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Investigating the regulatory roles of Platelet-derived Growth Factor in the dermal stem cell niche

by

Garrett Evan Moffatt

A THESIS

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Abstract

Skin-derived Precursors (SKPs) are multipotent, self-renewing adult dermal stem cells able to differentiate into functional neural and mesenchymal progeny. SKPs reside within a specialized niche including both the hair follicle dermal papilla and dermal sheath and function to induce hair follicle morphogenesis and cyclic regeneration. However, the factors regulating their behavior are not understood. My work demonstrates that Platelet-derived Growth Factor (PDGF) is a key regulator of SKP function within their hair follicle niche. Using adult SKPs I show that growth in the presence of PDGF-B promotes increased SKP proliferation and self-renewal *in vitro*, as well as increased hair follicle formation in an *ex vivo* hair growth assay. Finally, my work identifies the hair follicle epithelium (outer root sheath) and potentially dermal adipocytes as a source of PDGF in the skin. Understanding this regulation will improve our ability to expand SKP numbers and quality for therapeutic application following skin injury.

Acknowledgements

I would like to take this opportunity to thank Dr. Jeff Biernaskie for his easy going guidance through this whole Master's degree endeavour. I would also like to thank my Master's committee members including Dr. James Cross, Dr. Sarah Childs, and Dr. Dave Hansen for their prompt delivery of advice and encouragement. Furthermore, I would like to thank the Canadian Institute for Health Research (CIHR) for the financial support they provided during my experimentation. Last, but not least, I would like to thank my fellow lab members for their moral support and willingness to help with learning new experimental techniques.

Dedication

To G.H.M and G.H.M., both who exemplify what it means to work hard.

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List of Symbols and Abbreviations

ANOVA	Analysis of Variance
αSMA	alpha-Smooth Muscle Actin
B27	Serum free cell culture supplement
Bcl2	B-cell lymphoma 2 gene
BESCs	Bulge Epithelial Stem Cells
BrdU	Bromodeoxyuridine
c-Myc	Myelocytomatosis cellular oncogene
C57BL/6	C57 black 6 (a strain of mouse with a black coloured coat)
CD34	Cluster of Differentiation 34
CM	Conditioned Media
CNS	Central Nervous System
CSF-1	Colony Stimulating Factor-1
Dermo-1	Equivalent to TWIST2 (Twist-related protein 2)
DMEM	Dulbecco's Modified Eagle Medium
DP	Dermal Papilla
DS	Dermal Sheath
E	Embryonic day (followed by a number)
ECM	Extracellular Matrix
EdU	5-ethynyl-2'-deoxyuridine (a proliferation marker)
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
ESCs	Embryonic Stem Cells
F12	Ham's F-12 Nutrient Mixture
FACS	Fluorescence-activated Cell Sorting
FGF	Fibroblast Growth Factor
GAG	Glycosaminoglycans

GFP	Green Fluorescent Protein
Grb2	Growth factor receptor-bound protein 2
H2B	Histone protein 2B
HBSS	Hank's Balanced Salt Solution
iPS	Induced Pluripotent Stem
IRS	Inner root sheath
JAK	Janus kinase
K5	Keratin-5
K14	Keratin-14
K15	Keratin-15
Klf4	Kruppel-like factor 4
Lgr5	Leucine-rich repeat-containing G protein-coupled receptor 5
Lhx2	LIM/homeobox protein 2
McSC	Melanocyte Stem Cell
MEK	MAPK/ERK kinase
MAP	Mitogen-activated protein (a kinase, formerly named ERK)
Mitf	Microphthalmia-associated transcription factor gene
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
Oct-4	Octamer-binding transcription factor 4
ORS	Outer root sheath
P	Post-natal day (followed by a number)
Pax3	Paired box 3
PDGF	Platelet-derived Growth Factor
PDGFRα	Platelet-derived Growth Factor Receptor Alpha
PDGFRβ	Platelet-derived Growth Factor Receptor Beta
phospho-PDGFRα	Phosphorylated Platelet-derived Growth Factor Receptor Alpha
PI3K	Phosphoinositide 3-kinase

PLC-γ	Phospholipase C-gamma
SB	Stratum Basale
SC	Stratum Corneum
Sca-1	Stem cell antigen 1
SCF	Stem Cell Factor
SEM	Standard Error of the Mean
SG	Statum Granulosum
SH2	Src homology 2
SHOX2	Short stature homeobox 2
SKPS	Skin-derived Precursors
Slug	Zinc finger protein SNAI2 transcription factor
Snail	Zinc finger protein SNAI1 transcription factor
Sos1	Son of sevenless homolog 1
Sox2	SRY (sex determining region Y)-box 2
$Sox2^{+}$:EGFP	Sox2 positive expressing Enhanced Green Fluorescent Protein
Sox9	SRY (sex determining region Y)-box 9
Src	Sarcoma (a proto-oncogene)
SS	Statum Spinosum
STAT	Signal Transducer and Activator of Transcription
Tcf3	Transcription factor 3
TGF-β	Transforming Growth Factor Beta
Twist	Twist-related protein transcription factor
USD	United States Dollars
W.H.O.	World Health Organization
Wnt	Wingless-related integration site

Chapter 1: Introduction

1.1 General Rationale

In the United States, it costs on average $5000 USD per day for the primary treatment of burn patients (W.H.O., 2002). Similarly, approximately 60,000 patients are hospitalized for severe burn injury annually (W.H.O., 2002). Worst of all, burns are fourth in the rankings for cause of unintentional child death in the USA (W.H.O., 2002). Each year over 300000 people die from burn injuries world-wide, a number that excludes the millions more who suffer from burn related disabilities and disfigurements (Peck et al., 2009). These types of injuries can have devastating, life-long effects on function and psychological well-being. Patients suffering full thickness wounds want new skin that functions normally, can grow hair, has proper colouration and can properly repair itself on a regular basis. Therefore, development of therapies to improve skin regeneration and minimize scarring following burn injury or other forms of skin trauma is critically important to improving function and overall quality of life.

The overall aim of my project is to begin to understand the mechanism by which dermal stem cells, a cell source with therapeutic potential for application to burn injury, are regulated within the skin. It is hoped that manipulation of this regulation *in vitro* will improve cellular growth, self-renewal, and *in vivo* function. Ultimately, improvements in these areas will allow for collection and expansion of functional, autologous cells for use in therapeutic application (i.e. better skin engraftment), or potentially activation of endogenous cells to enable better healing.

1.2 The Skin

The skin is the largest organ in the human body (see Figure 1.1). It is comprised of two major layers: the epidermis (outermost layer) and the dermis (below the epidermis). In murine skin, the epidermis is primarily composed of keratinocytes, whereas the dermis is comprised of various mesenchymal lineage cells including fibroblasts and adipocytes. The epidermis can be divided into four layers including: the basal layer (bottom), the spinous layer, the granular layer and the stratum corneum (top). As a whole the epidermis provides an impermeable and mechanically stable barrier for the skin, specifically through the outermost stratum corneum layer. The dermis, however, contains fibroblasts residing in an extracellular matrix (ECM) composed of fibronectin, collagen, proteoglycans, and elastin (Wong and Chang, 2008). This mixture provides an ideal environment for nutrient, metabolite and hormone diffusion (Keene et al., 1997). Also, interfibrillar glycosaminoglycans (GAG), in combination with the ECM, are involved with the chemical signalling aspects of the dermis including the binding of enzymes, inhibitors and signalling molecules (Keene et al., 1997). The dermal layer also contains resident immune cells, blood vessels and sensory nerve fibres. As a whole the dermal layer supplies nutrients required by the epidermis as well as acts as a physical cushion for prevention of mechanical injuries. Together the epidermis and dermis comprise the "first line of defence" for the body.

In addition to the function of pathogen defence, the skin also has other functions such as: temperature regulation, prevention of water loss, protection of underlying organs, and sensation. Sensory function within the skin is provided by resident mechanoreceptors as well as hair follicles that cover the majority of the mammalian body (Blanpain and Fuchs, 2009). Other

appendages such as sebaceous and sweat glands provide lubrication and contribute to thermoregulation, respectively.

In order to maintain function integrity (i.e. protect against ongoing environmental insult), various stem/progenitor cells within the skin and hair follicles undergo continuous self-renewal and support constant cellular turnover and tissue regeneration. These constant processes of cycling, repair, and regeneration make the skin and the hair follicles both intriguing models to study stem cell biology.

1.3 The Hair Follicle

Hair follicles invaginate into the skin as a continuation of the epidermal layer extending deep into the underlying dermis (Figure 1.1). These structures are the origin from which hair, an appendage that also plays a role in thermoregulation and sensation, is generated (Schneider *et al*., 2009). The growth of hair is determined by repeated cycles of destruction and regeneration undertaken by the hair follicle (Figure 1.2). The hair growth cycle includes three main stages of follicle morphology. The first stage is anagen where hair growth is occurring and the keratinocytes of the hair follicle extend down into the dermis and encapsulate the dermal papilla, a dense group of specialized dermal cells believed to induce hair growth (Chase, 1954). Anagen is a highly proliferative phase, especially within the lower epithelial matrix portion of the follicle near the dermal papilla. The next stage, catagen, is considered to be a "regression phase" where keratinocyte apoptosis results in degeneration of the lower portion of the hair follicle (Parakkal, 1970). At catagen the dermal papilla is completely separated from the rest of hair follicle by a portion of dermis that starts to progressively thin (Hansen et al., 1984). As the dermis thins the

"transient" or non-permanent region of the hair follicle completely disappears. The resulting follicle consists of only the "permanent" or upper region of the follicle with the bulge and sebaceous gland still intact (Chase, 1954). Also, the dermal papilla rests against the lower portion of this short form of the hair follicle, directly adjacent to an area called the hair germ (Greco et al., 2009). This final stage is called telogen and it is termed the "resting" state because cells are rarely dividing until the initiation of a new cycle (Paus et al., 1990).

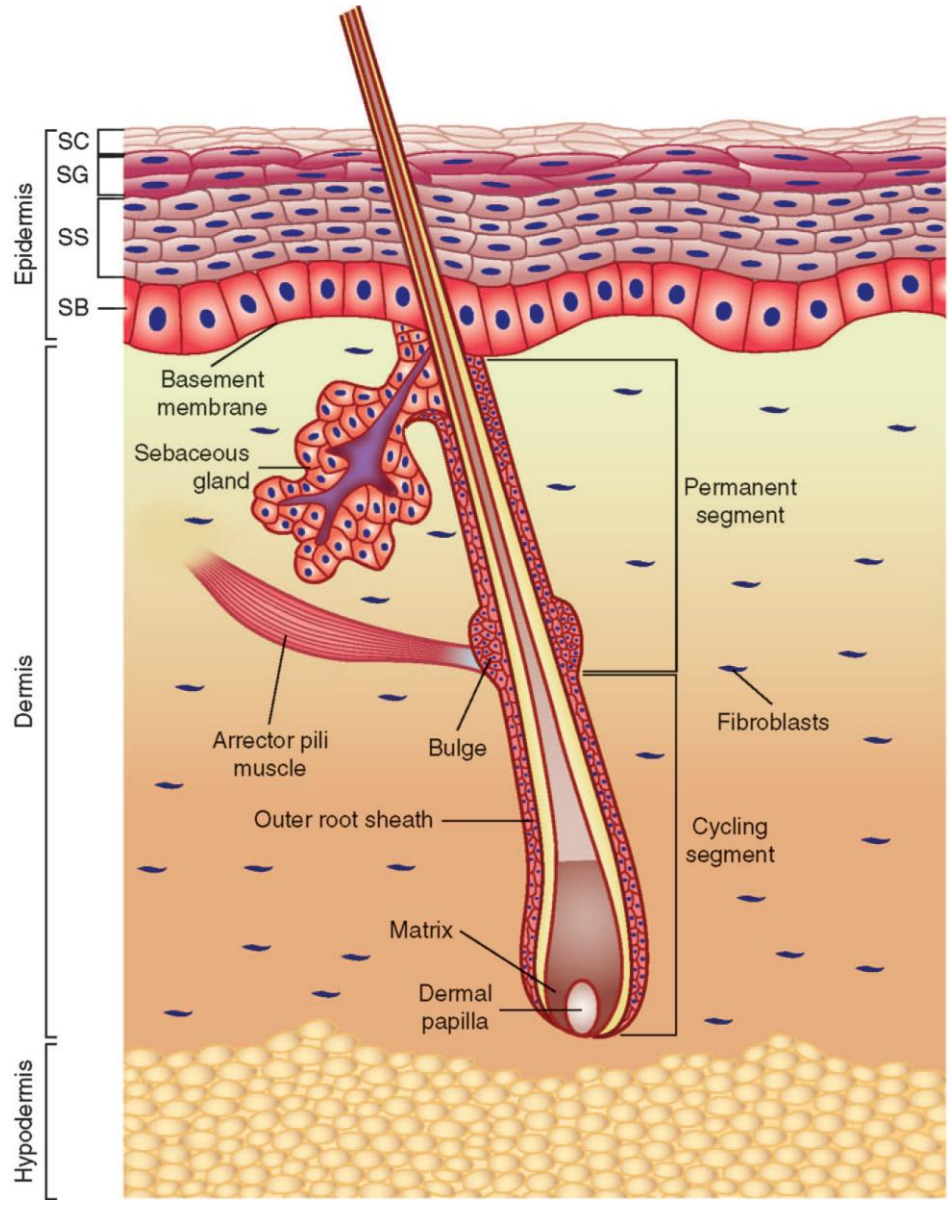

Figure 1.1 Skin Anatomy

Schematic representation of skin anatomy surrounding hair follicles including the two major layers: the epidermis and the dermis (with underlying hypodermis). The four divisions of the epidermis are also included: stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and the stratum basale (SB). The Arrector pili muscle and Sebaceous gland are also represented as they act as skin and hair follicle support appendages (Wong and Chang, 2008).

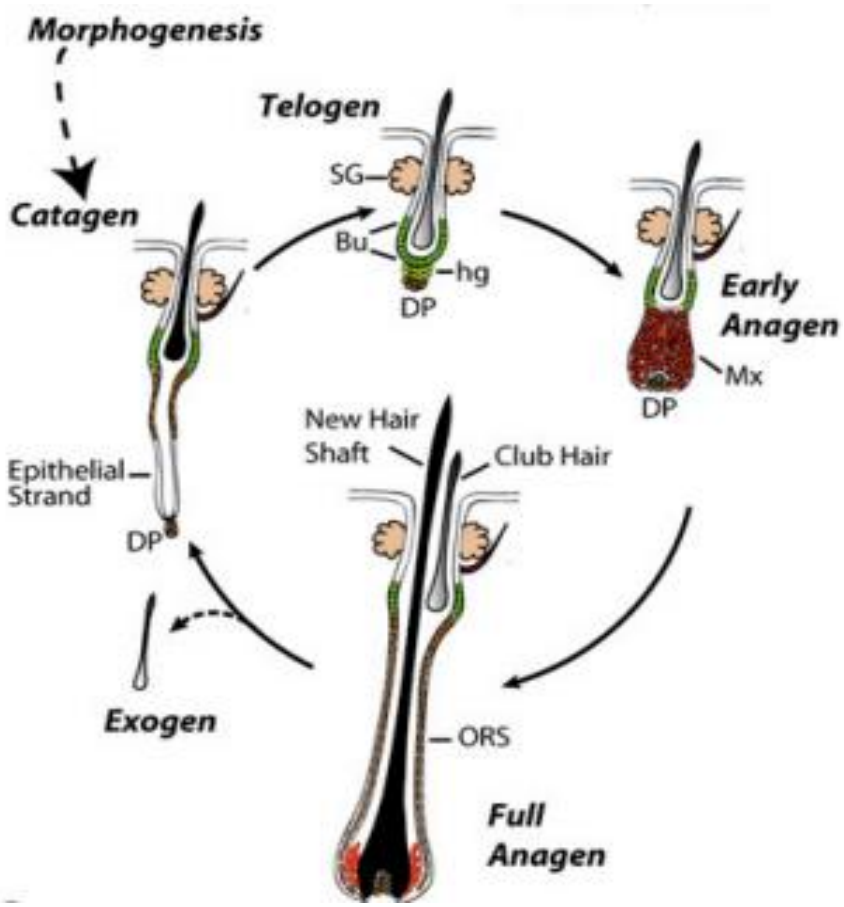

Figure 1.2 The Hair Follicle Cycle

Schematic representation of the hair follicle cycle with anagen (growth phase), catagen (degeneration phase) and telogen (dormant phase) displayed (Zhang et al., 2009). Components of the hair follicle are also displayed during each stage including: the bulge (Bu), dermal papilla (DP), hair germ (hg), matrix (Mx), and outer root sheath (ORS).

1.4 Stem Cells

Stem cells are proposed as cells that are unspecialized and able to divide and self-renew over long periods of time. Stem cells are also capable of differentiating into mature, terminally differentiated cells with specialized functions. Many forms of stem cells exist and they can be divided into categories based on potency. For instance, cells present within the inner cell mass of the developing blastocyst that are able to divide and produce every type of differentiated cell in an organism, are labelled as 'pluripotent'. Self-renewing cells residing in specific tissues that are capable of generating more than one specialized cell type or cell precursor are called 'multipotent'. Many multipotent cells fall into the category of an 'adult stem cell' meaning they are present to replace cells and repair tissue within certain organs of the body throughout an organism's lifetime.

The level of potency for stem cells, especially with respect to pluripotency, has traditionally been measured using assessment of *in vitro* differentiation, teratoma assays, molecular marker identification, and germline transmission. For instance, Takahashi and Yamanaka (2006) conducted experiments using transcription factors known to be expressed in pluripotent embryonic stem cells (ESCs) like Oct3/4, Sox2, C-Myc, and Klf4 to form induced pluripotent stem (iPS) cells from fibroblasts. To determine if these iPS cells were pluripotent like ESCs they performed the teratoma formation assays and in vitro differentiation with both cell types in parallel (Takahashi and Yamanaka, 2006). Pluripotency also includes germline transmission. However, as the potency level of a stem cell is reduced from pluripotent to multipotent, the tests become more limited and the use of molecular markers and *in vitro* differentiation is significantly increased. Isolation with specific markers (i.e. FACS) to obtain a certain population followed by *in vitro* growth, self-renewal and differentiation assessment has

become more readily used with respect to adult, multipotent stem cell populations. This has been exemplified through the field of adult, mesenchymal lineage stem cells extracted from the bone marrow (Jiang et al., 2002; Pittenger et al., 1999).

1.5 Skin Stem Cells

1.5.1 Epidermal Stem Cells

The skin contains many stem cell types that each have a particular role to play. This is due to the high turnover rate of cells in the skin and the constant cycling of hair follicles. For instance, interfollicular epidermal cells undergo rapid turnover where the stratum corneum will die and be sloughed off (Fuchs and Raghavan, 2002). Studies suggest that the epidermal turnover in mice occurs every 8-10 days while in humans it occurs every 40-56 days (Halprin, 1972; Potten et al., 1987). These cells are replaced by a population of basal epidermal stem cells proposed to either asymmetrically divide to send one "committed" daughter cell towards the outer layer while another daughter cell remains within the niche to self-renew and repopulate, or symmetrically to send both or none (Blanpain and Fuchs, 2009). It has been postulated that asymmetric division is the method through which these basal progenitors produce a suprabasal cell (flattening, non-dividing cell replacing stratified cell layers) and a new basal progenitor that is attached to its basement membrane niche through integrins to remain receiving the proper cell signals (Lechler and Fuchs, 2005). The committed, suprabasal daughter cells will begin to differentiate and flatten as they move outwards in a direction perpendicular to the basal layer (Lechler and Fuchs, 2005).

1.5.2 Bulge Epithelial Stem Cells (BESCs)

The unique ability of hair follicles to regenerate in part derives from stem cells that exist within their epidermal layers. The bulge of the hair follicle has been characterized as the niche (Figure 1.3) for a population of label-retaining, epithelial cells that contribute to the epidermal portion of the hair follicle structure throughout each cycle (Cotsarelis et al., 1990). They have been characterized as epithelial hair follicle stem cells due to their slow-cycling and quiescent nature, as well as their clonogenic growth behavior *in vitro* (Cotsarelis et al., 1990; Morris and Potten, 1994). It has been found that these bulge cells will retain their BrdU label through the cell cycle during pulse-chase experimentation, while cells that are proliferating around them tend to dilute this label quite quickly due to more rapid cell division (Tumbar et al., 2004). It has been shown through *in vivo* studies that a majority of hair lineage cells can be derived from these bulge cells and that they will even give rise to interfollicular epidermis post-injury (Morris et al., 2004). BESCs have the ability to aid in the formation of hair follicles and their accompanying sebaceous glands as labelled cells have been found in each location *in vivo* (Morris et al., 2004). During the initial stages of the hair follicle cycle, these cells will travel downward from the bulge to the hair bulb within the outer root sheath (ORS). Upon reaching the hair bulb they move towards the inner region and start to ascend the hair follicle allowing for formation and maintenance of the inner root sheath (IRS) and the matrix (Oshima et al., 2001). Some histological markers used to identify these cells in the hair follicle bulge of mice are Lgr5, Lhx2, CD34, K15, Sox9, Tcf3 and NFATc1 (Horsley et al., 2008; Jaks et al., 2008; Nguyen et al., 2009; Nowak et al., 2008; Rhee et al., 2006; Trempus et al., 2003). However, despite some overlap, these markers do not label all of the same cells and are not generally present at the same

time. This suggests that the bulge niche is composed of a heterogeneous population of cells with potentially different functions and potency.

1.5.3 Melanocyte Stem Cells

Another stem cell population present in the skin, and specifically within hair follicles, is the melanocyte stem cell (McSC) that inhabits the hair follicle bulge along with the previously mentioned keratinocyte lineage BESCs (Nishimura et al., 2002). These cells are slow-cycling, immature cells with the ability to generate progeny upon the initiation of anagen (Nishimura et al., 2002). Because melanocyte lineage cells contribute the pigmentation that is present in hair follicles giving the hair its colour, any factors that limit the function of the McSCs will lead to hair graying (Nishimura et al., 2005). The *Bcl2* and *Mitf* genes have been identified as essential in maintaining these stem cells with regards to their survival when becoming quiescent and prevention of premature instigation of cell differentiation, respectively (Levy et al., 2006; Nishimura et al., 2005; Steingrimsson et al., 2004). Furthermore, it has been shown that transforming growth factor β (TGF-β) signalling within the McSC niche is a key contributor to the arrest of cell cycling and entrance of these cells into a quiescent state (Nishimura et al., 2010). Induction of proliferation in McSCs during hair growth is initiated by an increase in Wnt signalling allowing these cells to contribute to the pigmentation of newly generated hair (Rabbani et al., 2011).

1.6 Skin-derived Precursors: The Dermal Stem Cell

Skin-derived Precursors (SKPs), as characterized by Toma *et al*. in 2001, are a dermal cell population present within their own dermal niche surrounding the hair follicle (Fernandes et al., 2004; Toma et al., 2001). They were later determined to be an adult dermal stem cell population due to their ability to self-renew *in vitro*, contribution to hair morphogenesis, and role in dermal maintenance and wound repair following transplantation (Biernaskie et al., 2009). These cells are multipotent, with the ability to form neural or mesenchymal progeny (Fernandes et al., 2004; McKenzie et al., 2006; Toma et al., 2001). In terms of neural progeny, they have been shown to differentiate into Schwann cells of the peripheral nervous system (McKenzie et al., 2006). When grown under specific conditions these are capable of forming dermal fibroblasts and adipocytes (Toma et al., 2001).

Dorsal SKPs derived from rodents have been shown to generate non-adherent, spherical colonies *in vitro* (Fernandes et al., 2004; Toma et al., 2001). The neural crest origin of these cells has been suggested by their expression of neural crest markers such as: SHOX2, twist, slug, snail, Pax3, and Dermo-1 (Fernandes et al., 2004). SKPs also express other markers such as fibronectin, vimentin, and nestin (Fernandes et al., 2004; Toma et al., 2001). However, fibronectin, unlike vimentin and nestin, is not expressed in CNS neurospheres, helping to distinguish the two cell types (Fernandes et al., 2004; Toma et al., 2001). SKP spheres derived from $Sox2^{+}$:EGFP isolated dorsal skin cells will also express the DP marker versican and the DS marker αSMA (Biernaskie et al., 2009). Clonal SKP spherical colony formation is not observed prior to embryonic day 15 (E15), indicating that this is the earliest point at which SKPs will be present in their dermal, hair follicle niche (Fernandes et al., 2004).

SKP-derived progeny not only hold characteristics that are similar to each endogenous cell type, but rather function in the same way and sometimes at an enhanced level to those progeny derived from other stem cell populations. For instance, it has been shown the SKP-derived Schwann cells have the ability to help with re-myelination of neurons within dysmyelinated brain tissue (McKenzie et al., 2006). In other instances, these cells displayed the ability to take part in the repair and re-myelination of axons within murine spinal cords that have been subjected to contusion injury (Biernaskie et al., 2007). Apart from their ability to differentiate and function as SKP-derived progeny, they are also able to contribute to dermal would repair and hair follicle formation directly (Biernaskie et al., 2009).

1.7 The SKP Niche

The SKP niche (Figure 2.1) is located specifically within the dermal papilla (DP) and the dermal sheath (DS) of the hair follicle (Biernaskie et al., 2009; Fernandes et al., 2004). Cells in the DP are positive for Sox2 while DS cells are positive for αSMA (Biernaskie et al., 2009). Sox2 has been implicated as an essential player in self-renewal of both adult and embryonic stem cells (Biernaskie et al., 2009; Graham et al., 2003; Yamanaka and Takahashi, 2006). This SKP niche provides key functional significance in terms of location, because these cells primarily function to allow for hair follicle regeneration (Biernaskie et al., 2009). SKPs also play a role in dermal repair following wounds (Biernaskie et al., 2009). This was shown using fluorescently labelled SKPs that were specifically moving out of both their DP and DS niche to contribute to this dermal repair (Biernaskie et al., 2009). However, it is still unclear whether the DS or DP contains the true "stem cell" that is able to repopulate both environments. Studies involving

micro dissection of the DP from whisker (vibrissae) hair follicles indirectly suggest that the DS is able to regenerate and repopulate the DP (Horne and Jahoda, 1992). These DS cells will also migrate and help to repopulate both the DS and DP upon transplantation (McElwee et al., 2003). This suggests that there is functional overlap between the two populations; however, the lack of specificity following gross dissection does not permit definitive conclusions. Moreover, the lack of a DS during the telogen phase of the hair cycle leads to postulation that perhaps these cells may disappear or even contribute to the ever-present DP, and in turn the DP is able to contribute to the regeneration of the DS during anagen. Previous investigation has shown a decrease in DP cells during late anagen and continuing into early catagen that occurs without apoptosis, suggesting that these cells were potentially migrating back into the DS (Tobin et al., 2003). Overall, these studies suggest that the dermal stem cell location may change depending on the stage in the hair cycle and some form of two-way travel may occur between the DS and DP, but this is not entirely clear.

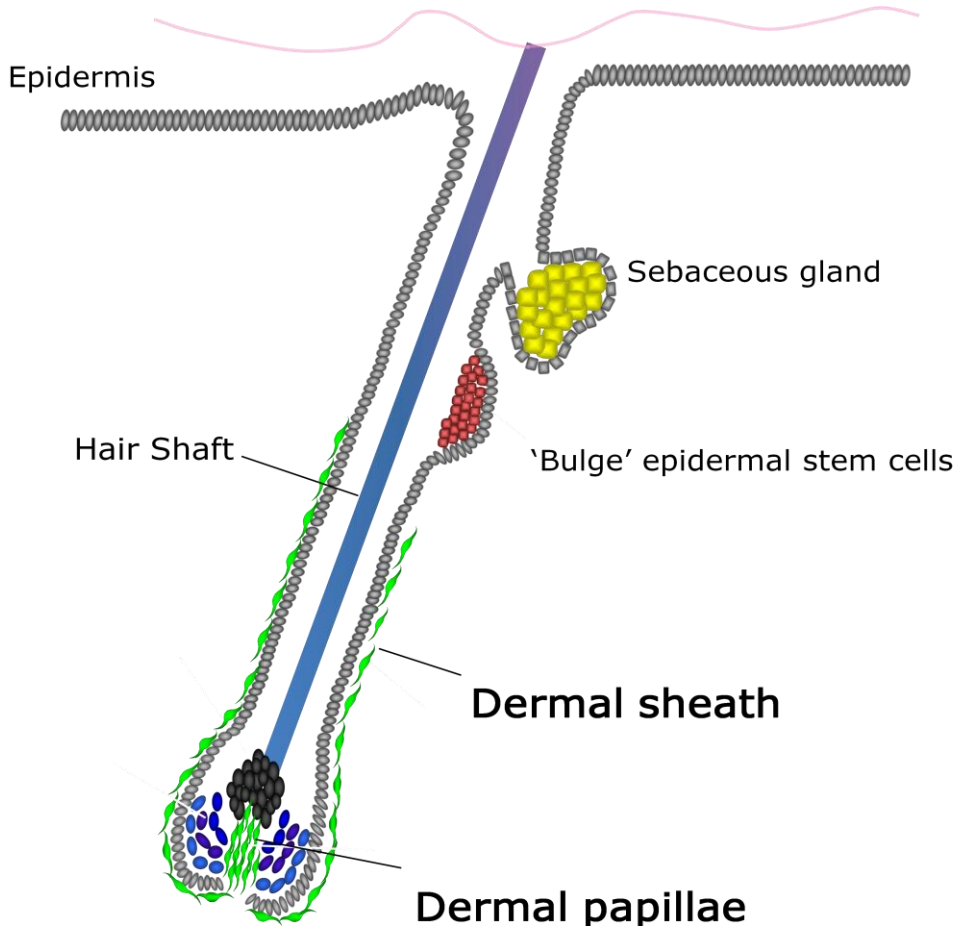

Figure 1.3 The Dermal Stem Cell Niche

Schematic representation of the hair follicle including the proposed dermal stem cell niche (dermal papilla and dermal shealth cells in green), the Bulge (red), and the Sebaceous gland (yellow). Adapted from J. Biernaskie.

1.8 Platelet-derived Growth Factor

Platelet-derived Growth Factor (PDGF) is a cell signalling protein (ligand) and is produced by both keratinocytes and fibroblasts in the skin (Ansel et al., 1993; Paulsson et al., 1987). Two key isoforms of PDGF, PDGF-A and PDGF-B, come as homodimeric entities (PDGF-AA or -BB) while in some cases a heterodimer exists (PDGF-AB) with a receptor binding affinity similar to that of PDGF-A (Heldin et al., 1979; Johnsson et al., 1982). PDGF signalling is transmitted by two specific PDGF receptors called PDGFRα and PDGFRβ. When bound by their ligand, these tyrosine-kinase receptors embedded in the plasma membrane will dimerize, autophosphorylate at their intracellular tyrosine regions, and initiate secondary signalling cascades within the cell. As a collective the PDGF receptors have been implicated in stimulating various effects including promotion of cell proliferation, motility, migration, growth, survival, differentiation, and actin rearrangement (Heldin et al., 1998). These functions are instigated through the docking of different Src homology 2 (SH2) domain containing molecules such as Phosphoinositide 3-kinase (PI3K), Phospholipase C-gamma (PLC-γ), the Grb2/Sos1 complex (leads to MEK/MAP kinase pathway), STAT (in the JAK-STAT pathway) and Src (Figure 1.4) to the autophosphorylated, intracellular tyrosine regions of the receptors (Heldin et al., 1998). The PDGF receptors hold similarities in structure to that of the colony stimulating factor-1 (CSF-1) and stem cell factor (SCF) receptors (Coussens et al., 1986; Yarden et al., 1987). PDGF-B has the ability to bind to both the PDGFRα and PDGFRβ with high affinity, whereas PDGF-A is only capable of binding to PDGFRα (Heldin and Westermark, 1999). Both PDGFRα and PDGFRβ have been found to be expressed by fibroblasts, vascular smooth muscle cells, neurons, mesangial cells of the kidney and Leydig cells, while various other cell types express only one or the other (Heldin and Westermark, 1999).

It has been postulated that because of its low blood concentration and half-life, PDGF tends to act on a local paracrine or even autocrine cell signalling basis (Ross *et al*., 1986). Furthermore, it has been described that PDGF has a chemo attractant ability with respect to human fibroblasts, setting it apart from other growth factors like fibroblast growth factor (FGF) or epidermal growth factor (EGF) that do not have this effect (Seppa et al., 1982). It has been said that this ability is cell and PDGF isoform dependent, meaning that in some cell types PDGF-AA will act contrary to its norm and inhibit the chemotactic effect of the other two isoforms (Siegbahn et al., 1990). Regardless, studies have suggested that the chemo attractant ability of PDGF could coincide with its mitogenic ability with regards to a role in wound repair (Ross et al., 1986). Cells would likely be attracted to the wound site and stimulated to proliferate and help with repair. Also, PDGF has been implicated in stimulating the production of collagen, fibronectin, and other matrix molecules, as well as instigating the contraction of collagen specifically during wound healing (Heldin and Westermark, 1999). However, these effects may result in pathogenesis as both the PDGF-AA and PDGF-BB ligands, as well as PDGFRα and PDGFRβ, have been shown to be expressed by various types of tumour cells (Heldin and Westermark, 1999).

Transgenic knock-outs for both the PDGF-A and PDGF-B ligands, as well as PDGFRα and PDGFRβ, have provided key insight into the importance of these factors during development. For instance, mice with a PDGF-B ligand deficiency (gene knock-out) die perinatally due to improper renal and cardiovascular (including blood vessels) development, as well as abnormalities in hematological components and characteristic internal bleeding (Leveen et al., 1994). Furthermore, PDGFRβ knock-out mutants also die before or at the point of birth with mirrored defects to those presented by the PDGF-B ligand mutants, but more specifically

due to renal and hematological abnormalities rather than cardiovascular defects (Soriano, 1994). It is thought that the increased severity of the PDGF-B ligand knock-out may be due to its ability to also bind and activate PDGFRα, and specifically α-receptors present in heart tissue (Heldin and Westermark, 1999). PDGF-A ligand knock outs display a phenotype that leads to death at approximately 3 weeks of age due to defects in alveogenesis and lung development including: poor smooth muscle cell development in the alveoli, an elastin fibre deficit in the parenchyma, and development of emphysema (Lindahl et al., 1997). PDGFRα knock-outs prove to be even more lethal as during embryonic development problems with the neural tube, improper cranial formation, and apoptosis of neural crest cells during their migration was exhibited in these mice (Soriano, 1997). A strain called Patch mice also exhibited a phenotype similar to that of the PDGFRα mice; however, their array of problems also included abnormal coat colour (Stephenson et al., 1991). It has been postulated that the increased severity of the PDGFRα null phenotype is due to the possibility that the PDGF-B may be the primary ligand of this receptor in certain tissue types (Heldin and Westermark, 1999).

One of my primary objectives is to understand the molecular mechanisms that regulate dermal stem cell fate, self-renewal, and proliferation within their hair follicle microenvironment. Previous work by Karlsson *et al* (1999) examined the effect of a less lethal PDGF-A ligand knock-out with respect to skin development and hair regeneration. They reported that during the first hair cycle the hair follicles and the skin developed normally, but from the second hair cycle (postnatal day 21) onward there is progressive dermal thinning and abnormal hair development (Karlsson et al., 1999). At onset of second anagen, these hair follicles seemed to lack the ability to move from the dormant telogen phase into the anagen growth phase. This pointed to a potential role of PDGF in the hair follicle cycle, and more specifically, in regulating dermal stem

cell behavior. Based on these studies, my thesis work examined the potential role of PDGF in regulating the maintenance and function of dermal stem cells within their hair follicle niche, as well as how this factor may contribute to the role they play throughout the hair cycle.

Another objective within my project is to help identify the source of the PDGF regulation acting upon the dermal stem cells within their niche and controlling their function within the skin. Festa *et al* in 2011 pointed to the adipocyte lineage as the source of the signal that controls the normal cycling of hair follicles within the skin. Their experimentation pointed to PDGF as a key regulator released by adipocyte lineage cells present below the follicles, and more specifically by preadipocyte cells and adipocyte precursors (Festa et al., 2011). It has been suggested that adipocyte conditioned media from cell cultures may increase the proliferation and migration of fibroblasts to wound sites (Schmidt and Horsley, 2013). Furthermore, adipocytes have been indicated to be a key regulator of wound healing *in vivo* by migrating into wounds and instigating the attraction of fibroblasts into the wound beds, rather than just the area surrounding the wound (Schmidt and Horsley, 2013).

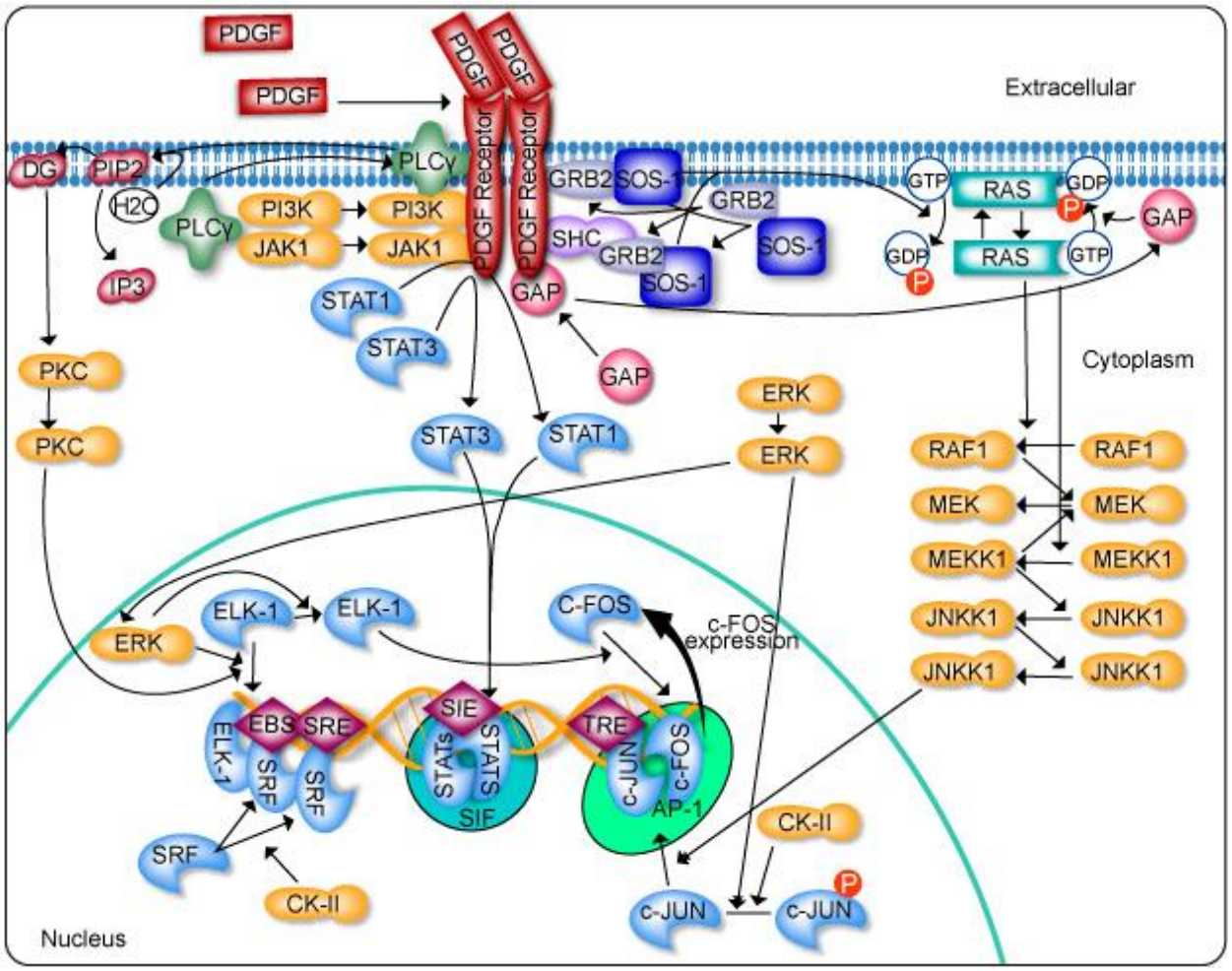

Figure 1.4 The PDGF Signalling Pathway

Schematic representation of the PDGF receptor signalling pathway. Adapted from website: http://www.genecopoeia.com/product/search/pathway/h_pdgfPathway.php. (GeneCopoeia, 2013)

1.9 Objectives and Aims

Hypothesis: I hypothesize that PDGF is a key regulator of dermal stem cell behavior within their hair follicle niche.

Aim 1: To determine if growth in the presence of PDGF plays a role in the ability of SKPs to proliferate and self-renew *in vitro*:

A) Does PDGF influence the growth of SKPs *in vitro* (i.e. cell proliferation)?
B) Does PDGF influence the ability of SKPs to form spheres in the longer term (i.e. self-renewal)?

Aim 2: To determine if growth in the presence of PDGF plays a role in the ability of SKPs to induce hair follicle formation *in vivo*.

Aim 3: To determine whether dermal adipocytes are the source of PDGF that promotes increased cell proliferation.

Chapter 2: Materials and Methods

2.1 SKPs isolation and *in vitro* expansion

SKPs were isolated from the back skin of adult (P30-33) GFP-expressing Sprague Dawley rats (SLC, Japan) as previously described by Biernaskie *et al* in 2007. Dorsal skin was dissected from each rat and attached hair fibres and subcutaneous fat were removed. Isolated skin was placed in dispase (5 mg/ml; StemCell Technologies) for approximately 1 hour at 37°C followed by mechanical removal of the epidermis. The remaining dermal layer was minced into 1-2 mm pieces and placed in collagenase (type XI, 1 mg/ml; Sigma Aldrich) for 1 hour at 37°C with agitation every 15 minutes. Supernatant containing isolated SKPs was removed, filtered (40 μm) and cells pelleted by centrifugation (1200 rpm for 5 minutes). Cells were washed in DMEM (Invitrogen) and HBSS (Invitrogen) twice and then total number of viable cells were quantified using a hemocytometer. Briefly, DMEM and F12 (3:1; Invitrogen) media containing basic fibroblast growth factor (bFGF; 40 ng/ml; BD Biosciences), epidermal growth factor (EGF; 20 ng/ml; BD Biosciences), B27 (2%; Invitrogen), and penicillin streptomycin (1%; Invitrogen) was used to maintain cell growth in culture (referred to as 'proliferation medium'). Primary cells were grown at an initial density of 50,000 cells/ml and subsequent passages were grown at 10,000-20,000 cells/ml to promote clonal colony formation. SKPs were passaged based on sphere size approximately every 8-12 days.

2.2 *In vitro* assessment of PDGF signalling on SKPs proliferation and self-renewal

Adult (passage two) dorsal rat SKPs were dissociated in collagenase (type XI, 1 mg/ml; Sigma Aldrich) to single cells. Cells were plated in 24-well plates at a density of 10,000 cells/ml

and grown in the control proliferation medium alone or with the addition of PDGF-A or PDGF-B ligands (50 ng/ml; both R&D Systems) for the duration of the experiment. Cells were fed every three days. Twelve days later, each well was imaged using an Axioplan inverted microscope (Zeiss) and the number (n = 4-5 wells per treatment) and size (diameter, n = 20 spheres per well, n=3 wells per treatment) of spherical colonies was quantified. In order to assess self-renewal, we performed a serial colony formation assay. Cells were grown at 20,000 cells/ml in either 24-well (n = 4-12 wells per passage) or 96-well plates (n = 4-80 wells per passage) in the presence or absence of PDGF-B. Upon plating, individual cells adhered to the plastic which enabled clonal growth of each colony. The number of colonies in each well was quantified 12-20 days after each passage for five consecutive passages.

2.3 *In vivo* hair follicle formation

To assess their inductive abilities *in vivo*, SKPs grown in the presence or absence of PDGF-A and -B were used. These cells were combined with epidermal aggregates extracted from neonatal mice (P0; C57BL/6; Charles River Laboratories). The extraction of the buds was achieved through placement of pup skin sections within trypsin enzyme (Invitrogen) and removal of the dermis rather than the epidermis. For each graft 10,000 aggregates were combined with 500,000 SKPs and subcutaneously injected under the back skin of adult nude mice (Charles River Laboratories). After a 2 week period, grafts were harvested for assessment of hair follicle number and morphology (n = 4-6 patches per treatment). Data was analyzed using one-way ANOVA and a p-value of < 0.05 was considered statistically significant. Adapted from Zheng *et al*., 2005; Biernaskie *et al*., 2009.

2.4 *In vitro* adipocyte conditioned media SKP growth assay

To assess whether adipocytes are a source of PDGF for the regulation of SKPs within the skin, adult adipose-derived cells were isolated (P32-33 rats) from the subcutaneous fat layer using the SKP isolation protocol (Section 2.1) and grown in flasks at a density of 50,000-100,000 cells/ml. Cells were passaged at least twice before the conditioned media was collected and passed through a 0.4 µm syringe filter. SKPs growing in the presence or absence of PDGF-B received 75% new SKP media and 25% conditioned SKP media while all other wells received 75% adipose precursor conditioned media and 25% SKP conditioned media (20,000 cells/ml, 24-well plates). To block PDGF signalling a subset of replicate wells were also administered Imatinib Mesylate (0.1%; Santa Cruz Biotechnology) or a PDGFRα neutralizing antibody (10 µg/ml; R&D Systems). Cells were fed every 3-4 days with Imatinib Mesylate and the PDGFRα neutralizing antibody being added and allowed to incubate for 30-45 minutes prior to the addition of the proper feeding regiment. Spherical colony size (diameter, $n > 38$ spheres analyzed per treatment) and number ($n = 3$ wells per treatment) were quantified 8 days later to assess differences in cell proliferation ($n = 3$ separate experiments, excluding the PDGFRα neutralizing antibody which is from one experiment).

2.5 ELISA analysis of adipocyte conditioned media for PDGF-AA and –BB ligands

In order to determine if the PDGF-AA and –BB ligands are secreted by adipocytes, conditioned media from hypodermal adipocyte cultures was analyzed using Quantikine ELISA kits (R&D Systems) according to their attached protocol. Supernatants were collected from rat (Sprague Dawley, age P32-34) adipocyte cultures ($n = 3$ rats, $n = 3$ wells per conditioned media

per ELISA) following the formation of secondary spherical colonies and stored at -20°C before use. The same age and breed of rat was used to generate the SKPs for growth in the adipocyte conditioning assays, as well as the SKPs conditioned media (n = 1 rat, n = 3 wells per ELISA) used for comparison in each ELISA. Positive and negative controls were also used in the assessment, as well as prepped standards included in each kit. Micro well plate contents were analyzed using an automated plate reader (PerkinElmer).

2.6 Immunostaining

To assess the location of PDGFRα positive cells within the skin we removed pieces of P8, P28 or adult telogen (P56) PDGFRαH2BGFP mouse (Jackson Laboratories) dorsal skin. PDGFRαH2BGFP mice were created through knock-in of the histone H2Be-GFP fusion protein (a reporter construct) into the PDGFRα locus to allow for nuclear expression of GFP in cells that express PDGFRα (Hamilton et al., 2003). The advantage of these mice is that they allow identification PDGFRα expression through nuclear GFP expression rather than cell surface expression, making visualization of cell location more obvious. The dorsal skin was cryosectioned into thin slices (20 µm) and adhered to 2% gel coated slides for staining. Epidermis was stained using keratin-5 (1:500; Covance) and cell nuclei were stained with Hoechst (1:5000, Sigma Aldrich) for orientation purposes. The cells of interest were stained using a chicken anti-GFP antibody (1:500, Millipore). To determine the source of PDGF-B ligand within the skin we immunostained for PDGF-B (1:200, Abcam) as well as used a phospho-PDGFRα (1:200; Santa Cruz Biotechnology) antibody to identify PDGFRα activation.

Images were taken using an Axioplan inverted microscope (Zeiss) or a confocal microscope (Leica TCS SP8).

To determine whether exposure to PDGF changes expression of signature SKPs markers, SKP spherical colonies cultured in the presence of control, PDGF-BB (50 ng/ml), and adipocyte conditioned media for 8 days were assessed using cytospin (800 rpm, 3 minutes) for the expression of phospho-PDGFRα (1:200; Santa Cruz Biotechnology). Cell nuclei were stained using Hoechst (1:5000, Sigma Aldrich) for orientation purposes. Cultures were fed 2 hours prior to cytospinning with their respective treatments. Spherical colonies were adhered to Shandon DoubleCytoslides (Thermo Scientific) before staining. Images were taken using an Axioplan inverted microscope (Zeiss).

2.7 Statistics

All data are represented as mean ± SEM. Data (with the exception of ELISA testing) were analysed with one-way ANOVA or one-tailed t tests where appropriate. $P < 0.05$ was considered statistically significant. SKP proliferation experiments were done with 2 biological replicates and 4-5 experimental replicates per treatment (Figure 2.1), however 2-3 extra biological replicates were done at different cell density (data not shown). SKP self-renewal experiments were done with 2 biological replicates in 24 well and 96 well plates with 4-12 and 4-80 experimental replicates per treatment, respectively (Figure 2.1). *Ex vivo* patch experiments were done with 4-6 replicates per treatment (Figure 2.2). Adipocyte-CM growth experimentation was done with 3 biological replicates (excluding PDGFRα neutralizing antibody with 1) and 3 experimental

replicates per treatment (Figure 2.3). ELISA assessment of Adipocyte-CM was done with 3 biological replicates (Figure 2.4).

Chapter 3: Results

3.1 Endogenous expression of PDGFRα in the DP and DS surrounding the hair follicle

To investigate whether the machinery required for PDGF-AA or -BB signalling is present within the SKP niche, I took advantage of a strain of PDGFRαH2BGFP mice that expresses GFP in cells that contain PDGFRα (Figure 2.1A-C). Immunostaining for anti-GFP and keratin-5 (K5) in post-natal day (P) 8 and 28 dorsal skin (anagen, hair follicle growth phase) confirmed expression of PDGFRα in cells located within the DP and DS as well as throughout the interfollicular dermis (Figure 2.1A and 2.1B). Within telogen (dormant hair follicle phase) dorsal mouse skin from the same strain, cells expressing PDGFRα were present within the DP located close to the hair germ as well as the interfollicular dermis (Figure 2.1C). Blood vessels labelled by αSMA in telogen dorsal skin also contained a few PDGFRα expressing cells (Figure 2.1C), which are likely vascular smooth muscle cells (Heldin and Westermark, 1999).

3.2 Growth in the presence of PDGF improves SKP proliferation *in vitro*

Since the PDGFRα is present within the dermal precursor niche, and is capable of binding both PDGF-A and PDGF-B, I then investigated the effect of PDGF on the growth of SKPs derived from dorsal rat skin *in vitro*. SKPs were isolated from ubiquitously expressing GFP rat back skin and grown in standard SKPs conditions or with the addition of PDGF-A, or PDGF-B for 12 days (a 50 ng/ml concentration was used for each PDGF as determined by optimal growth analysis). Quantification of spherical colonies at the end of a 10-12 day period showed an increase in the number of colonies when grown in the presence of PDGF-A (not statistically significant) and PDGF-B when compared to the control (Figure 2.1E). PDGF-B

showed the greatest effect, resulting in a >2-fold increase in the mean number of spherical colonies (Figure 2.1E) as compared to the control (470.4 ± 30.2 versus 261.8 ± 42.1; $p < 0.01$; n = 4-5 wells per treatment).

I also examined spherical colony size as an estimate of proliferative capacity and number of stem/progenitor cells in each colony. Addition of PDGF-A and PDGF-B enhanced colony size relative to control conditions (Figure 2.1D) and this was confirmed through measurement of spherical diameters after 10-12 days of growth in the presence of control, PDGF-A, and PDGF-B. A significant 50% increase in mean spherical colony size was found for SKPs grown in the presence of PDGF-A (Figure 2.1F) when compared with control (59.5 ± 1.4 μm versus 40.7 ± 1.7 μm; $p < 0.0001$). Also, a significant, almost 2-fold increase in sphere size was found for SKPs grown in the presence of PDGF-B (Figure 2.1F) when compared to control conditions (74.6 ± 4.7 μm versus 40.7 ± 1.7 μm; $p < 0.0001$; n = 20 spheres per well, 3 wells per treatment).

3.3 Growth in the presence of PDGF-B improves SKP self-renewal over the long-term *in vitro*

Because SKPs are proposed to be the dermal stem cell that surrounds the hair follicle, it is suspected that these cells are able to replenish their cell population within the DP and DS hair follicle niche over the lifetime of an organism. To examine their ability to self-renew, and therefore their potential to the replenish their population, I isolated dorsal SKPs from Sprague Dawley rats and seeded wells with equal densities of cells grown in the presence of either control or PDGF-B conditions. Each 12-20 day growth period I quantified the number of spherical colonies and then broke them down into single cells to be re-plated at the same original density. This was to ask whether these cells were able to continue to form spherical colonies (i.e.

continue to proliferate or self-renew) after repeating this process numerous times. In each assay, over successive passages, it was seen that SKPs grown under control conditions steadily declined in their ability to form spherical colonies over 5-6 successive passages (Figure 2.1G). SKPs grown in control conditions decreased in number of spherical colonies formed by more than 50% from passage 3 to 5 (Figure 2.1G). However, SKPs grown in the presence of PDGF-B maintained their ability to form spherical colonies with minimal decline all the way up to 6 passages (Figure 2.1G). Furthermore, SKPs grown in the presence of PDGF-B formed 2-fold or greater more spherical colonies than control in all rounds of treatment prior to passaging (Figure 2.1G).

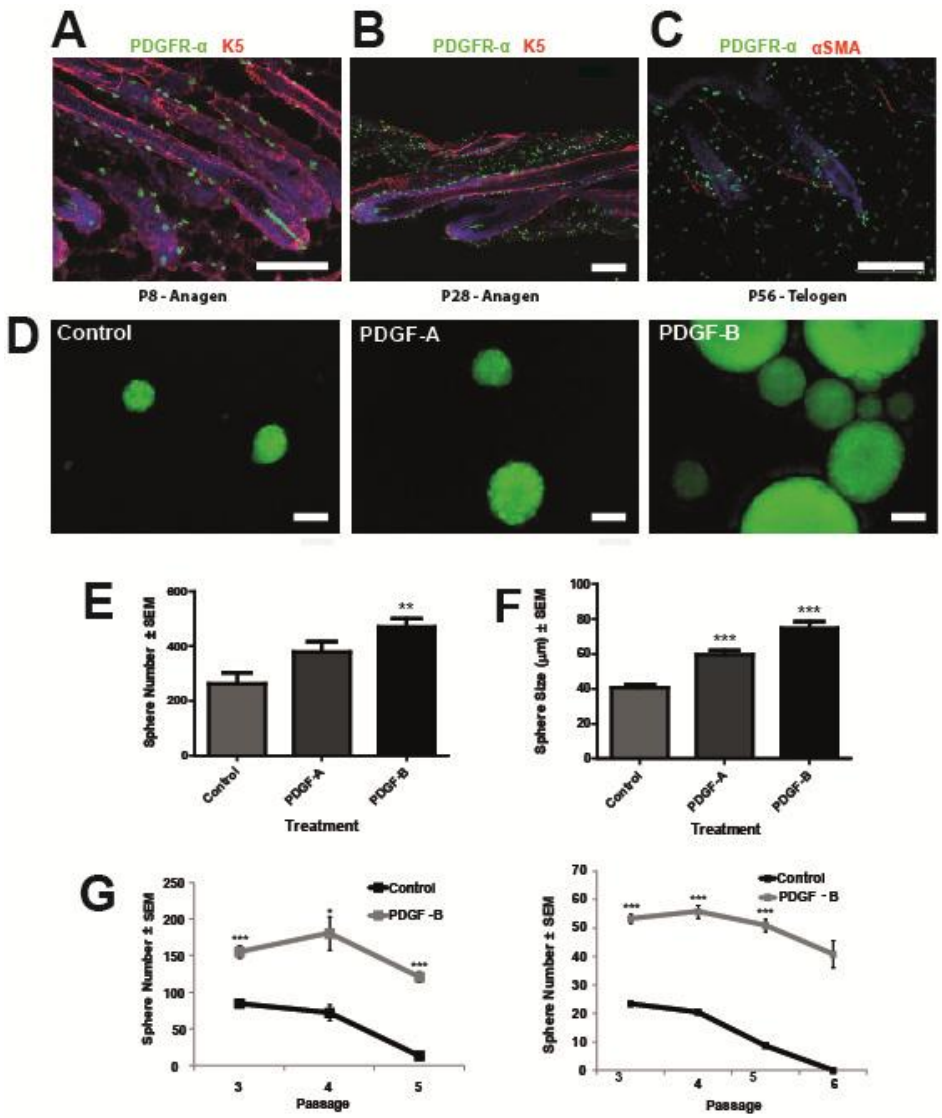

Figure 2.1 PDGF-B Improves SKP Proliferation and Self-renewal *in vitro*

(A-C) Immunohistochemical staining of anagen and telogen hair follicles from P8, P28 (anagen) and P56 (telogen) PDGFRαH2BGFP mouse skin, respectively (100 μm scale bars). (D) Qualitative representation of GFP labelled rat (P30-33) SKP spheres grown in the presence of control, PDGF-A or PDGF-B (50 ng/ml) treatment (100 μm scale bars). (E) Total sphere numbers were quantified and compared between treatment conditions (n = 4-5 wells per treatment). SKPs grown in the presence of PDGF-B formed ~2-fold more spheres than control. (F) Spherical diameters (i.e. size) were measured for colonies in four quadrants of each replicate well (n = 20 spheres per well, 3 wells per treatment). Both PDGF-A and -B showed a significant increase in spherical colony size (ANOVA ** = $p < 0.01$, *** = $p < 0.0001$). (G) SKPs (20,000 cells/ml) grown in the presence of control and PDGF-B were quantified for spherical colony formation over numerous passages (n = 4-12 wells per passage, 24 well plate, left chart; n = 4-80 wells per passage, 96 well plate, right chart). ± SEM is displayed. (t-test * = $p < 0.05$, *** = $p < 0.0001$)

3.4 Growth in the presence of PDGF-B improves the ability of SKPs to induce hair follicle morphogenesis

To ask whether growth of SKPs in the presence of PDGF improves their ability to induce hair follicle formation *ex vivo* I performed subcutaneous injections of conditioned rat GFP-expressing SKPs with mouse pup epidermal aggregates. These cell slurry injections produced numerous hair follicles as long as both cell types were present (Figure 2.2). More importantly, when the number of hair follicles formed was quantified it was found that growth of SKPs in the presence of PDGF-A did not alter the ability of these cells to induce hair follicles when compared to the control (Figure 2.2). However, when SKPs grown in the presence of PDGF-B were combined with the epidermal aggregates they showed a significantly greater hair follicle formation ability (Figure 2.2) of approximately 2-fold when compared to the control (380.8 ± 37.0 versus 188.0 ± 46.7; $p < 0.05$; n = 4-6 injections per treatment). GFP-expressing SKP cells were found to integrate into both the DP and DS of newly formed hair follicles following injection, irrespective of treatment type (Figure 2.2).

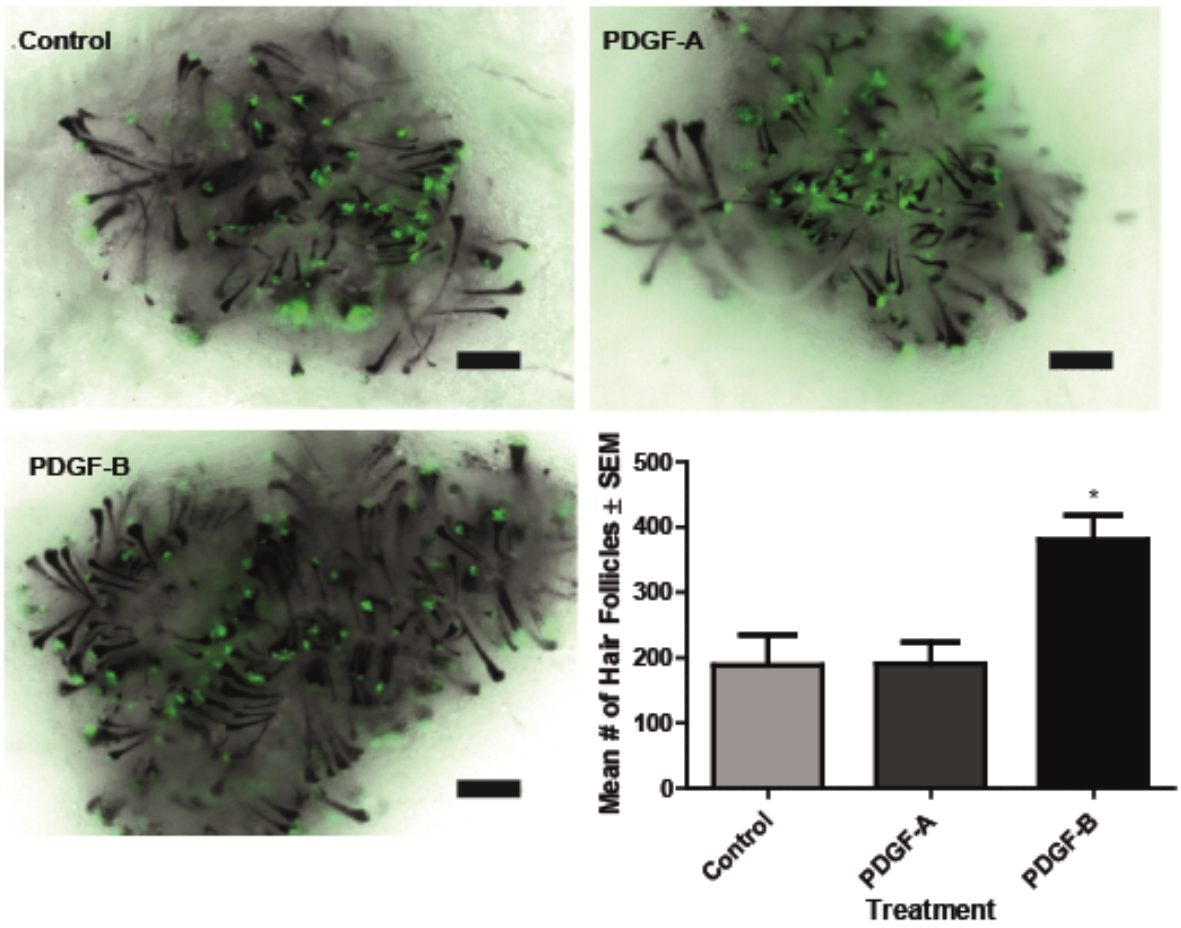

Figure 2.2 PDGF-B Improves SKP-induced Hair Follicle Morphogenesis

Hair follicle forming assays were performed with subcutaneous injections of 500,000 treated adult rat (P30-33) SKPs combined with 10,000 P0 mouse epidermal aggregates (500 µm scale bars). SKPs were on their first round (10-12 day period) of treatment in flasks with administration of the treatment occurring every 3-4 days. The treatments involved the use of a combination of 2 ml of 5× normal SKP media and either PDGF-A or PDGF-B (50 ng/ml). The SKPs were dissociated to single cells before being combined with the epidermal aggregates, and injections were made at a volume of 50 µl. Hair follicles were quantified after two weeks of growth *in vivo* (n = 4-6 transplants for each group). Data was analyzed using one-way ANOVA and a p-value of < 0.05 was considered statistically significant.

3.5 Adipocyte conditioned media partially enhances SKP proliferation

To examine whether hypodermal fat and the adipocyte cells within it represent a source of signalling, and specifically PDGF signalling, for SKPs within their hair follicle niche, I isolated and cultured cells from this layer to the point of secondary passage and extracted the cell supernatant (adipocyte conditioned media). Rat SKPs were then subjected to growth in this media with comparison to control conditions, growth in the presence of PDGF-B, and growth in the presence of tyrosine kinase inhibitor (Imatinib Mesylate) or PDGFRα neutralizing antibody (Figure 2.3A). Consistent with our previous experiments, PDGF-B treatment resulted in a significant, almost 2-fold increase in mean SKP spherical colony size (Figure 2.3B) compared to control (83.4 ± 2.9 μm versus 51.5 ± 1.2 μm; $p < 0.0001$; $n > 38$ spheres per well, 9 wells per treatment excluding PDGFRα neutralizing with 3 wells). It was also seen that the effect of PDGF-B on spherical colony size could be returned to the level of control through the addition of either Imatinib Mesylate or a PDGFRα neutralizing antibody (Figure 2.3B). When SKPs were grown in the presence of the adipocyte conditioned media a significant increase in mean spherical colony size of approximately 33% was seen (Figure 2.3B) when compared to the control (76.5 ± 3.1 μm versus 51.5 ± 1.2 μm; $p < 0.0001$). This effect was ameliorated by the addition of either Imatinib Mesylate or a PDGFRα neutralizing antibody (Figure 2.3B). Addition of Imatinib Mesylate and a PDGFRα neutralizing antibody to SKPs grown under control conditions caused no significant change in spherical colony size (Figure 2.3B).

Upon quantifying the results of growth under these specific conditions, I also found that the addition of Imatinib Mesylate or a PDGFRα neutralizing antibody to control conditions had no significant positive or negative effect on spherical colony number compared to the control (Figure 2.3B). SKPs grown in the presence of PDGF-B were found to produce approximately 3-

fold greater number of spherical colonies (Figure 2.3B) than those grown under control conditions (163.1 ± 13.9 µm versus 57.8 ± 5.2 µm; $p < 0.0001$; n = 3 wells per treatment, n = 3 rats excluding PDGFRα neutralizing with n = 1 rat). Furthermore, the addition of Imatinib Mesylate or a PDGFRα neutralizing antibody to SKPs grown in the presence of PDGF-B was able to block the growth effect provided by PDGF-B, whilst maintaining control conditions (Figure 2.3B). When SKPs were grown in the presence of adipocyte conditioned media a significant, almost 2-fold increase was seen with respect to the mean number of spherical colonies formed (Figure 2.3B) when compared to the control (92.3 ± 3.2 µm versus 57.8 ± 5.2 µm; $p < 0.05$). The addition of Imatinib Mesylate or a PDGFRα neutralizing antibody to SKPs grown in the presence of adipocyte conditioned media also repressed the effect of adipose-derived CM, resulting in a reduction in the mean number of spherical colonies to the level of those under control conditions (Figure 2.3B). Taken together, these results suggest that PDGF-B enhances dermal stem cell proliferation and self-renewal through the PDGFRα receptor. It may also be important to note that the effect on sphere size and number witnessed when SKPs were grown in the presence of adipocyte conditioned media was similar to that found with addition of PDGF-A (Figure 2.1).

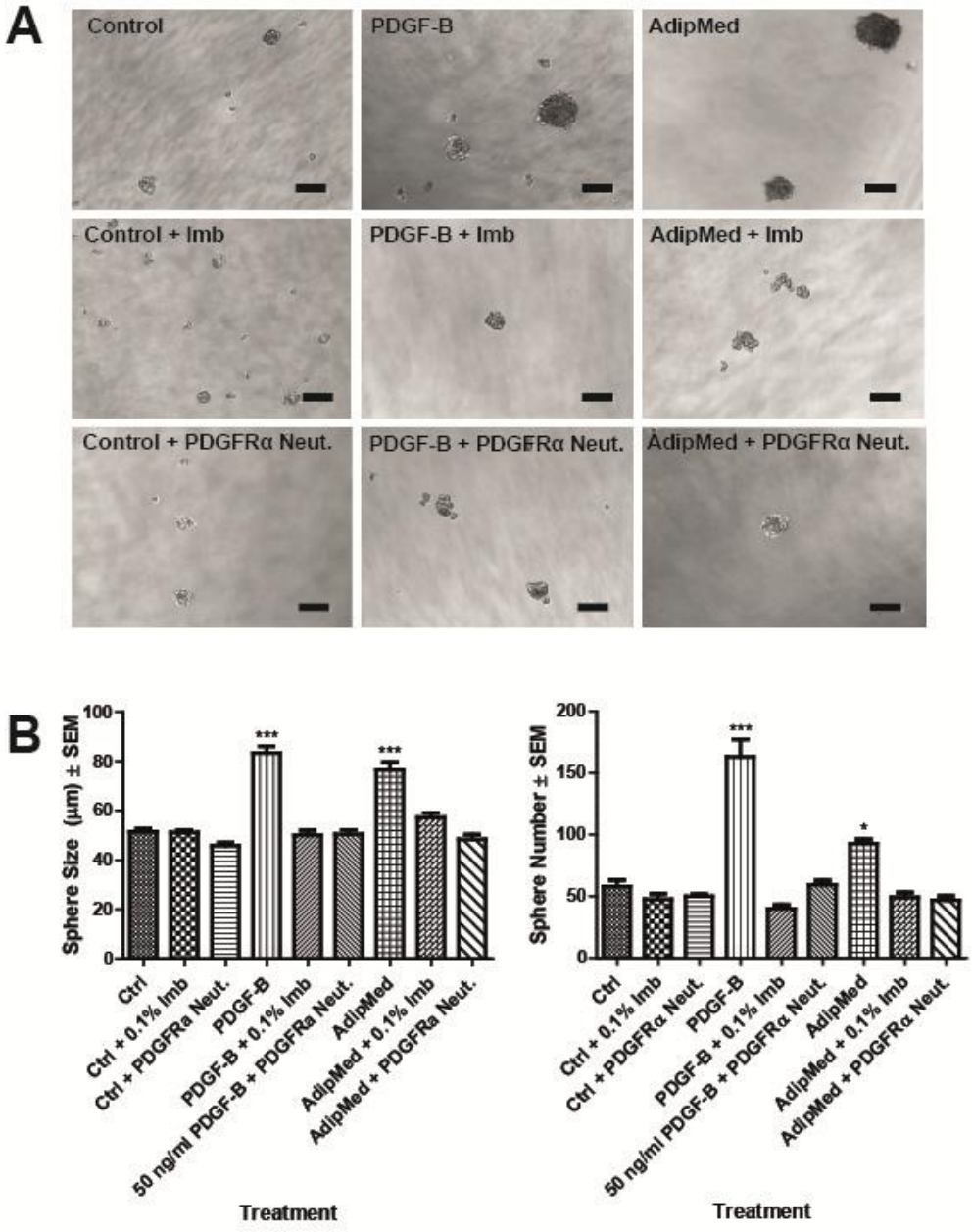

Figure 2.3 PDGF-B Induced Proliferation can be Blocked by Inhibition of PDGFRα

(A) Representative images of adult SKP colonies (day 8) grown under control conditions, or with the addition Imatinib Mesylate (0.1%), PDGFRα neutralizing antibody (10 µg/ml), PDGF-B (50 ng/ml), PDGF-B + Imatinib Mesylate, PDGF-B + PDGFRα neutralizing antibody, adipocyte conditioned media (replacing DMEM and F12 for control), adipocyte conditioned media + Imatinib Mesylate, and adipocyte conditioned media + PDGFRα neutralizing antibody (100 µm scale bars). (B) Quantification of both sphere size (diameter; n > 38 spheres per analyzed treatment) and sphere number (n = 3 wells) for each treatment type at day 8 (n = 3 separate experiments, excluding the PDGFRα neutralizing which is from one experiment). ± SEM is displayed. (ANOVA * = $p < 0.05$, *** = $p < 0.0001$)

3.6 Adipocyte conditioned media contains low levels of PDGF

We then asked whether PDGF-B activates PDGFRα in isolated dermal stem cells (SKPs). To do this, I performed immunofluorescence staining on cells grown in the presence of 1) control conditions, 2) addition of adipocyte conditioned media, or 3) addition of PDGF-B. Staining with a phospho-PDGFRα antibody, I found that PDGFRα is present on the surface of SKP spherical colonies and it is activated abundantly when cells are grown in the presence of PDGF-B (Figure 2.4A). Addition of adipocyte conditioned media caused a moderate increase in expression of phospho-PDGFRα, but there was less activation of the receptor than that from growth in the presence of PDGF-B (Figure 2.4A). However, growth in of SKP spherical colonies in the presence of adipocyte conditioned media still led to more abundant activation of PDGFRα than that found for SKP spherical colonies grown under control conditions (Figure 2.4A).

To ask directly whether PDGF-A or PDGF-B are present in adipocyte conditioned media and to quantify the amount, ELISA assays were performed for both ligands. Although robust PDGF was found in control samples (containing >2 μg/ml of PDGF-A), PDGF-A was not detected in the adipocyte conditioned media. (Figure 2.4B). This lack of PDGF-A was also confirmed in secondary passage SKP conditioned media (Figure 2.4B). However, when the adipocyte conditioned media was analyzed (n = 3 rats) for PDGF-B ligand content, it was found that small amounts were present averaging a level of approximately 40 pg/ml (Figure 2.4C). Interestingly, a similar amount of PDGF-B was found in the SKP conditioned media (n = 1 rats) that was also analyzed (Figure 2.4C) possibly suggesting that SKPs are able to generate the ligand as well, or that there was intradermal adipocyte or epithelial cell contamination during the SKP culturing process.

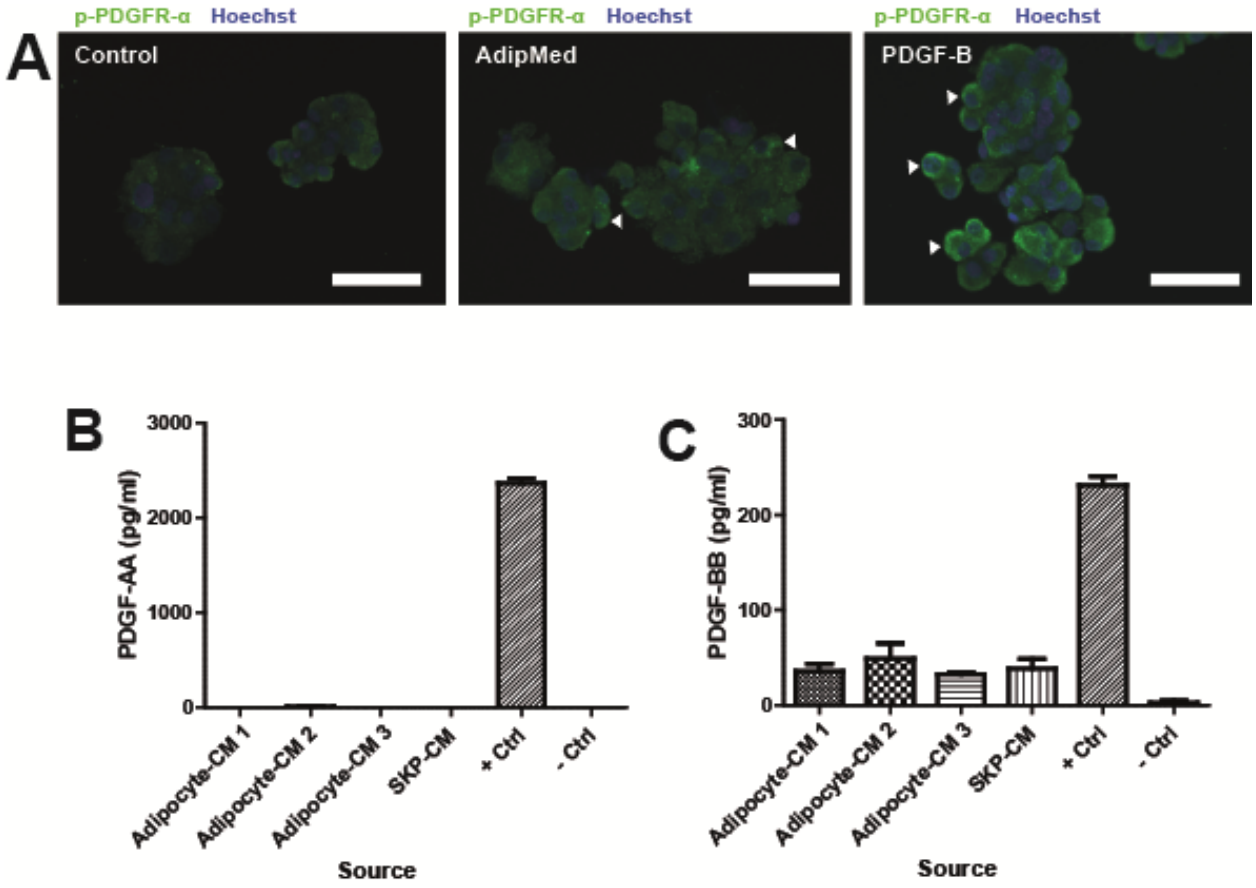

Figure 2.4 PDGFRα is Activated by PDGF-B in SKPs

(A) Immunohistochemical staining of SKP colonies (day 8) grown in the presence of control, adipocyte conditioned media, or PDGF-B for the activation and presence of PDGFRα on their cell surface (50 μm scale bars). Hoechst was used to stain nuclei. Exposure time was identical for all conditions. White arrowheads denote cells with high phospho-PDGFRα expression. (B) ELISA quantitative analysis of PDGF-A ligand content in adipocyte conditioned media (n = 3 rats; P32-33), SKP conditioned media (n = 1 rat; P32-33), and positive and negative controls (DMEM only). (C) ELISA quantitative analysis of PDGF-B ligand content in adipocyte conditioned media (n = 3 rats; P32-33), SKP conditioned media (n = 1 rat; P32-33), and positive and negative controls (DMEM only). ± SEM is displayed.

3.7 Expression of the PDGF-B ligand and activation of PDGFRα

In addition to investigating whether adipocytes from the hypodermis provide the PDGF regulation believed to be acting upon the SKPs in their niche, I also decided to pinpoint the expression of the PDGF-B ligand in the skin. To do this I performed immunofluorescence staining for the PDGF-B ligand on our PDGFRαH2BGFP mice, which would allow me to assess the location of PDGF-B in relation to dermal progenitors located in the hair follicle dermal sheath and papilla. It was found that the PDGF-B ligand is most strongly expressed within the epithelial compartments of the hair follicle (Figure 2.5A-C). In telogen skin it was found to be expressed within the epithelial bulge region and secondary germ, located directly adjacent to the dermal papilla (Figure 2.5C). Similarly, in anagen skin the PDGF-B ligand is expressed throughout the epithelial layers of the hair follicle, with greatest expression within the matrix surrounding the dermal papilla and the ORS which is directly apposed to the proliferative cells in the dermal sheath (Figure 2.5A-B). PDGF-B ligand expression seems to become weaker the further away the epithelial cells are from the hair bulb (Figure 2.5A-B). At all stages PDGFRα$^+$ dermal hair follicle cells appear to be directly adjacent or even touching PDGF-B expressing epithelium (Figure 2.5A-C). This is very clearly displayed by PDGFRα$^+$ dermal sheath cells of P8 and P28 anagen hair follicles (Figure 2.5A-B) that closely associated with PDGF-B expressing ORS, and in PDGFRα$^+$ dermal papilla cells of telogen follicles that appear to extend themselves towards the PDGF-B expressing hair germ.

To identify where PDGFRα receptor activation is occurring within the SKP niche, immunohistochemistry was completed using a phospho-PDGFRα antibody on P8, P28 and adult (P56) PDGFRαH2BGFP mouse dorsal skin. Findings show that a portion of the dermal sheath which is proximal to the dermal cup contains the highest amount of phospho-PDGFRα

expression in anagen follicles at both P8 and P28 (Figure 2.5D-E). PDGFRαH2BGFP dermal cells comprising the distal sheath at the base of the hair bulb do exhibit phospho-PDGFRα expression in P8 or P28 follicles, despite the presence of GFP-expressing PDGFRα^{+} cells (Figure 2.5A). This lack of expression is also the case in the dermal papilla of anagen hair follicles (Figure 2.5A). Not unexpectedly, PDGFRαH2BGFP^{+} cells in the dermal papillae and sheath of telogen hair follicles do not express phospho-PDGFRα (Figure 2.5C).

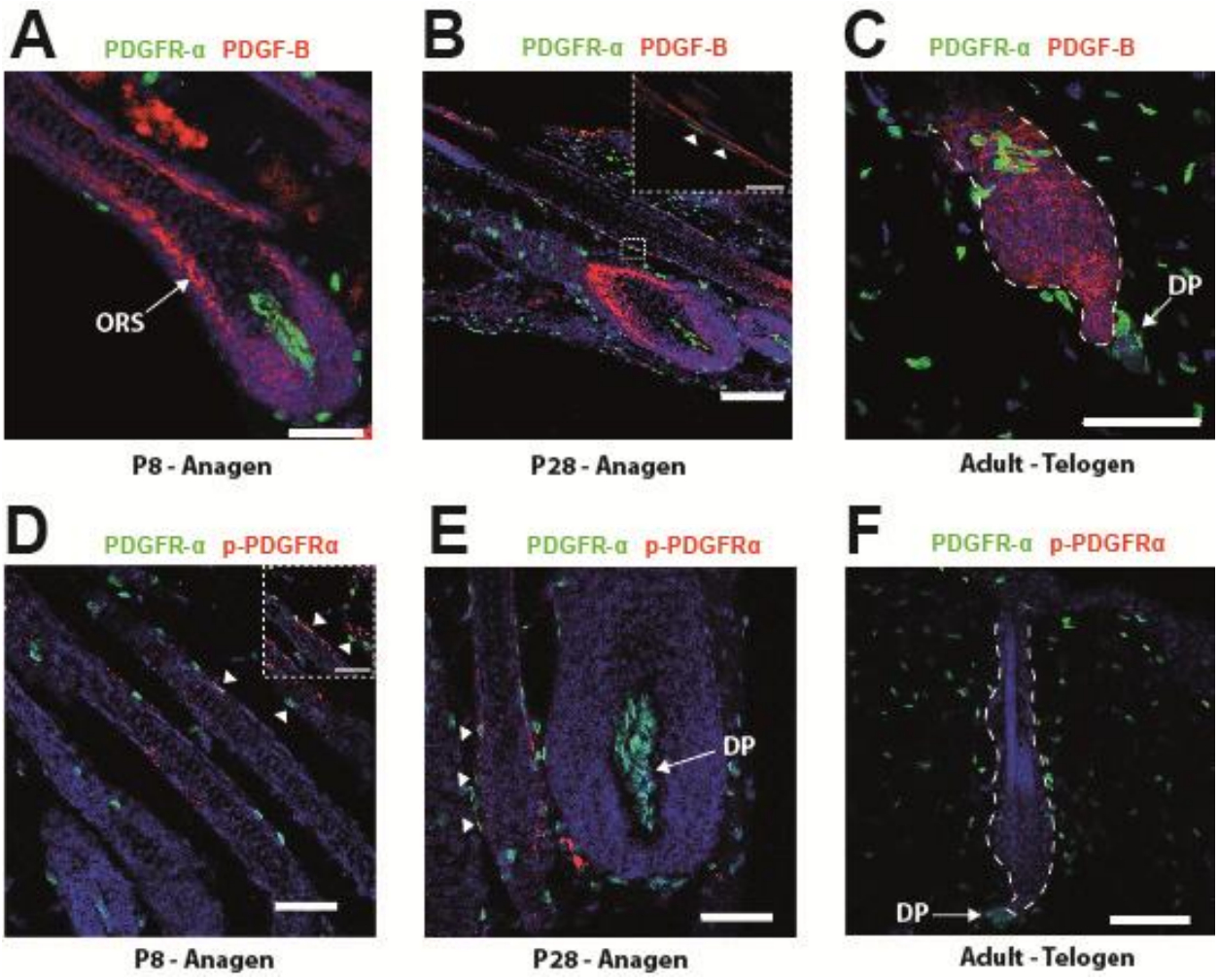

Figure 2.5 PDGFRα is Activated in Dermal Sheath Cells Directly Adjacent to Hair Follicle Epithelium Expressing PDGF-B

(A-F) Immunohistochemical staining of anagen and telogen hair follicles from P8, P28 and adult (P56 telogen) PDGFRαH2BGFP mouse skin, respectively. (A-C) Sections were stained using a PDGF-B ligand antibody (red) to assess the location of its expression with respect to the location of PDGFRα positive cells (green). White arrows (B) identify a pair of GFP-expressing PDGFRα^{+} cells within the dermal sheath that are closely associated with the epithelial layer expressing the PDGF-B ligand. (D-F) Stains with a phospho-PDGFRα antibody (red) were also completed to identify where PDGFRα receptor activation is occurring during each hair follicle stage. (D-E) White arrowheads denote GFP-expressing PDGFRα^{+} cells in the dermal sheath that co-localize with phospho-PDGFRα staining. (E) Dermal papilla cells (arrow) do not exhibit activated PDGFRα. (F) PDGFRα is not activated in the dermal papilla (white arrow) or sheath of telogen (P56) hair follicles (dashed lines border hair follicle epithelium). Scale bars represent 50 μm.

Chapter 4: Discussion and Future Directions

4.1 Discussion:

There are several main findings in these studies. First, growth of isolated dermal stem cells (SKPs) in the presence of PDGF-B, and to a lesser extent PDGF-A, enhances their proliferation and self-renewal *in vitro*. Second, SKPs grown in the presence of PDGF-B *in vitro* have an enhanced ability to form hair follicles *ex vivo* following subcutaneous transplantation into dorsal skin with epidermal aggregates. Third, SKPs grown in the presence of adipocyte conditioned media, that has been isolated from hypodermal fat, exhibit increased proliferation *in vitro*, and this effect is mitigated through blocking the PDGFRα receptor. Finally, based on my immunofluorescence staining of adult mouse skin at different stages of hair growth, it appears that the most likely source of the PDGF-B ligand is in the epithelial portions of the hair follicle closest to the SKP niche. Together, these experiments provide evidence of PDGF being a key regulator of SKP proliferation and self-renewal, and this pathway may be a valuable additive to stimulate mass expansion of SKPs for therapeutic use.

These findings help to propose various roles for PDGF-B in the regulation of SKPs within their hair follicle microenvironment. In confirming the location of the PDGFRα within the dermal stem/progenitor cells *in vivo* and in isolated cells, it is indicated that both the PDGF-A and PDGF-B ligands are at least able to act on these cells. Also, the ability of PDGF-B to robustly increase the size and number of SKP spherical colonies *in vitro* suggests that PDGF-B is a key regulatory factor keeping these cells proliferating and self-renewing within their hair follicle niche. Furthermore, the continued ability of SKPs grown in the presence of PDGF-B to produce an increased number of spheres over several passages suggests that PDGF-B is

maintaining these cells in a "stem cell" state with a persistent capacity to self-renew. This provides further evidence for the role of the microenvironment in maintaining the stem cell nature of SKPs as suggested by Biernaskie et al. 2009.

Next, the enhanced ability of SKPs grown in the presence of PDGF-B to produce numerous and properly formed hair follicles possibly suggests that a higher percentage of these cells are retaining their multipotency and more specifically maintaining their ability to become either dermal sheath cells or inductive dermal papilla cells (i.e. hair morphogenesis). My findings indicate that GFP-labelled SKPs grown in the presence of PDGF-B will reconstitute both the dermal papilla and dermal sheath of these new follicles (Figure 2.2), which constitutes maintained multipotency.

Initially, I proposed that PDGF regulation in part derives from adipocytes found below the hair follicles, as has been previously suggested (Festa et al., 2011). This was based on the finding that growth of SKPs in the presence of adipocyte conditioned media partially mirrored the effect of growth in the presence of PDGF-B with respect to an increase in spherical colony size, a growth pattern that is notably similar to that seen with PDGF-A (Figure 2.1F). These results indicate that the potential trace amounts of PDGF or some other protein within the adipocyte conditioned media are able to improve the proliferation of SKPs *in vitro*. The PDGF-C ligand, which also acts specifically through PDGFRα, expressed by mesenchymal cells in tissues of the heart and kidney *in vivo*, as well as human foreskin fibroblasts *in vitro*, is a potential candidate (Li et al., 2000). This would require that dorsal skin adipocytes are able to produce PDGF-C and that the appropriate proteolytic enzymes are present in the surrounding environment to cleave the full-length ligand down to its active form (Li et al., 2000). Nevertheless, addition of the tyrosine kinase inhibitor Imatinib Mesylate and a PDGFRα

neutralizing antibody prevented PDGF from binding to PDGFRα on the SKP cell surface, and subsequently blocked the adipocyte CM-enhanced SKPs growth as well as reduced the number and size of spherical colonies grown in the presence of PDGF-B (Figure 2.3). This indicates that PDGFRα is the definitive receptor being used by PDGF-B to improve the proliferation of the SKPs. Further confirmation of this comes from concomitant activation of PDGFRα on the surface of SKP spheres that were cultured in the presence of adipocyte conditioned media and PDGF-B, as shown by my phospho-PDGFRα antibody staining (Figure 2.4A).

ELISA analysis showed that adipocyte conditioned media does not contain PDGF-A, but does contain trace amounts of PDGF-B. This finding rules out PDGF-A, but makes it difficult to definitively conclude that such low levels of PDGF-B within the adipocyte conditioned media are able to improve the proliferation of SKPs alone. However, it is important to note that the adipocytes cultured to obtain the conditioned media were from hypodermal fat, meaning that intradermally positioned adipocytes were excluded. Also, no FACS was carried out to separate and isolate adipocyte precursors from mature adipocytes, indicating that the adipocyte cultures used to obtain the conditioned media likely contained a heterogeneous cell population. Recent studies have shown that subcutaneous adipose tissue (hypodermal fat) and skin adipose tissue (intradermal adipocytes) develop separately from one another, with intradermal adipocytes having been derived from cells within the dermis (Wojciechowicz et al., 2013). This indicates that due to their separate origins, hypodermal adipocytes and intradermal adipocytes may carry out different functions or secrete different factors *in vivo* as well as *in vitro*. Intradermal adipocytes, due to their close proximity to epithelial and dermal progenitor niches, may be a regulator of SKPs within their hair follicle niche. For instance, adipocytes positioned closer to SKPs may have the ability to produce more sufficient amounts of PDGF-B, or may even produce

PDGF-A as has been previously suggested (Festa et al., 2011). This would constitute a direct signalling relationship similar to that suggested between adipocytes and fibroblasts during wound healing (Schmidt and Horsley, 2013). However, hypodermal adipocytes are located further away, and therefore, may not function similarly or secrete the same factors as adipocytes imbedded in the dermis within a closer proximity of the SKP niche. It may also be important to note that the point of adipocyte cell harvest (P32-33 in this case) will likely affect the factors these cells secrete *in vitro*, as cells at the point of transition from telogen to anagen may secrete higher levels of proliferative factors, such as PDGF.

Given that PDGF-A was not present in adipocyte or SKP conditioned media, PDGF-B was found at low levels in both types of conditioned media, and PDGFRα is the key receptor through which PDGF-B is acting upon the SKPs, it has become clear that adipocytes, at least hypodermal adipocytes, may not be the primary source of endogenous PDGF controlling SKP function. Therefore, as a follow-up I chose to use a PDGF-B ligand antibody on dorsal skin from PDGFRαH2BGFP mice to assess the location of its expression with relevance to hair follicle dermal cells *in vivo*. This would highlight whether intradermal adipocytes or other cells play a part in the release of this proposed PDGF-B regulatory signal to the SKP cell niche, and more specifically would identify the range of action (i.e. paracrine). My findings show that the greatest expression of PDGF-B is coming from the epithelial layers of the hair follicle (i.e. outer root sheath cells) and specifically those close to the SKP niche such as the hair germ in telogen follicles or the matrix within the hair bulb of anagen follicles, rather than adipocytes. In all stages it was found that PDGFRα^{+} dermal hair follicle cells, such as those in the dermal sheath of anagen follicles or in the dermal papilla of telogen follicles, are directly adjacent or even touching PDGF-B expressing epithelium (Figure 2.5A-C). The absence of PDGF receptor

expression by the hair follicle keratinocytes, as well as a high expression of PDGF-B by these cells (Ansel et al., 1993), indicates that they are potentially the major contributors of this signalling to cells residing in the SKP niche. Furthermore, the finding that these cells are directly adjacent to the SKP niche provides evidence that short range, paracrine signalling may be their main mode of action. This leads to the notion that perhaps adipocyte-mediated PDGF may be more critical for attracting fibroblasts to wound sites (i.e. intradermal adipocytes) as has been previously suggested (Schmidt and Horsley, 2013). On the other hand, it has also been suggested that adipocytes may only produce PDGF when the SKP niche (i.e. DP) is closer to them (i.e. adipocytes below the hair follicle) during certain points of the hair cycle such as anagen (Festa et al., 2011). Both suggestions, in combination with those provided above, might explain why the adipocytes I isolated were found to produce no PDGF-A and low amounts of the PDGF-B in culture, while implicating cells within the epithelial portions of the hair follicle as more prominent and direct regulators of SKPs with respect to their actions during the hair follicle cycle *in vivo*.

It is important to point out that PDGF-B ligand expression is highest within the hair follicle shaft region, a portion of the hair follicle epithelium that is directly adjacent to a highly proliferative population of dermal sheath cells (Figure 2.5). It may be that there is a signalling gradient where PDGF-B expression in epithelial cells is stronger near the bottom of the hair follicle and begins to decline as you move progressively further away from the hair bulb. Along with images depicting PDGFRα expressing dermal precursors present in pairs along the dermal sheath of anagen hair follicles (Figure 2.5), this could indicate that as dermal precursors move upward along the dermal sheath and away from the dermal stem cell niche (i.e. surrounding the

base of the follicle bulb) as they differentiate. This conclusion would be in line with our *in vitro* PDGF-B experiments that demonstrate a role for PDGF-B in dermal progenitor proliferation.

Additionally, immunohistochemical staining of PDGFRαH2BGFP dorsal skin with a phospho-PDGFRα antibody indicates that there is higher expression, and therefore, activation of PDGFRα in the dermal sheath (Figure 2.5). The activation of the PDGFRα receptor is in PDGFRαH2BGFP cells that I have also shown to be closely associated with epithelial cells expressing the PDGF-B ligand, which further indicates a role for PDGF-B in regulating their activity. Furthermore, previous experimentation by our lab has indicated through the use of proliferation markers (Ki67 and EdU) that cells in this region of the dermal sheath are rapidly proliferating. This indicates the PDGF-B may be acting through PDGFRα to initiate proliferation of SKPs in the dermal sheath.

4.2 Future Directions

Although I have identified a role for PDGF signalling specifically in dermal stem cells, as well as one potential source of PDGF-B in the epithelial layer of the hair follicle, several additional experiments should be done in order to further clarify this relationship. Our lab has recently begun studies using a newly created strain of αSMACreERT2:PDGFRαFloxZEG mice that represent an inducible knock-out model for cells expressing both PDGFRα and αSMA (a marker of the DS). These mice were created through the breeding of a PDGFRα floxed mouse containing a Z/EG (GFP) reporter with a mouse containing a Cre recombinase construct (CreERT2) downstream of a αSMA promoter. It is expected that through conditionally knocking-out these PDGFRα expressing DS cells with tamoxifen we will be able to monitor their

function with respect to the hair follicle cycle and dermal wound healing. Assessing their function will also be aided by their expression of GFP following induction.

It will also be important to demonstrate a direct functional effect of epithelial cell-derived PDGF-B on SKP proliferation. This can be done using two different strategies: First, epithelial cells from the ORS of the hair follicle could be isolated by FACS to help verify that these cells are a major source of PDGF-B within the skin. A marker specific to epithelial cells in the ORS is Sox9 and fortunately our lab has a strain of Sox9:GFP knock-in mice. FACS could be used to isolate Sox9:GFP positive cells from these mice, which could be immediately placed into an ELISA plate to assess their secretion of the PDGF-B ligand. This would definitively confirm our expectation that these cells are the main source of the PDGF-B signalling that acts upon SKPs. Second, Sox9:GFP positive cells could be isolated by FACS and then co-cultured with SKPs to assess whether SKP proliferation and self-renewal is comparable to that of SKPs grown in the presence of PDGF-B (i.e. spherical colony size and number). We could again use a tyrosine kinase inhibitor (i.e. Imatinib Mesylate) or a PDGFRα neutralizing antibody to attempt to mitigate these affects. This would also definitively identify these cells as key regulators of SKPs through PDGF-B secretion.

As was previously mentioned, PDGFRα is a receptor that binds both the PDGF-A and PDGF-B ligands (Heldin and Westermark, 1999). Therefore, even with all the evidence I have provided indicating PDGF-B and PDGFRα as the most important mechanisms in SKP regulation, it cannot be ruled out that studying a strain of inducible PDGFRα knock-out mice does not necessarily implicate PDGF-B as the only potential ligand acting through the receptor. Therefore, to definitively examine the role of the PDGF-B ligand it may be ideal to create an inducible knock-out that pinpoints the epithelial cells that express PDGF-B and their role with

respect to SKPs and the hair follicle. This means that we would want to breed together a PDGF-B floxed mouse with a K14CreERT2 (K14 is an epithelial cell marker) mouse to create a strain of mice that through tamoxifen application would delete PDGF-B gene expression in skin epithelial cells. Upon doing so we could study the effect such a knock-out would have on hair follicles and their regenerative cycle, as well as wound healing. This would uncover the definitive role PDGF-B plays *in vivo*.

Future experimentation should also focus towards an assessment of PDGF-B and its regulatory function with respect to human SKPs. Although mice and rats represent good model organisms, it is always ideal to assess the merit of these findings as they pertain to humans. Furthermore, it is a crucial step before exploiting PDGF-B for therapeutic use in obtaining abundant and well maintained quantities of these dermal stem cells before transplant (i.e. wound healing and skin grafts). Interestingly, preliminary experiments in our lab have indicated that similar increases in proliferation and self-renewal are found when culturing human SKPs in the presence of PDGF-B (Kumar, Cheng and Biernaskie, unpublished results).

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