Properties of Vibrissa follicle cells during follicle development and regeneration, and their interactions with embryonic stem cells.

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"Properties of Vibrissa Follicle Cells During Follicle Development and Regeneration, and Their Interactions with Embryonic Stem Cells."

by

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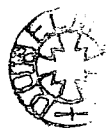
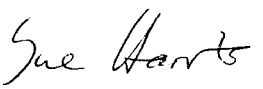


This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.



S. J. Harris

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ABSTRACT

Follicular dermal cells possess properties that regulate induction of follicle formation, hair growth, and follicle end bulb regeneration after amputation. While the molecular basis for these developmental interactions are being uncovered, it is evident that innervation, pigmentation and vascularisation of the follicle, together with its role as a stem cell repository, make interpretations of molecular function within the follicle, complex. This thesis examined aspects of vibrissa follicle development using immunohistochemistry, and demonstrated that segregation of the follicle dermis and epithelial differentiation were defined by lamin-A antibody. Versican, a proteoglycan recently implicated in dermal papilla induction, was absent from early dermal condensations, but its expression correlated with follicle innervation during development and the adult hair growth cycle. When lower follicle regeneration was studied with *in situ* hybridisation and immunohistochemistry, versican showed two distinct expression domains. These were the dermal components of the end bulb and the follicle neck. BrdU labelling of cell division showed regeneration of the epithelial component to be consistent with stem cell location. Paucity of dermal cell proliferation left the precise origin of the new dermal papilla unresolved, but α-smooth muscle actin expression showed that dermal sheath cells moved through the glassy membrane. *Sonic hedgehog* expression indicated that epithelial-mesenchymal interactions, evident in follicle development, were mirrored in regeneration.

A co-culture model investigated the capacity of follicular dermal cells to induce embryonic stem (ES) cell differentiation. Unexpectedly, follicle cells were seen to maintain ES cells in an undifferentiated condition. Differentiation assays demonstrated that ES cells remained pluripotent after co-culture. Interleukin-6 family cytokines, known to maintain ES cell pluripotency, were detected by RT-PCR in cultured cells and vibrissa follicles. Thus, since the follicle dermis produces these cytokines, they may be acting to inhibit the differentiation of follicular epithelial stem cells and/or maintain multipotent stem cell populations in the follicle.

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ABBREVIATIONS

AFP	Alpha-1-fetoprotein
APM	Arrector pili muscle
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BDMA	n-Benzyldimethylamine
BM	Basement membrane
BMP	Bone morphogenetic protein
bp	base pairs
BrdU	5-Bromo-2'-deoxyuridine
CF	Club fibre
CM	Conditioned media
CMV	Cytomegalovirus
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNTFRα	CNTF receptor α
CSPG	Chondroitin sulfate proteoglycan
CT-1	Cardiotrophin-1
DC	Dermal condensation
DDSA	Dodecenylsuccinic aldehyde
DEPC	Diethyl pyrocarbonate
DIA	Differentiation inhibitory activity
DIG	Digoxygenin
dNTP	deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid

DP	Dermal papilla
DS	Dermal sheath
DVN	Deep vibrissal nerve
EB	Embryoid body
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EM	Electron microscopy
EPU	Epidermal proliferative unit
ES	Embryonic stem
ESRF	Embryonic stem cell renewal factor
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GAP-43	Growth-associated protein-43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GE	Germinative epithelial
GF	Growing fibre
GFP	Green fluorescent protein
HABR	Hyaluronic acid-binding region
HS	Hair shaft
ICB	Inner conical body
IL	Interleukin
IRS	Inner root sheath

ISH	*In situ* hybridisation
kb	kilobase
l	litre
LEF	Lymphoid enhancer factor
LIF	Leukemia inhibitory factor
m	milli-
M	Molar
MEM	Minimal essential medium
min	minutes
n	nano-
NBT	Nitro blue tetrazolium
NCAM	Neural cell adhesion molecule
OCT	Optimum cutting temperature
ORS	Outer root sheath
OSM	Oncostatin M
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Petri dish
PDGF	Platelet derived growth factor
PF	Paraformaldehyde
RA	Retinoic acid
RNA	Ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse transcriptase-PCR
SF	Skin fibroblasts

Shh	Sonic hedgehog
α-sma	α-smooth muscle actin
STAT	Signal transducers and activators of transcription
SVN	Superficial vibrissal nerve
TAE	Tris-acetate-EDTA
TC	Tissue culture
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TRITC	Tetramethylrhodamine isothiocyanate
TVP	Trypsin-versene-PBS
T_3	Triiodothyronine
U	Units
μ	micro-
Z40	Zin40

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Chapter 1: Introduction

For many years the hair follicle has been the subject of extensive research with the physiological process of cyclic hair growth coming under close scrutiny (Chase, 1954; Hardy, 1992; Fuchs, 2001). More recently, it has become apparent that the hair follicle provides an effective means of studying a number of aspects of biology: the epithelial-mesenchymal interactions that are an integral part of hair follicle cycling provide an opportunity to investigate developmental interactions in an accessible system; the immune privilege demonstrated by hair follicles is an intriguing aspect for the immunologist; pigmentation of the hair fibre presents an opportunity to study melanogenesis; the regulatory mechanisms that initiate hair growth and the cessation of growth are potentially important in tumour biology; the complex innervation, particularly of the rodent vibrissa follicle, provides a model for studies on the detailed regulation of sensory innervation; and, over the last decade, research into hair follicle stem cells has identified the hair follicle as a source of multipotent cells, demonstrating plasticity *in vivo* and *in vitro*.

1.1. DEVELOPMENT OF THE HAIR FOLLICLE

1.1.1. Hair follicle morphogenesis

The hair follicle develops as a result of complex interactions between the epithelium and mesenchyme. Other skin appendages, including teeth and feathers, develop in a similar manner, sharing both morphological characteristics and signalling mechanisms, particularly in the earliest stages of development (Davidson & Hardy, 1952; Hardy, 1992; van Genderen et al., 1994; Thesleff et al., 1995; Kratochwil et al., 1996; Dassule & McMahon, 1998). A diagram of the developmental stages of the mouse vibrissa follicle (Figure 1.1) shows how the epidermis first thickens, then invaginates in response to signals from the underlying dermis. The basic process is the same for all hair follicles but slight differences occur in timing and rate of development

between different types of follicles. Structural differences between species are also evident, but the underlying principles of epidermal-mesenchymal signalling are believed to be universal in hair follicle development (Davidson & Hardy, 1952; Chase 1954).

Figure 1.1. The developmental stages of the rodent vibrissa follicle.

Stage 0 - thickening of the epidermis results in the epidermal placode forming.

Stage 1 - epidermis begins to invade the underlying dermis.

Stage 2 - the epidermal plug is now surrounded by the densely packed dermal cells that wil become the dermal papilla and sheath.

Stage 3 - the presumptive dermal papilla is surrounded by the invaginating epidermal downgrowth.

Stage 4 - the characteristic bulbous shape of the follicle starts to appear as does the keratinized inner root sheath.

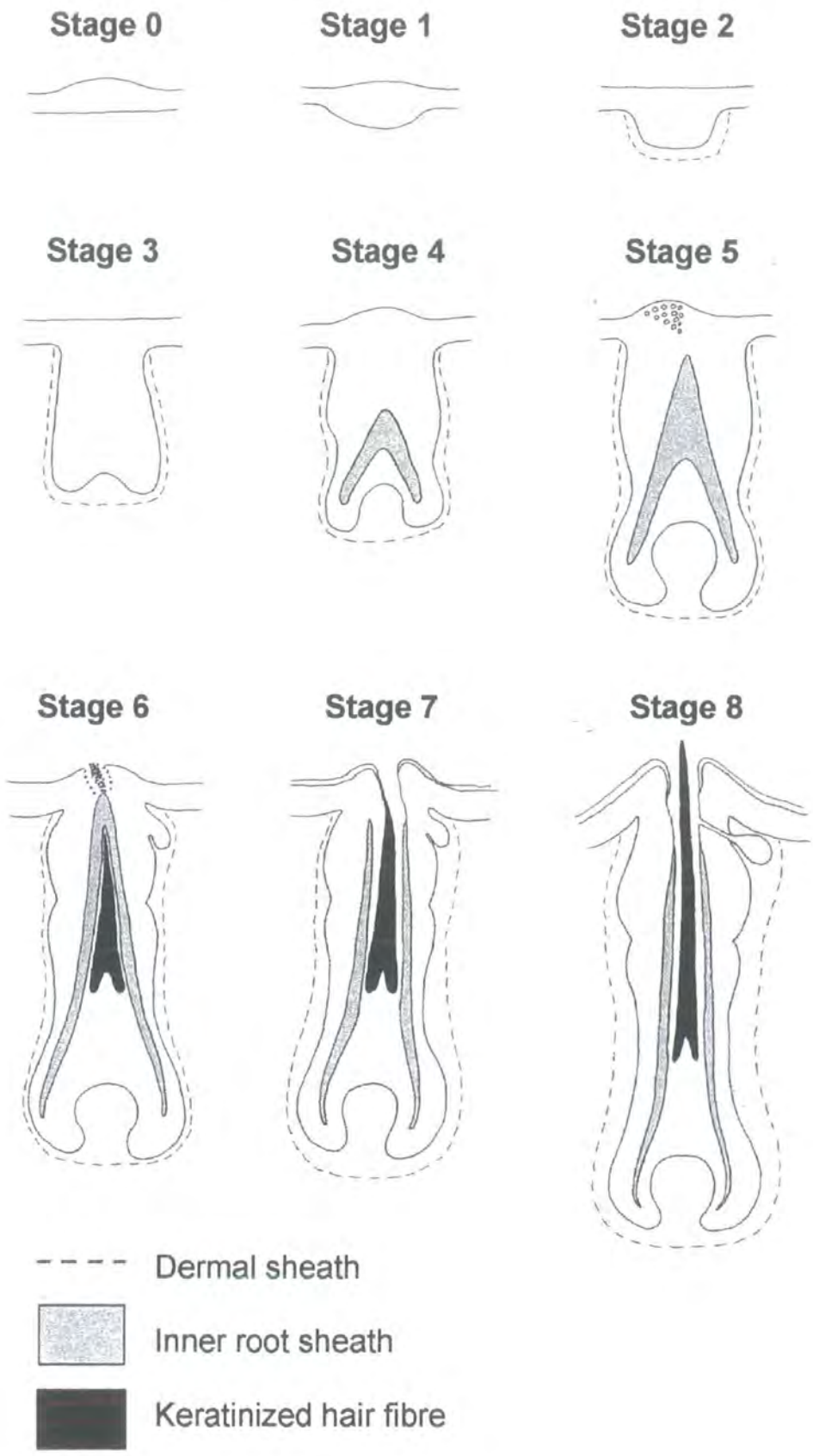
Stage 5 - Cells at the surface of the epidermis begin to differentiate in preparation for formation of the hair canal.

Stage 6 - Apoptosis occurs at the presumptive hair canal, the developing sebaceous gland is visible and keratinized hair fibre is present.

Stage 7 - The hair canal opens.

Stage 8 - Keratinized hair fibre emerges from the follicle.

Adapted from Davidson & Hardy (1952).


Stage 0
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
Stage 6
Stage 7
Stage 8
Dermal sheath
Inner root sheath
Keratinized hair fibre

1.1.2. Pattern formation

The precise nature of the epidermal-mesenchymal interactions that are involved in appendage formation has long been a subject of great interest, as such interactions are a fundamental part of the development of many organs including lung, liver, mammary gland, intestine and teeth (Thesleff et al., 1995; Chuong, 1998). Many of the cell signalling molecules involved are also implicated in patterning (Oro & Scott, 1998). That is they are thought to be responsible for regulating the arrangement of structures, in addition to influencing the morphogenesis of individual structures.

The most fundamental patterning processes include establishment of body axis and left-right asymmetry, but there are many more refined processes which are just as strictly regulated. For example, the vibrissa follicles develop in a particular sequence; pelage follicles initially develop in triads of primary follicles, around which secondary follicles develop at a later stage; and in chick skin, feather development spreads from the midline to the flanking regions of the tract, with feather buds developing in hexagonal arrays (Dhouailly, 1977; Sengel et al., 1980). In association with these appendages there are secondary structures such as vasculature and innervation that also require strict regulation of their development for successful function.

Such elaborate patterning in an organ (the skin) that is so accessible provides an excellent model for studying patterning during development (Sengel et al., 1980; Chuong & Widelitz, 1998). The molecular control of pattern formation is a complex process in which threshold levels, gradients and sequestering of molecules all play their part, but it is generally based on simple principles such as lateral inhibition and self-promoting feedback loops (for recent reviews see Freeman, 2000; Meinhardt & Gierer, 2000; Gurdon & Bourillot, 2001).

1.1.3. Dermal condensation

Elaborate tissue recombination experiments have established that the pattern of appendage development in an epidermis is determined by the underlying dermis (Sengel et al., 1980). 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 (Davidson & Hardy, 1952). This aggregation in the dermis is crucial in morphogenesis of teeth, feathers and hair, but the means by which it is achieved are still unclear (Thesleff et al., 1995). Cell adhesion, migration and morphogenesis are all likely to be contributing factors.

The physical process of aggregation is one point for discussion, but perhaps a more fundamental process is the segregation of these cells from those of the surrounding mesenchyme. The aggregative properties displayed in hair follicle development are maintained in adulthood and are evident in cultured dermal papilla cells both *in vitro* and, when implanted ectopically, *in vivo* (Jahoda & Oliver, 1984a; Messenger, 1984). This retention of embryonic properties makes the segregation of follicular dermal cells from mesenchymal cells all the more intriguing, as this differentiation results in a cell population that displays some remarkable properties throughout adulthood.

Identifying the signalling molecules expressed by these cells is an important task in hair biology as the dermal condensation is responsible for the message to the epidermis inducing follicle formation. One molecule, whose expression in developing skin is exclusive to the cells of the dermal condensation, is Noggin and in *noggin* -/- mice, follicle morphogenesis is significantly delayed (Botchkarev et al., 1999a). It has been demonstrated in other systems that Noggin induces developmental processes by antagonising the inhibitory influence of BMP signalling (Zimmermann et al., 1996). Botchkarev et al. (1999a) determined that by inhibition of BMP-4 signalling, Noggin

effectively upregulates LEF1, a transcription factor crucial to hair follicle morphogenesis (van Genderen et al., 1994; Zhou et al., 1995; Kratochwil et al., 1996), and, independent of BMP-4, Noggin also inhibits the expression of p75, a neurotrophin receptor that has been implicated in follicle morphogenesis (Botchkarev et al., 1999). Whilst Noggin is not essential to induction of hair follicle development, its expression clearly has an inductive effect.

The cells of the early dermal condensation will contribute to the dermal components of the follicle (dermal papilla and dermal sheath), but the small number of cells in the early condensation is clearly not sufficient to form these tissues without additional cells becoming involved. Studies have shown that little or no proliferation occurs in these dermal condensations (Wessels & Roessner, 1965) but it is unclear by what means further cells are recruited into these tissues or from where these cells originate. Investigations into the molecular profile of aggregating dermal cells, in comparison to the non-follicular dermal cells, are important to further our understanding of some of their characteristics in adulthood as well as development.

1.1.4. Key signalling molecules involved in hair follicle development

The expression patterns of various growth factors, extracellular matrix (ECM) molecules and transcription factors have been shown to change during follicle organogenesis (Table 1.1) (for review see Oro & Scott, 1998; Fuchs, 2001; Callahan & Oro, 2001). Whilst many molecules have been identified as being differentially expressed during hair follicle development, this is not necessarily sufficient evidence to conclude that such molecules are directly involved in hair follicle morphogenesis. The isoforms of TGF-β are a good example as they have all been found to change expression during follicle development, with significant differences between the distribution of TGF-β1, TGF-β2 and TGF-β3 (Heine et al., 1987; Lyons, Pelton &

Hogan, 1990; Pelton et al., 1991). However, a combined genetic and functional approach by Foitzik et al. (1999) indicated that whilst TGF-β2 was necessary for hair follicle development, TGF-β1 and TGF-β3 did not appear to be critical in follicle morphogenesis. This demonstrates how differential expression during development is not sufficient to imply a functional role. The development of other systems associated with follicles (innervation, vasculature, melanogenesis) should always be borne in mind when attributing function to a molecule based on expression patterns alone.

Table 1.1. Molecules which are implicated in hair follicle development by differential expression.

Molecule	Reference
ECM molecules	
Tenascin	Jiang & Chuong, 1992
	Kaplan & Holbrook, 1994
Fibulin-1 & -2	Zhang et al., 1996
Perlecan	Kaplan & Holbrook, 1994
Cell adhesion molecules	
NCAM	Scarisbrook & Jones, 1993
	Kaplan & Holbrook, 1994
E-Cadherin	Hirai et al., 1989
	Kaplan & Holbrook, 1994
P-Cadherin	Hirai et al., 1989
Growth factors & receptors	
PDGF-A	Karlsson et al., 1999
TGF-βR II	Paus et al., 1997
TGF-α	Luetteke et al., 1993
	Mann et al., 1993
BMP2 & BMP4	Lyons et al., 1990
	Bitgood & McMahon, 1995
	Wilson et al., 1999
EGFR	Green & Couchman, 1984
	Hansen et al., 1997
HGF	Lindner et al., 2000
Met (HGFR)	Lindner et al., 2000
Cell signalling molecules	
Notch	Kopan & Weintraub, 1993
	Favier et al., 2000
Sonic Hedgehog	Bitgood & McMahon, 1995
	Iseki et al., 1996
β-catenin	Huelsken et al., 2001
Retinoic acid receptor-α & -γ	Viallet & Dhouailly, 1994
Transcription factors	
LEF-1	van Genderen et al., 1994
	Zhou et al., 1995
	Kratochwil et al., 1996
	DasGupta & Fuchs, 1999
Msx1 & Msx 2	Stelnicki et al., 1997
Alx-4	Hudson et al., 1998

Transgenic animals have been used to identify signalling molecules involved in hair follicle organogenesis, an example being the *noggin* -/- mice used by Botchkarev et al. (1999a) in the studies described earlier (see 1.1.3). Often a role in hair follicle development corresponds to functions at other sites of epithelial-mesenchymal interactions (van Genderen et al., 1994, Hudson et al., 1998). The most widely studied molecules include those of the Wnt/β-catenin/LEF-1 pathway (outlined in Figure 1.2), BMPs and sonic hedgehog (Shh) (Millar et al., 1999, van Genderen et al., 1994, Gat et al., 1998, Chiang et al., 1999, St-Jacques et al., 1998, Wang et al., 2000). A significant number of growth factors, including platelet-derived growth factor, TGF-α and -β, FGF and EGF (du Cros, 1993; Luetteke et al., 1993; Danilenko et al., 1996; Karlsson et al., 1999; Betsholtz et al., 2001), have also been shown to exert an effect on hair follicle development by functional studies. A list of molecules that function in hair follicle morphogenesis is presented in Table 1.2.

Figure 1.2. Wnt canonical pathway.

(A) In the absence of Wnt signalling glycogen synthase kinase (GSK3) degrades intracellular β-catenin.

(B) Binding of Wnt to the receptor frizzled, signals dishevelled (Dvl) to inhibit GSK3 from destabilizing β-catenin. This results in an accumulation of β-catenin which is then transported into the nucleus to bind with TCF/LEF complexes and so regulate the target genes of Wnt. The membrane bound protein Notch is also capable of binding Wnt, as are cell surface glycosaminoglycans (GAG) and these are thought to act as co-receptors. Cerberus antagonizes Wnt signaling by binding the protein to a site which overlaps its antagonistic binding site for BMP. Such competition could result in Cerberus functioning to regulate the levels of Wnt and BMP in relation to each other.
Adapted from Dale (1998).

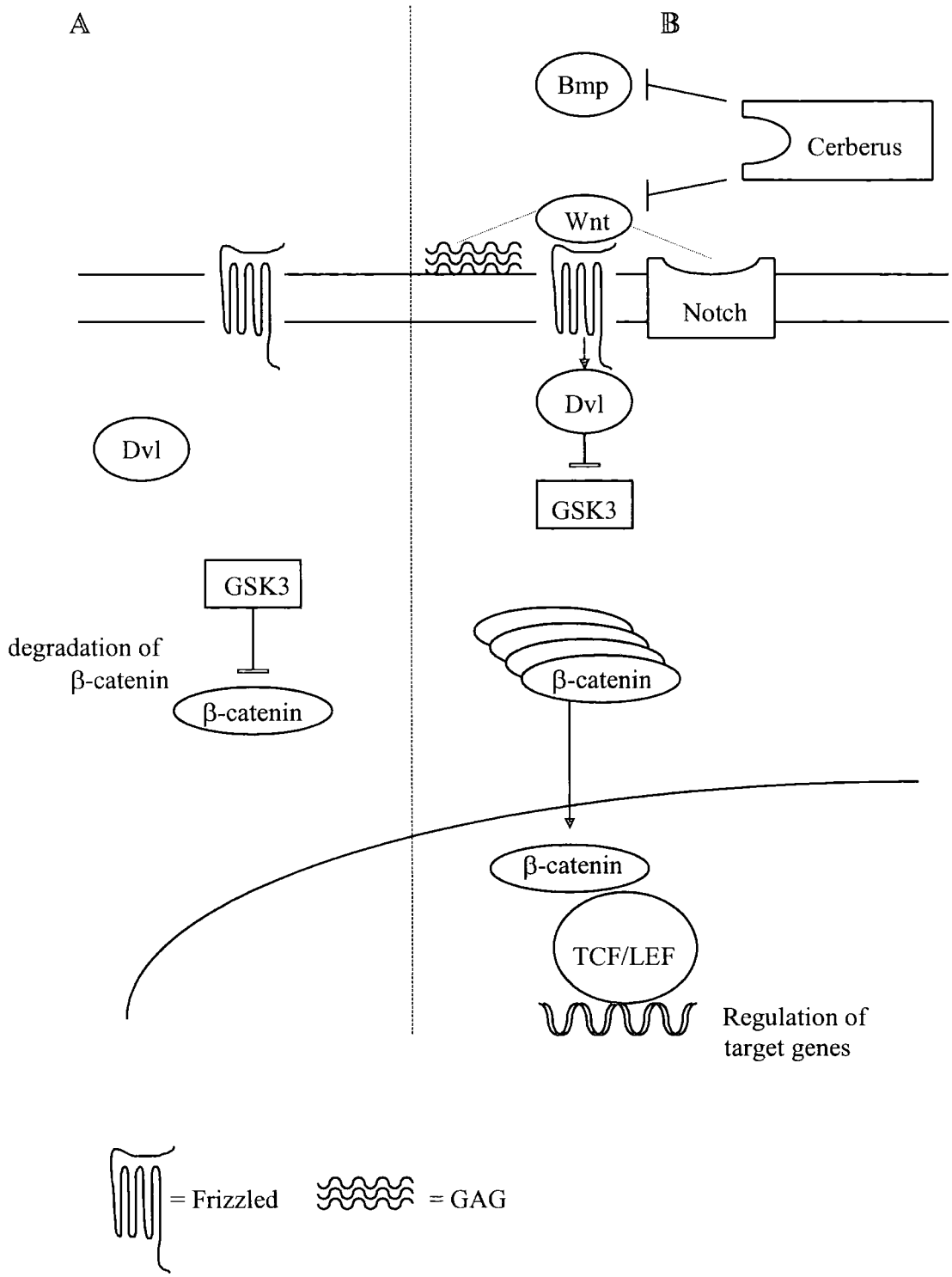

A
B
Bmp
Cerberus
Wnt
Notch
Dvl
Dvl
GSK3
GSK3
degradation of
β-catenin
β-catenin
β-catenin
β-catenin
TCF/LEF
Regulation of
target genes
= Frizzled
= GAG

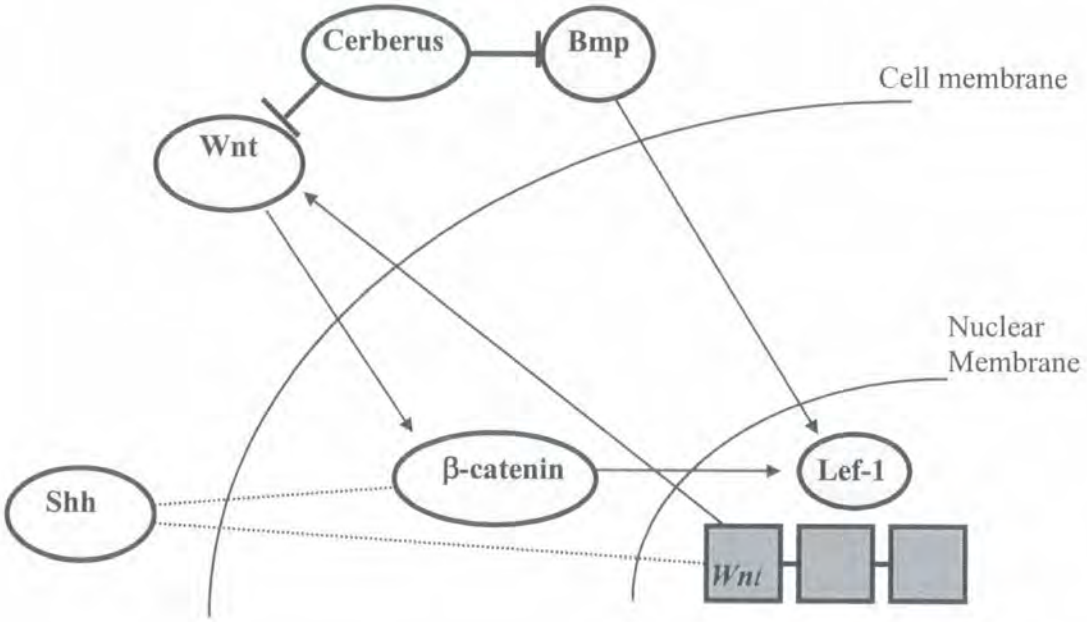
Table 1.2. Molecules which have been demonstrated to influence hair follicle morphogenesis by functional studies.

Molecule and method	Influence on follicle	Reference
Truncated β-catenin under K14 promoter	Interfollicular epidermal cells induced *de novo* follicle formation	Gat et al., 1998
β-catenin - mutant gene under K14 promoter cannot encode protein	β-catenin -ve skin has no hair follicles	Huelsken et al., 2001
β1 integrin	Crucial for follicle development to occur	Brakebusch et al., 2000 Raghavan et al., 2000
Sonic hedgehog -/- mice	Follicle development limited to hair germ formation, any further epithelial downgrowth resulted in epidermal cyst-like structures	St-Jacques et al., 1998 Chiang et al., 1999 Wang et al., 2000
Shh - transient expression	Premature onset of anagen	Sato et al., 1999
TGF-α -/- mice	Disorientated, misaligned follicles with curly whiskers and wavy hair	Luetteke et al., 1993 Mann et al., 1993
TGF-β2 -/- mice	Delayed HF morphogenesis and a 50% reduction in follicle number	Foitzik et al., 1999
TGF-β2 treatment of wildtype skin explants	Induction of HF development	Foitzik et al., 1999
FGF subcutaneous injection	Inhibition of follicle development and thickening of connective tissue layer beneath panniculus carnosus - restricted to area around injection site	du Cros, 1993
EGF subcutaneous injection	Inhibition of follicle growth and reduced skin thickness - affected whole body	du Cros, 1993
PDGF-A -/- mice	Disorganization of follicles and mishapen bulbs, reduced number of α-sma positive dermal sheath cells	Karlsson et al., 1999

Signalling molecules are often described in association with a particular intracellular pathway. However, cellular morphogenesis occurs as a result of a multitude of signals and the relationship between signalling pathways, both extracellularly and intracellularly, is important in understanding developmental signals. Feedback loops are often established by connections between pathways and provide an important mechanism by which developmental processes can be strictly regulated (reviewed by Freeman, 2000). In the developing hair follicle, *Wnt5a* is a downstream target of Shh (Reddy et al., 2001), β-catenin acts upstream of *Shh* and *bmp* (Huelsken et al., 2001), and BMP signalling is mediated by the transcription factor LEF1 (Kratochwil et al., 1996). Thus the signalling of Wnt, BMPs and Shh is linked in a fairly complex fashion (Figure 1.3). Unravelling the signalling mechanisms of hair follicle development is a complex but important topic of hair follicle research.

Figure 1.3. Relationships between Wnt, Shh and BMP signalling.

Cerberus potentially balances Wnt and BMP signalling by extracellular antagonism. Shh regulates *Wnt* gene expression. LEF-1 acts downstream of β-catenin (which is downstream of Shh and Wnt) and BMP.



1.2. THE ADULT HAIR GROWTH CYCLE

1.2.1. Anatomy and remodelling of hair follicles during 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 (anagen). When growth stops the follicle regresses (catagen) before entering a rest period (telogen) after which anagen restarts and the cycle is repeated. The process of shedding the fibre after the follicle has regressed is sometimes referred to as exogen (Paus & Cotsarelis, 1999).

The basic cycle of anagen-catagen-telogen-anagen is common to all types of hair follicle but there are significant differences in the anatomy, and consequently the cyclic remodelling process, of different types of hair follicle. An example of this is the rat vibrissa follicle (see Figure 1.4) when compared to the rat pelage follicle (Young & Oliver, 1976). The most obvious anatomical difference is in size, but perhaps the most significant cyclic difference is in the telogen phase of the cycle (Figure 1.5). The pelage dermal papilla regresses with the column of epidermal cells up to the level of the bulge, so reducing the length of the follicle to less than a third of its anagen length. In contrast, there is very little regression of the vibrissa follicle papilla. The epithelial matrix regresses and the bulb of the follicle shrinks in size but the papilla remains in the same place. These morphological differences have raised significant questions regarding the initiation of the next anagen phase.

Figure 1.4. Diagrammatic representation of an anagen vibrissa follicle.
Key structures of the follicle-sinus complex are shown excluding blood supply, innervation and external muscle attachments. DP - dermal papilla, DS - dermal sheath, GM - glassy membrane, IRS - inner root sheath, ORS - outer root sheath, C - capsule, SG - sebaceous gland, CF - club fibre, GF - growing fibre, RW - ringwulst.
Adapted from Oliver, 1966a.

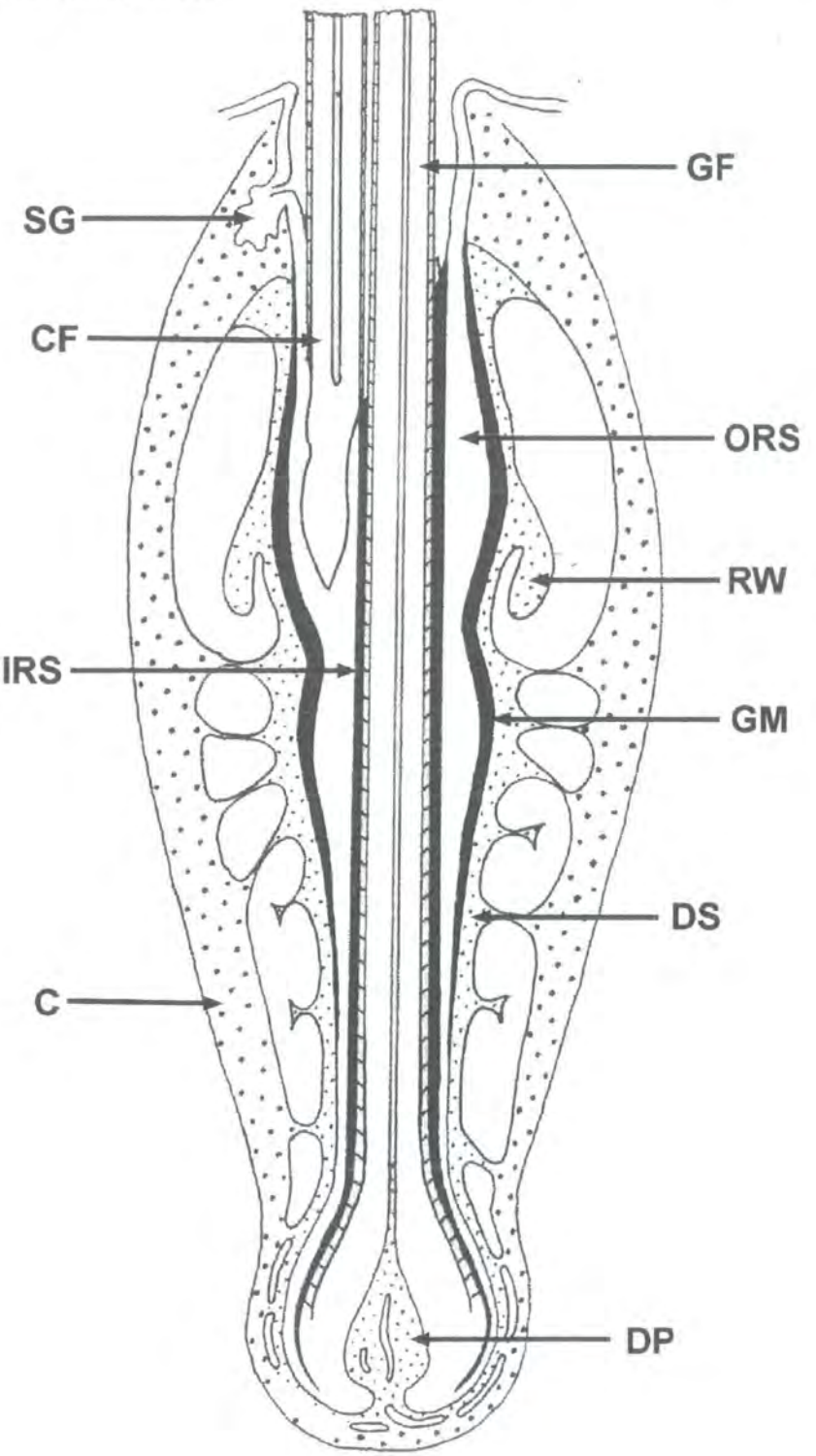
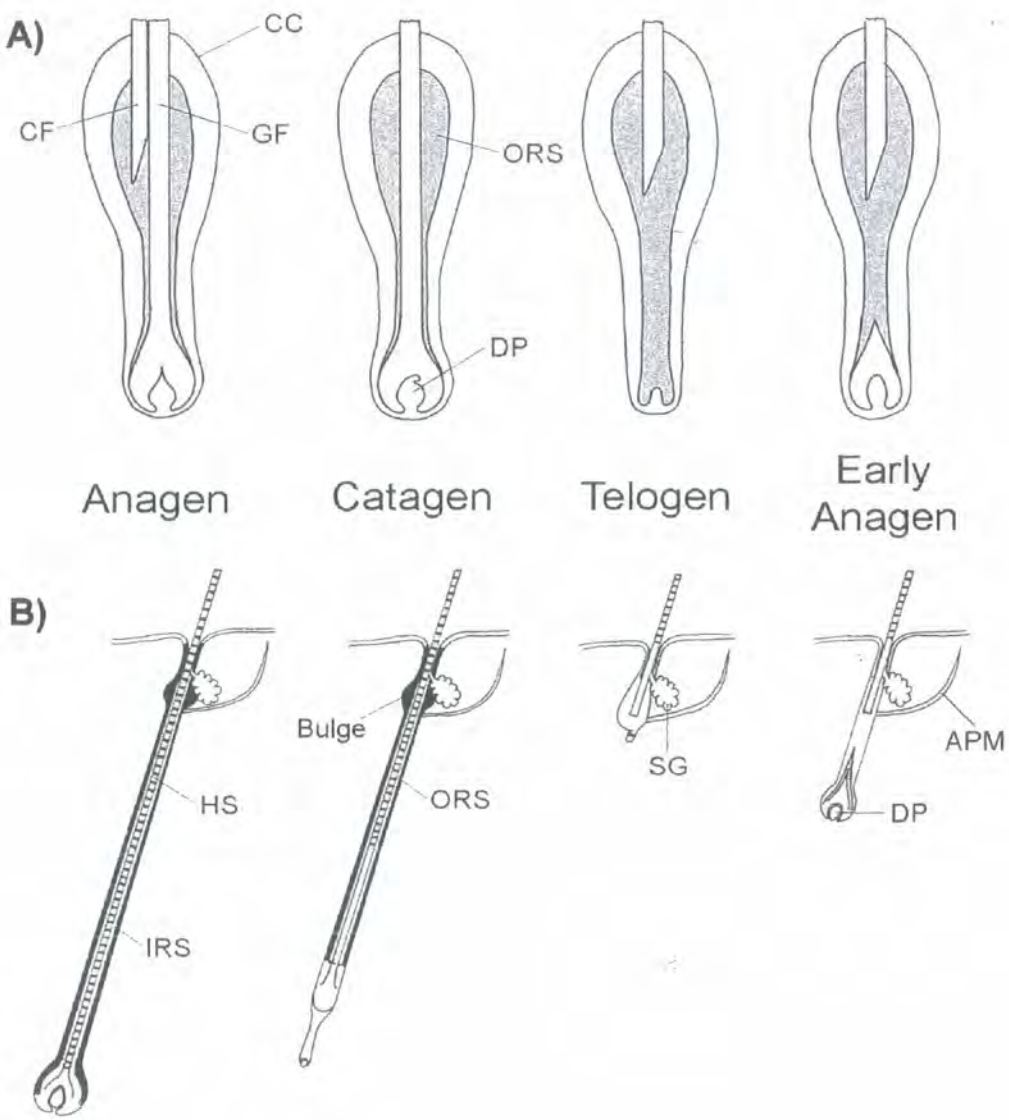


Figure 1.5. Comparison between vibrissa and pelage follicle growth cycles.
A) In the vibrissa follicle the shape of the dermal papilla (DP) and the position of the growing (GF) and club fibres (CF) are the key indicators of cycle stage.
B) The pelage follicle undergoes more dramatic changes during the growth cycle with the regression of the dermal papilla to the level of the bulge (catagen-telogen). Initiation of anagen results in regeneration of this temporary part of the follicle, a fibre is produced and the cycle repeats itself.
ORS - outer root sheath, IRS - inner root sheath, SG - sebaceous gland, CC - collagen capsule, HS - hair shaft, APM - arrector pili muscle.
Adapted from Paus et al., 1997.



The epithelial-mesenchymal interactions have always been regarded as the key interactions for regulating the adult cycle as well as follicle morphogenesis. Cotsarelis et al (1990) proposed that a population of slow-cycling cells in the bulge of the pelage follicle outer root sheath were putative stem cells. It was hypothesised that regression of the follicle end bulb during telogen provided the means by which dermal papilla cells gained contact with these follicular stem cells to initiate the next anagen phase. However, this did not provide a suitable explanation of how this was achieved in the vibrissa follicle where the dermal papilla remains at a significant distance from the bulge. This has since been resolved by some elegant experiments demonstrating a migration of stem cells through the outer root sheath into the vibrissa follicle end bulb (Oshima et al., 2001).

The vibrissa follicle (Figure 1.5) is a specialised hair follicle characterised by a blood-filled sinus surrounding the follicle itself and forming what is referred to as a follicle-sinus complex (Rice et al., 1986). The follicle-sinus complex is highly innervated commensurate with the vibrissa follicle's specialised role as a mechanoreceptor. Despite these differences the vibrissa follicle remains a valid model for hair biology research, as properties initially attributed to vibrissa follicle cells, such as the capacity to induce hair follicle formation, have since been identified in pelage follicles (Jahoda et al., 1984; Reynolds & Jahoda, 1992).

1.2.2. Molecular basis of the adult cycle

The anatomical changes that occur through the hair follicle cycle are well documented, but still relatively little is known about the signals involved in triggering the switch from one stage of the process to the next. In the search for this information, many studies have observed that specific molecules undergo cyclic changes within certain parts of the hair follicle, and subsequently proposed that these molecules are

involved with the regulation of the cycle. An extensive review of molecules associated with hair follicle cycling was carried out by Stenn, Prouty & Seiberg (1994) and this has recently been followed up by Stenn & Paus (2001).

Functional studies, either *in vivo* or *in vitro,* have provided information about a number of molecules which influence the adult hair growth cycle (Table 1.3). Clinical treatments can also provide clues to the mechanisms that regulate hair growth. The unexpected side-effects of cyclosporin A, a treatment for autoimmune conditions, includes hirsuitism and this has been attributed to promotion of follicular epithelial cell proliferation (Takahashi & Kamimura, 2001).

Despite the significant amount of research into the control of the hair growth cycle, there is still limited evidence as to which molecules are fundamental to hair growth and which are functional in systems linked to the follicle, such as innervation or melanogenesis. In a recent descriptive study, Ito & Kizawa (2001) found the cell-growth-associated, calcium-binding proteins S100A4 and A6, to be expressed in such a manner through the pelage follicle growth cycle, they proposed that these genes are associated with migration and proliferation of follicular stem cells. Whilst there is nothing to say this is not the case, and such work can only help to unravel the complex processes within the follicle, the evidence appears to be purely circumstantial. Findings such as these should be regarded as interesting observations to be followed up with more functional analysis, relating not only to the hair growth cycle but also to the other processes involved such as innervation and vascularization of this dynamic organ.

During the hair growth cycle, epithelial-mesenchymal interactions regulate the morphological changes occurring in the follicle (Hardy, 1992) with much of the developmental process being recapitulated. The hair follicle is therefore an excellent model for studying such interactions.

Table 1.3. Molecules affecting the adult hair growth cycle as shown by functional studies.

Molecule	Expression/function	Reference
Shh	Induction of anagen	Sato et al., 1999 Wang et al., 2000
β-catenin	Induction of anagen	Huelsken et al., 2001
Wnt3	Hair shaft differentiation	Millar et el., 1999
Dvl2	Hair shaft formation	Millar et al., 1999
BMPs	Matrix proliferation and hair shaft differentiation	Blessing et al., 1993 Kulessa et al., 2000
Fgf5	Transition from anagen to catagen	Hebert et al., 1994
EGF receptor	Transition from anagen to catagen	Hansen et al., 1997
β1-integrin	Matrix proliferation	Brakebusch et al., 2000 Raghavan et al., 2000
KGF receptor	Blocked signalling delays hair regrowth	Werner et al., 1994
Protein kinase A	Inhibition of matrix proliferation *in vitro*	Harmon & Nevins, 1997
IL-1α	Inhibition of matrix proliferation *in vitro*	Harmon & Nevins, 1993

1.3. METHODOLOGY IN HAIR BIOLOGY RESEARCH

The methods employed to study hair follicle biology have developed as both technology and knowledge of the subject have progressed. Early histological studies led to an anatomical understanding of hair follicle development and adult cycling and provided the basis for many subsequent expression and functional studies (Davidson & Hardy, 1952; Chase, 1954; Wessels & Roessner, 1965; Young & Oliver, 1976). The availability of antibodies specific to growth factors and their receptors, extracellular matrix components, and cell signalling proteins has enabled the expression of many molecules to be identified within the follicle (Green & Couchman, 1984; Couchman & Gibson, 1985; Jahoda et al., 1992b; du Cros et al., 1995; Paus et al., 1997). Relatively simple molecular techniques, such as RT-PCR (Lindner et al., 2000), have been followed by *in situ* hybridisation (van Genderen et al., 1994; Gat et al., 1998; DasGupta & Fuchs, 1999) and RNase-protection assays for detection of gene transcripts *in situ*. Such biological techniques are applicable to any experimental system, but the significance of the data can only be as good as the model system employed. Table 1.4 highlights the advantages and disadvantages of some of the experimental systems that are used in hair biology research.

Table 1.4. Experimental models used to investigate various aspects of hair biology. Applications, advantages and disadvantages are highlighted (disadvantages in italics), and some example studies that have used such models are listed.

Model system	Comments	References
Rodent skin	Postnatal hair growth synchronized Depilation induces synchronized growth Simple and inexpensive Factors can be investigated by topical application	Blessing et al., 1993 Botchkarev et al., 1999b Lindner et al., 2000
Comparative studies	Specialised hair follicles (eg. vibrissa) Deer stags show seasonal changes in response to androgens (mane follicles)	Mosconi & Rice, 1993 Thornton et al., 1996
Transgenic mice	Genetics of mice well known Effects throughout organism *Genetic redundancy can mask effects* *Knockout may be embryonically lethal*	van Genderen et al., 1994 St-Jacques et al., 1998 Yamanishi, 1998 Karlsson et al., 1999
Natural mutants	Altered hair growth, keratinization, orientation or pigmentation Establish genetic basis of human forms (eg. *hairless*) *Unknown genetic background*	Hebert et al., 1994 Nehls et al., 1994 Ahmad et al., 1998
Transgenes under K14 promoter	Restricts expression to basal cells of epidermis and outer root sheath cells Can manipulate genes of which knockouts prove embryonically lethal	Botchkarev et al., 1998 Millar et al., 1999 Charpentier et al., 2000 Huelsken et al., 2001
Cell culture	Distinct populations grown in isolation or in combination with other cell types Strict regulation of exogenous factors *Cells can change characteristics (eg. α-sma in DP cells)*	Messenger, 1984 Jahoda & Oliver, 1984a Calver et al., 1993 Takahashi & Kamimura, 2001
Organ culture	Maintains in vivo relationship between follicular cell populations Strict regulation of growth conditions cyclic effects of exogenous factors *Limited period of growth*	Philpott et al., 1989, 1992 Hirai et al., 1992 Tobin et al., 1993 Harmon & Nevins, 1997
Tissue recombinations	Spatial, temporal and species specificity Exogenous factors can be applied	Dhouailly, 1977 Sengel et al., 1980

1.3.1. The rodent vibrissa follicle

The rodent vibrissa follicle (Figure 1.4) is particularly suited to experimental manipulation and study due to its size and accessibility, making isolation, histological studies, surgical manipulation, stage identification (by fibre length), and organ culture all relatively straightforward. The ability to microdissect the vibrissa follicle has also enabled the production of cDNA libraries from different components of the end-bulb, leading to the development of subtractive libraries (Whitehouse et al., 2001 - in press).

The rat vibrissa follicle was the first to be used for the isolation of dermal papilla cells for cell culture purposes, and subsequently, dermal sheath and germinative epithelial cells have also been cultured from these large follicles (Jahoda & Oliver, 1981; Reynolds & Jahoda, 1991, 1996). Just as tissue recombination experiments revealed the inductive properties of embryonic dermis, recombinations of adult epidermis and vibrissa dermal papillae resulted in follicle formation indicating the inductive properties of the dermal papilla (Oliver, 1970). The advantage of the vibrissa follicle in such experiments is the phenotypically recognisable fibre that results from these induced follicles (Jahoda et al., 1993). This a) enables identification of the site of implant, and b) demonstrates the dermal papilla is responsible for the type of follicle formed, as was indicated by earlier work using embryonic tissue recombinations (Dhouailly, 1977; Sengel et al., 1980). These inductive properties were first identified using vibrissa follicle dermal tissue, but have subsequently been demonstrated in dermal papillae from human follicles (Reynolds et al., 1995), thus supporting the use of the vibrissa follicle as a valid model for hair biology research.

The vibrissa follicle has also been used as an experimental model for innervation due to the complex arrangement of fibres and the spatial arrangement of 'barrels' in the cortex corresponding to that of the vibrissa follicles in the mystacial pad (Woolsey & Van der Loos, 1970; Woolsey et al., 1975; Van Exan & Hardy, 1980; Munger & Rice,

1986; Rice et al., 1986; Arvidsson & Rice, 1991; Waite & Jacquin, 1992; Mosconi et al., 1993; Rice, 1993; Rice et al., 1993; Fundin et al., 1999). The proliferative capacity of the epithelial component of the follicle has also led to the vibrissa follicle being used as the paradigm for epithelial stem cell studies (Rochat et al., 1994; Oshima et al., 2001).

The wide variety of disciplines that have employed the vibrissa follicle as a model have led to a considerable body of literature on the subject. However, there are still fundamental questions that remain to be answered regarding the nature of the dermal-epithelial interactions that regulate the follicle cycle, the relationship between dermal papilla and dermal sheath cells, and the stem cell population within the follicle.

1.4 STEM CELLS

1.4.1. What are stem cells?

The term stem cells refers to a population of cells that have, amongst other characteristics, the potential for unlimited self-renewal, and the ability to give rise to large numbers of differentiated cells of a number of lineages (Miller, Lavker & Sun, 1997). There are progenitor cells in most tissue types that can multiply to replace cells when necessary, blood and epidermis being good examples. The stem cells of mammalian epidermis have been extensively researched due to the accessibility of the tissue for both *in vivo* and *in vitro* studies (Watt, 2001; Fuchs & Segre, 2000). Whilst studies of stem cells of adult tissues is an important area of research, both in relation to therapeutic possibilities and as a means of understanding the differentiation processes within a particular lineage, the opportunity to investigate very early differentiation is provided by embryonic stem cells. These are the ultimate stem cells as they can differentiate along any lineage and produce all the cells of an organism, including cells of the germ line.

1.4.2. Embryonic stem cells

Embryonic stem (ES) cells were first isolated and cultured by Evans & Kaufman (1981) and Martin (1981) who obtained them (by slightly different methods) 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. The totipotency of ES cells was demonstrated by injection into blastocysts which were then implanted into pseudo-pregnant, surrogate mothers so generating chimaeric mice (Bradley et al., 1984). These experiments highlighted the potential for generating transgenic mice from cultured ES cells which had been genetically manipulated. The first examples of animals generated from ES cells carrying a specific mutation were two lines of mice lacking the enzyme hypoxanthine phosporibosyl transferase (hprt), one generated by selecting for spontaneous mutants (Hooper et al., 1987) and one selecting from retrovirally infected ES cells for rare insertional mutants (Kuehn et al., 1987). Since these early experiments many more mutations have been studied, using gene targeting combined with ES cell technology (Koller & Smithies, 1992).

The generation of transgenic animals using ES cell technology is a great aid to research into specific genes, regarding their function in the development and function of an organ or system. However, studies of ES cells *in vitro* can also provide a wealth of information for the developmental biologist regarding cellular characteristics (review Rathjen & Rathjen, 2001). For example, how the ES cells interact with each other as well as other cell types (as they would in the developing blastocyst), what extracellular matrix they produce, if any, and what influences specific growth factors and cytokines may have on these totipotent cells. With regard to this last point the routine use of exogenous leukemia inhibitory factor (LIF) to maintain ES cell cultures results from

studies which isolated differentiating inhibitory activity (DIA), later identified as LIF, from culture media conditioned by buffalo rat liver cells (Smith & Rathjen, 1991). The action of LIF on ES cells *in vitro* appears to replicate its role in embryogenesis as LIF is expressed by extra-embryonic tissues, whilst the cells of the inner cell mass express LIF receptors in the pre-implantation blastocyst (Rathjen et al., 1990b; Nichols et al., 1996). Such investigations are key to fundamental questions in developmental biology, whilst the production of transgenic lines and chimaeric mice aim to answer more specific questions.

In recent years the potential of ES cell-derived populations of specific cell lineages for use in transplants has gained a great deal of attention both in the scientific community and in the world's media. The ethical issues involved in human ES cell research have 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). Meanwhile ES cell-derived glial cells have efficiently myelinated axons in the central nervous system (CNS) of myelin-deficient mice, demonstrating that such cells can function *in vivo* (Brustle et al., 1999; Liu et al., 2000). ES cell research holds great potential for such therapies, but there will always be an ethical issue with human ES cell research and therapy.

1.4.3. Adult stem cells

Stem cells are present in a wide range of adult tissues enabling these tissues to undergo a certain amount of self-healing/regeneration. Recently, adult stem cells have been exposed as more multipotent than was initially thought. For many years it was believed that the CNS had a decidedly limited regenerative capacity. It was then noted

that particular neuronal populations in the adult brain were replaced efficiently and neural stem cells have since been identified (for review see Kuhn & Svendsen, 1999). It has recently been 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 et al., 1999), but also have the capacity to undergo haematopoietic differentiation when injected into sublethally irradiated mice (Bjornson et al., 1999). Intriguingly, transplanted bone marrow cells have been shown to infiltrate the central nervous system and differentiate into cells which possess neuronal characteristics (Mezey et al., 2000). This is in contrast to the long-held belief that any neural cells lost due to toxins, disease or injury could not be replaced. Experiments such as those mentioned suggest that such cells may be replaced, albeit at a slow rate, by cells originating from outside of the CNS. It has also been proposed that this may be the route by which diseases spread from the haematopoietic system into the CNS (Mezey et al., 2000).

Another example of adult stem cells are those cells isolated from murine skeletal muscle which Jackson et al. (1999) demonstrated to have haematopoietic potential. 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 possiblity 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. This point was highlighted by a recent article asking "Can anything make anything?" (Morrison, 2001).

Recently the ever-increasing numbers of multipotent or "reprogrammable" cells found in adult tissues has led to suggestions that these cells may in fact become more plastic during the latter stages of development. Stem cells are typically found in a 'niche' distinct from the surrounding cell population. This isolation results in influence from only the immediate neighbours of these cells and so there is a limited number of signals affecting the stem cells. During development as cells are migrating, differentiating, proliferating and undergoing morphogenesis, all cells are exposed to a wide variety of signals. The suggestion is that during this time the future 'adult stem cells' are more differentiated than the same cells in adulthood to protect them from these influences. Once in the niche they are no longer at risk of encountering these signals and can revert to a state in which they are ready to fulfil their role as a stem cell. This plasticity is not evident *in vivo* as the cells are only required to provide cells of the lineage appropriate to their location. Only by manipulating these cells *in vitro* has the degree of multipotency become evident (review, Clarke & Frisen, 2001).

Research into adult stem cells has huge potential therapeutic benefits and despite many problems that need to be overcome before such treatment will become routine, the prospect of clinical use of adult stem cells is a very appealing one. Whilst ES cells have considerable potential, the ethical issues involved in obtaining human ES cells and deriving treatments from them are hugely complex and to many people would be totally unacceptable. Somatic nuclear transfer (Campbell et al., 1996; Wilmut et al., 1997) would still have some ethical arguments associated with it as an oocyte would be required. The ethics of using adult stem cells would be far simpler if methods can be developed to remove the adult stem cells from the patient's body and either implant them into the therapy site or culture them to change their properties to the required

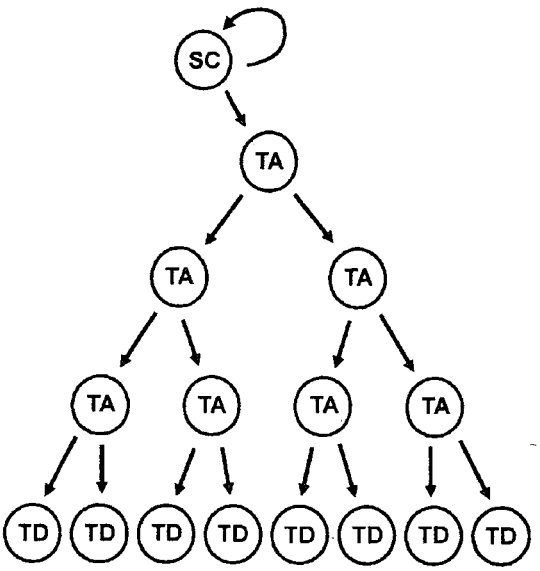
lineage prior to implantation. This would also have the added bonus that rejection of the graft would not be an issue as the cells would have originated from the recipient of the treatment. Locating multipotent cells in adult tissues which are easily accessible, and developing techniques to manipulate these cells along several lineages are research targets that could provide huge benefit for sufferers of many clinical disorders.

1.4.4. Epidermal stem cells

The skin is perhaps the most obviously self-renewing tissue of the human body with the uppermost layers of cells being constantly sloughed off. All cells of the epidermis derive from stem cells within the basal layer. These proliferate, producing cells which migrate through the layers of the stratified epidermis, and will ultimately repopulate the upper layers as they are shed. The cells change characteristics, or differentiate, as they progress through the layers to the outer surface of the skin (reviewed by Potten, 1981; Turksten & Troy, 1998).

The most widely accepted model for epidermal proliferation is for the stem cell daughter cells to be either stem cells themselves (self-renewal) or epidermal progenitor cells. These progenitors are transit-amplifying (TA) cells which have a limited proliferative capacity, but will undergo several rounds of division before committing to a specific fate and undergoing terminal differentiation. By this means, a single stem cell can give rise to numerous terminally differentiated cells from just one cell cycle (Figure 1.6). Hence, the stem cells are slow-cycling cells whilst TA cells are frequently dividing, a distinction that is often used to characterise cells in adult tissues (Watt, 2001).

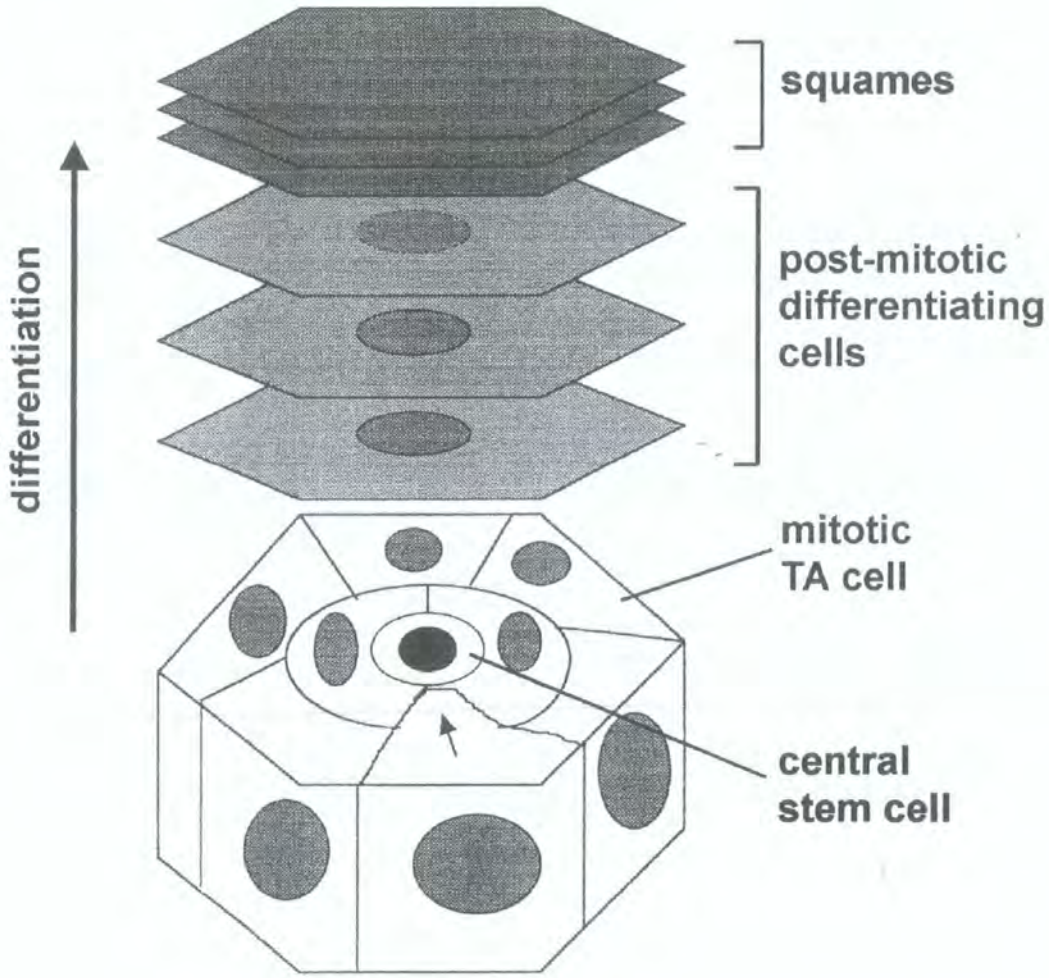
Figure 1.6. One stem cell cycle results in multiple terminally differentiated cells. Stem cell (SC) division results in self-renewal and a transient amplifying (TA) daughter cell. The TA cell undergoes a limited number of rounds of division before the progeny become terminally differentiated (TD) cells.
Adapted from Watt, 2001.



1.4.4.1. Location of epidermal stem cells

The morphology of the epidermis is highly diverse, differing in mechanical and biochemical properties, depending on the body site. Similarly the location of the stem cells is varied (reviews - Miller et al., 1997; Jones, 1997). For example, palm epidermis has clusters of stem cells in the rete ridges, whilst in the ear skin, stem cells are spread throughout the tissue with a single stem cell located at the centre of a group of TA cells in the basal layer of the thin epidermis. This second arrangement is described by the epidermal proliferative unit (EPU) theory which was first proposed by Potten (Potten, 1974; Potten & Allen, 1975; Allen & Potten, 1976). In the EPU model (presented in diagramatic form in Figure 1.7) the skin is divided into hexagonal columns based around a single stem cell in the basal layer. The stem cell of the EPU gives rise to between 8 and 10 TA cells. These proliferate in the surrounding basal layer before moving into the suprabasal layers as non-dividing, committed cells that progress through the upper layers of the epidermis, undergoing terminal differentiation in the process. Further evidence to support this model has come from a number of studies (Morris et al., 1985; Kam et al., 1986; Pellegrini et al., 2001; Ghazizadeh & Taichman, 2001; reviewed by Potten, 1981; Miller et al., 1997; Slack, 2000).

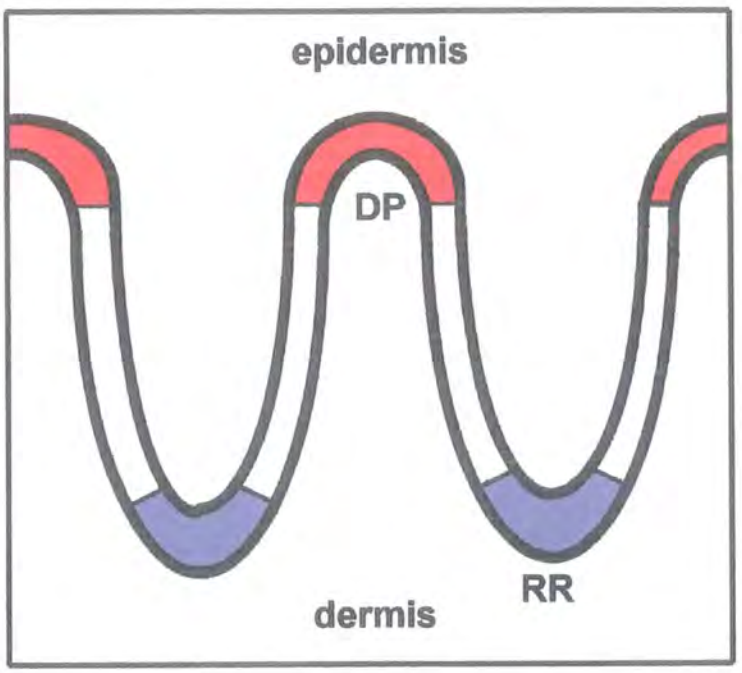
Figure 1.7. The epidermal proliferative unit.
According to this model of epidermal proliferation, a central stem cell is surrounded by 8 - 10 mitotic basal cells. A post-mitotic cell will move (indicated by small arrow) into the suprabasal layer and undergo differentiation, becoming enucleated during the process. Ultimately the cell will become a squame which will be lost from the skin after it has reached the surface.
Adapted from Allen & Potten, 1976.



There is considerable variation in skin structure between species, as well as areas of the body. Whilst the EPU model is widely accepted for the thin stratified epidermis of dorsal mouse skin, the majority of human epidermis shows no evidence of EPUs based on a single stem cell (Jones, 1997). Instead, the epidermal stem cells appear to be in clusters in anatomically defined sites, reminiscent of the corneal stem cell compartment. For example, the epidermis of both human and monkey palm is quite thick, and characterised by alternating deep and shallow rete ridges which contain stem cells and transit amplifying cells respectively (Lavker & Sun, 1982, 1983; Miller et al., 1997). In contrast, the epidermal stem cells in human scalp skin are at the tips of the dermal papillae (Jones et al., 1995) (Figure 1.8). Although the anatomical arrangement of epidermal stem cell and TA cell compartments varies, the model of stem cell division producing TA cells which give rise to numerous terminally differentiated cells (summarised in Figure. 1.6) is valid for all epidermis.

Figure 1.8. The location of epidermal stem cells differs between palm and scalp epidermis.

The stem cells of scalp epidermis (red) are located at the tips of the dermal papillae (DP), whilst the stem cells of palm epidermis (blue) are at the base of the rete ridges (RR).



1.4.4.2. Molecular characteristics of epidermal stem cells

Models such as the EPU provide anatomical definitions of epidermal stem cells whilst the molecular characteristics of these cells are slowly being revealed. A high level of cell surface β1-integrin expression correlates with rapid adhesion to type IV collagen, and has been described as a marker for keratinocyte stem cells *in vitro* and *in vivo* (Jones & Watt, 1993; Jones et al., 1995; Zhu & Watt, 1999). The expression of this integrin is thought to maintain cells in the stem cell compartment (Zhu et al., 1999). Whole mount staining of human epidermis determined that islands of cells with higher levels of β1-integrin expression than those around them, did not include proliferating cells (Jensen et al., 1999). A recent study by Pellegrini et al. (2001) identified epidermal stem cells with an antibody to the transcription factor p63. Strong expression was detected in individual cells along the basal layer of epidermis whilst the adjacent TA cells showed some expression, but this was significantly weaker. Building up a molecular profile for epidermal stem cells is likely to bring the greatest success in identifying pure stem cell populations, as individual markers will frequently include other cell populations such as TA cells. This fact was highlighted by a recent differential display study in which no molecules showed more than a two-fold difference in expression between stem cell- and transit amplifying cell-enriched populations (O'Shaughnessy et al., 2000).

1.4.5. Hair follicle stem cells

Stem cells of epithelial tissues, particularly epidermis, are amongst those that have been the most widely investigated (Fuchs & Segre, 2000). The two populations of the hair follicle, dermal and epidermal, have both been shown to possess regenerative and inductive capabilites (Oliver, 1966a, 1966b; Jahoda et al., 1984, 1992a, 1993; Reynolds & Jahoda, 1992, 1996; Reynolds et al., 1995) that enable the follicle to

recover from accidental loss of a hair fibre or a more severe insult such as a skin wound. The influence of follicular dermal cells on epithelial cell populations has been widely studied (Oliver, 1967, 1970; Ibrahim & Wright, 1982; Jahoda et al., 1984; Jahoda & Reynolds, 1993; Matsuzaki et al., 1996; Robinson et al., 2001).

Extensive studies on outer root sheath cells have led to proposals that this tissue harbours the stem cells of the follicular epidermis (Cotsarelis et al., 1990, Rochat et al., 1994; Oshima et al., 2001) and studies have also identified the contribution of the outer root sheath to interfollicular epidermis (Lenoir et al., 1988; Limat et al., 1991; Taylor et al., 2000). The multipotent germinative epithelial cells were only isolated from follicles fairly recently and therefore less experimental data is available on their characteristics both *in vivo* and *in vitro* (Reynolds & Jahoda, 1991, 1996). However, all these studies combined indicate that the anatomically distinct dermal and epidermal populations of the hair follicle contain cells that possess extensive proliferative potential, multipotency, inductive and regenerative properties, and in the case of GE cells have an undifferentiated cytoplasm.

All of the features described above are characteristic of stem cells and whilst there is still a great deal of work to be done, the idea that the hair follicle might provide a source of adult stem cells that may be exploited for therapeutic use is gaining evidence all the time (Reynolds & Jahoda, 1994; Jahoda & Reynolds, 2001). An additional feature of hair follicle cells that would be beneficial in clinical use is their apparent immune privilege which allows transplantation between immunologically incompatible individuals with no evidence of graft rejection (Reynolds et al., 1999). While the epithelial stem cells of the hair follicle are subject to intense study, there has been less interest in the possibility of a dermal stem cell population.

1.5. FOLLICULAR DERMAL CELL CHARACTERISTICS

The capacity to induce follicle formation is retained in low passage dermal papilla cells which display aggregative properties, crucial to follicle induction, *in vitro* and when implanted *in vivo* (Jahoda et al., 1984; Jahoda & Oliver, 1984a). This behaviour originates from the embryonic dermal condensation as discussed previously. Both dermal sheath and dermal papilla cells arise from the dermal condensation. This common origin is believed to account for the two cell types sharing characteristics and being able to compensate for each other in some instances.

Cultured follicular dermal cells can be distinguished from skin fibroblasts *in vitro* by their aggregative behaviour (although this is less evident in dermal sheath cells) and expression of α-smooth muscle actin (Jahoda et al. 1984a, Reynolds et al., 1993). In the same way that tissue recombinations have demonstrated properties specific to dermis and epidermis, cultures of various cell combinations have revealed some interesting relationships between different follicular cell populations. In particular, combinations of dermal and epidermal cells have demonstrated the trophic support provided by dermal papilla cells to be greater than that from skin fibroblasts (Reynolds et al., 1991). The germinative epidermal cells, that are located around the basal stalk of the dermal papilla in the follicle end bulb, display organotypic behaviour *in vitro* in the presence of dermal papilla cells. Discrete groups of germinative epidermal cells are separated from the surrounding dermal cells by a basement membrane (Reynolds & Jahoda, 1991). This is particular to dermal papilla cells as germinative epidermal cells showed no evidence of forming similar structures when exposed to skin fibroblasts. The ability to influence epidermal cell populations is therefore possessed by embryonic and adult follicular dermal cells and these can be demonstrated *in vitro*, by altering epidermal cell growth or behaviour, and *in vivo*, by inducing follicle formation.

As well as possessing inductive properties, the regenerative capacity of follicular dermal tissue is considerable. Amputation of the lower third of the vibrissa follicle, including the end bulb, results in the regeneration of a fully functional end bulb (Oliver, 1966a, 1966b). In these circumstances, the lower dermal sheath is believed to interact with the outer root sheath cells at the site of the amputation and subsequently a dermal papilla forms (Oliver, 1966b, Jahoda et al., 1992a). In follicle regeneration the dermal sheath apparently gives rise to dermal papilla cells, whilst in follicles induced by implantation of dermal papilla, these cells give rise to the dermal sheath of the resulting follicle. This interchangeable role not only reflects the common origin of dermal papilla and dermal sheath cells, but also demonstrates a degree of plasticity in these dermal cells that is intriguing. Whilst the hair follicle displays characteristics that suggest there is a multipotent epithelial cell population present and investigations into these progenitor cells have been extensive, the potential of the dermal component has largely been ignored.

1.6. OBJECTIVES

The work presented in the following chapters aims to investigate interactions between various cell populations relating to follicle development, cycling and regeneration.

1) I compared the expression of versican with follicle innervation to investigate a possible role for this extracellular matrix molecule, in the developing and adult follicle, other than regulation of follicle growth. The recruitment of mesenchymal cells into the dermal component of the developing follicle was observed using a marker of mitotically quiescent cells.

2) In vibrissa follicle regeneration, cellular proliferation and α-smooth muscle actin expression were investigated in an attempt to identify the source of the dermal papilla

cells in the regenerated end bulb. The process of epithelial regeneration, partly revealed by cell proliferation, was also studied by detecting Sonic hedgehog expression. I compared patterns of Sonic hedgehog and versican expression in regeneration to previously reported developmental expression patterns to determine whether aspects of development are reflected in follicle regeneration.

3) I co-cultured follicular dermal cells with embryonic stem cells to investigate if epithelial differentiation would be induced. This did not occur but some interesting observations were made which led me to further investigate the interactions between follicular dermal cells and embryonic stem cells in culture. Cytokine expression was studied in follicular cells *in vitro* and *in vivo*. I also studied the effect of ES cells on dermal cell expression of α-smooth muscle actin.

The work presented provides further insight into the properties of follicular cell populations and how they interact with their environment *in situ* and embryonic stem cells *in vitro*.

Chapter 2: Versican expression in relation to follicle innervation, and nuclear lamin A expression, during the development and adult growth cycle of rat vibrissa follicles.

2.1. INTRODUCTION

2.1.1. Extracellular matrix

2.1.1.1. The extracellular matrix in development

Extracellular matrix (ECM) has a crucial role to play in many developmental processes in which it facilitates cell growth, migration, differentiation and adhesion, in addition to stabilising established structures. The morphogenesis of the hair follicle requires significant changes in the ECM, biochemical and structural, particularly at the epithelial-mesenchymal interface, where the ECM forms a basement membrane. In appendage development, the basement membrane undergoes changes to allow the proliferation of epithelial cells to result in invasion of the dermis. The importance of the ECM components in this process were first demonstrated over 30 years ago. Collagen was shown to be a crucial factor in epithelial morphogenesis in a number of structures, whilst the differential turnover of glycosaminoglycans in regions of the basal lamina, correlated with the occurrence of morphogenetic change (Wessels & Cohen, 1968; Grobstein & Cohen, 1965; Bernfield & Banerjee, 1982; reviewed by Thesleff et al., 1995). Whilst ECM influences many developmental processes, it is important to keep in mind that integrity and composition of the ECM is directed by cells.

2.1.1.2. The extracellular matrix of the dermal papilla

Cell–cell interactions are clearly an important factor in the development and cycling of the hair follicle, but cell–matrix interactions may prove to be just as critical. The extracellular matrix provides structural support to the cells of a tissue but is also a key player in a number of regulatory mechanisms, mediated by the binding of cell surface molecules and signalling molecules to its components. For example, ECM proteoglycans have been shown to affect patterning and development by regulation of

Wnt, Hedgehog and BMP signalling (Dale, 1998; Capdevila & Belmonte, 1999; Selleck, 2000). The number of dermal papilla (DP) cells is believed to remain the same throughout the adult growth cycle, with no evidence for significant proliferation at any stage (Pierard & de la Brassinne, 1975; Young, 1980). However, the DP is known to be the major regulator of hair growth, and although these cells are mitotically inactive, they are very active in other areas, particularly in the synthesis of ECM. The amount of ECM in the DP fluctuates with the hair growth cycle and this is reflected in the changing volume of the DP, whilst the number of cells remains fairly constant (Young, 1980; Messenger, 1991).

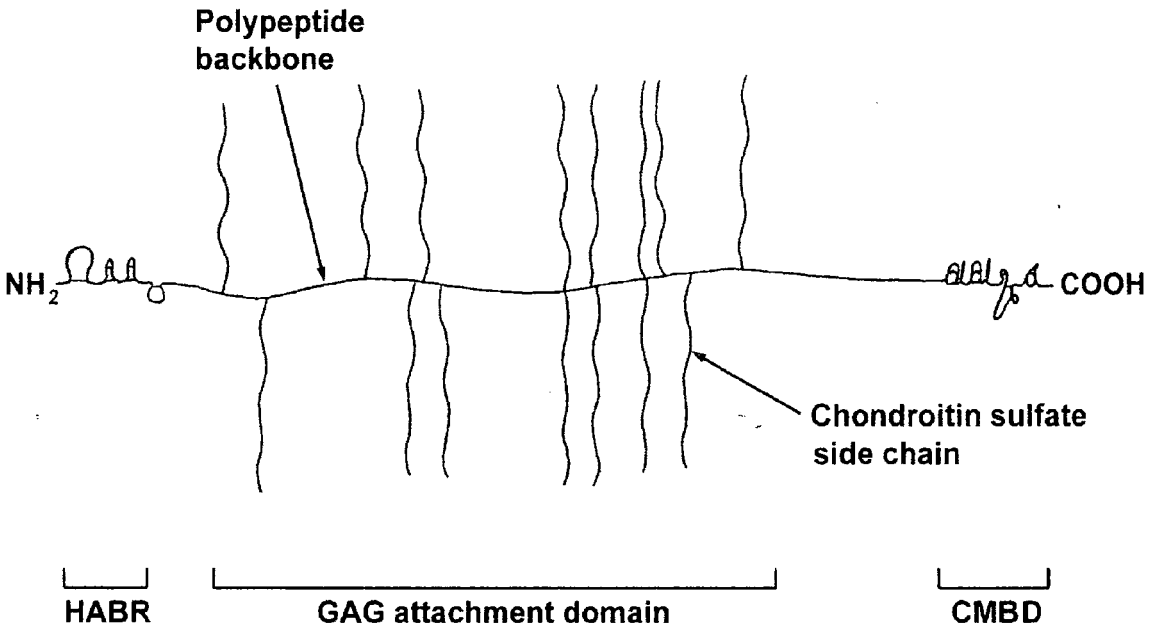
Various components of the ECM have been described in relation to hair follicle biology. Collagen, fibronectin and laminin are present in the basement membrane between the dermal and epidermal components of the follicle, and in the ECM of the DP at various stages of the growth cycle (Couchman & Gibson, 1985; Couchman, 1986; Messenger et al., 1991; Jahoda et al., 1992b). Proteoglycans make up a significant portion of the ECM and consist of a polypeptide backbone with glycosylated side chains. The nature of these side-chains distinguishes between heparan sulfate and chondroitin sulfate proteoglycans, both of which are found in the hair follicle ECM, along with smaller proteoglycans, the syndecans and decorin (Couchman & du Cros, 1995; Couchman, 1986 & 1993). Chondroitin-6-sulfate proteoglycan, with its cyclical expression in the lower part of the follicle, has been proposed to contribute to the immune privilege of the hair follicle by "screening" the bulb cells, which lack histocompatibility antigens, from natural cytotoxic cells (Westgate et al., 1991). Cyclical expression patterns together with extensive expression during follicle regeneration (Jahoda et al, 1992a) have led to proposals that components of the ECM might be responsible for regulating the follicle growth cycle.

2.1.1.3. Versican

The proteoglycan versican undergoes a dramatic change in expression levels during the hair growth cycle and has been proposed to play a role in its regulation (du Cros et al., 1995). Versican is a large extracellular matrix molecule from the family of aggregating chondroitin sulphate proteoglycans that includes aggrecan, neurocan and brevican (review, Schwartz et al., 1999). Neurocan and brevican are specific to the nervous system (Rauch et al., 1992; Yamada et al., 1994), whilst aggrecan and versican are expressed both in the nervous system and a number of other tissues (Bode-Lesniewska et al., 1996; Li et al., 1996; Schwartz, 1999).

The structure of versican (Figure 2.1) is characteristic of the aggrecan family with globular domains at either end of the glycosaminoglycan (GAG) attachment domain. At the N terminus, a hyaluronic acid-binding site is present, whilst at the C terminus there is a complex multiple binding site, including epidermal growth factor (EGF)-like and lectin-like motifs (Zimmermann & Ruoslahti, 1989). These domains allow versican to bind and interact with many other ECM components.

Figure 2.1. Schematic diagram of the structure of versican.
Versican consists of a core polypeptide backbone with multiple chondroitin sulfate chains attached to the central portion. At the N terminus the globular domains comprise the hyaluronic acid-binding domain (HABD) whilst at the C terminus, a complex multiple binding domain (CMBD) includes EGF-like, lectin-like and complement regulatory protein-like domains. Adapted from Zimmerman & Ruoslahti, 1989.



In expression studies in adult murine skin, versican was found to be strongly expressed in the hair follicle DP during anagen, but lost from the papillae of non-growing (telogen) follicles (du Cros et al, 1995). Due to this cyclic expression in the DP it was proposed that versican may play an inductive role or support the regulatory process of the follicle growth cycle. More recently, molecular techniques have been used to directly investigate the inductive properties of versican (Kishimoto et al., 1999). A transgenic mouse was made expressing green fluorescent protein (GFP) driven by the versican promoter. After versican-expressing DP cells, combined with keratinocytes, were implanted onto the skin of nude mice, they successfully induced hair follicle formation. However after the cells were passaged once, the fluorescent subpopulation of cells was lost, along with their inductive capacity. From this the authors concluded that versican may contribute to the induction of hair follicle formation.

2.1.2. Inhibition of neural growth

Proteoglycans play a wide range of roles as mediators of cell-cell and cell-matrix interactions and as structural components of the ECM (Perrimon & Bernfield, 2001; Rapraeger, 2001). They have also been shown to influence patterning of neural crest cell migration and axon outgrowth (reviewed by Margolis & Margolis, 1997, Perris & Perissinotto, 2000 and Yamaguchi, 2001). Snow et al. (1991) demonstrated that the chondroitin sulfate chains of a particular proteoglycan were responsible for altering the course of axon growth *in vitro*, and proposed that this molecule may play an inhibitory role in the development of retinal ganglion cell axons. The inhibitory properties of the chondroitin sulfate proteoglycan, phosphacan, on neurite outgrowth *in vitro*, were not attributable to the chondroitin sulfate side chains but to the core protein (Milev et al., 1994). In the aggrecan family, neurocan and brevican have both been shown to be potent inhibitors of neurite outgrowth *in vitro* and are proposed to regulate neuronal

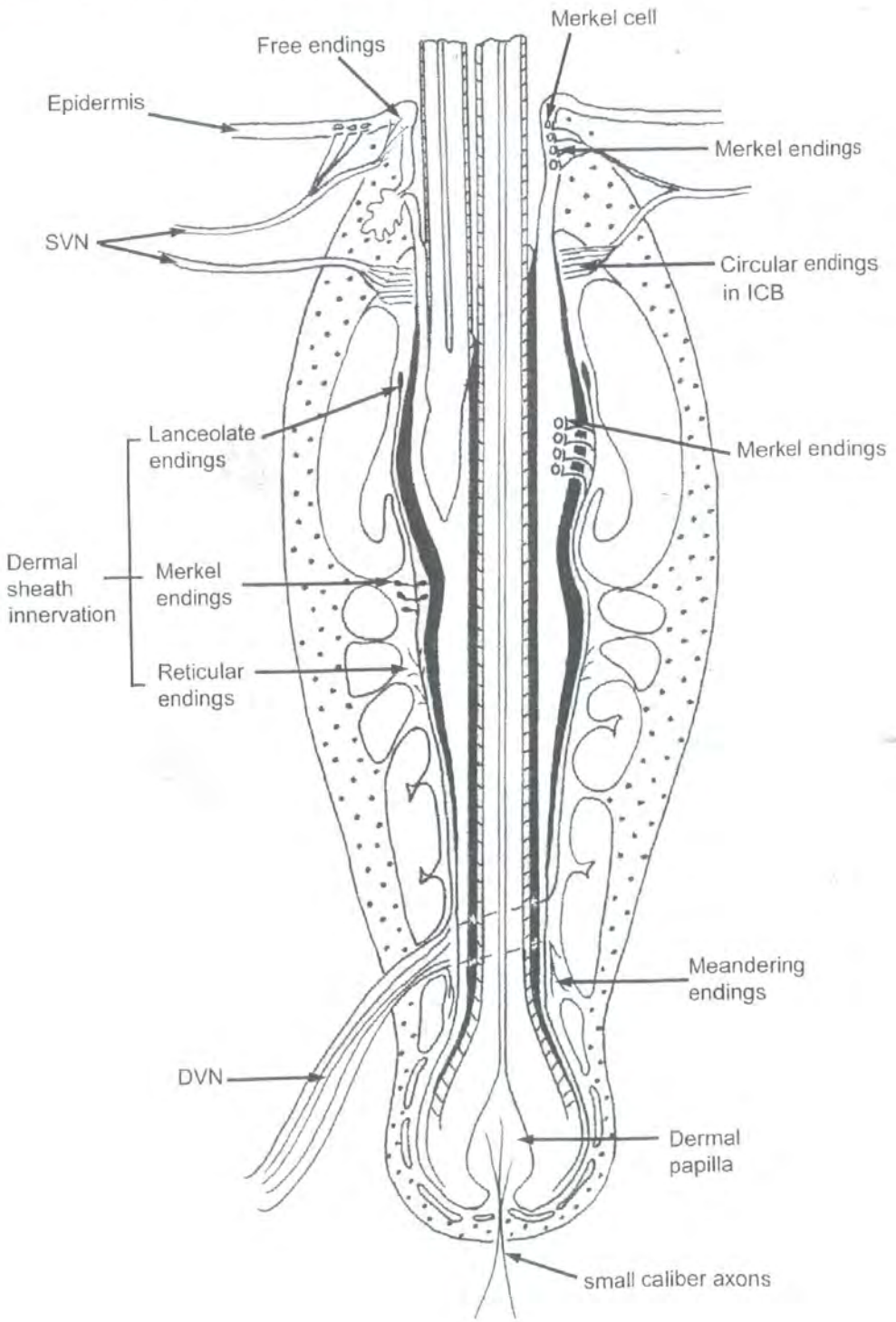
outgrowth *in vivo* (Friedlander et al., 1994; Margolis & Margolis, 1997 (review); Yamada et al., 1997). Whilst neurocan's core protein is responsible for this effect (Li et al., 2000), the chondroitin sulphate chains of brevican are the key to this role (Yamada et al., 1997). This is a good example of how, even within the same family of proteoglycans, there are significant differences in the manner by which they induce a similar effect.

In the development of both the chick (Landolt et al., 1995) and the mouse (Henderson et al., 1997) versican has been shown to influence neural crest cell migration. Versican has been thought to form part of the barrier to neural crest cell migration and axon outgrowth, so restricting their pathways (Landolt et al., 1995; Henderson et al., 1997). As versican has a more widespread distribution than neurocan and brevican, it is possible that such effects will influence the peripheral as well as the central nervous system.

2.1.3. Vibrissa follicle innervation

The vibrissa follicle is a well-innervated mechanoreceptor (Figure 2.2) whose adult innervation has been well-documented (Arvidsson & Rice, 1991; Mosconi & Rice, 1993; Rice, 1993; Rice et al., 1986, 1993; Waite & Jacquin, 1992) whilst developmentally, the link between patterning of whisker follicles on the face and cortical barrels in the brain has also been the subject of in depth research (Woolsey and Van der Loos, 1970; Woolsey et al, 1975; Munger & Rice, 1986, for reviews see Micheva & Beaulieu, 1997; Schlaggar & O'Leary, 1993). As such, it provides a good model for studies on development of peripheral innervation.

Figure 2.2. Innervation of the rodent vibrissa follicle.
The lower two thirds of the follicle are supplied by the deep vibrissal nerve (DVN) whilst superficial vibrissal nerves (SVN) converge at the neck of the follicle to innervate the inner conical body (ICB) and the epidermal tissues distal to this. Many different types of nerve endings are present in the follicle, both in the epidermal and dermal components.
Drawing after Oliver (1966a) and Rice et al. (1993).



Each vibrissa follicle is supplied by a single deep vibrissal nerve (DVN) and several superficial vibrissal nerves (SVN). These nerves are derived from the infraorbital branch of the trigeminal nerve which runs through the lower dermis of the mystacial pad. Van Exan & Hardy (1980) have described the early stages of murine vibrissa follicle innervation, and Fundin et al. (1999) studied the glial cell line-derived neurotrophic factor (GDNF) family of molecules in relation to the molecular control of murine vibrissa follicle innervation and nerve ending distribution. However, very little has been published about other molecular influences during the transition from the placode stage of follicle development, with a nerve plexus beneath it, to a highly innervated and evolved structure featuring many different types of nerve endings.

Pelage follicles are also well innervated, but to a lesser degree than vibrissa follicles. A series of papers (Botchkarev et al., 1997, 1999b; Peters et al., 2001) have described the changes in pelage follicle innervation during the follicle cycle, regarding the number of fibres associated with particular parts of the follicle as well as the type of fibres involved. Botchkarev et al. (1997) found that in rodent skin the number of nerve fibres innervating pelage follicles changed with the hair cycle, particularly around the isthmus of the follicle. This region corresponds to the inner conical body of the vibrissa follicle. These authors suggest that the cycle-linked variation of neural cell adhesion molecule (N-CAM) and growth-associated protein (GAP) 43 expression in pelage follicles may be related to change in innervation rather than hair growth mechanisms. In the original versican expression study on adult murine skin, du Cros et al. (1995) observed that versican was present at the neck of the murine pelage follicle in telogen, but absent from this region in anagen. Given that the changes in versican expression at the neck of the follicle are in synchrony with changes in the nerve supply, this suggests a possible link between versican and follicle innervation. No work has been published

on any cyclic changes in vibrissa innervation, despite detailed studies establishing the extensive and complex innervation of the follicle-sinus complex (Rice et al., 1993).

2.1.4. Nuclear lamins

Nuclear lamins are type V intermediate filament proteins which form the network that is the nuclear lamina, a structure lining the inner aspect of the inner nuclear membrane. The nuclear lamina interacts with many integral proteins of the nuclear envelope, as well as chromatin (reviewed by Gruenbaum et al., 2000) and is involved in a number of important processes: targeting nuclear membrane vesicles to chromatin after mitosis; regulating nuclear size and shape; and contributing to organisation of replication domains (reviewed by Vaughan et al., 2000; Moir et al., 2000).

These functions are a necessary feature of all cell types and hence lamins are present in all cells, but the subtypes of these filaments vary according to the cell type and the stage of the cell cycle. Lamins A and C are alternatively spliced products of the same gene and are expressed in differentiated cells and proliferating cells respectively (Rober et al.,1989; Vaughan et al., 2000; Venables et al, 2001). Mutations in the lamin A gene cause some types of Emery-Dreifuss muscular dystrophy (EDMD), a muscle-wasting disease (Bonne et al., 1999). Mice with a knockout in the lamin A gene develop an EDMD phenotype and also suffer from loss of fat cells, a characteristic of a familial lipodystrophy which has been traced to a missense mutation in the human lamin A/C gene (Cao & Hegele, 2000; Sullivan et al., 1999). The reasons why fat and muscle cells appear to be more affected by altered lamin A/C function are not fully understood although various theories have been proposed, all of which are yet to be substantiated (reviewed by Gruenbaum et al., 2000). Clinical studies have also revealed lamins to be a target of autoantibodies in a wide variety of autoimmune diseases, paticularly those associated with the liver (Gruenbaum et al., 2000). The components of the nuclear

lamina are evidently important in a number of processes and extensive work will be required to appreciate the full complexity of their interactions and regulation within the nucleus.

One important function of the lamin A protein is the structural and mechanical integrity of the nucleus. Studies have shown that if lamin A expression is disrupted, the nuclear structure is distorted and there is ectopic expression of emerin, a lamin-associated protein usually found within the nuclear membrane (Spann et al., 1997; Sullivan et al., 1999). Other intermediate filaments have been shown to function in mechanical stabilisation of cells and it is thought, from these studies and lamin A gene knockout mice (EDMD mice), that lamins A and C make a significant contribution to the structural integrity of the nucleus (reviewed by Moir et al., 2000). The differential expression of the splice variants between non-dividing and proliferating cells may be a means of maintaining or regulating this integrity whilst the major changes of cell division are occurring.

Jol-4 is a monoclonal antibody that is specific to lamin A and reacts with the nuclear envelope of quiescent (non-proliferating) cells (Dyer et al., 1997). Previous work has demonstrated that the cells of the developing hair follicle dermal condensation are essentially non-dividing and these cells apparently remain in this quiescent state through to DP formation (Wessels & Roessner, 1965). The Jol4 antibody was therefore used to observe the formation of the dermal condensation and subsequent establishment of the dermal components (dermal papilla and dermal sheath) of the vibrissa follicle.

2.1.5. Aims

Versican expression within the hair follicle is intriguing because it has been associated with induction of follicle formation and regulation of the hair growth cycle,

as well as regulating development of the nervous system. Using the vibrissa follicle as a model, the aim of this study was to establish the distribution of versican during follicle development, and investigate whether a relationship existed between versican expression and development of the complex vibrissal innervation. The population of non-dividing cells was also investigated as a means of identifying follicular dermal cells from the surrounding mesenchyme.

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 microscope (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 follicles was removed with fine forceps. The exposed follicles were cut transversely at the neck, placed in minimal essential medium (MEM) and sorted according to their hair cycle stage (Williams et al., 1994) prior to embedding. Snouts were dissected from decapitated embryonic (E14 to E18) and newborn (P0) Wistar rats and the lower jaws were removed and discarded. Dorsal skin was dissected from E18 rat embryos.

Specimens were embedded in TissueTek 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 versican, neurofilament and lamin A expression (including those that were double-labelled).

Tissue sample	Versican	Neurofilament	Lamin A
Embryonic snout	19	11	8
Newborn snout	2	2	0
Anagen vibrissa follicle	3	3	3
Catagen vibrissa follicle	2	3	2
Telogen vibrissa follicle	4	3	1
Embryonic skin	2	0	1
Cultured DP/DS	5	0	0

2.2.2. Immunohistochemistry

Frozen sections (7μm) of vibrissa follicles, embryonic and newborn rat snouts, and E18 dorsal skin were cut (on a Leica CM3050 S cryostat), thaw-mounted on poly-lysine coated slides and air-dried for 1 to 3 hours before being processed for immunohistochemistry. For comparison between versican and neurofilament expression, sections were mounted on alternate slides.

For basic immunohistochemistry, sections were washed three times in PBS before application of the primary antibody (Table 2.2) diluted in filter-sterilised PBS. The slides were then incubated for 1 hour at room temperature, or overnight at 4°C before removing the primary antibody with four 5-minute washes in PBS. Secondary antibody (Table 2.3), diluted in filter-sterilised PBS, with Evans blue counterstain (30μg/ml) in some cases, was then applied and incubated for 30 minutes at room temperature in the dark before four 5-minute washes with PBS. Sections treated with a biotinylated secondary antibody had streptavidin-fluorescein isothiocyanate (FITC) (1:80, Gibco)

applied and incubated as for the secondary before the final four 5-minute washes with PBS. All sections were mounted under coverslips in mowiol 4-88 (Calbiochem). Negative controls were performed by omitting the primary antibody and/or secondary antibody and incubating the sections in PBS instead.

For more direct comparison between versican and neurofilament expression, some sections were double-immunolabelled by the following method. The slides were air-dried as above then washed three times in PBS before applying the primary antibodies (12C5 and NF200) mixed and diluted in filter-sterilised PBS. The slides were incubated overnight at 4°C then the primary antibodies were removed with four 5-minute washes in PBS. Secondary antibodies were applied and incubated in a dark humidity chamber for 45 minutes at room temperature. The secondary antibodies were washed off as for the primary antibodies. Sections treated with a biotinylated secondary antibody were incubated in streptavidin FITC (1:80, Gibco) as for the secondary before the final four 5-minute washes with PBS. The stained sections were mounted in mowiol 4-88 (Calbiochem) under coverslips.

A Nikon Optiphot UFX-II microscope, equipped with epifluorescence and the appropriate filter sets for FITC and tetramethylrhodamine isothiocyanate (TRITC) fluorescence, was used for initial observation and analysis. Images were taken on 35mm film with a Nikon FX-35A camera. Further analysis was carried out and images recorded using a confocal laser-scanning microscope (MicroRadiance scanning system, BioRad). All images were processed using Adobe Photoshop 3.0 software.

Table 2.2. Details of primary antibodies used for immunohistochemistry.

Antigen	Host	Antibody	Dilution Factor	Source	Reference
versican	mouse	12C5 (monoclonal)	1:5	DSHB	Asher et al., 1991
neurofilament 68	mouse	NF68 (monoclonal)	1:100	Sigma	
neurofilament 200	rabbit	NF200 (polyclonal)	1:100	Sigma	
Lamin A	mouse	Jol4 (monoclonal)	1:10	Serotec	Dyer et al., 1997

Table 2.3. Details of secondary antibodies used for immunohistochemistry.

Antigen	Host	Conjugate	Dilution Factor	Source
mouse IgG	rabbit	FITC	1:30	DAKO
mouse IgG	goat	AlexaFluor546	1:100	Molecular Probes
mouse IgG	goat	biotin	1:80	Gibco
mouse IgG	goat	AlexaFluor488	1:100	Molecular Probes
rabbit IgG	goat	AlexaFluor546	1:100	Molecular Probes
rabbit IgG	goat	TRITC	1:100	KPL

2.2.3. Histology

8μm wax sections were stained with Weigert's hematoxylin, Curtis's ponceau S and Alcian blue. Photos were taken on Axiovert 135 microscope using Fuji Provia 400 film or a Spot RT digital camera (Diagnostic Instruments Inc.).

All images were processed using Adobe Photoshop 3.0 software.

2.2.4. Cell culture

Rat dermal papilla and dermal sheath cells were isolated and cultured as described in section 4.2.1.

2.3. RESULTS

2.3.1. Versican expression during the development of vibrissa follicles

The overall pattern of versican staining in the mystacial pad changed as the vibrissa follicles developed. A diagrammatic summary of the major changes is shown in Figure 2.3. Low magnification microscopy showed that at the earliest stages of follicle development (E14/15, stages 1-3) versican staining was present throughout the majority of the facial dermis, but it became progressively restricted so that by E16 (stage 4/5) versican was seen as a strip of strong immunoreactivity immediately below the epidermis.

Formation of a dermal condensation below the epithelial placode is one of the earliest cellular events in hair follicle development (Fig. 2.3, stage 1 and Fig. 2.4 A). Consistently at this stage of vibrissa follicle development, the condensed mesenchyme had significantly lower levels of versican expression than the neighbouring dermis, which revealed strongly positive immunolabelling (Fig. 2.4 B,C).

In the next stage of hair follicle development, the epithelium grows down into the dermis to form a bud (Fig. 2.3, stage 2, Fig. 2.4 D). During this process the lack of versican staining was maintained in the dermis adjacent to the epidermal tissue. However as the epithelial downgrowth became flattened at the plug stage (Fig. 2.3 stage 3), versican expression increased in a group of dermal cells at the base of this structure whilst the dermis to the side of the downgrowth remained weakly labelled relative to the interfollicular dermis (Fig. 2.4 E).

Figure 2.3. Schema of versican immunoreactivity through the developmental stages of the rat vibrissa follicle.
Intensity of shading reflects the intensity of staining seen.

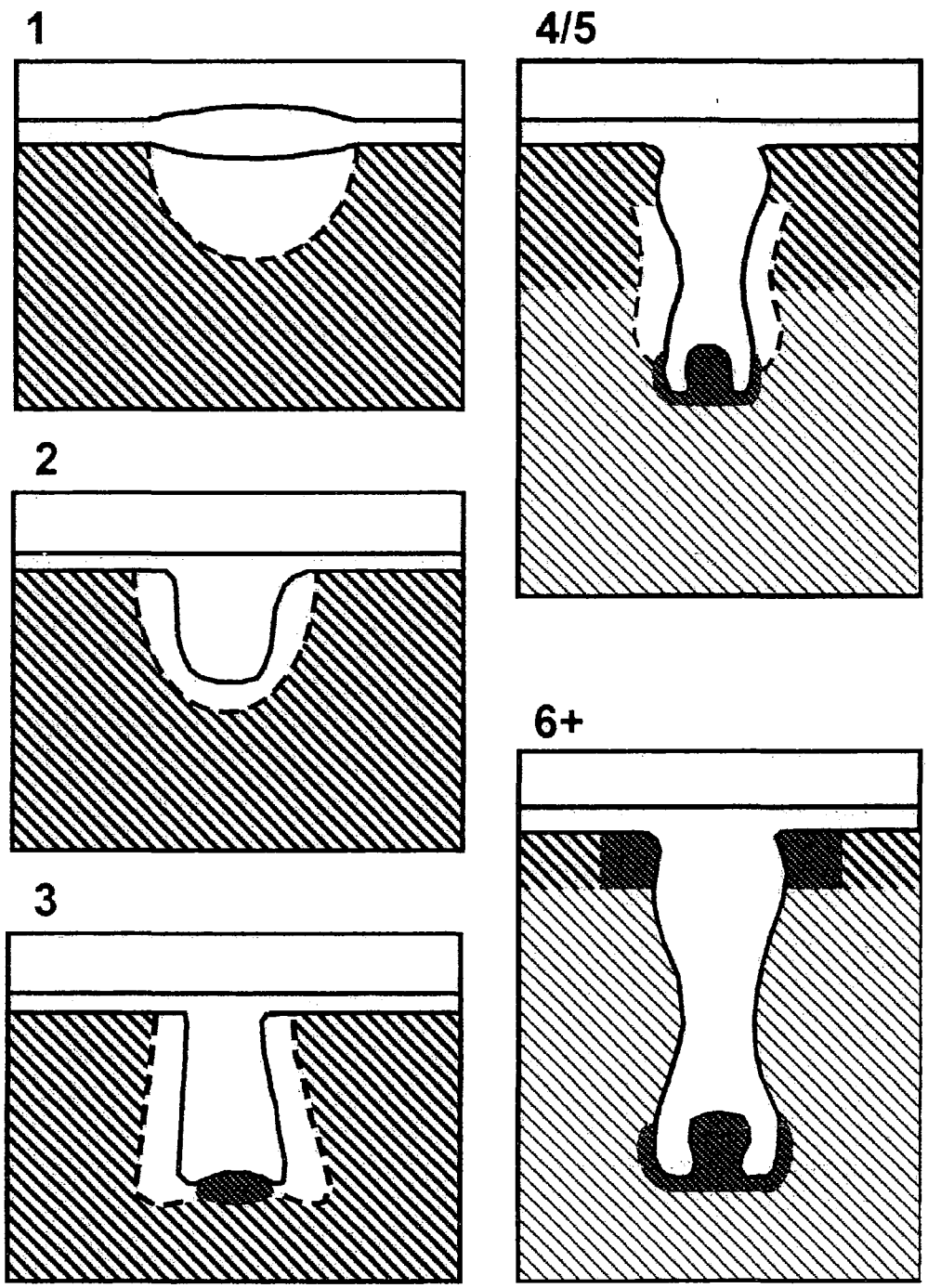
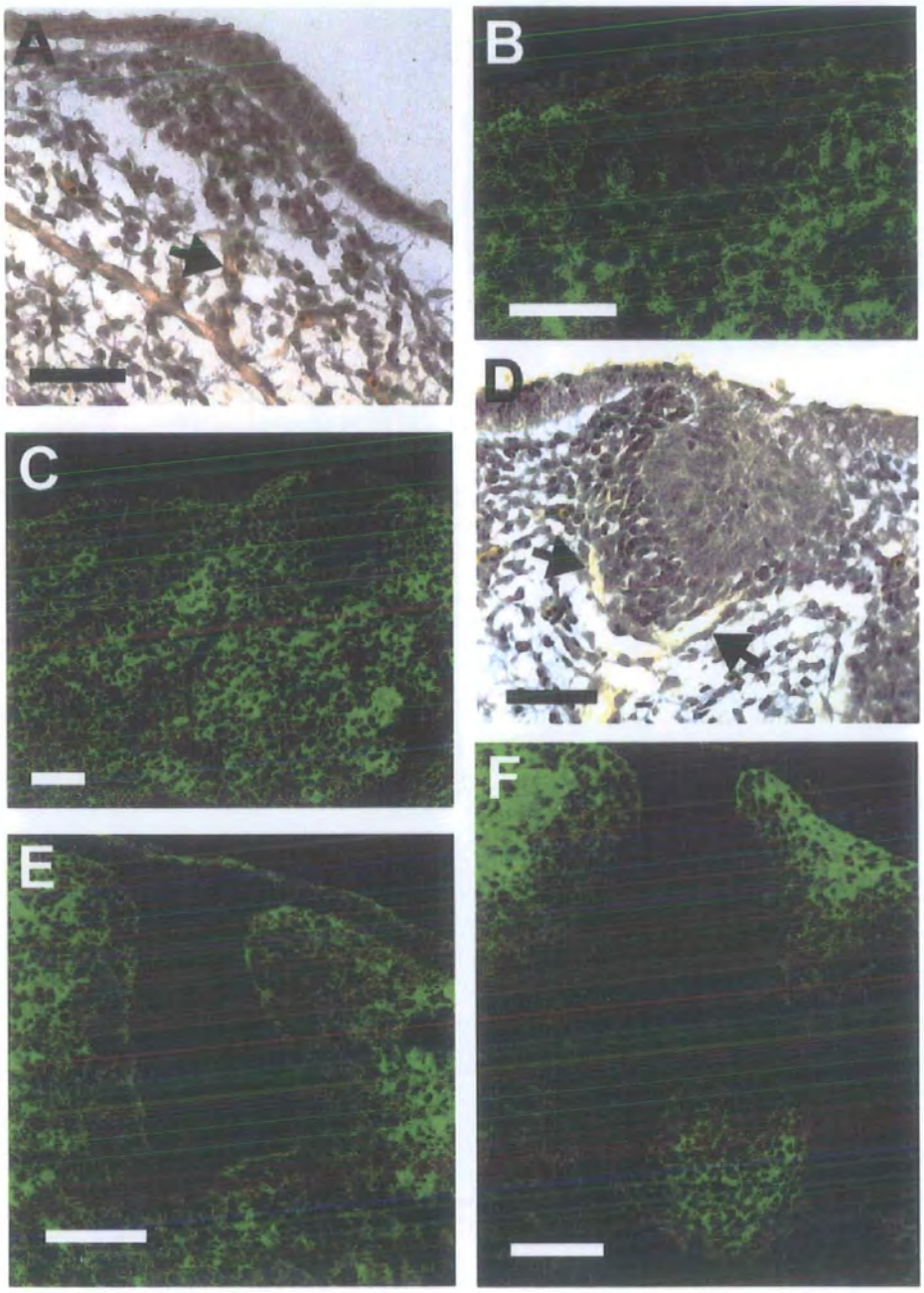


Figure 2.4. Versican expression from stage 1 to stage 4/5 of rat vibrissa follicle development.

Sections of embryonic (A-E, E15; F, E16) snout stained with Weigert's haematoxylin, Curtis' ponceau S and Alcian blue (A, D) or immunostained for versican (B, C, E-F). The nerve (arrow) approaches the follicle from the posterior aspect (A) and divides below the developing hair peg sending branches (arrows) around the sides of the mesenchymal condensation (D). Comparable stages show the mesenchymal condensation to be very weakly stained for versican relative to the surrounding dermis (B,C). Dermis around the epidermal downgrowth maintains this weak staining as cells at the base of the hair peg start to develop versican immunoreactivity (E). At a later stage the presumptive papilla and dermal sheath show strong versican labelling (F).

Scale bar = 50μm.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As the epithelial component of the follicle continues to grow into the dermis, it invaginates to surround the dermal cells at the base, which then constitute the presumptive dermal papilla. Versican labelling in this group of dermal cells developed from an initial low level (Fig. 2.4 E) to a strong presence in the developing papilla (Fig. 2.4 F). At E16, the dermal components of the follicle became more defined and the lower dermal sheath also displayed strong versican immunoreactivity. As the base of the hair follicle extended down into the weakly labelled lower dermis, the positively stained elements at the follicle base became increasingly sharply delineated (Fig. 2.3, stage 4/5). This restricted expression pattern was maintained through E18 and into newborn samples. Versican staining in the upper dermis changed from a layer of moderate staining at E16, to strong staining in the dermis at the neck of the vibrissa follicles with moderate expression in the interfollicular sub-epithelial dermis at E18 (Fig. 2.3, stage 6+). In newborn snout, pelage follicles were occasionally observed between the vibrissa follicles, highlighted by the strong versican expression in the dermal papilla.

Normal histology gave limited information regarding the innervation of the developing follicles but it was observed that the nerves were located on the periphery of the dermal condensation (Fig. 2.3 A,D). Comparable samples showed the dermal condensation to be very weakly stained for versican (Fig. 2.3 B,C). These observations prompted further investigation into the relationship between versican expression and follicle innervation.

2.3.2. Versican distribution compared with the innervation of developing vibrissa follicles

Examination of adjacent sections at stage 1 of follicle development revealed that nerves approached the follicle primordia from the posterior aspect and branched in the dermis beneath the epidermal placode (Fig. 2.5 A,E). By stage 2 fibres had diverged

and were cupping the dermal condensation below the developing follicle bud (Fig. 2.5 B,F). This was clearly not a simple case of the approaching nerve dividing in two, instead its fibres spread to create a basket-like network that encased the developing follicle. This branching occurred within the follicular dermis, which displayed the weakest versican staining, but interestingly nerves skirted around the edge of this region rather than entering the dermal condensation itself (Fig. 2.5 B,F).

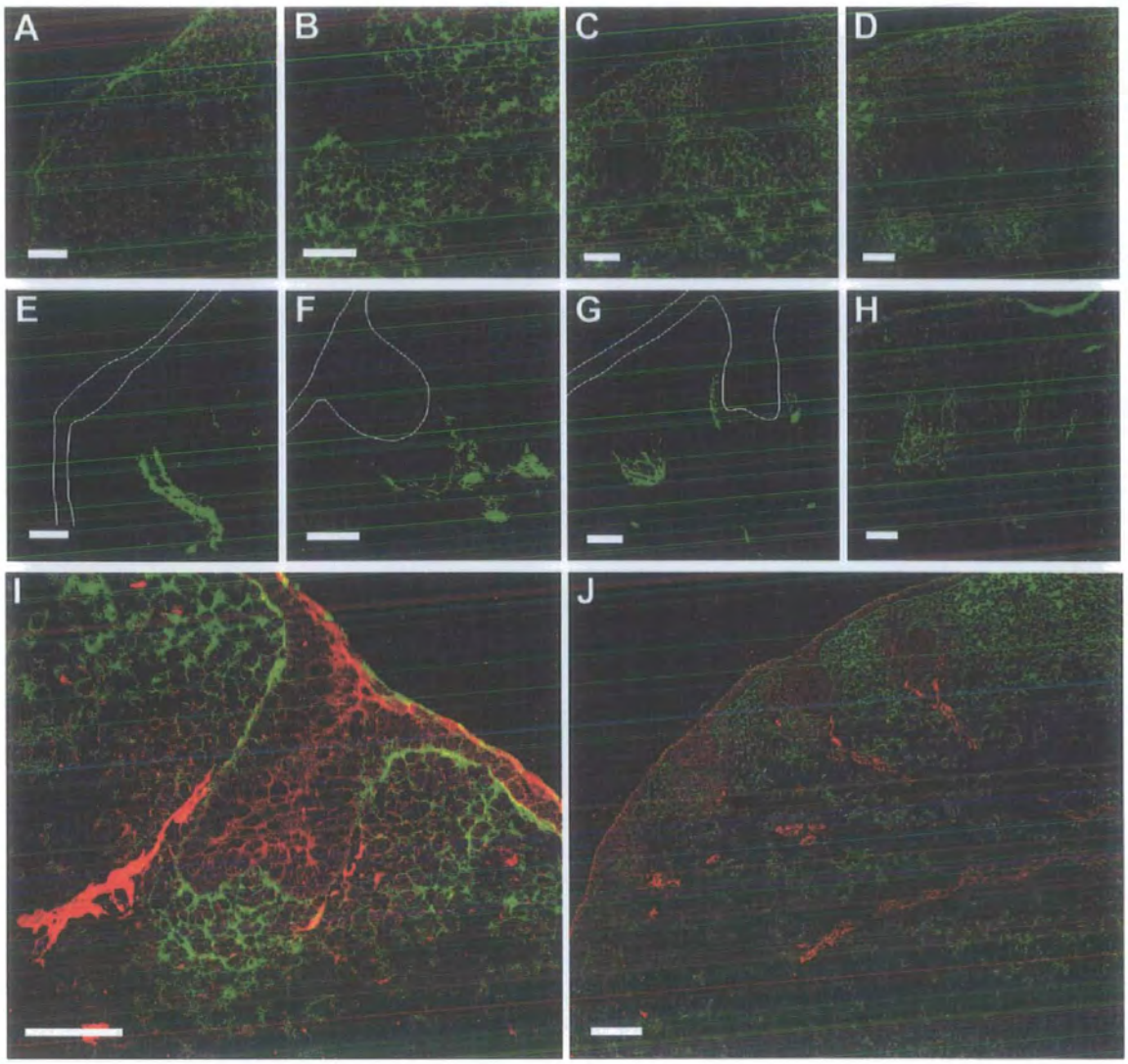
As the follicle elongated and versican staining became evident in the presumptive papilla (stage 3), nerve fibres were seen running up either side of the follicle alongside the epithelial downgrowth just above the level of the developing papilla (Fig. 2.5 C,G,D,H). The fact that they were located in the dermis with the weakest versican staining was most clearly demonstrated using double immunolabelling (Fig. 2.5 I). Occasionally when a follicle had been cut off-centre a network of fibres could be seen spreading over the length of the follicle (Fig. 2.5 G,H) which gives a clearer picture of the three dimensional network of fibres present. It should be noted that the red fluorescence seen in the epithelial tissue (Fig. 2.5, I, J) is non-specific autofluorescence which was also seen in negative controls in which the primary antibody was omitted. The strong labelling of the nerves was however specific as it was not seen in negative controls.

At lower magnification using double immunolabelling, the infraorbital branch of the trigeminal ganglion that innervates vibrissa follicles, could be seen running through the cheek pad sending out smaller branches to individual developing follicles (Fig. 2.5 J). The pathway of this large nerve bundle ran through the lower dermis of the mystacial pad, which displayed very weak versican labelling.

Figure 2.5. Versican and neurofilament expression patterns in developing vibrissa follicles.

Adjacent sections (A,E, E14; B-D, F-H, all E15) stained for versican (A-D) or neurofilament (E-H) show the correlation between weak versican expression and axonal pathways from the earliest stage of follicle development (A, E) through to stage 4 (D, H). Occasionally when a follicle is cut off-centre a network of fibres is seen spreading over the middle portion of the follicle (G, H) giving a clearer picture of the three-dimensional network of fibres present. Note that fibres do not associate with the follicle at the level of the end bulb where the dermal sheath and dermal papilla show versican immunoreactivity. Double immunolabelling (I, J , E15) clearly demonstrates the nerves (red fluorescence) following paths free of versican (green fluorescence) immunoreactivity.

Scale bar: A = 100μm, B – J = 50μm .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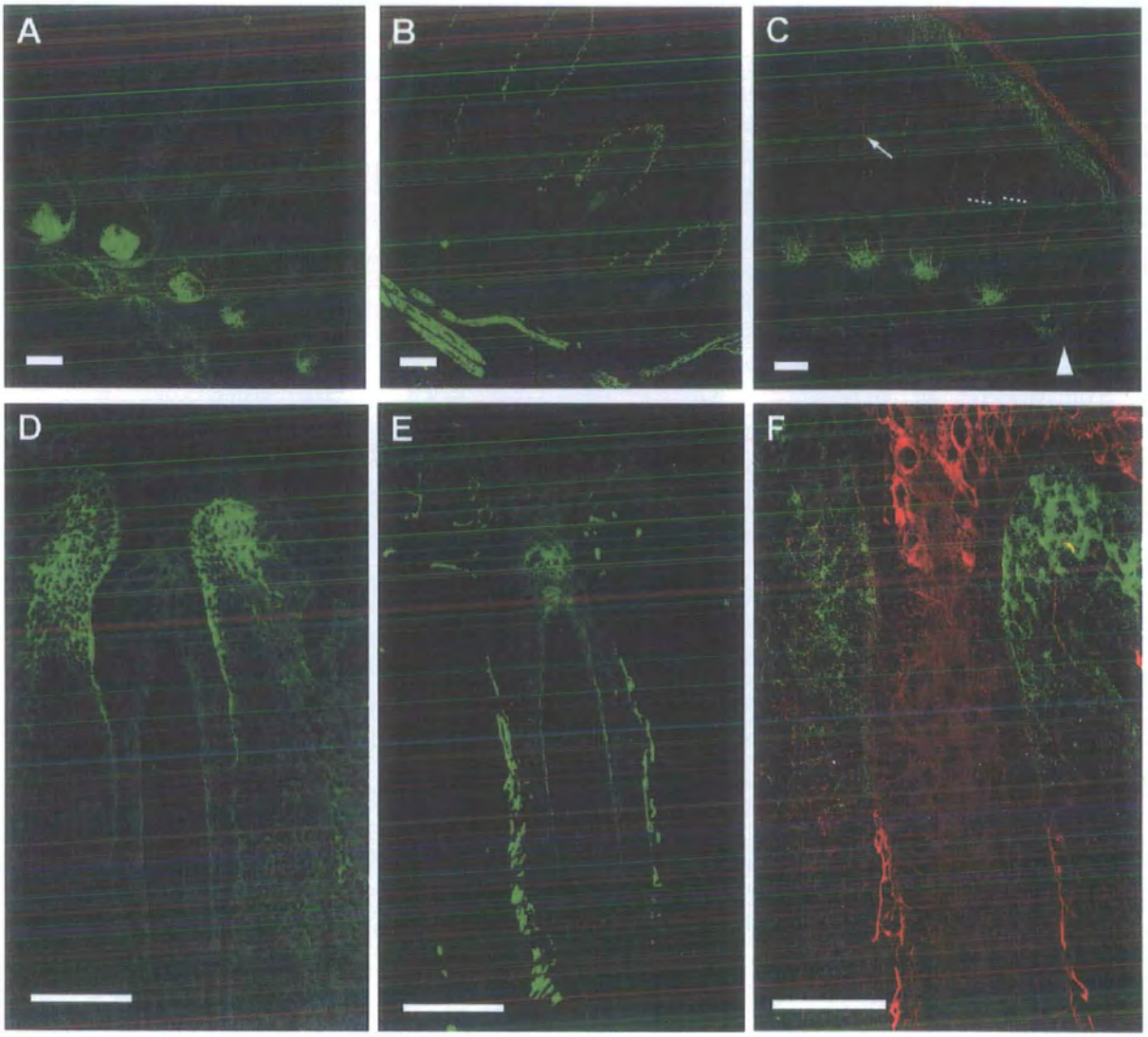
J

Sections of E18 and newborn rat snout (Fig. 2.6) showed rows of vibrissa follicles at stage 6+ with the collagen capsule developing around the follicle. When stained for neurofilament, fibres were seen running up the sides of follicles starting above the end-bulb. Fibres immediately caudal to the follicle were regularly observed to be slightly lower than those on the rostral aspect, consistent with the observation that the deep vibrissal nerve approached the follicle from the posterior aspect (Fig. 2.6 A-C). The restricted areas of strong versican labelling correlated inversely with the pattern of innervation. Thus, nerves were absent from the dermal papilla and lower dermal sheath. In the mesenchyme below the follicles, absence of versican staining appeared to correspond with the path of large nerves (Fig. 2.6 A-C). This reciprocity was highlighted in the follicle neck, where the level at which the main nerve stopped appeared to correspond precisely with the point at which strong versican labelling started (Fig. 2.6 D,E). This was confirmed by double labelling at a slightly earlier stage (E18) where the clear border between the two stains was evident (Fig. 2.6 F). As before the specific staining of the nerves was accompanied by red autofluorescence in the epithelial tissue (Fig. 2.6 C,F). Some green autofluorescence was also evident in epithelial tissue (Fig. 2.6 D,E). Small nerve fibres were present around the neck of the developed follicle within the area showing strong versican expression (Fig. 2.6 E). This was in contrast to the nerves in the lower part of the follicle, which were only observed in areas with weak versican staining.

Figure 2.6. Versican and neurofilament expression patterns in stage 6+ follicles. Newborn (A, B, D, E) and E18 (C, F) rat vibrissa follicles stained for versican (A, D) or neurofilament (B, E) or double immunolabelled (C, F). A strong inverse correlation between the expression of versican and the presence of nerve fibres is most clearly demonstrated at the point where neurofilament staining stops at the border of versican expression (A – C). Each follicle is innervated by a deep vibrissal nerve (arrowhead) whose fibres wrap around the follicle appearing more distal to the follicle bulb of the rostral aspect than the caudal aspect of the follicle (indicated by lines). Also visible are skin nerves (arrow) that run between the vibrissal follicles and branch to form the superficial vibrissal nerves (Rice et al., 1986).

Scale bar: A, B = 50μm, C – F = 100μm.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In summary, at key points in development, staining of adjacent sections with versican and NF68 antibodies, combined with double immunolabelling, demonstrated a notable absence of versican expression from the path of developing vibrissal innervation. This relationship was maintained from the early stage of dermal condensation to stage 4/5, during which time considerable morphogenesis had occurred and the pattern of versican and neurofilament expression had changed accordingly.

2.3.3. Versican expression and innervation at the follicle neck during the adult vibrissa follicle cycle

Neurofilament immunostaining of 20μm sections of an anagen follicle gave a good overall picture of the major components of the vibrissa follicle innervation with confocal microscopy (Fig. 2.7). Some non-specific staining was evident in the epithelial matrix. The deep vibrissal nerve (DVN) was clearly visible entering the collagen capsule and extending branches into the dermal sheath (Fig. 2.7 A,C). At the level of the ring sinus, nerve endings were observed penetrating the glassy membrane (boxed area - Fig. 2.7 C). Superficial vibrissal nerves (SVN) were also observed approaching the follicle distal to the level at which fibres of the DVN extend. The SVN extended fibres which encircled the follicle, this orientation is clearly seen in an oblique section (Fig. 2.7 C) and at high magnification (Fig. 2.7 B,D). Labelling was largely absent from the lower third of the follicle, below the nerve entry point, although in one anagen follicle very small nerves were visible entering the dermal papilla at its base (Fig. 2.7 E).

Immunostaining of follicles at different stages of the hair growth cycle with neurofilament antibody demonstrated a fairly consistent innervation. All the major innervation in the upper two thirds of the follicle remained constant, and the circular

arrangement of smaller nerves surrounding the neck of the follicle was also consistent at all stages of the hair cycle (arrowheads, Fig. 2.8 A-C).

Versican immunostaining showed that versican was abundant in the neck of the adult follicle in anagen, telogen and catagen (Fig. 2.8 D-F). Versican staining encircled the epithelial shaft just below the sebaceous gland and was also present in the dermal tissue around the deep vibrissal nerve within the collagen capsule (not shown).

Figure 2.7. Neurofilament staining of the adult rat vibrissa follicle.
Innervation by supericial vibrissal nerves (SVN) is evident above the dotted line whilst innervation below this is from the deep vibrissal nerve (DVN) (A). At higher magnification (B) SVN descend towards the circumferentially arranged fibres of the inner conical body. An oblique section of the follicle demonstrates the difference in orientation of the nerve fibres between the DVN whose fibres tend to run along the length of the follicle whilst those of the SVN are perpendicular to the follicle at the neck (C,D). Some nerve endings at the level of the ring sinus penetrate the glassy membrane (boxed area in C). Small axons are seen in the basal stalk of the dermal papilla (E).
Scale bar: A, C = 100μm, B, D, E = 20μm.

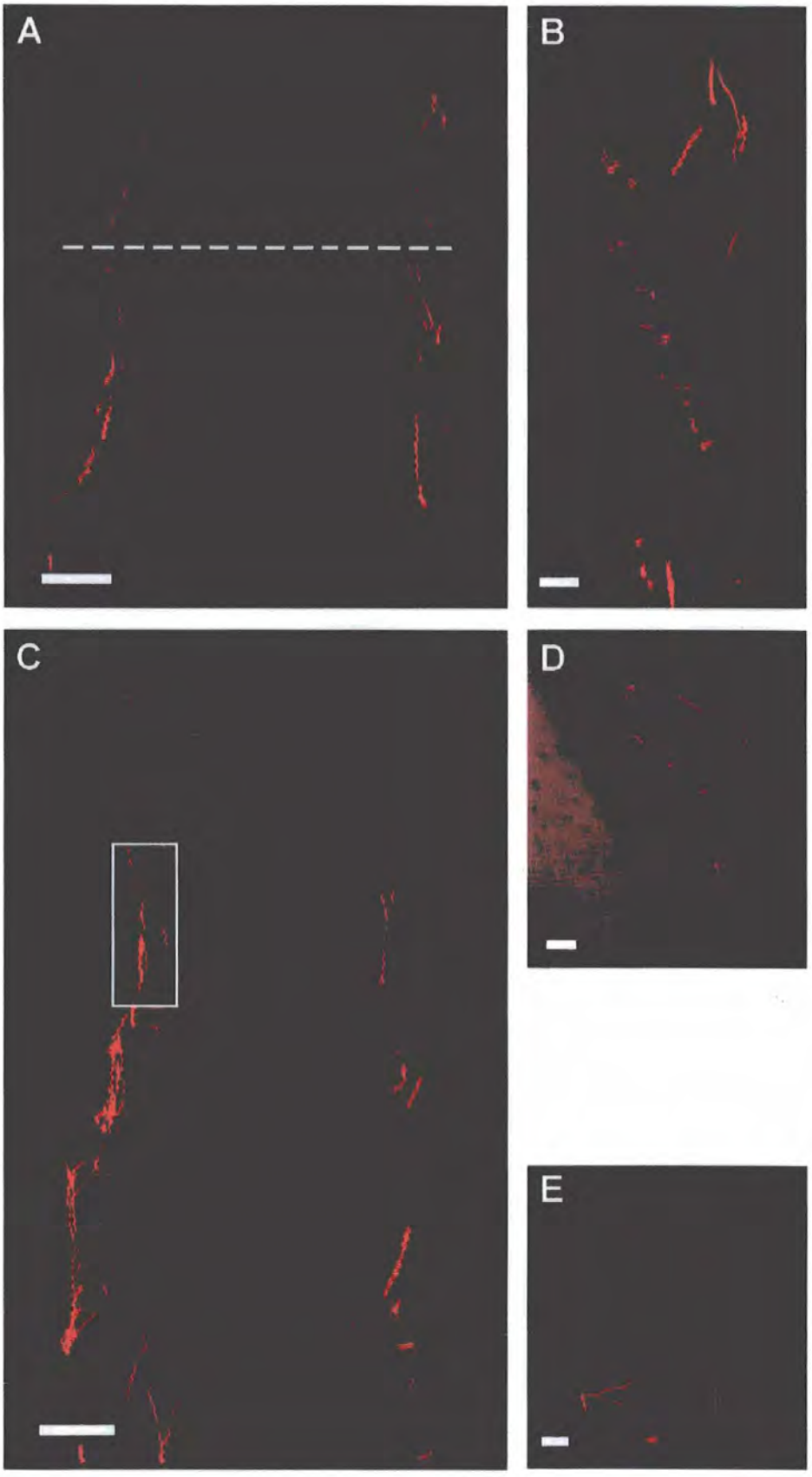
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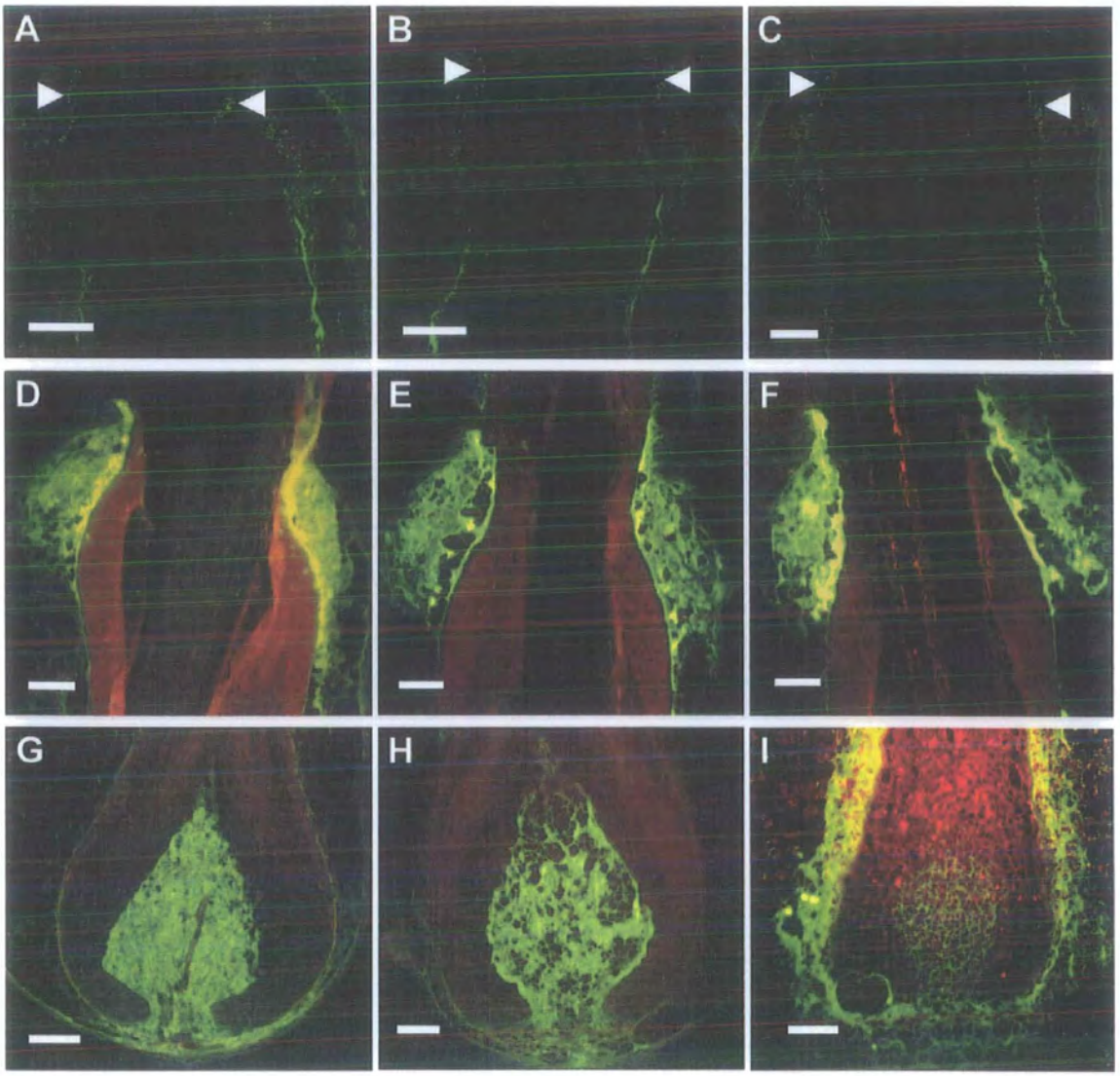
Figure 2.8. Versican and neurofilament staining through the adult vibrissa follicle cycle. Anagen (A, D, G), catagen (B, E, H) and telogen (C, F, I) follicles stained for neurofilament (A – C) and versican (D – I). Red fluorescence (D-I) is due to the Evans blue counterstain. Small calibre nerve fibres around the follicle neck (arrowheads) are present throughout the cycle (A – C) as is the high level of versican immunoreactivity (D – F). Versican is abundant in the anagen dermal papilla (G), starts to diminish in catagen (H) and is severely reduced in telogen (I).
Scale bar = 100μm.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In the bulb of anagen follicles there was strong versican labelling in the dermal papilla and lower dermal sheath (Fig. 2.8 G). This started to disappear from the papilla in catagen, progressing from the fallen apex to the base of the papilla, but remained quite strong in the sheath (Fig. 2.8 H). During telogen versican staining was dramatically reduced in the papilla but increased in the dermal sheath (Fig. 2.8 I). As the follicle switched back to anagen, versican expression increased from the base of the papilla upwards and the cycle was repeated.

2.3.4. Versican is expressed in the extracellular matrix of dermal papilla and dermal sheath cells *in vitro*

Versican staining of cultured dermal sheath and dermal papilla cells showed that the extracellular matrix produced by both types of cells contained versican (Fig. 2.9). The strength of versican immunoreactivity increased with cell density so in the same cultures there were areas which were relatively free of versican (Fig. 2.9 A,B) and areas where the proteoglycan was abundant (Fig. 2.9 C,D). The cells themselves were very weakly stained irrespective of passage number. This is in agreement with the observations of Zimmermann et al. (1994) who found that only a small proportion of versican was retained in skin fibroblast cells whilst a significant amount was secreted into the culture medium. However, in their cultures the versican expression levels decreased with confluency whilst any mitotic cells displayed a bright signal. The difference in findings could be attributed to the fact that papilla and sheath cells do not reach confluency as such but continue proliferating, forming clumps and layers on top of one another. Such aggregates are associated with a localised increase in ECM and this is again reflected in the versican immunoreactivity (Fig. 2.9 E,F) which highlights such patches. The expression of versican in the ECM of dermal papilla and sheath

cultures supports the *in vivo* findings and shows this proteoglycan is an important component of the dermal tissue of the hair follicle.

2.3.5. Jol-4 immunostaining pattern in vibrissa follicles

In the early stages of vibrissa follicle development Jol-4 immunoreactivity was evident in the early dermal condensation below the thickening epidermal placode of the stage 1 vibrissa follicle (Fig. 2.10 A). In the surrounding mesenchyme a few cells were also positive but the number of positively-stained cells decreased as the distance from the dermal condensation increased. As the epidermis became invaginated, the surrounding dermal cells maintained strong Jol-4 immunoreactivity and became more condensed (Fig. 2.10 B,C). At stage 3 when the epidermal downgrowth flattened at the base, the presumptive papilla cells were Jol-4 immunoreactive, as was a layer of dermal cells two to three cells thick, around the epidermal downgrowth which would presumably form the dermal sheath in the developed follicle (Fig. 2.10 D,E).

Figure 2.9. Versican expression in dermal papilla and dermal sheath cell cultures. Versican is expressed in the ECM of dermal sheath (A, C, E) and dermal papilla (B, D, F) cultures. Levels of expression change with cell density as does the level of ECM so low density culture (A, B) shows a low level of versican immunoreactivity whilst high density areas (C, D) have a much higher level of immunoreactivity. In both sheath and papilla cultures this fluctuation of expression in concert with cell density is emphasised by the raised expression levels in patches of aggregating cells (E, F).
Scale bar = 50μm.

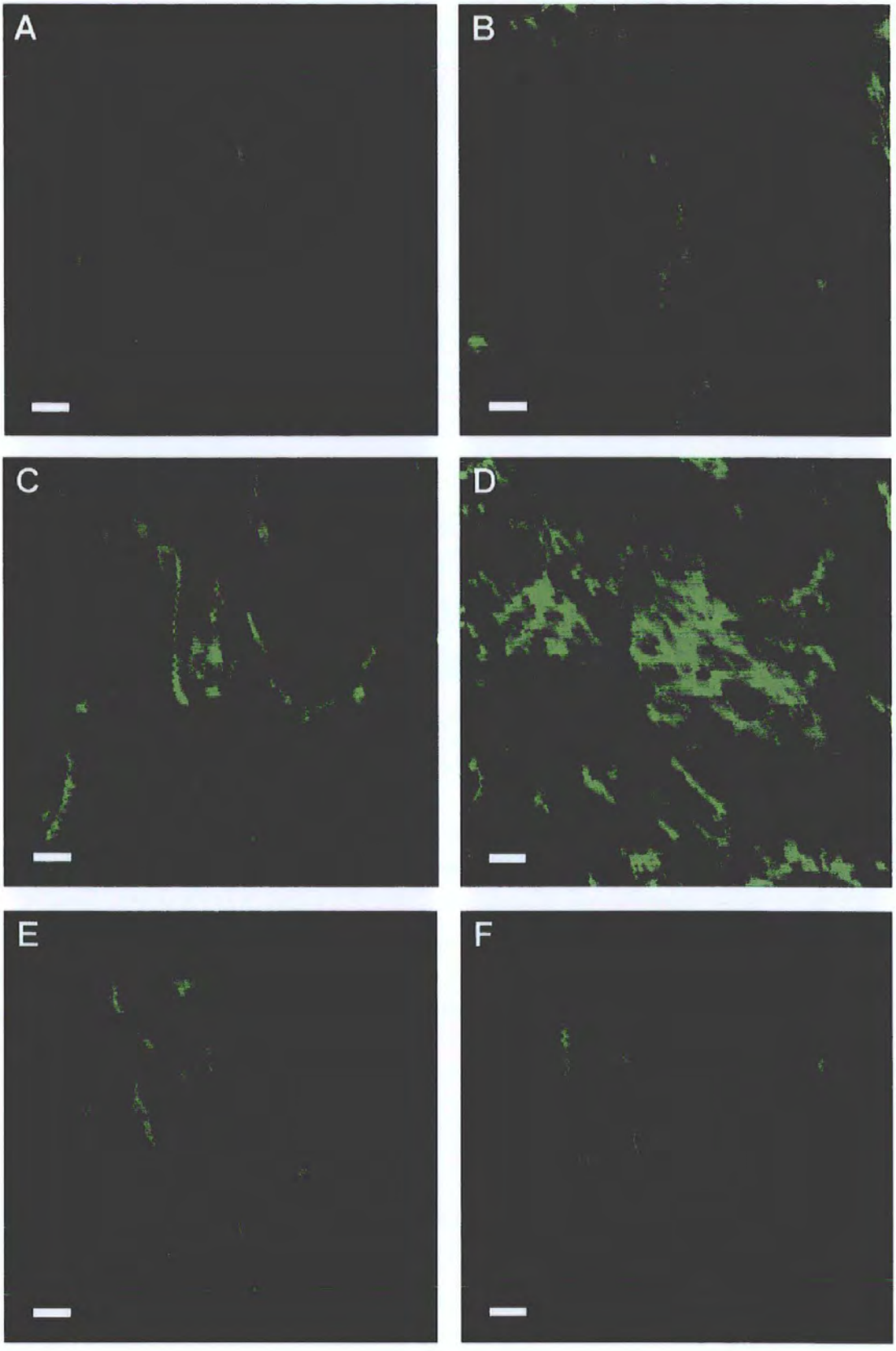
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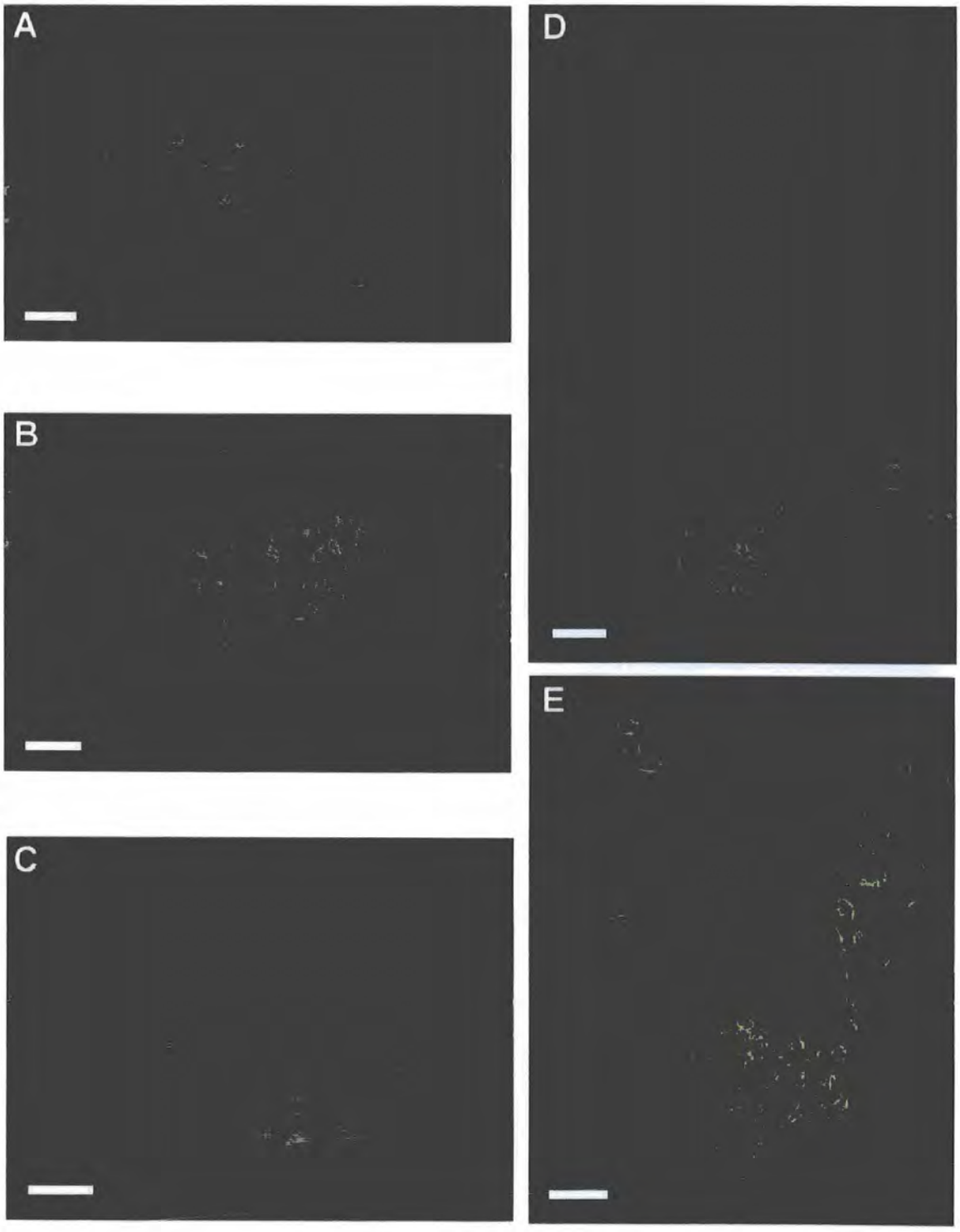
Figure 2.10. Jol4 immunoreactivity in the early stages of vibrissa follicle development. Sections of embryonic (E15) rat snout immunostained with Jol4 antibody show that from the earliest stage of follicle development the cells of the dermal condensation are immunoreactive (A–C). This staining is consistent as the epidermis thickens (B) and develops into the hair peg at which stage Jol4 staining is evident in the dermal cells adjacent to the epidermal downgrowth as well as beneath the leading edge (C). At stage 3 of follicle development Jol4 staining is evident in the presumptive papilla and sheath as well as in the central column of the epidermal downgrowth (D,E).
Scale bar = 50μm.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E18 rat back skin was also immunostained with the Jol-4 antibody. The frequency of Jol-4 positive cells appeared to be greater in the dermis than in mystacial pad dermis but dermal condensations were still evident and the pattern of staining in pelage follicles reflected that seen in vibrissa follicles (Fig. 2.11 A-C). There was also a significant number of suprabasal epidermal cells that were Jol-4 immunoreactive.

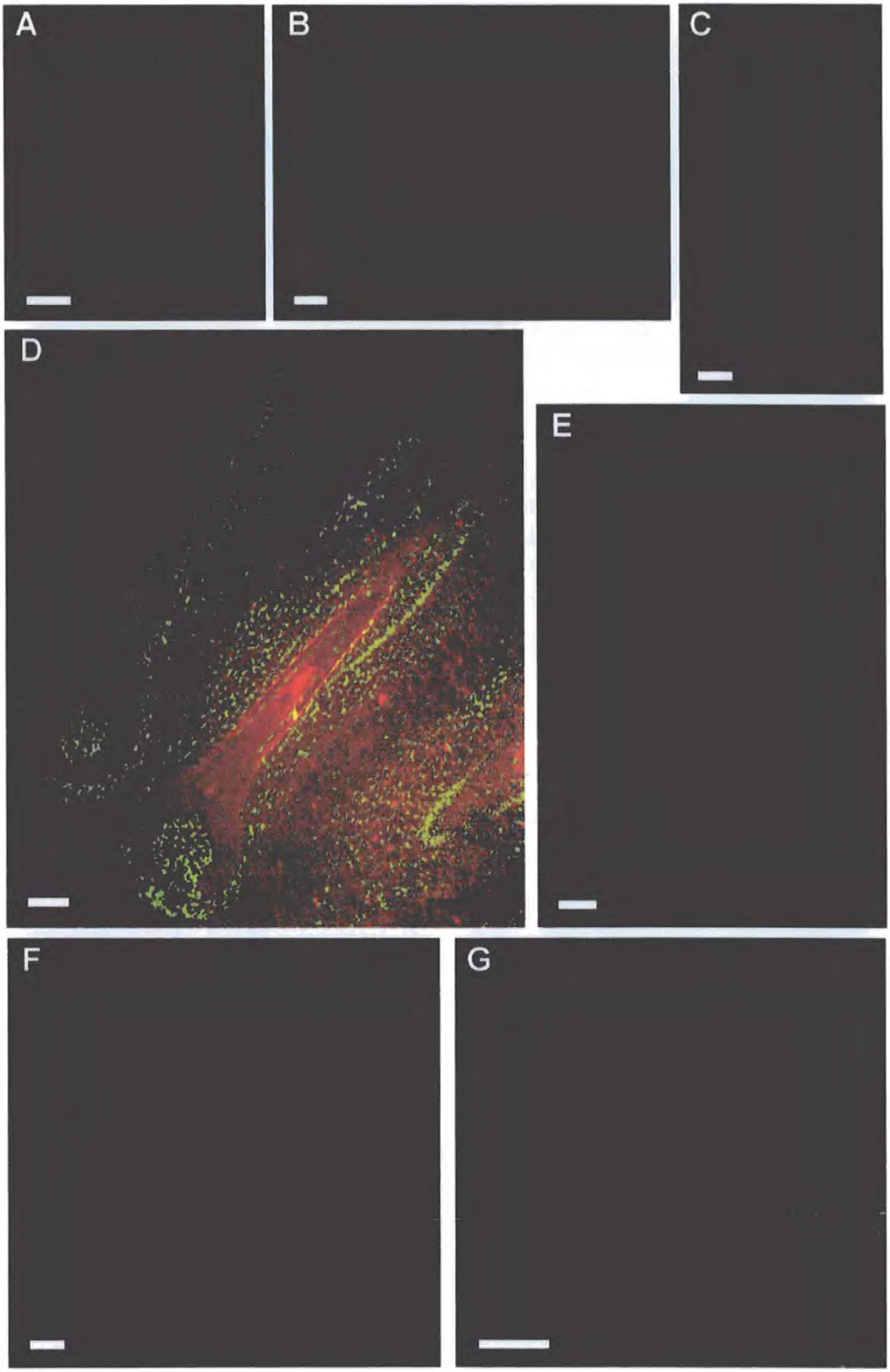
Jol-4 immunoreactivity was maintained by the vibrissa follicle dermal cells right through to adulthood and appeared to be consistent throughout the follicle cycle (Fig. 2.11 D-G). In anagen, strong staining was observed in dermal papilla and dermal sheath cells (particularly in the lower third of the follicle), and in the epithelial cells of the outer root sheath. There was also a layer of inner root sheath that displayed Jol-4 immunoreactivity, seen in newborn and adult follicles (Fig. 2.11 C,D). In catagen, dermal papilla, dermal sheath and outer root sheath staining was maintained and the germinative epithelial cells, and a layer of outer root sheath cells around the lower end bulb (Fig. 2.11 G) developed Jol-4 immunoreactivity.

Throughout development, Jol-4 immunoreactivity distinguished the densely packed dermal cells of the follicle from the dermal fibroblasts of the surrounding tissue, so providing a useful tool for early identification of dermal cells of the follicle. The Jol-4 staining pattern was almost the exact reverse of that of versican in follicular dermal tissue from the earliest stage of follicle development. As development progressed versican expression was upregulated in the Jol-4 stained dermal papilla and sheath, changing the relationship between the two staining patterns.

Figure 2.11. Jol4 immunoreactivity in pelage follicle development and the developed vibrissa follicle.

E18 pelage follicles at various stages of development (A-C) display similar Jol4 labelling to corresponding stages of vibrissa follicle development. Certain layers of the stage 6+ follicle (outermost layer of ORS and a layer of IRS) are highlighted by Jol4 immunoreactivity as well as the dermal papilla and dermal sheath (D). Red fluorescence is due to the Evans blue counterstain (D). The same structures remain highlighted by Jol4 labelling during anagen (E) and catagen (F,G) in the adult follicle. In catagen the germinative epithelial cells display a higher level of staining than the epithelial matrix (G).

Scale bar: A-C = 20μm, D-G = 100μm.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2.4. DISCUSSION

2.4.1. Summary

From the work described here I have made three principal observations: (A) versican has a distinct expression pattern through the process of follicle development as well as during the growth cycle of the adult follicle, which exhibits two independent areas of expression; (B) significant changes in versican expression coincide with changes in the expression of neurofilament, suggesting a relationship between the extracellular matrix proteoglycan and the path of axons, both during development and the adult follicle cycle; and (C) both versican expression and Jol-4 immunoreactivity highlight the early dermal condensation, indicating the withdrawal of these aggregated cells from the cell cycle and a change in their extracellular matrix, supporting previous proposals that the fate of dermal cells of the hair follicle is determined from a very early stage in development.

2.4.2. Versican in follicle development

Versican was first described in association with hair follicles by du Cros et al. (1995) who observed that expression of this proteoglycan changed with the hair growth cycle in two regions of the follicle - the dermal papilla and at the neck of the follicle. Intriguingly versican has been proposed to play a key role in the induction of follicles by transplanted dermal papilla cells. Kishimoto et al. (1999) demonstrated that adult dermal papilla cells that lost versican expression in culture, also lost their inductive properties when put into a hair follicle induction assay. In contrast to our work, these authors report strong versican expression in the initial dermal condensation using a versican promoter driven *lacZ* gene. The discrepancy may reflect the different methods used to detect versican expression at the gene or protein level, but it should be noted

that their focus is the pelage follicle, whilst the results presented here are focused on the vibrissa follicle. Kishimoto et al. (1999) state that at E13.5 (the age when they observe the earliest dermal cell expression of the *lacZ* gene in pelage follicles) the condensed mesenchyme of vibrissa follicles also expressed *lacZ*. The data to support this statement is not presented but as vibrissa follicles develop at an earlier embryonic age than pelage follicles it is likely that the vibrissa follicles in question are at a significantly later developmental stage. If this is the case and the *lacZ* gene expression occurred as follicles entered stage 3 (Fig. 2.3) this would correspond to our findings in rat vibrissa follicles. Assuming this to be true, pelage and vibrissa follicles appear to differ in their versican expression during the earliest stages of development. (There is a precedent for such an argument as Wang et al. (2000) observed that Sonic hedgehog signalling was crucial for development of body hair beyond the hair peg stage, whilst the vibrissa follicles were unaffected by interference in the Shh signaling pathway.) Whilst pelage follicles were evident in some of our sections of newborn snout, they were invariably at stage 6+ and noticeable due to the strong versican expression in the dermal papilla.

A more detailed and concentrated comparison between pelage and vibrissa follicle development regarding versican expression would be required to clarify this issue. Any difference in versican expression, between pelage and vibrissa follicles, should be considered in the light of their functional and anatomical differences, including different degrees of complexity in follicular innervation.

2.4.3. Regulation of innervation by versican

Like other aggregating chondroitin sulphate proteoglycans, versican has previously been implicated in directing neuronal outgrowth, but only in relation to neural crest cell migration and axon growth in the central nervous system. *Splotch* mice have mutations in the transcription factor *Pax3*, and exhibit significant neural crest cell

migration defects, despite the neural crest cells being intrinsically capable of migration. Henderson et al. (1997) found versican was over-expressed in neural crest migration pathways of the *splotch* mouse and proposed that this was due to the mutated *Pax3* gene being unable to down-regulate versican in these areas. Bovine versican has also been demonstrated to have inhibitory effects on axon growth in vitro (Schmalfeldt et al., 2000).

The investigation of versican distribution within and around the developing vibrissa follicle, revealed a distinct and progressively well-defined pattern of expression. Between stage 1 and stage 6+ of follicle development, versican expression changed from being very weak in the mesenchymal aggregation and the dermis surrounding the hair peg, to strongly positive in the dermal papilla and lower dermal sheath. In general, early on in development, its absence in the dermis immediately surrounding the developing vibrissa follicle corresponded with the presence of axons. It is also clear that the localised reduction of versican staining was not simply due to the axons (which do not express versican) covering particular areas, as nerve fibres occupied only part of the weakly labelled tissue.

Nerves do not appear to cross boundaries between strong and weak versican domains. The transition from a basket of nerve fibres encasing the entire stage 3 follicle to a tube of fibres running from just above the papilla up to the neck of the follicle is an interesting morphological change, the mechanics of which are not clear. If nerves are avoiding penetrating, or crossing over, areas of high versican expression, it is possible that the downwards movement of the presumptive papilla, which at the stage in question is beginning to express versican, results in the basket of fibres allowing the follicle to grow down through the network. The observations described here suggest that versican influences the complex peripheral innervation of the rat vibrissa follicle relatively late on in development.

2.4.4. Does versican act alone?

The idea that nerves may be channelled into areas with little or no versican, does not, on its own, account for all my observations. In particular, I observed that at the start of follicle development, nerves remained at the outer edge of a zone of dermis that had weak versican expression, but did not penetrate the actual dermal condensation at the centre of this zone. Other molecules expressed in the condensation may, therefore, act to prevent nerve fibres straying into this area. Coupled with relatively high versican expression outside of the condensation, this would provide a very specific growth path for innervation. The relative absence of versican inside the condensation does correlate well with cell activities that accompany the formation of this structure. Previous studies have shown that versican inhibits cell adhesion and has a positive role in cell proliferation (Yamagata et al., 1989, Zimmermann et al., 1994, Yang et al., 1999). Therefore its absence from the dermis immediately beneath the epidermal placode is consistent with increased cell adhesion (Kaplan & Holbrook, 1994) and absence of proliferation (Wessels & Roessner, 1965), both of which are characteristic of the dermal condensation.

Vibrissa follicle innervation is complex with many different types of nerve endings present in the different components of the follicle-sinus complex. A recent study has observed that the expression patterns of GDNF family ligands and receptors correlate with the development of specific sensory nerve endings in vibrissa follicles (Fundin et. al., 1999). This work highlights GFRα2 as having an expression pattern directly opposite to that of versican in the early stages of follicle development. For example, GFRα2 mRNA expression is seen in the mesenchymal condensation in stage 1, and in stage 2 it is visible around the sides of the epidermal downgrowth. Clearly the fine detail of follicle innervation is dependent on multiple molecular cues. As the

innervation of the vibrissa follicle is well-documented it could be a useful tool for further investigations into molecular controls of innervation in a similar manner to the work of Fundin et al. (1999).

2.4.5. Innervation at the neck of the vibrissa follicle is constant through the adult follicle cycle

Recent work has shown that peripheral innervation in skin is not constant. Intriguingly, it changes with the pelage hair follicle cycle, particularly the network of fibres around the portion of the follicle distal to the point of insertion of the arrector pili muscle, that changes in both number and type of fibres (Botchkarev et al., 1997; Peters et al., 2001). Previously du Cros et al. (1995) observed changes in versican expression in the corresponding region of the follicle neck, and when these two studies are compared the changes in versican expression and innervation correlate well. When versican is present at the neck of pelage follicles during telogen, the number of nerve fibres is reduced, whereas during anagen this versican expression is lost whilst nerve fibres are increased in number.

A significant difference between rodent vibrissa and pelage follicles is that vibrissa follicles retain their non-growing club hair while the next one grows, hence a fibre is always present. Pelage follicles lose their hair part way through the telogen phase of the hair cycle. As specialised mechanoreceptors, vibrissa follicles provide constant sensory input, therefore it is not surprising I observed constant innervation in the inner conical body, the region of the vibrissa follicle corresponding to that in which versican and innervation are cyclic in pelage follicles. Innervation in this part of the follicle arises from a number of superficial vibrissal nerves rather than the deep vibrissal nerve, which innervates the majority of the follicle (Mosconi et al., 1993; Rice et al., 1993). In their detailed study on vibrissa follicle innervation Rice et al. (1993)

made no specific observations regarding the cycle stage of the follicles, but due to the number of samples used it would be expected that these authors would also have mentioned any significant changes in follicle innervation.

The relatively constant innervation at the neck of the follicle is accompanied by consistently strong versican expression in the surrounding dermal tissue. Whilst the versican expression in the lower third of the follicle is similar between pelage and vibrissa follicles it is the expression around the neck that differs, as does the innervation. The relationship between innervation and versican is therefore maintained beyond birth.

2.4.6. Summary of adult vibrissa follicle innervation

Over the last 15 years detailed studies have been carried out on the neuroanatomy of the vibrissa follicle providing an excellent reference for those whose work depends on an understanding of the complexity of this organ's innervation (Figure 2.2). Six areas of the follicle are innervated: 1) the epidermal rete ridge collar, 2) the inner conical body, 3) the dermal sheath at the level of the ring sinus, 4) the outer root sheath at the level of the ring sinus, 5) the dermal sheath at the level of the cavernous sinus, and 6) the dermal papilla. All vibrissal innervation is derived from the infraorbital branch of the trigeminal nerve that gives rise to nerves that run through the dermis of the mystacial pad, dorsal to the rows of vibrissae that they supply. These are the row nerves, branches from which form the deep vibrissal nerves (DVN), of which there is one per follicle, and superficial vibrissal nerves (SVN), which are more numerous with several SVN supplying the distal portion of each vibrissa follicle (Rice et al., 1986).

The range of types of nerve fibres and nerve terminals has led to many proposals of function for the extensive neural network in the follicle. Nociception and proprioception seem to be the main roles attributed to the nerves (Arvidsson & Rice,

1991; Mosconi et al., 1993; Rice et al, 1986). This work has also emphasised the anatomical and physiological difference between the SVN and DVN networks. One example of this is the proposal by Rice et al. (1986) that the fibres innervating the inner conical body detect movements of the follicle-sinus complex with respect to the mystacial pad, whilst innervation from the DVN detects movements of the vibrissa with respect to the follicle-sinus complex. In later work, Rice (1993) used a lectin to identify a subpopulation of neurons within the vibrissa follicle. It highlighted the axons that are circumferentially arranged in the inner conical body and only a small number of DVN fibres in the cavernous sinus were labelled by this method, so providing further evidence that the superior and deep innervation of the vibrissal follicle are two separate networks within a complex structure.

2.4.7. Versican expression associated with fine nerve fibres

The area of strong versican expression at the follicle neck included the path of fine nerve fibres from the SVN which were observed consistently throughout the follicle cycle. The association of axons with strong versican expression is contradictory to the pattern seen in development where axons appeared with weak versican expression. However, this is in line with recent ideas suggesting that versican regulates innervation in a far more complex manner than was first thought. Initially versican was believed to simply have an inhibitory effect on neural crest cell migration and axon outgrowth (Landolt et al., 1995; Henderson et al., 1997), but there is recent evidence that in the mature central nervous system, versican acts as a stabilizing factor to limit neuronal plasticity (Niederost et al., 1999; Schmalfeldt et al., 2000). Versican's constant presence at the neck of the established (stage 6+) follicle may well be ensuring the stable innervation necessary for the follicle to successfully function as a sensory organ.

This leaves the question of how, developmentally, this nerve supply reached this region. The innervation at the follicle neck from the superficial vibrissal nerve appears to develop slightly later than the deep vibrissal nerve supply to the lower two thirds of the follicle. Fibres were not evident at the follicle neck until the newborn stage, despite a significant number of fibres being present in the subepidermal dermis in the interfollicular skin. Since the neck of the follicle has a ring of versican around it, (Fig 2.8 D - F) this must require fibres to move into an area abundant in versican.

A recent proposal is that versican positively influences neural crest cell migration if the gradient of versican expression is gradually increasing but any significant stepwise change in concentration, whether that be an increase or decrease, will negatively affect their migration (Perissinotto et al., 2000; Perris & Perissinotto, 2000 (review)). It is possible from the observations presented here that this is also the case with axonal growth. Axons run through areas of weak versican expression rather than enter adjacent dermal tissue that has considerably higher levels of the proteoglycan present (Figure 2.5). Axons appear to be directed along the correct path by sharp boundaries in versican expression. It is therefore possible that the superficial fibres grow toward the follicle neck with the increasing versican gradient which develops between E16 and E18. This is when the uppermost layer of dermis changes from uniform versican expression to moderate staining in the interfollicular area and strong staining immediately around the vibrissa follicles. Once the fibres have reached their target in the follicle the versican acts to maintain this innervation during the complex process of remodelling that occurs during the follicle cycle.

The relationship betweeen versican and neural pathways does not appear to be a black and white permissive or non-permissive one, more a case of guidance which, in combination with other factors, results in a delicate system being established and maintained to the best effect.

2.4.8. A conserved function within the aggrecan family

This work supports previous studies proposing that versican influences the outgrowth of axons in development and in adult neuronal remodelling. The mechanism by which it does this has not been elucidated. As mentioned earlier, other members of the aggrecan family have been shown to have a similar effect on neurite outgrowth and therefore it seems likely that a part of the gene that is highly conserved between all of these molecules (aggrecan, versican, brevican and neurocan) would be responsible for this effect. The binding of the glycosaminoglycan, hyaluronan, to the highly conserved NH_2 terminal region is a common characteristic of the aggrecan family, so hyaluronan may be the effector molecule. However, *in vitro* assays have demonstrated inhibition of neurite outgrowth by purified proteoglycans (Friedlander et al., 1994), and in one case hyaluronan was used in such an assay and shown to have no inhibitory effect on neurite outgrowth (Schmalfeldt et al., 1998). Nevertheless, it has recently been demonstrated that it is an NH_2-terminal fragment of neurocan containing an Ig loop and an HA binding domain that is responsible for the inhibition of N-cadherin and β1 integrin-mediated neurite outgrowth and cell adhesion, and that breaking this fragment down further leads to loss of the inhibitory activity (Li et al., 2000). It therefore appears that the inhibitory characteristics of the aggrecan family proteoglycans on neurite outgrowth, are encoded in the same highly-conserved region that imparts the hyaluronan-binding capacity. This is consistent with the fact that the smaller glycoproteins hyaluronectin and GHAP also appear to have an inhibitory effect on neurite outgrowth (Bignami et al, 1988).

Hyaluronectin is a hyaluronan binding glycoprotein that has been isolated from brain and is probably an alternatively spliced gene product that is produced by cells as well as resulting from proteolytic cleavage of the larger versican molecule (Zako et al.,

1995). It has been demonstrated that hyaluronectin is produced by oligodendrocytes and Schwann cells *in vitro* (Courel et al., 1998) so the glial cells produce a glycoprotein that binds with hyaluronan in the ECM and at the same time appears to regulate neurite outgrowth. Hyaluronectin is possibly the same as GHAP but this has not been confirmed in the literature to date.

The antibody used for these studies was originally designed against glial hyaluronate binding protein (GHAP) (Asher et al., 1991), which has now been found to be a product of proteolytic cleavage of versican (Zimmermann & Ruoslahti, 1989; Perides et al., 1995). Later work by the group showed the antibody to bind to versican as well as GHAP (Perides et al., 1995).

CD44 is the hyaluronan receptor and this is involved in the degradation of hyaluronan, that is it binds the glycoprotein to the cell surface so it can be internalised and degraded. A major function of hyaluronan appears to be maintenance of the extracellular space between cells, an example being the development of the cornea when hyaluronan loss results in condensation of the stroma of the cornea (Toole & Underhill, 1983). Using murine pelage skin, Underhill (1993) studied CD44 and hyaluronan distribution during development of follicles and observed an inverse correlation, with CD44 being present in the condensed mesenchyme beneath developing follicles and hyaluronan being present elsewhere in the mesenchyme but absent from these condensed areas. Hyaluronan therefore appears to have the same expression pattern as that described here for versican, which is not unexpected as versican binds hyaluronan (LeBaron et al., 1992). However, in adult pelage skin, hyaluronan (at low levels) is present in the dermal papilla in anagen, present but less so in catagen, but also present in telogen whilst versican is absent from the telogen papilla.

2.4.9. Further work to support the proposed relationship between versican and peripheral innervation

The data shown here is purely descriptive with no functional evidence to support the proposed functional role of versican in directing the innervation of the vibrissa follicle. Using the techniques employed here, further support for these proposals may be obtained by carrying out similar studies on other follicle systems which have different innervation patterns. The follicle-sinus complexes of different species are known to vary in the level of innervation particularly within the inner conical body (Rice et al., 1986; Mosconi & Rice, 1993) and hence a different versican expression pattern may support the correlation described here. Also, the development of other peripheral sensory organs, such as taste buds, may provide information about the relationship between versican and neural pathways. Expression of the hyaluronan receptor, CD44, has been studied in taste buds (Witt & Kasper, 1998) suggesting hyaluronan, a high-affinity ligand for versican, is likely to be present.

Transgenic techniques could be employed to incorporate the versican gene under the control of a promoter for a gene expressed specifically by dermal condensation (DC) cells. From this data lamin A is a possible candidate in that it is expressed by DC cells but the expression of lamin A is too widespread for this to be a viable option. Other options might include BMP, FGF and Wnt family members but again these are involved in a wide range of structures through development so may not be viable for the development of transgenic animals. However, if a suitable promoter could be identified and the technique employed, it would be possible to determine whether nerve fibres would be able to penetrate the outer part of the early dermal condensation and so facilitate the passage of the DVN into the dermal sheath of the vibrissa follicle. Based on the proposed role of versican in cell proliferation and adhesion, it may be the case

that the DC would not develop into such a defined structure and ectopic versican expression in this region may affect the overall development of the hair follicles.

Alternatively a mutation could be generated in the NH_2 terminal of the versican gene to see if a) the same developmental expression occurs and b) how innervation is affected. This would follow up the theory that it is the hyaluronan-binding domain that is responsible for the effects of versican on neural outgrowth.

Corthesy et al. (1999) established that partial denervation of the whisker pad resulted in regeneration of follicle innervation in the normal arrangement. There was no compensation by intact nerves from other follicles and regenerating fibres innervated specific follicles only. Studies on versican distribution in relation to such regenerating innervation would indicate whether the neuronal guidance mechanisms differ in the adult animal relative to those seen in development. The data presented here, along with that of du Cros et al. (1995) would suggest that versican is still involved in such a process in adulthood, but regeneration after denervation would involve a major nerve bundle, rather than the delicate nerve branches and endings that are involved in the cyclic remodelling of the hair follicle.

2.4.10. Versican as an indicator/regulator of cell proliferation

Versican is produced by dermal (skin) fibroblasts in culture but versican mRNA expression has been shown to be inversely proportional to the confluency of the cultures. Due to this and the localisation of versican in the proliferating basal epidermis it has been suggested to regulate cell proliferation along with the high-affinity ligand, hyaluronan (Zimmermann et al., 1994). The expression pattern of versican in the early stages of development appears to support this as the very weakly stained dermal condensation has been shown to consist of non-dividing cells (Wessells & Roessner, 1965). Cultured dermal papilla and dermal sheath cells do not reach confluency as such,

but continue to divide in culture. Versican forms part of the extracellular matrix of dermal papilla and dermal sheath cultures with levels of versican increasing as the cell density increases, as might be expected as the amount of ECM produced will increase with the number of cells. Zimmermann et al. (1994) observed versican expression was lost when keratinocytes were induced to terminally differentiate, providing another indication that versican expression may be associated with proliferation.

2.4.11. Nuclear lamin A expression

Cells labelled by the Jol-4 antibody are non-dividing cells as the epitope against which the antibody is designed becomes masked by lamin-protein or lamin-chromatin interactions when the cell prepares for proliferation (Dyer et al, 1997). The term "quiescent" has been used to describe Jol-4-positive cells (Dyer et al., 1997) but this gives the impression the cells are inactive, and is therefore very misleading in the case of follicular dermal cells that are actively producing ECM and growth factors throughout development and the adult cycle (Couchman & Gibson, 1985; Couchman, 1986; Messenger et al., 1991; Jahoda et al., 1992b; Kaplan & Holbrook, 1994; Kozlowska et al., 1998). The cells will therefore be described as non-dividing rather than quiescent.

Jol-4 immunoreactivity in the early dermal condensation supports observations made in feather as well as hair development that dermal condensation formation is not a result of cell proliferation but due to rearrangement of cells resulting from changes in cell adhesion (Wessells & Roessner, 1965; Noveen et al, 1995). In the absence of cell proliferation, the increase in the number of Jol-4 immunoreactive cells suggests recruitment from the surrounding mesenchyme continues through development.

The distinct staining pattern of dermal cells of the hair follicle from the earliest stages of follicle development suggests that the fate of these cells is predetermined from

a very early embryonic age. The lack of versican expression in the dermal condensation also identifies these cells, but whilst lamin A expression is a characteristic of follicular dermal cells at all stages of the follicles life, versican expression is more changeable through the developmental process. Osada & Kobayashi (2000) found dermal cells from the sites of developing murine vibrissa follicles have the ability to induce ectopic follicle formation from E14 (stage 2), confirming that the cells start to possess characteristics of the mature follicle dermal cells from very early on. However, this inductive capacity is not yet present in these cells at the stage at which the distinctive versican/Jol-4 expression pattern is first seen. The distinctive lack of versican expression coupled with Jol-4-immunoreactivity therefore identifies the dermal cells of the hair follicle from the surrounding mesenchyme in the earliest stages of follicle morphogenesis.

The presence of Jol-4 immunoreactive cells in the dermal papilla of the mature vibrissa follicle is no great surprise. Perhaps a more interesting observation is the number of quiescent cells present in the outer root sheath, particularly in the bulge area. Cotsarelis et al. (1990) identified a population of slow-cycling cells in this region, leading to proposals that the stem cells of the hair follicle reside in the follicle bulge. Oshima et al. (2001) have recently proposed a model by which the stem cells of the vibrissa follicle originate in the bulge of the follicle and migrate through the outer root sheath down to the follicle bulb where they differentiate under the influence of the dermal papilla. Prior to this, the bulge hypothesis appeared to be feasible in pelage follicles as anagen is initiated in such follicles by regression of the dermal papilla up to the level of the bulge bringing the necessary tissues into close proximity for the exchange of signals. However, in the vibrissa follicle such an extensive shortening of the follicle does not occur (Young & Oliver, 1976) and there appears to be no physical interaction between the dermal papilla and the cells of the bulge. My results

demonstrate that quiescent cells predominate throughout the length of the outer root sheath at all stages of the adult follicle cycle. This supports the proposal put forward by Oshima et al. (2001) that non-dividing stem cells migrate through the outermost epidermal layer to gain the position where division and differentiation can occur under the influence of the dermal component of the follicle.

2.4.12. Conclusions

Versican and Jol-4 immunoreactivity distinguish follicular dermal cells from the surrounding mesenchyme in the initial stages of vibrissa follicle development and have distinctive patterns of expression throughout follicle morphogenesis. During the adult follicle cycle, Jol-4 expression confirms the lack of proliferation in dermal papilla and dermal sheath, whilst demonstrating that a non-dividing population of cells is consistently present along the length of the outer root sheath. Versican expression in the adult follicle is cyclic in the dermal papilla, but consistently strong in the inner conical body. I propose a separate role for versican in regulation of vibrissa follicle innervation, that is distinct from versican's expression in the dermal papilla and its putative involvement in the inductive process of hair growth.

CHAPTER 3: The regenerative response of epithelial and dermal cells in the amputated vibrissa follicle.

3.1. INTRODUCTION

3.1.1. Regeneration of complex structures

The ability to regenerate complex structures that require orientation of multiple tissues is extremely limited in mammals. Deer display a naturally occurring regenerative process, the annual growth of antlers. This involves regeneration of cartilage and skin which develops into antler velvet (Li & Suttie, 2000). In response to injury however, there is only one known example of regeneration of a partial limb, that occurs in the absence of exogenous factors. Regeneration of the most distal tip of the murine limb is successful provided that the amputation is proximal to the nail bed (Borgens, 1982; Reginelli et al., 1995). However, the regenerative potential in urodele amphibians and teleost fish is more extensive, with the two species displaying successful limb regeneration and dermal fin regeneration, respectively (Brockes, 1997; Laforest et al., 1998).

Both limb and fin regeneration are preceded by the development of a blastema immediately below the wound healing epidermis at the site of amputation. This structure consists of relatively undifferentiated mesenchymal cells that are derived from the structures at the site of amputation. The interaction of these multipotent cells with the overlying epidermis induces formation of the muscular, neuronal and cartilaginous structures required for limb or fin regeneration (Brockes, 1997; Laforest et al., 1998).

The epidermal-mesenchymal interactions that facilitate regeneration involve some of the signalling molecules common to many developmental processes, including both limb and hair follicle development. Fibroblast growth factors (FGF) and Sonic hedgehog (Shh) are involved in a feedback loop in limb development (Niswander et al., 1994) as well as being involved in hair follicle development (du Cros, 1993; St-Jacques et al., 1998; Chiang et al., 1999). These signalling molecules have been identified as

key factors in regenerative processes in fish and amphibians (Laforest et al., 1998; Torok et al., 1999; Poss et al., 2000). In addition, the exposure of amputated chick limb buds to FGF-2 or FGF-4 induces a regenerative response which includes upregulation of *Shh* expression (Taylor et al., 1994; Kostakopoulou et al., 1996). These models of regeneration therefore provide excellent opportunities for further investigations into developmental signalling mechanisms.

3.1.2. Sonic hedgehog in hair follicle development

Sonic hedgehog (*Shh*) is expressed in epidermal cells at all stages of follicle development, initially in a group of cells in the epidermal placode. Expression continues in the epidermal cells at the base of the downgrowth, and subsequently, *Shh* expression is limited to the inner root sheath in the adult follicle (Iseki et al., 1996). Although *Shh* expression is detected in the epithelial placode, prior to formation of a dermal condensation (Iseki et al., 1996; Karlsson et al., 1999), the induction of follicle morphogenesis does not require Shh signalling, as demonstrated by the fact that hair germs form in the skin of *Shh* -/- mice (St-Jacques et al., 1998; Chiang et al., 1999). However, the expression of Shh is evidently important to follicle morphogenesis as a number of studies have demonstrated abnormal follicle development as a result of altered Shh signalling (St-Jacques et al., 1998; Chiang et al., 1999; Karlsson et al., 1999; Wang et al., 2000). Expression of this gene is therefore functionally important in the epidermal-mesenchymal interactions that regulate hair follicle development.

The regulation of the hair growth cycle in adulthood is also under the influence of Shh, as demonstrated by Wang et al. (2000) who effectively inhibited postnatal hair growth by blocking Shh signalling with a monoclonal antibody. Transient expression of *Shh* is capable of inducing telogen follicles to enter anagen, providing further evidence that this gene regulates follicle cycling as well as morphogenesis (Sato et al., 1999).

Whilst expression of *Shh* is limited to epidermal cells of the hair follicle, the signal is thought to act in a paracrine fashion on the mesenchymal cells. This is supported by functional studies in which the skin of *Shh* -/- mice, when grafted onto nude mice, failed to develop dermal papillae. The epithelial cells formed downgrowths but with no associated papillae they were simply columns of cells that displayed no signs of hair differentiation (Chiang et al., 1999). The expression of Shh is therefore a crucial factor in the development of the dermal components of the hair follicle, but is not essential for follicle induction.

3.1.3. Follicle induction

The induction of follicle development results from signals from the dermis conveying information to the epidermis regarding the size and location of the appendage that will form. The nature and orientation of the appendage is dependent on the epidermis. Each tissue therefore has a distinct role in successful appendage development. These properties were identified in the respective tissues by a series of elaborate dermis-epidermis recombination experiments (Sengel, 1976b; Dhouailly, 1977; Sengel et al., 1980).

Embryonic inductive properties are maintained beyond birth in follicular dermal cells. The inductive properties of adult follicular dermal tissue were first demonstrated by Oliver (1967b) who implanted vibrissa dermal papillae into the remaining upper segment of vibrissa follicles from which the lower half had been amputated. The subsequent production of a hair fibre determined that the dermal papilla of the hair follicle was responsible for inducing fibre production in the vibrissa follicle. The resilient nature of this inductive capacity was demonstrated by combining organ culture techniques with dermal implantation. Fibre growth ceases at 10-15 days of follicle organ culture. Robinson et al. (2001) cultured follicles for at least 35 days before

isolating the dermal tissue from the end bulb and implanting it into the base of an amputated follicle. In all cases, fibres were produced indicating that the cessation of fibre growth was no reflection on the inductive capacity of the follicular dermal tissue.

The inductive properties described demonstrate that, in association with follicular epithelial tissue, the dermal papilla induces hair fibre production (Oliver, 1967b; Kobayashi & Nishimura, 1989). It could be argued that this is an innate property of the epithelial tissue as well as the dermal tissue. However, Oliver (1970) implanted dermal papillae in association with ear, scrotal sac and keratinizing oral epithelium and demonstrated that afollicular epithelia could be induced by association with dermal papilla to produce cells of a follicular nature. This work was one of the first indications of the pluripotent nature of epithelial cells.

The capacity of the intact dermal papilla to induce follicle bulb formation and fibre growth was also found to be true of cultured dermal papilla cells of low passage number (Jahoda et al., 1984; Horne et al., 1986; Lichti et al., 1995). The cells were implanted as a pellet in amputated follicles in the same manner that intact dermal papilla had been implanted, and subsequently fibres were produced. Similarly, dermal papilla cells were capable of inducing follicle formation in non-follicular epidermis (Reynolds & Jahoda, 1992) as had been seen with intact dermal papilla (Oliver, 1970). In another model of follicle induction, low passage cultured dermal papilla cells combined with epithelial cells from neonates induced follicle formation and hair growth on the back of a nude mouse (Lichti et al., 1995). These experiments demonstrate that the embryonic inductive properties retained in the adult dermal papilla, are not lost in cultured dermal papilla cells of low passage number.

Later studies, in which dermal papilla cells were implanted in ear wounds, indicated that the dermal papilla was responsible for determining the type of fibre produced as large vibrissa-type fibres were evident amongst the fine hairs of the ear

(Jahoda et al., 1993). Pelage dermal papilla cells and human dermal papillae also induced follicle formation, supporting the theory that inductive properties of the dermal papilla are universal and are not limited to the vibrissa follicle dermal papilla (Reynolds & Jahoda, 1992; Reynolds et al., 1995).

The inductive properties of dermal sheath tissue, when implanted in the upper half of amputated vibrissa follicles, were initally demonstrated by Horne & Jahoda (1992). This tissue successfully formed fully functional dermal papillae in over 60% of recipient follicles. The formation of multiple papillae in some follicles resulting in multiple fibres, and a lack of distinct papillae corresponding to no fibre production, emphasised the crucial role of the dermal papilla in hair growth. More recently, dermal sheath dissected from the scalp of a male, was shown to induce follicle formation in the forearm of a female recipient (Reynolds et al., 1999). The nature of the fibre produced was markedly different from the native hairs, providing evidence that the dermal component of the follicle determines the size and type of the hair follicle.

Experiments with cultured dermal sheath cells have been less successful in inducing follicle formation. However, combining dermal sheath cells with germinative epithelial (GE) cells prior to implantation resulted in follicle formation (Reynolds & Jahoda, 1996). The effect was the same on high passage dermal papilla cells that had lost their inductive capacity. The fact that skin fibroblasts were incapable of follicle induction with or without the presence of GE cells demonstrated that follicular dermal cells were receptive to the influence of the GE cells, and it was not a universal influence on dermal cells. The maintenance of the inductive capacity, and the acceptance of epithelial instruction to induce follicle formation supports the idea that follicular dermal cells retain embryonic properties in adulthood.

When follicle formation is induced in adult epithelial tissue, the implanted dermal papilla is believed to give rise to the entire dermal component of the follicle, including

the dermal sheath (Reynolds & Jahoda, 1992). This implies a degree of multipotency in cells of the dermal papilla or at least a capacity to switch phenotype. Similarly, non-follicular epithelial cells demonstrate the ability to produce cells of a follicular lineage as a result of induction by dermal papillae (Oliver, 1970). The transition from one cell type to another is a process that requires investigation as the steps involved may provide important information regarding the lineage of cells and their developmental background. The transition between dermal papilla and dermal sheath cells, which appears to proceed in either direction according to which tissue is inducing follicle formation, provides an opportunity to investigate the relationship between these cell types which both appear to retain embryonic properties.

3.1.4. Follicle regeneration

Pioneering studies by Oliver in the 1960s demonstrated not only the inductive properties of the dermal papilla, but also the regenerative capacity of vibrissa follicles after a major insult. Removal of the dermal papilla or the lower third of the follicle resulted in the regeneration of these structures from the remaining follicular components, dermal sheath being the proposed source of the regenerated dermal papilla (Oliver, 1966a, 1966b). The idea that such regenerative properties were common to all hair follicles was supported by studies on terminal human hair follicles (Jahoda et al., 1996).

Amputation is an extreme insult to the hair follicle. Regeneration after less severe injury has also been investigated and revealed that loss of dermal papilla cells was rectified by recruitment of cells from the "local lower mesenchyme" (Jahoda & Oliver, 1984c). This again reflects the events seen in amputated follicles where the dermal papilla is believed to be reformed from lower dermal sheath cells (Oliver, 1966b; Jahoda et al., 1992a). The fact that dermal sheath is capable of producing dermal papilla

tissue is evident from the inductive studies described previously (Horne et al., 1986; Reynolds et al., 1999).

The vibrissa follicle will only regenerate if amputation is restricted to the lower third of the follicle. This has been attributed to properties of the lower dermal sheath that may be lacking in the upper two thirds. One known difference is the expression of α-smooth muscle actin which is evident in the lower third of the dermal sheath but absent from the upper part of the follicle. Investigators have used this lower portion of the tissue for follicle induction and this is also the portion generally used for cell culture experiments. Horne observed a behavioural difference *in vitro* between the cells of the lower third, which displayed a degree of aggregation, and those of the upper two thirds, which gave no indication of aggregative behaviour. This aggregative property is believed to be crucial in the induction and regeneration of hair follicles (Jahoda & Oliver, 1984a; Jahoda & Oliver, 1984c; Horne et al., 1986).

Unlike limb or fin regeneration in amphibians and fish, there is no distinct blastema in the regenerating vibrissa follicle. However, the principle of an undifferentiated mass of cells at the site of amputation that interact with epithelial cells to result in successful regeneration, is much the same. The source of the undifferentiated cells in the vibrissa follicle is open to question, the dermal sheath having been thought of as the origin of the new dermal papilla cells (Oliver, 1966b; Jahoda et al., 1992a), but there is also the possibility that the mesenchymal tissue around the follicle contributes to the regenerative process as well. Determining the exact origin of the newly formed tissues in the regenerated end bulb is fundamental to understanding the mechanisms involved in follicle regeneration.

3.1.5. Follicular epithelial cell regeneration

During follicle regeneration, the remodelling of the dermal components is accompanied by significant changes in the remaining epithelial component, the outer root sheath. The epithelial cells of the hair follicle have significant regenerative capabilities. These are evident both in the normal growth cycle, where proliferation and differentiation are continually occuring to generate a new hair fibre, and in a wound healing situation, whether this be end bulb amputation or an insult to the interfollicular skin. After experimental removal of the end bulb and the hair fibre, layers of outer root sheath cells are left *in situ*. These are thought to proliferate, to produce the epithelial cell population which will initially plug the hole created by removal of the fibre, and subsequently interact with dermal components to generate a new matrix and hair fibre (Oliver, 1966b; Jahoda et al., 1992a). If the interfollicular epidermis is mechanically separated from the dermis, hair follicle epithelial cells migrate from the upper region of the follicle out onto the dermis and regenerate an epidermis (Krawczyk, 1971; Pang et al., 1978). Outer root sheath cells therefore exhibit a degree of multipotency that is exploited in response to injury.

Lenoir et al. (1988) developed a method in which hair follicles were implanted vertically in a dermal substrate thus mimicking the *in vivo* conditions produced at the donor site of split-skin surgical grafts. From the outer root sheath cells of these follicles, a stratified epidermis was successfully generated *in vitro*. ORS cells have also generated a stratified epithelium when cocultured with dermal fibroblasts, which was comparable to that generated by normal epidermal keratinocytes in the same experimental conditions (Limat et al., 1991). The regenerative qualities of epithelial cells therefore, include considerable proliferative potential as well as multipotency that results in contributions to the epidermis as well as differentiated hair fibre.

3.1.6. Epithelial stem cells of the hair follicle

The proliferative capacity and multipotency displayed by follicular epithelial cells indicates the presence of a stem cell population within this tissue. The isolation of these stem cells could prove extremely useful as the hair follicle is a repository for stem cells that can contribute to the interfollicular epidermis as well as the epithelial components of the pilosebaceous unit (Taylor et al., 2000). Rochat et al (1994) determined that a single follicular keratinocyte colony-forming (stem) cell had the proliferative capacity to renew the epidermis of the whole human body. Such properties mean research into these cells is potentially relevant to clinical applications, ranging from tumour biology and gene therapy to skin replacement treatments (Lenoir et al., 1988; Cotsarelis et al., 1990; Lavker et al., 1993; Miller et al., 1993; Li & Hoffman, 1995; Jahoda & Reynolds, 2000). In the search for follicular stem cells two potential sources have been identified: a subpopulation of outer root sheath cells in the bulge, (an anatomically distinct area in rodent pelage follicles) situated at the arrector pili muscle attachment site; and the germinative epithelial cells, a population of cells at the base of the follicle bulb. The research into these two putative stem cell populations is outlined below.

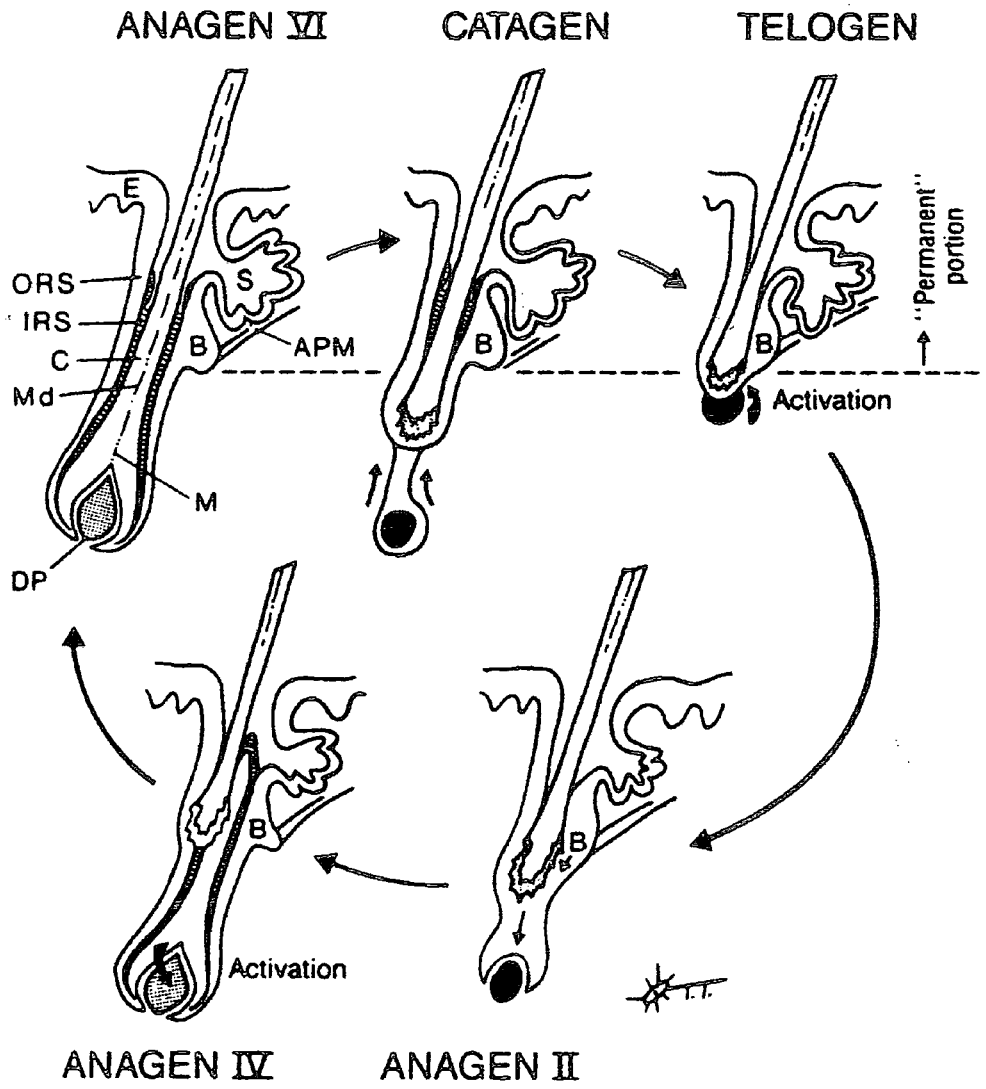
3.1.6.1. Stem cells in the follicle bulge

Cotsarelis et al. (1990) identified a slow-cycling population of outer root sheath cells in the bulge, by their capacity to retain a radio-isotopic label, and proposed that these cells constituted the stem cell population of the hair follicle. The bulge is at the level of the most proximal part of the permanent upper follicle during the growth cycle, and the fact that it remains *in situ* after plucking of the hair fibre, supports the proposal that epithelial stem cells of the follicle reside in this section of the ORS rather than the temporary portion of the follicle. Having identified the location of the putative stem cells, Cotsarelis et al. (1990) then went on to propose 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 (Figure 3.1).

Further evidence that stem cells reside in the bulge was gained from studies of neonatal follicles and interfollicular epidermis. Label-retaining cells which originated in the follicle bulge, contributed to the neonatal interfollicular epidermis as well as to the components of the temporary portion of the pelage follicle. The same cells were also observed to migrate into the adult interfollicular epidermis as part of the wound healing process (Taylor et al., 2000). Whilst it has long been known that outer root sheath cells contribute to reepithelialisation in wounded skin, this was the first demonstration that such cells originate from the bulge of the follicle.

Figure 3.1. The bulge activation hypothesis.
The bulge (B) and structures distal to it comprise the permanent portion of the follicle during the pelage follicle cycle. During catagen the lower portion of the follicle degenerates and the dermal papilla (DP) regresses to rest below the follicle bulge during telogen. This close association acitvates the epithelial cells of the bulge resulting in the initiation of anagen. This hypothesis also proposes that the growing epidermal matrix activates the dermal papilla in early anagen.
ORS - outer root sheath, IRS - inner root sheath, C - cortex, Md - medulla, M - matrix, S - sebaceous gland, E - epidermis, APM - arrector pili muscle.
Cotsarelis et al., 1990.



3.1.6.2. Stem cells in the follicle end bulb

The germinative epithelial (GE) 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. Their ultrastructural profile is decidedly stem cell-like with a large nuclear-cytoplasmic ratio and with minimum organelles visible in the cytoplasm, reflecting a slow metabolic rate (Reynolds & Jahoda, 1991). As well as being multipotent, the GE cells have the capacity to confer follicle inducing properties onto other cell types (Reynolds & Jahoda, 1996). Evidence of the high proliferative potential in the GE cell population is the observation that repeated plucking of a follicle results in repeated regrowth of a fibre. Gharzi et al. (1999) demonstrated that up to three times the normal length of fibre can be generated by such means, indicating GE cells are capable of proliferating indefinitely and the length of anagen is not regulated by a limited proliferative capacity of GE cells. These cells are protected when a fibre is removed prematurely (plucking) (Gharzi et al., 1999), as are the outer root sheath cells of the bulge. During catagen, the epithelial matrix visibly separates from the GE cells (personal observation, Fig. 2.11 G) indicating that GE cells remain in the epithelial follicular tissue during the catagen and telogen phases of the cycle. These cells therefore display a range of qualities that are common to stem cells: multipotency; high proliferative capacity; protected location; simple cytoplasm; and a high nuclear:cytoplasmic volume ratio.

3.1.6.3. Are stem cells restricted to one location within hair follicles?

There is evidence to support both locations as sites in which follicular epithelial stem cells reside, but there is also evidence to suggest that cells are not restricted to either one or the other. A distinguishing characteristic of stem cells is their colony-

forming ability *in vitro*, demonstrating their high rate of proliferation and capacity for self-renewal. 85% of the cells with clonogenic potential in human hair follicles, which lack an anatomically distinct bulge, were found to be in the lower outer root sheath (Rochat et al., 1994). In contrast, Kobayashi et al. (1993) found 95% of the colony-forming cells in the mid-anagen rodent vibrissa follicle were located in the section that contained the bulge. However, in late catagen vibrissa follicles, colony-forming cells were present in the intermediate section, between the bulge and the end bulb, where none had been observed in the mid-anagen follicle (Kobayashi et al., 1993). This was the first indication that cells migrate from the bulge to the end bulb as stem cells.

Oshima et al. (2001) used the constitutive expression of the *lacZ* reporter gene in Rosa 26 mice, to investigate the migration of cells from the vibrissa follicle bulge. Migration of the cells through the outer root sheath into the end bulb, combined with the changing distribution of clonogenic cells during the growth cycle provided strong evidence that stem cells migrate from the follicle bulge before dividing in the end bulb (Oshima et al., 2001). Other studies have proposed epithelial stem cells exist in multiple compartments in the pilosebaceous unit (Commo et al., 2000; Ghazizadeh & Taichman, 2001). Taking into account all the studies discussed it appears that follicular epithelial stem cells can be found at multiple sites in the hair follicle at any stage in the follicle cycle.

End bulb amputation deprives the regenerating follicle of the germinative epithelial cells as a potential source of epithelial cells. Regeneration of a functional end bulb can be induced by implanting dermal tissue at the base of the follicle in contact with the cut edge of the outer root sheath (Oliver, 1967; Horne & Jahoda, 1992), or the upper two thirds will spontaneously regenerate an end bulb by interactions between the remaining tissues at the site of amputation (Oliver, 1966b; Jahoda et al., 1992a). There have been no indications from previous studies on follicle regeneration, that the cells of

the bulge are specifically activated in the regenerative process. In the early stages of regeneration, cell numbers increase considerably to fill the central cavity. Such a rapid increase in cell numbers is likely to result from stem cell proliferation, thus providing an opportunity for analysing the distribution of epithelial stem cells in the regenerating vibrisaa follicle.

3.1.7. The role of the extracellular matrix in follicle regeneration

Extracellular matrix is known to play an important role in the morphogenesis of many structures that develop as a result of epithelial-mesenchymal interactions, including the hair follicle. The process of follicle regeneration has also been shown to involve considerable changes in the extracellular matrix. Jahoda et al. (1992a) studied the distribution of laminin, fibronectin and collagen type IV during follicle regeneration and, from morphological and biochemical changes, the initial events were described as a wound response. The significant difference to normal healing however, was the lack of scar tissue in the regenerated follicle. This was also true of follicles which had been wounded in the end bulb (Jahoda & Oliver, 1984c), and amputated follicles in which hair fibre growth was induced by implantation of dermal papilla cells (Horne et al., 1986). Significantly the implantation of skin fibroblasts in the same experimental model resulted in the deposition of scar tissue at the level of amputation (Horne et al., 1986). The specialised nature of the ECM produced by follicular dermal cells has been investigated *in vivo* and *in vitro* but the functional significance has only been described in terms of hair growth. Given the extensive role the ECM plays in wound healing (Martin, 1997), a specialised ECM could hold great potential for scar-free wound healing. The regenerating vibrissa follicle provides an excellent model for studying scar-free healing, as well as extracellular matrix remodelling in a morphogenetic process.

3.1.8. Aims

The regenerative properties of vibrissa follicles are well established but many questions remain unanswered (Oliver, 1966a, 1966b; Kobayashi & Nishimura, 1989; Jahoda et al., 1992a). A number of methods were employed in this study to investigate various aspects of the regenerative process. The degree of cell proliferation was investigated by BrdU incorporation, and α-smooth muscle actin, a marker for follicular dermal sheath *in vivo*, was detected by immunostaining. Both these approaches were intended as means to identify the origin of the dermal papilla. Epithelial regeneration was observed and the location of putative stem cells investigated by BrdU incorporation and Jol-4 immunostaining. The expression of a key signalling molecule in epidermal-mesenchymal interactions, Sonic hedgehog, was studied by *in situ* hybridisation to establish whether signalling in follicle regeneration reflected that seen in follicle development. Finally, versican and laminin, were immunolabelled to extend the studies of Jahoda et al. (1992a) on ECM remodelling during regeneration.

3.2. MATERIALS AND METHODS

3.2.1. Surgical procedure for end-bulb amputation

End bulb amputation was performed on up to 10 vibrissa follicles in one mystacial pad of 13 PVG rats of either sex, aged between 3 and 5 months, with the exception of two rats aged 12 months (used for 6d and 8d BrdU treated regenerating follicles). The method was essentially that of Oliver (1966a). Animals were initially sedated with Halothane and then anaesthetized by an intramuscular injection of Hypnorm (80μl) (Janssen Animal Health), immediately followed by an intraperitoneal injection of 75μl Valium (5mg/ml) (Phoenix Pharmaceuticals). The vibrissa follicles were then exposed by cutting an L-shaped incision around the anterior and ventral edges of the mystacial pad and, using fine forceps and spring scissors, cutting away the connective tissue to expose the end bulbs. Individual vibrissa follicles had the end bulb amputated and discarded before the vibrissae (club and growing fibres) were plucked from the follicle. The mystacial pad was then sutured back in place and the rat left to recover for a specific length of time before the regenerating follicles were biopsied.

The majority of follicles were in mid-late anagen at the time of amputation. However, in two rats the amputated follicles were in the very early stages of anagen and consequently the fine growing fibres were not always successfully removed. This was evident when the follicles were sectioned and these follicles were excluded from the results data.

3.2.2. Freezing down regenerating follicles

Follicles were dissected out of the mystacial pad of the rat 1, 2, 4, 6, 8, 10 or 12 days after the amputation surgery. They were either BrdU treated (see 3.2.3.1) prior to embedding or immediately embedded individually in TissueTek O.C.T. compound

(Agar Aids), snap frozen in liquid nitrogen and stored at -80°C prior to being sectioned for immunohistochemistry or *in situ* hybridisation.

3.2.3. Assessing cell proliferation

3.2.3.1. BrdU treating regenerating follicles

Follicles were dissected out of the mystacial pad of the rat 1, 2, 4, 6 or 8 days after the amputation surgery. They were immediately placed in 10μM BrdU in pre-warmed MEM and incubated at 37°C for 2 hours. This was followed by a 20min PBS wash at 37°C and the follicles were then individually embedded as described above.

3.2.3.2. Immunological detection of BrdU incorporation

6μm frozen sections were cut on a Leica CM3050 cryostat, and thaw-mounted on to poly-lysine coated slides. They were fixed in ethanol fixative (70ml absolute ethanol made up to 100ml with 50mM glycine, pH2) at -20°C for 20min followed by three washes in PBS. The sections were incubated in 10% lamb serum in PBS for 30min to block non-specific binding. A monoclonal antibody against BrdU was diluted (1:10), and a polyclonal antibody to laminin was diluted (1:40) in PBS for double-labelling of sections. The diluted primary antibody mix was applied to the sections and incubated at 37°C for 1hr. Unbound antibody was removed with four PBS washes before application of the secondary antibody mix (AlexaFluor 488 anti-mouse and AlexaFluor 546 anti-rabbit (Molecular Probes), both diluted 1:100 in PBS) in which the sections were incubated for 45min. Unbound antibody was removed as before and the sections were mounted in mowiol 4-88 (Calbiochem), containing an anti-fade agent, under glass coverslips.

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

3.2.4. Immunostaining

6μm frozen sections were cut, as described above. Sections were washed three times in PBS before application of the primary antibodies diluted in filter-sterilised PBS (see Table 3.1 for antibodies and dilution factors). The slides were then incubated for 1 hour at room temperature, or overnight at 4°C and the unbound primary antibody removed with four washes in PBS. Secondary antibodies (AlexaFluor 488 anti-mouse and AlexaFluor 546 anti-rabbit (Molecular Probes), both diluted 1:100 in PBS), were then applied and incubated for 30min at room temperature in the dark. Unbound antibody was removed with four PBS washes and sections were mounted in mowiol under glass coverslips.

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

Table 3.1. Details of primary antibodies, the source from which they were obtained, and dilution factor for immunostaining. All antibodies were monoclonal except NF200 & L9393.

Antigen	Host	Antibody	Dilution Factor	Source	Reference
Lamin A	mouse	Jol4	1:10	Serotec	Dyer et al., 1997
BrdU	mouse	anti-BrdU	1:10	Boehringer	
α-sma	mouse	anti - αsma	1:10	Prof. Gabbiani, Geneva University	Skalli et al., 1986
Versican	mouse	12C5	1:5	DSHB	Asher et al., 1991
neurofilament	rabbit	NF200	1:100	Sigma	
laminin	rabbit	L9393	1:40	Sigma	

Table 3.2. Numbers of regenerating follicles biopsied, at a range of time points after surgery, and immunostained with a panel of antibodies or hybridised with a Shh riboprobe.

Marker	1d	2d	4d	6d	8d	10d	12d
Jol4	5	2	1	2	2		5
BrdU	4	1	2	2	1		
Versican	2	1	2	1	1		1
α-sma			1				1
NF200							1
Laminin	5	1	2	2	2		5
Shh	1		2*[1]		1*	2	3

* no anagen positive control

3.2.5. Detection of *Shh* transcripts

3.2.5.1. Sonic hedgehog construct for riboprobe synthesis

1.6kb of human sonic hedgehog (Shh) cDNA inserted into the multi-cloning site (EcoRI) of pBluescript SK(+) was used as the template for synthesis of sense and antisense Shh riboprobes. This construct was a generous gift from Paul Hunt, Durham University, who received the construct from Colin Tabin, Harvard Medical School, Boston, U. S. A..

3.2.5.2. Synthesis of DIG-labelled riboprobes

Shh plasmid DNA was isolated from 1.5ml liquid culture of bacteria using a Qiagen miniprep kit according to the manufacturer's instructions.

Plasmid DNA was linearised with BamHI for the sense probe, or with XhoI for the antisense probe, and an aliquot electrophoresed on a 1.2% agarose gel to confirm that the digestion was complete.

Riboprobes were prepared by transcription of 0.5-1.0μg linearised plasmid (see above) in a 20μl reaction containing transcription buffer (Promega), 10mM DTT (Promega), 2μl nucleotide mix containing Digoxygenin (DIG)-labelled UTP (Boehringer), 0.5μl (20U) placental Rnase inhibitor (RNaseOUT, Gibco) and 1μl (10-20U) RNA polymerase (T7 for sense or T3 for antisense) (Promega). The reaction was incubated at 37°C for 2 hours.

A 1μl aliquot was run on a 1% agarose gel to determine the efficiency of the transcription. The remaining probe was treated with 2μl Dnase I (Rnase free) (Promega) and incubated at 37°C for a further 15min. 100μl DEPC-treated H_2O, 10μl 4M LiCl and 300μl 100% ethanol were then added and the reaction incubated at -20°C for 30min.

The precipitated RNA was then spun down for 10min at 14,000rpm, the supernatant removed and the pellet washed in 75% ethanol/25% DEPC-treated H_2O. The pellet was then air-dried before being resuspended in 30µl DEPC-treated H_2O. The optical density at 260nm was then read on a spectrophotometer and the RNA diluted to a final concentration of 0.1µg/µl before storing at -20°C.

3.2.5.3. *In situ* hybridisation and washing

Method based on that of Wilkinson (1992).

All incubations were done at room temperature unless otherwise stated and all solutions were DEPC-treated (or made up with DEPC-treated dH_2O in the case of Tris buffers).

6µm cryostat sections were cut and thaw-mounted onto RNase-free Polysine slides (BDH). The sections were immediately fixed in 4% paraformaldehyde in PBS for 20min. They were washed (3 x 5min) in PBT (DEPC-treated PBS with 0.1% Tween-20) before bleaching with 6% hydrogen peroxide in PBT for 1 hour. Three x 5min PBT washes were followed by treatment with Proteinase K (10µg/ml for 15min) then another 5min PBT wash before post-fixing the sections in 0.2% glutaraldehyde / 4% paraformaldehyde in PBS for 20min. The sections were then washed twice (5min each) in PBS before dehydrating them with 5min incubations in the following series of solutions (all made up with DEPC-treated H_2O): 0.83% NaCl; 25% methanol; 50% methanol; 75% methanol; 100% methanol. After a final 5min incubation in 100% methanol the slides were allowed to air-dry.

Hybridisation buffer was made up and the riboprobes diluted to 3ng/µl in hybridisation buffer (50% formamide, 1% SDS, 5x SSC (pH 5.0), 50µg/ml heparin, 50µg/ml yeast RNA) before applying 100µl of the relevant probe to each slide.

Sureseal™ frames (Hybaid) were used to prevent subsequent evaporation of the probe. Slides were placed on a flat rack within a humidity chamber and incubated overnight at 65°C.

The Sureseal™ frames were removed with a razor blade and the slides immediately placed in solution 1 (50% formamide, 5x SSC, 1% SDS warmed to 60°C) for 2 x 30min washes at 60°C. This was followed by 3 x 30min washes in solution 2 (50% formamide, 2x SSC warmed to 60°C) at 60°C. The slides were washed twice (10min each) in TBST followed by a 30min wash in TBST with 30mM levamisole (Sigma). Slides were now ready for detection of the DIG-labeled probe.

3.2.5.4. Preparation of Anti-DIG antibody

A small amount of follicle powder (prepared as described in Appendix I) was added to 1ml TBST (0.29M NaCl, 5.6mM KCl, 25mM Tris Hcl (pH 7.5), 0.1% Tween-20 in DEPC-treated H_2O) and incubated at 65°C for 1 hour. 50µl lamb serum (Gibco) plus 20µl anti-DIG-alkaline phosphatase antibody (Boehringer Mannheim) were added to the follicle powder mix which was then agitated at 4°C for 1 hour. The follicle powder was then pelleted by centrifugation at 14,000rpm for 2min and the supernatant containing the pre-adsorbed antibody (approximately 1ml) was decanted off.

3.2.5.5. Immunological detection of DIG-labelled probe

1ml pre-adsorbed antibody was diluted in 99ml TBST plus 1ml lamb serum. The slides were immersed in this solution and incubated at 4°C overnight. Unbound antibody was removed with 3 x 20min PBT washes followed by 2 x 30min NTMT (100mM Tris Hcl (pH 9.5), 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween-20) washes. A subsequent 30min NTMT wash included 2mM levamisole. Finally the colour substrate

was prepared by adding 11.25µl 30mg/ml nitro blue tetrazolium (NBT) plus 8.75µl 20mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) to 1ml NTMT. The slides were placed on a flat rack and 500µl colour substrate added per slide. They were then placed in the dark, in a humidity chamber, and incubated at room temperature for 1hr to 3 days with regular checks for colour change. The slides were sometimes incubated in NTMT overnight and fresh colour substrate added in the morning to enable better control of the development of background colour. When colour had developed to a sufficient level slides were washed twice in NTMT before being mounted under glass coverslips in gelatin glycerol (Sigma).

A sense probe synthesised from the same plasmid was used as a negative control, whilst mid-anagen vibrissa follicles from rats which had not been subjected to surgery were used as positive controls in the majority of experiments.

3.3. RESULTS

3.3.1. General observations

Follicle regeneration proceeded as described by Oliver (1966b) and Jahoda et al. (1992a), with no significant differences observed in the majority of samples. Variation in the rate of regeneration was previously noted by Jahoda et al. (1992a) who described the regeneration process in stages, identified by morphology, to clarify the description of results (Figure 3.2). These stages will be used to categorize the results presented here with one additional substage. The first stage in follicle regeneration results in epithelial 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 (Figure 3.2).

Table 3.3. Numbers of follicles, at different stages of regeneration, immunolabelled with a panel of antibodies.

Laminin was also labelled in follicles of all stages included in this data set.

	Jol-4	BrdU	Versican	α-sma
Pre-stage 1	4	4	2	
Stage 1	4	2	2	
Stage 2	3	2	1	1
Stage 3	4	2	2	
Stage 4	2		1	1

Figure 3.2. Key stages in lower follicle regeneration.

Stage 0: immediately after end bulb amputation the fibre is removed resulting in a hollow cylinder of epithelial tissue within the glassy membrane.

Pre-stage 1: the cavity caused by removal of the hair fibre is still considerable and the glassy membrane is distorted/irregular in shape at the site of amputation.

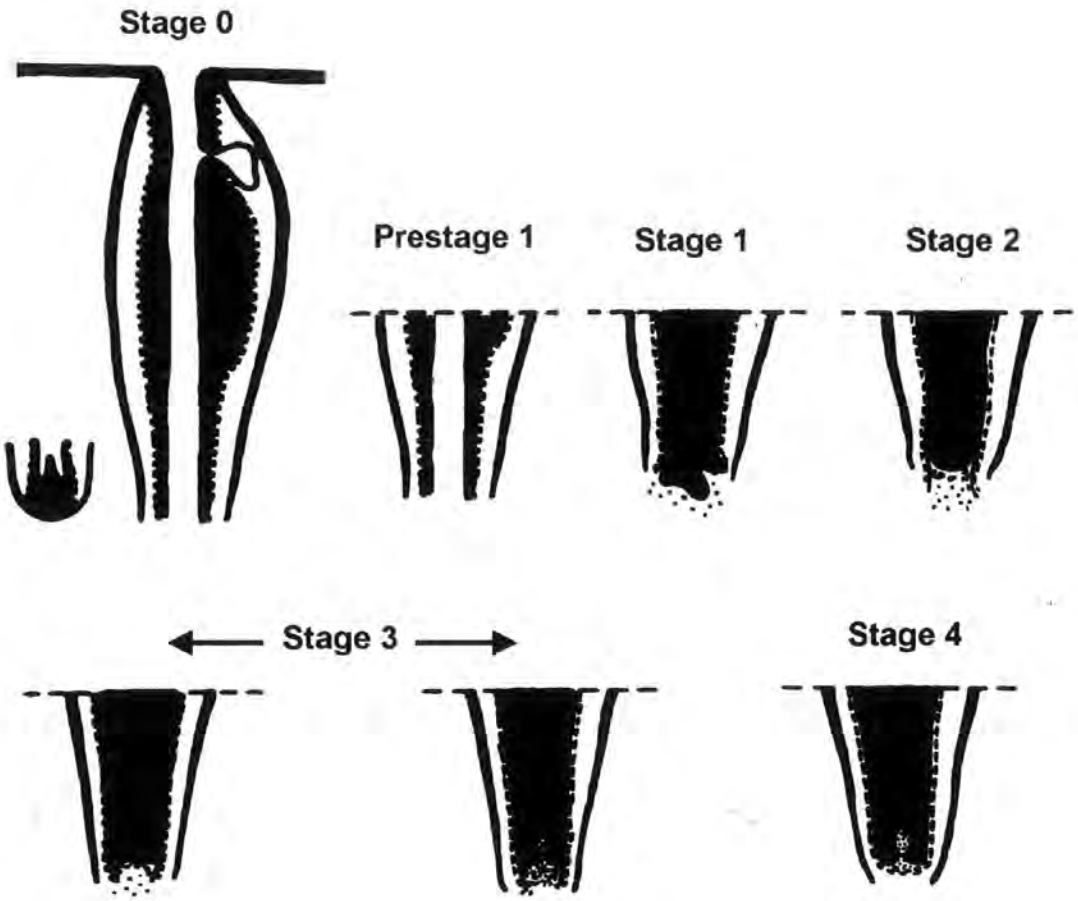
Stage 1: the epithelial cells fill the cavity and extend beyond the glassy membrane and the level of the original amputation in an irregular fashion. Dermal sheath cells accumulate around the base of the follicle.

Stage 2: the epithelial column evens out at its base and is enclosed within the extended glassy membrane along with dermal sheath cells that are accumulating below the epithelium.

Stage 3: the dermal cells enclosed by the extended glassy membrane indent the base of the epidermal column, this extends with time resulting in papilla formation.

Stage 4: the new end bulb develops further as the dermal papilla increases in size, hair differentiation occurs and the glassy membrane disappears from beneath the end bulb.

Adapted from Jahoda et al., 1992a.


Stage 0
Prestage 1
Stage 1
Stage 2
Stage 3
Stage 4

3.3.2. Determining areas of cell proliferation

PRE-STAGE 1

All follicles at pre-stage 1 had been biopsied 24 hours after the amputation of the end bulbs. 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 within the proximal end of the follicle (Fig. 3.3 A,B,D). Cells were occasionally present within the most proximal area of the cavity and these included a minority of cells that had incorporated BrdU (Fig. 3.3 D) whilst the majority were labelled with Jol-4 antibody (Fig. 3.3 B). Distal to this, the basal layer of the outer root sheath was adherent to the glassy membrane and this single cell layer was labelled with Jol-4 antibody (Fig. 3.3 A), whilst a minority of cells had incorporated BrdU (Fig. 3.3 D). These proliferating cells were spaced along the basal layer at fairly regular intervals. The tissue became less disrupted towards the distal end of the follicle and the layers of ORS cells increased in number. As they did so it was evident that BrdU incorporation had occurred within the suprabasal layers of this tissue as well as the basal layer (Fig. 3.3 D). The vast majority of cells however were determined to be non-dividing by their immunoreactivity to the Jol-4 antibody (Fig, 3.3 A).

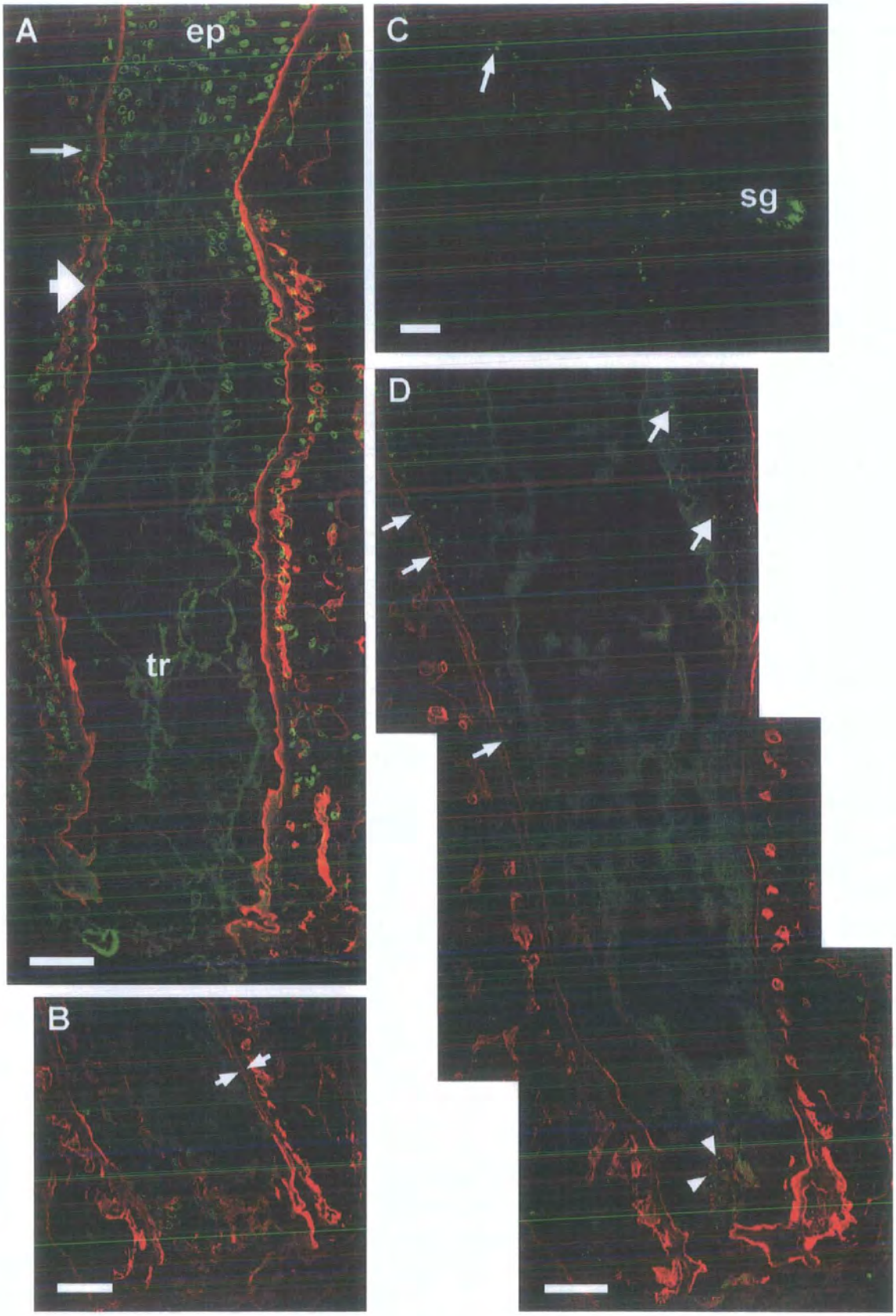
BrdU had also been incorporated into the nucleus of epithelial cells at the neck of the follicle. In one follicle in particular there was a distinct line of labelled cells running from the skin epidermis into the follicle infundibulum and down to the level of the bulge (Fig. 3.3 C). Cells of the sebaceous gland had also incorporated BrdU. In other follicles there was not such a distinctive line, but individual cells within the basal epithelial layers at the neck of the follicle were positively marked. The nuclear staining here was distinctly different in appearance to that seen in the lower, regenerating part of the follicle. Staining in the cells at the neck revealed considerably larger nuclei than were seen in the BrdU labelled cells in the proximal portion of the follicle.

Figure 3.3. Cell proliferation and cycle activity in pre-stage 1 regenerating vibrissa follicles.

Pre-stage 1 follicles immunostained with monoclonal Jol-4 antibody (A, B) or a monoclonal antibody against BrdU (C,D) (green fluorescence) and, with the exception of C, a polyclonal antibody against laminin (red fluorescence).

(A) Jol-4 staining highlights the nuclear envelope of cells in the dermal sheath (small arrow) and basal and suprabasal epithelial (ep) cells in the distal half of the follicle. The dermal and epithelial compartments are separated by the glassy membrane (large arrow). The proximal part of the follicle still contains tissue remnants (tr) from the removal of the hair fibre. (B) Close to the site of amputation a small number of epithelial cells are stained with the Jol-4 antibody but Jol-4 staining in the dermal sheath is minimal. The glassy membrane is indicated by arrows. (C) BrdU is incorporated by epithelial cells in the upper follicle in the infundibulum, the sebaceous gland (sg) and in the cells at the point where folicular outer root sheath is continuous with epidermis (arrows). (D) In the proximal follicle proliferation is occurring in a sub-population of basal (thin arrows) and suprabasal (wide arrows) epithelial cells. In the small cluster of epithelial cells at the site of amputation there are cells that have incorporated BrdU (arrowheads).

Scale bar = A, B, D = 50μm, C = 100μm.


A
ep
tr
B
C
sg
D

Dermal sheath cells were stained with Jol-4 antibody, although the most proximal section of the follicle had noticably fewer labelled cells than the rest of the dermal sheath, indicating a subpopulation of cells from this tissue had entered the cell cycle (Fig. 3.3 A, B). Jol-4 staining was evident in cells within the collagen capsule and in the loose mesenchymal tissue of the follicle sinus complex. There was little or no evidence of BrdU incorporation in the dermal tissue (Fig. 3.3 D).

STAGE 1

The epithelial cells formed a solid column filling the cavity that had been evident in pre-stage 1 follicles (Figure 3.4). At the base of the follicle, a deposition of laminin delineated the border between the epithelial compartment and the surrounding dermal tissue. Within the basal layer of epithelial cells BrdU incoporation was evident at fairly regular intervals throughout the lower follicle. The column of epithelial cells displayed a high level of Jol-4 staining in all areas except the proximal part of the early stage 1 follicle (Fig. 3.4 A). Dermal cells in close proximity to the laminin deposition were Jol-4 immunoreactive (arrows, Fig. 3.4 B). The nuclei indicated these cells displayed polarity, orientated perpendicular to the long axis of the follicle. At the neck of the follicle Jol-4 staining highlighted cells in the suprabasal layers only (Fig. 3.4 C).

The epithelial column extended beyond the level of the glassy membrane during stage 1 of regeneration and as it did so Jol-4 staining became evident in cells throughout this compartment (Fig. 3.4 D). During this stage a few cells were weakly labelled with BrdU in the proximal region of the epithelial compartment, and in the loose dermal tissue at the base of the follicle (Fig. 3.4 E). Very occasionally a dermal sheath cell was labelled but overall the level of proliferation was very low. This was emphasised by the high level of Jol-4 staining in the dermal tissue, including the dermal sheath, as well as in the epithelial column.

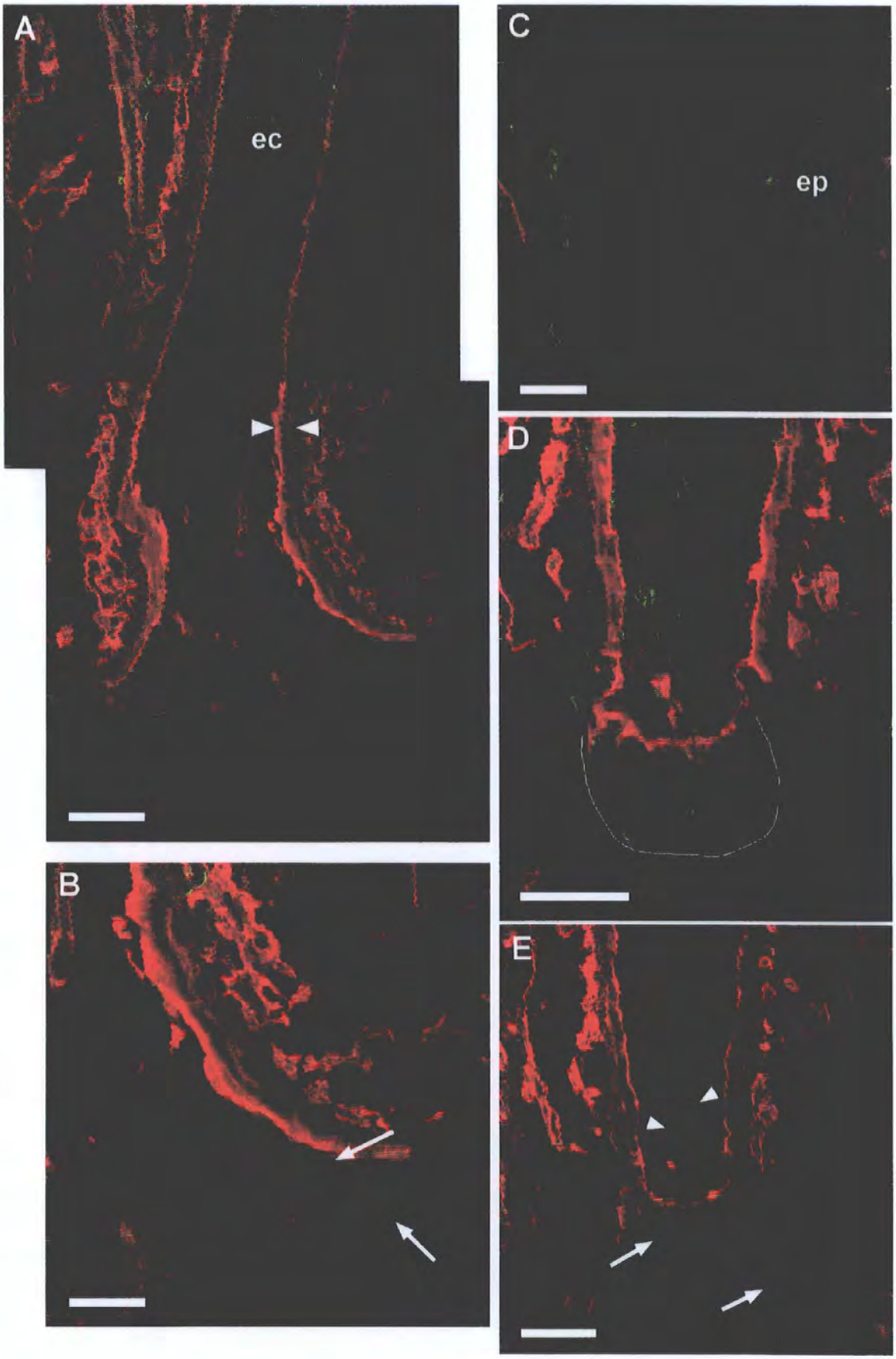
Figure 3.4. Cell cycle activity and proliferation in stage 1 regenerating vibrissa follicles.

Stage 1 follicles immunostained with Jol-4 (A - D) or a monoclonal antibody against BrdU (E) (green fluorescence), and a polyclonal anti-laminin antibody (red fluorescence).

(A) Jol-4 staining highlights cell nuclei in the distal epithelial column (ec) of early stage 1 follicles. Arrowheads mark the glassy membrane. (B) Dermal cells (arrows) in close proximity to the laminin deposit at the base of the epithelial column are labelled with Jol-4. (C) Jol-4 staining in the epidermal cells (ep) at the follicle neck highlights the stratified nature of the epidermis with non-dividing cells featuring in the uppermost layers only. (D) As the epithelial cells extend beyond the glassy membrane, Jol-4 staining is evident throughout the epithelial cell population, and Jol-4 stained dermal cells accumulate (cluster of cells is outlined) around the base of the epithelial column.

(E) BrdU staining is weak but incorporation is evident in proximal epithelial cells (arrowheads) and a few dermal cells (arrows) scattered at the base of the follicle.

Scale bar: A, C-E = 50μm, B = 25μm.


A
ec
C
ep
D
B
E

In particular Jol-4 staining showed a cluster of dermal cells at the base of the epithelial column that was reminiscent of the dermal aggregation seen in follicular development (Fig. 3.4 D). Cells within the surrounding mesenchymal tissue were also frequently labelled with the Jol-4 antibody.

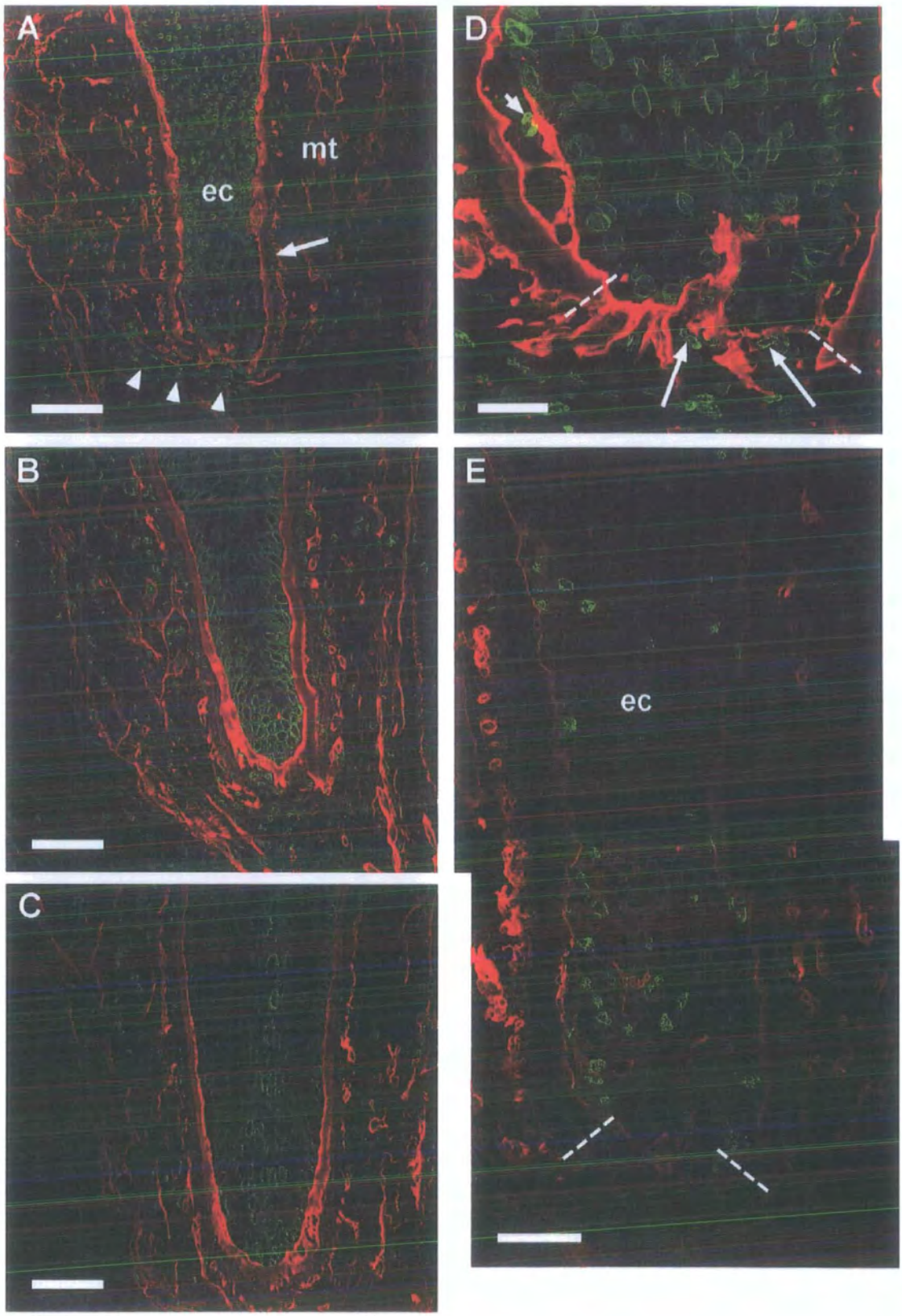
STAGE 2

The base of the epithelial column had retreated to within the extended glassy membrane of stage 2 follicles. At the cut base of the follicle, a laminin-containing basal layer of extracellular matrix separated the dermal and epithelial compartments (Figure. 3.5) as in stage 1. The epithelial compartment contained a large number of Jol-4-labelled non-dividing cells throughout, including along the length of the basal layer (Fig. 3.5 A,B). In one follicle however, a central column of cells exhibited a slightly different staining pattern that separated them from the surrounding suprabasal layers of epithelial cells (Fig. 3.5 B,C). The sections of this follicle were slightly oblique with that shown in Fig. 3.5 B being more midline than that in Fig. 3.5 C. It appears this follicle may be at a slightly later stage of regeneration than the follicle in Fig. 3.5 A. This is evident from the more organised arrangement of the cells. The shape of the nuclei, highlighted by Jol-4 staining, indicates that the cells are arranged radiating from a central column of cells aligned with the long axis of the follicle (Fig. 3.5 B,C). This is in contrast to the random arrangement of nuclei seen at a slightly earlier stage (Fig. 3.5 A,D).

BrdU incorporation was evident at regular intervals in the basal layer along the entire length of the follicle. In the most proximal part of the column, this was more pronounced and included all cell layers, so forming a plug of proliferating cells at the open base of the follicle (Fig. 3.5 E). Proliferation was also evident in a few dermal cells positioned centrally below the epithelial column and laterally

Figure 3.5. Jol-4 staining and BrdU incorporation in stage 2 regenerating follicles.

Stage 2 follicles immunostained with Jol-4 (A-D) or a monoclonal anti-BrdU antibody (E) (green fluorescence) and a polyclonal anti-laminin antibody (red fluorescence). (A) Jol-4 immunoreactive cells occupied all areas of the epithelial column (ec) and were evident throughout the dermal sheath (arrow) and loose mesenchymal tissue (mt). Jol-4 immunoreactive dermal cells were evident as a strip of cells (arrowheads) extending laterally from the dermal aggregation at the base of the epithelial column.
B and C show slightly oblique sections (B is the closest to the midline) of one follicle in which the shape of the epithelial cell nuclei indicated cells were becoming organised, with a central column developing within the epithelial compartment. (D) Dermal cells stained with Jol-4 (long arrows) were very closely associated with the laminin that separated the epithelial and dermal compartments across the cut end of the glassy membrane (indicated by dashed lines). The short arrow indicates a Jol-4 stained dermal cell between the inner aspect of the glassy membrane and the epithelial column. (E) BrdU incorporation was evident in cells at the proximal end of the epithelial column (ec) and in the basal epithelial layer in the more distal part of the follicle. Dashed lines indicate the end of the glassy membrane.
Scale bar: A = 100μm, B, C, E = 50μm, D = 25μm.


A
mt
ec
D
B
E
ec
C

within the loose mesenchymal tissue. These cells were frequently associated with laminin expression and appeared to be within vasculature. The dermal sheath cells lateral to the glassy membrane were strongly stained by the Jol-4 antibody (Fig. 3.5 A,B) as were dermal cells within the confines of the glassy membrane below the epithelial column (Fig. 3.5 A,B,D). Jol-4 staining highlighted a strip of mesenchymal cells extending across the base of the follicle from one side of the severed capsule to the other (Fig. 3.5 A). This was more evident in some follicles than others.

STAGE 3

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. BrdU staining revealled proliferating cells at regular intervals in the basal layer of the ORS along the length of the glassy membrane (Fig. 3.6 A). At the proximal end of the follicle the BrdU labelled epithelial cells were more numerous around the dermal indentation of the laminin-positive basement membrane (Fig. 3.6 A).

The majority of dermal cells that surrounded the base of the epithelial column were mitotically quiescent. Only one cell in this area was observed to have incorporated BrdU, in two follicles. An occasional cell in the loose mesenchymal tissue lining the follicle capsule had incorporated BrdU (Fig. 3.6 A) as had cells within the vasculature at the very base between the ends of the regenerating capsule.

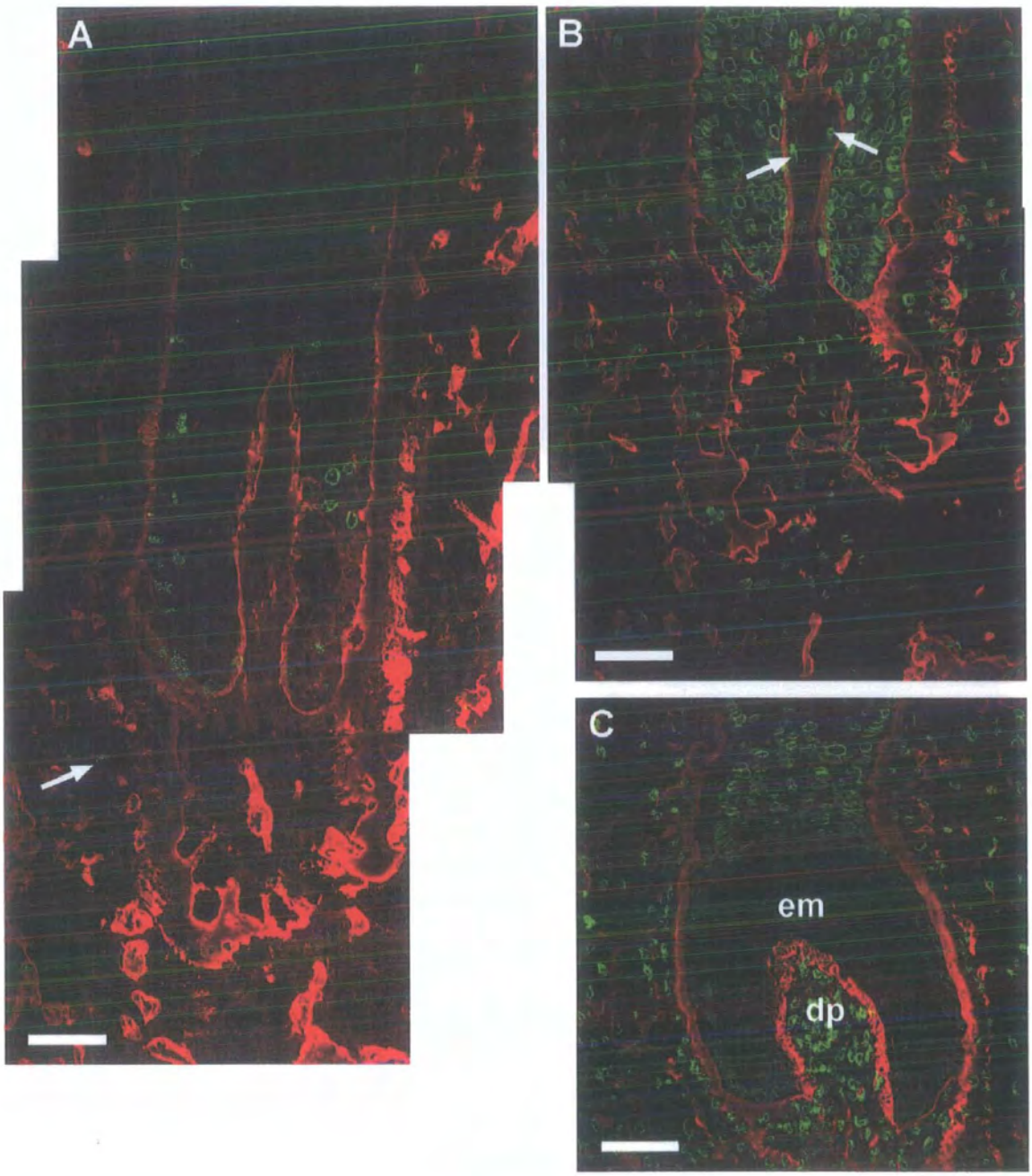
Staining with the Jol-4 antibody highlighted the nuclei of the vast majority of epithelial cells in the regenerating follicle as well as the lower dermal sheath cells (Fig. 3.6 B). Dermal cells within the confines of the extended glassy membrane and in the presumptive dermal papilla were also Jol-4 immunoreactive (Fig. 3.6 B).

Figure 3.6. Cell proliferation and dermal papilla formation during stages 3 and 4 of vibrissa follicle regeneration.

Stage 3 (A, B) and stage 4 (C) follicles immunostained with a monoclonal antibody against BrdU (A) or Jol-4 (B, C) (green fluorescence), and a polyclonal anti-laminin antibody (red fluorescence).

(A) BrdU incorporation was detected in the proximal epithelial cells and in the basal epithelial layer along the length of the follicle. Individual dermal cells were occasionally labelled with BrdU (arrow). (B) At stage 3 Jol-4 staining highlighted the nuclei of cells throughout the epithelial compartment and dermal sheath cells, including those within the area enclosed by the extended glassy membrane (arrows). (C) Once an end bulb had formed Jol-4 labelling was lost from the epithelial cells in the new follicle matrix (em) around the dermal papilla (dp).

Scale bar = 50μm.

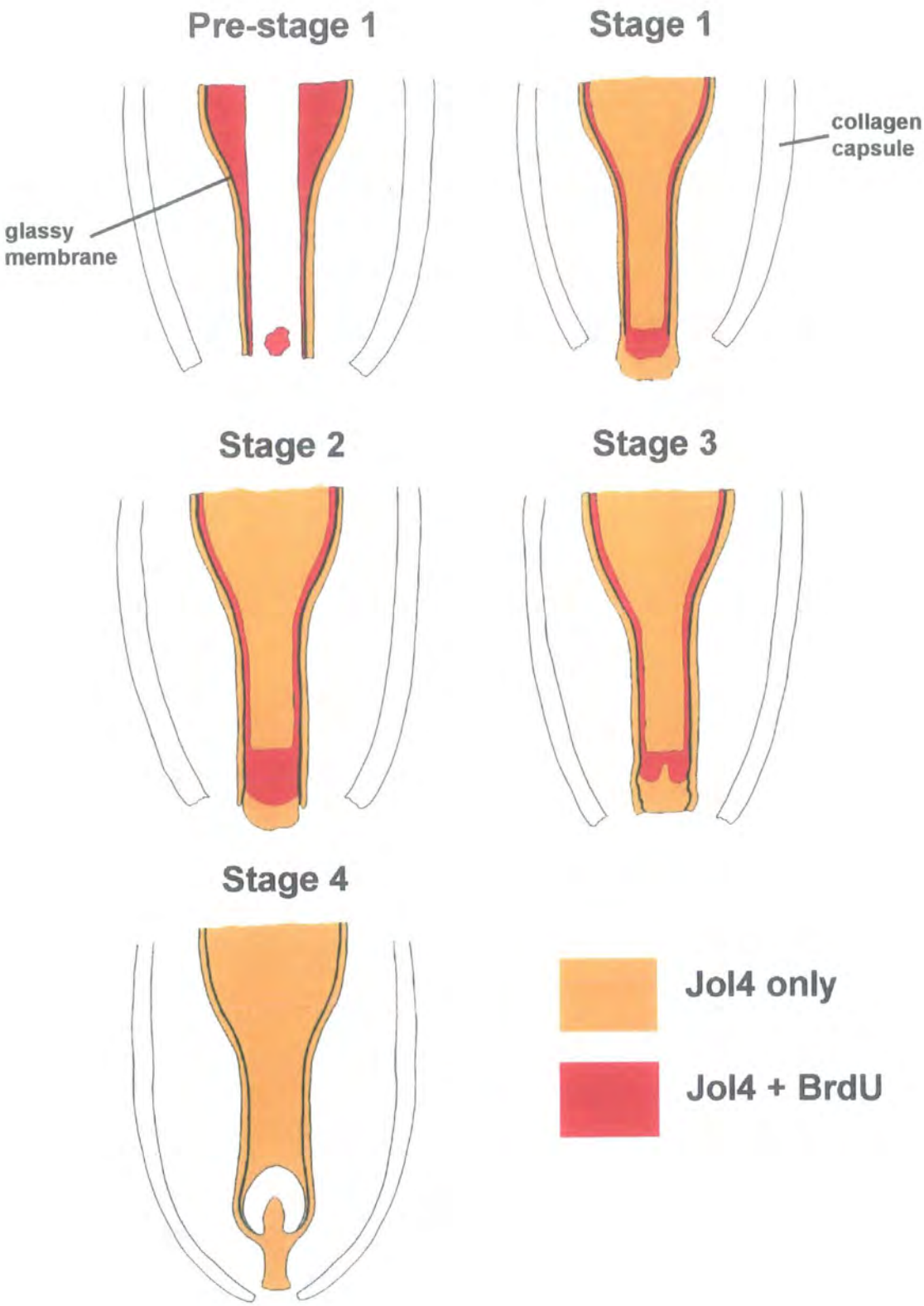

A
B
C
em
dp

STAGE 4

BrdU incorporation was not performed on any follicles that had reached stage 4 in the regeneration process. However, Jol-4 staining exhibited a labelling pattern similar to that seen in a late stage developing follicle (see chapter 2, Fig. 2.11 C,D). The newly formed dermal papilla and dermal sheath were heavily labelled whilst staining was noticeably weaker in the developing epithelial matrix (Fig. 3.5 C). Distally, cells of the outer root sheath were labelled by Jol-4 antibody as they had been throughout regeneration.

A summary of these results is shown in Figure 3.7 as a diagrammatic representation, highlighting regions in which proliferation was evident during the stages of follicle regeneration.

Figure 3.7. Schematic diagram indicating areas of Jol-4 staining and BrdU incorporation during regeneration of the vibrissa 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.


Pre-stage 1
Stage 1
collagen capsule
glassy membrane
Stage 2
Stage 3
Stage 4
Jol4 only
Jol4 + BrdU

3.3.3. α-smooth muscle actin expression

An extensive study of this protein's expression was not carried out but two follicles, one at stage 2 (Fig. 3.8 A-F) and one at stage 4 (Fig. 3.8 G, H), were immunostained with a monoclonal α-smooth muscle actin antibody. The stage 2 follicle was atypical in that it exhibited an unusual split in the centre of the epithelial column, that was delineated by the laminin antibody (see 3.3.4.1). Significant levels of α-smooth muscle actin expression were observed in the loose mesenchymal tissue lining the inside of the collagen capsule at stage 2, along with strong expression in the dermal sheath, particularly in the proximal half of the follicle. However, expression was downregulated in the dermal sheath, alongside the most proximal section of extended glassy membrane, on the nerve side (Fig. 3.8 A-C). In contrast, those dermal cells within the compartment created by the extended glassy membrane displayed high levels of α-smooth muscle actin expression. This extended into the convolutions of the basement membrane between the dermal and epithelial cells (Fig. 3.8 A,D - arrowheads). The α-smooth muscle actin staining highlighted dermal sheath cells extending processes into the glassy membrane itself (Fig. 3.8 C,F). Jahoda et al. (1992a) made similar observations by TEM of dermal sheath cells apparently passing through the disrupted lower glassy membrane in this stage of regeneration.

A degree of asymmetry was seen in this follicle with much more α-smooth muscle actin expression being evident in the loose mesenchymal tissue on the nerve side of the follicle (Fig. 3.8 D). In the most proximal section of the dermal sheath the asymmetry was the opposite, with higher expression on the side of the follicle opposite that at which the nerve enters (Fig. 3.8 A-D). Obliquely cut sections revealed that the circular arrangement of actin fibres, typically seen within

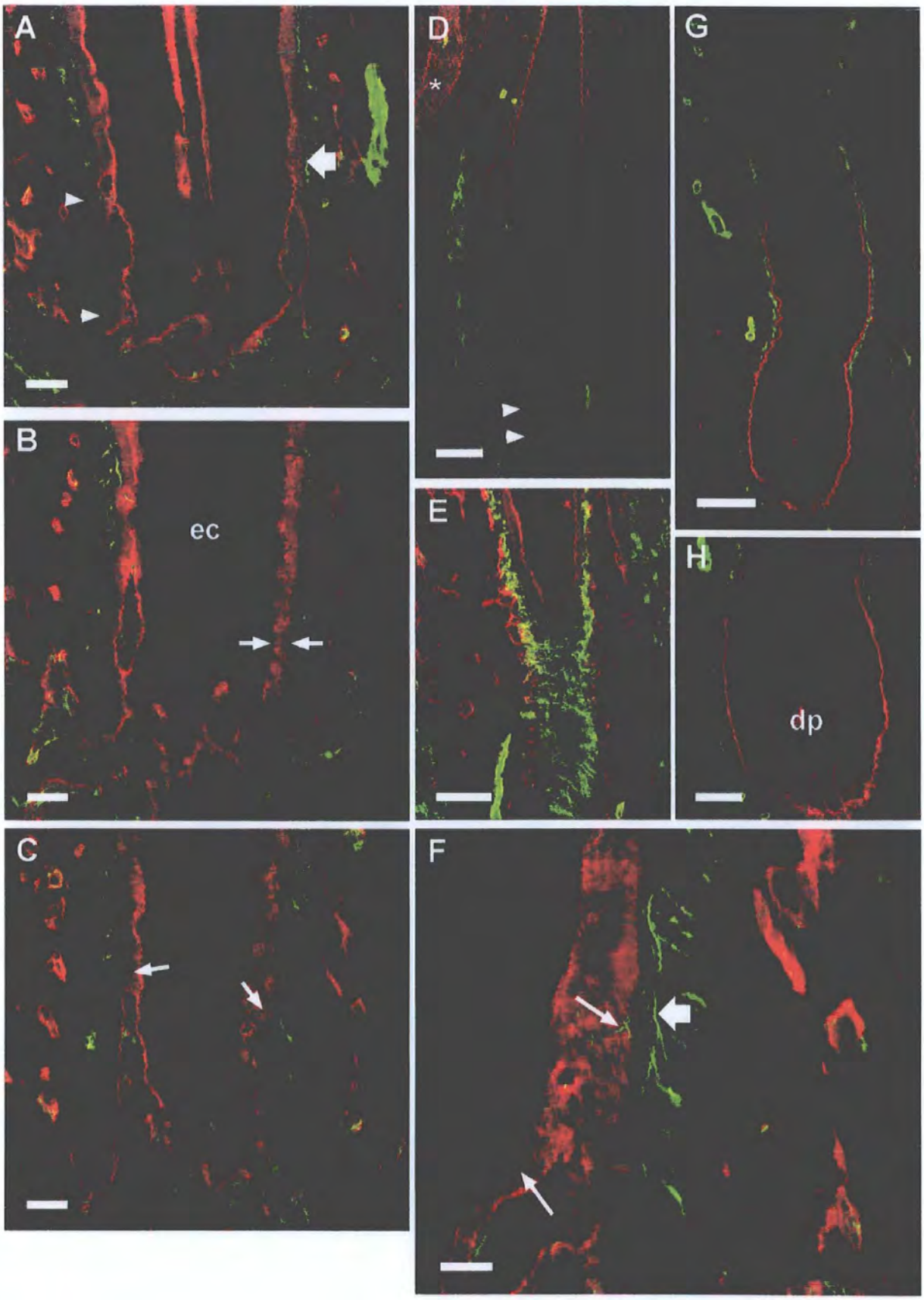
Figure 3.8. α-smooth muscle actin expression in vibrissa follicle regeneration.

Stage 2 (A-F) and stage 4 (G-H) follicles immunostained with a monoclonal antibody against α-sma (green fluorescence) and a polyclonal antibody against laminin (red fluorescence).

Stage 2: An unusual laminin deposition extends distally from the base of the epithelial column in a midline section of the stage 2 follicle (A). α-sma expression in the dermal sheath was strong in the lower third of the follicle (D) but diminished at the very base (between arrowheads in A & D), particularly on the side from which the deep vibrissal nerve entered the follicle. Successive sections from the middle of the follicle (A) towards the edge (C) demonstrate the base of the glassy membrane (arrowheads in B) separates from the epithelial column (ec) and α-sma-positive dermal cells often become sandwiched between the two structures (A-C). α-sma-positive cell projections also extend deep into the glassy membrane (small arrows, C, F). α-sma staining is also evident within the mesenchymal tissue lining the lower capsule (D). The characteristic circular arrangement of α-sma-positive fibres in the lower dermal sheath is clearly shown in an off-centre section (E). α-sma-positive fibres are also evident running perpendicular to the circular fibres, between these and the glassy membrane (large arrow, A & F).

Stage 4: Once the end bulb has reformed, α-sma expression is much the same as in a typical anagen vibrissa follicle with no expression in the dermal papilla (dp) and the lower dermal sheath expression ceases level with the top of the dermal papilla (G, H).

Scale bar: A-C = 20μm, D, G = 100μm, E, H = 50μm, F = 10μm.


A
B
ec
C
D
*
E
F
G
H
dp

the dermal sheath of vibrissa follicles, was also apparent in the regenerating follicle (Jahoda et al., 1991) (Fig. 3.8 E). However, in the dermal sheath cells immediately adjacent to the glassy membrane there were also α-smooth muscle actin fibres aligned with the long axis of the follicle, perpendicular to the circular fibres (Fig. 3.8 A,F - large arrows). Strong α-smooth muscle actin expression also highlighted small blood vessels within the follicle.

In a stage 4 regenerating follicle (Fig. 3.8 G,H), α-smooth muscle actin expression was similar to that seen in the anagen vibrissa follicle with distinct staining of the dermal sheath in the proximal part of the follicle but not around the end bulb. As before, blood vessels were effectively highlighted by the α-smooth muscle actin in the vessel walls.

3.3.4. Extracellular matrix remodelling

3.3.4.1. Laminin

Laminin was used as an effective means of delineating the glassy membrane and to aid orientation when viewing sections by fluorescence microscopy. The glassy membrane was highlighted (Fig. 3.8 B - arrows), along with other basement membranes, including those of blood vessels. These observations were consistent with those previously made by Jahoda et al. (1992). A layer of laminin was observed at the junction between the epithelial compartment and the dermal cells at the base of the regenerating follicle where the glassy membrane was 'open' (Figure 3.4). This was not an extension of the glassy membrane but a thin deposition of laminin, evident from stage 1 of regeneration, delineating the two separate compartments. The only follicles in which this was absent were those designated as pre-stage 1 of regeneration. As regeneration progressed, more laminin accumulated and by stage 4 the layer was of

similar thickness around the newly-formed dermal papilla as the lower glassy membrane in the same follicle.

One follicle in particular, believed to be in stage 2 of regeneration, showed an unusual laminin deposition which extended through the lower portion of the epithelial column. Initially, this was thought to be an extended indentation representing the early stage of dermal papilla formation, but the length of projection into the epithelial column was excessive for this to be the case (Fig. 3.8 A,D).

3.3.4.2. Versican

PRE-STAGE 1

Versican immunoreactivity was seen as normal in areas associated with innervation (see chapter 2), that is in the mesenchymal tissue about half way up the follicle and at the infundibulum (Fig. 3.9 A). In the lower third of the amputated follicle, versican was expressed within the glassy membrane on the nerve side of the follicle (Fig. 3.9 B). Occasional staining was seen in the glassy membrane on the opposing side but this was weak and inconsistent in comparison. This asymmetry was consistent in both follicles studied at this stage. The most striking staining however, was the strong labelling associated with the dermal sheath at the lowermost end of the open glassy membrane (Fig. 3.9 C). This was highly localised at this stage, limited to the dermal sheath within approximately 100μm of the cut end of the glassy membrane. Distal to this, versican expression in the dermal sheath was significantly reduced.

Figure 3.9. Versican expression in early regenerating vibrissa follicles.

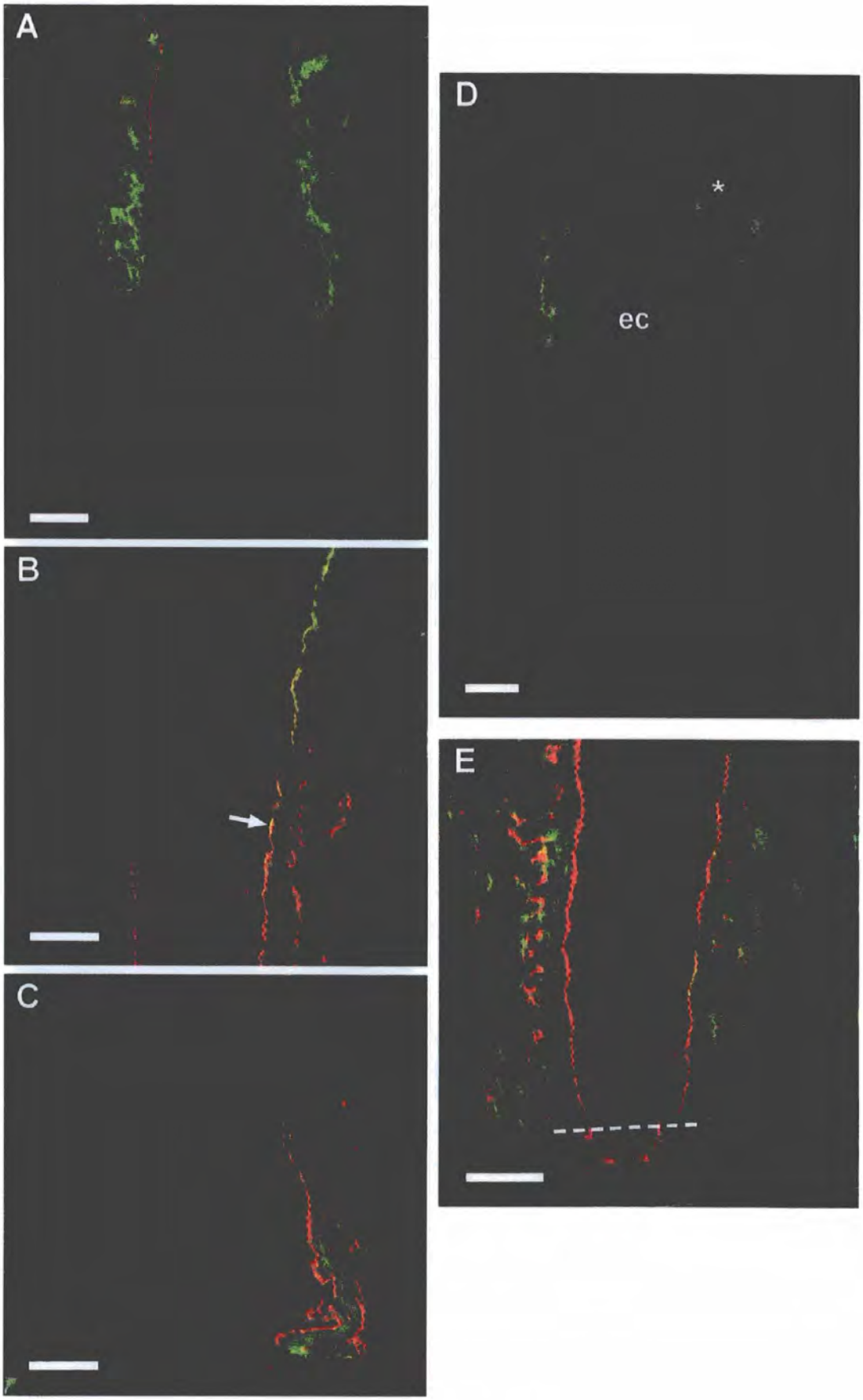
Pre-stage 1 (A-C) and stage 1 (D, E) follicles immunostained with a monoclonal antibody (12C5) against versican (green fluorescence) and a polyclonal antibody against laminin (red fluorescence).

(A) Pre-stage 1 follicles (A-C) display versican immunoreactivity at the infundibulum as in normal adult vibrissa follicles. (B) Asymmetry is evident within the glassy membrane half-way up the follicle with significant expression in the membrane (arrow) on the side of the follicle which is penetrated by the deep vibrissal nerve. (C) Versican is abundant within the dermal sheath at the open end of the glassy membrane.

(D) Stage 1 follicles show strong versican immunoreactivity in the dermal tissue corresponding to the point of entry of the deep vibrissal nerve (*). ec - epithelial column.

(E) There is also dermal expression in the lower region of the follicle extending laterally from the lower dermal sheath. The immunoreactivity stops abruptly at the lower edge of the glassy membrane, indicated by a dashed line.

Scale bar: A, D = 100μm, B, C, E = 50μm.


A
B
C
D
*
ec
E

STAGE 1

As in pre-stage 1 follicles, versican was abundant at the level of the deep vibrissal nerve entering the capsule and this staining extended throughout the dermal tissue in this region (Fig. 3.9 D). In the proximal portion of the follicle, versican expression remained strong in the lower dermal sheath and extended laterally into the surrounding dermal tissue with no evident asymmetry (Fig. 3.9 E). However, asymmetry was seen within the glassy membrane itself as versican expression was detected in this extracellular matrix (although at lower levels than in pre-stage 1 follicles) on the nerve side of the follicle but not the other (Fig. 3.9 E). This was consistently observed in the early stages of follicle regeneration. The lateral spread of versican from the dermal sheath, had a distinct border at the level of the proximal end of the glassy membrane. Dermal tissue below this lacked versican expression, in sharp contrast to the strong labelling evident in the dermal tissue alongside the glassy membrane, including the dermal sheath (Fig. 3.9 E).

STAGE 2

Versican expression was maintained in the proximal dermal sheath during this stage as was the expression within the loose mesenchymal tissue around it. The border extending laterally from the end of the glassy membrane was also still evident, with tissue proximal to the point of amputation remaining free of versican immunoreactivity (Fig. 3.10A).

STAGE 3

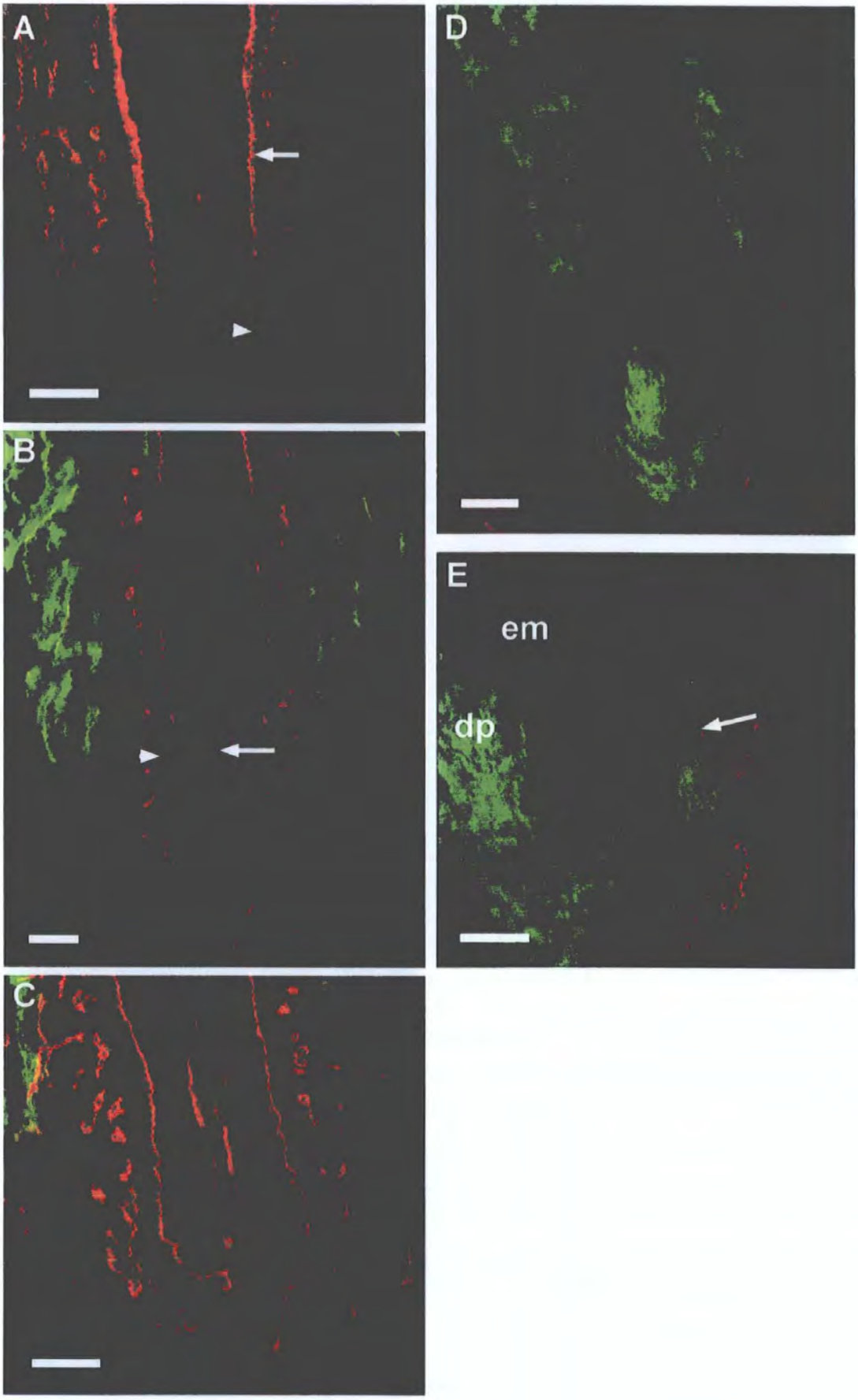
Versican staining in the dermal sheath during stage 3 was weaker than that seen in previous stages. Versican expression in the loose mesenchymal tissue of the proximal stage 3 follicle was now stronger than that of the dermal sheath (Fig. 3.10 B,C), in

contrast to previous stages. The loose dermal tissue, lateral and slightly distal to the base of the epithelial column, maintained a high level of versican expression. At the very base of the regenerating follicle where the dermal tissue had formed an indentation in the base of the epithelial column, only low levels of versican were evident, though some was present within the dermal tissue surrounded by the laminin-positive basement membrane (Fig.3.10 B,C).

STAGE 4

In the regenerated end bulb versican was abundant throughout the loose mesenchymal tissue, within the collagen capsule (Fig. 3.10 D). Strong expression was evident in the newly formed dermal papilla as in an anagen follicle. The glassy membrane and lower dermal sheath however, displayed a slightly weaker level of versican immunoreactivity than the rest of the mesenchymal tissue within the follicle. Double-labelling with a neurofilament antibody demonstrated that areas free of versican expression were frequently associated with the presence of nerve fibres (Fig. 3.10 E).

Figure 3.10. Versican expression during stages 2-4 of vibrissa follicle regeneration. Stage 2 (A), stage 3 (B,C) and stage 4 (D, E) follicles immunostained with a monoclonal antibody (12C5) against versican (green fluorescence) and a polyclonal antibody against either laminin (A-C) or neurofilament (D, E) (red fluorescence).
(A) Versican is expressed in the lower dermal sheath adjacent to the glassy membrane (arrow) but stops abruptly at the point where the glassy membrane ends (arrowhead). (B) At stage 3 versican is expressed at relatively low levels in the dermal sheath (arrowhead) compared to the surrounding dermal tissue. In the early dermal stalk penetrating the epidermis traces of versican are evident (arrow). (C) A higher magnification of the section in B. (D) By stage 4 versican is abundant throughout all dermal tissue enclosed by the collagen capsule. (E) The new dermal papilla (dp) is densely stained for versican. Double-labelling with neurofilament indicates breaks in the versican staining are commonly associated with the presence of nerve fibres (arrow). em - epithelial matrix. Scale bar: A-C, E = 50μm, D = 100μm


A
B
C
D
E
em
dp

3.3.5. Sonic hedgehog (*Shh*) expression

Pre-stage 1 follicles showed no evidence of *Shh* expression in any part of the follicle. At stage 1 the dermal tissue that enclosed the amputated end of the follicle as part of the wound response, displayed a high level of staining that extended to the edge of the capsule. This tissue was well vascularised and staining was particularly strong in association with blood vessels. In one particular follicle, punctate staining lined the outer aspect of the glassy membrane, suggestive of *Shh* expression in lower dermal sheath cells (Fig. 3.11 A). Sense controls indicated that this staining of the dermal sheath and the irregular, patchy staining seen in the mesenchymal tissue around the proximal end of the follicle was specific (Fig. 3.11 B). There was no evidence of *Shh* expression in the epithelial cells of stage 1 follicles. The same was true of stage 2 follicles with dermal tissue exhibiting patchy staining in the lower mesenchymal tissue and the proximal capsule, whilst epithelial cells displayed only background levels of staining in all areas of the epithelial column.

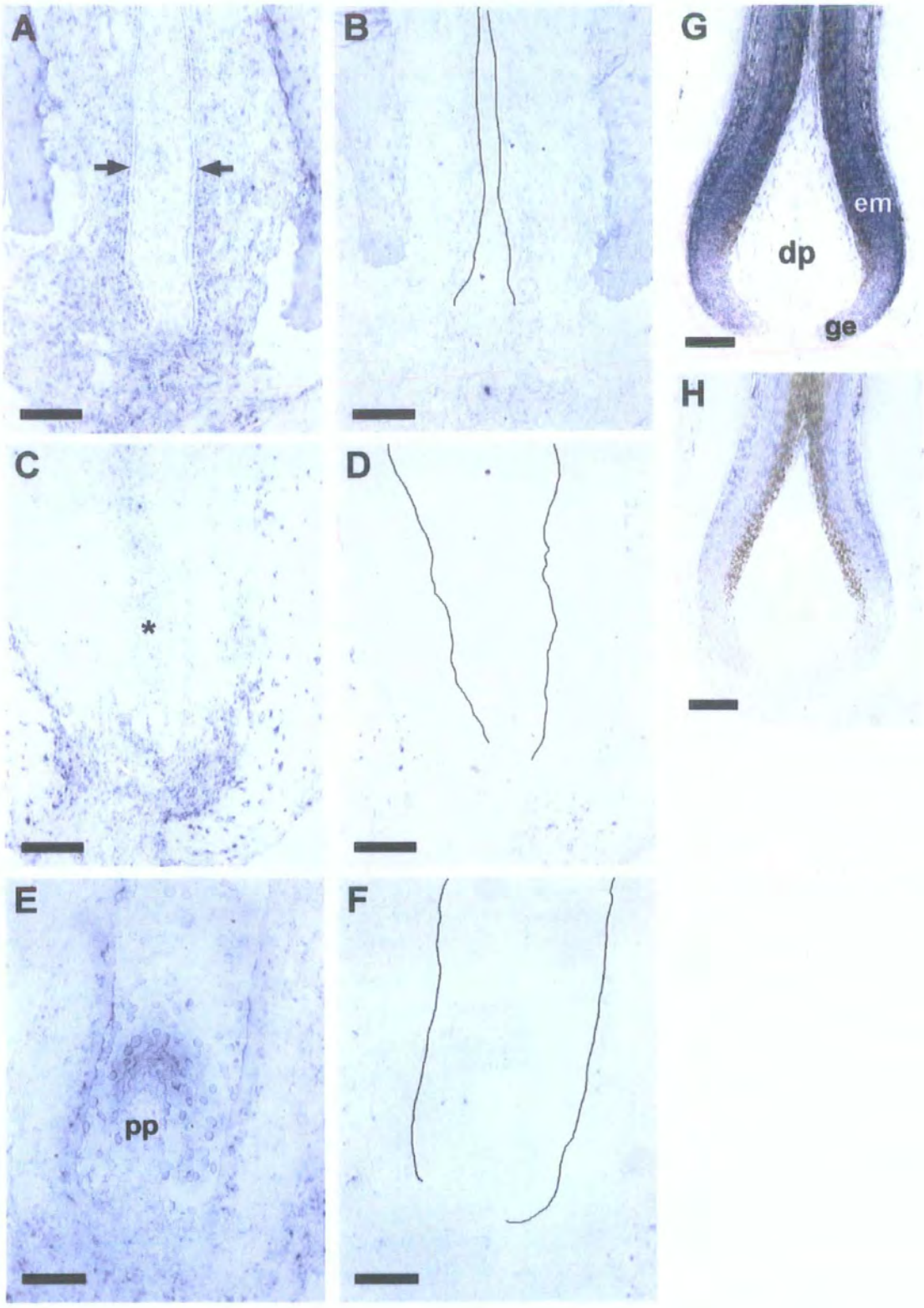
During stage 3 the dermal cells formed an indentation in the proximal end of the epithelial column. During this process *Shh* expression was evident initially as a weak band of staining in the most central cells of the epithelial column (Fig. 3.11 C). Subsequently, as the cells within the indentation developed a more typical papilla formation, a cap of epithelial cells situated around the developing papilla showed significantly higher levels of *Shh* expression (Fig. 3.11 E).

In all cases, hybridisation with a sense riboprobe acted as a negative control (Fig. 3.11 B,D,F), whilst for positive controls, anagen vibrissa follicle sections were hybridised alongside the experimental sections (except for two experiments which had no positive control), again with anti-sense and sense probes (Fig. 3.11 G,H). Strong expression was detected in the epithelial matrix of anagen follicles as previously reported (St-Jacques et al., 1998; Karlsson et al., 1999; Sato et al., 1999).

Figure 3.11. Expression patterns of *Shh* in vibrissa follicle regeneration and anagen, by *in situ* hybridisation.

Stage 1 (A,B) and stage 3 (C-F) regenerating follicles and an anagen vibrissa follicle (G,H) hybridised with anti-sense (A, C, E, G) or sense (B, D, F, H) *Shh* riboprobes. Lines indicate the position of the glassy membrane in B, D & F.

(A) During stage 1, *Shh* expression is only detected within the dermal tissue, particularly the lower dermal sheath (below the level indicated by arrows) and the mesenchymal tissue at the base of the follicle. (C) During stage 3, epidermal cells in the centre of the column (*) weakly express *Shh* and expression in the mesenchymal tissue is maintained. (E) A distinct cap of cells around the presumptive papilla (pp) express *Shh*. (G) The expression of Shh in anagen follicles was used as a positive control for the in situ hybridisation method. Cells of the epithelial matrix (em), but not the germinative epithelial (ge) cells, expressed *Shh*. dp - dermal papilla. (B, D, F & H) Sense controls demonstrate staining A, C, E & G represents specific hybridisation of riboprobes. Scale bar: A-D, G, H = 100μm, E, F = 50μm.


A
B
G
em
dp
ge
H
C
*
D
E
pp
F

3.4. DISCUSSION

3.4.1. Summary

Regeneration in the epithelial compartment resulted from extensive proliferation in the basal layer of outer root sheath cells. This proliferation extended along the length of the follicle and was also evident at the follicle neck and in the epidermis continuous with the outer root sheath. This supports proposals that stem cells are continuously migrating through the outer root sheath during anagen (Oshima et al., 2001). The lack of dermal cell proliferation suggested the dermal structures of the regenerated end bulb were derived from mesenchymal cells that migrated into the follicle during regeneration. α-smooth muscle actin expression however, supported proposals that dermal sheath cells formed the dermal papilla and migrated through the glassy membrane. Versican expression demonstrated that the dermal sheath responded to injury in a manner that differed from that of the other tissues within the follicle. In addition, the association of versican with innervation, described in chapter 2, was evident in follicle regeneration. *Shh* was expressed in epithelial cells associated with the regenerating dermal papilla, as it is expressed in association with the developing dermal papilla.

3.4.2. Where are the regenerated tissues derived from?

3.4.2.1. Cell proliferation

BrdU is incorporated into the nucleus of cells during DNA replication and can subsequently be detected immunologically, thereby providing a means for detecting cell proliferation (Gratzner, 1982). The observation that proliferation occurs in the outer root sheath cells of pre-stage 1 follicles, suggests that local cell division gives rise to the cells that subsequently fill the cavity created by removal of the hair fibre. Proliferation

is also evident in cells at the neck of the pre-stage 1 regenerating follicle, raising the possibility that cells originating from the surrounding epidermis migrate into the follicle and contribute to the initial stages of regeneration. Outer root sheath cells have been demonstrated to contribute to interfollicular epidermis in neonatal skin during tissue expansion and in adult skin during re-epithelialisation after wounding (Taylor et al., 2000). There is no evidence that the interfollicular epidermis would not be capable of reciprocating, in follicle regeneration.

The concentration of proliferative activity in the proximal end of the epithelial column, from stage 1 onwards, reflects the significant amount of remodelling occurring in this region of the follicle. The increase in cell numbers is likely to be a contributing factor in the initial projection of the epithelial column into the dermal tissue at the base of the follicle. The distribution of proliferating cells in stage 3 follicles is reminiscent of that seen in the end bulb of an anagen follicle (Gharzi et al., 1999; Rumio et al., 2000) indicating that these cells are forming the new epithelial matrix of the end bulb. Throughout the regenerative process, proliferating cells were identified at regular intervals along the inner aspect of the glassy membrane. This suggests epithelial stem cells are distributed throughout the follicle.

Epithelial cell proliferation in the basal layer of outer root sheath along the length of the follicle supports the proposals by Oshima et al. (2001) that stem cells migrate from the bulge through the outer root sheath during anagen. Under the "traffic light" hypothesis (Oshima et al., 2001) stem cell migration ceases in the lower outer root sheath at the onset of catagen. Migration from the bulge continues, resulting in the stem cells accumulating along the length of the outer root sheath during catagen right back to the bulge. Subsequently, cells stop migrating out of the bulge during telogen. Only when early anagen is initiated and the "queue" of cells moves downwards, do bulge cells begin to migrate again. Studying BrdU incorporation during the follicle cycle

would be a means of testing this hypothesis, whilst the observations described above on mid-anagen follicles support the proposal of stem cells migrating along the length of the outer root sheath at this stage.

The "traffic light" hypothesis would also suggest that regeneration of the follicle would be successful at all stages of the cycle as there are consistently stem cells along the length of the follicle, the density of these being the only variable. Investigating the regeneration of follicles amputated at different stages of the follicle cycle would therefore reveal any changes in the regenerative capacity of the dermal sheath according to the cycle stage.

In all stages of regeneration, the vast majority of epithelial cells displayed nuclear labelling with the monoclonal antibody, Jol-4. This antibody is specific to lamin A in the nuclear envelope of non-dividing cells (see chapter 2, Dyer et al., 1997) and therefore, identified the mitotically quiescent cell population within the follicles. The newly formed epithelial matrix of a stage 4 follicle was the only epithelial cell population in which Jol-4 staining was conspicuously absent, suggesting this follicle was in the early stages of fibre production. Whilst Jol-4 staining was intended to highlight non-dividing cells, the nature of the stain also revealed the shape of cell nuclei and in some instances this gave further insight into the changes occurring during regeneration. The nuclei of one stage 2 follicle in particular indicated that cells were becoming polarised within the epithelial column. Cell nuclei were aligned along the long axis of the follicle prior to indentation of the base of the epithelial column. This regular arrangement contrasted with the randomly arranged epithelial cells observed in follicles at earlier stages of regeneration. Such organisation indicates the epithelial cells are undergoing morphological changes during this stage of regeneration.

Rober et al. (1989) found that during development the expression of lamin A/C was only upregulated when tissues were differentiating leading to proposals that lamin

A/C expression limits the plasticity of cells. Whilst this may be true to a certain extent, the data presented here demonstrates that lamin A expression, detected by Jol-4 immunostaining, does not identify a cell as 'differentiated'. The fate of the cell may simply have been limited somewhat, for example, a dermal cell may have been designated as hair follicle-specific but still has the potential to contribute to papilla or sheath. Similarly a Jol-4 labelled epithelial cell can still potentially contribute to any of the seven follicular cell types.

Very few dermal cells had successfully incorporated BrdU, suggesting there was no surge of dermal cell proliferation in the regenerative process. The most extensive dermal staining was seen in a stage 1 follicle and this was restricted to less than 10 cells over a number of sections, only one of these cells being in the dermal sheath. The follicular mesenchyme and dermal sheath have been cited by previous authors as sources of additional dermal cells in regeneration of the dermal papilla (Oliver, 1966b; Jahoda & Oliver, 1984c; Jahoda et al., 1992a), but proliferation in these areas was not detected. Lack of cell proliferation implies that migration of dermal cells either from within the vibrissa follicle, or from the mesenchymal tissue at the site of amputation, is necessary to provide a considerable proportion of the dermal cell population for the new end bulb. However, there is also the possibility that the lack of BrdU incorporation in dermal cells may reflect a longer S phase than that of the epidermal cells. In the most proximal section of dermal sheath, in pre-stage 1 follicles, less Jol-4 immunoreactive cells were present than in the distal dermal sheath. The lack of BrdU incorporation in areas that were not stained by the Jol-4 antibody suggests these areas contained cells that were either just entering the cell cycle or had already completed the S phase by the time of BrdU treatment. Testing a range of BrdU incubation times, in combination with a detailed study of cell migration during the regenerative process, should provide more informed answers regarding the source of the dermal cells of the new end bulb.

3.4.2.2. α-smooth muscle actin expression

The inner cells of the lower dermal sheath display strong α-smooth muscle actin expression *in vivo* (Jahoda et al., 1991). Detection of this protein during regeneration therefore identifies the dermal sheath cell population, along with any cells in which this protein is upregulated as part of the regeneration process. The expression of α-smooth muscle actin was only studied in one follicle at stage 2 of regeneration and one follicle at stage 4, due to limited antibody stocks. This must therefore be borne in mind when considering the results. In the stage 2 regenerating follicle, expression of α-smooth muscle actin is evident in the dermal cells gathered below the epithelial column, prior to dermal papilla formation. Previous authors have proposed these cells to be of dermal sheath origin and the expression of α-smooth muscle actin supports these proposals.

Following wounding of the dermal papilla, Jahoda & Oliver (1984c) observed cells within the glassy membrane. This was followed by observations, at the ultrastructural level, of cell projections within the disrupted glassy membrane that were described as dermal sheath cells moving through the membrane (Jahoda et al., 1992a). I observed filamentous α-smooth muscle actin within the lower glassy membrane, providing further evidence of dermal sheath cells passing through this thickened ECM. Cells within the glassy membrane itself and those situated on the inner aspect, where it had detached from the epithelial column, displayed the same high level of α-smooth muscle actin expression as seen in dermal sheath cells distal to the site of end bulb regeneration.

The point at which dermal cells become dermal papilla cells in the regeneration process is unknown but, it appears the first interaction of dermal sheath cells with the epithelial cells, and subsequent cell movement into the confines of the extended glassy

membrane, does not result in downregulation of α-smooth muscle actin. The expression of this protein in stage 4 of regeneration is much the same as that of an anagen vibrissa follicle suggesting the switch to dermal papilla occurs in dermal cells during stage 3, presumably as they form the indentation that will develop into a functional papilla.

Expression of α-smooth muscle actin was not restricted to the tissue closely associated with the lower epithelial column. Expression extended into the loose mesenchymal tissue, particularly that immediately adjacent to the inner aspect of the collagen capsule. Jahoda et al. (1996) observed that regenerating human hair follicles, implanted beneath the skin of athymic mice, developed bulbous structures of similar appearance to the collagen capsules surrounding vibrissa follicles. It was proposed that these structures were formed from the outer layer of dermal sheath whilst the inner layer contributed to the new dermal papilla. The wider expression of α-smooth muscle actin in regenerating vibrissa follicles supports the proposal that dermal sheath cells may contribute to the regeneration of the collagen capsule as well as the dermal papilla. However, the distinction between the layers is unclear. The only indication from the results presented here is the orientation of α-smooth muscle actin filaments in the lower dermal sheath. The fibres lining the outer aspect of the glassy membrane lie along the long axis of the follicle whilst fibres external to these, appear to lie perpendicular to this, encircling the glassy membrane. The significance of these two layers is unclear in the regenerating vibrissa follicle and detailed analysis is needed to reveal if a similar arrangement is evident in the anagen vibrissa follicle.

The expression of α-smooth muscle actin within the mesenchymal tissue was lost by stage 4 of regeneration suggesting the functional requirement is transient, possibly as part of the wound response.

3.4.2.3. Possible ways of deducing the origin of the new dermal papilla

Previous studies proposed the dermal sheath gave rise to the regenerated dermal papilla. The expression of α-smooth muscle actin in dermal cells at the base of the stage 2 follicle, as well as in cells passing through the glassy membrane, appears to support these assertions. However, while the dermal sheath seems the most likely origin of these cells, migration of cells into the wound site from the surrounding mesenchyme, or delivery of cells via the blood supply are still possibilities in the regeneration of the end bulb. Indeed, the possibility of a distant source of dermal cells is made all the more likely by the evident lack of dermal cell proliferation in the regenerating follicle. The exact origin of the regenerated dermal papilla is still to be defined.

One means by which cells could be tracked during follicle regeneration would be to take advantage of transgenic mice in which all cells express marker genes, for example, Zin40 mice which express *lacZ* in all cells. Lengths of Zin40 mouse vibrissa follicle wall, consisting of dermal sheath and outer root sheath cells only (Oliver, 1967a), could be implanted under the kidney capsule of nude mice, a site that has been successfully used for ectopic follicle regeneration by Kobayashi & Nishimura (1989). The *lacZ* gene, expressed by Zin40 cells, could then be detected by X-Gal staining, thus determining which tissues derived from Zin40 cells. Any contribution from the host tissue would be identifiable by the lack of blue staining.

BrdU incorporation detected minimal dermal cell proliferation during regeneration. As mentioned previously the protocol for incorporation could be altered to address this issue but other techniques might also be employed to detect proliferating cells such as tritiated thymidine incorporation or using Ki67 antibodies which detect a nuclear protein only expressed by proliferating cells.

3.4.3. Do events in follicle regeneration reflect aspects of appendage development?

During the adult hair cycle the morphological changes and remodelling processes that occur are often regarded as a recapitulation of the epithelial-mesenchymal interactions of follicle development. The regeneration that occurs in the vibrissa follicle is likely to involve similar epithelial-mesenchymal interactions, to achieve the successful production of a functional end bulb.

3.4.3.1. Dermal papilla regeneration: aggregation

The formation of a dermal papilla is the key factor in successful hair fibre production by regenerated follicles. Disruption of the dermal papilla has demonstrated that the integrity of this group of specialised cells is crucial for normal hair growth in an otherwise intact follicle (Jahoda & Oliver, 1984b, 1984c). Dermal papilla cells possess innate aggregative properties both *in vivo* and *in vitro* (Wessells & Roessner, 1965; Jahoda & Oliver, 1981, 1984a; Jahoda et al., 1993). The fact that high passage dermal papilla cells lose both their inductive capacity and aggregative behaviour, indicates the importance of aggregation to follicle formation (Horne et al., 1986). The aggregation of dermal cells observed in hair follicle development would therefore be expected to occur in regeneration.

The most prominent aggregation of dermal cells was highlighted by Jol-4 staining in stage 1 follicles. A cluster of Jol-4 immunoreactive cells was situated beneath the epithelial column that extended below the glassy membrane. This extension of the epithelial cells provides an opportunity for the epithelial and dermal cells to interact without obstruction by the glassy membrane. In follicle development, epithelial-mesenchymal signals lead to dermal aggregation. Direct epithelial-mesenchymal interaction at this early stage of regeneration appears to result in a similar aggregation in the dermal tissue immediately proximal to the epithelial column. Dermal sheath cells

from the lower third of the follicle display aggregative behaviour *in vitro* whilst those from the upper follicle do not and it has been suggested this is related to the regenerative properties of the proximal third of the follicle (Horne et al., 1986), these cells sharing some of the key characteristics of dermal papilla cells.

The dermal cells that gather below the epithelial column are thought to act in a manner comparable to that of the dermal condensation observed in follicle development. Further study of the proteins expressed by these cells would enable the direct comparison of signalling mechanisms occurring in regeneration to those of development. In particular, determining the pattern of Noggin and BMP expression would be interesting, as these two signalling molecules are understood to play important roles in follicular morphogenesis. BMP exerts an inhibitory influence in development, preventing follicle induction. The expression of Noggin by the cells of the dermal condensation antagonises BMP signalling so providing a permissive signal, enabling the initiation of follicle development (Botchkarev et al., 1999a).

In the adult vibrissa follicle, Noggin is only expressed in the dermal papilla and would therefore be an interesting molecule to study during the process of regeneration. Establishing at what stage the cells of the new dermal papilla express Noggin, and the position of these cells when they do, may indicate the origin of these cells. For example, if the dermal papilla is shown to be a derivative of dermal sheath by the experiments described previously using Zin40 mice, the expression of Noggin might first be evident in the dermal sheath at the site of amputation, prior to cell aggregation. Alternatively, the cells may aggregate but not express Noggin until the dermal compartment creates an indentation in the base of the epithelial column. The pattern of Noggin and BMP expression, when compared to that seen in development, would indicate how the regeneration process relates to follicle organogenesis, in addition to providing some important information regarding the transition from dermal sheath to dermal papilla.

3.4.3.2. Sonic hedgehog expression

Sonic hedgehog (*Shh*) is expressed in the epithelial component of the developing follicle and the adult follicle, but has not been detected in follicular dermal tissue (Bitgood & McMahon, 1995; Iseki et al., 1996; Gat et al., 1998; Karlsson et al., 1999). In the regenerating follicle, however, transcripts of *Shh* were detected throughout the lower mesenchymal tissue at the wound site and in the lower dermal sheath. In a recent review, Callahan & Oro (2001) discussed the fact that *Shh* or its target genes were commonly expressed in association with multipotent progenitor cells at a time of proliferation and differentiation. The expression pattern observed in regenerating follicles correlates with this, as dermal sheath is the proposed source of dermal papilla cells and the mesenchymal tissue will ultimately contribute to the closure of the capsule below the newly formed end bulb. In the early stages of regeneration therefore, *Shh* expression does not reflect the pattern of expression seen in early stages of follicle development, but highlights areas in the process of extensive remodelling.

This association with areas of remodelling is continued into the later stages of follicle regeneration when *Shh* is expressed in the epithelial cells, as well as the loose mesenchymal tissue. Expression in the lower dermal sheath was transient. The weak expression in the central epithelial cells suggests these cells are undergoing some morphological change, possibly in preparation for migration distally, to enable indentation at the proximal end of the epithelial column by dermal cells. (This is also suggested by the orientation of the cells in one particular stage 2 follicle in which Jol-4 staining highlighted the nuclei of the central epithelial cells to be aligned with the long axis of the follicle (Fig. 3.5 C).)

During early papilla formation in stage 3 of follicle regeneration, a collection of cells around the apex of the new papilla express *Shh*. At this later stage of regeneration the expression of *Shh* resembles that seen in stage 3 developing follicles when

expression is detected at the base of the epithelial downgrowth in association with the presumptive dermal papilla (Iseki et al., 1996). Shh is not crucial to follicle induction but is essential for successful morphogenesis of the dermal papilla (Chiang et al., 1999). *Shh* expression in the epithelial cells associated with the dermal papilla suggest Shh is once again regulating the formation of this structure. Recently, *Wnt5a* has been identified as a target of Shh signalling in follicle morphogenesis (Reddy et al., 2001). Determining the expression of this and other target genes in follicle regeneration, would provide further evidence as to whether developmental processes were involved in regeneration of a functional end bulb.

The close interaction between epithelial-mesenchymal cells is crucial for follicle organogenesis, as is the aggregation of follicular dermal cells. As mentioned previously, the fact that epithelial cells extend into the dermal tissue at the same stage as dermal cells were seen to form a cluster of cells beneath the epithelial column suggests the inductive interactions of follicle regeneration occur at this early stage. Previous suggestions were that wound healing characterised the initial tissue response and inductive interactions were second to this (Jahoda et al., 1992a). Whilst some wound healing characteristics are evident, the direct interaction of dermal and epithelial cells without the barrier of the glassy membrane provides an opportunity for inductive signals to be relayed between the two tissues at this early stage of regeneration.

3.4.3.3. Lessons from other examples of regeneration

Regeneration in mammals is decidedly limited in comparison to that of urodele amphibians and teleost fish. These organisms are capable of regenerating amputated limbs or fins and the formation of a blastema is believed to be crucial for successful regeneration (Brockes, 1997). The blastema develops beneath the wound epidermis and is derived from the mesenchymal tissue at the site of amputation. The interactions

between the epidermis and the blastema have been shown to involve signalling molecules that participate in developmental epidermal-mesechymal interactions. As such some lessons may be learnt from studies on amphibian and fish models that are relevant to mammalian appendage regeneration.

Mesenchymal expression of *Shh* is not a feature of hair follicle development but is evident in other sites of epithelial-mesenchymal interactions in development, including the developing limb bud. Polarised *Shh* expression in the posterior mesenchyme of the limb bud is integral to pattern formation (Niswander et al., 1994). Similar polarisation was demonstrated in the epithelial matrix of hair follicles by Gat et al. (1998) who observed aberrant expression coincided with random orientation of the follicle in relation to the skin. Whilst there was no asymmetry or polarisation of expression in the regenerating follicles of this study, it would be interesting to see if polarised *Shh* expression was evident in the later stages of follicle regeneration.

Limb regeneration does not generally occur in mammals, but two examples have been demonstrated in particular circumstances. One displays regenerative properties in the presence of exogenous factors (Taylor et al., 1994), whilst the other is similar to the vibrissa follicle in that regeneration will occur provided the amputation is distal to a certain point (Borgens, 1982). In this second model, the distal tip of the murine toe was observed to regenerate provided the amputation was distal to the nail bed. The authors observed that there was no recognizable blastema formed but proliferation of fibroblasts and progenitors of osseous tissue was evident at the site of amputation (Borgens, 1982). The accumulation of a mass of mesenchymal tissue is therefore a feature common to all regenerative processes, including that of the vibrissa follicle. The precise nature of this tissue, with regard to cellular composition and origin is unclear and further investigations are required to establish the degree of multipotency contained within this

tissue. Extracting a sample of this tissue and establishing an explant culture *in vitro* could be a basic, but effective, means of determining the cells present within this tissue.

The sites of regenerative potential in chick limb and murine toes both express *msx* genes. Amputation beyond the region of *msx* expression does not result in regeneration (Reginelli et al., 1995; Kostakopoulou et al., 1996), suggesting expression of these genes is crucial to the regenerative process (Poss et al., 2000). Msx genes are involved in the development of feathers, teeth and hair, and expression has also been reported in the inner root sheath of adult hair follicles (Noveen et al., 1995; Chen et al., 1996; Stelnicki et al., 1997). A more detailed analysis of *msx* distribution within the follicle might prove informative as to the cause of the difference in regenerative potential between the upper two thirds and the lower third of the follicle.

The lack of regeneration in follicles from which more than the lower third has been amputated (Oliver, 1966b) has been attributed to a lack of inductive or regenerative properties in the upper follicle wall, comprising dermal sheath and outer root sheath (Oliver, 1967a). However, Matsuzaki et al. (1996) observed frequent generation of follicle bulb formation in the upper portion of folicles from which the lower half had been amputated. The fibres produced from these bulbs were pelage-like, reflecting the small size of the bulbs produced. Whilst bulbs were formed they were frequently embedded in the dermal tissue at the upper end of the follicle, raising the question of how induction occurred, as at the site of bulb formation dermal and epidermal components were separated by the glassy membrane. Further investigation is required to determine the nature of these follicle bulbs and the tissues from which they are derived.

3.4.4. The role of extracellular matrix in regeneration

3.4.4.1. Versican

The concentrated expression of versican, at the proximal end of the pre-stage 1 follicle, suggests dermal sheath cells are expressing versican as part of their initial wound response, possibly in association with cell proliferation (Zimmermann et al.1994). This is a reaction specific to dermal sheath cells as versican is not expressed in any other tissue at the site of amputation. From stage 2 to 3 the expression in the dermal sheath diminishes, becoming weaker than that of the loose mesenchymal tissue in the lateral aspects of the follicle. However, at no stage is versican expressed in the dermal tissue enclosing the base of the follicle. This indicates that a) versican is not expressed as a component of the universal wound response, and b) versican plays no direct role in the epithelial-mesenchymal interactions occurring during follicle regeneration as at all times, areas of versican expression remain separated from the epithelial cells by the glassy membrane. Indeed, the lack of versican in the aggregating dermal tissue is reminiscent of the expression pattern observed in development.

From the data presented in the previous chapter (see 2.3) and the results obtained by double-labelling with neurofilament antibody in a stage 4 regenerating follicle, it appears that versican has two separate roles in the regenerating follicle. The early localised versican expression may be related to a specific wound response, whilst the later widespread expression is likely to be associated with the regeneration or stabilization of follicle innervation during this period of remodelling. The expression in the upper half of the follicle during the early stages remains associated with the deep vibrissal nerve and the superficial nerves at the neck of the follicle. The expression throughout the mesenchyme of a stage 4 regenerating follicle and the presence of fine nerve fibres on the inner aspect of the collagen capsule, was reminiscent of the strong expression at the follicle neck in association with the delicate fibres of the superficial

vibrissal nerves. I propose versican stabilises the innervation of the vibrissa follicle during the later remodelling stages of regeneration.

It is well documented that the dermal components of the hair follicle produce a specialised ECM (Couchman & Gibson, 1985; Couchman, 1986; Messenger et al., 1991; Couchman, 1993; Couchman & du Cros, 1995) and the contribution of ECM to healing is significant (reviewed by Tuan & Nichter, 1998). Given that versican appears to be specific to the wound response of dermal sheath cells it may be a key factor in the scar-free healing and regeneration of follicle tissue (Jahoda & Oliver, 1984c). Limited data is available on the contribution of versican to wound healing and the expression of versican varies between different models of healing (Boykiw et al., 1998; Geary et al., 1998; Gutierrez et al., 1998; Plaas et al., 2000). Perhaps most intriguing though, is the observation that wound healing myofibroblasts, known to express α-smooth muscle actin and recently proposed to derive from dermal sheath (Jahoda & Reynolds, 2001), express versican at high levels (Hakkinen et al., 1996). There is no clear evidence of the role of versican in the wound healing process. However, it is expressed in cells associated with skin wound healing, upregulation of versican is rapid in the amputated dermal sheath, and hair follicles, which express versican throughout the dermal component (chapter 2; du Cros et al., 1995), are able to heal without scar tissue forming. All these facts provide a good basis for further investigation into the contribution of versican in successful healing.

3.4.4.2. Laminin

Laminin is expressed in all basement membranes and as such provided a useful marker to identify both the glassy membrane and the extracellular matrix laid down in stage 1 of regeneration to separate the epithelial and dermal compartments. It also highlighted the vasculature within the regenerating follicles, which was mostly

associated with the lower dermal sheath and the plug of dermal tissue that covered the amputated end of the follicle by stage 2. This is further evidence that a wound-type response has occurred during follicle regeneration as a rich blood supply is one of the key requirements for any such process.

3.4.5. Conclusions

The regenerating vibrissa follicle remains an excellent model for studying various aspects of hair follicle biology. 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 these cells.

Aspects of development are evident in follicle regeneration, including the physical aggregation of dermal cells at the base of the follicle. *Shh* expression is not evident in the initial stages of regeneration when epithelial and dermal cells first interact, but at the time of dermal papilla formation, *Shh* is expressed in the epithelial cells. The molecular induction of follicle regeneration is yet to be elucidated, but the morphogenetic *Shh* signal is evident in the later stages of regeneration, as it is in development.

The capacity to heal scar-free is an intriguing one and many possibilities have yet to be explored in asking what factors result in this flawless healing. The specialised ECM of the follicular dermal tissue is a likely source and from the expression observed in the early stages of regeneration, versican is a possible factor.

Regeneration models provide good opportunities to investigate both wound healing and developmental processes. The vibrissa follicle is an excellent example of such a model.

Chapter 4: Follicular dermal cells and embryonic stem cells: reciprocal interactions.

4.1. INTRODUCTION

4.1.1. Embryonic stem cells

Embryonic stem (ES) cells originate from the inner cell mass of early blastocysts and have two important properties. First, they are totipotent, that is when injected into a blastocyst they integrate into the developing embryo and contribute to all cell lineages including the germ line (Bradley et al., 1984; Saburi et al., 1997). Second, in an appropriate environment, they will proliferate in culture indefinitely without any change to their behaviour (Smith, 1990). Established lines of murine ES cells provide a very useful tool for investigating the differentiation process as, given the correct stimuli, in the form of additions to the culture medium and substrate, they can be induced to differentiate along a variety of cell lineages, including blood and neuronal cell types (Hole & Smith, 1994; Bain et al., 1995; Dani et al., 1997).

The manipulation of the genetic material of ES cells in culture provides a powerful tool for investigating specific genes, either in differentiation processes *in vitro* or a functional role *in vivo* by the creation of chimaeric mice (Capecchi, 1989; Koller & Smithies, 1992; Bagutti et al., 1996; Dani et al., 1998; Rohwedel et al., 1998; Gimond et al., 2000). For example, the requirement of β1-integrin expression, for keratinocytes to assemble an ECM, was initially described after β1-integrin null ES cells showed atypical characteristics when induced to differentiate along the keratinocyte lineage (Bagutti et al., 1996). These findings have been supported by recent *in vivo* studies in which targeted deletion of the β1-integrin gene in keratinocytes resulted in severe abnormalities in hair follicles and interfollicular skin (Brakebusch et al., 2000). The studies on ES cells therefore provided the basis for a more focused analysis *in vivo*.

When murine ES cells were first isolated they were cultured on a layer of feeder cells to maintain them in an undifferentiated, pluripotent state. These feeder cells were initially teratocarcinoma stem cells (Martin, 1981). Subsequently, buffalo rat liver cells were found to produce conditioned media which had the same effect (Smith & Hooper, 1987). The molecule involved was purified from conditioned media of the feeder layer cells and found to be a polypeptide, which was subsequently named differentiation inhibitory activity (DIA) (Smith et al., 1988). Independently, Williams et al. (1988) demonstrated that the pluripotency of ES cells could be maintained by leukemia inhibitory factor (LIF), and the work of Gearing et al. (1987) and Moreau et al. (1988) established that DIA and LIF were actually one and the same molecule, as suggested by Smith et al. (1988).

4.1.2. Interleukin-6 cytokine family

LIF is a member of the interleukin-6 (IL-6) cytokine family which also includes ciliary neurotrophic factor (CNTF), oncostatin M (OSM) and cardiotrophin-1 (CT-1). CNTF, OSM and CT-1 have all been shown to maintain pluripotent ES cells in culture, in the same manner as LIF (Conover et al., 1993, Rose et al., 1994, Pennica et al., 1995). These cytokines share a four helical bundle structure and exert their effects on the cell through receptor complexes that include the gp130 subunit, first identified and cloned by Taga et al. (1989) and Hibi et al. (1990).

The common signalling mechanisms of the IL-6 family result in these cytokines displaying equivalent function in a number of assays (Pennica et al., 1995; Piquet-Pellorce et al., 1994). In contrast, there are circumstances where family members exert opposite effects on the same process. For example, there is evidence for IL-6, LIF, CNTF and OSM to have both anti-inflammatory and pro-inflammatory responses. In neural tissue, LIF plays a key role in response to injury and possible infection, and there

is evidence to suggest that LIF is chemotactic for inflammatory cells within the nervous system (Gadient & Patterson, 1999).

As a family of cytokines, the effects of these molecules are very widespread, both *in vitro* and *in vivo*, affecting the nervous system (Yamamori et al., 1989; Li et al., 1995), haematopoietic system (Moreau et al., 1988), adipogenesis (Aubert et al., 1999), hepatic development and maturation (Baumann & Wong, 1989; Kamiya et al., 1999; Miyajima et al., 2000), kidney development (Barasch et al., 1999), maintaining pluripotency within the early embryo (Nichols et al., 1996), and the maternal expression of LIF is crucial to implantation of the blastocyst (Stewart et al., 1992). The functions of these molecules are complex, changing with the tissue involved and also the circumstances (reviews - Gomez-Lechon, 1999; Miyajima et al., 2000).

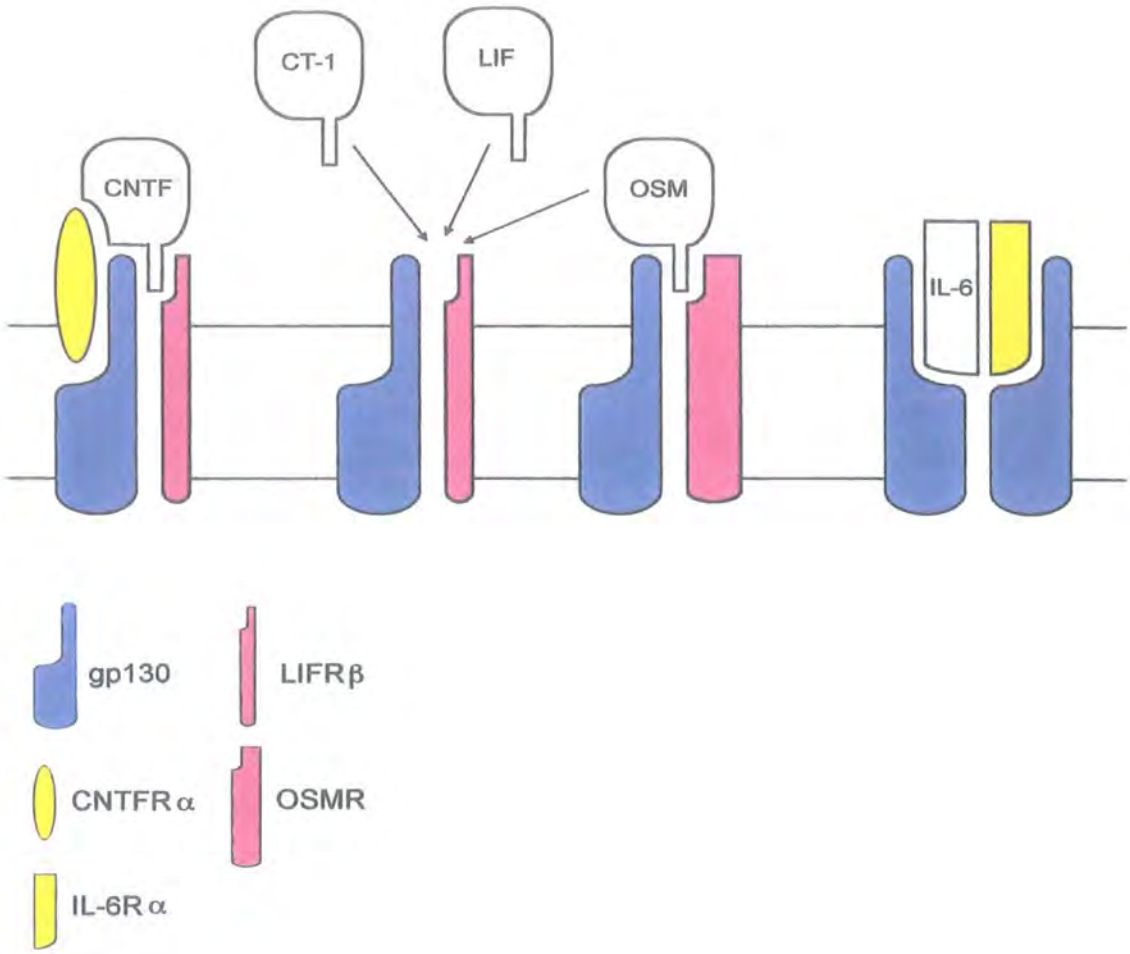
4.1.3. Cytokine receptor complexes and signalling pathways

4.1.3.1. Signalling at the cell membrane

The diversity of function within this family of cytokines is mediated to a degree by the various receptor components required to form functional complexes, the components and configurations of which are shown in Figure 4.1 (for reviews see Kishimoto et al., 1994; Bravo & Heath, 2000). When LIF binds with low affinity to the LIF receptor (LIFR) it induces this receptor to form a complex with gp130 so generating high-affinity binding sites for LIF (Gearing et al 1992). CT-1, like LIF, interacts directly with the LIFR and then gp130 is recruited (Pennica et al., 1995). OSM can bind directly to soluble gp130, so acting as an IL-6 antagonist (Sporeno et al., 1994), and interact with the LIFR/gp130 complex. There is also an OSM specific receptor which forms heterodimers with gp130. OSM therefore has two positive signalling mechanisms, potentially with separate signal transduction pathways (Gomez-Lechon, 1999), and an antagonistic mechanism, a good example of the diversity within

this family of cytokines. CNTF requires a third component, CNTFRα, to make a functional receptor complex (Conover et al., 1993).

Figure 4.1. gp130 receptor complexes for which the IL-6 family cytokines are ligands. IL-6 and CNTF require a specific α-subunit for signal transduction, as well as the gp130 homo- and hetero-dimer respectively. LIF, CT-1 and OSM all signal through gp130/LIFRβ heterodimers, and OSM can also signal via an OSMR/gp130 heterodimer.

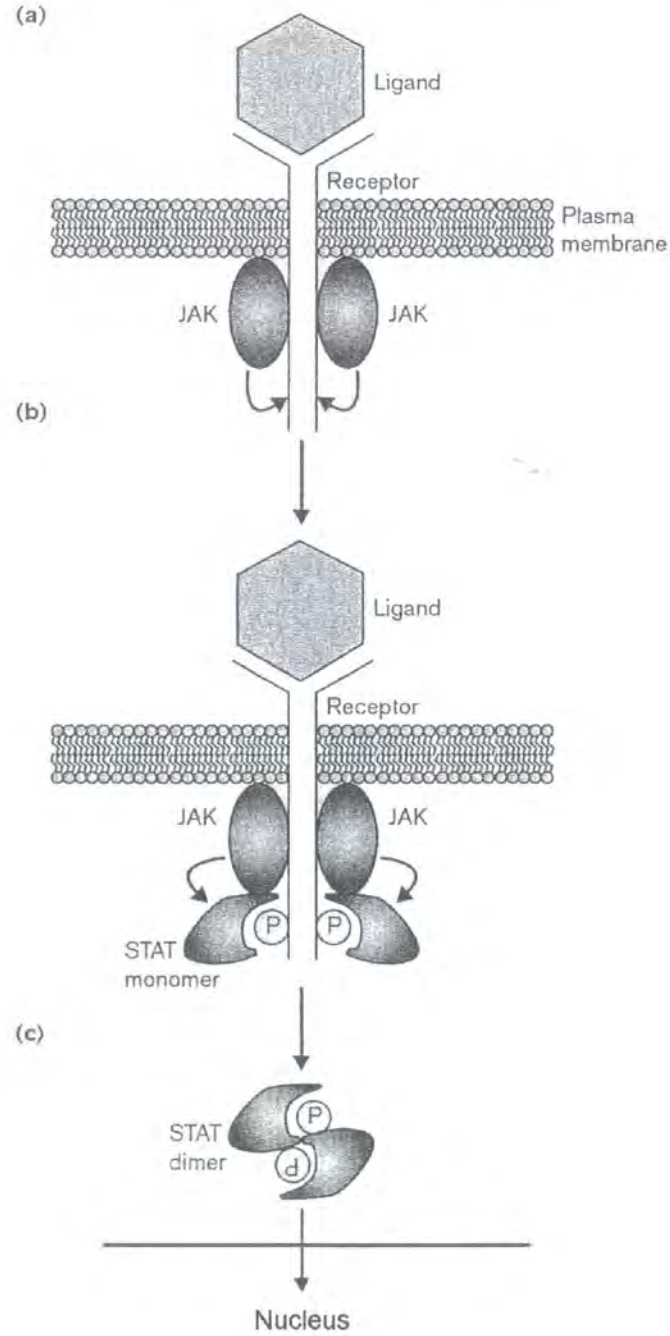


4.1.3.2. Intracellular signalling pathways

The signal from receptor complexes involving gp130 is transduced by two cascades independent of one another, the Ras/MAP kinase pathway, activated by the tyrosine kinase Hck (Ernst et al., 1994; 1996), and the Jak-STAT (STAT- signal transducer and activator of transcription) pathway (Ernst et al., 1996; 1999). The exact combination of events after gp130 activation varies hugely, depending on the ligand and the cell type, but in ES cells the result is maintenance of a pluripotent cell population. In ES cells, activation of gp130 by IL-6 bound to a soluble form of IL-6 receptor, is sufficient to maintain ES cells (Yoshida et al., 1994). ES cells do not express the membrane-bound IL-6 receptor, hence the gp130 molecules cannot form heterodimers (Saito et al 1992). The successful formation of gp130 homodimers initiated by the soluble IL-6 receptor, is a clear indication that gp130 is the key membrane bound receptor to initiate the intracellular signalling cascade that will maintain ES cells in culture.

Downstream of gp130, tyrosine-phosphorylation of STAT3 (Figure 4.2) is necessary for the DNA-binding activity of this transcriptional regulator (reviewed by Williams, 2000). ES cells consistently express STAT3, but tyrosine-phosphorylation of STAT3 only occurs in the presence of LIF (Boeuf et al., 1997). Expression of a dominant-negative mutant STAT3 protein in ES cells resulted in differentiation in the presence of LIF, providing the first evidence that tyrosine phosphorylation of STAT3 is crucial to LIF-dependent maintenance of ES cell pluripotency (Boeuf et al., 1997). Further work demonstrated a critical amount of STAT3 was not only essential, but its activation alone (at sufficient levels) is enough to maintain ES cells in an undifferentiated state (Raz et al., 1999; Matsuda et al., 1999; Ernst et al. 1999). Genes whose transcription is regulated by STAT3 are therefore potential candidates as effector molecules for self-renewal of ES cells.

Figure 4.2. The STAT signalling pathway.
The signalling process is simplified and broken down into three main steps: (a) ligand binding to the receptor results in activation of JAKs which tyrosine phosphorylate the receptor chains; (b) STAT monomers are recruited to these phosphorylated receptor chains and are subsequently tyrosine phosphorylated themselves by the JAKs; (c) phosphorylated STATs dissociate from the receptor, form dimers and translocate to the nucleus where they bind regulatory DNA elements. Williams, 2000.



4.1.4. Plasticity in adult cell populations

While the embryonic stem cell has the highest degree of plasticity, with the potential to differentiate along any lineage, it has been demonstrated that plasticity amongst adult somatic cells is more frequent than was previously thought (reviews - Seale & Rudnicki, 2000; Clarke & Frisen, 2001). The ability to direct cells along an alternative lineage enables the processes of cell specification and differentiation to be investigated. The ultimate aim of such research is to understand the mechanisms by which a blastocyst develops into an embryo, and how embryonic tissues differentiate to form complex structures.

The influence of neighbouring cells can dramatically affect a cell's phenotype and this is evident during development in many instances, the development of skin appendages being just one example (Hardy, 1992; Thesleff et al., 1995). During developmental processes, cells are understood to be receptive to inductive signals directing them along particular lineages. In adulthood however, the lineage of a cell is believed to be restricted within a specific tissue. Stem cells or progenitors give rise to transit amplifying cells that undergo a few rounds of proliferation, resulting in a number of tissue-specific, terminally differentiated cells. This belief has been challenged recently by the demonstration that the transit-amplifying population of the corneal epithelium is capable of producing epidermal keratins, glandular epidermis and hair follicles (Ferraris et al., 2000). Induced by exposure to embryonic dermal tissue, the basal epithelial cells lose their corneal phenotype before taking on other characteristics, suggesting there is a deprogramming step that occurs before reprogramming of the cells. Not only does this demonstrate the previously unrecognised plasticity of a somatic cell population, but provides further evidence of the inductive capabilities of embryonic dermal tissue.

4.1.5. Follicular dermal cell lineage

The inductive capacity retained by adult follicular dermal cells is one of a number of intriguing properties they possess. Whilst the lineage of the epithelial cells under their influence is relatively well understood, little attention has been paid to the development and differentiation of dermal papilla and dermal sheath cells. The dermis is derived from the mesoderm and experiments have shown that different regions of dermis derive from different mesodermal structures. The dorsal dermis, for example arises, from somites whilst the dermis of the head is a derivative of neural crest cells (Couly & Le Douarin, 1988; Oliver-Martinez et al., 2000). However, prior to somite formation, the unsegmented mesoderm has the ability to determine appendage patterning, indicating that cells are already fated to appendage lineages (Mauger & Sengel, 1970).

Patterning signals determine the site of formation of the dermal condensation, a crucial step in early follicle morphogenesis. The aggregative properties displayed by dermal papilla cells *in vitro*, and when implanted ectopically *in vivo*, have led to suggestions that cells of the dermal condensation retain their embryonic status through into adulthood (Jahoda & Oliver, 1984a). Further evidence to support this idea comes from the fact that a specialised ECM distinguishes these cells from other skin fibroblasts in development and adulthood, both *in vivo* and *in vitro* (chapter 2; Couchman & Gibson, 1985; Couchman, 1986; Messenger et al., 1991; Couchman, 1993; Couchman & du Cros, 1995). Indeed, many of the properties of dermal papilla and dermal sheath cells that have been demonstrated experimentally, can be explained by the cells being in a relatively undifferentiated state. Follicular dermal cells have the capacity to regenerate the end bulb of an amputated follicle (Oliver, 1966b; Jahoda et al., 1992a). They are also capable of inducing follicle formation when combined with an epithelium *in vivo* (Jahoda et al., 1993; Reynolds & Jahoda, 1992; Reynolds et al.,

1995). The type of follicle formed is determined by the dermal implant, a fact demonstrated by implanting vibrissal dermal tissue beneath ear epidermis for example, resulting in a thick vibrissa-type fibre being produced (Jahoda et al., 1993). At the neck of the follicle, the outer root sheath is continuous with the interfollicular epidermis, whilst the follicular dermal sheath is continuous with the dermal papillary layer of the skin (Jahoda & Reynolds, 2001). This has led to the proposal that dermal sheath cells play a role in wound healing, with follicular cells contributing to the interfollicular dermis, as outer root sheath cells contribute to interfollicular epidermis (Taylor et al., 2000; Jahoda & Reynolds, 2001). In a dermal sheath transplant it has been shown that the dermal papilla of the induced follicle was derived from the originally transplanted cells, showing that the cells are capable of changing phenotype (Reynolds et al., 1999). All these findings demonstrate that the follicular dermal cells possess stem cell-like qualities, and are able to instruct other "stem cells" to differentiate along a certain lineage.

4.1.6. Aims

The inductive and regenerative properties of the hair follicle dermal cells imply that they instruct cells in their immediate environment to undergo changes. ES cells are defined by their flexible, pluripotent nature and therefore provide an excellent tool to investigate inductive processes. I cocultured follicular dermal cells with ES cells, based on the hypothesis that the stem cells would be directed along an epithelial lineage. Early experiments demonstrated this was not the case so further investigation focussed on the nature of the reciprocal interactions between the cell populations, the level of differentiation that had occurred in the ES cell population, and the influence that the ES cells had on the dermal cells.

4.2. MATERIALS & METHODS

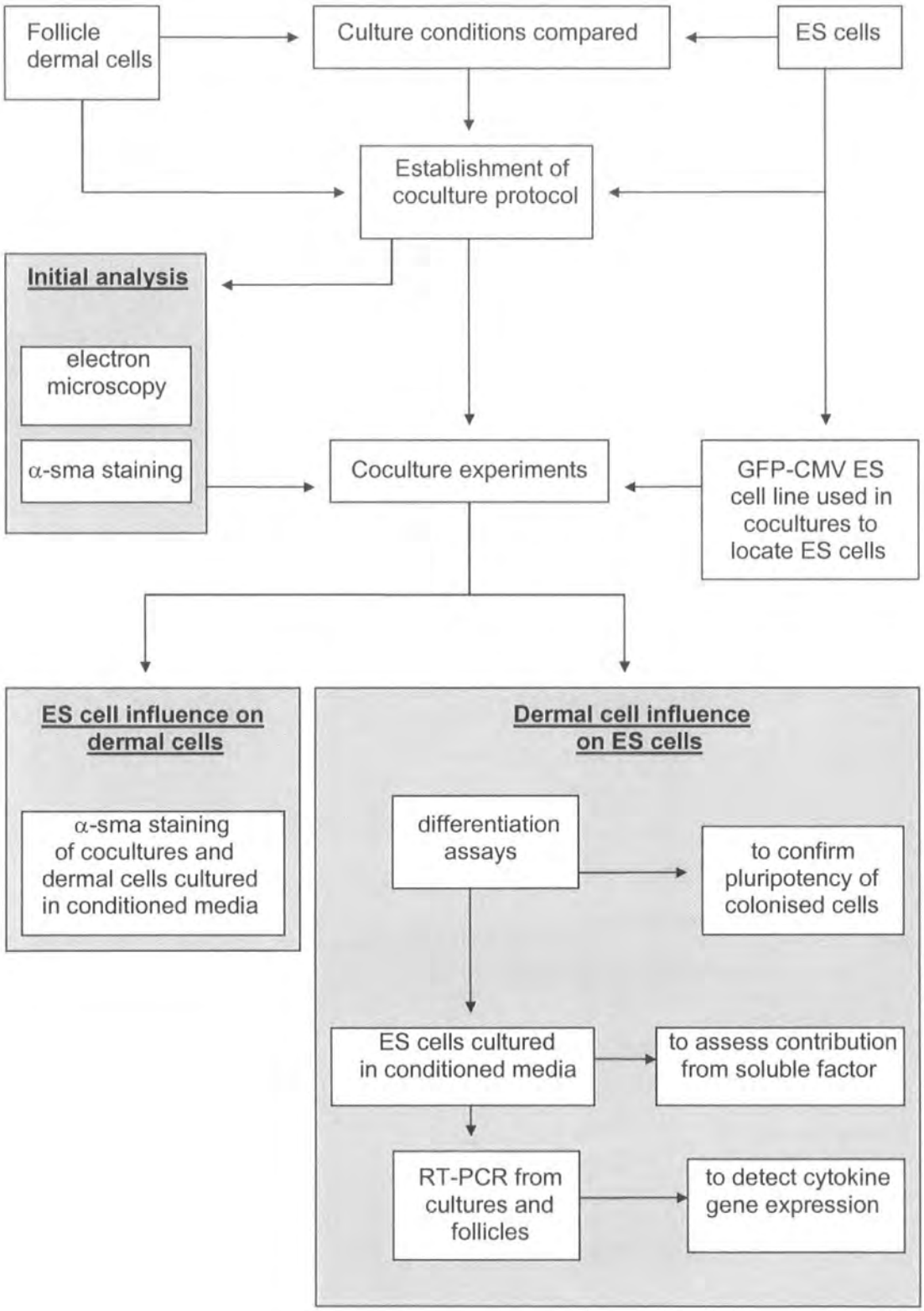
To investigate the interactions betweeen hair follicle dermal cells and embryonic stem cells, the two cell types were grown in culture together. Pilot experiments (described in Appendix II) were performed to determine culture conditions and to refine methodology. Figure 4.3 shows the progression from the pilot experiments to the more detailed analysis of cell interactions, characteristics and gene expression.

4.2.1. Cell culture

4.2.1.1. Vibrissa follicle dermal cells

Dermal cells were obtained from the vibrissa follicles of adult PVG rats or Zin40 mice, aged between 2 and 6 months, by the dissection method previously described (2.2.1). After exposure of the follicles and removal of surrounding connective tissue the follicle end bulbs were cut off with curved scissors and placed in MEM (Gibco) containing 50μg/ml gentamicin. (At this stage the follicles were sometimes kept at 4°C overnight before continuing with the next step.) Each individual follicle bulb was then placed in a drop of MEM on the lid of a petri dish and microdissected using watchmakers forceps under a Zeiss Stemi SV11 microscope. The bulb was inverted to expose the dermal papilla (DP) and dermal sheath (DS) which remained attached to the collagen capsule. The DP and DS were cleaned of epithelial cells as much as possible before separating them from the capsule. The DP was then separated from the DS and the components collected in two 35mm culture dishes containing 2ml MEM with 20% foetal bovine serum (FBS). The dermal tissue was then torn with forceps and pressed to the bottom of the dish to aid attachment and encourage cell growth. The cultures were incubated at 37°C in 95%O_2/5%CO_2 and left undisturbed for at least eight days. They were subsequently maintained in MEM with 20% FBS.

Figure 4.3. Flow diagram outlining the progression of experiments and the methods involved at each stage of the investigation.



4.2.1.1.1. Passaging dermal cells

Explant cultures of dermal cells were passaged to 25cm^2 flasks once cells were growing well, usually between 2 and 4 weeks after the initial explant was plated out. 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 2-3 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 1800rpm for 3 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.

4.2.1.2. Skin fibroblasts

Ear skin or footpad skin was used as the source of "skin" dermal fibroblasts. Ear skin was first shaved with a scalpel blade to remove as much hair as possible. The skin was scored with a scalpel to the depth of the connective tissue beneath and the layer of skin carefully removed with as little connective tissue associated with it as possible. Two to three pieces of skin were collected by this method and placed in MEM. In a dry 35mm culture dish the skin was then minced with fine scissors to give pieces no bigger than 1mm^2. These were spread over the surface of the dish and left to adhere for 1 minute before adding 2ml MEM with 20% FBS. The culture was then left to grow at 37°C in 95%O_2/5%CO_2. Skin fibroblasts were passaged as described in 4.2.1.1.1.

4.2.1.3. RDP-B cells

A spontaneously transformed cell line of rat dermal papilla cells, RDP-B (Reynolds et al., 1991), was maintained in MEM with 10% FBS. These cells were passaged as described in 4.2.1.1.1.

4.2.1.4. Embryonic stem cells

The established murine embryonic stem cell line, CGR8 (Mountford et al., 1994) was used for all investigations. Cells were grown on 0.1% gelatin-coated tissue culture plastic and maintained in CGR8 medium (Glasgow MEM containing non-essential amino acids, 0.25% $NaHCO_3$, 2mM L-glutamine, 1mM pyruvate, 100μM 2-mercaptoethanol and 10% FBS; Gibco BRL) supplemented with LIF as described by Smith (1991). At all times, unless specifically stated, the culture medium contained 5,000U/ml Penicillin and 5,000μg/ml Streptomycin (Gibco). CGR8 medium supplemented with LIF will be referred to as complete CGR8 medium. ES cells were passaged by washing with PBS, incubating with trypsin-versene-PBS (TVP) at 37°C for 5 minutes and stopping the reaction with CGR8 medium. The cell suspension was then transferred to a sterile tube, centrifuged at 1000rpm for 5 minutes and the cell pellet resuspended in CGR8 medium. The cell suspension was then transferred to gelatin-coated flasks for routine maintenance, or the cells were counted using a haemocytometer for setting up co-cultures.

For some studies green fluorescent protein (GFP) labelled ES cells were used to distinguish those of ES cell origin from dermal cells. To obtain labelled cells, CGR8 cells were transfected with a CMV-GFP vector (gift from John Mason, Edinburgh) by electroporation. Stable colonies were selected using 2μg/ml puromycin and expanded.

These cells are referred to throughout the text as CMV-GFP ES cells. This cell line was developed and provided by Majlinda Lako (Durham University).

4.2.1.5. ES/dermal cell co-cultures

Pilot studies demonstrated no difference in cell behaviour between two methods of setting up co-culture. To achieve a degree of consistency in dermal cell confluency and ES cell numbers the following method was used in all subsequent co-culture experiments.

4.2.1.5.1. Simple co-culture

Dermal cells of passage number 2 to 7 (see Table 4.1 for numbers) were grown in 35mm dishes to form a monolayer of 60-80% confluency. ES cells grown in culture flasks, as described above, were trypsinised using TVP, spun down, resuspended in CGR8 medium to give a single cell suspension and $3.1x10^5$ cells added per dish in a minimal volume. The total volume of media was made up to 2ml per dish with CGR8 medium (no exogenous LIF).

4.2.1.5.2. Exposure of dermal cells to co-culture media through porous membrane

Co-cultures were grown on porous membrane inserts (0.45μm pore size, Falcon) over 6-well plates containing dermal cells for up to 6 days. Z40 DS and DP, and PVG DP cells (all passage number 3) were used for these experiments, once for each cell type and each was done in duplicate with two positive controls cultured alone in CGR8 medium. The PVG DP cultures were exposed to co-culture medium for 5 days before comparing the α-smooth muscle actin expression in the cells grown in the well beneath by immunocytochemistry (see 4.2.2.2. for method). One of the control cultures was

used as a negative control (no primary antibody) for the α-smooth muscle actin immunostaining.

Table 4.1. Number of sets of ES cell co-cultures with each dermal cell type. Co-cultures were set up with dermal papilla cells (DP), dermal sheath cells (DS), and ear or footpad (FP) skin fibroblasts (SF) that had been passaged between 2 and 7 times. For each set of co-cultures the number of replicate dishes ranged from 2 to 9. The RDP-B cells are a transformed cell line so passage number is not applicable but was >10 at the time of use.

	PVG				Zin40		
Passage number	DP	DS	SF Ear	SF FP	RDP-B	DP	DS	SF Ear	SF FP
2	1		1	1	N/A	2	2		2
3	1	4			N/A	1	2		
4	4	3			N/A	3	3		
5	2	2			N/A			1	
6		1			N/A				
7				1	N/A				
Total	8	10	3		4	6	7	3	

4.2.2. Initial Analysis

4.2.2.1. Electron Microscopy

Cells were fixed in 50:50 Karnovsky's fixative:sodium cacodylate buffer for 1 hour at 4°C (Karnovsky, 1965). The fixative was then removed and 0.5% osmium tetroxide in sodium cacodylate buffer was added and the cells were left at 4°C for 50 minutes. Dehydration was then carried out through a series of alcohols starting with 70%, then 95% and finally 100%. Three 5 minute washes of each (10 minutes in 100%) were done before progressing onto the next alcohol.

During the dehydration period the embedding medium (10ml araldite CY212, 10ml DDSA, 1ml dibutyl pthalate and 0.4ml BDMA) was prepared with araldite,

dodecenylsuccinic aldehyde (DDSA) and n-Benzyldimethylamine (BDMA) pre-warmed to 40°C. The reagents were all mixed thoroughly to ensure even polymerization.

The final 100% ethanol wash was aspirated, replaced with a 1:1 mix of 100% ethanol and araldite mix and the dishes left open in a fume hood for 30 minutes to allow the ethanol to evaporate. The ethanol/araldite was then removed and approximately 0.5ml embedding medium added to form a very thin layer covering the cells which was incubated at room temperature for 1-2 hours. This was then replaced with fresh embedding medium and incubated at 60°C for 24 hours. At the end of this incubation the embedded cells were left in the dish for re-embedding at a later date.

The layer of embedded cells was removed from the plastic dish and cut into small strips (2mm wide) using a scalpel. These were washed in 70% ethanol and dried at 37°C. Each individual strip was then immersed in a labelled mould containing fresh embedding medium, before being incubated at 60°C for 24 hours.

Semi-thin (1μm) sections were cut on a C. Reichert (Austria) Om U3 ultramicrotome, mounted on slides and allowed to dry before staining with toluidine blue. Ultra-thin sections (60-90nm) were cut on an ultramicrotome (as for semi-thins) and mounted on a copper grid. The sections on the grid were then coated as follows in preparation for analysis by transmission electron microscopy (TEM). The grid was placed face down in 1% uranyl acetate in 70% ethanol for 10 minutes then washed thoroughly by dipping twice in dH_2O. The grid was then placed face down in Reynolds lead citrate (Reynolds, 1963) for 10 minutes before washing thoroughly as before.

Analysis of the semi-thin sections was carried out on a Zeiss Axiovert 135 microscope, whilst the ultra-thin sections were viewed by TEM (Philips EM 400T/ST Analytical TEM/STEM system).

4.2.2.2. Immunocytochemistry

Cultured cells, on plastic or glass coverslips, were washed twice with PBS. They were then fixed in either methanol (2 min., -20°C) or, for CMV- GFP ES cell co-cultures, 4% paraformaldehyde (20 min., room temperature). After two washes with PBS, primary antibody (Table 4.2) diluted in PBS sterilised by passage through a 0.2um filter, was applied to the cells and incubated at room temperature for 1 hour. Unbound antibody was removed with four 5 minute PBS washes before adding the secondary antibody (Table 4.2) diluted in filter-sterilised PBS. The slides were incubated at room temperature for 45 minutes in the dark then the unbound antibody was removed by PBS washes as before. The stained cells were mounted under glass coverslips in mowiol, a glycerol-based mountant containing an anti-fade reagent (recipe in Appendix I). Initial observations were done on a Zeiss Axiovert 135 microscope. Subsequent analysis was carried out on a confocal laser-scanning microscope (MicroRadiance scanning system, BioRad). All images were processed using Adobe Photoshop 3.0 software.

Table 4.2. Source and dilution factors of antibodies for immunocytochemistry

Antibody	Dilution	Source
anti-α-smooth muscle actin (monoclonal)	1:10	Prof. G. Gabbiani, University of Geneva
anti-albumin (polyclonal)	1:100	DAKO
anti-alpha-1-fetoprotein (polyclonal)	1:50	DAKO
NF200 (polyclonal)	1:100	Sigma
AlexaFluor 546 anti-mouse	1:100	Molecular Probes
AlexaFluor 546 anti-rabbit	1:100	Molecular Probes
FITC anti-mouse	1:80	DAKO

4.2.3. Differentiation assays

4.2.3.1. ESC plucking

This process is summarised by Figure 4.4. From each co-culture, at each timepoint, three lots of ES cells were plucked and plated out. Two of the three samples were then used for the differentiation assays (the third sample was in reserve and discarded once the differentiation assays were established).

To isolate ES cells from co-cultures the medium was removed and the cells washed twice with warmed PBS. 30µl TVP (see Appendix I) was taken up in a yellow pipette tip and repeatedly expelled and aspirated over a small area of the co-culture until small gaps could be seen in the cell layer. The contents of the pipette tip were then expelled into a 0.1% gelatin-coated 35mm tissue culture dish and the cells grown up in complete CGR8 medium until cell numbers had reached approximately $3.5x10^6$. At this stage the cells were trypsinised and started on the differentiation assays.

The methods for these differentiation assays are detailed below and summarised in Figure 4.5.

4.2.3.2. Blood Assay

$2x10^6$ cells were suspended in 10ml complete CGR8 medium, transferred to a $25cm^2$ culture flask (uncoated) and incubated for 2 days at 37°C. On day 2 the embryoid bodies (EB) (Martin & Evans, 1975) were collected by repeatedly pipetting medium over the layer of cells and this was then aspirated and centrifuged for 3 minutes at 1000rpm. The supernatant was discarded and the collected EBs were resuspended in 10ml fresh CGR8 medium (no exogenous LIF). They were then transferred to a bacteriological Petri dish for 2 days incubation. On day 4 the EBs were collected, spun down and resuspended in fresh CGR8 medium before incubation in a bacteriological Petri dish for a further two days. On day 6 the CFU-A assay was set up.

Figure 4.4. Method for isolating ES cells, from cocultures, for subsequent differentiation assays.

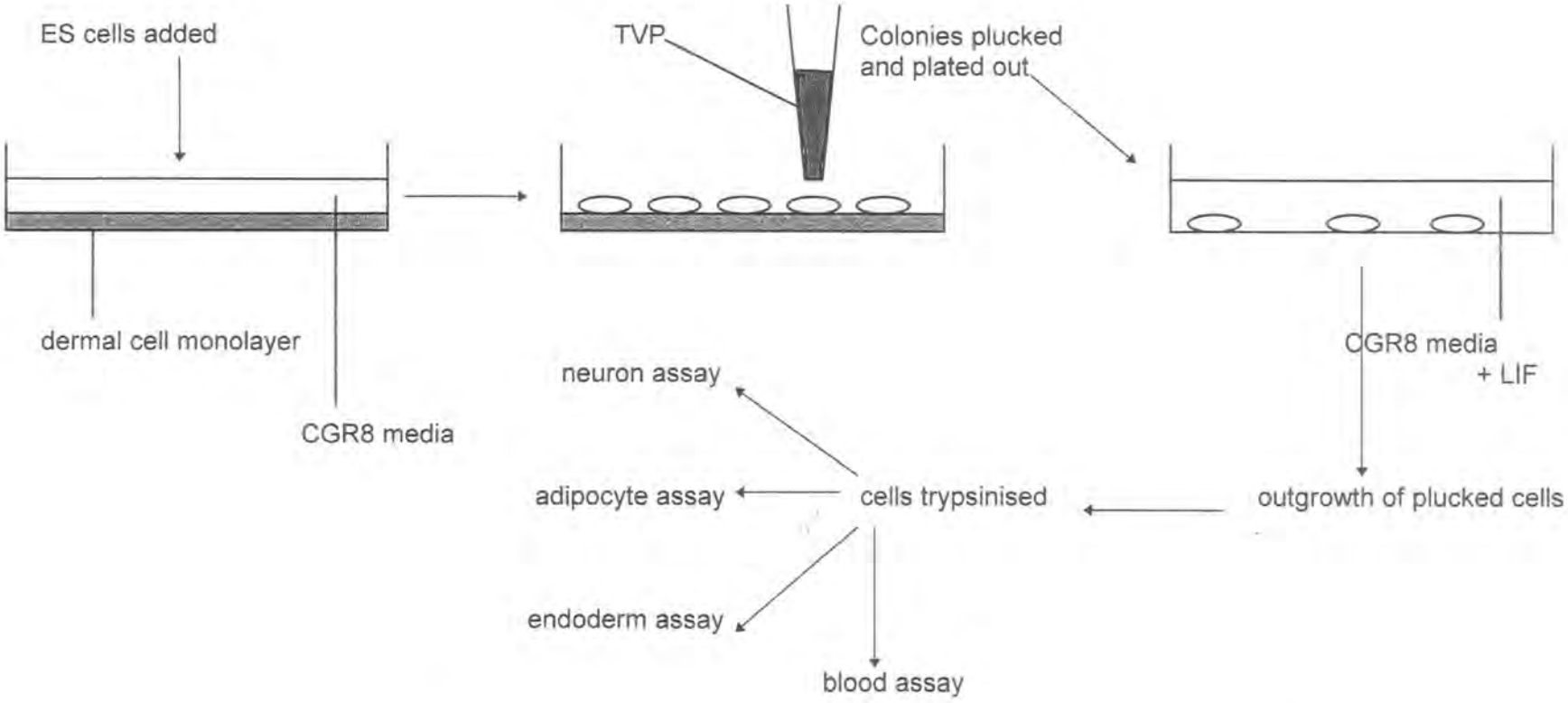
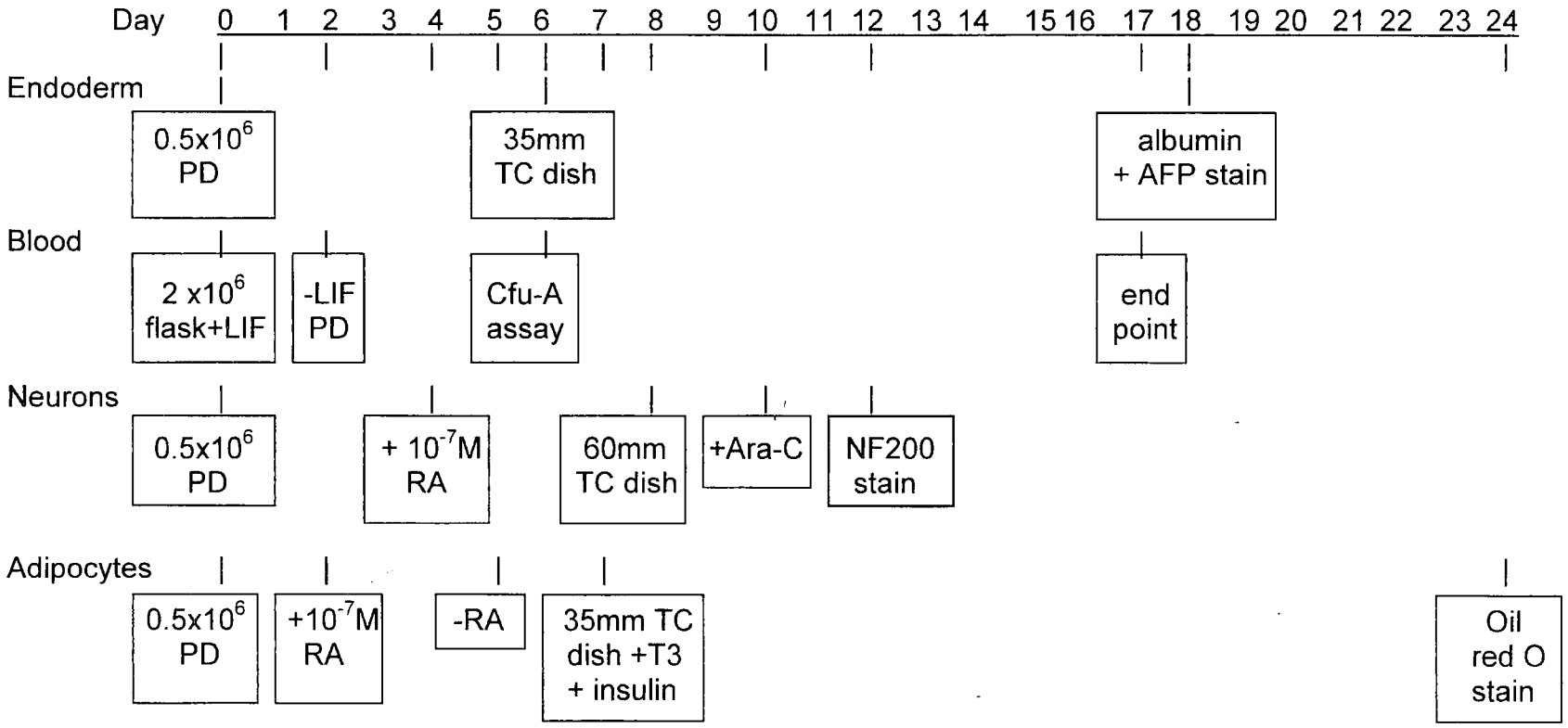


Figure 4.5. Schematic representation of protocols for differentiation assays.

Cells were counted and resuspended in CGR8 medium (+LIF for blood assay) in the vessel indicated on day 0. Changes in culture conditions are indicated. Medium was changed every 2 days between the steps shown, except day 10 of the neuron assay when cytosine arabinoside (AraC) was added to the medium present. PD - Petri dish, TC - gelatin-coated tissue culture, AFP - alpha-feta-1-protein, RA - all-trans retinoic acid, NF - neurofilament, LIF - leukemia inhibitory factor.



4.2.3.2.1. CFU-A assay

The assay is based on the method described by Pragnell et al. (1994). The assay was carried out in 35mm Petri dishes and consisted of a sandwich of Noble agar, the bottom layer at 0.6% with added cytokines, and the top layer, containing EBs, at 0.3%. Agar was melted at 60°C then cooled to 37°C. The mix for the bottom layer of the dishes consisted of 1.2% Noble agar in CFU-A media (see Appendix I) with L929 conditioned media and AF19 conditioned media (these provide CSF-1 and GM-CSF respectively) (Pragnell et al., 1994). 1ml of this mix was added to each of three dishes and allowed to set before making the mix for the top layer which consisted of approximately 150 Ebs in 0.3% Noble agar in CFU-A media. 1ml of this top layer mix was added to each dish to get an even distribution of EBs between dishes. The plates were left to set before being incubated in 10% CO_2 at 37°C. Dishes were left for 11 days and the results analysed by inspecting the dishes using low power microscopy.

4.2.3.3. Neuron Assay

This method is modified from Bain et al. (1995). $0.5x10^6$ cells were transferred to a bacteriological Petri dish and the volume made up to 10ml CGR8 medium. They were then incubated for 2 days at 37°C. On day 2 the EBs were collected by centrifugation (3min. 800rpm) and resuspended in 10ml fresh CGR8 medium. The EBs were incubated as before for a further 2 days. On day 4 the medium was changed as before and all trans-retinoic acid (Sigma) was added to a final concentration of 10^{-7}M (10^{-5}M stock made up in 100% ethanol and stored at -20°C). This was repeated on day 6. By day 8, the EBs were at the stage designated by Bain et al (1995) as 4-/4+. Approximately 50 EBs were transferred to gelatin-coated 60mm dishes (three plates were set up for each assay). These were then incubated with 5ml CGR8 medium -

2ME (2-mercaptoethanol prohibits differentiation so is left out of the medium from this stage) for 2 days. After this the majority of the EBs had attached and cells, including some neuron-like cells, were growing out of them. Cytosine arabinoside (Ara-C) was added to a final concentration of 18μM on day 10 and the dishes were incubated for a further 2 days to optimize neuron-like cell numbers.

Immunocytochemistry was carried out on day 12 on differentiated cells from a 6d PVG DP/ES cell co-culture (n = 1). The method was as described in 4.2.2.2., using NF200, a primary antibody specific to neurofilament.

4.2.3.4. Adipocyte Assay

This method was carried out according to Dani et al. (1997). $0.5x10^6$ cells were transferred to a bacteriological Petri dish and the volume made up to 10ml with CGR8 medium. They were then incubated for 2 days at 37°C. On day 2 the EBs were collected by centrifugation (3min. 800rpm) and resuspended in 10ml fresh CGR8 medium with 10^{-7}M all-trans retinoic acid (Sigma). This was repeated on days 3 and 4 giving the EBs three days exposure to retinoic acid. This was withdrawn on day 5 and the cells were resuspended in CGR8 medium. On day 7 the EBs were collected as described previously and approximately 40 EBs were plated out on each of three gelatin-coated 35mm dishes and incubated in 2ml CGR8 medium with 2nM triiodothyronine (T3) (Sigma) and 85nM insulin (Sigma). The cells were maintained in this medium which was changed every two days until day 24 when they were stained with Oil red O to detect lipid (Green & Kehinde, 1974).

4.2.3.4.1. Oil red O staining

Cells were washed twice in PBS then fixed in calcium formol (4% formaldehyde, 1% $CaCl_2$) for 1 hour. They were then incubated in 60% isopropanol for 15 minutes before being stained in filtered Oil red O stain (3 parts saturated solution of oil red O in 99% isopropanol, 2 parts distilled water) for 15 minutes. This was washed off with a brief (15 seconds) rinse of 60% isopropanol followed by washes with distilled water. The cells were then counterstained with haematoxylin (3 minutes), washed well in tap water and given a final rinse in distilled water before being mounted in aquamount (BDH).

4.2.3.5. Endoderm Assay

$0.5x10^6$ cells were transferred to a bacteriological Petri dish and the volume made up to 10ml CGR8 medium. They were then incubated for 2 days at 37°C. On day 2 the EBs were collected by centrifugation (3min., 800rpm) and resuspended in 10ml fresh CGR8 medium. The EBs were incubated as before and on day 4 the medium was changed as on day 2 before incubation at 37°C for a further 2 days. On day 6 the EBs were plated out on 35mm 0.1% gelatin-coated dishes and maintained in CGR8 medium. On day 18 the cells were stained with antibodies against albumin and alpha-1-fetoprotein (DAKO) according to the method in 4.2.2.2.

4.2.4. Conditioned Medium

In the routine maintenance of cultures, medium was removed from dermal cell cultures and dermal/ES cell co-cultures after 48 hours, centrifuged (30 min. at 3,300rpm) to remove cell debris, and the supernatant stored at -20°C for future use.

4.2.5. Microscopy

Live and oil red O stained cultures were viewed by phase contrast microscopy on a Zeiss Axiovert 135 microscope. Images were taken with a Contax 167MT camera on 35mm film, or a Spot RT digital camera and RT software (Diagnostic Instruments Inc.). Fluorescent immunostaining was analysed by confocal laser scanning microscopy (Microradiance scanning system, LaserSharp 2000, BioRad).

4.2.6. Molecular Analysis

4.2.6.1. RNA extraction from cultured cells

The RNA was extracted from cultured cells by the following method using Stratagene's microRNA kit according to the manufacturers protocol. RNA was extracted from either an entire dish of cells or a plucked "colony" of cells from the co-cultures. The cells were first washed twice with PBS/EDTA then colonies were plucked in 20µl trypsin (0.25%) and transferred to an eppendorf containing 0.5ml lysis buffer. For whole dishes 0.5ml lysis buffer was added to the dish to lyse the cells and the lysed cell solution transferred to a clean microcentrifuge tube. (In some instances 1ml lysis buffer was added to dishes with large numbers of cells and the lysed cells split between two microcentrifuge tubes.) 50µl 2M sodium acetate (pH4.0) was added to each tube followed by 0.5ml phenol (equilibrated to pH5.3-5.7 with succinic acid) and 200µl chloroform:isoamyl alcohol. The lid was then closed and the tube vortexed vigorously until a uniform white suspension was seen, followed by centrifugation for 5 minutes at 14,000rpm. The aqueous phase was then transferred to a clean microcentrifuge tube containing 0.5ml isopropanol (+ 5µg glycogen carrier for RNA extracted from single colonies). The tube contents were mixed by inversion and incubated at -20°C for several hours (ideally overnight).

After the required time the RNA was pelleted by centrifugation at 14,000rpm for 30 minutes at 4°C. The supernatant was removed and the pellet washed with 75% ethanol/25% DEPC-treated H_2O before being left to air dry. The RNA pellet was resuspended in DEPC-treated H_2O, then aliquoted and stored at -80°C.

RNA was extracted from 6d co-cultures, Zin40 dermal cell cultures, ES cells maintained by frequent passaging and addition of LIF, and ES cells that were cultured in the presence (+LIF) or absence of LIF (-LIF) for 6 days (no passaging).

4.2.6.2. RNA extraction from mouse vibrissa follicles

Initially RNA was extracted from vibrissa follicles that had not been sorted according to their cycle stage. Subsequently extractions were done from mid-anagen vibrissa follicles. The follicles were dissected out and placed in MEM. Once all the follicles were collected they were trisected with a scalpel into end-bulb, mid-follicle and upper follicle (Figure 4.6) and the pieces put into the appropriate glass homogenizers which were on ice and contained 330µl lysis buffer. The tissue was then homogenized over ice for 5 minutes, the homogenate transferred to a microcentrifuge tube and the RNA extraction carried out as described in 4.2.6.1.

4.2.6.3. DNase treatment of RNA

Ambion's DNase-free kit was used to remove any contaminating genomic DNA from the extracted RNA by the following method. 5µl 10xDNase I buffer and 3U DNase I were added to 50µl RNA in a microcentrifuge tube and incubated at 37°C for 25 minutes. 5µl DNase inactivation reagent was then added, mixed with the tube contents by flicking and incubated at room temperature for 2 minutes. This was then

pelleted by centrifugation (1 minute, 14,000rpm) and the supernatant containing the DNA-free RNA transferred to a clean microcentrifuge tube.

4.2.6.4. cDNA Synthesis

1μg random hexamers (Promega) was added to RNA in DEPC-treated dH_2O to a final volume of 15μl and incubated at 70°C for 5 minutes. The samples were then chilled on ice and the tubes briefly centrifuged. 5μl 5x RT buffer + 5μl 2.5mM dNTPs were then added followed by 200U M-MLV reverse transcriptrase (RNase H Minus, Point mutant) enzyme (Promega). For each RNA sample negative controls (no RT enzyme) were also performed. The reactions were then incubated at 37°C for 1 hour before being heated to 74°C for 15 minutes and finally chilled on ice.

4.2.6.5. Polymerase Chain Reaction (PCR)

Each PCR consisted of a sample of cDNA in PCR buffer with 0.2mM dNTPs, 1-2mM $MgCl_2$ (see Table 4.3), 0.5μM of each primer and 1U Taq polymerase in a final volume of 15μl. cDNA negative controls were tested with GAPDH primers to ensure the RNA was free of contamination. All PCR reagents were from Gibco except the primers which were from Cruachem Ltd (Genosys for CNTFRα and STAT3 primers). The samples were treated as follows:

Step 1: 94°C (2 min)

Step 2: 30 cycles of 94°C (20s), annealing temp. (Table 4.3) (30s), 72°C (40s)

Step 3: 72°C (10 min)

After the reaction the samples were cooled on ice then aliquots were electrophoresed on a 1.2 - 1.5% TAE/agarose gel depending on the size of the

expected PCR product. Images of agarose gels were recorded on BioRad's Gel-Doc System and the images processed using Adobe Photoshop 3.0.

Figure 4.6. Diagram indicating levels at which vibrissa follicles were cut to separate end bulb, mid-follicle and upper follicle sections for RNA extraction. The upper cut was made level with the ringwulst whilst the lower cut was made above the end bulb below the point of entry of the deep vibrissal nerve.

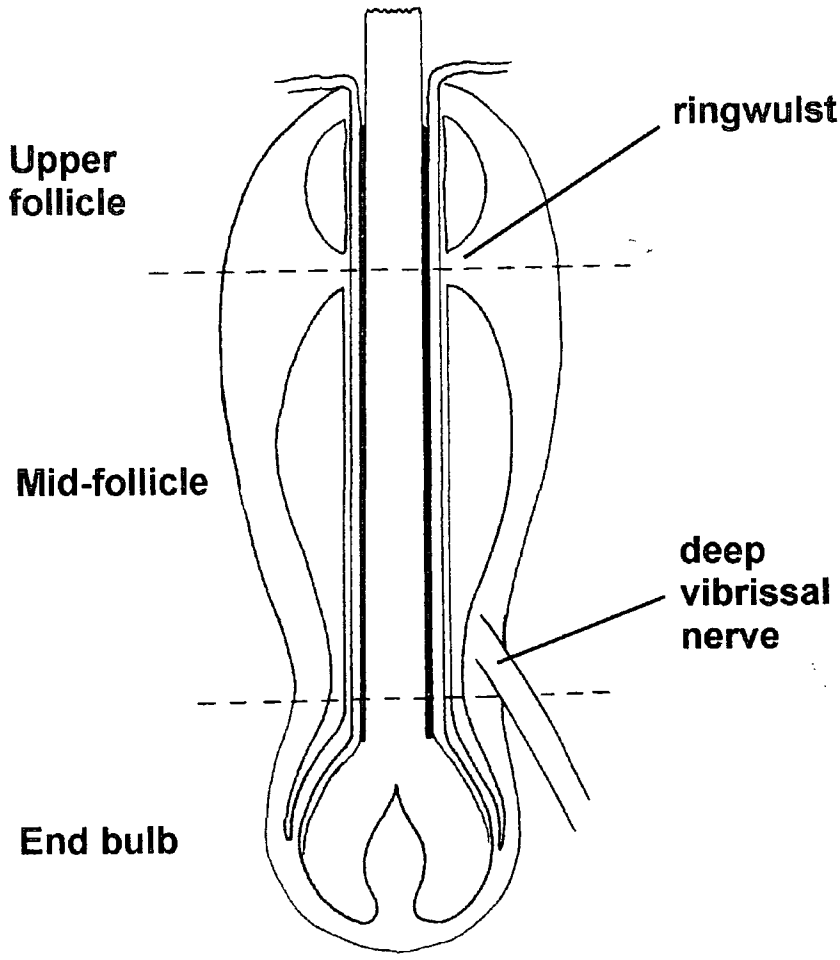


Table 4.3. Primers and PCR conditions

All primers were designed against published murine sequences except GAPDH1 and GAPDH2 which were designed against rat sequence but can be applied to mouse cDNA for RT-PCR.

Primer pair	Oligo (5'-3')	[$MgCl_2$] mM	annealing temp. (°C)	Product size
LIF for	ATT GTG CCC TTA CTG CTG CT	1.5	61	583bp
LIF rev	GCC TGG ACC ACC ACA CTT AT			
CT-1 for	GAG GAA TAC GTG CAG CAA CA	2.0	57	389bp
CT-1 rev	AGC ACC TTG GCT GAG AAG AT			
CNTF for	CTT TCG CAG AGC AAT CAC CT	1.5	61	579bp
CNTF rev	CCC CAT AAT GGC TCT CAT GT			
CNTFRα for	CTG TTT CCA CCG TGA CTC CT	2.0	59	802bp
CNTFRαrev	TGG GAC ACT GGT CAA GAA GA			
OSM for	CAC GGC TTC TAA GAA CAC TGC	2.0	59	547bp
OSM rev	CGA TGG TAT CCC CAG AGA AA			
GAPDH1	GCC AAA AGG GTC ATC ATC TC	1.5	61	379bp
GAPDH2	ACG GAT ACA TTG GGG GTA GG			

4.3. RESULTS

4.3.1. Observations from cell cultures

4.3.1.1. Dermal cell culture

PVG rat dermal papilla (DP) and dermal sheath (DS) cell cultures displayed typical morphology and behaviour in initial explant cultures and subsequent passages. In particular, characteristic aggregations were seen in post-confluent DP cultures (Jahoda & Oliver, 1984a). It was also observed that DP cells had a slower rate of growth than DS cells.

Zin40 mouse DP and DS cells cultured in the same conditions had a noticeably faster rate of growth than the corresponding PVG cells although the growth rate of DP was still slower than that of DS. The mouse cells were also more compact in culture than their PVG rat counterparts and the DP cell aggregation behaviour was less prominent. An example of each cultured cell type is shown in Figure 4.7.

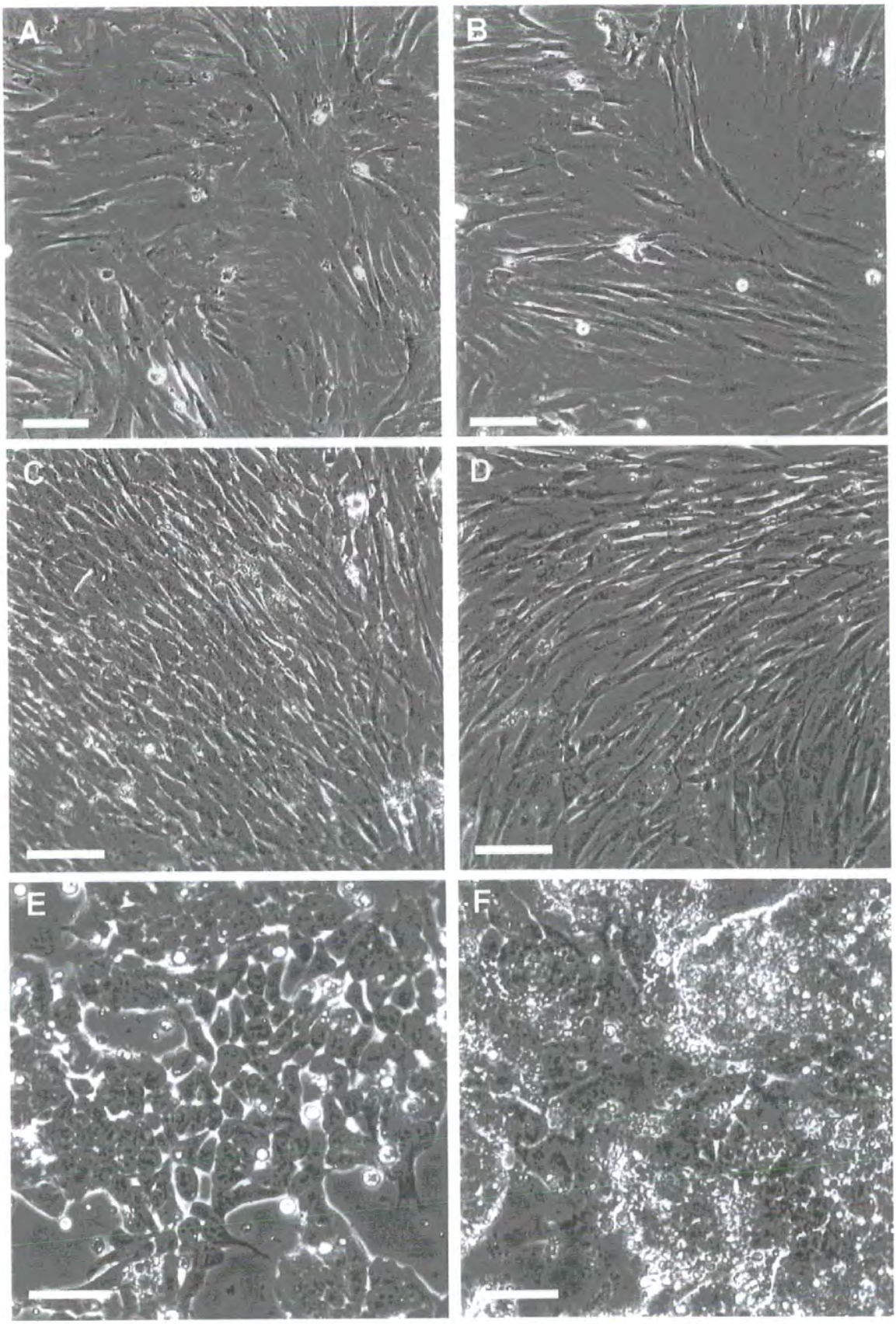
4.3.1.2. Embryonic stem cell culture

ES cells, cultured on gelatin-coated tissue culture plastic, grew in colonies and displayed typical morphology as small, compact cells with distinct nucleoli and a high nuclear:cytoplasmic volume ratio (Fig 4.7 E). LIF withdrawal resulted in these cells beginning to change characteristics and show evidence of differentiation in a very short period of time. As shown in Figure 4.7 F, within 6 days of LIF withdrawal considerable changes occurred as nucleoli became less pronounced, some cell death occurred, many cells were in suspension and multiple morphologies were evident in a single culture.

Figure 4.7. Typical morphology of cell types.

Dermal papilla (A & C) and dermal sheath (B & D) cells have fibroblast-like morphology with Zin40 cells (C & D) appearing more compact than the comparable PVG cells (A & B). ES cells maintained with LIF are small and compact with distinct nucleoli (E) compared to ES cells after 6 days without exogenous LIF when they show more varied morphology and nucleoli are less obvious (F).

Scale bar: A - D = 100μm, E & F = 50μm.


A
B
C
D
E
F

4.3.1.3. Co-cultures

The pilot experiments carried out to establish suitable culture conditions in which to conduct these experiments are described in Appendix II. From these preliminary studies several key observations were made: A) dermal cells were capable of supporting ES cell growth in otherwise unsuitable culture medium; B) medium conditioned by dermal cells was not sufficient to maintain viable ES cells; C) a characteristic morphology developed in ES/dermal cell co-cultures whether they were plated out as a mixed cell population, or ES cells added to an established dermal cell monolayer. All subsequent co-cultures were prepared by establishing a dermal cell monolayer prior to addition of ES cells in the form of a single-cell suspension, and experiments were carried out in CGR8 (ES cell) medium.

4.3.1.3.1. Morphology of co-cultures

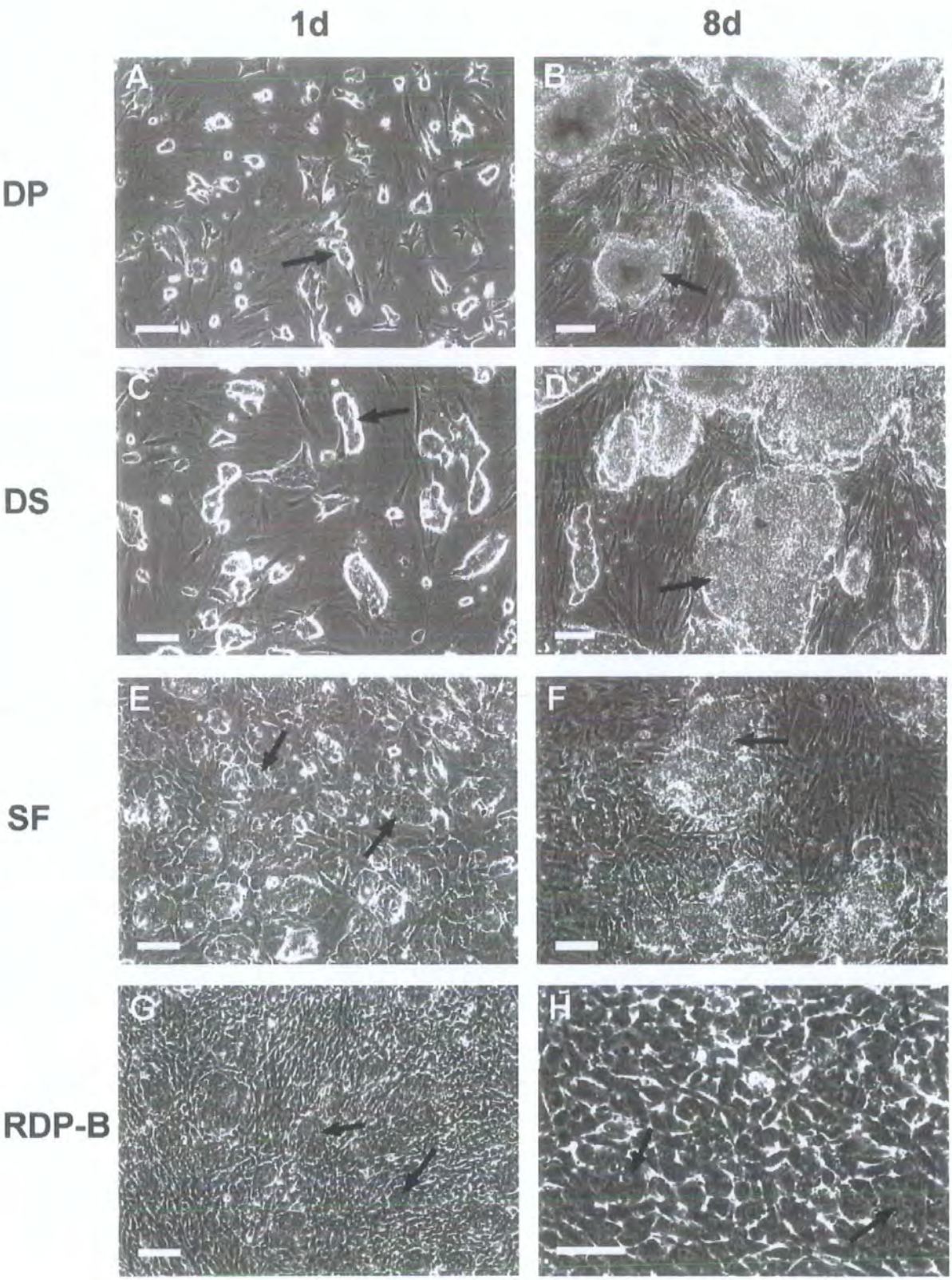
When ES cells were co-cultured with follicle dermal cells the differences in morphology were such that the two cell types were easily identifiable, with ES cells settling in discrete colonies on top of the dermal cell monolayer (Fig. 4.8 A-D). This was a very quick process as when ES cells were added to dermal cells and washed off instantly with medium, a good number of cells remained attached to the dermal cell layer. Distinguishing the two cell types was also straightforward when the cells were plated as a mix, rather than adding ES cells to an established monolayer of dermal cells. The ES cells remained small and compact within the colonies, whilst the dermal cells retained their fibroblast-like morphology.

The development of ES cell colonies was observed in co-cultures with DP and DS cells originating from both PVG rats and Z40 mice. To establish if this effect on ES cells was a feature common to all dermal cells, co-cultures were set up with PVG dermal skin fibroblasts from two body sites, footpad and ear. With both of these, whilst

Figure 4.8. Dermal cell/ES cell coculture morphology after 1d (A, C, E, G), 8d (B, D, F) and 4d (H).

ES cells form visible colonies (arrows) after 1d on PVG DP (A) and DS (C) cells. Colonies are maintained for at least 8d (B, D). On SF and RDP-B cells patches of ES cells (arrows) are evident after 1d (E, G). On SF the ES cell patches grow in colonies but these are not as compact or as distinct from the fibroblast layer as on follicular dermal cells (F compared to B & D). On RDP-B cells, the ES cells (arrows) blend into the rapidly proliferating fibroblast layer so after 4d ES cells can only be identified by high power microscopy (H).

Scale bar = 100μm

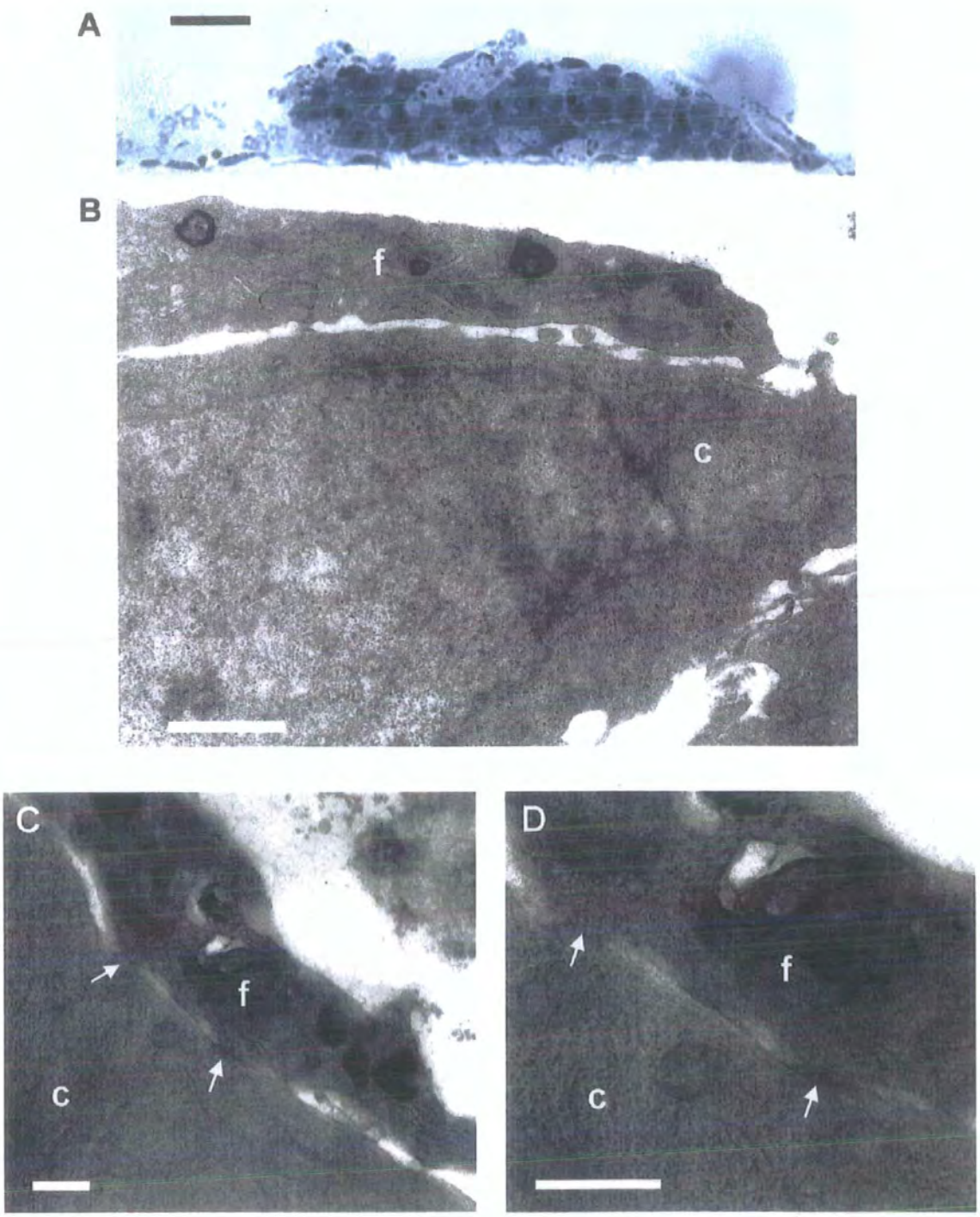

1d
8d
DP
DS
SF
RDP-B
A
B
C
D
E
F
G
H

ES cells again grew in clusters these did not remain as separate from the dermal cell layer. In DP and DS co-cultures the developing ES colonies stood proud of the dermal cells. The ES cell colonies in skin fibroblast cultures tended to be flatter and less distinct in appearance (Fig. 4.8 E,F). The difference was most evident in the first round of co-cultures. Subsequently, a considerable amount of variation was seen in ES cell behaviour in skin fibroblast co-cultures, with some colonies having almost identical morphology to that seen in follicular cell co-cultures. A transformed rat dermal papilla cell line, RDP-B, was also used for co-cultures and in these the ES cells, although distinguishable by their small compact morphology, tended to grow within the dermal cell layer (Fig. 4.8 G,H). This behaviour was consistent from one co-culture to the next (n = 4) and RDP-B cells were therefore used as a control rather than the inherently variable skin fibroblasts.

4.3.1.3.2. EM of co-cultures

Transmission electron microscopy (TEM) was used as an initial means of establishing if the colonised cells were maintaining their undifferentiated ES cell characteristics. Conventional microscopy of semi-thin sections stained with toluidine blue gave an overall perspective of the composition of the rounded colonies in co-culture (Fig. 4.9 A). Each colony was composed of tightly packed round cells, whilst thin, elongated fibroblast-like cells grew in a single cell layer over the colony. TEM through the colonies showed that the rounded cells within them had a very large nuclear:cytoplasmic volume ratio and few organelles were present in the cytoplasm (Fig. 4.9 B). The cells were also fairly tightly packed with minimal extracellular matrix. In contrast, the thin, flattened cells growing over the colony had many organelles in the cytoplasm and the cell nucleus was not always

Figure 4.9. Dermal cell/ES cell coculture processed for electron microscopy.
Toluidine blue staining of a semi-thin section shows a typical colony of closely packed, small, rounded cells with a layer of thin, elongated cells growing over the top in a single layer (A). Transmission electron microscopy reveals the difference in the cell types with a complex cytoplasm in the thin, fibroblastic cells, whilst the simple cytoplasm and high nuclear:cytoplasmic ratio of the colonised cells suggests they are maintaining stem cell characteristics (B). In places the contact between fibroblast-like cell (f) and the colonised round cell (c) was very close making it difficult to discern a clear border between the two (arrows - C, D).
Scale bars: A = 25μm, B = 1μm, C & D = 0.5μm.


A
B
f
c
C
f
c
D
f
c

evident, indicating a much smaller nuclear:cytoplasmic ratio in these cells (Fig. 4.9 B). In some sections the contact between the colonised, round cells and fibroblast-like cells was very close, with the border between the two cells being difficult to determine in places (Fig. 4.9 C, D). These observations supported an initial idea, not obvious from conventional phase microscopy, that the colonies consisted of ES cells whilst the dermal cells grew around them.

4.3.1.3.3. Green Fluorescent Protein (GFP) as a tool to identify cells of ES cell origin

The microscopical evidence suggested dermal cells were growing around colonies of ES cells, but the possibility that the cells on the outside of the colony were differentiated ES cells could not be ruled out. To investigate this possiblity and to distinguish betweeen ES and dermal cells in subsequent experiments, a CMV-GFP stably transfected line of ES cells was used, enabling cells of ES cell origin to be identified using fluorescence microscopy. This showed that the small compact colonised cells were indeed of ES cell origin whilst the larger bipolar cells were GFP-negative and therefore dermal cells (Figure 4.10).

4.3.1.3.4. Changes in co-culture morphology over time

For the first 2-3 days of co-culture there was no significant change in dermal cell morphology but subsequently these cells became more compact and bipolar (Fig. 4.8 B, D). As co-cultures matured the colonies of ES cells became larger, growing as mounds above the base layer of dermal cells. The dermal cells between colonies became more polarised whilst those at the edge of colonies started to grow over them. In all colonies, cells that maintained ES cell morphology were distinguishable. At the edge of colonies, particularly in more mature co-cultures, fluorescence microscopy revealed that a

minority of ES cells showed signs of differentiation (Figure 4. 10). In one particular culture, a large dividing cell was visible by phase contrast microscopy between two colonies of ES cells. Fluorescence microscopy revealed that this individual cell was expressing GFP, showing that a subpopulation of ES cells was moving out of the colonies, dividing and changing morphology (Fig. 4.10 A, B). Cells that originated from the ES cell population spread from the edge of the colonies at the base of the cultures. They displayed various morphologies ranging from very large, flattened cells, that appeared to be forming a layer on the dish under the rest of the cells, to small compact cells that appeared to be budding off the edge of colonies (Fig. 4.10 C, D). However, the vast majority of ES cells retained their undifferentiated morphology within the colonies.

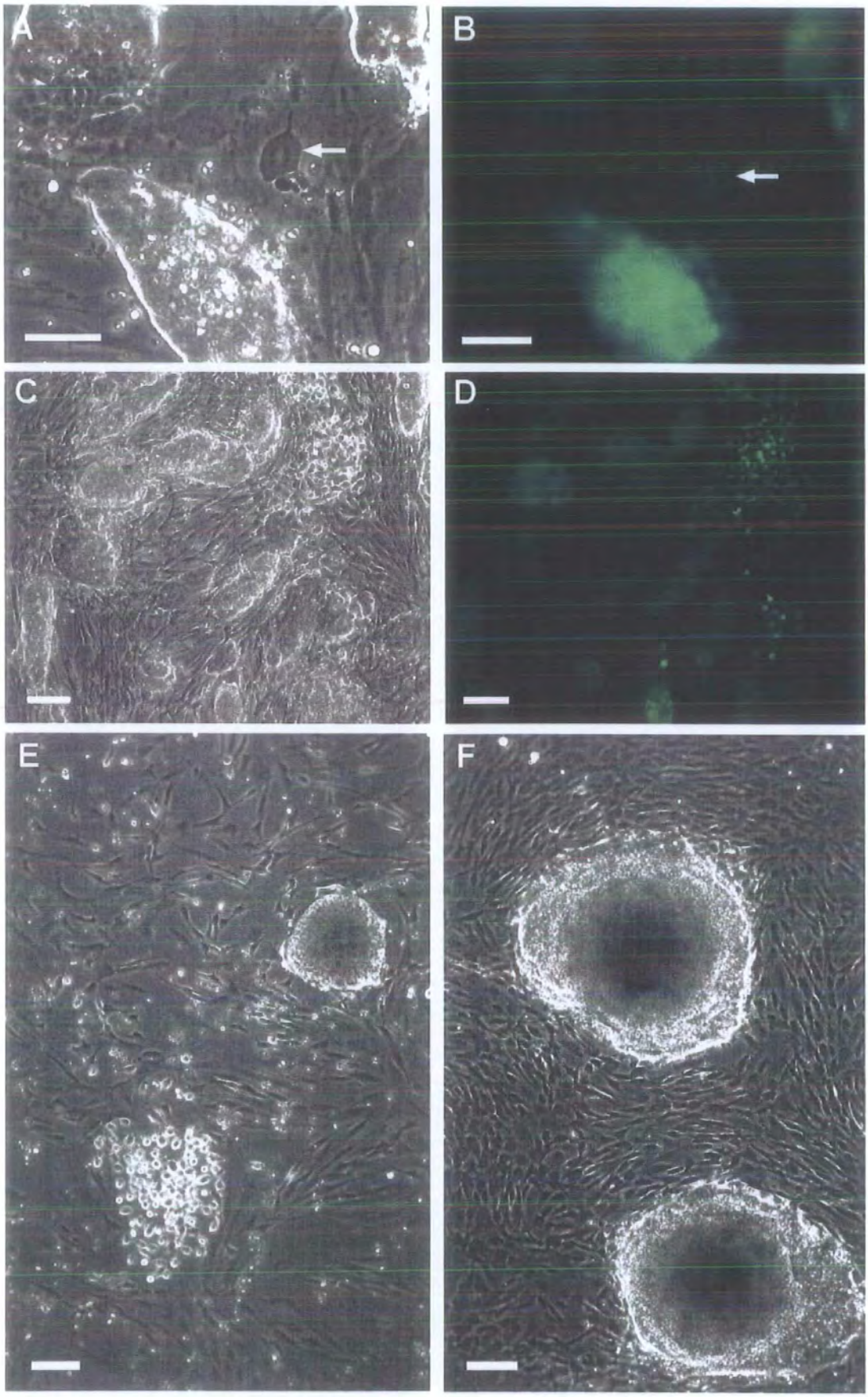
Another feature of co-cultures after a few days was the large number of rounded up cells seen floating in the media. It was confirmed in one culture that these were indeed viable cells by spinning down the supernatant and plating out the pellet in a flask. Dermal and ES cell morphology was evident in this culture and co-culture morphology developed in some areas (Fig, 4.10 E,F). These cells went on to produce more floating cells growing in clumps. The ES cells in this culture were of the CMV-GFP line and as a result it was possible to estimate that approximately 30% of the fibroblast-type cells in this culture were derived from the ES cell line.

In a small number of co-cultures of two weeks or more, the massed cell layers began to peel away from the dish and in some cases came away altogether. When this occurred a few cells were observed still attached to the dish and these were maintained. Proliferation of these cells produced a mostly dermal cell population but a few ES cells were also present resulting in ES cell colony formation, in a manner typical of co-cultures.

Figure 4.10. Identification of cells of CMV-GFP ES cell origin by fluorescence microscopy.

A 2d Z40 DS/ES cell coculture contains a large dividing cell (A) which by fluorescence microscopy is identified as being of ES cell origin (B) demonstrating that whilst the majority of GFP positive cells are within colonies there are a minority which undergo differentiation. An 8d PVG DP/ES cell coculture has many distinct colonies but some cells appear to be differentiating (C) and again the GFP enables identification of these rounded up cells, as well as the colonised cells, as ES cells (D). Cells spun out of the media removed from an 8d PVG DS/ES cell coculture grew in a characteristic coculture morphology (E & F) 24hr and 6d after plating respectively.

Scale bar: A & B = 50μm, C - F = 100μm.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4.3.2. Influence of follicle dermal cells on ES cells

To establish whether colonised ES cells had retained their pluripotency during a period of co-culture, it was necessary to isolate them from the dermal cells before assessing this characteristic. Ideally the whole co-culture would have been enzymatically dissociated and the cells separated to isolate as many ES cells as possible. Unfortunately we did not have access to a FACS machine that maintained the cells in a viable state after counting. In the absence of this, I therefore used a method by which a few colonies of ES cells were removed and these cells were then expanded in the presence of LIF (non-differentiating conditions). The cells were then exposed to conditions designed to induce differentiation along a number of lineages.

4.3.2.1. Plucked cells

Cells were plucked from co-cultures at successive time points, the earliest being 6 days, and the latest being 20 days, after ES cells were added to dermal cells. When ES cells were plucked from small areas of the co-cultures, some dermal cells were also usually removed resulting in a mixed culture. In cases where the dermal cells were closely associated with the plucked colonies the resulting culture developed as a co-culture once more and the dermal cells multiplied at a faster rate than the ES cells (Fig. 4.11 A,B). To prevent the ES cells being swamped by dermal cells the colonies were re-plucked. Plucking a colony clean from surrounding cells was much easier to achieve the second time around as these isolated colonies were clearly visible to the naked eye, so facilitating their easy removal with a micropipette tip.

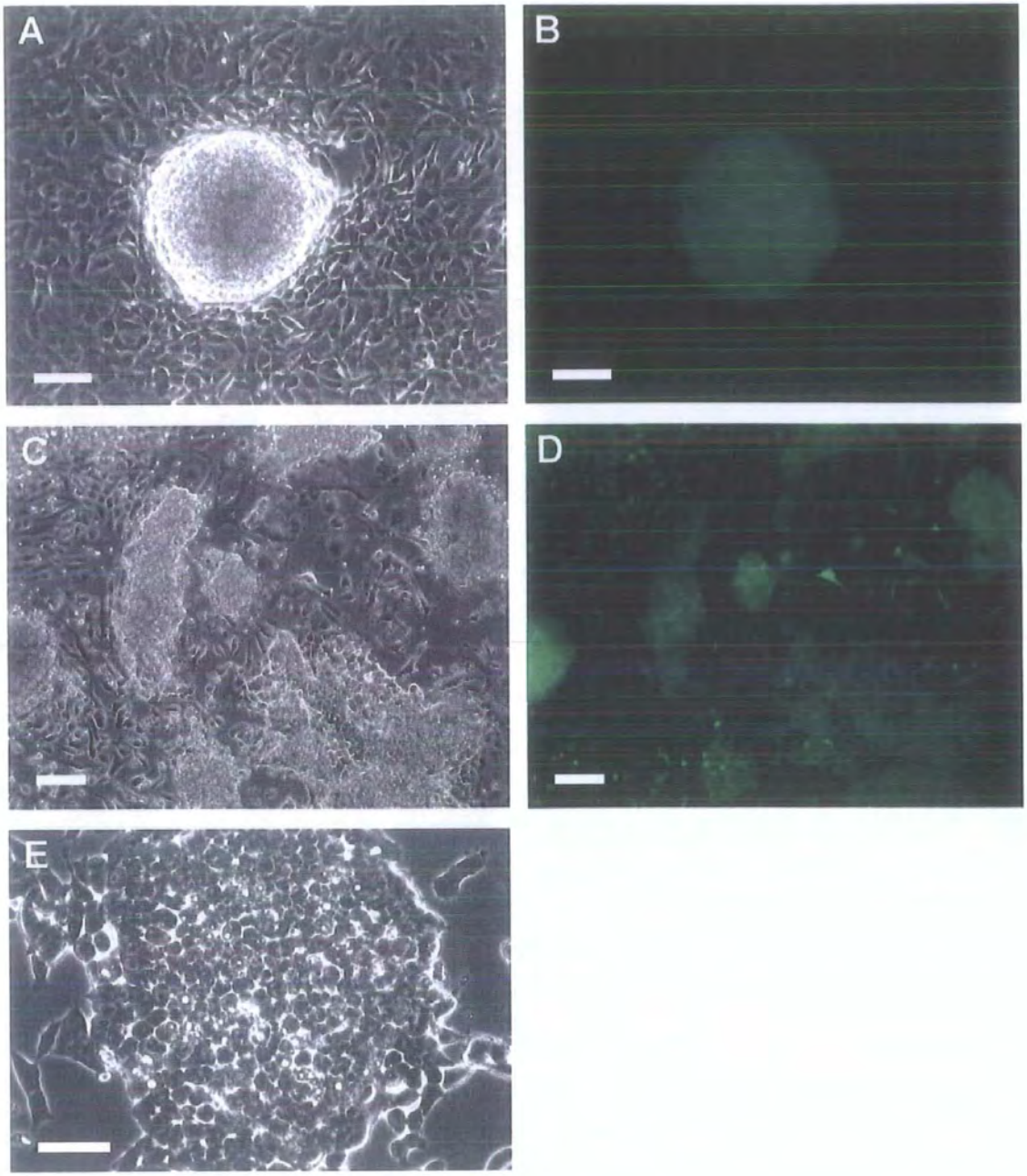
During the time in which the plucked cells were proliferating to provide sufficient numbers for the pluripotency assays, a number of cells underwent significant changes, resulting in a range of cell morphologies within these cultures (Fig. 4.11 C,D).

Figure 4.11. Plucked cells.

Plucked colonies were often accompanied by dermal cells which grew rapidly forming new 'cocultures' (A,B). Cells underwent differentiation to a degree whilst the majority remained in colonies (C, D) or grew in adherent patches (E).

Cells plucked from 6d PVG DS/ES (A,B), 20d Z40 DP/ES (C,D), 6d Z40 DS/ES (E).

Scale bar: A - D = 100μm, E = 50μm.


A
B
C
D
E

Despite this, the vast majority of cells of ES cell origin (determined by GFP) maintained the typical ES cell morphology whether in colonies or in patches of cells adhering to the culture substratum (Fig. 4.11 E).

When sufficient cells were available the cells were trypsinised and used to initiate the various differentiation assays. In the cultures where a few dermal cells remained, they were lost at this stage during the formation of embryoid bodies (EBs), the initial step for all assays, as cells of ES cell origin formed these aggregates whilst dermal cells adhered to the substratum.

4.3.2.2. Initial pluripotency assays

In the first set of assays, neuron-like cells were derived from all groups of ES cells on which the assay was performed (after 6, 10 and 14d co-culture with PVG DP; after 8d co-culture with Z40 DS; after 6, 10, 14 & 20d co-culture with Z40 DP). In all instances, except for 20d Z40 DP, experiments were performed in duplicate. A positive control was run for each differentiation assay using ES cells maintained with LIF.

CFU-A assays for haematopoietic activity were also carried out but were less successful. A very low percentage of EBs produced colonies (an indication of haematopoietic differentiation), but this was also the case with positive controls using ES cells maintained with LIF. The low result was therefore attributed to a failure of the assay rather than the potential of the cells. In support of the early indications that these cells were pluripotent, cardiomyocytes, clearly distinguishable by spontaneous rhythmic contractions, were observed in cultures of differentiating ES cells derived from all types of co-culture.

4.3.2.3. Endodermal, mesodermal and ectodermal pluripotency assays

To further test whether pluripotency of ES cells was maintained in co-culture, another round of co-cultures was set up and the cells plucked at 6 and 10 days. This round of assays was more extensive. Differentiation was directed along cell lineages representative of the three primordial tissues, the time points were more consistent, replicates were done for each culture at each time point, and there was less of a problem with contamination. All these improvements allowed a comprehensive set of data to be obtained. Four differentiation assays were carried out from each sample of plucked cells: neuron, adipocyte, endoderm and blood. Observations of cardiomyocytes were also noted.

Figure 4.12 shows cells derived from a 6 day Z40 DS co-culture at key time points in the three successful differentiation assays. This is a representation of the process and ES cells plucked from PVG DP and DS, and Z40 DP co-cultures underwent similar changes giving the same positive results. Once again the positive control for the CFU-A assay was unsuccessful, so the failure of the plucked cells to demonstrate evidence of haematopoietic differentiation is likely to be as a result of technical problems and does not reflect a limitation in pluripotency.

4.3.2.3.1. Neuron assay

Networks of neuron-like cells were visible by day 12 of the neuron assay (Fig. 4.13 A). Axon-like projections extended in networks from EBs, in association with other cell types of more varied morphology which adhered to the culture substrate (Fig. 4.13 B,C). The number of EBs that had neuron-like cells associated with them were counted on day 12 as by day 14 a large proportion of EBs had floated away and there was considerable cell death.

Figure 4.12. An example of the key steps in the differentiation assays.
Cells originating from a 6d Z40 DS/ES cell coculture formed embryoid bodies (EBs) (A, A') which were treated in the manner specific to each differentiation assay (B-D, B'-D') before being plated out on gelatin-coated tissue culture dishes. Cells grew out from the EBs and differentiated along a number of lineages (E, F) and the success of the assays were determined by either morphology (in the neuron assay - G) or by the relevant staining method (for adipocytes and endoderm - H, I). Images A'-D' are fluorescence micrographs which confirm the EBs seen by phase microscopy (A-D) originate from ES cells within the coculture. Similarly the green fluorescence in I is GFP whilst the red fluorescence is albumin showing the ES cells have differentiated into endodermal cells. Scale bars: A - F = 200μm, G - H = 100μm, I = 50μm.

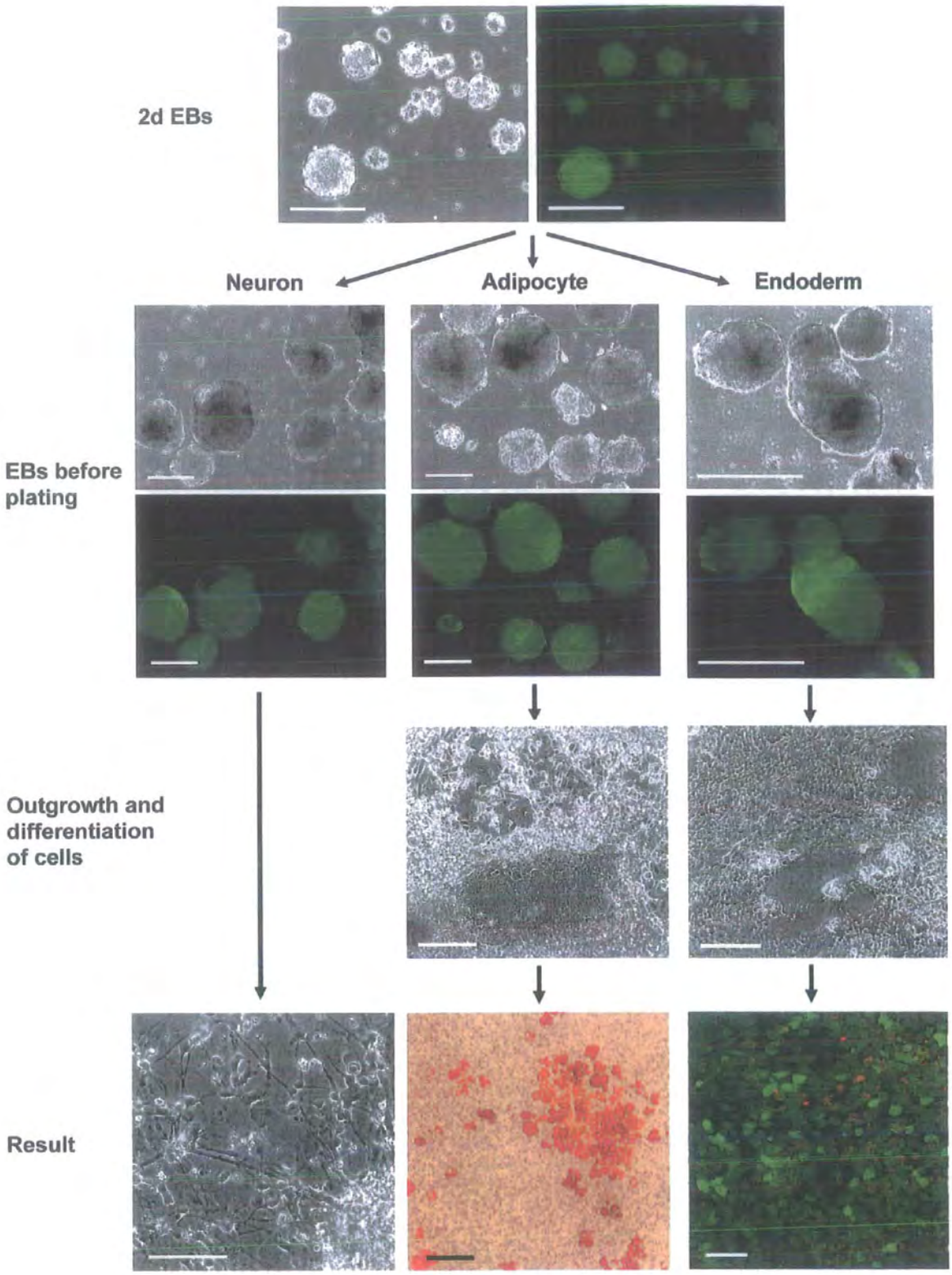
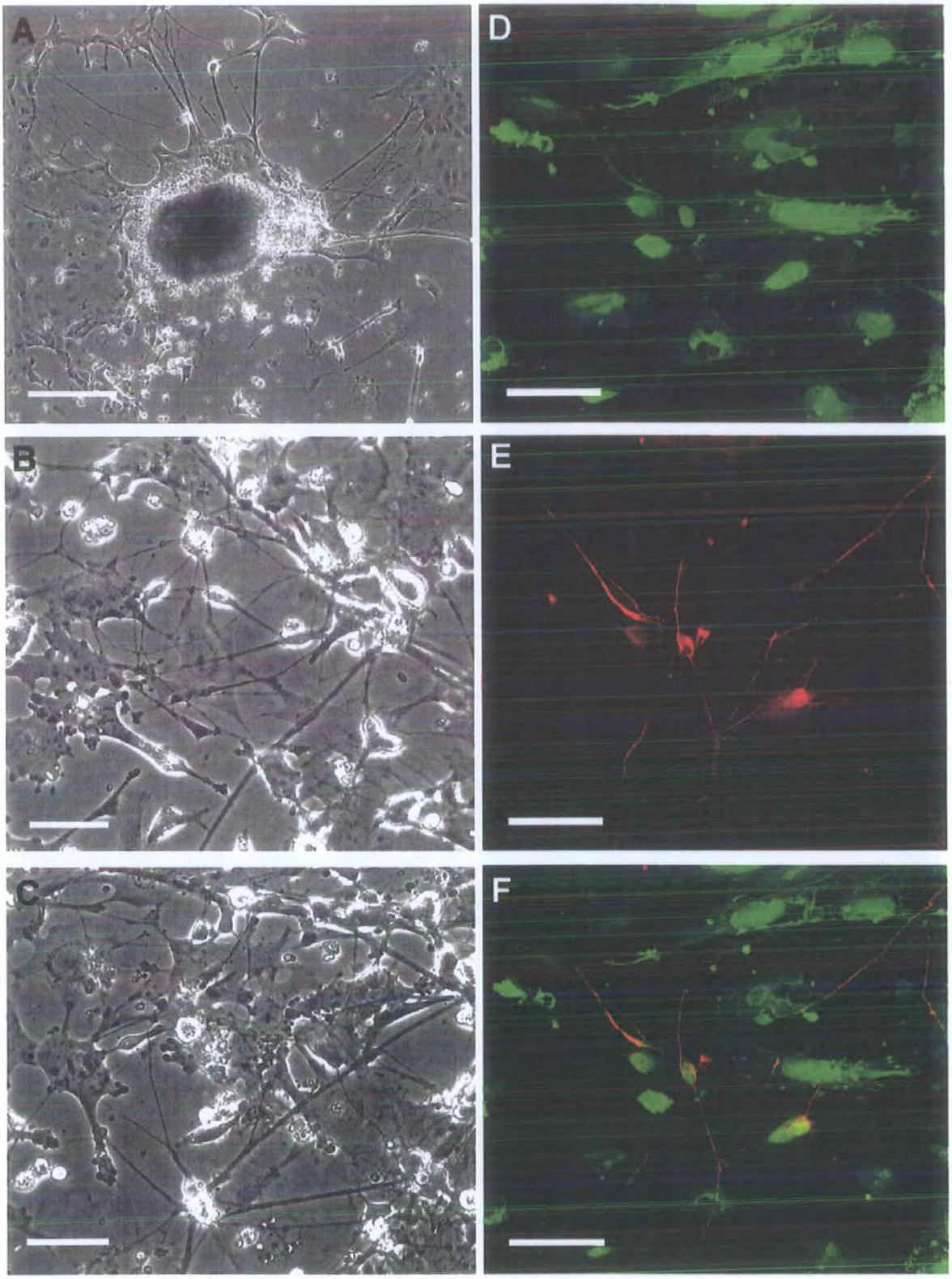

2d EBs
Neuron
Adipocyte
Endoderm
EBs before plating
Outgrowth and differentiation of cells
Result

Figure 4.13. Neuronal assay results.

Networks of neurons extend from EBs derived from ES cells after 6d coculture with PVG DS cells (A - C). CMV-GFP ES cells after 6d coculture with PVG DP cells produce neuron-like cells, the projections of which are visible by fluorescence microscopy (D). Immunostaining with NF200 demonstrates expression of neurofilament protein in this culture (E) and the colocalization with the GFP expression identifies the ES cell origin of these neuron-like cells (F).

Scale bar: A = 200μm, B - F = 50μm.


A
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B
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F

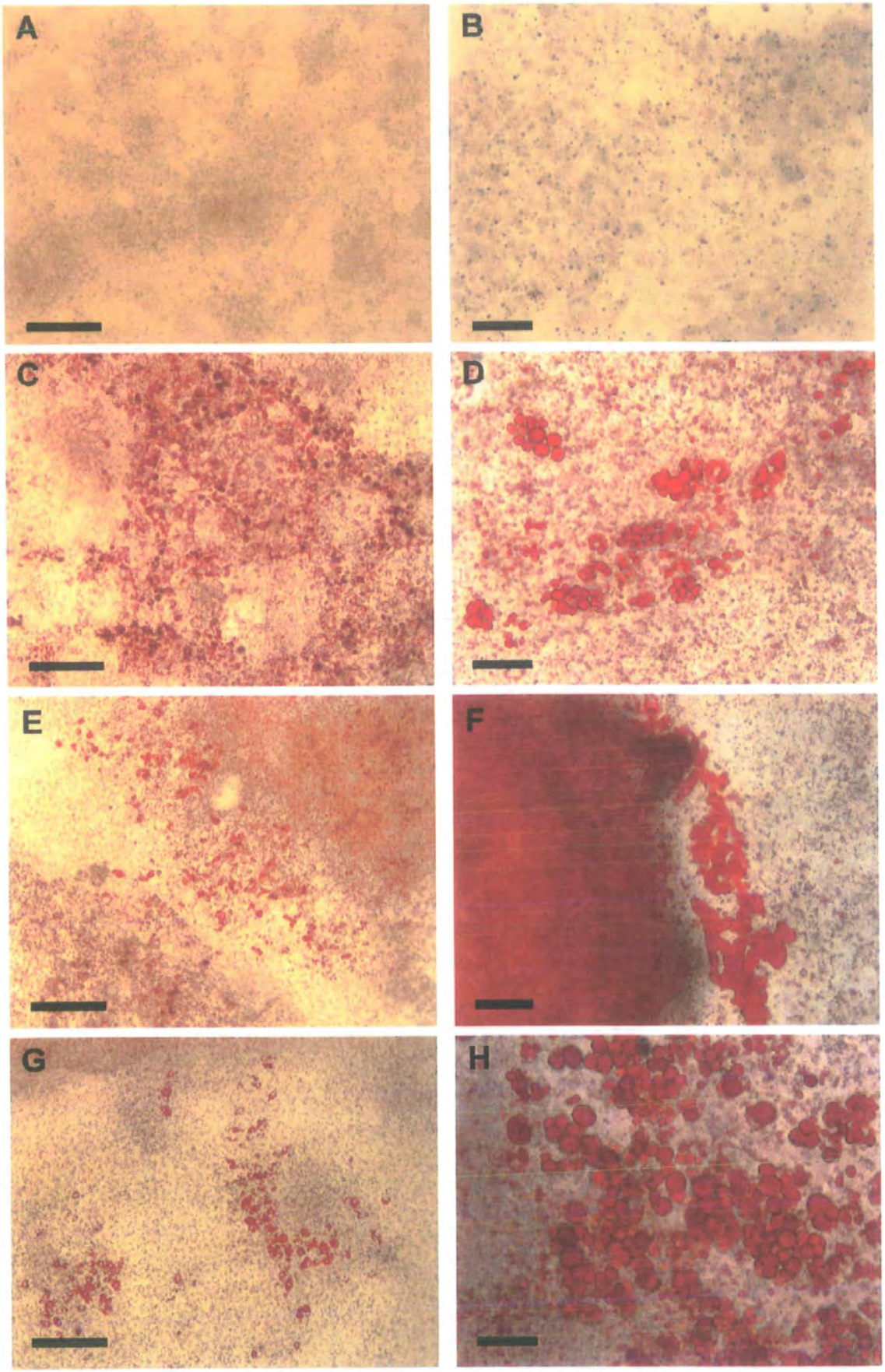
Cells originating from co-cultures with PVG and Z40, DP and DS cells gave positive results in this assay, with a minimum of 65% of EBs producing networks of neuron-like cells. The greatest numbers of neuronal outgrowths was observed in cells originating from 6d Z40 DS co-cultures where 90% of EBs had neuron-like cells associated with them (Table 4.4).

To characterise the cells further, immunocytochemistry was carried out, using an antibody specific to neurofilament, on a sample of differentiated cells (assay day 12) originating from a 6d PVG DP/ES cell co-culture. The GFP was expressed in axon-like processes (Fig. 4.13 D) which were then revealed to be immunoreactive for neurofilament (Fig. 4.13 E). When the two images were merged it was confirmed that the neurofilament was present in GFP-positive cell projections (Fig. 4.13 F).

4.3.2.3.2. Adipocyte assay

ES cells plucked from PVG and Z40, DP and DS co-cultures gave consistently positive results in this assay. This was in stark contrast to undifferentiated CGR8 cells that had tiny lipid droplets scattered around with no evidence of lipid-filled cells at all (Fig. 4.14 A,B). Throughout experimental cultures, oil red O staining showed small lipid droplets and large lipid-filled cells in patches ranging from a collection of just a few cells to up to 50 cells closely packed together (Fig. 4.14 C-H). One EB in particular was observed to be producing copious amounts of lipid (Fig. 4.14 F). Approximately 30% of each culture exhibited high levels of lipid deposition.

Figure 4.14. Adipocyte assay results determined by oil red O staining.
ES cells maintained with LIF have very little lipid present (A, B). ES cells plucked from 10d Z40 DS/ES cell coculture (C, D), 6d Z40 DP/ES cell coculture (E, F) and 6d PVG DS/ES cell coculture (G, H) all produced high levels of lipid (stained red) having been subjected to the adipocyte differentiation assay demonstrating their ability to differentiate along the adipocyte lineage.
Scale bar: A, C, E, G = 200μm, B, D, F, H = 50μm.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4.3.2.3.3. Endoderm assay

At the time when these assays were done there was no published method for inducing ES cell differentiation along an endodermal cell lineage and, as a result of spontaneous differentiation, less than 1% of cells were expected to express endodermal markers (personal communication Elizabeth Jones, Newcastle, U.K.). Cells originating from all co-cultures gave positive results in this assay. Small clusters of cells were immunoreactive for albumin and less for alpha-feta-1-protein (AFP) (Figure 4.15). As expected these comprised no more than 1% of the total cell population. The CMV-GFP ES cells confirmed that the cells expressing these proteins were of ES cell origin. As well as cellular expression of these proteins a significant amount of extracellular matrix staining was evident, particularly with the albumin antibody (Fig. 4.15 E,F).

The method adopted for this assay was based on allowing the EBs to differentiate without any exogenous factors and although many studies have identified stages at which the AFP and albumin genes are expressed in developing EBs, there is less data available on the detection of these proteins (Abe et al., 1996; Itskovitz-Eldor et al., 2000; Xu & Lim, 2000). Therefore for some of the samples tested, EBs that were in excess of the requirements for the CFU-A assays were maintained in CGR8 medium for a further 6 days before plating out (day 12 of the assay). The resulting cultures of cells were stained for AFP and albumin 6 days later. Although a detailed analysis was not carried out to compare these two methods, the results showed no apparent difference between the cultures with AFP and albumin expression detected in both.

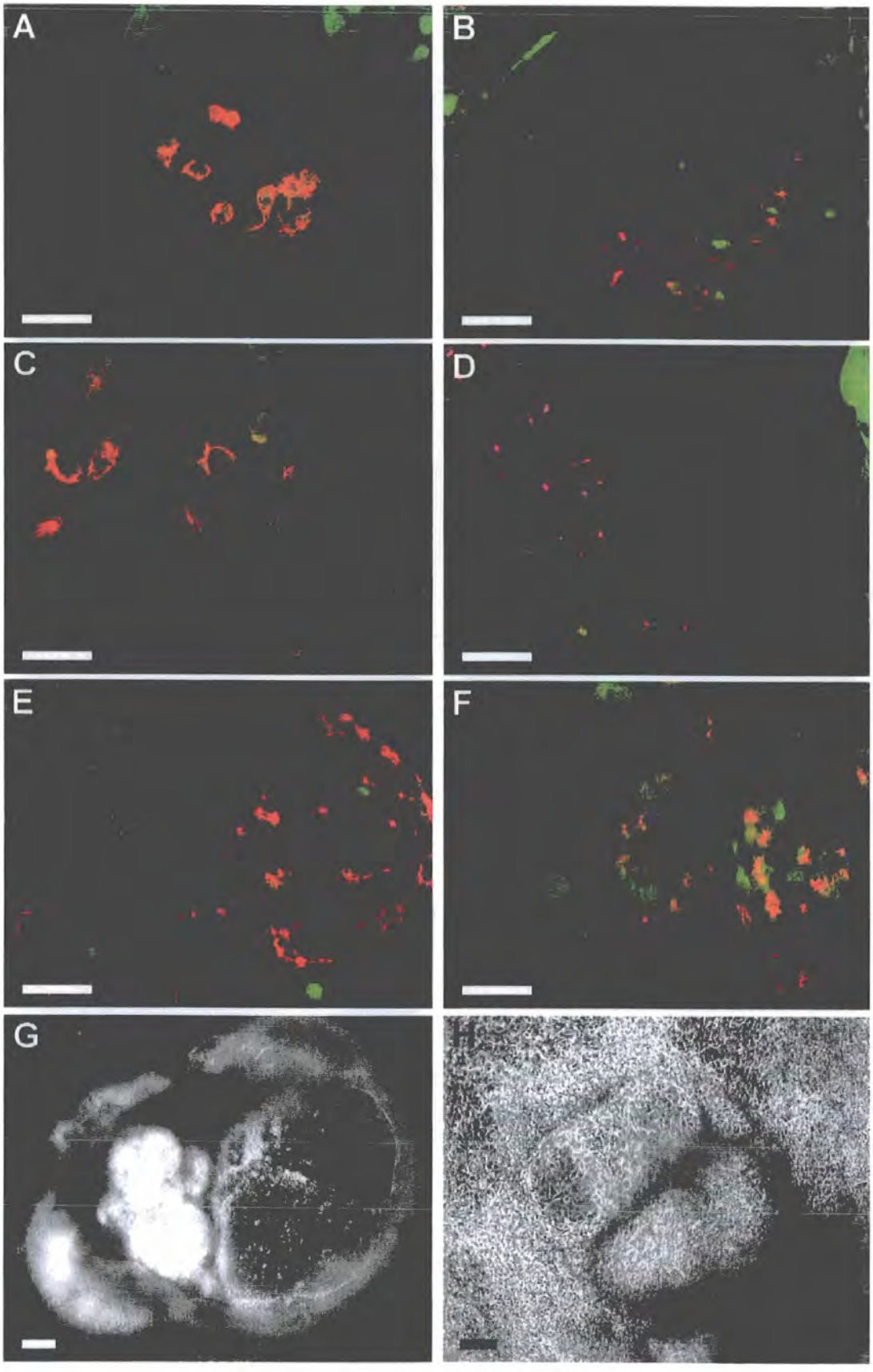
In some cases the spontaneous differentiation of EBs resulted in intriguing developments, as yolk sac-like structures formed. Within a number of these there were morphologically distinct cell types and complex structures formed in the shape of strands of tissue spanning the cavity, or two separate chambers developing. Others were simple bubble-like structures with no distinguishing features.

Figure 4.15. Results of endodermal differentiation assay.

Endodermal cells are present in cells plucked from 6d cocultures of GFP CMV-ES cells with Z40 DP (A), Z40 DS (B, D) and PVG DP (C, E, F). Green fluorescence identifies the cells as of ES cell origin whilst red fluorescence shows immunoreactivity for AFP (A, B) and albumin (C - F).

During the differentiation process some EBs developed highly complex structures with rhythmically contracting cavities (G) and 3D structures within multilayered cell cultures (H).

Scale bar: A, B, D = 25μm; C, E - F = 50μm; G = 200μm; H = 100μm.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One however was particularly noticeable as contractile muscle tissue was evidently present in three or four places in the wall of the "bubble" and also in a strand across the cavity (Fig. 4.15 G). This resulted in the whole structure contracting in a manner that was visible under a low power, dissecting microscope. Similar three dimensional bodies were evident in some of the developing adherent cultures (Fig. 4.15 H).

Even when such structures were absent, the potential of these cells to differentiate along many lineages was shown by the many different morphologies present in the outgrowth from EBs. Figure 4.16 shows examples of differentiated cells which originated from ES cells plucked off a 6d Z40 DP/ES co-culture.

4.3.2.3.4. Cardiomyocytes

During the period of outgrowth of differentiating cells from EBs many cultures contained groups of cells that were observed to spontaneously and rhythmically contract, a characteristic of cardiomyocytes. These groups were estimated to range from less than ten (Fig. 4.16 F) to over two hundred cells and were often organised as interconnected patches contracting as a synchronised network. Contractile cardiomyocytes were evident in cells derived from all co-cultures after both 6 and 10 days, with the exception of 10d PVG DP co-cultures (see below).

In summary, ES cells plucked from Zin40 and PVG, DP and DS co-cultures gave rise to cells of mesodermal, endodermal and neuroectodermal lineage (Table 4.4). The cells plucked from 10d PVG DP/ES co-culture became contaminated during the outgrowth period but within these cultures neuron-like cells and cells of preadipocyte morphology were observed suggesting these cells would also have resulted in multiple lineages.

Figure 4.16. Multiple cell types in one endoderm differentiation assay.
Cells derived from 6d PVG DP/ESC cocultures had differentiated into many morphologies by day 18 of the endoderm assay (A - E). On day 10 contracting muscle cells (arrow) were seen (F).
Sclae bar: A - E = 100μm, F = 50μm.

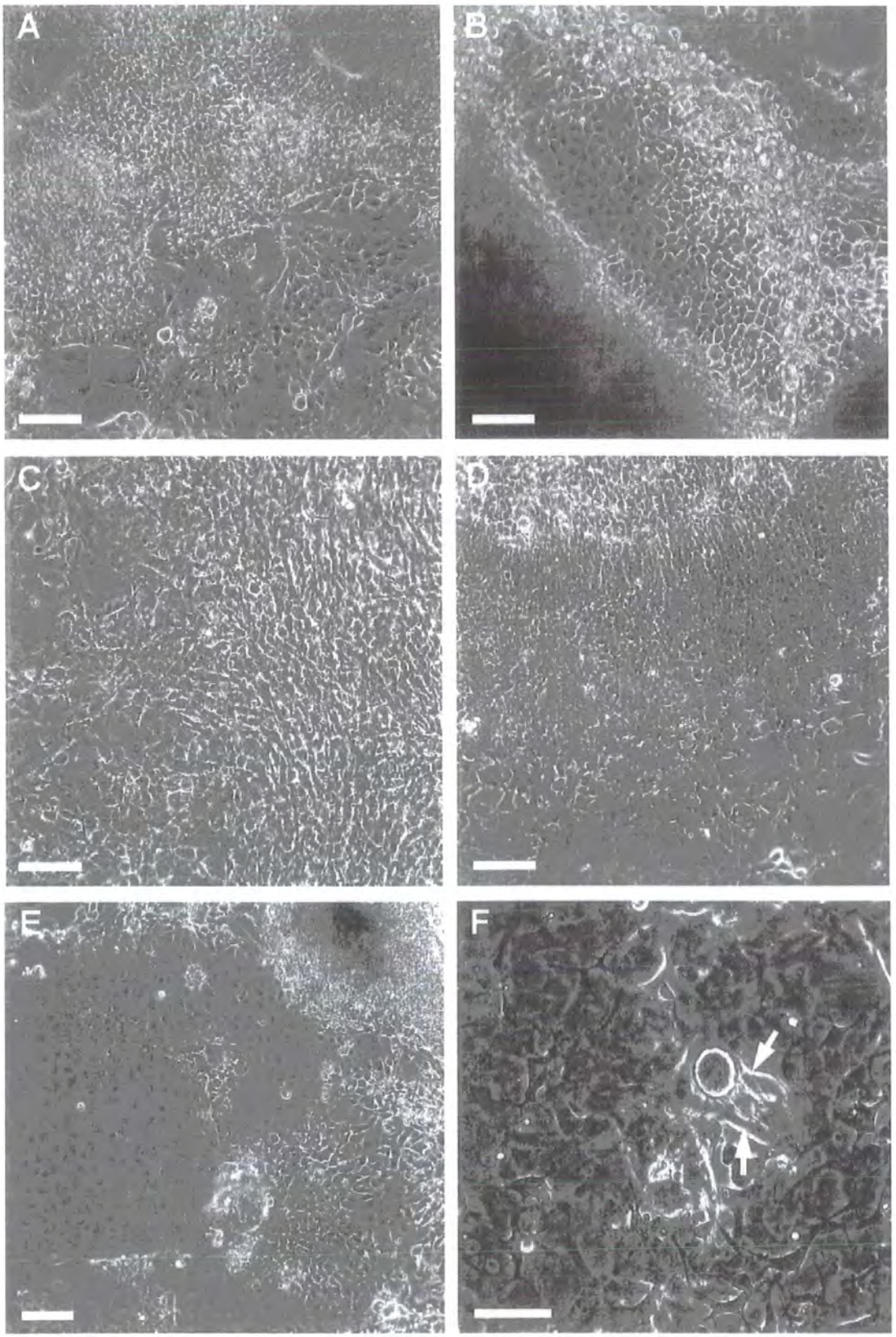
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Table 4.4. Summary of neuroectodermal, mesodermal and endodermal assay results.

Neuronal assay results are expressed as percentage of EBs that were associated with axons. Adipocytes were abundant in all completed assays and some cells with preadipocyte morphology were seen in cultures from 10d PVG DP co-cultures. AFP and albumin expression was detected in a small proportion of the total cell population. The number of cardiomyocytes observed was varied but they were evident in all completed assays.

Dermal cell type	Z40 DS		Z40 DP		PVG DS		PVG DP	
	6d	10d	6d	10d	6d	10d	6d	10d
Neurons	73% 90%	65% 65%	88% 84%	75% 80%	82% 87%	82% 70%	73% 72%	+
Adipocytes	++	++	++	++	++	++	++	+
AFP Endoderm	+	+	+	+	+	+	+	N/A
Albumin	+	+	+	+	+	+	+	N/A
Cardiomyocytes	+	++	+	++	+	+	++	-

4.3.2.4. The influence of conditioned medium on ES cells

Having established that the pluripotency of ES cells was maintained by follicular dermal cells in culture, some basic conditioned medium experiments were carried out to assess the contribution to this process by soluble factors. ES cells were grown in RDP-B conditioned MEM + FBS (conditioned over 24hrs and diluted 1:1 with CGR8 medium) for 6 days. Over this period these cells underwent considerable differentiation, although there was less cell death than occurred in a flask of ES cells in CGR8 medium with no added LIF (data not shown). The degree of differentiation suggested there was no soluble factor present, with LIF-like properties.

To determine if follicular dermal cells shown to maintain ES cells in co-culture, had the same capacity via factors secreted into the media, ES cells were cultured for 10 days in conditioned medium (CM) diluted 1:1 with CGR8 medium. The morphology of these cells was then compared to that of ES cells cultured for the same period in CGR8 medium with or without LIF (Figure 4.17). The cultures from which the CM was taken were as follows: 6d Z40 DP/ES cell co-culture, 8d Z40 DS/ES cell co-culture, DP and DS (for the first four days Z40 conditioned MEM/FBS, then switched to PVG conditioned CGR8 medium).

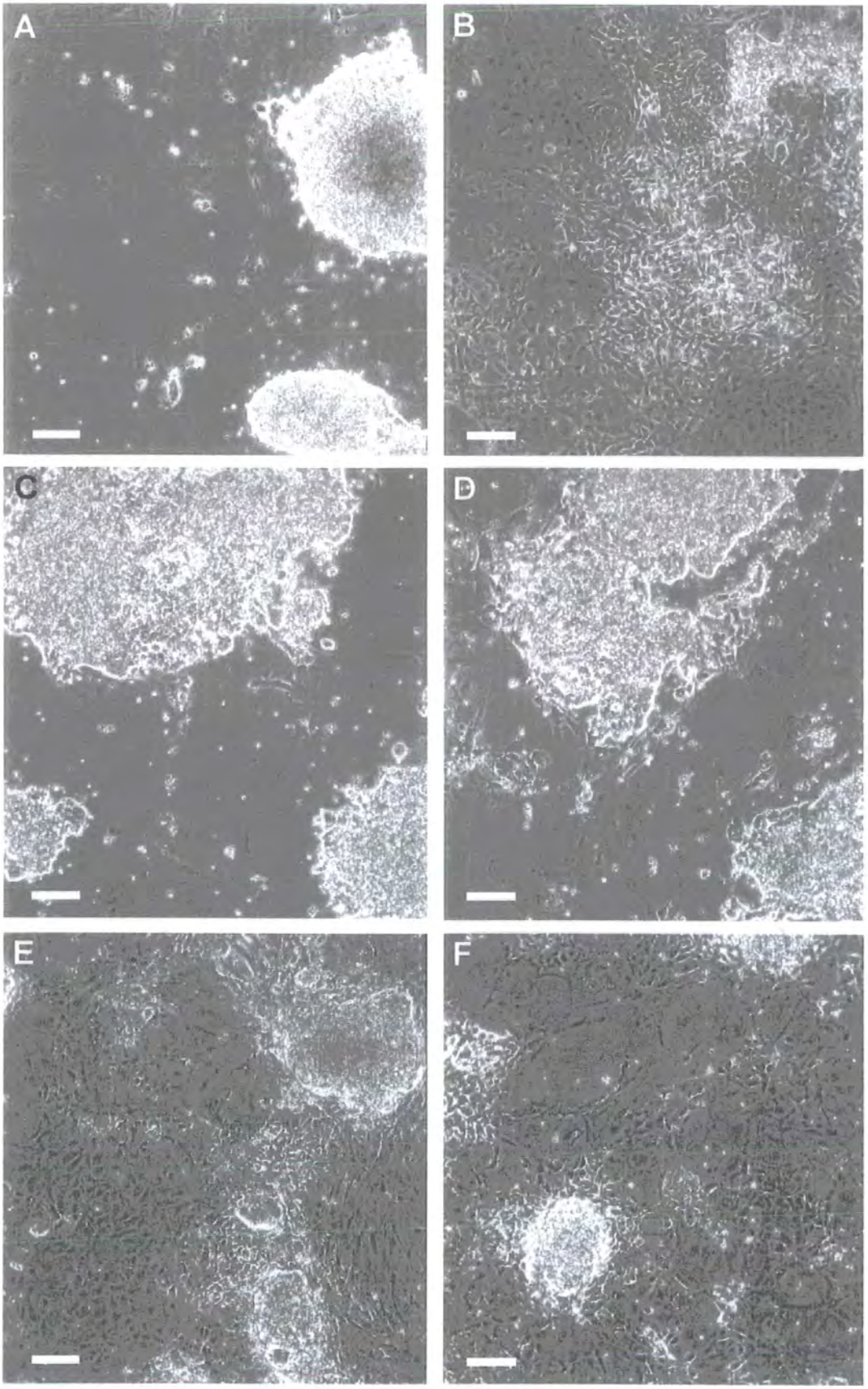
As found previously there was a marked contrast between the cells maintained in complete CGR8 medium and those cultured in CGR8 medium with no added LIF. The vast majority of cells maintained with LIF grew in dense colonies and just a few cells exhibited morphological changes at the edge of these colonies (Fig. 4.17 A). In contrast, many cells underwent morphological changes in the culture without LIF (Fig. 4.17 B). In all CM cultures, a larger proportion of the ES cells maintained a typical ES cell morphology than in the CGR8 medium (no LIF) culture, suggesting that there is a certain amount of soluble factor secreted by the dermal cells (both in the presence and absence of ES cells) that prevents differentiation of ES cells. The medium conditioned by co-cultures appeared to be more effective than CM from pure dermal cultures, and DP/ES co-culture CM (Fig. 4.17 C) more so than DS/ES CM (Fig. 4.17 D). Similarly the DP CM cultures contained more ES-like cells (Fig. 4.17 E) than the DS CM cultures (Fig. 4.17 F). My observations were independently validated by another individual who was unaware of the previous work.

Figure 4.17. ES cells cultured in conditioned media (CM).

Cultures were maintained in the relevant media for 10 days: complete CGR8 medium (A); CGR8 medium (no LIF) (B); DP/ES CM (C); DS/ES CM (D); DP CM (E); DS CM (F).

Coculture CM (DP/ES more so than DS/ES) appeared to maintain ES cells in a state closest to that seen in complete CGR8 medium, whilst DS cells were the least effective, although these cultures still retained more cells of ES morphology than CGR8 medium (no LIF).

Scale bar = 100μm.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4.3.2.5. Cytokine expression in cultured cells

Having established that soluble factors within the culture medium had the capacity to inhibit ES cell differentiation, RT-PCR was used to detect transcripts of four cytokines (LIF, CNTF, OSM and CT-1) known to maintain ES cells *in vitro*.

Results are shown in Figure 4.18 A. LIF, CNTF and OSM results are n = 2, CT-1 results are n = 1. The small sample numbers must be taken into account when interpreting this data. Dermal cells (DP, DS and RDP-B) all expressed LIF and CNTF. OSM expression was clearly detected in RDP-B cells but DP and DS cells had barely detectable levels of expression. Of the faint bands the full transcript was most evident in DP and the shorter transcript more highly expressed in DS. CT-1 was detected in DP and DS cultures but not in RDP-B cells. ES cells maintained in complete CGR8 media with frequent passaging ("ES+LIF") expressed LIF, OSM and CNTF but not CT-1. ES cells cultured for 6 days in complete CGR8 media without passaging became confluent and a degree of differentiation occurred despite the presence of exogenous LIF ("ES + LIF diff'd"). Transcripts of all four cytokines were detected in these cells as was the case with ES cells cultured in CGR8 media with no exogenous LIF over a period of 6 days ("ES diff'd"), although OSM expression appeared to be weaker in these cultures. When ES cells were co-cultured with dermal cells the transcripts of all four cytokines were detected in the co-cultures.

In summary, the DP, DS and RDP-B cells express the four cytokines we investigated at varying levels, as did undifferentiated ES cells. Co-cultures expressed all four cytokines as did differentiating ES cells, both in the presence and absence of exogenous LIF. These results raised the question of whether these cytokines were expressed by follicular cells *in vivo* and if so, where within the follicle?

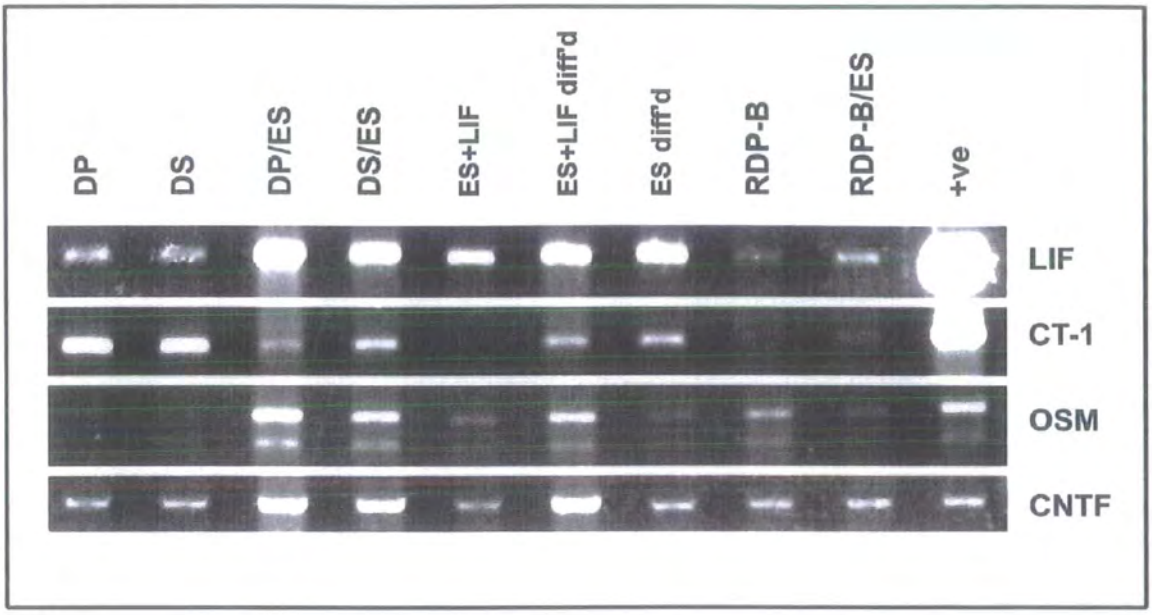
Figure 4.18. RT-PCR results.

Cytokine transcripts for LIF, CT-1 OSM and CNTF were detected in A) cultured cells and B) segments of mid-anagen vibrissa follicles.

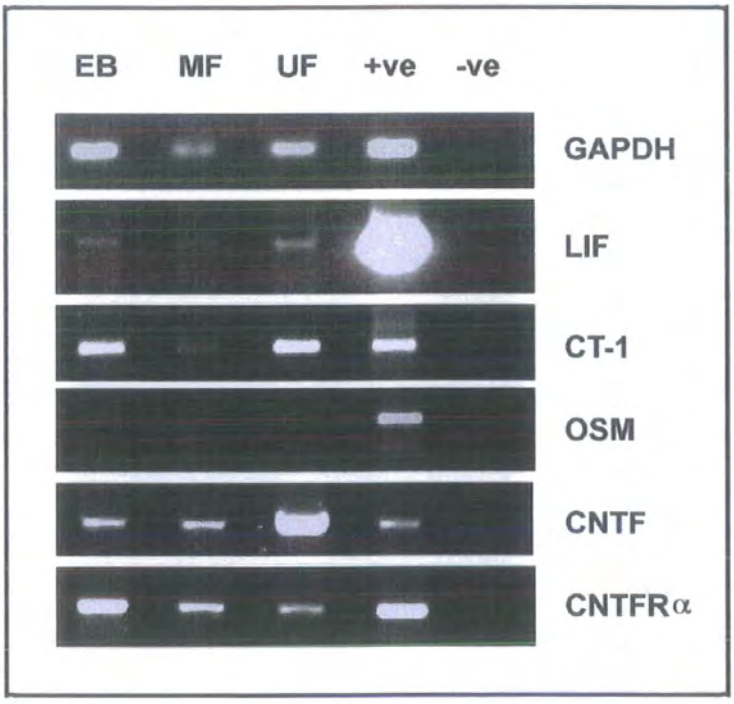
A) cDNA was synthesised from RNA extracted from dermal cell cultures (DP, DS, RDP-B), undifferentiated ES cells (ES+LIF), 6d cocultures (DP/ES, DS/ES, RDP-B/ES), and differentiating ES cells after 6 days in the presence or absence of exogenous LIF (ES+LIF diff'd, ES-LIF diff'd). LIF and CNTF were detected in all cell cultures, whilst CT-1 was not detected in undifferentiated (ES+LIF) cells or RDP-B cells, and OSM was barely detectable in DP or DS cells.

B) Mid-anagen Zin40 vibrissa follicles were trisected and RNA extracted from the end-bulbs (EB), mid-follicle sections (MF) and upper follicle sections (UF). LIF, CT-1 and CNTF transcripts were detected in all sections of the follicle whilst OSM was undetected. The α component of the CNTF receptor (CNTFRα) was also expressed throughout the follicle.

A


DP
DS
DP/ES
DS/ES
ES+LIF
ES+LIF diff'd
ES diff'd
RDP-B
RDP-B/ES
+ve
LIF
CT-1
OSM
CNTF

B


EB
MF
UF
+ve
-ve
GAPDH
LIF
CT-1
OSM
CNTF
CNTFRα

4.3.2.6. Cytokine expression in vibrissa follicles

Vibrissa follicles were dissected from three Zin40 mice (n=1) and follicles identified as being in mid-anagen of the growth cycle were used as a source of RNA. The results (n = 2) are shown in Figure 4.18 B and demonstrate that of the four cytokines under investigation, CNTF, LIF and CT-1 were expressed in upper follicle, mid-follicle and end bulb sections of mid-anagen follicles, whilst OSM was not expressed at comparable levels in any part of the follicle. Very weak OSM expression was however, evident in the end bulb, such expression being consistent with the weak expression of this cytokine in DP and DS cells *in vitro*. The receptor component CNTFRα was also expressed in all three sections of the mid-anagen vibrissa follicle. The *in vivo* data shows transcripts of three of these cytokines, LIF, CNTF and CT-1, are present in multiple regions of the mid-anagen vibrissa follicle.

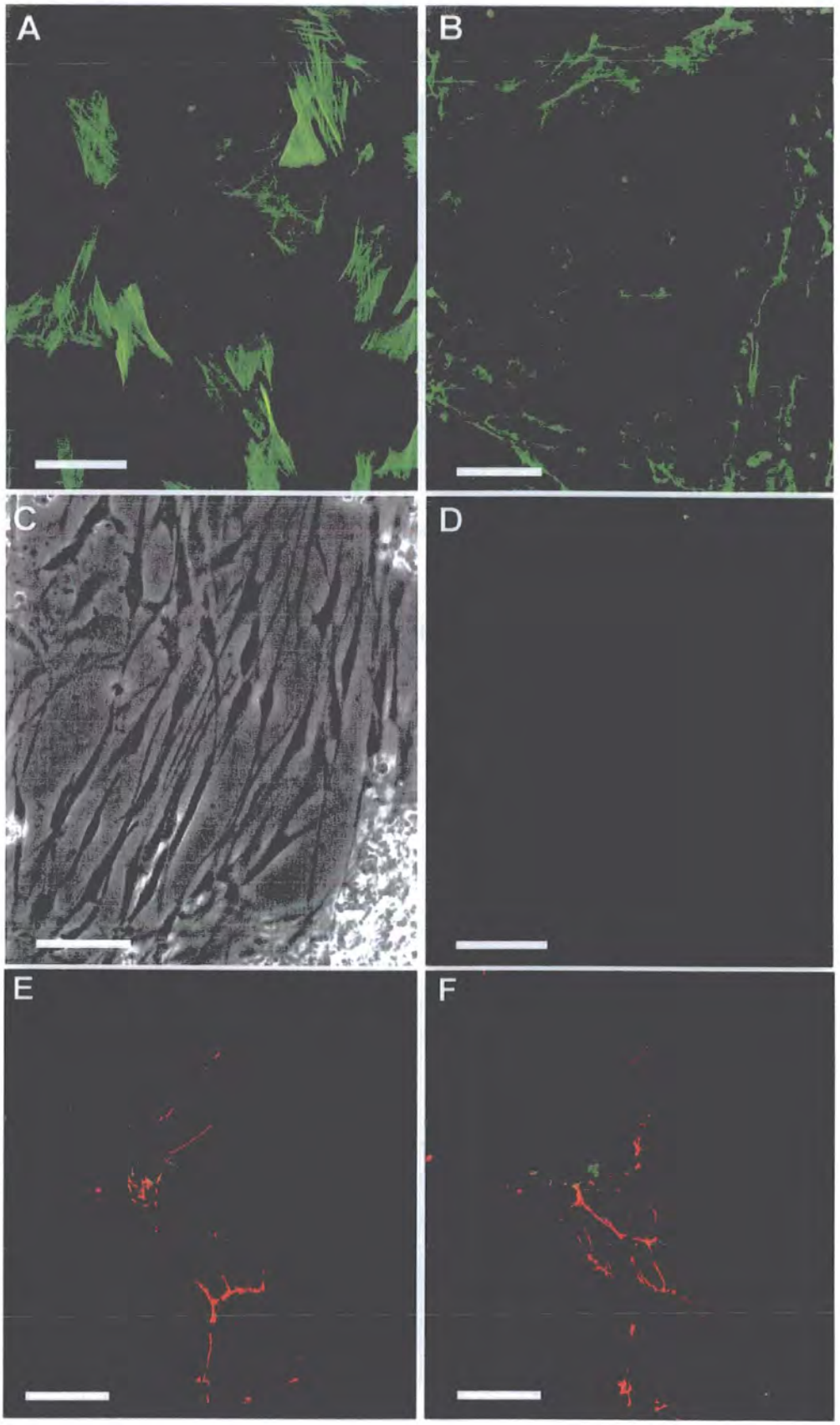
4.3.3. Influence of ES cells on dermal cells

4.3.3.1. α-smooth muscle actin expression

The expression of α-smooth muscle actin (α-sma) by DP and DS cells *in vitro* is a characteristic which distinguishes them from the non-follicular dermal (skin) fibroblasts (Jahoda et al., 1991). Co-cultures were immunostained with a monoclonal antibody for α-sma and two key observations were made (Figure 4.19). First, within the co-cultures only a minority of cells were positively labelled. Those that were stained were usually in groups and exhibited a distinctly different staining pattern to the typical stress fibre appearance seen in cultures of pure DP or DS cells (Fig. 4.19 A, B). The α-sma was located around the edge of these cells and only faintly expressed in the central part of the cell, suggesting cell junctions had developed between adjacent cells in a manner not normally associated with follicular dermal cells *in vitro*.

Figure 4.19. α-smooth muscle actin expression in cocultures.

α-smooth muscle actin is expressed in a typical stress fibre pattern by DP cells in vitro (A). In DP/ES cell cocultures significant changes to the DP cells occur. DP cells closely associated with ES cells change cytoskeletal structure as shown by the α-smooth muscle actin expression pattern (B). DP cells between the ES cell colonies change morphology becoming bipolar (C) and these cells also lose their α-smooth muscle actin expression, as shown in D which is the identical frame of C but viewed by fluorescence microscopy. GFP-CMV ES cell cocultures combined with red immunofluorescence demonstrate that α-smooth muscle actin expression is only associated with DP cells in close contact with ES cells (E - F). These are two different planes of focus of the same area of culture. Scale bar: A, B = 100μm, C - F = 50μm.


A
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The cells expressing α-sma in this manner appeared to be flat, squamous cells, collectively forming a pavement-type layer of cells which was generally associated with a colony of ES cells. The dermal origin of these cells was confirmed in CMV-GFP ES cell co-cultures as α-sma staining was not colocalised with GFP expression and was therefore not exhibited by ES cells. Confocal microscopy revealed this α-sma-positive layer of dermal cells wrapped around ES cell colonies (Fig. 4.19 E,F).

The second observation was the distinct lack of α-sma expression in many of the dermal cells. As mentioned previously dermal cells which were not associated with ES cell colonies changed morphology developing a characteristic bipolar appearance (Fig. 4.19 C). These cells had significantly reduced α-sma expression, in some cases losing it altogether (Fig. 4.19 D). The location of these cells between the ES cell colonies generally meant they were in the lower part of the co-culture but in longer-term co-cultures which had developed multiple layers of dermal cells, the bipolar morphology was evident in lower and upper layers of dermal cells, as was the lack of α-sma expression. These effects were seen consistently in ES cell co-cultures with PVG (n = 3) and Zin40 (n = 1) DP and DS cells.

To eliminate the possibility that the changes described were caused by culturing dermal cells in different media, α-sma expression was compared in dermal cells grown in CGR8 medium and cells grown in MEM + FBS. There was no significant difference in the level of α-sma expression (Fig. 4.20 A,B). The same was true when cells were cultured in complete CGR8 media.

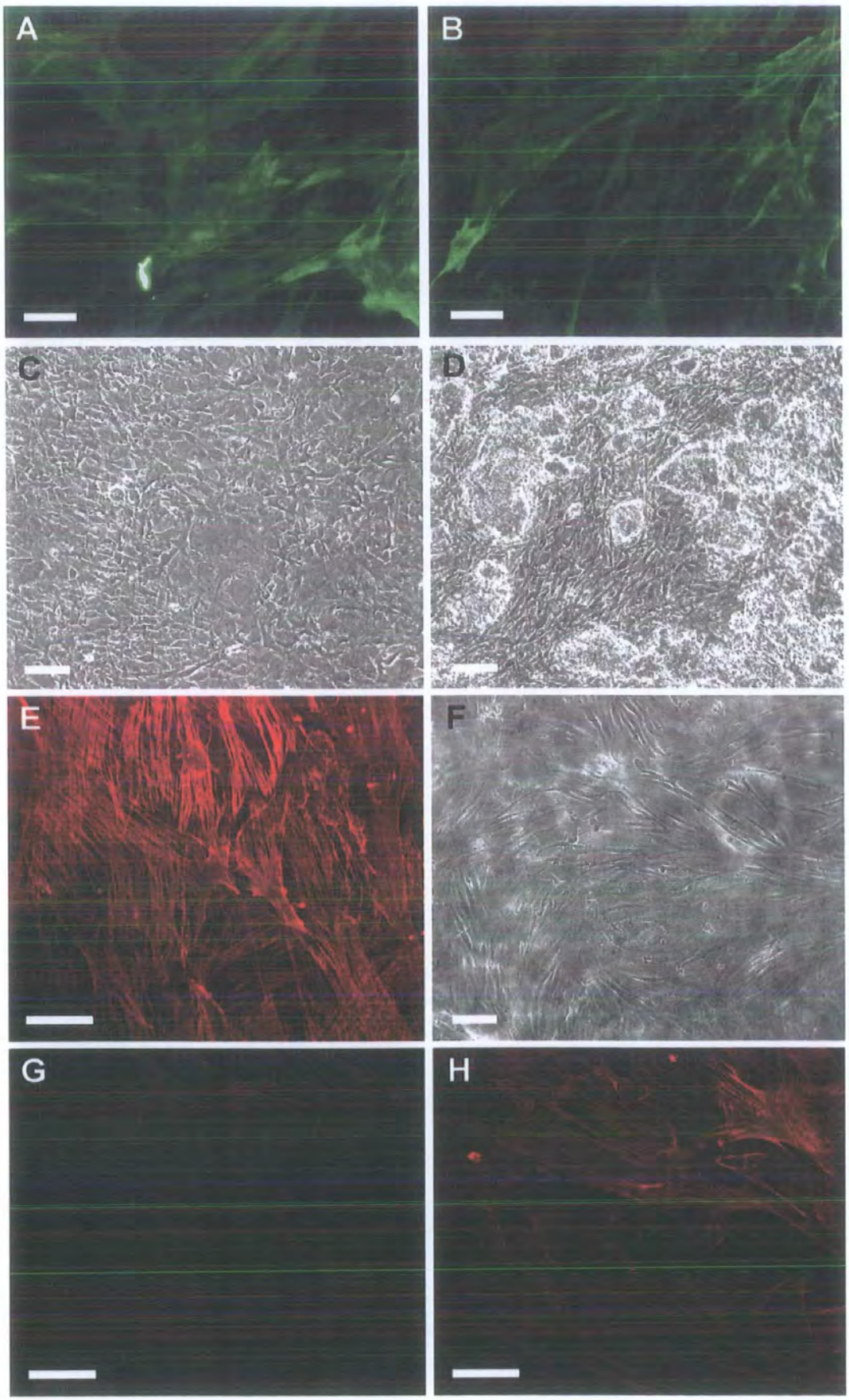
4.3.3.2. The influence of conditioned media on dermal cells

The above results suggested that ES cells were influencing dermal cells in two different ways. The change in morphology and downregulation of α-sma expression in

the dermal cells between the ES cell colonies, was either due to soluble factors or extracellular matrix produced within the co-culture. However, the redistribution of α-sma within the cells associated with ES cell colonies suggested a direct influence from ES cells. To investigate the influence of soluble factors an initial experiment was carried out using conditioned medium taken from a 10d Z40 DP/ES cell co-culture. After 4 days exposure to this medium, Z40 DP and DS cells showed no significant change in the level or pattern of α-sma expression (data not shown). (It should be noted that this medium had been stored at -20°C for 6 months so factors may have degraded in this time.) The most direct way to test the hypothesis that soluble factors resulted in a reduced α-sma expression was to expose dermal cells to the medium in which a co-culture was growing without allowing any contact. This was achieved by culturing dermal cells in the base of a culture well in which a co-culture was growing on a suspended porous membrane.

DP and DS cells of low passage number (P3) were plated out in wells which then had membrane inserts added and dermal cells were plated on the membrane. ES cells were subsequently added to form a co-culture on the membrane. By this method dermal cells on the base of the well (Fig. 4.20 C) were cultured in the same medium as the co-culture (Fig. 4.20 D), so were exposed to exactly the same soluble factors as if grown in co-culture but with no chance of direct contact with the ES cells. This was done with Z40 DS and DP cells, and PVG DP cells and in all cases over the 5 - 6 days exposure to co-culture medium, the dermal cells at the base changed from the normal morphology (Fig. 4.20 C) to the bipolar, spindly phenotype seen previously in co-cultures (n = 1 for each dermal cell type, two membranes for each) (Figure 4.20 F). When Z40 DP cells at the base of the well were fixed and stained for α-smooth muscle actin the expression level appeared weaker than controls (Fig. 4.20 G,H compared to E).

Figure 4.20. Influence of soluble factors on α-smooth muscle actin expression.
PVG DS cells cultured in MEM+FBS (A) or CGR8 media (B) for 8 days exhibit the same α-smooth muscle actin (α-sma) expression.
Zin40 DP cells cultured in wells separated from cocultures by a porous membrane demonstrated that soluble factors influence α-sma expression. A control culture of DP cells (C) stained positively for α-sma (E). The same cells cultured beneath a coculture (D) for 4d had changed morphology with many cells adopting a bipolar appearance (F) and these cells had considerably reduced α-sma expression (G) whilst a few maintained their fibroblast-like appearance and these tended to have slightly higher α-sma expression but this was still weaker than controls (H).
Scale bar: A, B = 50μm, C, D, F = 100μm, E, G, H = 25μm.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4.4. DISCUSSION

4.4.1. Summary

The work presented in this chapter shows that reciprocal interactions occur between dermal cells of the rodent vibrissa follicle and embryonic stem (ES) cells *in vitro*. Pilot experiments indicated dermal cells could support ES cell growth in media in which they would not normally survive. This early indication that dermal cells influenced ES cells, was confirmed by the finding that ES cells, maintained in co-culture with dermal cells, retained their pluripotency over a considerable length of time. Cytokines that are known to maintain ES cell pluripotency were found to be expressed at the mRNA level by follicle dermal cells *in vitro* and also in mid-anagen vibrissa follicles *in vivo*.

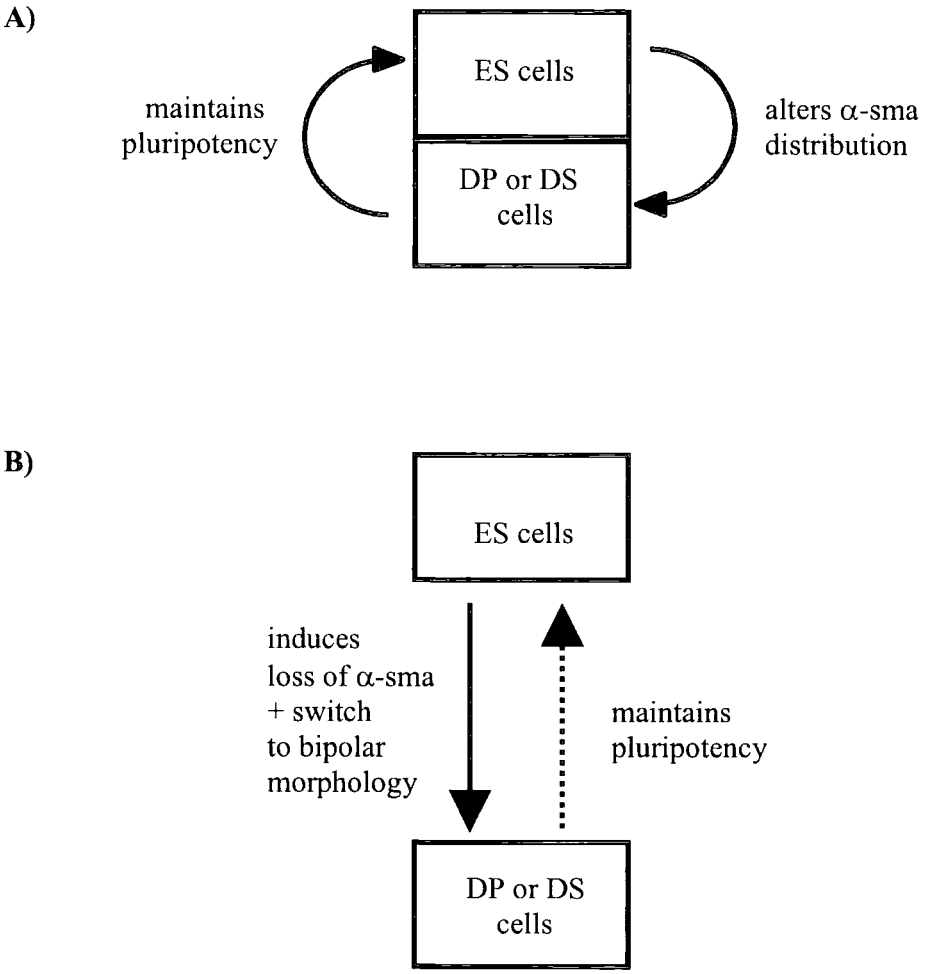
The influence of the ES cells on the dermal cells was observed using immunostaining for α-smooth muscle actin, combined with the use of GFP expressing ES cells. This demonstrated two major changes in the dermal cell characteristics. Dermal cells closely associated with ES cells were found to express the cytoskeletal protein, but the pattern of expression had changed considerably from the usual stress fibre pattern. In contrast, dermal cells in the lower part of the culture, in between ES cell colonies, had significantly reduced levels of α-smooth muscle actin expression and were frequently undetectable by fluorescence microscopy.

The interactions described are summarised in Figure 4.21 including both short and long range effects. Together these results indicate a complex relationship in dermal/ES cell co-cultures. The results have implications for dermal cell influences on other cell types within the hair follicle, and stem cell research in general with regards to how ES cells can influence more differentiated cells.

Figure 4.21. Interactions between follicular dermal cells and ES cells in co-cultures.

(A) Close contact interactions.

(B) Interactions mediated by soluble factors.



4.4.2 The influence of dermal cells on ES cells

4.4.2.1. Co-culture morphology

The morphology of the follicular dermal cell/ES cell co-cultures indicated ES cells were multiplying in such a way as to minimise contact with the dermal cells and so maintain their own characteristics. The colonies formed resembled those seen in cultures of ES cells maintained by LIF or by a feeder layer (Smith et al., 1988; Smith et al., 1992). TEM gave the first evidence that colonies comprised ES cells and this was confirmed when co-cultures were set up with ES cells expressing GFP. Some differentiation of CMV-GFP ES cells was evident for a small percentage of cells within the co-cultures but this is a normal feature of ES cell culture (Smith et al., 1992).

Electron microscopy of sections through co-cultures revealed a distinct similarity with another co-culture system that has used follicular dermal cells, that of dermal papilla and germinative epithelial (GE) cells. In such co-cultures, GE cells form organotypic structures surrounded by dermal papilla cells (Reynolds & Jahoda, 1991; Reynolds et al., 1993). A distinct basement membrane is laid down, separating the two cell populations, which is not evident in dermal/ES cell co-cultures but the general arrangement is very similar.

In some co-cultures with skin fibroblasts the ES cells appeared less distinct than in the follicular dermal cell co-cultures, resulting in a different overall morphology. Other skin fibroblast/ES cell co-cultures displayed a morphology quite similar to follicular dermal cell co-cultures. The identity of cells within skin fibroblast cultures is variable particularly with respect to numbers of cells expressing α-smooth muscle actin (Reynolds et al., 1993). An explanation for this is that cultures of dermal fibroblasts from rodent skin explants will almost inevitably include a contaminating population of follicular or glandular fibroblasts. Due to this the RDP-B cell line was used as a more consistent option for comparison to primary dermal cell cultures. This transformed cell

line does not express α-smooth muscle actin, does not display aggregative behaviour and has lost the inductive properties of primary dermal papilla cells. It therefore represents a dermal fibroblast population with none of the characteristics specific to follicular dermal cells.

4.4.2.2. Isolation and proliferation of cells

For the results of the pluripotency assays to be valid the cells committed to these assays needed to be as pure a population as possible. Cells plucked from co-cultures were therefore cultured in non-differentiating conditions for a period before starting the differentiation assays. This served two purposes: first, there were insufficient numbers of cells to start the differentiation assays and therefore a period of proliferation was required; and second, it is advisable to passage ES cells at least once if they have been maintained on feeder cells (that is essentially what the dermal cells are acting as in the co-cultures). This is to avoid any incorporation of the dermal cells into the embryoid bodies (EBs) (Hole & Smith, 1994), a possibility that I monitored by the use of CMV-GFP ES cells. Whilst GFP-positive EBs are not guaranteed to be purely ES cell-derived structures, the odd contaminating dermal cell being undetectable, at later stages in the differentiation assays the expression of GFP by cells of multiple morphologies, including the target cell populations, demonstrated these cells were derived from ES cells.

During the proliferation period, GFP was an invaluable tool for tracing the origin of cells. In some cases the morphology was straightforward and GFP expression simply confirmed what was already evident. However, for cells of a fibroblast morphology, fluorescence microscopy enabled differentiating CMV-GFP ES cells to be distinguished from contaminating dermal cells. The expression of GFP was observed in cells that had differentiated along a variety of lineages, as would be expected from the findings of

Hadjantonakis et al. (1998), who used the same vector to establish GFP-expressing ES cells and subsequently produced chimaeric mice which exhibited GFP expression in all cell lineages. GFP expression was therefore regarded as a means of identifying cells of ES cell origin, in all stages of differentiation.

Differentiation inevitably did occur to some extent in virtually all the cultures and more so in some than others, but there was no pattern as to the resulting cell lineage. In ES cell cultures there is always a background level of differentiation that is not inhibited by increasing the concentration of LIF (Smith et al., 1992). All indications during this proliferation stage were that the cells isolated from co-cultures were behaving in a manner typical of pluripotent ES cells.

4.4.2.3. Pluripotency assays

The fact that ES cells are pluripotent has been clearly demonstrated by their contribution to every cell lineage in chimaeric mice (Bradley et al., 1984, Saburi et al., 1997). Spontaneous differentiation upon LIF withdrawal *in vitro* results in differentiated EBs which frequently contain cardiac muscle, neuronal, chondrocytic and haematopoietic cells (Martin, 1981; Zhuang et al., 1992; reviews - Bradley, 1990; Rathjen & Rathjen, 2001). However, methods have only been established for inducing differentiation along a limited number of cell lineages including neuronal cells, adipocytes and chondrocytes (Dani et al., 1997; Bain et al., 1995; Kramer et al., 2000). Recently, growth factors have been identified which induce endodermal cells to differentiate further than occurs in spontaneous differentiation, resulting in mature hepatocytes (Hamazaki et al., 2001). All these differentiation procedures result in a mixed cell population with a relatively high percentage of the target cell lineage. However, it would be advantageous to achieve pure cell populations if these cells are to be used for investigations beyond the initial differentiation process. An endothelial cell

differentiation method has been described by Balconi et al. (2000) that utilises the Polyoma middle T virus in a selective immortalisation procedure, resulting in a pure endothelial cell population. Further methods to achieve pure differentiated cell populations will no doubt develop as the field of stem cell research is explored and more investigators make use of ES cells as a research tool.

The differentiation assays used in this study were chosen to produce a cell lineage derived from each of the three different layers of the trilaminar germ disc. Neurons are derived from the ectoderm (more specifically neuroectoderm), adipocytes are of mesodermal origin, whilst alpha-feta-1-protein (AFP) and albumin are markers of endodermal cells. The fact that ES cells plucked from co-cultures were capable of differentiating into all of these lineages is a strong indication that they retained the key characteristic of the embryonic stem cell, pluripotency.

The neuronal assay was based on the method described by Bain et al. (1995) to produce cells of a neuronal phenotype. In their article the authors referred to these cells as neuron-like cells, despite having detected tubulin and neurofilament proteins, expression of neuron-specific genes, and electrophysiological properties, including the capacity to generate action potentials and expression of a variety of ion channels. The authors declare caution on the basis that specialized functions such as the formation of synapses has not yet been demonstrated by these cells. On this basis the results of our work have also produced neuron-like cells, but as more and more evidence of ES cells resulting in differentiated, well-characterized cells comes forward, so the confidence that these cells are fully functional neurons increases. ES cell derived glial cells have certainly proved their functional capacity *in vivo* by myelinating host axons after transplantation into myelin-deficient or chemically demyelinated rat spinal cord (Brustle et al., 1999; Liu et al., 2000). There is a strong possibility that the adherent, fibroblastic cells seen associated with the neuron-like cells in this assay were glial cells,

as neuronal cell culture is rarely successful in the absence of glia. It would be interesting to establish the nature of these cells by immunostaining with a panel of glial cell markers.

Dani et al. (1997) describe the use of retinoic acid and adipogenic hormones, insulin and triiodothyronine (T_3) to induce differentiation resulting in large numbers of adipocytes. Retinoic acid (RA) is also crucial to neuronal differentiation, but at a later stage (4-8d) than in the adipocyte assay (2-5d), an example of how temporal expression patterns are as important as spatial expression patterns in developmental biology. Indeed, Dani et al. (1997) found treatment with RA before 2d to be cytotoxic whilst later treatment did not result in adipogenesis, revealing a strict window of time permissive for commitment to the adipocyte lineage. In a protocol which employed BMP-2 in induction of chondrocyte differentiation, also a mesenchymal lineage, the 2-5d period was also critical (Kramer et al., 2000). Kramer et al. (2000) conclude that their results, when combined with similar findings involving different mesenchymal lineages (Dani et al., 1997; Johansson & Wiles, 1995; Rohwedel et al., 1998), indicate that this early stage of differentiation is the time when mesenchymal cells have their fate determined along a particular lineage.

As expected only a small proportion of cells were stained positively for albumin and less cells stained for AFP. This difference between the two endodermal marker proteins could reflect the different temporal expression patterns seen in EB development. Whilst AFP mRNA is expressed in day 5 EBs albumin is not expressed until much later (day 13). Subsequently, AFP expression is downregulated as cells mature whilst albumin is strongly expressed at day 18 and *in vivo* is maintained in adulthood (Abe et al., 1996; Hamazaki et al., 2001). Differentiating cultures may contain endodermal cells that express albumin but have reached the stage at which AFP is downregulated and these cells are, therefore, undetected by immunostaining for AFP.

The presence of cardiomyocytes within many of the differentiated cultures, particularly those of the endodermal assay, provides further evidence of the pluripotency of these cells.

The CFU-A assay has been utilised as a standard assay for haematopoietic potential with a number of cell types (Pragnell et al., 1994). In my experiments however, the positive controls proved unreliable. Hence, the lack of cell bursts (positive indication of haematopoietic potential) in the experimental assays does not indicate that the cells from co-culture lack haematopoietic potential, simply that this assay was not working sufficiently well to deem whether the cells had haematopoietic potential or not.

The most rigorous test of pluripotency is to inject cells into a blastocyst, reimplant this into a surrogate mother and produce a chimaeric mouse. If the cells can be shown to have contributed to the germline cells, as well as somatic cells, then there is no question of their pluripotency. For our purposes however the resulting chimaeras would not have served any purpose beyond confirming pluripotency. In the light of the fact that the dermal cells expressed cytokines already proven to maintain ES cell pluripotency, the use of animals was deemed unnecessary.

4.4.2.4. Controls for pluripotency assays

Positive controls were carried out with LIF-maintained ES cells to ensure the differentiation assay methods were successful. The original papers for the differentiation assays detailed the proportion of cells expected to differentiate along the target lineage. Such high proportions are not seen in cultures of pluripotent ES cells undergoing spontaneous differentiation upon withdrawal of LIF (Bain et al., 1995; Dani et al. 1997). Therefore the data presented demonstrates the differentiation of the

majority of cells along a particular lineage was due to the protocol these cells were subjected to rather than a predetermined fate.

Dermal cells exposed to retinoic acid (to simulate the neuronal assay) or to retinoic acid followed by T3 and insulin (to imitate the adipocyte assay) showed no significant change, indicating that neuronal and adipocyte differentiation had resulted from ES cells. This was confirmed by the detection of GFP expression within the differentiating cultures.

4.4.2.5. Long term maintenance

The pluripotency of cells was determined after 6, 10, 14 and 20 days of co-culture. The 6 day time point was significant as after this length of time without any external influence ES cells would have begun differentiation. This is clear both from our own observations (controls for the conditioned media experiments) and published work (Smith & Hooper, 1987; Smith et al., 1988; Duval et al., 2000). Cells were also plucked after 10, 14 and 20 days from co-cultures and all that were subjected to the differentiation assays gave consistently positive results. The longest co-culture tested was a 20d PVG DP/ES co-culture from which neuron-like cells were derived. This indicates that follicular dermal cells were effective at maintaining ES cells for a significant length of time, despite the changes that the dermal cells themselves underwent in relatively early stages of co-culture.

4.4.2.6. Initial hypothesis

The inductive capacity of dermal papilla cells has been well-documented (see 3.1.3 and Jahoda et al., 1984; Horne et al., 1986; Reynolds & Jahoda, 1992; Jahoda & Reynolds, 1993). Non-follicular epithelium will form follicles when associated with DP cells, demonstrating the ability of DP cells to direct the differentiation of cells in close

proximity, as is believed to be their physiological role in the adult hair follicle (Hardy, 1992; Jahoda & Reynolds, 1996). The initial hypothesis for my work was therefore that DP cells, when co-cultured with pluripotent ES cells, would induce differentiation along the lineage of follicular epithelial cells. The dermal cells did exert an influence on the behaviour of the ES cells, but not what was expected. In contrast to the working hypothesis, the dermal cells maintained the ES cells in an undifferentiated state, an effect usually achieved by exogenous LIF or embryonic stem cell renewal factor (ESRF) (Smith, 1991; Dani et al., 1998).

4.4.2.7. By what means do dermal cells maintain ES cells?

The maintenance of ES cells by LIF can be mediated by two means. LIF is produced in a soluble form and a matrix-associated form (Rathjen et al., 1990a). The morphology of ES cells exposed to dermal cell or co-culture conditioned media indicated that a soluble factor with the capacity to inhibit differentiation was present in the media. Whilst the experimental conditions were not ideal (batches of conditioned media had to be changed in some cultures due to limited stocks), media conditioned by co-cultures proved to be the most effective. Differentiating ES cells express LIF enabling the self-renewing population to be maintained (Rathjen et al., 1990b). Therefore, a proportion of the inhibitory factor in the co-culture conditioned media may be LIF from a small number of differentiating ES cells. However, given that pure dermal cell conditioned media has the same effect, albeit to a lesser extent, and ES cells are maintained in co-cultures, it appears that co-culture of these two cell types upregulates cytokine expression in either dermal or ES cells. As there appears to be more changes occurring in the dermal cells than ES cells in co-culture, I propose any upregulation is likely to be in the dermal cell population.

The media for these experiments was conditioned by non-aggregated, confluent DP cells. The aggregative phenomenon of DP cells has important physiological relevance to the development and cycling of the hair follicle and is likely to be associated with significant changes in the biochemistry of the cells (Jahoda & Oliver, 1984a). As such the property of maintaining ES cell pluripotency may be more evident in cultures at a particular state of aggregation than another, a possibility that could be investigated with media conditioned by DP cells in various states of aggregation.

4.4.2.8. Potential candidates

Four members of the IL-6 family of cytokines (LIF, CNTF, CT-1 and OSM) have the capacity to maintain ES cells in their undifferentiated state *in vitro* (Smith et al., 1988; Williams et al., 1988; Conover et al., 1993; Rose et al., 1994; Pennica et al., 1995) and transcripts of all four of these cytokines were detected in co-cultures. When considering my results it must be noted that the sample numbers were very small so whilst they may give an indication of cytokine expression, further work would be required to confirm this expression profile.

Another potential member of this cytokine family is an as yet unidentified ligand for the gp130/LIFRβ receptor complex known only as sweat gland-derived differentiation activity. Whilst functional assays have determined that this factor acts through the same pathway as LIF, CNTF, CT-1 and OSM, its identity remains unknown (Habecker et al., 1997). Another potential candidate is ES cell renewal factor (ESRF), a soluble macromolecule, whose ES cell maintaining-activity is trypsin-sensitive and independent of the LIFR/gp130/STAT3 pathway (Dani et al., 1998). The expression of ESRF was not investigated due to the lack of available sequence for primer design.

Cytokine PCR products were of the expected size, with one additional smaller product from the OSM primers. This is likely to be the mOSM 13 alternate transcript which results from alternate splicing of exons 1 and 3, giving a product 130bp less than the full OSM transcript (Voyle & Rathjen, 2000). The two splice variants of OSM appear to be differentially expressed in DP and DS cultures, although both are at extremely low levels. DP cells express the full length transcript whilst DS cells display a higher level of the shorter transcript. This splice variant has been proposed to encode an intracellular OSM protein, the significance of this is not yet known. Further work would be required to confirm if the differential expression of OSM splice variants, and the expression of CNTF, CT-1 and LIF, detected in DP and DS cells is reflected in the individual tissues *in vivo*.

By comparison with expression in single cell-type cultures the expression of each cytokine in DP/ES and DS/ES cell co-cultures can probably be attributed to the dermal cells (CT-1), the undifferentiated nature of the ES cells (OSM) or both (LIF and CNTF). In the case of RDP-B co-cultures LIF, CNTF and OSM are expressed in both RDP-B cell cultures and undifferentiated ES cell cultures. However, neither of these express CT-1 at the level detected in the RDP-B co-cultures. This suggests that either the ES cells are differentiating in RDP-B/ES co-cultures, or the interaction between the two cell types has resulted in upregulation of CT-1 expression in a sub-population of cells. Given the morphology of RDP-B co-cultures, in which ES cells are difficult to identify by phase microscopy after a few days of co-culture, the first scenario seems likely whilst the second may also be true.

It would be interesting to know if the interaction between the cell types results in altered expression levels in co-cultures, as appears to be the case for CT-1 in RDP-B/ES co-cultures, but due to the nature of the material it would be extremely difficult to assess this in a truly quantitative fashion. Whilst the RT-PCR could be carried out semi-

quantitatively, an equal amount of RNA from one co-culture may have different proportions of dermal and ES cell RNA to another similar co-culture. Once again the ability to separate the two cell populations and then analyse them would prove far more useful. For our purposes however, determining the presence or absence of cytokine transcripts was sufficient.

A more intriguing case of upregulation appears to occur with OSM. Not only does it appear to be upregulated in cocultures with DP and DS cells, but in ES cells alone the expression level is altered dependent on the conditions in which the cells were cultured. These three samples were processed simultaneously and the GAPDH levels were comparable so the difference in expression levels are significant. Undifferentiated ES cells (ES+LIF) display a similar level of OSM expression to those which have differentiated upon withdrawal of LIF (ES-LIF diff'd). However, those that differentiated in the presence of LIF (ES+LIF diff'd) have significantly higher levels of OSM. This would suggest that differentiation in the presence of LIF is not indiscriminate, but preferential towards one or more cell lineages which characteristically express OSM.

4.4.2.9. Maintenance of stem cell populations within the hair follicle

The expression of these cytokines *in vitro* clearly has a relevance in experimental work, but the important question is whether they play a functional role *in vivo*? The expression of LIF, CNTF and CT-1 in all three segments of mid-anagen vibrissa follicles suggests they are physiologically relevant. The distribution within the follicle was assessed in an attempt to determine the function of these cytokines. Previous studies have cited them as promoters and inhibitors of both cell differentiation and cell proliferation (Gearing et al., 1987; Williams et al., 1988; Smith et al., 1988; Malik et al., 1989; Conover et al., 1993; Rose et al., 1994; Pennica et al., 1995; Aubert et al.,

1999), processes which occur predominantly in the follicle end bulb and are key to the cyclic nature of the hair follicle. The end bulb is also the location of the primitive germinative epithelial cells which are maintained in an undifferentiated state, as are cells within the bulge of the outer root sheath. An expression pattern restricted to the end bulb, or the end bulb and the upper follilcle segment containing the bulge, would have supported the idea that the cytokines function to maintain the epithelial stem cells. OSM expression, although at barely detectable levels, was exclusive to the end bulb, reflecting the weak expression detected in DP and DS cells *in vitro*. The expression of LIF, CNTF and CT-1 in all segments of the mid-anagen follicle however, indicates a widespread function within vibrissa follicles.

Whilst cytokine expression may act to maintain the stem cell population, other molecules have been identified which are believed to regulate exit from the stem cell compartment. In those epithelia in which stem cells are in clusters, the release of cells into the TA compartment is likely to require changes in cell adhesion. Delta-Notch signalling appears to be involved in this process with stem cells expressing high levels of the ligand, Delta, whilst the highest levels of its receptor, Notch, are detected in the suprabasal layers (Lowell et al., 2000). The authors postulate that Delta-Notch signalling at the border of stem cell clusters stimulates cells to enter the TA compartment, whilst within the cluster, cell cohesiveness is enhanced by Delta expression.

Notch expression within the hair follicle exhibits a similar pattern. It is absent from the putative stem cells of the bulge and end bulb, but is highly expressed in the adjacent cell populations of suprabasal outer root sheath cells and epithelial matrix cells, particularly precursors of the cortex, cuticle and inner root sheath (Kopan & Weintraub, 1993; Favier et al., 2000). Kopan & Weintraub (1993) proposed that Notch regulates cell fate decisions, a suggestion that is supported by the expression pattern

through the adult growth cycle. During catagen, *notch* expression extends into the basal layer of ORS and in early anagen, is evident in the epithelial matrix and throughout the epithelial column (Favier et al., 2000). Delta is not expressed in follicular epithelial cells, but the expression of other Notch ligands, those of the Serrate/Jagged family, supports proposals that within the hair follicle, Notch participates in determining the fate of epithelial stem cells (Powell et al., 1998; Favier et al., 2000).

Another molecule proposed to stimulate exit from the epidermal stem cell compartment is c-Myc. Ectopic expression of c-Myc, under the control of the K14 promoter, impaired wound healing in mice and, over time, depleted the epidermal stem cell population. Intriguingly, sebaceous differentiation occurred at the expense of hair differentiation in the pilosebaceous unit, suggesting cells were stimulated to become TA cells of a sebaceous lineage rather than epidermal or hair lineages (Arnold & Watt, 2001; Waikel et al., 2001). This supports a model by which the step from stem cell to TA cell is not purely for amplification purposes, but also narrows the range of a cell's multipotency. Further evidence of the role of c-Myc in cell differentiation comes from recent studies describing three discrete rings of c-Myc expression in the anagen hair follicle. Each ring of expression corresponds to a layer of the inner root sheath at the level where cells within that layer undergo terminal differentiation (Rumio et al., 2000; Barajon et al., 2001). The transfer from stem cell to TA cell, and TA cell to terminally differentiated cell, is an area of research with important clinical relevance as it is likely to be reflected in the control of tumour growth.

Identifying the location of the stem cell and TA cell populations within the hair follicle has been the subject of many studies but there seems to be no single 'niche' in which they reside. The follicular bulge and end bulb have been proposed as putative stem cell sites (Cotsarelis et al., 1990; Reynolds & Jahoda, 1991; Gharzi et al., 1999) but more recently there has been evidence to suggest stem cells are more widespread

throughout the follicle. Oshima et al. (2001) proposed that whilst the upper follicle contains the vast majority of cells with clonogenic potential, these cells migrate down the outer root sheath during anagen, and become germinative epithelial cells prior to differentiation into one of the epithelial lineages that contributes to the growing hair fibre. The presence of cytokines in all segments of the follicle could therefore be explained as a means for maintaining the primitive state of the cells during this migration.

The presence of stem cells throughout the follicle is also proposed by Ghazizadeh & Taichman (2001) though in a somewhat different manner. They describe the pilosebaceous unit as separated into discrete sections (IRS, ORS, sebaceous gland, infundibulum), each containing a stem cell population, and propose hair follicle stem cells may be organised in a manner similar to epidermal stem cells. Distinct proliferative units produce the cells of the sections described and only under certain circumstances (such as a wound response) do adjacent proliferative units compensate for each other. By such a means the stem cells of the infundibulum contribute to the interfollicular epidermis, as described by Taylor et al. (2000). In normal circumstances however, the hair follicle proliferative units remain separate from those of the interfollicular epidermis. The location of stem cells within the hair follicle remains a matter of some debate but with the expression of the cytokines LIF, CNTF and CT-1 in the upper, middle and end bulb sections of the vibrissa follicle, the potential for harbouring stem cells appears to exist throughout this organ.

Whilst epithelial stem cells within the hair follicle have been the subject of intense investigation, the dermal components of the hair follicle have not really been investigated as a source of stem cells. However, as the outer root sheath contributes to the epidermis in a wound-healing scenario, the dermal sheath has been proposed to contribute to regenerating skin (Jahoda & Reynolds, 2001). Further multipotency has

been demonstrated in both DP and DS cells using the CFU-A assay described in 4.2.3.2.1. Microdissected tissue fragments of dermal papilla and dermal sheath, and cultured DP and DS cells, gave positive results in this assay indicating the presence of cells with haematopoietic potential (M. Lako, personal communication). LIF and similar cytokines may be functioning within the follicle to maintain a suitable niche for dermal stem cells as well as epithelial stem cells.

4.4.2.10. Cytokine signal transduction in the hair follicle

Having established that the cytokines are widely expressed in the hair follicle there is the possibility that their signalling is restricted by extracellular modulation or the distribution of receptors. LIF is produced in both extracellular matrix-associated and diffusible forms by alternative splicing at the exon1/exon2 boundary. The two forms appear to be transcribed from different promoters and hence, could be differentially regulated (Rathjen et. al., 1990a). Expression of the immobilised extracellular matrix form will be spatially regulated and exert a more selective influence on the adjacent cells, whilst the diffusible form will influence any cells in the environment which express the appropriate receptors. Determining the expression of these alternate transcripts within the follicle may provide some clues as to the cells that are targeted by cytokine signalling.

Similarly, determining the expression of cytokine receptors might highlight the target cells. LIF, CT-1 and OSM operate through the gp130/LIFRβ complex whilst CNTF requires a third component named CNTFRα to make a functional CNTF receptor complex (reviewed by Kishimoto et al., 1994; Bravo & Heath, 2000). We found CNTFRα to be expressed in the vibrissa follicle which suggests hair follicles may be the location of the transcripts detected at low levels in skin by Ip et al. (1993).

The expression of CNTFRα in the follicle is a novel observation. When this receptor subunit was first identified and its expression investigated, it was thought to be restricted to the central nervous system and hence the biological function of CNTF restricted in the same manner (Davis et al., 1991). Since then its expression pattern has been found to be more widespread (Conover et al., 1993; Ip et al., 1993; Yang et al., 2001) and sites of expression can now include the vibrissa follicle where it may be associated with the follicle's complex peripheral innervation. However, it may be involved in the maintenance of a stem cell population within the follicle.

Although these cytokines were investigated due to their known role *in vitro*, (and *in vivo* in the case of LIF) in maintaining a stem cell population, the numerous roles that these cytokines play in a wide range of tissues must not be ignored. Further evidence of their function in relation to follicular stem cells is required and altering expression by using transgenes under the K14 promoter could prove very useful in achieving this objective.

4.4.2.11. Involvement of STAT3 in the hair growth cycle

A component of the intracellular signalling pathway downstream of LIF, CNTF and CT-1 signalling has already been identified as functionally important in the hair growth cycle. Targeted disruption of the *Stat3* gene in keratinocytes resulted in a failure to initiate second anagen (Sano et al., 1999). This was attributed to cells being unable to migrate from the bulge after telogen, as *Stat3* -/- keratinocytes displayed inhibited migration in an *in vitro* assay (Sano et al., 1999). Subsequently, it was demonstrated that anagen could be initiated in these mice by mechanical or chemical means via a STAT3-independent signalling pathway that promoted keratinocyte migration (Sano et al., 2000). The authors proposed HGF and EGF as candidates in regulating follicular

keratinocyte migration via a STAT3-dependent pathway, but LIF, CNTF and CT-1 signalling via STAT3-phosphorylation might also influence cell migration.

Another migration-related study found DP cell conditioned media contains chemoattractants for outer root sheath cells *in vitro* (Fujie et al., 2001). The presence of soluble factors with inhibitory properties on ES cell differentiation was detected in conditioned media experiments. The soluble factors present are likely to include one or more of the cytokines LIF, CNTF and CT-1, all of which transduce their signal via a gp130 receptor complex and intracellular phosphorylation of STAT3, a key step in the maintenance of ES cells (Matsuda et al., 1999; Raz et al., 1999). It is possible therefore, that these cytokines may be effective both in maintaining the multipotency of a stem cell population within the hair follicle, and influencing its migration. Further investigation will be required to elucidate the contribution of these cytokines to various aspects of hair follicle biology.

4.4.2.12. Other potential roles for cytokines within the hair follicle

Cytokines are known to play a role in inflammation. Whether they are pro-inflammatory or anti-inflammatory depends on the cell type and the cytokine but the IL-6 cytokines are no exception (reviewed in Gadient & Patterson, 1999). In such a response cytokines are extremely fast acting and there is always a possibility when working with cultured cells, or tissue that has been mechanically manipulated, that such an inflammatory response has been initiated and cytokine expression is upregulated. Although there is no reason to believe this is the sole reason for cytokine expression within follicular cells or tissue it must be considered as a possibility.

The IL-6 family of cytokines play a wide variety of roles which are often opposite on two different cell types, for example LIF inhibits differentiation of ES cells whilst inducing differentiation of myeloid cells, the role by which it was discovered (Gearing

et al., 1987). So whilst the focus has so far been on their potential role as 'protecting' the stem cell population of the hair follicle, it is also possible that these factors may be present to promote differentiation of hair follicle stem cells. Slack (2000) expressed the opinion that whilst epithelial stem cells may act as unipotent cells the majority of the time, when tissue regeneration was required as a result of damage or insult, the same cells were multipotent and signals would be expressed which would activate this dormant multipotency. Potentially the IL-6 cytokines are ideal candidates for such a role given their rapid upregulation as part of the inflammatory response and their diverse roles in regulation of cell proliferation and differentiation.

Of all the possible roles discussed it should be remembered that there may be multiple roles for these cytokines within the hair follicle that may be elucidated via different intracellular pathways. A review on FGF signalling highlighted two distinct pathways that were independently responsible for influencing cell migration and proliferation (Boilly et al., 2000). It may be a similar case for the LIF family cytokines as two intracellular pathways activated by gp130 dimers have been identified (Ernst et al., 1994; Ernst et al., 1996; Ernst et al., 1999).

A wide array of molecules have been detected within the hair follicle but for most the functional significance of their presence is unknown (Stenn et al., 1994; Stenn & Paus, 2001). The hair growth cycle is a complex, dynamic process and has several secondary processes associated with it, such as remodelling of the vasculature and innervation, as well as melanogenesis. The detection of LIF, CNTF, OSM and CT-1 in cultured dermal cells is a novel observation and from the data presented here, and the body of literature on the diverse actions of these cytokines, a functional relevance is apparent. Further investigation is required to determine the mechanisms to which these cytokines are relevant within the hair follicle, whether they are directly related to the

regulation of the hair growth cycle, or as appears to be the case for versican (discussed in 2.4), are involved in systems associated with the vibrissa follicle.

Determining the expression pattern of the cytokines and the components of the receptor complexes would indicate the tissues that are targets of cytokine signalling and from this further studies could be developed. If, for example, the outer root sheath appears to be a tissue involved in cytokine signalling, altering expression of LIF or one of the other cytokines under the regulation of the K14 promoter may demonstrate an effect on the proposed stem cell population. Interfollicular wounds in such a model would demonstrate the effect of a cytokine on the outer root sheath cells that are known to migrate into the epidermis during epidermal wound healing (Taylor et al., 2000; Arnold & Watt, 2001), thus indicating the influence LIF, CNTF, OSM or CT-1 has on the stem cells in the upper follicle. The function of cytokines within the hair follicle could also be investigated by blocking the signal with antibodies, either in follicle organ culture, or by subcutaneous injection.

4.4.3. ES cells influence dermal α-smooth muscle actin expression

The initial hypothesis was proved wrong regarding the influence of follicular dermal cells on ES cells, and whilst the ES cells maintained their own characteristics they appeared to influence the dermal cells within co-cultures. This was initially evident from the altered morphology observed in dermal cells in areas between colonies. Bipolar morphology was observed in these cells by phase microscopy. Immunostaining with an antibody against α-smooth muscle actin revealed changes in expression in all dermal cells. The exact nature of the change was apparently dependent on the relationship of the dermal cell with ES cells.

4.4.3.1. Two methods of influence: contact and soluble/ECM factor

The bipolar cells situated betweeen ES cell colonies had dramatically downregulated expression of α-smooth muscle actin, most cells being undetectable by fluorescence microscopy. In contrast, the follicular dermal cells that were situated on the surface of ES cell colonies expressed α-smooth muscle actin, but the pattern of expression at the intracellular level had changed. α-smooth muscle actin expression was altered from a stress fibre pattern, typically observed in cultured follicular dermal cells, to expression localised to the edge of the cell and points of contact with adjacent cells. This expression was reminiscent of that seen in ES cell-derived endothelial cells (Balconi et al., 2000). The lack of a similar intracellular α-smooth muscle actin expression pattern in dermal cells exposed to conditioned media, indicates that contact, or at least close association (possibly via the extracellular matrix), with ES cells is necessary for cells to retain their α-sma expression in ES cell co-cultures and switch to the squamous morphology observed.

In cells at the base of the co-cultures, away from the ES cells, α-smooth muscle actin expression was down-regulated whilst the cells underwent a visible change in morphology, becoming bipolar. The effector molecule resulting in this change was a soluble factor secreted into the media either by the ES cells, or by the dermal cells under direct influence of the ES cells by contact. Evidence for this comes from experiments in which co-cultures were grown on membranes above dermal cell cultures. Morphological changes occurred within 2-3 days and when the dermal cells were stained after 5 days, downregulation of α-smooth muscle actin was evident compared to controls. The fact that these changes occur in synchrony suggests the cells may have become more motile as the bipolar morphology was one of a less adherent cell, and α-smooth muscle actin has been connected with a retardation in cell motility

through organization of focal contacts (Ronnov-Jessen & Petersen, 1996). Why the cells should become motile is unclear. It is possible that the ES cells were producing a chemoattractant, resulting in dermal cells engaging with the ES cell colonies and changing morphology to the squamous cells. If this was the case it would be intriguing to be able to follow individual cells over a time course of these co-cultures, observing both morphology and α-smooth muscle actin expression.

The two changes in α-smooth muscle actin expression indicate there were two different influences exerted on the dermal cells, dependent on their relationship with the ES cells. Over a distance, soluble factors downregulated expression of this cytoskeletal protein. Close contact on the other hand, resulted in altered cytoarchitecture and accordingly, the distribution of α-smooth muscle actin was changed.

4.4.3.2. Reprogamming somatic cells

The changes observed in dermal cells indicate that the ES cells were effectively reprogamming the follicular dermal cells. Reprogramming a cell involves changes to the nucleus to direct expression of genes that were not previously expressed and suppression of some of those that were. The most direct reprogramming of nuclei has been achieved by somatic nuclear transfer. That is the injection of a somatic cell nucleus into an enucleated oocyte. The cytoplasm of the oocyte will reprogram the nucleus so the cell becomes totipotent as demonstrated by cloning experiments resulting in healthy mammals (Wilmut et al., 1997; Wakayama et al., 1998). These experiments have demonstrated the ability of cytoplasm to reprogram nuclei when direct contact has been established. Other experiments have suggested reprogramming can occur without such drastic interjection.

Non-follicular epidermis has been shown to transdifferentiate upon the induction of follicle formation by cultured dermal papilla cells (Reynolds & Jahoda, 1992). However, this is transdifferentiation as opposed to reprogramming, as the production of follicular epidermal cells could be attributed to the resident population of epidermal stem cells. The first clear evidence that transient amplifying cells can be reprogrammed came from Ferraris et al (2000) who combined central corneal epithelium, isolated from the corneal stem cell population which is restricted to the periphery of the tissue, with dermis from a variety of locations. Under the influence of embryonic dermis, corneal epithelium gave rise to hair follicles or sweat glands. The results suggested that the corneal epithelium first reverted to a stem cell-like condition before differentiating into the more complex epidermis. The down-regulation of α-smooth muscle actin in follicular dermal cells in ES cell co-cultures may well be a process of dedifferentiation prior to differentiation along another cell lineage, possibly that seen in dermal cells in contact with the ES cell colonies.

In culture, myotubes have been shown to be reprogrammed by a number of means (reviewed by Hughes, 2001). Induction of the transcription factor Msx1 resulted in a significant proportion of myotubes cleaving, to become mono-nucleated cells which were then capable of expressing markers of a number of lineages other than muscle (Odelberg et al., 2000). To investigate further the potential of dermal cells for reprogramming other cell types, it would be interesting to co-culture follicular dermal cells with these myotubes. Ferraris et al (2000) suggested corneal epithelia was dedifferentiated prior to production of hair follicle and glandular cells and the combination of follicular dermal cells with myotubes provides the opportunity to investigate if this property extends beyond epithelial tissues.

Mesenchymal tissue has been demonstrated to reprogram epithelial tissue, but there are also instances in which the reverse is true. Barasch et al. (1999) demonstrated

that LIF induces mesenchymal cells of the kidney to convert to epithelial cells and go on to form nephrons. The adjacent cells of the ureteric bud are the source of LIF whilst the kidney mesenchyme expresses the receptors gp130 and LIFR. Interestingly the authors found that the ureteric bud expresses CNTF, OSM and CT-1 as well as LIF. It seems a common theme that these cytokines are rarely expressed in isolation of each other, suggesting that the different cytokines can compensate for each other, or that a delicate balance between them is required to regulate processes in which they are influential.

Recent work by Jahoda & Dhouailly has indicated that ES cells have an inhibitory effect on appendage morphogenesis *in vivo* (C. A. B. Jahoda, personal communication). Where follicle formation would normally occur, the presence of ES cells appears to prevent this process. Combined with my results this would suggest that ES cells have a propensity to inhibit or reprogram dermal papilla cells in some way.

4.4.3.3. Interactions between cells at different stages of differentiation

From these studies it appears that the less differentiated cell type exerts a greater influence on more differentiated cells than *vice versa*. This is also the case in somatic nuclear transfer with the simple cytoplasm of the oocyte directing the somatic cell nucleus, resulting in the development of an embryo with the genotype of the donated nucleus (Wilmut et al., 1997; Wakayama et al., 1998). Embryonic dermis affects adult epithelia and embryonic stem cells affect adult dermal cells. If this is a general phenomenon, it would be interesting to investigate the potential of ES cells for reprogramming other cell types besides follicular dermal cells. By co-culturing ES cells with a selection of cell types which are well characterised and have good lineage markers the degree of de-differentiation or reprogramming could be established. For example, if ES cells were found to change expression of MyoD in muscle cells or

neurofilament expression in a neuronal culture, this would be an extremely interesting result demonstrating a significant level of reprogramming.

4.4.4. Conclusions

I have shown that follicular dermal cells are capable of maintaining pluripotent ES cells *in vitro*. The detection of LIF, CNTF and CT-1 expression in cultured follicular dermal cells, as well as intact vibrissa follicles, is a novel observation. I have demonstrated that ES cells exert strong influences, by soluble factors and close contact, on follicular dermal cells. This capacity of ES cells to influence other cell populations is an aspect of ES cell biology that has not previously been observed and merits further investigation. Under the influence of ES cells, the dermal cells display a degree of plasticity that results in significant changes in their phenotype, suggesting a level of multipotency that was previously unrecognised. The multipotent cell population within the dermal component of the follicle has the potential to contribute to tissues beyond the confines of the hair follicle, thus providing an accessible source of stem cells that could be exploited for therapeutic applications.

Chapter 5: Conclusions

In this thesis I describe work using the vibrissa follicle as a model for studying various aspects of hair follicle development, innervation, and regeneration *in vivo*, in addition to investigating the relationship between follicular dermal cells and embryonic stem (ES) cells *in vitro*. Central to the study is the character and properties of hair follicle dermal cells. Thus, in my first experimental chapter, amongst other things, I investigated the appearance of dermal condensations, the precursors of both dermal papilla and dermal sheath cells. One of the questions examined in the following chapter was the transition of dermal sheath to papilla phenotype during the process of follicle regeneration. I then set out to determine whether the well established inductive powers of follicle papilla and sheath cells could act in the context of ES cell differentiation.

In considering the results, one important point that emerged from the work concerns the complexity of molecular expression in the follicle, and an understanding that an individual molecule may play different functional roles in conjunction with different follicular activities. Another was that the role played by groups of molecules may be subject to multiple interpretation so the context in which molecular expression is examined in relation to the follicle is very important. Although the combination of follicular dermal cells with ES cells *in vitro* produced results that were totally unexpected, in terms of novelty and interest these data were intriguing, and may produce real insights into questions of cell lineage and reprogramming.

Versican was observed to have a relationship with follicle innervation throughout development, during the growth cycle and the regeneration of the adult follicle. Strong expression in the amputated dermal sheath indicated a role for versican in the early wound response of this tissue. Whilst versican may have dual or multiple roles in the hair follicle, I propose that a significant function of this proteoglycan is to regulate the complex follicular innervation.

My work highlights the point that differential expression of molecules within the hair follicle, should be interpreted on the basis that the follicle is comprised of more than just a hair growth mechanism. Innervation, vascularisation and melanogenesis are all systems that change with the follicle cycle and should be considered when attributing function on the basis of distribution.

Follicular dermal cells were co-cultured with ES cells, the hypothesis being that the ES cells would be induced to differentiate along a follicular epithelial cell lineage. These novel experiments proved the original hypothesis wrong, as differentiation was not induced. In fact, the ES cells remained pluripotent whilst exerting strong influences on the dermal cells. The maintenance of the ES cells in co-culture can be attributed to the production of cytokines previously demonstrated to maintain ES cells *in vitro*. I made the original observation that LIF, CNTF, CT-1 and OSM were expressed in follicular dermal cell cultures and the vibrissa follicle. In isolation, the expression of these cytokines within the follicle might have been viewed as an indication that they were involved in the follicle growth cycle. However, having observed the maintenance of ES cells in co-culture, I propose that these cytokines regulate the stem cell population within the follicle.

Follicular epithelial stem cells have been the subject of intense study for a number of years and there is an increasing body of literature that suggests they are not restricted to a single location within the follicle. Recently, Shimazaki et al. (2001) demonstrated that signalling via the CNTFR/LIFR/gp130 receptor complex supports the self-renewal of a neural stem cell population *in vivo*. Thus, the differentiation inhibitory activity of these cytokines is not restricted to ES cells. The expression of LIF and other IL-6 family cytokines throughout the length of the vibrissa follicle therefore, provides evidence that the follicular environment is suitable for widespread distribution of stem cells. This is supported by my observations of epithelial cell proliferation at regular

intervals along the regenerating follicle. I propose that IL-6 family cytokines act to provide an environment in which stem cells can migrate through the follicle in an undifferentiated state.

The maintenance of ES cells by dermal cells was reciprocated by the ES cells altering the phenotype of the dermal cells, demonstrating that follicular dermal cells have a greater degree of plasticity than was previously recognised. Follicular dermal cells possess a degree of multipotency, demonstrated *in vivo* during the induction of follicle formation and regeneration. In addition to this, recent evidence of haematopoietic potential within these tissues (M. Lako, personal communication), suggests the multipotency of follicular dermal cells extends to lineages beyond those of the follicle. Whilst the epithelial stem cells of the follicle have been the focus of many studies, the dermal cell population appears to have considerable potential as a source of multipotent stem cells. The extent of this multipotency is not evident *in vivo*, but *in vitro* experiments, such as the co-cultures with ES cells, provide an excellent opportunity to investigate the full potential of these cells.

My results could have a profound impact on stem cell research, raising new questions about the inductive capacity of ES cells. This characteristic has not previously been recognised in ES cells, as the focus of stem cell research has been to determine the mechanisms that induce ES cells to differentiate, rather than to investigate their influence on other cell populations. Having demonstrated that expression of a follicular dermal cell marker is altered by ES cells, the fundamental question of whether ES cells affect other somatic cell populations in a similar way, could be answered with co-cultures of other differentiated cell types, for example, muscle or glial cell cultures. The analysis of lineage markers in such co-cultures would demonstrate whether ES cells have an innate ability to reprogram somatic cells. A new perspective on ES cell biology

has been highlighted by my novel approach of co-culturing these cells with somatic cells.

APPENDIX I

Preparation of Materials

CELL CULTURE REAGENTS AND MEDIA

TVP: 250mg trypsin

372mg EDTA, disodium salt

10ml chicken serum

1litre PBS (Oxoid tablets)

filter-sterilize and store in aliquots at -20°C

Alpha medium stock: One 10 litre alpha medium powder pack + 200mg gentamycin sulphate + 100ml MEM 100x vitamins made up to 3 litres with dH_2O and filter sterilized.

2x CfuA medium:

42ml alpha medium stock

50ml donor horse serum

2ml glutamine

6ml $NaHCO_3$ (7.5%)

COS cell medium:

50% HAMS F-12 medium

50% Glasgow MEM

1% FBS

glutamine (1x)

ELECTRON MICROSCOPY

0.2M Sodium cacodylate buffer:

4.28g $Na(CH_3)_2AsO_2.3H_2O$ in ddH_2O made up to 100ml

pH adjusted with HCl to 7.3

Karnovsky's fixative: 40% solution A and 60% solution B

Solution A = 2g paraformaldehyde dissolved in 40ml ddH_2O

Solution B = 10ml 25% gluteraldehyde

50ml 0.2M sodium cacodylate buffer (pH7.3)

NB/ Solutions A and B are kept separate at 4°C until just before use

IMMUNOSTAINING AND IN SITU HYBRIDISATION

Mowiol:

2.4g mowiol 488 (Calbiochem) and 6g glycerol were added to 6ml dH_2O then mixed thoroughly and incubated at 37°C overnight. 12ml 0.2M TrisHCl (pH8.5) was added and the mix agitated at 50°C until everything had dissolved. The solution was then clarified by centrifugation at 5000xg for 15 minutes before adding 1,4-diazobocyclo-[2.2.2]-octane (DABCO) antifade agent (Sigma) to 2.5%. When this was thoroughly dissolved the mowiol solution was aliquoted and stored at -20°C.

Follicle powder

Vibrissa follicles were dissected from 3 adult PVG rats and put in MEM. The follicles were ground to a powder in liquid nitrogen using a pestle and mortar before adding ice-cold acetone. The suspension was poured into a 50ml plastic tube and kept on ice for 30 minutes. The powder was then pelleted by centrifugation (1 minute at 1700rpm) and the acetone poured off. 1ml ice-cold acetone was added, the pellet resuspended and centrifuged again (2 minutes at 2000rpm) before the supernatant was removed by pipetting and the pellet spread out on a clean surface to air dry. The dry powder was then stored at 4°C in a microcentrifuge tube.

APPENDIX II

Pilot experiments

ES cells are routinely cultured in CGR8 media whilst follicular dermal cells are maintained in MEM with FBS. When dermal cells were first cultured in CGR8 media it was observed that the cells remained healthy although the growth rate was slightly slower than in MEM with FBS. In contrast, when ES cells were cultured in MEM, with or without FBS (20%), the cells died. Cocultures were set up in both types of media and all cells appeared to be viable. This suggested that the dermal cells supported the ES cells in coculture in MEM + FBS. In an attempt to determine whether this was by contact or by secretion of factors into the culture media conditioned MEM + FBS was removed from a flask of PVG DS cells and diluted in CGR8 media as well as fresh MEM. ES cells were then cultured in the two dilution series of DS cell conditioned media and it was found that the cells died in all cases except those containing CGR8 media.

Summary of experiments to determine media requirements of cultured cells

Cell type	CGR8 Media	MEM + FBS	DS cond. MEM
DP	+	+	NA
DS	+	+	NA
ES cells	+	-	-
DP/ES	+	+	NA
DS/ES	+	+	NA

The conclusions from these preliminary experiments were: 1) dermal cells can be maintained in CGR8 media; 2) ES cells can not be cultured in MEM + FBS; 3) viable dermal cells can support the growth of ES cells in MEM + FBS; 4) factors secreted into the culture media are not sufficient alone to maintain ES cells in MEM.
On the basis of these experiments further investigations were carried out with all cocultures maintained in CGR8 media.

Dermal cells were cocultured with ES cells either as a mixed culture or a monolayer with ES cells added. For the mixed cultures, the two cell types were trypsinised, spun down and resuspended. Equal volumes of the cell suspensions were then mixed, the cells spun down again, resuspended in the appropriate growth medium and plated out in 35mm dishes. For the monolayer cultures, dermal cells were grown in 35mm dishes to form a monolayer. ES cells grown in 0.1% gelatin-coated culture flasks were trypsinised with TVP, spun down, resuspended and a volume of cell suspension added to each dish of dermal cells.

The two types of coculture did not appear to be any different morphologically. Colonies of cells developed in both mixed and monolayer cocultures. After this inital comparison subsequent cocultures were all set up using an initial monolayer of dermal cells and adding ES cells to this.

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