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K15 & Id3 expression in intact and regenerating adult vibrissae rodent hair follicles

By

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Abstracts

The hair follicle can renew itself through an intrinsic stem cell population and has the ability to undergo hair follicle growth cycle; its regenerative activities under normal and experimental conditions together with a feature of easy access make it an ideal model system to investigate adult stem cell activities.

The different stages of the rat vibrissae hair follicle cycle have been well documented and described; recent studies have provided an insight into the molecular control of hair follicle cycling and development. However, current understanding of stem cells activity during different stages of hair follicle cycle is limited and controversial. Keratin 15 is proposed as an epithelial stem cell marker, during follicle cycle. Keratin 15 is found predominately to cover the lower hair follicle, including the hair follicle matrix. Its overall expression varies depending on the different stages, these findings are consistent with the “traffic light model”, and i.e. epithelial stem cells migrate during follicle stages. However, we have proposed some corrections to this model. Id3 is a transcriptional regulator protein, expressed in both epithelial and dermal cells during hair follicle cycle, and the cellular translocation phenomenon especially in stage of Anagen were observed, and this may suggest the cells exchange the activities between proliferation and differentiation.

The regenerative properties of vibrissa follicle are well established but many questions were left especially in the cellular and molecular level. For example, the sources of regenerative dermal papillae together with epithelial stem cells activity during regeneration of hair follicles remain unclear. Both keratin and ID proteins have been previously reported to have roles in the skin wound healing process, but in the hair follicle regenerating process their roles remains unclear. The number of K15 positive epithelial cells significantly increased at the initial stages of regenerating follicles, this confirmed previous results, which showed the outside epithelial cells filling in at this stage. K15 was more specifically expressed in the outer root sheath cells along the glassy membrane in the later stages of regenerating follicle, suggesting that the epithelial stem cells had migrated down to interact with dermal sheath cells to reform dermal papillae. In contrast, the response of Id3 positive cells to the injured hair follicle is relatively late; epithelial cells gradually increased their expression of Id3 in mid-regenerating stage. Id3 highlighted dermal cell accumulation below amputation site in



the later regenerating stages. This result suggests that ID3 positive dermal cells accumulation may be mimicking the dermal condensation environment in the hair follicle development.

Finally, the explant end bulb of hair follicle and isolated dermal papillae differentiate into osteoclasts *in vitro* suggesting the stem cell multi-potential features. This work demonstrated that sufficient stem cell niche signalling conditions could mimic stem cell niche for cell differentiation.

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Chapter 1 Introduction

1.1 General biology of Hair follicle

The hair follicle is one of the most complex mini-organs of the animal and human body (Fig1.1). Hair follicles are exquisitely productive protein fibre factories which double as sensory organs and serve as an instrument of psychosocial communication, excretion and protection. There are two features, which contribute to making the mammalian hair follicle a unique, biological system. Hair follicles are the one of organs in the mammalian organism that undergoes life-long cycling, involving phases of rapid growth (anagen), apoptosis-driven regression (catagen), and relative quiescence (telogen) (Dry, 1926). They have a highly active neuroectodermal-mesodermal interaction system that incorporates numerous stem cells and that underpins their regeneration activity in normal and experimental conditions. Plenty of evidence has demonstrated that their exclusive ability to regenerate is due to the epithelial and mesenchymal interactions that drive waves of daughter cell populations, derived from resident epithelial progenitor cells, into defined strata of differentiation. These transformations are controlled by changes in the local signalling milieu, based on changes in expression/activity of a constantly growing number of known cytokines, growth factors, hormones, neurotransmitters and other key mediators of the hair follicle cycle.

1.1.1 Locations and environments

Mammalian hair follicles are part of the skin, and in most mammals they serve to augment the insulation the skin but they can also serve to provide secondary sexual characteristics or as camouflage.

Mammalian hair follicles are associated with different glands and tissues, and together with nerves and blood vessels located in the skin, they form a mesh that inter-reacts with the local environment (Fig1.1), and which guarantees that individual organs functional properly. There are two organs directly attached to hair follicles, sebaceous

gland and arrector pili. The sebaceous gland is a tiny sebum-producing gland found everywhere except specific locations such as palms and lips. The arrector pili contains a bundle of muscle fibre that is responsible for causing the follicle to become more perpendicular to the surface of the skin, and to protrude slightly above the surrounding skin. Stem cells are believed to be located at the junction of the arrector and the follicle, and are principally responsible for the ongoing hair production during the rapid growth stages of the hair follicle.

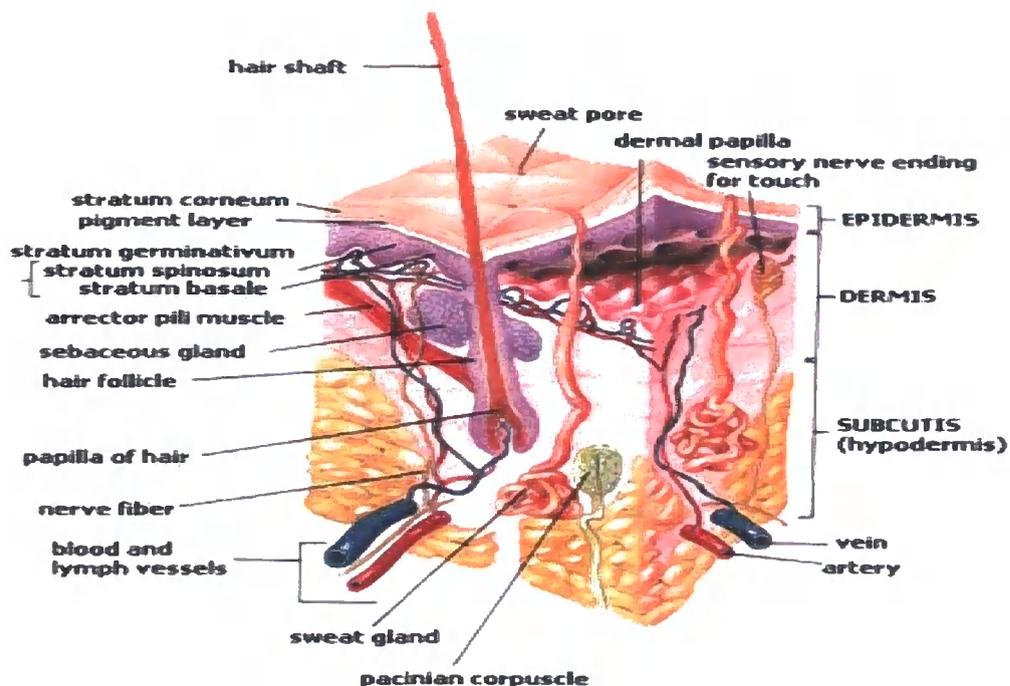


Figure 1.1 The locations of hair follicle and their environment

1.1.2 Rodent/murine vibrissae hair follicle

Vibrissae, or whiskers, are specialized hairs, usually employed for tactile sensation in most mammals, including rodents. Vibrissae hairs commonly grow around the nostrils and other parts of the face in most mammals, and this type of fibre is usually thicker and stiffer than other types. Vibrissae fibres like other hairs, consist of inert material and contain no nerves, but do have special sensory cells associated with them. Vibrissae are different from other hairs mainly because they are follicles sealed inside a collagenous capsule containing blood sinus. These play an important role in animal detection of

even extremely small deflections (Walter, 1960, Dehnhardt, 1999). Large nerve trunks penetrate the lower part of the follicle capsule and divide many times as they ascend and completely envelop the connective tissue sheath of the follicle. In the mid region of the largest follicles, more than 500 myelinated nerves have been observed, and other regions also contain the nerves around the hair follicle closer to the central region and terminated at the outer layer of the epithelial cells (Hollis, 1974).

Similar to other types of hair follicle, the vibrissae hair follicle both can renew itself through an intrinsic stem cell population and has the ability to cycle. In addition, vibrissae follicles have a regular pattern on the face, rapid growth rate large size, and long and very regular cycle times (Fig1.2), that make excellent models with which to study hair follicle stem cells, and epithelial /mesenchymal interactions.

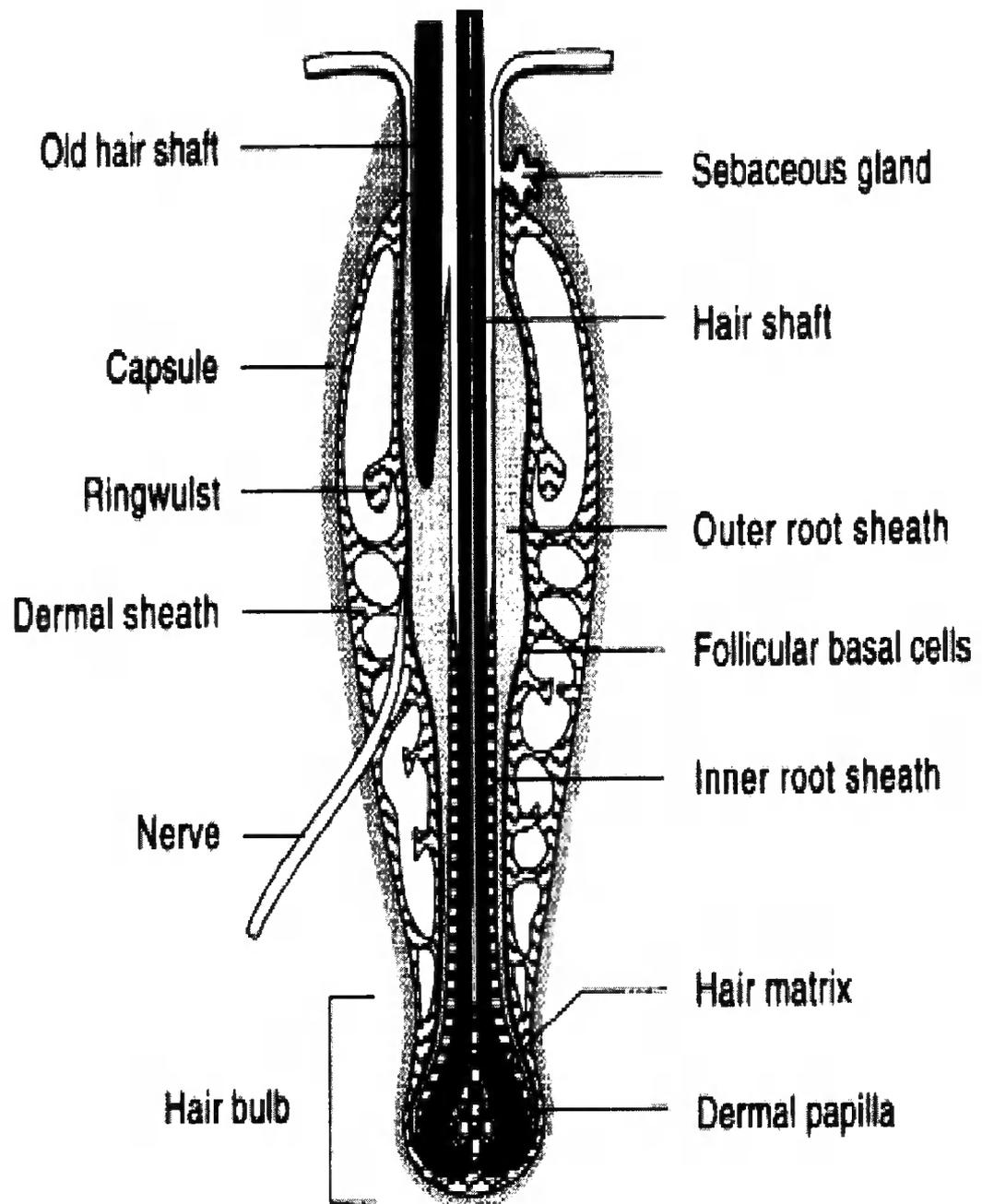


Fig.1.2 Schematic representation of an anagen vibrissa hair follicle

Key structures of the follicle-sinus complex are shown excluding blood supply, innervation and external muscle attachments.

Adapted from Matsuzaki *et al*, 1998

1.2 General development of hair follicles and the role of dermal cells roles in the development process

Hair follicle development is the result of neuroectodermal-mesodermal interactions, and can be divided into morphologically distinguishable stages, induction, organogenesis and cytodifferentiation. (Paus, 1999)

1.2.1 Hair follicle morphogenesis

The hair follicle develops as a result of complex interactions between the epithelium and mesenchyme. Other skin appendages, including teeth, feathers, develop in a similar manner, sharing both morphological characteristics and signalling mechanisms, particularly in the earliest stages of development (Davidson. 1952; Hardy, 1992; Van Genderen, 1994; Thesleff, 1995; Dassule, 1998; Schmidt-Ullrich, 2005). Morphologically, the hair follicle development has been classified into 8 stages. Initiation of hair follicle development begins with “crowding” of epidermal keratinocytes, which become enlarged and elongated, and get organized in microscopically easily recognizable hair placodes (Stage 1). Placodes formation is succeeded by a condensation of specialised fibroblasts with inductive properties in the underlying mesenchyme. The signalling cross talk between this mesenchymal condensate and the epithelial hair placodes drives proliferation in structures, shaping the follicular dermal papillae (DP) in the mesoderm and starting down growth of the ectodermal placodes.

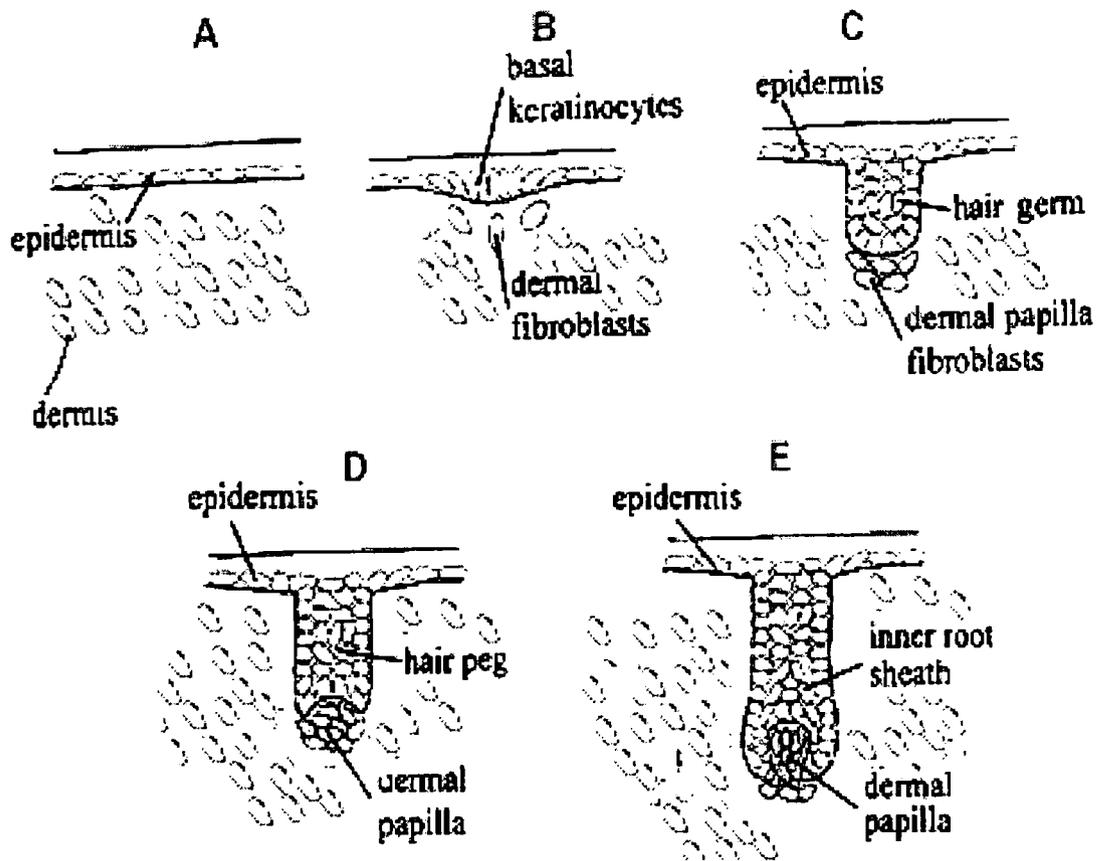


Fig.1.3 Morphogenesis of hair follicle

A: E13 undifferentiated epidermis (stage 0). Interactions gradients of activators and inhibitors create an inductive field in epidermis and dermis.

B: E14 Placode (stage 1). Promotion of placode proliferations

C: E15.5 Gem stage (stage 2). Dermal papillae formation process

D: E16.5-17.5 (Peg, stage 3-5). Polarity of hair follicle, hair shaft formation.

E: E18.5 (Bulbous peg Stage 6-8). Differentiation of inner root sheath, differentiation hair shaft, immigration of melanocytes and hematopoietic cells, innervation vascularization

Modified from Schmidt-Ullrich *et al* 2005

Already the earliest events of murine hair follicle development are characterized by intensive proliferative activity. The following stage of massive keratinocytes proliferation leads to the formation of the hair germ (Stage2). Further down-growth progresses to the *peg* stage (stage 3-4), where the most-proximally located keratinocytes begin to enwrap the DP, followed by the bulbous peg stage (stage 5-8) when distinct strata of epithelial differentiation within the HF become morphologically noticeable. During stage 5, hair follicle keratinocytes begin to form the inner sheath (IRS). Evidence shown that these epithelial cells in the follicles terminally differentiate, they slowly form a rigid tube (Paus R, 1999). In the centre of this tube, terminally differentiated trichocytes, which will form the hair shaft, become organized and compacted. The IRS is surrounded by increasingly distinct, cylindrical outer root sheath (ORS) cells. IRS formation is a crucial step in hair follicle morphogenesis, since it heralds that the follicle prepares to serve its key function: that of a hair shaft factory. (Philpott, MP, 1998, Schmidt-Ullrich, R, 2005).

1.2.2 First dermal signal

A series of signals sent between dermal cells and overlying surface epithelium cells that can cause fate changes in both cell populations, ultimately results in differentiation of the hair shaft, root sheath, and dermal papillae (Hardy, 1992; Wu, 2004; Jiang, 2004). The first dermal signals which unveil hair follicle development initiation are important and lead to formation of a circumscribed placode of functionally distinct keratinocytes. The first clue to identify the signal came from studies of subcellular localization of β -catenin (Normally, 1999). Consistent with that, Kratochwil *et al* have shown that *lef1*, the lymphoid enhancer-binding factor family of DNA binding factors is expressed in the mesenchyme of the mouse vibrissa pad prior to vibrissa follicle development (Kratochwil, 1996). In summary, evidence (Muller-Rover, 1999; Andl 2002 and Jamura 2003) suggests that the activation of Wnt signalling in the skin in mouse is required for the initiation of hair placode formation. However, up to date, it is not known exactly which member of the Wnt family is responsible for and whether it arises intra-dermally or intra-epidermally.

1.2.3 Dermal condensation

The earliest indication of appendage formation is a thickening of the epidermis and the formation of a dermal condensation from the mesenchymal cells immediately beneath the basement membrane. This aggregation is crucial for the hair follicle formation, and cell adhesion, migration and morphogenesis are all likely to be contributing factors.

Wnt signaling is likely to be required for the induction of dermal condensation (Huelsken, 2001), platelet-derived growth factor-A is expressed in the placode, whereas its receptor is expressed in the dermal condensation (Karlsson, 1999). Sonic hedgehog (SHH) proteins are also present in the follicular placode and play a major part in later epithelium-mesenchymal signalling (Bitgood, 1995). Wnt and PDGF-A molecules are strong candidates as components of the first epithelial signal inducing formation of the dermal condensate. SHH acts later in follicular morphogenesis, is dependent on WNT signalling and is required for proliferation of follicular epithelium and development of the dermal condensate into a dermal papilla.

1.3 The Adult Hair Growth Cycle

Unlike most organs, the hair follicle undergoes extensive remodelling throughout adulthood. All follicles cycle at regular intervals with the length of the cycle ranging from weeks to years depending on the species and type of hair follicle in question. During each cycle the dermal papilla signals the epithelial cells of the surrounding matrix to proliferate and differentiate resulting in hair growth.

1.3.1 Nature and theories of hair follicle cycle

The most unique feature of hair growth is its cycle. The hair growth cycle describes the changing morphological of the shaft and follicle over time. It involves the rhythmic change of the hair follicle through phases of growth (anagen), regression (catagen), and rest (telogen). Synchronized hair follicle cycling in mammals prepares the hair coat for seasonal changes in habitat conditions as well as procreational activities (Stenn, 2001).

The purpose of hair follicle cycling in mammals with individual (asynchronous) follicle waves may include cleaning the skin surface of debris and parasites, and excretion of deleterious chemicals by encapsulation within trichocytes (Stenn, 2001). In addition, follicle cycling might serve as a regulator of paracrine or even endocrine secretion of hormones and growth modulators produced within the follicle and secreted into the skin or circulation (Paus, 2004). Finally, hair follicle cycling may act as a safe-guarding system against malignant degeneration by protecting rapidly dividing keratinocytes from oxidative damage by deletion during catagen (Paus, 2004).

Theories proposing an overarching mechanism for hair follicle cycling must include the characteristics of the cycle, such as its periodicity; persistence; and autonomy; epithelial and mesenchymal interactions; the variation from site to site; and the exquisite sensitivity to numerous extra follicular growth modulating signals such as hormones and growth factors. There are at least seven theories have been proposed until now, which are shown in table 1.

Title	Concept	Reference
Epithelial theory	The cells reside in the bulge region initiate the cell cycle.	Stenn, 1999
Papilla morphogen theory	Papillae cells secrete morphogen that orchestrates the cycle, the follicle cycle is set up by the cell cycle of the papillae cells that secrete morphogen(s) only during G ₀ /G ₁ phase.	Stenn, 1999
Bulge activation theory	Factors in the papillae act on the stem cells of the bulge region to orchestrate the cycle.	Cotsarelis, 1985
Resonance theory	Hair follicle cycle set up not by a single cell compartment but resonating control by tissue, the interactions of diffusing and reacting morphogens	Stenn, 1999
Oscillating theory	Oscillation control in telogen, eg. Transcriptional factor levels within cell.	Stenn, 1999
Inherence embryonic theory	Controlled by the inherent cycle clock, which is first established during embryogenesis and continues through the whole lifetime.	Stenn, 1999
Inhibition-disinhibition theory	Inhibition control system in the anagen phase, together with dis-inhibition control system in telogen to modulate the hair cycle system.	Chase, 1954
BMP signalling regulates stem cell activation	BMPs are key protein to regulate local organ stem cell together with other proteins by a hierarchical way.	Chuong CM, 2008

1.3.2 Anagen

During anagen, many molecular key regulators of hair biology not only activate morphogenesis but also regulate anagen induction and duration.

The epithelial stem cells differentiates into at least 8 different cell lines, forming the ORS, companion layer, Henle's layer, Huxley' layer, cuticle of the IRS, cuticle of the

hair shaft, shaft cortex, and shaft medulla. As hair shaft cells terminally differentiate, they extrude their organelles and become tightly packed with bundles of 10-nm filaments assembled from cysteine-rich hair keratins, which become physically cross-linked to give the hair shaft high tensile strength and flexibility. The IRS also keratinizes so that it can rigidly support and guide the hair shaft during its differentiation process, but its dead cells degenerate as they reach the upper follicle, thereby releasing the hair shaft that continues through the skin surface. The duration of anagen determines the length of the hair and is dependent upon continued proliferation and differentiation of matrix cells at the follicle base.

1.3.3 Anagen-to-Catagen transition

The anagen period ends with a highly controlled involution of the hair follicle resulting in apoptosis and terminal differentiation. The matrix cells are referred to as transit-amplifying cells due to their limited cell division potential before differentiation. As the supply of the matrix cells declines, hair shaft and IRS differentiation slows and follicles enter a destructive stage called catagen.

In mice, the timing of the first catagen onset varies significantly from one skin region to another. In pigmented mice, the progression of catagen is evident from the colour of the skin, which changes from the dark gray to black of anagen to pale pink by telogen. As with morphogenesis, the first catagen begins in a wave, spreading from the top of the head caudally towards the tail and laterally down the sides of the animal. In back skin taken from the midline, the onset of the first catagen ranges from P14 at the upper back near the tail. Catagen lasts 3-4 days in mice (Steen 2001, Paus, 2004).

There is some evidence suggesting that growth factors and neurotrophin proteins play roles in the transition of anagen and catagen (Fortzik, 2000), however, how they work together to promote the transition have not been identified.

1.3.4. Catagen

The first sign of catagen is the cessation of melanin production in the hair bulb. Clinically, telogen follicles have a depigmented proximal hair shaft (club hair).

Melanocytes involved in apoptosis are recruited from melanocytic stem cells of the secondary hair germ (Tobin, 2001). The programmed cell death of these stem cells might be an important factor for hair graying (Nishimura, 2005). In contrast to the ORS and the hair matrix (with their huge numbers of apoptotic cells), there is no programmed cell death in the dermal papillae, possibly because of the expression of the apoptosis suppressor bcl-2 (Müller-Röver, 2005).

Catagen is the dynamic transition between anagen and telogen (Muller-Rover, 2001). During catagen, the bottom of the hair shaft seals off into a rounded structure (in pelage follicles) called a club, which moves upward, and come to rest beneath the bulge, where it remains anchored during telogen. The hairless gene (Hr) is responsible for the strong connection between the condensing DP and the diminishing hair follicle epithelium in catagen and telogen follicles. It functions as a safeguard of apoptosis control during catagen, (Panteleyev, 2000) Hr operates as a negative transcription repressor and insures that apoptosis only takes place in certain tissue in the correct order. The Hr gene encodes a zinc finger transcription factor whose disruption prevents the DP from ascending and interacting with stem cells of the bulge, resulting in permanent alopecia during the first catagen period in mice lacking a functional gene.

As the lower follicle recedes, a temporary structure forms, the epithelial strand, which is unique to the catagen stage of the hair follicle. This contains many apoptotic cells and is completely eliminated by the time the dermal papilla reaches the cells that surround the remnant club hair.

1.3.5 Telogen, telogen to anagen transition

Following catagen, follicles lie dormant in a resting phase, termed telogen. This is a phase of relative quiescence regarding proliferation and biochemical activity. The follicles remain in this stage until they receive the intracellular and extracellular signals. The unpigmented club hair often remains stuck in the hair canal. Follicle stem cells, which are believed to reside in the “bulge region” of hair follicle, are activated at the telogen-to-anagen transition. One or two quiescent stem cells at the base of the telogen

follicle, near the DP, are activated to initiate a new hair shaft. These cells then begin to proliferate rapidly, and become the transit-amplifying daughter cells that are fated to form the new hair follicle (Cotsarelis, 1990, Oshima, 2001). The new follicle forms adjacent to the old pocket that harbours the club hair, which will eventually be shed. This creates the “bulge” and adds a layer to the stem cell reservoir. The new hair emerges from the same upper orifice as the old hair. In many ways, the telogen to anagen transition resembles the activation of embryonic skin stem cells that are stimulated to make the follicle de novo. Signaling by Wnts (Gat, 1998) and Shh (Callahan, 2004) are indispensable for new anagen, whereas Bmps (Botchkarev, 1999) have been implicated in hair follicle differentiation. The molecular steps involved are likely to hold clues to understanding the activation and specification of stem cells.

1.4 Stem cells

1.4.1 Basic features of stem cells

Stem cells are primal cells found in all multi-cellular organisms that retain the ability to renew themselves through mitotic cell division and can differentiate into a diverse range of specialized cell types. Therefore, the rigorous definition of a stem cell requires that it possesses two properties: self-renewal, the ability to go through numerous cycles of cell division while maintaining the undifferentiated state; and unlimited potency, the capacity to differentiate into any mature cell type. In a strict sense, this makes stem cells either totipotent or pluripotent, although some multipotent and unipotent progenitor cells are sometimes referred to as stem cells. These properties can be illustrated *in vitro*, using methods such as clonogenic assays, where the progeny of single cell is characterized (Friedenstein, 1974). However, *in vitro* culture conditions can alter the behaviour of cells, making it unclear whether the cells will behave in a similar manner *in vivo*. Considerable debate exists whether some proposed adult cell populations are truly stem cells.

1.4.2 Embryonic stem cells

Embryonic stem cells were first isolated and cultured by Evans & Kaufman and Martin (1981), who obtained them from the inner cell mass of pre-implantation blastocysts. The key characteristic of these cells is the ability to differentiate along any cell lineage, including that of the germ cells, a property known as totipotency. This can be confirmed from experiments which highlight the potential for generating transgenic mice from cultured ES cells which have been genetically manipulated (Hooper, 1987 & Koller, 1992)

After more than 20 years of research, there are no approved treatment or human trials using embryonic stem cells. Their tendency to produce tumors and malignant carcinomas, cause transplant rejection, and form the wrong kinds of cells are just a few of the hurdles that embryonic stem cell researchers have been unable to overcome. The ethical issues involved in human ES cell research have also been extensively debated

whilst studies on murine ES cells have made huge steps towards developing the techniques required for such therapeutic approaches. Transplantation techniques to replace the damaged cells in Parkinson's disease have been developed using foetal neurons, so paving the way for similar treatment with stem cell-derived neurons (Bjorklund & Lindvall, 2000). However, somatic cells can be reprogrammed by transferring their nuclear contents into oocytes (Wilmut 1997), or by fusion with ES cells (Cowan, 2005), indicating that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells. More recently, Katahashi *et al* demonstrate induction of pluripotent stem cells from embryonic and adult mouse fibroblasts cells by introducing certain factors (Katahashi, 2006). This work may extend the application of stem cell in medicine and more importantly, this result confirmed the mimic stem cell niche environment *in vitro* could direct cell reprogramming and differentiation.

1.4.3. Adult Stem cells

Adult stem cells are undifferentiated cells found through the body that divide to replenish dying cells and regenerate damaged tissue. These adult stem cells are present in a wide range of adult tissues enabling replenishment and regeneration activities.

A great deal of adult stem cell research has focused on clarifying their capacity to divide or self-renew indefinitely and their differentiation potential (Gardner RL, 2000). Adult stem cells, like embryonic stem cells, have pluripotent potential and can differentiate into cells derived from all three germ layers. Recently, adult stem cells have been shown to have much greater potency than was initially thought. While embryonic stem cell potential remains theoretical, adult stem cell treatments are already being used to successfully treat many diseases. For many years it was believed that the CNS had a decidedly limited regenerative capacity. Until late 1990s, it was then noted that particular neuronal populations in the adult brain were replaced efficiently and neural stem cells have since been identified (Kuhn & Svendsen, 1999). For example, it has shown that ependymal cells from the ventricular walls of the brain are not only

neural stem cells, capable of producing neurons, astrocytes and oligodendrocytes (Johansson, 1999), but also have the capacity to undergo haematopoietic differentiation when injected into sublethally irradiated mice (Bjornson, 1999). Similarly, adult stem cells isolated from murine skeletal muscle have been demonstrated to have haematopoietic potential (Jackson *et al* 1999). Gussoni *et al.* (1999) took this a step further by demonstrating the reverse to be true with haematopoietic cells contributing to muscle fibres. This work also demonstrated the potential of the haematopoietic system as a delivery mechanism for stem cell therapy when targeting multiple sites around the body.

There is also the possibility that cells are not simply remaining as stem cells until they are required to differentiate, but that they remain plastic after a certain amount of differentiation. That is they are not terminally differentiated but have the capacity to be reprogrammed. As more and more studies find that adult somatic cells, previously thought to be irreversibly committed to a particular lineage, possess a degree of multipotency, the possibilities seem endless (Morrison, 2001).

Over the past ten years, more than 100 manuscripts have been published indicating that cells from a given tissue might be capable of differentiating into cells of a different tissue, and these have therefore been placed under the umbrella of stem cell plasticity. Evidence has shown that the adult stem cells plasticity in haematopoietic/mesenchymal bone marrow cells, skeletal muscle cells, neural cells and other tissue cells.

At least four different explanations exist for the perceived plasticity described. First it is clear that stem cells for a given tissue might exist in unrelated tissue. This has been elegantly shown for Haematopoietic stem cells in muscle (McKinney-Freeman, S.L, 1990, Laird 2008), and also has been shown for oval cells in bone marrow (Kikyo, N, 2000). In this instance, the apparent lineage switch is not caused by a single stem cell that can alter its differentiation behavior; rather, the presence of multiple stem cells gives the impression of plasticity. Second, the perceived plasticity might be caused by the transplanted cells fusing with a host cell of a different lineage, leading to transfer of

the genetic information of the transplanted cell to the host-derived cell (Gupta, 1999; Allici 2007). Third, since the cloning of Dolly, a large body of evidence has been created indicating that an adult cell from many mammalian species can be reprogrammed to acquire a pluripotent fate when the nucleus is introduced into the cytoplasm of a non-fertilized egg (Park, 2008; Rossant, 2008). De-differentiation and re-differentiation is also thought to underlie the ability of amphibians and fish to regenerate limbs spontaneously. Therefore, the perceived plasticity might occur via de- and re-differentiation or via nuclear reprogramming (Ferraris, 2000 Pearton, 2005). Finally, cells with pluripotent characteristics might persist even after the initial steps of embryological development.

Haematopoietic stem cells from bone marrow, blood or umbilical cord blood have been used clinically to re-establish the haematopoietic system following radiation and/or chemotherapy for the past three decades. More recently, use of epidermal stem cells and corneal stem cells is being explored in the clinic, and in pre-clinical models (Rama 2001; Santos 2005; Featherstone 2007; Myers 2007), as is the use of neural stem cells to correct defects of the central nervous system and spinal cord (Hatakeyama, J 2004; Yu D 2008). In short, research into adult stem cells has huge potential therapeutic benefits despite the many problems that need to be overcome before such treatment will become routine.

1.4.4. Epidermal stem cells

Interfollicular epidermal stem cells have an extensive self-renewal capacity and produce progeny that undergo terminal differentiation along the epidermal lineages. A frequently mentioned epidermal stem cell niche lies in a region of the hair follicle, known as the bulge region, but evidence has shown that other kinds of stem cell exist inside the hair follicle as reviewed somewhere elsewhere (Watt, 2006). Epidermal stem cells, like other adult stem cells, have progeny that are destined to terminally differentiate but that can first undergo a few rounds of division, during which time they are known as transit-amplifying cells. The stem cells are also thought to be slow-cycling cells or

rarely cycling cells, with a superior clonogenicity and proliferative capacity (Claudinot, 2005). Stem cells divide infrequently, so as to reduce DNA replication errors, and are also commonly protected from environmental and chemical assault by the niches they inhabit (Waters, J.M. 2007). These unique features of stem cells have allowed researchers to identify a likely anatomical location of epidermal stem cells in hair (Cotsarelis.G. 1990) and feathers follicles (Yue Z.C. 2005). Several research methods have been applied to identify the physical location of stem cells in epidermis, such as BrdU or ³H-thymidine staining to identify label retaining cells.

Since Cotsarelis (Cotsarelis, G.1990) has highlighted the bulge as an important anatomical niche for hair follicle epithelial stem cells in mice, more recent researchers have utilized new improved techniques such as transgenic expression of a histone-GFP label, dye-exclusion properties of the cells or molecular stem cell markers to illustrate the more precise physical localization of stem cells in mice, rat and human hair follicles as well as keratinocytes (Lyle, 1998; Trempus, 2003; Blanpain, 2004; Webb, 2004). The potential candidate epidermal stem cells markers include b1-integrin, keratin 15, keratin 19, CD71, and transcription factor. Double-labelling techniques have shown that bulge cells give rise to lower follicles structure, including the outer root sheath, matrix and medulla; and bulge cells contribute to the neonatal epidermis and the repair of the epidermis after a full thickness wound (Taylor, 2000). Furthermore, Liu *et al* (2003) and Tumber *et al* (2004) applied different approaches to explore hair follicle stem cell multipotency. Their results are similar, and these results indicate that bulge cells normally contribute to 3 major cell types of the cutaneous epithelium: the entire hair follicle, epidermis and sebaceous glands.

Human epidermal stem cells can be cultured *in vitro* in appropriate conditions and lead to the formation of cultured epithelium sheets. The epithelium sheets can be transplanted to re-epithelialise burn wounds, chronic wound and ulcers (Ronford,V. 2000). In addition, hair follicle stem cells have been used in preparing skin equivalents, and can form epithelium in deep burn wounds after implantation (Hoeller D, 2001).

1.4.5. Hair follicle dermal stem cells

The dermal cells in hair follicle contain two populations, the dermal papilla and the dermal sheath. Both populations of dermal cells are well-characterized particularly because of their remarkable inductive powers. The isolated and cultured dermal cells from mature adult follicles have been shown to be capable of inducing a range of epithelia to form follicles (Oliver, 1966a, b). Microsurgical manipulations have demonstrated that the hair follicle dermal cells shown to have stem or progenitor cell capabilities in regeneration experiments performed on individual whisker follicle (Oliver, 1966a, b). It was demonstrated that if the lower one third of the rodent vibrissae hair follicle was amputated, after three weeks, the amputated region would regenerate a functional replacement. Specifically, the dermal papilla was reformed from the adjacent dermal sheath cells, showing that within the follicle dermis they acted as a progenitor population. There is evidence that the sheath-papilla exchange is not entirely unidirectional, since in recent work using labelled dermal papilla cells in follicle induction experiments, the dermal papilla cells made the new dermal sheath in induced follicles. To some extent, therefore, these two cell compartments are interchangeable (McElwee, 2003; Inamatsu M 2006).

There is considerable evidence that dermal cells might serve as dermal stem cells within hair follicle (Gharzi, 2003; Jahoda 2003; Richardson 2005); and act in a parallel manner to epithelial stem cells. In particular, given the role of the follicle epithelium repopulating the epidermis during wound healing, it has been suggested that the dermal sheath may play a similar role in the dermis (Jahoda, 2001; Gharzi, 2003). Lako *et al* demonstrated that there is haematopoietic stem cell activity in the follicle dermis (Lako, M 2002), and further more, Jahoda *et al* used the clonal dermal papilla and sheath cells give rise to adipogenic and osteogenic lineages (Jahoda, C, 2003, Richardson 2005). However, though these interesting findings, to fully prove this point and also to clarify the exact role of the dermal sheath in hair follicles more investigations are required.

1.5 Objectives

The work presented in the following chapters aims to investigate the roles of hair follicle epidermal stem cells in the normal hair follicle and regenerative process after microsurgical amputation, and also the potential of isolated and cultured dermal cells in responsive to stimuli signals.

1) The different stages of rat vibrissae hair follicle cycle have been well documented and described (Paus R, 1999; Stenn KS, 2001 and Paus R, 2004); and recent studies have provided insight view of molecular control of hair follicle cycling and development (Lindner G, 2000; Foitzk K, 2003 and Bazzi H, 2007). However, studies of stem cells activity during different stages of hair follicle cycle are limited, due to the lack of specificity of hair follicle stem cell makers, and the various sources of hair follicle stem cells in different types of hairs.

My project focused first on normal vibrissae hair follicles, and investigating the epithelial stem cell and Id3 positive cell activities during different cycle stages, by comparing and contrast the different expression pattern in each stage.

2) The regenerative properties of vibrissa follicle are well established but many questions remain especially in the cellular and molecular level (Oliver 1966, Jahoda 1992). For example, the source of the regenerated dermal papillae is still a mystery, epithelial stem cells activity during hair follicles regeneration is little understood. In addition, though both keratin 15 and Id3 proteins expression have been previously reported their roles in skin wound healing processes; their roles in hair follicle regeneration remain unclear.

The second aim of my thesis is to investigate the expression pattern of K15 and Id3 in regenerating hair follicles after microsurgical amputation.

3) It is still debatable whether the isolated and cultured hair follicle cells from follicular end bulbs and dermal papillae are dermal stem or TA cells. My project was also interested to find if this group of cells possess the potential to become osteoclasts.

Chapter 2

K15 and Id3 expression in adult hair follicle cycle and the hair follicle end bulb cells plasticity *in vitro*

2.1 Introduction

2.1.1 Epidermal stem cells

Epidermis is stratified self-renewing tissue that continuously generates new cells to replenish the dead squames that slough off its upper surface.

It is known that cells are generated through proliferation that occurs only in the basal layer; therefore, stem cells must be located there. Approximately 5% of adult epidermal basal cells possess a “holoclone” phenotype characterized by high reproductive capacity and low level of terminal differentiation, and these cells are thought to represent stem cells. In mouse skin, individual basal cells divide less frequently compared to their surrounding cells, and these surrounding cells with slow cycling basal cells are organised in a hexagonal unit (Mackenzie,1970), which was termed as epidermal proliferated unit or EPU (Potten,1974). It was assumed that the central cell within the EPU generates the rapidly proliferating cells, termed transit amplifying (TA) cells, which are responsible for continual epidermal renewal.

2.1.2 Hair follicle stem cells

The proliferative capacity and multipotency displayed by follicular epithelial cells indicate the presence of a stem cell population within this tissue.

Adult hair follicles go through cyclic phases of hair production. In the mouse pelage, waves of hair growth occurs at specific times in the life of a mouse, and the timing of

the waves can vary according to different strains and ages. In the anagen stage, there is an enormous amount of cell proliferative activity in the germinal or matrix region at the base of the follicle. There is a strict spatial organization and migration pathways in this region, suggesting that the ancestral cells for all cell lineages are located in the very lowest regions of the follicle matrix. Legu, Cotsarelis et al (1990) and Taylor et al (2000) demonstrated that, at least in the early phase of skin development, both epidermal and follicular cells can be tracked back to a specialized region in the upper outer root sheath of the follicle called the bulge. The role of this reserve of stem cells in the normal adult hair cycle is less clear, although some data suggest that the matrix is repopulated at each cycle by stem from the bulge region (Oshima et al, 2001). More recently, Claudinot and Barrandon isolated single keratinocytes from the upper or lower regions of whisker follicles of adult rats, and then tested their clonogenicity. They showed that after more than 140 doublings, both upper and lower portion of the follicles cell still be able to develop a new hair follicle from a new born rat, though the number of clonogenic keratinocytes from lower region of the follicle depended on the stage of hair cycle (Claudinot 2005). In short, in searching for follicular stem cells two potential sources have been identified: a subpopulation of outer root sheath cells in the bulge, situated at the arrector pili muscle attachment site; and the germinative epithelial cells, a population of cells in the base of the follicle bulb.

2.1.3 Theories of epithelial stem cells in hair follicle cycle

Increasing evidence suggest the multiple locations of epithelial stem cells, and it is also commonly thought that committed progenitors and stem cells are key players during the hair follicle cycle and wound healing/regenerating processes.

Keratinocytes within the hair follicle stem cell niche are believed to be primed to respond to at least two sets of stimulatory signals to generate a bidirectional flow of stem cells (Christiano, 2004). An upward flow of stem cells out of the bulge into the epidermis is generated in response to epidermal injury. A downward flow is induced by

a periodic signal from a cluster of hair follicle dermal cells, at the beginning of each hair cycle. However, the mechanisms and the precise signals involved in stem cells to step out of the niche are largely unknown.

Cotsarelis et al (1990) proposed a model, described as the bulge activation hypothesis, whereby the stem cells in the bulge are activated to proliferate, so initiate anagen, when in close contact with the dermal papilla of the shortened, telogen follicle (Fig2.1).

2.1.4 Follicle end bulb cells in hair follicle

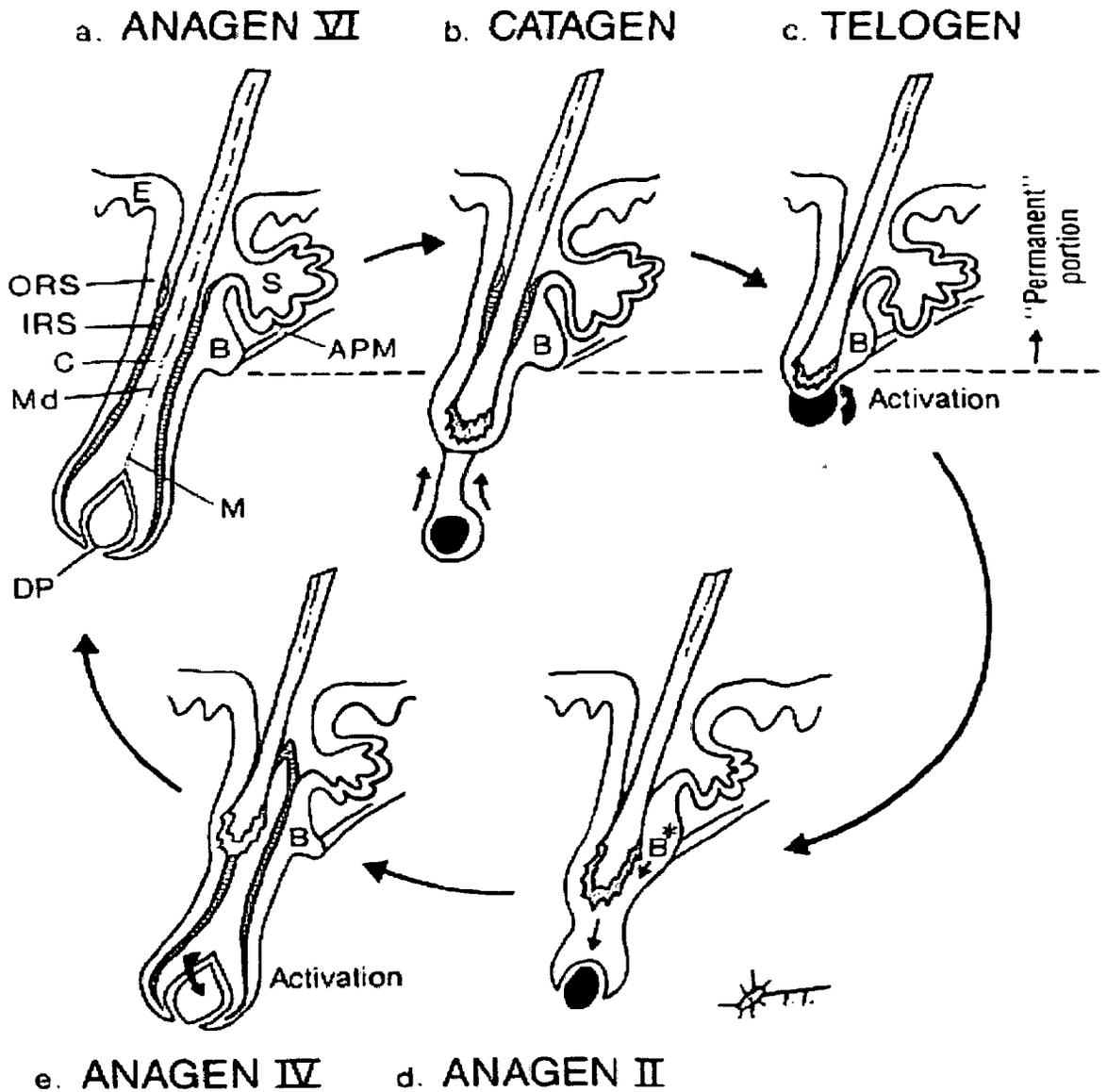
There are three cell types believed to be important at the very base end bulb of the hair follicle, dermal papillae, dermal sheath and the germinative epithelial (GE) cells. Dermal papilla cells are specialized mesenchymal cells that play pivotal roles in hair formation, growth and cycling, and are located at the bottom of hair follicle. The dermal sheath cells surround the outside of the hair follicle and contain progenitor cells that maintain and regenerate the dermal papillae. The germinative epithelial cells are those at the very base of the epithelial matrix, sandwiched between the dermal sheath and the dermal papilla. They differentiate to produce the epithelial cell layers of the hair follicle comprising the root sheaths and the fibre. Germinative cells have the capacity to confer follicle inducing properties onto other cell types (Reynolds, & Jahoda, 1996).

In most hair follicles the dermal papilla changes morphologically during the hair cycle. In anagen, the papilla is composed of a group of fibroblasts, embedded in a loose connective tissue stroma. It is encased by the epithelial cells of the bulb but separated from the epithelium by a well defined tri-laminar basement membrane. In early phase of catagen, the papillary stroma decreases, and the bulb epithelium withdraws to release the papilla. In telogen, the papilla rests at the proximal base of the follicle as tight cluster of cells containing virtually no ultra structurally recognizable extracellular matrix.

GE cells have been isolated that are capable of proliferating indefinitely, suggesting

that the length of anagen is not regulated by a limited proliferative capacity of GE cells (Gharzi et al 1999). During catagen and telogen in vibrissae follicles the GE cells may remain in the epithelial follicular tissue. Therefore, GE cells are believed to be stem cell like cells, which are predicated to play a crucial role in hair follicle cycle.

Fig2.1 The bulge activation hypothesis



The bulge and structures distal to it comprise the permanent portion of the follicle cycle. Bulge cells are normally slow cycling but can be stimulated by dermal papilla to undergo transient proliferation during early anagen. During anagen IV dermal papilla cells undergo transient proliferation and de-condensation possibly in response to a matrix signal. Matrix cells, being derived from the bulge (stem) cells, are transient

amplifying cells and thus have only a limited proliferative potential. The upward movement of DP during late catagen is crucial to allow the subsequent physical interaction of the resting bulge cells by DP cells, and to start a new cycle of hair growth.

ORS - outer root sheath, IRS - inner root sheath, C – cortex, Md – medulla, M – matrix, S – sebaceous gland, E – epidermis, APM – arrector pili muscle.

Gotsarelis et al., 1990

Dermal sheath cells connected to the dermal papilla at the very bottom of follicle in anagen, are developmentally related to dermal papillae cells. They have the same developmental origin and experimentally can turn into papillae cells (McElwee, 2003).

2.1.5 Keratin 15

Owing to their unique regulation in a pairwise, epithelial tissue-type, and differentiation-specific manner, keratin genes are very useful tools for studying epithelial differentiation. Keratin filament networks generally function as a structural scaffold, providing mechanical support not only for individual cells, but also for entire epithelial sheets.

The intermediate filament keratin, K15, is present in variable abundance in stratified epithelia. More recently, K15 has been used in identifying and isolating putative bulge stem cells for further analysis by various means (Lyle 1998).

Whitbread showed that the *K15* gene is expressed throughout the hair cycle in the basal layer of the outer root sheath that envelops the follicle in sheep. In the follicle bulb *K15* is expressed in cells situated next to the dermal papilla but not in the inner bulb cells (Whitbread 1998). Later in the same year, Lyle *et al* demonstrated that K15 protein level also highlighted the stem cell region in the hair follicle bulge region without staining the remaining hair follicle. They showed C8/144B monoclonal antibody which recognized K15, to be highly expressed in the bulge region, which has been demonstrated as a stem cell niche (Lyle 1998).

Although K15 mRNA and protein are reliably expressed at high levels in the follicle

bulge, it can also be found in lower follicle ORS and the interfollicular epidermis. Thus the use of K15 expression follicle as the sole criterion for defining a bulge stem cell has been cautioned against (Waseem 1999). However, the K15 promoter used for generation of transgenic mice possesses a pattern of activity restricted to the bulge in the adult mouse (Liu 2003), and this marker has been used in several studies looking at follicle stem cells activities (Lyle 1998).

2.1.6 ID3 biology

ID proteins are key regulators of development where they function in part to preserve the stem cell state and control lineage determination. ID proteins contain a relatively conserved helix-loop-helix (HLH) structural motif. The ID proteins inhibit transcription factors, which are no longer able to bind to the DNA target sequence and activate transcription. More recently, studies have uncovered additional partners of ID proteins and proposed new mechanisms, such as subcellular compartmentalization and ubiquitin-dependent proteasomal degradation, for the regulation of ID protein activity (Benezra, 2005; Jackson, 2006). More recently, the interest of biomedical research in ID biology has been heightened by the multifaceted activity of ID proteins in cancer and tumour cells. The third member of this class protein, ID3 has been implicated in many diverse developmental, physiological and pathophysiological processes. Expression and function of ID3 is under many complex layers of regulation (Figure 2.2).

It is becoming increasingly clear that the function of various bHLH (including ID proteins) proteins in regulating gene expression/cellular function is critically dependent on subtle changes in their cellular activities. Deed *et al* used human Id3 to demonstrate that E protein, by nuclear chaperoning Id, can regulate the available cellular pool of its own inhibitory partner (Deed 1996). This suggested that the Id3 proteins required E-protein as a partner to be stabilised and functional. Interestingly, Id3 has been reported to be localized to specific subcellular locations in some specific cell types (Chen, 1997). Id3 also contains several potential phosphorylation sites (Nagata, 1995),

and this has been suggested to block the heterodimers between E-protein and myo D. In the hair follicle during morphogenesis and during hair cycling there is complex cross-talk between various signalling pathways. During morphogenesis, BMP signalling has been shown to be important in inhibiting the placodal fate of cells surrounding newly developing follicles and is also involved in the differentiation of the hair shaft.

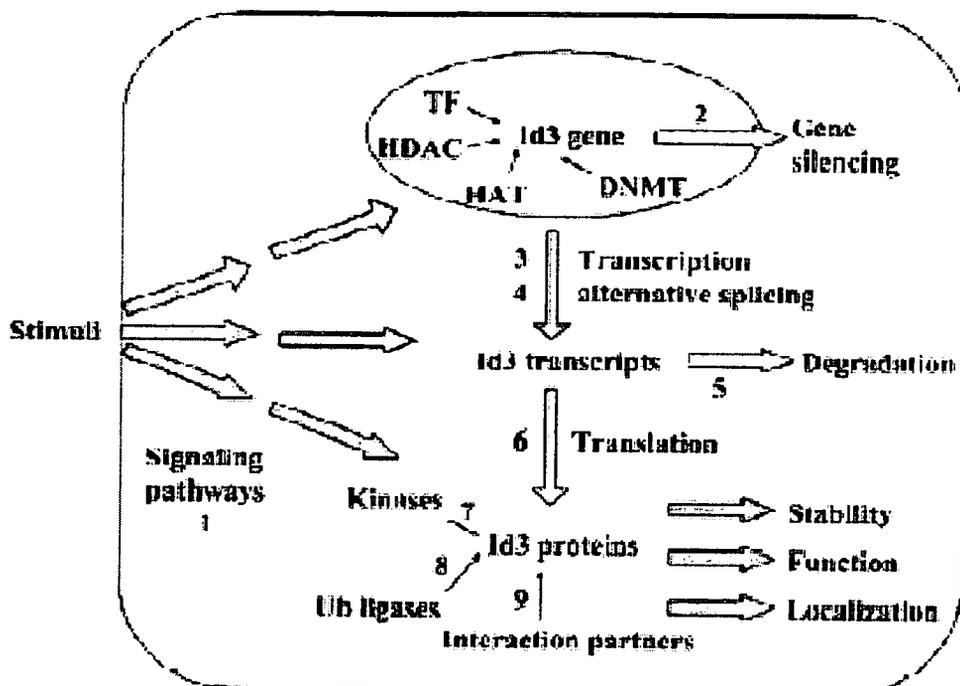


Figure 2.2 Multiple mechanisms regulating inhibitor of differentiation 3 (Id3) expressions and function.

A variety of stimuli and physiological state activate diverse signal transduction pathways

- (1) to regulate the expression and function of Id3 at multiple level
- (2) regulate the gene transcription
- (3) alternative splicing to form two isoforms
- (4) additional factors affect proteins stability and
- (5) translation
- (6) phosphorylation

- (7) ubiquitination
- (8) specific kinases and ubiquitin ligases that modulate its function and stability
- (9) subcellular location and ultimate biological effect of Id3 might be dependent on the availability, relative abundance and physiological significance of its various protein partners

Signalling via the TGF- β pathway has been linked to promotion of the placode and shape of the follicle (Schmidt-Ullrich and Paus, 2005). To date there has only been one report of Id3 in the hair follicle and this focussed on the role of BMP signalling in the control of Id3. O'Shaughnessy *et al* showed that the expression of the BMP target Id3 in the dermal papilla closely mirrors the BMP activation state in both pelage and vibrissa follicles. But interestingly during telogen when no BMP signalling is present, Id3 expression was found. This highlighted the possibility that not only BMPs are involved in regulating Id3, but also cell-cell contacts (O'Shaughnessy, 2004). Removed from the matrix by microdissection, freshly explanted dermal papilla cells expressed high levels of nuclear ID3, which fell dramatically after even a single passage in culture. However, this Id3 expression could be restored by adding BMP-4 (O'Shaughnessy, 2004).

2.1.7 Introduction to Osteoclasts

Bone remodelling is the predominant metabolic process regulating bone structure and function during adult life. Imbalances of remodelling can result in gross perturbations in skeletal structure and function, and potentially to morbidity and shortening of lifespan.

Osteoclasts are specialized cells derived from the monocyte/macrophage haematopoietic lineage that develop and adhere to bone matrix, then secrete acid and lytic enzymes that degrade it in a specialized, extracellular compartment. Therefore, most adult skeletal diseases are due to excess osteoclastic activity, leading to an

imbalance in bone remodelling which favours resorption, such as osteoporosis. Discovery of the RANK-signalling pathway (Lacey, 1998) in the osteoclasts has provided insight into the mechanisms of osteoclastogenesis and activation of bone resorption, and how hormonal signals impact bone structure and mass.

It is known that two haematopoietic factors, the TNF-related cytokine RANKL and the polypeptide growth factor, colony-stimulating factor-1, are necessary and sufficient for osteoclastogenesis (Fig.2.3), and for the subsequent activation of RANK on the surface of haematopoietic precursor cells (Lacey, 1998; Hsu,1999).

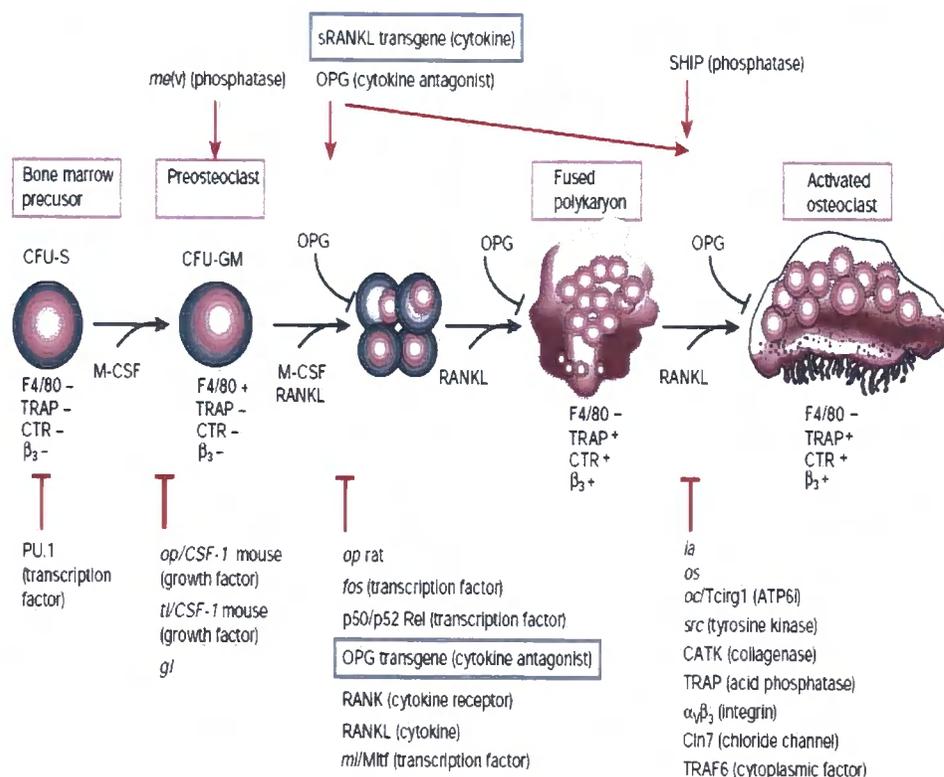


Figure 2.3 Osteoclastogenesis.

Development schema of haematopoietic precursor cell differentiation into mature osteoclasts, which are fused polykaryons arising from multiple (10-20) individual cells. Maturation occurs on bone from peripheral blood borne mononuclear cells with traits of the macrophage lineage shown above.

M-CSF and RANKL are essential for the osteoclastogenesis, and their action during lineage allocation and maturation is shown. OPG can bind and neutralize RANKL, and can negatively regulate both osteoclastogenesis and activation of mature osteoclasts.

Adapted from Boyle *et al*, 2003

Jahoda *et al* shown that hair follicle dermis-derived stem cells are capable of differentiating into fat and bone cells in culture, and that the same population can partially repopulate the blood system (Lako 2002; Jahoda 2003). Others have shown that cell derived from the follicle dermis display an even wider stem cell potential, being able to differentiate into neural lineages. Strikingly, the molecular profile of these hair follicle dermal-derived cells has many features in common with bone marrow mesenchymal stem cells, and one of the hallmark signatures of these cells is their expression of alkaline phosphatase. In short the hair follicle dermis is regarded as a key stem cell niche in skin, and interestingly, hair follicles also have unique hormonal responsiveness and can participate in both autocrine and paracrine hormonal activities (Hoogdujin, 2006). Although hair follicle dermal cells can be isolated discretely and are a relatively homogeneous population, questions remain as to whether all the observed stem cell activities originate from the same cell population. The blood stem cell activity, for example, could derive from multipotent progenitors within the tissue, or circulating stem cells.

Movement of precursor or progenitor cells within the body is being increasingly described as participating in beneficial processes –for example, homing of bone marrow cells to healing skin wounds. Recently, it has been shown that osteoblast progenitor cells are in the circulating blood of mammals including humans (Mochizuki, 2006), leading to the suggestion that osteoblast progenitors and/or osteoblasts could be supplied to regenerating bone via the circulation.

2.1.8 Aims

The hair follicle has unique features, because it can both renew itself through an intrinsic stem cell population and has the ability to cycle. To study the stem cell activity during different stages in the vibrissae hair follicle cycle, a number of proteins have been chosen to investigate their expression. K15, a proposed marker of hair epithelial stem cells, was used study the epithelial stem cells activity during hair follicle stages. Id3, which is a transcriptional regulatorly proteins, was used to look at dermal and epithelial cell differentiation and proliferation activities in different hair follicle cycles. The other aim is to focus on hair follicle dermal stem/TA cell activity *in vitro*. Treating explants dermal papillae and end bulb cells from hair follicle with osteogenesis signals, was intended to testify if these cells have the ability to differentiation into other types of cells *in vitro*.

2.2 Materials and methods

2.2.1 Animals

PVG rats of either sex (3-7 months) were killed and the vibrissa follicles isolated under a dissection microscopy (Nikon SMZ-10) using the method described by Robinson et al. (1997). An L-shaped incision was made postero-ventral to the mystacial pad and the flap of skin folded back. The connective tissue surrounding the vibrissa follicle was removed with fine forceps. The exposed follicles were pulled out from the face pad with their hair fibres intact for staging individually into the main follicle cycle categories. The follicles were then cut transversely at the neck and placed in minimal essential medium (MEM, Gibco).

2.2.2. Freezing down hair follicles

All the follicle samples were immediately embedded individually in Tissue Tek O.C.T. compound (Agar Aids), snap frozen in liquid nitrogen and stored at -80°C prior to being sectioned for immunohistochemistry. Table 2.1 shows the number of specimens sectioned and stained.

Table 2.1 Total numbers of specimens stained for K15, ID3 expression

Tissue sample	K15	ID3
Anagen	3	3
Catagen	2	2
Telogen	2	2
Early Anagen	2	2

2.2.3. Immunohistochemistry

Frozen sections (7 μ m) of rat vibrissae follicles and ear wound skin were cut on a Leica CM3050 cryostat, thaw-mounted on to poly-lysine coated slides, and air dried for 1 to 3 hours before being processed for immunohistochemistry. They were fixed in methanol at -20°C for 15-min and then a 5-min fixative in ethanol followed by three washes in PBS. The sections were incubated in 10% donkey serum in PBS for 30 min to block non-specific binding. The slides were then incubated in primary antibodies (Table 2.2) for 1 hour at room temperature or overnight at 4°C and then unbound primary antibody was removed with four washes in PBS. Secondary antibodies (Table 2.3) were then applied and incubated for 60 minutes at room temperature in the dark. Unbound antibody was removed with four PBS washes and sections were mounted in mowiol under glass cover slips.

Negative controls were performed by omitting the primary antibody and/or secondary antibody and incubating the sections in PBS instead.

Table 2.2 Details of Primary antibodies used for immunohistochemistry.

Antigen	Host	Antibody	Dilution Factor	Source	Reference
K15	Mouse	Anti-K15	1:100	Lab Vision	Waseem,1999
ID3	Rabbit	Anti-ID3	1:100	Santa Cruz	Sun, 1991
Integrin	Mouse	Anti-Integrin	1:50	Cambridge	Takata, 1989

Table 2.3 Details of Secondary antibodies used for immunohistochemistry

Antigen	Host	Antibody	Dilution Factor	Source	Reference
Anti Mouse	Donkey	Donkey anti-mouse	1:500	Santa Cruz	Fifre, 2006
Anti Rabbit	Donkey	Donkey anti-rabbit	1:500	Santa Cruz	Jones, 2004

2.2.4 Histology

7 μ m Tissue Tek sections samples were fixed in 95% methanol and 5% acetone for ten minutes, followed by a distilled water wash. Samples were stained with haematoxylin (Sigma-Aldrich) for 1-1.5 minutes, washed again with distilled water and 70% ethanol, and then placed in eosin (Sigma-Aldrich) stain for 30 seconds to 1 minute. The sample slides were dipped into 95% ethanol twice and absolute alcohol twice; left in the histoclear (Sigma-Aldrich) for 10 minutes and then mounted with DPX mounting media (Agar scientific).

2.2.5 Explant culture

Primary cultures of adult and new born rat dermal papilla cells, was started by carrying out the above micro-dissection in rats and exposing vibrissa follicles (Section 2.2.1 and 2.2.2). The exposed hair end bulbs were then cut off and transferred to separate media droplets in Petri dishes. The excess connective tissue blood vessels around the end bulbs were further cleaned using dissection tools, and the capsule was then turned inside out to reveal the papilla. The isolated papilla was then transferred to a well in a four well dish that contained 20% FBS (Gibco) MEM. After all papillae had been transferred, normally 7-10 per well, a needle was used to push them down to the plastic base of the well to increase the chance that they would adhere. The cells were left in the 37°C, 5% CO₂ incubator for several days.

2.2.6 Cell culture

Explant cultures of dermal cells were passaged to 25cm flasks once cells were growing well, usually between 2 and 4 weeks after the initial explant were established. Flasks of cells were passaged when confluency was reached and prior to aggregation in the case

of DP cells.

Dermal cells were washed twice with warmed PBS/EDTA before addition of trypsin (0.25% in PBS/EDTA). The cells were incubated in trypsin for 1-2 minutes, until cells had begun to detach. The flask was tapped gently to detach the cells and 1ml MEM+FBS was added to inactivate the trypsin. The cell suspension was transferred to a sterile tube, the cells spun down at 1000rpm for 5 minutes, the supernatant discarded and the cell pellet resuspended in 1ml MEM+FBS. The cell suspension was then transferred to flasks or dishes and volume made up with MEM+FBS.

2.2.7 Osteoclast differentiation *in vitro*

The dermal papilla primary cultures were incubated at 37°C and 5% CO₂ for 1-2 weeks to allow cells to grow out from the explant, and reach 20-40% confluency in the 35-mm dishes. Cells was washed with PBS twice and then cultured in MEM (Gibco) containing 10% FBS with 100ng/mL recombinant mouse M-CSF (R&D Systems) together with 25ng/mL RANKL (R&D system) for 2-3 weeks, and the media was changed every twice a week.

2.2.8 TRAP assay

After 2-3 weeks the TRAP assay was applied to cultured dermal cells in conditioned media, to check for the presence of osteoclasts signified by Acid Phosphatase Leukocyte staining (Sigma-Aldrich). Images of the cells were firstly recorded before the assays. A fixative solution was prepared by combining 25ml citrate solution, 65ml, methanol and 8ml of 37% formaldehyde. Cells in a 35-mm dish were treated with fixative solution and left in room temperature for 30 seconds, followed by several rinses with filtered PBS. Cells were then incubated in solution combining 45ml

deionized water pre-warmed to 37°C, 1ml diazotized Fast Garnet GBC solution, 0.5ml Naphthol AS-BI Phosphate Solution, 2ml Acetate solution and 1ml Tartrate solution in a 37°C, 5%CO₂ humidity incubator for one hour. They were then rinsed several times with alkaline tap water to blue cell nuclei.

2.3 Results

Vibrissa hair follicles showed distinct distribution of individual proteins through the different hair cycle developmental stages. Minor variations in expression were also observed among follicles at the same stage. Hair follicle stages were used to categorize the results here.

We also categorized the follicle into three parts according to their physical structure and location in the body. The proximal region described the region from the end bulb to the region before widening of the epithermal column. The centre region described the region that from the wide epithelial column to the neck of hair follicle. The rest of the top region of the follicle was named the distal region (Figure2.4).

In addition, a cartoon picture was produced to show the general K15 and Id3 expression pattern during hair follicle stages (Fig2.9) in the end of this section.

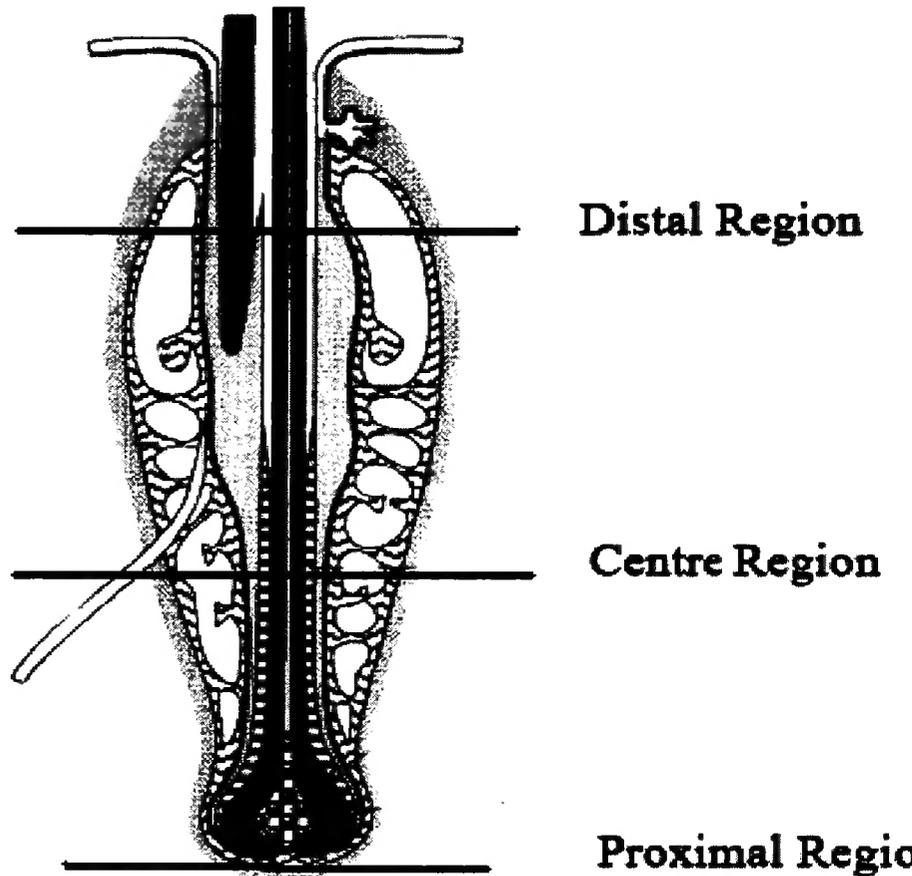


Figure 2.4 Categorized regions of hair follicle

The proximal part describes the region that from the amputation site to the region before the wider epithelial column starts to widen.

The centre part describes the region from the wider epithelial column to the neck of the follicle.

The rest of the top region of the follicle is named the distal part.

2.3.1 K15 expression in adult rat vibrissae hair follicle

Anagen

Anagen follicles are long and very straight morphologically, as during this growth stage, when cells undergo differentiation and proliferation, it is clearly observed that a fibre is growing out from the bottom of the follicle towards the skin, and the club fibre is pushed up to the upper central part of the follicle.

K15 protein expression was clearly strong in outer root sheath cell, along the glassy membrane from the distal region to the proximal region but not continuous in the end bulb area (Figure 2.5A). K15 staining was particularly strong in the bulge region in the centre, and K15 immuno reactivity was also associated with cells around the club fibre (Fig2.5A). K15 staining of ORS cells lining the glassy membrane was asymmetrical on the two sides of the follicle. There was more labelling in both quantity and intensity on the club hair/nerve entry side rather than its mirror opposite (Fig2.5A).

In the proximal region of early anagen hair follicle, K15 was strictly expressed discontinuously along outer root sheath. On the club hair side, these K15 positive cells reached the hair matrix, at the very bottom of follicle. Newly formed hair shaft cells on the top of dermal papillae (Fig3.5B), were also K15 positive cells. Higher magnification images showed that the staining of outer root sheath was not universal when DAPI labelled outer root sheet cells were overlapped with K15 positive cells (Fig 3.5C).

In the central region of follicles, K15 positive cells increased in numbers along the glassy membrane. K15 labelled epithelial cells gradually increased from one single cell layer to several layers thick. The new hair shaft cells appeared elongated and were completely negative for K15. Dermal cells outside the glassy membrane were also K15 negative (Fig2.5E). K15 strongly stained the bulge region cells, which appeared larger and round shaped. Cell located inside glassy membrane showed two distinct cell types, tightly organised, narrow shaped cells and large disorganised round shaped cells. Interestingly, cells around club fibre which was in its final position still showed

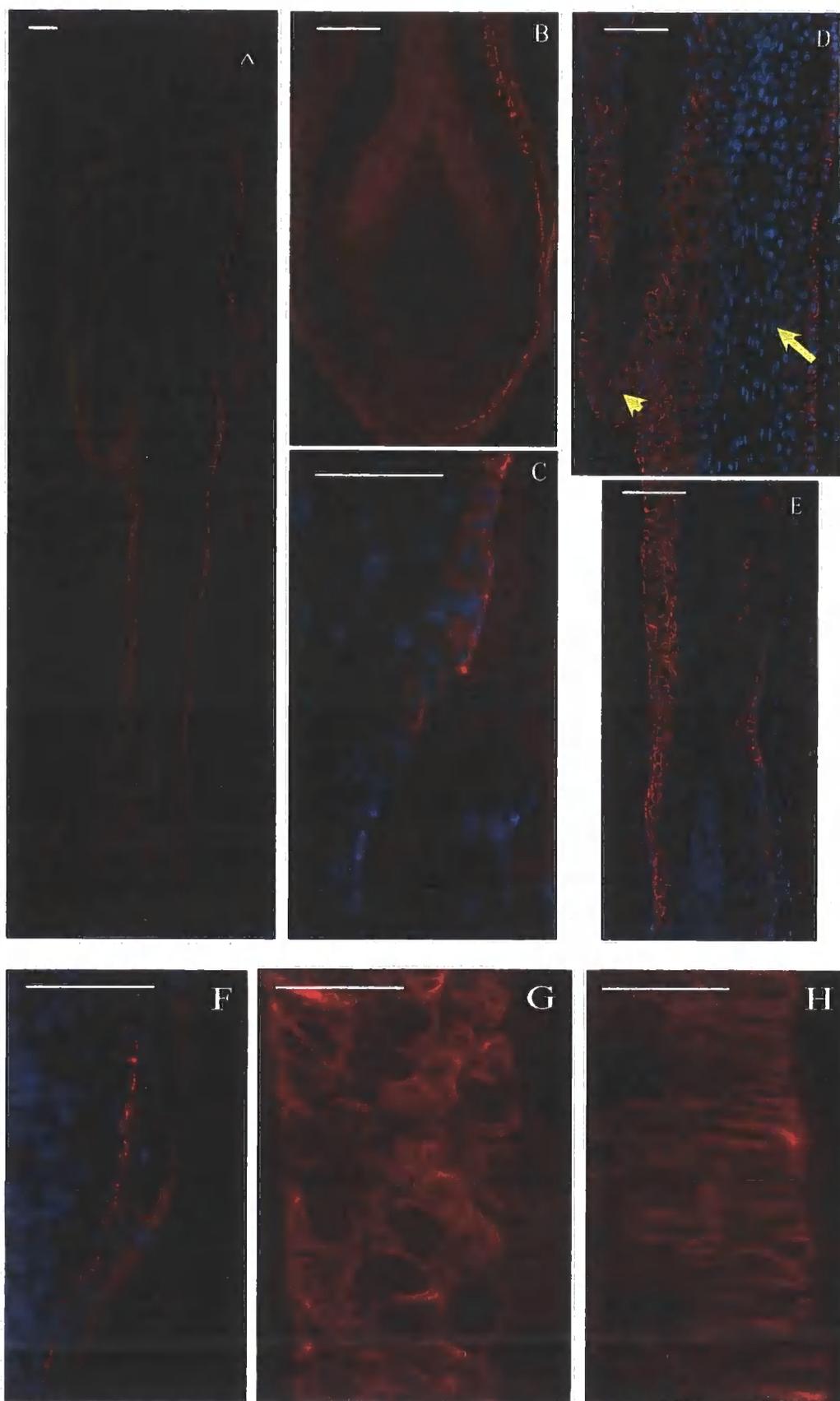


Fig2.5 K15 expression in the Anagen hair follicle

A: Overall view of K15 staining in an anagen hair follicle, K15 proteins are expressed along the glassy membrane through the whole follicle.

B: The proximal region of follicle, K15 is not continuously expressed along the outer root sheath cells, and the melanocytes which are located on the top of the dermal papilla are weakly stained with K15.

C: Higher magnification (x63) view of the proximal region of a follicle, showing the outer root sheath cells discontinuously expressed, nuclei are counterstained with DAPI bind which are a blue colour.

D: In the bulge region, K15 cells are particularly strong; the old fibre has been pushed away (Arrowhead), and left an empty gap. Cells inside the glassy membrane showing differences in morphologically (Arrow)

E: In the centre region of the hair follicle, DAPI shows the hair shaft cells and dermal cells are K15 negative.

F: Cells located in the proximal region of the follicle, the K15 cells are elongated and very small in size.

G: Cells located in the centre region, are larger in size and round in shape.

H: Cells located in the distal region, appeared in round shaped and medium sized.

In this section all scale bars shown represent 100µm.

quite strong K15 staining. Even when the club was lost at late anagen, positively stained cells were still visible around the void (Fig2.5D).

K15 highlighted cells showed clear morphological differences depending on their location in the follicle. In the proximal part, K15 stained cells were more elongated and smaller in size (Fig2.5F). Central region K15 positive cells were round in shape, and increased dramatically in their size (Fig2.5G). In contrast, the distal region K15 positive cells were narrow, applanate shaped, medium sized cells (Fig2.5H).

Anagen is the longest phase of the hair follicle cycle, and K15 protein expression pattern showed some variation in individual follicles. In general, K15 proteins were upregulated in this stage and all three regions of hair follicle, proximal, central and distal showed quite strong expression, especially in the bulge area. In addition, asymmetrical K15 staining in cells on the glassy membrane and on opposite sides of the hair follicle was also a repeatable observation.

Very late Anagen/Catagen

Catagen is the dynamic transition between anagen and telogen. During catagen, dermal papillae remain a similar size to that seen in anagen; however, the club fibre located near the bulge region has disappeared. Melanin production in the hair bulb is stopping, but there are still some melanocytes cells sitting on top of the dermal papillae, making most of the catagen follicles appear black at the bottom.

Overall K15 expression decreased dramatically at catagen. K15 positive cells were concentrated in ORS cells adjacent to the glassy membrane in the distal and central regions of follicles. The proximal region appeared to have limited staining, however, matrix cells above the dermal papillae in the end bulb also contained K15 positive cells, similar to the anagen follicle. The K15 expression pattern also showed asymmetry, with the nerve entry side displaying more K15 positive ORS cells along the glassy membrane than its opposite side (Fig2.6A). In the proximal region, some punctuated K15 positive cells were located along the line of ORS, on the outside of bulb matrix. All the dermal cells were K15 negative.

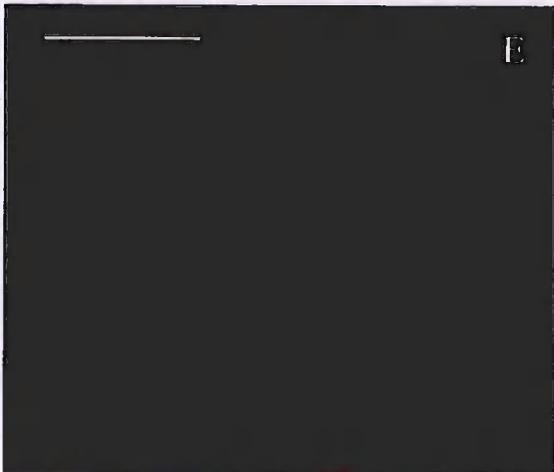
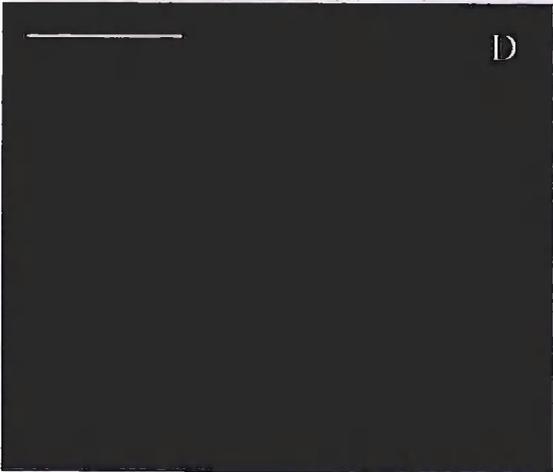
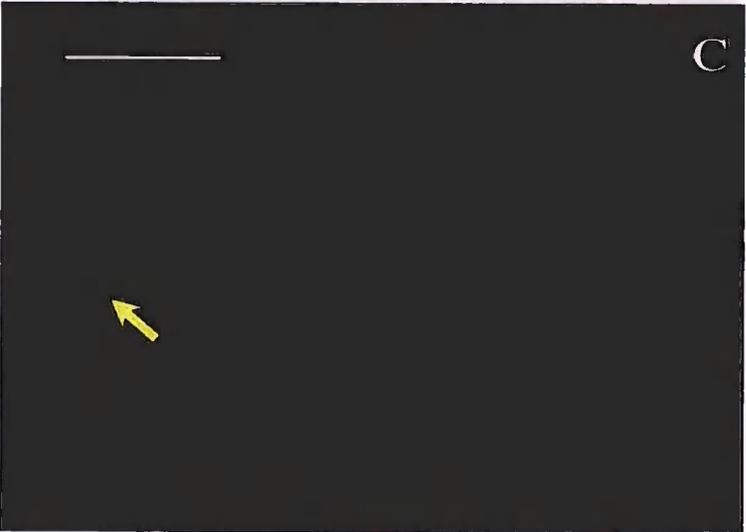


Fig2.6 K15 expression in late anagen and catagen vibrissae hair follicle

A. The overall expression of K15 in early anagen catagen hair follicle. The K15 expression is dramatically decreased and most of it is focused only in follicular basal cells, and in these staining is also asymmetric.

B. The view of the follicle end bulb. K15 staining of epithelial basal cells is discontinuous from the central region, and some of the matrix cells are cytoplasm highlighted K15 positive cells. Dermal papillae are K15 negative.

C. The upper part of proximal region showing K15 staining of follicular basal epithelial cells. The terminally differentiated hair shaft cells are K15 negative (Arrowed).

D and E. In the central region, numbers of K15 positive cells are limited, and the positive cells along the glassy membrane show differences in morphology as well as intensity of K15 expression.

In this section all scale bars shown represent 100 μ m.

The tightly regulated elongated hair shaft cells in the epithelial matrix above the dermal papilla were weakly K15 positive and this result was a repeatable observation (Fig2.6B and C). In the upper part of proximal region, K15 staining continuing in the cell cytoplasm along the glassy membrane and the hair shaft began to lose K15 staining (Fig2.6C).

In the central region, K15 labelling was continuous in basal outer root sheath cells in a single cell line along the glassy membrane on the both sides of follicle. Interestingly, the cellular shape of K15 positive cells located on either side was different at the same horizontal level (Fig2.6D and E). Some variation was seen in the central region; at this stage with some specimens having more limited expression (data not shown).

Telogen

In telogen the unpigmented club hair remains stuck in the hair canal. The dermal papilla had reduced significantly in size, and the whole hair canal also appeared smaller.

Overall expression of K15 remained limited in telogen follicles. Along the ORS, K15 positive expression was still strongest in the central region but the staining extended down more proximally than in catagen follicles, but discontinuously. The asymmetrical distribution of K15 expression was not clearly apparent at this stage, however, the K15 expression was severely disrupted in the distal and lower central regions, and staining was not seen in the epithelium above the follicle end bulb (Fig 2.7A).

In the end bulb, some intensely K15 stained dermal sheath cells were found in the outermost part of the follicular canal (Fig2.7A). DAPI staining illustrated that the new hair shaft had not been formed (Fig2.7A). The upper part of the proximal region formed a single cell line attached to the glassy membrane, and both sides K15 expression ended at nearly same level (Fig2.7B and C).

In the central region, “gaps” appeared in the K15 positive single cell line (Fig2.7D), and the members of K15 negative cell “gaps” increased in the upper part of this

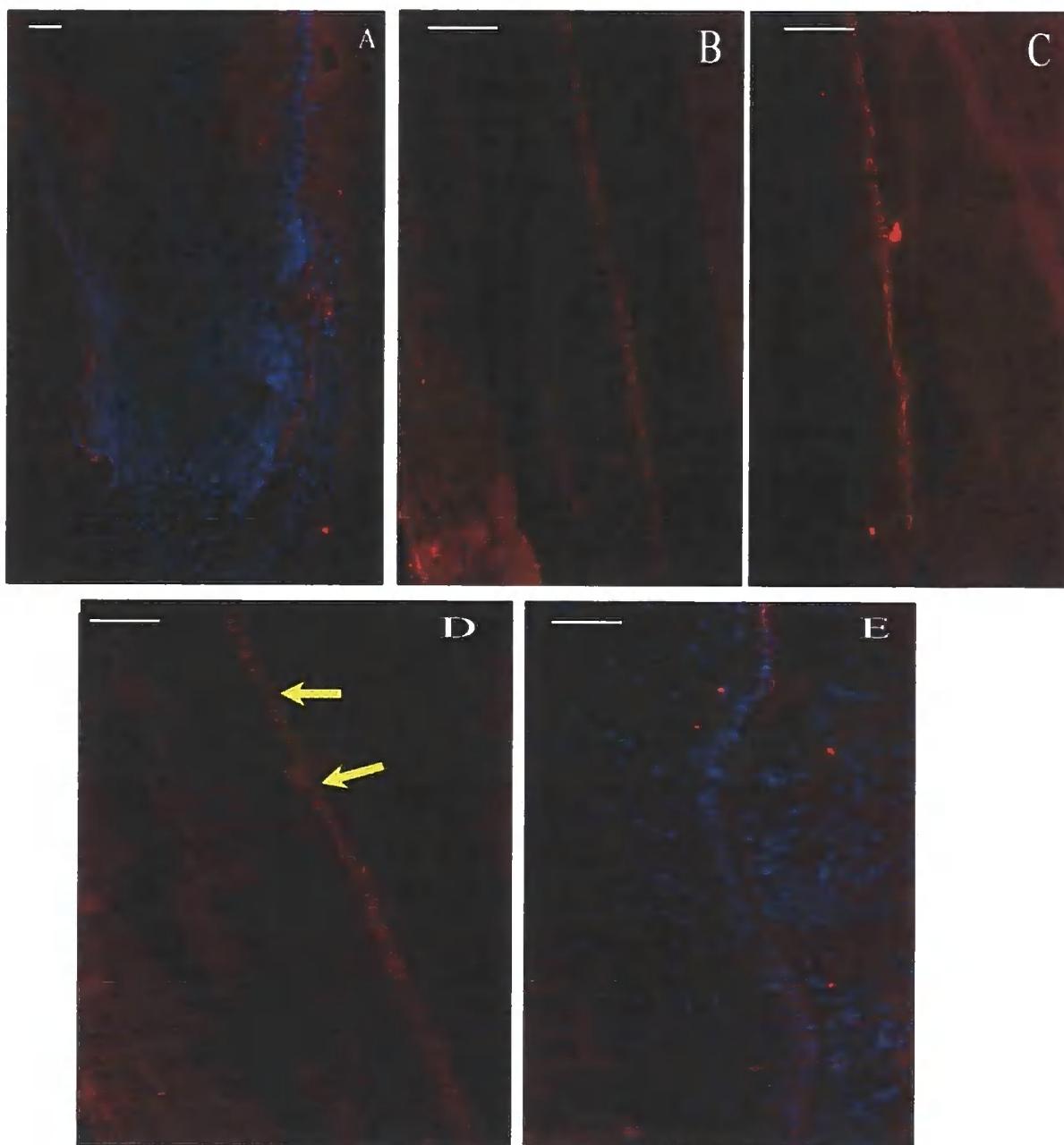


Fig2.7 K15 expression in telogen vibrissae hair follicle

A: Proximal region of hair follicle, K15 positive cells are discontinuously stained along the glassy membrane, and the old fibre is moving upwards.

B and C: the same horizontal level of K15 expression along the glassy membrane.

D: "Gaps" (arrows), as a result of weak or absent staining are observed in central region of the follicle.

E: In the central region, DAPI staining (Blue) showed the absence of K15 positive epithelial basal layer cells and longer "gaps" in labelling.

In this section all scale bars shown represent 100 μ m.

region (Fig2.7E). In contrast to other stages, K15 positive cells in different regions of the Telogen follicles did not have noticeable differences in morphology (Fig2.7 A, B, and D).

Early-Anagen

During the transition between telogen to anagen stages, the new dermal papilla and fibre had not been fully formed *i.e.* dermal papillae were still small in size and the second fibre have not reached the skin surface. The club fibre was positioned away to the side leaving a new canal for the upcoming newly formed fibre.

Overall expression of K15 increased from the distal to the central region. In the proximal part of the follicle, the dermal papilla was still quite small and the hair shaft cells were K15 negative. From lower part of the proximal region to the distal region strong K15 expression was seen in epithelial cells along the glassy membrane, especially in the bulge region which showed 3-4 cell layers of positive cells (Fig2.8A).

In the end bulb region, K15 positive cells were faintly stained along the outer root sheath. The expression pattern was asymmetrical, with more continuous expression on the bulge region side. A cluster of cells in the middle of the end bulb, were K15 positive. Above the end bulb a region of the ORS showed little or no K15 labelling (Fig2.8B). In the upper part of the proximal region, the K15 staining become positive and showed significantly increased in cell numbers, with several cell thicknesses (Fig2.8C).

In the central region, the newly formed hair shafts had not reached the skin epidermis, and DAPI showed clear shaft cell polarity. The K15 positive cells highlighted the cell cytoplasm on the two sides of glassy membrane, and showed a quantitative asymmetry (Fig2.8D). K15 staining was very strong on the bulge region side and, nearly all of cells in this region were K15 labelled, except the old fibre canal, which was hollow (Fig2.8E).

The cells began to show morphological differences depending on their locations (Fig2.8F and G), which was also a feature of mid-anagen follicles.

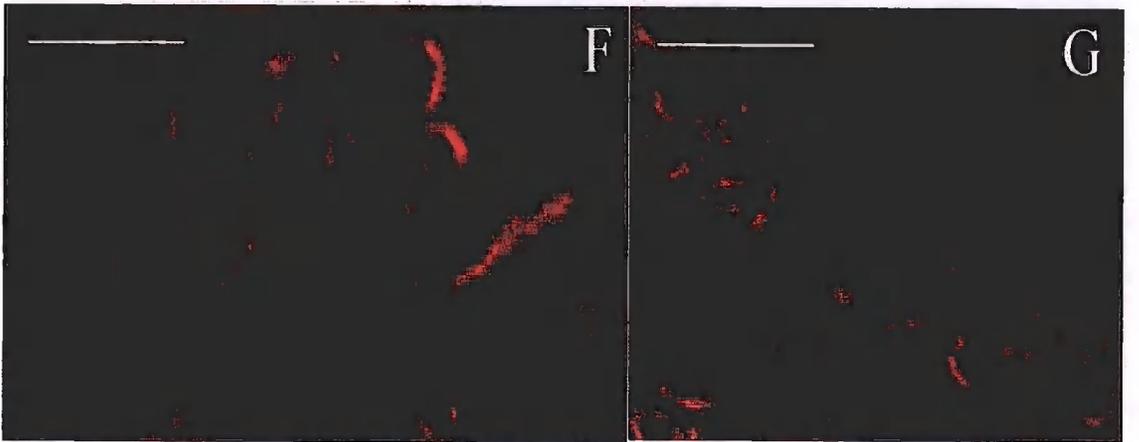


Fig2.8 K15 expression in early anagen rat vibrissae hair follicle

A: The overall view of K15 expression pattern in the early anagen follicle. K15 positive cells have increased significantly from telogen throughout the entire follicle, especially in the bulge region. Both follicular basal cells and outer root sheath cells are K15 positive.

B: End bulb of hair follicle. A small cluster of K15 positive cells is located in the middle of the end bulb, basal cell layers are continuously stained and surround the whole end bulb.

C: The upper part of the proximal region, showing the depth of K15 positive cells increasing, gradually from the basal cell layer along to rest of the outer root sheath cells, and the staining intensity also increasing.

D: In the central region, terminal differentiated hair shaft cells are K15 negative. Although epithelial cells on both sides of the glassy membrane are K15 positive, the nerve entry side has greater numbers of K15 stained cells.

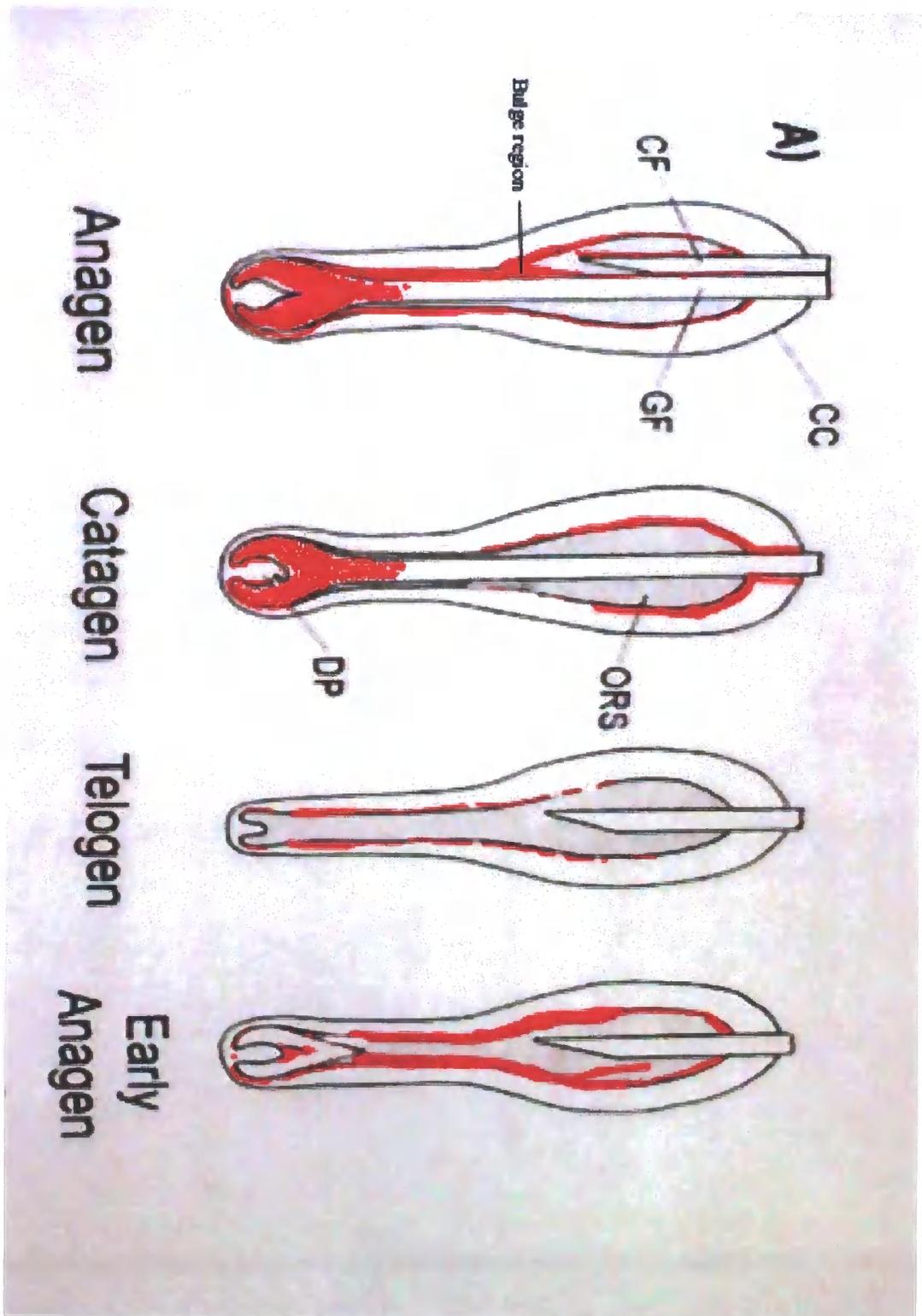
E: In the bulge region of hair follicle, beneath the club hair, K15 staining is strong with the bulge region cells having prominent cytoplasmic labelling.

F: K15 positive cells from the upper proximal region and central region

G: K15 positive cells located in the distal region.

In this section all scale bars shown represent 100 μ m.

Fig 2.9 A diagrammatic summary of K15 expression during the stages of follicle cycle



2.3.2 ID3 expression during the adult rat vibrissae hair follicle cycle

Anagen

Id3 showed strong labelling in epithelial cells inside the epithelial column throughout the entire anagen hair follicle (Fig2.10a). The majority of the dermal cells around the club hair, which was located adjacent to the bulge region in the central region of hair follicle, were also Id3 positive. The dermal sheath cells, outside of the follicle, showed clear Id3 positive staining wrapped around the proximal region of hair follicle. In the proximal region, the large dermal papilla cells were Id3 positive, which highlighted the nuclei of these specialised cells; however, neither the cell matrix nor melanocytes were Id3 positive (Fig2.10b). The Id3 stained dermal sheath cells also showed direct contact with dermal papilla cells in the extreme bottom of hair follicle (Fig2.10b). In the middle of proximal region, the epithelial cells began to express Id3 protein; starting from single cell layer and gradually increasing to 5-6 cell layers thick; with Id3 protein mainly localised in the cytoplasm (Fig2.10c).

The boundary between the proximal region and central region can be artificially drawn by the Id3 cellular translocation changes. In the proximal region, Id3 labelled the epithelial cells' cytoplasm, whereas in the central region, Id3 expression spread whole cells were labelled (Fig2.10 d and e). The bulge region, located beneath the club hair, showed relatively strong Id3 staining both in dermal and epithelial cells (Fig2.10a). Interestingly, the mirror image side, opposite the bulge region showed Id3 positive epithelial cells but limited staining in dermal cells (Fig10.a).

In the distal region of the anagen follicle, Id3 positive cells showed Id3 gathered in the cytoplasm if they were epithelial cells; and in the nuclei if dermal (Fig2.10f and g), with both types showing dramatically decreased cell size in this region.

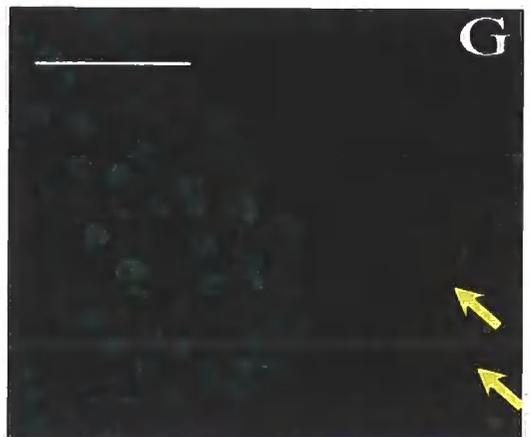
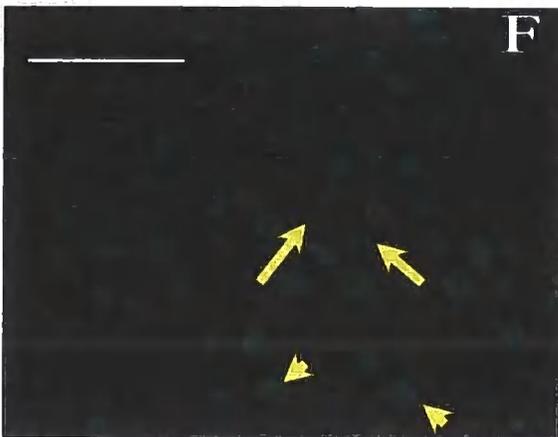


Fig2.10 Id3 staining in anagen vibrissae hair follicle

A: Overall view of Id3 staining. Id3 staining is located in both dermal and epithelial cells.

B: At end bulb of the hair follicle, dermal papilla cells are Id3 positive, and restrict to the cell nuclei.

C: In the upper part of the proximal region, epithelial basal layer cells and outer root sheath cells are both Id3 positive, and these cells have cytoplasm highlighted.

D: In the proximal region of the hair follicle, the Id3 staining highlighting the cytoplasm only.

E: In the central region, Id3 staining highlighting the whole cell.

F: Id3 positive cells in distal region, showing a smaller size, and with some of these positive cells with labelling highlighting the cytoplasm (arrows), and others are showing whole cell staining (arrowheads).

G: Dermal cells in distal region also are Id3 positive, but they only show nuclei staining (arrow).

In this section all scale bars shown represent 100 μ m.

Catagen

The overall Id3 expression during catagen was relatively low. Although the proximal region maintained a similar staining level, the central and distal region revealed significantly decreased labelling, notably in the epithelial cells of the hair shaft canal (Fig2.11a). Dermal sheath cells were still found in the proximal region with clear contact with dermal papilla cells.

In the proximal region of catagen follicles, the enlarged dermal papillae were Id3 positive, and the Id3 protein strictly expressed in the nucleus. Dermal sheath cells also showed nuclear staining, and were in contact with dermal papilla cells at the base of the follicle (Fig2.11b). Catagen dermal papilla cells became more condensed if compared with anagen dermal papillae cells. Matrix cells and melanocytes above the dermal papillae were Id3 negative, as well as epithelial cells (Fig2.11b). In the middle of the proximal region, outer root sheath cells were Id3 positive, and associated dermal cells outside the glassy membrane were nuclear stained (Fig2.11c).

In the central region, increasingly numbers of dermal cells turned Id3 positive. Their distributions was disorganized, but most of showed strictly nuclear labelling (Fig2.11d). In contrast, the rod shaped glassy membrane cells showed cytoplasmic Id3 labelling, and epithelial cells inside the glassy membrane showed Id3 expression in entire cells (Fig2.11d). Interestingly, the terminally differentiated hair shaft cells, in the centre of the hair shaft canal were Id3 negative (Fig2.11d).

In the distal region, the glassy membrane cells showed fainter cytoplasmic Id3 staining and were discontinuously distributed, with several non-labelled “gaps” along the membrane (Fig2.11e). Unlike Anagen follicle, both dermal and epithelial cells in the distal region did not show a noticeable cell size change (Fig2.11e)

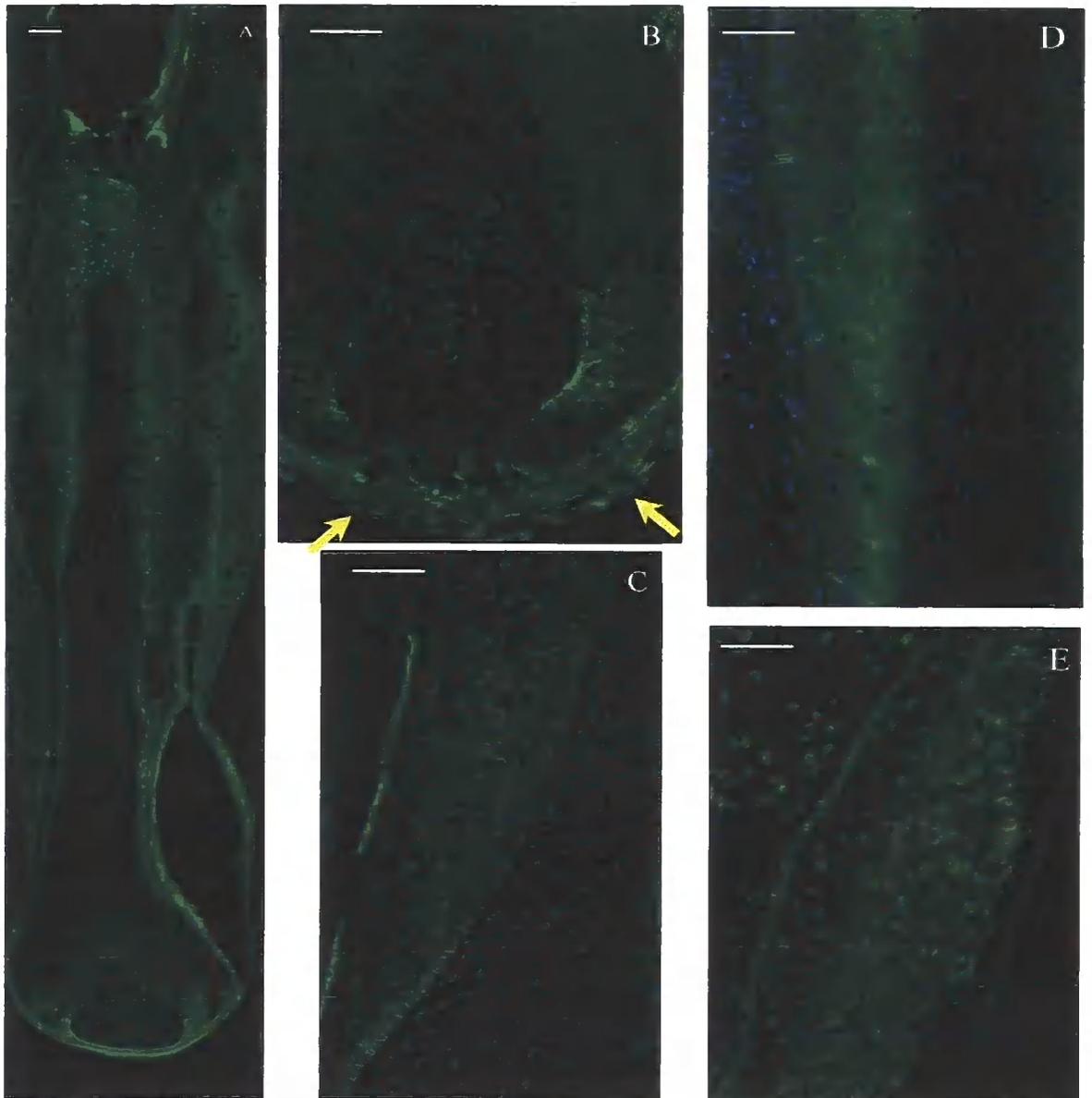


Fig2.11 Id3 staining in Catagen follicles

A: Overall view of Id3 staining, revealing that Id3 stained cells decrease in numbers.

B: End bulb of the hair follicle. Dermal papillae cells are Id3 positive, and dermal sheath cells outside the hair follicle also highlighted with Id3 (Arrow).

C: Middle part of the proximal region. Dermal cells are small and nuclei highlighted by Id3, epithelial cells are relatively large, and Id3 stains their cytoplasm.

D: In the central region of the hair follicle, hair shaft cells, which show elongated shaped are Id3 negative, inner and outer sheath cells show a round shape together with narrower basal layer cells that are Id3 positive cells. Dermal cells out side the hair shaft canal, have nuclear highlighted Id3 positive cells.

E: Epithelial and dermal Id3 stained cells in the distal region of a catagen hair follicle.

In this section all scale bars shown represent 100 μ m.

Telogen

A study of Id3 expression in telogen vibrissae were not extensively performed due to the limited telogen follicle available, but one follicle was immunostained with Id3 antibody.

The overall Id3 expression in telogen hair follicle decreased dramatically from the distal region to the proximal region, and in both epithelial and dermal cells. Dermal papillae decreased in size, but these specialised cells were still Id3 labelled in their nuclei. The Id3 positive cells were confined to around the glassy membrane of the proximal and central regions, higher in the distal region, only some randomly distributed outer root sheath cells were also Id3 positive. The Id3 highlighted dermal sheath cells, which had been observed in the other cycle stages were absent in telogen follicles (Fig2.12a and b).

In the central region, Id3 expression was closely associated with epithelial cells on the glassy membrane, but dermal sheath cells were Id3 negative. The Id3 staining intensity reduced along follicle basal cell layers (Fig2.12a and c).

In the distal region, the epithelial basal layer cells were completely lost their Id3 expression, but Id3 expression found in some epithelial outer root sheath cells located inside the hair shaft canal. However, no dermal cells were found to express Id3 (Fig2.12a).

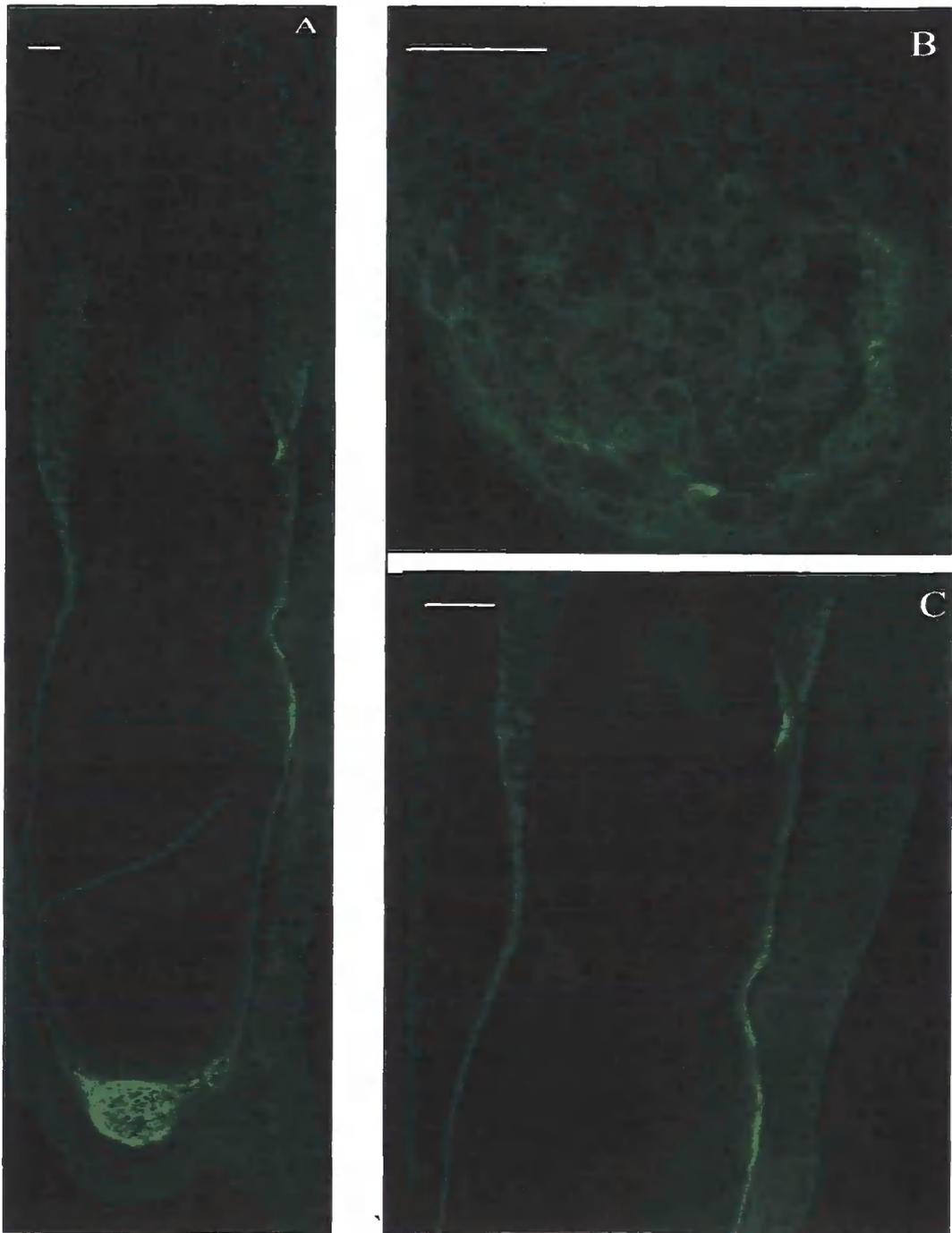


Fig 2.12 Id3 staining in Telogen vibrissae hair follicle

A: Overall view of Id3 staining in a telogen follicle. Id3 cells labelling has decreased dramatically.

B: The end bulb of a follicle, showing a dermal papillae that has become smaller in size, and dermal papilla cells are still Id3 positive.

C: In the central region, Id3 proteins are expressed in the follicular basal epithelial layer cells only.

In this section all scale bars shown represent 100 μ m.

Early-Anagen

The overall Id3 expression in early anagen follicle showed a marked increase from mid-anagen and catagen follicles, with expression in previously unlabeled locations. It was evident that both epithelial and dermal cells throughout the entire early anagen follicle were strongly Id3 labelled.

In the proximal region, the angle of sectioning through the follicular end bulbs was not ideal so that partial dermal papillae were present in this specimen (Fig2.13a). The dermal papilla was still small in size, and the cell nuclei were stained with Id3 antibody (Fig2.13b). Dermal sheath cells, with strong Id3 fluorescence, surrounded the proximal region of early anagen follicles (Fig2.13a), and were directly associated with dermal papillae cells at the extreme end of hair bulb. Epithelial basal cells, were cytoplasmically labelled with Id3, and were visible from the proximal region to distal region of follicle (Fig2.13a).

In the central region, both follicular basal layer cells epithelial cells and 3-4 layers of outer root sheath cells showed Id3 expression particularly in the cell cytoplasm (Fig2.13c). The basal layer and outer root sheath cells were morphologically different (Fig2.13c) as the basal cells were elongated and outer root sheath cells were rod shaped. In the middle of the central region, the club hair was pushed on the top of the nerve entry side, and nearly all the outer root sheath cells were cytoplasmically highlighted Id3 positive (Fig2.13d).

In the distal region, the newly formed hair shaft had not reached the outer skin epidermis, since some epithelial cells still appeared in the hair shaft canal in the distal region, and those hair shaft cells were Id3 negative (Fig2.13e). Unlike the central and proximal region epithelial cells, those in the distal region epithelial cells showed Id3 proteins probably located more in the cell nuclear envelope (Fig2.13e). Under higher magnification, Id3 highlighted several small loops, which surrounded the DAPI stained blue nuclei (Fig2.13f). Furthermore, double staining with K15, which labelled the cytoplasm of basal cells in the distal region, together with DAPI staining, gave a clear

illustration of Id3 bound in or around the nuclear envelope (Fig.2.13g).

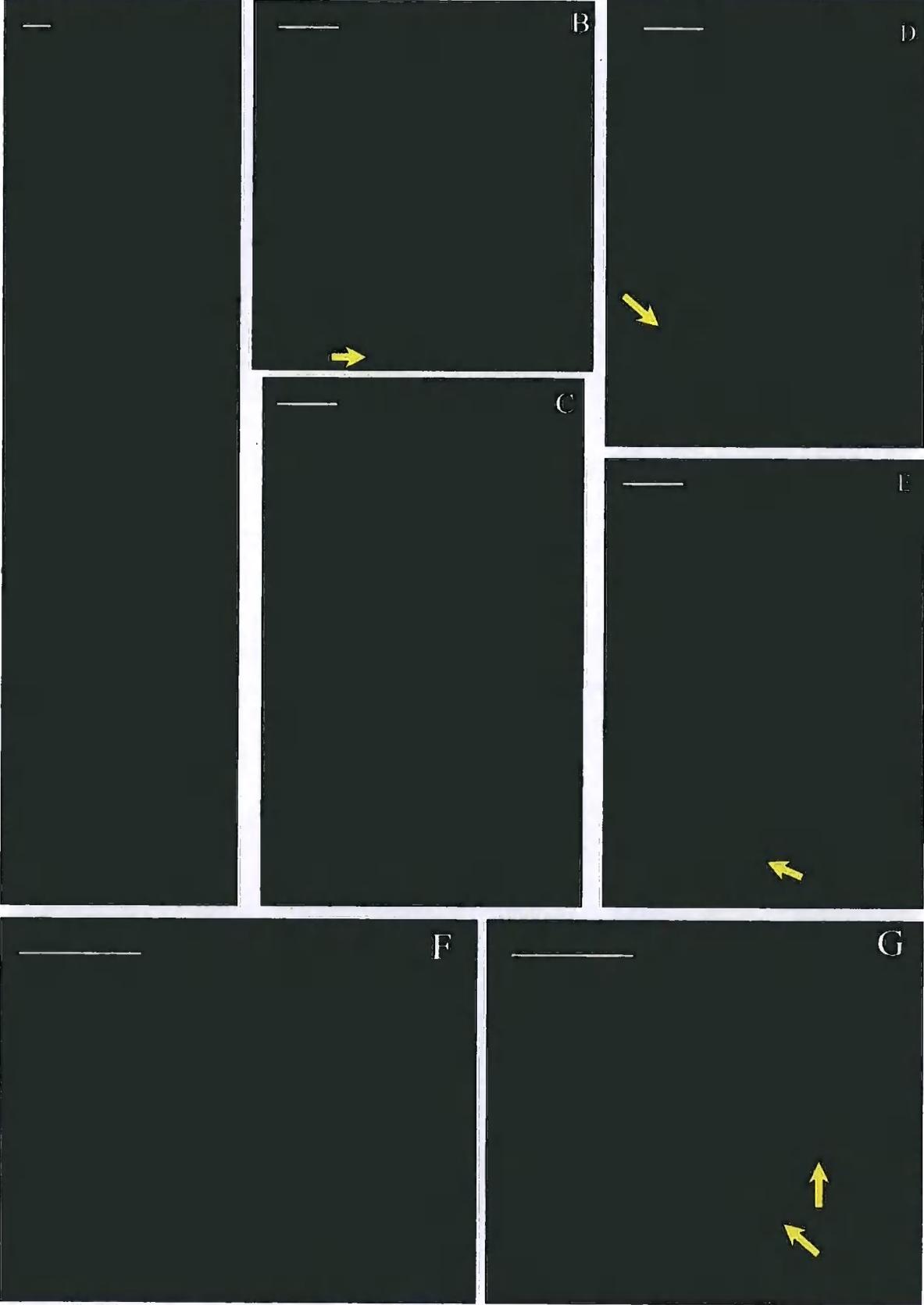


Fig2.13 Id3 staining in Early anagen rat vibrissae hair follicle

A: Overall view of Id3 staining in an early anagen hair follicle. The club hair has moved into the central region, and numbers of Id3 positive epithelial cells have increased significantly inside the hair shaft canal. Dermal cells along the glassy membrane are also Id3 positive.

B: The end bulb of follicle. Newly formed dermal papillae with Id3 highlighted nuclei. The epithelial basal layer cells surrounding the dermal papillae are Id3 positive, together with outside follicular dermal sheath cells (Arrow).

C: In the upper of proximal region, outer root sheath cells also show Id3 staining in their cytoplasm, and Id3 staining is asymmetric in quantity on the two sides of the follicle.

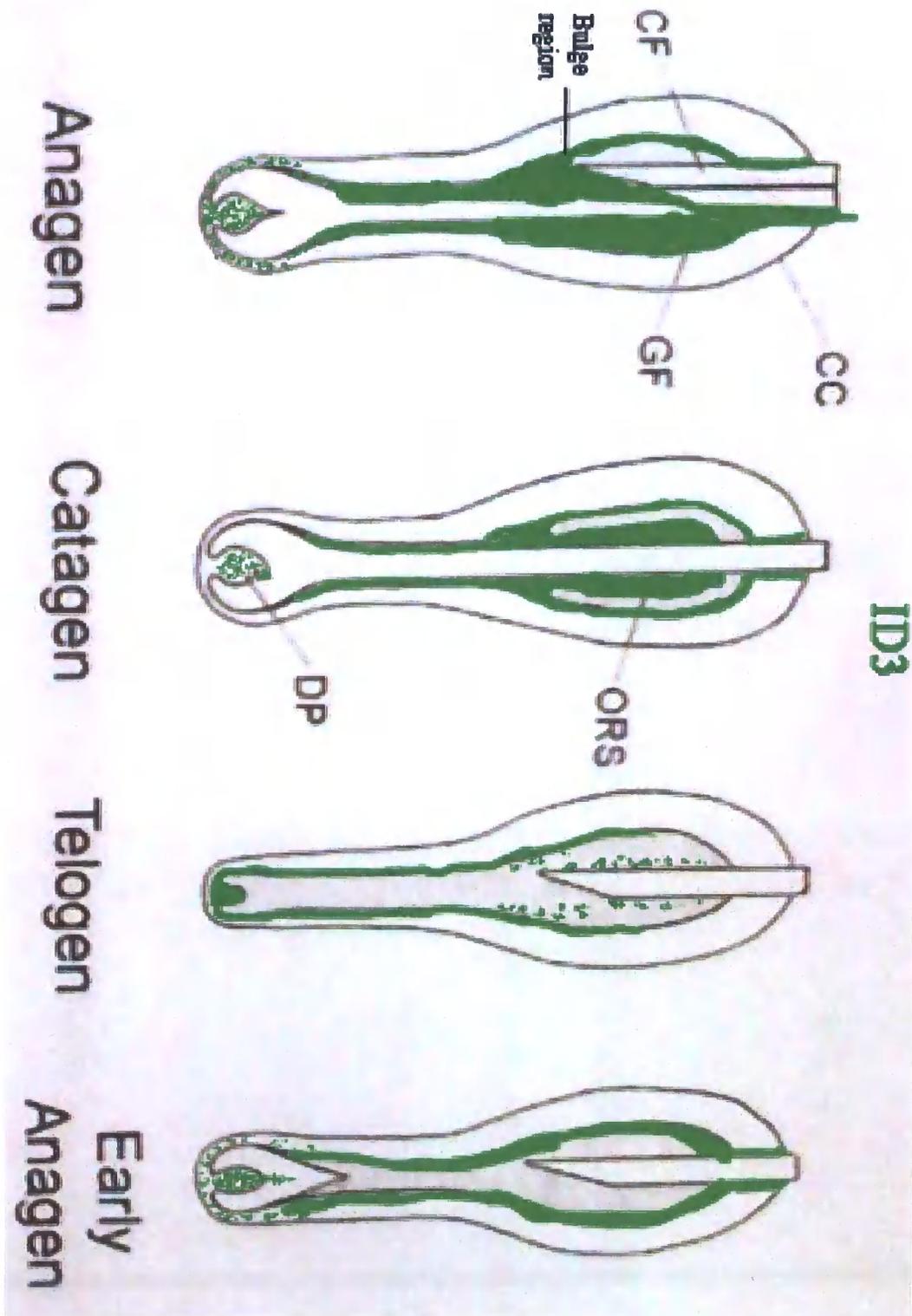
D: In the bulge region, Id3 highlights epithelial cells, and a clear dermal cell line along the glassy membrane (Arrow).

E: Distally, Id3 staining mainly surrounds the nuclei. The terminally differentiated hair shaft cells are Id3 negative (Arrow).

F: Distally, at higher magnification, Id3 proteins wrap tightly around the nuclei (DAPI stained Blue), and in contrast with follicular basal epithelial layer cells.

G: Id3 double staining with K15, K15 usually occupies the cytoplasm, DAPI stains the nuclei and ID3 is only present in the area around the nuclei, and not the cytoplasm (Arrow).

Fig 2.14 A summary of results as a diagram, highlighting regions in which Id3 expression occurs during the stages of the follicle cycle.



2.3.3 Directed differentiation of hair follicle end bulb cell cultures

The differentiation assay was applied to primary cultures of new born rat hair follicle end bulb explants.

After 14 days incubation of rat new born end bulbs, cells extended out from the end bulbs to a confluency of 30-40% (Fig2.15a). These explanted cells showed fibroblasts cell type morphology, and the cell size was relatively small (Fig2.15a). Osteoclastogenesis signals M-CSF and TRANKL were both applied to the 14-day explanted end bulb cultures- that had been periodically checked the appearance of cell types and numbers in primary cell culture.

There was no significant change in cell density after treatment with the osteogenic signals, but after 14-days of treatment, comparatively “huge” cells were observed in dishes, surrounded by fibroblasts-like cells (Fig2.15b). These huge cells showed multiple, large nuclei, and a rounded shape, with smooth edge. After 23 days, continue treatment with osteogenic differentiation signals, the differentiated cells increased in size, and numbers. Furthermore, the edges of these cells were no longer smooth multiple small projection-shaped extrusions appeared attached to one side of these differentiated cells (Fig2.15c and d).

The treatment of the mouse newborn hair follicle end bulb, showed negative results. No huge or differentiated cells were found in M-CSF and RANKL signal treated populations after 30 days (Data not shown).

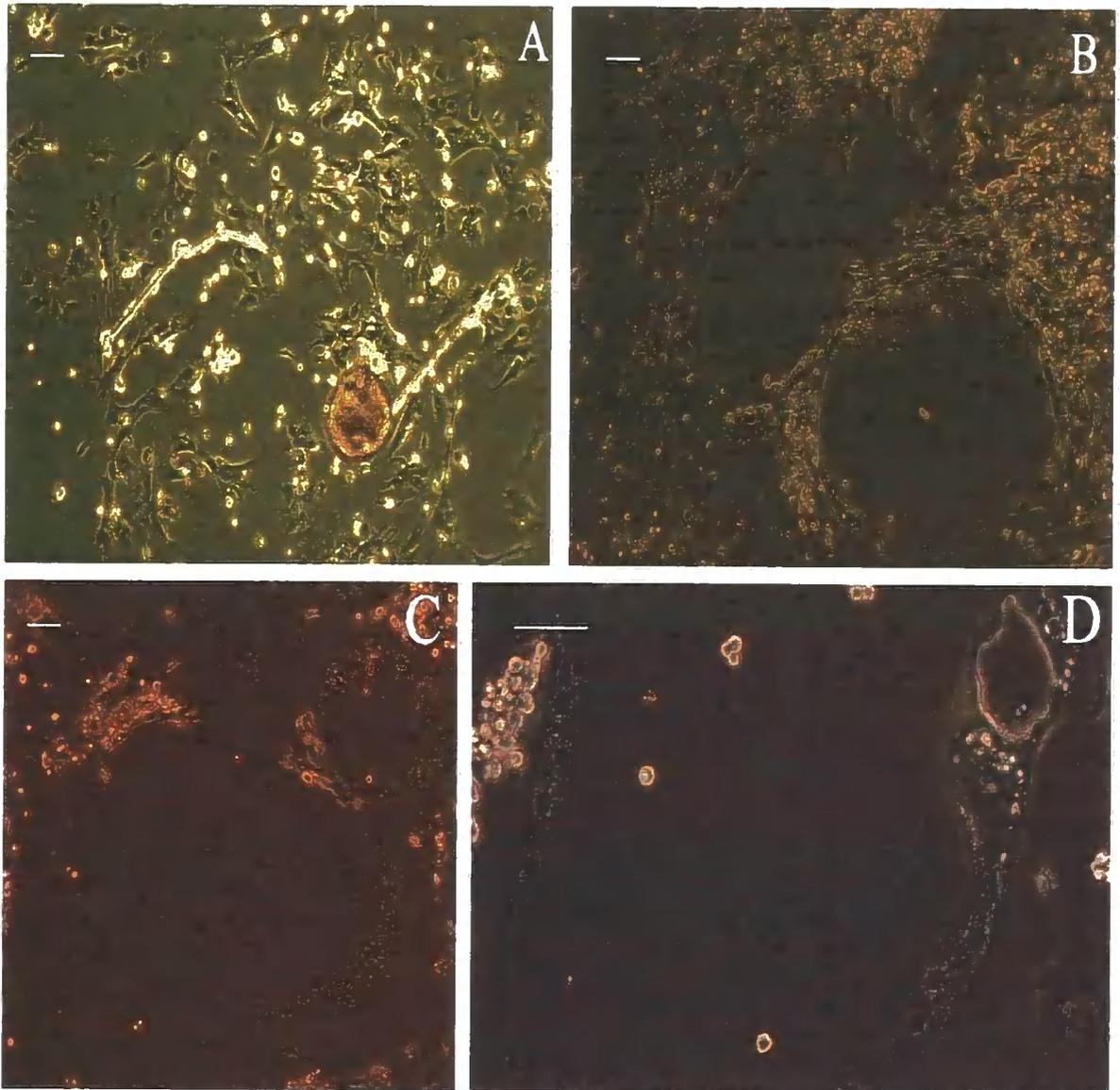


Fig 2.15 End bulb cell culture and differentiated osteocalsts

A: 14 days incubation of rat newborn end bulb cells. The cells covered 30-40% of the culture substrate.

B: After 14-day treatment with RANKL and M-CSF, osteoclasts-like cells were observed.

C: 23 days after osteogenesis signal treatment, osteocalsts have increased in size.

D: Higher magnification of Osteocalsts cells.

In this section all scale bars shown represent 100 μ m.

2.3.4 Directed differentiation of hair follicle dermal papilla cell culture

Similar to the primary newborn end bulb cell cultures, primary adult rat follicle dermal papillae were also incubated for 14 days, until the dermal cells growing out from dermal papillae reached 30-40% confluence (Fig2.16a). M-CSF and TRANKL were then added into the dermal papillae cell cultures, and periodically observed.

The presence of the “giant” cells was detected after 11 days treatment with osteogenic signals (Fig2.16b), which was slightly earlier than in primary newborn end bulb cell culture. This new cell type was as previously described above in end bulb cultures, large in size with multiple cell nuclei, and with smooth cell edges (Fig2.16b and c). The only difference was that the projector shaped extrusions were not found in the dermal papilla explant cell cultures, even after 30 days treatment with osteogenic signals (Fig2.16 d and e).

Mouse bone marrow mesenchymal cells was cultured and treated with osteogenic signals as a positive control. After 7 days treatment, inactivated osteoclasts were observed; 11 days after treatment, activated osteoclast were observed (Fig2.16f). Considering the notable morphological and size differences between dermal cells and osteoclasts, confirmation of these results required no TRAP assay, but one was still performed on the differentiated cells for confirmation, which gave a positive result (Data not shown).

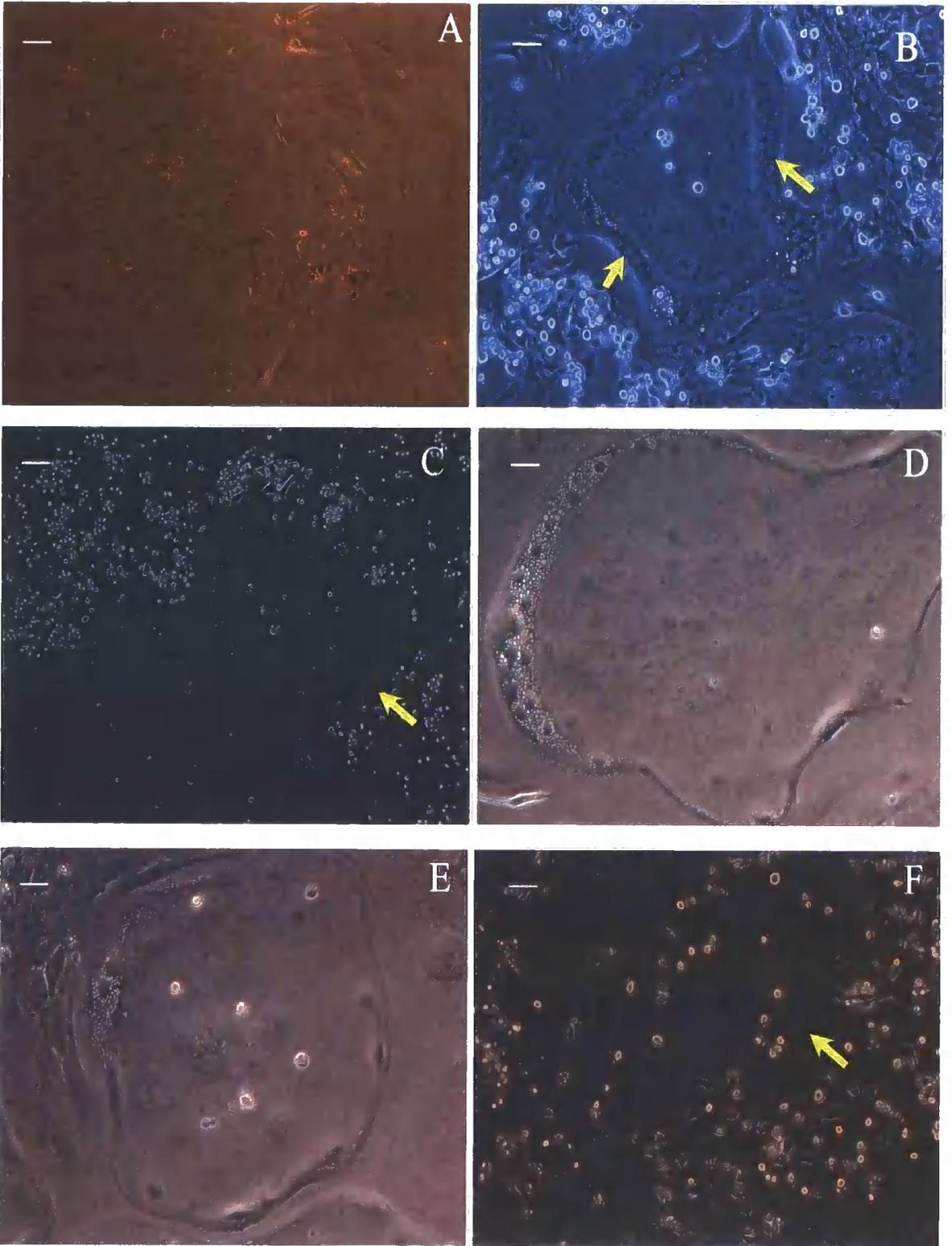


Fig2.16 Dermal papillae cell culture and osteoclast differentiation

A: Explant dermal papillae cell culture after 14 days. Cells were at 30-40% confluence.

B: 11 days after dermal papilla cell culture treating with RANKL and M-CSF. An Osteoclasts-like cell shown in the centre has multiple nuclei (arrows) distributed around the cell edge.

C: Another image showing osteoclast-like cells (Arrow).

D: 30 days cultured dermal papilla cells after treating with osteogenesis signals. Cells have increased in size, and nuclei have moved to the side of the cell.

E: Osteoclasts in 30-days osteogenesis treatment media, no projections are seen around the cell edge.

F: Bone marrow cells, as positive control, left in osteogenesis media over 11 days, a typical osteoclast is shown (arrow).

In this section all scale bars shown represent 100µm

Chapter 3

K15 and Id3 expression in regenerating adult rodent vibrissae hair follicle

3.1 Introduction

3.1.1 Regeneration process in forming complex structure

In biology, regeneration is an organism's ability to replace body parts. Invertebrates such as sponges, hydra, planarians and starfish, can all regenerate the entire organism from a conglomeration of cells or small pieces. Some degree of regeneration occurs in many, if not all-vertebrate embryos. Among adult vertebrate urodele amphibians, newt and salamanders, are able to regenerate their amputated tail and limbs.

The regeneration process in the axolotl and newt has been extensively studied (Mullen, 1996, Bryant, 2002). After amputation, the epidermis migrates to cover the stump in less than 12 hours, forming the apical epidermal cap (AEC). Over the next few days, the blastema forms lying beneath the stump tissue and within this tissue the pattern formation genes- such as HoxA and HoxD are reactivated, just as they were when the limb was formed in the embryo. The distal tip of the limb is formed first in the blastema, and the intermediate portions of the pattern are filled in during growth of the blastema by the process of intercalation. Motor neurons, muscle and blood vessels grow with the regenerating limb and re-establish the connections that were present prior to amputation. The time that this entire process takes varies according to the age of the animal, ranging from about a month to around three months in the adult until the limb becomes fully functional.

Recently, as understanding of stem cells and regenerative science has increased, it is believed that stem cells, progenitor cells or even reprogrammed other cells might take part in the regeneration processes, The adult cells or transient progenitor cells could de-differentiate into a stem cell state similar to embryonic cells (Ferraris, 2000).

Therefore, understanding the role of progenitor cells in regeneration activities could lead to better treatments for individuals with nerve injuries, missing limbs, and damaged or destroyed organs.

However, regenerative abilities are rarely exhibited in mammals with a few exceptions including antlers, fingertips, minor skin injury and hair follicles.

3.1.2 Hair Follicle regeneration

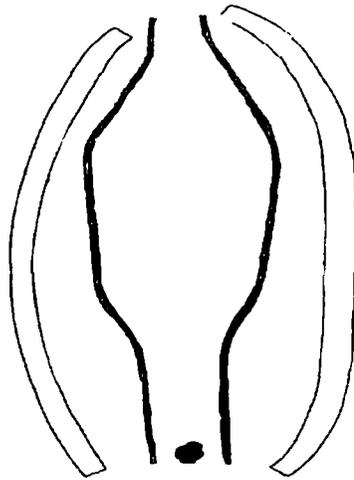
Pioneering studies by Oliver in the 1960s (1966a, b) demonstrated the remarkable recuperative power of the hair follicle after experimental ablation. His work showed the inductive properties of the dermal papilla and the regenerative capacity of vibrissa follicles after a major insult. Removal the lower third of the vibrissae follicle stimulates the remaining follicle tissue to participate in the regeneration process, and finally to form a completely restored lower follicle structure, including a dermal papilla and epithelial matrix. Oliver (1966a, b) proposed that the dermal sheath cells are the source if the regenerated dermal papilla. It is generally thought that the whiskers regenerate in rat vibrissae follicles only if amputation is restricted to approximately the lower third of the follicle is situ. Regeneration of hair bulbs was observed in follicles, the lower third of which had been severed, but when the lower half of the follicle was removed, no regeneration occurred (Horne.K.A, 1986). However, one group has reported the regeneration of small follicles in the upper regions of vibrissae follicles after higher level amputation (Matsuzaki.T, 1996). Jahoda *et al* (1992) used microsurgical amputation of the lower follicle bulb to observe changes to extracellular matrix and the basal lamina during the sequence of events leading to reconstruction of a fibre-producing hair follicle. The results were detailed by immunohistology and electron microscopy. The initial response was essentially found to be a wound reaction, in that hyperproliferative follicle epidermis quickly spread to below the level of amputation, associated with downward movement of mesenchymal sheath cells. Once a new basal line of epidermis and a complete basement membrane were established, a

new papillae was formed. The authors subsequently summarized these regenerative activities into four stages, indicated in the figure below (Figure 3.1).

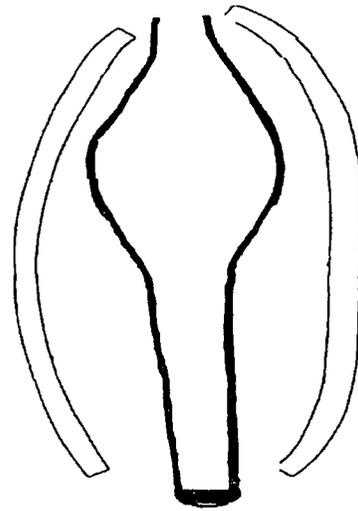
The result show that dermal sheath cells in the lower dermal sheath contributed to regenerative properties that may be lacking in the upper two thirds. This also consistent with alpha-smooth muscle actin proteins expression, which is evident in the lower third of the dermal sheath but absent from the upper part of the follicle. McElwee *et al* used GFP-expressing mouse vibrissa follicle cells to show both cultured dermal papillae and peribulbar dermal sheath (the proximal region dermal sheath) are equally capable of hair follicle induction after transplantation (McElwee 2003). In addition, the lower portions of the tissue have also been used for follicle induction and cell culture experiments.

Although the stages of hair regeneration have been studied in detail by Jahoda *et al* (Jahoda, 1992), many aspects of the regeneration process have yet to be described at the cellular and molecular levels. More specifically, the precise source of the replacement cells in the regenerating vibrissa remains unclear, It has been proposed the dermal sheath cells is the origin of the new dermal papilla cells (Oliver, 1966b Jahoda, 1992). More recently, Mayumi Ito *et al* (Ito M, 2007) used *Tg(Krt1-15-cre/PGR)22Cot; R26R* transgenic mice, which permanently express *LacZ* in bulge cells and in all progeny of labelled bulge cells to demonstrated that non-hair-follicle bulge cells were the primary source of regenerated follicles in a wound healing model. This research group also utilized the *Krt1-15-CrePR; R26R* transgenic mice, which approximately 70% of bulge and 50% of non-bulge epidermal cells expressed *LacZ* before wounding, to indicate that new follicles originated from cells outside of the hair follicle stem cell niche. However, the exact origin of stem cells could give rise to a new hair follicle awaits elucidation.

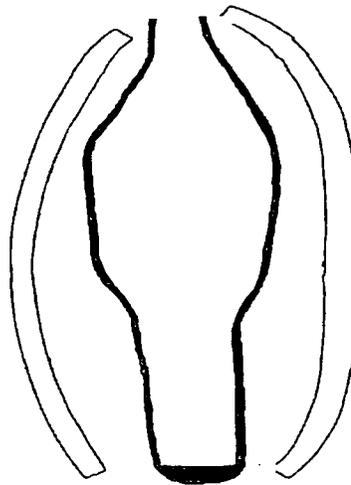
Pre-Stage



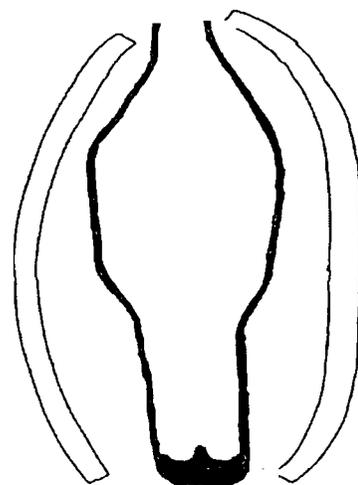
Stage 1



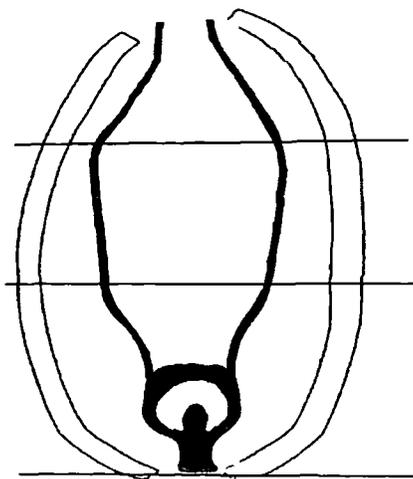
Stage 2



Stage 3



Stage 4



Distal Region

Centre Region

Proximal Region

Figure 3.1 The stages of regeneration

Stage 0: with the whole bulb structure and hair shaft removed, the remaining follicular epithelium is left as a hollow cylinder, still separated from encircling lower mesenchyme cells by the glassy membrane.

Stage 1(0-6 days): epidermal cells fill up the space inside the hair shaft forming a solid column of cells whose base is irregularly shaped and which pushes out to lie below the end of the glassy membrane and beneath the original level of amputation. Dermal sheath cells become active, and move to surround the base of follicle.

Stage 2(7-10 days): the follicle epidermis moves up and regularizes, so that its base forms a level surface within the confines of the glassy membrane, which now extends below the bottom of the epidermis as an overhanging “skirt” structure. Dermal sheath cells accumulate beneath the epidermis.

Stage 3 (11-16): papilla formation takes place with progressive independent of the epidermis by dermal cells still enclosed within the membranous skirt.

Stage 4 (17-30): the new dermal papilla increase in size, as hair differentiation becomes apparent and a fibre is produced. The extension of glassy membrane beneath the new fibre producing follicle bulb gradually disappears.

3.1.3 Keratins and ID proteins in wound healing process

Keratin genes are very useful tools for studying epithelial differentiation, and departure therefrom. In normal skin tissue, K16 and K17 genes are expressed in specific subsets of epithelial cells within epithelial appendages but generally excluded from interfollicular epidermis (McGowan, 1998), K15 proteins are mostly located both in the epidermis and also the follicle epithelium including the bulge region (Cotsarelis, 1990).

After wounding, restoration of epithelial continuity is defined as wound closure, and is achieved by the combined action of two distinct mechanisms: keratinocytes migration into the wound site, and the contraction of specialized fibroblasts in the granulation tissue underneath the wound site.

Examination of the wound edge tissue at the ultrastructural level has revealed that keratinocytes undergo progressive hypertrophy and changes in cytoarchitecture during this initial period. Thus, keratinocytes located in the inner half of the epidermis adopt a polarized and elongated morphology, with many individual cells sending cytoplasmic processes towards the wound site (Odland, 1968; Paladini 1996). One of the markers of activated epidermal keratinocytes is the expression of keratin 6 and K16. In human keratinocytes, K6 is activated by cytokines and growth factors (Komine *et al*, 2001). It has been proposed that cytoskeleton changes that include the expression of K6 and K16 keratin filaments provide plasticity and flexibility while maintaining the resilience of the intracellular scaffold that is important for migration in mouse (Wong, 2003).

The presence of ID proteins in the epidermis and dermis of skin both human and murine have been reported (Langlands K 2000, Pammer J 2004); however, there is limited information about the expression of ID proteins role in wound healing processes. Rotzer *et al* recently showed that Id1, Id2 and Id3 are all strongly down-regulated in keratinocytes *in vitro* and *in vivo* by activin, which plays a crucial role in skin morphogenesis and wound healing. Furthermore, the authors found that in 3-D organotypic cultures, Id1 over expressing keratinocytes formed a hyperthickened

and disorganised epithelium that was characterised by enhanced keratinocyte proliferation, abnormal differentiation and an increased rate of apoptosis. Their results showed suggested that ID proteins play a role in the regulation of epidermal homeostasis (Rotzer 2006).

3.1.4 Aims

The regenerative properties of vibrissa follicle are well established but many questions remain about the control of this process at both the cellular and molecular level (Oliver 1966, Jahoda 1992). My projects used Immunohistochemistry to illustrate the expression of K15 and ID3 during the regenerating process. K15, as a putative epithelial stem cell marker, applied here was intended to study and help describe the epithelial stem cells activity in regenerating hair follicles. Id3, showed an changing expression pattern in normal hair follicle cycle stages as described in the previous chapter, suggesting it is worthy of further study. Investigate the Id3 protein expression was intended to compare and contrast this protein in normal and experimental regeneration since its expression changes may be closely associated with control of cell differentiation and proliferation.

3.2. Materials and Methods

3.2.1. Animals

Vibrissa follicles were isolated from PVG rats of either sex (6-10 months) as described in section 2.2.1., chapter 2.

3.2.2. Surgical procedure for end-bulb amputation

End bulb amputation was performed on up to 6 vibrissae follicles in one mystacial pad of 10 PVG rats of either sex, aged between 4 and 10 months. The method was essentially that of Oliver (1966a). Animals were anaesthetised by an intramuscular injection of Hypnorm (80 μ l) (Janssen Animal Health), immediately followed by an intraperitoneal injection of 75 μ l Valium (5mg/ml). The vibrissa follicles were then exposed by cutting an L-shaped incision around the anterior and ventral edges of the mystacial pad (Fig3.1a) The skin flaps were then gripped with forceps to explore the follicles. Using fine forceps and spring scissors, connective tissue was cut away and to explore the end bulbs of follicles (Fig3.1b, c). Individual hair follicle end bulb were then amputated and discarded, before the vibrissae fibres were plucked from the follicle (Fig3.1d). The mystacial pads were then sutured back in place and the rats left to recover to different time lengths before the regenerating hair follicles were biopsied. (This operation is performed by Kristian Sorenson).

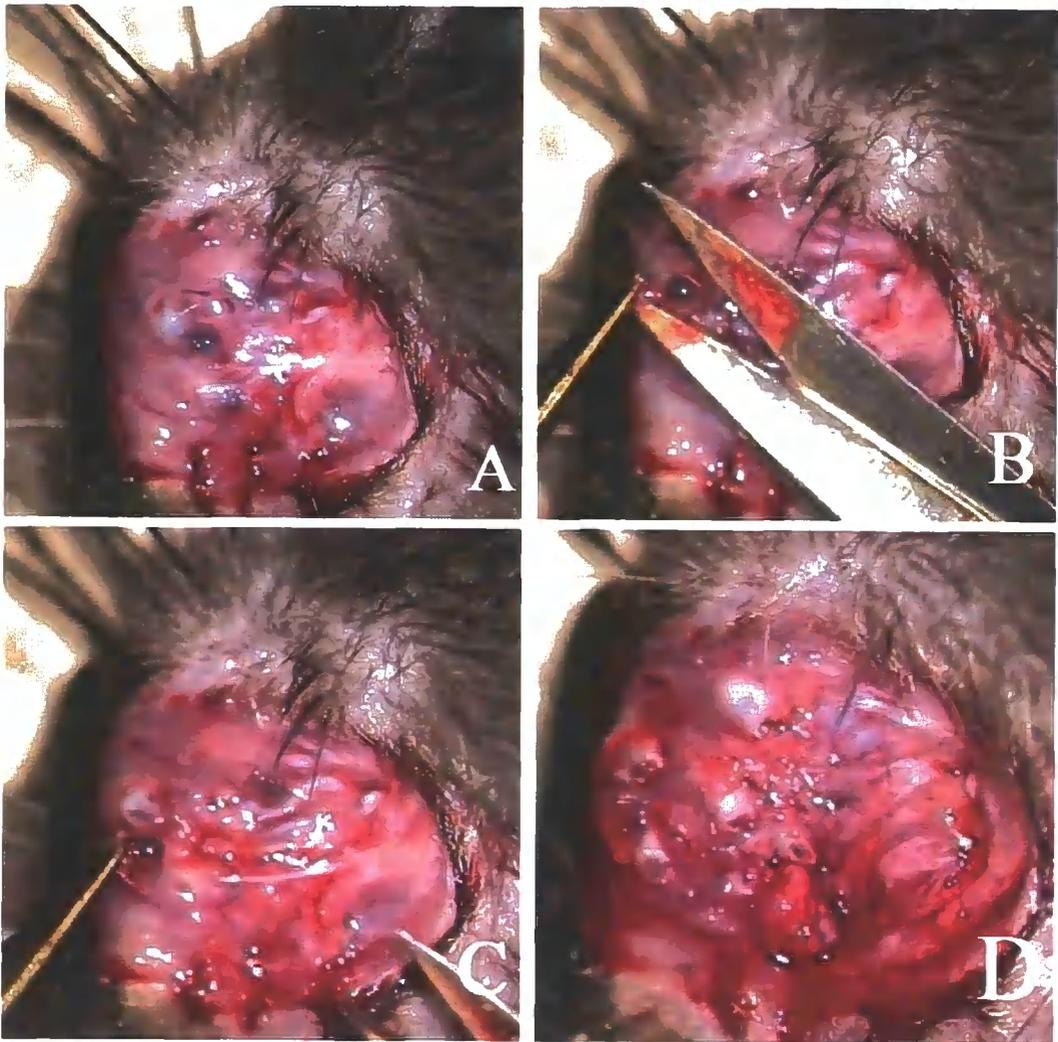


Fig3.2 Phases of micro-surgical rat vibrissa follicle end bulb amputation

(A) An L shaped incision has been opened, and hair follicles are seen embedded in connective tissue. (B and C) Connective tissue has been cleaned away around the follicle base, and end bulbs of vibrissa follicles are cut. (D) Around six follicle end bulbs were removed in each mystacial pad.

Table 2.2 Total numbers of specimens of regeneration hair follicles stained for ID3, K15, Integrin, PPAR λ , α -smooth muscle actin and P63 expression.

Tissue Sample	K15	ID3	Integrin
Day 1	2	0	1
Day 4	3	3	1
Day 6	2	2	0
Day 8	4	4	1
Day 10	3	3	0
Day 11	3	3	0
Day 14	3	3	0
Day 21	3	3	0

3.2.3. Freezing down regenerating follicles

Follicles were dissected out of the mystacial pad of the rat 1, 2,4,6,8,10,11,14 and 21 days after the amputation surgery; and the ear wound areas were excised 3,5,8,11 and 14 days after the injury.

3.2.4. Immunostaining

Staining was performed according to the methods described Chapter 2, 2.2.3

3.3 Results

3.3.1 General Observations

Follicle regeneration proceeded as described by Oliver (1966) and Jahoda *et al* (1992), with no significant differences observed in the majority of samples. The hair follicle regeneration stages that Jahoda *et al* (1992) described will be used as a chronological order to present the results obtained from experiments. The first stages in follicles regeneration results in epithelium cells filling the hollow tube created by removal of the fibre at the time of amputation. In some follicles biopsied after 24 hours, this space had not yet been occupied and such follicles will therefore be referred to as being pre-stage 1.

However, there were some variations in each stage, and the regeneration rate of each individual follicle was different.

3.3.2 Keratin 15 in Regenerating hair follicles

Pre-stages 1 (Day1)

All follicles at pre-stage 1 had been biopsied 24 hours after amputation of the end bulb. The epithelial cells had not yet filled the space created by removal of the hair fibre and there was a significant quantity of disrupted tissue remnants, particularly in the proximal and centre of hair follicles. In the proximal regions epithelial cells were disorganised and the majority of them were keratin 15 negative (Fig3.3A).

In the central section of the follicle the basal layer epithelial cells of the outer root sheath were adherent to the glassy membrane and this single cell layer was labelled with K15. This layer of k15 positive cells was particularly strongly fluorescent just above the proximal region where the epithelial column began to widen (Fig3.3A). Additionally the two sides of basal layer ORS cells along the glassy membrane expressed K15 with spatial asymmetry. It was clearly seen that on the side of the

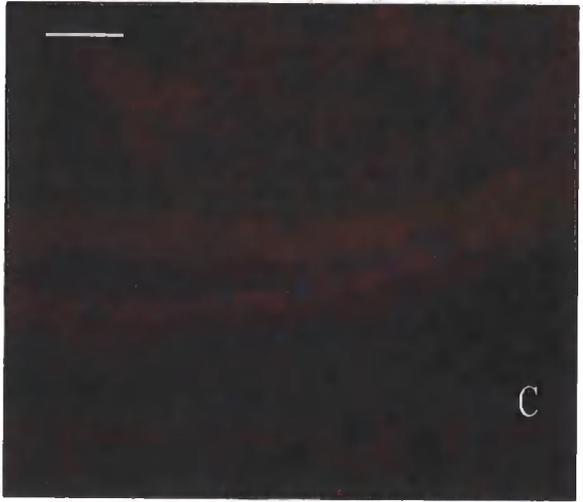
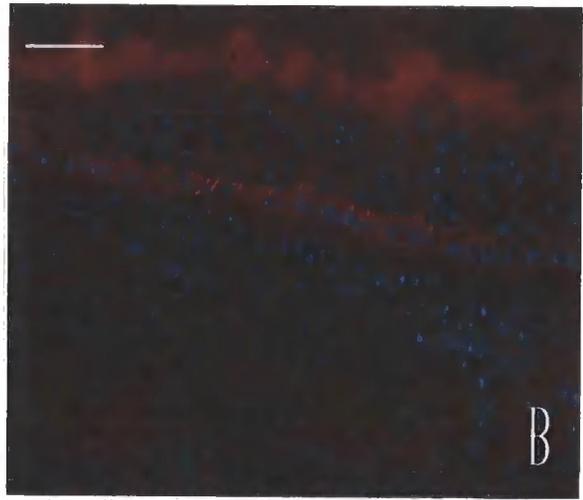


Figure 3.3 K15 and Integrin α 6 β 1 incorporation in pre-stage regenerating vibrissae follicles.

Pre-stage 1 follicles immunostained with monoclonal K15 (A, B and C) or a monoclonal antibody against Integrin α 6 β 1 (D and E)

(A) Overall view of K15 expression pattern of pre-stage regenerating vibrissae follicles. The positive K15 cells along two sides of glassy membrane show asymmetry, and arrows shown the interruptions gap along the basal layer.

(B) In the centre of the hair follicle, K15 highlights the cytoplasm of cells along the glassy membrane on one side, cell nuclei in DAPI stained (blue) cells overlapped with nearly all of K15 positive cells, and nuclear staining shows that cells in the suprabasal layer and inside are K15 negative

(C) On the other side of hair follicle in the central region of hair follicle, the K15 staining is discontinuous and interrupted

(D) Integrin α 6 β 1 expression continues from the proximal end to the centre of each follicle along the glassy membrane. In this region its expression pattern is similar to K15. It clearly illustrates that the integrin expression is asymmetric between the two sides, with more labelling on the nerve entry side.

(E) Integrin α 6 β 1 staining highlights the cytoplasm of cells around the proximal end of follicle towards to the amputation site, where positively labelled cells have gathered to form a cluster which has detached from the glassy membrane.

In this section, all scale bars shown represent 100 μ m.

follicle corresponding to main the entry point of the nerve, expression along the glassy membrane was more extensive and more continuous, whereas labelling on the opposite side was more restricted and with more gaps (Fig3.3A). Higher magnification and the use of DAPI staining confirmed these observations. DAPI stained cells overlapped with all the k15 positive cells on the basal layer of the “nerve entry” side of the follicle, and DAPI staining also showed that the suprabasal layers were present but unstained (Fig3.3B). Similarly on the opposite side, staining was weaker and discontinuous even where DAPI showed that basal layer cells were present (Fig3.3C).

Around the neck of the follicles in the upper segments, only a few K15 positive cells were visible suggesting that only limited numbers of basal layer cells were expressing K15 in this part of the follicle. Integrin expression paralleled that of K15 in the central portion of the follicle, with basal outer root sheath cell labelling on the nerve entry side of the follicle extended further proximally and distally compared to its mirror image (Fig3.3D).

At the proximal end of the follicle the basal epidermal cells appeared to have detached from the glassy membrane and were “floating” inside the cavity unstained for K15. By contrast some labelling of cells with integrin was observed in these detached epithelial cells near the amputation site (Fig3.3E).

Stage 1 (Day 2-Day 6)

The epithelial cells formed a solid column filling the cavity that had been evident in pre-stage 1 follicle. In the upper part of the regenerated hair follicle this column was wider, and then reduced to form a narrower tube extending from the central region to the lower sections of the follicles. K15 expression was mostly concentrated in the epithelial cell column in the proximal and central part of hair follicles (Fig3.4A). The dermal cells were K15 negative. At the extreme proximal end of the follicle, an irregular shaped or tongue shaped epithelial structure, had pushed out to lie below the end of the glassy membrane and beneath the original level of amputation. In some specimens, K15 protein was not uniform in this lowest region, and the expression

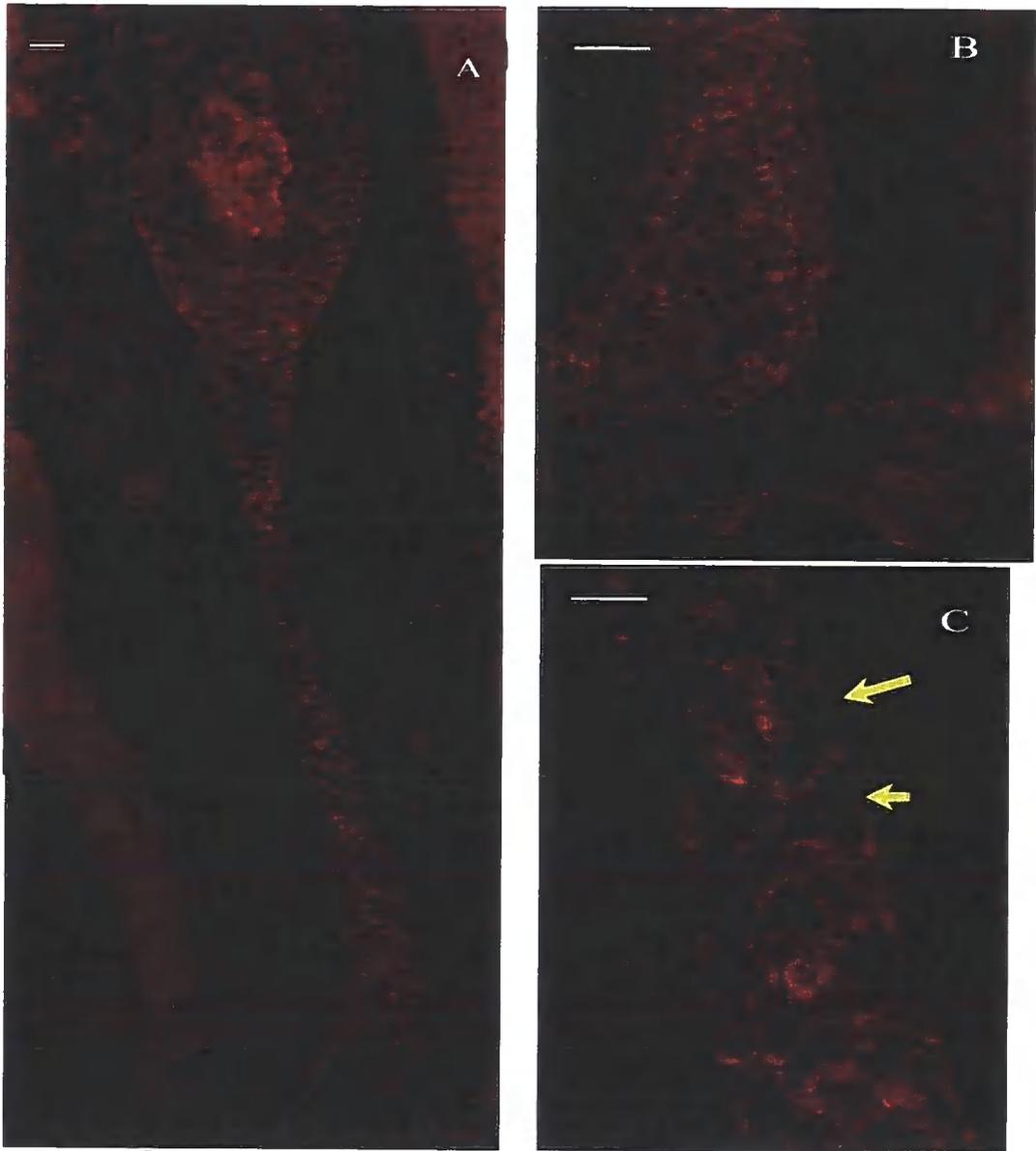


Figure 3.4 K15 positive cells activity in Stage 1 regenerating follicle

(A) K15 expressions in lower half of regenerating follicle. The epithelial column comprises two parts, the wide column located in the centre of follicle, linked to a narrow column. There are some K15 stained cells in the mesenchymal tissue, but the majority of them are concentrated within epithelial column.

(B) Close to the amputation site, the epithelial column pushes out of the original amputations site, and has formed a hollow triangle structure. Beneath the extended epithelial cells, dermal cells are K15 negative.

(C) In the narrow tube, K15 highlighted cytoplasm cells show a polarity (arrow), this may suggest that the cells may migrating.

In this section, all scale bars shown represent 100 μ m.

pattern shown gaps (Fig 3.4b). Within the basal layer of epithelial cells in the central regions of follicles, K15 was generally negative and only few randomly distributed positive cells. Below this, epithelial cells within the column centrally and proximally displayed high level of K15 expression (Fig3.4A). These cells were closely packed, and the staining indicated possible polarity, with the basal surfaces of the cells having reduced staining (Fig 3.4c). K15 positive cells in the narrower proximal epithelial “tube” were more tightly packed and the more internal cells appeared more intensely stained. This contrasted with the K15 positive cells higher up in the wide column where more expression was seen towards the edge (Fig3.4A)

Stage 2 (Day 7-Day 10)

By this stage, the base of the epithelial column had withdrawn to within the extended glassy membrane. The basal layer of the epithelial column from the centre to the bottom of the hair follicles mostly contained highly marker K15 positive cells. However within the epithelium compartment the more central cells only rarely showed staining for K15, with the exception of cells towards its proximal end (Fig 3.5a). Integrin was more widely expressed and was seen both in the basal and central epithelial cells of the epithelial column (Fig 3.5b). Later in this stage, K15 positive cells, increased in number and occupied more central space within the epithelial column.

At the base of follicles, a five to six cell layer of K15 positive cells occupied the amputation site. Outside of the basal layer in dermal tissue, a few individual weakly stained K15 positive cells were observed (Fig 3.5d). K15 cells were clearly distributed along the basal layer of the follicle thoroughly from the proximal to the distal and finally reach the outside skin epidermis (Fig 3.5a). Two days later, in the regenerating hair follicles showed increased number of K15 positive cells at the proximal end part of the follicle. These cells were shown to be in close contact with each other in the base of follicle, but slightly higher up individual randomly distributed K15 positive cell were observed (Fig 3.5e). However, no cells outside the basal layer were stained for K15

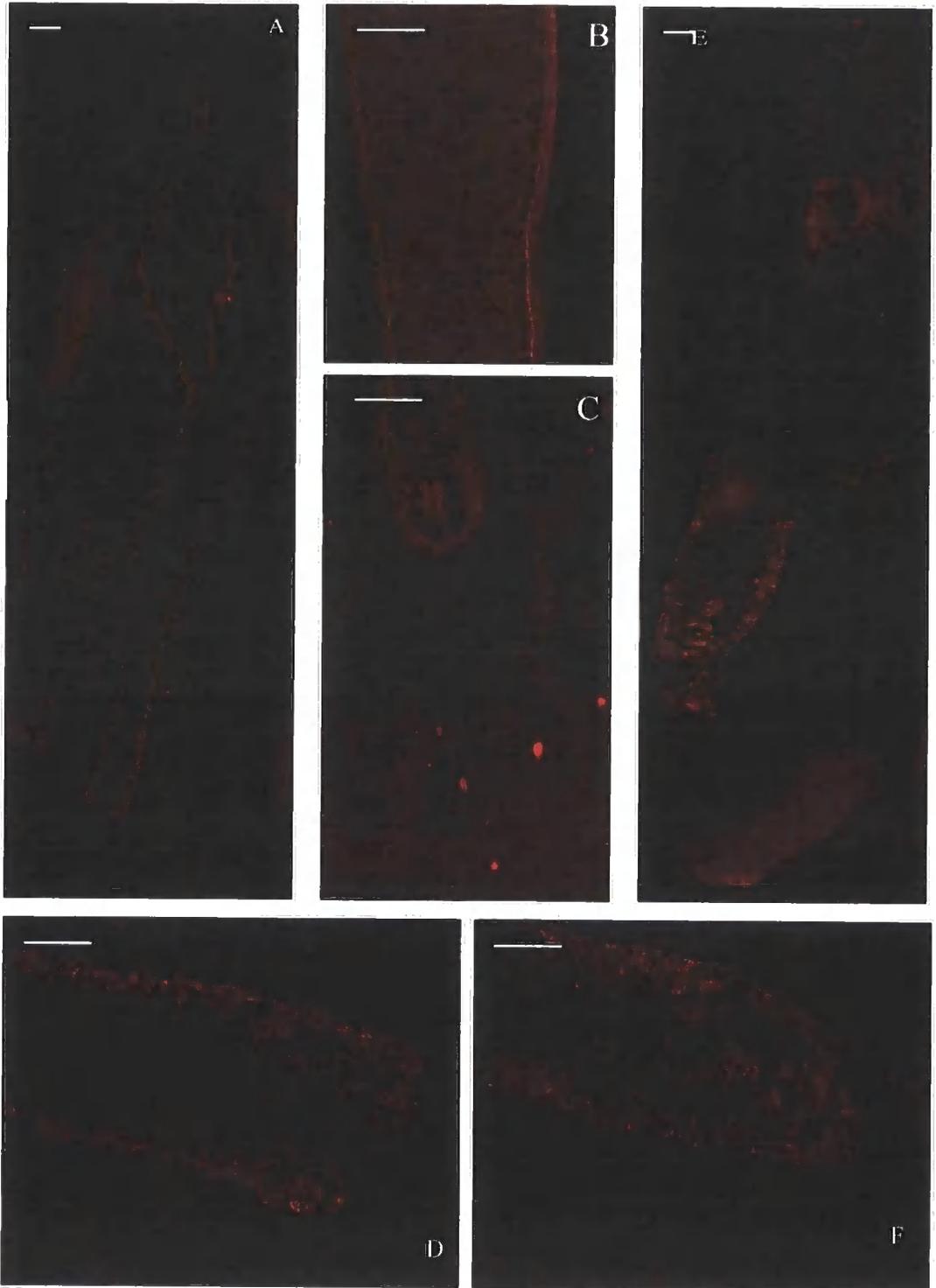


Figure 3.5 K15, and Integrin $\alpha6\beta1$ expression in stage 3, regeneration vibrissae follicles.

Stage 3 follicles immunostained with monoclonal K15 (A, D, E and F) Integrin $\alpha6\beta1$ (B) and P63(C). All of them are red fluorescence.

- (A) Overall view of K15 staining in a Day 8 regenerating hair follicle. K15 is mostly located along the glassy membrane to forming a complete loop wrapping the epithelial column. K15 expression is limited to cells inside the column cells, but at the end of follicle, there are some cells were K15 positive cells.
- (B) Integrin $\alpha6\beta1$ staining in the centre part of a regenerating follicle. Integrin $\alpha6\beta1$ highlights the cytoplasm of cells along the glassy membrane and also inside the epithelial column.
- (C) P63 staining in the distal part of regenerating hair follicle. Both the dermal and epidermal cells show negative results of P63 staining.
- (D) The proximal part of a Day 8 regenerating hair follicle, stained with K15. It clearly shows that some cells inside the epithelial column are K15 positive.
- (E) Overview of K15 staining in Day 10 regenerating vibrissae follicle. K15 positive cells appear in the epithelial column, but most of them are located in the proximal part, and they lose the K15 expression around the centre of the follicle.
- (F) The proximal part of a Day 10 regenerating vibrissae follicles, showing the K15 positive cells are mostly gathered in the distal region.

In this section, all scale bars shown represent 100 μ m.

(Fig 3.5f). Numbers of K15 positive cells along the basal layer decreased dramatically in the central and distal segments of the hair follicle.

Stage 3 (Day 11-Day16)

The proximal tip of the epithelial column was no longer flat. The basement membrane was indented upwards into the body of epithelial cells as the initial stages of dermal papilla formation took place. The Keratin 15 positive cells showed discontinuous staining along the length of the basal layer, however, in the epithelial column increased numbers of K15 positive cells were seen, especially at the proximal region of the regenerating follicles, with some occasional staining in upper regions (Fig 3.6a).

The majority of dermal cells that surrounded the base of the epithelial column were K15 negative cells, and DAPI staining showed these polarized cells around the extreme end of proximal region of epithelial column (Fig 3.6b). The majority of epithelial cells in the proximal region of follicles were K15 positive cells and these cells were considerably condensated (Fig 3.6c). These K15 highlighted cells showed small circular shaped in morphology. The size of the K15 positive epithelial cells appeared bigger, and the distance between K15 stained cells higher up in the proximal region also increased (Fig 3.6c). Interestingly, inside the epithelial column, K15 highlighted epithelial cells showed various shapes, *e.g.* triangle and rod shapes (Fig3.6c). In the centre region of regenerative follicle, cells inside the epithelial column were beginning to gradually lose their K15 expression, but the two sides of basal layer cells still expressed K15 at day 11(Fig.3.6d).

In day 11 regenerating follicle, K15 expression was weakly stained along the basal layer in the central of regenerating follicle (Fig3.6a), but the follicular basal cells showed stronger staining of K15 in the distal region and continuous reached the outside skin epidermis

In day 14 regenerating follicle, The K15 expression shared a similar expression pattern with Day 11 regenerating follicle; however, the epithelial cells began to lose K15 expression in the epithelial column in the bottom of the proximal region (Fig3.6e).

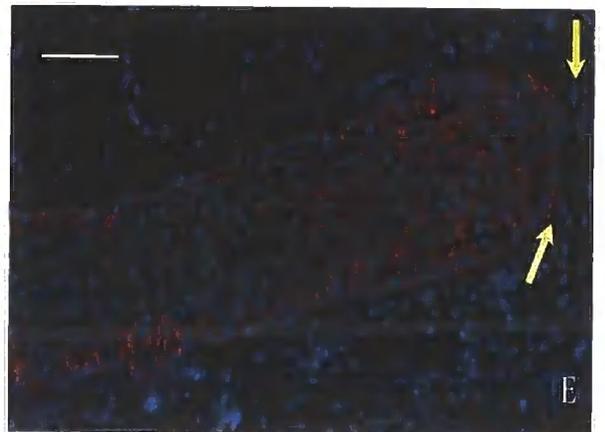
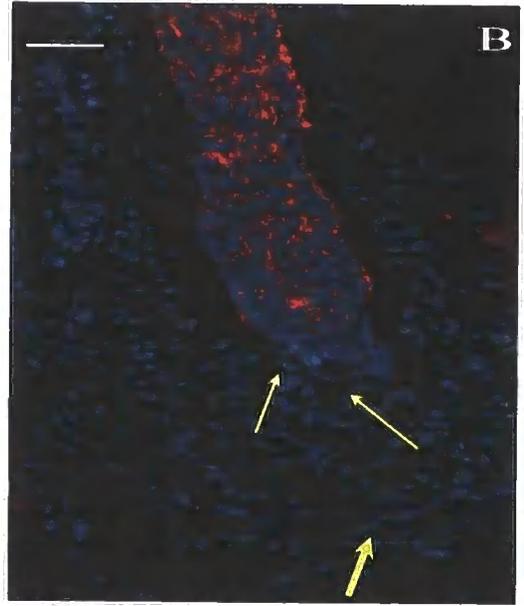


Fig 3.6 K15 staining in regenerating vibrissae follicles

- (A). Overview of K15 expression in a Day 11 regenerating hair follicle.
- (B). The proximal end of the follicle is counterstained with DAPI (Blue), and arrows show where the dermal cells and epidermal cells are interacting (Arrow)
- (C). Lower third of regenerating follicle, showing K15 positive cells throughout the epithelial column that are in contact and appear triangle shaped (Arrow).
- (D). Centre of a regenerating follicle, K15 expression has been lost in the central epithelium but still can be seen in cells along the glassy membrane.
- (E) Day 14 regenerating follicle, a small dermal papillae is observed, and some dermal cells have penetrated into the epithelial column (Arrow), proximally (DAPI blue).

In this section, all scale bars shown represent 100µm.

Stage 4 (Day 17-25)

21 days regenerating vibrissa follicle showed an enlarged dermal papillae, and a newly formed hair shaft began to extend from the dermal papillae, while the hair epithelial cells column was also widened. The amputated site in the proximal region was completely sealed (Fig3.7a). More importantly, cells around the dermal papilla were stratified, since several different cell layers have wrapped dermal papillae (Fig.3.7a and b).

In the proximal region, K15 positive outer root sheath cells, which were distributed along the glassy membrane, reached the very bottom of hair follicle bulb, and in contrast, dermal papillae cells and matrix cells were K15 negative. Dermal papillae cells were polarized and elongated, and they directly contacted with dermal sheath cells. In addition, these dermal cells showed morphological differences from the epithelial cells (Fig3.7b). In the upper part of proximal region, K15 highlighted the cytoplasm of inner root sheath cells inside the epithelial column (Fig3.7c).

In the central region of 21-day regenerating follicle, numbers of K15 positive epithelial cells had decreased dramatically, except for the follicular basal cells along the glassy membrane. Outer root sheath cells in the central region had begun to lose their K15 proteins, with very few exceptions (Fig3.7d). In the distal region, K15 was very weakly labeled in the cells along the glassy membrane only (Fig3.7e).

A summary of these results is shown in Figure 3.8 as a diagrammatic representation, highlighting regions in which K15 expression occurs during the stages of follicle regeneration.



Fig 3.7 K15 staining in Day 21 regenerating vibrissae follicles

Stage 3 follicles immunostained with monoclonal K15 only, which shows the red fluorescence.

(A) Overview of the proximal region of a day 21 regenerating vibrissa hair follicle. The amputation site is sealed and dermal papilla enlarged in size (Arrow); several cell layers appeared wrapped the dermal papillae (Arrow head).

(B) Dermal papillae cells lose their K15 expression, and dermal sheath cells (Arrow) around the bottom of the newly formed dermal papilla, showed the direct contact with it. Outer root sheath cells along the GM reached the proximal region (Arrow head).

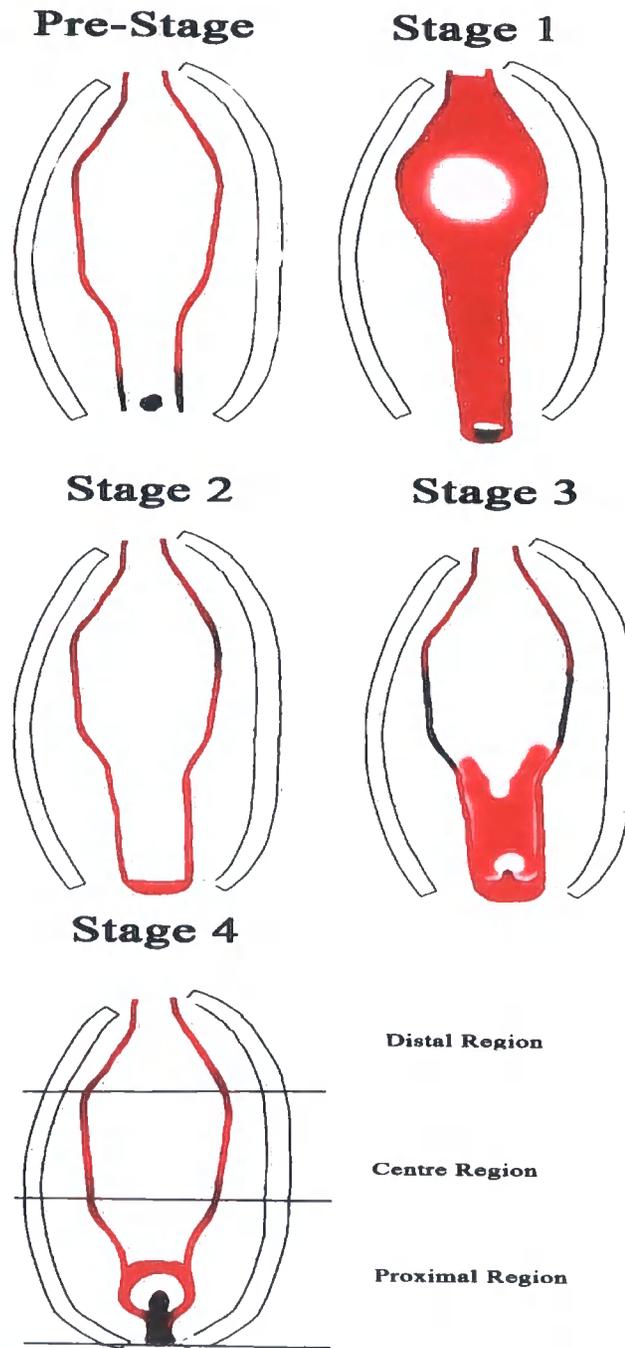
(C) Keratin 15 express in the cytoplasm of the upper part of proximal region in day 21 regenerating hair follicle.

(D) and (E) K15 expression gradually lost in the higher up of the hair follicle.

In this section, all scale bars shown represent 100 μ m.

Figure 3.8 Schematic diagram indicate areas of K15 staining during regeneration of the vibrissae follicle end bulb.

The areas highlighted are the dermal sheath (external to the glassy membrane), the epithelial column and the dermal cell aggregation that develops at the base of the regenerating follicle.



3.3.3 ID3 expression during regeneration process

Stage 1

ID3 immunoreactivity was seen both in dermal and epithelial cells. In the base of early stage regenerative follicles, the epithelial column were consists full of Id3 positive cells. Cellular transition of ID 3 positive cells were another repeatable observation during hair follicle regenerating process, both cytoplasm and nuclei staining was observed (Fig3.9a). Dermal cells in regenerating follicle showed nuclear staining. Id3 incorporation was evidence at regular intervals in the basal layer along the lower half of the follicle.

In the proximal region of stage I regenerating follicles, Id3 positive cells showed mainly inside the epithelial column (Fig 3.9a). Id3 highlighted the cytoplasm of epithelial cells cover the whole epithelial column. Outside the basal layers, the dermal cells showed nuclei staining along the glassy membrane, but in the most proximal part of the column, the nuclear highlighted cell line was interrupted down to the amputation site (Fig3.9a).

In the centre region of regenerating follicle, Id3 positive cells inside the epithelial column showed less condensate, and majority of the staining were still located in cytoplasm (Fig 3.9b). In dermal tissue, the Id3 stained cells remained in the nuclear and their distribution was thinly spread (Fig3.9b). The staining intensity both in epithelial and dermal cells was relatively weaker than proximal region (Fig3.9b).

Slightly later in this stage, there was no significant changes occurred in the proximal epithelial column, with exception of the regular interval nuclei highlighted outer root sheath cells arrived down to the narrowed prominence at the amputation site (Fig3.9c). In the centre of follicle, Id3 positive epithelial cells were seen staining through a whole cell, including nuclear and cytoplasm, which were different from earlier in this stage (Fig3.9d).

In the distal region, Id3 labelling pattern exhibits similar to that seen in a normal catagen follicle, and its expression reached the skin epidermis (Fig3.9e).

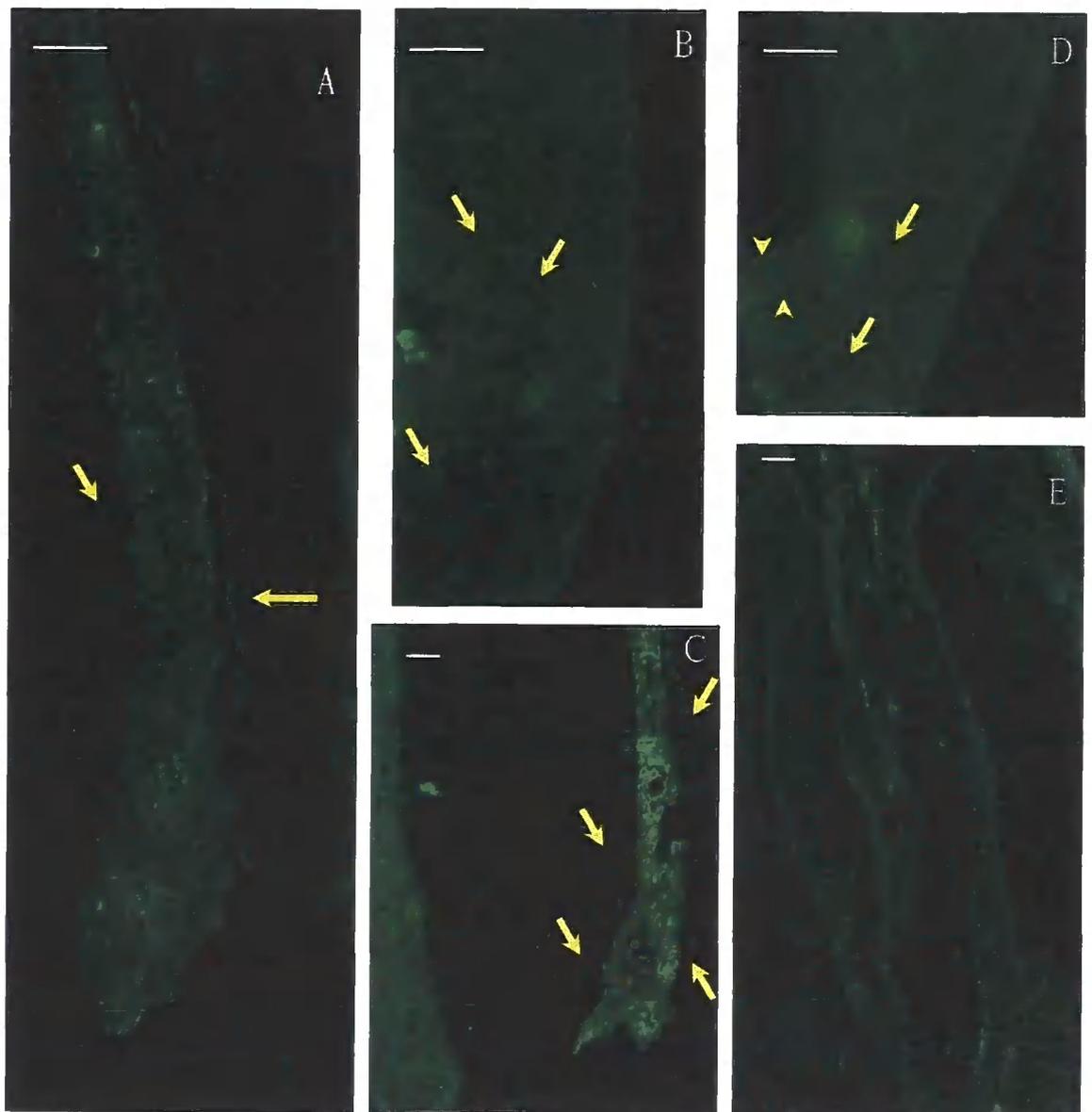


Fig3.9 ID3 expression in Day 4 and Day 6 regenerating vibrissae follicles

(A) Proximal part of Day 4 regenerating follicle. Cells in epithelial column were cytoplasm highlighted Id3 positive cells, and dermal cells were nuclei. The Id3 positive cells formed a single cell line along the glassy membrane in dermal tissue (Arrow).

(B) Centre of Day 4 regenerating follicle, the overall ID3 staining reduced dramatically, and the epithelial cells are Id3 cytoplasm highlighted (Arrow).

(C) Proximal part of Day 6 regenerating follicle, the Id3 positive dermal cell lines reached the bottom with regular interval along the GM (Arrow).

(D) The central region of day 6 regenerating hair follicle, some epithelial cells showed Id3 staining also inside the nucleus (Arrow), in contrast with cytoplasm Id3 highlighted cells (Arrowhead).

(E) First half of follicle Day 6 regenerating follicle.

Stage 2

In day 8 regenerating hair follicle, at the cut base of hair follicle, the Id3 positive cells were increased in numbers in dermal region, the nuclei highlighted single regular interval dermal cell line which had been observed in earlier stage, increased Id3 expression quantity in the lower half of follicle, and Id3 stained dermal cells formed a complete loop around the entire proximal epithelial column (Fig 3.10a). Inside the epithelial column, epithelial cells were condensate; majority of epithelial cells were showed Id3 florescence both in nuclei and cytoplasm. On day 10, the overall Id3 positive cell numbers in the proximal region were continue increasing; interestingly, some of these nuclear highlighted Id3 marked dermal cells which located direct beneath the epithelial amputation site showed perpendicular to the epithelial column, which did not show in two days earlier (Fig 3.10b). In addition, cells inside the epithelial column in the proximal region were Id3 positive, and expressed in cytoplasm only (Fig3.10b).

In the centre of hair follicle, Id3 highlighted epidermal cells remained the level of quantity and intensity with earlier stage. Inside the epithelial column, most of Id3 positive cells were concentrated in two sides of column at Day 8 regenerating follicle (Data not shown), and Id3 expression found both in nuclear and cytoplasm in majority of them. In the Day 10 regenerating follicle, in the edge of epithelial column, Id3 expression intensity became weaker, in contrast with Id3 positive epithelial cells located inside the column (Fig3.10c). The overall Id3 positive dermal cells were increased in numbers from day 8 to day 10 regenerating follicle, especially dermal cells along the glassy membrane, with highlighted nuclear (Fig3.10b and c).

In the distal region of regenerating follicle, dermal cells remain in the nuclear with large quantity of Id3 positive cells, however in epithelial cells, the cytoplasm highlighted Id3 cells expressed restricted along the glassy membrane, and further reach the outside skin epidermis (Fig3.10d).



Fig3.10 Id3 expression in Stage 2 regenerating vibrissae follicle

- (A) Overview of Id3 expression in lower half of day8 regenerating follicle. Cells along the epithelial column show horizontal direction, towards to the amputation site (Arrow).
- (B) Overview of Id3 expression in lower half of day10 regenerating follicle. Id3 marked dermal cells located direct beneath the epithelial amputation site showed perpendicular to the epithelial column (Arrow); and epithelial cells expressing Id3 in cellular cytoplasm only (Arrow head).
- (C) In central region, dermal cells were nuclei stained which overlap with DAPI staining (blue,

Arrow), which in contrast with cytoplasm stained epithelial cells (Arrowhead).

(D) In the neck of Day 8 regenerating follicle.

Stage 3

The epithelial column canal appeared reduce in size in this stage. Overall cell numbers staining of Id3 proteins was decreased both in epithelial and dermal tissue (Fig3.11a). It is clear that many dermal cells lost their Id3 expression especially in the central and distal regions, but in the proximal region, considerably large numbers of Id3 positive cells observed though their intensity decreased dramatically (compare with previous stages (Fig3.11a). Id3 highlighted outer root sheath cells along the glassy membrane with a regular interval, from distal region along the epithelial column reached to the amputation site, and it is noticeably that large numbers of Id3 positive cells were gathered beneath the amputation site (Fig3.11a and c). The cellular location of Id3 were separated by glassy membrane, *i.e.* epithelial cells were cytoplasm staining; dermal cells were stained in nucleus (Fig3.11a and c). Similarly, later in this stage, the dermal cells that surrounded the base of the epithelial column were Id3 nuclei highlighted cells (Fig3.11b), whereas the epithelial cells were cytoplasm Id3 highlighted.

In the proximal region of day 11 regenerating follicles, Id3 positive cells inside of epithelial column showed different in morphology. Two single cell layers which just located the edge of both sides epithelial column, showed weak staining, and they were rod shape towards to the glassy membrane (Fig3.11c), in contrast, cells between these two single cell layers showed considerably condensate, and majority of them were sphere shaped. At day 14, cells located in the proximal end of epithelial column, Id3 expression highlighted both cytoplasm and nuclear (Fig 3.11b).

Both quantity and intensity of Id3 staining in dermal cells were decreased in the central region of this stage, their appearance were barely could be seen (Fig3.11a and d). Inside the epithelial column, the cytoplasm highlighted Id3 positive epithelial cells expressed from proximal region to the central region; however, the rod shaped follicular basal cells which along the glassy membrane were weakly stained (Fig3.11e), higher up in central region, both epithelial and dermal cells began to lose their Id3 staining.

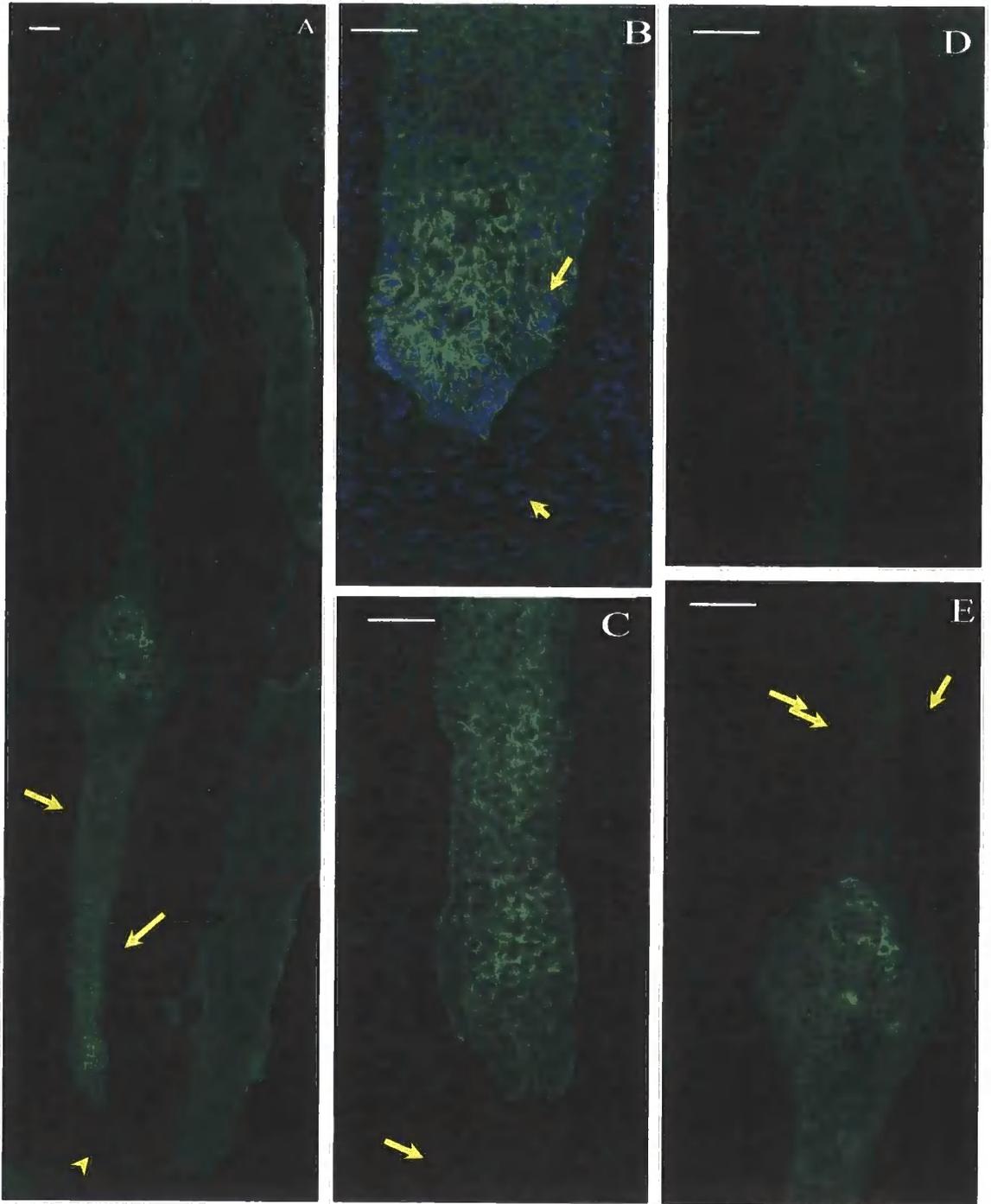


Fig 3.11 Id3 expression in Day11 and Day 14 regenerating vibrissae follicles

(A) Overall view of Id3 staining in Day 11 regenerating follicle. The Id3 highlighted outer root sheath cells along the epithelial column with regular interval from distal reached to the amputation site (Arrow). Large numbers of Id3 labelled dermal cells accumulated underneath the amputation site (Arrowhead).

(B) Proximal part of Day 14 regenerating follicle, cells inside the epithelial column are showing Id3 positive (Arrow), and dermal cells outside the epithelial column Id3 negative (Arrowhead).

(C) Proximal part of Day 11 regenerating follicle. Id3 weakly stained the dermal cells underneath the amputation site (Arrow).

(D) and (E) Central regions of Day 11 regenerating follicle, both dermal and epidermal cells began to lose the Id3 expression (Arrow).

However, in the neck of hair follicle, the Id3 staining was reappearance in both dermal and epithelial column, and both of them were shown nuclear staining (Fig3.11a).

Stage 4

Id3 expression in 21 days regenerating follicle were very strictly located in the dermal cells through the entire hair follicle. However, the newly formed dermal papillae cells were Id3 negative cells, but the outer root sheath and dermal sheath cells were Id3 positive (Fig3.12a and b).

Proximally, follicular basal cells were noticeably Id3 highlighted cytoplasm, which different with Id3 nuclei highlighted dermal sheath cells (Fig3.12c and d).

A summary of these results is shown in Fig3.13 as a diagrammatic representation, highlighting regions in which Id3 was evidence during stages of follicle regeneration.

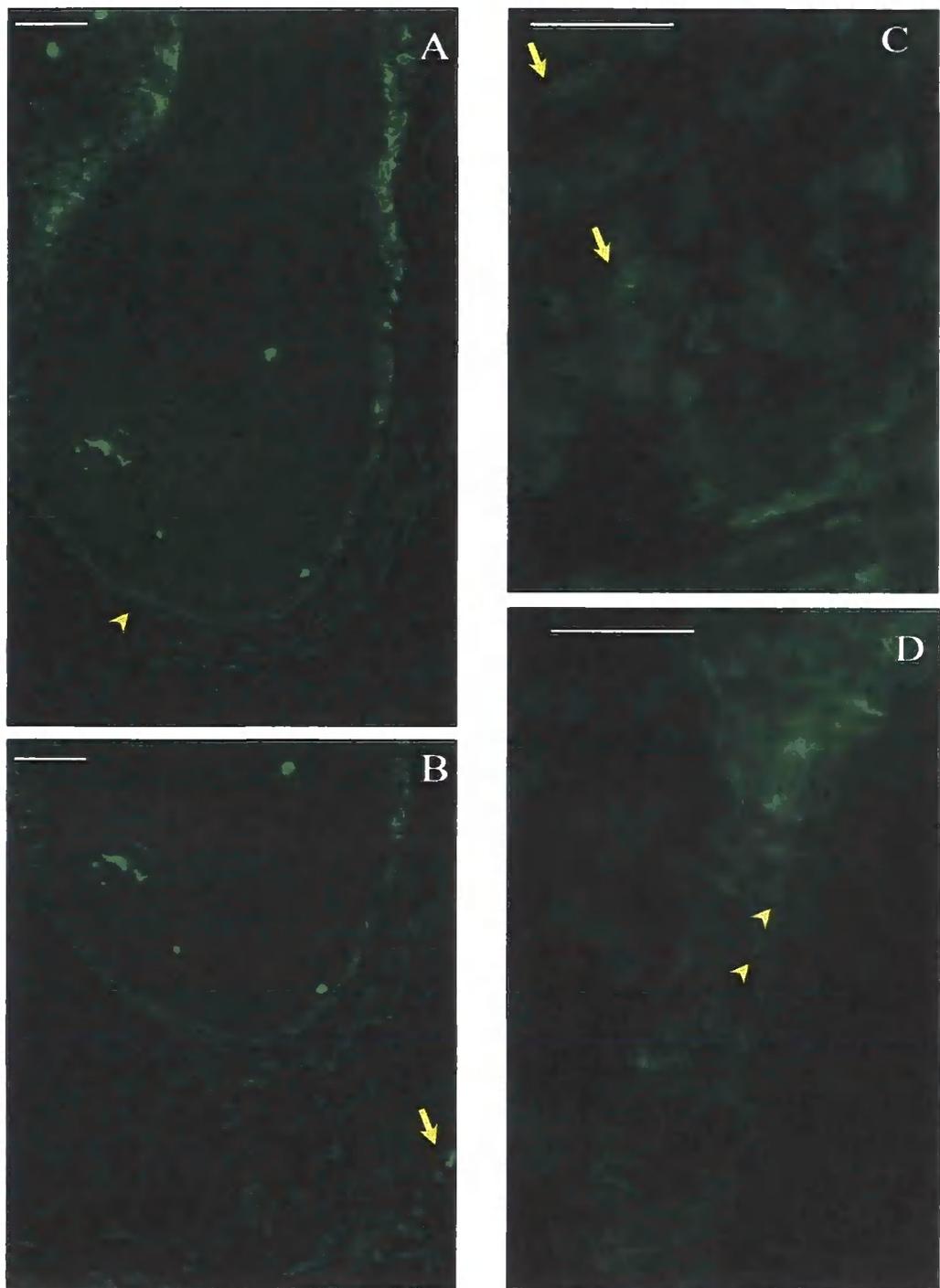
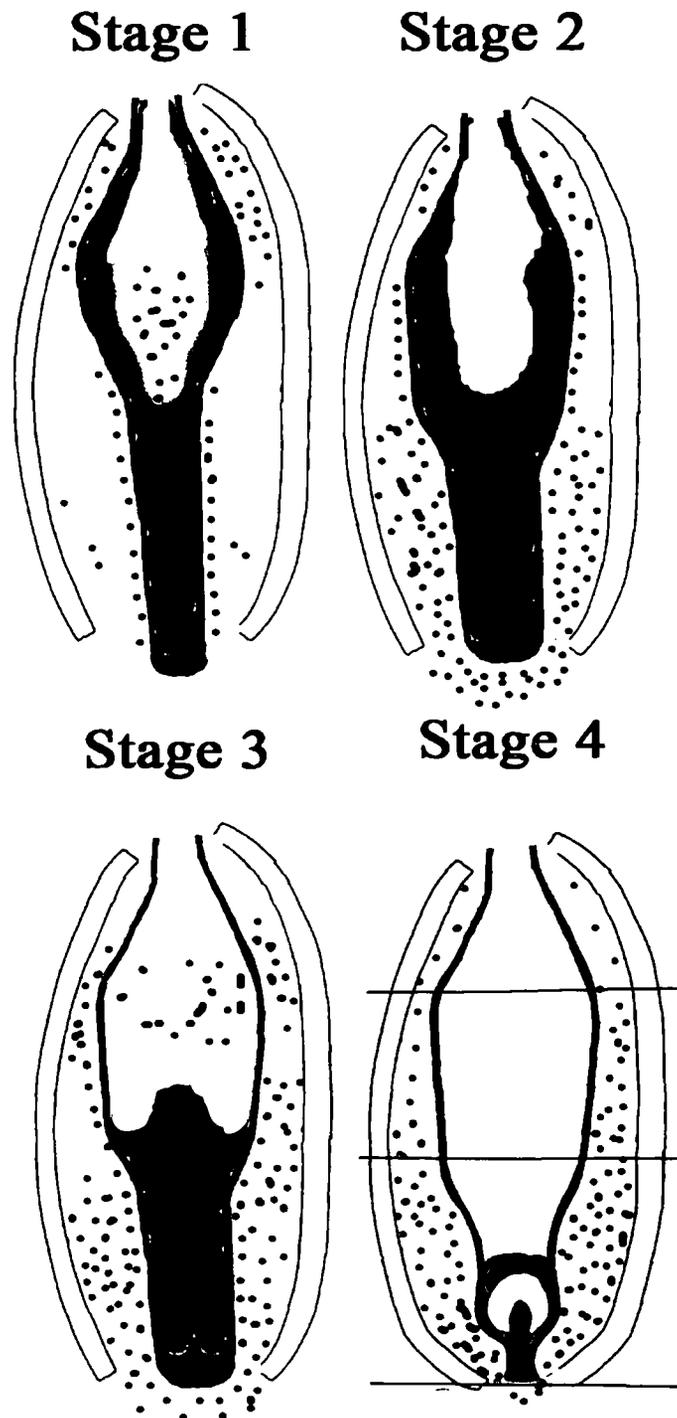


Fig3.12 Id3 expression in Day21 regenerating vibrissae follicles

- (A) The proximal region of 21 days regenerating follicle, newly formed dermal papillae are Id3 negative, but outer root sheath cells along the GM are Id3 positive (Arrowhead).
- (B) Dermal sheath cells, are Id3 positive (Arrow)
- (C) Proximally, dermal sheath Id3 nuclear highlighted (Arrow)
- (D) Outer root sheath cells are Id3 cell cytoplasm highlighted (Arrowhead).

Fig 3.13 Schematic diagram indicating areas of Id3 staining during regeneration of the vibrissae follicle end bulb

The areas highlighted are the dermal sheath (external to the glassy membrane), the epithelial column and the dermal cell aggregation that develops at the base of the regenerating follicle.



Chapter 4 Discussion

4.1 Summary

4.1.1 Ordinary Hair follicle

Ordinary hair follicles produce multiple differentiated epithelial cell layers in different cycle stages resulted from epithelial stem/ TA cells. K15 protein expression during the vibrissae hair follicle cycle showed different patterns and locations especially in the proximal region of follicles, possibly due to cell migration. This has been proposed in the hair follicle predetermination hypothesis (Panteleyev, 2001). K15 positive cells were found around matrix cells in some certain stages also suggested the possibility of stem cells existence in hair follicle matrix, which also supports the predetermination hypothesis (Panteleyev, 2001) and the Barrandon group's work on matrix cells (Claudinot S, 2005). The observation about K15 expression in Catagen hair follicle was inconsistent with the idea that stem cells migrate down along the outer root sheath during anagen, and are arranged in straight continuous cell lines along the glassy membrane in outer root sheath cells during catagen (Oshima,H 2001). In addition, my results also showed that K15 positive cells appeared in the distal region of hair follicle, and in some anagen hair follicles, the K15 positive cells even continued to be linked to the outside interfollicular epidermis. This result suggested various sources of epidermal stem cell also might be involved in the hair follicle cycle.

Id3 protein expression patterns in different stages of the hair follicle cycle were more complex, and involved both epithelial and dermal cells. One very interesting feature of Id3 positive cells was the cellular staining translocation, where the majority of epithelial cellshad predominantly cytoplasmic/perinuclear staining but dermal cells were more restricted to nuclei staining. Since Id3 has been reported to label the



proliferating, undifferentiating cells in several mammalian cell types, and be down regulated in terminal differentiated cell types (Altherton GT, 1996 Moldes M, 1997, Persengive SP, 1997 and Chassot AA, 2007), this could offer an explanation about the differences in expression pattern between epithelial and dermal cells, especially during anagen. However, this is not consistent with the fact that dermal papilla cells are not proliferative at any time of the hair cycle, Therefore although the Id3 function and the mechanisms for cellular translocation remained unclear in rat vibrissae hair follicle, our results may suggest the epithelial Id3 positive cells undergo cell cycle processes, and I also suspect these Id3 proteins might be involved in cell de-differentiation and reprogramming processes.

4.1.2 Regenerating hair follicles

Regeneration in the epithelial compartment resulted from extensive proliferation in the basal layer of outer root sheath cells. K15 positive cells increased dramatically in the early stages of regenerating hair follicle, and these cells occupied the whole epithelial column in first 8 days. This support proposal that stem cells are continuously migrating through the outer root sheath during anagen (Oshima, H, 2001), and this suggests that other sources of epidermal stem/TA cells may be involved in hair follicle epithelial cell regeneration processes, as was demonstrated by Cotsarelis's group early this year (Mayumi Ito, 2007). In the later stages, K15 positive cells showed direct connection with dermal cells in the amputation site, which suggested the likelihood that dermal sheath cells become dermal papilla cells, as has been proposed decade ago by Jahoda, and also been recently demonstrated by MacElvee *et al* in culture conditions (Jahoda 1992 and MacElvee 2003). Dermal and epithelial Id3 positive cells showed their response to the injury to hair follicle were almost immediate, and although evidence suggested the dermal cell accumulation in amputated hair follicles involved migration from mesenchymal cells, my results also suggested the dermal cells may replicate

themselves at same time during later stages of regeneration.

4.1.3 Differentiation of osteoclasts in culture conditions

Osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursor cells at or near the bone surface by two important haematopoietic factors, RANKL and CSF-1. Our result showed that newborn rat end bulb cells and adult rat dermal papillae cells under treatment with these factors also could be induced into osteoclasts. More recently, new facets of hair follicle dermal cell activity have been observed, including the creation of fat, bone muscle and cartilage (Jahoda, 2003, Hoogduijn, 2006 and Rufaut, 2006). However, there has no work showing that rodent vibrissa follicle dermal cells can be directed into specific haematopoietic cell types such as osteoclasts. Considering the similarity of bone marrow mesenchymal stem cells and hair follicle dermal stem cells (Hoogduijn, 2006), the current work has actually demonstrated that these two stem cell types are exchangeable in certain circumstances. Environmental factors could provide a network of cell signalling which is usually named as the stem cell niche, and much work has shown that TA cells in a stem cell niche could de-differentiate back to a stem cell state and even differentiates into other cell types (Pearton, 2005). Our work may also suggest mimicking the osteoclast precursor cell niche signals *in vitro*, can direct stem/TA cell de-differentiation and further differentiation into a specific cell type.

4.2 K15 and Id3 positive cells activity in ordinary hair follicle

4.2.1 K15 positive cell migration

Keratins were some of the earliest markers used to define the anatomical location of the epidermal niche, particularly K15 and K19. More recently, K15 has been used as a hair follicle bulge stem cell marker in various ways, including for microarray gene expression studies (Morris, 2004). Observation in anagen showed up-regulated stage of K15 protein staining, especially in the bulge region, which suggested a stem cell/TA cell rich region. During anagen, K15 highlighted anagen outer root sheath cells from the bulge region to the end bulb in the proximal region (Fig2.5). Asymmetric staining of K15 on two sides of the glassy membrane together with occasional “gaps” along the K15 positive cell lines suggested that K15 positive cells may migrate from the bulge region to the proximal region during growth stages, as suggested by many others (Panteleyev, AA 2001, Oshima, H 2001). The different cell types were found according to the different regions of anagen hair follicle, and in the extreme bottom of hair follicle, cells wrapped dermal papillae were clearly showed stratified, these evidence suggested cell differentiation occurred in this stage.

Catagen hair follicles showed more interrupted K15 staining along the glassy membrane, but stronger staining around the dermal papillae region especially in matrix cells. Barrandon’s group demonstrated that stem cell migration started from anagen, and the migration should be terminated at catagen, which suggests that during catagen, stem cells should be aligned along the glassy membrane from the bulge region to the bottom of the hair follicle. However, based on our observations, K15 positive cells were absent in the lower central and upper proximal region, and they reappear in the matrix cells and outer root sheath cells surrounding the dermal papilla. Our results were inconsistent with Barrandon’s previous work on cell clonogenicity and replicative potential in different parts of the catagen hair follicle (Cloudinot, 2005).

K15 positive cells lose their staining dramatically during telogen, but K15 labelled cells can be found along the glassy membrane in the whole proximal region, while the club hair was moving upwards and a new bulge region forming (Fig2.7). In addition, occasionally K15 positive cells around the dermal papilla suggested some stem cell activity still ongoing even during telogen. The nearly continuous K15 staining of cells from the matrix to the proximal region along the glassy membrane may suggest the possibility of stem cell migration starting from telogen, which is earlier than expected (Roh 2005); and this may also explain the asymmetric staining on the two sides of the glassy membrane.

K15 positive cells in early anagen hair follicle increased significantly; in fact, cell numbers in general are increased significantly. The club hair is located on the top of the bulge region, where K15 labelled cells covered the whole region. The K15 positive cells appeared both above and below of the bulge region, which conforms with the suggestion that bulge region stem cells migrate in both directions in early anagen (Christiano, 2004). In the downward movement, K15 cells reached the region just above the dermal papilla (Fig2.8), and were relatively weakly stained in the lower part of the end bulb. Still, these results suggest stem cell migration had reached the very bottom of the hair follicle in the early anagen. The matrix cells also appeared K15 positive in early anagen; however, there is no direct connection between them, suggesting that the matrix cells had probably not been replenished by outer root sheath cells. Cell differentiation was probably initiated from this stage since different cell types have been observed.

The traffic light hypothesis (Oshima, 2001) does not seem to perfectly match my observations although I also believed that cell migration might take an important role in the hair cycle. The results can be explained in a few ways. One, the timing of stem cell migration may start and cease earlier than originally proposed (Oshima, H 2001, Roh 2005). Stem cells might start their migration from telogen and terminate at mid/late anagen. Outer root sheath cells in catagen may still contain great clonogenicity and

replication potentials, like late TA cells. This result also may be due to the existence of stem cells in matrix cells. Matrix stem cells may require to be activated during early anagen, and these activated cells could then consequently play a role in different cell layers and new hair shaft formation in later stages. This “Relay” hypothesis may offer an explanation for the matrix cells high clonogenicity and also their ability to form new hair follicles (Cludinet, 2005). This may also explain the reason the bulge region was not the stem cell source for newly formed hair follicle in wounded skin, but their appearance was observed during new hair follicle formation (Mayumi Ito, 2007). However, my results could not offer any clue for mechanisms involved in this. It is possible that the bulge region stem cells migrated down and released some activation signals such as BMP signalling since this BMP signalling was found to be restricted in anagen and telogen (O’Shaughnessy, 2004, Chuong CM, 2008), and the activated quiescent stem/TA cells could move down for differentiation into new cell layers. Finally, there is also the possibility that cell motility activity would compromise stem cell potential; therefore, the migrated cells will lose their stem cell features gradually.

4.2.2 Id3 expression in ordinary hair follicle

The functions of Id3 proteins in rodent vibrissa hair follicles cycling remain to be solved. Id3 protein expression patterns were much more complex than K15, since its expression changed in both dermal and epithelial cells, as well as in different cellular locations. My results showed that this protein is expressed in all stages of hair follicle dermal papillae cells, which is not consistent with previous reports (O’Shaughnessy, 2004), though the expression intensity was relatively fainter in catagen and early anagen (Fig2.11 and Fig2.13). Therefore, these results suggested that Id3 roles in the adult hair follicle cycle could involve other cellular processes apart from being a BMP targeting protein as O’Shaughnessy *et al* suggested (O’Shaughnessy, 2004).

My results showing large numbers of epithelial Id3 positive cells in anagen hair follicles, together with limited Id3 staining in telogen suggested the possibility that Id3 might be involved in cell proliferation. More recently, Chassot *et al* demonstrated that Id3 protein is required for cell cycle progress by regulating p27^{kip1} mRNA in early G1 phase (Chassot, 2007). In catagen, these Id3 positive cells lost their Id3 activity in the proximal region and gradually became less Id3 labelled in the central region (Fig2.11). These observations may be due to the cells undergoing differentiation processes, as Id3 proteins, similar to other Id proteins, can be inactivated by cytoplasmic sequestration during the growth phase (Kurooka, 2005 and Kim 2006). The catagen hair follicle outer root sheath cells were terminally differentiated, and prepared for the apoptosis process, so limited Id3 proteins were detected. The hair shaft cells which gave a negative Id3 result suggests that the level of Id3 expression is down-regulated when cells undergo terminal differentiation, something which has also been described by other researchers (Atherton, 1996 and Persengiev 1997).

However, this does not explain why the Id3 protein highlighted the cytoplasm of follicle basal layer epithelial cells through the whole follicle cycle even in telogen. One possible explanation is that epithelial basal layer cells were through the whole hair follicle cycle, and some pathway may link these closely contacted basal layer cells from central region down to the proximal region. O'Shaughnessy proposed that cell-cell communication may induce BMP4 expression that in turn induces Id3 expression, and they also mentioned the possibility of a notch signalling pathway since it has been found in hair follicle cycle (O'Shaughnessy, 2004, Reynaud-Deonauth, 2002 and Powell, 1998).

Considering Id3 expression also tends to be higher in immortalized cell lines, maintaining the self-renewal capacity of embryonic stem cells (Ying, 2003), special attention was paid to Id3 expression pattern in the bulge region of the hair follicle. During early anagen, bulge region cells showed Id3 labelling nearly covering the whole region (Fig2.13). Id3 highlighted the cytoplasm of these bulge region cells suggesting

that they were undergoing cell differentiation. These could be due to the stem cells turning into TA cells along the outer root sheath, and Id proteins being required to be transported from cell nuclei to cytoplasm for degradation, prior to these TA cells moving down to the end bulb region. Anagen and catagen follicle bulge region cells had cell nuclei or the whole cell stained with Id3 (Fig2.10 and Fig2.11), and this may suggest the bulge cell differentiation process (transition to TA cells) had terminated. Interestingly, Id3 highlighted only limited number of bulge region cells in telogen, and these were also nuclear or entire cell labelled, and more restricted to the basal layer. This might suggest that bulge region cells had began to prepare for cell proliferation and cell migration at this stage.

Id3 staining in dermal cells was appeared to be nuclear only, including the dermal papillae and dermal sheath cells. These suggested that hair follicle niche is incapable of providing dermal cells differentiation. In early anagen, Id3 stained dermal cells were found in the neck of hair follicle, and these Id3 labelling cells accumulated beneath the bulge region and formed a single cell line with regular intervals along the glassy membrane and finally extended to the extreme bottom of the hair bulb (Fig2.13). Id3 is a short lived protein with 20mins half-life, without binding partners; Id3 will be degraded by ubiquitination dependent manner (Bounpheng, 1999). This probably offers an explanation as to why, in telogen, dermal cell numbers reduced dramatically and the Id3 staining also disappeared quickly.

Dermal papilla cells in all stages showed nuclear stained Id3, and in early anagen and anagen showed dermal papilla in contact with dermal sheath cells in the bottom of the end bulb suggested dermal papillae and dermal sheath cell contact is involved in new hair shaft formation. Dermal sheath cells are proposed as dermal progenitor cells during wound healing processes (Jahoda, 2001), and dermal papillae and dermal sheath cells are exchangeable in vivo and in vitro (Jahoda 1992 and McElwee, 2003). During early anagen, dermal sheath cells moving down could activate the dermal papilla cells

or directly replace them, and these activated dermal papilla cells may involve in helping produce the new hair shaft.

There are some unusual or “unexpected” observations for which I could not give a logical explanation or suggestion. Firstly, since Id3 is a transient protein with so short a half-life, how could these proteins survive in the epithelial cell cytoplasm in early anagen and anagen? In the cell nucleus, a dimerization partner is crucial for Id3 protein stability since E proteins and interferon inducible protein p204 have been shown to have key roles in Id protein stability (Thatikunta, 1999 and Liu, 2002), and Id proteins have been shown to be predominantly expressed in the cytoplasmic/perinuclear region in COS cells, an immortalising a cell line derived from African green monkey kidney with virus for produce large T antigen. However, so far no Id3 protein-binding partners have been found to stabilize Id proteins in epithelial and dermal cell cytoplasm. Secondly, there are clusters of epithelial cells in the distal region in early anagen showing nuclear envelope staining (Fig2.13). Other Id proteins have been shown to transport through the nuclear pore by NES (Lasorella, 2005 and Makita, 2006); however, there has no report for Id3 transport through the nuclear pore, though they share a similar DNA sequence (Lasorella, 2005 and Makita, 2006). It is also possible that these cells undergo de-differentiation, since in tumour cells cytoplasmic Id3 proteins have been consistently observed (Vandaputte, 2002).

4.3 Plasticity of end bulb cells and dermal papilla cells

4.3.1 End bulb cells differentiation into osteoclasts

My results showed that cells from the new born rodent hair follicle end bulb can differentiate spontaneously *in vitro*. This adds to the earlier report that cells present within the hair follicle dermis support haematopoietic reconstitution *in vivo* and the more recent discovery that hair dermal cells differentiate into adipogenic and osteogenic lineages (Lako, 2002 and Jahoda 2003).

The end bulb cells differentiation timing (14 days) took longer than the bone marrow cells (4-7 days), this suggested that the mesenchymal cells *in vitro* environment might negatively influence the multipotent stem cells differentiation in response to the osteogenic signals. It has been proposed that mesenchymal stem cells might govern by activation/inhibition of ERK (Jaiswal, 2000).

The first observed osteoclasts-like cells were after 14 days treatment with RANKL and MCS-F. The smooth edge suggested these osteoclasts were inactivated. Osteoclasts increased in size 23 days after treatment, and the projection shaped extrusions were found in 30 days treatment (Fig2.14). These observations showed the osteoclasts were gradually becoming activated.

4.3.2 Dermal papilla cell differentiation to osteoclasts

Adult rodent dermal papilla explant cells shared a similar morphology with new born mice end bulb cells, as well as a similar cell growth rate. However, osteoclasts were firstly observed after 11 days treatment with M-CSF and RANKL. This could be because the end bulb microenvironment could provide a more protective stem cell niche rather than isolated dermal papilla, since the end bulb is mixed with several other cells types including epithelial, melanocyte, mesenchymal cells or even blood cells.

The osteoclasts after 30 days treatment with osteogenic signalling interestingly showed smooth edges, which in contrast with the rough edge of osteoclasts differentiated from new born mice end bulbs (Fig2.14 and Fig2.15). *In vivo*, the mature multinucleated osteoclasts are activated by the signal RANKL (Bugess, 1999), which leads to the initiation of bone remodelling. Since the activated osteoclasts were found in newborn explant cells after treating them with exactly same amount of osteogenic signals, this suggested that dermal papillae explant cells might hinder the RANKL signalling pathways; alternatively, it is also possible that the new born stem cells were more sensitive and efficient with signal responsiveness.

4.3.3 The origin of osteoclast precursors, and differentiation

Hair follicle dermal sheath cells have been proposed as a progenitor dermal cell source during the wound healing process (Jahoda, 2001), dermal papillae have been shown in many cases to be capable of differentiation into several different cell types outside the hair follicle (Gharzi, 2003 and Jahoda, 2003). However, the hair follicle dermal stem/TA cells differentiation mechanisms and processes have not been fully understood, in fact, the processes of osteoclast precursor cell differentiation have also remained to be solved. Mochizuki *et al* showed that precursor cells committed to osteoclasts are induced by RANKL, which could further activate the Nuclear Factor kappa B (NF- κ B) mediated signalling pathway (Mochizuki, 2006). This is a pathway that is crucial for all eukaryotic cells, since it is involved in 150 gene transcriptions and also involved in hair follicle development. Bone marrow mesenchymal stem cells have been proposed as sharing many similar features with hair follicle dermal stem cells (Hoogdujin, 2006), with sufficient RANKL molecule stimulation, it is possible that hair follicle dermal stem cells could accumulate and differentiate into osteoclasts. In addition, dermal papillae showed strong Id3 staining in hair follicle cycles, and Id3 has been

demonstrated to be involved in the osteogenesis process (Maeda Y, 2004).

My results shows that cells at 30-40% confluence will initiate dermal stem cells becoming osteoclasts, interestingly, higher confluence cells would prevent osteoclasts formation (data not shown). This may due to the cytokines that are released by the dermal papilla cells that keep the cells proliferating rather than differentiating, hence the density of the cells matters to cell differentiation. It is possible that the stem cell niche, can be applied in a competition mode in order to control stem/TA cell differentiation and proliferation.

Although we restricted our experimental question as to whether follicle derived cells could directed into osteoclasts, it still possible that other sources of cell types could contribute in this differentiation process. In electron microscopy, it has been shown that blood cells can be attached to the dermal papilla cells (data not shown), and melanocyte cells and epithelial matrix cells can also attach to the top of dermal papillae due to difficulty in their removal during dissection procedures. Therefore, it is difficul to draw a conclusion on this project and future work still need to be done for further confirmation. For example, Wnt1-cre labelled mice would be a better model for further confirmation of dermal papilla cells differentiation into osteoclasts.

4.4 K15 and Id3 positive cell activity in regenerating hair follicles

4.4.1 K15 expression in the regenerating hair follicle

The observations of K15 expression pattern in the pre-stage illustrated that the K15 positive cells did not immediate change in response to hair follicle amputation. K15 proteins still showed asymmetry and discontinuous expresion along the glassy membrane (Fig3.3) as observed in the non-amputated hair follicles. K15 highlighted distal region epithelial cells (Fig3.3) raising the possibility that cells from the surrounding epidermis migrate into the follicle and contribute to the initial stages of regeneration. In the proximal region, no DAPI staining has been detected suggesting that cells did not reach the bottom of amputation site (Fig3.3). Recently, lineage analysis of *de novo* formed hair follicle after wounding demonstrated that the nascent hair follicle arise from epithelial cells outside the hair follicle stem cells niche, the bulge region (Ito, 2007). This work demonstrated that outside epidermal cells in the wound assume a hair follicle stem cells phenotype.

The K15 positive epithelial cells soon occupied the whole narrowed epithelial column (Fig3.4) in stage 1. These increased K15 positive epithelial cells could migrate from outside epidermal cells, since K15 has been reported in the epidermal skin in human and mouse (Liu, 2003, and Webb, 2004). Together with self-proliferating cells within interfollicular epithelial cells, the increase in cell numbers is likely to be a contributing factor in the initial projection of the epithelial column into dermal tissue at the base of the follicle. The distribution of K15 positive cells in stage 2 moved along the glassy membrane, with a cluster of cells accumulated in the base of hair follicle. This accumulated cluster cells increased in numbers over time. This observation indicated that stem cells from outside epithelial cells and from the bulge region may undergo differentiation into epithelial cell types in the lower hair follicle, as has been suggested in other cases (Morrison, 2004). In stage 3, the wider epithelial column may be due to a

dramatically increased cell number, indicating that the K15 negative epithelial cells inside the column have proliferated rapidly. In the end bulb cells, the K15 highlighted cells directly in contact with DAPI stained dermal cells outside the column suggest the mesenchymal and epithelial cell interaction may be involved in epithelial matrix and dermal papilla formation. Epithelial cells have shown morphological differences (Fig3.6) suggesting different epithelial cell type differentiation is also occurring. In the final stage, a new dermal papillae was formed, and newly formed hair shaft cells were moving upwards. K15 cells inside the epithelial column began to lose their K15 expression (Fig3.7) suggesting that the multiple epithelial cell layers wrapped around the dermal papillae are nearly formed.

K15 expression pattern in the epithelial column along the length of the follicle supports the proposals by Oshima (Oshima, 2001) that stem cell migration through hair follicle cycles, and also support the findings by Ito (Ito, 2007) that outside epidermal stem cells migration into hair follicle can play a role in hair follicle regeneration. Interestingly, through the entire hair follicle regenerating stages, there is no evidence showing K15 positive cells accumulating near the bulge region. This may indicate that the roles which bulge region stem cells played may not be as much as would be expected during the regeneration process. In the early stages, the epithelial column accumulated large number of K15 positive cells, and these cells soon lost their K15 expression within 2 days. This might suggested the interfollicular microenvironment is providing de-differentiation signals. These migrated outside epidermis in pre-stage, may be reprogrammed into stem/TA cell at stage 1, and these cells in turn become interfollicular epithelial cells in stage 2. Similar work has been demonstrated *in vitro* (Ferraris 2000 and Pearton 2005). Later stages, would involve the outside epithelial cells stopping their migration, and the lower hair epithelial cells beginning to differentiate into different cell types (Jahoda, 1992). Interestingly, K15 highlighted cells accumulated in the bottom of the follicle, and gradually moved up. This may suggest stem cell differentiation might start proximally and then spread distally. The

gradual formation of an upward indentation by mesenchymal cells has initiated the mesenchymal and epithelial cell interaction (Fig 3.6) and is a sign of new dermal papillae formation, which is a key factor in successful hair fibre production by the regenerating follicle. Dermal papilla cells possess innate aggregative properties both *in vivo* and *in vitro* (Jahoda, 1981; 1984 and 1993). This may offer an explanation as to why the bottom cells were extremely compactable starting from stage 3 (Fig 3.6 and Fig3.7). The newly formed dermal papillae cells in stage 4 were K15 negative, their present to replace the K15 positive epithelial cells occupied the epithelial column in earlier stages, this suggested that mesenchymal cells may invade into the epithelial column to form a new dermal papilla, and the epithelial cells were pushed away to the sides forming the cell layers wrapped around the newly formed dermal papilla cells.

4.4.2 Id3 expression in regenerating hair follicle

Id3 was detected in both epithelial and dermal cells in the regenerating hair follicle, similar to the adult hair follicle. Id3 expression also showed cellular translocation differences according to their locations. However, the regenerating hair follicles did not share the Id3 expression patterns with ordinary hair follicles, and Id3 expression in regenerating hair follicles may involve up-regulated Id3 protein expression especially in the proximal region of regeneration, which also has been reported in other tissue regeneration (Shimizu-Nishikawa, 1999 and Wang, 2003).

Id3 staining in epithelial cells showed cellular translocation changes during the regeneration stages, as well as altering their expression patterns. In the stage 1 of hair follicle regeneration, since it has no evidence shown that epidermal cells outside hair follicle are Id3 positive, so few Id3 staining detected from outside epidermis to the distal region of hair follicle inside the epithelial column (Fig3.9). The numbers of Id3 highlighted nuclear epithelial cells increased in the central region (Fig3.9). It is possible that these newly migrated skin epithelial cells were initially Id3 negative, and cells

undergo differentiation process into follicular epithelial cells in response to the interfollicle cell niche signals. In the proximal region, Id3 staining mostly focused on cytoplasmic; therefore, it may suggest Id3 protein are transferring to cytoplasm and these epithelial cells in proximal region began to differentiation. Id3 highlighted cells cytoplasm in the central and proximal region with a few exceptions at the extreme bottom of epithelial column at early stage 2 (Day 8), and Id3 expression also gradually labelled cytoplasmic in the proximal and lower central regions at late stage 2 (Day 10) (Fig3.10). These cells may undergo cell proliferation and leading to rapidly increasing numbers of epithelial cells in stage 2 may offer an explanation of widen epithelial column. Consistent with K15 expression, this also may indicate the epithelial cells differentiation initiated from bottom of the follicle, and moving upwards. Higher up in distal region, negative results were found, this results may suggest the differentiation had not reached the distal region. In stage 3, proximal region showed cytoplasmic Id3 staining, and this might a sign that these cells would lose Id3 protein expression as similar observations have been found in outer root sheath cells in anagen hair follicle (Fig2.10), and also suggests that epithelial cells differentiation process is going to be aborted as this has been described in other researches (Shimizu-Nishikawa, 1999). Some weakly nuclear stained epithelial cells were found in the central region suggested that possible of cell differentiation still ongoing in the central and distal region. In stage4, epithelial cells lost their Id3 expression except the follicular basal layer cells, suggests the epithelial cells differentiation have accomplished.

Id3 highlighted dermal cells increased rapidly from stage 2 regenerating hair follicle, and in all stages of regenerating, the vast majority of dermal cells displayed nuclear labelling with Id3 antibody. The regenerating of dermal cells have cited by other authors that involve in other source of dermal cells replacement during regenerating hair follicles, such as mesenchymal and dermal sheath cells (Oliver, 1966b, Jahoda & Oliver, 1984 and Jahoda 1992). The limited dermal Id3 staining in the early stage indicated that the majority of dermal cells migration may occur in later stages of

regenerating process, and since dermal cells present is close associated with dermal papillae regeneration (Jahoda, 1992), therefore it is also consistent with the timing of dermal papillae formation.

In stage 1 regenerating follicle, regular interval dermal cells along the glassy membrane started from distal region continuous expressed till the proximal region (Fig3.9) is evidence that some dermal cells migration from proximal region to distal region in early regenerating process. There is no evidence to show participate of dermal sheath cells or mesenchymal cells in the end bulb region in this stage. Id3 highlighted dermal cells in proximal region, especially beneath the epithelial column increased rapidly, and there were some Id3 positive dermal cells from outside follicle connect with the dermal cells inside the follicle (Fig3.10). These accumulated dermal cells beneath epithelial column are thought to act in a manner comparable to that of the dermal condensation observed in follicle development. Id3 have been reported as targets of BMP signalling (Hollnagel, 1999), and also has been investigate *in vitro* (O'Shaughnessy, 2003). These Id3 highlighted dermal cells might enhance the possibility that the regeneration of hair follicle is mimic the dermal condensation process in development stages, and this also enhance the possibility that external dermal cells source penetrated into epithelial column in order to complete dermal papillae regeneration as proposed half century ago (Oliver, 1966). In stage3, direct connections between dermal cells outside the epithelial column and epithelial cells were observed in several specimens, which suggested the dermal cells penetration. However, the observation of dermal cell penetration only observed restrict located beneath the epithelial column (Fig3.11), and based on my results, the dermal cell source is difficult to distinct since all the dermal cells from distal, central regions and outside hair follicle were all accumulated beneath epithelial column. In contrast of epithelial cells in stage4, dermal cells were still Id3 highlighted, although their expression intensity was gradually become weaker (Fig3.12). The newly formed tiny dermal papilla was Id3 negative, and newly formed hair shaft-like cells were found. This may suggested the newly formed dermal papilla

was premature, and they may be undergoing differentiation process.

The point at which dermal cells become dermal papilla cells in the regeneration process is unknown but, we observed the dermal cells and epithelial cells interaction in stage 3, around 11 and 14 days. The newly formed tiny dermal papilla was seen in day 21. This suggested the dermal papilla cells transformation would occur within day 14 and day 21. However, the dermal papilla cells might undergo longer differentiation process to become mature than we thought.

The exact source of dermal cells is also unknown, but evidence demonstrated that regeneration of hair bulbs also occurred in the upper halves of rat vibrissae follicles when they were implanted under the kidney capsule (Matsuzaki, T. 1996). Immunohistochemical studies revealed that the newly formed small hair bulbs were induced in the outer root sheath by the dermal papilla cells that were converted from the dermal sheath cells surrounding upper vibrissae follicles. This evidence further confirmed the dermal sheath cell type conversion, and also suggested the certain conditions (kidney capture) are required since the hair bulb formation was not detected in the upper half of follicles that were kept at their original site by cutting their lower halves in situ (Horne, K.A, 1986)

4.5 Conclusion

Vibrissa hair follicles provide a good model to study stem cell activity because of their easy accessibility and self-renewal. Although the argument of stem cell sources in the cycling hair follicle has lasted for several decades, the full picture is still unknown. The existence of multiple stem cell sources, migration hypotheses together with the involvement of skin epidermal stem cells make the hair follicle, although a tiny organ, still one with unexplained questions. My results suggest the hair follicle stem cell migration is the most likely model to explain the K15 expression pattern, and I personally make some new suggestions following observations that are inconsistent with other researchers.

The regenerating vibrissa follicle remains an excellent model for studying various aspects of hair follicle biology and wound healing process. The stem cell source for regenerating hair follicles required further investigation, though increasing evidence showed the outside skin epidermal stem cells are likely to play an important role. It is also interestingly to look at bulge region stem cell activity in the skin wound healing process. The differentiation of dermal papilla cells from the existing cell population is an area that requires further study, but the dermal sheath remains the most likely source of new dermal papilla cells.

However, we have to bear in mind is that the exact roles of K15 and Id3 is still unknown in some aspects, especially in the wound healing process. There are limited research reports involving K15 and Id3 and their roles in wound healing process and although they have been said to participate in stem/TA cell differentiation, these mechanisms are still largely unknown. Therefore, knockout/knockdown experiment would probably provide new information on the functions of these proteins in this context. Interestingly, our ongoing project, investigating K15 and Id3 expression pattern of skin wound healing process might offer more details about this.

Hair dermal stem/TA cells differentiation into osteoclasts suggested that these cells might have multi-potential features *in vitro*. This work demonstrated that sufficient stem cell niche signalling conditions can mimic the stem cell niche in order to initiate cell differentiation. Considering the easy access of hair follicle stem cells and their specific niches, they have the potential to provide future medical applications in tissue engineering.

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