1-3 are from cells cultured in high calcium; samples 4-6 are cultured in low calcium, both for 72 hours (M = marker). Samples 1 and 4 are untransfected, 2 and 5 are control siRNA transfected and 3 and 6 are K15 siRNA transfected. In low calcium media, a slight increase in expression of the 59kDa keratin can be observed in K15 siRNA transfected cells (lane 6). This increase is also observed in untransfected, control siRNA transfected and K15 siRNA transfected cells cultured in high calcium media (lanes 1, 2 and 3). Few differences can be observed here, although this assessment was carried out using cytoskeletal extract from only one replicate of the initial assay.

Figure 3.18 demonstrates that a slight increase in a 59kDa keratin (possibly K10) can be seen in K15 siRNA transfected cells compared to control siRNA transfected and untransfected cells in low calcium media; such a difference is not observed after cells are cultured in high calcium media; the 59kDa band is more prominent in control cells here, suggesting that there may be an increase in K10 expression following exposure to high calcium media. K10 expression is associated with differentiating keratinocytes, and has been shown to be expressed in differentiating HaCaT cells (Micallef *et al*., 2009).

3.3.5.4: Differentiation of HaCaT Cells in Organotypic Cell Culture

Recently, a scaffold has been utilised to aid 3D culture of keratinocytes. This is thought to be an improvement on the previously used technique of culturing cells on collagen gels. The scaffolds allow cells to infiltrate the scaffold before growing out of the top of the scaffold to the air-liquid interface.

The ability of the HaCaT cell line used in this study to differentiate was investigated by using 3D culture to examine K14, K15 and K1 expression following exposure to the air-liquid interface.

After 14 days at the air-liquid interface, K14 expression was observed in cells throughout the stratified layers and in the scaffold (*Figure 3.19* A). This suggests that K14 expression remains active in HaCaT cells regardless of any differentiation occurring. In contrast, although some K15 was observed throughout cells after 14 days at the air-liquid interface (*Figure 3.19* B), most of the K15-positive cells observed were either in the scaffold or just above the surface of the scaffold. This is as expected of a basal layer keratin. It also demonstrates the ability of HaCaT cells to reduce K15 expression during differentation.

As for K14 expression, K1 was expressed in HaCaT cells throughout the 3D culture. Less K1 was observed in those cells in the scaffold, whilst most cells above the scaffold expressed K1. *Figure 3.19* C shows that K1 expression through the culture varied between cells, and was not only present in those cells above the basal layer.

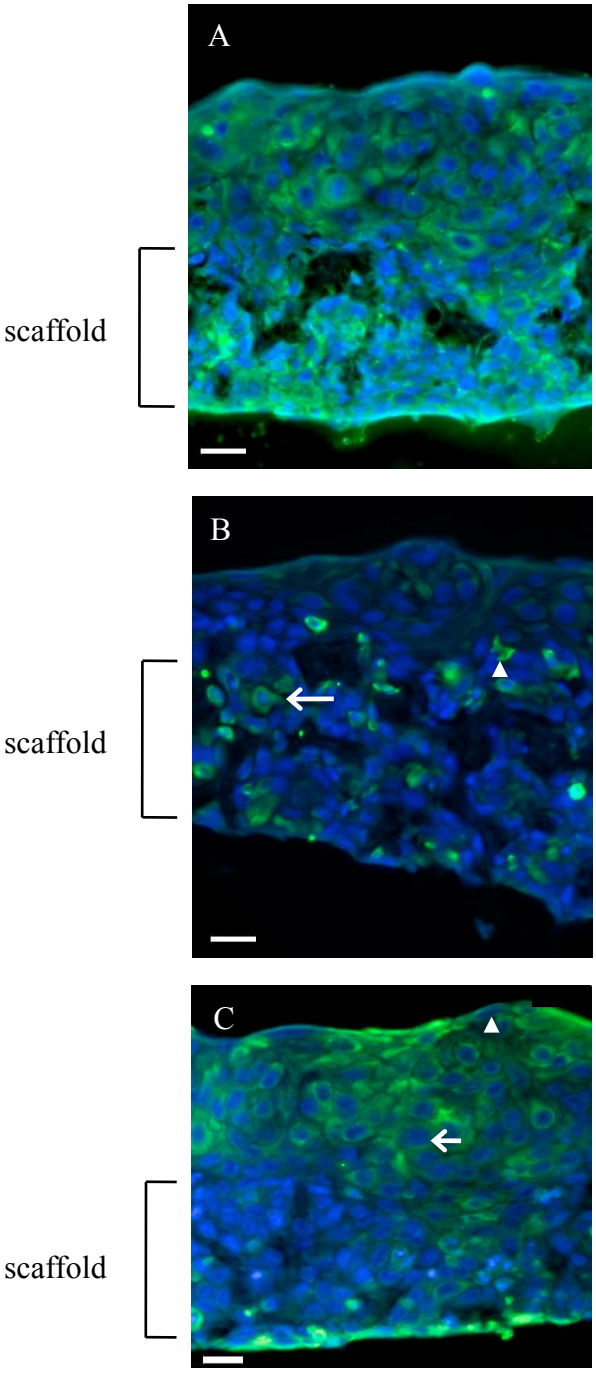


Figure 3.19: K14 and K15 in organotypic HaCaT cell cultures grown on scaffolds, raised to air-liquid interface for 14 days. All sections have been stained with the nuclear marker DAPI (scale bar = 20μm).
As characteristic in HaCaT cells, K14 (A) is expressed throughout the section, including cells within the scaffold. K15 (B) is expressed to some extent in those HaCaT cells within the scaffold (arrow). It is also expressed in those cells just outside the scaffold (arrow head). Some residual K15 is observed throughout the rest of the stratified cells. K1 (C) appears to be expressed throughout the cells in the scaffold, and to some extent in those cells above. Most K1 however appears to be present in those cells above the surface of the scaffold (arrowhead), with less K1 expressed in the cells above. Increased levels then appear again towards the surface of the culture. DAPI staining clearly shows the larger nuclei of the cells above the scaffold, and flattened nuclei towards the top of the culture (arrowhead).

3.4: Discussion

3.4.1: K15 in Mammalian Skin

This study used immunofluorescence to examine the appearance of K15 in mammalian whole skin. A similar pattern was seen using different antibodies to stain mouse, rat and human skin. It had been previously demonstrated that differential expression of K15 could be observed in either frozen or paraffin-embedded human skin sections (van der Velden *et al*., 2009). In human skin, K15 was observed in the basal layers, as expected (and previously observed in, for example, Waseem *et al*., 1999 and in intestinal epithelium by Zhan *et al*., 2007). The intensity of K15 staining reduced towards the skin surface, suggesting a reduction in the amount of K15 in suprabasal, differentiating cells. It is likely that the remaining protein is residual, as opposed to actively expressed (for example, as described in epidermolysis bullosa simplex skin sections by Peters *et al*., 2001). Waseem *et al*. (1999) demonstrated using *in situ* hybrdisation that K15 mRNA was primarily located in the basal keratinocytes, suggesting that K15 mRNA is downregulated in activated cells. In a study examining epidermolysis bullosa simplex mutations, Werner *et al*. (2004) suggest that although free keratin subunits have a half-life of less than 15 minutes, filaments were far more static. These publications support the conclusion that K15 is actively expressed in the basal layers, whilst residual, stable K15 (K14 and K5) keratin filaments can be observed in more differentiated, stratifying cells.

3.4.2: K15 siRNA Transfection in HaCaT Cells

3.4.2.1: Keratin Expression in K15 Ablated HaCaT Cells

One method of assessing the function of K15 expression in cells is to knock-down the protein using transient transfection methods and carry out further investigations.
Cells were fixed at various time points and immunofluorescence carried out to assess the effectiveness of the transfection (24 hours, 48 hours, 72 hours and 96 hours). HaCaT cells showed the most effective K15 knock-down at 48 hours, with almost full recovery by 72 hours. This recovery is swifter than a mesenchymal cytoskeletal protein, vimentin, where optimum knock-down using siRNA transfection is 72 hours post-transfection in MCF-7 cells (McInroy and Määttä, 2007). Also in MCF-7 cells, siRNA transfection of K8 and periplakin was also optimal at between 48 and 72 hours (Long *et al*., 2006). Experiments using RNAi techniques to knock-down K18 have also shown to be optimal (in HeLa cells) between 48 and 72 hours (Claser *et al*., 2008), whereas K6 knock-down in HaCaT cells has been shown to be optimal (up to 100%) between 72 and 96 hours post-transfection (Smith *et al*., 2008). This suggests that the optimal knock-down of K15 at 48 hours is comparable, if slightly earlier than the optimal time scale for other cytoskeletal proteins, and may indicate a faster turnover of the protein. In NHEK cells, the optimum K15 knock-down was at 72 hours post-transfection, again comparable with similar techniques used to knock-down similar proteins in keratinocytes. The rapid recovery makes it difficult to analyse long-term effects of, for example, differentiation using siRNA transfections. More stable transfections (e.g. lentiviral transfection) would be useful to investigate longer term effects of K15 ablation.

Post-transfection HaCaT cells at 24, 48, 72 and 96 h were observed for K14, K15 and involucrin using immunofluorescence. The amount of K14 expressed did not appear to change following K15 ablation (*Figure 3.5* and *Figure 3.6*). Nijhof *et al*. (2006) have demonstrated that K14 is expressed in keratinocytes in the basal layer of the IFE; this study has shown that K15 is also present in this basal layer. This indicates that both K14 and K15 are expressed simultaneously. Although in K14 knockout

mice K15 was not shown to be upregulated (Jonkman *et al*., 1996), Waseem *et al*. (1999) demonstrated that K15 expression was upregulated in human subjects with EBS, where both alleles for K14 were inactivated. In this study, no changes in K14 expression are observed in K15 siRNA transfected cells. However, some change in K14 organisation did occur. Image J was used to demonstrate that K14 was not expressed to the cell edge in K15 knock-down cells, unlike control cells. This could be due to cell spreading; this is further investigated in Chapter 4.

As well as K15, other keratins were observed using immunofluorescence in order to assess any effect either the transfection procedure or K15 ablation had on expression and/or localisation. K15 knock-down had no effect on K17 or K6. This is a further indication that this siRNA transfection process does not affect other keratins. It also suggests that these other keratins are not affected by a reduction in K15. There was some small effect observed in K8 expression however, although this is possibly due to the nature of K8 expression in HaCaT cells, which can be variable (Kazerounian, Uitto and Aho, 2002); this can be seen in *Figure 3.8*. This suggests that the effect may not then be due to the transfection method or K15 knock-down, but more likely to be a result of using HaCaT cells (rather than primary keratinocytes). Although K8 has previously been shown to have an effect on cell migration and wound healing (Long *et al*., 2006), it is unlikely that the heterogeneity observed in HaCaT cells will affect migration or wound healing to a great extent (as examined in Chapter 4).

3.4.2.2: The Effect of K15 Ablation on Differentiation Markers

Involucrin expression was greater in K15 knock-down cells compared to control siRNA transfected cells. This was most obvious after 48 h in the granular appearance of involucrin around the nucleus. Involucrin mRNA expression in HaCaT cells has been shown to increase when exposed to differentiation-inducing stimuli (such as increased extracellular calcium) (Micallef *et al*., 2009); however, this was suggested to occur after 5 days in high-calcium media (0.12M). Here an increase in involucrin protein is observed after just 48 h of K15 ablation, and can be seen to continue to increase up to 96 h post-transfection. As previously noted however, more conclusive evidence would be required to more accurately assess involucrin expression. This could include immunoblotting or RNA analysis.

Involucrin is useful as a differentiation marker as it is associated with cornified envelope formation. Involucrin is expressed when differentiation is induced, and is eventually cross-linked to the insoluble cornified envelope by transglutaminase (Bikle *et al*., 2001). Differentiation in keratinocytes is regulated by many transcription factors, for example the AP-1 family being the most prominent. *In vivo* differentiation occurs as a result of the intracellular calcium gradient that forms, with calcium levels being higher in more differentiated cells (Zbytek *et al*., 2005). The response of the involucrin gene to calcium in keratinocytes is affected by the transcription factor AP-1 (Ng *et al*., 1996; Tran and Crowe, 2004); this also occurs in *KRT1*. In the promoter region of involucrin resides the calcium response element (CaRE), which responds to both calcium and 1,25 dihydroxyvitamin D ($1,25(OH)_2D$). It is this region that contains the AP-1 site and an Sp1 site, which has also been shown to have a role in tissue-specific involucrin promoter activity (Bikle *et al*., 2001; Tran and Crowe, 2004). (The response of HaCaT cells to calcium will be discussed in further detail in section 3.4.6.) AP-1 has been shown to be important in upregulating differentiation-specific proteins involved in converting basal cells to spinous and granular cells (Rossi *et al*., 1998; Rorke *et al*., 2010). The CCAAT-enhancer binding protein (C-EBP) transcription factors are also known to affect involucrin (Oh and

Smart, 1998; Crish *et al*., 2006); C-EBPs can interact with AP-1, which adds a further effect to (in this case) involucrin upregulation. Disruption to the C-EBP binding site has been shown to result in discontinuous involucrin expression (Crish *et al*., 2006). Zbytek *et al*., (2005) demonstrated that involucrin was upregulated in HaCaT cells in response to corticotropin-releasing hormone (CRH); this was supported by Niderla-Bielinska *et al*. (2009) who demonstrated that as well as involucrin, filaggrin and keratinocyte differentation associated protein (Kdap) are also upregulated following EGF treatment. Involucrin upregulation in ORS cells of the hair follicle was also observed following EGF treatment.
Although no calcium levels were changed during this initial experiment, involucrin levels were increased following K15 ablation. This indicates that involucrin production can be induced via a pathway other than by increased intracellular calcium (for example, as in one of the pathways described above). It is not yet known what it may be that causes involucrin induction as K15 levels are reduced, although it suggests that differentiation is occurring. Involucrin RNA analysis or immunoblotting could be used to confirm these results.

3.4.3: The Effect of K15 Ablation on Cell Viablity

The MTT assay was used to assess the viability of HaCaT cells following K15 siRNA transfection. The results of this study suggest that K15 ablated cells proliferate to a greater extent than control siRNA transfected (i.e. K15-positive) cells. Tiede *et al*. (2009) carried out various experiments using K15-GFP+ cells (i.e. cells which have the *KRT15* promoter region labelled with GFP), including a viability assay. K15-GFP+ cells demonstrated greater viability compared to ORS keratinocytes following exposure to butyric acid. When Ki67 was used to identify proliferating cells, the percentage of Ki67-positive cells was far lower than in ORS keratinocytes. This suggests that K15-negative cells proliferate to a greater extent than K15-positive cells; the same result as observed in this study using the MTT assay.

3.4.4: The Effect of K15 Ablation on Junctional Proteins

To further investigate the differentiation of HaCaT cells following K15 knock-down, the expression of cell junction proteins were also investigated. Both desmoplakin and E-cadherin are expressed at similar levels in both control siRNA transfected cells and K15 siRNA transfected cells which were not cultured in high calcium media. The observation that desmoplakin remains unchanged is important, as desmoplakin has previously been found to affect cell proliferation in addition to its role mediating epithelial integrity or differentiation (Wan, South and Hart, 2007). Moreover, the targeting of desmoplakin to desmosomes is not dependent on K15. Since no change in desmoplakin is observed here, any effects on cell proliferation seen are not as a result of changes in desmoplakin expression. No ZO-1 was observed associated with cell-cell junctions; as ZO-1 is associated with highly differentiated cells, which would explain its absence in HaCaT cells cultured in low calcium media.

Desmosomes link to keratins (desmosome/intermediate filament complex [DIFC]) in order to confer mechanical stability to tissues (Cirillo and Prime, 2009); Cirillo and Prime did not observe that K15 was associated with desmosomal adhesion sites in HaCaT cells. The difficulty with assessing desmosome formation in transfected HaCaT cells is that transfection efficiency is reduced as cells become more confluent, and confluency is required to assess desmosome formation. For this reason, cells were allowed to become approximately 50% confluent before the transfection procedure was carried out. This ensured that some regions of HaCaT cells in culture

were confluent 48 h post-transfection and therefore it would be reasonable to assume that desmosome formation had the opportunity to occur. This said, if it were possible to ensure a higher confluency percentage for a longer period of time, this would aid in confirming this conclusion.
E-cadherin is associated with adherens junctions in epithelia, where it can bind to cytoskeletal and signalling proteins; it is thought that E-cadherin 'primes' the cell membrane, promoting adherens junction formation by altering its activity (Perez-Moreno, Jamora and Fuchs, 2003), a process linked to β–catenin (Faux *et al*., 2010). Cadherin levels possibly have a role in spindle positioning, epithelial sheet movement, cell morphogenesis and intracellular adhesion; it has also been suggested that E-cadherin may have a role in maintaining the stem cell niche, as adherens junctions may promote asymmetrical division by multipotent cells (Perez-Moreno, Jamora and Fuchs, 2003). It was therefore considered important to assess the effects of K15 ablation (if any) on adherens junctions and E-cadherin. There appears to be little difference between control and K15 siRNA transfected cells with regards to E-cadherin localisation. This suggests that adherens junctions are not affected by K15 ablation; in turn this suggests that K15 ablated cells are not differentiated to an extent where adherens junctions are no longer present. Furthermore, since it has been suggested that the presence of E-cadherin may be linked to asymmetrical cell division in tissue, it is possible that this may have relevance with regard to the HaCaT cells used in this study.
ZO-1 was observed as a representative marker of tight junctions. Tight junctions are observed in the granular cell layer of normal epidermis (Peltonen *et al*., 2007). No cell junction-specific ZO-1 staining was observed in either control siRNA transfected or K15 knock-down HaCaT cells; this is as expected as HaCaT cells in monolayer cultures are not representative of the granular layer.

In order to further investigate any differentiation which may have been occurring, additional exploration of this phenomenon was carried out using the calcium switch assay and organotypic cell cultures.

3.4.5: Responses to Changes in Extracellular Calcium

The 'calcium switch' assay has been used to assess differentiation in cells (for example, Kolly *et al*., 2005), although K15 expression has not previously been studied. Increased levels of extracellular calcium induce differentiation *in vivo* and simple experimental methods have been established to mimic the effects of this *in vitro* for further study.

K14 expression remains unchanged throughout this experiment, despite K14 being affected by differentiation (Nelson and Sun, 1983; Lena *et al*., 2010). This is a relatively short experiment however, and the effects of high extracellular calcium on K14 may not be observed after only 72 hours. Micallef *et al*. (2009) carried out a prolonged calcium switch assay which established that most changes occurred after 5 days in increased extracellular calcium.
However, Yuspa *et al*. (1989) carried out a calcium switch assay over a short period, and observed the effects on K1 and K10 (which would normally be expressed coinciding with repression of K14 expression [see also Lena *et al*., 2010]). After 24 hours a change can be observed in K1 expression, although only a slight change in K10 expression can be observed after 48 hours. No change in K14 mRNA could be detected throughout in Yuspa *et al*.'s 1989 experiments, although K14 mRNA soon disappears in the suprabasal layer *in vivo* (Roop *et al*., 1988). This may explain the

lack of change in K14 expression observed here (also observed by Paramio *et al*., 1999). K10 expression inhibits cell proliferation, although this may only contribute slightly to the increased proliferation observed in K15 knock-down cells (*Figure 3.11*); a small increase in K10 can be observed in the calcium switch in this study, although this again may be due to the relatively short time scale used.

Following exposure to low levels of calcium, involucrin expression is similar in control siRNA and K15 siRNA transfected cells after 6 hours, although after 72 hours, more involucrin is observed in K15 knock-down cells (the expression pattern remains the same). An increase in involucrin is observed in K15 knock-down cells following exposure to high extracellular calcium for 6 hours, despite reports that the involucrin response in HaCaT cells is slower than observed in NHEK cells (Micallef *et al*., 2009). However, after 72 hours, involucrin expression is lower in K15 knock-down cells than in controls. This may be due to the cross-linking of involucrin by transglutaminase, making the protein unavailable for immunofluorescence staining.
Involucrin has previously been observed to increase following exposure to high levels of extracellular calcium (for example, Micallef *et al*., 2010). The transcription factor AP-1 has been associated with inducing involucrin expression in response to an increase in intracellular calcium, where it is effective at the CaRE (Bikle *et al*., 2001). RT-PCR studies using the calcium switch method have demonstrated that involucrin mRNA expression is greater in high calcium media compared to low calcium media (Micallef *et al*., 2009), although this was after 6 days in HaCaT cells (and between days 1 and 6 in NHEK cells). This correlated with increases in the amount of protein. However, after 6 days both the NHEK and HaCaT cells became confluent; this was not allowed to occur in this study. Post-confluent HaCaT cells have previously been shown to express genes usually associated with more differentiated cells in the upper layers of the skin (Garach-Jehoshua *et al*., 1998), as well as markers of differentiation following exposure to high extracellular calcium levels (Micallef *et al*., 2009). As a measurable amount of involucrin (and involucrin mRNA) was observed at the first kinetic point of this study, it suggests that involucrin may be present in a mosaic pattern in *in vitro* monolayers. Other studies (such as Papp *et al*., 2003) have shown that the greatly upregulated involucrin response in confluent HaCaT cells could be in response to PKC activation, which regulates the transition of spinous to granular cells in HaCaT cells induced to differentiate in confluent *in vitro* conditions. Recently, expression of involucrin (and filaggrin) in HaCaT cells has been shown to be upregulated in cells expressing S100A8/A9 (via promotion of NADPH oxidase and KFκB activation) – usually produced in response to stress (Voss *et al*., 2011). Voss *et al*. (2011) suggest that this demonstrates the importance of the S100A8/A9 response in tissue remodelling and repair.

Cytoskeletal protein extracts from HaCaTs exposed to high and low extracellular calcium were run on a protein gel in order to separate the proteins in the extract. This demonstrated that K15 siRNA transfection does not affect the expression of other keratins. The keratins observed using this method were not affected by exposure to high levels of extracellular calcium for 72 hours (although as previously suggested, keratins are long-lasting proteins, and their presence in the cell extract could be residual). It is also possible, as previously suggested, that HaCaT cells express a slightly abnormal compliment of keratins, which can vary in the same cell population. This could explain why subtle differences which would occur normally after being exposed to high calcium for 72 hours in (for example) NHEK cells, may not be as

obvious in HaCaT cells. As both K15 and K16 are similar in size, it is not possible to distinguish between them using this method.

3.4.6: Utilising the Organotypic Cell Culture System

Using organotypic culture techniques, HaCaT cells were cultured for various time points at the air-liquid interface. This helped to establish changes in cell morphology and protein expression as the cells stratify and flatten. Using the organotypic culture system should give a more accurate representation of normal skin than HaCaT cells grown in a monolayer. This said, HaCaT cells are an immortalised cell line, not a primary cell line, and have a slightly different protein expression pattern. This suggests that some protein expression profiles may also be different in 3D culture. This was observed with K14 and K1 expression.

3.4.7: Conclusions and Further Work

3.4.7.1: Summary of Findings

HaCaT cells were a suitable cell line to use for K15 siRNA transfection as the normal K15 expression observed in these cells was reduced to a low level. HaCaT cells, although not primary cells, were considered a useful alternative to NHEK cells which, although normally expressed K15 and could be successfully transfected with K15 siRNA, could only be cultured to approximately passage 4. This would not yield the large cell numbers required for the experiments use in this study.

The proliferation assay suggested that K15-positive cells proliferate less than K15-negative cells; this finding has also been described elsewhere (Tiede *et al*., 2009).

The only keratin affected by K15 siRNA transfection was K10, which appeared slightly increased following K15 ablation. This indicated some HaCaT differentiation, particularly when considered alongside the increases in involucrin expression observed (which was comparable to that seen after HaCaT cells were cultured in high extracellular calcium). The differentiation occurring however is not to a great extent, as expression of cell-cell junction markers and filaggrin remain unchanged.

Organotypic cell cultures were used to more accurately assess the ability of HaCaT cells to differentiate and to compare keratin expression with those observed in monolayer cultures. Keratin expression suggested that the slightly altered keratin profile of HaCaT cells remained.

3.4.7.2: Further Work

It was not possible to observe in this study whether the effects of increased extracellular calcium or K15 ablation had a great effect on involucrin levels in HaCaT cells. A future study could culture HaCaT cells in various concentrations of extracellular calcium and compare this to K15 ablated cells at specific time points. If the amount of involucrin (or involucrin mRNA) could then be quantified, this may give a measurable indication of the amount of differentiation K15 ablated cells undergo (since the differentiation of cells in increased levels of extracellular calcium is a known quantity). Quantification of other data, such as expression of other keratins or transcription factors, would also be useful.

Development of a more stable K15 knock-down (or knock-out) would also increase the amount of time cells could be cultured and experiments carried out. For example, a stable transfected cell line (such as using lentiviral techniques) could be used to assess the longer term effects of high (or low) extracellular calcium exposure. K15 ablated cells could also be used to establish longer term (i.e., 35 day) organotypic cultures, which would give a useful indication of the effects of K15 ablation on skin

differentiation. The technique has been shown to work well using HaCaT cells and immunofluorescence staining (*Figure 3.19*) and similar techniques could be used with more stable transfections.
Likewise, if overexpression of K15 could be induced, and the experiments carried out here repeated, further information about the effects of K15 on cell proliferation and differentiation may be elucidated.

Chapter 4: Effects of K15 Downregulation on Differentiation and Proliferation

4.1: Introduction

4.1.1: Aims

Keratinocyte adhesion, attachment and migration are important *in vivo*, particularly during stratification and wound healing. The mechanics of cell adhesion and cell spreading and volume regulation are also important in circumstances such as osmotic perturbation, proliferation, cell-cell contacts and cell shape change (Schön *et al*., 1996; Blase *et al*, 2009). *In vivo* epidermal cells would not only be attached to each other (through, for example, desmosomes) but also attached to proteins in the extracellular matrix at the basement membrane. As in *in vivo* conditions this would be the case particularly for K15-positive cells in the hair follicle bulge and the basal layer of the interfollicular epidermis, it was deemed useful to investigate the effect of K15 ablation on cell attachment. Moreover, several intermediate filaments have been shown to influence adhesion and migration, such as vimentin (Ivaska *et al*., 2007), K14 and K5 (Morley *et al*., 2003), K6 (Wong and Coulombe 2003) and K8 (Long *et al*., 2006). As other IF proteins had previously been demonstrated to affect adhesion and motility, it was considered important to assess the effect of K15 ablation on these properties.

4.1.2: Types of Cell Migration

Three types of distinct cell migration have been described (for example, Sahai, 2005). Firstly, individual mesenchymal cells (and those cancer cells which undergo epithelial to mesenchymal transition), for example, move slowly through a series of steps in a process termed mesenchymal or crawling cell migration (for a recent review, see Augello, Kurth and De Bari, 2010). Lamellipodia, filopodia or pseudopodia form from actin protrusions, which adhere to the extracellular matrix (mediated via integrins at podosomes and focal adhesions) (for a review, see Bugyi and Carlier, 2010). This allows the contractile force of actomyosin to be transmitted to the extracellular matrix, moving the cell body from the leading edge. Proteases, such as MMPs, degrade extracellular matrix proteins to aid movement through the matrix. Secondly, migration of some tumour cells has been described as amoeboid movement, a method also utilised by leukocytes, where cells with weak extracellular matrix interactions appear to crawl via pseudopodia; this is much faster than mesenchymal motility and does not require proteolysis of the matrix. Instead, cells squeeze through gaps in matix in an amoeboid fashion (see Guck *et al*., 2010 for a review). Finally, in wound healing, embryonic morphogenesis and primary cancers, collective migration is important. Cells move together as a 'sheet', and not individually. The cells maintain their integrity through cell-cell adherence junctions, whilst at the wound edge, leading cells use actin ruffles to pull sheets or clusters of cells. MMPs again act to form a pathway for cells to move through. Although further research is required, it is likely that this type of movement is similar to that which occurs during the development of epithelial sheets, utilising myosin- and actin-mediated protrusions guided by chemotactic cues (Sahai, 2005; Friedl and Gilmour, 2009).

4.1.2.1: The Role of Actin in Cell Migration

Changes in cell shape are mediated by the cytoskeleton. The biophysical and molecular basis of these changes is still unclear, although it has been shown that actin microfilament rearrangement, assembly, severing and cross-linking occurs at the leading edge of migrating cells (for example, Theriot and Mitchison, 1991; Connelly *et al*., 2010).

Lamellipodia

Actin assembly is converted into a protrusive force during cell migration; one such way this occurs is through the development of lamellipodia. Lamellipodia are made up of branched actin filaments which form a thin mesh at the cell's leading edge; lamellipodia are generally free from capping proteins (Miyoshi *et al*., 2006). These networks of branches can vary in number, leading to varying breadths (usually between 1 and 5μm) (Small *et al*., 2002).

The assembly of lamellipodia (and filopodia) are regulated by Rho family small GTPases; Rac1 signals the formation of lamellipodia (Nobes and Hall, 1995). In order for the actin filament branching processes to be regulated, migrating cells nucleate actin filaments at 70° angles from existing filaments; the nucleating factor of lamellipodia is the Arp2/3 (actin related protein 2/3) complex, activated by WASP (for a review, see Goley and Welch, 2006). Arp2/3 is also important in directing the lamellipodium protrusion (Le Clainche and Carlier, 2008). Arp2/3 is a stable complex comprising of seven subunits: ARPC1, ARPC2, ARPC3, APRC4, ARPC5, Arp2 and Arp3; Arp3 is important for nucleation whilst ARPC1, ARPC3 and APRC5 are activated by WASP (Wiskott-Aldrich syndrome protein) (Gournier *et al*., 2001).

Recently, Watanabe (2010) has demonstrated that AIP1-associated filament disruption occurs 15 times more frequently than Arp2/3 nucleation, further adding to the processes which affect lamellipodia dynamics. However, Watanabe (2010) admits that even when AIP1 disruption is considered, the rate of disruption still falls short of the predicted frequency, leading to the conclusion that other factors must have a role in the regulation of actin turnover (Tsuji *et al*., 2009). VASP (vasodilator-stimulated phosphoprotein) has also been shown to have a role in accelerating filament elongation; *in vitro* TIRF microscopy has confirmed that this occurs through the delivery of monomeric actin to the (growing) barbed end (Breitsprecher *et al*., 2008).

Using FRAP (fluorescence recovery after photobleaching), it has been possible to analyse fibroblast lamellipodium; this demonstrated that as actin filaments polymerise at the leading edge, the filaments are depolymerised at the rear (although it has since been demonstrated that FRAP data is inconsistent with current studies showing fast dissembling of actin) (Watanabe and Mitchison, 2002; Lai *et al*., 2008). This process is known as 'treadmilling' (as described by Small *et al*., 2002). Some of these effects are cell specific; for example, in keratinocytes, it was demonstrated that depolymerisation was occurring at the same rate as polymerisation (Theriot and Mitchison, 1991; Watanabe and Mitchison, 2002). Calculations of the critical concentrations involved in ATP hydrolysis during polymerisation of actin has suggested that (on average) the filaments can move forward whilst maintaining its length (for more detail see Small *et al*., 2002). This treadmilling process alone is too slow to account for the speed at which some cells migrate. Some proteins however have been identified which are capable of accelerating the rate of treadmilling; these include cofilin (also known as ADF), profilin and capping proteins.

Cofilin is present through lamellipodia apart from at the leading edge (Svitkina and Borisy, 1999). It binds to ADP-actin resulting in a change in structure, leading to an increased rate of depolymerisation (Carlier *et al*., 1997; Carlier, Ressad and Pantaloni, 1999). This, in turn, increases the growth of the barbed end. Profilin has also been shown to increase the treadmilling rate, a process whereby subunits simultaneously polymerise at the barbed end and depolymerise from the pointed end (Wegner, 1976) (for a review, see Yarmola and Bubb, 2006). Unlike cofilin, profilin/actin complexes form exclusively at the barbed end. Together, the combined effect of cofilin and profilin have been demonstrated to increase the treadmilling effect 125-fold (Didry,

Carlier and Pantaloni, 1998; Le Clainche and Carlier, 2008). The effect of capping proteins is to block further polymerisation at barbed ends (reviewed by Pantaloni, Le Clainche and Carlier, 2001). This process also aids in controlling the density of actin filaments (Wiesner *et al*., 2003).

Filopodia

Filopodia are long bundles of actin, which initiate from branches of lamellipodia at the cell's leading edge. These protrusions contain between 15 and 20 parallel filaments arranged into a bundle (Lewis and Bridgman, 1992).

Filopodia formation is signalled by Cdc42, a small GTPase of the Rho family (Nobes and Hall, 1995). Miki *et al*. (1998) demonstrated that N-WASP potentiates the ability of Cdc42 to induce filopodia; although this suggested that Arp2/3 may also have a role, it was later demonstrated that Arp2/3 is not observed in the tight, unbranched actin filament bundle of filopodia (reviewed by Le Clainche and Carlier, 2008). Instead, formins have been proposed to have a role in filopodia regulation. Formins have two conserved domains: FH (forming homology) 1 interacts with profilin whilst FH2 stabilises actin dimers by nucleating the filaments (Higashida *et al*., 2004; reviewed by Le Clainche and Carlier, 2008). Pellegrin and Mellor (2005) demonstrated that mDia2 (diaphanous-related formin) localises at the tip of filopodia, and has been shown to respond to Cdc42 in NIH 3T3 cells (Peng *et al*., 2003). Rho was also observed to have a role (Pellegrin and Mellor, 2005). Rho signalling determines the ratio of 'open' (active) to 'closed' mDia1 (Watanabe, 2010).

4.1.2.2: The Scratch Wound Assay

Several *in vitro* models have been developed to investigate the molecular mechanisms involved in migration. Scratch wound assays can be used as a way of investigating epithelial and mesenchymal cell migration (Wong and Gotlieb, 1984), and has been utilised by several groups (for example Wong and Gotlieb, 1988; Long *et al*., 2006).

When a confluent monolayer is scratched, cell-cell contacts are disrupted and growth factors in cell culture media aid in healing through proliferation and migration (Yarrow *et al*., 2004). Scratch wound assays have been used to study the role of, for example, p53 (Sablina, Chumakov and Kopnin, 2003), Rac (Fenteany, Janmey and Stossel, 2000), Rho (Nobes and Hall, 1999) (all important for actin dynamics) and intermediate filament and cytoskeletal organisation (Long *et al*., 2006, Boczonadi *et al*., 2007). The importance of the cytoskeleton in wound repair was shown to be important thirty years ago (Kreis and Bircheier, 1980), which has been elaborated on since; the role of K15 however has not been examined in this way previously.

Other intermediate filament proteins have been shown to play an important role in wound healing. For example, in immortalised cell lines with EBS mutations in K14 or K5 migrate faster than control keratinocytes (Morley *et al*., 2003). Morley *et al*. conclude that this is as a result of known upregulation of stress-activated kinase pathways in epidermolysis bullosa simplex (EBS) keratinocytes. Vimentin has previously been shown to influence integrin recycling and hence regulate adhesion and migration (Ivaska *et al*., 2007). Wong and Coulombe (2003) describe skin explant cultures from K6α/K6β-null mice to mimic the *in vivo* behaviour of keratinocytes at a wound edge. Outgrowth of K6α/K6β-null cells was greater (1.8-fold increase) than wild-type skin explants (and shown to be via migration as opposed to mitosis or increased cell size). Wong and Coulombe also report altered F-actin content in K6α/K6β-null mouse skin. Defective wound healing of these mice was observed *in vivo*. Long *et al.* (2006) and Boczonadi *et al*. (2007) demonstrated that

keratin organisation at the wound edge is required for effective wound closure (aided by IF-binding proteins such as plectin and periplakin). Boczonadi *et al*. (2007) used MCF-7 cells expressing the periplakin N-terminus to accelerate keratin reorganisation in scratch-wounded cells. siNRA knock-down of plectin in these MCF-7 cells resulted in reduced keratin re-organisation at the wound edge. In MCF-7 cells, keratin staining was generally increased 30 minutes after the *in vitro* scratch wound assay (particularly in forming a cage-like structure around the nucleus), and K8/K18 fibres produced cable-like bundles at the wound edge within 2 hours (Long *et al*., 2006).

4.1.3: Keratinocyte Adhesion and Spreading

4.1.3.1: Focal Adhesions in Keratinocytes

Formation of focal adhesions is required for cell adhesion, whilst co-ordinated release is needed for cell migration. Co-ordinated actin microtubule dynamics are likely to be important in mediating spatiotemporal regulation of focal adhesion dynamics during cell migration. Microtubules can specifically target focal adhesions and promote turnover, possibly through microtubule motor-mediated supply of disassembly factors (Kaverina, Rottner and Small, 1998; Kaverina, Krylyshkina and Small, 1999; Krylyshkina *et al*., 2002; Krylyshkina *et al*., 2003; Wu, Kodama and Fuchs, 2008). Ezratty, Partridge and Gundersen (2005) have further demonstrated the involvement of actin in focal adhesion turnover by showing that FAK (focal adhesion kinase) and dynamin have roles. The co-ordination of actin and microtubule involvement in focal adhesion dynamics is possibly through + end tracking proteins, which mediate the cytoskeletal crosstalk (Carvalho *et al*., 2003; Wu *et al*., 2006; Akhmanova and Steinmetz 2008; Wu, Kodama and Fuchs, 2008).

The ECM has an active role in maintaining focal adhesions with cells, and also providing a migration-initiating signal; this effect can be enhanced by growth factors. As a cell migrates, focal complexes form at the leading edge. These focal complexes develop into focal adhesions (Gumbiner, 1996). This allows the cell to polarise and to translocate across the focal adhesion, which is then released from the ECM (Mitchison and Cramer, 1996). This polarisation occurs through clustering of integrins at the focal adhesion site. These integrins are also capable of activating the Rho-GTPases, Rac and Cdc42, which aid in actin reassembly with lamellipodia and filopodia formation at the cell's leading edge (Schwartz and Shattil, 2000; Ridley, 2001).

Adhesion sites are complex, comprising many different structural and signalling molecules and regulatory proteins (Schober *et al*., 2007; Möhl *et al*., 2009). One such protein is vinculin; vinculin is a structural protein that has a role in polymerisation of the actin cytoskeleton where it localises at focal adhesions. Vinculin is comprised of a globular head domain connected to the tail domain by a proline-rich region (Möhl *et al*., 2009). When the head and tail domains are linked by intramolecular interactions, many binding sites are unavailable. Activation releases the head and tail domains, making binding sites available; the mechanism of activation still remains unclear, although interaction with actin can make this occur in talin. Phosphorylation has also been shown to have a role in activation, ligand binding and stabilisation (Möhl *et al*., 2009; Küpper *et al*., 2010).

4.1.3.2: The Role of Filopodia in Cell Adhesion

As well as at focal adhesions, vinculin is also present in filopodia, particularly at the tip, which is believed to be involved in actin bundling, elongation and substrate sensing. Filopodia have a fundamental role in formation of adhesions, determining

the shape and localisation of most adhesion sites (Schäfer *et al*., 2009). Schäfer *et al*. (2009) have also suggested that filopodia are similar to lamellipodal focal complexes (without the late focal adhesion protein zyxin). Vinculin immunofluorescence highlights focal contacts (Mahadi Abdul Jamil *et al*., 2008). In motile cells, focal complexes are produced as integrins cluster at specific regions of the cell edge. These are bound to matrix proteins such as laminin, collagen and fibronectin, and indirectly to F-actin via talin, paxillin and vinculin (Spinardi and Marchisio, 2006; Möhl *et al*., 2009). Such structures have been compared to the podosomes as observed in mesencymal cells (Spinardi and Marchisio, 2006). Filopodia focal complexes increase in size following contact with lamellipodium, forming focal adhesion sites in the direction of the filopodia (Schäfer *et al*., 2009), incorporating proteins such as α-actinin, zyxin and focal adhesion kinase (FAK) (Möhl *et al*., 2009). These structures can mature further, into fibrillar adhesions. These elongated, stable structures have a slightly different protein composition, which includes a lower level of vinculin; in addition, proteins are modified, where, for example FAK phosphorylates focal adhesion proteins. For vinculin, this phosphorylation stabilises an open confirmation required to increase the binding affinity for other focal adhesion proteins (Möhl *et al*., 2009).

4.2: Materials and Methods

The materials and methods used in investigation of the effect of K15 on differentiation and proliferation are described in Chapter 2. No changes or variations were made to the methods described.

4.3: Results

4.3.1: Experimental Approach

Chapter 3 demonstrated that K15 ablation in HaCaT cells may affect differentiation. The aim of this chapter is to further investigate other effects of K15 ablation in HaCaT cells. In this chapter, using the same siRNA methods utilised in chapter 3, further investigation of the effects of K15 ablation was carried out. The effects on other cytoskeletal proteins were examined, as well as how this affected cell size and shape. Following the result that K15 ablation had little effect on cell-cell junctions, the effect of K15 ablation of cell adhesion was observed in this chapter. Previous studies have shown that expression of adhesion molecules in the skin is regulated by several factors (such as calcium concentration) and are capable of affecting differentiation (Hennings *et al*., 1980; Lewis, Jensen and Wheelock, 1994; Denning *et al*., 1998; Denning *et al*., 2000; Szegedi *et al*., 2008); since the previous chapter indicated that K15 affected cell differentiation (although not cell-cell adhesion), it was considered useful to assess the effects of the same K15 ablation on cell adhesion and spreading.

In addition, it was thought important to assess any effect of K15 knock-down on cell migration, since this has also been previously shown to be affected by differentiation in keratinocytes (for example, Hoffman, 2007).

4.3.2: Adhesion of K15 Depleted HaCaT Cells

4.3.2.1: Actin and Vinculin Expression and Localisation

Using the same siRNA procedure as previously described, HaCaT cells were cultured on glass coverslips, siRNA transfected, and subjected to immunofluorescence staining. Staining of filamentous actin with fluorescently labelled phalloidin

highlighted the actin arrangement in control siRNA transfected and K15 siRNA transfected cells. There is little difference between K15 ablated cells and control siRNA transfected cells 48 h post-transfection in HaCaT cells (as also observed in NHEK cells – *Figure 3.10*). This indicated that K15 ablation has no effect on actin expression or localisation in HaCaT cells. Since cell spreading in HaCaT ablated cells appeared abnormal however, it was considered useful to use immunofluorescence to examine actin arrangement in attaching cells. In addition, vinculin staining was used to examine focal adhesion sites in control siRNA and K15 siRNA transfected cells.

Cells were siRNA transfected, trypsinised 48 h later and re-seeded onto glass converslips. The coverslips were then fixed at various timepoints following seeding, and immunofluorescence staining was carried out. Actin, as expected in both control siRNA transfected and K15 siRNA transfected cells, was more prominent at the cell edges as the cells adhered and began to spread (*Figure 4.2*). The thickness of the bundles at the cell edge was measured using Image J; this indicated that the bundles were thicker in K15 siRNA transfected cells compared to control siRNA transfected cells. Statistical analysis demonstrated that the difference in thickness was significant ($p=0.001$). This was complimented with vinculin staining, carried out at the same timepoints following seeding in transfected cells (*Figure 4.1*; *Table 4.1*).

Vinculin, as a focal adhesion protein, would mark any focal adhesions produced in the HaCaT cells; Möhl *et al*. (2009) has previously demonstrated that low levels of vinculin can be observed throughout the cytoplasm whilst distinct bright spots indicated regions of a high concentration of bound vinculin (and therefore focal adhesions). For comparison, the number of focal adhesions per cell were counted; the average number of adhesions per cell were higher in control cells (61.78) compared to K15 siRNA transfected cells (41.58) (*Table 4.1*). Using Student's T-test, these results are shown to be significantly different ($p=0.001$).

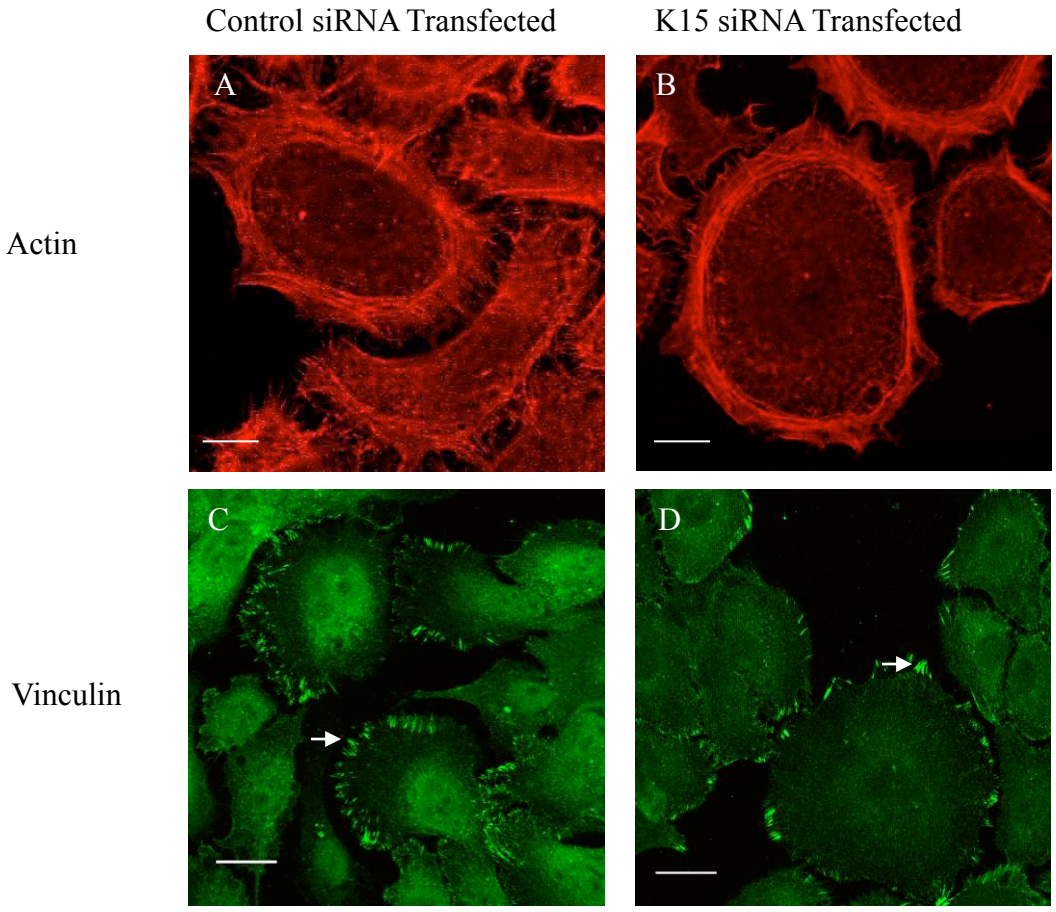


Figure 4.1: Expression of Actin and Vinculin in K15 siRNA Transfected HaCaT Cells
Single plane confocal image of immunofluorescence showing actin expression at 180 minutes post-seeding in control siRNA tranfected HaCaT cells (A), K15 siRNA transfected HaCaT cells (B), and single plane confocal images of vinculin immunofluorescence in control siRNA transfected HaCaT cells (C) and K15 siRNA transfected HaCaT cells (D) (scale bar = 10μm). *Arrows*: examples of vinculin-positive focal adhesions.

There is bundling of actin fibres at the cell periphery, most apparent in K15 knock-down cells (B). Both control siRNA transfected (C) and K15 siRNA transfected cells (D) produce vinculin-positive focal adhesions.

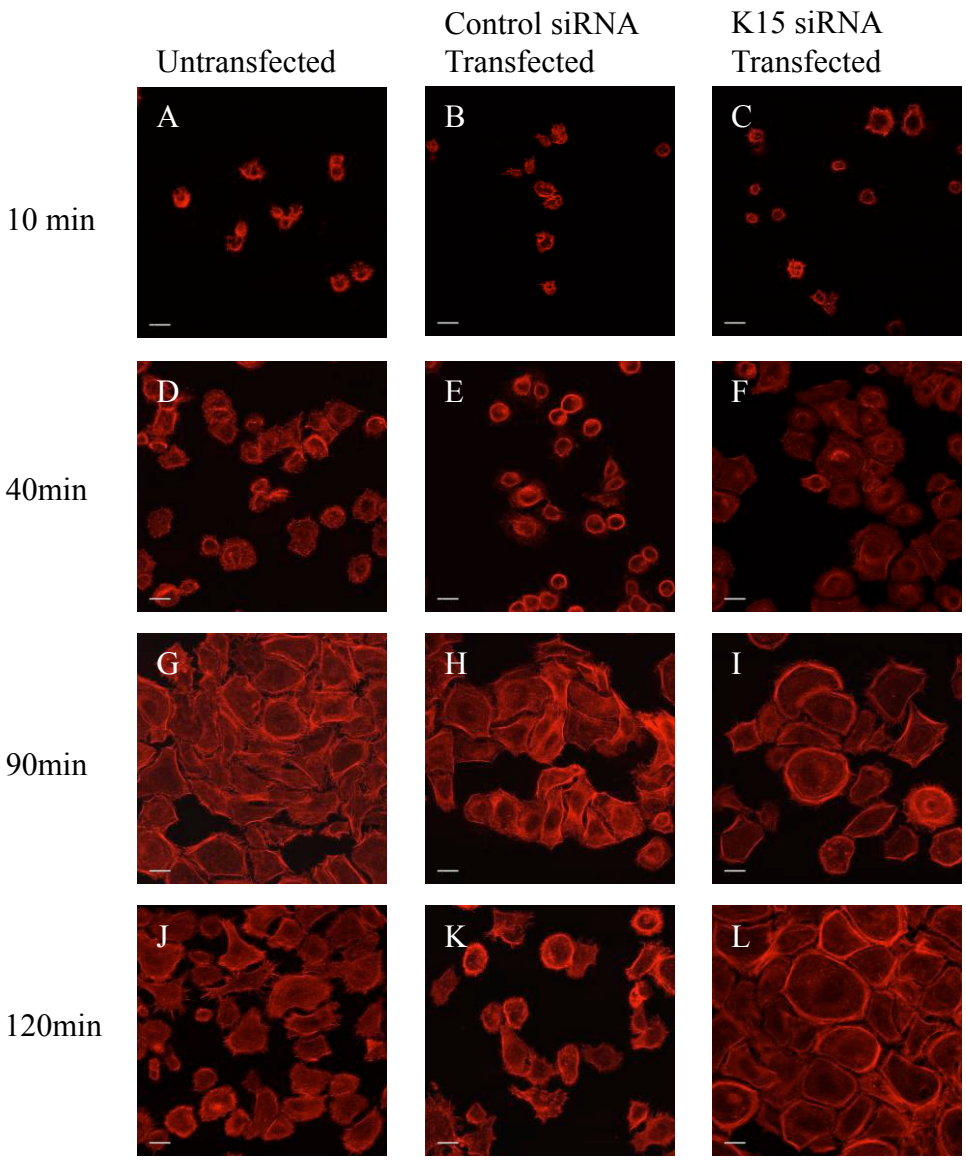


Figure 4.2: Actin organisation during cell spreading.
Actin immunofluorescence in untransfected (A, D, G, J), control siRNA transfected (B, E, H, K) and K15 siRNA transfected (C, F, I, L) HaCaT cells (scale bar = 10μm). The cells were allowed to spread for 10, 40, 90 and 120 minutes after seeding onto glass coverslips. Bundling of actin at the cell periphery can be seen at 90 minutes post seeding in untransfected (G), control siRNA transfected (H) and K15 siRNA transfected cells (I). The bundling persists in K15 siRNA transfected cells until at least 120 minutes post-seeding (L); this is less obvious in untransfected (J) and control siRNA transfected cells (K) at the same timepoint.

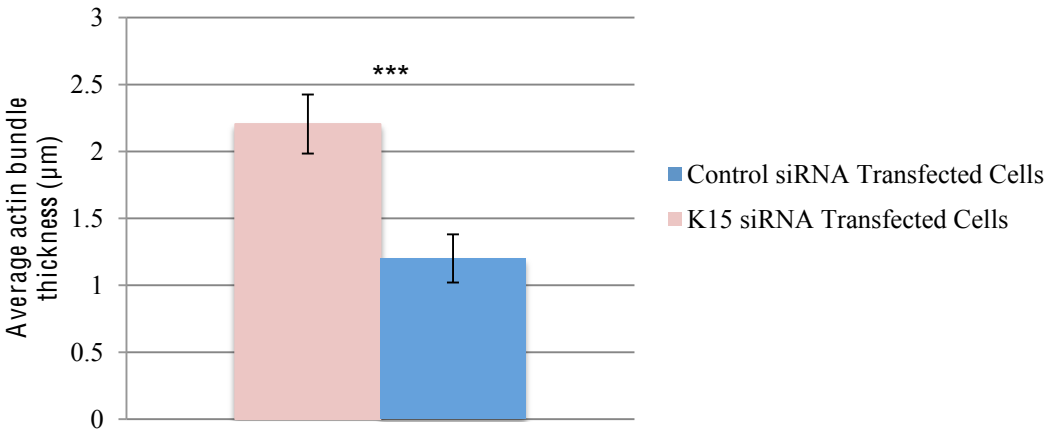


Figure 4.3: Actin bundle thickness in K15 ablated HaCaT cells
Measurements of actin bundle thickness at the edges of cells were made using Image J. 10 measurements were made per cell, and 10 cells were measured. Mean and standard error are shown. The difference in bundle thickness between control siRNA transfected and K15 siRNA transfected cells is statistically significant (*t*-test, $p<0.001$) at 180 minutes post-seeding (***).

Table 4.1: Focal adhesions in K15 ablated HaCaT cells

	Number of focal adhesions	Number of cells	Average number of focal adhesions per cell
Control siRNA transfected	3089	50	61.78
K15 siRNA transfected	2079	50	41.58

The average number of focal adhesions per cell were calculated from the immunofluorescence images. K15 knock-down cells had, on average, fewer focal adhesions than control siRNA transfected cells.

4.3.2.2: Live Cell Imaging of HaCaT Adhesion

An adhesion assay was carried out to observe actin arrangement in HaCaT cells during cell spreading and adhesion.

Live cell imaging was used to observe initial cell adhesion. Cells were seeded into wells of six-well plates and immediately mounted onto the Zeiss Axiovert live cell imaging microscope. The chamber maintained a constant temperature and controlled CO_2 levels. Phase contrast images were then taken between 140 seconds post-seeding and 600 seconds (*Figure 4.4*). Although a few cells can be seen to move little, no cell spreading can be observed up to 10 minutes post-seeding. The cells seeded are still relatively small and spherical with a low cytoplasm:nucleus ratio. This experiment was used to establish the time-point at which enough cells would be adhered to the glass coverslips for useful immunofluorescence. As observed in *Figure 4.4*, cells did not begin to adhere prior to 10 minutes post-seeding; this was the time-point deemed appropriate to begin fixing cells for immunofluorescence.

The marked difference observed in actin bundle thickness at the cell edge during cell spreading required further investigation; both K14 and K15 immunofluorenscence was carried out using the same adhesion assay.

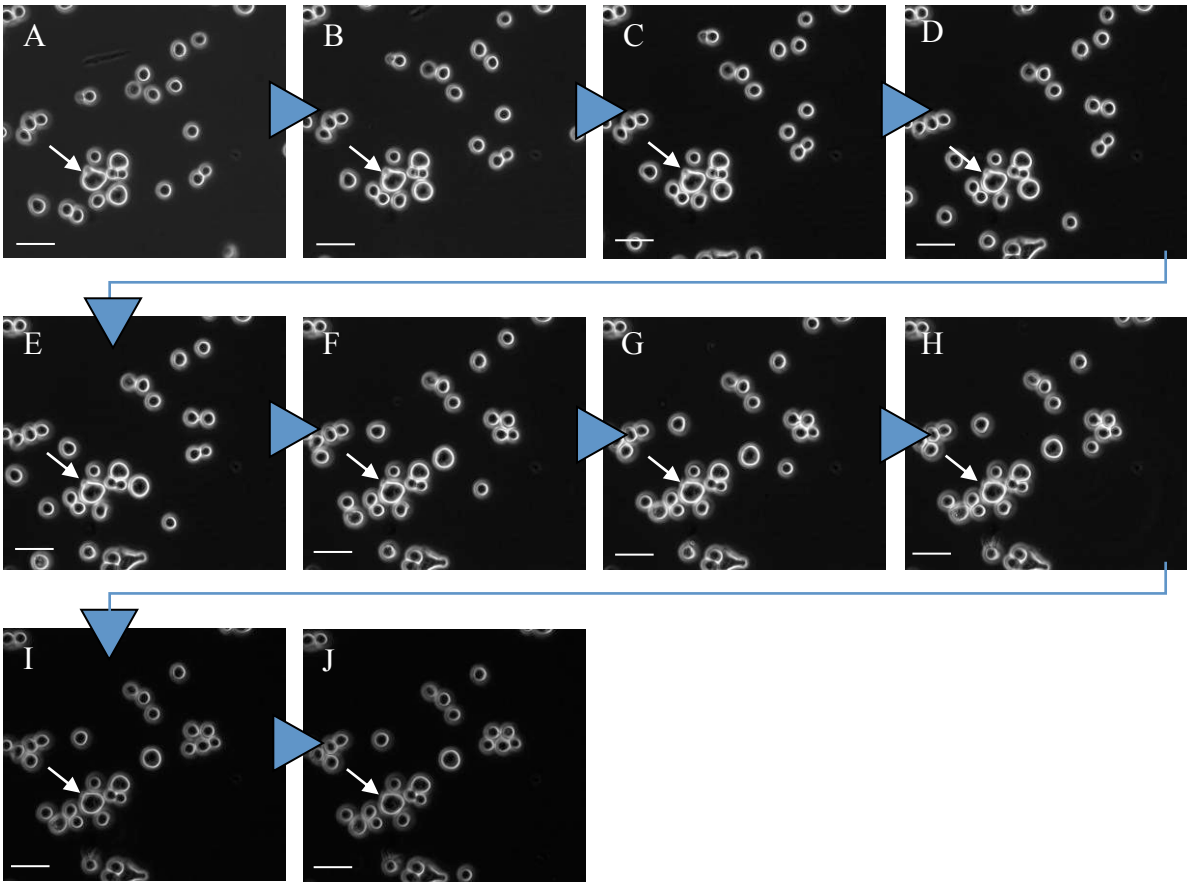


Figure 4.4: Live cell imaging of HaCaT cell adherence and spreading
Untransfected HaCaT cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). As shown by the example cell (arrow), little cell spreading occurs within the first 10 minutes post-seeding.

4.3.2.3: Keratin Localisation in HaCaT Cell Adhesion

K15 expression in siRNA transfected cells is minimal (*Figure 4.5* I). There is normal K15 expression in untransfected and control siRNA transfected HaCaT cells. K15 can be observed throughout the cytoplasm up to the edge of the cell.

K14 expression is observed as soon as cells begin to attach to the glass coverslip in untransfected, control siRNA transfected and K15 knock-down cells (10 minutes) (*Figure 4.6*). As soon as the attached cells begin to spread, K14 arrangement can be observed and this appears normal in untransfected, control siRNA transfected and K15 knock-down cells. In K15 siRNA transfected cells, K14 expression towards the spreading cell edges appears lower than in control cells (*Figure 4.7*). This could be due to the abnormal spreading observed in K15 knock-down cells, as previously suggested by actin immunofluorescence (*Figure 4.2*).

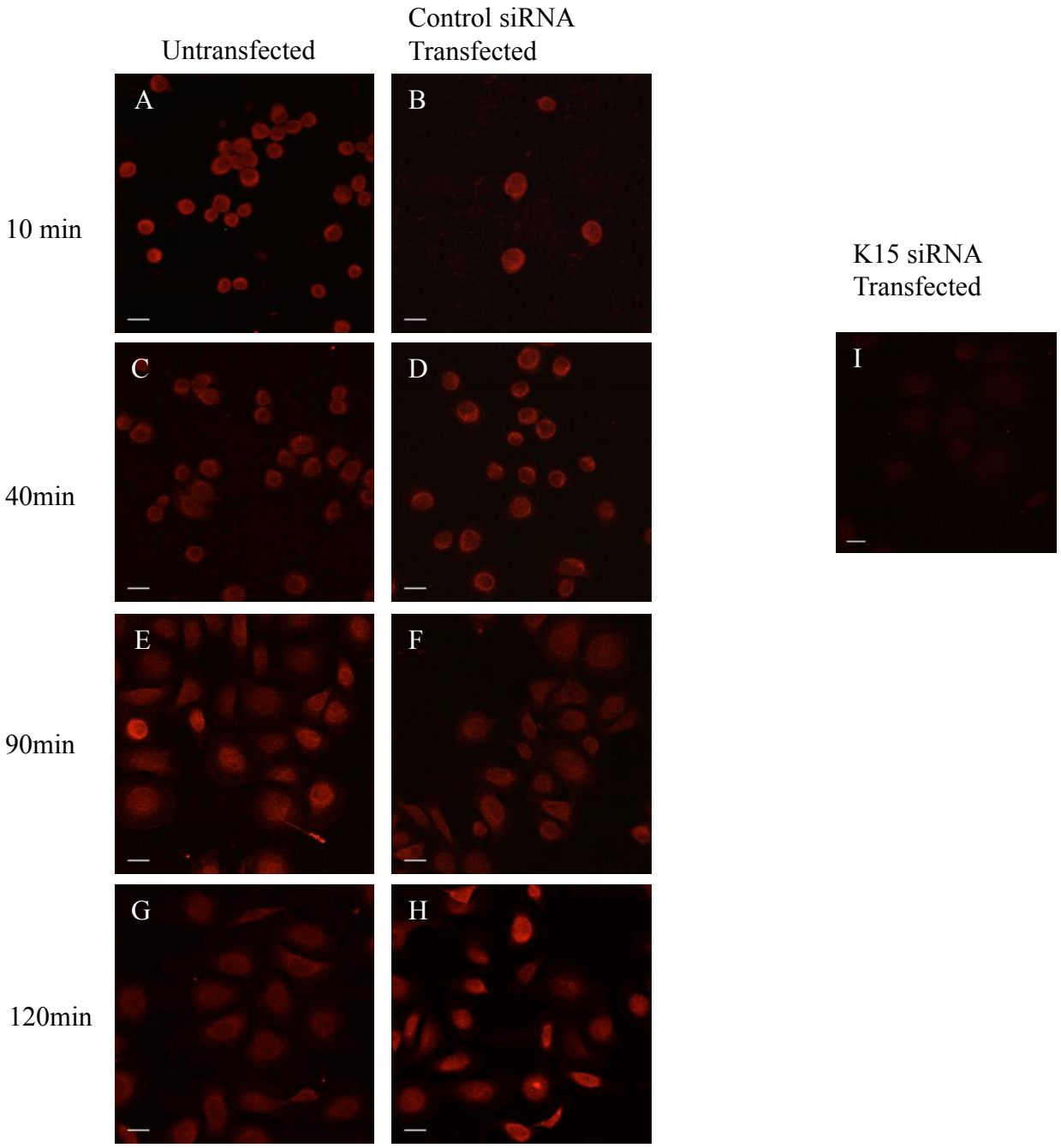


Figure 4.5: K15 organisation during cell spreading.
K15 immunofluorescence in untransfected (A, C, E G), control siRNA transfected (B, D, F, H) and K15 siRNA transfected (I) HaCaT cells. The cells were seeded onto glass cover slips and fixed at 10, 40, 90, and 120 minutes (scale bar = 10μm). K15 expression is similarly observed throughout the cells in both untransfected and control siRNA transfected HaCaT cells, with very low levels expression in K15 siRNA transfected cells.

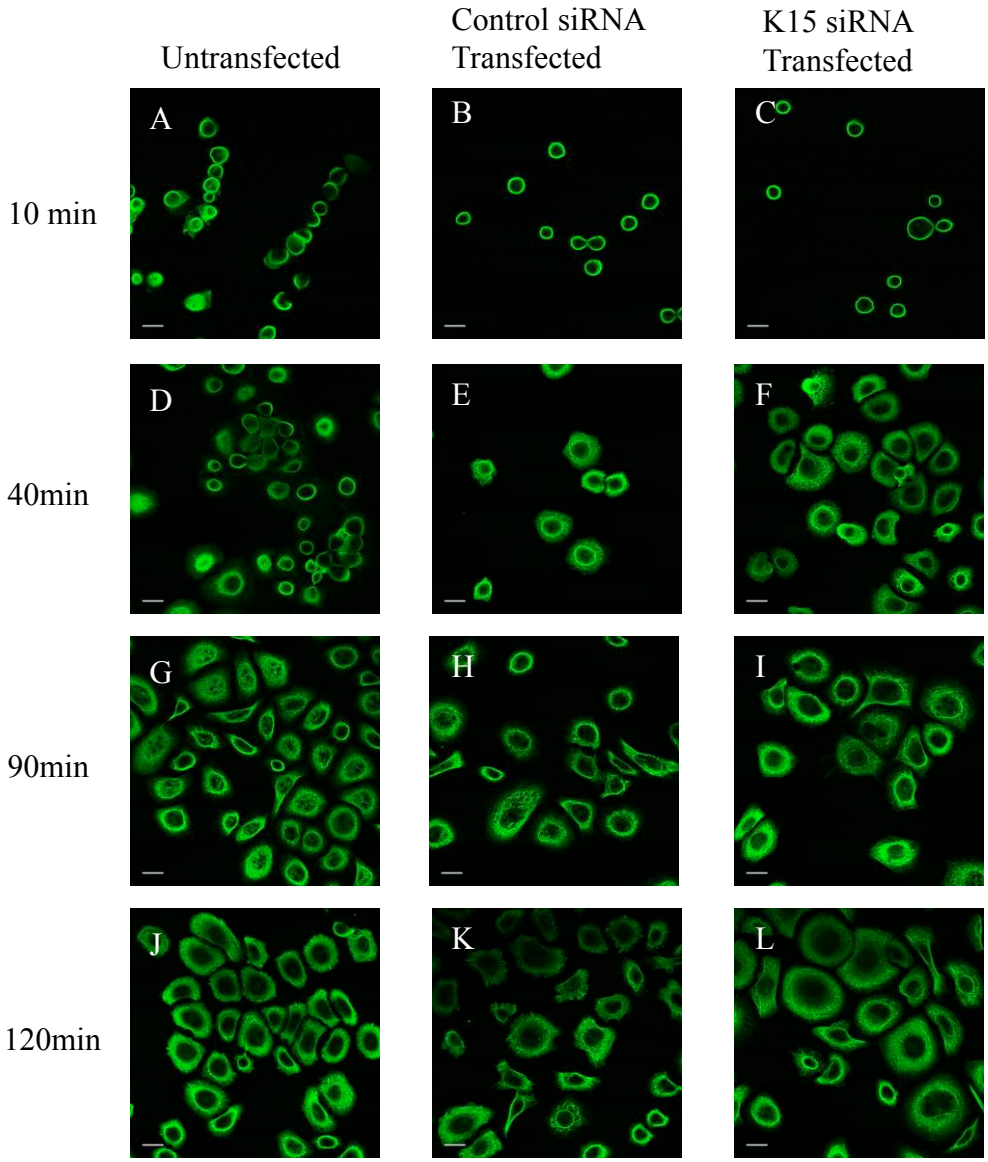


Figure 4.6: K14 organisation during cell spreading.
K14 immunofluorescence in untransfected (A, D, G, J), control siRNA transfected (B, E, H, K) and K15 siRNA transfected (C, F, I, L) HaCaT cells. The cells have been seeded onto glass coverslips and fixed after 10, 40, 90, and 120 minutes (scale bar = 10μm). K14 expression in control siRNA transfected and untransfected cells appears similar, suggesting that the transfection procedure does not affect K14 expression. K14 expression at the cell edge appears reduced in K15 ablated cells (L).

It was thought useful to investigate whether any correlation existed between the absence of K14 at the cell edges and the apparent increase in actin bundling at the cell edges in K15 ablated cells. Using Image J, measurements between the cell edge and the K14 cytoskeleton were made. Using Student's T-test, it was established that the difference in absence of K14 at the cell edge was significantly different in K15 ablated cells compared to control siRNA transfected cells ($p=0.05$).
The distance between the edge of the K14 cytoskeleton and the cell edge in K15 ablated spreading HaCaT cells is, on average, 2µm (*Table 4.1*). Likewise, the thickness of actin bundling at the cell edge is 2.2µm (*Figure 4.3*). Similarly, in control siRNA transfected cells, the distance between the K14 edge and the cell edge is 1.3µm (*Table 4.1*) and the thickness of the actin bundles are 1.2µm (*Figure 4.3*). The similarity between these measurements suggests some correlation between the two. It is possible that the lack of K14 could be due to the increased amount of actin at the cell edges (i.e. no K14-positive, structural, IF cytoskeletal fibres are required because of the increased presence of actin in the region). The opposite may also be true: that the lack of K14 at the cell edges requires structural support from the actin cytoskeleton. It is more likely that the former, not the latter, is what is occurring here, since actin has already been shown to have a role in cell spreading (see, for example, section 4.1.3.2). Since this occurs in control siRNA transfected cells as well as K15 ablated cells (albeit to a lesser extent), it is likely that where this phenomena is observed in K15 ablated cells, it is an exaggeration of an event which occurs in normal keratinocytes.

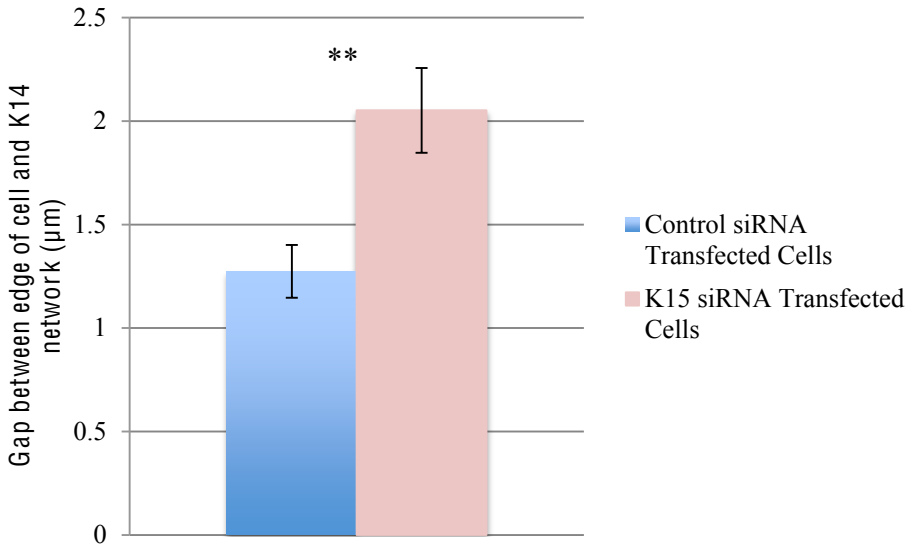


Figure 4.7: K14 network in HaCaT cells

Measurements of the gap between the edge of the cells and the K14 network were made using Image J. Ten measurements were made per cell and 10 cells were measured. Mean and standard error are shown. The difference between the average gaps in K15 siRNA transfected and control siRNA transfected cells is statistically significant (*t*-test, $p<0.05$) at 180 minutes post-seeding (**).

4.3.2.4: Changes in Size and Shape of K15 Ablated HaCaT Cells

To establish whether the cytoskeleton rearrangement had an effect on cell area and circularity, Image J was used to quantify these parameters (*Figure 4.8*). In control siRNA transfected HaCaT cells, average cell area increased as the cells attached to the glass coverslip and spread; this peaked at approximately 90 minutes post-seeding. This increase in cell area is even greater in K15 knock-down HaCaT cells, where cell area can be seen to increase up to approximately 120 minutes post-seeding; the cell area also appears to increase at a faster rate compared to controls.

As HaCaT cells attach, they begin to spread in order to form cell-cell contacts. Cells without K15 spread to a greater extent than control siRNA transfected cells. K15 has not been previously shown to be directly involved in cell spreading or migration, although it must have an effect to affect cell size to the extent observed here (at 120 minutes, control average cell area was 205μm^2; K15 knock-down average cell area was 409μm^2). These results are significantly different ($p=0.001$), suggesting that the larger cell area observed in K15 siRNA transfected cells is as a result of K15 ablation.

As well as cell area, cell circularity could be calculated; this was deemed useful as cell shape may be affected by the differences observed in cytoskeletal arrangement. In both control siRNA transfected and K15 knock-down cells, cell circularity decreased as cells spread. Despite a significant difference between cell size, there is no significant difference in circularity between control siRNA and K15 siRNA transfected cells (*Figure 4.9*), as established using Student's T-test. This indicates that despite differences in cell area and cytoskeletal arrangement, K15 ablated cells do not differ in their shape.

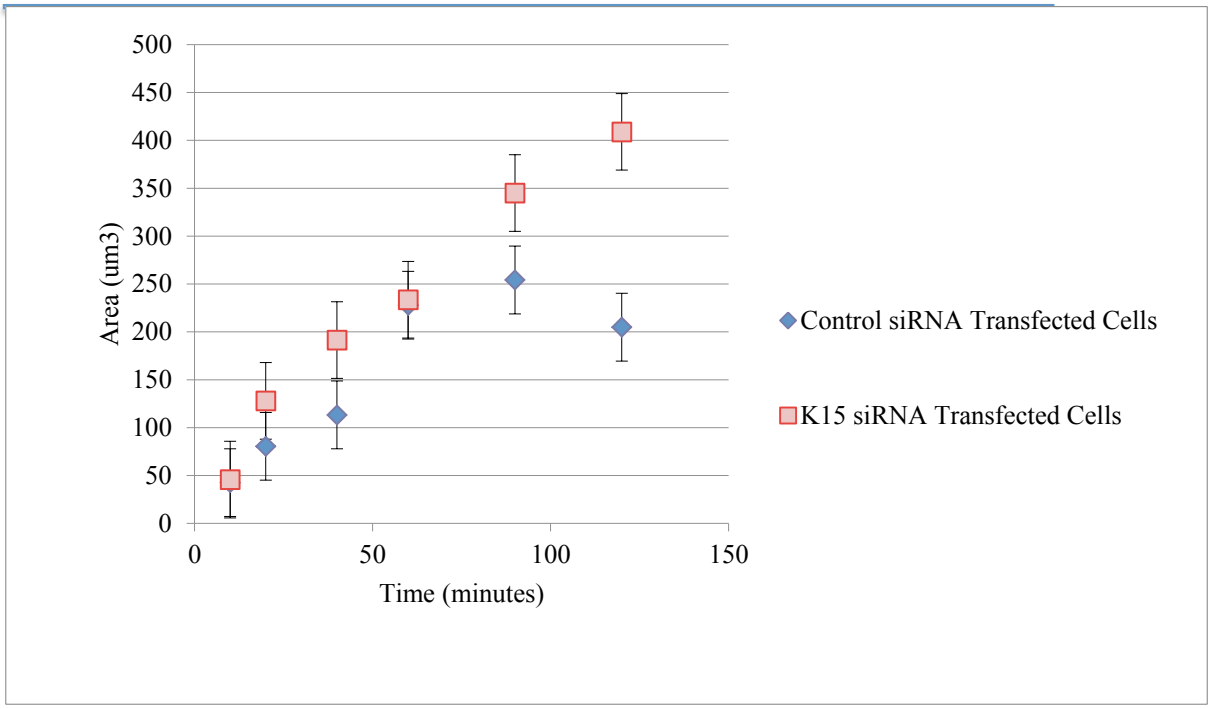


Figure 4.8: HaCaT cell area during adhesion to uncoated glass coverslips. Calculations were made using Image J software. K15 siRNA transfected cells have a larger average area 180 minutes post-seeding than control siRNA transfected HaCaT cells. Error bars = standard error.

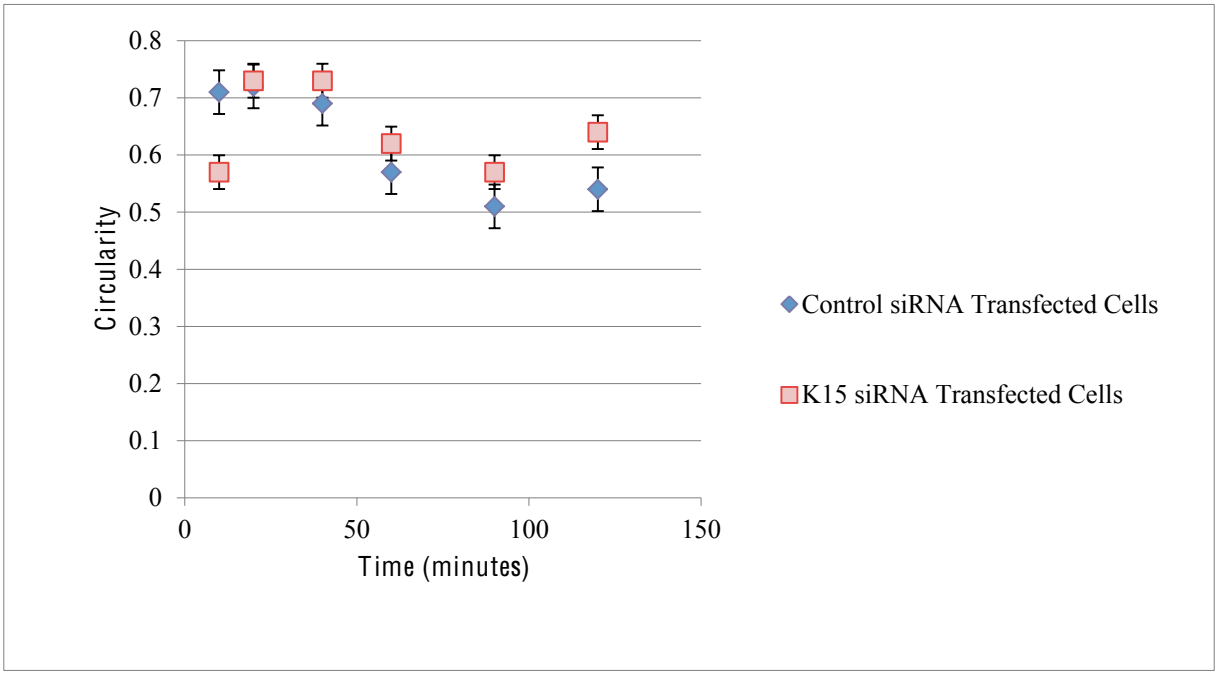


Figure 4.9: HaCaT **circularity, calculated using the 'form factor'** formula.
In both K15 knock-down cells and control transfected cells, circularity decreases slightly as cells spread (1=perfect circle). There is no significant difference between control siRNA transfected HaCaT cells and K15 siRNA transfected HaCaT cells. Error bars = standard error.

4.3.2.5: NHEK Adhesion

For comparison, the adhesion assay was also carried out on untransfected NHEK cells. These cells took longer to initially adhere, which prevented useful immunofluorescence being carried out at 10 minutes post-seeding. Between 40 and 120 minutes post-seeding however, similar actin bundling at the cell edge can be observed (*Figure 4.10*, C, F, I). K15 and K14 expression appears normal throughout the cells (*Figure 4.10*, A, B, D, E, G, H). These results then are comparable with the results observed for untransfected and control siRNA transfected HaCaT cells. Cell size and circularity in attatching cells were also calculated for NHEK cells. Circularity of the cells varied little during spreading (*Table 4.2*). Cell area, as expected, increased during cell spreading, which indicates that the actin bundling observed is involved in increasing cell size following adhesion (*Table 4.3*).

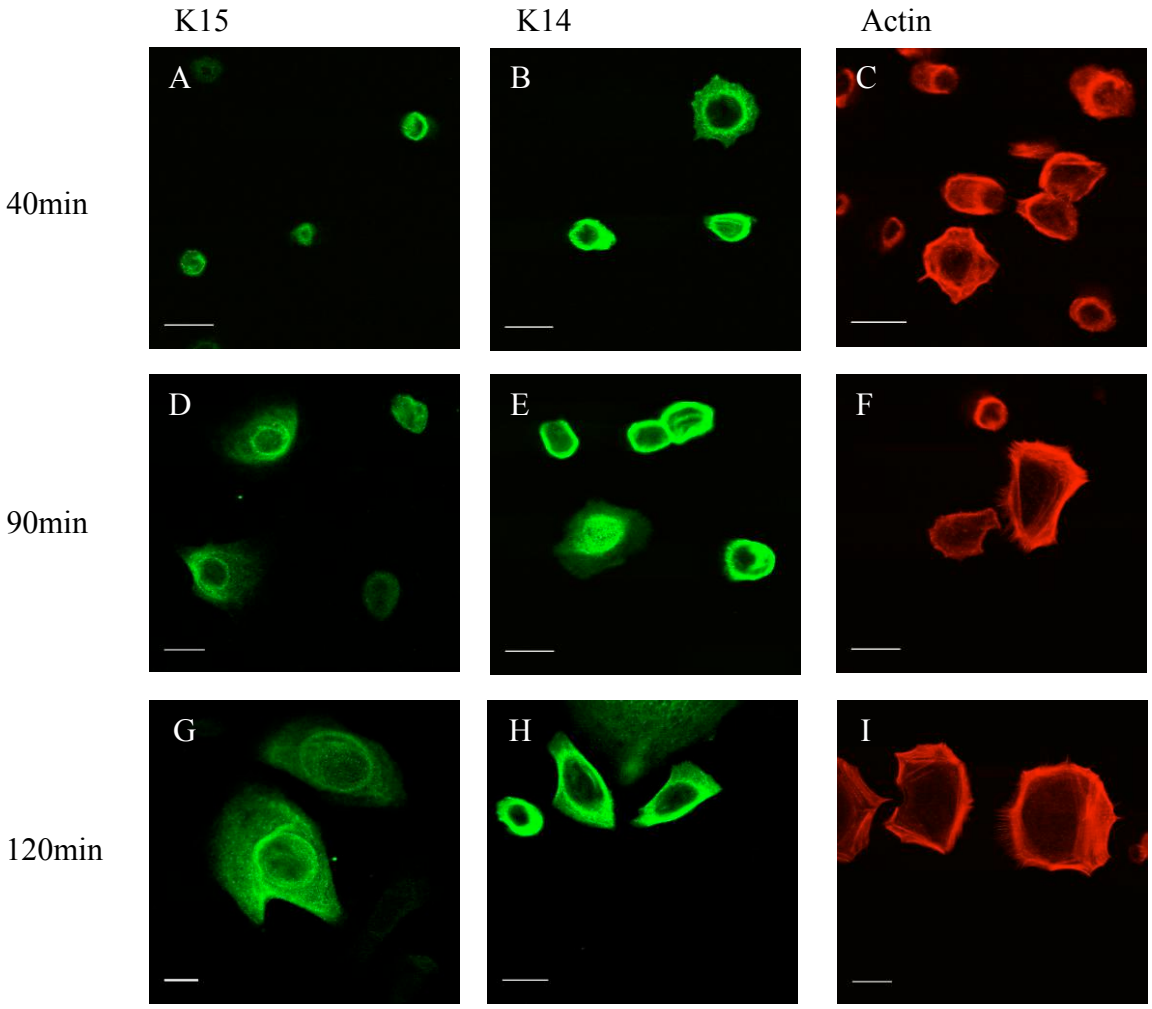


Figure 4.10: K15, K14 and actin organisation in spreading NHEK cells
NHEK cells were seeded onto glass coverslips and allowed to attach and spread for 40 (A, B, C), 90 (D, E, F) and 120 minutes (G, H, I). This allowed K15, K14 and actin expression and configuration to be observed at different stages of attachment and spreading (scale bar = 10μm). As in HaCaT cells, some bundling of actin at the edges of cells is observed (I). No changes in K14 (H) or K15 (G) expression were noted at 120 minutes post-seeding.

Table 4.2: Table of NHEK cell circularity during attachment.

	NHEK Cells
Minutes	Untransfected
40	0.75
90	0.77
120	0.73
180	0.71

Cell circularity decreases little between 40 and 180 minutes post-seeding of the untransfected NHEK cells (1=perfect circle).

Table 4.3: Table of NHEK cell area during adhesion to uncoated glass coverslips

	NHEK Cells
Minutes	Untransfected
40	49.9
90	68.2
120	170.7
180	257.2

As the cells adhere, the NHEK cells spread and the area increases from 50μm^3 to 257μm^3 180 minutes post-seeding (calculated using Image J software, measured in μm^3).

4.3.2.6: Quantitation of HaCaT Adhesion

The MTT assay was used to quantify the number of adhesive cells at various time points post-seeding (*Figure 4.11*). Upon initial seeding of $5x10^5$ K15 siRNA transfected and control siRNA transfected cells, approximately 5% of these had adhered after 40 minutes. At 90 minutes post-seeding, a significant difference can be observed ($p<0.05$), with fewer control siRNA transfected cells adhering compared to K15 siRNA transfected cells. By 120 minutes post-seeding, the number of adhering K15 ablated cells has reached a plateau, whereas the number of control siRNA transfected cells are still adhering.

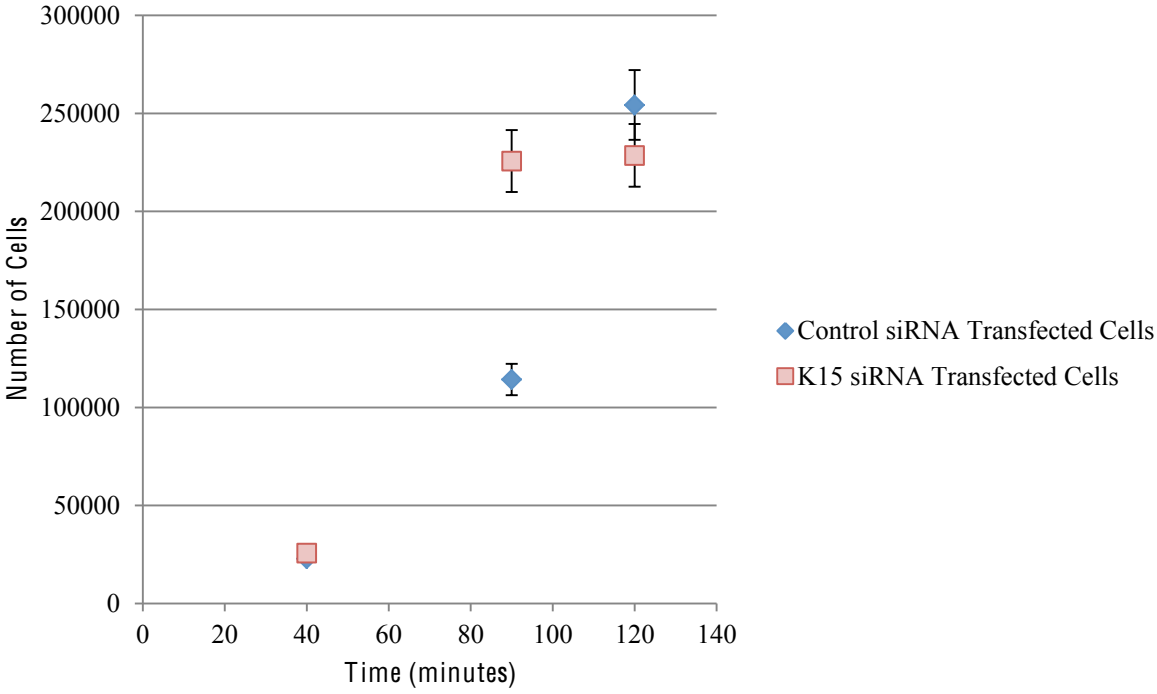


Figure 4.11: Cell numbers up to 120 minutes post seeding.
This graph shows that K15 siRNA transfected cells initially adhere faster than control siRNA transfected cells (90 minutes post-seeding). However, by 120 minutes more control siRNA transfected cells have adhered compared to K15 knock-down cells. (500000 HaCaT cells were initially seeded.) Error bars = standard error.

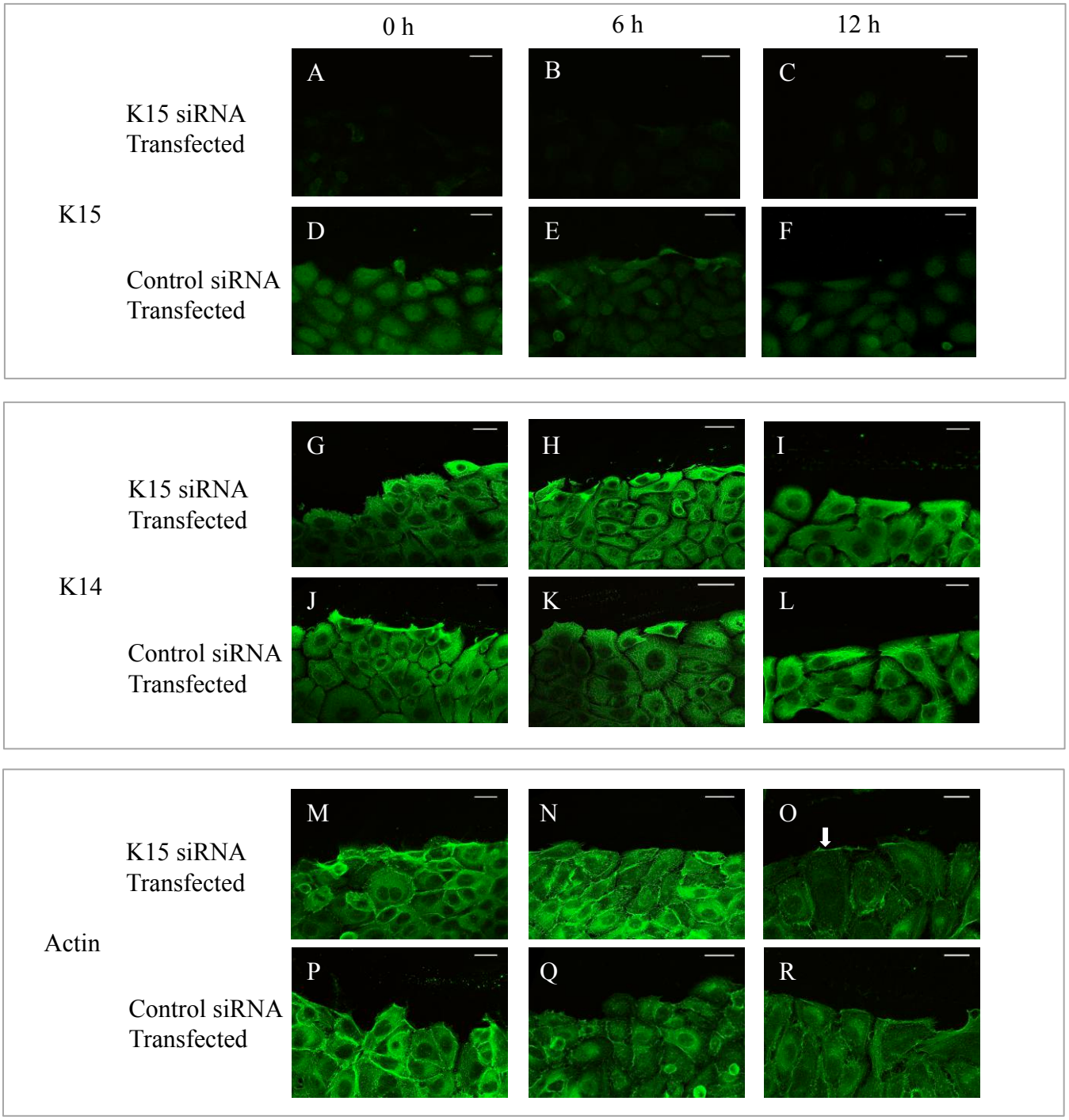


Figure 4.12: Immunofluorescence of HaCaT scratch wound assay at 0 hours, 6 hours and 12 hours following scratching

Little K15 is observed in K15 siRNA transfected cells (A, B, C) compared to control siRNA transfected cells (D, E,F). No changes in K15 expression are observed in control siRNA transfected cells following wounding at the wound edge. Similarly, no changes to K14 organisation are observed in either K15 siRNA transfected cells (G, H, I) or control siRNA transfected cells (J, K, L) after wounding. Actin expression appears normal throughout in control siRNA transfected cells (P, Q, R) and K15 siRNA transfected cells at 0 h and 6 h post-wounding (M, N). At 12 h post-wounding in K15 siRNA transfected HaCaT cells (O), some bundling of actin fibres can be observed at the wound edge (*arrow*). Scale bar = 25µm.

4.3.3: How Cell Migration is Affected By K15 Ablation

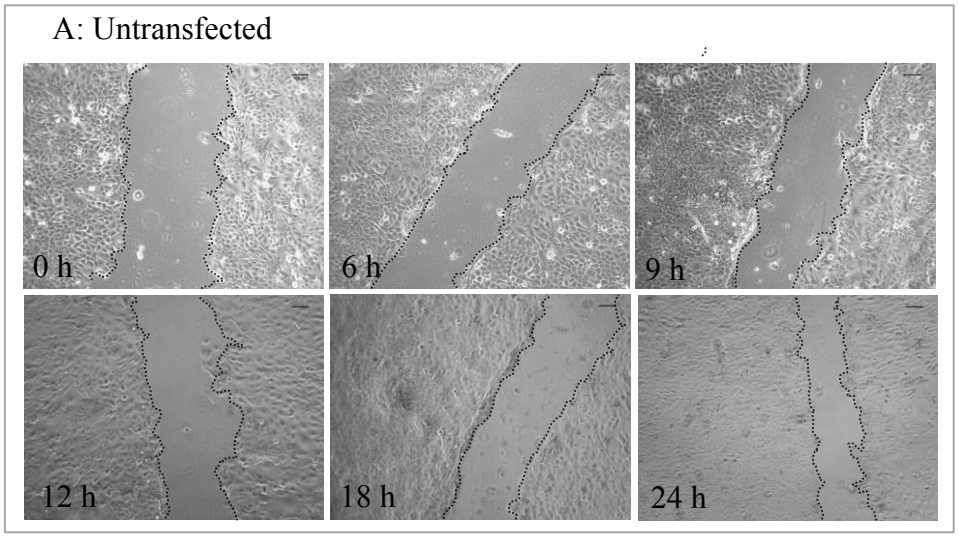
It has been suggested that cells from the hair follicle bulge (i.e., K15-positive cells) differentiate before migrating (Roh *et al*., 2005; Roh *et al*., 2008); this suggests that K15-positive cells would be less motile than K15-negative cells. Considering this, it was deemed useful to assess the motility of HaCaT cells following K15 ablation by siRNA transfection. To assess cell motility, a scratch wound assay was employed. Cells to be transfected were seeded onto glass coverslips and transfected 48 hours prior to scratching. This assay required cells to be 100% confluent, however this produces two difficulties. Firstly, confluent HaCaT cells have been shown to differentiate and stratify (for example, Capone *et al*., 2000). Secondly, for successful siRNA transfection, cells must be between 30% and 50% confluent. For these reasons, HaCaT cells could not be at 100% confluency before the scratch assay was carried out. Instead, areas where cells were most confluent (without beginning to stratify) were identified before the scratch was made. Cells were 'scratched' with a 200µl pipette tip, and fixed at various timepoints following the scratch. Live cell imaging techniques (as previously described) were used to produce phase contrast images of wound healing. Coverslips were also fixed and immunofluoresence techniques used to enable the effects of K15 ablation to be observed in 2D *in vitro* wound healing.

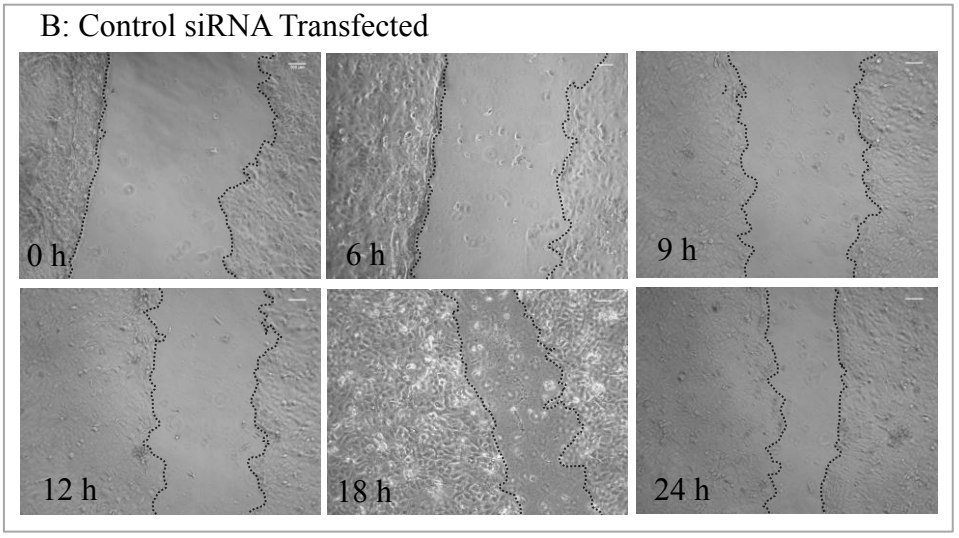
K15 siRNA transfected cells express very low levels of K15, as expected (*Figure 4.12*, A - C). Immediately following the scratch, K15 expression and organisation appear normal in control siRNA transfected cells (*Figure 4.12*, D - E). No bundling of K15 can be observed at the wound edge 12 hours post-wounding (*Figure 4.12*, F), suggesting that K15 does not have the same role in organising collectively migrating epithelial sheets as K8 has in simple epithelial cells (Long *et al*., 2006).

The immunofluorescence staining of actin and K14 in HaCaT cells shows that expression of these proteins are comparable in transfected and untransfected cells. *Figure 4.12* G - L shows K14 expression in K15 knock-down and control HaCaT cells. The arrangement of K14 in the confluent cells and at the wound edge appears similar; there is no bundling of K14 at the edge of the wound as seen in some other keratins (for example, K8: Long *et al*., 2006). There does however appear to be an increase in K14 in the cells at the edge of the wound 6 h after wounding; this appears in both control HaCaT cells and K15 knock-down cells (expression and localisation of K14 appears similar to control cells in K15 knock-down cells, suggesting that K15 siRNA transfection does not affect this K14 localisation). In an investigation into the role of TGFβ in wound healing, Werner, Werner and Munz (2000) demonstrated that K14 was upregulated by this cytokine during wound repair in HaCaT cells. K14 levels were also observed to increase continually for 5 days post-injury in mice. In sections of wounded skin, K14 immunofluorescence was observed in all keratinocytes around the wound. It may be a similar effect which has been observed in *Figure 4.12*, on a reduced scale (due to the monolayer of HaCaT cells used as opposed to *in vivo* testing).

Actin expression appears normal throughout the cells in both control siRNA transfected and K15 siRNA transfected HaCaT cells, and at both 0 h and 12 h post-scratch (*Figure 4.12* M - R). At 12 h post-scratch in K15 knock-down cells, some bundling of actin can be observed at the wound edge (*Figure 4.12*, O, arrow); such a phenomenon has previously been described (for example, Long *et al*., 2006 described this in MCF-7 cells). Long *et al*. however used electron microscopy techniques to identify structures such as lamellipodia (the images in *Figure 4.12* are a much lower magnification so such structures may not be seen). This was also observed at 30 minutes after wounding, compared to 12 h after wounding in this study. This may be

an effect similar to that observed previously in spreading cells; actin bundling at the cell edges may indicate migration. For this reason, the speed at which wound closure occurred was measured from microscope images of the wound region (*Figure 4.13*).





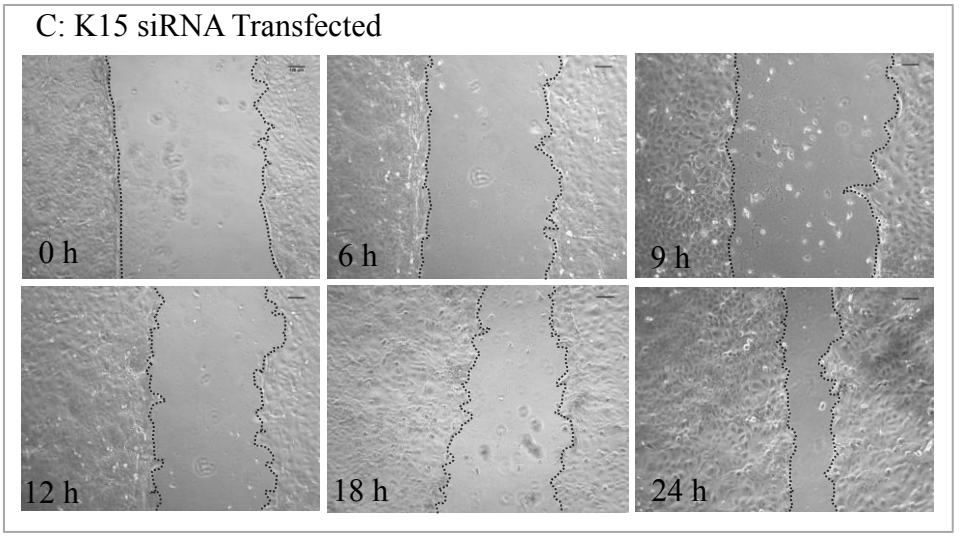


Figure 4.13: Wound healing in HaCaT cells. Phase contrast images of HaCaT wound healing in untransfected (A), control siRNA transfected (B) and K15 siRNA transfected (C) cells. The time post-wounding is noted.
The wound edges become more undulated over time in all samples. After 24 h, no wound had closed completely.
(Scale bar = 100μm.)

..... = edge of wound

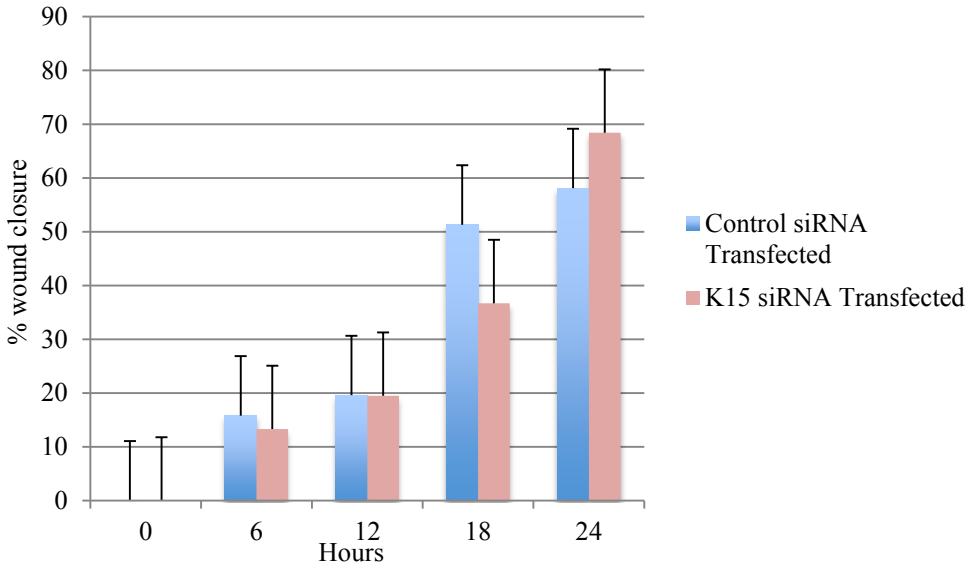


Figure 4.14: Percentage wound closure in K15 siRNA transfected and control siRNA transfected HaCaT cells

Locations of three control siRNA transfected and K15 siRNA transfected HaCaT wounds were photographed at 0, 6, 12, 18 and 24 h. Open wound distance at the start of the experiment is designated 0% and closure of the wound shown relative to this (i.e. % reduction of the wound area). Ten measurements were made per wound at each time point at each of the three wound locations photographed. Error bars denote standard error. The difference between the average wound closure at 18 h in control siRNA transfected cells and K15 siRNA transfected cells is not statistically significant.

Measurements were made from the phase contrast images produced from live cell imaging (*Figure 4.13*). The percentage wound closure was then calculated. *Figure 4.14* shows the percentage wound closure of control siRNA transfected and K15 knock-down HaCaT cells (where 100% indicates a completely closed wound).
Statistical analysis suggests that no significant difference is observed between wound closure times of K15 ablated and control siRNA transfected cells. No conclusive results were observed here, although as only monolayer cultures were used in this study, it may be that findings *in vivo* are be different (as bulge cells migrate from the bulge to the injured IFE). For example, Roh *et al*. (2008) suggested that more differentiated cells migrate faster than their stem cell counterparts.

4.4: Discussion

4.4.1: K15 Ablation Affects Keratinocyte Spreading

4.4.1.1: The Role of Actin in Keratinocyte Spreading

Using immunofluorescence, this study demonstrated that the actin bundles which form at the cell edge during cell spreading were significantly thicker in K15 ablated cells. A well-defined actin arrangement can be observed as soon as cells have adhered, and filopodia-like protrusions seen; the difference between control siRNA transfected and K15 siRNA transfected cells can be observed at 90 minutes post-seeding, where actin bundles at the cell edge to a greater extent in K15 knock-down cells. Measurements of actin bundle thickness were made using Image J. The average thickness of actin bundles in K15 knock-down cells was 2.2µm, compared to 1.2µm in control siRNA transfected HaCaT cells. This trend continues up to at least 180 minutes post-seeding, although cells were not examined after this time point since the MTT assay demonstrated that cells were beginning to proliferate (*Figure 4.12*). In a study concerned with establishing how cell shape is formed and maintained in epithelial cells, Zhang *et al*. (2005a) demonstrated that peripheral bundles of actin form upon induction of cell-cell contacts. These peripheral bundles were important in establishing the cuboidal morphology of epithelial cells. However, Zhang *et al*. (2005a) used established, confluent cultures of keratinocytes to establish the actin reorganisation which occurred after the formation of cell-cell junctions. Since in this study the actin bundles were observed soon after seeding, a different mechanism may be in place to produce the peripheral bundles seen here. Connelly *et al*. (2010) suggest that just as cytoskeletal tension controls mesenchymal stem cell differentiation, G-actin may have a similar effect in keratinocytes. It has been noted that during trypsinisation in where cell formation changes from flat to round in a small amount of time, there is no change in total cellular F-actin (Bereiter-Hahn *et al*., 1990), suggesting that organisation changes with altered adhesion (Ingber *et al*., 1994). Mooney *et al*. (1995) suggest that cell extension occurs through the resistance of the internal cytoskeleton and ECM tethers to the inward pull of the cytoskeleton; Mooney *et al*. (1995) refers to this as a 'tensegrity' mechanism. Using rat hepatocytes induced to adhere to ECM, Mooney *et al*. (1995) demonstrate that microfilaments and microtubules function together to change form in response to changes in cell-ECM binding (see also Luo *et al*., 2008).

4.4.1.2: Formation of Focal Adhesions during Keratinocyte Spreading

As vinculin has been shown to be incorporated into structures important in keratinocyte adhesion and migration, vinculin expression (assessed using immunofluorescence) was investigated in K15 knock-down cells to investigate any effect this protein may have on focal adhesions and migration. Despite no effect on

desmoplakin, E-cadherin or ZO-1 by K15 ablation (section 3.3.5.2), suggesting cell-cell attachments are not affected, cell-ECM adhesions and migration are different conditions for keratinocytes, and required further study.
Initial immunofluorescence of vinculin as a marker of focal adhesions highlighted these regions in attached HaCaT cells (*Figure 4.1*). Staining was observed as described by Möhl *et al*. (2009): distinct intense staining highlighted high levels of bound vinculin whilst a low concentration can be observed throughout the cytoplasm. When the number of focal adhesions were counted, more adhesions were present in control siRNA transfected cells. Since focal adhesions are important in actively migrating cells, this result suggests that cells with low levels of K15 migrate differently to K15-positive cells.

4.4.1.3: K14 and K15 Expression in Spreading Keratinocytes
K14 arrangement following K15 ablation in attaching cells was also investigated using immunofluorescence. K14 had been previously shown not to be affected by K15 siRNA transfection (*Figure 3.5, Figure 3.6*), although when cells were initially seeded and spreading, K14 could not be observed at the edge of the cell in K15 ablated cells at 120 minutes post-seeding. Morley *et al*. (2008) suggest that seeding of keratinocytes in culture induces a keratin expression pattern analogous to a wound healing reaction. K14 expression has been shown to increase during wound healing (Werner, Werner and Munz, 2000); this is not occurring here. Other studies have been concerned with whether cells are K14-positive or K14-negative, without closer observation of K14 arrangement. It may be that K14/K5 fibres are normal in K15 ablated cells, although can not form quickly enough to extend to the cell edge during the rapid expansion occurring as soon as cells are seeded. It may also be the case that the increased thickness of the actin bundles at the cell periphery prevent K14 filaments reaching the cell edges. K14 appearance is normal in K15 knock-down cells in cells which have been seeded at least 48 h prior (*Figure 3.5*) suggesting that the effect observed immediately post-seeding is not long-term. Although K14 fibres did not expand to the edge of the cell, it is possible that this would not have affected the function of the cell. Where cytoskeletal proteins were disrupted in newly-adhered hepatocytes, the cells remained smaller than controls for at least 48 hours (Mooney *et al*., 1995); similar work has not yet been carried out on keratinocytes.
K14 is known to affect cell junctions and cell junctional protein expression; Liovic *et al*. (2009) observed that in K14 mutant cells (mimicking severe EBS), different K14 abnormalities resulted in reduced amounts of connexin 43, desmoglein 3, desmoplakin and plakoglobin expression. If this effect is observed in EBS cells, it is possible that cell junctional proteins may be affected in the short-term after seeding in K15 ablated cells, where K14 does not reach the cell edge. As K14 arrangement is normal in K15 knock-down cells >48 h post-seeding, then this explains the normal expression of cell junctional proteins observed in this study (*Figure 3.13*). If cell-cell adhesion is at low levels shortly after seeding, this aids in explaining the motility of cells observed in the live cell imaging, where cells could be seen to move relatively swiftly (*Figure 4.4*); cells were seen to move towards each other, typical behaviour for keratinocytes which do not thrive at low confluencies.

4.4.1.4: Effects of K15 Ablation on Keratinocyte Size and Shape during Spreading
Both control siRNA transfected and K15 siRNA transfected cell size increased post-seeding. However, cell size increased to a greater extent in K15 ablated cells.
Connelly *et al*. (2010) showed that actin polymerisation inhibited cell spreading; cell size analysis in this study demonstrated that K15 knock-down cells were larger than

their control counterparts, suggesting that actin was not polymerising (or at least not to the same extent) as in control siRNA transfected cells. Previously, actin had been shown to polymerise following hypotonic challenge and volume changes (Blase *et al*., 2009). Depolymerisation has also been shown to enhance the symptoms of the skin blistering disease pemphigus vulgaris, similarly demonstrating the role of polymerised actin in preventing cell dissociation (Gliem *et al*., 2010). Where cell spreading is restricted and G-actin levels are reduced, JunB expression is stimulated, causing differentiation (Mehic *et al*., 2005). If this could occur *in vitro*, then K15 knock-down cells would become confluent more quickly (as they spread at an increased rate), and therefore induce JunB expression and differentiation stimulation. This could at least partially explain some of the differentiation observed in K15 siRNA transfected cells in chapter 3.

It has been shown that cell shape can control initiation of terminal differentiation (Connelly *et al*., 2010). Cell circularity and cell area were calculated from immunofluorescence images using Image J. As previously mentioned, cell area in K15 knock-down HaCaT cells is greater than in control siRNA transfected cells. Cell size has previously been used as a measure of differentiation in keratinocytes; smaller cells with a larger nuclear-to-cytoplasmic ratio are considered less differentiated (for example, Zbytek *et al*., 2005; Wan *et al*., 2007b). Cell shape has also been shown to be a factor: keratinocytes cultured in a manner which prevented cell-cell contact (such as suspension culture) lost the ability to divide and assemble cornified envelopes. Although inhibition of proliferation in spread cells was not found to produce a sufficient signal for terminal differentiation, the area of cell contact with the substratum aided in regulation of proliferation and differentiation. Cell size increases as cells terminally differentiate, as part of a positive feedback system whereby confluent cells are induced to leave the basal layer and differentiate (Watt and Green, 1981; Barrandon and Green, 1985; Watt, Jordan and O'Neill, 1988; Sevilla *et al*., 2008; Charest *et al*., 2009).

4.4.2: Effect of K15 Ablation on Keratinocyte Migration

Cell migration is important in many vital processes, such as embryogenesis and tissue repair and regeneration, and in pathological conditions such as cancer and inflammation (Webb *et al*., 2002). As it is possible that K15-positive cells from the hair follicle bulge differentiate (i.e. become K15-negative) before migrating (for example, Roh *et al*., 2008), it was considered important to assess the effect of K15 ablation on motility. For this, a scratch wound assay was employed; this has been previously utilised elsewhere (for example Wong and Gotlieb, 1988; Long *et al*., 2006).

Only small changes were noted in K14 arrangement during migration in both control siRNA transfected and K15 siRNA transfected cells. The increase in K14 observed in the cells at the leading edge may be as a result of increases in TGFβ, as described in section 4.3.2 (Werner, Werner and Munz, 2000). Importantly for this study however was the indication that no difference in K14 expression occurred following ablation of K15. This indicates that K15 does not affect the role of K14 during wound healing. This suggests that K15 does not have a role in organising collective migration, as K8 does for example (Long *et al*., 2006). K8 however is a simple epithelial keratin, found in single-layer epithelia; K14 and K15 are not expressed in single-layer epithelia but in complex epithelia consisting of several layers at different stages of differentiation. This could explain the absence of keratin bundling in HaCaT cells. K14 expression has been observed in re-epithelialised epithelium following injury, suggesting that basal keratinocytes have an important role in wound healing (Hosoya

et al., 2008); this study did not carry out any investigation of K14 arrangement in the migrating cells however.
Likewise, actin expression is similar in migrating cells following K15 ablation. At 12 h post-scratch in K15 siRNA transfected cells however, some actin bundling is observed at the cell edge. During cell migration, focal contacts form which develop into focal adhesions. The clustering of integrins, VASP and vimentin at the focal contact site converges alongside actin cables, or stress fibres (Reinhard *et al*., 1992). Actin bundles, perpendicular to the cell membrane, are also associated with puncta: clusters of β-catenin, α-catenin and E-cadherin which form at the early stages of cell-cell adhesion (Adams *et al*., 1996; Adams *et al*., 1998). These sites are important in actin polymerisation and reorganisation (Vasioukhin *et al*., 2000).
It is possible that this is a similar effect to that observed in spreading cells. Since in spreading cells this actin bundling coincided with an increased cell area, it was considered useful to further investigate the rate of wound closure following K15 ablation.
No significant differences were observed in the rate of migration between K15 siRNA and control siRNA transfected HaCaT cells. Previous studies have suggested that more differentiated cells migrate faster than stem cells (Roh *et al*., 2008). The results of this study suggest that K15 expression has little effect on keratinocyte migration. However, it has also been demonstrated that K15-positive bulge cells can contribute to wound repair *in vivo* (for example, Nakrieko *et al*., 2011). This suggests that although the K15 protein may not have a direct functional role in keratinocyte migration, the K15-positive undifferentiated cells may have a role in wound healing.

4.4.3: Conclusions and Further Work

4.4.3.1: Summary of Findings

K15 ablation had a significant effect on cell spreading and adhesion, resulting in increased actin bundling at the cell edge. In turn, this increased cell size (although did not affect cell shape). This is considered important as Zbytek *et al*. (2005), Wan *et al*. (2007b) and Connelly *et al*. (2010) observed that small cells, with a large nuclear-to-cytoplasmic ratio, are considered less differentiated. In this study, this would indicate that K15-negative cells are more differentiated than K15-postive cells, reinforcing the work which suggests that K15 is a marker of epithelial stem cells.
In addition, the effect of K15 ablation on keratinocyte migration was examined. It was established that no significant differences were observed in K15-negative keratinocytes compared to K15-positive keratinocytes. This indicates that the K15/K5 filaments have no direct function in HaCaT cell motility.

4.4.3.2: Further Work

As previously mentioned, K14 has been demonstrated to affect cell junctional protein expression (for example, Liovic *et al*., 2009). It was demonstrated that immediately after seeding, K14 was not expressed to the edge of K15 ablated HaCaT cells. However, this was not observed in cells which had been seeded for at least 50 h (as demonstrated by immunofluorescence in chapter 3). Likewise, normal expression of E-cadherin, desmoplakin and ZO-1 were observed in K15 ablated cells after this time. It may be useful to carry out further investigation of the development of cell-cell junctions (and localisation of junctional proteins) immediately following cell seeding, to investigate whether the absence of K14 at the cell periphery in K15 ablated cells has an effect.
To further investigate the role of K15 in wound healing, it would be useful to reproduce the *in vitro* wounding method using organotypic cell cultures following

control and K15 siRNA transfection. This would be more similar to conditions observed *in vivo*, however a more stable transfection may be required first.
Previously, electron microscopy has been used to identify laemellipodia at cell edges (for example, Ahmed, 2011). This may also be useful in this study to more closely observe the organisation of the actin cytoskeleton during cell adhesion and spreading in K15 ablated cells.

Chapter 5: Effects of K15 Ablation in Squamous Cell Carcinoma Cells

5.1: Introduction

5.1.1: Aims

The aims of this chapter are to elucidate the effect of K15 ablation on a squamous cell carcinoma cell line, 'MET' (Proby *et al*., 2000). Three of the four cell lines isolated by Proby *et al*. (2000) will be used to establish whether K15, identified as being expressed in at least poorly differentiated SCCs (for example, Abbas *et al*., 2011), effects cell motility, differentiation, adhesion or spreading. These will be tested as HaCaT cells were in chapters 3 and 4. Although Proby *et al*. (2000) suggested that these cells did not produce good organotypic cell cultures, MET 1, 2 and 4 cells were cultured in this way to establish whether a different method may be more successful. It would also help to establish whether the MET cells were capable of organised stratification and differentiation.

5.1.2: The MET Cell Lines

Whereas normal human primary cells have a finite lifespan (proliferating for a limited number of generations before no longer dividing), isolated tumour cells can divide indefinitely (i.e. are immortal). This occurs in the squamous cell carcinoma cell lines MET, isolated by Proby *et al*. (2000). The MET 1-4 cells were isolated from a progressive primary epidermal tumour and distant metastasis from one patient. The MET cell line cells were from an invasive, recurrent and metastatic squamous cell carcinoma (SCC), the most aggressive type of non-melanoma skin cancer. MET 1 cells were isolated from an SCC on the back of the left hand, MET 2 and 3 cells from two recurrent SCC from the same site, and MET 4 cells from the metastatic SCC from the lymph node (isolated at the same time as MET 3 cells). As MET 2 and 3 cells were from similar tumours, MET 3 cells were not used in this study. Although SCC sections isolated from the back of the hand were human papillomavirus (HPV) positive, all of the MET cell line cells were negative for HPV DNA. No external immortalising agents were required to immortalise the MET cell lines. Isolated cells were cultured in DMEM with 10% FCS, initially with 3T3 fibroblast feeder layers and EGF. All of the cell lines were dependent on adhesion for growth (no colony formation was observed in soft agar) (Proby *et al.*, 2000).

5.1.3: Intermediate Filament Expression in SCC Cells

5.1.3.1: Keratins

In the original publication describing the MET cell line cells, Proby *et al*. (2000) demonstrated that the keratin profile of the *in vitro* MET cell lines were maintained from the *in vivo* sections of each tumour.

Previous studies have observed the expression of keratins in SCC sections. Watanabe *et al*. (1995) observed that the keratin expression profile of well-differentiated SCCs was similar to that observed in normal epidermis. However, expression of differentiation-specific keratins was reduced in the immature tumour cells (in proportion to the malignancy of the SCC). In poorly-differentiated SCCs (and in most lymph node metastases), keratins typical of simple and squamous epithelia were expressed. Typical of this is the expression of simple epithelial keratin K7 in MET 3 and MET 4 cells – abnormally expressed in advanced cutaneous malignancies (Markey *et al*., 1991). As the SCC became malignant, the keratin profile also changed; this led Watanabe *et al*. (1995) to suggest that the expression of non-cornifying stratified squamous epithelial or simple epithelial keratins could be a marker for metastatic or invasiveness potential.

In their 2000 publication, Proby *et al*. define the keratin expression profile of the MET cell line cells, where it is described as typical of the keratin phenotype of the

tumour of origin. Of the epidermal keratins, Proby *et al*. suggested that K14 has panepithelial staining in normal skin. This was also observed in sections of the same SCC the MET cells were isolated from (including the lymph node). In MET 1-3 cells, strong K14 expression was observed; K14 expression was also observed in MET 4 cells, to a lesser extent. No heterogenous staining was noted. This is an unexpected result, as downregulation of K14 (and K15) has previously been reported in undifferentiated tumours (Markey *et al*., 1991; Morgan and Su, 1994).

K1/10 staining, observed in the suprabasal layers of normal skin, had a different profile in the SCC sections. There was no staining in the poorly differentiated regions of the primary SCC section or recurring SCC sections, with only a small amount of staining in the metastatic section. All of the MET cell line cells were negative for K1/10 staining. Likewise K4/13 staining was also negative in MET 1-4 cells and all sections (including normal skin).

K6, despite no expression being observed in normal skin, was observed to some extent in the SCC sections. In the SCC sections taken from the back of the hand, panepithelial K6 staining is observed. Lymph node metastasis sections were also K6 positive. K16 staining was observed separately from its usual partner K6, and a different staining profile was established. Suprabasal K16 staining was observed in the primary and reoccurrence SCC sections and strong K16 staining was seen in the metastatic lymph node section and all MET cell line cells (normal skin sections were, as expected, K16-negative).

Lastly, K17 expression was examined. Like K16, this was strongly positive in MET 1-4 cells and the lymph node section. In the reoccurring SCC section, K17 panepithelial and basal layer staining was observed (this was panepithelial and suprabasal in the primary SCC section). These results suggest some hyperproliferation.

Proby *et al*. (2000) also established the staining pattern of some simple epithelial keratins: K7, K8, K18 and K19. Despite pairing occurring between K7 and K18 or K19 and K8 with K18, different profiles were identified for all these keratins. K7, negative in normal skin and the primary SCC sections, displayed some heterogenous staining in the reoccurring section and the lymph node metastatic section. MET 1 and 2 cells were negative, whist a low number of MET 3 and 4 cells were positive. In the SCC reoccurring section, the SCC region was K18 positive, as were metastatic sections. MET 2 and 3 cells were also K18 positive. Some increase in limited K18 staining was observed in higher passage MET 1 and MET 4 cells compared to low passage cells. All sections (including normal skin) and MET cells were negative for K8 and K19. This is an interesting result as previous work had suggested that SCC cells are K8/K18 positive (Markey *et al*., 1991). Overall, the *in vivo* keratin profile is maintained *in vitro*.

Whilst Proby *et al*. (2000) did not specifically look for K15 expression in the MET cell lines, other publications have described their considerations of K15 expression in SCC cells. Recently, Abbas and Bhawan (2011) have described a study observing K19, K15 and nestin in SCC, basal cell carcinoma (BCC) and Merkel cell carcinoma (MCC) cells. Whilst K15 was observed in 30% of BCC cases, only one SCC case was K15 positive (5%) (all MCC cases were negative). In addition, SCC *in situ* K15 expression was observed in 53% of cases investigated, and only 3% of SCCs (well differentiated) (Abbas *et al*., 2011). These results suggest that K15 may be present in less differentiated SCC cells.

5.1.3.2: Actin

Actin, central to cell motility, invasion and metastasis, has previously been investigated in SCCs (for example, Kelley, Shahab and Weed, 2008). Actin polymerisation at the leading edge and the formation of lamellipodia, filopodia and podosomes (or invadopodia) are essential for tumour cell migration and invasion (Pollard and Borisy, 2003). Podosomes, the structures which allow cells to migrate through the ECM, are comprised of several proteins including actin, adhesion molecules, matrix degradation enzymes, membrane remodelling proteins, signalling proteins and actin regulatory proteins (Buccione, Orth and McNiven, 2004; Even-Ram and Yamada, 2005). The chemoattractants Wiskott-Aldrich syndrome protein (WASP) and Arp2/3 complex, for example, have previously been identified as being overexpressed in cancers and breast tumours (Wang *et al*., 2004; Sahai, 2005; Yamaguchi and Condeelis, 2007; Yamada *et al*., 2010). The WAVE-Arp2/3 complex for example is involved in the EGF pathway that promotes lamellipodia formation. Both N-WASP and cortactin are also components of podosomes. The actin-associated scaffold protein cortactin has recently been shown to be upregulated by expression of CRKII (CT10 regulator of kinase II) in aggressive oral SCC (Yamada *et al*., 2011). Also in aggressive oral SCC cells, Iwai *et al*. (2010) demonstrated that the rearrangement of actin (and redistribution of E-cadherin) was induced by cytoplasmic accumulation of β-catenin; EMT was also induced, enhancing the ability of SCC cells to invade and migrate.

Kelley, Shahab and Weed (2008) suggested that the amplification of chromosome segments 3q26-29, 8q23-24 and 11q13, as observed frequently in head and neck SCCs, contained actin regulatory regions (such as FAK, PI3-kinase and cortactin). This then promoted invasion (and metastases) in head and neck SCCs.

5.1.4: Differentiation Marker Expression in MET Cell Line Cells

5.1.4.1: Involucrin

Involucrin, as a marker of differentation, has been previously observed in human SCCs, and was oxygen-regulated (for example, Raleigh *et al*., 2000; Chou *et al*., 2004). This oxygen-regulation is most likely to be via the AP-1 sites in the promotor region of the involucrin gene (Crish *et al*., 2002; Chou *et al*., 2004). Chou *et al*. (2004) suggest that as involucrin is an early marker of terminal differentiation, hypoxia in tumours may arrest differentiation just short of the end stage. As expected, involucrin expression in poorly differentiated regions of SCC tumours has been demonstrated to be relatively low (Azuma *et al*., 2003; Chou *et al*., 2004). Commandeur *et al*. (2009) describe involucrin expression in SCC sections as prominent throughout the suprabasal layers, with intense staining of squamous nests.

Involucrin expression has also been observed in explant cultures. In normal human skin, involucrin is present in the upper layers of the epidermis; in explants however, involucrin is expressed as low as the stratum corneum (Commandeur *et al*., 2009). In primary SCC explants, the involucrin staining pattern was similar to that in primary SCC sections (i.e. all suprabasal layers were stained as well as staining in the squamous nests) (Commandeur *et al*., 2009).

Generally, involucrin expression is higher in normal cells compared to SCC cultures (Gasparoni *et al*., 2004). This again is thought to be due to changes in differentiation in SCC cultures compared to normal cell cultures. Rice, Rong and Chakravarty (1988) (and later Chou *et al*., 2004) however demonstrated an increase in involucrin expression in postconfluent SCC9 cells (a moderately differentiated cell line). Little involucrin staining was observed in the more poorly differentiated SCC4 cell line (Chou *et al*., 2004). In another partially differentiated SCC cell line (SCC12B2),

Commandeur *et al*. (2009) demonstrated that involucrin staining occurred throughout a 3D skin model. Also using SCC12B2 cells, Yang, Ng and Bikle (2003) demonstrated that calcium concentration had some effect on PKCα (and therefore involucrin expression). No similar effect was observed in SCC4 cells. Other SCC cell lines however are affected by extracellular calcium; Nakayama *et al*. (2005) demonstrated that filaggrin and involucrin expression could be induced by increasing the extracellular calcium concentration in two oral SCC cell lines (SAS and Ca9-22). IKKα (IκB kinase α), despite being recognised as a putative differentiation signal in normal keratinocytes, has also been shown to inhibit induction of involucrin (and filaggrin) expression by extracellular calcium in SCC cells (Nakayama, Ikebe and Shirasuna, 2005). Nakayama, Ikebe and Shirasuna (2005) suggest that this may be due to serine phosphorylation of IKKα by PKC in SCC cells. PMA (phorbol 12-myristate 13-acetate) was also shown to inhibit involucrin expression in SCC cells (Nakayama, Ikebe and Shirasuna, 2005); this is again unexpected as the opposite effect has been observed in normal keratinocytes (for example, Efimova and Erkert, 2000).

5.1.4.2: Filaggrin

The absence of the cornified envelope protein filaggrin in squamous cell carcinomas was first noted by Klein-Szanto *et al*. in 1984, who suggested that this absence could be used in the differential diagnosis of cutaneous tumours. This is in contrast to benign lesions of the oral mucosa, where normal filaggrin distribution is observed (Grosso *et al*., 1990). More recent studies have suggested that filaggrin is present in squamous cell carcinomas, but not lung squamous cell carcinomas (Miédougé *et al*., 2001). Cutaneous squamous cell carcinomas have also been shown to contain filaggrin-positive regions (for example, Akgül *et al*., 2011), and filaggrin-positive cells were identified in cultured SCC cells *in vitro* (Nakayama, Ikebe and Shirasuna, 2005).

Since filaggrin is a cornified envelope protein (and therefore a marker of late differentiation), it may be observed in the MET cell line cells used in this experiment. This will give an indication of how differentiated the cells of each MET cell line are.

5.1.5: Adhesion Marker Expression in MET Cell Line Cells

5.1.5.1: E-Cadherin

As previously described, calcium-dependent E-cadherin is associated with desmosomes and adherens junctions; these structures are important in maintaining tissue integrity. Proby *et al*. (2000) discuss the tendency of MET 1-4 cells to dissociate from each other (unlike normal keratinocytes), suggesting that cell-cell adhesion (and therefore E-cadherin expression) is likely to be different to that observed in normal keratinocytes. In 1999, Koseki *et al*. examined E-cadherin expression in SCC tumour samples. E-cadherin was reduced or absent in 70.9% of SCCs and 91.3% of lymph node metastasis. Koseki *et al*. (1999) suggest that E-cadherin is more frequently associated with well-differentiated SCCs, and may be useful as a metastatic marker. A previous study (Fuller *et al*., 1996) had also shown that of 16 SCCs examined, E-cadherin was absent in four samples and attenuated in another eight. Unlike Koseki *et al*. (1999), Lyakhovisky *et al*. (2004) suggest that E-cadherin expression does not correlate with tumour differentiation, however correlation was observed between E-cadherin (and β–catenin) and SCC morphology.

Alt-Holland *et al*. (2008) used 3D cultures to study E-cadherin in the SCC cell line HaCaT-II-4. The group found that individual, E-cadherin-deficient cells undergo transepithelial migration before becoming invasive.

5.1.5.2: Desmoplakin

Downregulation of desmosomal proteins is expected in metastasising cells as a reduction in cell adhesiveness occurs. However, this does not appear to always be the case; Kurzen *et al*. (2003) observed that although still highly regulated, only desmoglein 2 expression correlated with metastasis risk. In the same study, desmoplakin was observed in most SCC tumour cells, with the most intense staining observed in larger, more differentiated cells. Similar results were observed by Bosch *et al*. (2005), where more desmoplakin was observed in tumour stage IV tumours compared to stage I-III. A later study however reports a decrease in desmoplakin immunoreactivity in oral SCCs compared to normal epithelium (Narayana *et al*., 2010). Dysplasic tissues were observed to have disrupted desmoplakin localisation, with desmoplakin observed in the cytoplasm as opposed to the cell borders; the overall intensity of staining was also reduced. In oral SCC samples, desmoplakin was localised to the cell borders, but with lower protein expression than controls. Some SCC samples had no desmoplakin immunoreactivity.

As yet, there has been little published material referring to desmoplakin in MET cell line cells. As the above work on other SCC sections suggests however, desmoplakin is usually observed in SCC sections, suggesting that desmoplakin immunoreactivity should also be observed in the MET cell lines.

5.1.6: MET Cell Line Genetics

In a further study, MET 1-4 cells were shown to have chromosomal abnormalities, including loss of 3p, 8p, 5q, 17p and gain of 3q, 8q, 5p and 11q in most lines (Popp *et al*., 2000). A further amplified region is 17q24-25, as previously observed in the original tumour which had metastasised, is also observed in the cell lines. This also occurs with 10p and 20q gain, observed in all cell lines, was not observed in any of the original tumours (Popp *et al*., 2000). A similar study was carried out by Welkoborsky *et al*. in 2003, using human cell lines isolated from SCCs on the head and neck. The most frequent chromosomal anomalies in these lines were gains on 15q, 7p, 3q, 5p, 11q and 17q; losses were noted on 3p, 18q, 19p and 7q. Similar losses and gains (although not identical) were observed in primary lung SCCs by Boelens *et al*. (2009). This group also noted that gains at 7q, 8p and 10q only occurred in SCCs with lymph node metastases; none of these were noted by Popp *et al*. (2000) despite MET 4 cells being isolated from lymph node metastases.

As previously mentioned, Kelley, Shahab and Weed (2008) noted gains on 3q26-29, 8q23-24 and 11q13, shown to contain actin regulatory regions. This includes cortactin, a component of podosomes and therefore important in SCC cell invasion. Furthermore, McCaughan *et al*. (2010) observed 3q amplification in squamous lung cancer, noting that two known oncogenes, *SOX2* and *PIK3CA*, are coded for in this region (*SOX2* was demonstrated to be associated with the malignant progression of squamous lung carcinomas). The oncogene *c-Myc* (8q24) has also been identified in melanomas, and amplification is likely to be associated with advanced cutaneous melanomas (Kraehn *et al*., 2001). *L-Myc* (related to the *c-Myc* proto-oncogene) has also been identified on another region amplified in MET cells – the 5p region. Normal skin has no *L-Myc* expression, whereas it is expressed in MCCs (Paulson *et al*., 2009). Gain of 8q has also been reported in lung SCCs (Boelens *et al*., 2009). Overexpression of this region has been reported in other epithelial cancers, and target

genes of metastatic potential have been identified, including *CDH17*, *SPAG1*, *HAS2*, *MTBP*, and *RAD21* (Goeze *et al*., 2002; Oue *et al*., 2004; Thomassen, Tan and Kruse, 2008). Boelens *et al*. (2009) also suggest that a positive regulator of angiogenesis, *ANGPT1*, coded for in this region, may have a role in the development of distant metastases.

Although MET cells are not isolated from an individual with a head and neck SCC, head and neck SCCs are also characterised by amplification of the 11q region (Parikh *et al*., 2007). 11q amplification has also been reported in oesophageal SCC (Hu *et al*., 2009) and oral SCC (Reshmi *et al*., 2007). The breakage-fusion-bridge cycle mechanism which results in the amplification however means that some genes are lost; Parikh *et al*. (2007) suggest that this includes three genes (*MRE11A*, *ATM* and *H2AFX*) important in the DNA damage response pathway. Parikh *et al*. (2007) also demonstrated that SCC cells with 11q13 loss had a defective DNA damage response. Unlike MET cell lines however, several SCCs are reported to have losses at 11q; for example, lung SCCs (Rydzanicz *et al*., 2008) and cervical SCCs (Huang *et al*., 2007; Wilting *et al*., 2008).

The short arm of chromosome 3 (3p), reported as lost in MET cell lines (Popp *et al*., 2000) includes tumour suppressor genes which have a role in tumourigenesis (Li *et al*., 1994). Hogg *et al*. (2002) found that 3p loss occurs at all stages of head and neck SCC, suggesting that 3p loss is an early event in the development of SCC. More recent studies have highlighted several tumour suppressor genes involved, for example P300/CBP-associated factor (*PCAF*) (Zhu *et al*., 2009) and fragile histidine triad (*FHIT*) (Purdie *et al*., 2009). Since 3p deletion occurs so frequently in (head and neck) SCCs, it has been suggested that, if tests demonstrate 3p deletion, active intervention (such as chemoprevention and regular check-ups) are required (Abou-Elhamd *et al*., 2008).

Tumour suppressor genes have also been found at 8p, which Popp *et al*. (2000) also reported as lost in MET cells; Qin *et al*. (2008) reported 8p deletions in oesophageal SCCs whilst Ono *et al*. (2003) and Zhou *et al*. (2005) also reported this deletion in oral SCCs. Ono *et al*. (2003) observed that the tumour suppressor gene *FEZ1* had absent or reduced mRNA expression in the oral SCCs examined. Head and neck SCCs have also been shown to be affected by 8p loss (Braakhuis *et al*., 2004). Another tumour suppressor gene, mitochondrial tumor suppressor gene 1 (*MTUS1*) (located at approximately 8p21.3-22) has been shown to be downregulated in head and neck SCCs (Ye *et al*., 2007). Examination of chromosomal abnormalities in lung SCCs suggested that 8p23 loss was associated with lymph node metastases (Boelens *et al*., 2009).

Loss of 5q, reported in MET cells (Popp *et al*., 2000) has also been reported in other SCCs, including oesophageal (Li *et al*., 2004; Su *et al*., 2006), head and neck (Rybicki *et al*., 2003; De Schutter *et al*., 2006) and anus (Gervaz, Hirschel and Morel, 2006). Boelens *et al*. (2009) report that approximately 440 genes at 5q14.2-23.3 and 5q31.1-35.2 which are lost in 41-68% of SCCs.

The most important gene lost from 17p loss in MET cells and other SCCs is *TP53*; there are also other tumour suppressor genes in this region (Götte *et al*., 2001; Allegra *et al*., 2009). This has been observed in oesophageal SCCs (Hu *et al*., 2009) and head and neck SCCs (for example, De Schutter *et al*., 2006; Ye *et al*., 2007). In its role as a tumour suppressor, p53 works in several ways, including inducing growth arrest, activating DNA repair and initiation of apoptosis (Borrás, Gómez-Cabrera and Viña, 2011). Mutations or deletions of *TP53* are present in approximately 50% of tumours (Hollstein *et al*., 1991; Vermeij *et al*., 2011).

5.1.7: Migration in SCC Cells

The ability and likelihood of a tumour to become invasive relies, in part, to the cells' ability to migrate. SCC cells become motile and move through the basement membrane into the dermis, resulting in an invasive SCC. The ability of these cells to migrate depends to a great extent on the rearrangement and phosphorylation of the actin cytoskeleton (see section 5.1.3.2.). Recently, Yamashiro *et al*. (2010) have demonstrated that ectopic K8/18 co-expression and filament formation is associated with increased migration and invasion of some cancer cells (Hendrix *et al*., 1992; Chu *et al*., 1997), including LY cells (cutaneous SCC cells). The same effect was not observed with Pam212 cells.

Likewise, cadherins have been shown to have a role. In SCC cell line A431, silencing of T-cadherin resulted in elongated, disorganised cells with increased motility. This included an increase in invasive potential. *In vivo*, SCCs classified as poorly-to-moderately differentiated express higher levels of T-cadherin compared to well differentiated SCCs (Pfaff *et al*., 2010). Loss of E-cadherin is also associated with increased motility in an SCC cell line (HaCaT-II-4) (Alt-Holland *et al*., 2008). An increase in metastatic potential is observed in SCCs which lack functioning Type VII collagen (ColVII); mutations in the ColVII gene (*COL7A1*) (resulting in recessive dystrophic epidermolysis bullosa) increase an affected individual's susceptibility to aggressive SCCs (Martins *et al*., 2009).

Although little work on migration of MET cells has been carried out, the previous publications which refer to other SCCs and cell lines suggest that actin rearrangement, a change in keratin expression profile and changes in cell-cell adhesions would indicate that MET cells are more motile than normal keratinocytes.

5.1.8: Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is a series of events that convert epithelial cells to a more motile phenotype (Zeisberg and Neilson, 2009), through alteration of cell-cell and cell-ECM junctions and cytoskeletal reorganisation (Radisky, 2005). This process occurs under normal conditions, mostly in embryogenesis (such as in gastrulation and neural crest formation in amniotes) and is also a mechanism utilised by cancerous cells to disperse from the primary tumour (Acloque *et al*., 2009), via the Wnt and TGFβ signaling pathways. Snail and Slug transcription factors are also influential (Hay, 2005). As the above suggests, EMT may occur in SCCs, as changes occur in keratin expression and cell-cell adhesions are lost. In order to elucidate whether MET cells were capable of undergoing EMT *in vitro*, organotypic cultures were produced and immunofluorescence carried out.

5.1.9: Three-dimensional Culture of SCC Cells

Squamous cell carcinoma cell lines (MET cells) have been previously studied using the organotypic method. In the original paper describing these cells, Proby *et al*. (2000) observed 3D growth on de-epidermised dermis (DED), although no accounts of invasion were recorded. The original tumours from which the MET cell lines were derived were described as 'deeply invasive' so it is possible that these cells may act similarly *in vitro*. More recent work studying SCC cells have used the 3D culture method to demonstrate invasiveness; for example, Brusevold *et al*., (2010) demonstrated that hypoxia could induce invasion in oral SCC 3D culture, whilst Grugan *et al*. (2010) have shown that fibroblast-secreted hepatocyte growth factor has a role in oesophagal SCC invasiveness.

When cultured on de-epidermised dermis (DED), Proby *et al*. (2000) did however demonstrate that MET cell line cells did not stratify. When cultured in this way, MET

1-4 cells were disorganised and appeared abnormal. A previous study (Gioeli *et al*., 1997), using another human SCC cell line (A388.6TG.c2) produced organotypic cultures as a method of investigating tumour suppression. Again, the 3D cultures appeared abnormal, but some stratification did occur.

5.2: Materials and Methods

The materials and methods used in investigation of the effect of K15 on differentiation and proliferation are described in Chapter 2. No changes or variations were made to the methods described.

5.3: Results

5.3.1: Experimental Approach

The aims of this chapter were to observe the normal expression and appearance of K15 in the squamous carcinoma cell lines MET 1, 2 and 4. The original paper describing these cells (Proby *et al*., 2000) did not describe K15 expression in these cells. Once it had been established that K15 was expressed in MET cell line cells, the effects of K15 ablation on adhesion, spreading and migration was assessed. These results could then be compared with HaCaT cells.

5.3.2: Characterisation of K15 in MET 1, 2 and 4 Cells

As previously carried out in HaCaT cells, double staining of K15 and K14 was carried out in all three MET cell lines used in this study (*Figure 5.1*). The K14 observed in all three MET cell lines suggest a poorly organised IF network, with little filamentous staining observed. Some non-specific nuclear staining can also be observed in MET 2 cells (*Figure 5.1* E). Although Proby *et al*. (2000) stated that MET 1, 2 and 4 cells were K14-positive, no immunofluorescence images were included in the paper to indicate the arrangement or localisation of K14.

The appearance of K15 is also different in the MET cell line cells compared to HaCaT cells. K15 does not appear filamentous in any of the MET cell lines (*Figure 5.1* B, F, J), instead having a more granular appearance. This suggests that K15, although present in MET 1, 2 and 4 cells, is not filamentous. This is particularly observed in MET 2 cells (*Figure 5.1* F).

The DAPI staining of the MET cell line cells suggest that the nuclei of the MET cells are also abnormal. Nuclear abnormalies (such as polymorphism) has previously been described in cancer cells and more specifically in squamous cell carcinomas (Kurokawa *et al*., 2005).

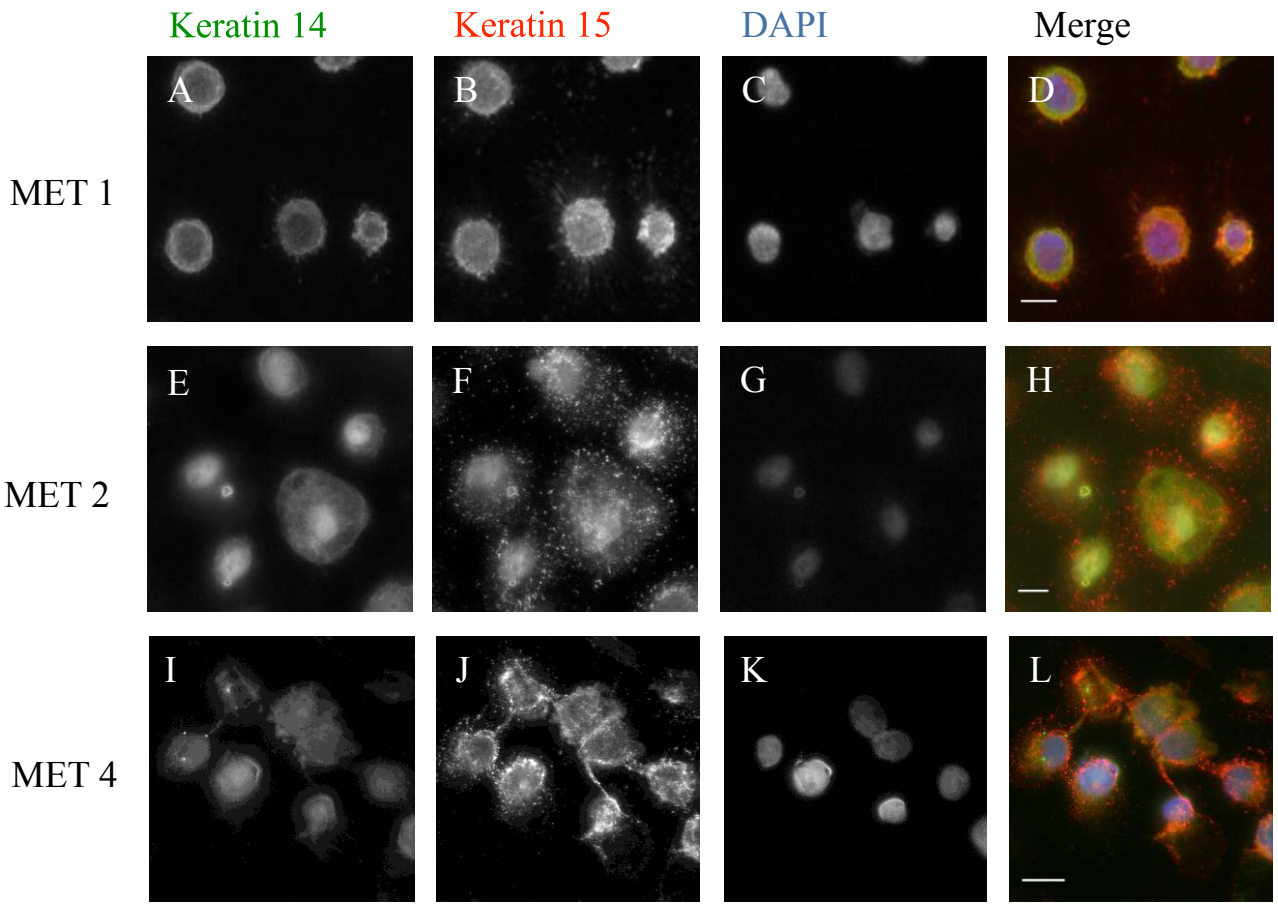


Figure 5.1: K14 (green), K15 (red) and nuclear DAPI immunofluorescence in MET cell line cells.

Single plane confocal images of immunofluorescence showing both K14 and K15 in MET 1 cells (A, B, C, D), MET 2 cells (E, F, G, H) and MET 4 cells (I, J, K, L) (scale bar = 10μm).

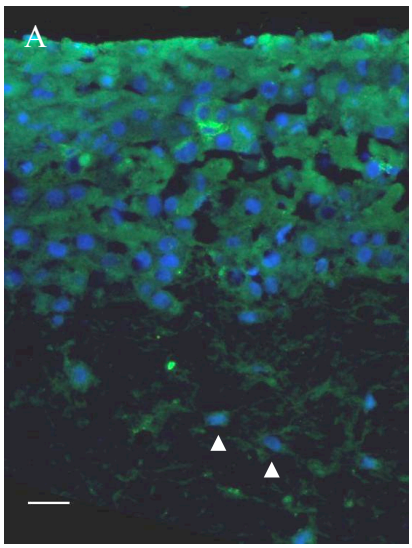
The immunofluorescence shows that cells can vary in the amount of K14 expressed. K14 appearance is not filamenous, as in HaCaT cells. The arrangement of K14 is more concentrated around the nucleus, and does not appear throughout the cytoplasm to the cell edge.

K15, although present, has not formed filaments in the MET cells, instead appearing granular, particularly in MET 2 cells (F). K15 is present through the cytoplasm, up to the edges of the cells.

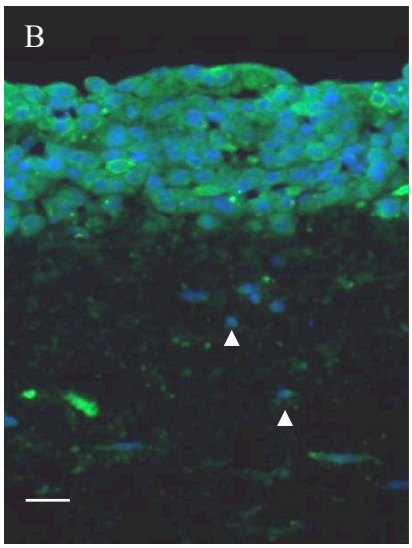
In addition, 14 day organotypic cell cultures were also created for each of the MET cell lines. Although Proby *et al*. (2000) had difficulty culturing MET cells on DED scaffolds, it has been possible to culture MET cells on collagen gels (*Figure 5.2*). All three MET cell lines were cultured for 14 days at the air-liquid interface and some stratification was observed; this was to the greatest extent in MET 1 cells and to a lesser extent in MET 4 cells. MET 1 cells also demonstrated a slight change in cell morphology between those cells closest to the collagen gel and those at the surface; flatter cells are observed closer to the surface of the culture (*Figure 5.2*, A). Some similar morphological changes were observed in MET 2 cells (*Figure 5.2*, B), although no changes were seen in MET 4 cells (*Figure 5.2*, C).

K15, expressed in MET 1, 2 and 4 cells in monolayer culture, was also expressed throughout in 3D culture (*Figure 5.2*, A, B, C). In MET 1 and 2 cells (*Figure 5.2*, A, B), K15 expression appears similar in all cells throughout all layers of the culture. There are cells throughout the MET 4 cell culture however (*Figure 5.2*, C) that appear brighter, indicating increased K15 expression. This does not occur at any specific layer (as in normal skin sections), suggesting that the expression pattern here is due to the heterogenous K15 expression of MET 4 cells and is not occurring as a consequence of 3D culture. This could also be a consequence of the aggregation of K15 observed in monolayer cultures (*Figure 5.1*, J).

Further examination of MET 1 and MET 2 cell organotypic cultures demonstrated that cells had migrated into the collagen cells (no similar observation was made in MET 4 cells). This was demonstrated by DAPI staining; these cells were K15-negative (*Figure 5.2*, A, B; arrowheads). This suggested that these cells had become invasive, and possible EMT had occurred.



collagen gel



collagen gel

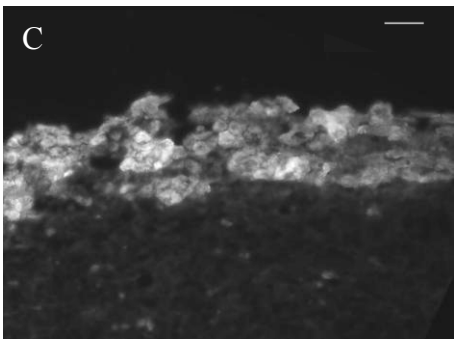


Figure 5.2: Organotypic MET cell line cultures grown on collagen gels, raised to air-liquid interface for 14 days.
K15 immunofluorescence in MET 1 (A), MET 2 (B) and MET 4 (C) cells. Sections A and B also show the nuclear marker DAPI (scale bar = 20µm). In MET 1 cells (A), K15 expression appears uniform throughout the stratified layers. Cell morphology changes in a similar fashion to HaCaT cells. Similarly, uniform expression of K15 is noted throughout the stratified MET 2 layers (B), although fewer changes in morphology can be seen. In MET 4 cells, K15 expression appears disorganised throughout the cell layers, with regions of low and high brightness observed. Arrowheads in A and B indicate where K15-negative cells have invaded the collagen gels. No similar observations were made in MET 4 cells (C).

5.3.3: Differentiation of MET 1, 2 and 4 Cells

The results of the organotypic culture experiments suggested that MET 1 and 2 cells were still capable of some differentiation, resulting in morphological changes toward the surface of the 3D culture. In order to investigate the differentiation potential of MET 1, 2 and 4 cells, cells were exposed to high and low calcium media for varying lengths of time.

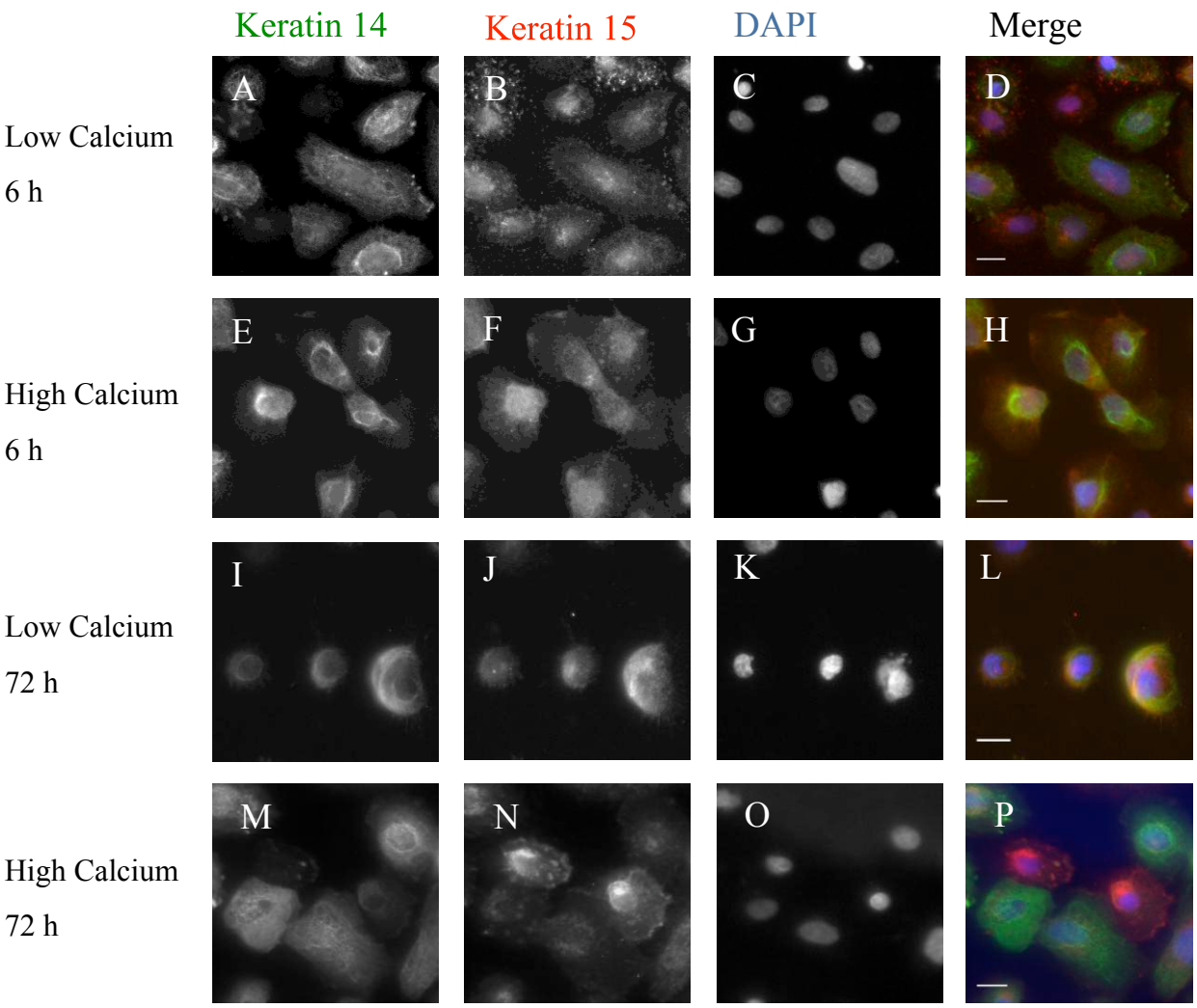


Figure 5.3: K14 (green), K15 (red) and nuclear DAPI immunofluorescence in MET 1 cells following exposure to different levels of extracellular calcium.

Single plane confocal images of immunofluorescence showing both K14 and K15 in MET 1 cells following exposure to low calcium media for 6 h (A, B, C, D), high calcium media for 6 h (E, F, G, H), low calcium media for 72 h (I, J, K, L) and high calcium media for 72 h (M, N, O, P) (scale bar = 10μm).

K14 and K15 expression varies in MET 1 cells, particularly with exposure to high calcium media. K15 (red) can be observed at the cell edges throughout, whereas K14 expression (green) is concentrated around the nucleus; this spreads further into the cytoplasm after 72 h exposure to high calcium media, although does not reach the cell edge (as K15 does).

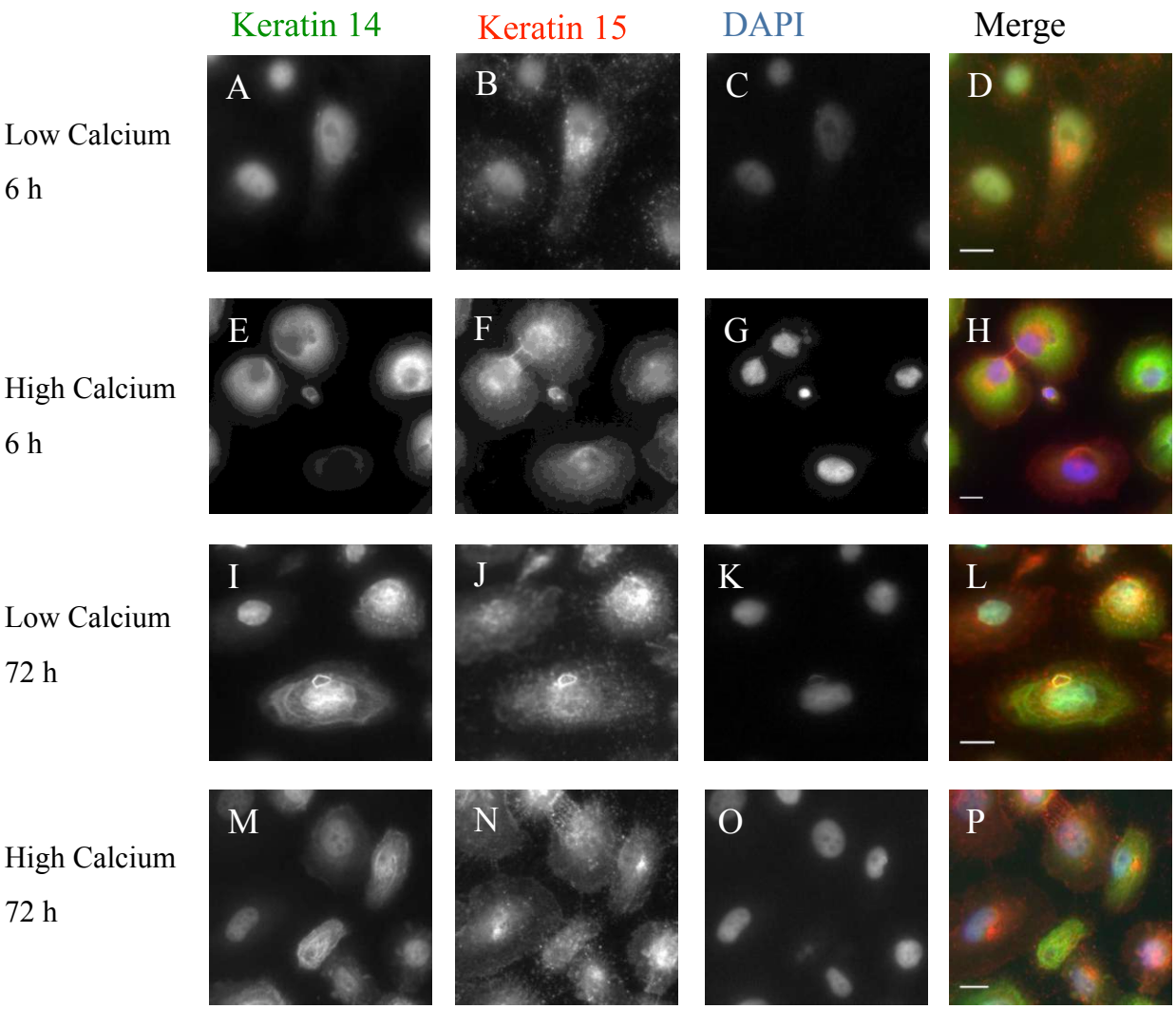


Figure 5.4: K14 (green), K15 (red) and nuclear DAPI immunofluorescence in MET 2 cells following exposure to different levels of extracellular calcium.

Single plane confocal images of immunofluorescence showing both K14 and K15 in MET 2 cells following exposure to low calcium media for 6 h (A, B, C, D), high calcium media for 6 h (E, F, G, H), low calcium media for 72 h (I, J, K, L) and high calcium media for 72 h (M, N, O, P) (scale bar = 10μm).

Immunofluorescence indicates that both K14 and K15 expression changes little in response to changes in extracellular calcium.

Some heterogeneity can be observed in the expression of K14, with some cells expressing higher levels than others. The merge panel also indicates that K14 expression is not observed to the edge of the cell, whereas K15 is.

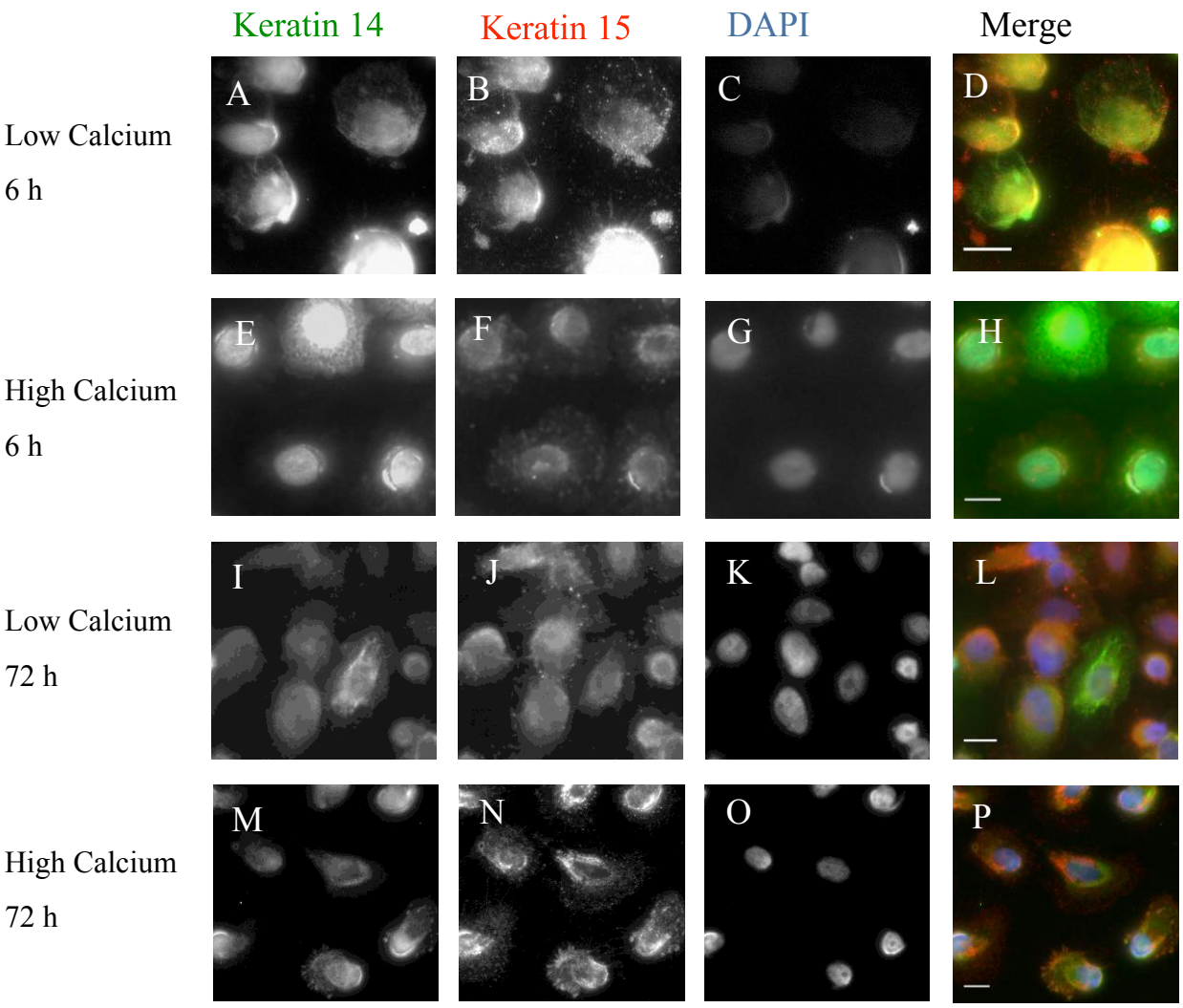


Figure 5.5: K14 (green), K15 (red) and nuclear DAPI immunofluorescence in MET 4 cells following exposure to different levels of extracellular calcium.

Single plane confocal images of immunofluorescence showing both K14 and K15 in MET 4 cells following exposure to low calcium media for 6 h (A, B, C, D), high calcium media for 6 h (E, F, G, H), low calcium media for 72 h (I, J, K, L) and high calcium media for 72 h (M, N, O, P) (scale bar = 10μm).

As observed with other MET cell lines, there is little change between K15 and K14 expression following culture in different concentrations of extracellular calcium.

There is some variation in K14 expression between MET 4 cells; K14 is also lacking at the cell edge (unlike K15).

5.3.3.1: K14 and K15 Expression in MET Cell Lines Exposed to High and Low Extracellular Calcium Concentrations

As in *Figure 5.1*, the K14 network again appears poorly defined in MET 1 cells following exposure to varying levels of extracellular calcium. This suggests that the irregular IF network observed is not as a result of changes in extracellular calcium concentration. Some heterogeneity can be observed in K14 expression in MET 1 cells after exposure to high calcium for 72 h (*Figure 5.3* M, P), where some cells express low levels of K14. Proby *et al*. (2000) observed keratin expression in their paper describing the four MET cell lines isolated, and no heterogeneity of K14 expression is described. Since Proby *et al*. (2000) used immunoblotting to establish the presence of keratins in the MET cell line cells, no comment on arrangement could be made or inferred from the results. K15 expression however appears to remain around the nucleus more than towards the edge of the cells; it is possible that the K15 network has collapsed into aggregates around the nucleus (for example, *Figure 5.3* N). This said, K15 staining can be observed throughout the cytoplasm to the edge of the cells, whereas this is not always observed in K14. An example of this can be seen at 6 h after exposure to high calcium, where K15 can be observed at the cell edges, unlike K14 (*Figure 5.4*, E, F).

Similarly, in MET 2 cells, K15 can be observed throughout the cytoplasm up to the cell edges after 6 h in high calcium media, whereas K14 cannot (*Figure 5.4, D*). The keratin staining in MET 2 cells following exposure to high and low calcium media again does not appear as expected for IF filaments, with K15 in particular forming aggregates (for example *Figure 5.4* N). Heterogeneity in K14 expression can also be observed; this can be seen in merge panels highlighting K15 staining in the absence of K14 staining (*Figure 5.3* H, L, P), where K15-positive cells can be observed with little K14 staining.

In MET 4 cells, K14 is not present at the cell edges in cells cultured in either high or low calcium (*Figure 5.5* A, E, I M). K14 and K15 staining in these cells again suggest that the normal IF network is not in tact; this is particularly the case in MET 4 cells exposed to high calcium media for 6 h (*Figure 5.5* E, F). DAPI staining after 6 h exposure to low calcium media appears abnormal, which is likely to be an error during the staining process.

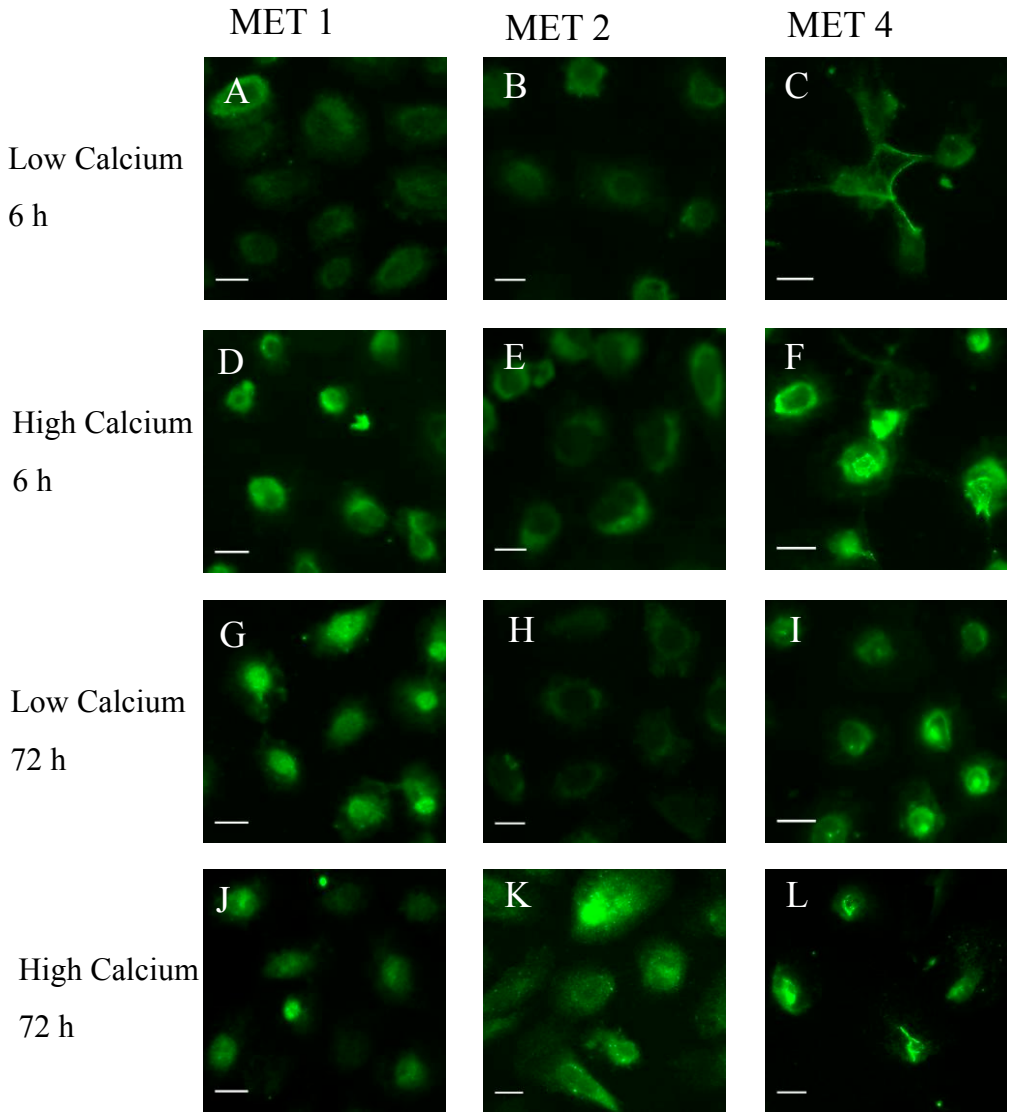


Figure 5.6: Involucrin expression in MET cell line cells following exposure to different levels of extracellular calcium.

Single plane confocal images of immunofluorescence showing involucrin expression in MET cell line cells (scale bar = 10μm).

Expression in MET 1 and 4 cells is similar, with low levels observed at 6h exposure to low calcium, and increased levels observed on exposure to high calcium. Levels are expected to be high following 72 h exposure to high levels of extracellular calcium, although as cells differentiate involucrin be begin cross-linking other proteins, making it unavailable to immunofluorescence antibodies for staining. Staining intensity of involucrin in MET 2 cells is slightly lower than in MET 1 and 4 cells, although is still increased when cells are cultured in high calcium media.

5.3.3.2: Involucrin Expression in MET Cell Lines Exposed to High and Low Extracellular Calcium Concentrations

It was considered useful to use immunofluorescence to establish whether exposure to varying levels of extracellular calcium would affect involucrin expression, since this had been established in HaCaT cells.

In MET 1 cells, low levels of involucrin can be observed after 6 h exposure to low levels of calcium; this is as expected, as low calcium would not induce differentiation. Involucrin levels are raised after 6 h in high calcium media, with expression concentrated around the nucleus. Lower levels of involucrin can be observed after 72 h in high calcium media compared to 6 h; this could be because the involucrin has become cross-linked, preventing the protein from being available for immunofluorescence (a similar effect had been observed in K15 ablated HaCaT cells). Levels of involucrin are still relatively high following 72 h exposure to low calcium media; this could indicate that some spontaneous differentiation is occurring in MET 1 cells, despite the presence of K15. This suggests that the K15 expression in MET 1 cells may not correspond with a degree of differentiation. Some involucrin staining appears nuclear in the MET 1 cells (for example, *Figure 5.6* D), suggesting that some cross-reaction of the antibody may be occurring.

Lower levels of involucrin can be observed in MET 2 cells compared to MET 1 and MET 4 cells (*Figure 5.6*, B, E, H, K). Involucrin levels of cells cultured in low calcium media are low, suggesting that no differentiation is occurring. Unlike MET 1 cells, this indicates that these cells are less likely to spontaneously differentiate. Relatively low levels of involucrin can be observed after only 6 h in high calcium media, whereas more involucrin can be observed after 72 h. This suggests that longer exposure to high levels of extracellular calcium induces some differentiation in MET 2 cells.

In MET 4 cells, involucrin is expressed at varying levels following exposure to high or low calcium media (*Figure 5.6*, C, F, I, L). After 6 h in low calcium media, low levels of involucrin are observed (*Figure 5.6*, C); this is as expected, as involucrin is a marker of differentiation, which is not induced by low levels of extracellular calcium. At 72 h in low calcium media, an increase in involucrin can be observed, although levels have not increased to those seen when MET 4 cells are exposed to high calcium media (*Figure 5.6*, I). After 6 h exposure to high calcium media, there is an increase in involucrin expression. Involucrin is expressed throughout the cell, although more intense staining can be observed around the nucleus of some cells. High levels of involucrin can also be observed after 72 h in high calcium media. Again, this is mostly focused around the nucleus. This would indicate that exposure to high levels of extracellular calcium does induce differentiation in MET 4 cells. The slight reduction in involucrin observed after 72 h in high calcium media is possible due to the cross-linking of involucrin, rendering it unavailable for staining. This is similar to the involucrin expression observed in MET 1 cells. These results suggest that despite the disorganised arrangement of MET 4 cells in organotypic culture, they are capable of differentiation.

5.3.4: K15 Ablation of MET 1, 2 and 4 Cells

5.3.4.1: Cytoskeletal Protein Expression in K15 Ablated Cells

K14 expression in MET cell line cells has previously been shown to vary between cells. Following K15 siRNA transfection, K14 expression was again observed using immunofluorescence. In MET 1, 2 and 4 cells, K14 expression appears to vary little between control siRNA transfected and K15 siRNA transfected cells (*Figure 5.7*).

As actin expression changed with K15 ablation in HaCaT cells, actin expression was observed in MET cell line cells. No changes can be observed in actin arrangement in K15 siRNA transfected cells compared to control siRNA transfected cells (*Figure 5.8*). Actin fibres can be seen throughout the cell, typically reaching the edge of the cells (and some filopodia-like actin protrusions can be seen). This suggests that K15 ablation has no effect on actin arrangement in MET cell line cells.

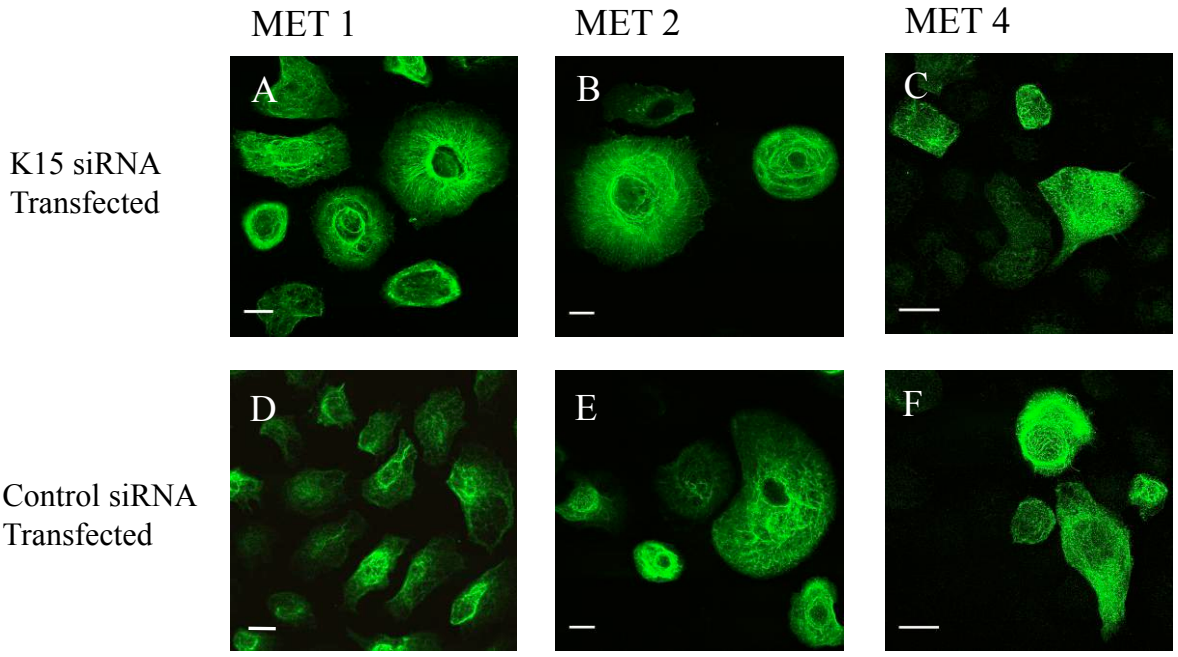


Figure 5.7: K14 expression in MET cell line cells 48 h post-transfection.
Single plane confocal images of immunofluorescence showing both K14 in control siRNA transfected (D, E, F) and K15 siRNA transfected cells (A, B, C) (scale bar = 10μm).
As previously observed, K14 expression is heterogeneous. K14 expression in MET 1, 2 and 4 cells is similar in control siRNA transfected and K15 knock-down cells.

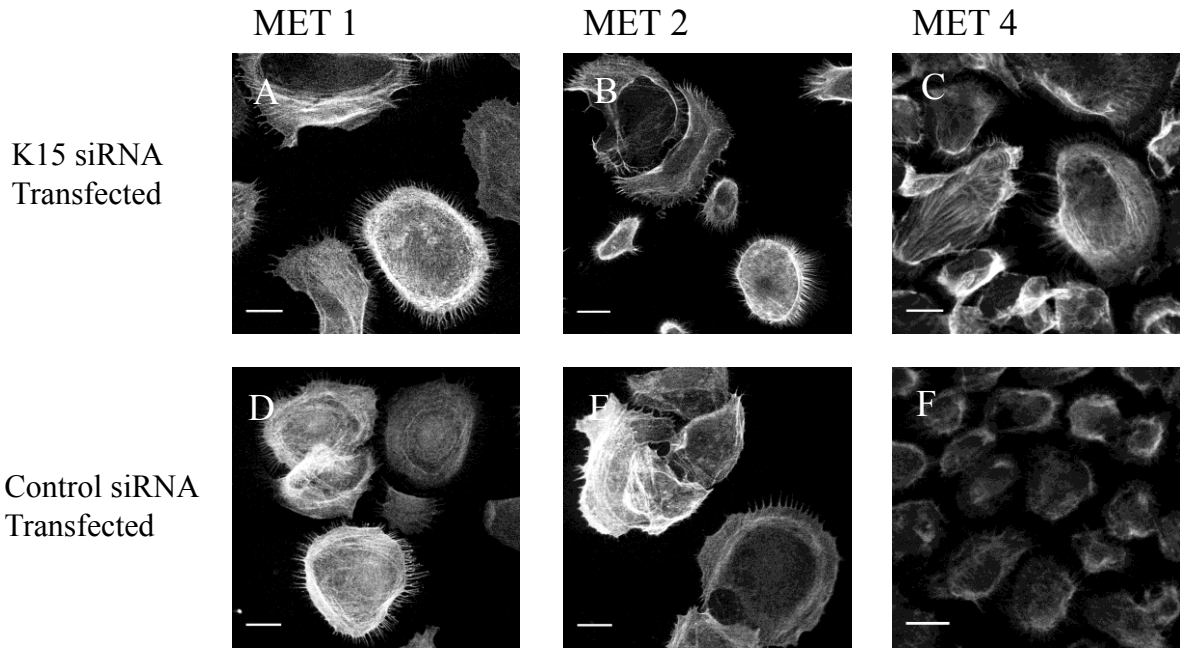


Figure 5.8: Actin expression in MET cell line cells 48 h post-transfection.
Single plane confocal images of immunofluorescence showing actin in control siRNA transfected (D, E, F) and K15 siRNA transfected (A, B, C) cells (scale bar = 10μm).
Actin expression appears normal in all three cell lines, with expression throughout the cells. Some filopodia-like protrusions can be observed, concurrent with the increased likelihood of cell dissociation in MET cell line cells.

5.3.4.2: Cell-Cell Adhesion Markers in K15 Ablated Cells

Desmoplakin staining (data not shown) suggested that no desmosomes formed between any cells, in either control siRNA transfected and K15 siRNA transfected MET cell line cells. This is possibly due to the low cell density; it is possible that MET cell line cells do produce desmosomes at increased cell densities.

Control siRNA transfected MET 1, 2 and 4 cells demonstrate some E-cadherin-positive cell-cell junctions, despite cells being somewhat dissociated (*Figure 5.9*, D, E, F). Cells were more dissociated in K15 siRNA transfected MET 1 and MET 4 cells, where few E-cadherin-positive cell-cell junctions can be observed due to the low cell density (*Figure 5.9*, A, C). However, where cell-cell contact has been established, E-cadherin-positive adherens junctions do form. The cell-cell adhesions in K15 siRNA transfected MET 2 cells are comparable to control siRNA transfected cells (*Figure 5.9*, B). Fuller *et al*. (1996) and more recently Canavese *et al*. (2007) have suggested that lack of E-cadherin correlates with invasive potential. MET 1 and MET 2 control siRNA transfected and K15 siRNA transfected cells all produce E-cadherin-positive adherens junctions, suggesting that their invasive potential is limited. Control siRNA transfected MET 4 cells appear to have more adherens junction formation compared to K15 siRNA transfected cells, suggesting that K15 ablated MET 4 cells have an increased invasion potential. However, the low cell density may reduce the number of adherens junctions in K15 siRNA transfected cells; increased cell densities may allow more cell-cell contact and increased opportunity to produce adherens junctions.

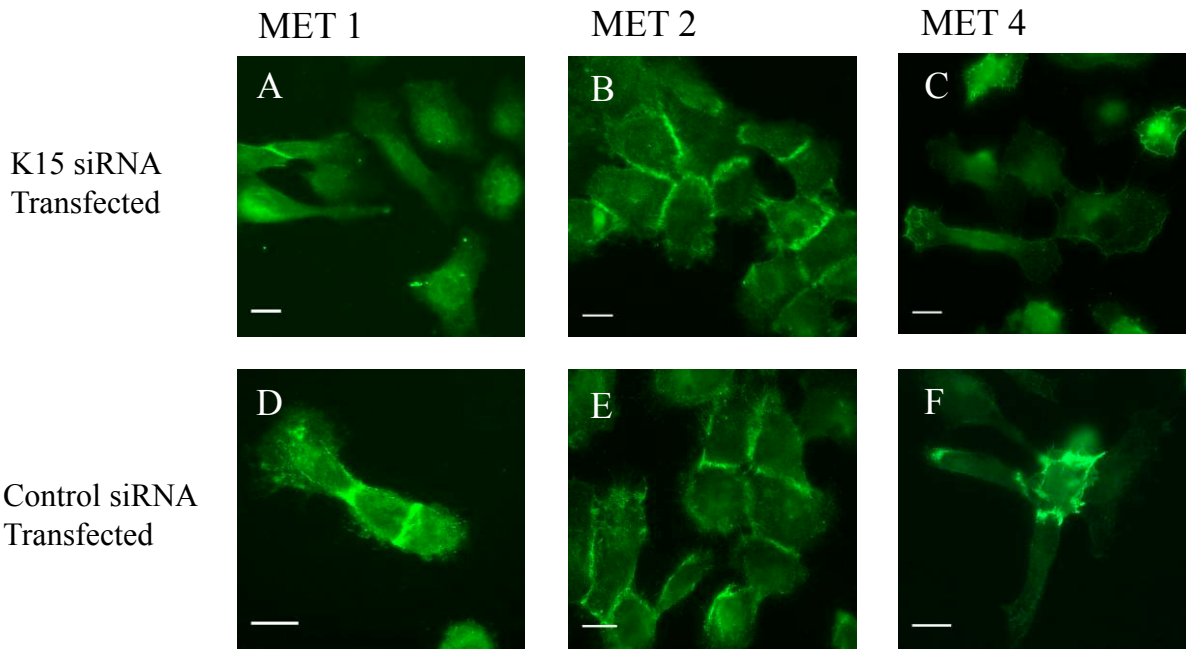


Figure 5.9: E-cadherin expression in MET cell line cells 48 h post-transfection.
Single plane confocal images of immunofluorescence showing E-cadherin in control siRNA transfected (D, E, F) and K15 siRNA transfected cells (A, B, C) (scale bar = 10μm).
Control siRNA transfected cells of all MET cell lines have some E-cadherin-positive cell-cell junctions. A similar staining pattern is observed in K15 siRNA transfected MET 2 cells, suggesting that K15 ablation does not affect formation of cell-cell junctions. Fewer junctions are identified in MET 1 and 4 K15 siRNA transfected cells, possibly due to increased cell dissociation.

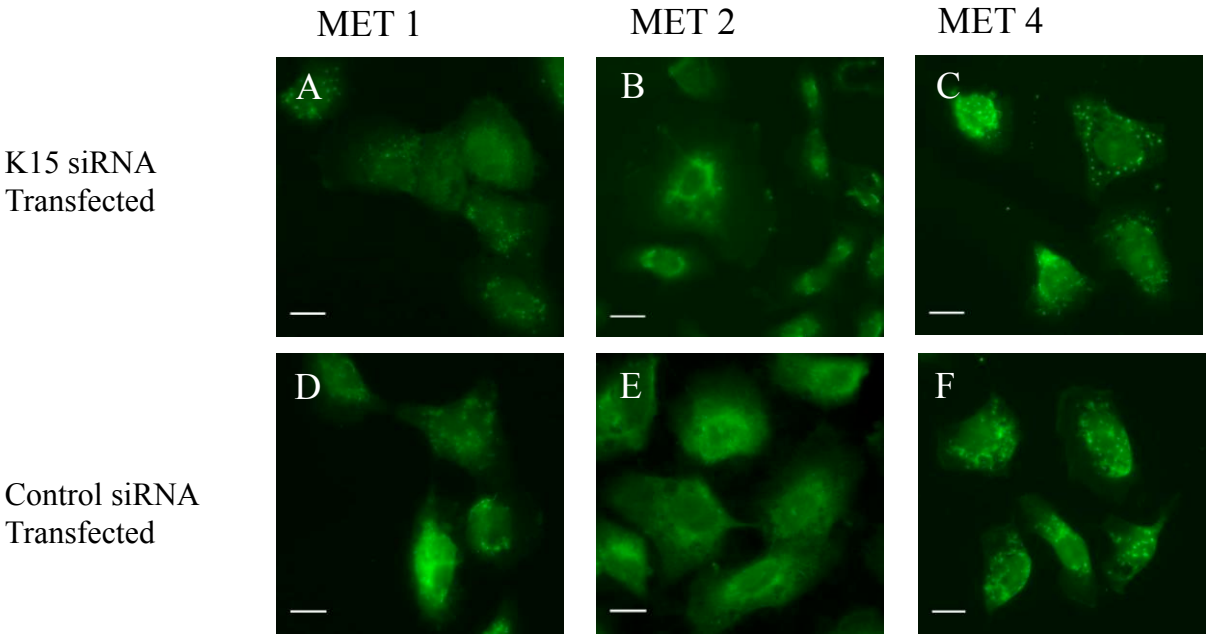


Figure 5.10: Filaggrin expression in MET cell line cells 48 h post-transfection.
Single plane confocal images of immunofluorescence showing filaggrin in control siRNA transfected (D, E, F) and K15 siRNA transfected cells (A, B, C) (scale bar = 10μm).
All cells appear to have a similar level of filaggrin present, in both control siRNA transfected and K15 siRNA transfected cells. Filaggrin appears around the nucleus in the cytoplasm, and in some cells has a granular appearance.

5.3.4.3: Filaggrin Expression in K15 Ablated Cells

In monolayer cell culture, filaggrin expression in MET cell line cells was assessed. The calcium switch experiment had already established that all three MET cell lines used in this study were capable of differentiation. Filaggrin expression, assessed following K15 ablation, would demonstrate whether K15 ablated cells could differentiate to the extent that filaggrin is produced above levels in undifferentiated cells. Expression levels appear similar in both K15 ablated cells (*Figure 5.10*, A-C) and control siRNA transfected cells (*Figure 5.10*, D-E), suggesting that K15 expression does not affect filaggrin expression in MET cells. In all cells, filaggrin appears localised around to the nucleus in the cytoplasm. In addition, filaggrin in MET 4 control siRNA and K15 siRNA transfected cells appears granular (*Figure 5.10*, C, F). These results indicate that K15 ablation does not initiate differentiation of MET cell line cells to an extent where filaggrin is upregulated.

Table 5.1: Charts of MET 1 and MET 4 circularity, calculated using the 'form factor' formula (1=perfect circle).

	MET 1 Cells	
Minutes	K15 siRNA Transfected	Control siRNA Transfected
10	0.75	0.71
20	0.57	0.61
120	0.49	0.43

	MET 4 Cells	
Minutes	K15 siRNA Transfected	Control siRNA Transfected
40	0.59	0.62
120	0.58	0.63

Like HaCaT cells, MET 1 cells (both K15 knock-down and control siRNA transfected) decrease in circularlity during spreading, although there is little difference between K15 knock-down cells or control cells. MET 4 cell circularity changes little between 40 and 120 minutes post-seeding, and there is also no significant difference between K15 knock-down cells and control siRNA transfected cells.

5.3.4.4: Cell Circularity of K15 Ablated MET Cell Line Cells

Although filaggrin immunofluorescence suggested that K15 ablation did not induce MET 1, 2 or 4 cells to differentiate, another indicator of differentiation (cell circularity) was tested. Since MET 1 cells appeared to have the most differentiation potential (as demonstrated by the change in morphology in organotypic cultures) and MET 4 the least, measurements of these cell lines were used to calculate circularity (Table 5.1). At 120 minutes post-seeding, there is no significant difference between K15 siRNA transfected and control siRNA transfected MET 1 cells. Likewise, no significant difference was noted between control siRNA and K15 siRNA transfected MET 4 cells 120 minutes post seeding. This suggests that cells were no more circular prior to K15 ablation than following it, which correlates with the unchanged actin distribution previously discussed. Using Student's t-test, the difference between MET 1 and MET 4 control siRNA transfected cell circularities were found to be significantly different ($p<0.05$). This would suggest that the MET 4 cells are less differentiated than MET 1 cells.

5.3.5: Adhesion of K15 Ablated MET 1, 2 and 4 Cells

To obtain live cell imaging images of MET cell line cells adhering and spreading, cells were suspended in media, and seeded into wells of a six-well plate. As soon as the cells are seeded, the plate is mounted on a live cell imaging microscope and phase contrast images taken automatically at intervals of 46 seconds.

Due to the small region which can reasonably observed at the magnification used, only a small number of cells were observed. This prevented useful statistical analysis being carried out.

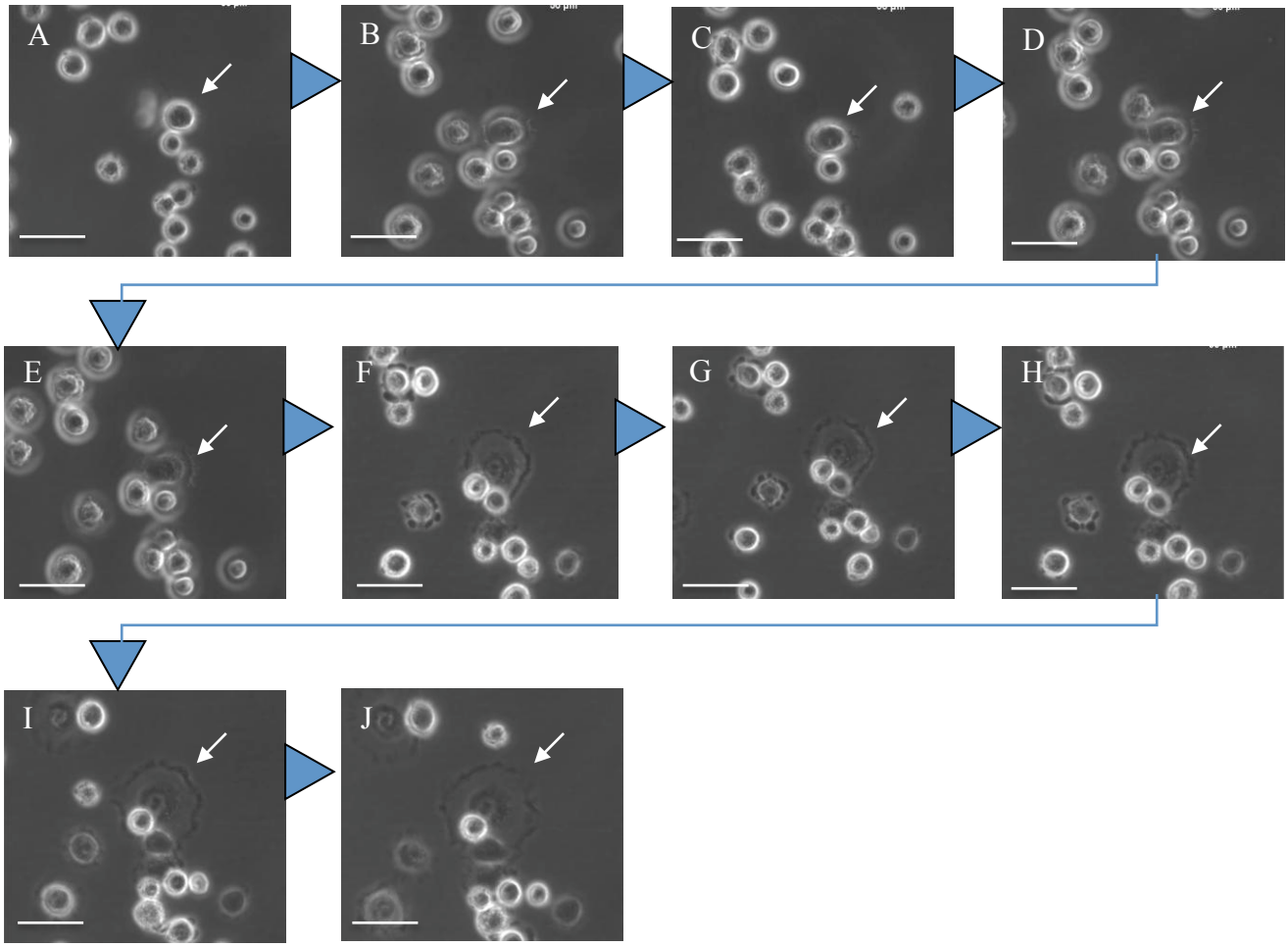


Figure 5.11: Live cell imaging of MET 1 cell adherence.
Ten images of control siRNA transfected MET 1 cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). Arrowhead = time-elapsed images.
As shown by the example cell (arrow), little cell spreading occurs within the first 10 minutes post-seeding. As cells are seeded, some cells begin to adhere to the plate immediately (for example, the cell highlighted by the arrow). As the cell spreads the nucleus can be observed, as can the thicker cell edge produced as the cell is spreading.

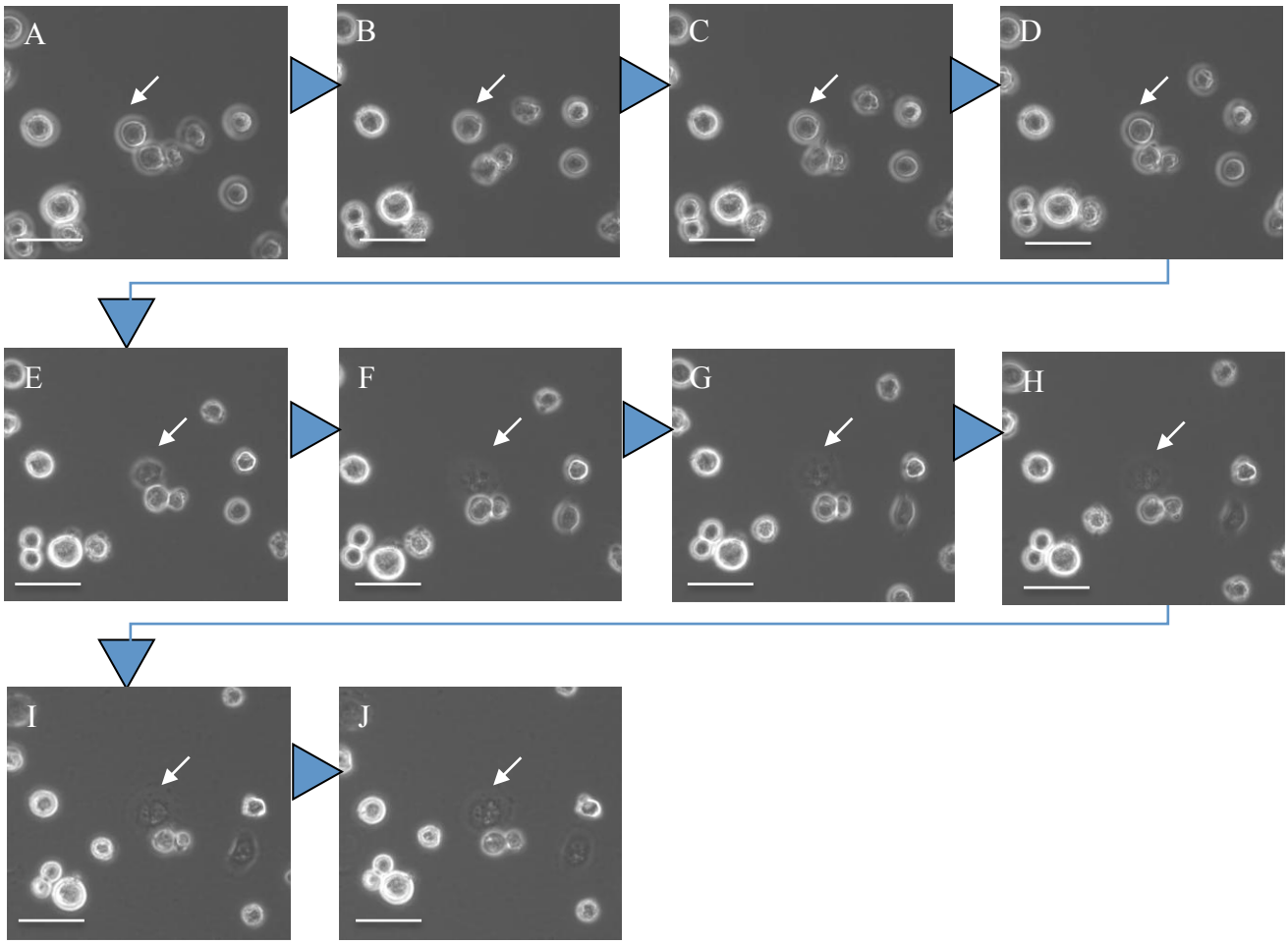


Figure 5.12: Live cell imaging of MET 1 cell adherence.
Ten images of K15 siRNA transfected MET 1 cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). Arrowhead = time-elapsed images.
As shown by the example cell (arrow), little cell spreading occurs within the first 10 minutes post-seeding. As cells are seeded, some cells begin to adhere to the plate (for example, the cell highlighted by the arrow). As the cell spreads the nucleus can be observed, and by 600 seconds (J) the thicker cell edge can also be observed.

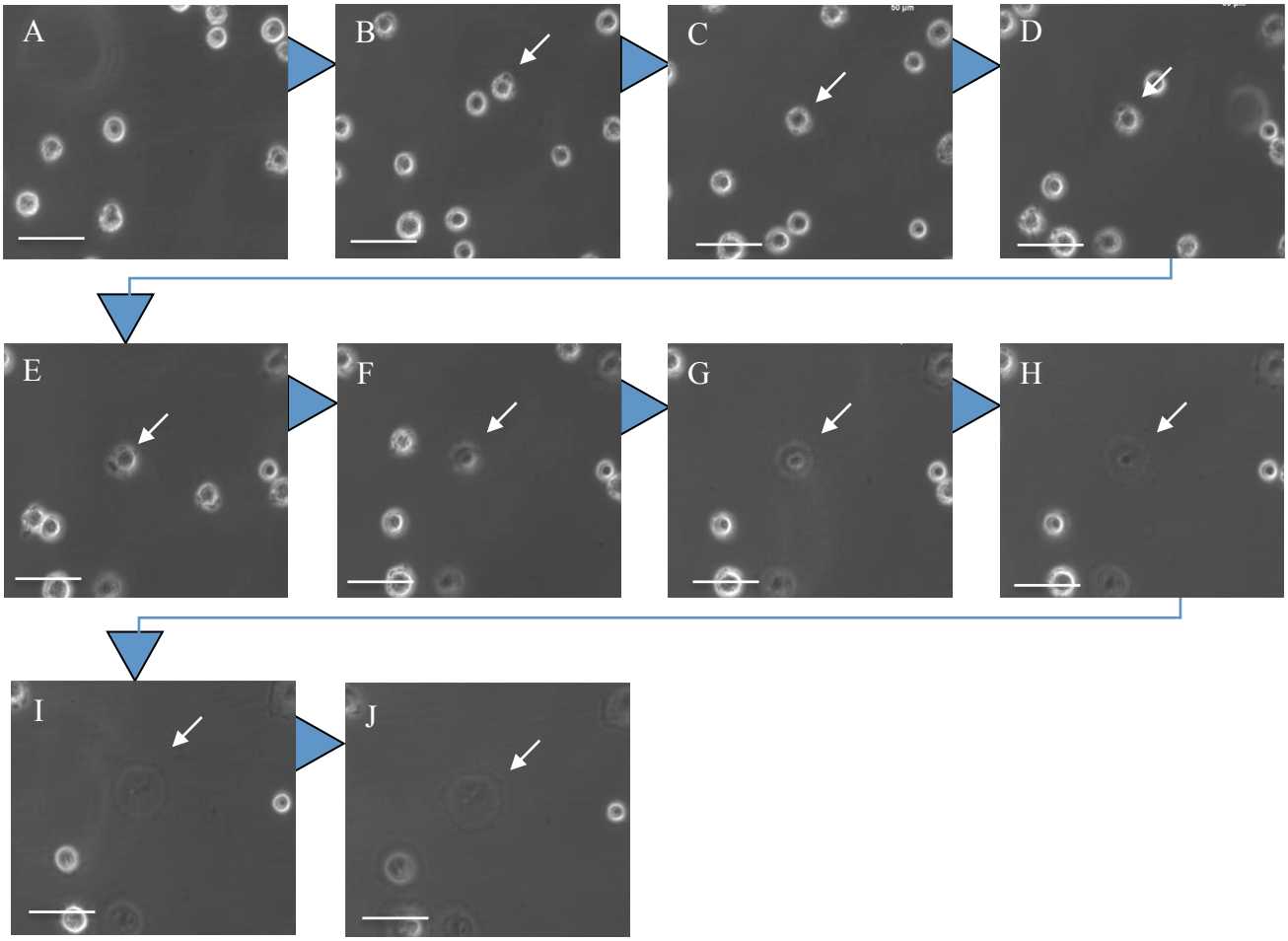


Figure 5.13: Live cell imaging of MET 2 cell adherence.
Ten images of control siRNA transfected MET 2 cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). Arrowhead = time-elapsed images.
The cell highlighted by the arrow begins to attach and spread approximately 232 seconds post-seeding (C). As the cell spreads the nucleus can be observed, as can the thicker cell edge.

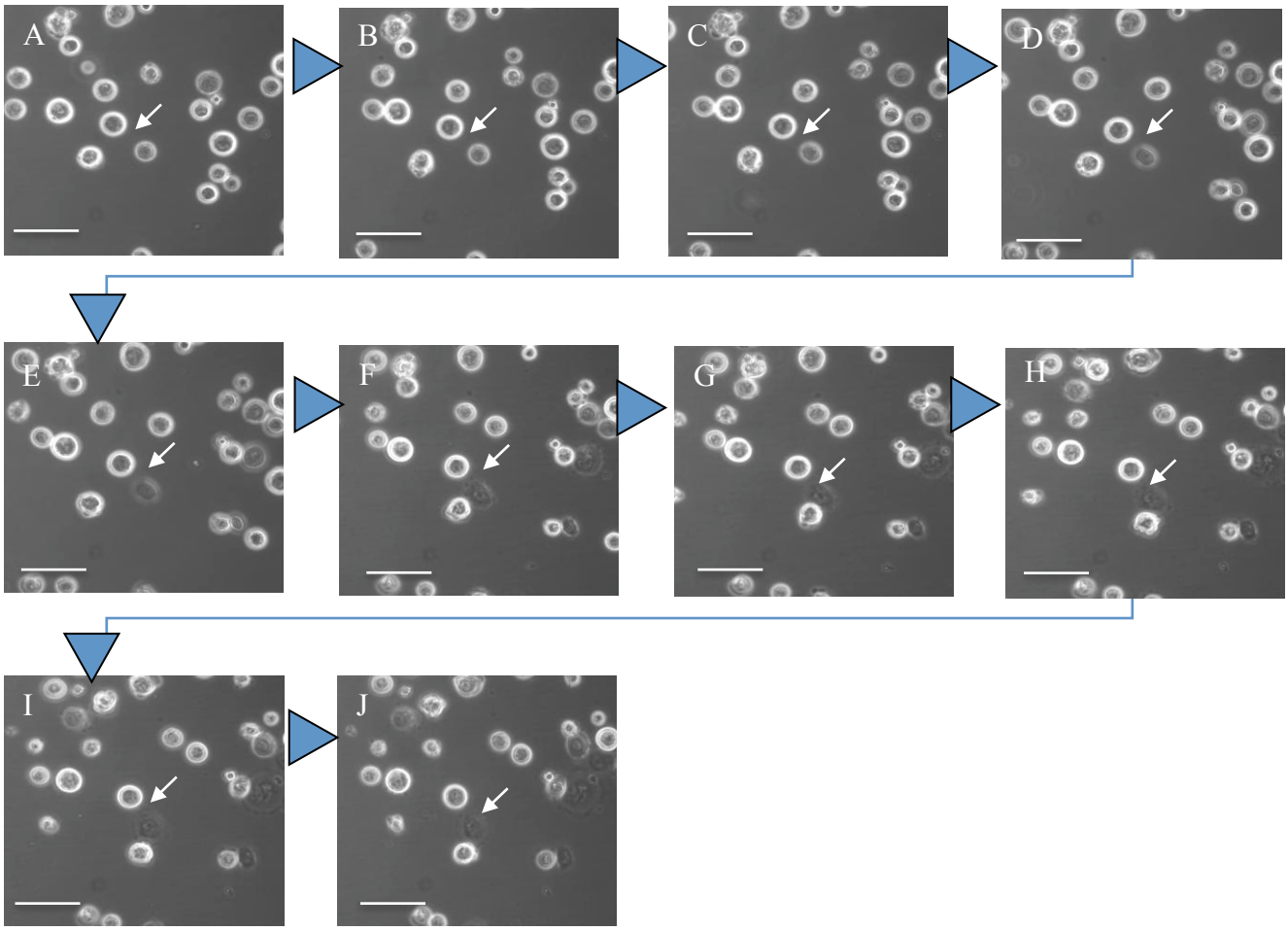


Figure 5.14: Live cell imaging of MET 2 cell adherence.
Ten images of K15 siRNA transfected MET 2 cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). Arrowhead = time-elapsed images.
Although the cell highlighted by the arrow begins to attach and spread approximately 140 seconds post-seeding (A), the cell does not spread to a great extent as observed, for example, in MET 2 control siRNA transfected cells by 600 seconds post-seeding (J). The nucleus of the cell is visible, however the spreading cell edges are less obvious.

Control siRNA transfected MET 1 cells begin to adhere to the plastic of the six-well plate soon after seeding. *Figure 5.11* shows several cells attaching and spreading (for example, the cell highlighted by the arrow). Some cells have attached and begun spreading within 10 minutes. In these cells, the nucleus can be identified, as well as the thick contours of the cell edge. Fewer numbers of cells have not yet begun to spread (smaller, very bright cells on the microscopy images).

Similarly to control siRNA transfected cells, K15 siRNA transfected cells begin to attach soon after seeding (*Figure 5.12*). However, when compared to control siRNA transfected MET 1 cells, fewer cells have begun to spread within 10 minutes, and most cells still appear to be small and round compared to spreading cells. Those cells which are spreading appear similar in morphology to control siRNA transfected MET 1 cells (i.e. the nucleus and defined cell edges can clearly be seen). Morphologically however, spreading K15 siRNA transfected MET 1 cells are still smaller than their control counterparts 10 minutes post-seeding.

Fewer control siRNA transfected MET 2 cells adhere within 10 minutes compared to MET 1 control siRNA transfected cells, and take slightly longer than MET 1 cells to adhere (*Figure 5.13*). As this is the case, MET 2 control siRNA transfected cells have not spread to the same extent as MET 1 control cells 10 minutes post-seeding. The nucleus and cell edge can clearly be observed in spreading cells (such as the cell highlighted by the arrow). K15 siRNA transfected MET 2 cells appear to attach at a similar time to MET 2 control siRNA transfected cells (*Figure 5.13, 5.14*). Nuclei and cell edges are also clearly defined although spreading cells have not spread to the same extent as MET 2 control siRNA transfected cells 10 minutes post-seeding. Many cells have adhered but have not yet begun to spread (these appear small and round in microscopy images).

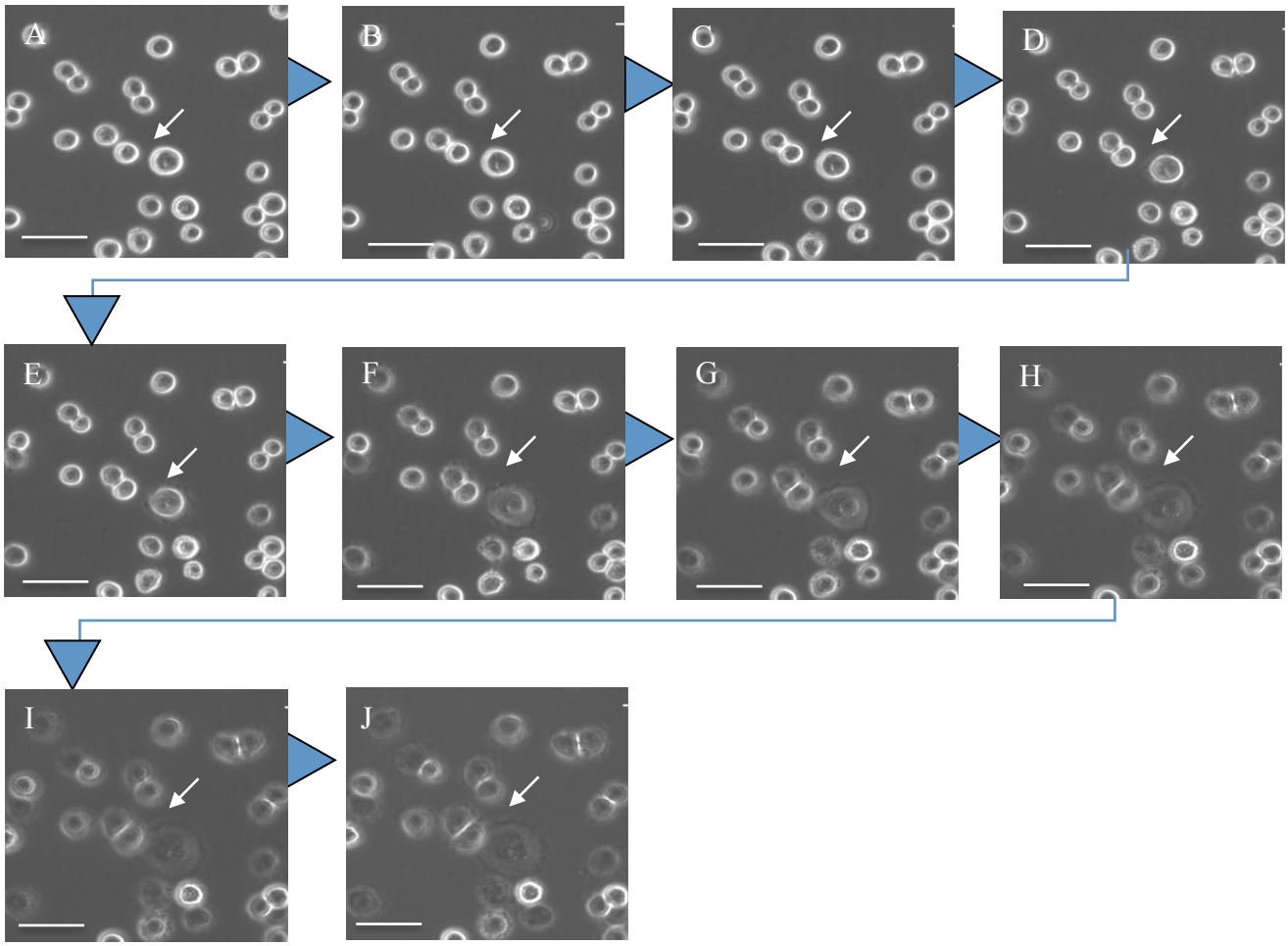


Figure 5.15: Live cell imaging of MET 4 cell adherence.
Ten images of control siRNA transfected MET 4 cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). Arrowhead = time-elapsed images.
Although a single cell is highlighted here, most cells seeded begin to attach and spread by 370 seconds post-seeding (E). By 600 seconds post-seeding the cells are almost all adhered and spreading; the cell highlighted again shows a thicker cell edge.

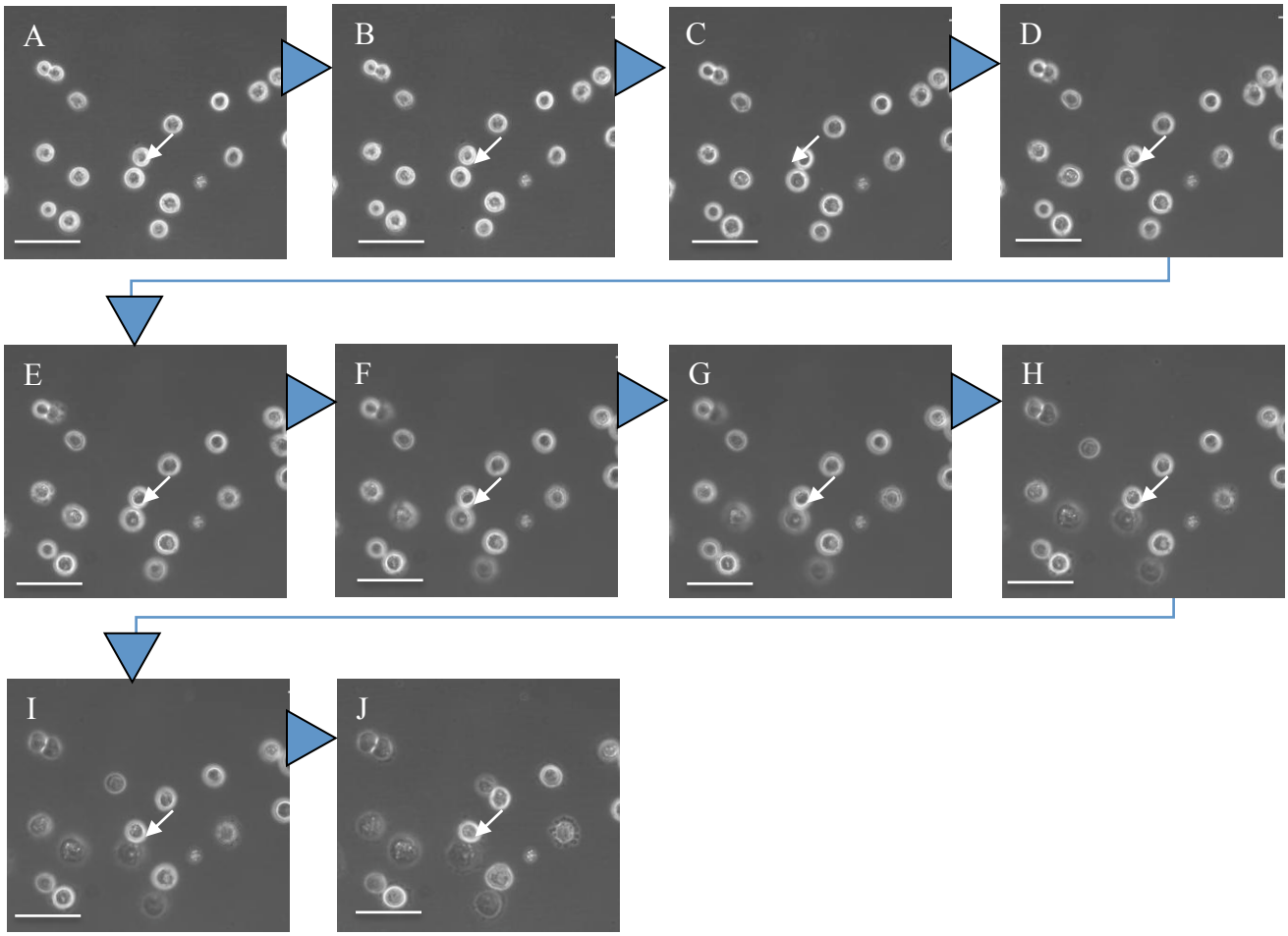


Figure 5.16: Live cell imaging of MET 4 cell adherence.
Ten images of K15 siRNA transfected MET 4 cells between 140 seconds (A) and 600 seconds (J) post-seeding (scale bar = 50µm). Arrowhead = time-elapsed images.
Similarly to MET 4 control siRNA transfected cells, MET 4 K15 ablated cells appear to adhere by 600 seconds post-seeding, although do not appear to have spread to the same extent. As highlighted by the arrow, the adhered cells can be seen to have a thicker spreading cell edge.

Control siRNA tranfected MET 4 cells adhere and begin to spread earlier than control siRNA transfected MET 1 and MET 2 cells; by 10 minutes post-seeding, most cells have adhered and started to spread (there are few small, round cells – *Figure 5.15* J). The nucleus and defined cell edge can be seen in cells that have been spreading (for example, see arrow). Similarly, K15 siRNA transfected MET 4 cells adhere swiftly (*Figure 5.16*). These cells however take longer than their control siRNA transfected counterparts to begin spreading. As this is the case, the average cell size is smaller 10 minutes post-seeding than control cells. The defined nucleus and cell edge are still visible however.

5.3.6: Migration of K15 Ablated MET 1 Cells

A scratch wound assay was used to assess the rate of sheet migration in MET cell line cells, as had been carried out in HaCaT cells (Chapter 4). MET 1 cells were used for this assay as these cells would grow in a confluent monolayer in culture whereas MET 2 and MET 4 cells were more dissociated in monolayer cultures. For the scratch would assay, a monolayer is required which is as close to 100% confluency as possible, without stratification. MET 2 and MET 4 cells however would begin to stratify in regions of the well or flask before a confluent monolayer was established. For this reason, only MET 1 cells were available for this assay. MET 1 cells were transfected and cultured as for HaCaT cells. As soon as the 100% confluent monolayer was scratched, the six-well plate was mounted onto a live cell imaging microscope, and phase contrast images taken every 10 minutes.

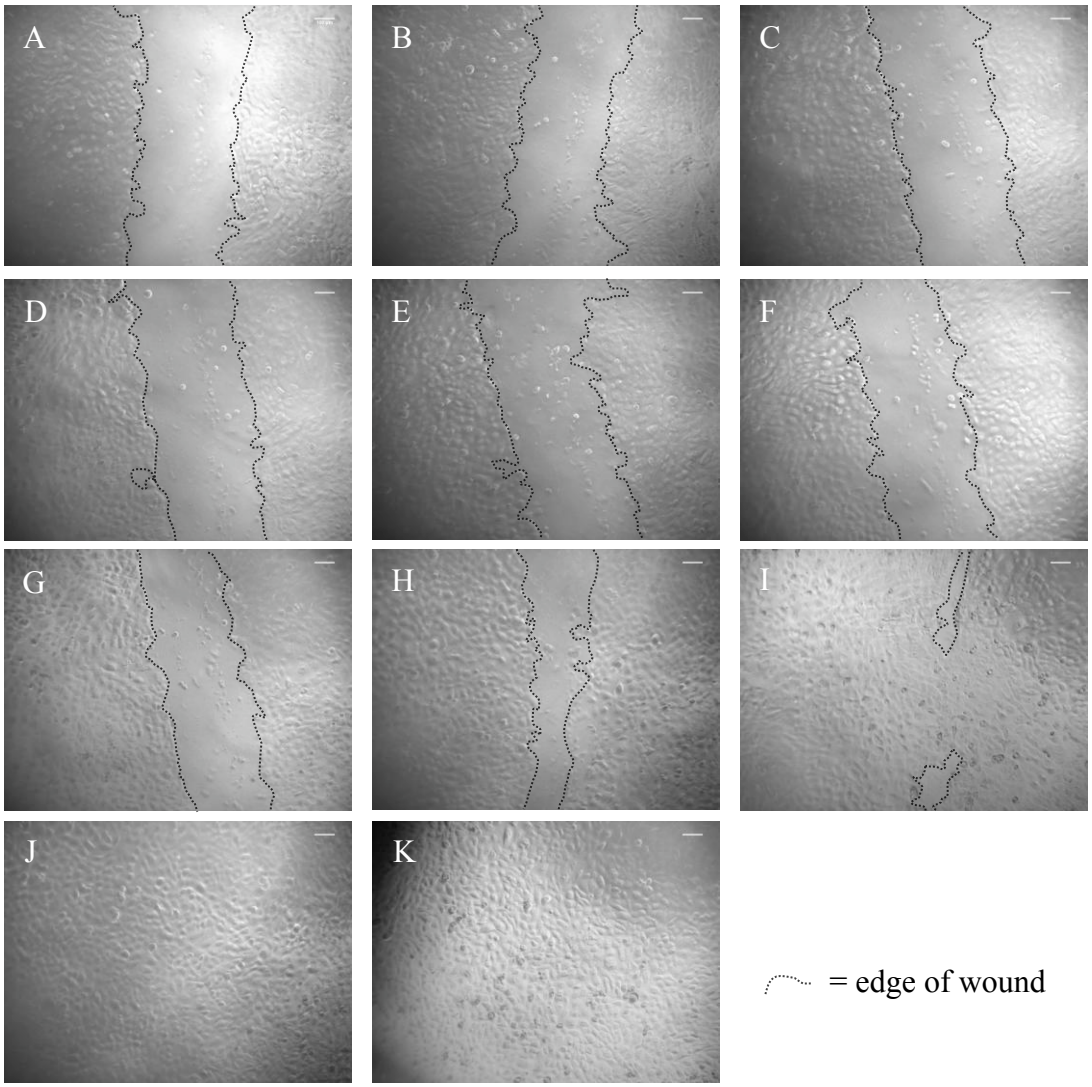


Figure 5.17: Live cell imaging of a scratch wound assay

MET 1 control siRNA transfected cells. The 10 images are taken at the following timepoints post-scratch: 0 mins (A), 10 mins (B), 20 mins (C), 40 mins (D), 60 mins (E), 90 mins (F), 120 mins (G), 180 mins (H), 240 mins (I), 300 mins (J) and 360 mins (K). At this time, the wound is completely closed.

Scale bar = 100μm.

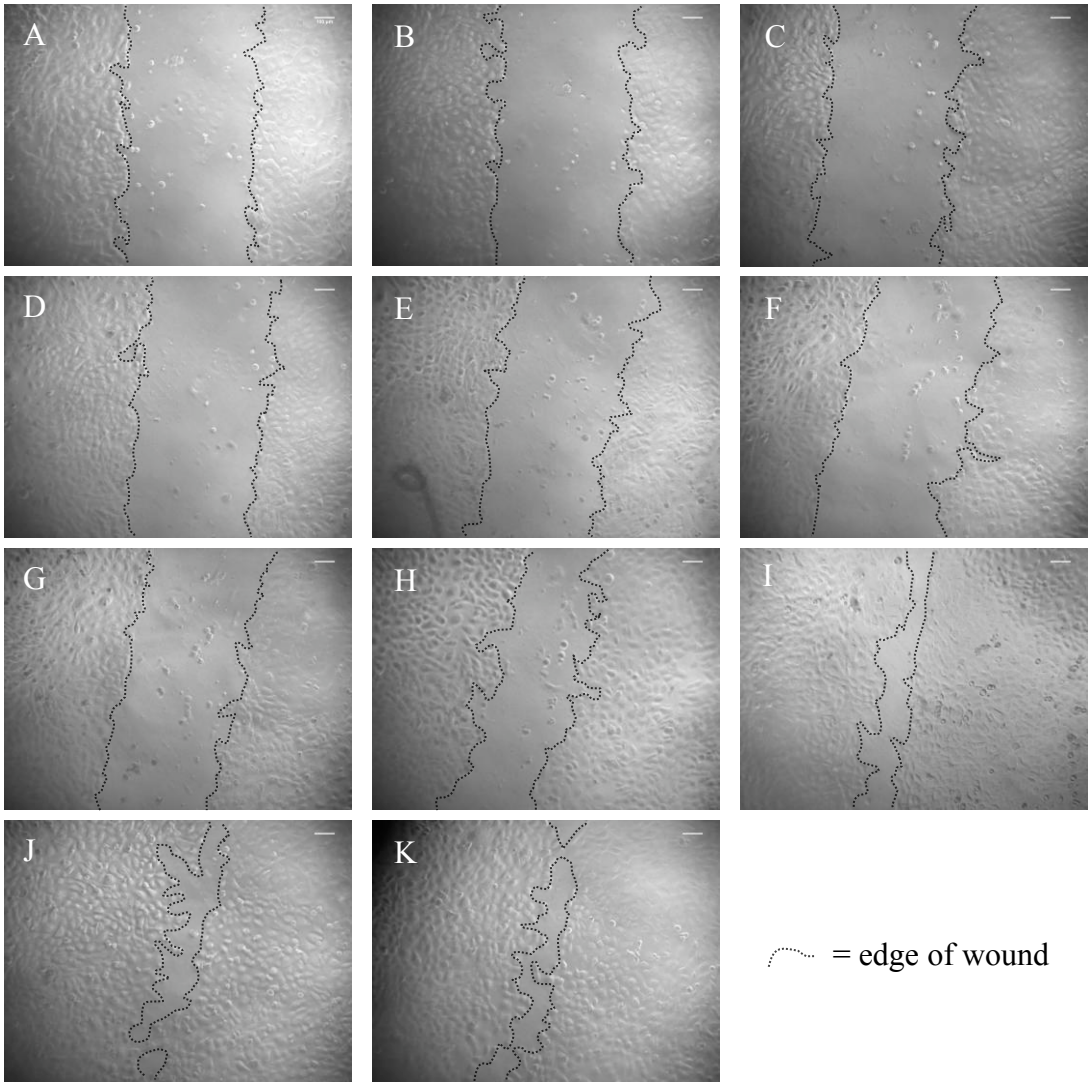


Figure 5.18: Live cell imaging of a scratch wound assay

K15 siRNA transfected MET 1 cells. The 10 images are taken at the following timepoints post-scratch: 0mins (A), 10 mins (B), 20 mins (C), 40 mins (D), 60 mins (E), 90 mins (F), 120 mins (G), 180 mins (H), 240 mins (I), 300 mins (J) and 360 mins (K). At this time, the wound is not yet completely closed, unlike control siRNA transfected MET 1 cells. This suggests cells are collectively migrating at a slower rate.

Scale bar = 100μm.

In MET 1 cells, the wound healed faster than in HaCaT cells (as expected from tumour-derived cell lines). Similar undulation of the wound edge can be observed. In control siRNA transfected cells, the wound is closed by 300 minutes post-scratch (*Figure 5.17*). This does not occur in the K15 siRNA transfected cells, where the wound is still open at 360 minutes post-scratch (*Figure 5.18*). A graph showing percentage wound closure (*Figure 5.19*) suggests that initially, the K15 knock-down monolayer wound begins to heal more swiftly. However, by 300 minutes the control siRNA transfected monolayer wound is closed and the K15 knock-down wound is not. The effect of K15 ablation in HaCaT cells is opposite to the effects observed in MET 1 cells: in K15 ablated HaCaT cells, the wound heals slightly quicker than control siRNA transfected cells. In MET 1 cells however, the control siRNA transfected wound heals quicker.

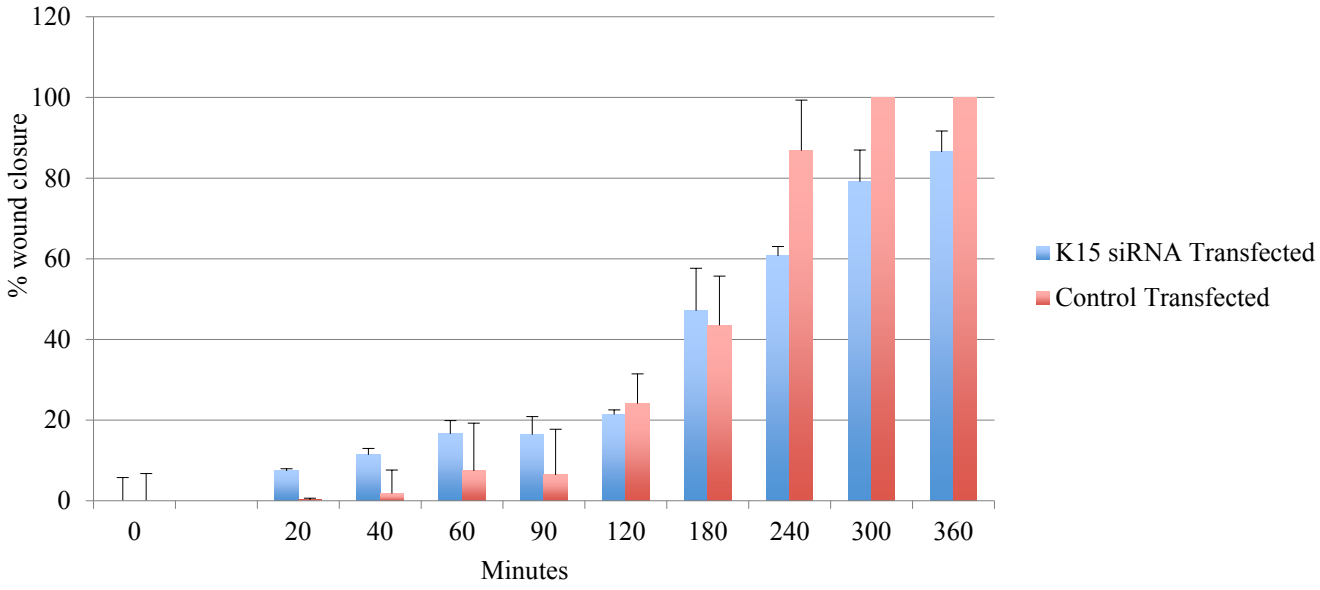


Figure 5.19: A graph to show the percentage wound closure of K15 siRNA transfected and control siRNA transfected MET 1 cells.

Wound closure was measured using the live cell imaging images (from *Figure 5.17* and *5.18*) using Image J. Wound closure was calculated at 0% at 0 mins, and closure calculated relative to this (i.e. 100% indicates a completely closed wound). Error bars = standard deviation.

5.4: Discussion

5.4.1: Keratin 14 Expression in MET Cell Lines

In the initial study characterising MET cell line cells, Proby *et al*. (2000) carry out immunoblotting to establish the presence or absence of various keratins. This however does not examine the appearance or localisation of these proteins in the cell. This study has used immunofluorescence techniques to observe the appearance of K14 (and K15) in MET 1, 2 and 4 cells. K14 did not appear as expected in normal keratinocytes; instead, the filaments in the MET cell line cells were not clearly defined. In addition, K14 was not expressed throughout the cytoplasm to the cell edge (for example, *Figure 5.1* H). These results suggest that K14 has a limited structural role in these cells.

In control siRNA transfected cells (*Figure 5.7*), K14 appears as in untransfected cells (*Figure 5.1*); heterogeneous staining is observed in MET 2 and 4 cells; low levels of K14 are observed in MET 1 cells. Following K15 knock-down, K14 expression in MET 1, 2 and 4 cells are comparable to control siRNA transfected cells. Werner *et al*. (2000) demonstrated that after skin injury, K15 mRNA levels were reduced whilst K14 levels increased. This suggests that such compensation is possible. Since levels of K14 appeared generally higher in MET cells, it is possible that no such compensation was required due to loss of K15 in these cells.

However, since the staining produced is not as expected (i.e. K14 does not appear filamentous and some nuclear staining has occurred), caution must be exercised when considering these results. Further work could be carried out to confirm these possible findings; these will be discussed in section 5.4.7.2.

5.4.2: Keratin 15 Expression in MET Cell Lines

Unlike K14 expression, K15 levels are similar in all three MET cell lines examined. K15 expression has not been previously examined in MET cell line cells, although expression in SCC sections has been examined. Kanoh *et al*. (2008) demonstrated that SCCs were K15-positive, nestin-negative and CD34-negative. From these results, Kanoh *et al*. (2008) suggested that SCCs developed from keratinocytes in the basal layer of the epidermis. In this study, K15 is expressed throughout to the edge of the MET cells, and in some regions has a granular appearance – this is most obviously seen in MET 4 cells. A concentration of K15 can be observed around the nucleus.

This abnormal appearance of K15 has been briefly described elsewhere. Jonkman *et al*. (1996) demonstrated that K15 was upregulated in some EBS suffers with K14 mutations. However, the K15 assembled into protofilaments with K5, but not into larger intermediate filaments or higher order keratin bundles. Aggregation of keratin has been described in murine squamous cell carcinomas: Frontelo *et al*. (1998) demonstrated that following exposure to TGFβ1, the entire cytoskeleton became disorganised, with keratins forming focal juxtanuclear aggregates. Although TGFβ1 expression has not been confirmed in MET cell lines, other SCCs are known to express this growth factor; alongside EGF, TGFβ1 can induce EMT in SCCs (Walsh *et al*., 2011; Richter *et al*., 2011). Since MET 4 cells are established from a metastatic tumour, it is possible that these cells are capable of EMT. This study also demonstrated that MET 1 and 2 cells were capable of invasion (*Figure 5.2* A, B), suggesting that these cell lines are also capable of EMT. Confirmation of TGFβ1 and EGF expression in these cells may aid in confirming this (see also section 5.4.7).

As previously mentioned, Proby *et al*. (2000) were not successful in creating organotypic cultures of MET cell line cells using DED. It has however been possible to culture MET 1, 2 and 4 cells on collagen scaffolds. After 14 days at the air-liquid

interface, all MET 1, MET 2 and MET 4 cells were K15-positive, although variation was observed between and within cell types. The change in morphology in MET 1 and MET 2 cells cause the cells at the top of the culture to flatten; these cells are K15-positive; this is not observed in normal skin sections. The expression of K15 in MET 4 cells varies, with some cells expressing K15 to a greater extent than others. This aids in confirming the previous observation seen in monolayer cultures: i.e. keratin expression in MET cell line cells is heterogenous.

5.4.2.1: Effect of K15 Ablation on MET Cells

To assess the effect of K15 ablation in MET cell line cells, K15 siRNA transfection was used to reduce K15 expression. The optimum knock-down of K15 in MET cell line cells occurred at 48 h post-transfection, as in HaCaT cells. As previously mentioned, K14 expression does not appear to change in MET 1, 2 or 4 cells. It is unlikely any compensatory mechanism occurs to increase the stability of the cell structure, since the granular appearance of K15 suggests that it's role in MET cells in not structural. In addition, it is unlikely that K14 has an important structural role, again considering the unusual expression pattern. Windoffer and Leube (1999) demonstrated that keratin hetrodimers and tetramers (referred to as 'granules') are located at the cell membrane, since microfilaments or focal contacts or adhesions at the membrane may initiate keratin filament formation at the periphery and centre of the cell. Modifications of keratins are capable of affecting filament structure, including glycosylation, deimination, phosphorylation and dephosphorylation, for example. Phosphorylation of keratins can enable or prevent interaction with other keratins (Woll *et al*., 2007), signaling molecules, receptor molecules (Kirfel, Magin and Reichelt, 2003) and other proteins (Liao *et al*., 1995b; Strnad *et al*., 2002). In addition, phosphorylation affects the solubility and organisation of filaments (Liao *et al*., 1995a; Strnad *et al*., 2002). As phosphorylation has such an effect on keratin remodelling (Owens and Lane, 2003; Pekny and Lane 2007), it is possible that in the MET cells have defects in phosphorylation or dephosphorylation which may cause the effect on K15 and K14 observed. Another explanation may be that where mature K14 and K15 keratin filaments were present, actions such as deimination or proteolytic cleavage has resulted in conformational changes. Deimination for example has been shown to cause conformational changes to K1 and K10 during terminal differentiation (Senshu *et al*., 1996). Proteolytic cleavage has also been shown to occur as part of the keratinisation of stratified epithelia (Presland and Jurevic, 2002).

There were also no changes in actin expression or arrangement in MET 1, 2 or 4 cells following K15 knock-down. Since some filopodia-like protrusions can also be observed in both K15 siRNA transfected and control siRNA transfected cells, it is likely that cells are spreading in both control and K15 ablated MET 1, 2 and 4 cells. Although Iwai *et al*. (2010) demonstrated that actin is rearranged in SCC cells (induced by accumulation of β-catenin), increasing the potential of the cells to invade and migrate, no evidence of actin rearrangement was observed in this study. This said however, it was noted that cells were more dissociated in K15 ablated cells (*Figure 5.8*, A, B, C) and fewer E-cadherin-positive cell-cell adhesions were observed compared to control sRNA transfected cells in MET 4 cells. Lack of E-cadherin has previously been linked with invasive potential (Fuller *et al*., 1996; Canavese *et al*., 2007). This lack of E-cadherin-positive adherens junction formation however may be as a result of low cell density, so caution must be taken with interpretation of this result. In the original publication describing the MET cell lines however, Proby *et al*. (2000) describe the dissociation of MET cells, resulting in fewer cell-cell junctions. This result of this study then is consistent with a previous result.

Although it appears that K15 ablation may affect migration (see also section 5.3.6), it did not appear to affect differentiation (see also section 5.3.4). Neither filaggrin levels nor appearance changed following K15 knock-down in all MET cell lines examined. Likewise cell circularity calculations suggest that there is no significant difference in cell shape between control siRNA transfected cells and K15 siRNA transfected cells 120 minutes post seeding. Some changes were observed however soon after seeding; in MET 1 cells, K15 knock-down cells did not adhere as quickly as control siRNA transfected cells. In MET 2 and 4 cells, K15 siRNA transfected cells appear to adhere at the same time as control siRNA transfected cells, but spread more slowly. It has previously been suggested that K15-positive cells (i.e. stem cell-like cells) adhere rapidly *in vitro* (Liu, Zhou and Gao, 2008; Roh *et al*., 2008). K15-negative cells then adhere more slowly, as observed in MET 1 cells. Since it is possible that the K15 observed in MET 2 and 4 cells is not having a substantial effect on differentiation (as suggested by the involucrin results in control siRNA and K15 siRNA transfected MET cells), it is also possible that K15 expression is not a marker which would indicate rapid cell adhesion either. It is also likely that K15 is only a 'marker' of this phenomenon, and not directly involved in cell adhesion. Further work is required to identify the mechanisms that highlight K15 as a marker of rapidly attaching cells. In addition, further investigation the number of cells adhering is required using, for example, an MTT assay to quantify the number of attached cells at given timepoints (see section 5.4.7.2).

5.4.3: Differentiation of MET Cell Line Cells

Similar K14 and K15 arrangement was also seen in cells exposed to different levels of extracellular calcium. Although a calcium switch assay has not previously been carried out on MET cells, some studies have been made with regards to differentiation. In normal skin, as intracellular calcium levels increase, keratin expression changes from K14/K5 to K10/K1 (section 1.1.1.2.; Yuspa *et al*., 1989). Watanabe *et al*. (1995) observed that differentiation-specific keratins were expressed at reduced levels in immature SCC cells, whereas expression of simple epithelial or non-cornifying stratified squamous epithelial keratins could be a marker for metastatic or invasiveness potential. Proby *et al*. (2000) noted that MET 1-4 cells were K14-positive, K10/K1 negative. In this study, some reduction in K14 levels are observed following 72 h exposure to high extracellular calcium, suggesting that some differentiation may be occurring. In MET 1 cells, a change in both K14 and K15 arrangement can be observed after 72 h in high calcium media. Both keratins switched from being concentrated around the nucleus to being spread throughout the cell cytoplasm. To further examine differentiation in MET cell line cells following exposure to high or low levels of extracellular calcium, involucrin immunofluorescence was carried out. In MET 1 cells, increased levels of involucrin were observed after 6 h exposure to high calcium; lower levels of staining were observed after 72 h exposure, possibly due to involucrin crosslinking (making the protein unavailable for immunofluorescence). After 72 h exposure to high calcium levels, involucrin levels in MET 2 cells are increased, again suggesting that some differentiation may be occurring.

Image J was used to calculate cell circularity in MET 1 and 4 cells. It can be seen that in both cell types, at 120 minutes post-seeding, there is little difference between K15 siRNA transfected cells and control siRNA transfected cells in both MET 1 and MET 4 cell lines. Control siRNA transfected MET 1 cells are slightly less circular than MET 4 cells (and has been shown to be statistically significant); this also occurs in

K15 ablated cells (although this is not statistically significant). As circularity is an indicator of more undifferentiated cells, it is expected that K15-positive cells are more circular than K15 ablated cells. Although the calculations would suggest that this occurs in MET 1 cells and not MET 4 cells, no statistical significance between control siRNA transfected and K15 siRNA transfected cells occurs in either cell type. The reason that MET 4 cells are more circular could be related to actin arrangement and therefore E-cadherin expression. Lack of E-cadherin in MET 4 cells may led to rearrangement of the actin cytoskeleton (Alt-Holland *et al.*, 2008), typical of invasive tumour cell phenotypes (however, the actin cytoskeleton appeared normal in this study: *Figure 5.8* F). It could be then that the most invasive cell type (MET 4) are smaller and more circular than the other MET cell line types.

5.4.4: Actin Arrangement and Cell Adhesion Marker Expression in MET Cells

As actin arrangement was affected following K15 ablation in HaCaT cells, actin immunofluorescence was carried out following K15 siRNA transfection. As with K14, levels of actin appeared to vary within cell populations. In MET 4 cells, actin levels in both control siRNA transfected and K15 siRNA transfected cells are similar. In MET 1 and 2 cells, some heterogeneity can be observed, although the arrangement is similar. In all MET cell line cells, actin can be observed up to the edge of the cell. Filopodia-like protrusions can also be observed at the cell edges, as in HaCaT cells. Actin arrangement and expression is linked to E-cadherin. Alt-Holland *et al*. (2008) describe a lack of E-cadherin in HaCaT-II-4 cells leads to reorganisation of the actin cytoskeleton, including motility structure aquisition. E-cadherin can be observed in both K15 siRNA transfected and control siRNA transfected MET 1 and 2 cells, in regions of cell-cell adhesion. This is also seen in MET 4 control siRNA transfected cells, although not K15 ablated MET 4 cells. As noted by Proby *et al*. (2000), these cells are predisposed to dissociate; this may be the reason for the absence of E-cadherin-positive cell-cell adhesions in these cells. It does however suggest that K15 knock-down MET 4 cells dissociate to a greater extent than control siRNA transfected MET 4 cells (and also to a greater extent than K15 ablated MET 1 and 2 cells). Alt-Holland *et al*. (2008) note that E-cadherin suppression is linked to HaCaT-II-4 migration in 3D culture; if low levels of E-cadherin are linked to a more motile cell phenotype, this would, in theory, be more obvious in MET 4 cells. It has previously been suggested that K15-negative cells would be more motile than K15-positive cells (Roh *et al*., 2005; Roh *et al*., 2008); this study also observed that after 24 h, K15 knock-down HaCaT cells (cultured as a monolayer) had closed a wound to a greater extent than control (K15-positive) cells. It may be that this is also the case in the MET 4 cells. In unaltered MET 4 cells, K15 expression (although high) is relatively granular in appearance compared to, for example, MET 1 cells (*Figure 5.1*); this may be either as an effect of increased motility, or a regulatory factor in this feature. However, as it is difficult to culture MET 4 cells in a confluent monolayer, it was not possible to complete a good scratch wound assay in order to assess MET 4 motility, either with or without K15.

5.4.5: Migration of MET 1 Cells

MET 1 cells could be grown to form a confluent monolayer, allowing wound closure speed (as a measure of motility) to be assessed (*Figure 5.17, 5.18*). As is typical of cancer cells, SCC cells are more motile than normal keratinocytes. Proby *et al*. (2000) suggest that MET 1-4 cells dissociate, and are therefore less likely to form junctions which would allow movement as a migratory sheet. When cultured as a monolayer, HaCaT-II-4 cells had low levels of E-cadherin, induction of β–actin

production and reorganization of the actin cytoskeleton (Alt-Holland *et al*., 2008). Alt-Holland *et al*. (2008) suggest that these changes are likely to contribute to the activation of the invasive tumour cell phenotype from the precursor to the carcinoma, as observed *in vivo*. Collagen has also been shown to have an effect on migration. Type VII collagen is the main component of attachment structures within the lamina densa of the basement membrane, called the anchoring fibrils. Mutations to the Type VII collagen gene can cause a type of epidermolysis bullosa. When the same mutation is inserted into MET 1 cells using RNAi, migration and invasion are promoted whilst differentiation is reduced; migration was increased two-fold (Martins *et al*., 2009).
Although initially K15 siRNA transfected cells appeared to move to a greater extent, control siRNA transfected cells were the first to completely close the wound. This could be as a result of the sheet of cells being 'over stretched' in the swift migration of K15 ablated cells at the wound front and needing to then compensate; this is unlikely however as control siRNA transfected cells have reached similar levels of wound closure by 120 minutes post-scratch. It may also be an artifact of the MET 1 cells' tendency to dissociate. If this is more likely in K15 ablated cells, then MET 1 K15 siRNA transfected cells are less likely to 'strive' to close a wound compared to control cells; K15 ablated MET 1 cells need to be in contact with other cells to a lesser extent, therefore having a reduced requirement for wound closure.

5.4.6: Conclusions and Further Work

5.4.6.1: Summary of Findings

These results suggest that MET cells do not differentiate normally. The indicators of differentiation also produce different results in each of the cell lines tested. For example, morphological changes (almost identical to those observed in HaCaT cells) could be observed in MET 1 cells, to a lesser extent in MET 2 cells and to no extent in MET 4 cells. Some changes were observed in expression levels of involucrin following exposure to different concentrations of extracellular calcium, however these results were not as conclusive as those observed in HaCaT or NHEK cells. Similarly, K15 ablation generally had little or no effect on MET cells which would indicate that without K15, cells being to differentiate.
Since K15 is expressed in MET 1, 2 and 4 cells, it would suggest that these SCC cells arise from normal cells which already express K15 (i.e. are relatively undifferentiated).

5.4.6.2: Further Work

As previously indicated in section 5.4.3.1, several other experiments could be carried out. To assess the structural integrity of cells with and without K15, cell stretching tests could be used to observe the ability of cells to sustain stretching with and without K15; this would indicate whether K15 had a role in maintaining the structure of the cell, and, if so, how influential this role was. Such tests have been carried out before to assess remodeling of the keratin network in EBS (Russell *et al*., 2004), to establish phosphorylation of PKB and EGFR in keratinocytes (Kippenburger *et al*., 2005) and more recently to demonstrate differences in mechanical stability in K10-mutant keratinocytes (Obarzanek-Fojt *et al*., 2011).
It would also be useful to establish whether cell-cell junctional proteins are expressed in more confluent monolayers of MET cell line cells. This would be more difficult in successfully siRNA transfected cells, however only regions of high confluency are required to establish cell-cell contact and therefore cell-cell junctions. Immunofluorescence of desmoplakin, E-cadherin and ZO-1 would aid in establishing

whether MET cell line cells are capable of forming various cell-cell junctions, and whether K15 ablation has any effect. Furthermore, to quanitatively assess whether K14 is upregulated in K15 ablated MET 1 cells (or MET 2 and 4 cells), western blotting could be carried out following K15 siRNA transfection in these cells. Image J can be used to quantify these results. In addition, the same antibody could be used as Proby *et al*. (2000) used in their study, allowing results to be more directly compared.

It would also be useful to calculate cell circularity after a longer period post-seeding (for example, 24 h) to give a further indication of any changes in cell shape that occur in K15 ablated MET cells. Although it appears that these cells are not necessarily undergoing 'normal' differentiation (section 5.4.8.1), cell shape is an indicator of how undifferentiated a cell may be. It may also be useful to calculate nuclear:cytoplasm ratio as a further indication (for example, as in Roh *et al*., 2008). As previously mentioned, additional work could be carried out to identify the mechanisms which highlight K15 as a marker of rapidly attaching cells. Immunofluorecence images of cross sections of attaching cells may indicate whether K15 has a role at the cell periphery. To further investigate the number of adhering cells, an MTT assay could be used to quantify the number of cells at various timepoints following seeding.

It was also noted in section 5.4.2 and 5.4.6 that EMT may be occurring in MET 1 and 2 cells. To further examine this, it would be useful to use immunofluorescence to identify E-cadherin and β–catenin in organotypic cultures. Cells undergoing EMT would demonstrate reduced E-cadherin levels whilst β-catenin levels increase; alteration may also be observed in actin arrangement (Alt-Holland *et al*., 2008), which could also be observed using immunofluorescence.

This study observed that K15-negative MET 1 cells migrated more slowly than K15-positive MET 1 cells. This is similar to the initial results observed in HaCaT cells, where control siRNA transfected HaCaT cells migrate faster than K15 ablated HaCaT cells. Proby *et al*. (2000) suggest that MET cells dissociate, form fewer cell-cell junctions, and therefore are not efficient as a migratory sheet. In order to further investigate the motility of MET cells, organotypic cultures, as used in this study, could be scratched and immunofluorecence used to establish where K15 and cell-cell junctional proteins are expressed. Since Martins *et al*. (2009) also demonstrated that collagen can have an effect on MET 1 cell migration, it may also be possible to culture monolayers of MET cell line cells onto collagen-coated plates prior to scratching to assess the effects of collagen on MET cell motility.

Chapter 6: Conclusions and Future Outlook

6.1: Aims

The overall aim of this study was to establish the effect of K15 ablation on keratinocytes. This was considered important as K15 has previously been shown to be expressed in primary keratinocytes and has been suggested as a marker of hair follicle stem cells. To achieve this, siRNA techniques were used to transiently transfect five cell lines: the immortalised keratinocyte cell line HaCaT, the SCC cell lines MET 1, 2 and 4, and normal human epithelial keratinocytes (NHEK).

6.2: Normal Expression of K15

6.2.1: Expression of K15 in Whole Skin

Normal K15 expression was observed in chapter 3 (mammalian skin, HaCaT and NHEK cells) and chapter 5 (SCC cells). Some similar work had previously been carried out by, for example, Waseem *et al*. (1999), who identified K15 expression in the basal layers of human skin with a reduction in intensity towards the surface; a similar expression pattern was observed in this study (*Figure 3.10*). Waseem *et al*. (1999) further confirmed this observation by using tissue *in situ hybridization* to demonstrate that K15 mRNA was located only in the basal cells. This would suggest that the K15 observed above the basal layer is not actively expressed but is residual protein. It has previously been shown that keratin subunits have a short half-life, whereas filaments are more stable (Werner *et al*., 2004); this suggests that K15/K5 filaments are present in the cells above the basal layer, closer to the surface of the skin. This also gives an indication of the functions of K15: since keratinocytes require more mechanical stability as they differentiate, the absence of K15 in the uppermost layers of the epidermis suggest that K15 is not a keratin which provides a significant amount of mechanical stability.

6.2.2: Expression of K15 in HaCaT Cells and NHEKs

Since K15 is expressed in basal cells, it was expected that HaCaT cells, as immortalised keratinocytes, and NHEK cells would also actively express K15. This was found to be the case. Previously, Werner, Werner and Muntz (2000) had also demonstrated that K15 was actively expressed in HaCaT cells by identifying K15 mRNA expression, and several studies have demonstrated K15 expression in primary human keratinocytes in tissue sections (for example, Kloepper *et al*., 2008). As can be seen in *Figure 3.10*, K15 expression appears similar to K14 expression when using immunofluorescence. This suggests that the K14 staining (and therefore K15 staining) in this study are comparable to other previously published works.

6.2.3: K15 Expression in MET 1, 2 and 4 Cells

K15 expression was not investigated in the original paper describing the SCC cell lines MET (Proby *et al*., 2000). It has been demonstrated elsewhere however (for example, Kanoh *et al*., 2008) that SCCs were K15-positive, suggesting that SCC develop from the basal layers of the epidermis. This study found that MET 1, 2 and 4 cells were all positive for K15. The existence of a cancer stem cell population, which express stem cell markers, has been suggested. For example, Harper *et al*. (2010) have demonstrated that tumour-initiating cells in the skin are resistant to apoptosis. It is possible that these cells are also K15-positive, although further investigation is required to establish this. Furthermore, the presence of a cancer stem cell population suggests that K15 may be expressed not to confer mechanical stability to the MET cells, but to allow migration; in cancer cells, this may be an indicator of invasive potential.

As previously noted, keratin subunits have a shorter half-life than keratin filaments (Werner *et al*., 2004). It was noted in MET cell line cells that K15 in particular was granular, and did not appear as filaments. Granular staining of keratins has been previously noted: Windoffer and Leube (1999) and Windoffer *et al*. (2004) demonstrated that heterodomers and tetramers were observed at cell membranes, before forming filaments. It is possible that this explains the appearance of K15 in MET cells. Another possible explanation is that the granules are the remnants of K15 filaments, which have been reduced to granules through processes such as deimination or proteolytic cleavage (see section 5.4.3.1). This explanation is more likely, as in some cell populations few K15 filaments can be observed, which would be expected if the filaments were being degraded inside the cell. It is possible that K15 filaments may form, but have a faster turnover than in non-carcinoma cells. Functions of keratins are to provide mechanical stability, to interact with signaling or receptor molecules (Kirfel, Magin and Reichelt, 2003) and to interact with other proteins (Liao *et al*., 1995b; Strnad*,* Windoffer and Leube, 2002). If only a few, thin K15 filaments are formed, their contribution to these functions may be limited.

6.3: K15 Knock-down using siRNA

K15 siRNA was successfully used to knock-down K15 expression in all cell lines used in this study. Since this technique would only transiently transfect the cells, an optimum time point was established for each cell line. Previous studies including McInroy and Määttä, 2007, Long *et al*., 2006, Claser *et al*., 2008 and Smith *et al*., 2008 suggested that an optimum time point for keratin ablation in similar cell lines would be between 48 and 96 hours post-transfection. It was established that the optimum time for K15 ablation in HaCaT and MET 1, 2 and 4 cells was 48 h post-transfection. In NHEK cells the optimum was slightly later at 72 h post-transfection; all of these time scales are comparable to other studies using the technique to knock-down similar proteins in other cell lines.
Once these optimums were established, they were used for all following experiments.

6.3.1: The Effects of K15 Ablation on Other Cytoskeletal Proteins

6.3.1.1: Effect of K15 Ablation on Other Keratins

It has previously been demonstrated that K14 is expressed in basal layer keratinocytes (Nijhof *et al*., 2006), suggesting that both K14 and K15 are expressed simultaneously in the IFE. In this study, the amount of K14 expression does not appear to have changed, however an organisational change does occur. It was observed that K14 was not expressed up to the cell edges in HaCaT cells following K15 ablation (*Figure 4.7*). This may be due to the abnormal spreading which has been seen to occur in K15 ablated HaCaT cells. Recently, Lee and Coulombe (2009) have shown that K14 (paired with K5) can 'self-organise' into filaments in fibroblasts. It has previously been shown that cytoskeletal bundling can generate an inward tension, which can prevent cell spreading; Gross and Kinzy (2005), Karakozova *et al*. (2006) and Kotadiya*,* McMichael and Lee (2008) demonstrated this with F-actin and Lee and Coulombe (2009) demonstrated a similar effect with keratins. Lee and Coulombe (2009) observed that thicker bundles resulted occurred in cells with a reduced area. However the opposite appears to be occurring here with K14 – i.e. the apparent restriction of K14 from the outer edges of the cell occurs in cells which are spreading to a greater extent than control cells. It is possible that the increased actin bundling observed at the edge of K15 ablated cells may prevent K14/K5 filament formation in this area.

This effect was not observed in MET cells however. In MET 1, 2 and 4 cells, no change in K14 expression was noted following K15 ablation. Western blotting could be used to further quantify whether any compensation of K14 for K15 was occurring. McGowan *et al*. (2002) and Fan and Yoon (2003) demonstrated compensation for K17 by other keratins can occur in both murine and human skin. Since this can occur in other keratins, it is feasible for this to occur between K14 and K15, particularly since both produce dimers with K5.
Neither K17 nor K6 were affected by K15 siRNA transfection in HaCaT cells. K8 expression in K15 ablated cells was variable, however this has previously been observed in HaCaT cells (Kazerounian *et al*., 2002).
These results suggest that K15 does not affect the expression of other keratins in normal skin or in SCCs. Furthermore, any results which demonstrated an effect following K15 ablation was likely to be as a result of K15 knock-down, and not as part of a secondary effect via another keratin affected by K15 knock-down.

6.3.1.2: Effect of K15 Ablation on Actin
Immunofluorescence of actin in NHEK cells suggested that actin arrangement was not affected by K15 ablation. Similar effects were noted in HaCaT cells and MET cells, despite suggestion from Iwai *et al*. (2010) that actin rearrangement occurs in SCC cells transfected with mutant β-catenin. This change was also suggested to increase the invasion and migration potential of these cells.
The transfected NHEK cell results were obtained from cells which had been seeded for at least 72 h (i.e. at least 24 h prior to transfection). Immediately following seeding, actin arrangement was shown to be different in K15 ablated cells (compared to control siRNA transfected cells). This is discussed in section 6.6.2.

6.4: K15 Ablation Results in Increased Differentiation

Involucrin expression was assessed as a marker of differentiation. Expression was greater in K15 knock-down HaCaT cells compared to control siRNA transfected cells 48 h post-transfection. Although involucrin levels have previously been shown to increase following exposure to extracellular calcium (for example, Bikle *et al*., 2001; Tran and Crowe, 2004), this study did not change the extracellular calcium levels for this experiment, demonstrating that a rise in involucrin expression can also be initiated by other pathways. This result however indicated that since involucrin expression is initiated following K15 ablation, the cells appear to be differentiating.
Filaggrin, usually expressed in cells of the granular layer and above, is only expressed in very low levels in HaCaT cells. The level however increases once the cells become confluent and begin to stratify (Papp *et al*., 2003). In this study, where confluent cells could not be efficiently transfected, levels of filaggrin were low in controls (as expected) and in K15 ablated cells. This indicates that although some differentiation may be occurring (as indicated by the increase in involucrin), differentiation has not occurred to the extent that filaggrin levels increase. For confirmation, western blotting could be used to demonstrate whether any profilaggrin processing was occurring (which can not be demonstrated using immunofluorescence), and to confirm the involucrin immunofluorescence results.

6.4.1: Additional Effects of the Calcium Switch Assay

Similar levels of K15 were observed in control siRNA transfected cells in both high and low calcium conditions. This is most likely to be due to the slightly abnormal keratin expression pattern observed in HaCaT cells. It may also be due to the relatively short length of time the cells were exposed to high extracellular calcium for.

Likewise, MET cell line cells tested also suggest that little change is occurring in K15 expression following exposure to increased levels of extracellular calcium. Again this could be due to the slightly altered keratin expression pattern observed in MET 1, 2 and 4 (Proby *et al*., 2000).
Although K14 expression has previously been shown to change during differentiation (Nelson and Sun, 1983; Lena *et al*., 2010), no changes in K14 expression were noted following the calcium switch assay. This may have been due to the relatively short length of the experiment; in longer experiments, K14 expression was demonstrated to be affected after 5 days (Micallef *et al*., 2009). This is supported by the findings of Yuspa *et al*. (1989), who demonstrated that no change in K14 mRNA levels could be detected after 48 h. Lack of K14 mRNA is observed in the granular layer *in vivo* (Roop *et al*., 1988); it has been demonstrated by lack of filaggrin expression that differentiation in HaCaT cells does not occur in monolayer cultures, supporting the observation that no changes in K14 expression occur. Despite no changes in K14 expression occurring in HaCaT cells, a change was observed in MET 1 cells following exposure to high extracellular calcium for 72 h. A slight reduction was observed, suggesting some differentiation. Like K15, K14 localisation also changed, reducing the amount of keratin around the nucleus and becoming spread throughout the cytoplasm.
As expected, involucrin levels in control siRNA transfected HaCaT cells exposed to high levels of extracellular calcium increased. The levels of involucrin in K15 siRNA transfected HaCaT cells increased to a greater extent when exposed to high levels of extracellular calcium and continued to rise. The opposite occurred in MET 2 cells, as expected since SCCs have previously been shown to express involucrin (for example, Zhou *et al*., 2011). However, it is more likely that continuing differentiation in the MET 2 cells meant that the involucrin present was cross-linked by transglutaminase, which occurs as cells begin to form the cornified envelope. This would make the involucrin unavailable for immunofluorescence, reducing the staining observed. A similar effect was noted in MET 1 cells.
This suggests that less involucrin was observed in MET 2 cells compared to MET 1 cells. Previously it has also been suggested that involucrin is downregulated in progressing oral carcinomas (Tseng *et al*., 2007). These results are different to those observed in mice; Prince *et al*. (2007) demonstrated that K14 and involucrin were expressed in different populations of head and neck SCCs.

6.5: Reduced K15 Expression Correlates with Increased Proliferation

In this study, the MTT assay was used to assess cell viability as an indicator of proliferation. Although the findings discussed suggest overall effects on proliferation, the MTT assay does not differentiate between proliferation, cell death or differentiation. These results suggest that K15 ablated HaCaT cells proliferate to a greater extent than K15-positive cells. However, the results were not statistically significant. More replicates and larger cell numbers could be used to confirm this result and independent methods, such as FACS anaylsis of the DNA content could be employed. Although the MTT assay was a different method than that utilized by Tiede *et al*. (2009), the result was similar (i.e. K15-negative cells proliferate to a greater extent than K15-positive cells). Since K15-negative cells proliferate to a greater extent than K15-postive cells, it is also possible that this will have an effect on migration as assessed by the scratch wound assay (discussed in section 6.7.1).
Over longer periods however (for example, several passages), K15-positive cells have been shown to have a greater colony formation efficiency than K15-negative cells.

For example, Liu, Zhou and Gao (2008) demonstrate that in K15-positive stem cells from adult goat skin, a high colony forming efficiency value can be achieved between passages 2 and 8.
Stem cells need to be capable of high levels of controlled proliferation. It is likely that in the short-term (such as the time scale investigated in this study), proliferation is generally slower. However, over the longer term (such as that investigated by Liu, Zhou and Gao, 2008), proliferation of these cells can continue, producing, for example, a greater colony forming efficiency than more differentiated (K15-negative) cells. These cells may be capable of higher levels of proliferation initially, however their ability to proliferate reduces as the cells begin to terminally differentiate. Over the longer term then these cells will have low colony forming efficiency values.

6.6: K15 Ablation Affects Cell Adhesion and Spreading

Using live cell imaging, HaCaT and MET 1, 2 and 4 cells were seeded and phase contrast images taken approximately every 42 seconds. The HaCaT cells (both control siRNA transfected and K15 siRNA transfected) began to adhere after approximately 10 minutes. The MET 2 and 4 control siRNA and K15 siRNA transfected cells also adhered at approximately the same rate (although spread more slowly); K15 ablated MET 1 cells however adhered slightly slower than control siRNA transfected cells. Liu, Zhou and Gao (2008) and Roh *et al*. (2008) have previously suggested that K15-positive cells adhere rapidly *in vitro*; this appears to occur in MET 1 cells.
Hormia *et al*. (1995) demonstrated that it took up to 24 h for HaCaT cells to adhere to plastic; HaCaT cells used in this study were the slowest of the cell lines to adhere but had begun to adhere after 10 minutes. This meant that it was possible to start fixing cells to carry out immunofluorescence from this point. This was carried out to establish how the cytoskeleton changed as the cells spread.

6.6.1: K15 Ablation Does Not Affect Cell-Cell Junctions

Initial immunofluorescence of cell-cell junctional proteins in HaCaT cells was carried out on cells which had been seeded for at least 72 h (i.e. 24 h prior to transfection). Desmoplakin was expressed at similar levels in control siRNA transfected cells and K15 siRNA transfected cells. It has previously been shown that DIFCs do not associate with K15, suggesting that K15 does not have a role in linking into desmosomes to increase the mechanical stability of tissues (Cirillo and Prime, 2009). Wan, South and Hart (2007) demonstrated that changes in desmoplakin expression can affect proliferation; this suggests that the increase in proliferation identified in K15 ablated cells in this study was not as a result of changes to desmoplakin expression or localisation. Further work using more confluent monolayers or organotypic cultures would be useful to confirm these findings.
E-cadherin levels also remained unchanged in K15 ablated cells. It has been suggested that E-cadherin, associated with adherens junction formation, has a role in maintaining the stem cell niche by allowing asymmetric division in multipotent cells (Perez-Moreno, Jamora and Fuchs, 2003). ZO-1, associated with highly differentiated cells (of the granular layer *in vivo*), was not expressed in either control siRNA transfected or K15 siRNA transfected HaCaT cells. Marthiens *et al*. (2010) suggest that the theory which states that adhesion molecules retain cells in the stem cell niche alone is outdated, and that more recently findings have demonstrated that proteins such as E-cadherin have other important roles in the stem cell niche, such as retention, division and exit. Cadherins have also been shown to be important in

regulation of centrosome positioning and spindle angle formation, demonstrating the wide variety of functions these proteins have. For example, ZO-1 (as part of adherens junctions) may be important in asymmetric cell division; Kadowaki *et al*. (2007) have demonstrated that both daughter cells inherit significant levels of ZO-1 (Konno *et al*., 2008), but only one will inherit the fate determinants associated with adherens junctions in embryonic neural stem cell division. Similarly, it is likely that E-cadherin promotes self renewal of neural stem cells (Karpowicz *et al*., 2009). Jin *et al*. (2008) have furthermore demonstrated that those cells expressing lower levels of E-cadherin are displaced from stem cell niches, suggesting that cadherin expression levels provide a mechanism for the removal of dysfunctional stem cells from a niche. E-cadherin knock-outs have also been shown to have reduced numbers of stem cells after aging compared to controls (for example, Boyle *et al*., 2007). Although no changes in cell-cell junctional proteins were observed in K15 ablated cells *in vitro*, 3D culture or tissue studies of a K15 knock-out mouse may provide further information as to whether cell-cell junctional proteins around the stem cell niche (in particular the hair follicle bulge) have any effect on, for example, ejecting cells from the niche. Label retaining studies alongside study of adhesion molecules may also help to establish where asymmetrical division is occurring relative to K15 expression.

6.6.2: K15 Ablated Cells Spread More Quickly than Controls

Vinculin, a focal adhesion protein, was examined in HaCaT cells 180 minutes post-seeding. Möhl *et al*. (2009) previously demonstrated that low levels of vinculin can be observed throughout the HaCaT cell cytoplasm whilst 'distinct bright spots' indicate regions of a high concentration of bound vinculin. Fewer focal adhesions were observed in K15 ablated HaCaT cells, suggesting that these cells were still spreading. Focal adhesions are dynamic, forming at the leading edge of migrating cells (Schäfer *et al*., 2009; Möhl *et al*., 2009). Since fixing and using immunofluorescence produces a 'snapshot' of cells, the fewer focal adhesions observed may be supportive of the observation that focal adhesions are dynamic. Möhl *et al*. (2009) further demonstrated that vinculin exchange occurs in focal adhesions of moving cells. Schäfer *et al*. (2009) also demonstrated that vinculin-containing focal adhesions form as maturations of filopodial focal adhesions; some filopodia and lamellipodia can be seen in spreading HaCaT cells (*Figure 4.1*) (see section 4.4.2).

As expected in spreading cells, actin bundling can be observed at the cell edge in HaCaT cells up to 120 minutes post-seeding in both control siRNA transfected and K15 siRNA transfected cells. In control siRNA transfected cells, the thicker bundles at the cell edges then dissipate and actin arrangement appears similar to that of control siRNA transfected cells which had been seeded for a longer period of time (for example, *Figure 4.1*). In the absence of K15 however, the thicker actin bundles at the cell edges remain (*Figure 4.2*, *Table 4.2*). As this is observed in control cells, it is likely that this is a normal part of cell spreading, however the continued arrangement of actin bundles in K15 ablated cells suggested that these cells may be spreading to a greater extent than controls – this has been demonstrated in this study (section 4.3.2.3). In a study observing changes in the actin cytoskeleton following cell volume changes, it was established that changes in keratinocyte cell volume resulted in rapid reorganisation of the actin cytoskeleton (Blasé *et al*., 2009). This demonstrates the ability of the actin cytoskeleton to reorganise swiftly as and when required (such as, for example, following adhesion and subsequent cell spreading). In addition, further studies have demonstrated the importance of filopodia and lamellipodia. Since cell adhesion is required for cell function and movement, Schäfer *et al*. (2009) suggest

that this depends on focal complexes connecting the extracellular matrix to the actin cytoskeleton. Using keratinocytes, Schäfer *et al*. (2009) demonstrate that once the VASP-containing tip makes contact with the substrate, a filopodal focal complex forms. When these are reached by lamellipodia, the filopodal focal complexes increase in size resulting in focal adhesion (smaller focal complexes have a faster turnover, which allows more motility). Larger focal adhesions increase stability, and are important in anchoring cells, reducing motility. Vinculin, as a focal adhesion marker, was used in this study to highlight any focal adhesions formed (as previously used by, for example, Möhl *et al*., 2009). On average it was observed that more focal adhesions were in place in control siRNA transfected HaCaT cells than in K15 siRNA transfected HaCaT cells (section 4.3.2.2). It is possible that fewer focal adhesions were formed in K15 ablated cells due to the continued spreading of the cells at 180 minutes post-seeding (*Figure 4.2*).

It was also noted that changes in the arrangement of the K14 cytoskeleton occurred during cell adhesion and spreading in K15 ablated cells compared to control siRNA transfected cells. In the absence of K15, reduced localisation of K14 is observed at the cell edges (*Figure 4.7*). Alongside the abnormal actin localisation and the increased cell size measured in K15 ablated cells, it appears that in the absence of K15, HaCaT cells spread abnormally following seeding. Using keratinocyte cell lines established from EBS patients, Morley *et al*. (1995) established that, following heat shock treatment and replating, cells containing mutant keratins were slower to spread than controls. Morley *et al*. (1995) suggested that this was due to a delay in the restoration of the normal IF network. If spreading is slowed by disruption of the normal IF network, it indicates that K15 can not have an important structural role in normal keratinocytes, since the rate of spreading increases following K15 ablation. If K15 had a structural role, as K14 does, then the rate of spreading would be slower than controls (i.e. K15-positive cells), as demonstrated in K14-mutant keratinocytes by Morley *et al*. (1995). It has also been demonstrated that keratin filament precursors (KFPs) move along actin stress fibres to focal adhesion sites (Windoffer *et al*., 2006). This study has demonstrated that K15 ablated cells produce fewer focal adhesions than control siRNA transfected (i.e. K15-positive) cells. If fewer focal adhesion sites results in a decrease in KFP formation (as postulated by Windoffer *et al*., 2006), it is possible that this is at least partially responsible for the initial lack of K14 expressed at the cell edges in K15 ablated cells.
Webb, Li and Kaur (2004) have suggested that keratinocytes which are K10-negative and CD71-positive and have a greater cell size are transit amplifying cells; however Webb, Li and Kaur (2004) also found this population of cells to be K15-positive, suggesting a different underlying factor contributing to cell size. In addition, smaller cells (which have been shown to occur following *Myc* knock-out in the epidermis) have been observed in the bulge, expressing K15, CD200 and not CD34 or CD271 (Zanet *et al*., 2005; Inoue *et al*., 2009). It is possible that the K15 ablated cells used in this study were undergoing differentiation, increasing their cell size as part of this process. Garzia *et al*. (2011) have also noted that K15-positive cells are smaller than K15-negative cells in human hair follicles. This provides additional support for the theory that K15 does not provide mechanical support for the cell; larger cells express a different keratin profile based on their requirement for more structural support.

6.7: The Effects of K15 Ablation on Migration can Vary

6.7.1: Effects of K15 Ablation on Migration in HaCaT Cells and MET 1 Cells

Roh *et al*. (2005) and Roh *et al*. (2008) have demonstrated that hair follicle bulge cells differentiate prior to migrating, suggesting that K15-negative cells are more motile than K15-positive cells. In this experiment, K15 ablated cells initially migrated faster than control siRNA transfected cells, similar to those results observed by Roh *et al.* (2005) and Roh *et al*. (2008). After 24 h however, K15-positive cells had migrated more than K15 ablated cells; it is possible that results *in vivo* would be more useful, for example, observing bulge cell migration using label-retaining studies followed by *in situ* hybridization to detect cells which produce K15 mRNA.
In addition, no bundling of K15 or K14 was observed at the leading cells of the migrating sheet. This is in contrast to K8/K18, which has been observed to bundle at the cell edge (Long *et al*., 2006). Long *et al*. (2006) speculate that this may be a strengthening response to protect the migrating epithelial sheet against traction forces (as described by du Roure *et al*., 2005). Since no bundling of K15 or K14 was observed, it is possible that these keratins are not required to protect migrating sheets against traction forces. This would be logical since K14 and K15 are expressed in the basal layers, where cells have a lesser requirement for strength and need a more pliable keratin filament network to allow migration. Furthermore, Long *et al*. (2006) suggest that IFs are involved in subcellular targeting of desmosomal proteins. In K8 knock-down cells, cell-cell adhesions were broken down and desmoplakin and periplakin were redistributed to the cytosol from the cell borders. The absence of K15 did not appear to affect the localisation of cell adhesion proteins desmoplakin, E-cadherin, or ZO-1. This suggests that although K8 may have a role in subcellular targeting for these proteins, K15 does not. However, K15 ablated cells were found to produce fewer focal adhesions than K15-positive control cells.

Actin bundling at the leading edge however was observed in K15 ablated HaCaT cells. This has also been demonstrated in MCF-7 cells, where lamellipodia were also identified (Long *et al*., 2006). This is likely to occur at the leading edge of the migrating monolayer since lamellipodia are an important mechanism used in migration and cell spreading. In migrating keratinocytes cultured from K6-null mice, Wong and Coulombe (2003) also observed the increased intensity of actin staining following immunofluorescence (see also section 6.6.2).

This study has demonstrated that K15 has little effect on differentiation in MET cell line cells, but did have some effect on migration in MET 1 cells. Migration in control siRNA transfected MET 1 cells was quicker than in K15 ablated cells. This suggests that K15 has an active role in migration in MET 1 cells. In turn then, this implies that the function of K15 in MET 1 cells is not (only) to provide mechanical stability, but can affect other cell functions which results in increased migration capacity. It has previously been demonstrated that transfection of vimentin, K8 and K18 to melanoma cells results in an increased migratory and invasive phenotype. It was thought that this was due to an increase in the number of focal adhesions and actin stress fibre formation (Chu *et al*., 1996). Chu *et al*. (1996) postulate that this is due to increased cytoskeletal interactions with extracellular matrices at focal adhesion sites. It is possible that as K15-positive cells move more quickly than K15-negative cells, interaction with the extracellular matrix may also have an effect on cell migration as Chu *et al*. (1996) describe following expression of K8/18.
Proliferation of MET 1 cells was not investigated, however the proliferation capacity of these cells may have affected the overall migration rate of MET 1 cells. This said, the contribution may not be large as wounds were 100% closed in control siRNA transfected cells after 360 minutes, and approximately 85% closed by the same time

point in K15 siRNA transfected cells. This would leave only a short period of time for cell proliferation to have a significant effect on wound closure times.
This is opposite to the effect observed in K16 knock-down murine keratinocyte explants (Wawersik and Coulombe, 2000), likely to be due to reduced proliferation as opposed to impairment of migration (Wawersik *et al*., 2001). Also using the scratch wound assay, Morley *et al*. (2003) demonstrated that migration was significantly faster in cells with severe EBS mutations (i.e. cells without fully functioning K5 or K14). It was observed that this occurred independently of proliferation. Chu *et al*. (1991) hypothesized that additional IF expression in melanoma tumour cells may allow cells to be more migratory. The disruption of keratin in these cells resulted in disruption of keratin filament organization and a decrease in invasive (and metastatic) ability. The same group carried out work in a human melanoma cell line (A375P), which normally only expresses vimentin. By transfecting these cells it was possible to induce expression of K8 and K18, which increased the rate of migration in a cell line with low metastatic ability (Chu *et al*., 1993). Murine fibroblasts transfected in the same way also demonstrated an increase in migration (Chu *et al*., 1993).
It is suggested that increased migration is due to the upregulation of stress-activated kinase pathways, which has been known to occur in EBS-affected keratinocytes (see also section 6.7.2.6). However, this is an effect observed in cells with mutant keratins, not in keratinocytes where siRNA has been used. It has also been shown that the loss of intracellular junctions may affect migration in EBS-affected keratinocytes (Liovic *et al*., 2009). In addition, using keratinocytes from K6-null mice, Wong and Coulombe (2003) observed enhanced outgrowth, resulting from migration (as opposed to mitosis). A similar study using K6 knock-out mice demonstrated that a delay in the re-epithelialisation of hair follicles occurred, despite no similar delay in the migration or proliferation of keratinocytes (Wojcik, Bundman and Roop, 2000). Upregulation of K16 in HaCaT cells has been shown to have the opposite effect (i.e. reduced migration) (Trost *et al*., 2010). A reduction in tyrosine phosphorylation in K16 knock-down HaCaT cells was also observed (see also section 6.7.2.4). It was demonstrated that the reduced migration observed in HaCaT cells which overexpress K16 was not mediated by any change in the morphology of the actin cytoskeleton, nor changes in cell-cell junctions or focal adhesions (Trost *et al*., 2010). However, Roth *et al*. (2009) have demonstrated that reduction in K5 expression may affect the actin cytoskeleton via p120-catenin.

6.7.2: Mechanisms Which May Cause Keratins to Affect Cell Migration

6.7.2.1: p120-Catenin

Roth *et al*. (2009) suggest a molecular link between K5 ablation, p120-catenin signaling and NFκB-targeted gene expression, possibly regulated through the activity of Rho family GTPases. In wild-type basal keratinocytes, p120-catenin has been shown to colocalise with E-cadherin at adherens junctions (Roth *et al*., 2009), suggesting that as cells dissociate, p120-catenin levels are reduced. This occurred in K5 knock-out cells (Roth *et al*., 2009). This finding also demonstrated for the first time a regulatory function of an epidermal keratin which contributes to the EBS pathology (Roth *et al*., 2009). Furthermore, p120-catenin is capable of regulating the actin cytoskeleton via modulation of RhoGTPase activity and transport and stabilization of cadherins at adherens junctions (Perez-Moreno and Fuchs, 2006; Perez-Moreno *et al*., 2008). Roth *et al*. (2009) suggest that the keratin cytoskeleton (and therefore K15) may be an important regulator between adhesion and migration and connecting the epithelium and associated tissues to the immune system by regulating p120-catenin localisation and activity.

6.7.2.2: Stress-Activated Protein Kinase (SAPK) Pathways

The SAPK pathways (and p38 reactivating kinase pathways) are part of the cellular response to physical stress, inflammatory cytokines and toxins. Although the pathways are similar, the SAPK pathways are distinct from the MAPK pathway. Isoforms of SAPK have been identified; these are the Jun N-terminal kinases (JNKs) (Derijard *et al*., 1994; Kallunki *et al*., 1994; Tibbles and Woodgett, 1999; White *et al*., 1999; Xia and Karin, 2004; Huang *et al*., 2004). It has been suggested that JNK regulates migration by phosphorylating paxillin in the focal adhesion complex, through regulating assembly of microtubules (again by phosphorylation) and by phosphorylating c-Jun (a member of the AP-1 family of transcription factors) (White *et al*., 1999; Huang *et* al., 2003; Chang *et al*., 2003; Xia and Karin, 2004). It has been observed that a TGFβ-activin signal leads to JNK activation, c-Jun phosphorylation and the formation of actin stress fibres. This also results in epithelial cell migration (Zhang *et al*., 2003). Furthermore, keratinocytes isolated from keratinocyte-specific c-Jun knockout mice have demonstrated a reduced migratory response to EGF (Li *et al*., 2003; Zenz *et al*., 2003). Phosphorylation of c-Jun at the leading edge of a migratory tissue may also contribute to upregulation of the TGFα-EGFR-ERK pathway (Behrens *et al*., 1999). Javelaud *et al*. (2003) demonstrated that JNK phosphorylation of c-Jun resulted in impaired migration in human fibroblasts. More recently, it has been shown that the JNK1 isoform is important in mouse wound healing; the absence of this isoform delays the repair of mice injured by tape-stripping. A similar effect is not observed for JNK2 or JNK3 ablation (Koehler *et al*., 2011). The same study used human keratinocyte organotypic cultures to demonstrate that JNK1 activity increased during differentiation. In JNK1-null mice, full-thickness wound repair was initially faster than in wild-type mice. However, between seven and 14 days post-wounding, wound healing was significantly delayed compared to controls. Koehler *et al*. (2011) remark that this demonstrates the importance of JNK1 in full-thickness wound healing. Since in this study no significant difference was observed in migration rates between K15 siRNA and control siRNA transfected HaCaT cells, further work is required to establish whether there is any connection between these findings.

6.7.2.3: Tyrosine Phosphatase

Tyrosine phosphatase inhibition has been shown to disrupt the keratin filament network in a few minutes, although takes longer to recover (approximately half an hour) (Strnad, Windoffer and Leube, 2002). Inhibition of tyrosine phosphatase by orthovanadate results in the disappearance of the keratin filament network, which is replaced by numerous small granules, mediated by p38 MAPK (see section 6.7.2.2). This resulted in cells becoming more rounded and an increase in the number of actin stress fibres, which eventually concentrated in the cell cortex (Strnad, Windoffer and Leube, 2002). Strnad, Windoffer and Leube (2002) used time-lapse fluorescent microscopy to establish how the breakdown of the keratin network occurred. Bundles of filaments straightened, then fragmented and formed small rods or granules. This also caused disruption to cell adhesion. This said, it was postulated that these granules were still anchored to other parts of the cytoskeleton. Removal of the orthovandate resulted in a reversal of this process, where granules were observed to elongate and fuse, initially creating thin filaments. These filaments then increased in length and thickness, re-establishing the keratin network. It is possible that such remodeling of the keratin filament network is occurring in some of the experiments described in this study. K15 expression in MET cells appears more granular, similar

to some of the images of keratins produced in Strnad, Windoffer and Leube (2002). It may be that K15 is undergoing modification by a tryrosine phosphatase resulting in this unexpected appearance in the cell. Likewise, tyrosine phosphatase may also have a role in reducing the K14 network at the cell edges during cell adhesion in K15 ablated cells, although no evidence of K14 granules were observed.

6.7.2.4 Molecular Ratios and Compromise

Julien (1999) suggested that disruption of the molar ratio between light, medium and heavy subunits of neurofilaments has consequences for assembly and therefore function in neurons. Wawersik *et al*. (2001) suggest then that disruption of the balance of keratins may be responsible for changes in migration observed following artificial knock-down of some keratins. This could particularly be the case when wound healing requires a compromise between mechanical stability and the plasticity required for cell migration; Bernot *et al*. (2002) and Wong and Coulombe (2003) suggest that K16 is an ideal candidate for this. This is supported by the finding of Mazzalupo *et al*. (2003), who suggest that mouse embryos (~E11.5) have the ability to induce K6, K16 and K17 in response to injury. Furthermore, Trost *et al*. (2010) have demonstrated that increased K16 expression alone reduces migration, however alongside a similar increase in K6, migration levels increase. It has been suggested that K15 undergoes posttranslational modification (for example, *Figure 3.6*), which may affect the molar ratio of K15/K5 filaments and therefore affect migration. This may particularly be the case in MET cells, where K15 expression was more granular and less filamentous than that observed in HaCaT cells.

6.7.2.5 Cell Adhesions and Actin

Cell-cell interactions have previously been shown to have a role in cell migration, both directly and indirectly. For example, Kim and Joo (2002) have demonstrated that Cas (Crk-associated substrate), localised at focal adhesions, is involved in cell migration and induction of gene expression. Tyrosine phosphorylation of Cas is induced by TGFβ; TGFβ signaling in turn is mediated by E-cadherin. An intact actin cytoskeleton is also required.

Focal complexes and adhesions are highly dynamic; alongside actin reorganization, these processed are vital for cell migration (Zamir *et al*., 2000; Webb, Parsons and Horwitz, 2002; Pollard and Borisy, 2003; Gupton and Waterman-Storer, 2006; Schäfer *et* al., 2009). Recently, work has shown that newly-formed adhesions at the leading edge of a cell have different vinculin dynamics compared to more mature adhesions at the back of the cell. As phosphorylation of vinculin decreases, the strength of focal adhesions increases (Möhl *et al*. 2009).

The formation of filopodia is also important in keratinocyte migration, a process which begins in the embryo. The migration process has been described as a four-step cycle: protrusion, formation of stable adhesion sites, contraction of the cell and rear release (Lauffenburger and Horwitz, 1996; Sheetz, Felsenfeld and Galbraith, 1998). Filopodia and lamellipodia are important during protrusion. Formation of filopodia has been shown to occur as exact extentions of lamellipodal focal adhesion sites. Where they are not able to attach stably to a substrate, the filopodia collapse and retract, and no focal adhesions form (Schäfer *et* al., 2009). There are two models which describe possible mechanisms for the formation of focal complexes. In the first, filopodia act as sensors, detecting appropriate substrate conditions for adhesion (Wood and Martin, 2002). In the second, small adhesion sites are already present along the filopodia which become more stable over time (Zaidel-Bar *et al*., 2004). This would explain the presence of VASP, vinculin, paxillin, talin, zyxin and tensin

which form filopodial focal complexes (Zaidel-Bar *et al*., 2004; Schäfer *et* al., 2009). Schäfer *et* al. (2009) suggest that filopodia move over the substrate until stable contact with VASP occurs at the tip spot. Following this, filopodial focal complexes form directly behind this tip, and filopodia regain their ability to elongate. Focal adhesion sites form once the cell body has moved enough for lamellipodia to reach the site. As well as cell guiding, filopodia have been shown to contribute to the formation of actin stress fibres (Nemethova, Auinger and Small, 2008). It has been shown that c-Jun, one of the SAPKs, is involved in forming filopodia.
It has also been demonstrated in endothelial cells that inhibition of actin polymerisation and assembly of focal adhesions reduces migration (Cezar-de-Mello *et al*., 2006). This was shown to involve the p38 (also known as stress-activated protein kinase 2) pathway. In control cells, FAK and actin were shown to colocalise at focal adhesions along the cells periphery. It is likely that the decrease in migration observed are also due to the lack of reorganization of actin and therefore defects in focal contact formation.

6.8: Conclusions

This study follows from the work of Werner, Werner and Munz (2000), which described the suppression of K15 expression *in vitro* by TGFβ and TNFα in HaCaT cells, and by cutaneous injury *in vivo*. Werner, Werner and Munz (2000) observed that levels of K15 mRNA were reduced in activated keratinocytes following skin injury. This paper also suggested that K15 regulation may be important in migration, differentiation, proliferation and wound healing.
All cell lines used in this study were found to be suitable for K15 siRNA transfection – i.e. all cells expressed K15 under control conditions and a marked reduction in K15 expression was observed following transfection. This allowed K15 ablation to be studied in primary keratinocytes, an immortalised keratinocyte cell line, and in SCC cells of different grades.
It was found that in the absence of K15, cells proliferate to a greater extent, cells begin to differentiate, upon seeding cells spread faster than controls (a process which involves changes in K14 and actin localisation, and a reduction in the circularity of cells) and produce fewer focal adhesions, and has some effect on cell migration. However, further experiments are required for confirmation of these results.
These results suggest that the structural role of K15/K5 filaments may not be as important as its role in signaling. It has previously been suggested that K15 expression in basal cells may prevent apoptosis, even under GVHD (graft-versus-host disease) conditions (Zhan *et al*., 2007). Expression of K15 in human bulge cells has also been shown to be unaffected by aging (Rittié *et al*., 2009), which suggests that K15-positive cells are slower cycling.

6.9: Future Outlook

The following discusses several possible methods which would aid in further establishing the function of K15 in cells and tissues.
Initially, additional markers of differentiation could be tested for. Immunofluorescence staining of K1 and K10, often used as differentiation markers (for example, Zhu *et al*., 1999), could be carried out on K15 siRNA transfected, control transfected and untransfected HaCaT cells. It would also be useful to carry out K1 and K10 staining on skin sections, since this would show the transition of basal cells to the spinous layer (as described in Zhu *et al*., 1999, for example). In

addition another useful technique would be the use of reverse transcription (RT)-PCR to validate the immunofluorescence data reported here. This would demonstrate the abundance of each RNA of interest (i.e. the keratins in particular) to demonstrate relative gene expression.
Long-term transfection could be used to carry out longer versions of the experiments carried out in this study (for example, longer exposure to high or low levels of extracellular calcium). This would also make culture of K15-negative organotypic cell cultures more feasible, since the transfection would ablate K15 for the entire air-liquid interface phase of culture growth.
The creation of a K15-null mouse model would further indicate the importance of K15 function in development and the effect of K15 ablation in the IFE.
Since keratins have also been shown to be important in signaling, posttranslational modifications of K15 would also aid in establishing which cellular responses K15 is involved in.

6.9.1: Gene Targeting

6.9.1.1 Genetically Engineered Mouse Models

Genetically engineered mice, have, in recent years, allowed researchers to investigate the importance of genes and proteins in a mammal that shares a significant amount of its physiology and pathology with humans. Animal models of disease have been produced which allow exploration of disease development and therapy testing. Several techniques have been developed for the creation of GEMMs (genetically engineered mouse models). Transgenic GEMMs are created by microinsertion of recombinant DNA into the pronucleus of the oocyte and transplantation of the oocyte into a foster female. The transgene(s) is inserted randomly into the mouse genome. Targeted mutation can be used to either knock-out or knock-in a gene. This procedure requires the homologous recombination of embryonic stem cells, which are inserted into blastocysts before transplantation into a foster female. These techniques allow the most control over genetic manipulation, compared to a technique such as random mutagenesis, where animals are exposed to DNA-altering chemicals or radiation. However, these techniques are not without their drawbacks. It is possible that interfering with the expression pattern of one gene may affect the expression pattern of genes near the inactivated gene locus or surrounding the (often randomly) inserted transgene.
In particular, many targeted mutation GEMMs have been produced for examination of skin conditions such as EBS (for example, as utilised by Lu *et al*., 2007), and keratins in particular have been targeted using GEMMs, such as expression of mutant *KRT6α* leading to changes in skin and hair phenotype (for example, Wojcik, Bundman and Roop, 2000) (Wojcik *et al*., 1999). In addition, tumour development has been studied using oncogene activation; *ras* has previously been shown to be involved in skin tumour initiation and several GEMMs are available to study this (Kemp, 2005). GEMMs of SCCs have also been developed (for example, Gatesman Ammer *et al*., 2011).
This study has demonstrated that K15 expression in murine skin is similar to that in human skin (chapter 3). The production of a targeted mutation GEMM with a K15 knock-out would demonstrate how the expression of this protein would affect skin development in the embryo and the effect on the IFE as an adult. It would also indicate whether any other keratins may have a compensatory effect following K15 ablation (for example, perhaps increased K14 levels). In addition, further studies could be carried out such as assessing the effect of K15 knock-out on wound healing *in vivo*. Primary keratinocytes from K15 knock-out mice could also be cultured *in*

vitro for further examination, such as immunofluorescence staining to establish any changes to the cytoskeletal structure of the cell. Another possible area of research would be the insertion of a reporter gene (such as *lacZ*). This would provide more information on when and where K15 is actively expressed, which would also give further clues to its function. Many *lacZ* reporter mouse models already exist, including for *lacZ* reporters for keratins. For example, Gu and Coulombe (2007) describe using a *hK6a-lacZ* reporter mouse model to analyse the regulation of *mK6a* mRNA during hair follicle cycling. Using this transgenic mouse eluded specificity issues related to the high sequence homology between K6a and other type II keratins. *KRT7-lacZ* transgenic mice have also been developed. Pujal *et al*. (2009) developed and used these mice to analyse *in vivo* activity of the gene. If a similar *KRT15-lacZ* mouse could be developed, information about when and where K15 is actively expressed could be elucidated.

6.9.1.2: Gene Targeting in Vitro

Gene targeting can also be used to create cell cultures *in vitro*, which can alter alleles in transduced cells. Recently, for example, Petek, Fleckman and Miller (2010) have produced human keratinocytes which have had one *KRT14* allele altered using a gene-targeted vector. Petek, Fleckman and Miller (2010) used a vector which used promoter trapping; this desgn can shift the balance of detection towards the recombinants, since integration of the vector at random locations does not 'trap' the activity of an active promoter. It was demonstrated that this system worked particularly well in keratinocytes. A downside of this type of transduction is that the vector will integrate at random locations in up to 10% of cells (Hirata *et al*., 2002; Petek, Fleckman and Miller 2010). This type of manipulation is useful for *in vitro* studies on human keratinocytes, such as 3D organotypic culture (it may also be possible to then graft these cultures onto athymic mice).

6.9.1.3: Conditional Knock-out Experiments

A common procedure for producing conditional knock-out mice is using the Cre/lox system. A genetically modified mouse if produced in which the gene of interest is flanked by two lox sites. This is then bred with a mouse expressing Cre recombinase; Cre recombines the two lox sites, removing the gene of interest from the genome. A similar procedure is followed when using the Flp/Frt recombination system, whereby Flp recombinase target (Frt) sequences flank the gene of interest, and flippase recombinase (Flp) excises the gene. When used in combination with, for example, the ER/tamoxifen system, the timing of the knockout can be controlled by delivering the drug tamoxifen. This technique would be particularly useful if the knockout of a gene would result in lethality during development, since the timing of the knockout can be delayed until adulthood if required. Investigation of the use of such methods has been carried out with regards to keratins (for example Ramirez *et al*., 2001; Means *et al*., 2005; Fujioka *et al*., 2011).

For investigation of K15 function, this would be a useful technique to employ if K15 expression significantly affected IFE development in the embryo (making the study of the adult epidermis difficult to compare to normal (i.e. K15-positive) controls. The development of a GEMM whereby K15 could be knocked-out in the adult would demonstrate how K15 affects normal skin turnover in the adult. It may also aid in the study of alopecia, which has recently been linked to K15 in some studies (for example, Hoang *et al*., 2009). In addition this may also demonstrate whether any compensatory mechanisms may be in place following K15 ablation.

6.9.1.4: Generation of Stable Knock-out Cell Lines and the use of Lentiviral Vectors

The generation of long-term knock-out lines of a gene of interest can be achieved in several ways. Generally this involves the integration of plasmid DNA into the genome. To select those cells which have successfully incorporated the plasmid into the genome, antbiotic selection can be used. Plasmid DNA, adenoviral, retroviral, and lentiviral techniques have all been used to produce stable knock-out (or knock-in) cell lines.

Lentiviral transfection has previously been used in epidermal stem cells (for example Di Nunzio *et al*., 2008), to introduce oncogene expression (for example Siwko *et al*., 2008) and importantly, to identify the activity of the K5 promoter (Endo *et al*., 2008). This latter injected lentiviral vectors encoding GFP into the K5 promoter during embryonic development, which allowed GFP production to be examined throughout development and in the adult. Again, if a similar technique could be employed to examine K15 promotor activity, insights could be gained into the conditions required for K15 expression. It would also indicate those conditions whereby K15 expression does not occur.

6.9.2: Study of Posttranslational Modifications

Part of the role of IFs is to modify cellular processes, such as the stress response, through their ability to regulate signaling molecules (Pallari and Eriksson, 2006). Posttranslational modifications (PTMs) are the key to functional diversity of IF proteins, affecting their structure and properties. PTMs have been shown to control some aspects of IF functions as well as maintain their dynamic properties (as reviewed by Hyder *et al*., 2008). Phosphorylation has so far been the most widely studied PTM, which is still considered to be the most consequential regulator of IF dynamics and function. More recently, O-linked glycosylation has been investigated as an additional regulator of phosphorylation in IFs.

It has been more than twenty years since phosphorylation was shown to be important in IF reorganisation during mitosis (Chou, Rosevear and Goldman, 1989); since then it has been demonstrated that the N-terminal domain of IFs (also important in polymerisation) is polymerised during mitosis (Ralton *et al*., 1994; Beuttenmuller *et al*., 1994). K18, phosphorylated on Ser33 during mitosis (Sihag *et al*., 2007), is also phosphorylated at the same point during interphase (Ku, Liao and Omary, 1998), suggesting that phosphorylation can be modulated at several sites during different cell phases.

O-linked glycoslylation (the addition or removal of saccharides onto or from the hydroxyl atoms of threonine or serine residues) has also been demonstrated to have a role during mitosis (Chou and Omary, 1993), whilst Slawson *et al*. (2006) demonstrated that it may have a role as a nutrient sensor and therefore signal transduction. It has also been suggested that O-linked glycosylation may compete with phosphorylation, which Wells *et al*. (2004) calls the 'yin-yang' hypothesis. Tao *et al*. (2006b) have shown for example that Ser48 O-linked glycosylation and phosphorylation on Ser52 of K18 could regulate each other.

Structural modifications of IFs, regulated by phosphorylation, occurs during stresses and disease states (Magin, Reichelt and Hatzfeld, 2004; Pekny and Lane, 2007; Godsel, Hobbs and Green, 2008). For example, Ridge *et al*. (2005) have demonstrated that K8 reorganisation is at least in part mediated by Ser73 phosphorylation by PKCδ, a downstream effector of activated PLC (which acts as a signal transducer of extracellular stimuli). Hyperphosphorylation (and the following modification) can indicate a loss of mechanical integrity. Phsphorylation of Ser24 of K8 determines where desmoplakin is deposited in the desmosome. This process than

is important in maintaining membrane integrity and signaling scaffolds during cell stress (Loranger *et al*., 2006). Furthermore, phosphorylation of keratins has also been shown to be important in keratin turnover. For example, phosphorylation of multiple sites on K8 and K18 aids in protecting against ubiquitination (and therefore degradation) of K8/18 (Ku and Omary, 2000).
PTM can also act as a regulator of signaling function. For example, the members of the 14-3-3 family can modulate the function of several proteins by interaction with phoshorylated IFs. For example, Ser33 phosphorylation of K18 during mitosis enhances K18 interaction with 14-3-3ζ, affecting K18 organisation and localisation (Liao and Omary, 1996; Ku, Liao and Omary, 1998). In addition, 14-3-3σ has been shown to be involved in hyperproliferation and an increase in keratinocyte size in K10-null mice (Reichelt and Magin, 2002). Reichelt and Magin (2002) have also shown that 14-3-3σ accumulates in the nucleus and binds to phosphorylation sites on K17. This activates the mTOR/Akt pathway, important in cell growth.

Several methods can be utilised in the study of PTMs. Initial examination using 2D gel electrophoresis can indicate whether phosphorylation is occurring. If used in combination with, for example, FACS, this could indicate particular cell stages where K15 phosphorylation was occurring. Furthermore, immunofluorescence has also been used to demonstrate where keratins colocalise with proteins associated with phosphorylation, such as 14-3-3 (such as in Ku, Liao and Omary, 1998). This is a simple method that would establish which proteins which may affect PTM of K15 by association. Pittenger *et al*. (2008) used site-directed spin labeling and electon paramagnetic resonance (SDSL-EPR) to examine the structural changes to vinculin following PKA phosphorylation *in vitro*. This established where phosphorylation was occurring, which meant that the effect this phosphorylation had could be hypothesised. If SDSL-EPR could be used to investigate K15 phosphorylation, it may suggest where phosphorylation occurs, giving clues to the effect this PTM has on K15 structure and function. Omary *et al*. (2006) also suggest that GEMMs are a useful way of studying phosphorylation. For example, transgenic mice have been created which overexpress K18 Ser33Ala (Ku *et al*., 2002), K18 Ser52Ala (Ku, Liao and Omary, 1998) and K8 Ser73Ala (Ku and Omary, 2006); the phenotype of these 'phosphorylated' mutant mice demonstrate the importance of phosphorylation in filament organisation, mitosis and cytoprotection. Similar work, once phosphorylation sites have been identified, may elucidate similar functions for K15.

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