Defining and Targeting Differentiation of Non-melanoma Skin Cancer

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Doctor of Medicine

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Declaration

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Abstract

Human cancer stem cells are proposed to play a critical role in tumour initiation and maintenance by their exclusive ability to regenerate the tumour. Thus cancer stem cells share many of the properties of normal stem cell including self-renewal and ability to give rise to progeny which undergo tissue-specific differentiation. Thus we hypothesised that by determining the normal patterns of tissue differentiation within cancer we could identify tumour type specific factors that promote differentiation, for therapeutic development. Therefore the aim of this study is to define patterns of human hair follicle differentiation in human basal cell carcinoma (BCC) in order to elucidate potential drug-able targets that can promote tumour specific differentiation. To test this hypothesis we analysed 20 different hair follicle specific differentiation markers, which define distinct layers within the normal adult hair, in six different human BCC samples using RT-PCR with normal hair follicle tissue as control. For the 12 specific keratin genes expressed in the BCC, we analysed expression by immunofluorescence on 20 different BCC samples, using hair follicle samples as positive controls. Our findings suggest that human BCC demonstrates both inward and upward differentiation patterns similar to the hair follicle, with expression of: outer root sheath (K5,14,16,and k17), companion layer (K75), inner root sheath (K26,27,28,71,72,and k74), and cuticle (K32,35,82,and k85); but not hair shaft (K31) markers. Consistent with these findings we observed the mutually exclusive relationship between expression of the early differentiation marker K19 and cell proliferation in the hair follicle and BCC. Similarly, expression of the outer root sheath keratins coincided with nuclear translocation of both GLI1 and NFIL-6, suggesting that BCC also share normal hair follicle tissue regulatory pathways. To further test the hypothesis that normal tissue factors observed in the hair follicle regulate BCC differentiation we have developed an *in vitro* BCC assay. Using this tissue culture model we hypothesised that BCC's are stuck in the telogen part of the hair follicle cycle, resulting from autocrine expression of bone morphogenic proteins 2 and 4. Inhibition of BMP signalling by addition of noggin as well as addition of TGF-β to BCC colonies in tissue culture led to further induction of inner root sheath, cuticle and medulla keratins. In summary we have shown that BCC exhibit hair follicle differentiation, which is similarly regulated, but is stuck in telogen arrest and can be rescued by addition of noggin and TGF-β2.

List of Presentation and Publication Arising From This Work

Publications

A. Original papers

Colmont CS, **BenKetah A**, Errington RJ*, et al.* (2014) Human basal cell carcinoma tumor-initiating cells are resistant to etoposide. *J Invest Dermatol* 134:867-70.

Colmont CS, **Benketah A**, Reed SH*, et al.* (2013). CD200-expressing human basal cell carcinoma cells initiate tumor growth. *Proc Natl Acad Sci U S A* 110:1434-9.

B. Abstracts (Oral and Posters Presentation)

A.BenKetah, P. Bowden, S. Reed and G. Patel. Human basal cell carcinoma demonstrates telogen arrest *British Journal of Dermatology (2014) 170, ppe6–e18.* (Oral presentation at BSID Meeting, Newcastle, 7-9 April, 2014).

A BenKetah, SH Reed, PE Bowden and GK Patel. Defining and targeting differentiation pathways in non-melanoma skin cancer. Carcinogenesis and Cancer Genetics. *Journal of Investigative Dermatology (2013); 133 (P 439),*
(Poster).
A. Benketah, G. Patel, S. Reed and P. Bowden . Human Basal Cell Carcinoma Differentiate Along Normal Hair Follicle Lineages. European Cancer Stem Cell Research day, Cardiff 26 January 2012 ; (Secured the best poster presentation)

A. Benketah, G. Patel, S. Reed and P. Bowden. Defining and targeting differentiation pathways in basal cell carcinoma. *Br.J.Dermatol* 2011 Jul;165 Suppl 1: pp 82-92 (PD19). (Poster presentation at the 91st British Association of Dermatologists)

A Benketah, Girish Patel, Paul Bowden. Defining and targeting differentiation pathways in basal cell carcinoma. *Journal of Investigative Dermatology* (2011), Volume 131 (p152), (poster presentation at 41st Annual Meeting of the European Society for Dermatological Research (ESDR), Barcelona, Spain; 7-10 September 2011.

A. Benketah, G. Patel, S. Reed and P. Bowden. Defining and targeting differentiation pathways in non-melanoma skin cancer. (Poster presentation) at the 25th Annual Postgraduate Research Day, School of Medicine, Cardiff University; 19th November 2010.

Dedication

To my sweetest most dedicated and truly beloved husband for his time, assistance and patience, to my parents for their love, support and prays, and to my lovely kids for keeping me joyful.

Acknowledgements

Abbreviation

Ab	Antibody
APRT	Adenine phosphoribosyltransferase
APM	Arrector Pili Muscle
ATP	Adenosine triphosphate
BCC	Basal Cell Carcinoma
Bcl-2	B-cell CLL/lymphoma 2
BCN	Basal cell nevus syndrome
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
BMP	Bone morphogenic protein
BMPr	Bone morphogenic protein receptor
BMPihh	Bone morphogenic protein inhibitor
cDNA	Complementary deoxyribonucleic acid
CL	Companion Layer
ORS	Outer root sheath layer
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
dH$_2$O	Distilled sterile water
DHH	Desert Hedgehog
Disp	Dispatched
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
PBS	phosphate buffered saline
DPX	Di-N-Butyle Phthalate in Xylene
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFr	Epidermal growth factor receptor
END2	Endothelin 2
FBS	Foetal Bovine serum
GAPDH	Glyceraldehyde3- Phosphate Dehdrogenase
GFP	Green Fluorescent Protein

GLI1	GLIoma-associated oncogenes homologue 1
GLI2	GLIoma-associated oncogenes homologue 2
GLI3	GLIoma-associated oncogenes homologue 3
GLIA/GLIact	GLI activated
GLIR	GLI repressor form
Grk2	G protein–coupled receptor kinase 2
GSK3β	Glycogen synthase kinase 3 beta
HaCaT	Keratinocyte cell line
HCA2	Fibroblast cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HH	Hedgehog
HDAC	Histone deacetylase inhibitors
HS	Hair shaft
IgG	Immunoglobulin
IHC	Immunohistochemistry
IHH	Indian Hedgehog
IRS	Inner root sheath
JAG-2	Jagged 2
JUN	Forms the AP-1 early response transcription factor
KAPs	Keratin-associated proteins
KDa	KiloDalton
KHG	Keratohyalin granules
KIT	Keratinocyte (melanocytes)
KRT	Keratins
KV	Kilo volt
mRNA	Messenger ribonucleic acid
NF-ķB	Nuclear Factor Kappa-light chain enhancer of activated B-cells
ORS	Outer root sheath
P53	Protein 53
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFRα	Platelet-derived growth factor receptor α
PTCH	Patched
qPCR	Quantitative Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	Interference ribonucleic acid

Rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcription-Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SHH	Sonic Hedgehog
SMO	Smoothened
SMAD	mother against decepetapegic homologue
S-S	Sulfide-sulfide
STAT	Singnal Traaaansducer and Activator of Transcription
SUFU	Suppressor of Fused
NOD-SCID	Non-Obese Diabetic – Sever Compromise-Immune-Deficient mice
TAE	Tris base, acetic acid and EDTA
TBS	Tris buffered saline
TBP2	Thyrodxine binding protein 2
TGF-β	Transforming growth factor β
TIC	Tumour initiating cells
TNF	Tumour Necrosis Factor
Tris	Tris (hydroxymethyl)aminomethane
UV	Ultraviolet
Wnt	Wingless/MMTV integration site factor

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Chapter 1. Introduction

1.1 Cancer Stem Cells

1.1.1 Stem cells

Stem cells are defined as non-specified (undifferentiated) cells that are capable of perpetuating themselves as stem cells and of undergoing differentiation into more specialized types of cells (Till and McCulloch, 1961; Weissman, 2000a). As such, stem cells are present in all multicellular organisms and in mammals are broadly divided into two types: embryonic stem cells and adult stem cells (Figure 1.1). Embryonic stem cells were first isolated from mouse embryos in 1981 and are able to give rise to all lineages apart from conception descendant (Evans and Kaufman, 1981; Martin, 1981; Thomson *et al.*, 1998).

After embryonic development cells lose their plasticity, but within tissue there remain adult tissue stem cells (Figure 1.1). In contrast to embryonic stem cells, under physiological conditions adult tissue stem cells only give rise to tissue specific lineages (are lineage restricted). The best characterised of these adult tissue stem cells is the haematopoietic stem cell, which is able to give rise to all the blood forming lineages (Dzierzak and Speck, 2008). In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing adult tissues (Weissman, 2000a). Thus, embryonic stem cells and adult tissue stem cells represent two distinct subgroups of normal tissue stem cells (Figure 1.1).

Adult tissue stem cells are relatively small in number but have now been identified in many tissues. They are believed to reside in specific areas within organs, the stem cell niche, and often they are quiescent for a long period of time until they become activated following disease or injury. The adult tissues in which stem cells have been demonstrated, include skin (Kaur and Potten, 2011; Potten and Hendry, 1973), hair follicle (Blanpain *et al.*, 2004; Cotsarelis, 2006; Ohyama *et al.*, 2006), brain (Clarke *et al.*, 2000; Weissman, 2000b), liver (Horb *et al.*, 2003), pancreas (Gmyr *et al.*, 2000), skeletal muscle (Asakura *et al.*, 2002), corneal limb (Pellegrini *et al.*, 1999), mammary gland (Shackleton *et al.*, 2006; Stingl *et al.*, 2006) and heart (Beltrami *et al.*, 2003). Thus, adult tissues contain rare and long-lived adult tissue stem cells that have the capacity to maintain and repair the tissue.

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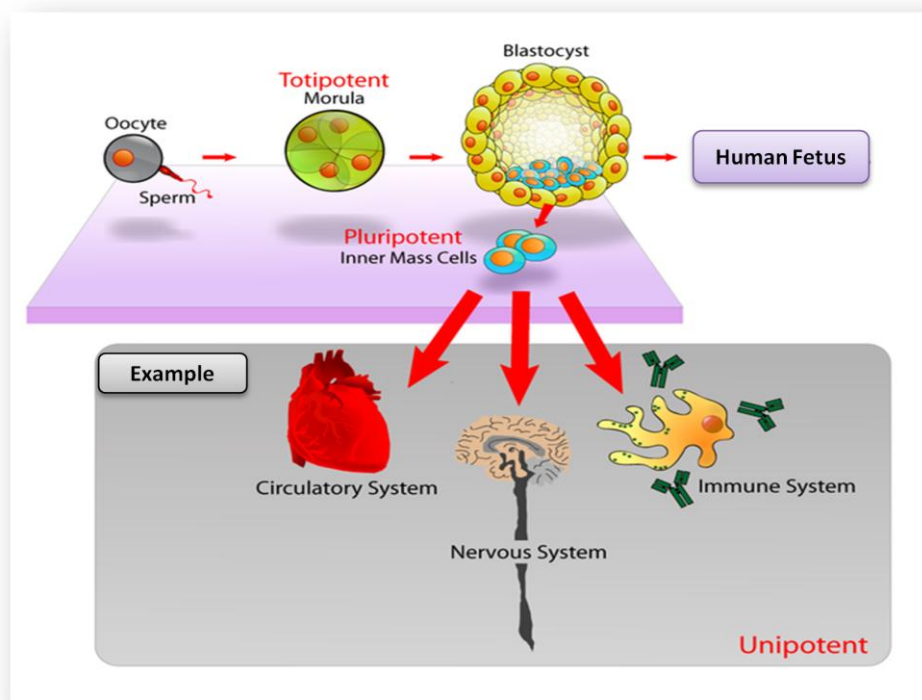
Figure 1.1: Embryonic and adult stem cells.
Adapted from online source (Wikipedia). Reprinted with permission of Wikipedia. Copyright © 2014.

1.1.2   Symmetric and asymmetric stem cell division

Stem cells possess the unique ability to self-renew and simultaneously generate differentiated progeny (Morrison and Kimble, 2006). Stem cells can divide symmetrically, whereby each daughter cell retains the properties of the parental cells, or asymmetrically, whereby one daughter cell retains the properties of the parental stem cell and the other daughter cell begins the process of differentiation (Figure 1.2) (Sherley, 2002).
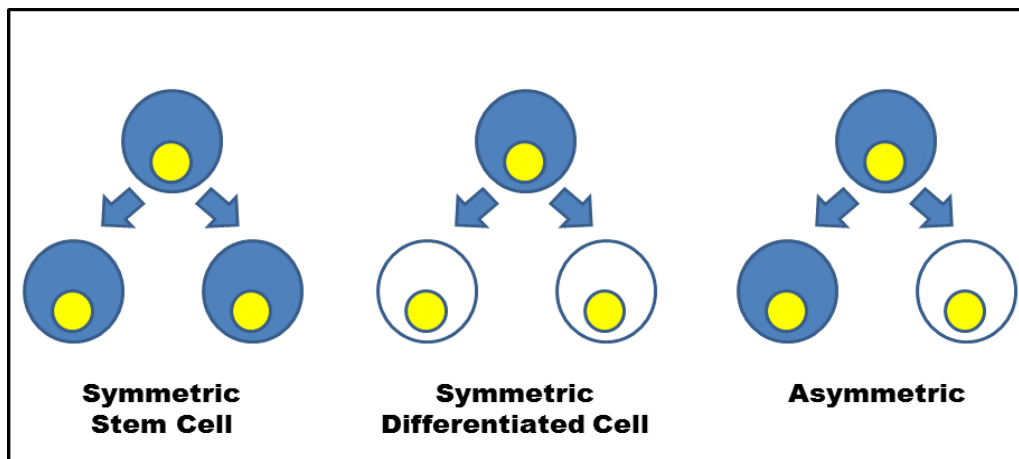
Figure 1.2: Diagram illustrating symmetric and asymmetric division of stem cells.
Blue cells are stem cells, and white cells are differentiated cells.

For example embryonic stem cells divide symmetrically during early embryonic growth, such that each daughter cell remains a pluripotent stem cell; resulting in a logarithmic expansion of cells (Evans and Kaufman, 1981). During germ layers formation in the early embryo and later, cells predominantly proliferate asymmetrically, as one daughter cell continue the process of self-renewal and the other daughter cell starts the process of differentiation.

Two different mechanisms for asymmetric stem cell division have been described (Betschinger and Knoblich, 2004; Clevers, 2005; Horvitz and Herskowitz, 1992). Intrinsic asymmetric stem cell division relies on the apical-basal relationship (planar polarity) between cells, characteristically evident in epithelia, to orientate mitosis. During mitosis in intrinsic asymmetric stem cell division the daughter cell programmed to undergo differentiation develops asymmetric protein localisation, which can be used to determine its subsequent fate. Although the same protein asymmetric localisation determines cell fate decisions during extrinsic asymmetric stem cell division, the external cues are derived from the location of the stem cell niche (Li and Xie, 2005). Thus both embryonic and adult tissue stem cells utilise symmetric and asymmetric division.

1.1.3 Adult tissue stem cells and cellular organisation of tissues

The presence of stem cells because of their epigenetic differences, establish a cellular hierarchy in which the stem cell is atop and is responsible for maintaining and replenishing the heterogeneous population of differentiated tissue cells. The best characterised and easily appreciable illustration of the relationship between adult tissue stem cells and differentiated progeny is during haematopoiesis (Fernández and de Alarcón, 2013). In a normal healthy adult, approximately 10^{12} new blood cells are produced daily in order to replenish and maintain steady state levels in the peripheral circulation (Reya *et al.*, 2001). Haematopoietic adult tissue stem cells located in the bone medulla (bone marrow) have the ability to form all the various types of blood cells (Till and McCulloch, 1961). Haematopoietic adult tissue stem cells have the capacity to self-renew and upon proliferation at least some of their daughter cells remain as haematopoietic adult tissue stem cells, to maintain and not deplete the haematopoietic

adult tissue stem cell pool (Morrison and Kimble, 2006). The other daughters of haematopoietic adult tissue stem cell, however commit to any of the alternative differentiation pathways that lead to the production of one or more specific types of blood cells (Alenzi *et al.*, 2009). Thus cells committed to differentiation represent the bulk population in the haematopoietic tissue, but cannot self-renew (Figure 1.3). The frequency of these progenitor, haematopoietic stem cells has been estimated by the ability to colony forming units to be 0.01% of the total number of bone marrow cells (Goodell *et al.*, 1996).
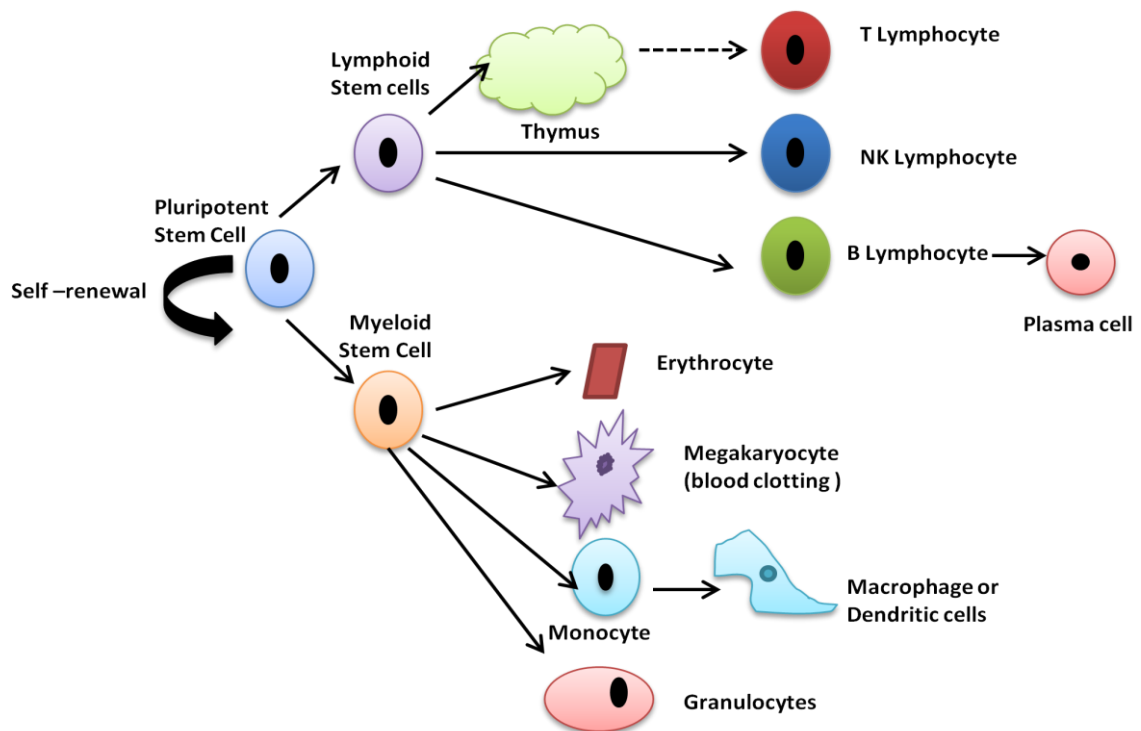
Figure 1.3: The haematopoietic stem cell division. Adapted from (Smith, 2003).

Adult tissue stem cells also reside in the skin to maintain epidermal cell turnover and repair after injury (Ghadially, 2012). Human skin epidermis is organised so that cells of the basal layer proliferate and give rise to suprabasal layers of differentiated cells (Mackenzie, 1970; Mackenzie, 1969).

In vitro colony forming assays have determined that the proliferative capacity of epidermal keratinocytes fall into three categories, based upon the pattern of the colony formed and ability to passage these cells serially: large smooth colonies with less than 5% of small abortive colonies are termed holoclones; small and abortive colonies of terminally differentiating cells called paraclones; and any colonies with intermediate proliferative capacity called meroclones (Rheinwald and Green, 1975). These findings mirror *in vivo* data labelling proliferating cells, with short pulse and long pulse-washout experiments, which illustrate the existence of both a label-retaining basal keratinocyte population of stem cells and a short lived rapidly proliferating population of transient amplifying cells (Morris *et al.*, 1985; Morris *et al.*, 1988; Potten and Morris, 1988). Hence these findings suggest a hierarchy of epidermal stem cells beginning with an epidermal stem cell, which gives rise to a continuum of cell populations with diminished capacity to proliferate and self-renew.

More definitive studies have been able to identify keratinocyte stem cells in the hair follicle, an epidermal mini-organ that throughout life undergoes cyclical cell turnover. Earlier studies had alluded to the presence of conventional label retaining stem cells in the bulge region of the hair follicle (Cotsarelis *et al.*, 1990). However the subsequent isolation of viable label retaining cells was made possible with transgenic mouse models, which directly evidenced by the ability of these cells to recreate the hair follicle (Tumbar *et al.*, 2004). Additional studies have substantiated the hair follicle bulge as the anatomical location of hair follicle stem cells (Cotsarelis, 2006), both in the mouse and human (Ohyama *et al.*, 2006).

Thus hair follicle bulge keratinocyte stem cells maintain and replenish the hair follicle epithelial cell lineages during hair follicle cyclical growth.

1.1.4 Tumour heterogeneity

Tumour cells exhibit heterogeneity as they progress in malignancy (Miller, 1982). Even though cancers are widely accepted to be initiated by one single cell and are genetically clonal in origin, they continue demonstrate cellular diversity during growth (Heppner, 1984). Tumour heterogeneity can be observed by variation in cell surface markers (Dexter *et al.*, 1978; Raz *et al.*, 1980), including tumour antigens (Miller and Heppner, 1979). But there is also intra-tumoural heterogeneity in genetic abnormalities (Mitelman *et al.*, 1972; Shapiro *et al.*, 1981). And so many tumours display genetic heterogeneity (Burrell *et al.*, 2013; Snuderl *et al.*, 2011). These differences result in differential growth rates within the tumour (Danielson *et al.*, 1980) and response to therapy (Barranco *et al.*, 1972; Yung *et al.*, 1982). Thus tumour heterogeneity represents a scientific and clinical problem.

The cellular heterogeneity within tumours raises an important question, do all tumour cells possess the same capacity to propagate or initiate new tumours? Initial observations in spontaneous murine cancer models suggested not all cancer cells could initiate new tumour growth (Bruce and Van der Gaag, 1963). Indeed similar observations were made in human cancers, using auto-transplantation (Southam *et al.*, 1962). These studies suggested that tumour heterogeneity also reflected heterogeneity in the cancer cell tumour initiating capacity and since over 1 million tumour cells were required to propagate tumour growth, it suggest that the frequency of tumour initiating cells within cancers was rare.

1.1.5 Tumour initiating capacity and cancer stem cells

It has been postulated for some time that in renewing tissues, such as the skin, only long-lived cells, i.e. stem cells, have sufficient time to acquire transforming mutations (Arwert *et al.*, 2012; Owens and Watt, 2003). In the classical model of skin two-step carcinogenesis model , benz (o) pyrene, the initiator binds to DNA causing a permanent genetic alteration (Friedewald and Rous, 1944). If the site is stimulated to proliferate by a tumour promoter or wounding epithelial cancer appears, yet the interval can be days, or even months or years in length (Boutwell, 1964). The location of benz(o) pyrene and the mutation induced was subsequently found to be in epidermal keratinocyte stem cells (Kangsamaksin *et al.*, 2007). Thus it is clear that the only way in which the initiated cells could still be present, if months or years have passed since initiation, is for initiation to have occurred in the self-renewing progenitor cell population.

Similarly, leukaemia was thought of as arising from cells of the haematopoietic system (Till and McCulloch, 1980). Suggesting that may be the heterogeneity that is an integral part of development of normal tissue could also be responsible for generating tumour cell heterogeneity. In accordance, it was reported that the majority of leukaemia blasts were post-mitotic and needed to be replenished from a small population of proliferative cells, some of which were dividing slowly (Clarkson and Fried, 1971). These studies raised the possibility that leukaemia, akin to the normal haematopoetic system, exhibits a hierarchical organization.

A hierarchical tumour organization contrasts starkly against the stochastic model of cancer growth. In the stochastic model, tumours are considered as a clonally derived collection of cells, with proliferation due to fluctuations in substrates for biochemical

reactions, genetic aberrations, or in response to stimuli from the microenvironment (Elowitz *et al.*, 2002; Swain *et al.*, 2002). The effect of these internal and external variables culminates in changes in cell proliferation, tumour cell phenotype and ultimately tumour morphology. The stochastic model of tumour growth is the basis of the Darwinian model of tumour cell evolution (Nowell, 1976). In contrast, to normal organ hierarchies that determine organs, stem cells generate progeny that differentiate along particular lineages. Thus if cancers are to be considered in the context of tissues, then cancer stem cells need to be considered at the apex of a hierarchy (Pierce and Cox, 1978).

The hierarchical or cancer stem cell model predicts that tumours are comprised of cells with differing tumour growth potential and importantly that not every cell within a tumour possesses tumour initiation potential. Due to a hierarchically organized system, where cancer stem cells are the only cells that contain long-term self-renewal potential, then cancer stem cells are a requisite for clonal maintenance (Dick, 2008).

1.1.6 Cancer stem cell theory

The cancer stem cell theory proposes that the bulk of tumour cells are in a state of terminal differentiation, along normal tissue lineages (Valent *et al.*, 2012). Malignant cells therefore demonstrate a unidirectional hierarchy in which cancer stem cells constitute a biologically unique subset. Cancer stem cells by their ability to promote tumour growth indefinitely maintain their numbers and give rise to differentiated cells (Nguyen *et al.*, 2012; Reya *et al.*, 2001). It is after all the presence of differentiation which pathologists appreciate when diagnosing and grading tumours, such as stratification in cutaneous squamous cell carcinoma (so called "keratin pearls").

Even in tumours where malignant cells are architecturally indistinct, they still demonstrate normal tissue patterns of differentiation that can often be elicited by immunohistochemistry. While the majority of cells within a cancer exhibit differentiation, their presence and the growth of the tumour is dependent upon a small population of cancer stem cells.

The stem cell theory therefore proposes that cancer stem cells have the capacity to drive tumour growth, by their exclusive ability to proliferate long-term, give rise to progeny that undergo differentiation, as well as maintain its own population (self-renewal) (Gil *et al.*, 2008). Indeed the central tenet of the cancer stem cell theory enticingly promises cure for cancer patients if cancer stem cells are efficiently killed. In contrast, the classical stochastic model of cancer growth predicts that cancer cells proliferate extensively and give rises to sub-clones as they exhibit a proliferative advantage. However the cell autonomous random growth predicted by the stochastic model is based on the assumption that most, if not all, cancer cells maintain the capacity to drive tumour growth. This in turn has led to the cancer stem cell hypothesis (Dick, 2009; Hanahan and Weinberg, 2011), whereby the presence of cancer stem cells within a tumour can be determined if it can be shown that a small sub-population of tumour cells is able to recreate tumour growth in an *in vivo* model, while at the same time demonstrating that the remainder of the tumour cells are unable to perpetuate cancer growth (Figure 1.4). To date 16 human cancers have been determined to show cancer stem cell driven growth, including the three common skin cancers (Colmont *et al.*, 2012) using *in vivo* model (Table 1.1).
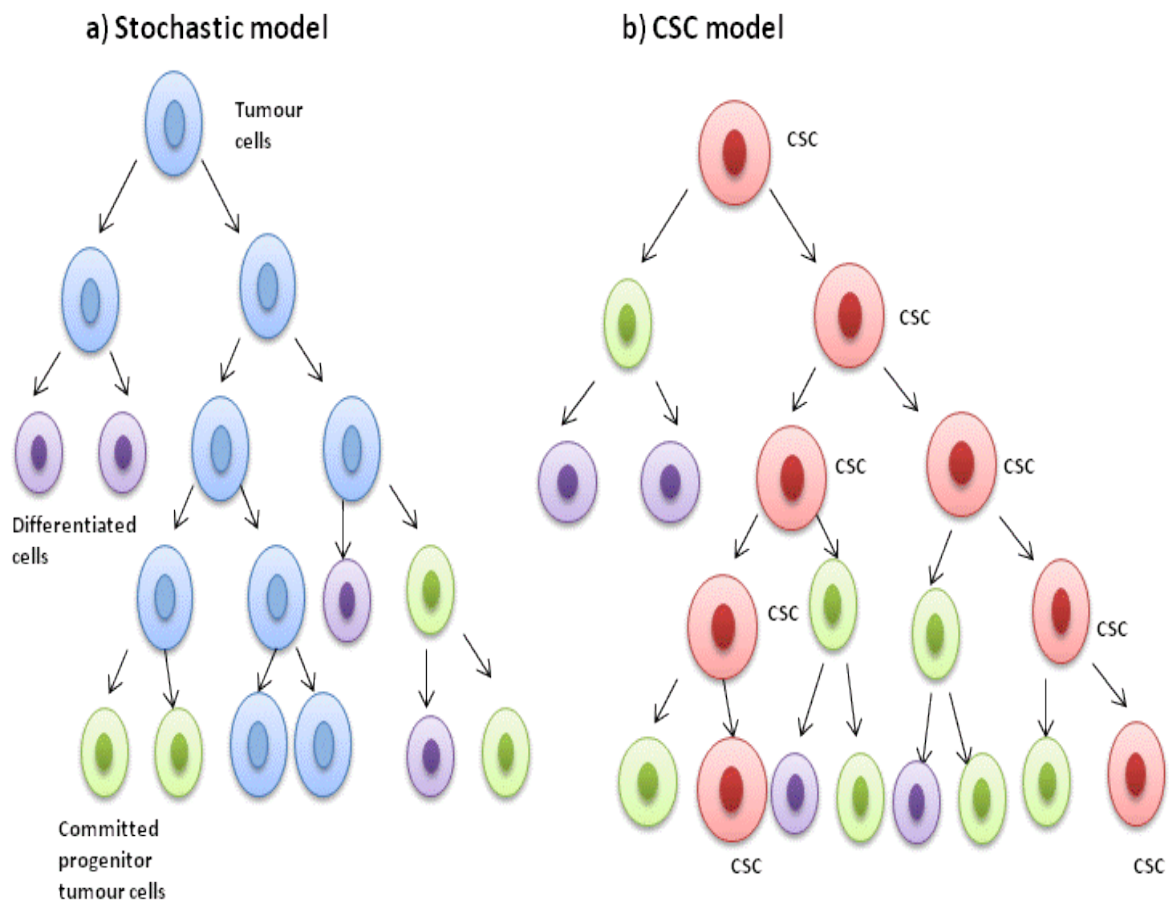
Figure 1.4: Schematic diagram of different growth models.

 (a) Stochastic model: all tumour cells are divided in hierarchy manner to keep its own population or differentiate: (b) cancer stem cell model: a limited number of tumour cells have the proliferative capacity and long term self-renewal capacity to give rise to committed progenitor with a limited proliferative advantage and terminally differentiate adapted from (Beck and Blanpain, 2013).

Table 1.1: The demonstration of cancer stem cells by several tumours, including skin cancers.

Human tumour	Normal adult tissue stem cell marker	Tumour initiating cell marker	Model used for validation	Percentage of marker +ve cells in tumour	References
Acute Myeloid Leukaemia	CD34+ CD38- CD90+ CD45RA-Lin-	CD34+ CD38-	SCID & NOD SCID	0.1%	(Bonnet and Dick, 1997; Doulatov et al., 2012; Lapidot, 1994)
Chronic Myeloid Leukaemia		CD34+ CD38-	NOD SCID	0.1%	(Doulatov et al., 2012; Wang et al., 1998)
Breast Cancer	CD49f+ CD24-	ESA+ CD44+ CD24-/low	NOD SCID	12-35%	(Al-Hajj et al., 2003; Petersen and Polyak, 2010)
Meduloblastoma	CD133+	CD133+	NOD SCID	6-21%	(Singh et al., 2004; Uchida et al., 2000)
GLIoblastoma Multiforme		CD133+		19-29%	
Colon Cancer	Lgr5+	CD133+	NOD SCID	1.1-24.5%	(Barker,2007; O'Brien et al., 2007a; Ricci-Vitiani, 2007)
Colorectal Cancer		EpCAM^{hi} CD44+	NOD SCID	0.03-38.7%	(Barker, 2007; Dalerba et al., 2007)
Pancreatic Cancer	Not defined	ESA+ CD44+ CD24+	NOD SCID	0.2-0.8%	(Kajstura et al., 2011)
Head and Neck SCC	CD29^{hi} CD44+	CD44+ Lin-	NOD SCID	<10%	(Jensen et al., 2008; Prince et al., 2007)
Melanoma	Not defined	ABCB5 CD271+ CD20+HMW-MAA+	NOD SCID & Rag2-/-γc-/-	1.6-20.4% 2.5-41%	(Boiko et al., 2010; Boonyaratanakornkit et al., 2010; Schatton et al., 2008; Schmidt et al., 2011)
Lung	CD117+	CD133+	NOD SCID	0.3-22%	(Eramo et al., 2008; Li et al., 2007)
Ovarian	Not defined	CD133+	NOD SCID	0.3-35%	(Curley et al., 2009)
Hepatocellular Cancer	CD90+ CD34+	CD90+ CD45-	SCID beige	0.03-6.2%	(Masson et al., 2006; Yang et al., 2008)
SCC	CD49^{hi} CD71^{lo}	CD133+ CD45-	Nude with stromal bed	0.1-1.7%	(Patel et al., 2012)
BCC	CD200	CD200	Nude with stromal bed	0.1-3.9%	(Colmont et al., 2013)

1.1.7 Cancer stem cells and chemotherapy resistance

Cancers such as prostate cancer, multiple myeloma, bladder, kidney, pancreatic and the common skin cancers are typically resistant to conventional chemotherapy and more recent targeted therapies (Morgan *et al.*, 2004). In recent years, there have been a number of studies published in a variety of solid tumour xenograft models demonstrating radiation and chemotherapeutic resistance of the cancer stem cell subset (Bao *et al.*, 2006; Li *et al.*, 2008; Ma *et al.*, 2008). Likewise, we have shown that BCC cancer stem cells are resistant to etoposide through constitutive and induced expression of multidrug resistance gene 1 (Colmont *et al.*, 2014).

1.1.8 Targeting cancer stem cells

Cancer stem cells share many characteristics with normal stem cells, notably researches have used normal stem cell markers to identify and enrich cancer stem cells; such as CD133 marker. Intriguingly, CD133+ cancer stem cell enriched populations have been identified in primary squamous cell carcinoma, medulloblastoma, glioblastoma multiforme, hepatocellular, ovarian, lung and colon cancer (Collins *et al.*, 2005; Eramo *et al.*, 2008; O'Brien *et al.*, 2007b; Patel *et al.*, 2012). Although CD133 and other such cell surface proteins used to isolate cancer stem cells may form the basis for therapy, often these cell surface proteins are also expressed by normal tissue cells, including stem cells (Richardson *et al.*, 2004; Yu *et al.*, 2002). Thus targeted therapies using cell surface proteins risk killing normal tissue stem cell. Hence there is a need for alternative approaches to targeting cancer stem cells.

In contrast to post mitotic differentiated cells, stem cells posses the ability to proliferate. In normal tissues cell, proliferation means that cell divide in size and number and therefore increase in cell mass. But also cells with proliferative capacity, including tumour cells, are able to migrate and secrete factors that facilitate blood supply and enzymatic degradation of tissue basement membrane (Weidner *et al.*, 1993; Weidner *et al.*, 1991). The normal process of proliferation is closely connected to the state of differentiation both during development and in adult life. Both proliferation and differentiation are regulated by several factors including transmembrane receptors, intercellular signalling and transcriptional factors. In most cancers the mutually exclusive properties of differentiation and proliferation favour proliferation and so excessive growth of tissue (Rheinwald and Beckett, 1980; Sugawara *et al.*, 1998).

As described above, cancer stem cells may divide symmetrically to give rise to two cancer stem cells or differentiated cell. More frequently cancer stem cells demonstrate asymmetric division, to give rise to one differentiated cell and through self-renewal maintenance of the CSC populations. The degree of differentiation is of prognostic value, for example pathologists graded well differentiated tumour, in which tumour maintain a normal tissue histology, is associated with good prognostic fate (Brantsch *et al.*, 2008). Conversely, cancers exhibiting limited numbers of differentiated cells, deemed poor or de-differentiated tumours, are associated with poor prognostic clinical outcome (Morgan *et al.*, 2008). As expected poorly differentiated tumours demonstrate a higher mitotic rate (Lewis and Weinstock, 2004), and so may have a higher number of cancer stem cells.

Although many experts believe that cancer stem cells represent the backbone of tumours and suggest that eliminating them can result in cure; the targeting of cancer stem cell presents a huge challenge.

Two conventional cancer therapies affect outcome by promoting differentiation: retinoid (Breitman *et al.*, 1981; Göttlicher *et al.*, 2001) and histone deacetylase inhibitors (HDAC) (Butler *et al.*, 2000; Butler *et al.*, 2002). Retinoid are routinely used in the treatment of promyelocytic leukaemia, all-trans-retinoic acid induces the differentiation of leukemic cells forcing cell death (de Thé and Chen, 2010). In addition, retinoid in combination with interferon-α has a promising role in treatment of sequamous cell carcinoma of skin and cervix (Chabner, 1993). But recent experimental findings have also shown retinoid to promote cancer stem cell differentiation by influencing cell fate decisions (Campos *et al.*, 2010; Ginestier *et al.*, 2009; Massard *et al.*, 2006).

In recent years, suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, both in *in vitro* and *in vivo* assays has also been shown to promote tumour differentiation in a number of different cancers: prostate carcinoma, myeloma, breast carcinoma, and murine erythroleukemia (Butler *et al.*, 2000; Huang and Pardee, 2000; Richon *et al.*, 1996). As with retinoid, the induction of differentiation by HDAC inhibitors is not the only mode of action. For example, SAHA in prostate carcinoma induces expression of thioredoxin-binding protein-2 (TBP-2) a gene found to inhibit thioredoxin gene in transformed cells of prostate tumour (Butler *et al.*, 2002). Thus these therapies that induce differentiation can be effective in the treatment of cancer and the recent identification of cancer stem cells provides a basis for their mode of action, by changing cancer stem cell division cell fate decisions.

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Cancer stem cells possess the ability to resist conventional therapies and so may be responsible for tumour recurrences. The lack of targetable molecules that distinguish them from normal tissue cells, the lack of reliable models and difficulty maintaining cancer stem cells in culture present technical difficulties to find new treatments. However, the identification of proliferation and differentiation pathways that can induce cell fate decisions in more rapidly dividing cancer stem cells, relative to normal tissue stem cells, may add new opportunities for specific therapies.

1.2 The Skin and Skin Cancer

1.2.1 The integumentary system

To understand the process and morphology of skin cancer, as well as its cellular components, it is first necessary to appreciate the structure and function of the skin. The skin is composed of two distinct embryological layers, the overlying epidermis and supporting dermis (Figure 1.5). Multiple cell types are present in both layers, although the abundant cell types in the epidermis are keratinocytes, which constitute the major barrier and protective part of the skin against exogenous physical and chemical agents, and in the dermis are fibroblasts, which provide structural support by production of the extracellular matrix. The skin also has within it appendages that represent invaginations of this basic alignment of layers, but with distinct functional characteristics. Eccrine glands regulate temperature by releasing sweat, sebaceous glands waterproof the adjoining hair as well as the skin, and apocrine glands (Urmacher, 1990). Hair, as discussed in detail later, is present to facilitate temperature regulation and provide protection over all the skin except the palms and sole.
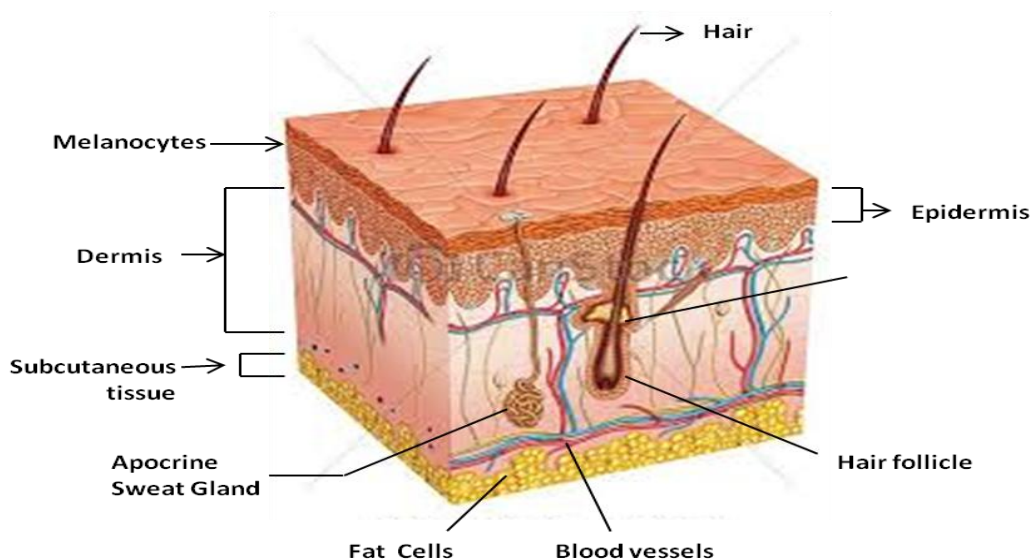
Figure 1.5: Cross section of skin.
Adapted from online source (AmericanSkinAssociation, 2012)

1.2.2 Skin cancer

The skin is the largest organ in the human body and is constantly exposed to carcinogens, most notably ultraviolet (UV) solar irradiation; hence skin cancer is the commonest malignancy worldwide. In the UK, skin cancer accounts for more than one quarter of all new cancer diagnoses, whilst where there is greater UV irradiation such as in Australia, more than 80% of all cancers are skin malignancies (Globocan, 2012).

The incidence of skin cancer is set to continue to increase as cultural trends favour less clothing and social behaviour supports sun bathing.

Over 95% of all skin cancers fall into three types: melanoma, basal cell carcinoma (BCC) and squamous cell carcinoma. The term non-melanoma skin cancer often relates to only BCC and squamous cell carcinoma, but should in fact include all the other types of "non-melanoma" skin cancers such as cutaneous T-cell lymphoma, dermatofibrosarcoma protruberans, as well as other rarer skin cancers such as Merkel cell carcinoma and sweat gland adenocarcinoma.

1.2.3 Basal cell carcinoma

BCC was first described by Jacob in 1827 (Jacob 1827) is the most common malignant neoplasm of humans and named after its morphological similarity to the keratinocytes of the epidermal basal layer. Although it presumably develops from keratinocyte stem cells of the hair follicle bulge, the precise origin of BCC is still unknown (Youssef *et al.*, 2010).

1.2.3.1 Clinical characteristics of basal cell carcinoma

Early BCC lesions commonly appear as small, translucent or pearly lesions, with small dilated blood vessels (telangectasia) distributed over the surface (Figure 1.6). BCC can develop at different anatomical locations, although approximately 80% appear on the head and neck (particularly on the face) (Rubin *et al.*, 2005). The remainder occur on the trunk and limbs, however BCC are only rarely described on palms and soles. Nodular BCC occurs most frequently on the face, usually located around the inner canthus of the eye, the nose and on the forehead. Lesions on the chin and outer aspects of the cheeks are relatively less common.

Usually BCC grow asymptomatically as a slow solitary translucent raised nodule. The lesion may reach a diameter of half a centimetre over a period of 1-2 years, before more rapid growth ensues leading to central ulceration (Figure 1.6); giving rise to the common name "rodent ulcer". At this point the BCC becomes symptomatic, as it bleeds easily, for example when washing of face, characteristically patients describe a lesion that "heals" and re-bleeds after minor injury.

1.2.3.2 Basal cell carcinoma classification

There are 4 main categories of BCC: nodular, superficial, infiltrative and morphoeic (Lang and Maize, 1986; Rippey and Rippey, 1997). The classification can be related to the pattern of the tumour growth. Nodular BCC is the most common type (~50% of cases), in which the lesion appears as a raised papule or nodule with telangectasia on the surface (Figure 1.6 a.). It sometimes forms a central depression that may ulcerate, bleed, or crust. Histologically, nodular BCC has small and large rounded nests of tumour cells with peripheral palisading growing downward from epidermis to dermis (Figure 1.6 d).

Superficial BCCs (~15% of cases) are particularly slow growing and tend to occur on the trunk, where they are often dry, scaly, flat erythamatous plaque which can mimic psoriasis, discoid eczema, and Bowen's disease (Figure 1.6 b). Microscopically, superficial BCC consists of multiple small islands of basaloid cells attached to the epidermis and confined to the papillary dermis (Figure1.6 e). Patients with a BCC located on the trunk, where superficial BCC's occur, are more prone to develop multiple BCCs, and these tumours develop at a faster rate than BCCs located elsewhere in the body (Lear *et al.*, 1998).

Morphoeic BCCs (10%) present as a flat, atrophic, indurated white or red plaque. Overlying telangectasia may be present. The edges appear unclear and the real size of the tumour is often much larger than what is clinically apparent (Figure1.6 c). Histology shows small root like projections that extend deep in the dermis, surrounded by a dense cellular stroma (Figure 1.6 f).
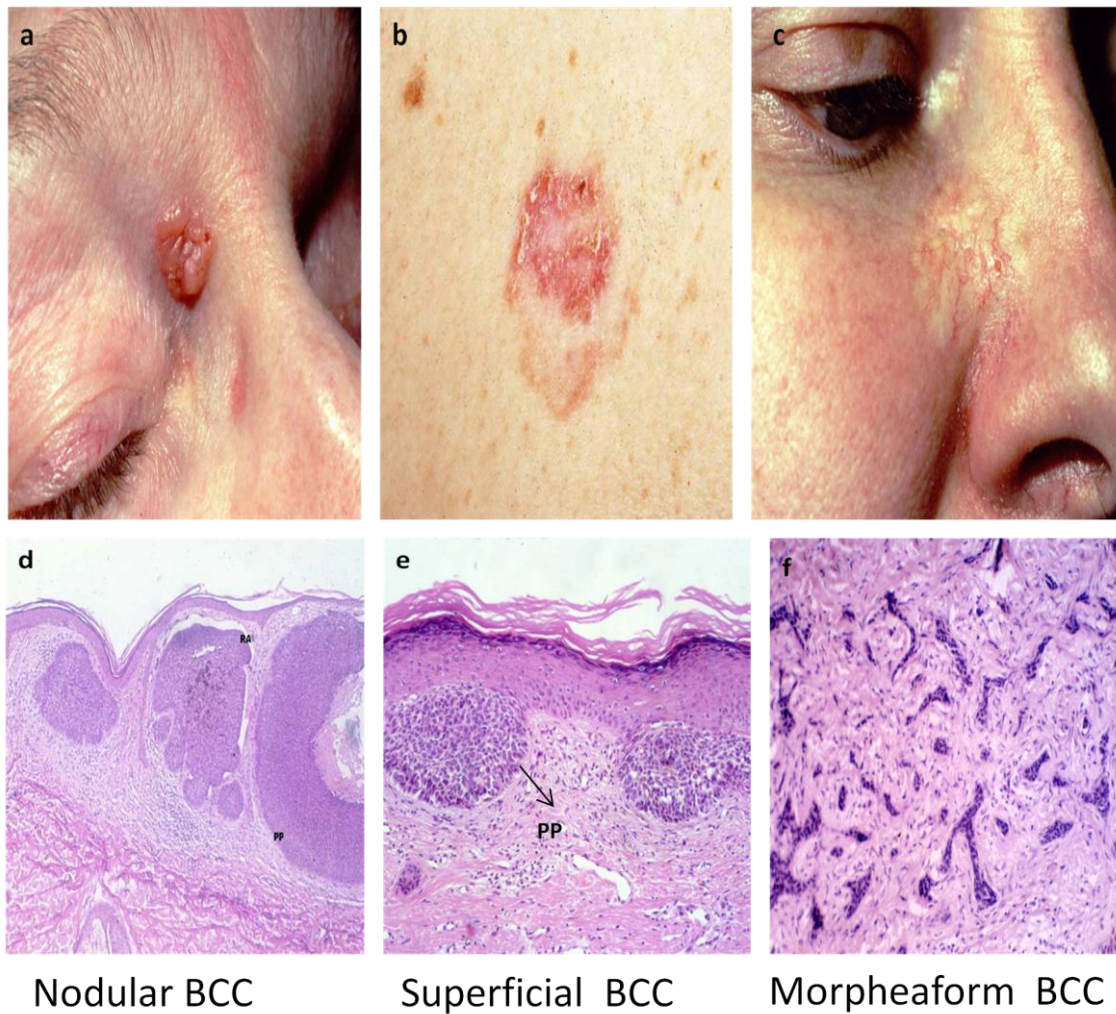
Figure 1.6: Three of four main categories of BCCs (nodular, superficial, and morpheaform).
Clinical and histological appearance of BCCs. Nodular (a,d), superficial (b,e) and morpheaform (c,f). PP (peripheral palisading), RA (retraction artifact). Adapted from online source (Medscape :drug & disease ,2014).

1.2.3.3 Epidemiology

BCC is the most common skin cancer among people with fair-skin colour, in contrast black-skinned individuals rarely develop non-melanoma skin cancer (Halder and Bang, 1988). It has been estimated that the lifetime of risk of BCC among Caucasians is between 28% and 33% (Miller and Weinstock, 1994). Men have a slightly higher incidence of BCC, consistent with the increased likelihood of outdoor work. In the UK

BCC occurred 56% in men and 44% in women, giving a male: female ratio of 1.5:1 (Lear *et al.*, 2007) and data provided by the office for national statistics (National Statistics, 2012). Though the rates are still highest for the naturally sun exposed skin of elderly men, the trend over the past decade is clearly towards an increasing incidence of BCC in younger women due to excessive tanning and sun bed use (Christenson *et al.*, 2005).

The reported average age-standardised rate per 100,000 is 98.6 and 22.7 for BCC and sequamous cell carcinoma respectively, although as many as one third of BCC and sequamous cell carcinoma diagnoses may go unregistered (Andl *et al.*, 2004; Lomas *et al.*, 2012; Musah *et al.*, 2013). The recorded incidence of BCC increased by around a third (36% in males and 32% in females) between 2000-2002 and 2008-2010 in England, Scotland, Northern Ireland and Ireland combined (National Cancer Intelligence Network). In contrast, there has been only a modest increase in overall cancer diagnoses over the last decade, 3% in men and 6% in women (CR UK). As the cancer registry statistics do not collect data pertaining to BCC the true socioeconomic burden is not known (Goodwin *et al.*, 2004).

Morbidity is more often accompanied with local tumour extension into vital structures such as cartilage and bone, causing cosmetic disfigurement. In instances of neglect, BCC tumours maybe very large indeed and have eroded into structures such as the orbit and cranium (Madan *et al.*, 2010). Only rarely do BCC metastasise, estimated to occur less than 0.0028% of occasions, when they can spread to lymph node, bones, and lung (Rubin *et al.*, 2005). Hence the overall mortality from BCC remains exceptionally low.

Patients who develop a BCC are at increased risk of developing further BCC, a meta-analysis found that the three year cumulative risk varied from 33% to 77% (Stern and Lange, 1988). In addition, studies showed that the risk of developing squamous cell carcinoma and malignant melanoma are also increased slightly after BCC (Boyd *et al.*, 2002; Gallagher *et al.*, 1995).

1.2.3.4 Risk factors

Recent literature reports an increase in the incidence of BCC in the 30-39 age group, perhaps correlated with the use of ultraviolet (UV) light sun beds for cosmetic tanning purposes, especially among younger women (de Vries *et al.*, 2004; Epstein, 2008; Karagas *et al.*, 2002). Exposure to the UV radiation is the main causative factor in the pathogenesis of BCC (Gallagher *et al.*, 1995), Consistent with the role of UV light exposure in the pathogenesis of BCC, the incidence of BCC also varies geographically and globally. In America, states that are close to the equator, such as Hawaii, there is incidence of almost 3 times that of states in the Midwest, such as Minnesota, while Australia has the highest rate of BCC in the world, with certain regions reporting an incidence of up to 2% per year (Marks *et al.*, 1993). However, the precise relation between BCC development and the amount, pattern, and timing of exposure to UV radiation remain unclear (Armstrong and Kricker, 2001) . Where there is debate, this relates to the timing of UV exposure, since adulthood cumulative UV exposure is not as well correlated as for squamous cell carcinoma (Corona *et al.*, 2001). Instead, several studies have shown that there is a strong correlation between cumulative sun exposure and the development of BCC during childhood and adolescence (Corona *et al.*, 2001; Rigel, 2008).

Exposure to other known carcinogens is also linked with the development of BCC. The risk of BCC is associated with exposure to arsenic, coal tar and psoralin as well as ionizing radiation (Diepgen and Mahler, 2002). Also, there is an association of smoking and skin cancer development, notably among young women (Boyd *et al.*, 2002).

In addition to the direct cumulative carcinogen exposure (e.g, UV light) in adulthood, intrinsic factors to the individual are equally important in determining who is at risk of developing a BCC. Patients at greatest risk tend to be fair skinned individuals (skin type 1) with red or blonde hair and light coloured eyes are , a risk which increases with childhood freckling and severe sunburn (Daya-Grosjean and Couvé-Privat, 2005; Rubin *et al.*, 2005; Wong *et al.*, 2003). Also it has been reported that there is a strong relationship between development of BCC and a positive family history of skin cancer (Corona *et al.*, 2001). BCC is also more common among individuals that consume a high dietary intake with a low intake of vitamins (Gallagher *et al.*, 1996; Yamada *et al.*, 1996).

Patients who have been on long-term immunosuppressive therapy have an increased risk of BCC, where the incidence of BCC was reported as 10 times more than in general population in renal transplant recipients and 21 times more in heart transplant recipients (Ong *et al.*, 1999). Immunosuppressant drugs are not only impair T cell function to prevent graft rejection, but also impair tumour surveillance allowing mutated cells to transform to progress to cancer (Tilli *et al.*, 2005). Likewise, disorders that impair DNA repair, so that increase numbers of mutations become fixed, also increase the risk of BCC; these include: xeroderma pigmentosa and Bazex's syndrome. Similarly, disorders

where there is a loss in skin pigmentation leading to an increase of risk of sunburn such as in cutaneous albinism, also increase the risk of BCC (Miller, 1991a, b).

The genetic analysis of Gorlin or nevoid basal cell carcinoma (NBCC) syndrome has elucidated the genetic basis of BCC, leading to the identification of abnormalities in the Sonic Hedgehog signalling pathway and resulted in therapeutic breakthroughs. Gorlin's syndrome is a rare autosomal dominant condition in which patients develop multiple BCC, pitting of the palms and the soles, jawline cysts, spine and rib anomalies, calcification of the falx cerebri, and cataracts (Gorlin, 1987). In addition to BCC, Gorlins patients are at higher risk of developing medullobasltoma and childhood rhabdomyosarcoma as well as other cancers (Gorlin, 1995). Gorlins has been shown to be caused by patched (PTCH) gene germline inactivation mutations, a tumour suppressor gene which is located to chromosome 9q22.3, with frequent loss of heterozygosity at 9q22.3 (loss of the remaining wild-type allele at 9q22.3) (Grachtchouk *et al.*, 2000; Johnson *et al.*, 1996; Oro *et al.*, 1997). Similarly, PTCH inactivated mutations occur frequently in sporadic BCCs (60-70%) and in BCCs associated with xeroderma pigmentosum (80-90%), as well as allelic loss of the remaining wild-type patched gene (53%) (loss of heterozygosity of PTCH) (Epstein, 2008).

1.2.3.1 Molecular genetics of basal cell carcinoma

The majority of BCCs occur sporadically. However, basal cell nevus syndrome (Gorlin syndrome) is a rare inherited disorder in which patients are more susceptible to develop BCCs. It has been thought that mutation in the P53 tumour suppressor gene and the patched gene (PATCH) play important roles in BCC development via targeting of UV.

The mutation of these genes leads to cell proliferation and BCC development (Kastan *et al.*, 1991).

I. *P53 gene*

The tumor suppresser gene, P53 is the most common genetic aberration found in more than half of all human cancers. Inactivation or mutation of this gene has been implicated as an important step in development of non-melanoma skin cancer, including BCC (around 65%) (Kastan *et al.*, 1991). P53 encodes for a phosphoprotein that regulates the cell cycle and induces apoptosis in cells in response to cellular stress, for example DNA damage (Katayama *et al.*, 2004).

II. *Sonic Hedgehog (SHH) Signal Transduction Pathway and Mutations in BCC Lesions*

The hedgehog (HH) signalling name came from a family of three HH ligands found in vertebrates, sonic hedgehog (SHH), indian hedgehog (IHH) and desert hedgehog (DHH). DHH is expressed mostly in gonads. IHH expression is found in primitive endoderm during bone formation. SHH is largely expressed in many mammalian tissues (Ingham and McMahon, 2001). It is required for both embryonic and adult hair follicle development (Bitgood and McMahon, 1995). SHH has been shown to prematurely induce the growth anagen phase of the hair follicle in the resting telogen follicle (Paladini *et al.*, 2005). It is also important in maturation of hair follicle dermal papilla (Karlsson *et al.*, 1999). Importantly, mutation of sonic hedgehog signalling pathways is found to be associated with hair follicle tumours and BCC (Oro *et al.*, 1997).

The SHH signalling pathway involves two transmembrane proteins, Patched (PTCH) and Smoothened (SMO). PTCH binds SHH ligand, whereas SMO acts as a signal

transducer. In the absence of SHH ligand, PTCH, at the cell surface, interacts with and inhibits intercellular SMO. This inhibition activates a transcriptional repressor (e.g. GLI in vertebrates). Once pathway activation occurs, then SMO can transport to the cell surface membrane and initiate signalling. On the other hand, in the presence of SHH ligand, the interaction of PTCH and SMO is altered and SMO is no longer inhibited. This will activate GLI zinc finger transcriptional proteins and these then enter the nucleus allowing transactivation of target genes (Ingham *et al.*, 2011; Wang *et al.*, 2007) (Figur1.7).

It has become well known that hyper-activation of the SHH pathway plays an important role in initiating some cancers including BCC. Mutations in the SHH signalling pathway has been demonstrated in both inherited BCC (basal cell nevus syndrome or Gorlin's syndrome) and the sporadic form. It has been shown that around 90% of sporadic BCCs have PTCH1 mutations and around 10% have SMO mutations (Epstein, 2008).

PTCH and SMO mutation have also been shown to cause BCC in patients with xeroderma pigmentosum, a genetic disease which is characterised by its inability to repair UV-induced DNA damage (Daya-Grosjean and Couvé-Privat, 2005), suggesting that repair of UV- associated DNA damage can reduce BCC (Bodak *et al.*, 1999).
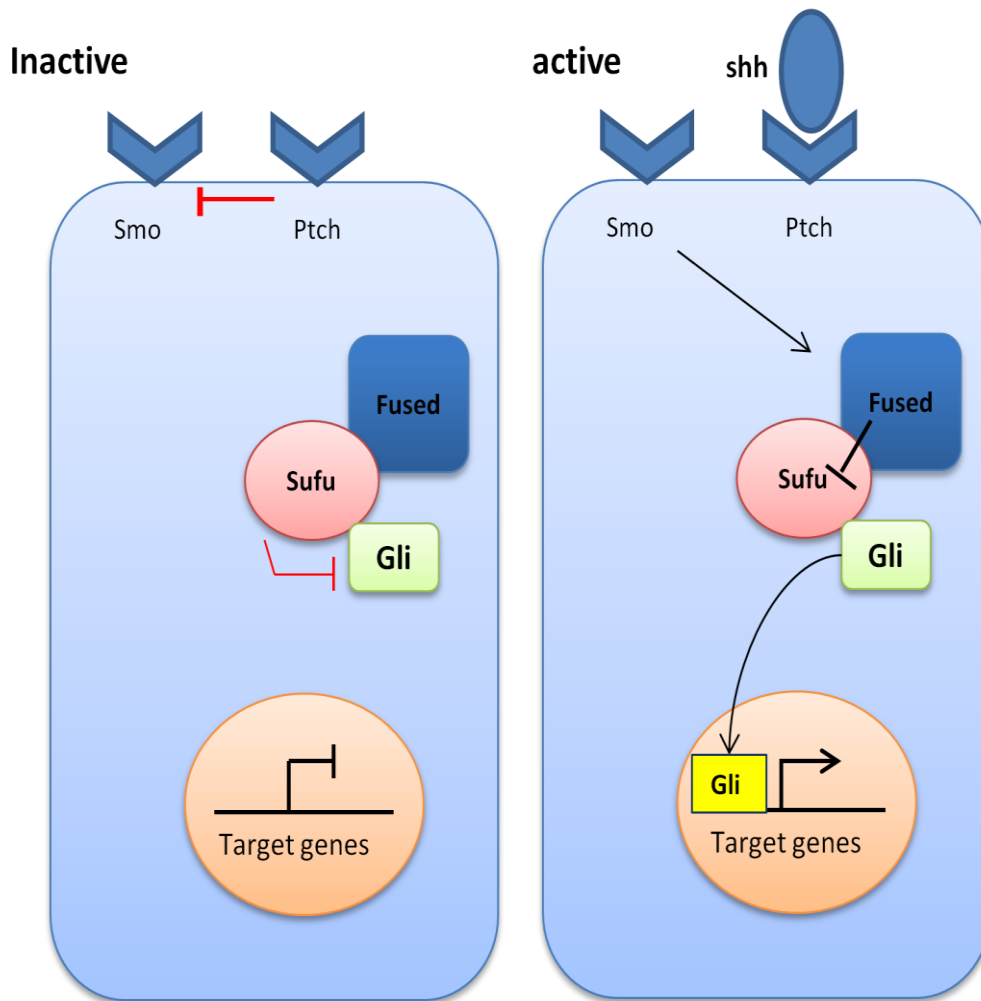
Figure 1.7 : The Sonic hedgehog (SHH) signalling pathway.
In the absence of the SHH ligand, the patched (PTCH) transmembrane receptor inhibits the protein smoothened (SMO) which may stay in intracellular vesicles. Binding of SHH to PTCH stimulate intracellular kinases, including G-protein coupled receptor kinase 3 , and protein kinase A . These kinases tighter with fused (SUFU) can stop SMO inhibition via phosphorylation. Which then will activates the translocation of GLI in to nucleus and activate the target gene.

1.2.3.2   BCC management

Generally, non-melanoma skin cancer, including BCC, are responsive to treatment at early stages. The management of those tumours is mostly depending on both patient features such as ability to manage the topical treatment, tolerability as well as concern regarding to the cosmetic result after surgery, and tumour characteristic such as size, depth of the lesion, and tumour recurrence. A variety of therapies have been described in the management of BCC (Ceilley and Del Rosso, 2006). The main aim of the treatment is to eradicate tumour with satisfactory cosmetic outcome to the patient.

1.2.3.2.1   Surgical modalities for BCC

Surgical excision is considered the preferred mode of treatment because it is a highly effective form of therapy for primary BCC, with an opportunity to confirm complete excision of the tumour by histology. After surgery with clear histological margins there is less than 2% recurrence rate after 5 year (Griffiths *et al.*, 2005; Walker and Hill, 2006). A complete excision with peripheral margins about 4-5mm is usually advisable and it is found to increase the cure rate to 95% (Breuninger and Dietz, 1991). However, incomplete excision, where one or more surgical borders excised with or very close to tumour, is frequently associated with recurrences (Sussman and Liggins, 1996). This can occur in tumours with indistinct clinical margins. At sites where skin preservation is important, often referred to the "T" zone of the face (Figure1.6), incomplete excision rates amongst surgeons tends to be higher; particularly with lack of experience of the operator (Kumar *et al.*, 2000). In this case, or where re- excision is necessary, Mohs micrographic surgery (MMS) is the treatment of choice. Although MMS is a very good treatment for high risk BCC with extremely high cure rates, it remains costly and time consuming and therefore is reserved for management of high-risk sites and in cases of

recurrence where the observed surgical margins may not adequately reflect the true extent of the tumour growth.

In contrast, tumour clearance with other techniques such as cryosurgery, curettage, and photodynamic therapy, where conventional histology is not used to demonstrate eradication, higher recurrence rates exist (Chiller *et al.*, 2000; Holt, 1988; Johnson *et al.*, 1991).

1.2.3.2.2 Radiotherapy

Radiotherapy has been shown to be an effective therapy for primary and metastatic BCC (Al-Othman *et al.*, 2001), and may be used in cases of recurrent BCC (Caccialanza *et al.*, 2001). Radiotherapy is useful for patients unable to tolerate surgery (Finizio *et al.*, 2002). For primary BCC, 170KV may be used for lesions up to 6mm depth. Electron beam radiotherapy maybe used for tumours invading deeper. Although radiotherapy is an effective treatment option, the dose has to be carefully titrated to avoid radionecrosis of underlying tissues, particularly where the skin is thin such as the eyelids and bridge of the nose (Telfer *et al.*, 2008). The overall 5-year cure rates following RT for treatment of primary BCC is approximately 91.3%, and for treatment of recurrent disease is 90.2% (Rowe *et al.*, 1989). As radiotherapy induces additional mutations in the surrounding tissues, it should be avoided in patients with xeroderma pigmentosa and basal cell nevus syndrome (Caccialanza *et al.*, 2004).

1.2.3.2.3 Topical therapy

Topical imiquimod is an immune-modulator which activates the immune system through stimulation of toll like receptors resulting in the release of pro-inflammatory

cytokines (Vidal *et al.*, 2004). Imiquimod has been successfully used to treat superficial BCC, with >90% clearance achieved after twice-daily treatment for 6-12 weeks (Marks *et al.*, 2001). Use of imiquimod is often limited by a marked inflammatory reaction, which can be painful. Although topical imiquimod has shown effectiveness for treatment of superficial BCC, it is not suitable for invasive BCC.

The management of BCC as described above is wholly reliant upon skin surgery, since alternative approaches are not as effective in all cases. In the setting of increasing BCC incidence, as well as cases of inoperable and metastatic disease, alternative approaches to manage BCCs are still needed. This project explores a potentially novel mechanism by targeting BCC cancer stem cells.

1.3   The Hair Follicle

Hair is a fundamental component in the evolution of the mammalian species and serves many functions: sensory, thermoregulation, and physical protection. Hair follicle development takes place *in utero* alongside foetal skin development and continues to undergo morphological changes in adulthood. It remains to be determined whether basal cell carcinoma (BCC) resemble an embryonic or adult hair follicle as both have been postulated (Colmont *et al.*, 2013; Youssef *et al.*, 2012). To understand the relationship between BCC and the hair follicle, it is essential to understand the biology of both its embryonic morphogenesis and adult hair follicle morphological changes that occur during the hair growth cycle.

1.3.1 Morphogenesis of the hair follicle

Human hair development begins at 9 to 12 weeks *in utero*, mainly of the eyebrows, upper lip and chin (Dawber, 1988). General hair development occurs approximately from the fourth month onward in a cephalocaudal arrangement (Goodhart.CB., 1960). Studies of murine hair development have characterised the three steps involved in the formation of a functional hair follicle: hair placode formation, hair follicle organogenesis and cytodifferentiation (Figure 1.8)(Schmidt-Ullrich and Paus, 2005).

As shown in figures 1.8 and 1.9 below, the formation of the hair placode begins as epidermal keratinocytes form clusters, above a dermal condensate that will eventually become the dermal papilla (mouse age: E14.5). Within the placode hair follicle keratinocyte stem cells begin to express Sox 9 (Fuchs and Nowak, 2008). Inductive signalling crosstalk between the placode and the underlying dermal condensate result in

expansion and elongation of the placode keratinocytes resulting in the formation of a hair germ (E15.5) and subsequent hair peg (E17.5).

Hair follicle keratinocyte stem cells residing above the basal layer of the hair germ also expand and then remain proximal to the base within the outermost layers of the hair peg (Woo and Oro, 2011). The outer root sheath (ORS) starts to form a cylinder around the inner root sheath (IRS), as hair bulb keratinocytes are triggered to terminal differentiation and a bulbous peg structure is formed (E18.5) (Millar, 2002). Timelines begin later for guard hairs, Awl, Auchene and zigzag hair follicles (Stenn and Paus, 1999). The process of hair follicle formation is spatially and temporally controlled, with multiple signals involved in this process (Mikkola, 2007). The key signalling pathways (Figure 1.9) are described below and include wingless (Wnt), sonic hedgehog signalling (SHH), transforming growth factorβ/bone morphogenic protein (TGF-β/ BMP), fibroblast growth factor (FGF), and tumour necrosis factor (TNF) families (Schmidt-Ullrich *et al.*, 2006; Zhang *et al.*, 2009).
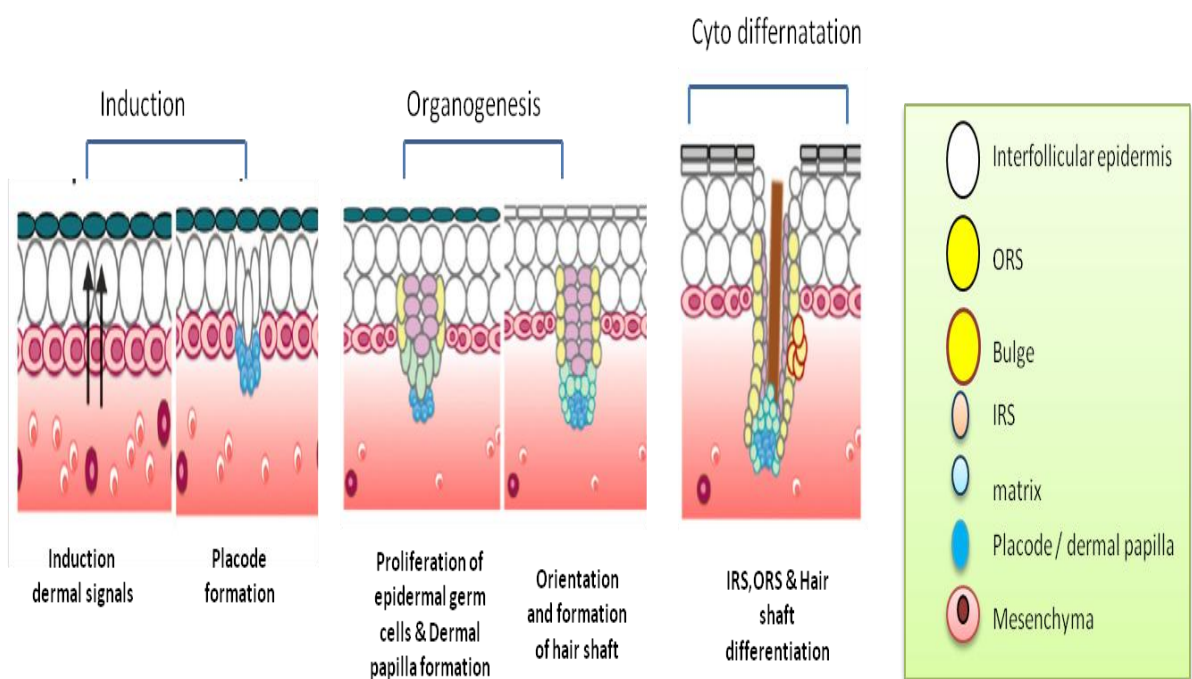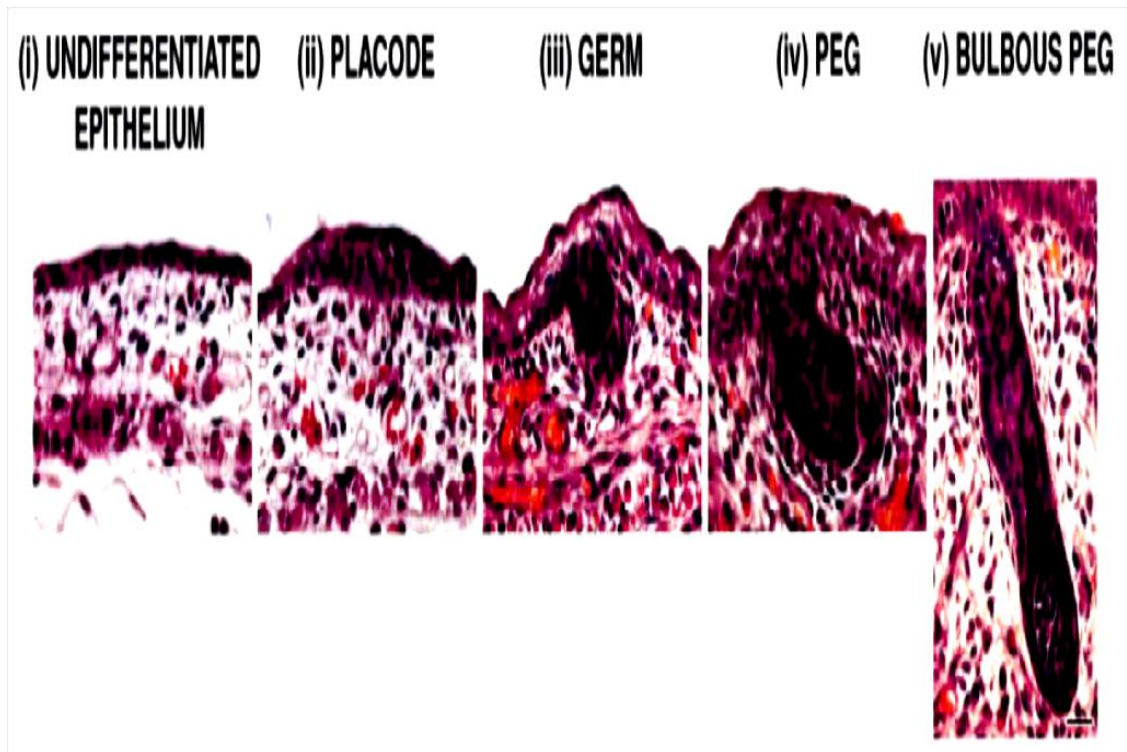
Figure 1.8.: Molecular mechanisms regulating hair follicle development.
Adapted from (Forni *et al.*, 2012; Millar, 2002).
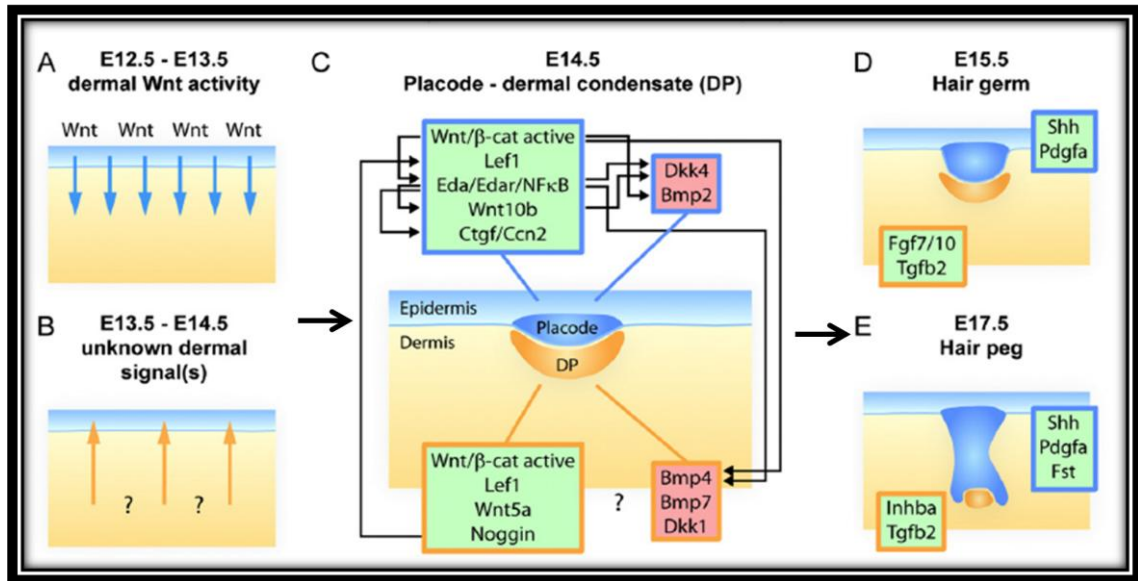
Figure 1.9: Mesenchymal–epithelial signal crosstalk during hair follicle induction.
Developmental stages (A–E) are illustrated schematically. (A) Epidermal Wnt signals to the dermis. (B) Unknown dermal signal(s) induce an epidermal response leading to placode formation. (C) Activating (green) and inhibitory (red) signals from placodes and dermal condensates (DP precursors) consolidate pattern formation through reinforcing placode/DP fate and lateral inhibition on neighboring epidermis. The network diagram depicts known hierarchies and regulatory connections between signaling pathways (as described in text). (D and E) signals regulate hair downgrowth at hair germ and peg stages. Adapted from (Sennett and Rendl, 2012).

1.3.1.1   Inductive phase – Wingless (Wnt) signals

The canonical Wnt growth factor pathway (Figure1.10) is active during hair follicle development and is of vital importance for dermal condensation, hair follicle development and stability (Chen *et al.*, 2012; Hardy, 1992; Millar, 2002). Murine studies reveal that epidermal Wnt is essential for the formation of the dermal papilla and in addition that over expression leads to excessive hair follicle induction and development of ectopic follicles (Collins *et al.*, 2011; Silva-Vargas *et al.*, 2005). Placode keratinocyte's releases the Wnt ligand Wnt10b, which forms a gradient that in turn participates in the patterning of hair follicle distribution.

Dermal cells expressing the Wnt receptor receive this signal, resulting in nuclear translocation of beta-catenin and recruitment of lymphoid enhancer binding factor (Lef1) and T Cell factor (TCF4) as part of the transcription complex. The expression of Wnt10b at E15.5 is associated with a marked reduction in epidermal Wnt inhibitor Dickkopf (Dkk4) expression (Bazzi *et al.*, 2007).
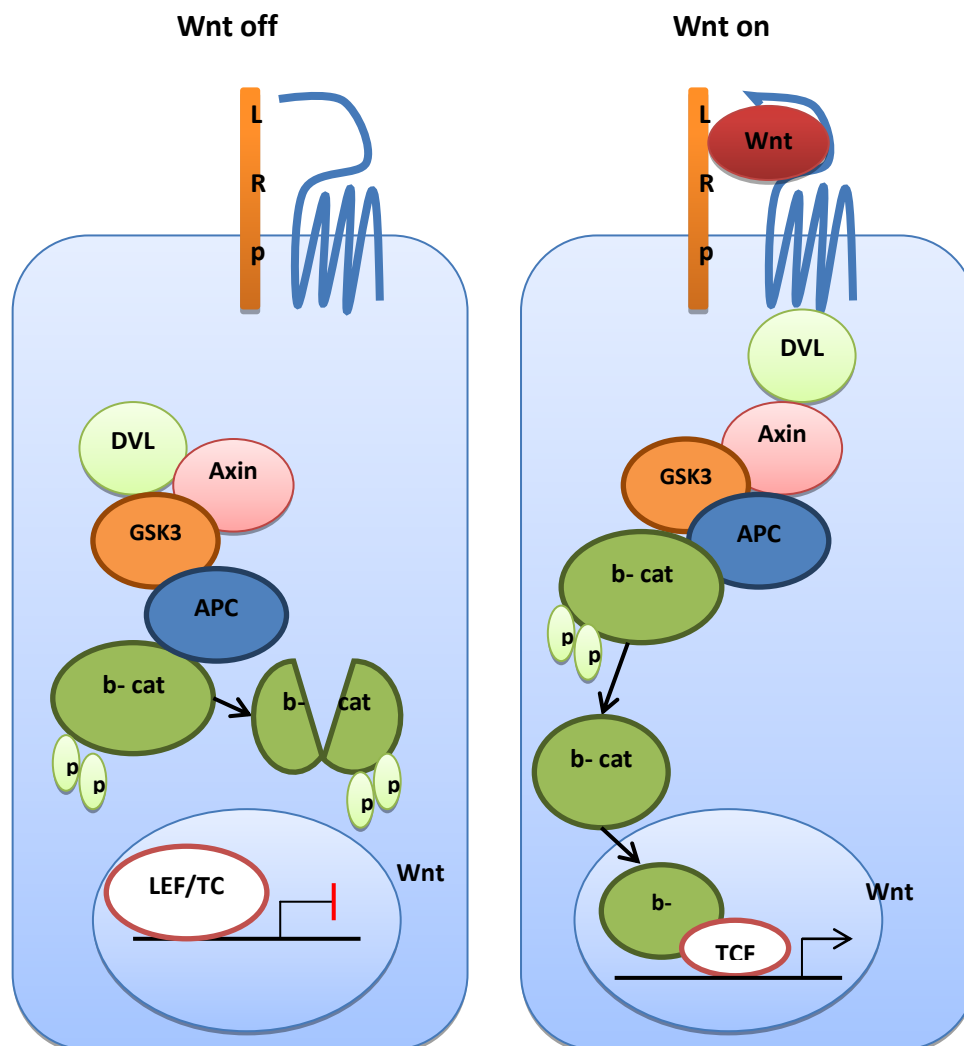
Figure 1.10: Wnt signalling pathway.
In the absence of wnt signal, cytoplasmic phosphorlyted β-catenin is degraded by a complex of proteins (axin, adenmatous polyposis coli tumour suppressor protein (APC) and glycogen synthase 3-b (GSK3-b). In the presence of Wnt, the degradation mechanism is inhibited and β-catenin translocates into the nucleus and bind to DNA binding factors aided by the lymphoid enhancer binding factor/T cell factor (LEF/TCF) family to regulate transcription of target genes.

1.3.1.2 Inductive phase – Fibroblast growth factor (FGF) signals

FGF ligands (Figure 1.11) are also essential in early stage of hair embryogenesis (Bergsland *et al.*, 2011; Mason *et al.*, 1994). Transgenic mice expressing a negative isoform of the common FGF receptor FGFr2IIIb exhibited failure of hair growth (Celli *et al.*, 1998), similarly FGFr2IIIb null mice showed delay of hair growth (Revest *et al.*, 2001). FGFs are observed throughout the epidermis in early stage of hair development (Richardson *et al.*, 2009).
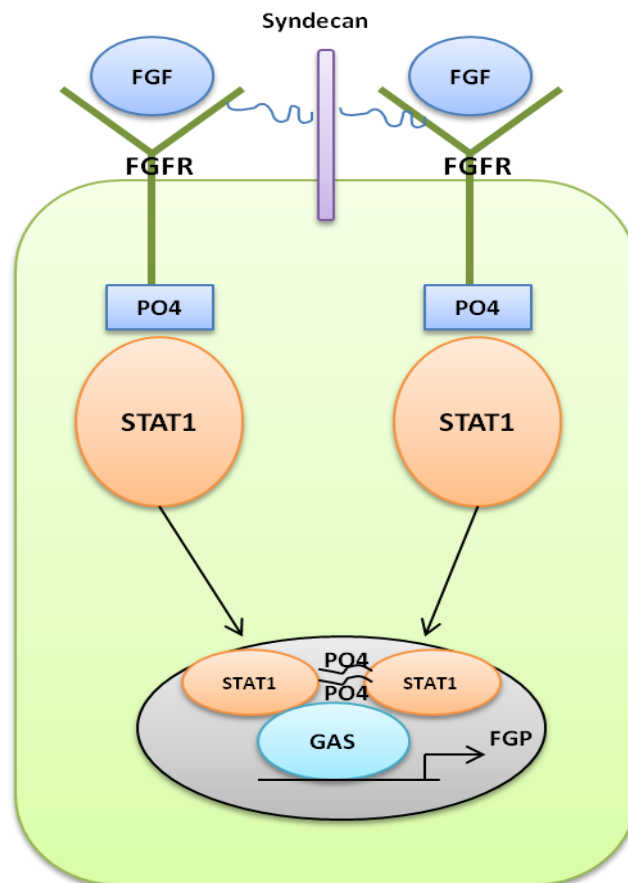
Figure 1.11: Fibroblast growth factor signalling pathway.
Fibroblast growth factor (FGF) binds to Fibroblast Growth Factor receptor (FGFR) with aid of syncdecan, phosphorylated FGFR attach and allow phosphorylation of the Signal Transducer And Transcription(STAT1), two phosphorylated STAT form a dimer that goes inside the nucleus and bind to Gamma Activator Sequences (GAS) promoter and impact gene expression.

1.3.1.3   Inductive phase – Transforming growth factor-β (TGF-β) signals

TGF-β2 expression (Figure 1.12) is present in both the placode and dermal condensate (Paus *et al.*, 1997). Exogenous TGF-β2 can induce dermal papilla formation sufficient to promote hair growth (Fuchs and Nowak, 2008). Consistent with this, TGF-β null mice showed arrested growth of hair follicle (Foitzik *et al.*, 1999).
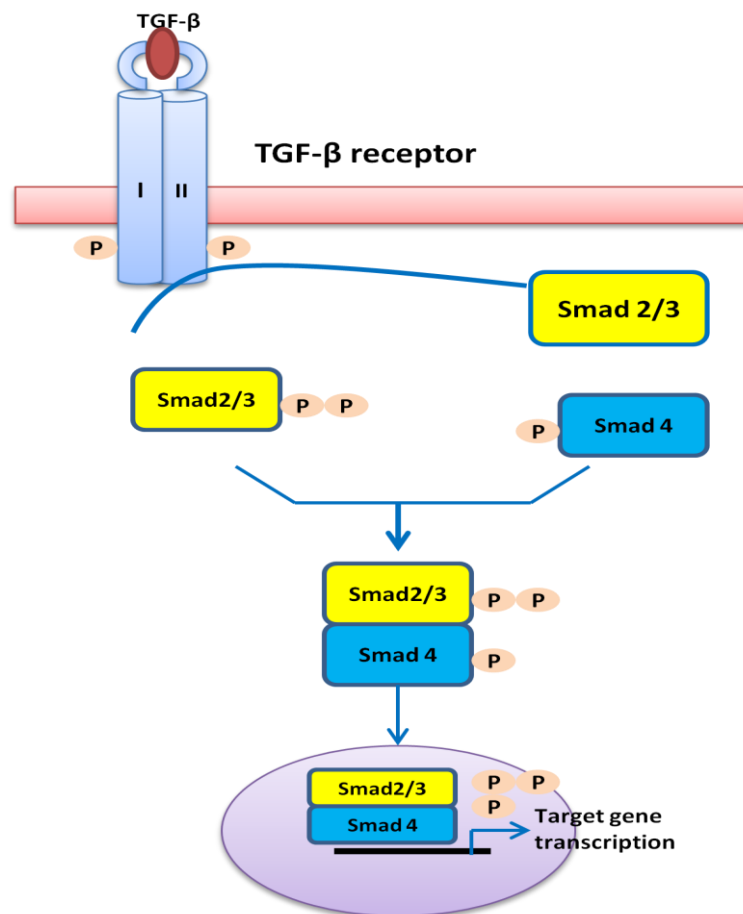
Figure 1.12: TGF-β signalling pathway.
TGF-β ligand binds to TGF-β receptor I,II which will lead to activation of smad 2 and smad 3. Phosphorylated smad 2,3 together with smad 4 transport into the nucleus to activate transcription of TGF-β target genes.

1.3.1.4   Inductive phase – Ectodysplasin (Eda) signals

Ectodysplasin (Eda) ligand and receptor (Edar), members of the TNF family (Figure 1.13), are also involved in establishment of the hair follicle placode (Naito *et al.*, 2002; Schmidt-Ullrich *et al.*, 2001). Inherited mutations, which impair the signalling of its receptor or downstream components, of the NFκB cascade result in ectodermal dysplasia syndromes characterised by hair, nail and tooth abnormalities (Laurikkala *et al.*, 2002; Monreal *et al.*, 1999). Eda and Edar are expressed throughout the foetal epidermis prior to placode formation, while after placode formation Eda expression remains ubiquitous, Edar is expressed by placode keratinocytes only (Headon and Overbeek,1999).
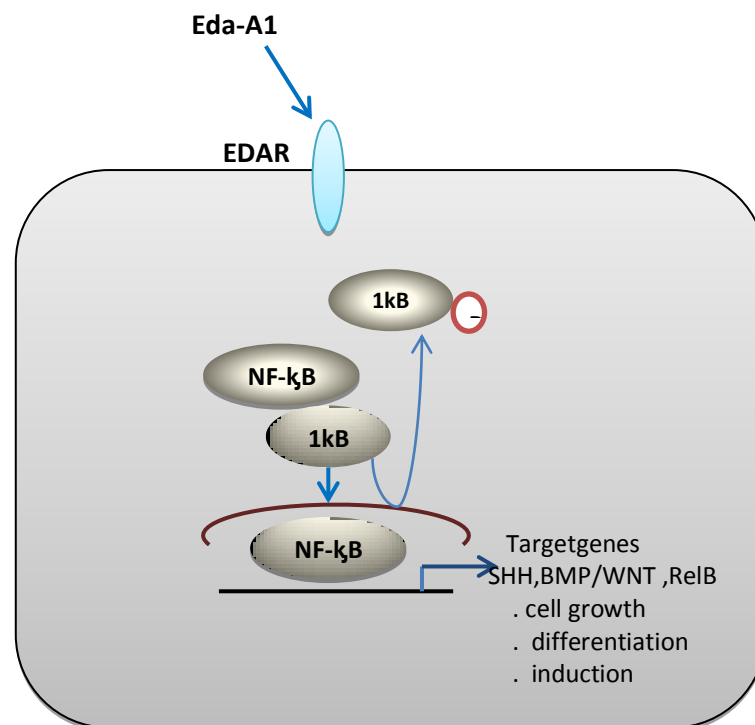
Figure 1.13: Eda signalling pathway.
 Ectodysplasin (Eda) binds to its receptor Edar, which leads to the activation of the transcription factor NF-κB. The degradation of the repressor IκBα allow the transport of NF-κB to the nucleus where it induces the expression of SHH (for epithelial growth), Rel B (for differentiation), and inhibit wnt/bmp (induction).

1.3.1.5 Inductive phase – Inhibitory bone morphogenic protein (BMP) signals

BMPs provide an inhibitory signal that blocks Wnt pathway induced formation of the placode (Jamora *et al.*, 2003) (Figure 1.14). BMP 2 and 4 is initially expressed diffusely throughout the epidermis and dermis, with formation of the placode expression diminishes within the area of placode (Kulessa *et al.*, 2000). In contrast the inhibitory BMP, BMP7, is expressed by the placode (Jung *et al.*, 1998). Inhibition of BMP signalling in receptor null mice led to accelerated placode formation (Andl *et al.*, 2004). Thus together with Wnt, BMP's orchestrate patterning of hair follicles.

BMP's together with Noggin, an endogenous BMP inhibitor, coordinate the localisation of the mesenchymal condensate in relation to the epidermis to induce follicle formation**.**

Neutralisation of BMP signalling by noggin over expression results in excessive placode formation (Plikus *et al.*, 2004), and noggin deletion results in failure of follicle induction (Botchkarev *et al.*, 2002). There also exists a complex interdependent relationship between BMPs, SHH and Edar signalling to regulate hair follicle growth following induction phase (Pummila *et al.*, 2007).
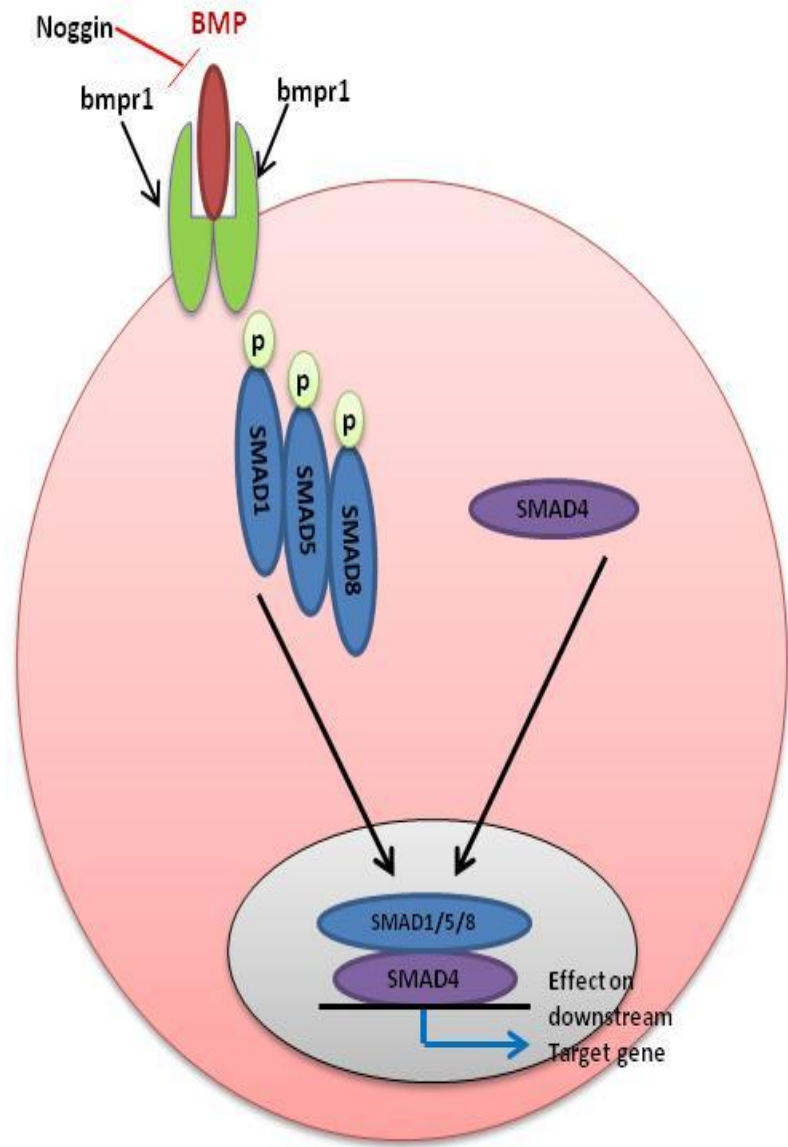
Figure 1.14: BMP signalling pathway:
BMP bind to the receptors (BMPr1) to promote activation of SMAD 1/5/8 proteins, which together with SMAD4 translocate to the nucleus where they regulate BMP's target gene transcription.

1.3.1.6 Initiation growth of hair follicle after inductive phase

Sonic Hedgehog (SHH) signalling (Figure 1.15) has an essential role after the induction phase, when the placode starts to be visible as a central down growth. SHH expression is first seen in the placode and as the development progress, it's expression becomes localised to the tip of bulb invagination above the dermal papilla (Iseki *et al.*, 1996). Smoothened knockout mice in which SHH signalling is blocked, initiate hair follicle development and formation of the dermal papilla, but hair follicle maturation fails (Chiang *et al.*, 1999; Woo *et al.*, 2012). Patched1 (Patch1), a receptor for SHH, and GLI1, a transcriptional effecter of SHH signalling, are expressed in the follicular epithelium and in the dermal condensate, consistent with the idea that SHH signals are required for the development of both components of the follicle (Chiang *et al.*, 1999; Woo *et al.*, 2012). Thus it seems likely that SHH orchestrate the "second dermal signal", regulating proliferation and down-growth of the follicular epithelium.
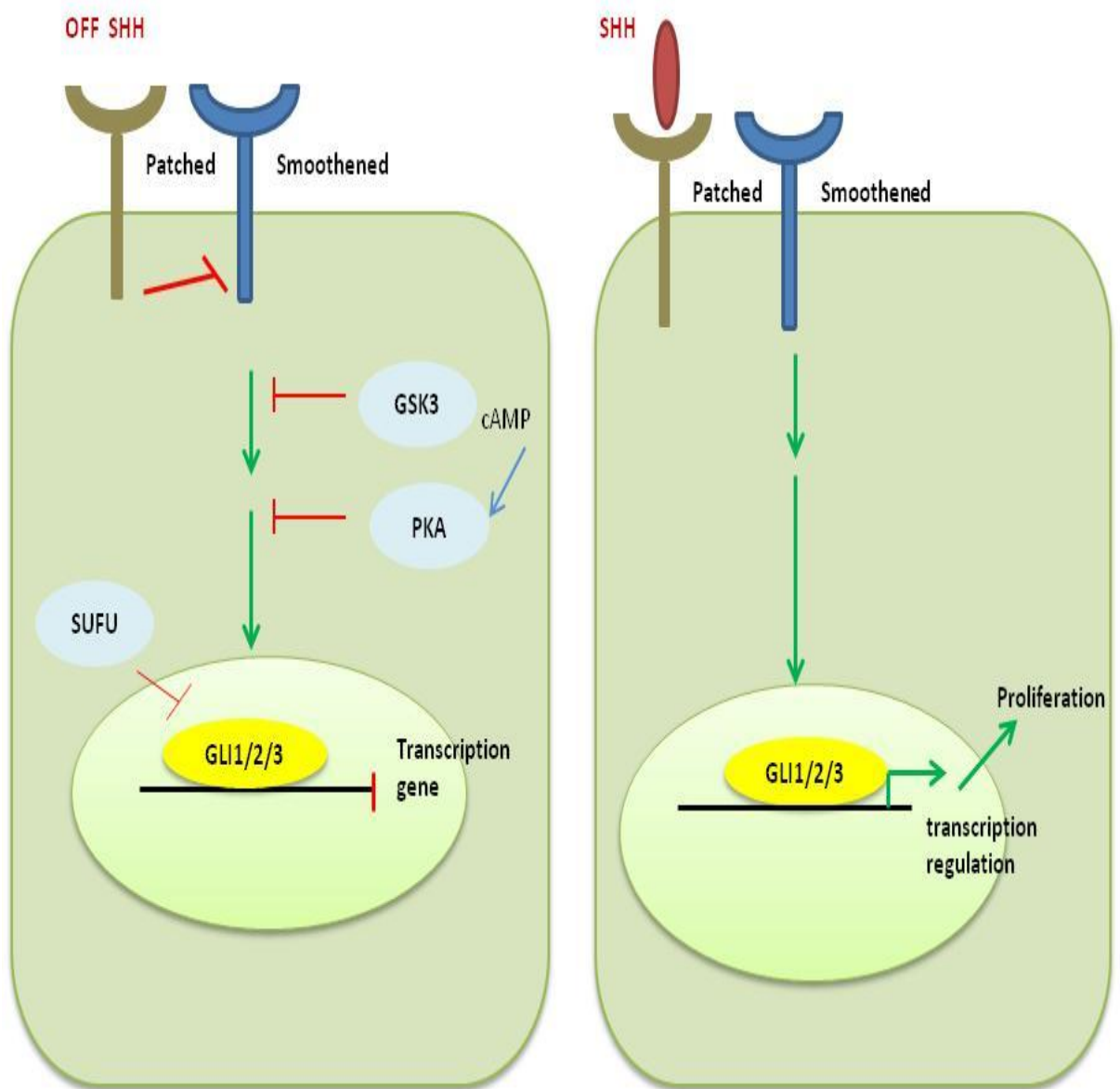
Figure 1.15: SHH signalling pathway.

SHH signal is activated by binding a receptor complex that includes patched and smoothened. Patched acts as an inhibitor of smoothened, whereas smoothened act as signal transducer. In the absence of a shh signal, smoothened is inhibited by patched protein. Kinases including GSK3, Protein Kinase A (PKA), and Suppresser of Fused (SUFU) also inhibit propagation of the shh signal. In the presence of a shh signal, the suppression of smoothened stops leading to translocation into the nucleus of the GLI transcription factors to effect direct gene regulation.

1.3.2 Maturation of the dermal papilla

The dermal papilla resides within the invagination at the base of the hair bulb. It is a continuation of the dermal sheath that surrounds the hair follicle, and shares the same mesenchymal embryonic origin. Multiple studies have shown that the dermal papilla has an inductive activity on overlying epithelia. Micro-dissected mouse and rat dermal papilla were able to induce a new hair follicle when implanted into glabrous skin of the foot pad (Oliver, 1970; Reynolds and Jahoda, 1992). In addition, isolated pure dermal papilla cells from postnatal back skin showed the ability to produce a new hair when transplanted together with postnatal epidermal cells (Driskell *et al.*, 2009; Rendl *et al.*, 2005). Interestingly, the type of hair growth was similar to the type of the hair origin from which the dermal papilla was isolated (Reynolds and Jahoda, 1992). Thus the combined interactions between mesenchymal and epithelial signals are essential for hair follicle formation and type specification (Driskell *et al.*, 2009).

SHH and Platelet-derived growth factor (Pdgf) signalling jointly mediate dermal papilla development (Karlsson *et al.*, 1999; Reddy *et al.*, 2001). The Pdgf-α signalling pathway has shown an important role in the later stage of hair follicle growth but it is not necessarily involved in hair follicle induction stage. Pdgf-α is expressed in the placode, whereas its receptor is expressed in the dermal condensate (Karlsson *et al.*, 1999). Consistent with this, mice lacking Pdgf-α have small dermal papilla, dermal sheath abnormalities, and thin hair, compared with wild-type siblings (Karlsson *et al.*, 1999). Hence Pdgf-α is also required for normal cross-talk between the follicle epithelium and its mesenchyme.

1.3.3 The adult hair follicle

The functional anatomy of the mature hair follicle in growth phase can be divided into two parts longitudinally (Figure 1.16 A-C). One is the upper permanent part which does not cycle visibly, which is composed of infundibulum that includes the opening of the hair canal to the skin and the isthmus. The other is the lower part, which is continuously remodelled in each hair cycle and represents the actual hair factory, the bulb. The infundibulum joins the isthmus region of the outer root sheath at its proximal end, where the arrector pili muscle is attached. The lower isthmus also has epithelial and melanocytic hair follicle stem cells in the so called bulge region. The bulge is the end of a permanent and non-cycling region. Bulge and the active bulb i.e., lower part of the hair follicle, are separated by suprabulbar hair follicle epithelium. This active bulb contains the matrix keratinocytes and hair follicle pigmentary unit. Activated matrix keratinocytes are rapidly proliferating cells. Their number determines the size of hair bulb and hair shaft diameter. As matrix cells differentiate they form the various cell lineages of the hair shaft and IRS (Schneider *et al.*, 2009).

The adult hair follicle in cross section consists of 8 distinctive layers (Figure 1.16D). The hair follicle ORS is a continuation of the overlying epidermis and demonstrates inward stratification. At the hair bulb, the basal and suprabasal keratinocytes above the dermal papilla are called matrix cells. The matrix cells, upon receipt of signals from the dermal papilla, undergo active proliferative differentiate upward to form the IRS and hair shaft. The IRS surrounds the hair shaft and it is composed of four histologically distinct layers: Companion, Huxley, and Henley and Cuticle layers. The hair shaft is located in the centre of the hair follicle and has three layers: Cuticle, Cortex and Medulla layers (Schneider et al., 2009).
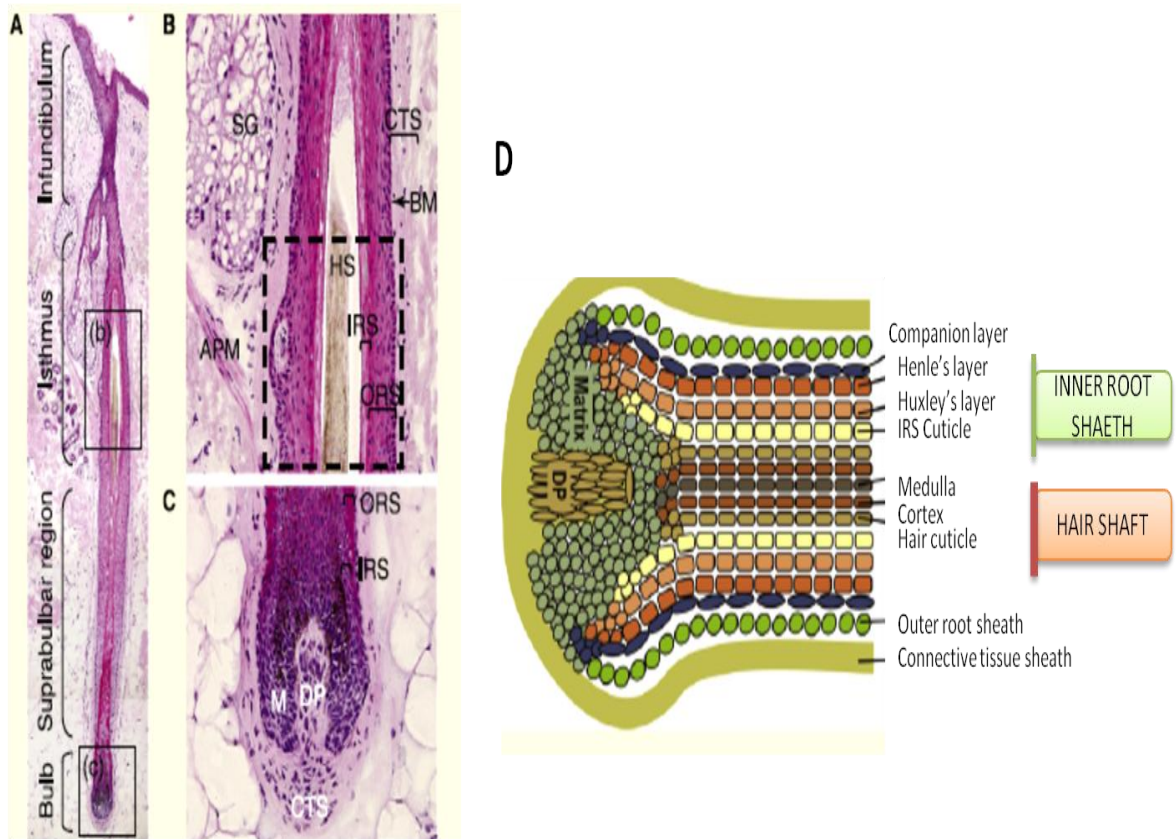
Figure 1.16: Histomorphology of the hair follicle.
A) demonstrate mature hair follicle section and its components, B) magnifying image of the isthmus in A, the dashed square indicate the site of the bulge, C) magnification of the bulb region in B, D) schematic diagram of the hair follicle layers, SG (sebaceous gland), CTS (connective tissue sheath), BM (basement membrane), HS(hair shaft), IRS (inner root sheath), ORS (outer root sheath), APM (arrector pili muscle), DP (dermal papilla), M (matrix). Adapted from (Schneider *et al.*, 2009).

1.3.4 Hair growth cycle

The hair follicle cycle describes the morphological events of the rhythmically occurring growth, regression and tissue remodelling processes in a complex neuroectodermal - mesodermal interactive system (Figure1.17) (Paus and Foitzik, 2004). It involves organ regeneration, rapid growth (anagen) and apoptosis driven regression (catagen). From catagen, the hair follicle goes back to anagen through an intermediate stage of relative quiescence (telogen) (Fuchs *et al.*, 2001; Paus and Foitzik, 2004). These transformations are controlled by changes in local signalling mechanisms, based upon changes in the activity of different mediators of hair follicle cycling (Krause and Foitzik, 2006). In this regard, different experiments have shown that the driving force of the hair follicle clock is located in the hair follicle itself (Paus and Foitzik, 2004).

Several regulatory mechanisms exist to orchestrate the hair growth cycle, from the immediate microenvironment and also the macroenvironment. The immediate microenvironment is composed of dermal papilla, dermal sheath and keratinocyte bulge stem cells, whereas macroenvironment consist of dermal fibroblast, surrounding stromal blood vessels, nerve plexus, adipocytes, exogenous hormonal factors and also the immune system (Plikus, 2012; Plikus *et al.*, 2008). Inherent growth factors which have been critical during hair follicle embryogenesis, such as Wnt and sonic hedgehog signalling, continue throughout life to control hair cycle (Fuchs *et al.*, 2001; Millar *et al.*, 1999).
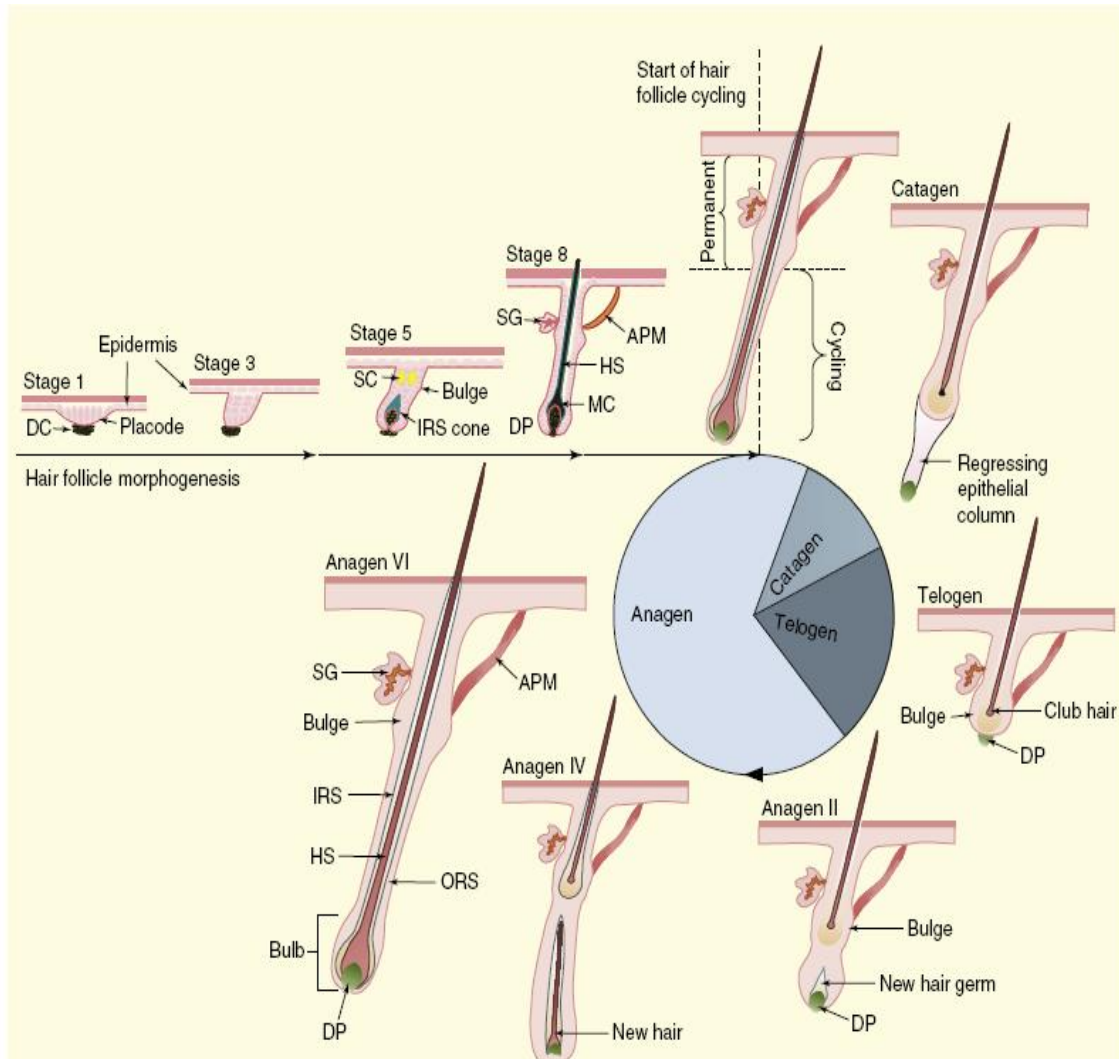
Figure 1.17: Key stages of the hair cycle.

The hair cycle is divided into three phases: Anagen (growth phase), catagen (regression phase) and telogen (resting phase) based on histological analysis. Postnatal hair morphogenesis leads to elongation of the follicle and production of the hair fibre, which emerges from the skin. Once the hair follicle has matured, it enters the regression phase, during which the lower, cycling portion of the hair follicle undergoes apoptosis. This process brings the dermal papilla into close proximity of the bulge, where the hair follicle keratinocyte stem cells reside. The molecular interaction between the hair follicle keratinocyte stem cells and the dermal papilla are essential to form a new hair follicle. The proximity between bulge and dermal papilla is maintained throughout telogen. Only when a critical concentration of hair growth activating signals is reached, anagen phase is entered and a new hair is regrown. (APM: arrector pili muscle; DC, dermal condensate (green); DP: dermal papilla (green); HS: hair shaft (brown); IRS: inner root sheath (blue); MC: melanocytes; ORS: outer root sheath; SC: sebocytes (yellow); SG: sebaceous gland). Adapted from (Schneider *et al.*, 2009). Reprinted with permission of the Current Biology. Copyright© 2014.

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1.3.4.1 Anagen

Anagen, the growing phase, is the longest segment of the hair cycle and its duration determines the length of hair. The duration of anagen varies across the body, human scalp anagen is estimated to last from 2 to 6 years, while leg hair anagen lasts between 19 to 26 weeks, and arm anagen is 6 to 12 weeks (Kligman, 1959). Hair matrix keratinocytes are believed to be transient amplifying cells, but recent findings suggest that they may persist and form the basis of hair growth during multiple hair cycles (Fuchs, 2007; Müller-Röver *et al.*, 2001). Anagen is divided into six sub-stages in which the dermal papilla gradually increases in size from anagen I to VI, associated with the rapid proliferation of matrix cells in the bulb leading to formation of the IRS and hair shaft (Müller-Röver *et al.*, 2001) (Figure1.17).

Epithelial-mesenchymal environment underpins the hair growth cycle, follicular matrix cells are maintained during anagen by reciprocal growth factor signalling with the mesenchymal dermal papilla (Oliver and Jahoda, 1988; Wang *et al.*, 2000). Fibroblast growth factors FGF7, FGF10 and FGF18, which are released by the dermal papilla, promote hair growth. By binding specific FGF receptors, FGF7 and FGF10 maintain anagen by promoting epithelial proliferation, (Greco *et al.*, 2009; Zhang *et al.*, 2006). Loss of FGF 10 results in loss of hair growth, while FGF7 null mice exhibit a milder hair loss phenotype (Wang *et al.*, 2000). In contrast, FGF18 appears to inhibit cell proliferation of hair bulge keratinocyte stem cells during anagen, since FGF18 null mice are unable to switch off keratinocyte stem cell proliferation in the hair follicle bulge that eventually leads to exhaustion of these cells (Blanpain *et al.*, 2004; Foitzik *et al.*, 2000; Hansen *et al.*, 1997).

1.3.4.2 Catagen

Catagen is the regression phase that is characterised by profound apoptosis of epithelial cells in the hair bulb and ORS, associated with an overall reduction in keratinocyte cell cycling (Figure 1.17). The level of anti-apoptosis gene, Bcl-2 and Bcl-X_L, decreases gradually during catagen (Lindner *et al.*, 1997). Bcl-2 and Bcl-X_L provide compensatory protection against hair follicle apoptosis and levels of both of these need to drop for catagen to proceed (Müller-Röver *et al.*, 1999). During catagen, hair shaft differentiation ceases, resulting in the formation of a "club" hair. The dermal papilla remains tethered to the hair bulb and moves upward toward the permanent non cycling portion of the hair (Blanpain and Fuchs, 2009; Nishimura, 2011). Towards the end of catagen, ORS keratinocytes expand resulting in a relatively undifferentiated hair bulb containing the hair germ transit amplyfying cells until the next anagen (Hsu *et al.*, 2011). Elevated TGF-β1 levels perpetuate catagen, as TGF-β1 null mice exhibit a retarded catagen phase (Foitzik *et al.*, 2000).

1.3.4.3 Catagen to telogen transition

Catagen leads to a loss of the lower portion of the hair follicle with relocalisation of the hair follicle bulb in proximity to the hair follicle bulge, which may provide a trigger for telogen to occur. In addition, the switch maybe further supported by a fall in TGF-β1 levels (Foitzik *et al.*, 2000).

1.3.4.4 Telogen

Telogen is the resting phase of the hair cycle during which hair shaft is no longer produced and is characterised by an absence of hair shaft-specific keratin mRNAs

(Bowden *et al.*, 1998) (Figure 1.17). The telogen follicle is maintained by a variety of signals from the dermal papilla and surrounding stroma. BMP2 and 4 expression is high during refractory telogen but fall dramatically during competent telogen in readiness for re-entry into the anagen phase (Plikus *et al.*, 2008). Consistent with this, over expression of the BMP antagonist noggin leads to a shortened telogen phase (Plikus *et al.*, 2008). Simultaneously factors associated with anagen are downregulated, including FGF18 (Greco *et al.*, 2009), and TGF-b2 (Oshimori and Fuchs, 2012) . The effect of Wnt expression is as a potent activator of anagen and is inhibited by increased expression of wnt antagonists, DKK1 and Sfrp4, in the surrounding stroma (Plikus *et al.*, 2011).

1.3.4.5   Telogen to anagen transition

The telogen to anagen transition, also known as the exogen phase, occurs when stem cells at the base of the telogen follicle are activated to proliferate by the adjacent dermal papilla (Hsu and Fuchs, 2012). Anagen can be initiated spontaneously by artificially wounding such as plucking, vigorous shaving or chemical exposure (Argyris , 1956). BMPs are important regulators of telogen and their levels together with endogenous inhibitors, such as noggin and follistatin, fluctuate during the hair cycle (Figure 1.18).

 BMP2 and 4 expressed during hair cycle reach a maximum level at the end of anagen and then levels fall at the end of telogen ahead of re-entry into anagen. BMP2 is predominantly released by surrounded mesenchymal cells and by subcutaneous adipocytes. In contrast, BMP4 is released from the hair follicle keratinocytes and dermal papilla, and similarly negatively regulates the transition to anagen (Plikus *et al.*, 2009).

The BMP antagonist noggin negates the effect of BMP by stoichiometric binding in the extracellular space (Zimmerman *et al.*, 1996), and is expressed by both the dermal papilla and hair follicle keratinocytes. As anagen follicles reach the last sub-stage, noggins expression disappears in the mesenchyme, but it remains expressed in the bulge (Plikus *et al.*, 2009).
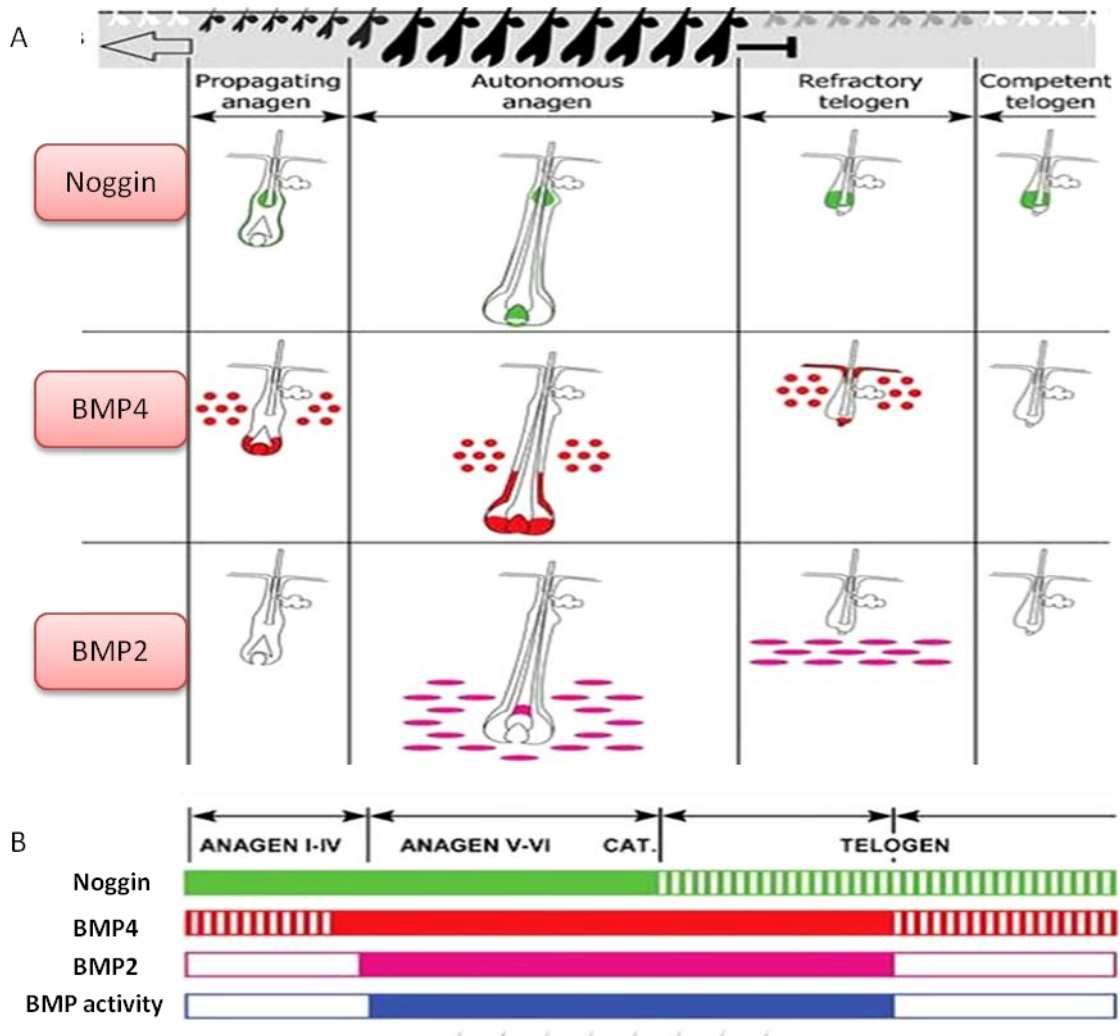
Figure 1.18: Fluctuation of BMP2-4 and BMP antagonist (noggin) during hair cycle.
A) Schematic representation of multiple expression sites of BMP2-4 and noggin throughout hair growth cycle. B). Showed strong expression demonstrated in solid areas, and absence expression of some (not all) sites in striped areas. Adapted from (Plikus *et al.*, 2009).

Transforming growth factor (TGF-β) is a growth factor secreted by hair dermal papilla cells during telogen phase. More recent evidence has implicated a crucial role of TGF-β in hair cycle regulation. Oshimori and Fuchs (2012) found that the activating mechanism of TGF-β in hair cycle regulation is mediated by its counter effect on BMP signalling pathways in hair germ cells. TGF-β2 null mutant mice showed delay in anagen initiation, therefore TGF-β2 is an essential mediator in the anagen initiation process (Oshimori and Fuchs, 2012).

Sonic hedgehog signalling (SHH) plays an important role in hair follicle embryogenesis but it also has an important signalling pathway during hair follicle cycling. Paladini *et al* have shown that hair keratinocyte proliferation during early anagen is dependent upon active SHH signalling (Paladini *et al.*, 2005). Consistent with this finding, over expression of SHH in postnatal skin of mice can initiate the anagen phase of the hair cycle and the addition of SHH agonists can trigger the transition from telogen to anagen (Paladini *et al.*, 2005). Despite these findings, anagen-initiation itself can occur in the absence of SHH signalling (Chiang *et al.*, 1999; Wang *et al.*, 2000). Thus the SHH signalling pathway has a collaborative role, with other signalling pathways, in early anagen.

1.3.4.6   Role of dermal papilla in regulating hair growth cycle

During catagen, the epithelial cells at the base of the follicle undergo apoptosis, but the dermal papilla remains intact and comes to rest next to the hair follicle bulge keratinocyte stem cells. As anagen progresses, cells at the base of the follicle start to proliferate, which results in downward growth of the follicle and the dermal papilla is both enveloped and pushed downwards. The dermal papilla cells themselves do not

divide, although the number of cells in the dermal papilla increases during anagen, possibly due to replenishment from neighbouring dermal sheath cells (Chi *et al.*, 2010; Tobin *et al.*, 2003).

In the telogen hair follicles, BMP4 is produced by dermal papilla fibroblasts to bind the BMPr1a receptor selectively expressed in the hair bulb keratinocytes, thus preventing the onset of anagen. BMP4 treatment prevents anagen development in hair follicles, while administration of the BMP4 antagonist noggin promotes the transition of telogen hair follicles to anagen (Botchkarev *et al.*, 2001). As discussed earlier, the dermal papilla produces and receives both BMP and FGF signals to modulate telogen-anagen transition.

During anagen the dermal papilla is involved in mediating a number of signalling pathways that are inductive for the overlying hair follicle epithelium. FGF7 and FGF10 are expressed in the dermal papilla and stimulate proliferation of the adjacent epithelial cells of the hair follicle (Greco *et al.*, 2009). In turn wnts released from the hair follicle keratinocytes induce activation of the dermal papilla (Enshell-Seijffers *et al.*, 2010). Interruption of β-catenin signalling in the dermal papilla results reduced proliferation of cells at the base of the follicle, which induces catagen and prevents anagen induction (Enshell-Seijffers *et al.*, 2010). In addition, BMP signals arising from hair follicle keratinocytes are essential to maintain the hair-inductive properties of dermal papilla cells during anagen (Rendl *et al.*, 2008) (Figure 1.19). Thus there are reciprocal epithelial mesenchymal signals between the macroenvironment, dermal papilla and hair follicle throughout the cell (Botchkarev and Kishimoto, 2003).

1.3.4.7 Role of macro-environment in regulating the hair cycle

In addition to short range signalling molecules, a number of growth factor hormones have also been implicated in regulating the hair growth cycle. Androgens play an important role in normal human growth (Randall *et al.*, 2001) as they transform vellus hair (non-pigmented, fine, short hair) on the body to terminal hair (pigmented, thicker, long hair) during puberty. The response of hair follicles to androgens varies according to the body site, for example, hair at axillary and pubic regions occur first during puberty and peak at third decade, whereas hair at the chest reaches peak later. Beard hair grows maximally at the fourth decade and remains the same afterwards (Randall *et al.*, 2001). Androgen receptors are expressed by dermal papilla fibroblasts, which in turn signal to release paracrine factors that induce local growth factors (Randall *et al.*, 1993).

The dermal papilla fibroblasts also express the oestrogen receptor (Chanda *et al.*, 2000). Exogenous administration of oestrogens (17 β- oestradiol) can induce telogen hair follicle to enter anagen (Ohnemus *et al.*, 2005).

Recently, adipocytes have been shown to have an important role in the hair follicle growth cycle. Adipocytes in the more local macroenvironment secrete Pdgf resulting in follicular proliferation (Figure 1.19), suggesting that adipocytes also have a stimulatory effect on hair follicle cycle (Festa *et al.*, 2011).

APM

NPNT

Nerve fibre

Quiescence induction
Proliferation induction
Differentiation
Survival
Lineage late choice

SHH

BMP4
FGF1

Keratin 6⁺

Basement Membrane

TGFβ

WNT

EDN2

Bulge stem cell

BMP4

BMPinh
FGF7
FGF10

Melanocyte stem cell

BMPinh
FGF7
FGF10

PDGFα

Adipocyte
progenitor

BMP6

KIT

BMP2

Dermal Papilla

Secondary
hair germ

Adipose Tissue
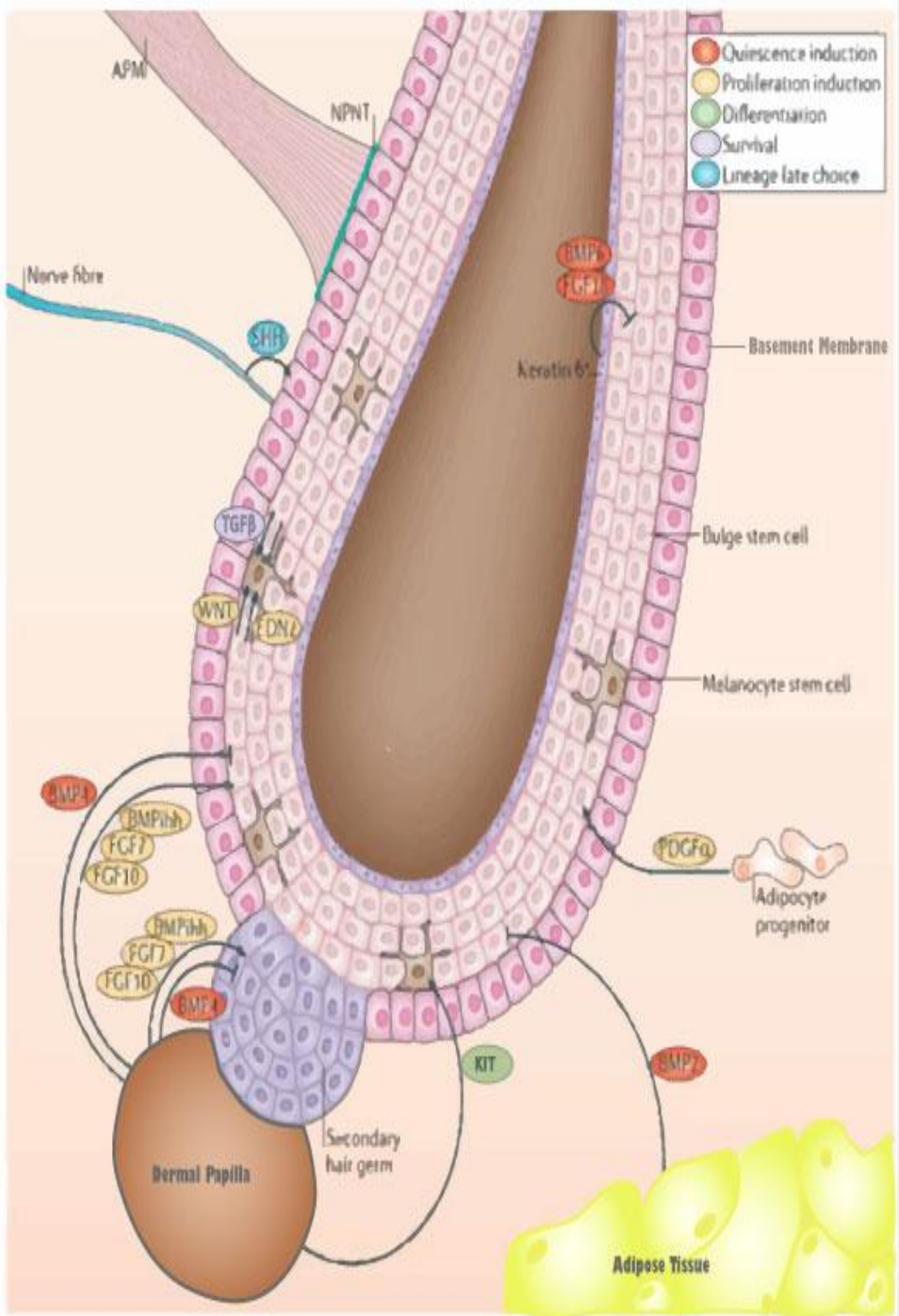
Figure 1.19: Macro and micro-environment surrounding hair follicle stem cells.
The diagram shows the interactions between hair follicle keratinocyte stem cells and neighbouring cell populations. Signals emanating from structures adjacent to the bulge or from the dermal papilla or adipocytes control the behaviour of bulge keratinocyte stem cells and consequently affect hair follicle cycling. The dermal papilla maintains bulge keratinocyte stem cells and secondary hair germ cells quiescent during telogen by producing bone morphogenetic protein 4 (BMP4) and triggers their proliferation during anagen through BMP inhibitors (BMPihh), fibroblast growth factor 7 (FGF7) and FGF10. Adipocytes maintain bulge keratinocyte stem cell quiescent through BMP2 secretion, whereas adipocyte progenitors promote their proliferation through platelet-derived growth factor-α (PDGFα) secretion. Adjacent keratinocytes promote quiescence through the secretion of BMP6 and FGF18. Hair follicle stem cell lineage determination is also dictated by microenvironmental cues, such as sonic hedgehog (SHH) signalling. The crosstalk is mutual, as signals generated by hair follicle keratinocyte stem cells affect other cell types, including muscle cells and melanocyte stem cells. Positional cues for the correct attachment of the arrector pili muscle (APM) to the bulge depend on nephronectin (NPNT) deposition by bulge keratinocyte stem cells. The survival (transforming growth factor-β (TGFβ)), quiescence, proliferation (WNT and endothelin 2 (END2) or differentiation (KIT) of melanocyte stem cells relies on signals from hair follicle stem cells and dermal papilla fibroblasts, which ensure their coordinated behaviour with the rest of the hair follicle cells during each stage of the hair cycle. Adapted from (Solanas and Benitah, 2013).

1.3.5 Hair growth cycle and differentiation

As described above, many mechanisms involved in the genesis and cycling of the hair follicles have been elucidated. The primary function of the hair cycle is to produce hair follicular keratinocytes undergo terminal differentiation to ensure the production of a tough emergent structure, hair. While light microscopy can be used to define the distinct layers/lineages of hair follicle differentiation and so define the hair follicle mini-organ, researchers frequently use expression of keratins as a surrogate marker to determine hair follicle lineages. Notably, many of the keratins expressed are unique to the hair follicle. Since this project utilises keratin expression to define differentiation patterns in human BCC knowledge of keratin biology is implicit.

1.4 Keratins

1.4.1 Intermediate filaments

Eukaryotic cell structure is dependent upon three types of cytoskeletal proteins, which are present in all cells to varying amounts dependent on the cell type: microtubules (23nm diameter), intermediate filaments (9-11nm diameter), and microfilaments (6nm diameter) (Frixione, 2000). The cytoskeletal proteins are typified by their ability to self-assemble into elongated macromolecules. Microtubules combine to form hollow cylinders, and in addition to providing structural support are notable for their roles in forming the mitotic spindle and intracellular protein transport. Microfilaments, or actin filaments, in addition to providing support are integral to cytokinesis, through interaction with myosin to form contractile molecular motors. The intermediate filament family consists of more than 70 different gene products, including the type I (acidic)

and type II (basic) keratins that provide the structural basis for all epithelial cells.

Keratins form a branched filament network within epithelial cells, which insert into cell surface structures, desmosomes and hemidesmosomes, which contribute to the stability of the epithelia and also to the attachment of the underlying extracellular matrix and connective tissues below (Figure1.20) (Moll *et al.*, 2008). Keratins are distinguished from the other intermediate filament proteins since they are preferentially expressed in epithelial cells and are obligate non-covalent heteropolymers including at least one type I and one type II keratin. Although the two keratins types share only 25-30% amino acid homology, the individual members of a single class are very closely related; with near perfect homology of the rod domains. However the structural organization of keratins is similar to all other intermediate filaments, as these proteins consist of a central coil-coil α-helical rod domain that is flanked by non–α-helical head and tail domains. (Coulombe and Omary, 2002).
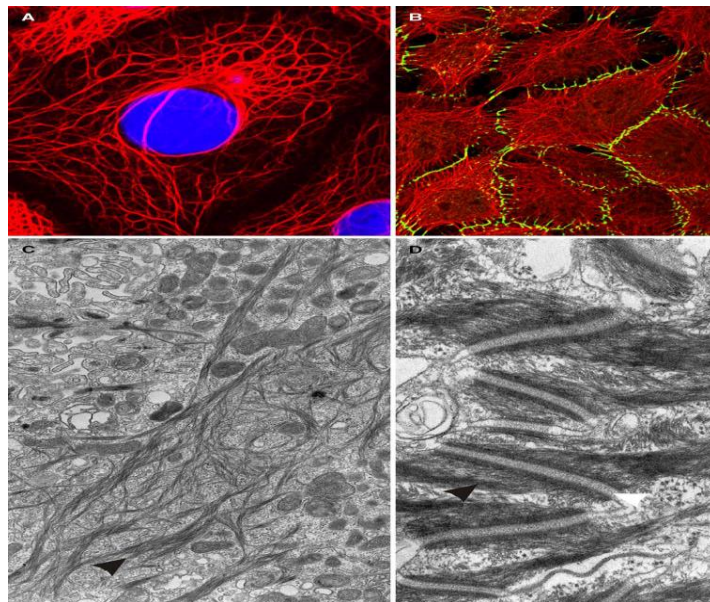
Figure 1.20: Cytoskeleton of the epithelial cell.
The cytoskeleton of the keratinocytes showed keratin seen in red and desmosomes in green (A and B). C) showed tonofilament bundles of keratin (black arrow), D) keratin intermediate filaments indicated with black arrow with insertion at the desmosomes of cell-cell contact sites of the keratinocytes (Moll *et al.*, 2008).

1.4.2 Nomenclature and chromosomal location

The human genomic organisation (HUGO) committee has given the keratin gene the prefix KRT (Rao *et al.*, 1996), resulting in a new nomenclature. Almost all the keratin genes are clustered at two different chromosomal sites: chromosome 17q21.2 (type I keratins, except KRT18) and chromosome 12q13.13 (type II keratins including KRT18) (Figure1.21). Type 1 keratin genes have 8 exons and 7 introns (except KRT18 and KRT19) and type 2 keratin genes have 9 exons and 8 introns (except KRT8) (Rogers *et al.*, 2000; Rogers *et al.*, 1998). Based on the HUGO, of the 54 human keratins and their genes, a four groups have been established; epithelial keratins/genes, hair keratins/genes, keratin pseudogenes, non-human epithelial and hair keratins of other mammalian species. The range of number of keratin group system will illustrate in the table 1.2 below.
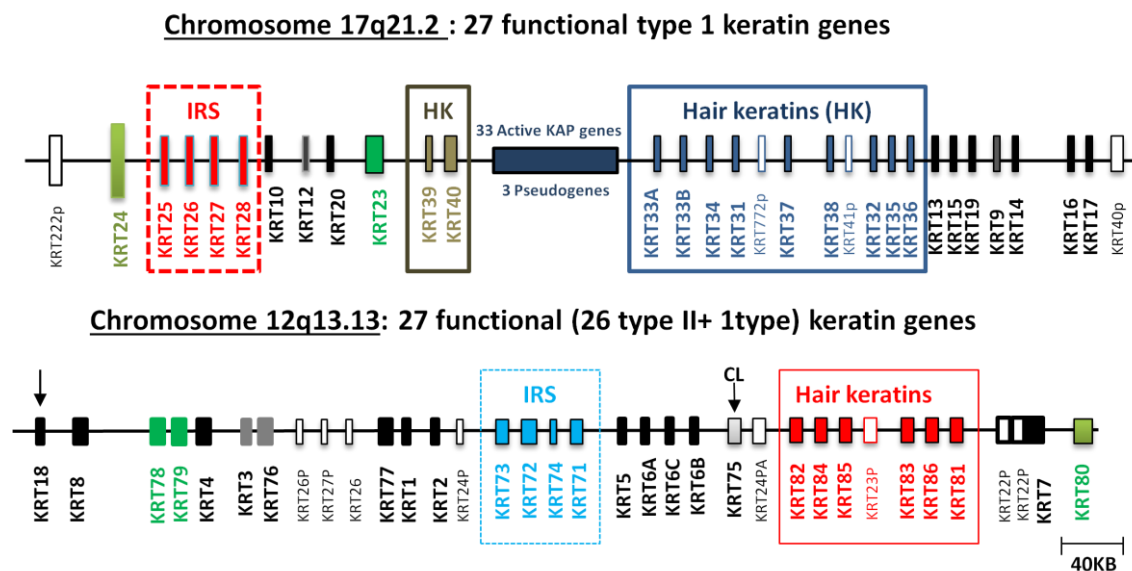
Figure 1.21: Genomic Organisation of Human Keratin Genes.
Type I and type II keratin gene subdomains are located on chromosomes 17 and 12, respectively. They include the hair follicle specific keratins, the "soft" epithelial IRS and "hard" hair shaft keratins (Hair keratins). The only known exception to the rule is the type I keratin (K18) that is located in the type II cluster on chromosome 12 (arrow). Adapted from (Moll *et al.*, 2008).

Keratin groups	Number of genes
Human type I epithelial keratins	**9–28**
Human type I hair keratins	**31–40**
Nonhuman type I epithelial and hair keratins	**41–70**
Human type II epithelial keratins	**1–8 and 71–80**
Human type II hair keratins	**81–86**
Nonhuman type II epithelial and hair keratins	**87–120**
Type II keratin pseudogene	**121–220**
Type I keratin pseudogenes	**221** ⟶

Table 1.2: Numbering system of keratin groups.
Adapted from (Schweizer *et al.*, 2006b).

1.4.3 Keratin protein structure

Keratin intermediate filaments are strict obligate heteropolymers, consisting of a strict pairing between type I (28 members) and type II (26 members) keratin proteins; which requires coordinated co-expression in epithelial cells (Moll *et al.*, 1982). Thus most keratin genes must be regulated in a pair wise manner, dependent upon the epithelial cell type in a differentiation-dependent fashion.

All keratin types have a common domain structure, which they share with all other intermediate filament proteins (Steinert, 1993; Steinert *et al.*, 1993). They contain an alpha helical central rod domain of conserved length, approximately 310 amino acids, comprising of four heptad repeat–containing segments (1A, 1B, 2A and 2B). The alpha helical rod domain is interrupted by three short linker sequences (L1, L12 and L2), at conserved locations, and flanked by amino terminal head and carboxy-terminal tail domains (Figure. 1.22 a,b).

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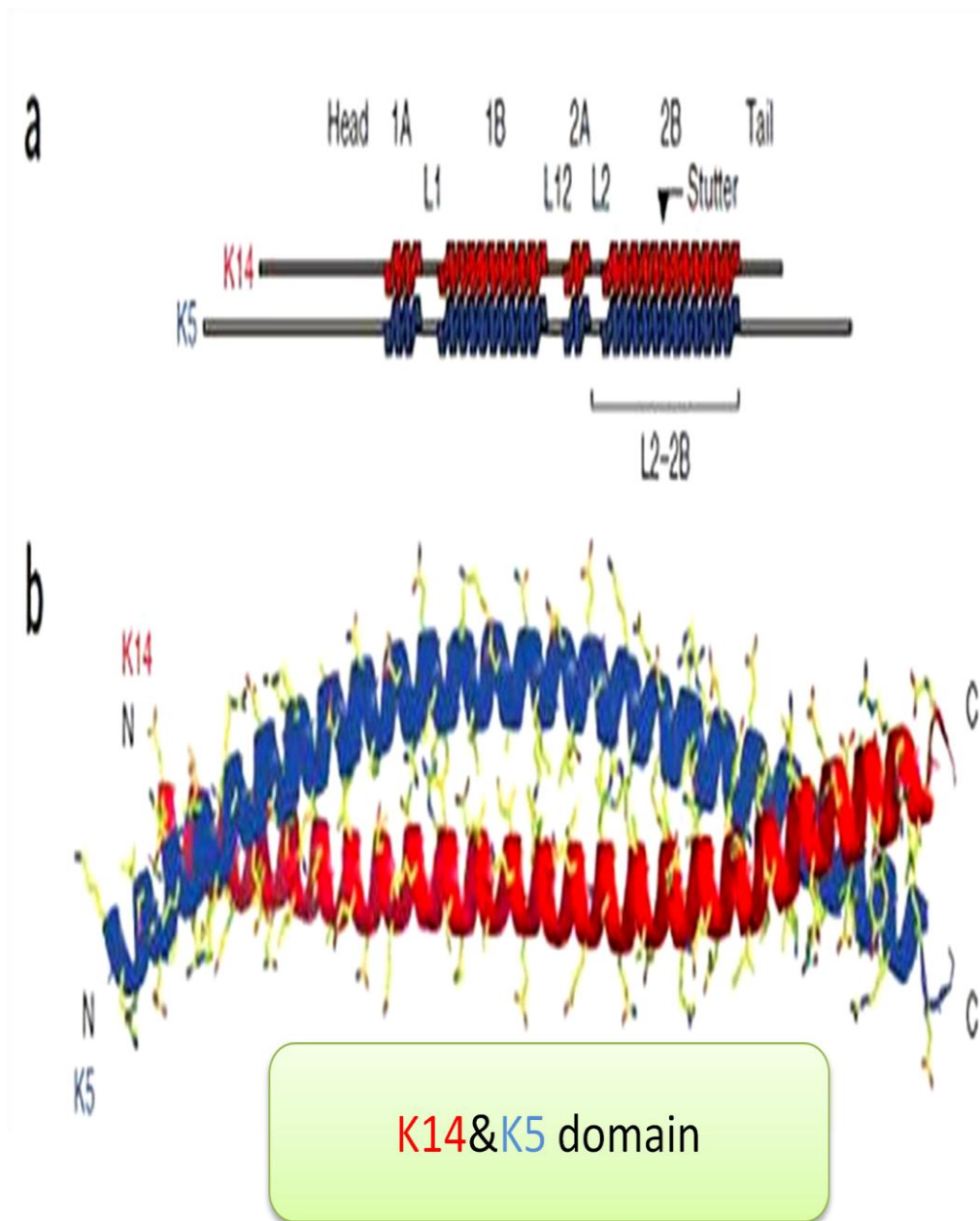
Figure 1.22: K5 and K14 domain structure.

showing crystal structure of the K5–K14 2B heterocomplex. (a) Schematic diagram of the domain structure of K5 and K14. (b) Crystal structure of the K5–K14 coil 2B heterocomplex. Adapted from (Lee *et al.*, 2012).

The central rod domain is the driver that sustains self-assembly into filaments and is composed of seven amino acid sequence heptad repeats, of which the first and fourth residues are occupied by hydrophobic amino acids that are crucial for the coils (Lee *et al.*, 2012). In the middle of the 2B domain the heptad pattern is interrupted, is "stuttered", as the two aligned parallel chains polymerize. The heterocomplex is favoured and is markedly more stable in solution relative to homodimers (Coulombe and Fuchs, 1990). The stuttered alignment facilitates tetramer formation by enabling coiled-coil dimers to interact along their lateral surfaces in an antiparallel orientation; so that their coil 1B subdomains overlap (Bernot *et al.*, 2005). The intermediate filament structure consists of eight tetramers, with four distinct subfibrils per filament (Figure1.23).
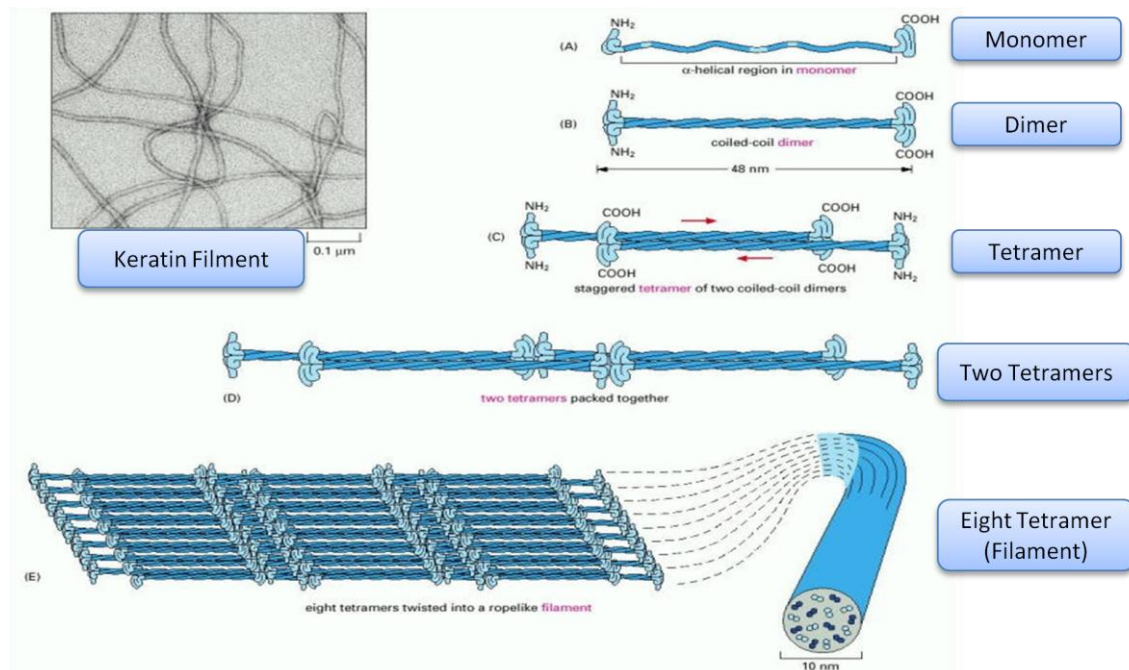
Figure 1.23:The structure of keratin intermediate filaments.
Adapted from (Bruce Alberts, 2002).

1.4.4 Keratin expression patterns in health and disease

Epithelial tissues have evolved into successful barriers that protect against the environment. To achieve this, epithelia undergo constant turnover to prevent microbes gaining a foothold and carcinogen acquisition. To ensure epithelial cell integrity, keratinocyte terminal differentiation is a precisely curated programme. Keratinocyte structure is dependent upon the expression of keratins, which in the skin can constitute 80% of the total protein mass. For example inherited keratin mutations can dramatically undermine the mechanical viability of the tissue, leading to skin and hair fragility with secondary reactive hyperplasia of skin and nails (Arin, 2009). This role of keratins in epithelial structural integrity is functionally integrated into the process of epidermal differentiation, so that the changes in keratin expression patterns represent differentiation landmarks.

In stratified epithelia with an overlying cornified layer such as the epidermis, keratins are abundant and densely bundled as tonofilaments. During the process of keratinisation, pairs of keratins are expressed that are highly specific for the state of differentiation. In the basal layer, keratins K5 and K14 are expressed, while in the suprabasal layers keratins K1 and K10 are synthesized (Fuchs and Green, 1980). In the uppermost layers K2 is expressed instead of K1 as the partner for K10. Within mucous membranes, such as the oral mucosa, the suprabasal layers constitutively express K6 and K16 instead. The epidermal suprabasal layer of the palms and soles express K1 and K9. Upon injury to the skin epidermis, K6, K16 and K17 are transiently induced in the suprabasal keratinocytes (Freedberg *et al.*, 2001). In simple epithelia of the internal organs, where mechanical stress is less pronounced, the loosely formed filaments contain K7, K8, K18, K19, K20 and K23 (Anderton, 1983; Moll *et al.*, 2008). This

relationship between keratinocyte differentiation and pattern of keratin expression is also overtly evident in the hair follicle, as described below.

1.4.5 Human hair follicle specific keratins

Over the last decade, a large number of hair follicle-specific epithelial keratins have been discovered. This began with the identification of K6hf (now KRT75), which was only expressed in the hair follicle companion layer (Winter *et al.*, 1998b). KRT75 was the first epithelial keratin to be identified to be specifically expressed in the hair follicle.

Many more epithelial keratins with hair follicle specificity have since been described, namely the type II keratins K6irs1, K6irs2, K6irs3 and K6irs4 (now KRT71–KRT74) and type I keratins K25irs1, K25irs2, K25irs3 and K25irs4 (now K25–K28), all of them specifically expressed in and closely restricted to the defined compartments of the hair follicle IRS (Langbein *et al.*, 2006). In addition to the epithelial "soft" cytokeratins, hairs are built up from "hard" or "trichocytic" hair keratins (Heid *et al.*, 1988a, b). The "hard" keratins differ from the epithelial keratins by their considerably higher sulphur content in their non-α-helical head and tail domains, which is mainly responsible for the extraordinary high degree of filamentous cross-linking by keratin-associated proteins (Table 1.3).

Keratin types	Type 1 Keratins		Type 2 Keratins	
	New name	Old name	New name	Old name
Epithelial keratins	KRT9	K9	KRT1	K1
	KRT10	K10	KRT2	K2
	KRT12	K12	KRT3	K3
	KRT13	K13	KRT4	K4
	KRT14	K14	KRT5	K5
	KRT15	K15	KRT6a	K6a
	KRT16	K16	KRT6b	K6b
	KRT17	K17	KRT6c	K6e/h
	KRT18	K18	KRT7	K7
	KRT19	K19	KRT8	K8
	KRT20	K20	KRT76	K2p
	KRT23	K23	KRT77	K1b
	KRT24	K24	KRT78	K5b
			KRT79	K6l
			KRT80	Kb20
Hair follicle-specific epithelial (root sheath) "soft" keratins	KRT25	K25irs1	KRT71	K6irs1
	KRT26	K25irs2	KRT72	K6irs2
	KRT27	K25irs3	KRT73	K6irs3
	KRT28	K25irs4	KRT74	K6irs4
			KRT75	K6hf
Hair follicle-specific shaft "hard" keratins	KRT31	Ha1	KRT81	Hb1
	KRT32	Ha2	KRT82	Hb2
	KRT33a	Ha3-I	KRT83	Hb3
	KRT33b	Ha3-II	KRT84	Hb4
	KRT34	Ha4	KRT85	Hb5
	KRT35	Ha5	KRT86	Hb6
	KRT36	Ha6		
	KRT37	Ha7		
	KRT38	Ha8		
	KRT39	Ka35		
	KRT40	Ka36		

Table 1.3: The Keratin Nomenclature.
The "Keratin Nomenclature Committee" established a new consensus nomenclature for mammalian keratin genes and proteins in accordance with the nomenclature of the Human Genome Organization (HUGO) for both the gene and protein names (Schweizer *et al.*, 2006b).

1.4.5.1 Hair follicle-specific epithelial "soft" keratins

It is now clear that some of the epithelial root sheaths of the hair follicle, the IRS, demonstrate unique expression of a number of very specific keratins (KRT25, KRT26, KRT27, KRT28, KRT71, KRT72, KRT73, KRT74, KRT75). Their quantitative "under-representation" when compared to the masses of hair or epidermal keratins in the tissue had hindered earlier attempts at detection by biochemical methods (Schweizer *et al.*, 2007).

Originally called K6hf, KRT75 was the first hair follicle specific keratin to be described. K75 is specifically expressed in the companion layer of the hair follicle, a thin layer between the outer and the inner epithelial root sheath. The companion layer, which adjoins the suprabasal cells of the ORS, forms a unique histological cell layer, extending upwards from the hair bulb matricial cells. Since mRNA expression of KRT75 is observed in the matricial cells of the bulb and ceases within the companion layer within which the KRT75 protein is evident throughout, these findings imply that the companion layer is the first of the hair follicle sheath layer demonstrating upwards differentiation (Wang *et al.*, 2003a). Explicitly, the companion layer is its own individual compartment of the hair follicle and not the innermost extension of outer root sheath inward differentiation.

In addition KRT75 has also been detected in the normal nail bed and fungiform papillae of the tongue (Perrin, 2007; Schweizer *et al.*, 2007). Consistent with its restricted expression, mutations in K75 appears to predispose to the common hair disorder pseudofolliculitis barbae (Winter *et al.*, 2004), which is characterized by inflammation from ingrown beard hairs, and to the loose anagen hair syndrome (Chapalain *et al.*,

2002). KRT75 expression has already been observed in tumours, trichoblastomas and basal cell carcinomas (Kurzen *et al.*, 2001).

A set of four type I keratins (K25–K28) and four type II keratins (K71–K74) is similarly highly specific for the hair follicle inner root sheath (IRS). These IRS keratins are differentially expressed in the IRS Henle layer, the Huxley layer, and cuticle layers. The keratinocytes of all three compartments express KRT71, KRT25, KRT27 and KRT28. However, KRT74 is restricted to the Huxley layer, whereas three keratins are sequentially expressed in the cuticle layer, (KRT73, KRT72 and KRT28) (Langbein *et al.*, 2003; Langbein *et al.*, 2004). Intriguingly normal epithelial keratins are not observed in the inner root sheath (Schweizer *et al.*, 2007). As yet human hair disorders related to the IRS keratins have not yet been discovered.

1.4.5.2 Hair follicle-specific "hard" hair shaft keratins

All keratin structural rigidity results from its double helix structure, similar to the structurally unrelated protein collagen, as well as the high glycine content. There is also a preponderance of amino acids with small nonreactive side groups, for which H-bonded close packing allows chemical specificity. Additional strength and rigidity is conferred by the presence of large amounts of the sulphur-containing amino acid cysteine, required for the disulphide bridges that result in thermally stable cross linking. The human hair shaft keratins (KRT31, KRT32, KRT33a, KRT33b, KRT34, KRT35, KRT36, KRT37, KRT38, KRT39, KRT40, KRT81, KRT82, KRT83, KRT84, KRT85, KRT86) have high cysteine content. The more flexible of the epithelial cells have fewer inter-chain disulphide bridges than the keratins in the mammalian hair shaft.

The sulphur-rich keratin proteins constituting the "hard" or "trichocytic" hair shaft keratins originally consisted of eight "major" (type I: Ha1-4, type II: Hb1-4) and two "minor" (Hax, Hbx) keratins (Heid *et al.*, 1988a, b; Heid *et al.*, 1986). 17 members of the hair shaft keratin subfamily have been identified, eleven type I hair keratins (KRT31–KRT40; previous designations Ha1–Ha8, Ka35, Ka36 and six type II hair keratins (KRT81– KRT86; previous designations Hb1–Hb6) (Schweizer *et al.*, 2007). Hair shaft keratins are also expressed in the nail matrix and nail bed where they contribute to the formation of the hard tissue of the nail plate (Perrin *et al.*, 2004). Expression of hair shaft keratins has also been observed in the filliform papillae of the tongue and within Hassall's corpuscles of the thymus (Heid *et al.*, 1988b).

The congenital hair disease monilethrix, characterized by fragile beaded hair shafts, has been associated with mutations in KRT86, KRT81 and KRT83 (Winter *et al.*, 1997). A distinct mutation in KRT85 has been associated with ectodermal dysplasia of hair and nail type, which is characterized by total alopecia and severe nail dystrophy(Naeem *et al.*, 2006).

Hair shaft keratins have also been detected in the hair follicle tumour pilomatricoma, which are believed to originate from hair matriacial cells (Cribier *et al.*, 2001).

1.4.6 Lineage specific hair Follicle differentiation patterns are mirrored by keratin expression

Keratin expression patterns in the hair follicle recreate the lineage specific differentiation patterns outlined earlier, such that each of the anatomical microscopic hair follicle layers can be defined by expression of specific keratins (Figure1.24). Furthermore, keratins expressed in hair follicle IRS and hair shaft are unique and not expressed elsewhere in the body. Thus it is possible to outline patterns of hair follicle differentiation that may be evident in BCC using keratin expression as a surrogate, however additional analysis would still be required to ensure that keratin expression in BCC is similarly tied to the process of differentiation.
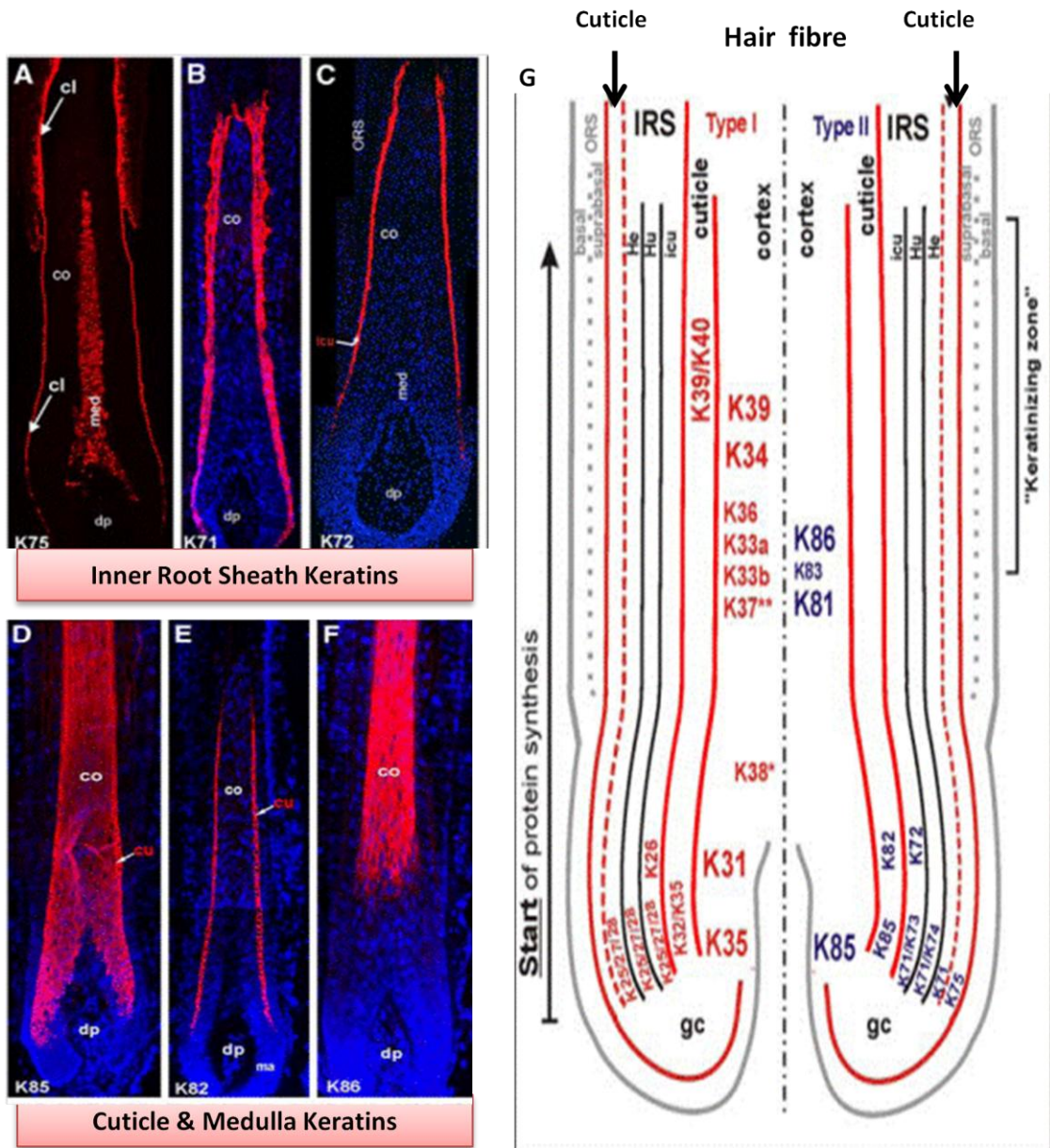
Figure 1.24: Immunofluorescence labelling diagram shows the hair follicle-specific keratins.

A) K75 is specifically expressed in the companion layer (cl) of hair. (B) K71 is expressed in all compartments. (C) K72 is found in the cuticle (cu) of the hair inner root sheath (IRS). (D)K85 is expressed in hair matrix, the upper cortex and the hair cuticle (cu). (E) K82 is demonstrated in the hair cuticle. (F) K86 is found in the mid-to-upper hair cortex (co). (G) showing the summary of the expression of all hair and hair follicle-specific keratins Adapted from (Schweizer *et al.*, 2006b).

Hence, in this project we hypothesised that by determining the normal patterns of tissue differentiation within cancer we could identify tumour type specific factors that promote differentiation, for therapeutic development. Therefore the aim of this study is to define patterns of human hair follicle differentiation in human basal cell carcinoma (BCC) in order to elucidate potential drug-able targets that can promote tumour specific differentiation.

Chapter 2. Materials and Methods

2.1 Skin Samples

Normal human skin and human BCC tissue was obtained after approval by the NHS R&D (Project ID: 08/DMD/4425) and ethics committee (reference: 09/WSE02/10). Anonymous skin samples were collected from patients undergoing surgery for skin cancer at the Welsh Institute of Dermatology, Cardiff and Vale University Health Board after written informed consent was obtained. All patients aged 18 years or over with basal cell carcinoma were eligible to participate in the study. The only exclusions were patients that could not or were unwilling to give consent. All human tissue samples were anonymous and processed in three potential ways: (1) frozen for immune-labelling, (2) treated with Trizol™ (Life Technologies, UK) for RNA extraction , or (3) cells were dissociated for primary culture.

Tissue samples frozen for immune-labelling were orientated, embedded and mounted onto a cork board using Tissue-Tek® OCT™ compound and snap-frozen in hexane cooled on dry ice. Tissue samples were stored in cryo-tubes immersed under liquid nitrogen (-196°C) until used.

Tissue for total RNA extraction was micro-dissected in which removed of overlying epidermis from the tumour bearing dermis, by sterile scalpel in the tissue culture hood under sterile conditions. The samples of tumour containing tissue were homogenised in Trizol™ , 1 ml of trizol was added to approximately 0.1 gram of tissue , followed by RNA isolation with an RNeasy kit (Qiagen, UK) using the manufacturer's instructions.

Tissue for cell culture was processed as previously described (Colmont et al, 2013). Briefly, human BCC tumour tissue was first mechanically dissociated using a sterile scalpel in a tissue culture hood and then incubated for 2 hours with dispase (BD Biosciences, UK) and ultrapure collagenase type IV (Sigma, UK) at 37°C. The supernatant was removed after brief centrifugations (1000g for 5 minutes), and the remaining tissue was incubated at 37°C for 5 minutes with 5ml trypsin 0.05% with EDTA to create a single cell suspension. Tissue remnants were removed by filtration through a 40 µm cell strainer.

2.2   RT-PCR

2.2.1   RNA extraction from cultured cells

Total RNA was also extracted from cultured cells using trizol reagent (Life Technologies, UK). Culture media was removed and the cells washed with 2ml phosphate buffered saline (PBS) per well three times before adding 1 ml of trizol reagent per well of a 6-well plate. The trizol wwas pipetted vigorously to detach all the cells and the contents were transferred to a sterile 1.5ml eppendorf tube and vortexed for 5 minutes before placing on ice. An aliquot (0.25 ml) of chloroform (Fisher Scientific, UK) was then added to each eppendorf, mixed by vigorous shaking for 15 seconds and then left to stand on ice for 15 minutes. The samples were the centrifuged at 12,000 rpm for 15 minutes at 4ºC and the top aqueous layer containing RNA was carefully transferred to a new sterile eppendorf tube by pipetting. The mRNA was isolated from the sample with an RNeasy kit (Qiagen, UK) using silica spin columns following manufacturer's instructions. In all cases, mRNA quantity was determined using a Nanodrop™, using 2 microlitres of RNA for the spectrophotometer reading.

RNA samples were run on an 2 % agrose gel to ensure RNA quality, by determining the intensity of the 18s and 22s RNA bands relative to the background. Where these were weak or if there was a substantial RNA smear then such samples were discarded. Samples of good quality were stored at -80°C.

2.2.2 Reverse transcription to create cDNA

cDNA was created using an iScript™ cDNA synthesis kit (Biorad, UK) as per manufacturer's instructions, the kit uses both oligo(dT) and random hexamer primers to ensure the broadest coverage of mRNA. Briefly, 500ng of RNA was added to a 20 μl reaction volume containing reverse transcriptase and typically yielded 20 μg cDNA. The total cDNA generated from each reaction was quantified by Nanodrop™, and a working solution of 100 ng cDNA per μl in RNAase and DNAase free sterile water. All cDNA samples were stored at -20°C until required.

2.2.3 Polymerase chain reaction (PCR)

High fidelity platinum Taq (Life technologies, UK) was used for all PCR reactions, as per manufacturer's instructions. A standardised quantity of cDNA (1 μg) was used for each 25 μl reaction volume to determine the expression of low yield keratins for both hair follicle and BCC samples. However, lower levesl of cDNA (200ng) were used per reaction when determining *in vitro* responses to treatment with noggin and TGFβ. Standard protocols were used to programme the thermal cycler (MJ Research PTC-200 Peltier).

Taq was active at 94 ℃ for 5 minutes and the products were denatured at 94 ℃ for 30 seconds. The annealing temperature was varied based on the properties of the individual primers used but the time was constant (30 seconds). Finally, extension (synthesis) was continued for 1 minute at 72 ℃ with a final extension of 10 minutes at 72 ℃.

In cases where multiple bands occurred at the theoretical annealing temperature (e.g for K16, K25, K75, K31, K34 primers) annealing temperatures were adjusted. In addition, to improve annealing specificity for DNA containing GC rich regions, Q solution (Qiagen, UK) was added as per manufacturer's instructions and the denaturation temperature increased to 98 ℃.

We designed primers to amplify low complimentary sequence regions between 200bp and 400bp in size (Table 2.1). Primers were 18-23 nucleotides in length, had a 60% GC content, and matching melting temperatures (TMs). To ensure specificity, primers pairs were entered into the National Centre for Biotechnology Information (NCBI); primer blast search engine.

Searches were made against both to the Homo sapiens (taxid: 9606) and mus musculus (taxid: 10090) Refseq mRNA databases to counter against false positives in mixed cultures. Furthermore, standard Sanger sequencing of PCR products was used to ensure amplification of the expected product. All primers were synthesised by Life technologies.

Table 2.1 : Summary of PCR Custom Primers for Keratins, BMPs, FGFs and Housekeeping Genes.

Gene (Accession number)	Nucleotide sequence	Exon	Product Size
Keratin 5 (NM_000424.3)	Forward: 5'-CCCAGTATGAGGAGATTGCCAACC-3' Reverse: 5'-TATCCAGAGGAAACACTGCTTGTG-3'	Exon 5 Exon 9	475bp
Keratin 14 (NM_000526.4)	Forward: 5'-GATCTCGGAGCTCCGGCGCACCATG-3' Reverse: 5'-GGCCTCCTAGGCCTGAGCGGGG-3'	Exon 5 Exon8	462bp
Keratin 15 (NM_002275.3)	Forward: 5'-GGAGTTCAGCAGCCAGCTGGCC-3' Reverse: 5'- CGGCCAGTGAGTTCTCCAGCCC-3'	Exon4 Exon6	308bp
Keratin 16 (NM_005557.3)	Forward: 5'-GCATGCAGTAGCGGCCTTT-3' Reverse: 5'-TCCAACAGCGAACTGGTACAGA-3'	Exon4 Exon5	151bp
Keratin 17 (NM_000422.2)	Forward: 5'-TTCCGCACCAAGTTTGAGACA-3' Reverse: 5'-AAGAACCAATCCTCGGCATCC-3'	Exon 2/3 Exon4	323bp
Keratin19 (NM_002276.4)	Forward:5- CCAGCTGGGCGATGTGCG-3' Reverse:5- - GAAGAGCCGGGGGTAAGGG-3'	Exon4 Exon6	253bp
Keratin 75 (NM_004693.2)	Forward: 5'-ACCGAGAGGGGCAGGCTTGAAG-3' Reverse: 5'-GAGTGGATGAAGTTGATCTCCTCGGG-3'	Exon2 Exon4	212bp
Keratin 26 (NM_181539.4)	Forward: 5'-GCGAACTGGGTCTGGTAGG-3' Reverse: 5'-AGTTCATCCAACACTCTGCGAAGA-3'	Exon1 Exon3	558bp
Keratin 27 (NM_181537.3)	Forward: 5'-CTGAGTACGAAGCCCTCGCAGAGC-3' Reverse: 5'- GTTTGATTTCCTGGGCCTCCATAGCC-3'	Exon1 Exon8	451 bp
Keratin 28 (NM_181535.3)	Forward: 5'-TAATGACCGCTTGGCATCCTAC-3' Reverse: 5'-TTGTTCTGTCTTGCCGTTGG-3'	Exon1 Exon8	1102bp
Keratin 71 (NM_033448.2)	Forward:5'- CGCACCCAGTATGAGGAGATTGCC-3 Reverse: 5'- GCCGGAAGCCATAGACACTGCC-3	Exon5 Exon9	493bp
Keratin74 (NM_ 175053.3)	Forward:5' CGCATGCATTATGAGGAGATCGCCC-3' Reverse:5' GGTCAACACCCGCAGAGCTGG-3'	Exon5 Exon9	513bp
Keratin 32 (NM_002278.3)	Forward: 5'-GTCAGTACGAGGCCATGGTGGAGG-3' Reverse: 5'-GGGGCACGCATGGGGAGGG-3'	Exon4 Exon7	474bp
Keratin 35 (NM_002280.4)	Forward: 5'-GGTGCCAGTATGAAACCCTGGTGG-3' Reverse: 5'-GGTGAGTAGTCAGGTGCACATGGG-3'	Exon4 Exon7	448bp
BMP2 (NM_ 001200.2)	Forward:5' CCTTAAGTCCAGCTGTAAGAGACACCC-3 Reverse:5' CACAACCCTCCACAACCATGTCC-3'	Exon3 Exon3	308bp
BMP4 (NM_ 130851.2)	Forward:5' GGAATTGGGCCCAGCTCCGG-3' Reverse:5' GGAGATGGCACTCAGTTCAGTGGG-3'	Exon4 Exon4	359bp
FGF7 (NM_ 002009.3)	Forward:5' GAACTGTTCCAGCCCTGAGCG-3' Reverse:5' CCCTCCGTTGTGTGTCCATTTAGC-3'	Exon2 Exon4	352bp
FGF18(NM_ 003862.2)	Forward:5' CCGCCTCCCTCCCGCCC-3' Reverse:5' GTCCCCATCCTCGCCGCGG-3'	Exon1 Exon3	264bp
BMPr1a (NM_004329.2)	Forward:5' GCCCCCTGTTGTCATAGGTCCG Reverse:5' CGCCACGCCATTTGCCCATCC	Exon6 Exon9	353bp
GAPDH (NM_001256799.1)	Forward: 5'-GACCCCTTCATTGACCTCAACTACA-3' Reverse: 5'-GCCTTCTCCATGGTGGTGAAGAC-3'	Exon2 Exon3	221bp

2.2.4 DNA agarose gel electrophoresis

PCR products were analysed by agarose gel electrophoresis which separates the products according to size. The size of products was estimated by comparison to a DNA ladder (Fischer, UK). A 2% agarose gel was prepared by adding 1g of agarose (Sigma-Aldrich, UK) to 50ml 2x Tris-Acetate-EDTA (TAE) buffer (see appendix 1) mixing and heating in a microwave. Ethidium bromide (5 μl of a 10mg/ml stock solution (Sigma-Aldrich, UK) was added to the mix. The gel was cast in an AGT1 tank (VWR International, UK) and combs to make 20 or 40 wells were used. After the gel had set, it was immersed in 50ml of 2x TAE electrophoresis buffer. DNA samples were mixed with 2 μl DNA loading dye and 10 μl of each sample was pipetted into each well. One or two lanes were loaded with a DNA ladder (Fischer, UK). The gel was then run at 50V for approximately 1 hour. The DNA bands were visualised on a UV transilluminator and images acquired using UV trans-illumination Alpha Innotech Multi Image II (Alpha Imager HP). The molecular weight of the product was determined by the location relative to the DNA ladder markers of known molecular weight. The predicted molecular weight of the PCR products was used to assess the presence of the correct cDNA, and therefore the correct mRNA, within the cells from which they were derived.

2.2.5 Sequencing of PCR products

The PCR products were cleaned before sequencing using a PCR purification kit (Qiagen, UK), following manufacturer's instructions, then submitted to Central Biotechnology Services (CBS), Cardiff University for ABI BigDye automated

sequencing. Sequencing data was analysed using alignment to the theoretical sequence using Vector NTI software.

The Modified Automated Sequencing Method using Big Dye v 3.1 & ABI 310 was applied. The forward and the reverse primer used for sequencing are listed in primer (Table 2.1). Briefly, the PCR product was run in 2% Agarose gel to check for strong and clear bands and with the appropriate size. The following components were added to a nuclease-free 500 µl microtube: 20 µL of PCR product was mixed with 24 µL of PEG (Polyethylene glycol) (26% PEG 8000, 6.6 Mm $MgCl_2$, 0.6 M NaOAc, pH5.2), the mixture was allowed to precipitate at room temp for 10 minutes then centrifuged at 13.000 rpm for 25 minutes at room temperature, the supernatant was carefully pipetted out (invisible pellets) The pellets were washed two times with 200 µL of ice cold 70% ethanol then centrifuged for 2 minutes at 15.000 rpm. The supernatant was carefully removed and the samples were left to dry at room temperature for 5 minutes max. At this time point the sequencing oligonucleotide dilutions were made up (7µL primer + 13 µL of nuclease free water) and kept on ice and the master mix was prepared (2 µL of x5 Big Dye buffer, 2 µL Big Dye (v3.1), 1 µL of nuclease free water). The reaction mix was prepared on ice by mixing 4 µL of the DNA sample, 1 µL of primer, and 5 µL of master mix. The reaction mix was transferred to the PE Applied Biosystems 9700 thermocycler.

2.3 Immunofluorescence

Tissue sections (8µm) were cut using a cryostat set at -21°C to maintain the frozen tissue. Sections were air dried at room temperature (RT) for 30 minutes and then either used immediately or stored for up to 8 weeks wrapped in tin foil at -80°C .

Indirect immunofluorescence was used, where the primary antibody binds to antigens in the tissue and the secondary antibody labelled with a fluorochrome binds to the primary antibody. Cryostat sections were air dried at RT for 15 minutes and encircled with a silicon grease pen (PAP pen from Dako, UK). Slides were brought to RT, fixed with dried acetone (Fisher Scientific, UK) for 20minutes at RT and then washed 3 times in phosphate buffered saline (PBS, see appendix 1) at pH 7.2 for 15 minutes. After that, the slides were incubated with a donkey serum block at a dilution of 1:50 for 30 minutes at room temperature, after which the slides were once more washed in PBS three times for 5 minutes. The equivalent of 1μg of primary antibody protein (Table 2.2) was added to each section for one hour at RT or overnight at 4°C, and then the slides were once more washed in PBS three times for 5 minutes. Sections were then incubated with species specific (murine antibodies also IgG subclass specific) conjugated flurochrome antibodies as per manufacturer's instructions together with 5 μl of pre-diluted (1mg/ml) 4', 6-diamidino-2-phenylindole (DAPI a nuclear DNA stain)(Roche diagnostic GmbH, Germany) in the dark for 30 minutes at RT. Slides were washed three times in PBS for 5 minutes each and coverslips mounted using fluorosave aqueous mounting media (hydromount with 1, 4-Diazabicyclo-octane, National diagnostics, UK). Slides were stored at 4°C and then examined under a fluorescent microscope.

Primary antibody	Clones	Host species	Company	Dilution and incubation period	Secondary antibodies
Keratin 5	PH607	sheep	Binding Site, UK	1:400 (1 hour)	1:200 anti-goat, Alexa fluor (AF) 594
Keratin 14	LL002	Mouse monoclonal	Abcam, UK	1:50 (1 hour)	1:200 Anti-mouse, AF 594
Keratin 16	LL025	Mouse monoclonal	Abcam, UK	1:10 (1 hour)	1:200 anti-mouse, AF 488
Keratin 17	CKE3	Mouse monoclonal	Sigma, UK	1:400 (1 hour)	1:200 anti-mouse, AF488
Keratin 17	N.D	Rabbit	Gift from Dr Rebecca Porter	1:100 (1 hour)	1:200 anti-rabbit AF 488
Keratin 19	RCK108	mouse	DAKO, UK	1:25 (1 hour)	1:200 Anti-mouse, AF488
Keratin 75	GP-K6hf	Guinea pig polyclonal	ProGen, GER	1:5000 (1 hour)	1:200 anti-guinea- pig, Alexa flour 594
Keratin 73	N.D	Rabbit	Gift from Dr Rebecca Porter	1:10 (1 hour)	1:200 anti-rabbit AF 488
Keratin 26	N.D	Rabbit		1:500 (1 hour)	1:200 anti-rabbit? AF488
Keratin 31	Hha1	Mouse	Gift from Prof M Phillpot	Neat	1:200Anti-mouse AF488
Keratin 32	Hhb1	Mouse		Neat	1:200Anti-mouse AF488
Ki67	Mib1	Mouse monoclonal	DAKO, UK	1:100 (1 hour)	Anti-rabbit AF594
Ki67	SP6	Rabbit polyclonal	Abcam, USA	1:100 (1 hour)	Anti-mouse AF 594
NF-IL6	C-19	Rabbit polyclonal	Santa Cruz, USA	1:50 (1 hour)	Anti-rabbit AF 594
GLI1	GTX27523	Rabbit polyclonal	GeneTex,Inc, USA	1:250 Overnight at 4 $^{\circ}$c	Anti-rabbitAF488
GLI2	GTX27195	Rabbit polyclonal	GeneTex,Inc, USA	1:500 Overnight at 4 $^{\circ}$c	Anti-rabbitAF488

Primary antibody	Clones	Host species	Company	Dilution and incubation period	Secondary antibodies
CD31(PECAM)	PECAM	Mouse monoclonal	BD Pharmingen, UK	1:250 (1 hour)	Anti-mouse AF 488
CD56 (NCAM)	NCAM	Mouse monoclonal	BD Pharmingen, UK	1:1000, 1:200 (1 hour)	Anti-mouse AF
Versican	2-B-1	Human monoclonal	Ams biotechnology (Europe) Ltd	1:1000 (1 hour)	Anti-mouse AF594
GADD153	B3	Rabbit polyclonal	Santa Cruz, USA	1:100 (1 hour)	Anti-rabbit AF594
GATA	Ab61168	Rabbit polyclonal	Abcam, UK	1:1000 Overnight 4°C	Anti-rabbit AF488
B-Catenin	E5	Mouse monoclonal	Santa Cruz Biotechnology,INC	1:500 (1 hour)	Anti-mouse AF488
BMPr1a	Ab38560	Rabbit polyclonal	Abcam, UK	1:50 Overnight 4°C	Anti-rabbit AF488
BMP2	AHP960	Human recombinant polyclonal	AbD seroTec, UK	1:1000 Overnight at 4 °c	Anti-rabbit AF488
BMP4	AB39973	Rabbit polyclonal	Abcam, UK	1:1000 Overnight at 4 °c	Anti-rabbit AF488

Table 2.2: List of Antibodies.
The table contains details of the protein or signalling pathway studied, the specific antigen, the antibody clone, the company where the antibody was purchased, the species in which the antibody was made and the dilution used.

2.4 Haematoxylin and Eosin Staining

Sections (7µm) of normal human hair bearing skin and BCC samples were cut using a cryostat and air dried onto superfrost plus slides (Fisher Scientific, UK), as described above.

Slides were brought to RT, fixed with dried acetone (Fisher Scientific, UK) for 20

minutes at RT and then washed 3 times in PBS for 5 minutes per wash. Sections were immersed in Mayer's haematoxylin (Fisher Scientific, UK) for 5 minutes, then rinsed in tap water for 5 minutes and then immersed in 1% eosin for 1 minute. The sections were then dipped in tap water before dehydration through graded alcohols for 5 minutes each (70%, 90%, 100% and 100%). After which the slides were then dried and a cover slip mounted over the section using DPX (di-n-butyl phthalate in xylene) mounting media (Fisher Scientific, UK).

2.5 Alkaline Phosphatase Staining Method

Precut cryostat sections of BCC samples were taken from the freezer and allowed to reach room temperature. Slide sections were fixed in acetone (Fisher Scientific, UK) for 15 minutes and then air dried to allow total evaporation of acetone before washing for 10 minutes in tris buffer pH 8.2. The alkaline phosphatase substrate kit (Vectrastain, UK) consisted of three reagents. Two drops of reagent 1 were mixed with 5 ml of tris buffer, followed by addition of 2 drops of reagents 2 and 3. The sections were then incubated in the above substrate mixture for 30 minutes, followed by a 5 minute wash in tris buffer and then the slides were rinsed in water. The slide was counterstained with haematoxylin (Sigma Aldrich, UK) for 5 minutes and washed again in tap water. Finally, all sections were dehydrated in serial alcohols, mounted in non-aqueous media and left to dry overnight.

2.6 Visualisation and Photography

Slides were observed under a Nikon optiphot microscope the next day and red staining denoted a positive reaction. Digital images were captured at different magnifications

using three objectives (x10, x20, and x40) using an Axiocam camera system controlled by Axiovision software (Zeiss). Standardised settings were used on the microscope and image capturing software to obtain accurate comparisons.

2.7   Cell Culture

2.7.1 BCC cell culture

BCC cells were plated onto a feeder layer (50 Gy irradiated 3T3 murine fibroblasts) in 6-well tissue culture plates. They were fed withkeratinocyte serum free media (Gibco, UK) supplemented with 20 ng/ml EGF, 10 ng/ml FGF-2 and 0.15 ng/ml bovine pituitary extract, 25 units/ml of penicillin, 25 µg/ml streptomycin and 10 µg/ml amphotericin. The media was changed every 3 days and after 2 weeks BCC colonies were evident under an inverted microscope (Figure 2.1). BCC colonies could be passaged after trypsin treatment (see below) onto a fresh irradiated 3T3 murine fibroblast feeder layer or preserved in cell freezing media (Gibco, UK).

Spheroidal BCC colonies within the centres of each well were photographed using an inverted light microscope with a 2x objective lens. Photographs were converted into binary images and colony number and size determined using Image J software (NIH, USA).
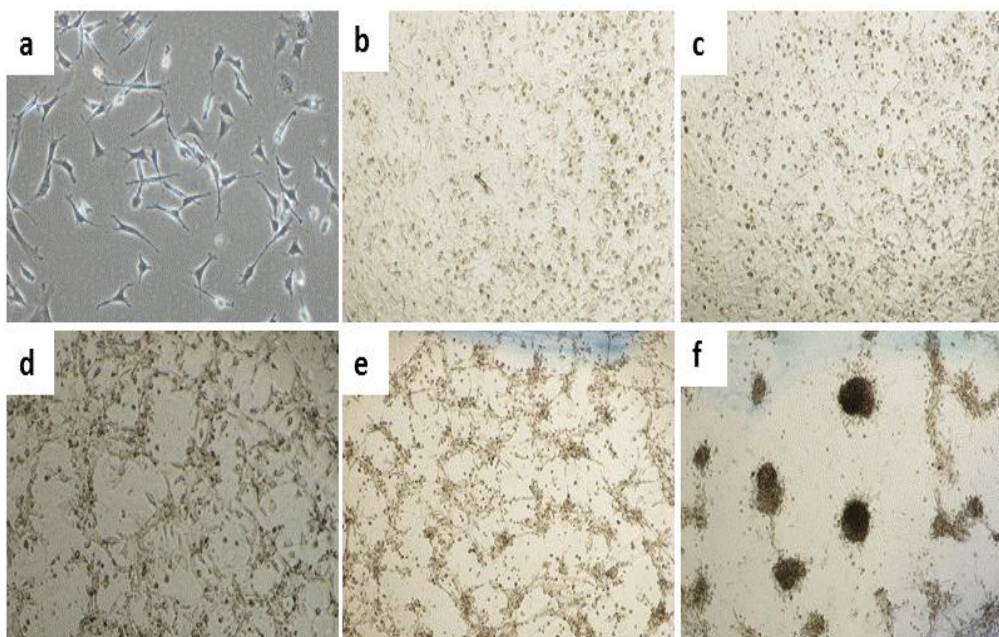
Figure 2.1: Images of BCC Colonies in Culture.
BCC cells were plated onto irradiated 3T3 fibroblast feeder layers, fed with keratinocyte serum free media and examined at different time points: **a**, plated 3T3 cells alone; **b**, BCC at day 1; **c**, BCC at day 3; **d**, BCC at day 6; **e**, BCC at day 9 and **f**, BCC at day 14.

2.7.2 Maintenance of NIH 3T3 feeder cells

NIH 3T3 murine fibroblasts were cultured in 80 cm^2 BD Falcon™ delta surface flasks (BD Biosciences, UK) containing 12 ml of Dulbecco's modified Eagles medium (DMEM) containing 4.5 g/L glucose, L-glutamine but without sodium pyruvate and supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, UK), 50 U/ml penicillin (Gibco, UK), 50 µg/ml streptomycin (Gibco, UK) and 2.5 µg/ml fungizone (amphotericin B from Gibco, UK). The cells were incubated at 37°C with 5% CO_2, and the culture medium was routinely replaced every 3 days.

2.7.3   Trypsinisation of cells

To detach cells from the tissue culture plate, the culture media was removed and cells were washed with sterile PBS. 5ml 0.05% Trypsin was added and the cells incubated at 37 ℃ for up to 5 minutes. To aid cell detachment, the tissue culture flask was lightly "smacked" a few times and once most cells were detached, the suspension was added to 30ml Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS in order to inactivate the trypsin. The 50 ml falcon tube was then centrifuged at 1,000 rpm for 5 minutes at RT and the supernatant discarded. The cell pellet was then re-suspended in either in culture media or cell freezing media for storage.

2.7.4   Cell counting

Cells were counted in order ensure that the correct number were plated and that there was parity between experiments. This was achieved with a haemocytometer grid and a cell counter (for ease of noting the number of cells observed).

A haemocytometer is a thick glass microscope slide, which is indented to a depth of 0.1mm in the shape of a rectangle and engraved with lines, defining an area of known volume (see below). A cover slip is placed on top.

A small volume of cells in media was pipetted onto the edge of the haemocytometer and 0.9mm^3 was taken up by capillary action. The cells were counted and those touching a line at the edge of the set area were included for two of the edge.
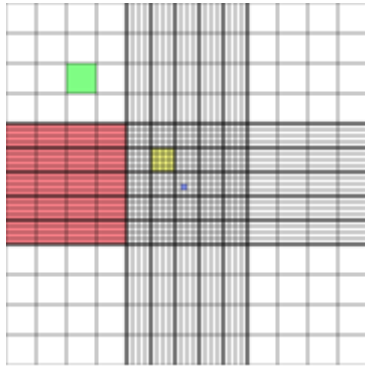
Square	Area	Volume at 0.1 mm depth
red	1 mm^2	100 nl (1 x 10^{-4}mm)
green	0.0625 mm^2	6.25 nl
yellow	0.04 mm^2	4 nl
blue	0.0025 mm^2	0.25 nl

Example:

The number of cells in each of four 'red squares' was counted and an average calculated. This gave the number of cells in 1mm^2 at a depth of 0.1mm.This number was multiplied by 10^4, and then by the total volume of the original flask. This gave an estimate for the total number of cells in the flask.

Square	Cell Number
1	40
2	31
3	28
4	41

Average cell number = 35

35 x 10^4 = 350,000

350,000 x 20 ml = 7 x 10^6 cells

2.7.5 Treatment of BCC colonies with noggin and TGF-β2

25 µg of lyophilised noggin (R&D systems, USA) was re-suspended in 100 µl of PBS containing 0.1% bovine serum albumin and 2 µg of lyophilised TGFβ2 (R&D systems, USA) was re-suspended in 100 µl of 4 mM HCl with 0.1% bovine serum albumin and a working solution made by adding 1 µl to 99 µl PBS.

Three different human BCC samples were dissociated and primary cell co-cultures established over 2 weeks (as described earlier) were used for this experiment. Each primary BCC culture was treated with trypsin and equal numbers of cells were plated into 15 wells of three 6-well plates onto freshly irradiated NIH 3T3 murine fibroblasts (feeder layer). Once the BCC colonies had re-established themselves, groups of 5 wells for each BCC sample were treated in triplicate as follows: fresh media, fresh media with 250ng/ml Noggin, fresh media with 500ng/ml Noggin, fresh media with 10µM TGFβ2, and fresh media with 100µM TGFβ2. Each well was photographed under an inverted light microscope with a 2x objective lens and a digital camera system (Zeiss Axiocam). Photographs were converted into binary images and both colony number and size were determined using Image J software (NIH, USA). After 48 hours RNA was extracted from each well and reverse transcribed into cDNA.

Chapter 3. Determination of Hair Follicle Specific Keratin Expression in Human Basal Cell Carcinoma

3.1 Introduction

The hedgehog signalling pathway is essential during embryogenesis but generally becomes quiescent during adulthood and only a few renewing adult tissues, including hair follicles, bone marrow and intestinal crypts remain active. However, a ligand-independent and ligand-dependent (autocrine and paracrine) reaction occurs in many tumours including gastrointestinal, prostate, haematological and neural cancers (Bhardwaj *et al.*, 2001; McMahon *et al.*, 2003). Sonic hedgehog signalling induces the proliferation of primitive human hematopoietic cells via BMP regulation (Bhardwaj *et al.*, 2001; Jiang and Hui, 2008; Scales and de Sauvage, 2009). Because of the abundance and accessibility of tumour tissues, BCC represents an ideal model to study therapeutics targeting the SHH pathway.

The signal transducer SMO is a key component of the SHH pathways. A number of SMO inhibitors are under development and at least three are currently in phase 2 clinical trials: GDC-0449/vismodegib (Genentech), LDE225/erismodegib (Novartis), and IPI-926/saridegib (Infinity). Pre-clinical studies show that these drugs are potent SMO antagonists, blocking both ligand dependent and ligand independent activation (De Smaele *et al.*, 2010; Tremblay *et al.*, 2009; Tremblay *et al.*, 2010).

However, recent clinical studies have shown that BCC cells persistduring treatment and retained the potential to regrow, leading to the suggestion that BCC tumour initiating

cells (TICs) exist and that they are resistant to elimination by SMO antagonists (Metcalf C, 2011; Skvara *et al.*, 2011; Von Hoff *et al.*, 2009).

BCC typically arise on hair bearing skin and by histology resemble basal cells of the hair follicle ORS from which they get their name and are thought to arise (Ghadially, 1961; Shimizu *et al.*, 1989). During hair follicle development in the embryo, epithelial-mesenchymal signalling results in the creation of a mesenchymal cellular condensation (emergent dermal papilla) and subsequent formation of an overlying epithelial hair follicle bud, consisting of an expansion of epidermal basal cells. This is followed by further expansion of the epidermal component with invagination into the underlying dermis and formation of a linked sebaceous gland epithelium. The distribution and ultimate hair follicle density is established *in utero*.

Although during puberty there is an increase in terminal hair numbers at specific sites, these form by maturation of pre-existing vellus hair follicles. The fully formed human hair follicle mini-organ demonstrates both inward and upward differentiation resulting in the structural formation of the hair follicle sheath and emergent hair shaft that arises from within. Unlike the original inter-follicular epidermal keratinocytes, differentiating keratinocytes in the hair follicle also express unique hair follicle specific keratin proteins. The process of hair growth is carefully choreographed and the hair follicle consists of concentric cell layers characterised by distinct patterns of hair follicle specific keratin heterodimer expression during each step of this complex differentiation process (Schweizer *et al.*, 2007). BCC tumour initiating cells (TICs) would be expected give rise to progeny that differentiated along hair follicle lineages and expression of specific keratins that suggested hair follicle differentiation, if present, would support the cancer stem cell model.

3.1.1 Design of Human Hair-follicle Specific Keratin Primers

To determine the presence or absence of differentiation within human BCC we sought to define the expression of hair-follicle differentiation specific keratins using RT-PCR. Hair-specific keratin gene mRNA sequences were identified using the National Centre for Biotechnology Information nucleotide search engine (http://www.ncbi.nlm.nih.gov/nuccore/).

Sequences for type I and type II keratin genes were aligned using Vector NTI Advance 10 (Invitrogen) to determine regions of sequence heterogeneity (Figure 3.1). Primers were designed to amplify low complimentary sequence regions of between 200-400bp (Table 3:1), with primers where possible of: 18-23 nucleotide length, 60% GC content, matching melting temperature (TM). To maximise specificity, primers pairs were entered into the National Centre for Biotechnology Information; primer blast search engine.

Searches were made against both *Homo sapiens* (taxid: 9606) and *Mus musculus* (taxid: 10090) using Refseq mRNA databases (pubmed) to ensure human specificity because of mixed cultures. Furthermore, standard Sanger automated sequencing of PCR products was used to ensure amplification of the expected sequences.

For each primer pair an optimal annealing temperature of the reaction was identified using positive and negative controls.
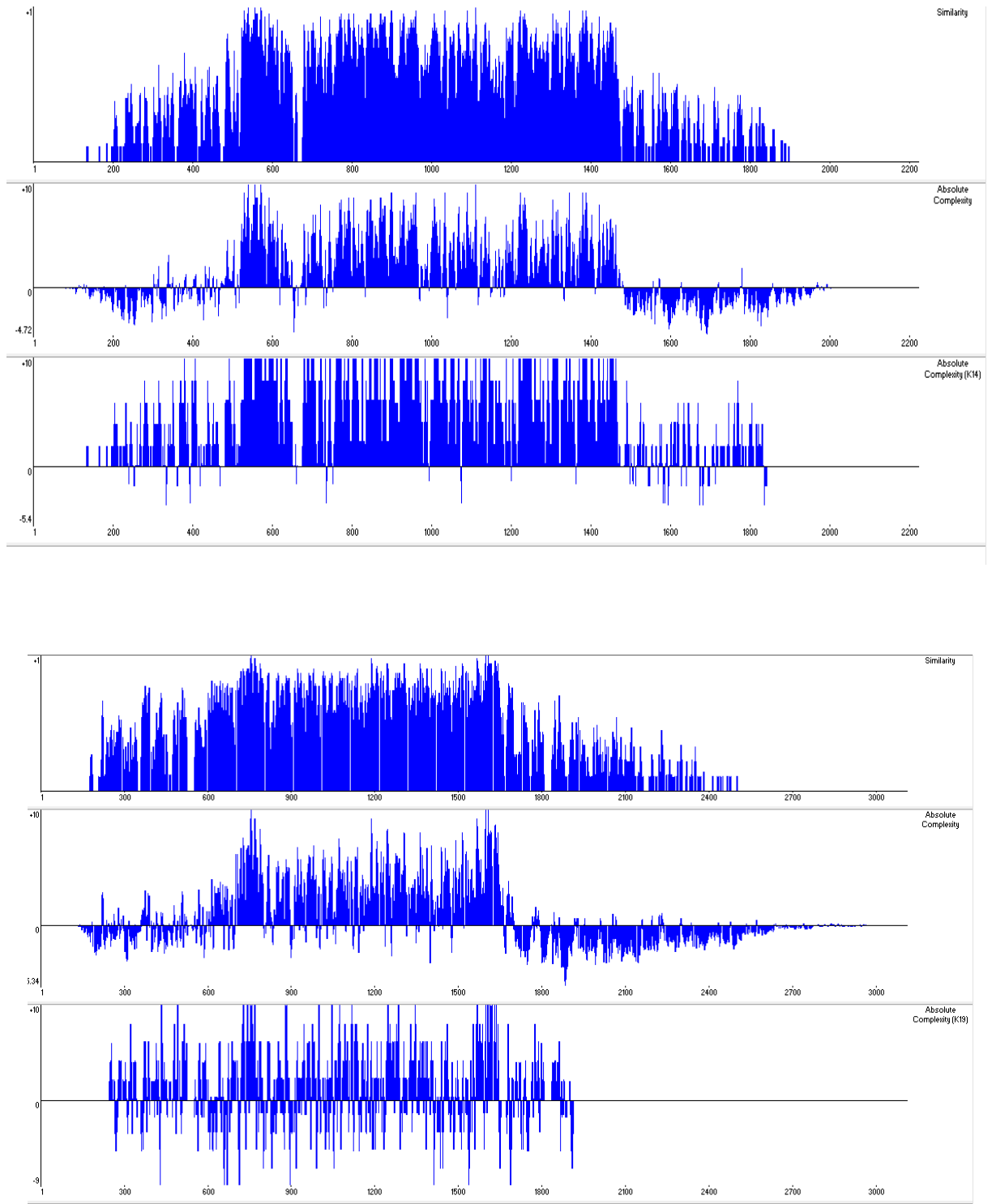
Figure 3.1: Sequence alignment complementary.
Plot of type I (upper), and type 2 (lower) Keratins. The greatest differences in sequence were observed in 5' and 3' ends of the gene. PCR sequences were selected from the 3' end to maximise sequence differences in the cDNA generated from polyA$^+$ mRNA during amplification.

3.2   Results

3.2.1 BCC expresses outer root sheath (ORS) keratins

Keratinocytes of the ORS are divided into basal and suprabasal layers. The basal layer keratinocytes characteristically express heterodimers of keratin K14 (type 1) and K5 (type 2). Variable basal cells in some individuals also express another type 1 keratin; K19. Stem cells, located within the hair follicle bulge, during anagen give rise to progenitor cells that are committed to terminal differentiation. Basal keratinocytes of the hair follicle ORS differentiate inwards to form the suprabasal layers. Three distinct patterns of keratin expression are observed within the hair follicle ORS suprabasal layers: (1) keratinocytes within the hair follicle infundibulum express K1 and K10, (2) at the level of the hair follicle bulge up to the mid portion of the hair follicle keratinocytes express K6 and K16, and (3) suprabasal keratinocytes below the mid portion of the hair follicle express K6 and K17.

We determined keratin expression by RT-PCR in human basal cell carcinoma tumour cells *in situ* and those in cultured colonies and compared with expression pattern to that of hair follicle ORS differentiation. Ten different human BCC samples were collected, trimmed to remove the overlying epidermis and subcutaneous fat, then immediately frozen and stored in liquid nitrogen. Total RNA was extracted from cryostat sections of frozen tissue samples and cDNA made using reverse transcriptase. Likewise, cDNA was made from hair bearing scalp tissue, which was used as a positive control and total RNA was also extracted from *in vitro* BCC colonies and used to make cDNA. Using human keratin-specific primers, PCR was simultaneously performed on cDNA made from   hair   follicle,   human   BCC   tissue   samples   (BCC),   cultured   BCC

colonies (BCC culture), NIH 3T3 fibroblasts total RNA extracts and a control PCR done in the absence of cDNA (Figure 3.2). In addition to the keratin genes, PCR of a house keeping gene GAPDH with equal amounts of cDNA was used to demonstrate the relative equivalence of starting cDNA levels.

Hair follicle tissue extracts were used as a positive control (lanes 1, 7), and NIH3T3 fibroblasts as a negative control (lanes 5, 11). In this particular experiment, the results for K5 and K17 were analysed on one gel (Figure 3.2a) and for K14 on another (Figure 3.2b). The basal cell keratin K5 of the correct amplicon size (475bp) was expressed in hair follicle samples, both BCC tissue samples and BCC in culture (Figure 3.2a). However, expression was high in one Primary BCC (lane 2) and more moderate in the other primary BCC and cultural BCC (lanes 3, 4). An additional PCR band was seen in the hair follicle extract (lane1). This corresponds in size (2500bp) to amplification of genomic DNA, which must have been a contaminant in this sample.

The expression of K17 (Figure 3.2a) was expressed in hair follicle tissue extracts and in both BCC samples and cultures. In this experiment, no keratin PCR products were obtained from NIH 3T3 fibroblast c DNA, only the presence of Primer dimers.

K14 expression was observed in both hair follicle tissue extracts and both primary BCC samples and BCC cultures (Figure 3.2b). However, higher level of K14 expression was present in BCC1 (lane 2) and BCC cultures (lane 4). Level of GAPDH expression were similar in all samples but not identical as levels were slightly lower in BCC tissue extracts (lane2, 3) (Figure 3.2c).
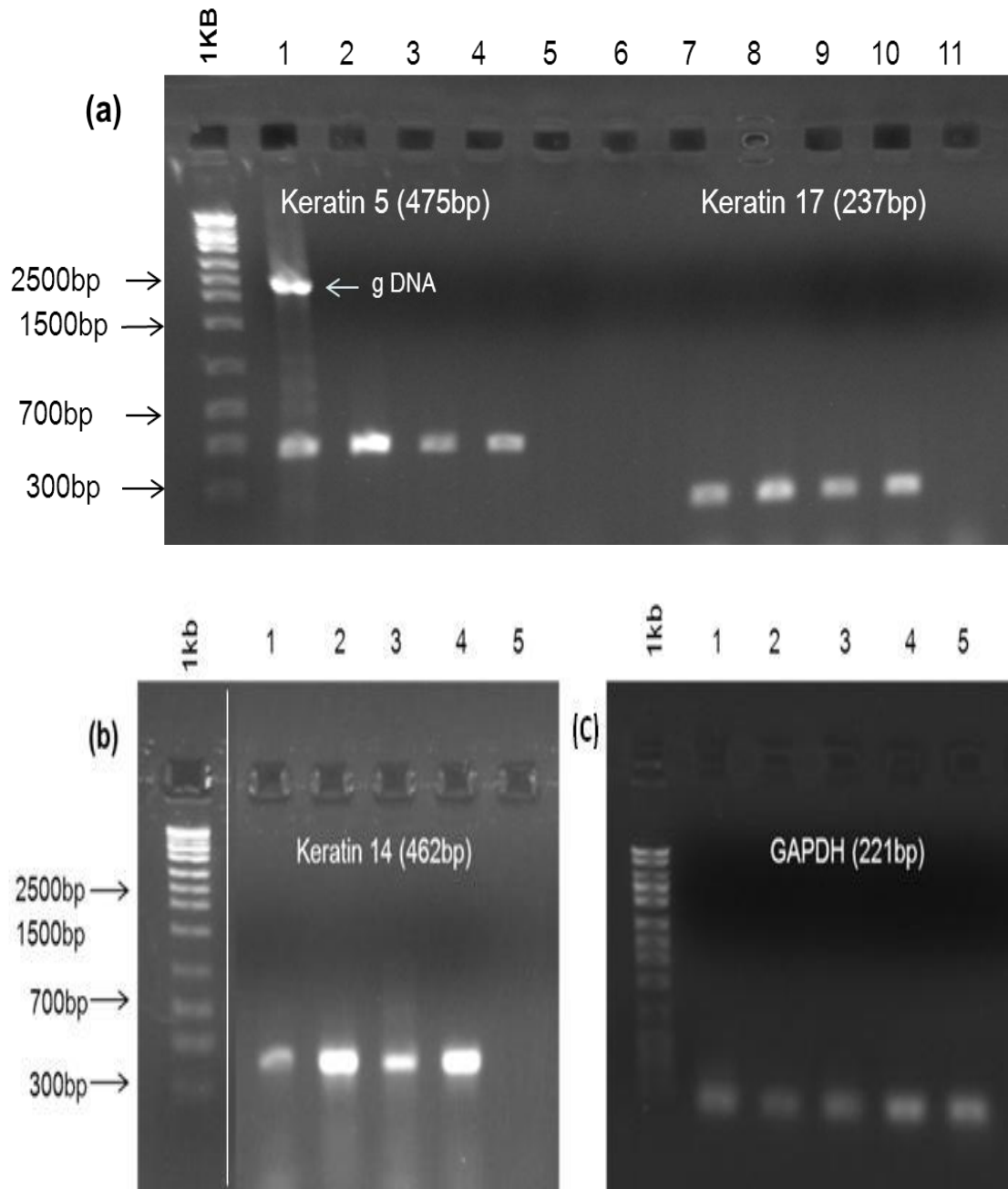
Figure 3.2: Expression of outer root sheath Keratins in Human BCC.

Amplified PCR products amplified with keratin or GAPDH primers were separated on 2% agarose gels, stained with ethidium bromide and viewed under UV light. (a) K5 (475bp), K17 (237bp), (b) K14 (462bp), (c) GAPDH (221bp). Samples were shown for hair follicle extracts positive control (**lanes 1, 7),** primary BCC number 21 **(lanes 2,8)** primary BCC number 22, (**lanes 3, 9**), BCC culture, (**lanes 4,10),** 3T3 NIH negative control(**lanes 5,11**). 1kb DNA ladder on left of each gel. In gel (a) g DNA indicated for genomic DNA. The DNA ladder was run with all samples, but where the ladder was separated from the data shown, we have separated it from the data gel shown.

3.2.2 BCC express the hair follicle companion layer keratin (K75)

The companion layer, which adjoins the suprabasal cells of the ORS, forms a unique histological cell layer extending upwards from the hair bulb matrix cells. The companion layer separates the ORS and IRS and is characterised by the unique expression of K75, the first hair follicle specific keratin to be described. K75 expression has already been observed in tumours, trichoblastomas and BCC (Kurzen *et al.*, 2001), Thus, we sought to determine K75 expression in our collection of human BCC samples.

RT-PCR of equal amounts of the house keeping gene, GAPDH was used to demonstrate the relative equivalence of input cDNA levels. All human scalp samples (positive control, n=3) demonstrated K75expression (Figure 3.3). Likewise, all BCC tissue samples (n=7) and cultured colonies (n=5) also demonstrated expression of K75. RT-PCR of NIH 3T3 Fibroblasts (n=3 samples) failed to produce a PCR band.
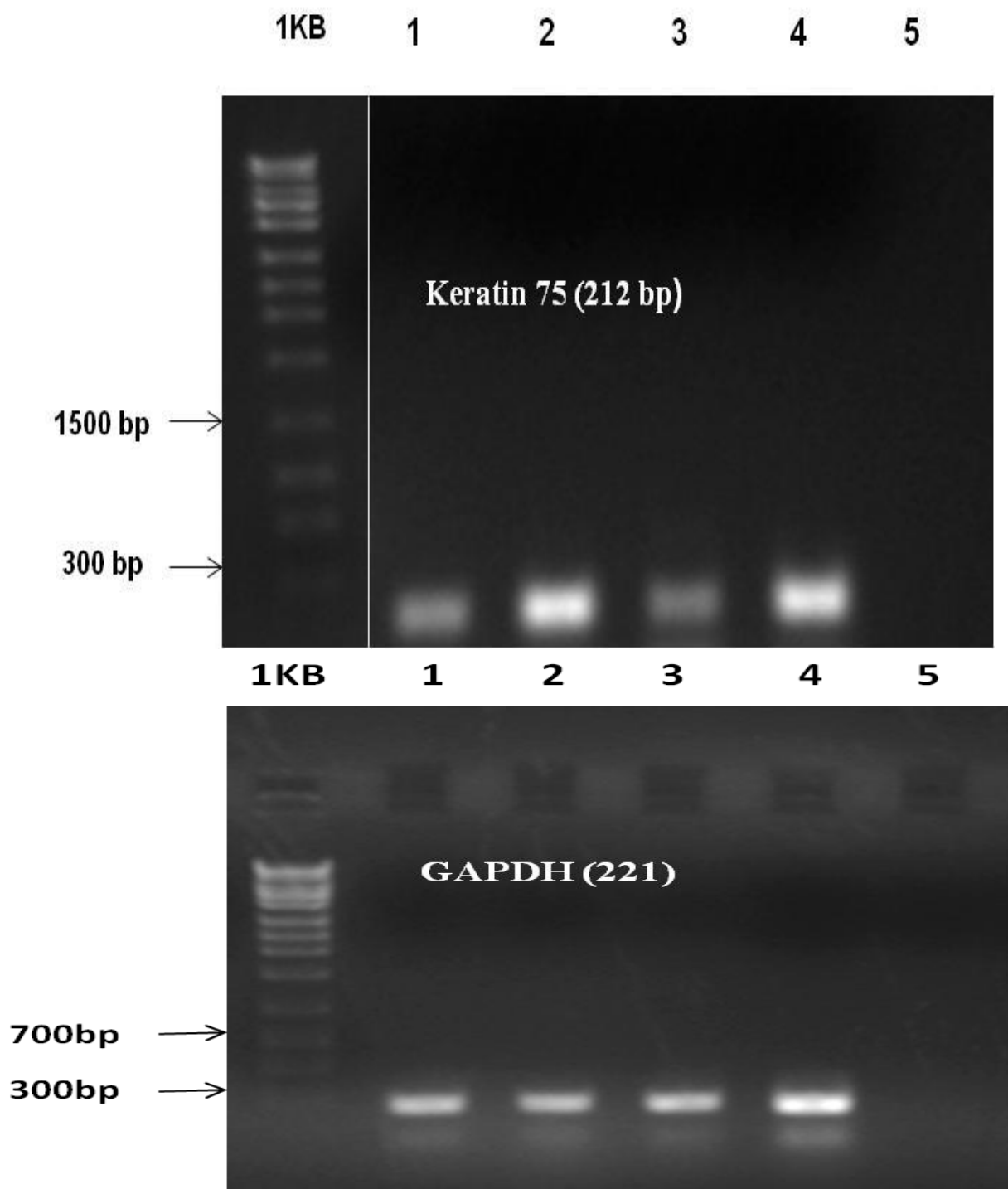
Figure 3.3: Expression of the Companion Layer Specific Keratin K75.
PCR amplified products of KT75 cDNA(212bp) or GAPDH (221bp) were separated on a 2% agarose gel, stained with ethidium bromide and visualised under UV light**. Lane1)** HF, **Lane 2)** Primary BCC1, **Lane 3)** Primary BCC2, **Lane 4)** BCC in culture. The DNA ladder was run with all samples, but where the ladder was separated from the data shown, we have separated it from the data gel.

3.2.3 BCCs Express Hair Follicle Inner Root Sheath (IRS) Keratins

The hair follicle IRS consists of three concentric layers: Henle layer, the Huxley layer, and IRS cuticle. Each layer of the IRS defines a specific lineage of differentiated hair follicle keratinocytes that emanate from basal matrix cells of the hair follicle bulb and migrate upwards. A set of four type I keratins (K25–K28) and four type II keratins (K71–K74) are expressed within the IRS. These IRS keratins are also differentially expressed in the IRS layers. The keratinocytes in all three compartments express KRT71, KRT25, KRT27 and KRT28. However, KRT74 is restricted to the Huxley layer and three other keratins (KRT73, KRT72 and KRT28) are sequentially expressed in the cuticle layer (Langbein *et al.*, 2003; Langbein *et al.*, 2004).

Intriguingly, epidermal keratins are not expressed in the IRS (Schweizer *et al.*, 2007). Therefore, we sought to determine the presence of IRS cell lineages in BCC by examining expression of these IRS specific keratins (Figure 3.4). All hair follicle samples expressed all of the IRS keratins. Despite equivalent amounts of input cDNA, which resulted in similar levels of GAPDH expression, not all of these genes were expressed in BCC tissue or cultured colonies.

All BCC tissue samples expressed the type 1 IRS specific keratins, but at lower levels compared to hair follicle tissues. BCC colonies in culture also expressed type 1 IRS specific keratins, with the exception of K25 and K27 in all cases. Of the two type 2 IRS keratins studied, K71 was consistently expressed by all BCC samples as well as BCC cells *in vitro*, and the level of expression was greater than that in hair follicle tissues. In contrast, the Huxley layer specific keratin, K74, was only expressed at low levels in a

few BCC tissues (2 out 7) and not at all in BCC cells in culture. These findings support

the hypothesis that BCC cells differentiate along IRS lineages.

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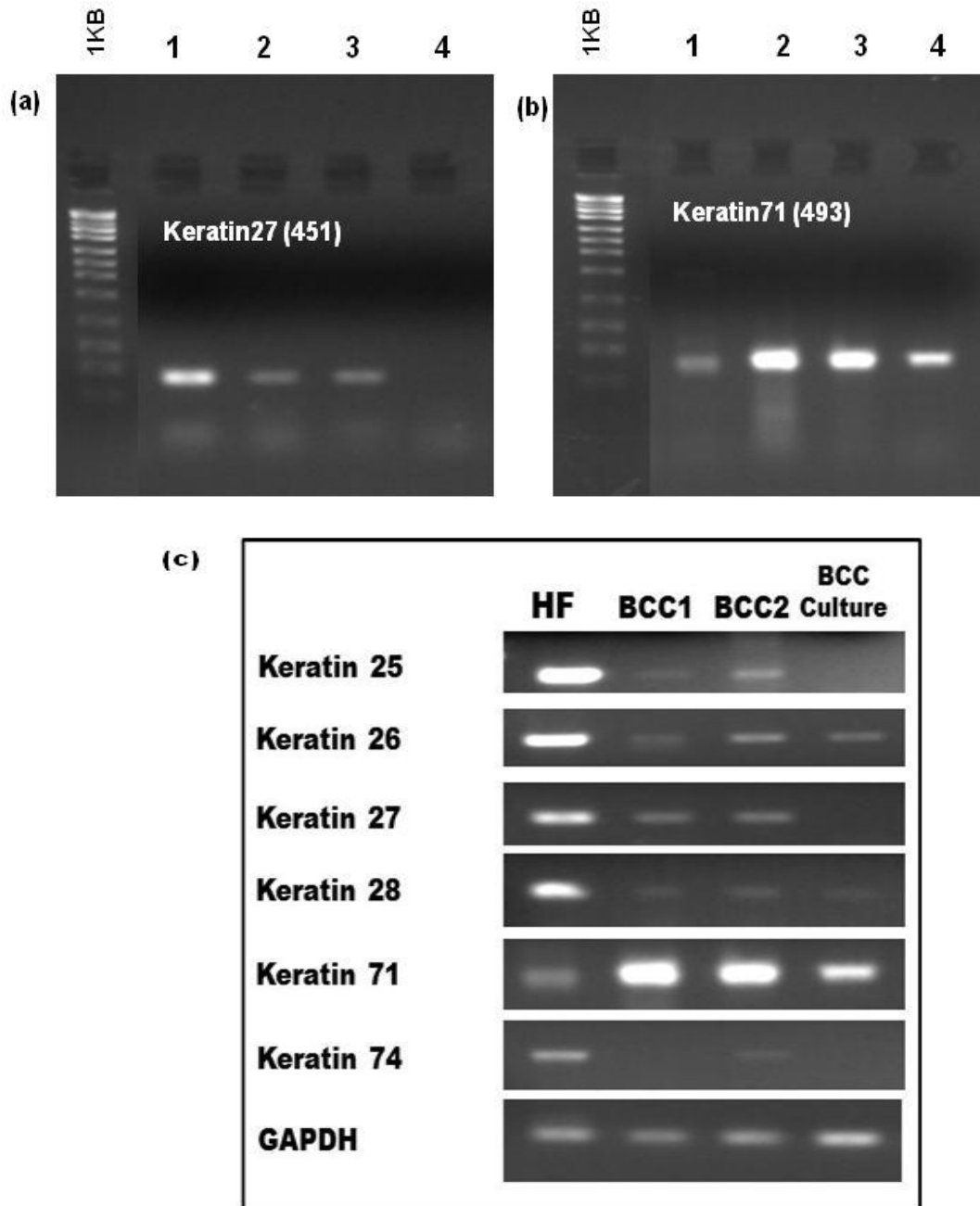
Figure 3.4: Expression of hair follicle IRS specific keratins in BCC.
PCR amplification product stained by Ethidum bromide and separated by 2% agarose
gel analysis of keratins in two different primary BCCs (BCC1, BCC2) and BCC in
culture. (a) and (b) full gels of K27 and K71 respectively. **Lane 1)** HF positive
control, lane2) primary BCC1,, **lane3**)primary BCC2, **lane 4**) BCC culture . (c) The
summary portions of inner root sheath keratins examined, includes GAPDH (internal
control).

3.2.4 BCCs express cuticle and matrix keratins of the hair shaft

The hair shaft is a keratin rich structure and unlike the other layers of the hair follicle contains multiple sulphur-rich "hard" keratins. K35 and K85 are already expressed in the hair-forming matrix of the cortex and the hair cuticle, but also combine with other hair shaft keratins (type I: K31, K33a, K33b, K34, K36, K38 and K39; type II: K81 and K86). K32 expression appears to be specific for the hair shaft cuticle layer. We next sought to determine the presence of hair shaft differentiation within BCC (Figure 3.5).
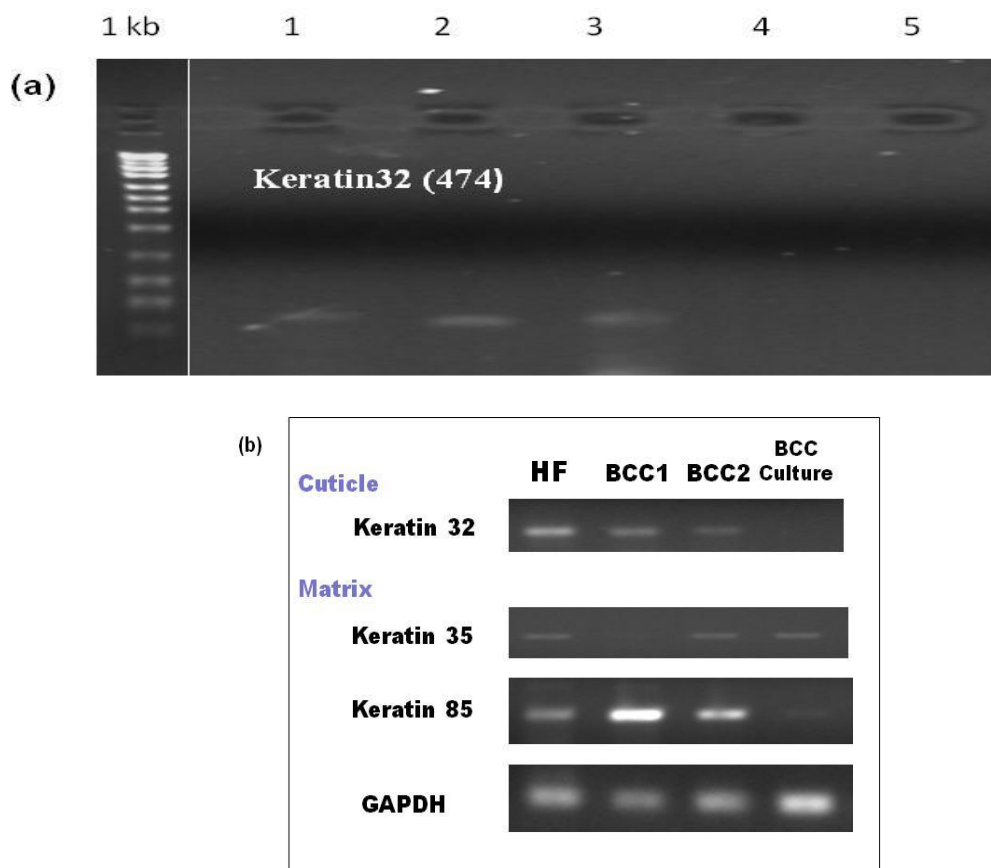
Figure 3.5: Expression of hair follicle cuticle and matrix specific keratins in BCC. Amplification product stained by Ethidum bromide and separated by 2% agarose gel analysis of keratins in two different primary BCCs (BCC1, BCC2) and BCC in culture. (a) Represent a full gel of K32 mRNA. **Lane 1)** HF positive control, **lane2)** primary BCC1, lane3**)** primary BCC2, **lane 4)** BCC in culture, **lane 5)** 3T3 NIH, negative control. The DNA ladder was run with all samples, but where the ladder was separated from the data shown, we have separated it from the data gel. (b) The summary portions of hair shaft keratins K32, K35, K85 and include GAPDH (internal control).

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As shown in Figure 3.5 (a,b) , in this particular experiment, the result for K32 was analysis in one full gel (Figure 3.5a), and the expression of other keratins including K32,K35,K85 is showing in a summary gel (Figure3.5b). Cuticle and matrix keratins were expressed within all hair follicle samples with correct amplicon sizes (lane 1).

However, the three keratins examined (K32, K35, K85) showed variable expression in BCCs (3 out of 7). Although K85 was robustly expressed in all primary BCC samples and in BCC cultures, only K35 and K85 expression was reproducibly observed in BCC cultures.

3.3   Summary

In summary, RT-PCR analysis performed on biopsies obtained from 20 BCC cases, hair bearing tissues and cultured BCCs demonstrated that hair follicle keratin lineages with expression typical of the outer root sheath (K5, K14, K16, and K17), companion layer (K75), inner root sheath (K26, K27, K28, K71, K72,and K74), and cuticle (K32, K35, K82,and K85) but not hair shaft (K31) markers. Our findings suggest that human BCC demonstrates both inward differentiation characteristic of the hair follicle ORS layers and upward differentiation which is characteristic of the hair follicle IRS and hair shaft. These findings support the hypothesis that BCC cells differentiate along hair follicle cell lineages.

Chapter 4. Distinct Patterns of Human Hair Follicle Keratin Expression in Basal Cell Carcinoma

4.1 Introduction

The human hair follicle, from which BCCs are thought to arise, is a mini-organ that demonstrates complex patterns of differentiation, regulated by the surrounding stroma via cytokine and growth factor signalling. Inward differentiation is evident in the hair follicle ORS layers, while the IRS and hair shaft demonstrate upward differentiation from the hair bulb (Fuchs, 1995).

Hair follicles consist of concentric cell layers that are characterized by distinct patterns of specific keratin expression. These concentric cell layers represent specific lineages of differentiated keratinocytes that can be clearly identified by mapping specific keratin expression (Langbein *et al.*; Moll *et al.*, 2008). The spacio-temporal pattern of differentiation and thus keratin expression in the hair follicle makes it an ideal mini-organ to study epithelial differentiation. Two distinct compartments of differentiation are evident, the inward differentiation of the ORS, which in fact represents the epidermis that has been plunged in to a hole in the dermis to make a tube to accommodate hair growth, while the remaining hair follicle layers demonstrate upwards differentiation from the germinative matrix (gm) upon receipt of inductive signals from the dermal papilla (dp) (Figure 4.1).

		Type I	Type II
Hair forming Compartment	Mid-/upper cortex	K31,33a,33b K34,35,36,37* K38	K81,83,85, K86
	Matrix/precortex	K35	K85
	Cuticle (cu)	K32, 35	K82, 85
	Medulla (currently investigated)	K16, 17... K33,34,37*... K25,27,28*...	K6, K75,... K5,81,...
Inner Root Sheath	Cuticle (icu)	K25,26,27,28	K71,72,73
	Huxley (Hu)	K25,27,28	K71,74
	Henle (He)	K25,27,28	K71
Comp. Layer (cl)	Companion layer	K16, 17	K75, 6
Outer Root Sheath	Suprabalsal layer	K14, 16, 17	K5, 6
	Basal layer (bORS)	K14, 16, K15	K5

BasalORS
cl
He
Hu
icu
cu
ma/co
med

dp

gm

Figure 4.1: Schematic illustration of hair follicle differentiation as defined by keratin expression.

Each distinctive layer of the hair follicle is characterised by specific keratin expression. Some keratins, such as the basal and suprabasal layers of the outer root sheath (ORS), are observed in other epithelia. The hair follicle ORS therefore demonstrates inward differentiation, in which basal cells give rise to differentiated suprabasal progeny. The keratins expressed in the companion layer (cl), inner root sheath (IRS) and hair-forming compartment are specific to the human hair follicle. These layers emerge from the germinative matrix (gm) and differentiate upwards. Adapted from (Langbein et al 2008).

BCCs are the most common type of skin cancer and mostly arise on hair bearing skin. Though the origin of BCCs is still not fully elucidated, it has long been believed that BCCs are derived from stem cells in the bulge area of the hair follicle ORS (Reis-Filho *et al.*, 2002). Recent transgenic mouse studies however have shown that BCCs can also arise from transformed interfollicular keratinocytes (Youssef *et al.*, 2010).

Whether BCCs arise from hair follicle bulge stem cells or interfollicular keratinocytes, all studies to date suggest that BCC cells demonstrate hair follicle keratinocyte morphology. Differentiation along hair follicle lineages is consistent with the observation that almost all BCCs demonstrate an activating mutation in the sonic hedgehog signalling pathway, a growth factor pathway that is fundamental in hair follicle development (Rubin *et al.*, 2005; Xie *et al.*, 1998). While the hair follicle elegantly demonstrates complex patterns of differentiation, BCC differentiation and its regulation remains unclear.

We have shown in Chapter 3 that BCCs express a variety of hair follicle specific keratin genes, providing circumstantial evidence that supports the presence of hair follicle differentiation in BCCs. Therefore, we hypothesised that the presence of these hair follicle keratin mRNAs would translate into keratin proteins in BCCs, which could be used to define hair follicle differentiation patterns within BCCs using antibody detection of hair follicle keratins.

4.2   Histological Characterisation of Basal Cell Carcinoma

After appropriate regulatory approval from NHS R&D and the Local Ethics Committee, we collected 20 human BCC tissue samples, which were obtained from patients in the Dermatology Department of Cardiff University Hospital. We initially undertook haematoxylin and eosin staining of tissue sections to determine the histologic type of basal cell carcinoma and 17 nodulocystic, 2 nodular, and 1 micronodular were identified (Figure 4.2). These samples did not include superficial BCCs.

Figure 4.2
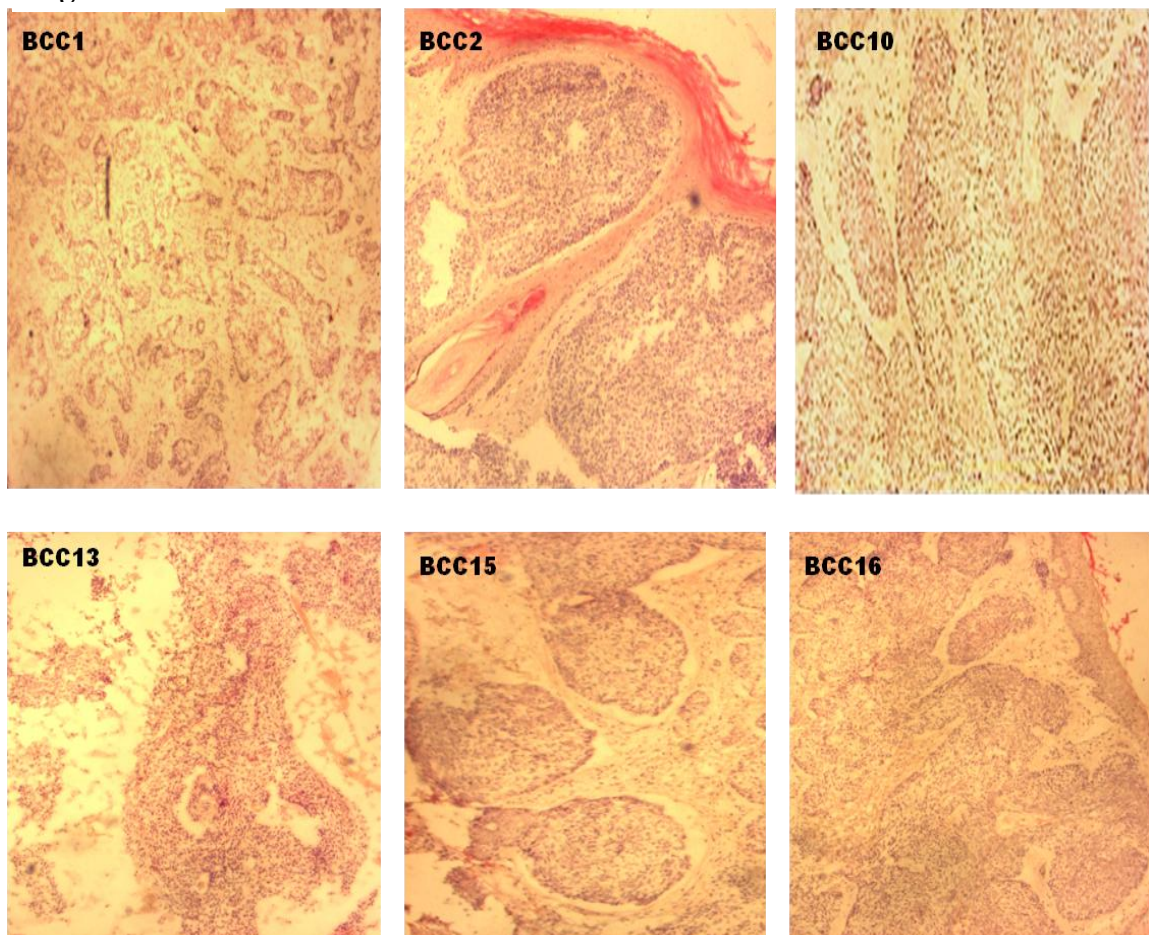
Figure 4.2: Human basal cell carcinoma tissue sections.
Representative human basal cell carcinoma sections stained with haematoxylin and eosin showing various histological subtypes: micronodular (BCC1), nodulocystic (BCC2, BCC10, BCC13) and nodular (BCC15, BCC16).

4.3 Expression of Hair Follicle Outer Root Sheath Keratins in BCC

As described in greater detail in Chapter 3, ORS keratinocytes are divided into basal and suprabasal layers. Basal layer keratinocytes characteristically express K5 (type 2 keratin), and K14 (type 1 keratin) which form the primary keratin pair of epidermal basal keratinocytes. These keratins are normally expressed in the undifferentiated basal cell layer and mRNA expression is absent in the differentiated suprabasal layers (Fuchs and Green, 1980). Occasionally, some basal cells also express another type 1 keratin (K19), which is a type 1 acidic keratin that unlike other family members is not paired with a basic type 2 keratin. It   is notably expressed in the periderm during embryogenesis (Schweizer *et al.*, 2006a). Together with K8 and K18, expression of K19 is often used to enumerate epithelial tumour cells in peripheral tissues where metastases are suspected (Allard *et al.*, 2004).

In contrast to suprabasal keratinocytes of the interfollicular epidermis, suprabasal keratinocytes of skin appendages including the hair follicle express three type 1 acidic keratins (K10, K16, K17) and two type 2 basic keratins (K1, K6). The distribution of keratin heterodimers can also vary depending on the location along the hair follicle and can be mutually exclusive. Within the infundibulum, suprabasal keratinocytes in the region of the hair follicle that transects the epidermis express the epidermal suprabasal keratin heterodimer of K1 and K10. Below the infundibulum, including the region of the sebaceous gland and hair follicle bulge extending to the mid portion of the lower follicle, suprabasal keratinocytes express a heterodimer of K6 and K16. In the lowermost portion of the hair follicle including the hair bulb, the suprabasal keratinocytes are characterised by expression of K6, K16 and K17.

The normal expression range of these keratins both in terms of the hair follicle and other tissues (including nail and tongue), manifests in the clinical presentation of pachonychia congenitia and steatocystoma multiplex that arise from loss of function mutations in some of these keratins (McLean *et al.*, 1995; Smith *et al.*, 1997).

Our RT-PCR data had already hinted at the expression of KRT5, KRT14, KRT19, KRT16 and KRT17 mRNAs. We next sought to confirm expression at the protein level by immunofluorescence, using previously characterised monoclonal antibodies, and to determine if their distribution architecturally within BCCs supported the presence of keratinocyte differentiation. Tumour differentiation, if present, would indicate a hierarchical growth pattern, which would be consistent with the cancer stem cell hypothesis.

4.3.1 Expression of outer root sheath basal layer keratins K5, K14, and K19

Previously characterised monoclonal antibodies to K5, K14 and K19 were used to label human hair follicle bearing skin samples, which acted as both positive and negative controls, in all BCC samples.

Hair follicle

As expected, K5 and K14 were expressed within the cytoplasm of keratinocytes in the basal layer of the hair follicle ORS and other skin appendages (Figure 4.3 a, b). Expression was however observed in all layers of the epidermis and ORS because these proteins are extremely stable and turnover is slow (Fuchs and Green, 1980).

K19 was also expressed in the basal layer of the ORS (Figure 4.3c). However, the pattern of expression was unlike the confluent pattern of expression of the other hair follicle basal keratins (Figure 4.3 d-g). Instead, K19 expression was absent in cells above the hair follicle bulge (Figure 4.3 h: white arrows), and was observed intermittently in basal cells and immediate suprabasal keratinocytes of the hair bulge extending to and including the hair bulb (Figure 4.3 i, j).

Figure 4.3
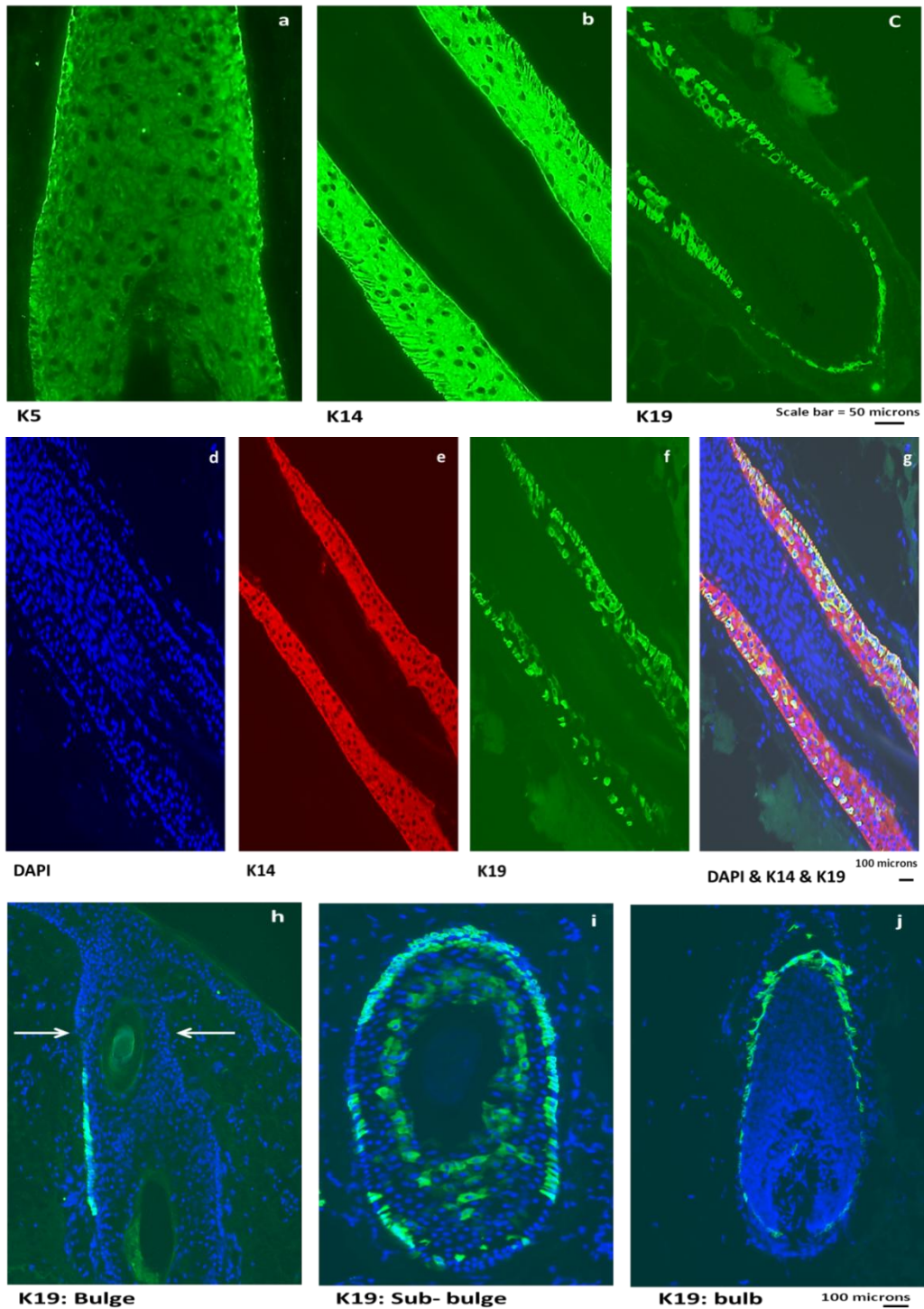
Figure 4.3: Immunofluorescence staining analyses expression of basal keratins K5, K14 and K19 in hair follicle.

Hair follicle frozen sections were labelled with K5, K14 or K19 antibodies. Top images represent expression of K5 (a), K14 (b) and K19 (c) in hair follicle (green). The middle image show the localisation of K19 in basal layer when co- labelled with K14, DAPI (d), K14 (e), K19 (f), and merge (g). Bottom images show the presence of K19 (green) in hair follicle bulge (h) and sub-bulge (i) extending to bulb (j) areas, the blue colour represented nuclear staining by DAPI. The white arrows displayed the supra-bulge area. The top images captured with 20x, scale bar 50 microns. Other images captured with 10 x, scale bar 100 microns.

Basal cell carcinoma

Surrounding each tumour nodule, there was a layer of K5 and K14 positively labelled tumour keratinocytes in all 20 BCC samples studied (Figure 4.4 a, b). We suspect that as in normal tissues, the persistence of K5 and K14 protein cells within the tumour nodule represented the continued presence of these proteins rather than their *de novo* expression, although *in situ* hybridisation experiments were not undertaken to confirm this. Hence, K5 and K14 could only be used to delineate BCC tumour nodules within the dermis.

18 out of 20 BCC tumour samples demonstrated K19 expression. In the majority of BCC samples, K19 positive cells were scattered amongst large numbers of negative cells, but occasional tumour nodules demonstrated expression of K19 in all tumour keratinocytes (Figure 4.4 c). While K19 expression was evident among basal keratinocytes, in all 18 cases of BCC samples K19 expression was most abundantly expressed by tumour keratinocytes within the tumour nodule (Figure 4.4 d-g). Thus, almost all BCC samples studied exhibited K19 expression, but unlike expression in the hair follicle, this was not restricted to basal and immediate suprabasal keratinocytes.

Figure 4.4
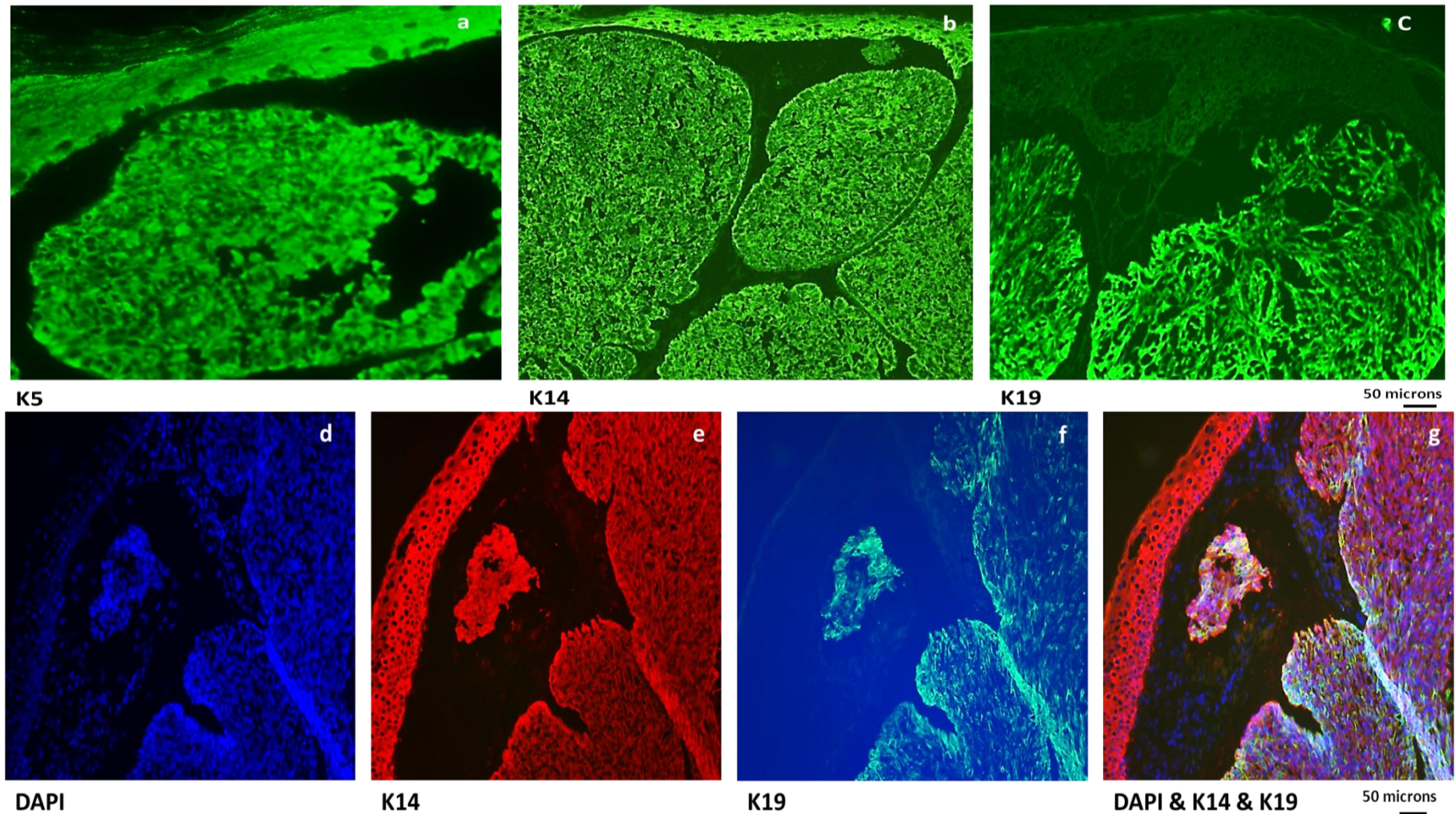
Figure 4.4: Immunofluorescence analysis of K5 and K14 expression in BCCs.
BCC sections were taken from different patients with 4 mm Surrounding margin, and then treated with K5, K14, or K19 antibodies. Top panel represents expression of K5 (a), K14 (b) and K19(c) in green. Bottom panel shows localisation of K19 in BCC samples when co-labelled with K14, DAPI (d), K14 (e), K19 (f), and merge (g). The slides were then visualised with fluorescent microscope. Images were captured with x20 objective, scale bar 50 microns.

4.3.2 Expression of outer root sheath supra-basal layer keratins K16, and K17

The expression of K16 and K17 was analysed by immunofluorescence on frozen sections of HF and BCC obtained from different patients. First, single labelling to identify the expression, and then double labelling of both keratins to demonstrate the relationship between them in terms of the specific location in both hair follicle and BCC.

Hair follicle

In the hair follicle, K16 and K17 are expressed within the cytoplasm of a well-defined population of keratinocytes that encompass all suprabasal keratinocytes of the ORS (Figure 4.5 a-h). K16 expression was restricted to the suprabasal layer of ORS between the infundibulum and the hair follicle bulb, whereas K17 expression was extended to include the hair follicle bulb. In addition, keratins demonstrated a mutually exclusive pattern of expression in the hair follicle (Figure 4.6 i-l).

Figure 4.5
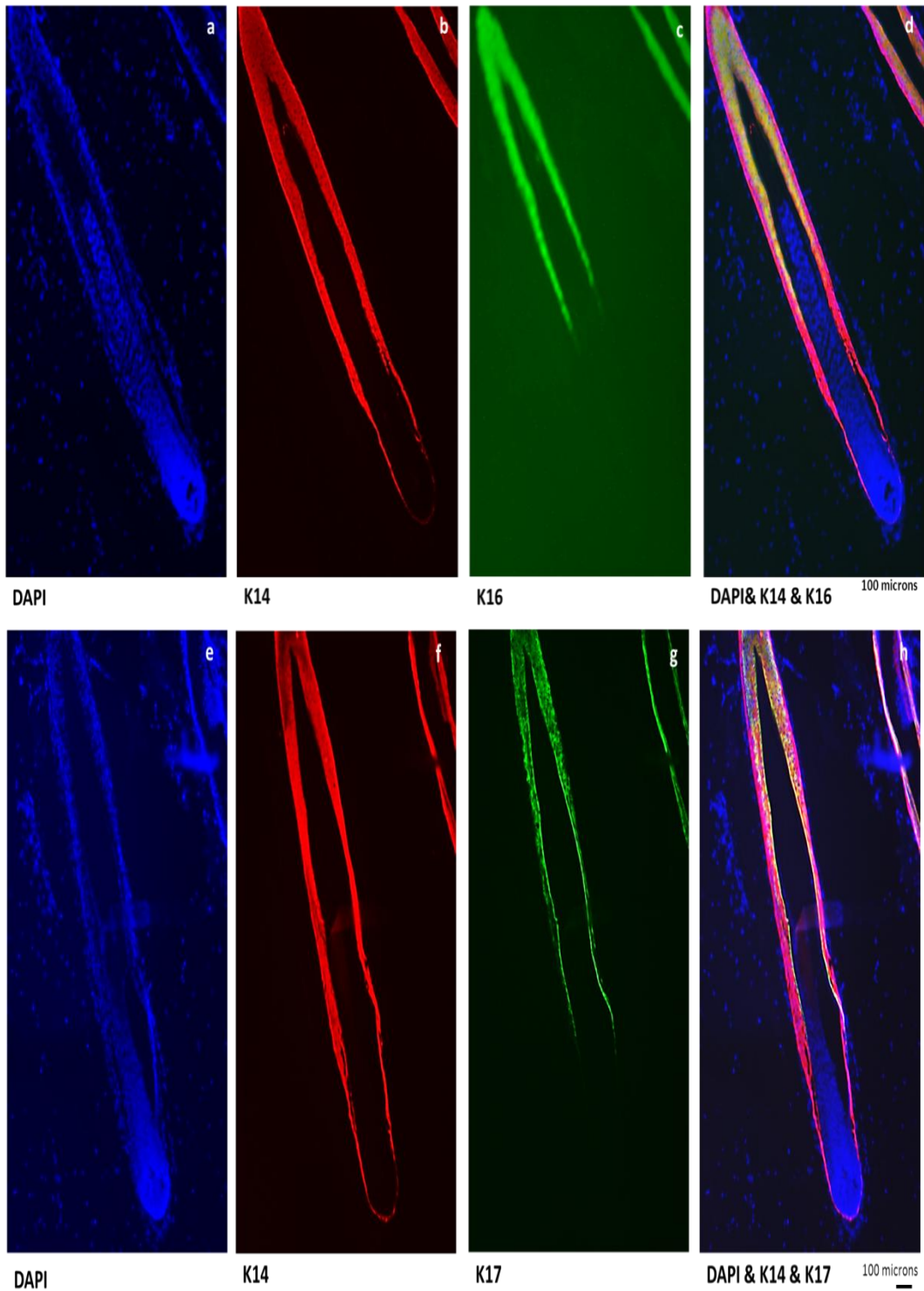
Figure 4.5: Immunofluorescence labelling of hair follicle supra-basal keratins K16 and K17 in hair follicle.

Hair follicle sections were treated with K14 (red) and K16 or K17 antibodies (green). K16 represented in top panel, DAPI (a), K14 (b), K16 (c) and merge (d). K17 showed in bottom panel, DAPI (e), K14 (f), K17 (g) and merge (h). Images were captured with x4 objective, scale bar (100 microns).

Figure 4.6
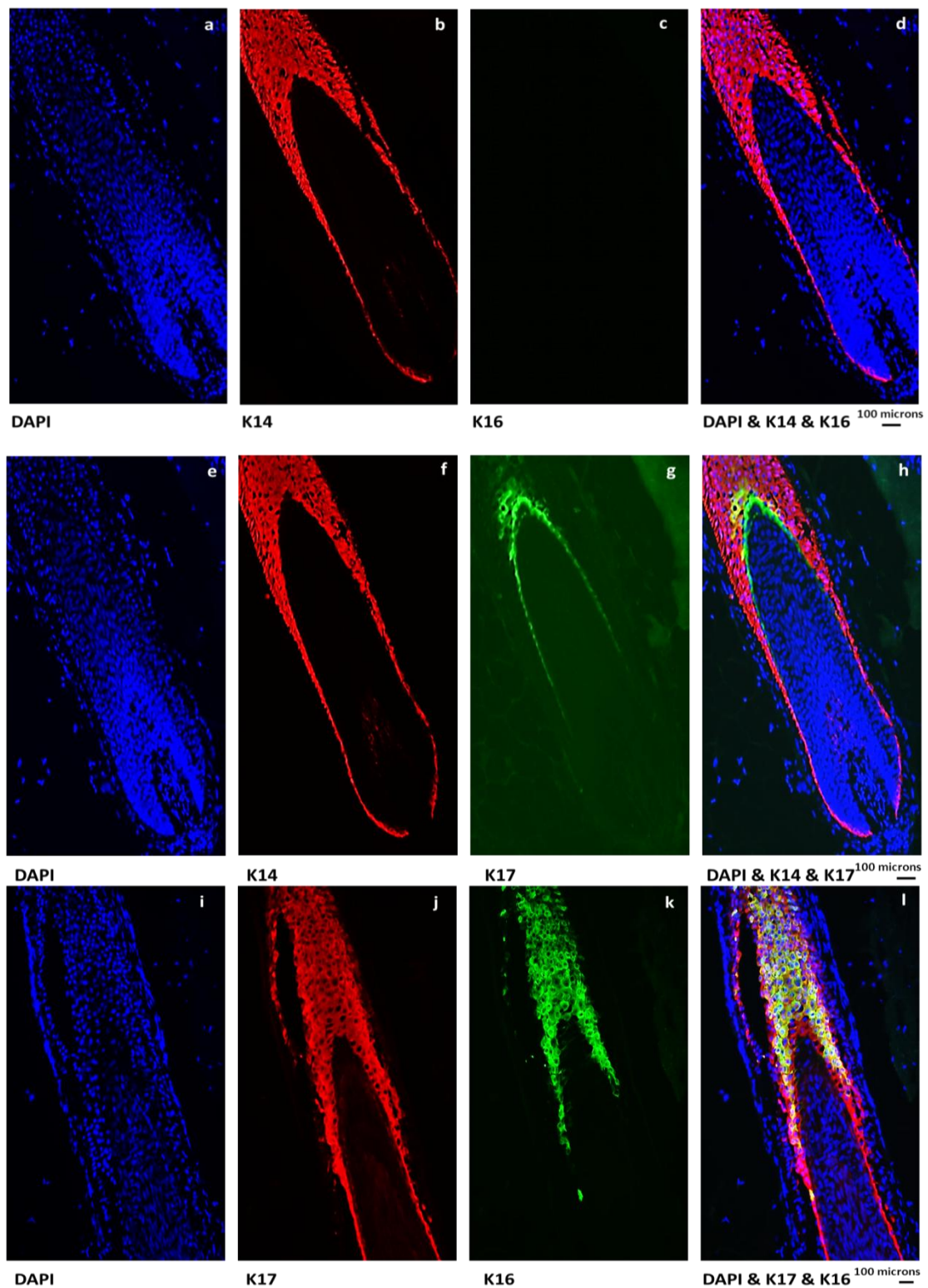
Figure 4.6: Immunofluorescence staining of hair follicle supra-basal keratins K16 and K17.

The labelling shows the pattern of expression in the hair follicle (bulb area). Top panel demonstrated the absence of K16 in hair bulb area, DAPI (a), K14 (b), K16(c), and merge (d). Middle panel demonstrated the extension of K17 in bulb area of the follicle, DAPI (e), K14 (f), K17 (g), and merge (h). Bottom panel showed double labelling of K16 and K17 and expression of K16 and K17 in follicle bulb area. Images were captured with x10 objective, scale bar (100 microns).

Basal cell carcinoma

K16 expression was observed in 13 out of 20 BCC samples, and in all such cases, this was restricted to a population of tumour keratinocytes within the tumour nodule itself (Figure 4.7d). K16 expression in BCC was similar to that found in the hair follicle in that expression was observed only in suprabasal keratinocytes, however, not all suprabasal keratinocytes within BCC samples expressed K16 and not all tumour nodules had K16 positive tumour keratinocytes (Figure 4.7 a-d). In contrast, K17 expression was observed throughout all tumour nodules in all samples studied (Figure 4.7 e-h). This was unlike K17 expression in the hair follicle and was not restricted to suprabasal keratinocytes. In addition, in BCC, unlike the hair follicle, co-incident expression of K16 and K17 was observed (Figure 4.7 i-l).

Thus, K17 exhibited a unique expression pattern within BCC, while K16 maintained expression in only suprabasal keratinocytes, albeit not uniformly. Furthermore, unlike the hair follicle, cytoplasmic expression of the K16 and K17 was coincident in all cases.

Figure 4.7
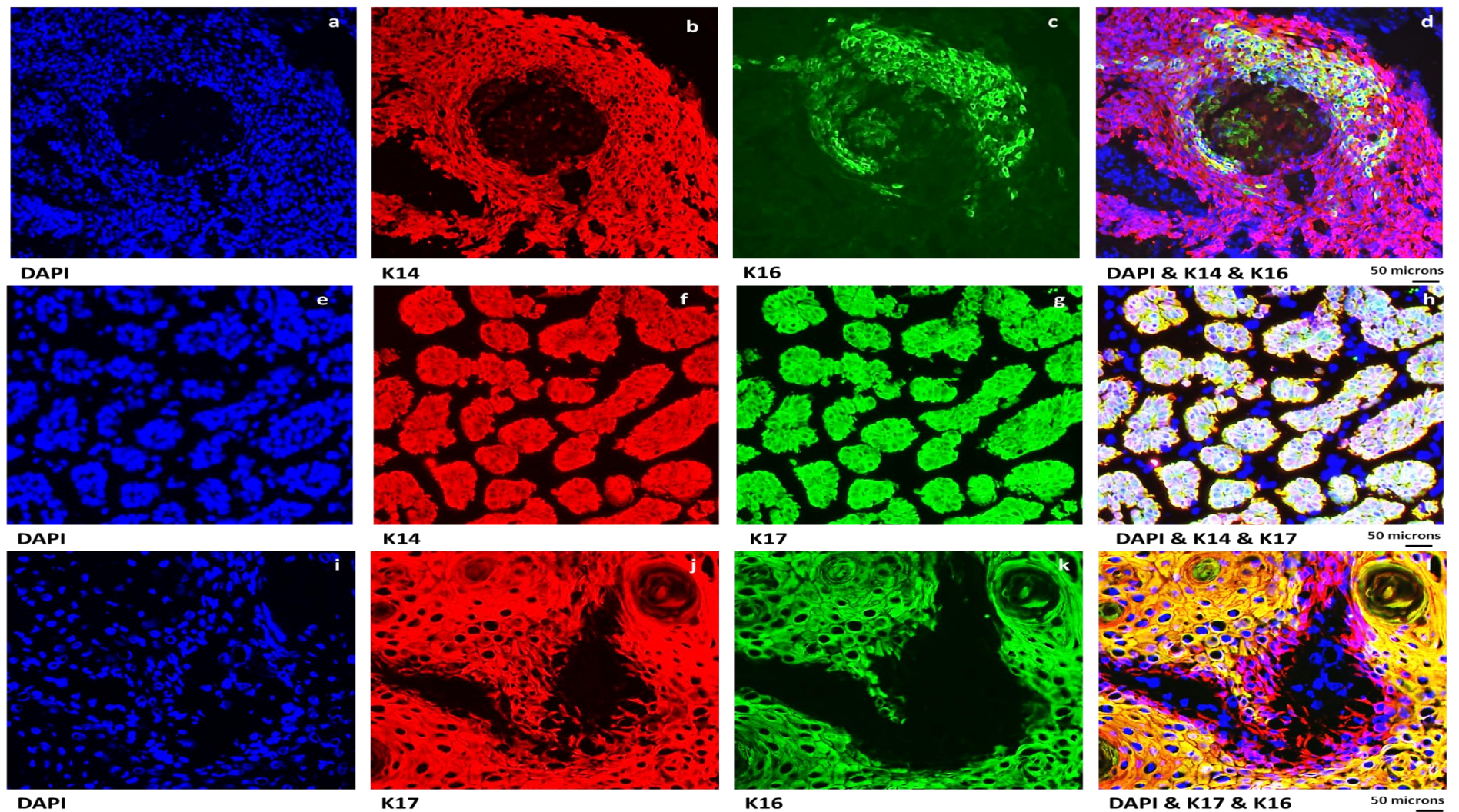
Figure 4.7: Immunofluorescence staining of K16 and K17 in BCC sections.
The staining shows the pattern of expression of hair follicle supra-basal keratins K16 (green) and K17 (red) in BCCs. Sections were treated with K14 and K16 or K17 antibodies. Top panel demonstrated the exclusive expression of K16, DAPI (a), K14 (b), K16(c) and merge (d). Middle panel showed the mutual expression of K17, DAPI (e), K14 (f), K17 (g) and merge (h). The bottom panel showed the co-expression of K16 and K17, DAPI (i), K17 (j), K16 (k) and merge (l). Images were captured with 20x objective, scale bar 50 microns.

4.3.3 Expression of hair follicle companion layer keratin K75 in BCCs

The companion layer is a thin cell layer between the ORS and IRS that extends upwards from the hair bulb matrix cells close to the dermal papilla and represents a distinct layer of differentiation (Wang *et al.*, 2003b). Keratinocytes of the companion layer express more than one keratin heterodimer, consisting of keratins K16, K17, K75 and K6. Human K75, a type 2 basic keratin, is specifically expressed within keratinocytes of companion layer of the hair follicle (Winter *et al.*, 1998a). Our RT-PCR data demonstrated expression of KRT75 within BCC, leading us to investigate the pattern of expression by immunofluorescence.

Hair follicle

In human hair follicles, K75 defined a single cell layer, with characteristic cytoplasmic labelling (Figure 4.8 a-c). We co-labelled samples with K75 and K14 antibodies and confirmed that K75 labelling was both distinct and medial to that of K14 (Figure 4.8 d-f), consistent with labelling of the companion layer.

Basal cell carcinoma

In 6 out of 20 BCCs, K75 was expressed within BCC tumour keratinocytes (Figure 4.9). K75 expressing tumour keratinocytes formed small clusters within occasional tumour nodules, but K75 was not expressed by basal keratinocytes at the tumour periphery (Figure 4.9 d-f). This central pattern of K75 expression is consistent with the pattern of expression observed in the hair follicle companion layer.

Figure 4.8
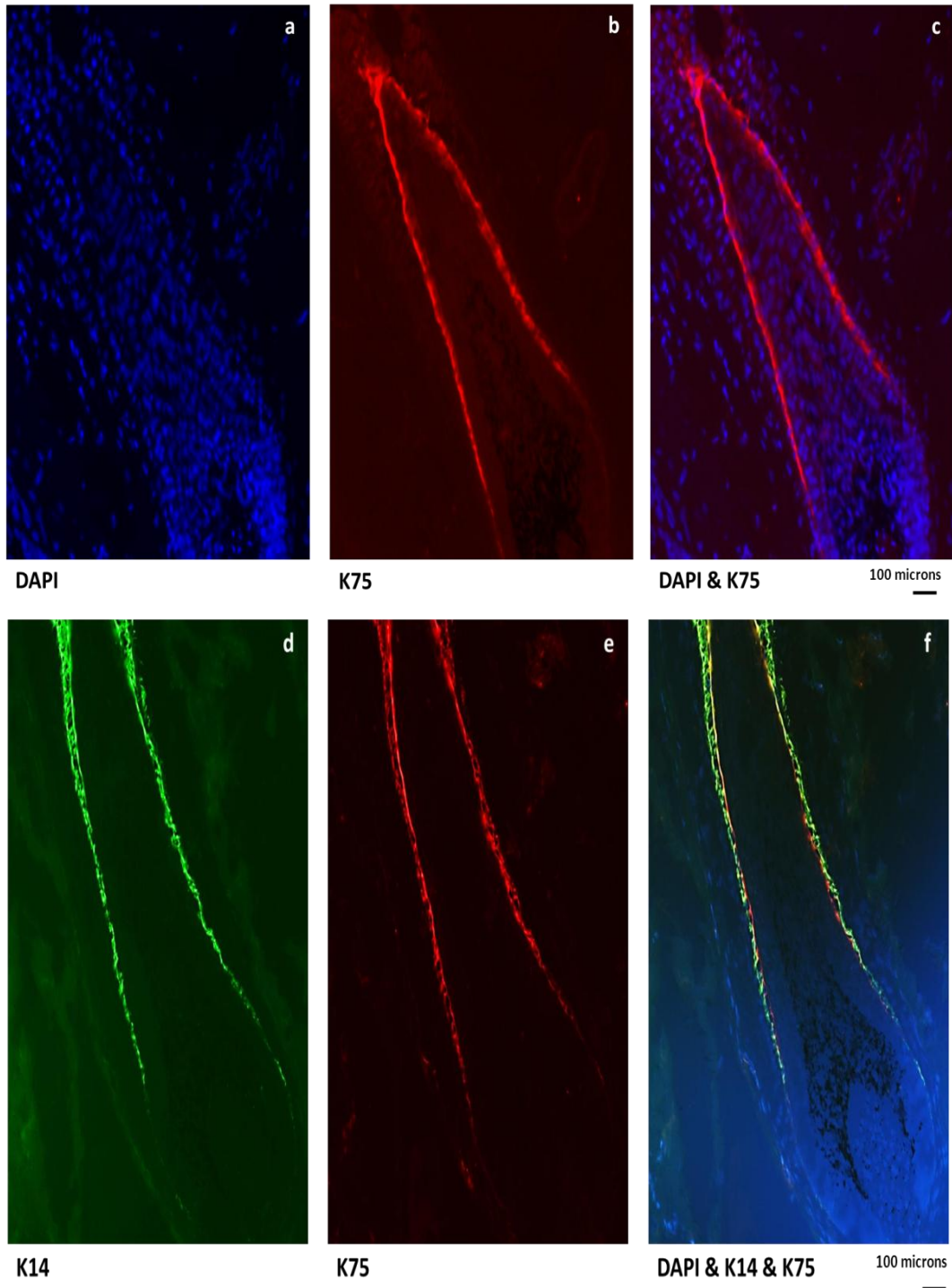
Figure 4.8: Representative Immunofluorescence of K75 expression in hair follicle.

Hair follicle tissue samples were treated with K75 (red) alone or with K14 (green). Top group showed single labelling of K75, DAPI (a), K75 (b) and merge (c). Bottom group demonstrated the localisation of K75 when co-labelled with K14, K14 (d), K75 (e), and merge (f). Images were photographed with x10 objective, scale interval 100 microns.

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Figure 4.9

DAPI	K14	K75	DAPI & K14 & K75 100 microns
DAPI	K14	K75	DAPI & K14 & K75 100 microns
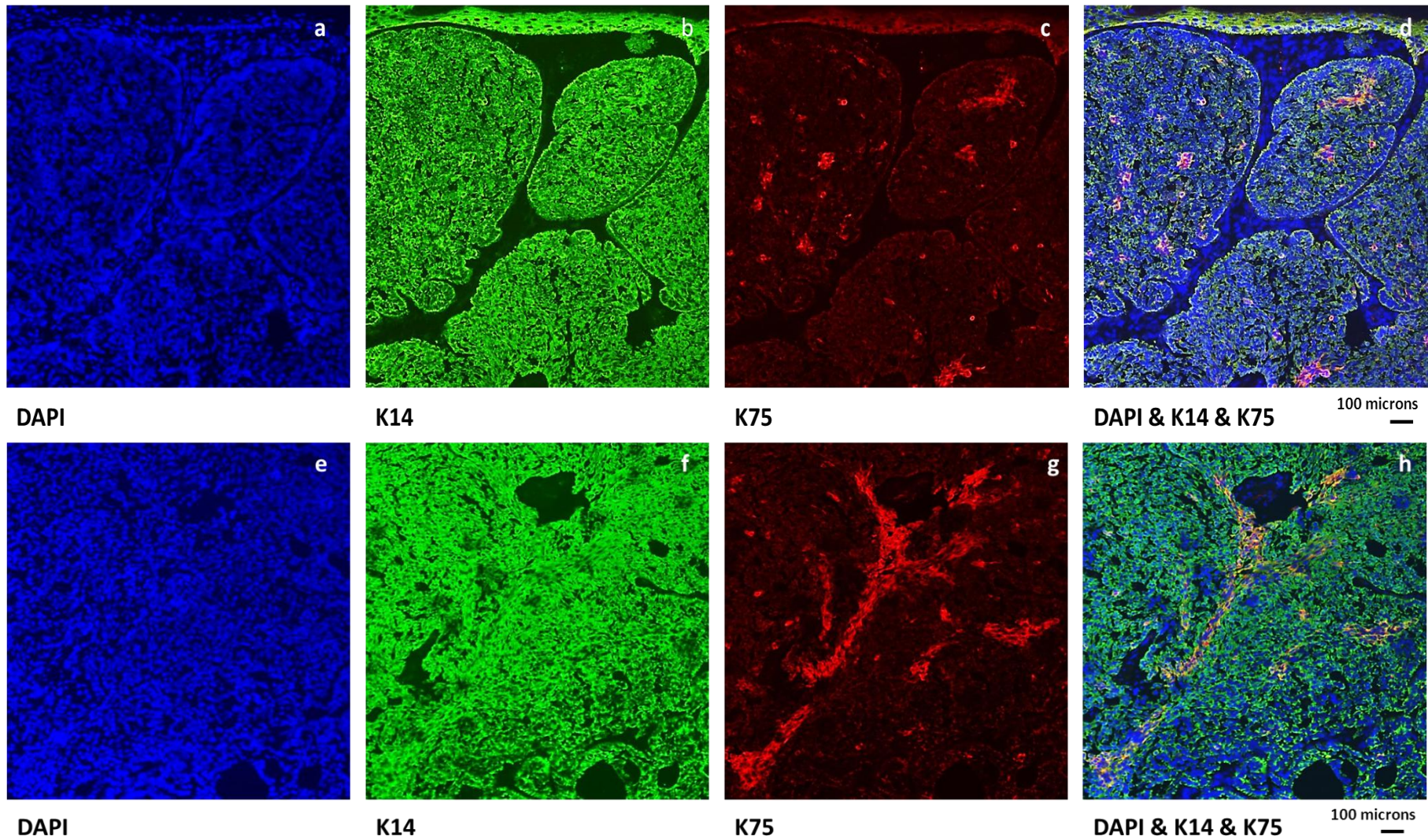
Figure 4.9: Representative immunofluorescence of hair follicle companion layer keratin K75 pattern of expression in BCCs.
Two different sections of BCC tumour were treated with K14 (green) and K75 (red) antibodies and visualised under fluorescent microscope. DAPI (a,e), K14 (b,f), K75 (c,g) and merge (d,h). Images were captured with 10x objective, scale interval 100 microns.

4.3.4 Expression of hair follicle inner root sheath keratins in BCCs

The IRS consists of three distinct histologically defined layers: the Henle layer, the Huxley layer, and the cuticle. Each layer is morphologically distinct, representing a specific differentiation lineage and is characterised by specific keratin expression. Some IRS keratins are expressed in all three layers (K25, K27, K28, and K71), while K74 is only expressed in the Huxley layer and K26, K72 and K73 are only expressed within the cuticle. By RT-PCR, we determined expression of a number of IRS keratins in human BCCs: KRT25, KRT26, KRT27, KRT28, KRT71 and KRT74. To confirm this data at the protein level, we used previously characterised antibodies to K26 and K28.

Hair follicle

The K28 antibody identified all three layers of the IRS in samples of human hair follicle. When hair follicle sections were co-labelled with K14, to define ORS keratinocytes, the IRS layers could be clearly distinguished by the presence of the intervening unlabelled companion layer (Figure 4.10 a-d).

K26 expression defined the cuticle layer, the innermost layer of the human hair follicle IRS. Hair follicle sections co-labelled with K17 was used to distinguish ORS from K26 antibody labelled IRS cuticle keratinocytes (Figure 4.11 a-d).

Basal cell carcinoma

Only 3 out of 20 BCC samples demonstrated K28 expression and this was cytoplasmic and restricted to very small clusters of 1-4 cells residing at the tumour periphery (Figure4.10 e-h).

In contrast, K26 expression was not detected in the BCC samples examined (Figure 4.11 e-h), suggesting a limit to the extent to which BCC keratinocytes exhibit IRS differentiation.

Figure 4.10
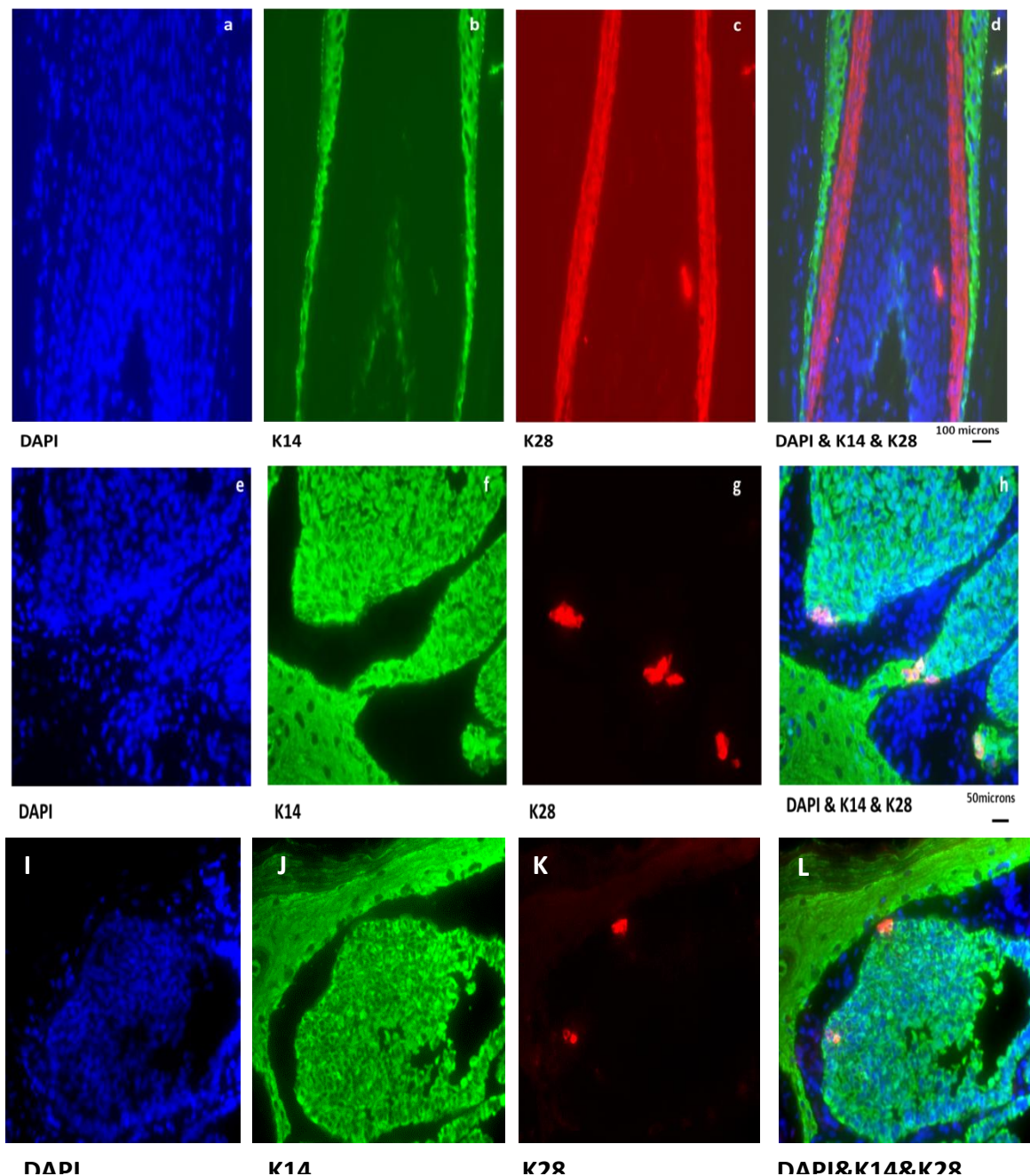
Figure 4.10: Double immunofluorescence staining showing K28 pattern of expression in both hair follicle and BCC tissue samples.

Hair follicle and BCC sections were treated with K14 (green) and K28 (red) antibodies. Top panel demonstrated expression of K28 in hair follicle, DAPI (a), K14 (b), K28 (c), and merge (d). Middle and bottom panels showed expression of K28 in BCC tumour sample, DAPI (e,i), K14 (f,j), K28 (g,k), and merge (h,l). Top images were captured with x10 objective, scale bar 100 microns, middle & bottom ones captured with x20 objective, scale bar 50 microns.
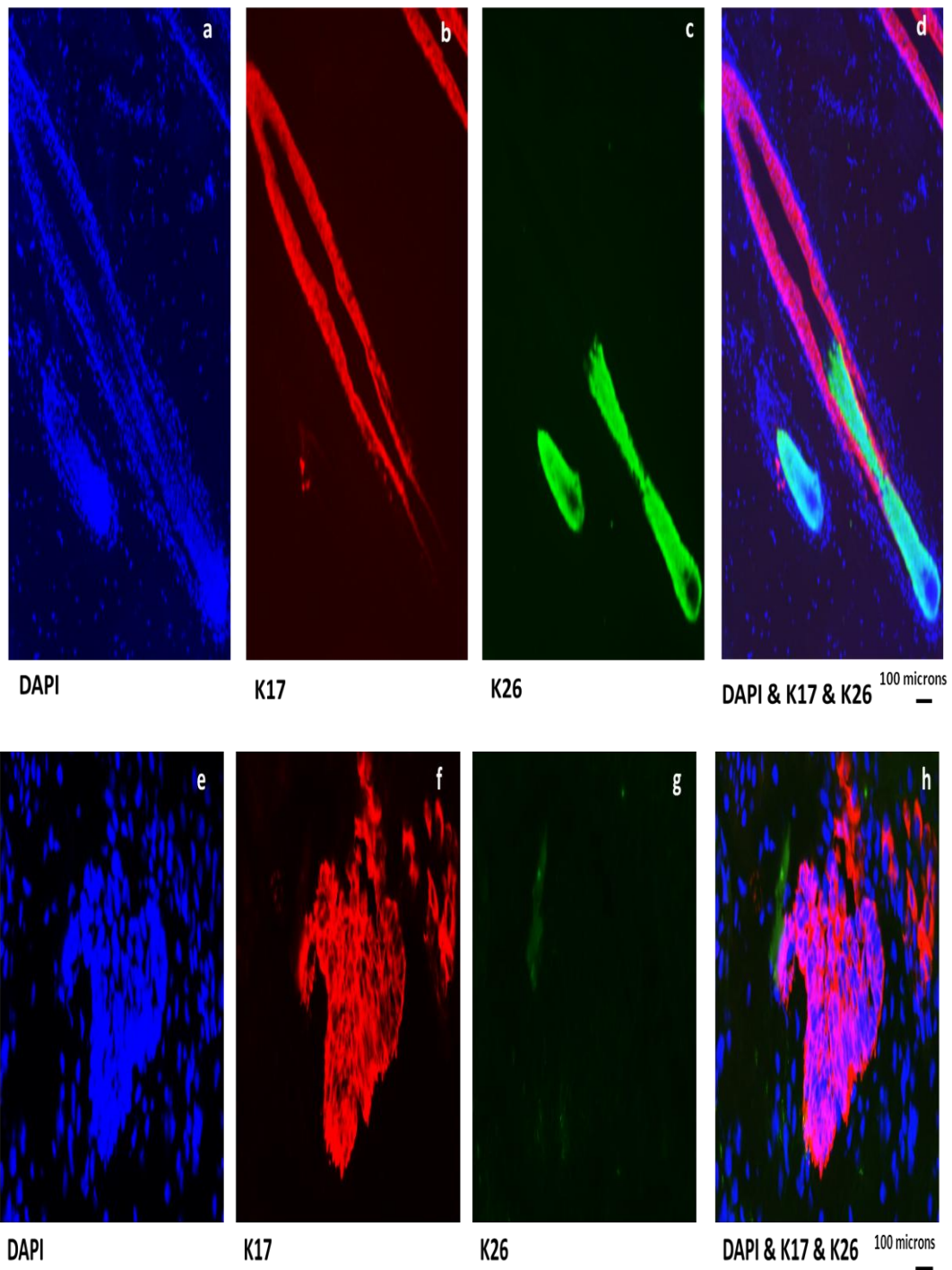
Figure 4.11: Double immunofluorescence staining showing K26 pattern of expression in both hair follicle and BCC tissue samples.

Hair follicle and BCC sections were treated with K17(red) and K26 (green) antibodies. Top panel demonstrated expression of K26 in hair follicle, DAPI (a), K17 (b), K26 (c), and merge (d). Bottom panel showed expression of K26 in BCC tumour sample, DAPI (e), K17 (f), K26 (g), and merge (h). Images were captured with x10 objective, scale bar 100 microns.

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4.3.5 Human BCCs do not express hair follicle shaft keratins

The human hair follicle shaft layer emerges from the hair bulb matrix cells and undergoes upward differentiation. The hard keratins expressed in trichocytes, which eventually are cross-linked to form the hair shaft, give the emerging hair shaft structural integrity. At the level of the trichocytes emerging from the hair bulb, three distinct histological compartments are evident each with distinct keratin heterodimers: Cuticle (K32, K35, K82 and K85), matrix or pre-cortex (K35 and K85), and mid/upper cortex (K31, K33, K34, K35, K38, K81, K85, and K86). The localisation of some of these keratins is described below.

In the hair follicle, K32 is uniformly expressed by cells of the cuticle (Figure 4.12 b), (white arrows show the cuticle cell layer) as well as the early hair cortex. While K31 labels the entire hair cortex (Figure 4.12 a) and K81 labels the mid cortex (Figure 4.12 c). However, none of these keratins were identified in BCC (n=20).
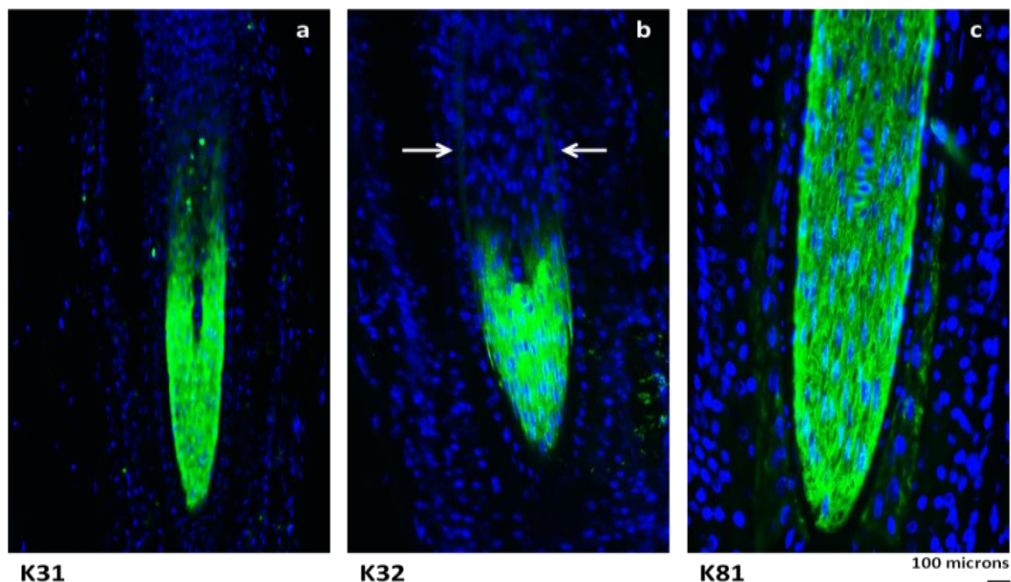
Figure 4.12: Expression of hair shaft keratins in hair follicle.
Hair follicle and BCC sections were single labelled with K31, K32 , and K81 antibodies top panel shows expression of K31(a), K32 (b) and K81 (c), white arrows indicate the hair shaft cuticle layer. Bottom panel shows the negative expression of K31(d) , K32(e) and K81(f), Images were captured with x10, x20 , scale bar 100 ,50 microns respectively .

4.3.6 Summary of hair keratin expression in human BCC

In summary, together with RT-PCR, we have analysed expression of 17 different hair follicle keratins that are normally expressed at various stages of hair follicle differentiation (Figure 4.13). ORS basal and suprabasal keratins are the most broadly expressed keratin heterodimers, as all BCC samples expressed K5 and K14. As in the human hair follicle, K5 and K14 expression was observed in all tumour basal cells by immunofluorescence. The other basal keratin, K19, was also expressed frequently within BCC (in 18 of 20 samples studied) and was observed both within basal and as well as the BCC inner cell mass keratinocyte cytoplasm.

The expression of suprabasal ORS keratins within BCC was intriguing in that all BCC keratinocytes (all 20 BCC samples) regardless of their location within the tumour nodule expressed K17, whose expression is normally restricted to suprabasal keratinocytes in the lower hair follicle ORS. However, K16 which is distributed in the hair follicle mid-portion suprabasal ORS exhibited more restricted expression (14 of 20 BCC samples) and defined keratinocytes within the tumour nodule core. Unlike in the hair follicle, K16 positive keratinocytes also expressed K17.

In addition to demonstrating keratin expression consistent with inward hair follicle differentiation, BCC tumour samples also expressed hair follicle specific keratins associated with upward differentiation. In 6 of 20 BCCs, K75 was expressed by keratinocytes in the inner cell mass of the tumour. Intriguingly, the IRS keratin K28 was also expressed by BCC (3 of 20 BCC samples) but only by small clusters of

keratinocytes at the tumour periphery. In contrast, BCC did not express hair shaft keratins.

Figure 4.13

	BCC Histological Type	Basal Outer Root Sheath		Supra-basal Outer Root Sheath		Companion Layer	Inner Root Sheath		Hair Shaft		
		K14	K19	K17	K16	K75	K28	K26	K31	K32	K81
BCC 1	Micronodular										
BCC 2	Nodulocystic										
BCC 3	Nodulocystic										
BCC 4	Nodulocystic										
BCC 5	Nodulocystic										
BCC 6	Nodulocystic										
BCC 7	Nodulocystic										
BCC 8	Nodulocystic										
BCC 9	Nodulocystic										
BCC 10	Nodulocystic										
BCC 11	Nodulocystic										
BCC 12	Nodulocystic										
BCC 13	Nodulocystic										
BCC 14	Nodulocystic										
BCC 15	Nodular										
BCC 16	Nodular										
BCC 17	Nodulocystic										
BCC 18	Nodulocystic										
BCC 19	Nodulocystic										
BCC 20	Nodulocystic										

Legend:
- Yellow: Throughout tumor
- Orange: Small clusters
- Black: Not expressed
- Red: Large clusters
- Blue: Occasional cells

Figure 4.13: Expression of hair follicle specific keratins in 20 samples of BCC examined.

In conclusion, these findings substantiate the presence of multiple tumour sub-populations within BCC and unveil the potential for BCC tumour keratinocytes to differentiate along pre-programmed lineages, which correspond to patterns observed in normal hair follicles. These findings also show that BCC growth demonstrate a cellular hierarchy. Additional experiments were required to ensure that the functional cellular attributes associated with expression of these keratins in hair follicle keratinocytes are retained in BCC.

4.4   Keratin Expression in the Hair Follicle and BCC Defines Cellular Function

In the hair follicle, keratin expression is associated with cellular differentiation that in turn is linked to cellular functions. For example the hard hair shaft keratins create more rigid intermediate filaments, by encoding a greater number of cysteine residues resulting in increased inter-chain disulphide bonds; hence the keratinocytes are less flexible. Likewise ORS keratins facilitate keratinocyte proliferation, while terminally differentiated keratinocytes that contain IRS and hair shaft keratins do not proliferate.

Multiple lines of evidence suggest that the processes of keratinocyte terminal differentiation and proliferation are mutually exclusive. In epithelia, the process of terminal differentiation is responsible for generation of the environmental barrier, so restricting proliferation to less differentiated means that they are safeguarded by layers of more differentiated progeny from potential environmental mutagens. Studies of epidermal proliferation, using immunohistochemical staining, all demonstrate that proliferating keratinocytes reside in the basal or immediate suprabasal keratinocytes.

The process is analogous to the haematopoietic system, in which long-term proliferative potential resides in a small population of bone marrow stem cells, subsequent non-stem cell progeny are committed to a pathway of programmed terminal differentiation, limited longevity and exhibit restricted proliferative capacity. Similarly the interfollicular epidermal keratinocyte stem cells that have

long-term proliferative capacity reside in the basal layer, while in the hair follicle keratinocyte stem cells reside in the ORS. Therefore, we hypothesised that if keratins in BCC reflect normal tissue patterns of differentiation, then similarly proliferation should be restricted to ORS keratin expressing BCC keratinocytes.

To study the relationship between proliferation and keratin expression we used double label immunofluorescence. The keratin primary antibodies were derived from different host species to the proliferation specific antibody (Ki67), to avoid cross labelling and thus maintain specificity. Furthermore, distinction could also be made since keratin antibodies labelled the cytoplasm whereas Ki67 antibodies labelled the nucleus.

The Ki67 antigen is a protein that in humans is encoded by the MKI67 gene, and is a cellular marker of proliferation(Scholzen and Gerdes, 2000). During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0) (Scholzen and Gerdes, 2000).

We used human hair follicle bearing skin samples as positive controls and negative controls, with sections in which the primary antibody was omitted. To determine whether BCC keratinocyte function and so phenotype was determined by expression of differentiation specific hair follicle keratins, we sought to define keratinocyte proliferation in relation to keratin expression. Hair follicle bearing

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normal human skin samples and BCC tissue were co-labelled with keratin specific and Ki-67 antibodies.

Proliferation in the hair follicle

In the hair follicle, Ki-67 labelling was restricted to keratinocytes within the basal and immediate suprabasal layers of the ORS. Consistent with this pattern of expression, proliferating keratinocytes were observed within keratinocytes expressing K14, K16, K17 and K19 (Figure 4.14 a-p). However, Ki-67 labelling was not observed in keratinocytes that expressed the companion layer (K75) (Figure 4.14 q-t) or IRS keratins (K28) (Figure 4.14 u-x).

Proliferation in BCC

To determine whether keratin expression in BCC tumour keratinocytes also determined cell phenotype we similarly compared keratin expression and proliferation using double labelled immunofluorescence (Figures 4.15 a-l).

Similarly in BCC Ki-67 labelling was co-incident with ORS keratins K5, K14, K19 and K17, but not K16 (Figure 4.15a-l). K16 expression in BCC was observed at the centre of tumour nodules, often adjacent stratified cells, well away from proliferation at the periphery of tumour nodules (Figure 4.15 e-h). Consistent with our hypothesis, proliferation was not observed in BCC keratinocytes that expressed companion layer K75 (Figure 4.15 m-p).

Figure 4.14

DAPI	K14	Ki67	K14 & Ki67 100 microns
DAPI	K16	Ki67	K16 & Ki67 100 microns
DAPI	K17	Ki67	DAPI & K17 & Ki67 100 microns
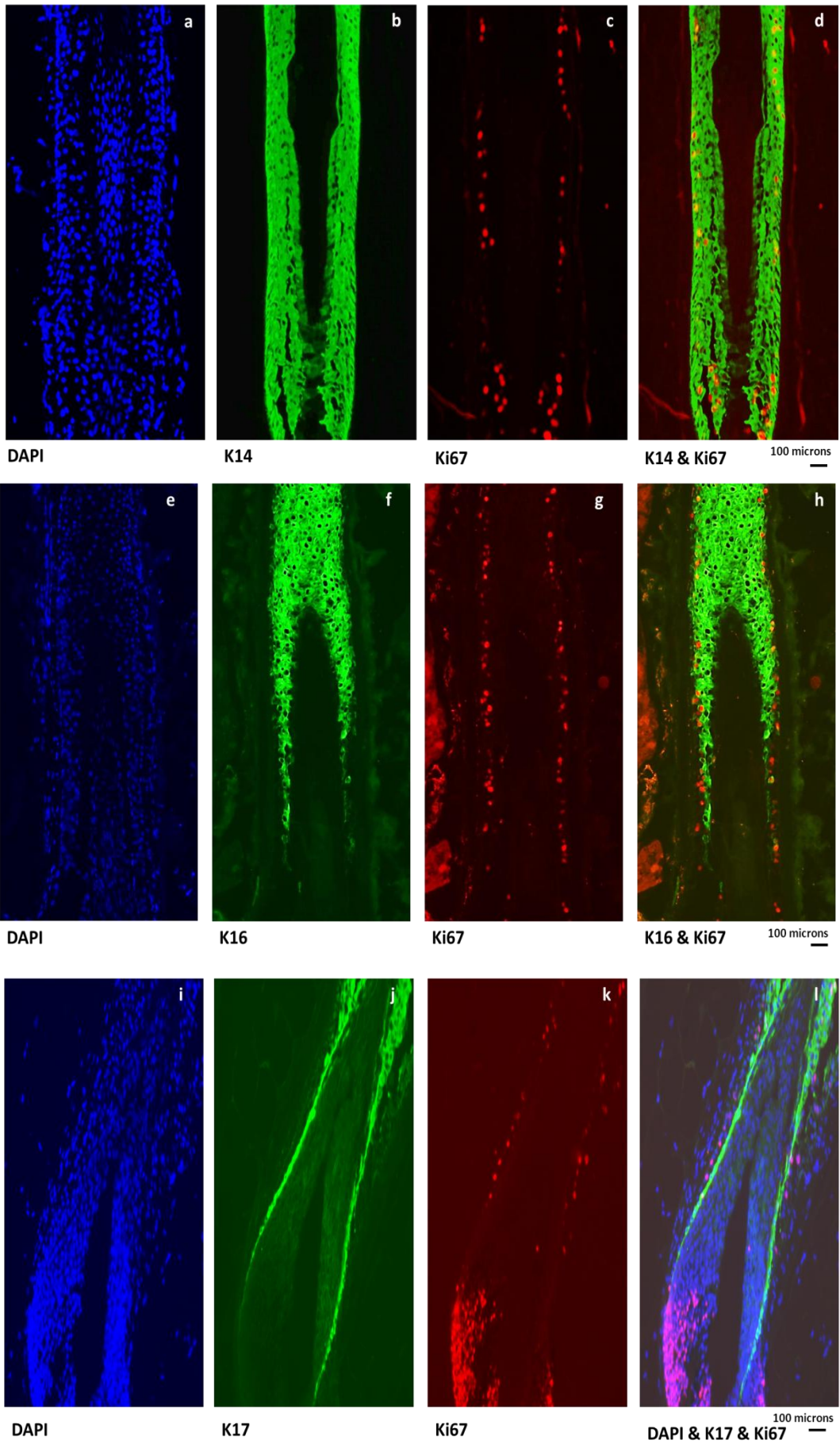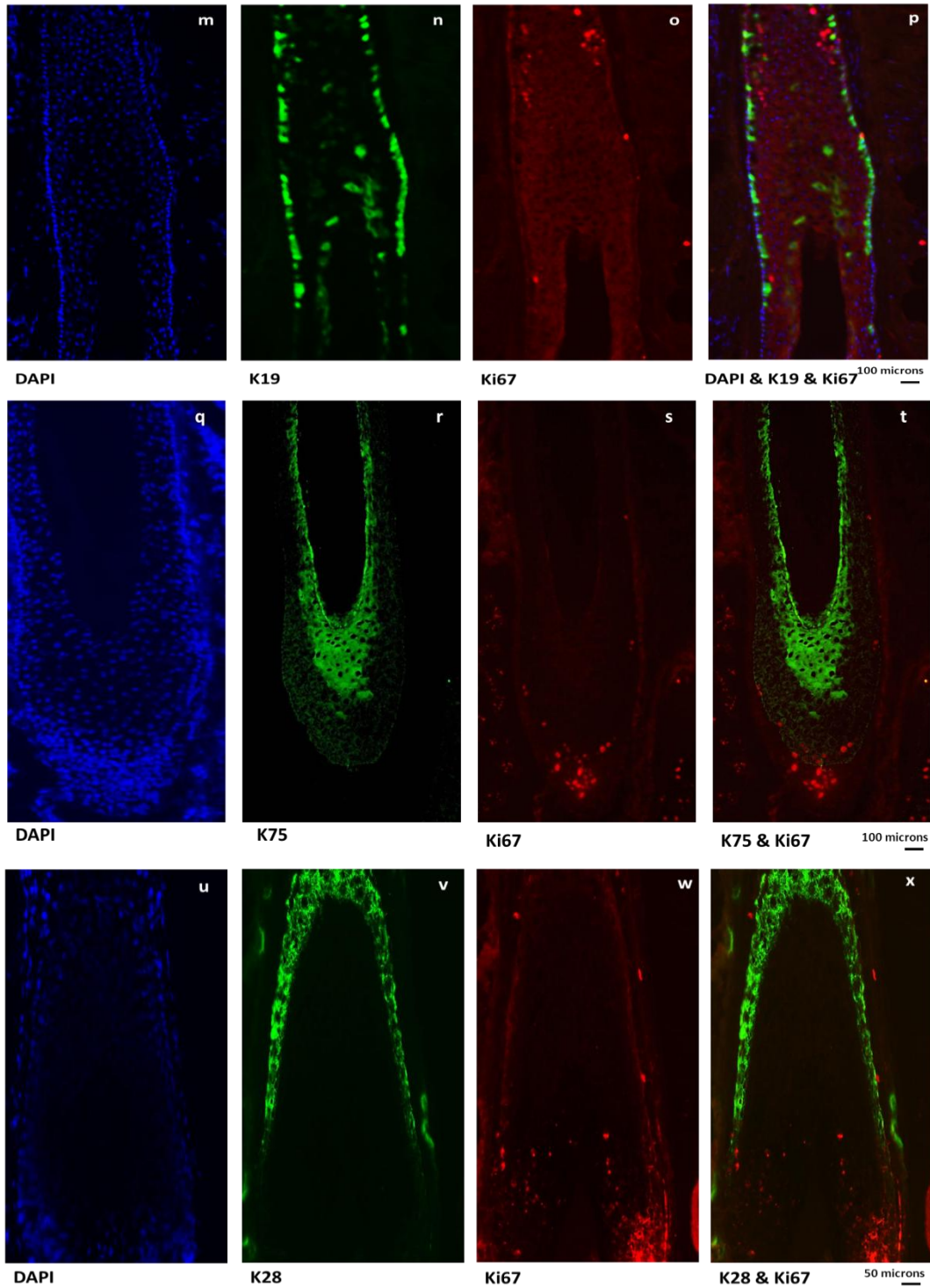
Figure 4.14: Double immunofluorescence analysis of co-expression of Ki67 and hair follicle keratins K14, K16, K17, K19, K75, and K28 in hair follicle sections.

For all images, left columns represented DAPI, left middle columns showed keratins expression; right middle columns showed Ki67 expression, and right columns represented merge. Co-expression of K28 and Ki67 image was captured with 20x objective, scale interval 50 microns. Other images were captured with 10x objective, scale bar 100 microns.

Figure 4.15
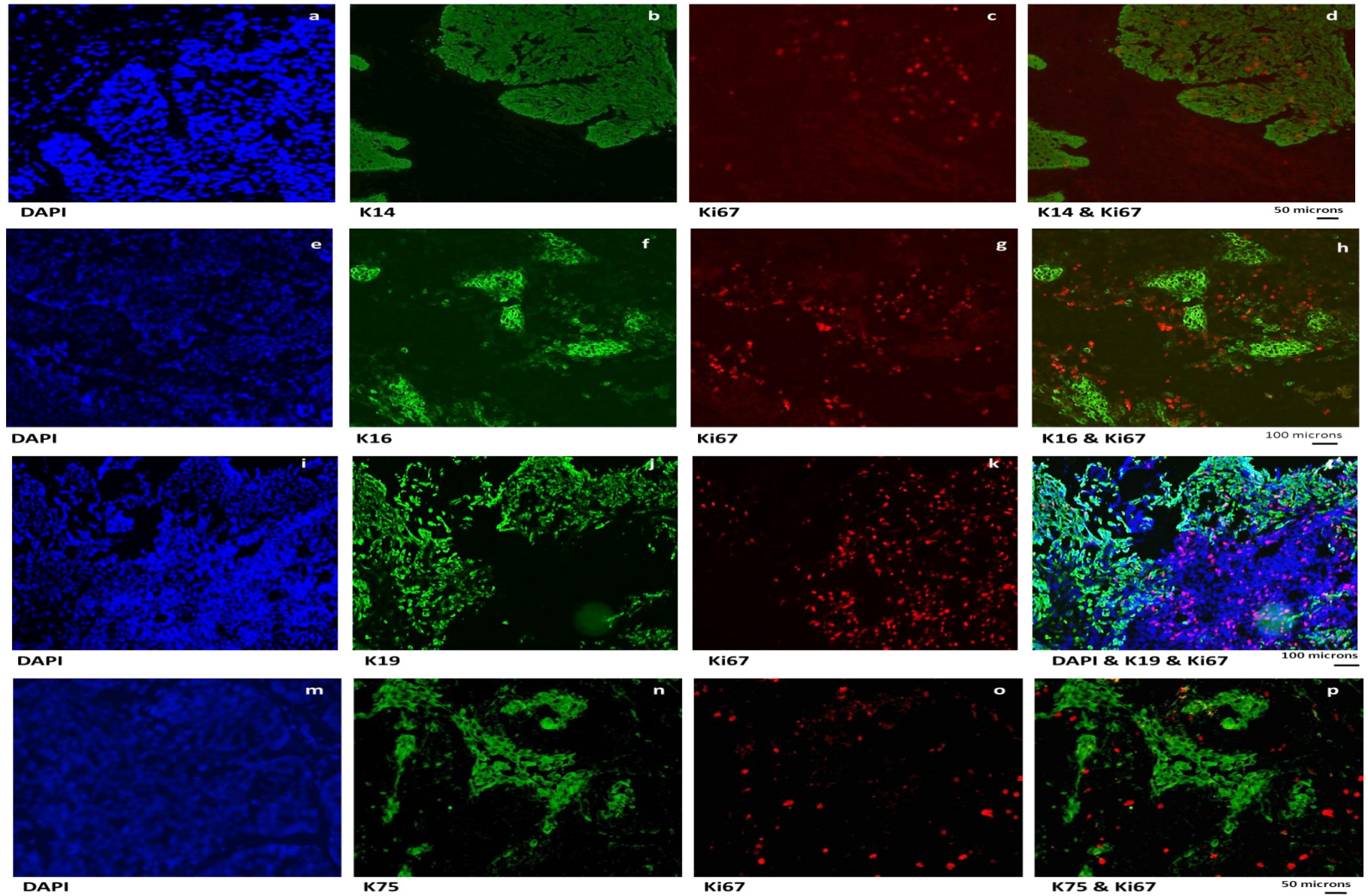
Figure 4.15: Expression and co-localisation of Ki67 and hair follicle keratins in BCC.
For all images left columns donate nuclear expression of DAPI in blue, left middle columns denote keratins expression in BCC samples, K14 (b), K16 (f), K19 (j), and K75 (n) in green. Right middle columns denote expression of Ki67 in red and right columns denote merge. Images were captured with 10x and 20x objectives, scale intervals 50 and 100 microns.

4.4.1 Statistical analysis

Hair follicle

Of the proliferating hair follicle keratinocytes 75±13% (Mean ± SEM) expressed K14, 12±2% expressed K16, 4±1% expressed K19 and 2±2% expressed K17 (Figure 4.16 a). There was a relationship between proliferation and keratin expression in that 16± 3% of K14 positive keratinocytes were proliferating, while only 4% of K16 positive keratinocytes were proliferating, 1% of K19 positive keratinocytes were proliferating and 1% of K17 keratinocytes were proliferating (Figure 4.16 b). Thus, the basal layer was the main site of hair follicle proliferation in which keratinocytes that express K19 were relatively quiescent. Similarly in the suprabasal layers where proliferation was less common, keratinocytes that express K17 were relatively quiescent.

Figure 4.16
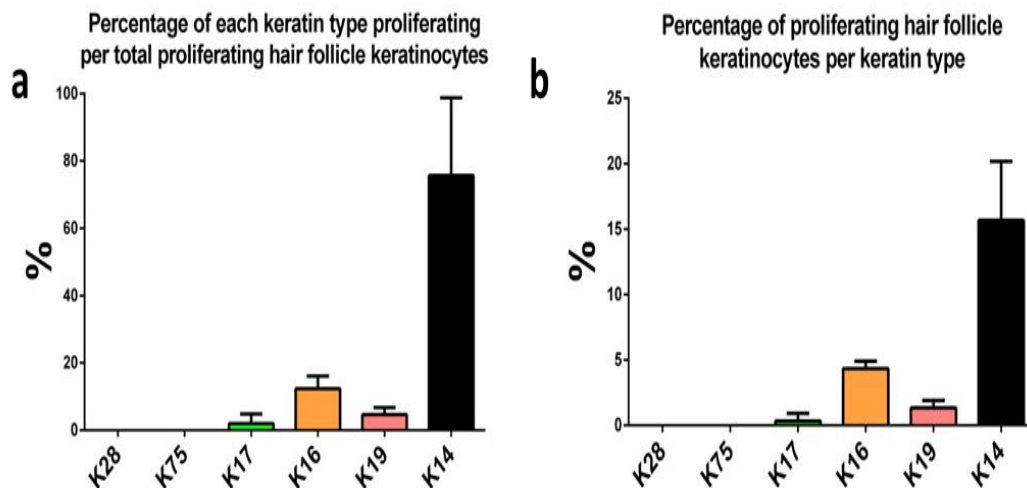
Figure 4.16: Representative bar chart showing the percentage of co-expression of Ki67 and hair follicle keratins in hair follicle tissue samples.

Basal cell carcinoma

Of the proliferating BCC tumour keratinocytes; 79±10% (Mean ± SEM) expressed K14, 14±2% expressed K16, 8±4% expressed K19, 92±4% expressed K17, and 2±1% expressed K75 (Figure 4.17 a). The relationship between proliferation and keratin expression varied in the different cell populations 23±13% of K14 positive keratinocytes were proliferating, 6±3% of K16 positive keratinocytes were proliferating, 3±1% of K19 positive keratinocytes were proliferating, 33±11% of K17 positive keratinocytes were proliferating and 4±3% of K75 positive keratinocytes were proliferating (Figure 4.17 b). Proliferation was restricted to BCC tumour keratinocytes that expressed keratins of the basal and suprabasal ORS layers with the exception of occasional K75+ BCC tumour keratinocytes that were proliferating. BCC tumour keratinocytes expressing K28, the IRS keratin, as in the hair follicle did not proliferate. Also, as in the hair follicle ORS, K19 expressing BCC tumour keratinocytes were quiescent relative to K14 tumour keratinocytes. Likewise the K16 tumour keratinocytes proliferated at similar rates to that observed in the hair follicle.

The only major difference between the proliferative rates of hair follicle and BCC keratinocytes relative to their keratin expression was the great increase in K17 positive BCC keratinocytes that were proliferating, which comes from over expression of this keratin as a direct consequence of changes in hedgehog signalling resulting from the genetic basis of the disease. Thus, keratin expression in BCC as in the hair follicle defines cellular function, with respect to proliferation, with the exception of K17 and occasional K75 positive keratinocytes.

Figure 4.17
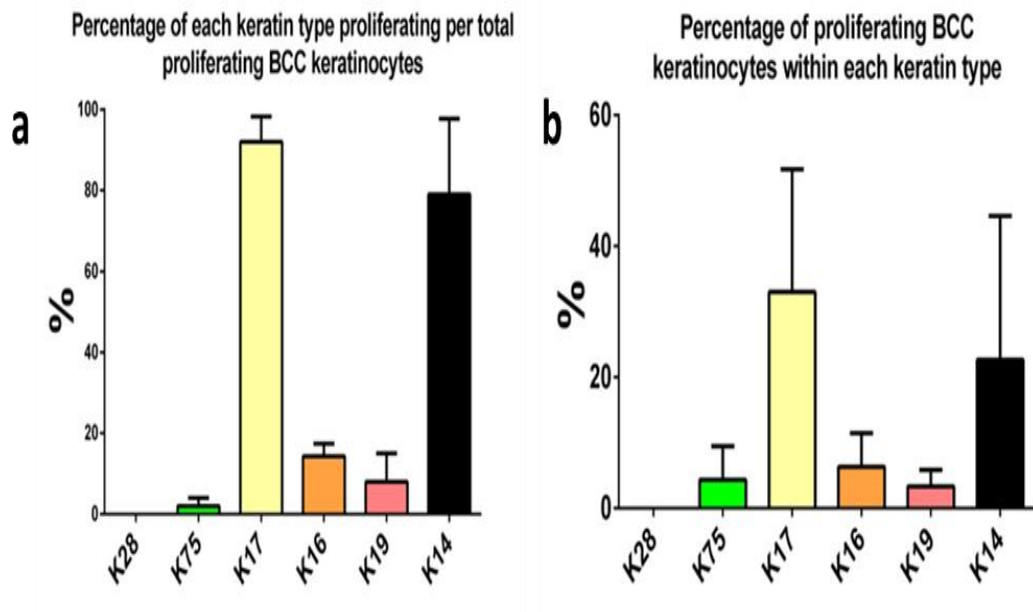
Figure 4.17: Representative bar chart showing the percentage of co-expression of Ki67 and hair follicle keratins in hair follicle tissue samples.

4.5 Regulation of Keratin Expression

The pattern of keratin expression in human BCCs was similar to that observed in human hair follicles. Furthermore, keratin expression within BCC and hair follicle keratinocytes was associated with similar functional phenotypes. We next hypothesised whether expression of keratins in both tissues is similarly transcriptionally regulated. If keratin expression in BCC could be shown to be regulated along the same lines as in normal tissue, it would strengthen our case for the existence of normal tissue differentiation within BCC.

While there have been great advances in hair follicle biology and the understanding of hair follicle keratin transcriptional regulation, many of these observations have been established in murine systems and are not therefore readily transferable to human samples. Expression of K16 has been described in association with nuclear translocation of NF-IL6 (also known as LAP, CRP2, TCF5, IL6DBP, C/EBP-beta) (Vyas *et al.*, 2001), a downstream component of many signalling pathways including the MAP kinase cascade. The expression of K17 has been shown to be regulated by GLI transcription factors as part of hedgehog signalling (Callahan *et al.*, 2004).

Since BCCs demonstrate constitutive over-expression of hedgehog signalling, this provides a basis for the expression of K17 in all BCC keratinocytes as we have observed. Thus, we sought to study these regulatory pathways using double-labelled immunofluorescence techniques to determine whether nuclear translocation of NF-IL6 and GLI transcription factors were co-incident with the expression of K16 and K17 respectively.

4.5.1   Regulation pattern of outer root sheath keratins

4.5.1.1   NF-IL6 regulation of human keratins in the hair follicle and BCC

Double labelling immunofluorescence was used to detect nuclear translocation of NF-IL6 in keratinocytes expressing various keratins.

Hair follicle and epidermis

The transcription factor, NFIL6, was expressed within the epidermis (Figure 4.18 a-b) and hair follicle (Figure 4.18 c-d). Within basal keratinocytes, NF-IL6 expression was within the cell cytoplasm in a diffuse pattern showing no obvious subcellular localisation. However, suprabasal keratinocytes, both within the epidermis and hair follicle, demonstrated nuclear staining (Figure 4.18 a-d). The nuclear localisation of NF-IL6, representing activation of the upstream signalling pathway, results in phosphorylation and hence translocation of this transcription factor to the nucleus, was observed within the ORS, companion layer and IRS layer of the hair follicle. The nuclear localisation of NF-IL6 was coincident with both K16 and K17 expression within the suprabasal ORS (Figure 4.19 e-l), suggesting that the signalling pathway, and in particular the transcription factor NF-IL6, may regulates expression of these keratins. Similarly, nuclear NF-IL6 expression was observed with K75 expression in companion layer keratinocytes (Figure 4.19m-p).

Figure 4.18
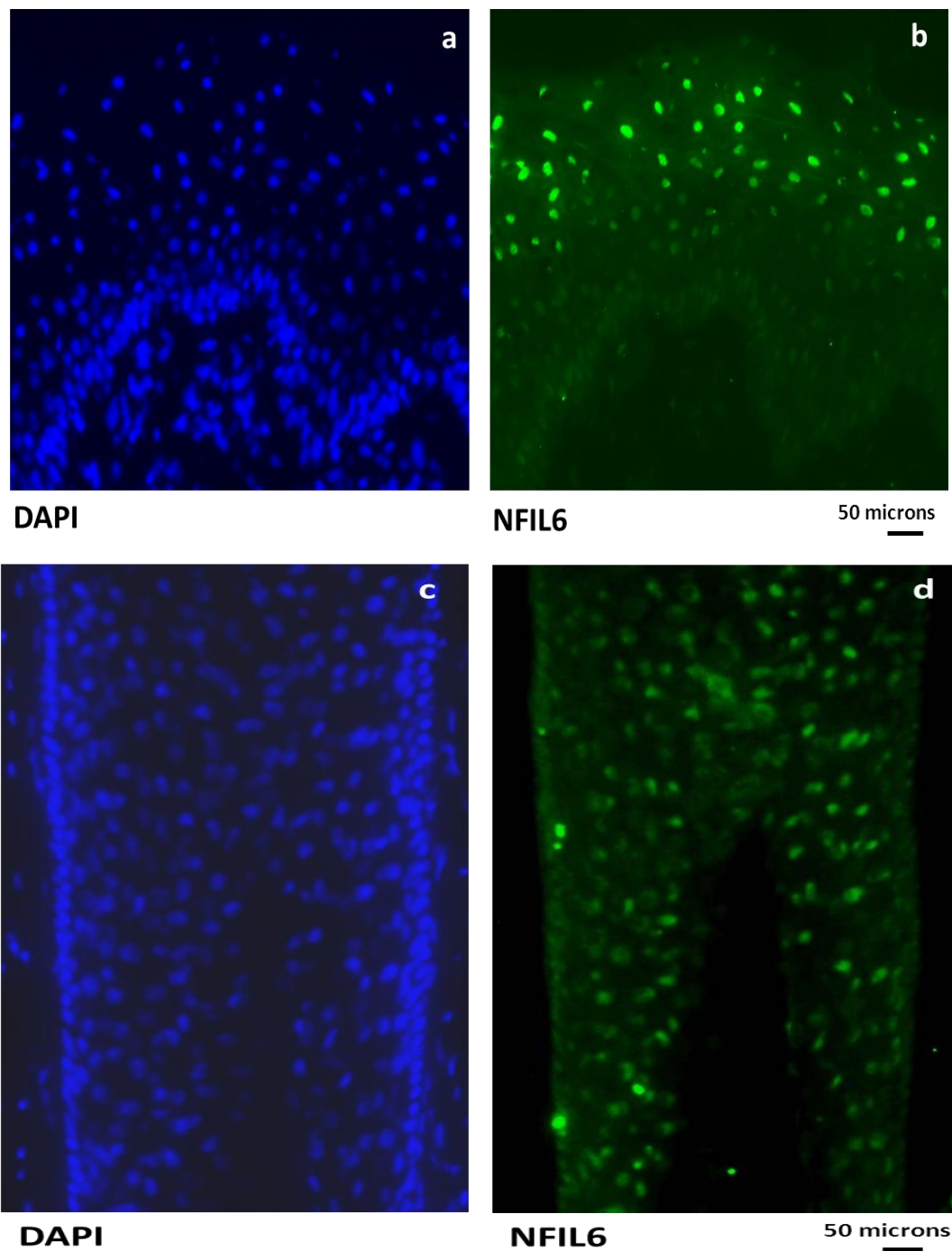
Figure 4.18: Immunofluorescence staining of NFIL6 and DAPI in epidermis and hair follicle.

The cytoplasmic expression of NFIL6 in basal layer of the epidermis, DAPI (a), and NFIL6 (b) and nuclear expression of NFIL6 in hair follicle suprabasal layer, DAPI (c), and NFIL6 (d). Images were captured with 20x objective, scale bar 50 microns.

153
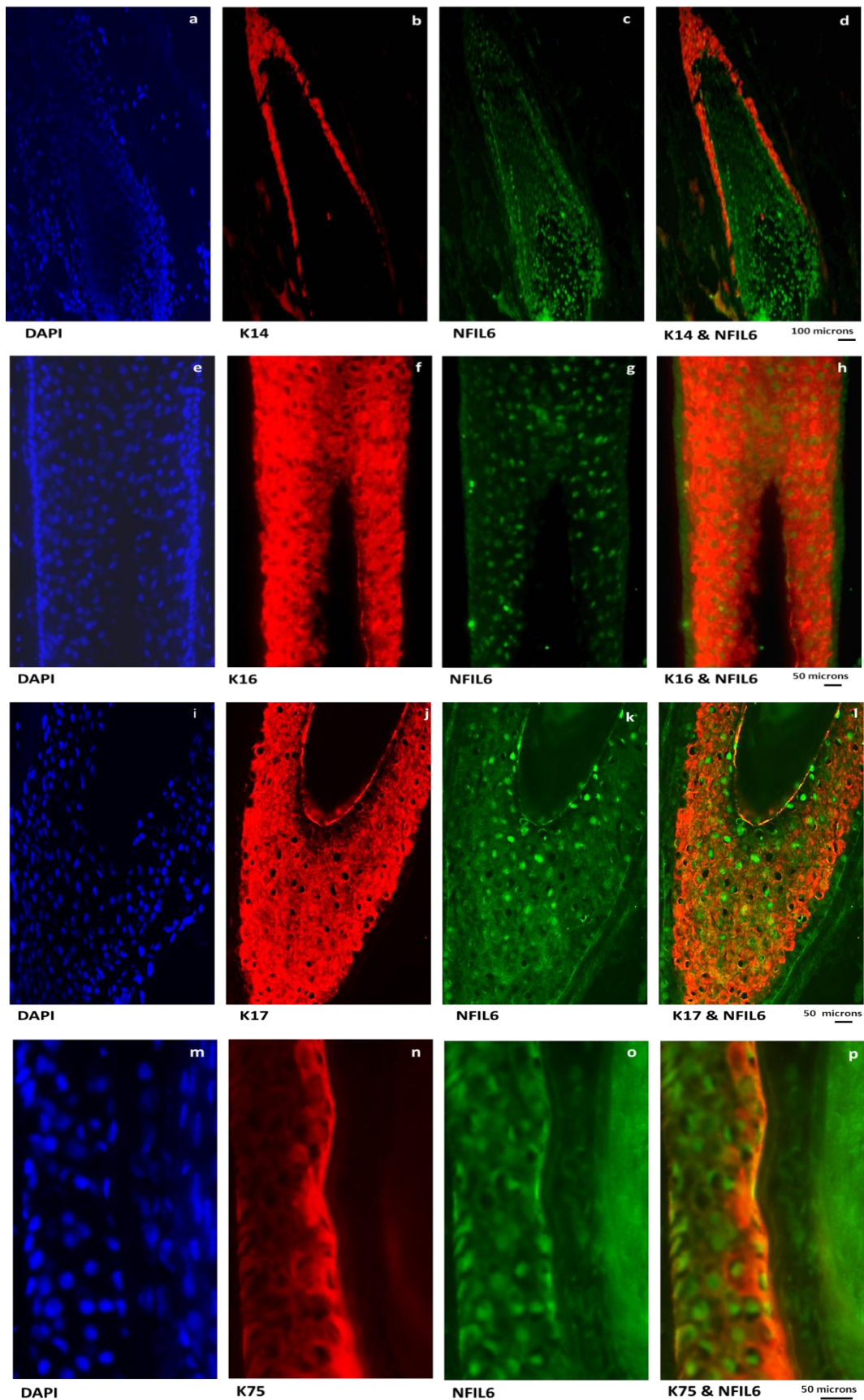
Figure 4.19

Figure 4.19: Representative immunofluorescence of NFIL6 expression and keratins in HF.

Hair follicle sections were treated with NFIL6 antibody (green) and keratin antibodies (red). For all images, left columns represented DAPI expression in blue, left middle columns showed keratins expression K14, K16, K17 and K75in red, right middle columns showed NFIL6 expression in green and the right columns demonstrated the merge. Images were captured with 10x, 20x, 40x objective, scale interval 100, 50, 50 microns respectively.

Basal cell carcinoma

NF-IL6 expression was also observed in BCC keratinocytes, (Figure 4.20a-d). However, unlike the situation in normal epithelial cells, NF-IL6 expression was not observed in all BCC keratinocytes. Within the BCC tumour samples examined, NF-IL6 expression was observed within clusters of BCC cells either diffusely distributed within the cell cytoplasm or alternatively localised within the nucleus (Figure 4.20 c, g). Expression of K16, but not K17 or K75, coincided with nuclear localisation of NF-IL6 within BCC keratinocytes nuclear localisation of NF-IL6 was exclusively associated with K16 expression and similarly K16 expression was exclusively associated with nuclear NF-IL6 (Figure 4.20 a-d), suggesting K16 expression in BCC was transcriptionally regulated and dependent upon NF-IL6.

Figure 4.20
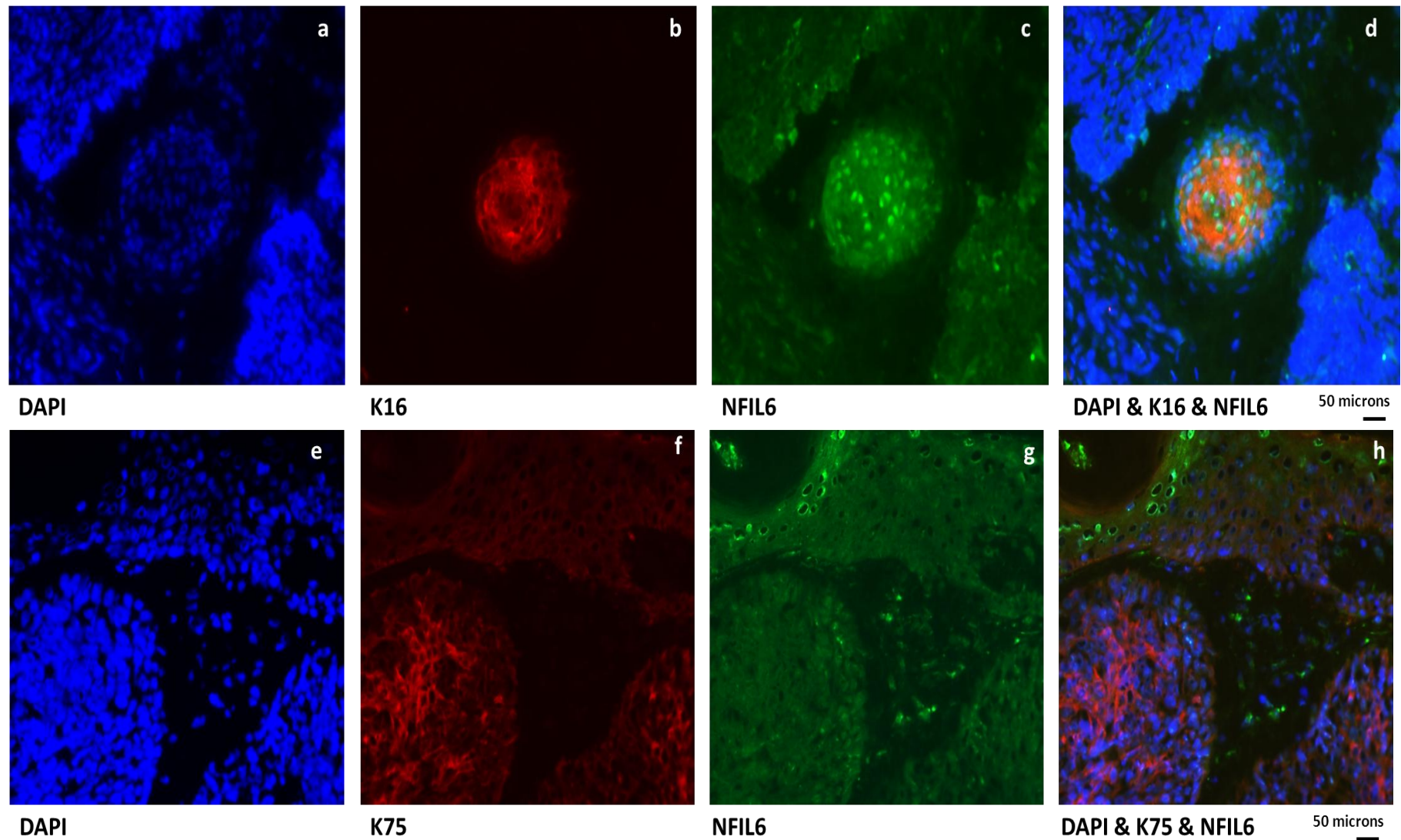
Figure 4.20: Double immunofluorescence staining analyses the expression of NFIL6 in BCCs.

BCC sections were treated with NFIL6 (green) and K16 or K75 (red). Top panel demonstrated the positive co-expression of NFIL6 and K16, DAPI (a), K16 (b), NFIL6 (c), and merge (d). Bottom panel showed the negative co- localisation of NFIL6 and K75, DAPI (e), K75 (f), NFIL6 (g), and merge (h). Images captured with x20 objective, scale bar 50 microns.

4.5.1.2 GLI regulation of human keratins in the hair follicle and BCC

4.5.1.2.1 Expressions of GLI transcriptional factor and K17, K75

Constitutively active sonic hedgehog signalling underpins the development of all human BCCs and is sufficient to give rise to BCC in murine models (Epstein, 2008). Furthermore, DNA binding sites for two downstream transcription factors (GLI1 and GLI2) are found in the promoter sequence of the K17 gene (Bianchi *et al.*, 2005). Hence, if K17 is regulated by GLI transcription factors and since K17 is expressed by all BCC keratinocytes, we hypothesised that all BCC keratinocytes would demonstrate nuclear translocation of GLI1 and/or GLI2 transcription factors.

Hair follicle

In the hair follicle, GLI1 was expressed within the cytoplasm of non-proliferating keratinocytes in a single cell layer extending up from the hair bulb. Expression of GLI1 was distinct and was not coincident with ORS keratins (Figure 2.21 a-d). Instead, cytoplasmic GLI1 expression coincided with expression of the companion layer keratin K75 (Figure 2.21 e-h). Similarly, GLI2 was also expressed by a single cell layer emanating from the hair bulb, with both cytoplasmic and nuclear labelling. GLI2 expression was coincident with K75 expression (Figure 2.22 a-d) and did not label keratinocytes expressing other hair follicle keratins (Figure 2.22 e-h).

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Figure 4.21
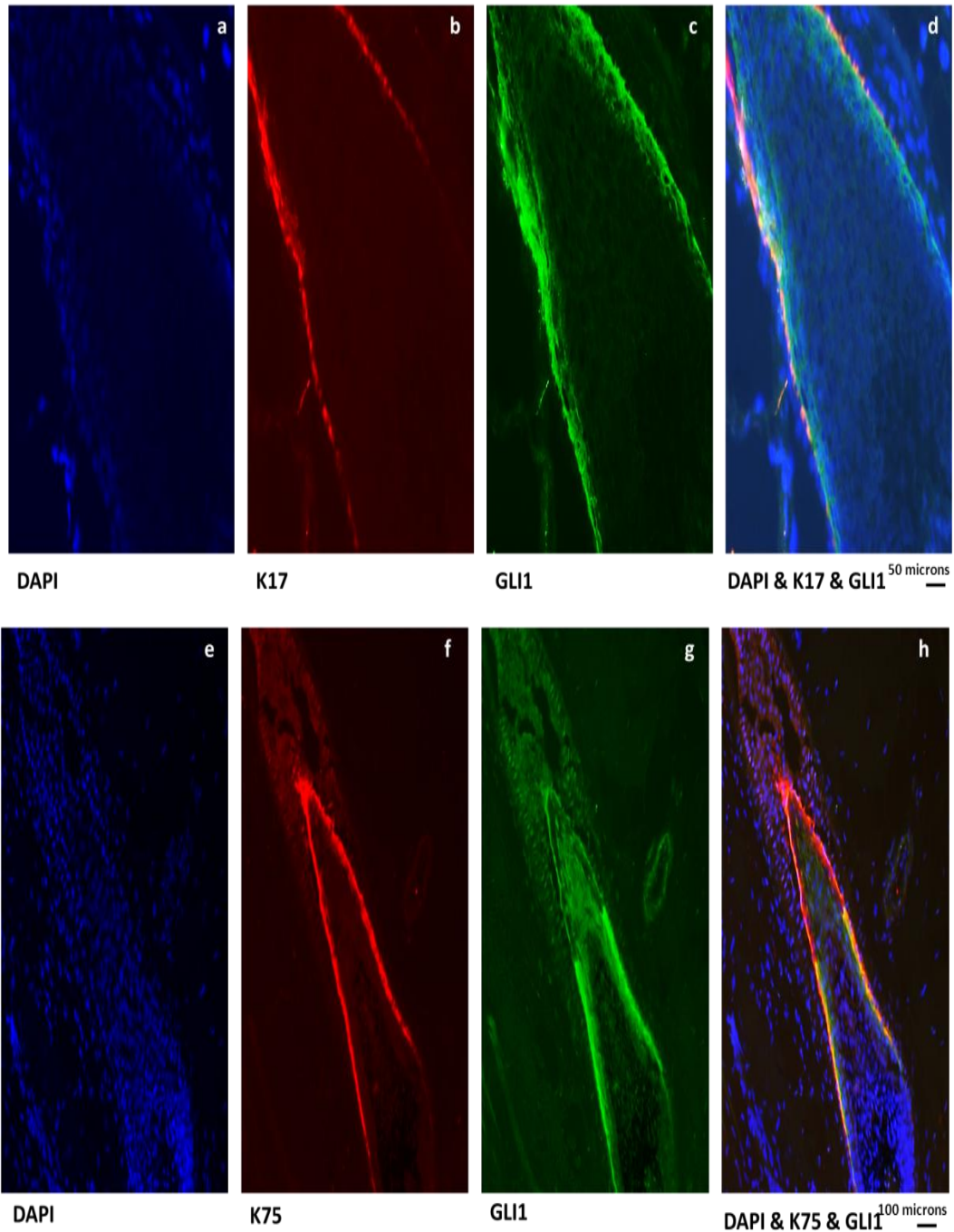
Figure 4.21: Immunofluorescence labelling analyses the expression of GLI 1 transcriptional factors and keratins in hair follicle.

Hair follicle sections were stained with GLI1and K17, or K75. Top panel indicated the localisation of GLI1 and K17, DAPI (a), K17 (b), GLI1 (c), and merge (d). bottom panel showed the co-localisation of GLI1 and K75, DAPI (e), K75 (f), GLI1 (g), and merge (h).top images were captured with 20x objective , scale interval 50 microns, bottom images were captured with x10, scale interval 100 microns.

Figure 4.22
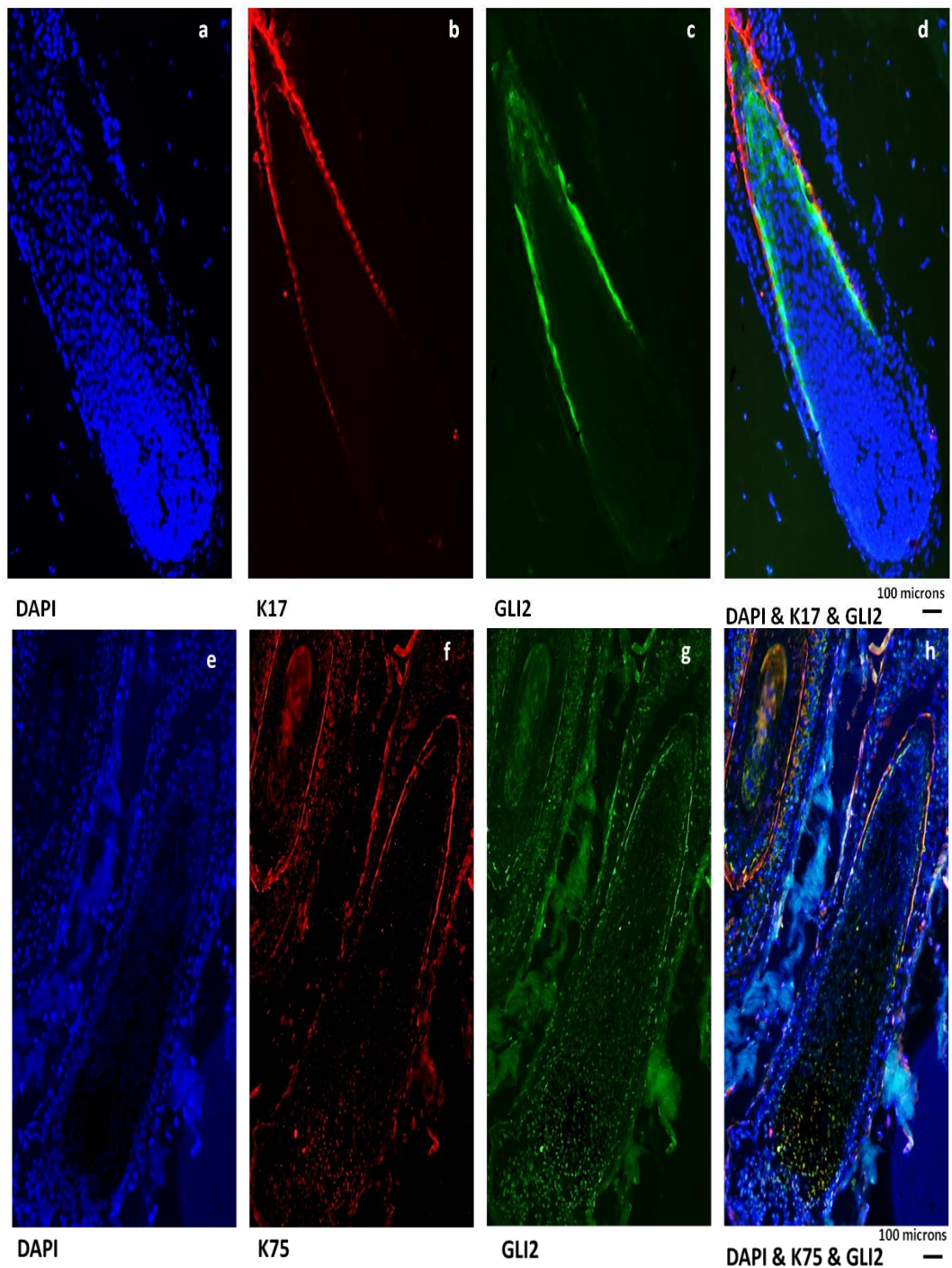
Figure 4.22: Immunofluorescence labelling analyses the expression of GLI 2 transcriptional factors and keratins in hair follicle.

Hair follicle sections were stained with GLI2and K17, or K75. Top panel indicated the pattern of expression of GLI2 and K17, DAPI (a), K17 (b), GLI1 (c), and merge (d). Bottom panel showed the co-localisation of GLI2 and K75, DAPI (e), K75 (f), GLI1 (g), and merge (h). Images were captured with x10 objective, scale interval 100 microns.

Basal cell carcinoma

In BCC samples, GLI transcription factors were not ubiquitously expressed throughout all BCC keratinocytes. Instead, we observed sporadic expression with both cytoplasmic and nuclear labelling. Similar to the hair follicle, diffuse cytoplasmic GLI1 expression was restricted to K75 expressing BCC keratinocytes (Figure 4.23 e-h), but not at all in BCC keratinocytes expressing K17 (Figure 4.23 a-d). On the other hand, GLI2 expression was more promiscuous and was evident in large clusters both with diffuse cytoplasmic and nuclear expression. GLI2 neither cytoplasmic nor nuclear expression coincided with expression of K17 (Figure 4.23 i-p), instead as with GLI1, GLI2 expression was coincident with K75 expression in BCC (Figure 4.23 e-h, m-p).

Figure 4.23
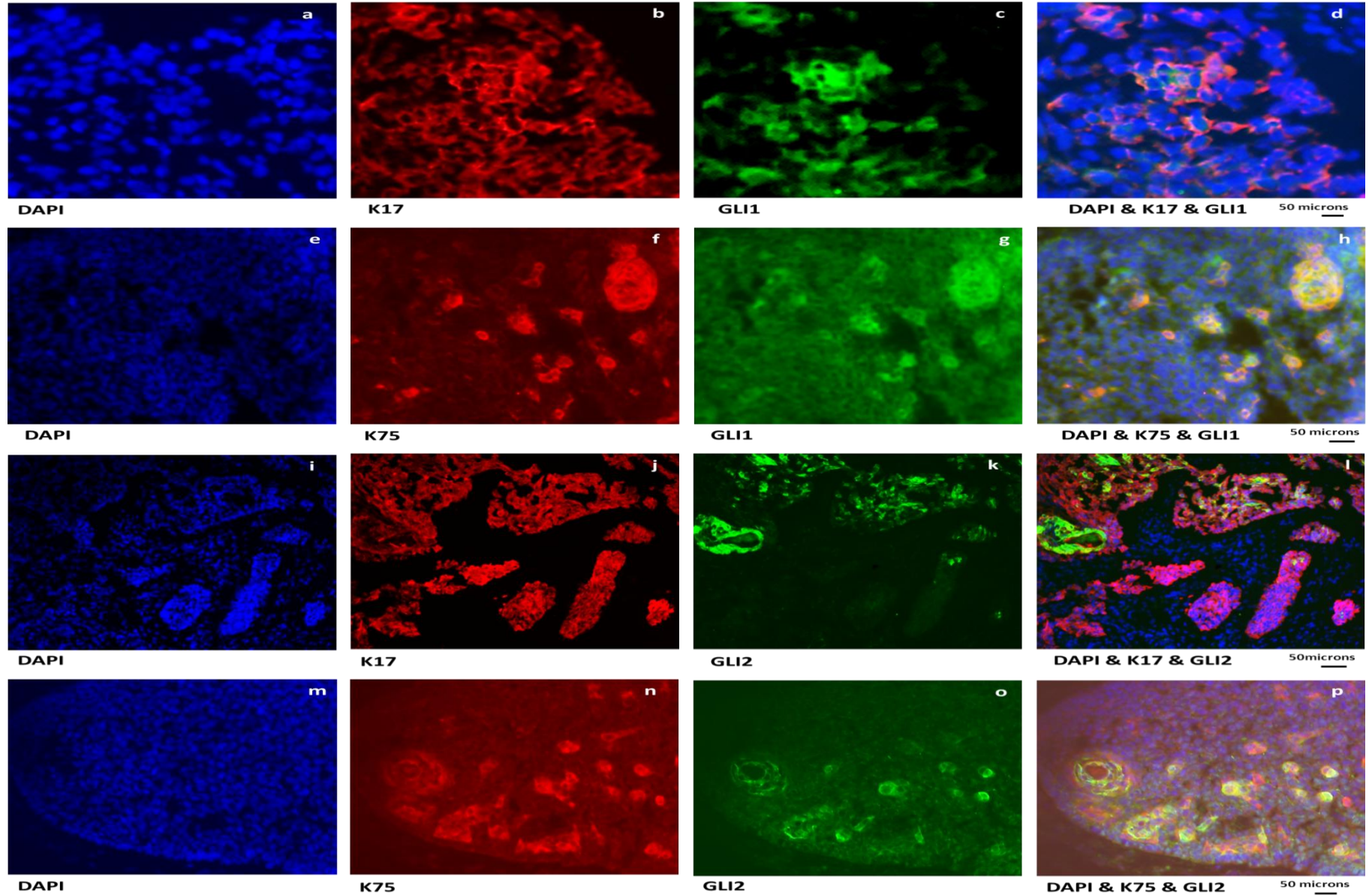
Figure 4.23: Immunofluorescence labelling analyses the association between GLI and keratins K17, and K75 in BCCs.
BCC sections were labelled with GLI1 or GLI2 (green) and K17 or K75 (red) antibodies. Left columns showed DAPI, left middle showed keratins staining, right middle showed GLI staining, and right columns showed the merge. Images were captured with 20x objective, scale bar 50 microns.

4.5.1.3 Expression of other transcription factors

Other transcription factors described as being associated with hair follicle keratin expression were also studied, including β catenin, Sox9, Gata3, and Gadd153. Labelling with antibodies to these transcription factors did not coincide with distinct hair follicle lineages in normal human skin and this precluded interpretation in BCC samples.

4.5.2 Regulation pattern of hair follicle inner root sheath in BCCs

The hair follicle is composed of epidermal (epithelial) and dermal (mesenchymal) compartments and their interactions play an important role in the morphogenesis and growth of the hair follicle (Millar, 2002). Effective cross-talk between these two compartments is also essential for post-natal proliferation and differentiation of the hair follicle IRS and hair shaft (Sennett and Rendl, 2012). For example, Wnt signalling, with nuclear localisation of β-catenin, regulate hair keratin expression (Fuchs *et al.*, 2001). We therefore hypothesised that epithelial-mesenchymal cross-talk regulating hair follicle differentiation may exist in BCC, sufficient to induce expression of companion layer and IRS keratins.

The mesenchymal portion of the hair follicle can be divided into two compartments, the dermal papilla and dermal sheath (Boiko *et al.*, 2010). The dermal papilla is located at the base of the hair follicle and is surrounded by the hair bulbs. The DS, or connective tissue sheath, lines the epithelium of the hair follicle from the bulge level downward and is contiguous with the base of the dermal papilla through a stalk. A basement membrane

separates the epithelial portion of the hair follicle from the dermal papilla and dermal sheath. Cells within the dermal papilla are specialized fibroblasts of mesenchymal origin, although markers and transcriptional profiles suggest that they are neural crest derived (Fernandes *et al.*, 2004; Rendl *et al.*, 2005).

BMP signalling from the dermal papilla is required to stabilise Wnt signalling for a proper epithelial maturation and differentiation. For example deletion of BMP receptor in the epithelia leads to failure of the matrix cells and differentiation (Andl *et al.*, 2004). Over expression of the BMP inhibitor (noggin) leads to excessive proliferation of matrix cells and prevents hair shaft maturation (Kulessa *et al.*, 2000). Similarly, postnatal morphogenesis of hair follicles is also dependent on FGF 7 and FGF10 ligands (Petiot *et al.*, 2003). Other transcriptional factors are also involved in hair follicle differentiation, but whether epithelial-mesenchymal signalling is involved is not known, they include Gata3, Hoxc13, Cutl1, Foxn1 and Msx2 (Schlake, 2007).

Cells within dermal papilla express specific enzymes and molecules, which are widely used to identify dermal papilla cells. Alkaline phosphatase (ALP) activity has been used as a marker to detect the presence of dermal papilla and regarded as an indicator for hair inductivity (Iida *et al.*, 2007; McElwee *et al.*, 2003). In mice, dermal papilla express ALP throughout the entire hair cycle (Handjiski *et al.*, 1994), expression is maximal in early anagen and decreases in the proximal half of the dermal papilla after the mid-anagen growing phase (Iida *et al.*, 2007). Other markers of the dermal papilla are less specific, for example α-Smooth muscle actin (αSMA) is present in the mid- to lower dermal sheath in human hair follicles but not in dermal papilla (Jahoda *et al.*, 1991).

Versican is expressed in the human hair follicle dermal papilla during anagen but also shows weak immunoreactivity for the dermal sheath outside K15-positive bulge epithelial cells. Corin is expressed specifically in the dermal papilla from the earliest stage in mice, but appears functionally unrelated to hair morphogenesis (Enshell-Seijffers *et al.*, 2008). The hair bulb and dermal papilla are rich in BMPs and BMP receptor 1a, but neither is specific for the dermal papilla (Rendl *et al.*, 2008). In summary, based on the available literature, only ALP appears to be a specific marker for hair follicle dermal papilla.

4.5.2.1   Role of dermal papilla cells in IRS keratin regulation in BCCs

To determine the presence of dermal papilla cells within BCC stroma, we first sought to identify dermal papilla cells by staining with dermal papilla markers versican, CD56, bmpr1a, and ALP. Versican labelled the dermal papilla mesenchymal cells but also stained the dermal sheath (Figure 2.24 a). CD56 was not specific to the dermal papilla; the stain was nuclear with wide expression throughout the HF (Figure 2.24 b).

In addition, BMPr1a was not a very specific marker for the dermal papilla and was widely expressed throughout the hair follicle (Figure 2.24c). Only ALP appeared to be relatively specific for the dermal papilla (Figure 2.24 d).

Figure 4.24

Versican   CD56   bmpr1a   ALP   100 microns
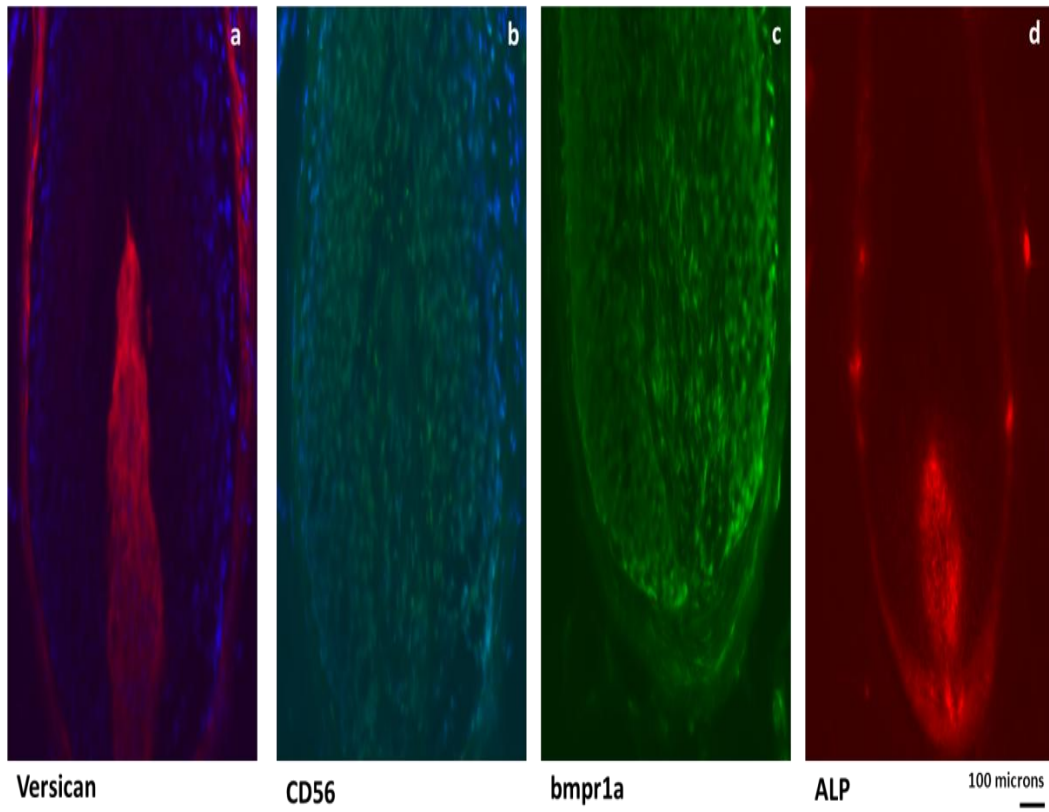
Figure 4.24: Immunofluorescence staining of hair follicle bulb including the dermal papilla.

Hair follicle sections were labelled with a) Versican, b) CD56, c) BMPr1a, and d) ALP. Images were photographed with x10 objective, scale bar 100 microns.

Therefore, we chose ALP to stain the dermal papilla on hair follicle tissue sections, which had the added advantage that it could be visualised both by conventional light microscopy and by immunofluorescence (Figure 4.25 a-i). BCC samples were stained to determine the expression and localisation of ALP labelling in hair follicles as well as in the tumour stroma of BCC samples.

Figure 4.25
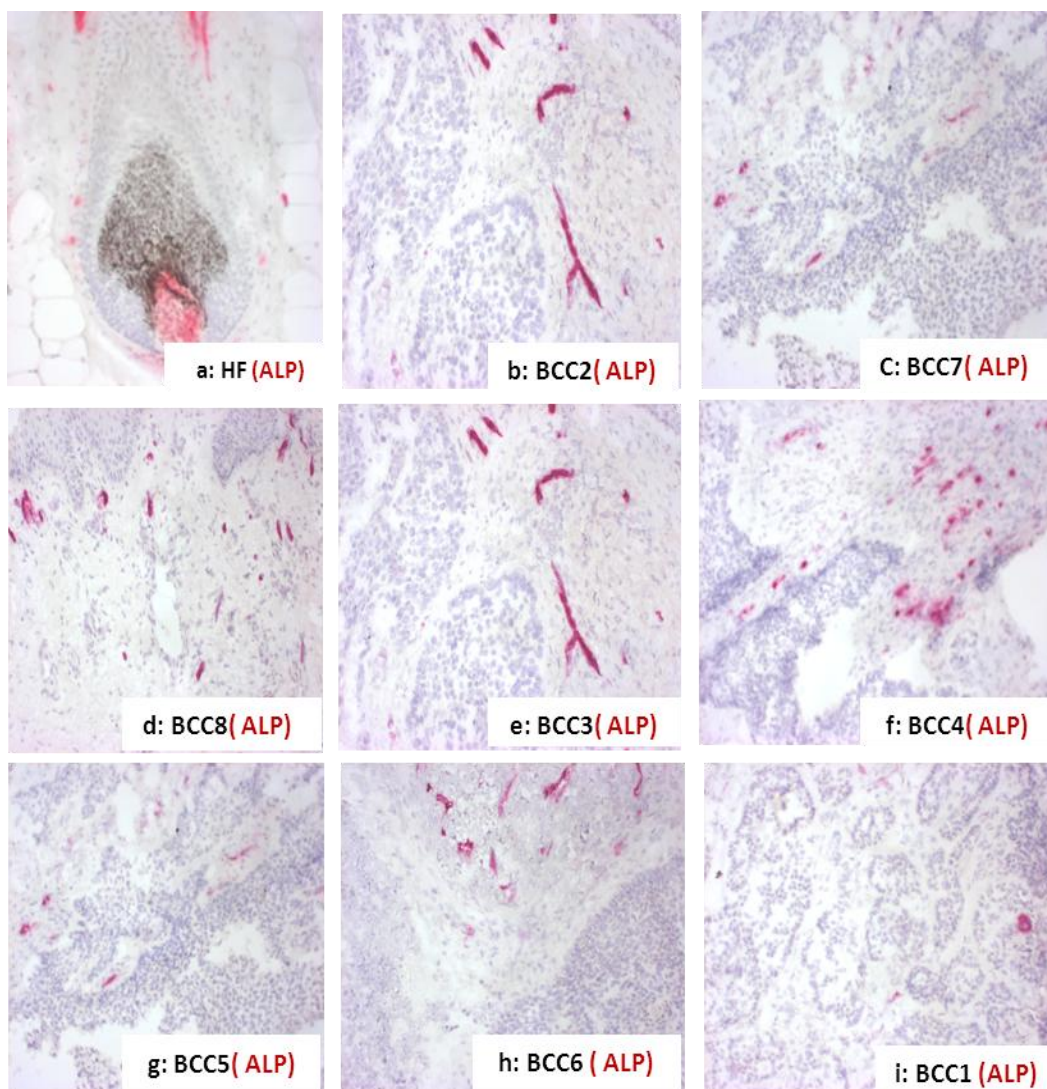
Figure 4.25: Immunohistochemical staining examines the expression of ALP in Hair follicle (HF)and BCC .
HF(a) and BCCs (b-i). All BCC samples expressed ALP in their stroma, represented in red. But ALP also detected blood vessels in some BCC sections.

Although ALP accurately defined the anatomical location of dermal papilla in the hair follicle, additional positive cells were observed within the surrounding stroma prominently surrounding the hair follicle within the dermal sheath, but not exclusively. Therefore, we could not determine whether the ALP positive cells in the BCC stroma were dermal papilla like cells or not.

Since the ALP positive cells in the BCC samples were arranged in linear patterns we hypothesised that dermal blood vessel both in the BCC and normal hair follicle tissue section may also be ALP positive. To confirm this we co-labelled hair follicle sections with the endothelial cell marker, CD31 and used the red auto-fluorescence from ALP staining. Using immunofluorescence, ALP did indeed simultaneously detect dermal papilla, which remained CD31 negative, but dual positive labelling was observed elsewhere in the stroma (Figure 4.26).

Figure 4.26

DAPI ALP CD31 DAPI & ALP & CD31   100 microns

DAPI ALP CD31 DAPI & ALP & CD31   50 microns
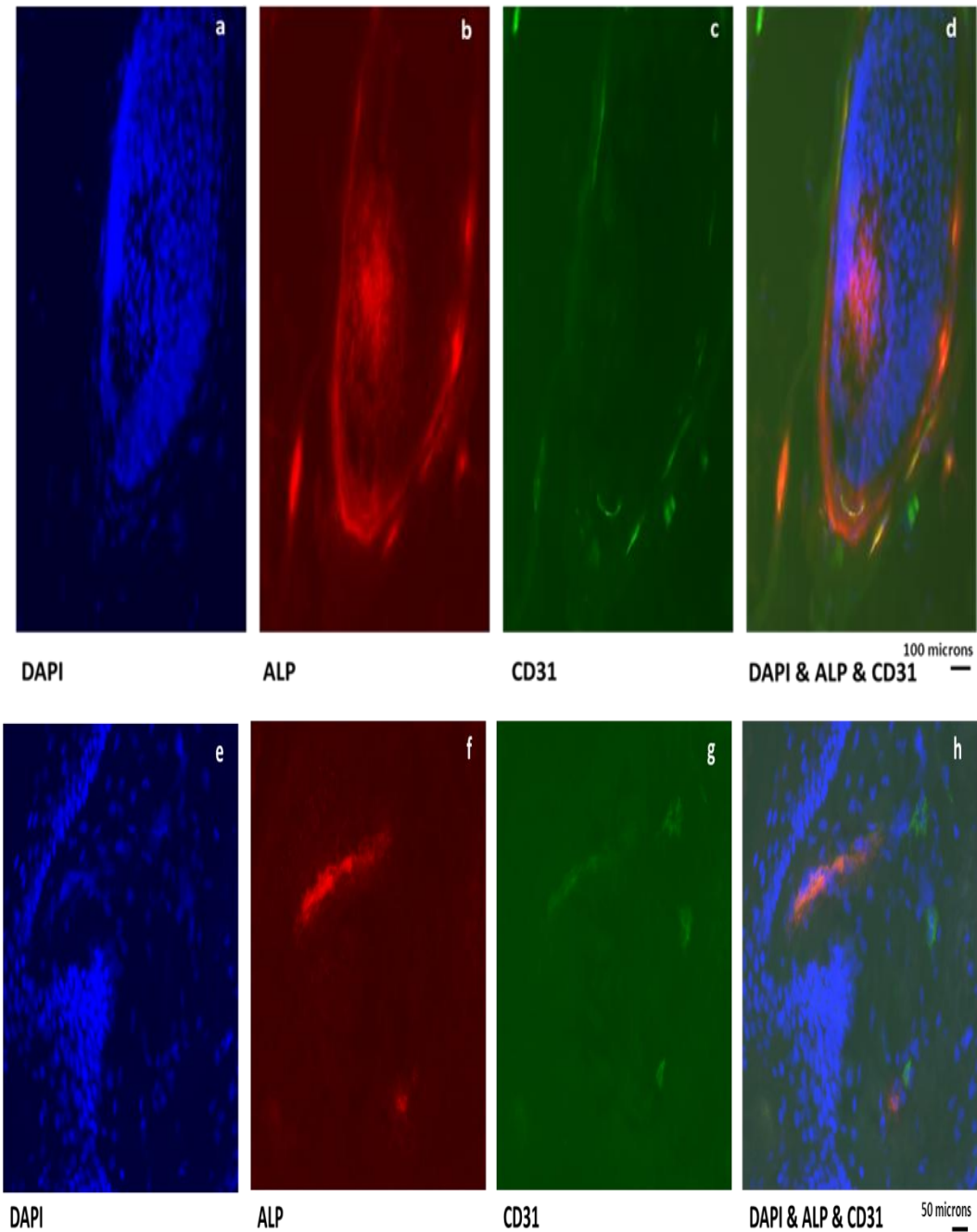
Figure 4.26 Immunofluorescence labelling showing the localisation of ALP and CD31 in both hair follicle and epidermis.

Top panel demonstrated the localisation of ALP and CD31 in hair follicle dermal papilla and surrounded stroma, DAPI (a), ALP (b), CD31 (c), and merge (d). Bottom panel showed the localisation of ALP and CD31 in epidermis, DAPI (e), ALP (f), CD31 (g), and merge (h). top images photographed with x10 objective, scale bar 100 microns, bottom panel captured with x20 objective, scale interval 50 microns.

Co-labelling normal skin structures with DAPI indirectly defined the epithelial structures (epidermis and hair follicle), however epithelial cells need to be labelled to define BCC tumour nodules. Thus, we triple-labelled hair follicle and BCC samples with CD31 and K17 antibodies , together with ALP staining (Figure 4.27). As expected, ALP defined the dermal papilla in the hair follicle bulb, as well as surrounding endothelial cells that were also CD31 positive.

In BCC sections, all ALP positive cells were within stroma, and ALP staining was always co-incident with CD31 positive endothelial cells. Although not all CD31 positive endothelial cells were ALP positive.

Thus, we surmised that dermal papilla was ALP positive, but so were blood vessel endothelial cells. In order to determine whether BCC stromal ALP positive cells could be dermal papilla like, we next triple labelled BCC samples with ALP, K17 (to determine tumour nodules) and CD31 (to detect blood vessels). We observed that in BCC samples, all ALP positive cells were CD31 positive (Figure 4.27). These findings suggest that ALP positive dermal papilla cells were absent in BCC stroma and so were not responsible for the companion layer and IRS differentiation patterns observed.

Figure 4.27
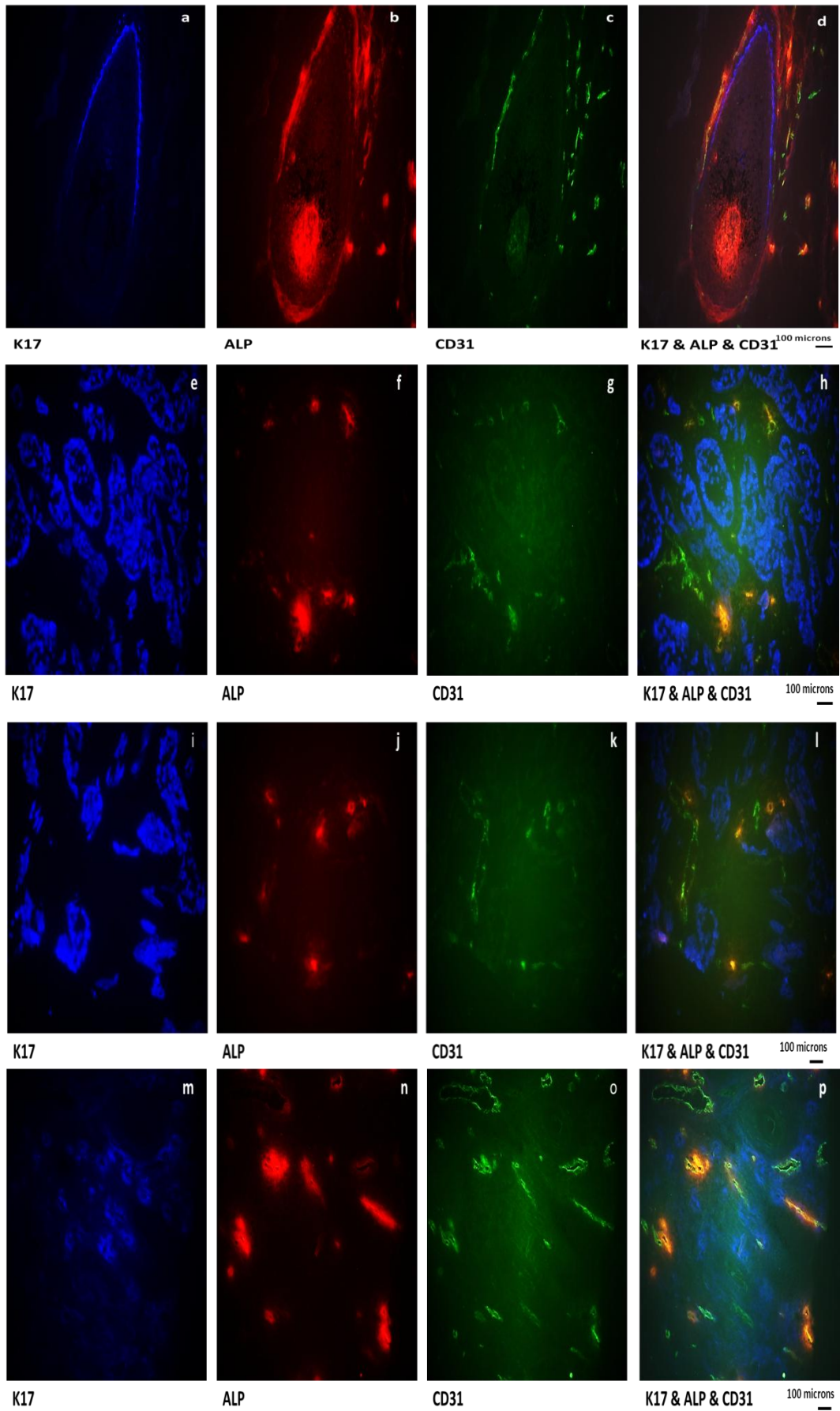
Figure 4.27: Triple immunofluorescence staining analysis the co-localisation of ALP and CD31 in both hair follicle and BCCS.
Left columns illustrated expression of K17 in blue, left columns showed the expression of ALP in red, right middle columns showed CD31 stained the blood vessels in green, and right columns showed the merge. All images were captured with x10 lens, scale bar 100 microns.

4.6   Summary

In summary, we have substantiated our RT-PCR data using antibody labelling of tissues to demonstrate both the presence and patterns of normal hair follicle differentiation within BCC keratinocytes. In BCC samples, we demonstrated ORS, companion layer and IRS differentiation. In line with keratin expression, BCC keratinocytes also demonstrate phenotypes attributed to certain keratin expression, with the exception of ubiquitous expression of keratin K17 resulting from dysregulation. The mutually exclusive relationship between differentiation and proliferation was most marked with K16 and K19 in both the hair follicle and BCC, expression of these keratins was associated with a distinct lack of proliferation.

The regulation of keratins in the human hair follicle remains to be completely elucidated, but again we were able to demonstrate overlap and provide explanation for the expression of keratins in BCC. For example K75 expression coincided with nuclear localisation of both NF-IL6 and GLI1. However, our studies failed to identify dermal papilla in the BCC stroma that may be responsible for moderating these differentiation patterns. These findings clearly demonstrate that BCC cells are heterogeneous and strongly suggest the presence of differentiation favouring the cancer stem cell hypothesis. But it remains to be determined whether differentiation patterns in BCC can be exogenously modulated and if so, how this could be translated into a potential therapeutic.

Chapter 5. Human Basal Cell Carcinoma Demonstrate Telogen arrest

5.1   Introduction

The study of the human hair cycle is difficult because of the asynchronous growth pattern of adult hair cycling, however mice have two synchronous hair growth cycles before adulthood that has led to a breakthrough in understanding of human hair follicle cycle and its regulation.

Studies undertaken at these coordinated periods of the hair cycle have shown that the telogen to anagen transition relies on communication between the stroma (macroenvironment, including dermal fibroblasts, adipocytes, vasculature and neural plexus), dermal papilla & stem cell niche (microenvironment) and the hair follicle keratinocyte stem cells. Within the context of the first and second postnatal hair cycles, neighbouring hair follicles also communicate to each other through release and receipt of various morphogens (BMP's and WNT signals). A refractory microenvironment consisting of high BMP levels may prevent a wave of WNT signalling from inducing the telogen to anagen transition (Blanpain *et al.*, 2004; Garza *et al.*, 2011; Greco *et al.*, 2009) .

During telogen, bulge keratinocytes secrete high levels of FGF18 to maintain a refractory phenotype (Blanpain *et al.*, 2004; Garza *et al.*, 2011; Greco *et al.*, 2009) . Deletion of FGF18 secreting keratinocyte from the hair follicle bulge dramatically shortened the telogen phase from 1 month to 1 week (Hsu *et al.*, 2011; Kimura-Ueki *et al.*, 2012) . FGF18 has an anti-proliferative effect of keratinocytes (Hsu *et al.*, 2011) .

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In addition to cell autonomous regulation of refractory telogen, high levels of BMP2 and 4 from the macroenvironment also induce refractory telogen (Plikus *et al.*, 2008).

In contrast, mice over expressing noggin, a natural BMP antagonist, demonstrate fast hair cycling (Plikus *et al.*, 2008). Exogenous noggin is sufficient to promote competent telogen by inhibiting the effects of BMP 2 and 4, although it remains to be determined if there is a reciprocal diminution of cell autonomous FGF18 signal.

In addition to the inhibitory pathways controlling telogen, TGF-beta2 can actively induce anagen (Oshimori and Fuchs, 2012). TGF-beta2 was observed to be released by the dermal papilla (microenvironment) during competent telogen, resulting in a transient activation of intracellular Smad 2 and 3 proteins in adjacent hair follicle keratinocytes (Oshimori and Fuchs, 2012). This counterbalances the inhibitory BMP signalling and is sufficient to induce anagen, adding yet a further layer of regulation.

Wnt signalling is also important in regulating the transition between telogen to anagen, but are not sufficient to achieve the switch alone (Plikus, 2012). During telogen the macroenvironment release Wnt antagonists Dkk1 and sfrp4 (Plikus *et al.*, 2011). The reduction in BMP levels is accompanied by a simultaneous reduction in Wnt antagonists, leading to transient Wnt activation (Plikus *et al.*, 2011; Plikus *et al.*, 2008) and transition to anagen, which was also accompanied by increased FGF7 and FGF10 (Greco *et al.*, 2009). These finding are summarised in figure 3.1.
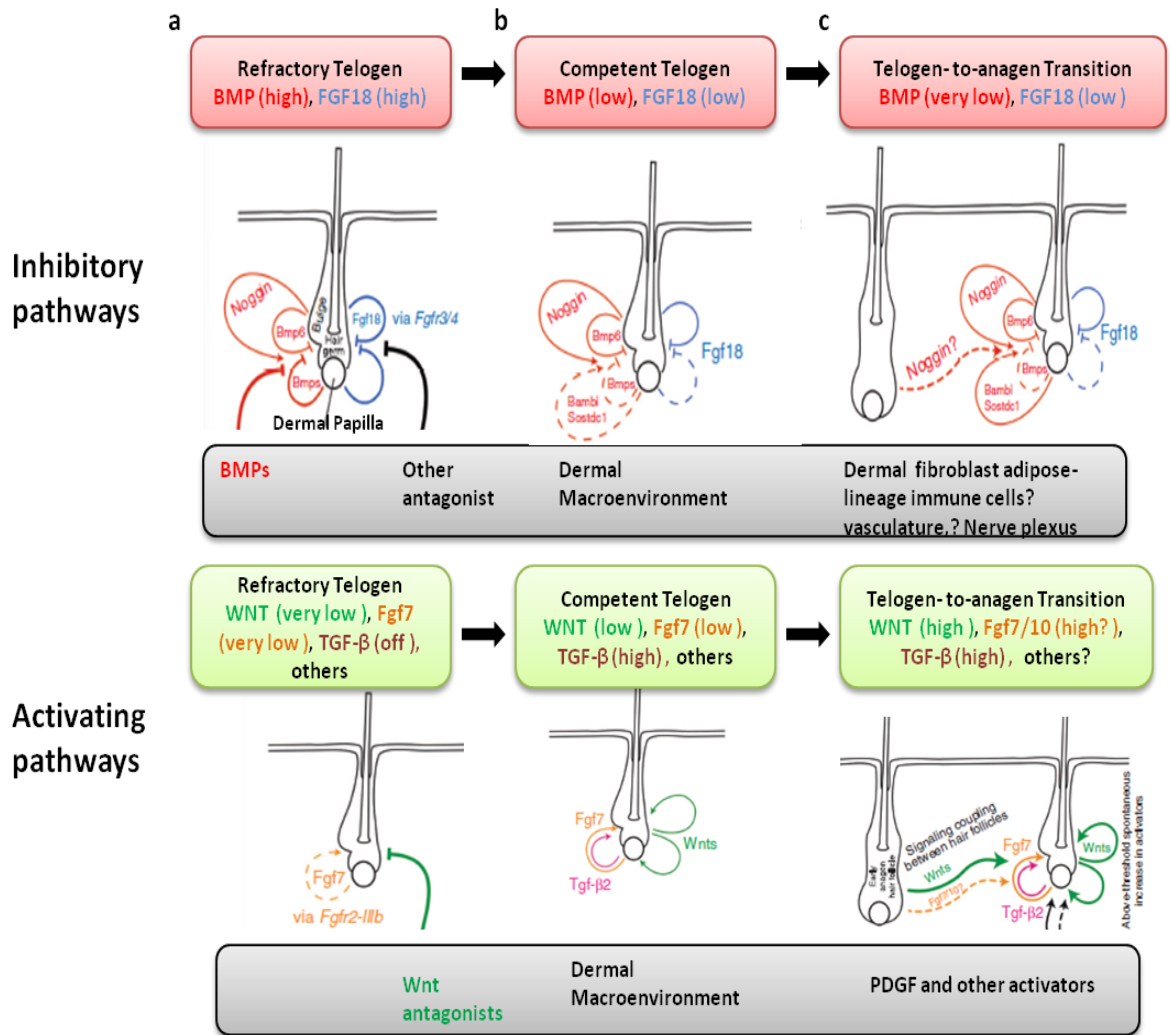
Figure 5.1: Hair follicle signalling during telogen to anagen transition.
(a) In refractory telogen, the inhibitory bmp2/4 and fibroblast growth factor 18 (FGF18) signalling are high and the activator Wnt and FGF7 signalling are very low. (b) Up on transition to competent telogen the inhibitory BMPs, FGF18 and FGF7 markers decrease and a small increase of WNT signalling. (c) During transition from telogen to anagen, there is an increase of WNT and TGF-b signalling from dermal papilla, and inhibition in the expression of BMPs and may be FGF18. Adapted from (Plikus, 2012).

Together our results in Chapter 3 and 4 suggest that human BCC potentially differentiate along the hair follicle ORS, companion layer, and IRS and medulla lineages; but do not complete the hair shaft differentiation (see Figure 4.13- Chapter 4). Thus, BCC differentiation is reminiscent of the pattern observed in hair follicle telogen.

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As described in the introduction chapter, in the hair cycle telogen is the resting phase in which all layers of the hair follicle exist with the absence of hair shaft formation. By contrast the various stages of anagen progress toward hair shaft production, while catagen is associated with apoptosis of the hair follicle. Furthermore telogen, unlike the other phases of the hair cycle, represents a relatively fixed state; which would be compatible with the complete absence of hair shaft keratins and also cell apoptosis. The slow growth of BCC tumours would certainly be compatible with a subtle perturbation in slow turnover, in contrast to the rapid growth that may be expected if the transformation occurred in an already proliferating anagen phase.

Hence the aim of this chapter is to determine whether BCC differentiation mirror telogen arrest that is refractory to competent transition into anagen, and also if this could be therapeutically overcome.

5.2   BCCs Express BMP2 and BMP4

The pattern of differentiation in BCC resembles that of hair follicles in refractory telogen. In the hair follicle refractory telogen is maintained predominantly by increased levels of BMP 2 and 4, released by the stroma as well as autonomously by hair follicle keratinocytes. BMP 2 and 4 bind and activate the BMP receptor 1a (BMPr1a) on hair follicle kertainocytes in the bulge and bulb regions of the hair follicle to suppress keratinocyte proliferation. Therefore we first sought determine if BCC have high levels of BMP 2 and 4, and if BCC keratinocytes express the reciprocal receptor BMPr1a.

20 different human BCC samples were collected and immediately frozen and stored in liquid nitrogen. RNA was extracted from cryostat sections of the frozen tissue samples and used to create cDNA for PCR analysis. Likewise cDNA was created from hair bearing scalp tissue, which was used as a positive control. RNA was also extracted from *in vitro* BCC colonies and NIH 3T3 fibroblast cultures and cDNA was made. Using primers to BMPr1a, BMP2, and BMP4 RT-PCR was performed on hair follicle samples, human BCC tissue samples, cultured BCC colonies, NIH 3T3 fibroblasts and sample with no cDNA (negative control). In this experiment, HF tissue sample was used as a positive control and H_2O as a negative control. A representative gel of the data (Figure 5.2) shows that equal amounts of cDNA were used for the experiments and quantitation was further validated using a house keeping gene (GAPDH).
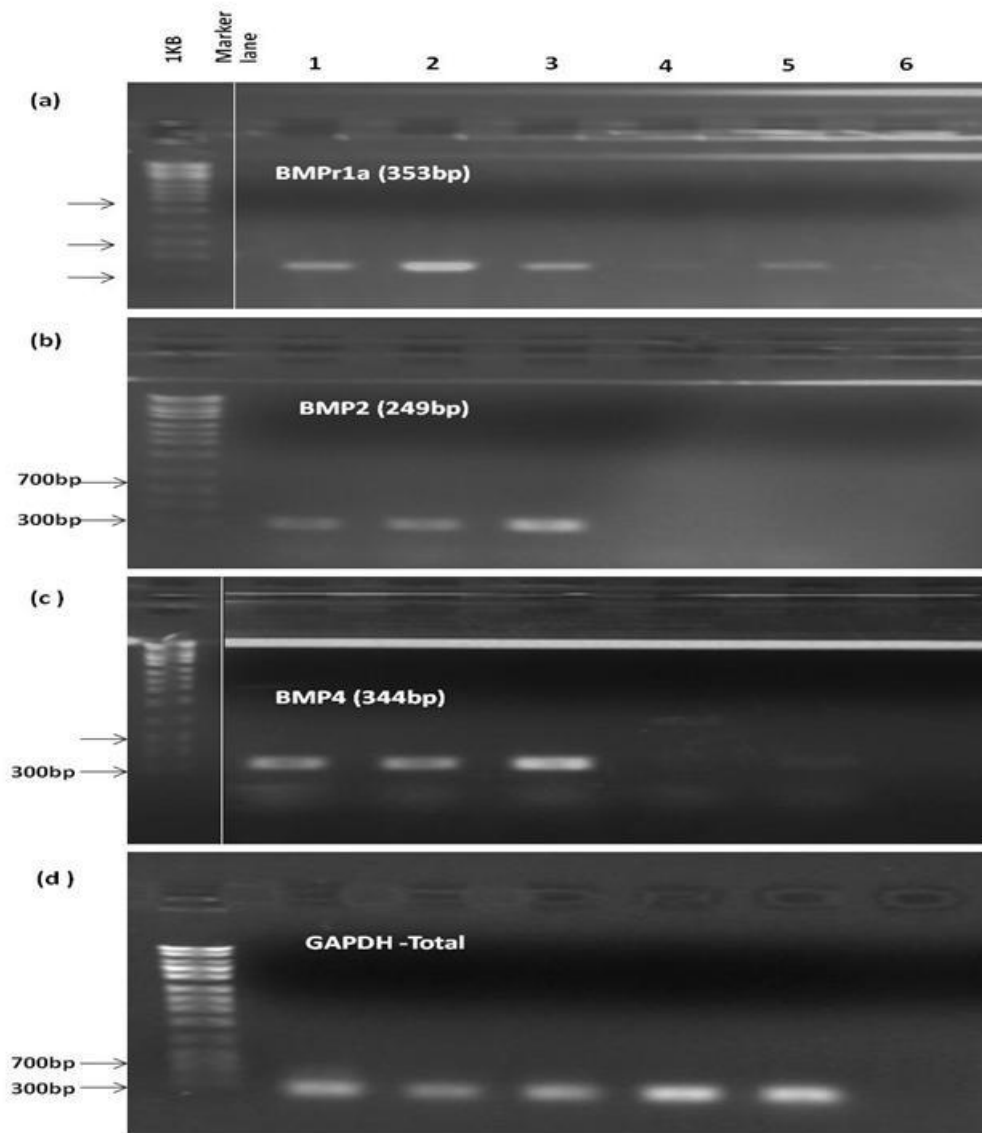
Figure 5.2: Expression of BMPr1a and BMP 2 and4 mRNA in human BCC.
PCR products amplified with bmpr1a or bmp2 or bmp4, primers were separated on 2% agarose gels, stained with ethidium bromide and viewed under UV light. (a) BMPr1a (353bp), (b) BMP2 (249bp), (c) BMP4 (344bp), and (d) GAPDH. Samples were shown for hair follicle extracts positive control **(lane 1),** primary human BCC1tissue **(lane2)** primary human BCC2 tissue **(lane 3),** BCC from cultured colonies on a NIH 3T3 feeder layer **(lanes 4),** NIH 3T3 fibroblasts **(lanes 5),**water negative control **(lane6)**. 1kbDNA ladder shown on left of each gel. The DNA ladder was run with all samples, but where the ladder was separated from the data shown, we have separated it from the data gel shown.

A BMP receptor (BMPr1a) of the correct amplicon size (325bp) was expressed in hair

follicle tissue, primary BCC sections and BCC cultural colonies (Figure 5.2 a.).

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BMPr1a was highest in primary BCCs (lane 2 and 3) and was lower but still present in BCC culture (lane 4). As PCR primers for BMPr1a also recognised the murine BMPr1a, a PCR band was also observed in the NIH 3T3 Fibroblast samples (lane 5). Whereas BMPr1a expression was absent in water (negative control) (lane 6).

BMP2 expression (with correct amplicon size, 249bp) was also observed in hair follicle tissue samples, primary BCC samples and BCC culture (Figure 5.2b). Levels of BMP2 expression were similar in primary BCC1, 2 (lanes 2 and 3), but no expression was observed in BCC cultures (lane 4). BMP2 was absent in NIH 3T3 fibroblasts (lane 5) and in the absence of cDNA (lane 6).

BMP4 expression (correct amplicon size 344bp) was also observed in hair follicle tissue samples, primary BCC samples and BCC cultures (Figure 5.2c). BMP2 expression levels appeared to be lower than BMP4 in BCC culture colonies (lane 4), where an additional non-specific PCR band (1500 bp) was seen in BCC culture colonies, which could be a contamination by genomic DNA. Similarly, a BMP4 PCR band was weakly observed with NIH 3T3 cells (lane 5) but not the water negative control (lane 6).

In summary, we observed mRNA consistent with expression of BMPs 2 and 4 in both primary BCC and to some extent in cultured BCC colonies. Since BMP4 was expressed by NIH 3T3 fibroblasts, we could not be certain that BCC cells in our culture were specifically responsible for generating the mRNA. Encouraged by these results we next sought to determine protein levels by immunofluorescence on BCC tumour tissue samples.

We next sought to confirm expression of BMP2, BMP4 and BMPr1a in BCC tissue samples at the protein level by immunofluorescence, using previously characterised monoclonal antibodies. However, as discussed earlier (section 4.5.2.1-Chapter 4), we were unable to optimise BMPr1a labelling in hair follicle tissue sections. The immunofluorescent technique was undertaken on 20 human BCC samples obtained from patients in the department of dermatology at Cardiff University. Samples were co-labelled with K17, in order to delineate BCC keratinocytes, and hair scalp tissues were used as a positive control and samples labelled without a primary antibody were used as negative controls.

In the hair follicle, BMP2 was observed within suprabasal keratinocytes of the ORS, companion layer and IRS (Figure 5.3 c, d). The expression was below the level of the bulge and throughout the bulb area. Cellular BMP2 expression was cytoplasmic and brightest around the nucleus, consistent with the expression pattern of a protein that is secreted from the cell. Likewise BMP4 was similarly expressed within suprabasal keratinocytes of the ORS, companion layer and IRS (Figure 5.3 g, h). Cellular BMP4 expression was also brightest around the nucleus.

BMP2 expression was observed in all BCCs samples. Within the tumour tissues there were areas with keratinocytes demonstrating both high levels of BMP2 expression (Figure 5.4 c,d) and absence (Figure 5.4 h). BMP2 expression was also observed to be expressed at high levels by BCC stromal cells (Figure 5.4 g,h). This focal pattern of BMP2 expression was observed throughout the tumour sample, with a reciprocal and mutually exclusive relationship between tumour cells and the stroma, so that at all sites

within the tumour either the BCC keratinocytes or the stromal cells were observed to express BMP2.

BMP4 expression was also observed in all BCCs samples (Figure 5.4 e-p). While BMP2 expression appeared to be uniform, expression of BMP4 was equitable distributed between both BCC keratinocytes (Figure 5.4 k, l) and stromal cells (Figure 5.4 o, l). Thus, BMP4 expression was also observed at high levels throughout the BCC tissue.

Figure 5.3
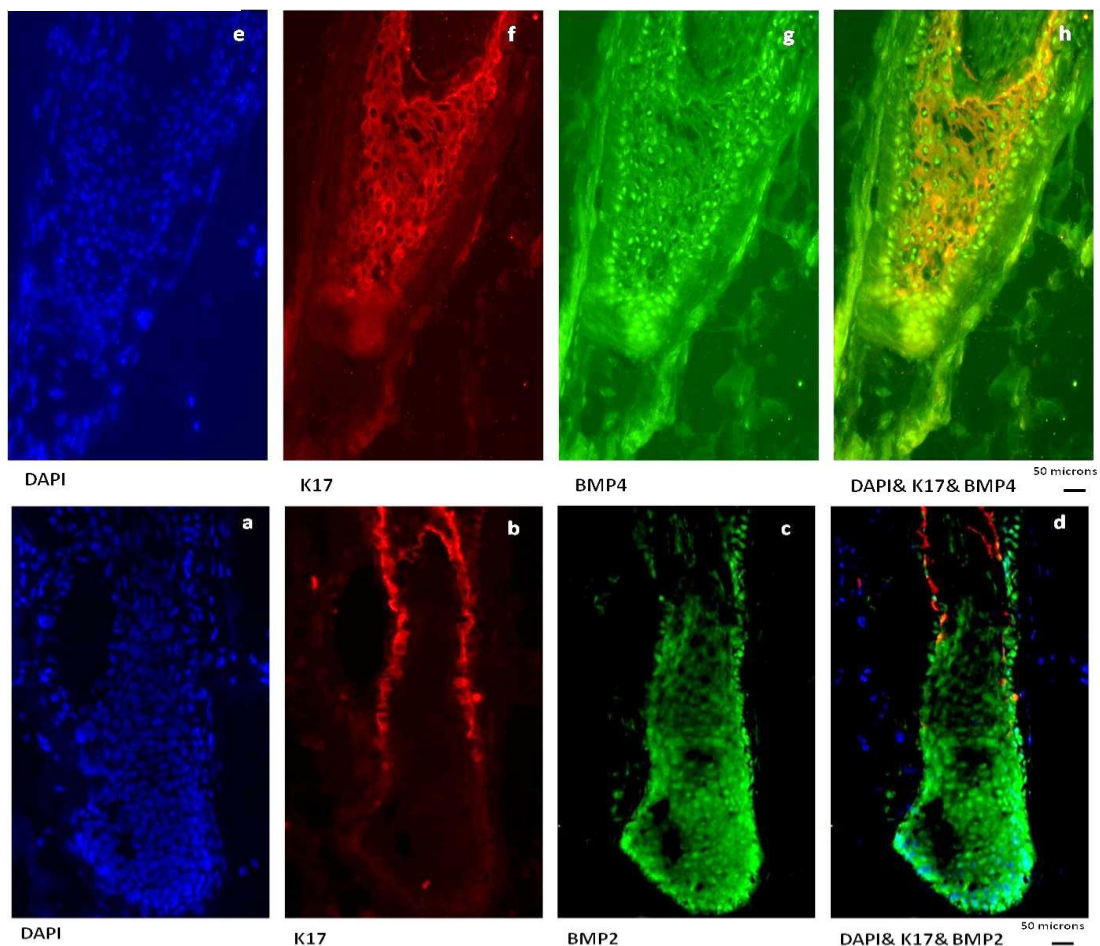
Figure 5.3:Immunofluorescence staining showing expression of BMP2,4 in hair follicle.

Hair follicle sections were labelled with BMP2 or BMP4 (green) and K17 (red) antibodies. Top panel shows expression of BMP2 in hair follicle. DAPI (a) , K17 (b) BMP(c) and merged image (d). Bottom panel demonstrates expression of BMP4 in HF: DAPI (e), K17 (f), BMP4 (g) and merged image (h). Images were captured with 20x objective (scale bar at 50 microns).

Figure 5.4
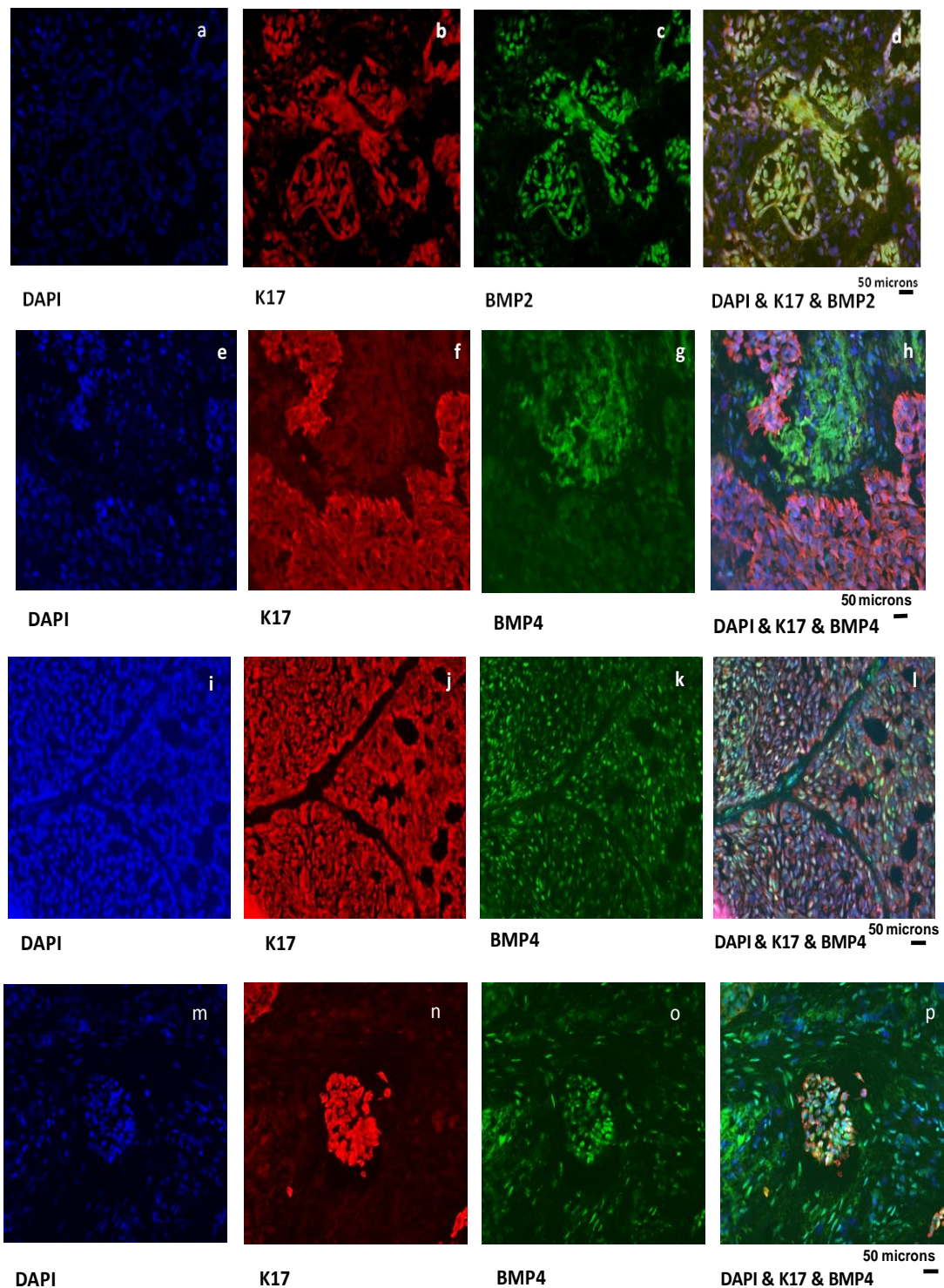
Figure5.4: Immunofluorescence staining showing expression of BMP2,4 in BCCs.
BCC sections were treated with BMP2 or BMP4 (green) and K17 antibodies (red). Top panel represents BMP 2 staining, a) DAPI , b)K17, c)BMP2, and d) merge. Other panels represent BMP4 labelling, e, i, m) DAPI, f, j, n) K17, g, k, o) BMP4, and h, i, p) merge. . All images were captured with 20x objective (scale bar 50).

In conclusion, we have observed that human BCC tissues express high levels of BMPs 2 and 4 both at the mRNA and protein level of examination. Although primary human BCC also express the BMPr1a receptor, we were not able to confirm this by immunofluorescence. BMP 2 and 4 expression was observed in both BCC keratinocytes themselves and surrounding stromal cells, which appears collectively to be relatively uniform across the entire tumour mass. Although we have not shown that there is corresponding active BMP signalling within BCC tumour keratinocytes, the findings are entirely consistent with our hypothesis that BCC may be held in a refractory telogen pattern of differentiation.

5.3 BCCs Express FGF 18 and FGF 7

Refractory telogen in the hair follicle is also maintained by autocrine FGF18 signalling, a factor that reduces keratinocyte proliferation (Hsu *et al.*, 2011). By contrast, levels of FGF7 increase upon entry into anagen signalling (Hsu *et al.*, 2011; Kimura-Ueki *et al.*, 2012). These two autocrine factors bind distinct keratinocyte cell surface receptors, FGF18 binds FGFR3 while FGF7 binds FGFR2/IIIb, and also have a distinct signal transduction pathways and gene regulation (Haque *et al.*, 2007; Niu *et al.*, 2007).

To determine whether FGF18 and FGF7 expression in BCC samples would be consistent with refractory telogen, RT-PCR was undertaken to determine expression of FGF18 and FGF7 on primary human BCC tissue samples, together with cultured BCC and NIH 3T3 Fibroblasts. In these experiments, hair follicle tissue was used as a positive control and H_2O as a negative control. Preliminary gel data are shown in figure 5.5 a, b, and c using equal amounts of cDNA validated using the house keeping gene.
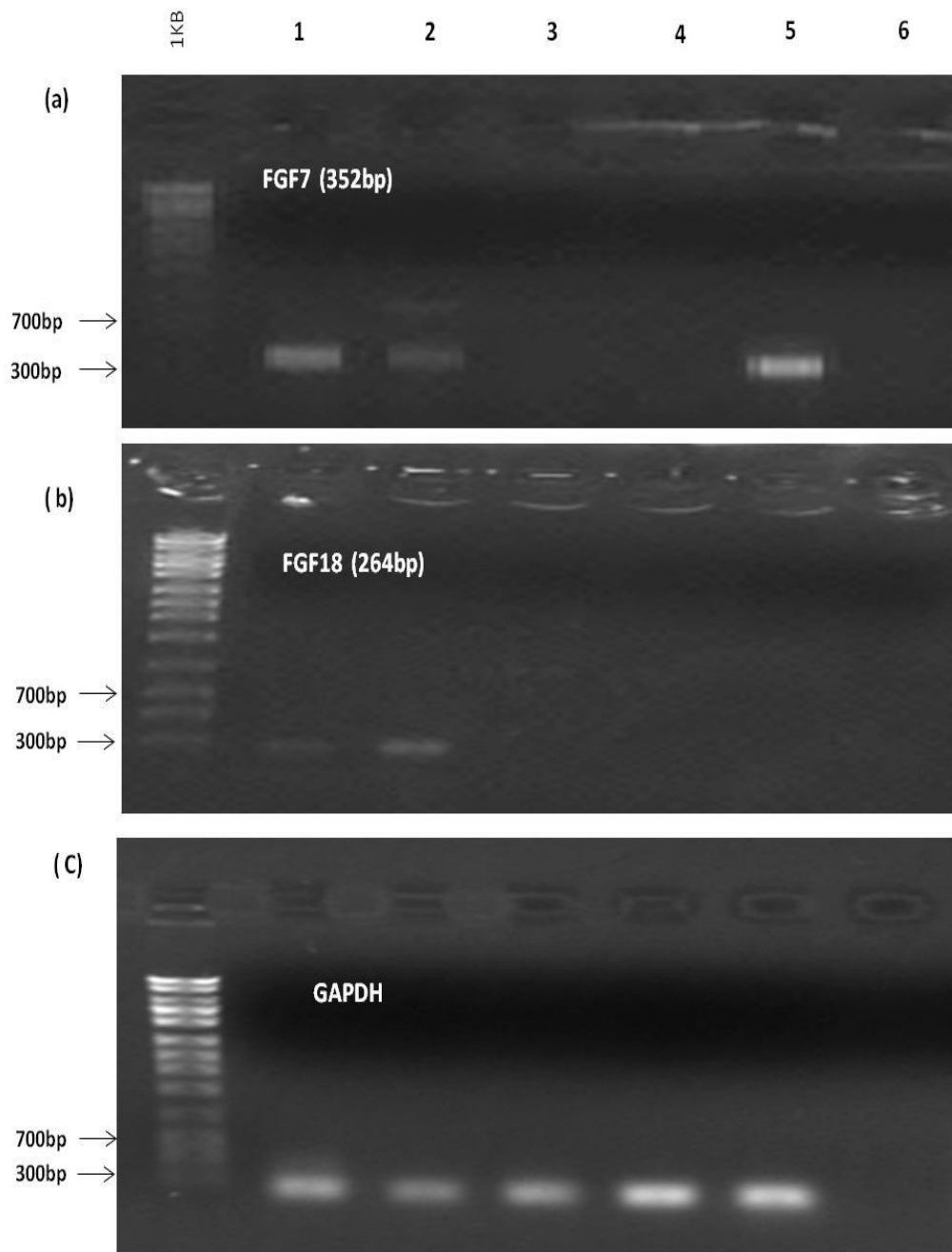
Figure 5.5: Expression of FGF7and FGF 18 mRNA in Human BCC Samples.
Amplified PCR products with FGF7or FGF18 primers were separated on 2% agarose gels, stained with ethidium bromide and viewed under UV light. (a) FGF7 product (352bp), (b) FGF18 product (264bp, and (c) GAPDH. Samples of hair follicle extracts (positive control) **(lane 1),** primary BCC1 **(lane 2),** primary BCC2, **(lane 3),** BCC cultures **(lane 4),** 3T3 control **(lane 5)** and H_2O negative control **(lane 6)**. 1kbDNA ladder shown on left of each gel.

Hair follicle bearing scalp skin demonstrated high levels of FGF7 expression (Figure 5.5 a lane 1) and lower expression of FGF18 (Figure 5.5 b lane 1), consistent with the prevalence of anagen hair follicles in the tissue, at the correct amplicon sizes (352bp for FGF7 and 264bp for FGF18). Relative to the hair follicle, BCC sample 1 (Figure 5.5 a, b, lane 2) had lower levels of FGF 7 and higher levels of FGF18 respectively. Unfortunately in the samples tested, the second BCC sample and the cultured BCC cells failed to demonstrate any expression of either FGF7 or FGF18 (Figures 5.5 a, b- lanes 3 and 4). Intriguingly, FGF7 (Figure 5.5 a, lane 5) but not FGF18 (Figure 5.5b, lane 5) was also expressed by NIH 3T3 fibroblast cells.

The preliminary data exhibits some internal inconsistencies that need further explanation. These are: (1) why BCC sample 2 did not demonstrate expression of either FGF7 or FGF18, suggesting variability between samples? (2) There was an absence of either FGF7 and FGF18 in the cultured BCC and yet we observed FGF7 expression in the NIH 3T3 samples that should be representative of the NIH 3T3 fibroblasts that are used in the BCC co-cultured samples. In addition, it will be important to determine FGF7 and FGF18 expression at the protein level (ideally using immunofluorescence or ELISA) to show which cells in the BCC tumour tissue express these factors.

5.4 *In* vitro BCC Colonies also Demonstrate Refractory Telogen

High levels of BMPs 2 and 4, as well as the expression of FGF18, in BCC tissues indicate that differentiation in BCCs may have stalled at a stage similar to refractory telogen in the hair follicle. To substantiate these findings and test our hypothesis, we

sought to overcome the factors that induce refractory telogen, using BCC colonies in culture, and to assess changes in differentiation.

To determine the presence of BCC cancer stem cells (Colmont *et al.*, 2013) and study BCC differentiation, we developed an *in vitro* assay to culture human BCC and assess colony formation efficiency and evaluate changes in gene expression (Colmont *et al.*, 2013). Primary human BCC suspensions formed colonies when grown in an adapted keratinocyte colony forming efficiency assay that incorporated a NIH3T3 feeder layer to support growth, and that had previously been used to detect and evaluate normal human keratinocyte stem cells (Terunuma *et al.*, 2007). During culture, primary human BCC cells attached selectively to NIH 3T3 cells in the feeder layers and not the tissue culture plate surface. After 14 days in culture, compact spheroidal colonies formed with > 100 cells tethered to NIH 3T3 feeder cells (Figure 5.7).

In contrast, normal human keratinocytes attached to the tissue culture plate and formed adherent monolayer colonies (Figure 5.6). Increased numbers of tethered sphere colonies formed when increased numbers of BCC tumour cells were cultured, demonstrating a dose response relationship. The cultured tethered spheres could also be serially passaged with approximately 50 tethered sphere colonies formed from an initial inoculum of 10^5 human BCC cells. Furthermore, the serial passage studies demonstrated that only 1-2 colony-forming cells were present on average for each tethered sphere. Importantly, these colonies contained tumour derived keratinocytes (Colmont *et al.*, 2013) and to confirm that these tethered sphere colonies contained cancer stem cells, we also demonstrated that xenografts of tethered sphere colonies recreated human BCC

tumours when implanted in immune-compromised mice (these experiments were undertaken by Dr G K Patel (Colmont *et al.*, 2013).

Similar to human BCC containing tissues, we observed that BCC colonies in culture also express differentiation specific keratins, representative of the hair follicle ORS, companion layer, IRS and medulla. Again, similar to BCC tissues, *in vitro* BCC colonies failed to express hair shaft keratins. Thus, the BCC *in vitro* assay could be used to test if competent telogen could be induced by overcoming factors that promote a refractory telogen pattern of differentiation.
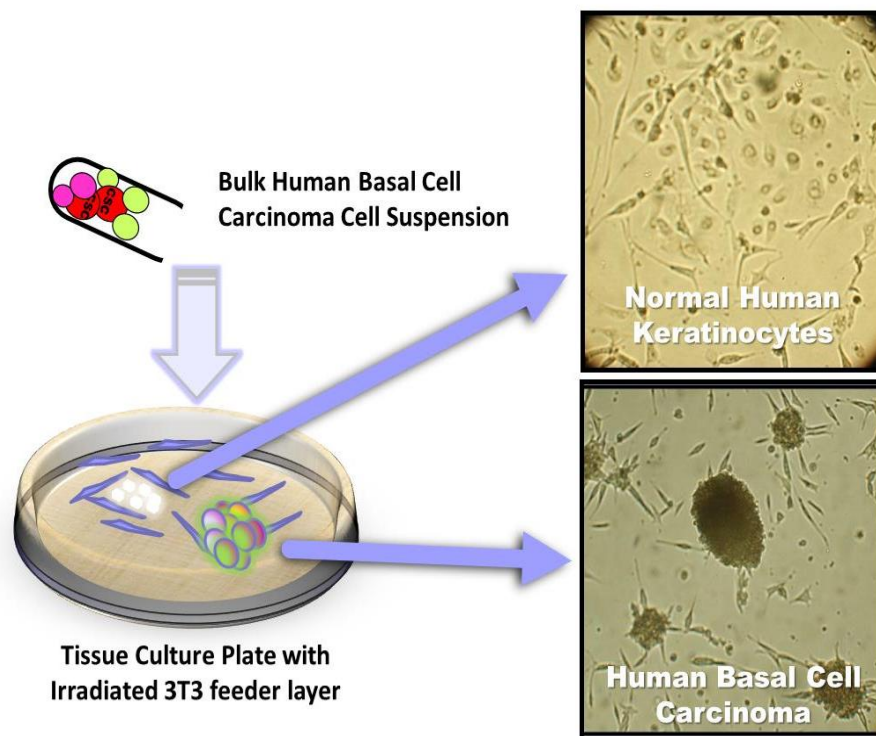
Figure 5.6: *In vitro* human BCC form tethered spheroidal colonies.
When normal human keratinocytes are added to a tissue culture plate with an irradtiated 3T3feeder layer they form adherent cobblestone colonies. In contrast when human BCC single cell suspensions are similarly placed in tissue culture they form spherical colonies that are tethered to the underlying 3T3 fibroblast.

5.5 Can Noggin Induce BCC Differentiation?

To substantiate these findings and test our hypothesis we first sought to block BMPs in BCC colonies in culture and assess changes in differentiation. Noggin is a naturally secreted polypeptide, encoded by the NOG gene, which binds and inactivates members of the (TGF-beta) superfamily. By diffusing through extracellular matrices more efficiently than members of the TGF-beta superfamily, noggin preferentially prevents BMPs from binding their receptors. It does so by directly binding several BMPs with very high affinities, at picomolar concentration, with a marked preference for BMP2 and BMP4 (Zimmerman *et al.*, 1996).

Noggin has a principal role in creating morphogentic gradients that plays an important role for induction of hair follicle growth by neutralising BMP inhibitory signalling *in vivo* (Botchkarev *et al.*, 2001; Botchkarev *et al.*, 1999). Over expression of noggin can overcome refractory telogen induced by BMPs 2 and 4 dramatically shortening the period between successive postnatal hair cycles (Plikus *et al.*, 2008). Recombinant human noggin is a bioactive protein that can be used in cell culture (Mieno *et al.*, 2004), therefore we sought to determine the effect of noggin on human BCC in culture.

Initially we determined whether native noggin was expressed by BCC colonies, using RT-PCR. Using noggin specific primers, RT-PCR was performed in parallel on hair follicle, human BCC tissue samples, cultured BCC colonies, NIH 3T3 fibroblasts and in the absence of cDNA. Representative gel of the data is shown in figure 5.8. Equal amounts of cDNA was used and validated with a house keeping gene (GAPDH).
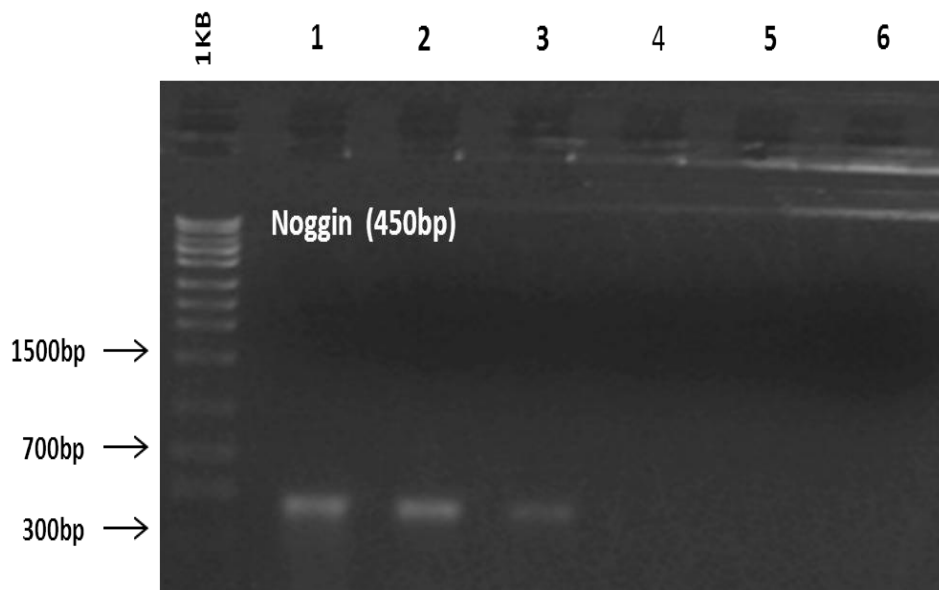
Figure 5.7: Expression of BMP antagonist (noggin) in human BCC.
Amplified PCR products with noggin primers (450bp) separated on 2% agarose gels stained with ethidium bromide and viewed under UV light. Samples of hair follicle extracts as positive control (**lane 1**), primary BCC1 (**lane2**), primary BCC2 (**lane 3**), BCC cultures lane **4**), 3T3 (**lane 5**) and H$_2$O as negative control (**lane 6**). A 1kbDNA ladder shown on left.

As shown in figure 5.7, hair follicle containing tissue was used as a positive control (lane 1), and the absence of cDNA was used as a negative control (lane 6). Noggin was expressed in all hair follicle bearing tissue samples (lane 1) and primary human BCCs (lanes 2 and 3). But native expression of noggin was absent in BCC colonies (lane 4) and in NIH 3T3 fibroblast samples (lane 5). The absence of native noggin in BCC cultures meant that recombinant noggin could be added to overcome BMP signalling and so test our hypothesis.

Three different human BCC samples were dissociated and primary cell co-cultures established over 2 weeks. Each primary BCC culture was treated with trypsin and equal numbers of cells were added to 6-well plates onto freshly irradiated NIH 3T3 murine fibroblasts (feeder layer). Once the BCC colonies had established themselves, groups of three wells were treated as follows: fresh media, fresh media with 250ng/ml noggin and fresh media with 500ng/ml noggin. Each well was photographed under an inverted light microscope with a 2x objective lens and a digital camera system (Zeiss Axiocam). Photographs were converted into binary images and both colony number and size were determined using Image J software (NIH, USA). After 48 hours, total RNA was extracted from each well and reverse transcribed into cDNA.

Based on previous studies, noggin directly binds BMPs 2 and 4 thus preventing BMP signalling. Treatment with noggin did not influence colony number or size (data not photographed). To test if exogenous noggin could also alter the expression of factors associated with refractory telogen we undertook RT-PCR of BMP 2, BMP4 and FGF7 (Figure 5.8).
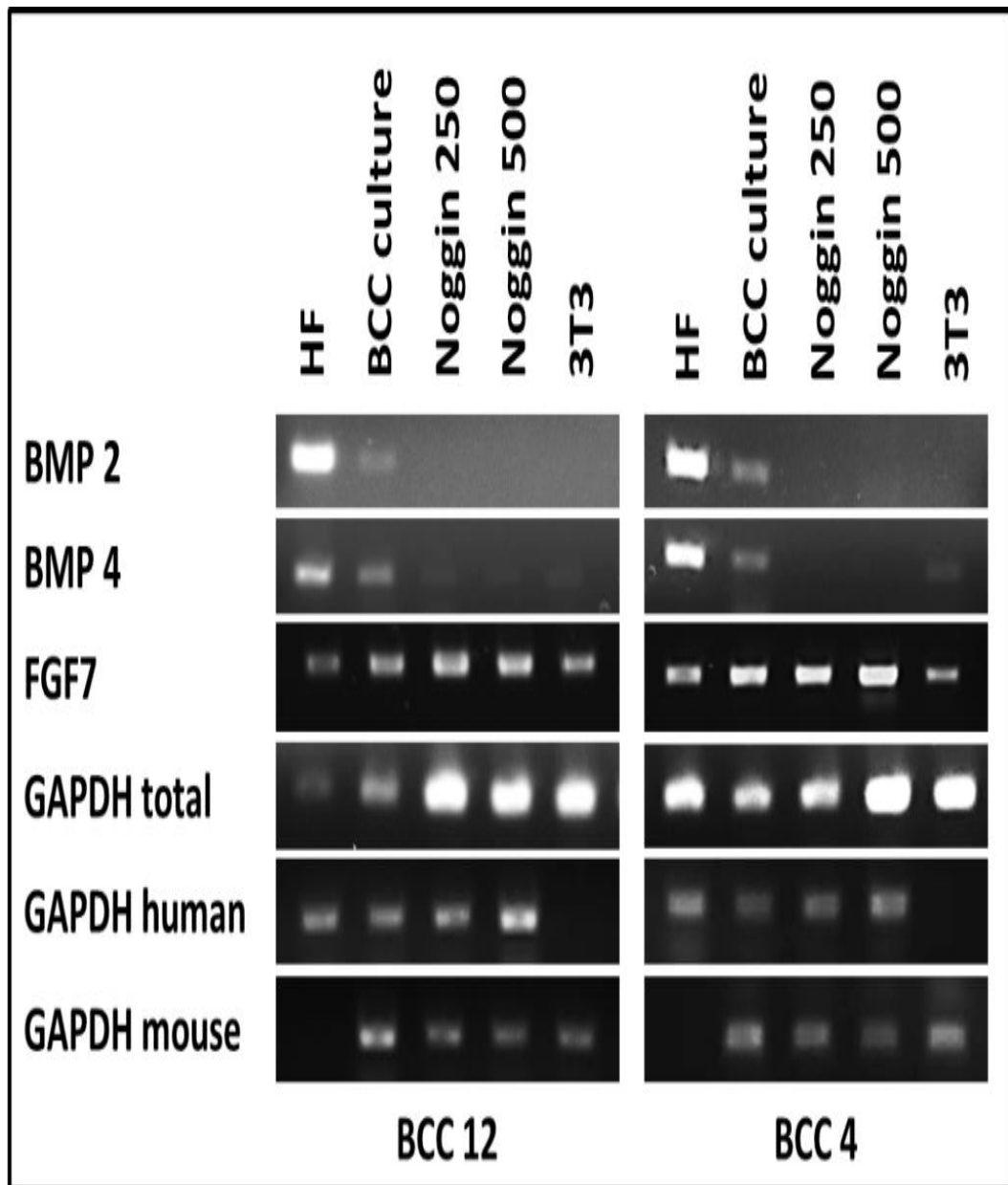
Figure 5.8: Expression of BMP2, BMP4 and FGF7 in BCC colonies exposed to noggin.

PCR products with primers on GAPDH were separated on 2% agarose gels, stained with ethidium bromide and observed under UV light. BMP2 (249 bp), BMP4 (344bp), FGF7 (352bp) on BCC12 (left gel) and BCC4 (right gel). Lane 1) hair follicle positive control (HF), Lane 2) BCC culture without noggin (+ve control), lane 3) BCC culture plus 250 noggin, lane 4) , BCC culture plus 500 noggin, lane 5) NIH3T3 The first lane contains a 1kb DNA ladder (not shown).

As expected, BMP2 and BMP4 was expressed in hair follicle tissues and untreated BCC culture colonies (BCC culture), but was absent in NIH 3T3 fibroblasts (3T3) (Figure5.8). Rather surprisingly BMP2 and BMP4 expression was down-regulated in BCC colonies treated with noggin when compared with non-stimulated BCC colonies at both doses of noggin (250 ng/ul and 500 ng/ul). In contrast noggin treatment did not influence FGF7 expression. Although these findings are preliminary, they would suggest that BMP expression in BCC cells is self-regulated by BMP signalling. Although this is one potential explanation for the reduction in BMP expression after noggin treatment, additional experiments would be needed to confirm this finding.

Since noggin treatment of BCC colonies in culture would inhibit BMPs 2 and 4, as well as seemingly diminish their expression, we next determined the effect on keratin expression. As described before, hair follicle IRS and cuticle layers emanate and migrate upward from matrix cells in hair follicle bulbs and are defined hair follicle differentiation patterns. Since our previous data determined the expression of some of the hair follicle IRS and cuticle specific keratins was not evident in BCC cultured colonies, we therefore determined whether noggin administration could induce these keratins and so support of our hypothesis.

Figure 5.9: Expression of hair follicle IRS and cuticle specific keratins in BCC colonies given noggin.

Amplified PCR products with noggin primers were separated on a 2% agarose gel stained with ethidium bromide and viewed under UV light. K25 (493 bp), K27 (451bp), K35(448bp), K85 (469bp), total GPADH (221bp), human GAPDH (268bp) and mouse GAPDH (272bp). BCC12 culture samples (left gels) and BCC4 culture samples (right gels): **lane 1)** hair follicle positive control(HF), **Lane 2)** BCC culture without noggin (+ve control), **lane 3)** BCC culture plus 250 ng/ml noggin, **lane 4)** , BCC culture plus 500 ng/ml noggin, **lane 5)** NIH 3T3 fibroblasts .

Both BCC12 and BCC4 cultured colonies when incubated with noggin for 48 hours were able to induce expression of hair follicle IRS specific keratins K25 and K27 compared to non-stimulated BCC colonies (Figure 5.9). K35 and K85, hair shaft cuticle sepsfic keratins, were also investigated but did not demonstrate obvious induction.

In summary, our preliminary results suggest that noggin treatment of BCC colonies could alter keratin gene expression and so promote differentiation in cultured BCC colonies, but in a restricted manner. The table below will summarise the impact of noggin on BCC differentiation.

GENES	BCC 12					BCC4				
	HF	BCC cul.	BCC+ 250 noggin	BCC+500 noggin	3T3	HF	BCC cul.	BCC+250 noggin	BCC+500 noggin	3T3
BMP2	+VE	+VE	-VE	-VE	-VE	+VE	+VE	-VE	-VE	-VE
BMP4	+VE	+VE	-VE	-VE	± VE	+VE	+VE	-VE	-VE	±VE
FGF7	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE
K25	+VE	-VE	+VE	-VE	-VE	+VE	-VE	+VE	-VE	-VE
K27	+VE	+VE	+VE	-VE	-VE	+VE	-VE	-VE	+VE	-VE
K35	+VE	+VE	-VE	+VE	-VE	+VE	+VE	-VE	-VE	-VE
K85	+VE	+VE	-VE	-VE	-VE	+VE	+VE	+VE	+VE	-VE
GAPDH total	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE
GAPDH Human	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE	+VE	-VE
GAPDH mouse	-VE	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE	+VE

Table 5.1: The effect of noggin on BMP2,4 , FGF7, keratins in human BCC culture colonies.

5.6 Can TGFbeta2 Induce BCC Differentiation?

BMPs and TGF-beta both belong to a TGF-beta superfamily. TGF-beta functions in tissue morphogenesis, homeostasis, and cancer by regulating diverse biological processes including proliferation, apoptosis, differentiation, and extracellular matrix production (Li *et al.*, 2006; Siegel and Massagué, 2003). Skin epithelial cells express distinct kinase receptors for both BMPs and TGF-beta, so that differentially they propagate their respective signals by phosphorylating distinct Smad proteins resulting in differential gene expression (ten Dijke and Arthur, 2007).

Although BMPs have an inhibitory effect on hair follicle differentiation, gene knockout studies of TGF-beta demonstrates differing functions: TGF-beta1 enhances hair development, TGF-beta2 delays hair development and TGF-beta 3 had no discernible effect (Foitzik *et al.*, 1999). Consistent with these findings, it has been recently shown that TGF-beta 2 is sufficient to induce competent telogen in murine studies (Oshimori and Fuchs, 2012). Therefore, we sought to determine if TGF-beta 2 was sufficient to reverse the refractory telogen pattern of differentiation when administered to our BCC cultured colonies.
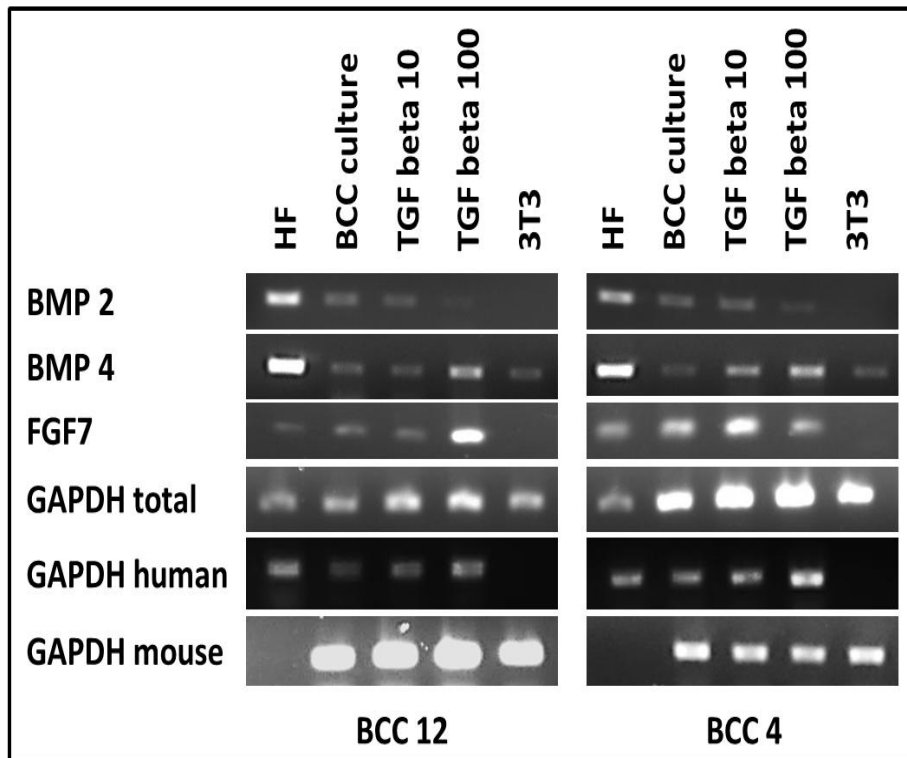
Figure 5.10: Expression of hair follicle BMP2, BMP4 and FGF7 in BCC colonies treated with TGF-beta2.
Amplified PCR products were separated on a 2% agarose gel, stained with ethidium bromide and observed under UV light. BMP2 (249 bp), BMP4 (344bp), FGF7 (352bp) total GPADH (221bp), human GAPDH (268bp) and mouse GAPDH (272bp). BCC12 culture samples (left gel) and BCC4 culture samples (right gel). **Lane 1)** hair follicle positive control(HF), **Lane 2)** BCC culture without TGF-b (+ve control), **lane 3)** BCC culture plus10 μM, **lane 4)** , BCC culture plus 100μM TGF-b, **lane 5)** NIH3T3 .

Once more we began by evaluating whether addition of the ligand would alter the regulation of BMP2, BMP4 and FGF7 in our culture assay, using RT-PCR. The addition of TGF-beta2 appeared to affect BMPs 2 and 4, by relatively decreasing BMP 2 and increasing BMP 4; FGF7 levels were unaffected (Figure 5.10). Although these observations are somewhat speculative, further experiments would be useful (e.g. repetition, quantitative RT-PCR and ELISA).

Next, we examined the ability of TGF-beta2 to induce expression of hair follicle IRS (K25, K27) and cuticle (K35, K85) keratins (Figure 5.11).
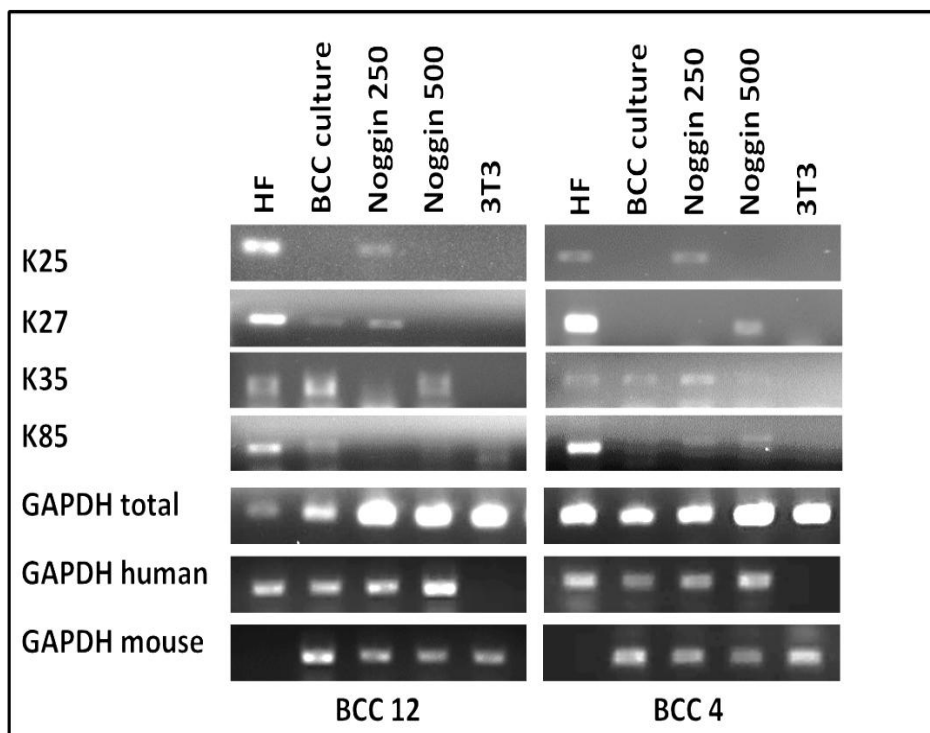
).
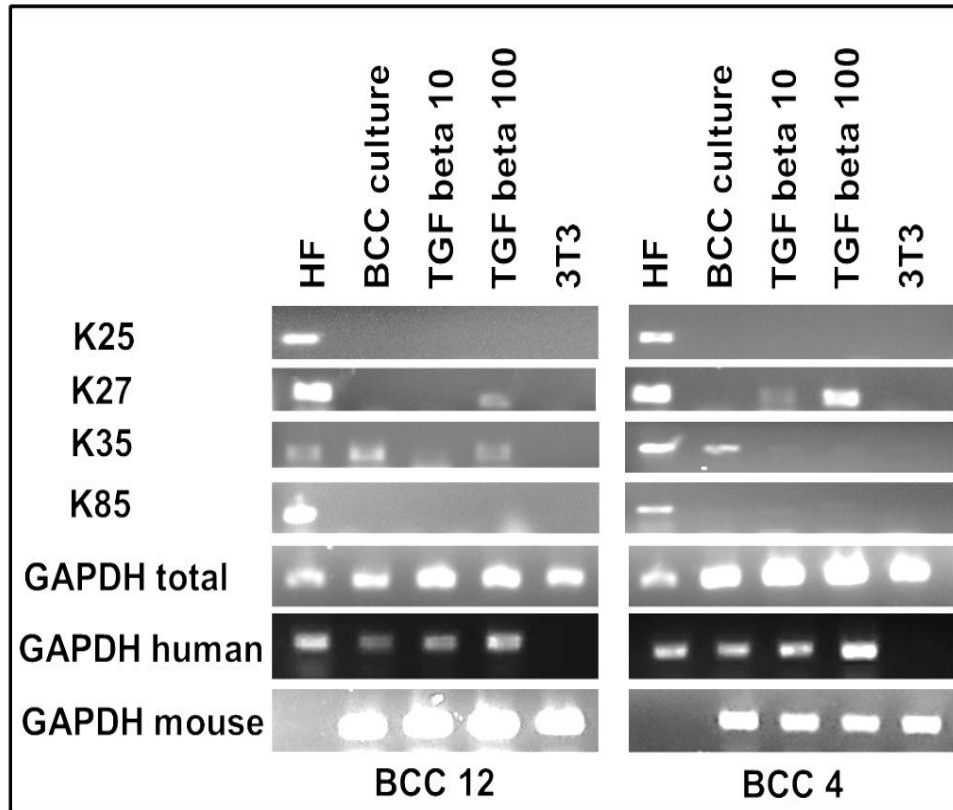
Figure 5.11: Expression of hair follicle IRS and cuticle specific keratins in BCC colonies treated with noggin.
Amplified PCR products with primers for GAPDH were seprated on a 2% agarose gel stained with ethidium bromide and visulaised with UV. K25 (493 bp), K27 (451bp), K35 (448bp) and K85 (469bp) plus total GPADH (221bp), human GAPDH (268bp) and mouse GAPDH (272bp). BCC12 culture samples (left gel) and BCC4 culture samples (right gel). **Lane 1)** hair follicle positive control(HF), **Lane 2)** BCC culture without TGF-b (+ve control), **lane 3)** BCC culture plus10 μM TGF-b, **lane 4)**, BCC culture plus 100μM TGF-b, **lane 5)** fibroblast NIH3T3cells.

Unlike noggin, TGF-beta2 induced some, but not all, hair follicle IRS and cuticle keratins. For both hair follicle IRS keratins examined (K25, K27), K27 was not expressed BCC culture free of TGF-beta2. But the expression appeared to be up

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regulated when BCC cells were treated with TGF-beta2 (Figure 5.11). In BCC12 samples, K27 expression was only seen with the higher dose (100 μM) of TGF-beta2, whereas in BCC4 samples K27 expression was induced in both TGF-beta2 treated BCCs (at 10 and 100 μM) when compared with non-treated BCC colonies. However, the expression was higher in BCC cells treated with 100 μM and more moderate in those treated with 10 μM (Figure 5.11).

K35 and K85, the cuticle keratins, were also expressed in BCC cultures treated with TGF-beta2. K35 and K85 expression were observed in all hair follicle tissue samples (positive control). Although K35 expression was observed in un-stimulated BCC cultures, the level of expression was absent in BCC cultures treated with low doses of TGF-beta2 but was expressed in BCC culture treated with high doses of TGF-beta2.

Likewise, K85 was also detected in both BCC culture colonies with and without TGF-beta2. However, the expression was weak to absent in BCC culture colonies free of TGF-beta2 which is consistent with our previous data on K85 expression in BCC cultures (Figure 5.11). The level of expression was only detected in one sample of BCC culture (BCC4) treated with TGF-beta2, and it was very weak. Both K35 and K85 expression was not observed in NIH3T3 tissue extracts.

In summary, unlike noggin, TGF-beta2 induced some, but not all, hair follicle IRS and cuticle keratins. K27 expression was induced by TGF-beta2, whereas K25 was not. However, expression of the cuticle associated keratins were not induced by TGF-beta2.The table below will summarised the effect of TGF-b on BCC differentiation.

GENES	BCC 12					BCC4				
	HF	BCC cul	BCC+ 10 TGF-β	BCC+100 TGF-β	3T3	HF	BCC cul	BCC+ 10 TGF-β	BCC+100 TGF-β	3T3
BMP2	+VE	+VE	+VE	±VE	-VE	+VE	+VE	+VE	+VE	-VE
BMP4	+VE	+VE	+VE	+VE	+ VE	+VE	+VE	+VE	+VE	±VE
FGF7	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE	+VE	-VE
K25	+VE	-VE	-VE	-VE	-VE	+VE	-VE	-VE	-VE	-VE
K27	+VE	-VE	-VE	+VE	-VE	+VE	-VE	+VE	+VE	-VE
K35	+VE	+VE	-VE	+VE	-VE	+VE	+VE	-VE	-VE	-VE
K85	+VE	-VE	-VE	-VE	-VE	+VE	-VE	-VE	-VE	-VE
GAPDH total	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE
GAPDH Human	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE	+VE	-VE
GAPDH mouse	-VE	+VE	+VE	+VE	+VE	-VE	+VE	+VE	+VE	+VE

Table 5.2: The effect of noggin on BMP2,4 , FGF7, keratins in human BCC culture colonies.

5.7 Summary

In this chapter, we have attempted to rationalise our earlier data detailing keratin expression within BCC tumour tissues. We had previously shown that keratin expression in BCC was associated with differentiation, similar to that observed in the hair follicle, by showing the relationship to proliferation and regulation by similar signalling pathways. We had been able to exclude the role of potential dermal papilla cells in the BCC stroma, as the driver for this phenotype. Instead, we hypothesised that BCC differentiation mirrored refractory telogen, a hair follicle cycle differentiation state induced by the macro-environment and cell autonomous signalling. Consistent with our hypothesis, we identified increased expression of BMPs 2 and 4, and FGF18 within BCC tumour tissues, as well as reduced levels of FGF7. These expression patterns were maintained in cultured BCC colonies, most notably BMPs 2 and 4. In preliminary experiments, we were able to induce differentiation, consistent with our hypothesis, by inhibiting BMP signalling (by addition of noggin) and addition of TGF-beta2. Further experiments with greater numbers of replicates would be good to substantiate this preliminary data. In addition, it would be useful to demonstrate active signalling in BCC cells by documenting SMAD phosphorylation and nuclear translocation.

Albeit preliminary, our findings provide encouragement to undertake additional experiments to support our hypothesis, including evaluating the combined effect of noggin and TGF-beta2 on differentiation.

Chapter 6. Discussion

6.1   Cancer Stem Cell Theory and Tumour Differentiation

Human BCC typically arise on hair-bearing skin, and BCC cells resemble basal cells of the hair follicle ORS, explaining the name and presumed origin of this tumour. In addition to conventional microscopy, investigators have also used immunohistochemistry with antibodies against hair follicle associated keratins to assertain the hair follicle origin of BCC (Kurzen *et al.*, 2001; Markey *et al.*, 1992). Further support has been provided by linage tracing transgenic mouse studies in which BCC appeared to arise from the hair follicle (Wang *et al.*, 2011). Although more recently, transgenic mice studies have shown that interfollicular keratinocytes can also give rise to BCC, intriguingly such tumours demonstrate hair follicle keratinocyte profiles (Youssef *et al.*, 2012). Despite controversy regarding the cell of origin, all authors agree that BCC cells show hair follicle morphology. Recently, our group has identified the presence of cancer stem cells in BCC using a human hair follicle stem cell marker called CD200 (Colmont *et al.*, 2013).

Over the last decade, cancer stem cells have been detected in several human cancers, such as leukaemia, melanoma, cutaneous squamous and basal cell carcinoma, breast, brain, ovarian and colon cancer. In these cancers a relatively small percentage of tumour cells are responsible for initiating and maintaining tumour growth, within this enriched population identified by specific cell surface protein expression that reside on these cancer stem cells. These cells demonstrate the capacity for self-renewal, and similar to normal adult tissue stem cells, they are able to resist killing by radiotherapy and chemotherapy (Colmont *et al.*, 2014; Gupta *et al.*, 2009; Sakariassen *et al.*, 2007). Rather enticingly, cancer stem cell theory proposes that cancer may be cured by the development of therapies that effectively target

cancer stem cells alone. In contrast, the remainder of tumour cells (non-cancer stem cells) go through terminal differentiation, similar to patterns observed in the normal tissue of origin, limiting their proliferative capacity.

Cancer stem cells divide to maintain their own population, through self-renewal, and give rise to a differentiated cell population (Pastrana *et al.*, 2011). To achieve relative proportions of cancer stem cells and differentiated progeny, to sustain and maintain tumour growth, cancer stem cells undergo both symmetric and asymmetric cell division (Morrison and Kimble, 2006). One strategy by which cancer stem cells can accomplish these two tasks is asymmetric cell division, whereby each cancer stem cell divides to generate one daughter with a cancer stem-cell fate (self-renewal) and one daughter that differentiates (Lechler and Fuchs, 2005). Asymmetric division can manage both tasks with a single division but this would leave cancer stem cells unable to expand in number, notably at the earliest stages of tumour growth. Cancer stem cells can also use symmetric divisions to self-renew and to generate differentiated progeny. Symmetric divisions are defined as the generation of daughter cells that are destined to acquire the same fate. It is plausible that cancer stem cells can rely completely on either symmetric divisions or on a combination of symmetric and asymmetric divisions. For example, normal skin homeostasis is maintained by both symmetric and asymmetric keratinocyte stem cell division (Kaur and Potten, 2011).

In the mature form, within an established tumour, our data for BCC and squamous cell carcinoma (SCC) suggest that cancer stem cell numbers are maintained at a relatively low but constant frequency (Colmont *et al.*, 2013; Patel *et al.*, 2012). The frequency of BCC cells expressing CD200, a hair follicle bulge stem cell marker, was $1.63 \pm 1.11\%$ (range 0.05-3.96%), within which the cancer stem cell frequency determined by limiting dilution analysis

was 1 in 822 (95% confidence interval 1 in 548 to 1,234). These findings would suggest that the frequency of BCC cancer stem cells relative to the differentiated progeny appears tightly regulated and that the vast majority of cancer cells are committed to differentiate. Thus, therapies that influence cell fate decisions by inducing cancer stem cells and give rise to mostly differentiated progeny would in time lead a relative reduction of cancer stem cells (Shahriyari and Komarova, 2013). Consequently, therapies that force the tumours to differentiate eventually would lead to exhaustion of cancer stem cell numbers potentially making the tumour non-viable (Pham *et al.*, 2011).

In this thesis, I have sought to define the differentiation pattern within BCC, to elucidate signalling pathways that may be specifically targeted to induce differentiation and so test our hypothesis. At first glance, it is hard to reconcile differentiation within BCC, since histologically BCC cells appear relatively monomorphic within tumour islands. In contrast, the hair follicle displays an intricate level of differentiation specifically choreographed to yield a hair shaft. Although investigators agree that BCC exhibit hair follicle morphology, to what extent does the tumour display hair follicle differentiation? Furthermore, can normal hair follicle differentiation programmes be used to manipulate BCC cancer stem cells?

6.2   Basal Cell Carcinoma Differentiate

Hair follicle differentiation, characterised by keratin expression, demonstrates both inward and upward differentiation. The hair follicle sheath, consisting of the ORS basal (K5, K14, K15 & K19) and suprabasal (K6, K16 & K17) layers, demonstrates inward differentiation. Upward differentiation from the matricial cells (K75) of the hair bulb comprises multiple distinct lineages giving rise to the IRS and hair shaft. Moving from ORS to central fibre, the

IRS is made up of companion (K6, K14, K16, K17 & K75), Henle (K25, K27, K28 & K71), Huxley (K25, K27, K28 & K74), and IRS cuticle (K25, K26, K27, K28, K71, K72 & K73) layers. The hair shaft consists of the hair shaft cuticle (K30, K32, K35, K40, K82 & K85), matrix (K35 & K85), cortex (K31, K33, K34, K35, K36, K37, K38, K39, K81, K83, K85 & K86), and medulla (K6, K16,K17, K25, K27, K28, K33, K34, K37, K75, K81 & K85). Highlighted in red are those keratins that are relatively specific for the layer outlined, which we have used to investigate differentiation in BCC. Keratin expression in the hair follicle is aligned to lineage specific differentiation programmes, their roles are closely tied to ensuring structural integrity of the keratinocyte layer. Added to which many of the IRS and hair shaft keratins are unique to the hair follicle and are not expressed in other tissues. However, keratin expression in BCC may or may not be a related to differentiation and additional data are required to prove this association.

We have used both RT-PCR and immunofluorescence (shown in blue below) to define the keratin expression in BCC. We have determined that BCC express keratins consistent with multiple hair follicle lineages: ORS (K5, K14, K16, K17, K19), companion (K75), Henle (K25, K27, K28, K71), Huxley (K25, K27, K28, K74), IRS cuticle (K25, K26, K27, K28, K71), hair shaft cuticle (K32, K35, K85), hair shaft matrix (K35, K85), hair shaft cortex (K35, K85), and hair shaft medulla (K35 and K85). Although K32 expression was evident by RT-PCR, expression of K32 and other hair shaft keratins (K31 and K81) were not apparent by immunofluorescence. All tumour samples studied expressed the basal keratin K14, throughout the tumour nodule, consistent with the persistence of the protein within cells that no longer express K14 mRNA (Lee *et al.*, 2012).

K19 expression was also evident in most BCC samples (18 of 20) examined, and similar to expression in the hair follicle, expression that was observed in the tumours was both within

the basal layer and inner cell mass. K16 expression, observed in 13 of 20 BCC samples, similar to the hair follicle where it was only expressed in the suprabasal layer, was restricted to the tumour inner cell mass. In contrast, K17 which is also restricted to the hair follicle ORS suprabasal layer was evident in all BCC cells in all tumour samples examined; consistent with being a sonic hedgehog signalling pathway regulated gene. These findings add too and support earlier observational studies reporting ORS morphology in BCC (Kurzen *et al.*, 2001; Markey *et al.*, 1992).

Intriguingly, our findings also show that BCC express IRS keratins. K75 was observed in 6 of 20 BCC samples studied; small clustered K75 positive keratinocytes were within occasional tumour nodules. K75 was not expressed by basal keratinocytes at the tumour periphery. In 3 out of 20 BCC K28 was expressed. Unlike K75, very small clusters of K28 expressing cells were observed residing at the tumour periphery. The presence of IRS keratins in BCC led us ask how these keratins were being regulated, since in the hair follicle, their expression is induced by the dermal papilla. Especially as hair follicle associated keratin expression in BCC has a similarly defined cellular proliferative capacity, consistent with their association with hair follicle keratinocyte differentiation.

6.3   Regulation of Keratin Expression

For keratin expression in BCC to be associated with differentiation, it was also important to show that keratins were regulated in a similar way to that in the hair follicle. Building on earlier studies of human hair follicle keratin regulation (Vaidya and Kanojia, 2007), we used nuclear translocation of transcription factors to indicate active signalling pathways involved in keratin regulation.

Despite the wealth of knowledge pertaining to hair follicle differentiation and keratin expression in the mouse, relatively little is known about human keratins. After a careful review of the literature, we began by studying the regulation of keratins 16 and 17 (K16 & K17). Both are ORS suprabasal keratins and are known to be up-regulated in proliferating and inflammatory skin diseases (Freedberg *et al.*, 2001). Yet counter to expectation they demonstrate markedly distinct patterns in BCC tissue. Expression of K16 was localised to the inner cell mass, distinctly absent in proliferating BCC keratinocytes, very much in line with its role in hair follicle differentiation. In contrast K17 was expressed throughout the BCC, consistent with it being a downstream hedgehog target gene. Moreover, K16 and K17 exhibited a mutually exclusive pattern of expression in the hair follicle, which is lost in BCC.

Hyperproliferation-inducing agents, EGF and TGF-α, induced K16 expression in normal human epidermal keratinocytes via an EGF-responsive element (Jiang *et al.*, 1993). Downstream of the EGF-responsive element, Sp1 directly interacted with the Sp1 binding site of the promoter (Magnaldo *et al.*, 1993). Sp1 together with c-Jun and c-Fos synergistically activated K16 (Wang and Chang, 2003). The coactivators p300/NFIL-6 could collaborate, by integrating the MAP kinase signal, with Sp1 and c-Jun in the activation of the K16 promoter. NFIL-6 is a member of the CCAAT/Enhancer-Binding Protein (also known as CEBP) transcription factor family and expression of NFIL-6 (CEBP-beta) is localised to the suprabasal ORS and interfolliclular epidermis consistent with its role in epidermal terminal differentiation (Bull *et al.*, 2002). As would be consistent with its role in keratinocyte differentiation nuclear localisation of NFIL-6 was congruent with K16 expression in both the hair follicle and BCC.

K17 is expressed in the suprabasal layer of the hair follicle ORS, but akin to K16, its expression is induced within the interfollicular epidermis in a broad range of conditions:

epidermal injury (Paladini *et al.*, 1996), viral infection (Proby *et al.*, 1993) psoriasis (de Jong *et al.*, 1991; Leigh *et al.*, 1995), and different types of cancers (Smedts *et al.*, 1992). The induction of K17 is specific for the inflammatory reactions associated with high levels of IFNγ, with activation of STAT1, such as psoriasis (Komine *et al.*, 1996), Interleukin-6 and leukemia inhibitory factor, which can induce phosphorylation of STAT1, can also induce K17 expression. But rather intriguingly, K17 is expressed ubiquitously throughout BCC keratinocytes (Grachtchouk *et al.*, 2000) and the K17 promoter harbours Gli binding sites that are responsive to Gli2 and potentiated by missing in metastasis (MIM) (Callahan *et al.*, 2004). Moreover, K17 promotes the growth of basaloid skin tumours in part by polarizing the immune response through fostering Th1/Th17 cytokine and chemokine expression (Depianto *et al.*, 2010). Since BCC demonstrate constitutive over-expression of hedgehog signalling, we sought to study these regulatory pathways using double-label immunofluorescence to determine whether nuclear translocation of Gli transcription factors was co-incident with expression of K17.

We hypothesised that since over expression of sonic hedgehog signalling induces BCC formation and BCC also express K17, there would be nuclear translocation of a Gli transcription factor throughout BCC tissue. Instead, we observed concomitant labelling of K75 with Gli1 nuclear translocation in the companion layer of the hair follicle. Similarly, Gli1 translocation was associated with K75 expression in BCC. The regulation of K75 by hedgehog signalling has not been previously described to our knowledge, and so this warrants further investigation. Although surprising, this finding compliments our assertion that hair follicle keratins are similarly regulated in BCC, consistent with their use to define differentiation in BCC.

6.4   The Search for Dermal Papilla Cells in BCC

We have shown that human BCCs demonstrate hair follicle differentiation, including the expression of IRS keratins. Since the immunofluorescence results presented in Chapter 4 have shown that expression of a hair follicle IRS keratin (K28) at the periphery of BCC tumours, this has led us to hypothesise that dermal papilla cells in the BCC stroma may be regulating its expression. The interdependence between the dermal papilla and hair follicle tissues made this an enticing proposition, especially as it has been established for some time that BCC growth is dependent upon stromal cells (Hernandez *et al.*, 1985).

To identify the presence of dermal cells in BCCs, immunostaining was undertaken using a variety of dermal papilla markers: versican, CD56, BMPr1a and alkaline phosphatise (ALP). However, none of the markers were specific individually but triple immunofluorescence with K17 (to detect the tumour cells), ALP (dermal papilla marker) and CD31 (blood vessels marker) could be used to identify dermal papilla cells in the hair follicle. Thus, although each of the dermal papilla markers showed positivity in BCC stroma, it was only after the BCC samples were triple labelled that we were able to exclude the presence of ALP positive dermal papilla cells in the BCC stroma. Thus, we have disproved our hypothesis that dermal papilla cells proliferated alongside BCC keratinocytes in a co-dependent manner, sufficient to induce IRS differentiation.

6.5   Refractory Telogen Arrest in BCC

Accepting that BCC demonstrates a hair follicle pattern of tissue growth, we once more asked why the pattern of differentiation was stalled; why was it that BCC do not grow hair shafts?

This led us back to the hair cycle, since hair growth occurs during anagen it is possible that maturation of BCC tissue is stalled either in catagen or telogen. Since catagen is a dynamic phase of the hair cycle that is characterised by hair follicle apoptosis, we hypothesised that BCC must be stuck in a telogen resting phase. Furthermore, if the hair cycle phase could be progressed to anagen this would in turn increase differentiation in line with our overall hypothesis.

The refractory telogen phase of the hair follicle cycle has recently been elucidated in hair follicle biology, albeit in mice and not humans. Refractory telogen is characterised as the molecular switch necessary for anagen to ensue, which is dependent upon a reduction in BMP 2 and 4 and an increase in WNT signalling from the environment. In turn, anagen in the hair follicle epithelium is characterised by increased expression of FGF7 and reduced expression of the inhibitory growth factor FGF18 (Blanpain *et al.*, 2004). In line with this, we observed that BCC expressed high levels of inhibitory signals (BMP2, BMP4 and FGF18). The expression of BMP 2 and 4 was observed in both BCC keratinocytes themselves and surrounding stromal cells, which was relatively uniform across the entire tumour mass. Thus, we hypothesised that reversing "refractory telogen" in BCC could induce further hair follicle differentiation.

6.6 Influencing Cell-Fate Decision in BCC

Refractory telogen in the mouse can be reversed by blocking receipt of BMP signals from the surrounding environment (Plikus, 2012), which can be achieved by increasing noggin levels. Noggin is a natural inhibitor of BMP's, in particular BMP 2 and 4, via stoichiometric binding of the substrate in the extracellular space. Similarly TGF-beta2, which is secreted by the

dermal papilla during anagen induction, if administered exogenously can induce anagen in refractory telogen hairs (Oshimori and Fuchs, 2012). Thus, it was possible to see if BCC "refractory telogen" could be reversed and simultaneously attempt induced differentiation.

To test this hypothesis we used an *in vitro* BCC culture assay, which facilitated BCC spheroidal growth on top of an irradiated NIH 3T3 fibroblast feeder layer. We initially observed the effect of adding noggin and TGF-beta 2 on expression of BMP 2, BMP 4, FGF7 and FGF 18. To our surprise noggin blocked BMP expression, suggesting that BMP expression in BCC may result from autocrine positive feedback signalling; this finding has important implications and warrants further investigation. For the purposes of our experiment, this did not impinge on our ability to examine BMP blockade as initially intended. Both noggin and TGF-beta 2 in our preliminary data showed evidence of keratin expression suggesting induced differentiation. With only two BCC samples examined, albeit with three replicates, we would need to examine a greater number of samples using quantitative analysis to verify these preliminary findings.

6.7   BCC Hair Follicle Growth Model

Our findings have shown that BCC demonstrate a hair follicle pattern of growth by intracellular expression of hair follicle specific keratins and appear to be regulated in a similar way to hair follicles (Figure 6.1). The following model is illustrating the hair follicle differentiated markers detected by our BCC tumour sample. The basal keratins K14 and K17 were expressed throughout the tumour nodule (Figure 6.1 - blue cells), the hair follicle suprabasal keratin K16 was exclusively expressed by the inner cell mass and the expression was consistent with the nuclear translocation of NFIL6 and KLF4 transcriptional factor

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(Figure 6.1- purple cell with light blue nucleus), the companion layer keratin K75 was expressed by a small clusters of BCC cells within the inner cell mass and its expression was dependent on gli1 transcription factor (Figure 6.1 - red cell with green nucleus). One hair follicle inner root sheath keratin (K28) was expressed by a small number of tumour cells toward the periphery (Figure 6.1 - pink cells). In addition, a human hair follicle bulge stem cell marker (CD200) was expressed by a small subpopulation of relatively undifferentiated BCC cells. Furthermore, BCC and its stroma showed expression of refractory telogen hair follicle inhibitory signals (BMP2, BMP4 and FGF18), supporting our hypothesis that BCC may be stuck in refractory telogen. However, BCC growth seems not to be dependent on hair follicle dermal papilla cells as in the hair follicle, as BCC stromal cells did not show expression of ALP positive dermal papilla cells, instead ALP stained the surrounding blood vessels.
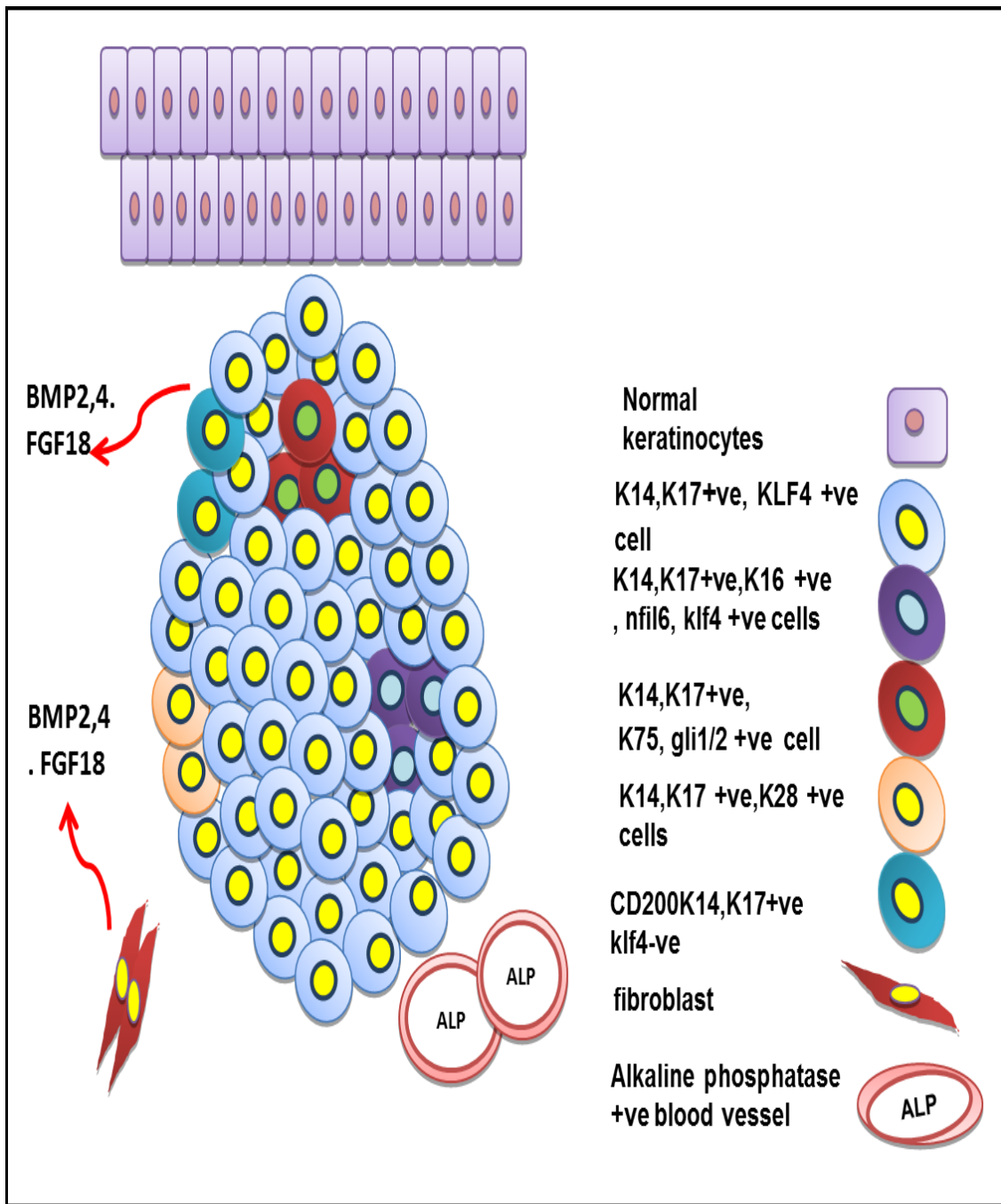
Figure 6.1: Schematic illustration of proposed BCC hair follicle growth model.
All markers expressed by BCC tumour in this project. K14, K17 +ve cells displayed in green, K16 +ve cells in purple with nuclear translocation of NFIL and klf4 displyed in light blue nucleus, K75+ve cells in dark red with nucluer translocation of gli1,2 green nucleus, K28 +ve cells at periphery displayed in pink, CD200 k14k17 +ve, klf −ve cells in dark blue, fibroblasts cells secret BMP2,4 and FGF18 , blood vessles ALP +ve cells.

6.8   Future Work

As alluded too throughout the thesis, there are a number of avenues of additional research that can be suggested as a result of this work:

1. The studies undertaken were on nodular BCC, although the most common histological subtype, additional studies would be required to extrapolate these findings to all BCC types.

2. It remains to be determined if and how K75 is regulated by hedgehog signalling.

3. We have only glimpsed at the BCC refractory telogen, additional experiments would be needed to substantiate these findings. It would be important to determine which cells produce FGF 7 and 18, as our data only relate to mRNA expression studies. Also which cells in the BCC environment receive these signals, since we have assumed this to be BCC keratinocytes and so additional studies would be important to substantiate the location of receptors.

4. The induction of "anagen" needs further BCC samples to be studied, with quantitative evidence of keratin expression. Similarly, it would be interesting to determine if noggin combined with TGF-beta 2 augmented differentiation.

5. To fully test our overall hypothesis, it would be important to undertake long-term culture or *in vivo* studies to determine if the induction of "anagen" in BCC's does lead to an overall reduction in BCC cancer stem cells.

6.9   Conclusions

 In conclusion, our work has shown that BCCs differentiate along hair follicle lineages characterised by keratin expression similar to that observed in the human hair follicle. These findings demonstrate a complex pattern of tumour biology, hitherto unseen by conventional light microscopy. Assuming that BCC growth mirrors perturbed hair follicle tissue growth,

our findings lead us to believe that BCC are stuck in "a refractory telogen state" a novel stage of the hair follicle cycle that represents the switch to anagen. In seeking to induce differentiation, we have attempted to promote "anagen" in BCC by targeting BMP and TGF-beta pathways. If substantiated, then this work will provide the basis for determining if induction of tumour specific differentiation can exhaust cancer stem cells and hence lead to novel therapeutic targets.

Appendix

7.1 Buffer Solutions

7.1.1 Preparation of Phosphate Buffered Saline (PBS) Solution

Stock 10x PBS (phosphate buffered saline at pH 7.2) for immune labelling was prepared and stored at room temperature.

10x PBS was made by dissolving 400g sodium chloride (NaCl), 179g disodium hydrogen phosphate dodecahydrate ($Na_2HPO4.12H_2O$), 12g anhydrous potassium dihydrogen phosphate (KH_2PO_4) in 4.5 litres of double distilled sterile water using a magnetic stirrer. 0.1M Hydrochoric acid or 0.5M sodium hydroxide was added to achieve a pH of 7.2 after which the volume was made up to a total of 5 litres.

A working solution (1x PBS) was prepared by diluting the stock 10x buffer with sterile water (1:10). In addition, 0.25g bovine serum albumin (BSA) was added to 25ml of 1x PBS buffer to create a diluent buffer (PBS-BSA) for primary and secondary antibodies.

7.1.2 Preparation of Tris HCl Buffer (pH 8.2)

One litre of stock of tris buffer (hydroxymethyl methylamine, pH 8.2) was prepared by adding 121.1g of tris reagent (Fisher Scientific, UK) to 1 litre of double distilled sterile water. Hydrochloric acid or sodium hydroxide were added to achieve the correct pH of 8.2. This tris buffer was used for preparation of the alkaline phosphatase solution to detect dermal papilla cells in the hair follicle and BCC samples.

7.1.3 Tris-Acetate-EDTA (TAE) Buffer

Tris-acetate-EDTA (TAE) buffer was used for agarose gel electrophoresis both as gel and running buffer. A 50x solution was made by dissolving 242g tris and 57.1g glacial acetic acid in 350ml double distilled sterile water, then adding 100ml 0.5M EDTA pH 8 and mixing before making up to a final volume of 500ml.

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