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Characterisation of Embryonic Dermal Precursor Cells



Sarah Naomi Blincko

MRes

Department of Biosciences

Durham University

2017

Characterisation of Embryonic Dermal Precursor Cells by Sarah Blincko

Abstract

Skin is an attractive organ for the acquisition of stem cells due to its accessibility, size and potential for autologous transplants. Research into skin development has implications for the isolation of stem cell populations, for example skin-derived precursors (SKPs), as well as the treatment of skin conditions, such as fibrosis.

This study centred on the early development, differentiation and stem cell potential of the dermis in embryonic mouse skin. Based on microarray data, the expression of specific Wnt family members was examined using RT-PCR and immunohistochemistry. No evidence of Wnt protein expression was observed in the dermis, but more embryonic stages and Wnt family members need to be explored to better understand Wnt signalling and its role in dermal development.

Our main focus was to investigate the plasticity of the common dermal fibroblast precursor population present at E13.5. We hypothesised that the dermis contains a common precursor capable of producing all cell types of the dermis and could harbour a high proportion of mesenchymal stem cell (MSC)-like precursors.

This E13.5 dermal cell (DC) population was investigated by exploring its differentiation potential when cultured in adipogenic and osteogenic media. These experiments indicated the E13.5 DCs contained a small subpopulation of MSC-like progenitors. However, when E13.5 DCs were cultured to produce SKPs and, subsequently, pushed to adipogenic and osteogenic lineages, they differentiated less than expected.

Most research regarding SKPs has used adult and older embryonic skin, therefore the findings here are novel in that SKPs were not expected at a younger age. However, RT-PCR revealed differences between the gene expression profiles of early and late embryonic dermal SKPs. Moreover, neither displayed the expected differentiation potential. The possible reasons for these unexpected findings include the potential role of hair follicle induction and/or a later migration of neural crest progenitors into the dermis.

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Abbreviations

2D	2-dimensional
3D	3-dimensional
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
Cbx5	Chromobox 5
"CD"	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
C/EBP α	CCAAT enhancer binding protein alpha
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DC	Dermal cell
DIK1	Delta-like 1
DMEM	Dulbecco's modified eagle's medium
DP	Dermal papilla
DS	Dermal sheath
dSKP	Dermal skin-derived precursor
DWAT	Dermal white adipose tissue
"E"	Embryonic day
EBF1	Early B-cell factor 1
ECM	Extracellular matrix
FABP4	Fatty acid binding protein 4
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GFP129	Green fluorescent protein 129
GSK-3	Glycogen synthase kinase-3
H3K9me3	H3 tri methyl K9
LEF-1	Lymphoid enhancer binding factor-1

MAP2	Microtubule-associated protein 2
MEM	Minimal essential medium
miR-124	microRNA-124
MSC	Mesenchymal stem cell
Myf5	Myogenic factor 5
MYT1L	Myelin Transcription Factor 1 Like
NeuN	Neuronal nuclei
OCT4	Octamer-binding transcription factor 4
"P"	Passage
Pax	Paired box
p75NTR	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PPAR γ	Peroxisome proliferator activated receptor gamma
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
rMSC	Rat mesenchymal stem cell
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
Sca-1	Spinocerebellar ataxia 1
SHOX2	Short stature homeobox 2
SKP	Skin-derived precursor
Sox	SRY-Box
SWAT	Subcutaneous white adipose tissue
TCF	T-cell factor
Wnt	Wingless-related integration site
Zfp	Zinc finger protein
-ve	Negative

Statement of Copyright

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1. Introduction

1.1 The Skin

Skin is an essential organ of the body and one of its key functions is to be a barrier by protecting the body from dehydration, microorganisms, toxins, chemicals, temperature, light and other hazards. It also has a role in temperature regulation, sensation and immunity (Sriram, Bigliardi, and Bigliardi-Qi 2015). The skin consists of a surface layer, largely made up of keratinocytes (the epidermis), and a mainly extracellular compartment containing heterogeneous fibroblasts and adipocytes (the dermis), separated by a basement membrane (Figure 1.1) (Wickett and Visscher 2006, Driskell et al. 2013, Forni et al. 2015). The dermis has been subdivided into three compartments: the upper, known as the papillary dermis, merging with the reticular dermis below, and an underlying hypodermis, which has a high proportion of adipocytes, now known as dermal white adipose tissue (DWAT) (Figure 1.2) (Driskell et al. 2014). Hair follicles are appendages found in most anatomical regions of the skin and one process they play a role in (among others) is thermoregulation, along with sweat glands and blood vessels (Fore 2006, Romanovsky 2014). The blood vessels also provide a source of nutrients to the skin. Nerve endings (which extend as far as the epidermis) are present to carry out the sensory function of the skin, including temperature and pain detection, and immune cells (such as monocytes, Langerhans cells and eosinophils) stand as an additional defence to the outside world if the skin should be broken (Fore 2006, Romanovsky 2014).

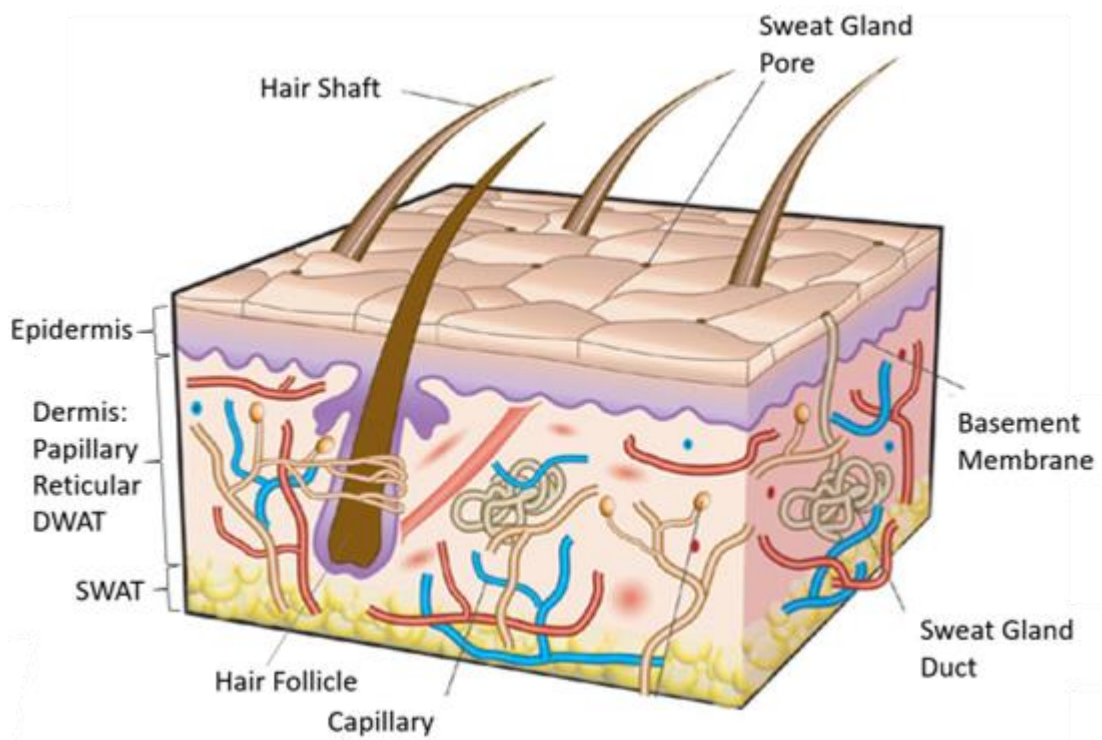


Figure 1.1: Structure of skin. The surface layer of the skin is called the epidermis and is divided from the dermis beneath it by the basement membrane. Blood vessels, hair follicles and sweat glands are all shown to be present in the skin, as well as the subcutaneous white adipose tissue (SWAT) below the skin. Adapted from (MacNeil 2007).

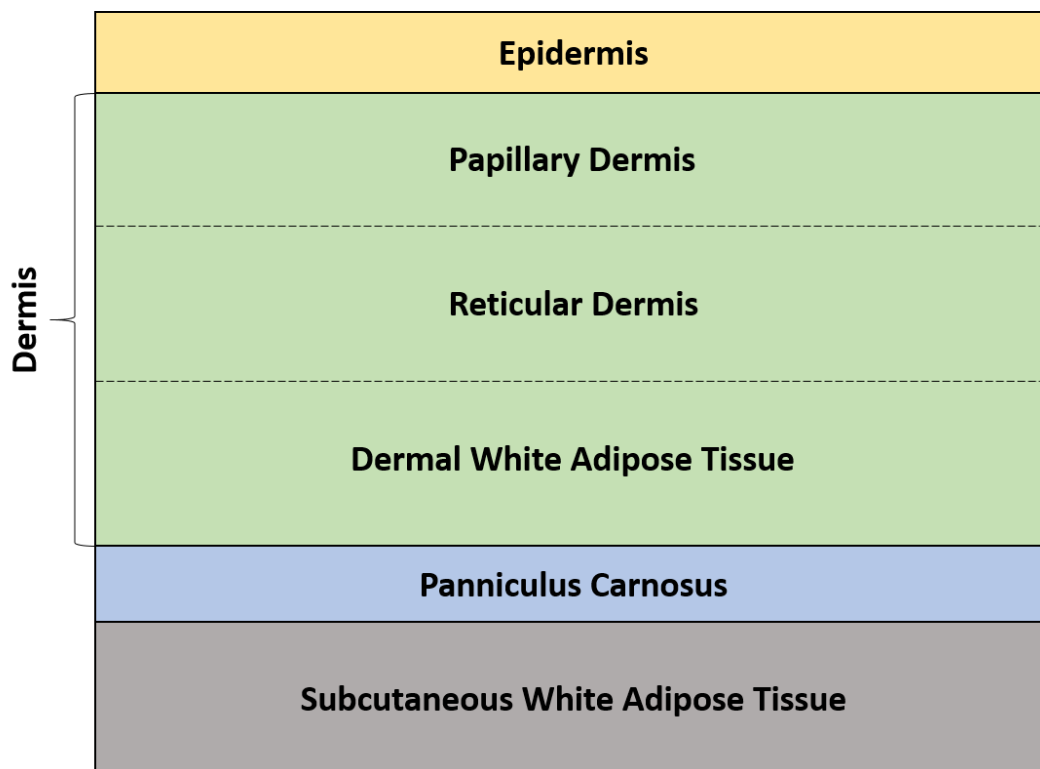


Figure 1.2: Simplified schematic of the structure of skin. The surface layer of the skin is called the epidermis and is followed by the dermis, which is split into the papillary dermis, reticular dermis and dermal white adipose tissue (DWAT). In mice, the panniculus carnosus (a muscle layer) separates DWAT from SWAT. Figure based on (Driskell et al. 2014).

1.1.1 The Dermis: Structure and Function

The dermis has a wide range of functions and cell types due to its division into three layers: the papillary dermis, reticular dermis and DWAT (Wojciechowicz et al. 2013, Mastrogiannaki et al. 2016).

In human skin, the papillary dermis is divided from the reticular dermis by the rete subpapillare (a vascular plexus) and the reticular dermis lies between this and the rete cutaneum (a deeper vascular plexus). However, they are also defined as distinct parts of the dermis by their extracellular matrix (ECM) composition. The papillary dermis has thin Collagen fibres that are loosely organised, a higher ratio of Collagen type III compared to type I and lower Versican levels. By contrast, the reticular dermis has well-organised, thick Collagen bundles, a lower ratio of Collagen type III to I and higher Versican levels (Sriram, Bigliardi, and Bigliardi-Qi 2015).

Fibroblasts (the main cell type of the dermis) have historically been considered to have more structural and supportive roles, as they are responsible for the synthesis of the ECM, particularly Collagen (Driskell and Watt 2015, Sriram, Bigliardi, and Bigliardi-Qi 2015). However, recent research has led to a new understanding of their role. They have been shown to have more dynamic functions in multiple skin-related processes, such as ageing, wound healing, fibrosis and epidermal:dermal communications (Maas-Szabowski, Shimotoyodome, and Fusenig 1999, Werner and Smola 2001, Eming, Krieg, and Davidson 2007, Werner, Krieg, and Smola 2007,

Bhattacharyya, Wei, and Varga 2011, Quan and Fisher 2015, Sriram, Bigliardi, and Bigliardi-Qi 2015).

The papillary and reticular dermis both consist of fibroblasts but have distinct functions due to the heterogeneous nature of the dermal fibroblast population and their distinct environments. For example, the dermal papilla (DP) cells in the hair follicle have a role in controlling the hair cycle (discussed later in this chapter) and the arrector pili muscle has a role in piloerection. However, both cell types are derived from papillary fibroblasts (Driskell and Watt 2015). In addition, the reticular dermis has a key role in the repair of the dermis during wound healing: these fibroblasts are involved in ECM deposition and degradation, and differentiating into myofibroblasts (Baum and Arpey 2005, Velnar, Bailey, and Smrkoli 2009, Rolin et al. 2014).

Recently an adipocyte population, termed DWAT, has been discovered to be of dermal origin and, thus, distinct from the subcutaneous white adipose tissue (SWAT) layer that resides below the skin (Wojciechowicz et al. 2013). In mice, DWAT and SWAT are clearly distinguished as separate fat depots by the panniculus carnosus (a striated muscle). However, in humans this is not the case because there is no panniculus carnosus and, consequently, the two depots are harder to define (Driskell et al. 2014, Alexander et al. 2015, Naldaiz-Gastesi et al. 2016). Previously, the main function of adipose tissue was thought to be an energy store but it is now known to have secretory, endocrine, insulating, inflammatory and metabolic roles (Trayhurn and Beattie 2001). The specific functions of DWAT are not fully understood at present but there is evidence to suggest a regulatory role in the hair follicle cycle and a role in the immune response to wound healing (Zhang et al. 2015).

1.1.2 Dermal Development

During embryogenesis, the dermis undergoes changes which eventually give rise to the adult dermis. Lineage tracing experiments, in mice, have been conducted to elucidate this process of dermal development. Figure 1.3 summarises this data and shows the dermis begins with a common 'fibroblast' progenitor cell which diverges to the papillary and reticular fibroblast progenitors which are then responsible for differentiating into the papillary fibroblast, DP, arrector pili muscle, reticular fibroblast and adipocyte cells. Driskell et al. (2013) proposed that fate restriction of the dermis takes place at embryonic day 16.5 (E16.5) when there is a divergence in the expression of markers (Driskell et al. 2013). This data led to the hypothesis that the dermis contains a common precursor responsible for producing all cell types of the dermis.

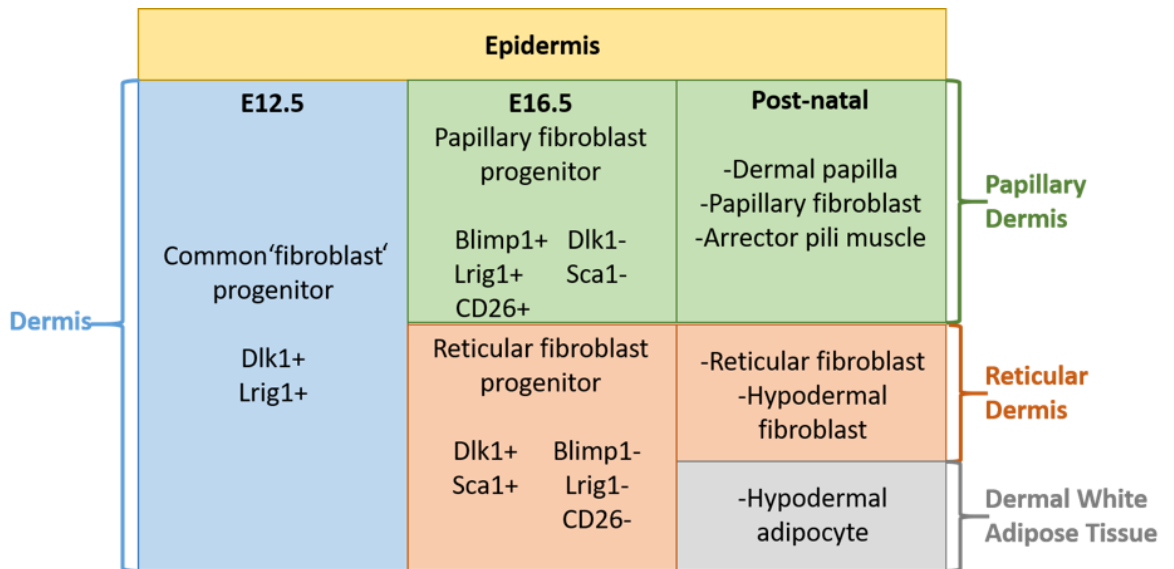


Figure 1.3: A summary of the lineage tracing data from Driskell et al. (2013) showing the dermis begins with a common 'fibroblast' progenitor which undergoes progressive differentiation to ultimately produce the final cell types found in the adult dermis. Figure based on Driskell et al. (2013).

Specifically in relation to DWAT, Wojciechowicz et al. (2013) used the pre-adipocyte marker FABP4 to investigate the timings of adipocyte development. FABP4 was shown to mark only the lower dermis of dorsal skin from E16, agreeing with the fate restriction proposed by Driskell et al. (2013). Therefore, cells of the lower dermis eventually give rise to DWAT (Driskell et al. 2013, Wojciechowicz et al. 2013). However, Rinkevich et al. (2015) showed that migration of Engrailed-1 positive cells (CD26+) from the papillary dermis to the reticular dermis takes place at E16.5. This may indicate that adipocytes are produced from cells originally of papillary dermis origin (Rinkevich et al. 2015). Therefore, the mechanism of dermal development is still a subject of interest and debate.

Further work has shown that adipogenesis of DWAT goes through a progressive series of commitment starting with a precursor cell, then a preadipocyte and finally a mature adipocyte (Figure 1.4). The general process of adipogenesis involves the expression of the early markers EBF1 and Zfp423 and the late markers C/EBP α and PPAR γ (Camp, Ren, and Leff 2002, Stephens 2012).

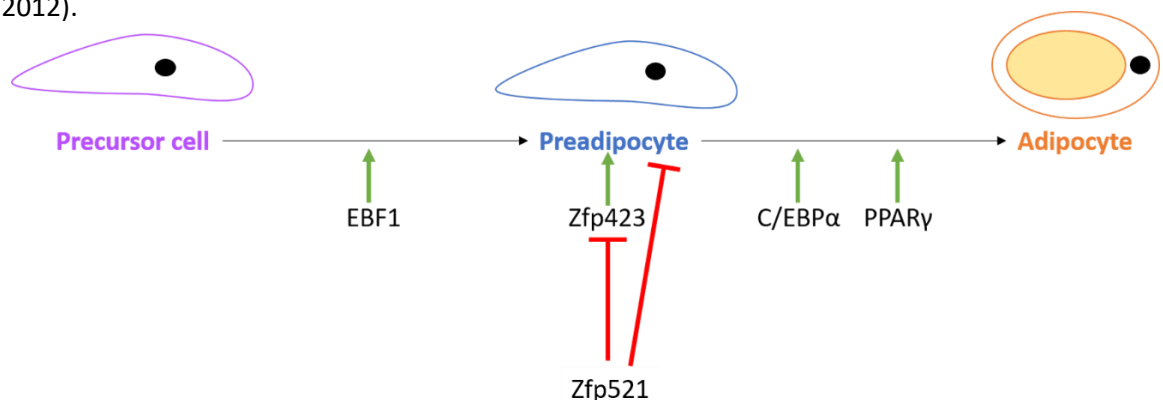


Figure 1.4: The stages of adipogenesis. The diagram highlights the 3 key stages involved in DWAT development: precursor cell, preadipocyte and adipocyte. Many transcriptional regulators are involved in this process and a few are shown here for each stage. Green arrows=activation; Red lines=inhibition. Based on Stephens (2012).

One of the questions addressed in this project revolves around the plasticity of the early embryonic dermal cells (DCs; thought to be the common 'fibroblast' progenitors), since further knowledge of this will hopefully improve our understanding of dermal development. Therefore, E13.5 mouse dermis was chosen as the focus of these studies for a number of reasons. Firstly, on a technical level, it is physically easier to separate the epidermis and dermis at this earlier stage of skin development. Secondly, again on a technical level, it is easier to remove the underlying muscle layer from the dermis than at earlier ages. And, finally, E13.5 is the last period before mouse pelage hair follicle formation is observed (E14.5) and, thus, represents the dermis before cells are morphologically segregated and before it becomes a more divergent, differentiated population (Sennett and Rendl 2012, Fu and Hsu 2013).

1.2 Hair Follicle Initiation and Cycle

Hair follicles are fascinating to study as they are composed of both epidermal and dermal elements and provide a great model to study regeneration as they repeatedly regenerate from a stem cell niche that resides within the follicle (Morgan 2014).

In mice, pelage hair follicles first become morphologically visible at E14.5 because of the placode: an epidermal accumulation of cells that subsequently initiates the dermal condensate (a dermal accumulation of cells). This initiation process involves cross-talk between the epidermis and dermis and Wnt signalling is thought to be involved in this communication (Sennett and Rendl 2012, Fu and Hsu 2013). Epidermal Wnts signal to the dermis leading to expression of a dermal signal (pre-placode stage). The epidermal Wnts aren't able to act on the epidermis due to an inhibitor but when the dermal signal is expressed this inhibitor is repressed, thus allowing Wnt activity in the epidermis. This leads to placode formation in the epidermis (placode stage), followed by the dermal condensate formation as a result of placode signalling (dermal condensate stage). Further hair follicle induction is subsequently achieved via dermal Wnt signalling to the placode (dermal condensate stage). Other signals (such as Shh) are involved in hair germ formation at E15.5 (hair germ stage) (Zhang et al. 2008, Chen et al. 2012, Sennett and Rendl 2012, Fu and Hsu 2013, Rishikaysh et al. 2014). Further to this step, epidermal growth occurs down into the dermis and surrounds the dermal condensate which ultimately forms the DP (hair peg stage). During this stage, the cells undergo differentiation which is under the regulation of many signals (for example, Wnt, BMP and Notch). Once follicle morphogenesis is complete, the hair follicles undergo cycling (Figure 1.5) (Stenn and Paus 2001, Fu and Hsu 2013, Rishikaysh et al. 2014).

The hair follicle cycle consists of three main stages, anagen (growth), catagen (regression) and telogen (rest), characterised by their distinct morphology (as depicted in Figure 1.5).

When the hair follicle is in telogen, the cells in the bulge area (highlighted in pink) are quiescent, the DP is not surrounded by the epithelial component of the follicle and there is minimal Wnt/ β -

catenin signalling. The balance between BMP and Wnt/ β -catenin signalling is key to the transition from telogen to anagen as BMP signalling inhibits anagen and Wnt/ β -catenin signalling promotes it. As anagen approaches, BMP signalling is reduced in the dermis and Noggin is released by the DP which inhibits BMP in the bulge area so that these cells are able to upregulate Wnt/ β -catenin signalling and proliferate. This leads to follicle elongation so that the DP and bulge area are further apart which regulates anagen from lasting too long (less Noggin from the DP will reach the bulge area as they become further apart). In anagen, the DP is surrounded by the epithelial component and the follicle itself extends further into the dermis (Lim and Nusse 2013). In catagen, the follicle regresses and brings the DP closer to the bulge area again so it is at its shortest during telogen (Figure 1.5) (Huelsenken et al. 2001, Sieber-Blum et al. 2004).

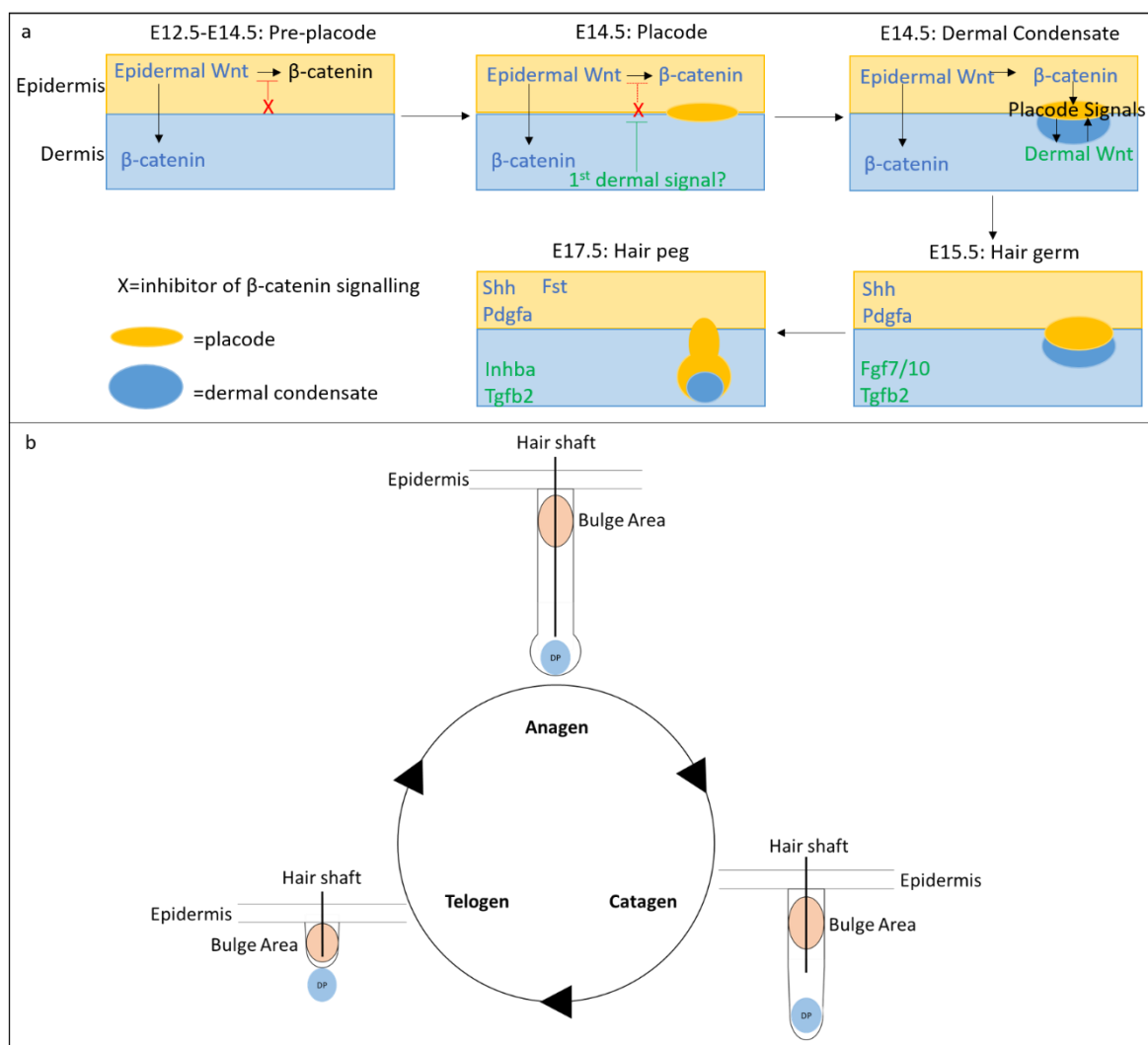


Figure 1.5: (a) A schematic of the stages of hair follicle initiation: E12.5-E14.5: pre-placode; E14.5: placode; E14.5: dermal condensate; E15.5: hair germ; and E17.5: hair peg. (b) A schematic of the three key stages of the hair follicle cycle-anagen (growth), catagen (regression) and telogen (rest). The DP, depicted in blue, is the dermal component of the hair follicle. The epidermal components are shown in white, but also include the bulge area shown in pink. Figure based on Stenn and Paus (2001), Sennett and Rendl (2012), Fu and Hsu (2013), and Lim and Nusse (2013).

1.3 Wnt/ β -catenin Signalling

Wnt/ β -catenin signalling (along with many other pathways such as, Hedgehog, Notch and BMP signalling) is one of the key developmental pathways (Nie, Luukko, and Kettunen 2006, Willert and Nusse 2012, Collu, Hidalgo-Sastre, and Brennan 2014, Lerner and Ohlsson 2015, Muñoz-Descalzo, Hadjantonakis, and Arias 2015, Edeling 2016).

Wnt signalling is involved in many different systems for example, bone formation, kidney development, neural development, cell fate decisions in embryogenesis and skin development (Mulligan and Cheyette 2012, Lim and Nusse 2013, Lerner and Ohlsson 2015, Muñoz-Descalzo, Hadjantonakis, and Arias 2015, Edeling 2016). There are 19 family members of Wnt ligands in mice and humans (Willert and Nusse 2012). These Wnt family members can be categorised into either canonical Wnts (acting through β -catenin; for example, Wnt 1, Wnt 3a) or non-canonical Wnts (acting independently of β -catenin; for example, Wnt 5a, Wnt 11) (Sinha et al. 2015). However, this categorisation is not definitive, as non-canonical Wnts may also act through the β -catenin-dependent pathway in certain contexts (Fathke et al. 2006). β -catenin-dependent signalling has been associated with targeting genes involved in proliferation, cell fate decisions and differentiation. β -catenin-independent signalling has been linked to cell migration and organ morphogenesis (Solis, Lüchtenborg, and Katanaev 2013). Interestingly, the pluripotency of a cell has also been linked to Wnt/ β -catenin signalling and this was shown (in a particular cell line of mouse embryonic stem cells) to be independent of LEF-1, indicating an alternative mechanism of action for β -catenin (Sato et al. 2004, Lluís et al. 2008, Marson et al. 2008, Kelly et al. 2011).

Of particular interest for this project is Wnt's key role in dermal development. This has been shown by the distinct localisation of Wnt-related gene expression in the papillary but not in the reticular dermis in neonatal skin. Its role in hair follicle initiation and cycling, as mentioned above, has also indicated this (Fu and Hsu 2013, Lim and Nusse 2013, Mastrogiannaki et al. 2016). Wnt/ β -catenin signalling is a short- and long-range signalling pathway for intercellular communication

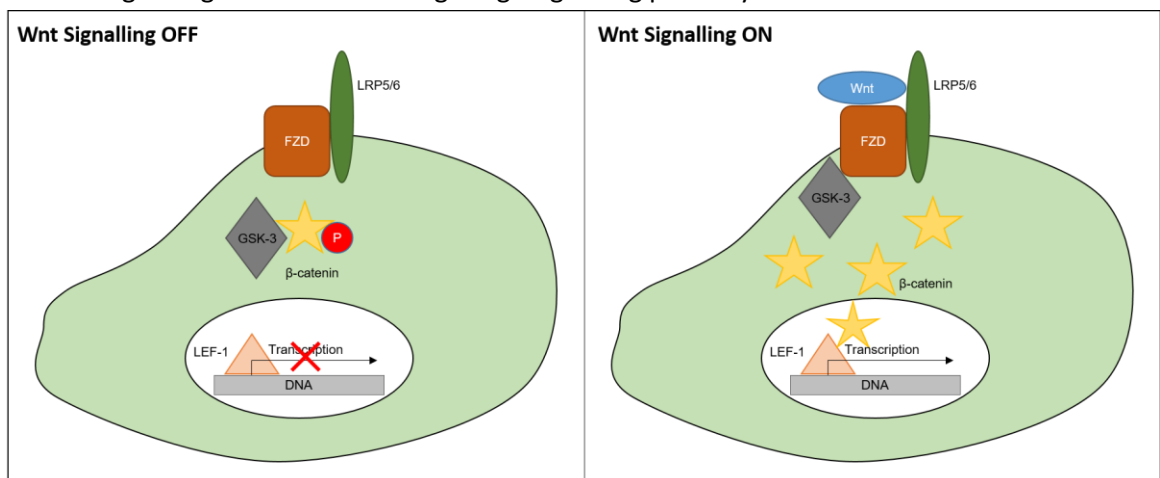


Figure 1.6: Schematic of the simplified Wnt/ β -catenin signalling mechanism. In the absence of Wnt signalling, β -catenin is phosphorylated by GSK-3 (part of the destruction complex) and subsequently degraded, so can't enter the nucleus to turn on transcription. In the presence of Wnt signalling, β -catenin is not degraded and, thus, it enters the nucleus to turn on transcription. FZD=Frizzled. Based on (Saito-Diaz et al. 2013).

and involves secreted Wnt glycoproteins which bind to the Frizzled and LRP5/6 co-receptors (Mikels and Nusse 2006, Saito-Diaz et al. 2013). When Wnt is bound, β -catenin is free and is therefore able to enter the nucleus and activate transcription of specific genes via the transcription factor LEF-1. However, in the absence of Wnt, β -catenin is phosphorylated by GSK-3 (a component of the destruction complex) and is consequently unable to enter the nucleus and, thus, the transcription of those specific genes does not take place (Figure 1.6) (Saitoh et al. 1998, Grigoryan et al. 2008, Saito-Diaz et al. 2013).

In addition to the role Wnt/ β -catenin signalling has in hair follicle initiation and cycling, it has been linked to lineage commitment. For example, it has been shown to be involved in promoting osteoblast development and maturation and plays a much-debated role in adipogenesis (Grigoryan et al. 2008). Wnt/ β -catenin signalling was thought to have a role in inhibiting adipogenesis due to the following: Wnt is downregulated in preadipocytes; Dkk1 (a Wnt inhibitor) is upregulated in adipogenesis; and Wnt signalling has been shown to block the induction of PPAR γ and C/EBP α (factors involved in adipogenesis) (Longo et al. 2004, Christodoulides et al. 2006, Christodoulides et al. 2009). However, there have been contradictory findings where ectopic expression of epidermal Wnt/ β -catenin signalling resulted in an expanded adipocyte layer (Donati et al. 2014). This may imply that the different family members of Wnt have different roles. Previous work (in the Jahoda lab) investigating RNA expression levels of the dermis and epidermis, has had limitations in deciphering the proposed individual functions of the Wnt family members due to lack of localisation. Hence, this project utilises immunofluorescent labelling of particular Wnt family members to elucidate their localisation and aid the understanding of their functions. This is particularly useful during hair follicle induction, for example, due to many of the Wnt family members being involved (Fu and Hsu 2013).

1.4 Stem cells

Stem cells are defined as cells capable of self-renewal (the ability to divide and retain their undifferentiated state) and differentiation (He, Nakada, and Morrison 2009, Chen, Ye, and Ying 2015). Stem cell populations are generally considered to expand via symmetric division (which produces two identical stem cells) but maintain their population via asymmetric division (which produces one stem cell and one cell that goes on to differentiate). This maintenance of stem cells is usually aided by a specialised microenvironment, termed a stem cell niche, which provides signals to control division and differentiation, as well as physically limiting the population size (He, Nakada, and Morrison 2009).

Due to skin being such an accessible organ, research has been done to find stem cell sources within the adult skin that can be used for autologous treatments (Wong et al. 2006, Vapniarsky et al. 2015, Kwon et al. 2017). Previous research has extensively explored the multiple stem cell

niches in the epidermis, for example the interfollicular epidermis, hair follicle, sebaceous gland and sweat gland (Watt 1998, Ghazizadeh and Taichman 2001, Janes, Lowell, and Hutter 2002, Uzarska et al. 2013, Kretzschmar and Watt 2014, Tadeu and Horsley 2014, Chen, Wang, and Shi 2015). The hair follicle is particularly interesting due to the multiple stem cell niches it contains from both the epidermis and dermis: for example, the bulge, the hair germ, the isthmus (all of epithelial origin) and the dermal sheath (DS) and DP (both of dermal origin) (Jahoda et al. 2003, Jaks, Kasper, and Toftgård 2010, Goldstein and Horsley 2012, Rahmani et al. 2014, Rompolas and Greco 2014). In contrast to the epidermal stem cell niches, dermal stem cells have only relatively recently become significant when Toma et al. (2001) identified a niche in the form of skin-derived precursors (SKPs) (Toma et al. 2001, Chen, Wang, and Shi 2015). Consequently, research has shown the dermis to contain mesenchymal stem cells (MSCs)/MSC-like cells in the dermis due to their broad differentiation potential and similar expression and functional properties to bone marrow MSCs (Goldring, Jones, Sewry, et al. 2002, Goldring, Jones, Thiagarajah, et al. 2002, Fernandes et al. 2004, Chen et al. 2007, Riekstina et al. 2009, Ambasudhan et al. 2011, Vaculik et al. 2012).

1.5 SKPs

SKPs represent a multipotent stem cell population derived from the skin which characteristically express Nestin, Fibronectin and Vimentin. They are observed *in vitro* and have the potential to produce neuronal and mesodermal lineage cell types: for example, neurons, glia, smooth muscle cells and adipocytes (Toma et al. 2001, Fernandes et al. 2004, Toma et al. 2005). The extent of the multipotency of this stem-like population is exciting as, previously, adult stem cells were thought to only differentiate to cell types found in their tissue of origin which severely limited their wider use (Joshi and Enver 2002).

However, there is controversy surrounding SKPs concerning the unclear nature of their origin (Hunt, Jahoda, and Chandran 2009). One potential origin for SKPs has been hypothesised to be the neural crest which is an embryonic, transient population of precursor cells that are derived (during gastrulation) from the dorsal border of the neural plate as a result of ectodermal and mesodermal signalling (Fernandes et al. 2004, Liu and Cheung 2016, Coste et al. 2017).

Subsequently, epithelial to mesenchymal transition occurs and so, these cells are able to migrate to different peripheral locations where they differentiate to a wide range of cell types (for example, bone, neurons, melanocytes, adipocytes and glial cells) (Liu and Cheung 2016, Coste et al. 2017). Interestingly, the dermis has different developmental origins in different anatomical locations. For example, the facial dermis is neural crest-derived, whereas the dorsal dermis is somite-derived (Fernandes, Toma, and Miller 2008, Jinno et al. 2010). This is an important factor to consider when choosing which skin sections to study and when drawing conclusions regarding the possible origin(s) of SKPs.

In addition to the question of SKP origin, there are questions as to whether SKPs are a skin-resident multipotent stem cell; whether they are a cell type with the ability to transdifferentiate; and whether they are something seen exclusively *in vitro* (Hunt, Jahoda, and Chandran 2009).

Research has mainly focused on producing SKPs from the adult dermis, but some work has been done on older embryonic skin (Toma et al. 2001, Fernandes et al. 2004). This work has attempted to answer some of the key questions surrounding SKPs and has provided evidence in favour of a neural crest origin for SKPs (discussed further in Chapter 5) and found that they do not arise due to transdifferentiation (Fernandes et al. 2004).

In contrast to previous literature, this project will investigate the properties of the SKPs produced from embryonic dermis as young as E12.5 and E13.5 with the hope that this will shed more light on the origin(s) of SKPs, how early they can be produced and provide information about the dermal population at these early embryonic stages, particularly regarding their plasticity.

1.6 Significance of Research

Dermal development is a significant area of research for a number of reasons.

First, understanding the way in which the different cell types develop can aid our understanding of the signals (for example, Wnt) that influence the behaviour of these cells and this can be applied to therapeutic treatments of conditions such as, fibrosis, diabetic ulcers and obesity (Trayhurn 2005, Wei et al. 2011, Quondamatteo 2014, Lafyatis et al. 2017).

Second, the fact that skin is an accessible organ has meant it is a very attractive source of stem cells-especially as there is the potential for it to provide an autologous source of stem cells for transplantation and tissue engineering applications (Wong et al. 2006, Vapniarsky et al. 2015, Kwon et al. 2017). For example, SKPs have been shown to innervate aganglionic intestine (a symptom of Hirschsprung's disease) via differentiation to enteric ganglia when injected *in vivo* (Wagner, Sullins, and Dunn 2014). Here, the key advantage is the autologous property of the transplanted cells which would reduce immune rejection. Additionally, the transplantation of autologous SKPs could have applications for the improvement of chronic wounds, for example diabetic ulcers (Shu et al. 2015) and burns (Bayati et al. 2017); as well as treat corneal endothelial disease (Inagaki et al. 2017); Parkinson's disease (Toma et al. 2005); type II diabetes mellitus; alopecia (Kellner and Coulombe 2009), Hirschsprung's disease (Wagner, Sullins, and Dunn 2014), de novo regeneration of hair follicles (Wang et al. 2016) and multiple sclerosis (Toma et al. 2005). However, more research is needed to establish the risk of tumour formation (Chen, Wang, and Shi 2015).

The downside to using adult samples is the fact that there will not always be enough tissue available to produce enough cells quickly (can be an issue for burn wounds). The culture time and

the cost are also disadvantages. A favourable alternative to autologous transplantation would be to use a bank of foetal DCs which have low immunogenicity and higher proliferative and regenerative properties compared to adult cells (Akershoek et al. 2016). However, the ethics of using foetal cells limits this approach and returns us to the preference for an accessible adult stem cell source (Kang et al. 2011). Thus, research into the optimal age for collecting skin cells in adults is being conducted with the view to cryopreserving them for future use. This is due to the discovery of a decline in the number and/or differentiation potential of SKPs correlated with the increased age of adult human skin. Having cryopreserved autologous cells would eliminate the issue of not having enough tissue available and quickly enough, while still using a source with reduced risk of rejection (Gago et al. 2009).

Third, in addition to transplant applications, SKPs can be used to produce specific cell types of an individual for screening as well as, be used to research neurodegenerative genetic disorders due to SKPs link with the neural crest (Toma et al. 2005, Fernandes, Toma, and Miller 2008).

Finally, SKPs may have been linked to 'cancer stem cells' so could have applications in testing and optimising cancer treatments by producing them *in vitro* from biopsies (Fernandes, Toma, and Miller 2008).

1.7 Thesis Aims

The first aim of this project was to investigate the localisation of particular Wnt family members in E13.5 skin, our embryonic stage of choice, in order to shed more light on the complex role Wnt/ β -catenin signalling has on dermal development.

The second aim was to investigate the plasticity of early embryonic DCs: firstly, by looking at their differentiation abilities in 2D cell culture and, secondly, by pushing them to a more primitive state to see if they could produce SKPs. This would lead to an understanding of how many cells of the E13.5 dermal population are stem-like. Is the population dominated by stem-like cells, as the lineage tracing predicts, or are these stem-like cells just a subpopulation (Driskell et al. 2013)? In addition, the production and differentiation of embryonic SKPs would allow the plasticity of this dermal population to be investigated, showing whether it is restricted in its ability to differentiate to particular lineages. It would also allow us to challenge the literature which say SKPs cannot be produced using skin earlier than E14 (Fernandes et al. 2004). These two experimental approaches were used to test the hypothesis, that the dermis contains a common precursor cell capable of producing all cell types of the adult dermis, to further understand the properties of this early dermal precursor population.

2. Materials and Methods

Please see (at the end of this chapter) Table 2.1 for the components of the culture media used, Table 2.2 for information on the primary antibodies, Table 2.3 for information on the secondary antibodies, Table 2.4 for other solutions used, Table 2.5 for information on the number of replicates of SKPs and Table 2.6 for primer sequences. Unless otherwise stated, the incubator used for cell culture was a SANYO MCO-18AIC incubator with 20% O₂, 37°C, 5% CO₂ conditions. All centrifugation steps used an Eppendorf Centrifuge 5810 R.

2.1 Mice

BALB/c and GFP129 mice were from Durham University Life Sciences Support Unit. Mice were used as animal models due to their similarities with the human genome; the fact that they produce litters of embryos; have a short gestation period of around 20 days; are easy to maintain; and have a fully sequenced genome allowing transgenic/knockout strains to be produced. At present, they are a good alternative to using human skin (Perlman 2016).

The presence of a vaginal plug was used to indicate pregnancy and to age the embryos (embryos=0.5 days old when vaginal plug forms). The animals were sacrificed using cervical dislocation. The embryos used were E12.5, E13.5, E14.5 or E15.5 (ages confirmed by microscopic analysis of features). Embryos were snap frozen by placing in a base mould filled with Tissue Tek O.C.T. (Sakura), put over liquid nitrogen until frozen, and stored at -80°C.

2.2 Isolation of the Dermis

Stages of embryos were confirmed by microscopic analysis. Figure 2.1 summarises the method of dermal isolation. Embryos were pinned on agar, and two pieces of the dorsolateral skin were cut per embryo, using ophthalmic scissors, and transferred into an enzymatic solution (1.5% pancreatin (Sigma), 1.25% trypsin (Sigma) in 1X Earle's) for 30-60 minutes at 4°C to separate dermis and epidermis. The epidermis was then removed from the dermis using a needle. For RNA extraction, the dermis or epidermis were put straight into RLT Buffer (the first step of RNeasy Mini Kit (Qiagen)). For cell culture, the epidermis was mechanically dissociated, and the dermis was put into an enzymatic solution of hyaluronidase (Sigma) and collagenase-2 (Worthington Biochemical Corporation) (100 units and 290 units per 1 ml PBS (Severn Biotech), respectively) for 15 minutes at 37°C. Then the dermis was mechanically dissociated into single cells, the enzymatic reaction stopped by adding DC media. Both epidermal cells and DCs were passed through a cell strainer (70 µm, Fisher Scientific) and finally, centrifuged in DC media for 10 minutes at 1000 rpm. This method was similarly used on whisker pad skin. Once isolated, the cells were either cultured in 1X SKP proliferation media, differentiation media or DC media.

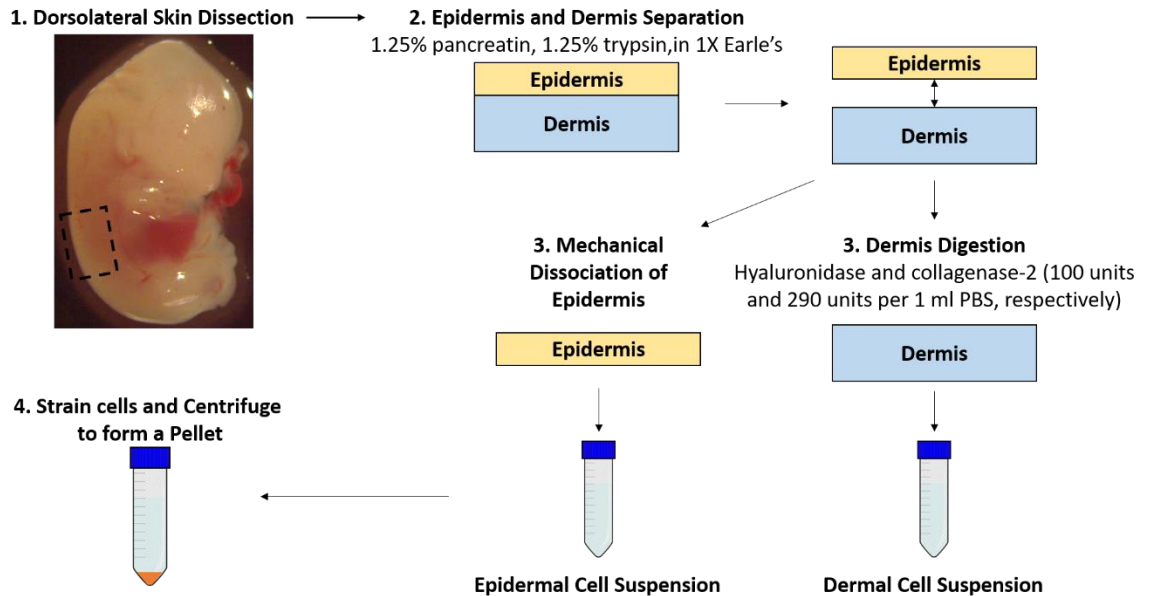


Figure 2.1: Schematic describing the method of dermal and epidermal cell isolation for use in cell culture. Cartoon images courtesy of Clipart.

2.3 DC Differentiation

The DCs were resuspended and seeded at 20,000 cells on coverslips in 24-well plates with adipogenic or osteogenic media. Cells were then cultured in adipogenic media for 7 days and then in DC control media for 7 days or osteogenic media for 21-28 days in the dark. The media was changed every 3-4 days and 2-3 days, respectively.

2.4 DC Adipogenic Differentiation Under Different Oxygen Conditions

The cells were resuspended and seeded at 20,000 cells on coverslips in 24-well plates. Cells were cultured in adipogenic media for 4 days and then in DC media for 4 days in either hypoxic (5% O₂, 37°C, 5% CO₂, Baker In vivoO₂ 400 incubator) or normoxic conditions (20% O₂, 37°C, 5% CO₂, SANYO MCO-18AIC incubator) in the dark, and the media was changed every 3-4 days.

2.5 Rat Mesenchymal Stem Cells (rMSCs)

rMSCs were isolated from Wistar rats as previously described (Croft and Przyborski 2006). They were maintained in culture with rMSC control media and seeded at 14,000-20,000 cells/ml in 24-well plates as a control for the osteogenic differentiation assays. Only rMSCs less than passage 8 (P8) were used.

2.6 3T3-L1 Cells

3T3-L1 cells were maintained in culture with 3T3-L1 control media and seeded at 20,000 cells/ml in 24-well plates as a control for the adipogenic differentiation assays. Only 3T3-L1 cells less than P12 were used.

2.7 Production of Dermal Skin-Derived Precursors (dSKPs)

The DCs were resuspended in 1X SKP proliferation media, seeded between 100,000 and 500,000 cells/ml into T12.5 flasks with 4 ml of 1X SKP proliferation media and incubated at 37°C and 5%

CO₂. The flasks were agitated once a day and 10X media was added every 3-5 days. Flasks were imaged using Zeiss Axiovert 40C microscope and Canon PowerShot A620 camera.

Once dSKPs had formed they were snap frozen by pipetting them into a base mould filled with Tissue Tek O.C.T. (Sakura), placing in liquid nitrogen until frozen and were stored at -80°C. Alternatively, SKPs were also put on coverslips with 1X SKP adherence media for 48 hours.

2.8 Passaging dSKPs

dSKPs were passaged by spinning the dSKP suspension at 250g for 4 minutes, saving the supernatant, and incubating in collagenase XI (1 mg/ml) for 10 minutes at 37°C. The enzymatic reaction was stopped with FBS and the dSKPs were triturated using a pipette. The cells were pelleted by centrifuging at 250g for 5-7 minutes and then resuspended in 2 ml of 2X SKP proliferation media. 1 ml of 2X SKP proliferation media cell suspension was added to 2 ml of conditioned media and 1 ml of 2X SKP proliferation media in a T12.5 flask.

2.9 Quantification of dSKPs

dSKPs were imaged in a 12-well plate using a Zeiss Stemi SV 11 microscope and AxioCam ERc 5s camera and the cell counter plugin on ImageJ was used to quantify the number of dSKPs per flask.

2.10 dSKP Differentiation

For adipogenic and osteogenic differentiation, 5-15 dSKPs (5 µl suspension) were plated in a 24-well plate with SKP adherence media overnight to allow adherence. Cells were then cultured in adipogenic media for 7 days (37°C, 5% CO₂) or osteogenic media for 20-28 days in the dark and the media was changed every 3-4 days and 2-3 days, respectively. Coverslips were imaged using Zeiss Axiovert 40C microscope and a Canon PowerShot A620 or A630 camera.

2.11 3D DC Culture-Hanging Drop Method

The isolated DC suspension was resuspended in DC media and then approximately 10,000 cells were seeded in 10-15 µl drops on the lid of a 90 mm petri dish. The hanging drops were kept hydrated by incubating with deionised water in the bottom of the petri dish. The media was changed on day 3 (a spheroid should have formed at this point) and day 7 and they were cultured for 8 days in total. The spheroids were imaged using Zeiss Stemi SV 11 microscope and AxioCam ERc 5s camera.

2.12 Staining

For immunofluorescent and Haematoxylin and Eosin staining of frozen tissue, 7 µm cryosections of the sample were cut on a Leica CM3050 S cryostat and air-dried at room temperature until dry.

2.12.1 Immunofluorescent Staining

1X PBS was used for 5 minutes at room temperature in all wash steps. Specimens were washed 3 times and then fixed using Methanol (Emsure) for 10 minutes at -20°C, or 4% PFA (Sigma) for 30

minutes at 4°C for the Cbx5 antibody. After 3 washes, 0.1% Triton X-100 (Sigma) was used for 30 minutes at room temperature for permeabilisation. Specimens were washed 3 times and then blocked with 2% BSA (Sigma) for 30 minutes at room temperature. Specimens were incubated either overnight at 4°C or at room temperature for 2 hours with the primary antibodies. After 3 washes, the samples were incubated for 1 hour at room temperature with the secondary antibodies, either alone, and followed by 3 washes and DAPI staining (KPL; 1:1000) for 5-10 minutes at room temperature, or in combination with DAPI (KPL; 1:1000). The samples were washed 2 or 3 times and were mounted with coverslips using Mowiol 4-88 (Sigma). A ZEISS AXIO Imager M1 microscope and a Hamamatsu C4742-95 camera were used to capture images.

2.12.2 Haematoxylin and Eosin Staining

All steps were done at room temperature. Mayer's Haematoxylin (VWR) was used to stain the nuclei of sections (5 minutes). Blueing was done with distilled water followed by alkaline alcohol, 70% ethanol and 95% ethanol, each for 30 seconds. Eosin Yellowish (Sigma) was used for 30 seconds to counterstain and the dehydration steps were 95% ethanol (15 seconds), 100% ethanol (45 seconds) and then Histoclear (National Diagnostics) for 5 minutes. DPX (Sigma) was used to mount the slides. A ZEISS Axiovert 40C microscope and Canon PowerShot A620 or A630 camera were used to capture the images.

2.12.3 Oil Red O Staining

Cells on coverslips were initially washed with 1X PBS at room temperature and then fixed for 1 hour in 10% Neutral Buffered Formalin at room temperature. Subsequently, cells were washed with deionised water 1-2 times and covered with 60% Isopropanol (Sigma) for 5 minutes and then Oil Red O working solution (3:2, Oil Red O stock:deionised water) for 30 minutes, all at room temperature. Specimens were then rinsed with tap water 2-3 times before being counterstained with Mayer's Haematoxylin (VWR) for 1 minute at room temperature. Cells were rinsed again in tap water and then mounted in Glycergel (Dako). Coverslips were imaged using Zeiss Axiovert 40C microscope and Canon PowerShot A620 or A630 camera.

2.12.4 Von Kossa Staining

Cells were initially washed with 1X PBS, then fixed for 30 minutes in 10% Neutral Buffered Formalin at room temperature. This was followed by 10 washes with deionised water, making sure to wash under the coverslip. Subsequently, cells were covered by 2% silver nitrate (BDH) for 1 hour in the dark at room temperature, and then for another hour under UV at room temperature. After washing three times, the deionised water was replaced with 2.5% sodium thiosulphate (Sigma) for 5 minutes at room temperature. Then washed twice with deionised water and mounted in Glycergel (Dako). Coverslips were imaged using a Zeiss Axiovert 10

microscope and AxioCam ERc 5s camera or Zeiss Axiovert 40C microscope and Canon PowerShot A620 or A630 camera.

2.13 RT-PCR

RNA extraction was done using the RNeasy Mini Kit (Qiagen). RNA quality and quantity were calculated using a NanoDrop Spectrophotometer. RNA was converted to cDNA using Superscript III reverse transcriptase (Invitrogen). All work was done under RNase free conditions. RT-PCR was performed on a Biometra thermal cycler. Each individual PCR reaction (10 μ l total volume) had 50-100 ng/ μ l of cDNA, primer mix of 10 μ M, 2X MyTaq mix (Bioline) and sterile water (Gibco). The conditions used were: step one (95°C for 3 minutes), then 35 cycles of step two (95°C for 45 seconds), step three (specific annealing temperature for 30 seconds) and step four (72°C for 30 seconds). Then step five (72°C for 7 minutes), after which the products were kept at 4°C until ready to run on the 2% agarose gel (Severn Biotech Ltd). Gel was imaged using a Syngene InGenius machine. See Table 2.5 for primer sequences.

2.14 Microarray

The processing of samples and microarray was carried out by the Jahoda lab in collaboration with the Christiano lab, New York. Dorsal epidermis and dermis from C57BL/6J embryos between E12.5 and E15.5 (embryos=0.5 days old when vaginal plug forms) were used and total RNA isolated using RNeasy Minikit (Qiagen). Affymetrix reagents and protocols were used to amplify and label the samples which were then hybridised to a MOE430A microarray chip (Bazzi et al. 2007). The data output was normalised and analysed using Expression Console Software and then Transcriptome Analysis Console Software by Adam Gilmore (Affymetrix). The normalised values were then plotted without statistical parameters.

Table 2.1: Details of the media used for cell culture.

Reagent	Concentration	Company	Catalogue Number
1X SKP Proliferation Media			
DMEM (low glucose):F12 (3:1)	-	Gibco	DMEM: 21885-025 F12: 31765-027
Epidermal growth factor	20 ng/ml	Peprotech	AF-100-15
Basic fibroblast growth factor	40 ng/ml	Peprotech	100-18B
B27	2% v/v	Gibco	17504-044
Fungizone	0.5 µg/ml	Gibco	15290-026
Pen/Strep	100 units/100 µg/ml	Gibco	15070-063
1X SKP Adherence Media			
FBS	5% v/v	Gibco	10270; 10099-141
1X SKP Proliferation Media	See above	See above	See above
Osteogenic Media			
10% MEM	-	Gibco	MEM:42360-024 FBS: 10270; 10099-141
Dexamethasone	100 nM	Sigma	D4902-100MG
Ascorbate-2-phosphate	50 µM	Sigma	A8960-5G
β-glycerophosphate	10 mM	Sigma	G-9891
Fungizone	0.5 µg/ml	Gibco	15290-026
Pen/Strep	100 units/100 µg/ml	Gibco	15070-063
Adipogenic Media			
StemPro Adipogenesis Differentiation Kit	-	Gibco	A10070-01
Pen/Strep	100 units/100 µg/ml	Gibco	15070-063
Dermal Cell Media			
MEM	-	Gibco	42360-024
FBS	10% v/v	Gibco	10270; 10099-141
3T3-L1 Control Media			
DMEM (high glucose)	-	Gibco	31966-021
FBS	10% v/v	Gibco	10270; 10099-141
Dermal Cell Control Media			
DMEM (low glucose)	-	Gibco	21885-025
FBS	10% v/v	Gibco	10270; 10099-141
rMSC Control Media			
DMEM (high glucose)	-	Gibco	31966-021
FBS	10% v/v	Gibco	10270; 10099-141
Glutamine	1:100	Gibco	25-030-081

Table 2.2: Details of the primary antibodies used.

Target	Company	Catalogue Number	Dilution
Anti-histone H3 (tri methyl K9)	Abcam	ab8898	1:100
Cbx5	ProteinTech	11831-1-AP	1:100
C/EBP α	Cell Signalling	8178S	1:100
C/EBP α	Santa Cruz	sc-61	1:100
Dlk1	PTG	10636-1-AP	1:100
FABP4	R&D Systems	AF1443	1:50
Fibronectin	Institut Pasteur de Lyon	-	1:200
LEF-1	Cell Signalling Technology	2230	1:200
Nestin	DSHB	rat-401	5 μ g/ml
Vimentin	Abcam	ab92547	1:250
Wnt 2	ProteinTech	11160-1-AP	1:100
Wnt 4	Santa Cruz	sc-5214	1:100
Wnt 5a	R&D Systems	MAB645	1:100
Wnt 11	R&D Systems	AF2647	10 μ g/ml

Table 2.3: Details of the secondary antibodies used.

Type	Company	Catalogue Number	Dilution
Donkey anti-goat 594	AlexaFluor	A11058	1:500
Donkey anti-goat 488	AlexaFluor	A11055	1:500
Donkey anti-mouse 594	AlexaFluor	A21203	1:500
Goat anti-mouse 488	AlexaFluor	A11029	1:500
Donkey anti-rabbit 594	AlexaFluor	A21203	1:500
Goat anti-rabbit 594	AlexaFluor	A11012	1:500
Donkey anti-rabbit 488	AlexaFluor	A21206	1:500
Donkey anti-rat 594	AlexaFluor	A21209	1:500
Donkey anti-rat 488	AlexaFluor	A21208	1:500

Table 2.4: Details of the solutions used for staining.

10X PBS (in dH₂O)			
Chemical	Concentration	Company	
NaCl	80 mg/ml	Sigma	
KCl	2 mg/ml	Sigma	
Na ₂ HPO ₄	14.4 mg/ml	Honeywell	
KH ₂ PO ₄	2.4 mg/ml	BDH	
10X Earle's Solution (in dH₂O; A and B mixed in equal quantities)			
Chemical	Concentration	Company	Solution A or B
NaCl	71.6 mg/ml	Sigma	A
KCl	4.2 mg/ml	Sigma	A
NaH ₂ PO ₄	1.5 mg/ml	Sigma	A
Glucose	10.5 mg/ml	BDH	A
NaHCO ₃	66.7 mg/ml	BDH	B
10% Neutral Buffered Formalin (in dH₂O)			
Chemical	Concentration	Company	
37% Formaldehyde	10% (v/v)	Sigma	
Na ₂ HPO ₄	6.5 mg/ml	Honeywell	
NaH ₂ PO ₄	4 mg/ml	Sigma	
Oil Red O Stock (in 99% Isopropanol (Sigma))			
Chemical	Concentration	Company	
Oil Red O	3 mg/ml	Sigma	
Mowiol			
Chemical	Concentration	Company	
Mowiol [®] 4-88	2.5 g	Sigma	
Glycerol	6 g	Sigma	
Tris (200 mM pH 8.5)	12.5 ml	Sigma	
dH ₂ O	6 ml	-	

Table 2.5: Number of replicates of the dSKPs made.

Type of SKP	Number of times made
E12.5 dSKP	4
E13.5 dSKP	5
E14.5 dSKP	1
E15.5 dSKP	2
E14.5 Whisker Pad SKP	1

Table 2.6: Details of the primers used. Primers taken from papers (Fernandes et al. 2004, Chen et al. 2006, Kim et al. 2010).

Gene	Forward primer	Reverse primer	Anneal Temp (°C)	Product (BP)
Pax3	GGAGGCGGATCTA GAAAGGAAGGA	CCCCCGAATGAG ATGGTTGAA	71.4	374
Slug	CGTCGGCAGCTCCA CTCCACTCTC	TCTTCAGGGCACC CAGGCTCACAT	75.8	348
Twist	CTTCCGCCACCCA CTTCCTCTT	GTCCACGGCCTG TCTCGCTTTCT	75.05	334
Snail	CGGCGCCGTCGTCC TTCT	GGCCTGGCACTGG TATCTCTTAC	72.05	398
Sox9	CCGCCATCACCCG CTCGCAATAC	GCCCTCCTCGCT GATACTGGTG	76.9	544
P75	GTGCGGGGTGGGC TCAGGACT	CCACAAGGCCAC AACCACAGC	75.3	422
SHOX2	CCGCCGCCGCAAG ACCAC	TCCCAAACCCGC TCCTACAAA	75.65	355
Dermo-1	GCGGCGCTACAGC AAGAAATC	CCATGCGCCACAC GGAGAAGG	72.95	356
Nexin	CCACGCAAAGCCAA GACGAC	GAAACCGGCCTGC TCATCCT	69.95	289
Versican	TGGAAGGCACAGC AGTTTACC	TCATGGCCACAC GATTAC	67.8	427
Wnt-5a (for SKPs)	CCCCCTCGCATGA AGAAGC	CAGCCGCCACACA ACCAAGT	72.5	552
Nestin	GAGAAAGAGAATC AGGAGCC	CAAGAGACCTCAG AGATTCC	58.75	430
Sox2	GGCAGCTACAGCAT GATGCAGGAGC	CTGGTCATGGAGT TGTA CTGCAGG	72.05	130
Wnt 2	CGACACCCAGATGT GATGC	GGCCGATTCCCGA CTACTT	65.3	159
Wnt 2b	CTGCACGCTCTTGG GAAC	ATAGCTCCCCGCA GACTCC	65.2	177
Wnt-5a (for skin)	AGTCCTTTGAGATG GGTGGTA	CCTCTGGGTTAGG GAGTGTC	62.4	175
Wnt 5b	CCAGTGCAAGAGACC GGAGATG	GTTGTCCACGGTG CTGCAGTTC	70.35	201
Wnt 7a	GGCTTCTCTCGGT GGTAGC	TGAAACTGACACT CGTCCAGG	65.15	155
Wnt 7b	ACCGTCTTCGGCA AGAACT	CCTGGCGTTCTTTT TGATCT	65.05	261
Wnt 9a	GGTGGGCAAGCAC CTAAAC	GTACAAGCTCTGG TGTTCCGGG	65.65	157
Wnt 10b	GAAGGGTAGTGGT GAGCAAGA	GGTTACAGCCACC CCATTCC	65.3	158
Wnt 11	CAGCCTTCGTGTAT GCCCT	CCGTAGCTGAGGT TGTCCG	65.5	160

3. The Skin

3.1 Introduction

The development of the epidermis and hair follicles has been studied extensively (Fu and Hsu 2013, Lim and Nusse 2013, Liu, Zhang, and Duan 2013, Rishikaysh et al. 2014). However, dermal development (resulting in a subdivided dermis consisting of the papillary dermis, reticular dermis and DWAT) has only recently been explored and the mechanisms involved still need further elucidation (Driskell et al. 2013, Driskell et al. 2014, Driskell and Watt 2015). It has been hypothesised that the dermis contains a common precursor cell from which all these DC types derive and Wnt/ β -catenin signalling (a key pathway in development) has been associated with the lineage decisions of the dermis (Fu and Hsu 2013, Donati et al. 2014, Driskell and Watt 2015, Mastrogiannaki et al. 2016, Plikus et al. 2017). In particular, Wnt/ β -catenin signalling from the epidermis to the dermis is thought to be involved in specifying the papillary dermis fate (Driskell and Watt 2015). However, the timing and localisation of this pathway in skin, particularly in relation to the specific Wnt family members, is less well known. In addition, little is known about the common precursor population in the early dermis. A lot of research on SKPs (a multipotent stem cell population derived from the skin) has been done in adult skin but less research has been conducted on embryonic skin, especially at the early stages of skin development (Toma et al. 2001, Fernandes et al. 2004, Toma et al. 2005). Consequently, little is known about the characteristics and localisation of these early dermal precursor cells *in vivo*.

In this chapter, the changes in skin development between E12.5 and E15.5 were tracked using immunofluorescent staining for LEF-1, a transcription factor downstream of the Wnt signalling pathway, and Dlk1, a non-canonical Notch ligand associated with progenitor cells and the negative regulation of adipogenesis (Smas and Sul 1993, Vorotnikova et al. 2010, Falix et al. 2012, Driskell and Watt 2015, Traustadottir et al. 2016). E14.5 skin, one of the most characterised stages of mouse skin development, is the stage at which pelage hair follicle morphogenesis is first observed in the form of placodes and, from the literature, the earliest time-point at which SKPs could be isolated from the skin (Fernandes et al. 2004, Sennett and Rendl 2012, Fu and Hsu 2013). Therefore, E13.5 skin is the final stage of skin development before hair follicles begin to form and, according to lineage tracing, is thought to have a relatively primitive dermis (Driskell and Watt 2015). In addition, microdissection and enzymatic dermal-epidermal separation is cleaner and easier at E13.5 than at younger stages. This chapter will focus on characterising the expression of a selection of Wnt family members in E13.5 skin, as well as Nestin, to investigate the location of progenitor cells.

3.2 Methods and Approaches

Figure 3.1 summarises the methodical approaches taken for this chapter of the project (See Chapter 2 for detailed methods), involving immunofluorescent staining of whole skin and RT-PCR of the dermis and epidermis.

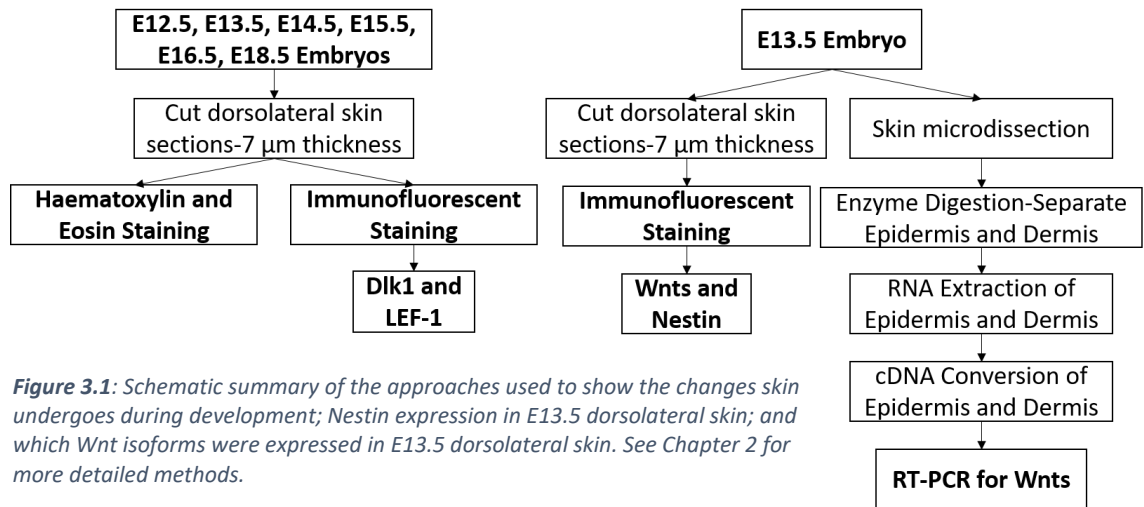


Figure 3.1: Schematic summary of the approaches used to show the changes skin undergoes during development; Nestin expression in E13.5 dorsolateral skin; and which Wnt isoforms were expressed in E13.5 dorsolateral skin. See Chapter 2 for more detailed methods.

3.3 Results

3.3.1 The Changes in Skin During Development

Basic histology of the skin at E12.5 showed a thin epidermal layer of 1-2 cells deep and a morphologically uniform dermis. By E13.5, the epidermis thickened to 2-3 cells deep but the dermis remained the same. At E14.5, the epidermis thickened, became more defined and displayed the first sign of hair follicle induction: the emergence of epidermal placodes (an accumulation of epidermal cells, indicated with an arrow head). The dermis remained the same but a denser layer of cells at the bottom of the dermis formed (indicated by the dashed lines). This layer is where the panniculus carnosus is shown to form in later development. At E15.5, the epidermal placode had expanded to become an early follicle bud and started to push down into the dermis where a dermal condensation (accumulation of DCs) had increased in size. Again, the dermal border was defined by the cells predicted to develop into the panniculus carnosus (indicated by the dashed lines; Figure 3.2.a-d).

At E12.5, LEF-1 was expressed in most of the dermis and in the basal layer of the epidermis (although less intensely). By E13.5, this expression was restricted more to the papillary dermis (indicated by double ended arrows) and the epidermis, where the brightest expression was in the basal layer. At E14.5, this papillary dermal and epidermal LEF-1 expression was maintained but was also expressed in the placode and reticular dermis. By E18.5, LEF-1 was only expressed in the DP and intermittently in the basal epidermis, most likely representing the last round of follicle initiation which takes place around this stage (Figure 3.2.e-h) (Andl et al. 2002).

Dlk1 was expressed in a few cells at E12.5, in a region at the bottom of the dermis where, later, the panniculus carnosus develops. The number of cells expressing Dlk1 in this region increased at E13.5 and E14.5. At E14.5, Dlk1 was also expressed in regions further below the skin, that were also suspected to be muscle due to the morphology. Finally, at E16.5, the panniculus carnosus had undoubtedly formed and thickened in this same region and continued to strongly express Dlk1. The epidermis, follicular downgrowths and the reticular dermis also expressed Dlk1 at E16.5 but with less intensity than the panniculus carnosus (Figure 3.2.i-l; regions of suspected muscle indicated with dashed lines).

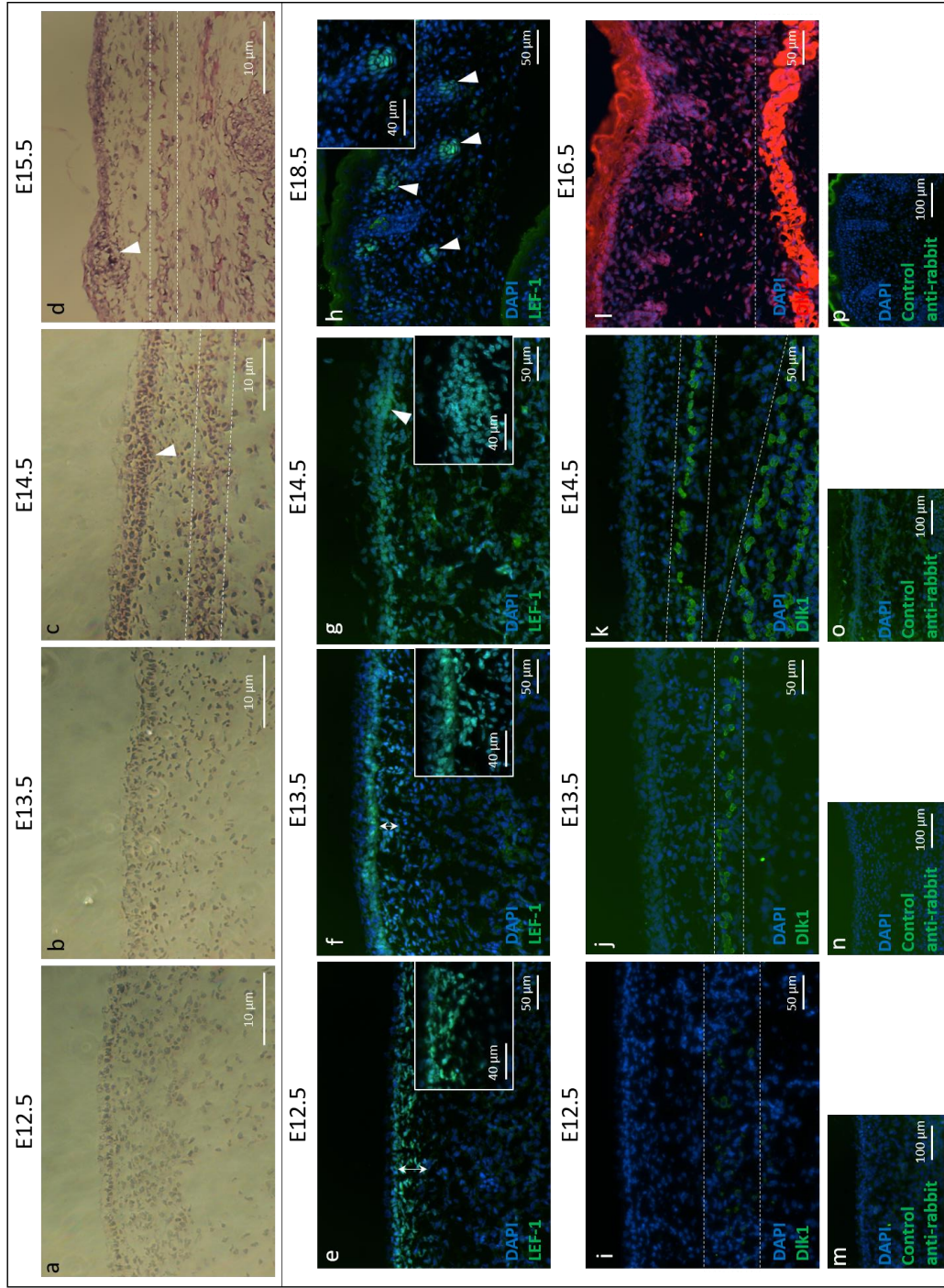


Figure 3.2: A summary of the changes that occur in skin between E12.5 and E18.5 using Haematoxylin and Eosin staining (a-d), LEF-1 (e-h) and Dlk1 immunofluorescent staining (i-l). E12.5 control (m), E13.5 control (n), E14.5 control (o) and E18.5 control (p) are also shown. Dashed lines indicate the suspected panniculus carnosus or other muscle; arrow heads indicate hair follicles and double ended arrows mark the region of the dermis expressing LEF-1. Image 1 courtesy of Adam Gilmore. No repeats.

Selected data from an Affymetrix microarray comparing the gene expression of the dorsal epidermis and dermis of C57BL/6J embryos ranging from E12.5 and E15.5, previously conducted by the Jahoda lab in collaboration with the Christiano lab (New York), is illustrated in Figure 3.3. This showed the expression of Wnt 2, Wnt 2b, Wnt 4, Wnt 5a, Wnt 5b, Wnt 7a, Wnt 7b, Wnt 9a, Wnt 10b, Wnt 11 and Nestin, all of which were expressed in the epidermis at E12.5, E13.5, E14.5 and E15.5 (Figure 3.3.a); and in the dermis at E13.5, E14.5 and E15.5 (Figure 3.3.b). Expression levels of most of the genes in both compartments, were remarkably constant, although Wnt 2 and Wnt 7a did decrease over time in the epidermis.

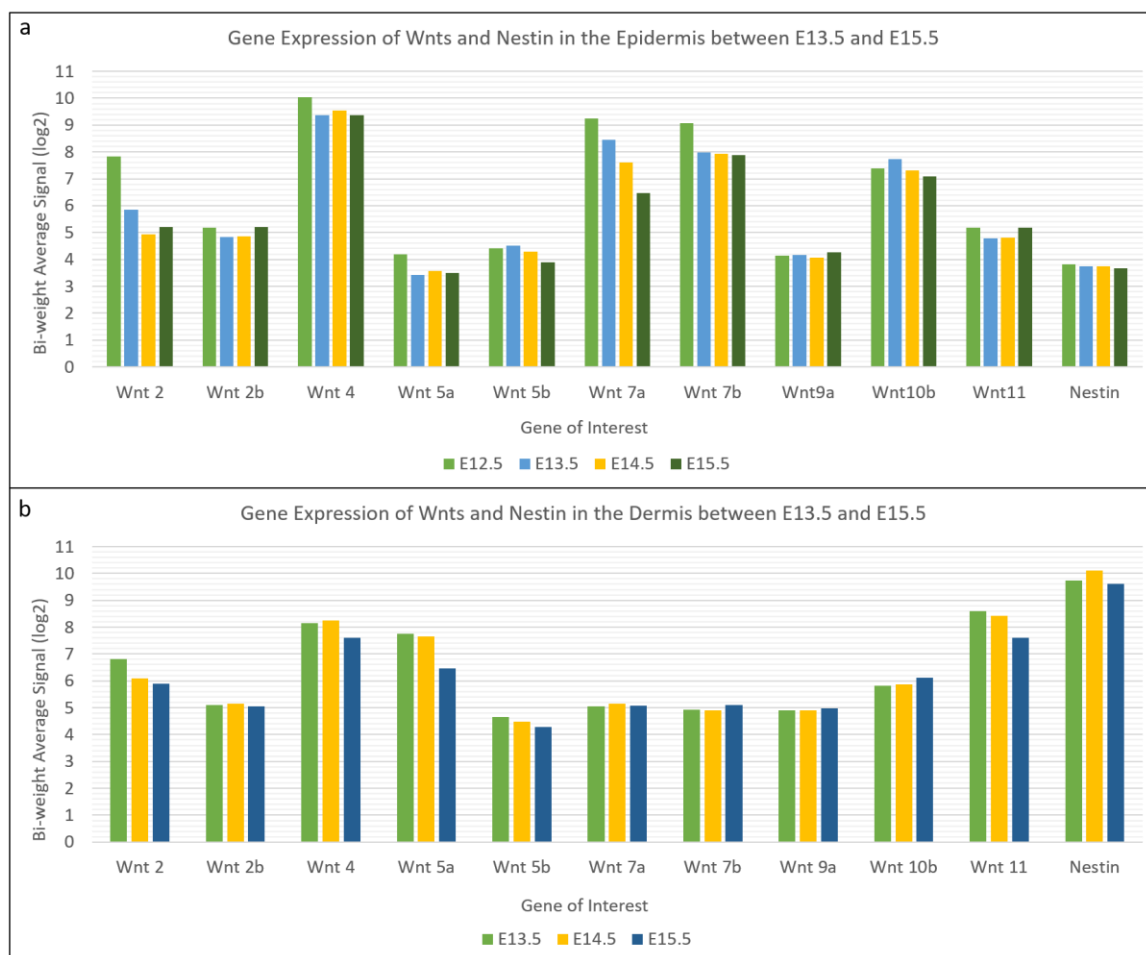


Figure 3.3: Gene expression profiles of a selection of Wnt isoforms and Nestin in the epidermis (a) and dermis (b) of different age points during embryogenesis. Data is from a microarray done previously by the Jahoda lab in collaboration with the Christiano lab.

3.3.2 E13.5 Skin

To further analyse the expression of Wnt isoforms in E13.5 skin, RT-PCR analysis was performed on E13.5 dermis and epidermis. Figure 3.4 showed that Wnt 5b, Wnt 11, Wnt 7a, Wnt 10b, Wnt 2b, Wnt 9a and Wnt 5a were present in both the dermis and epidermis in E13.5 skin. In contrast to the array data, Wnt 2 was expressed only weakly in the epidermis, and Wnt 7b was not expressed in either the epidermis or dermis.

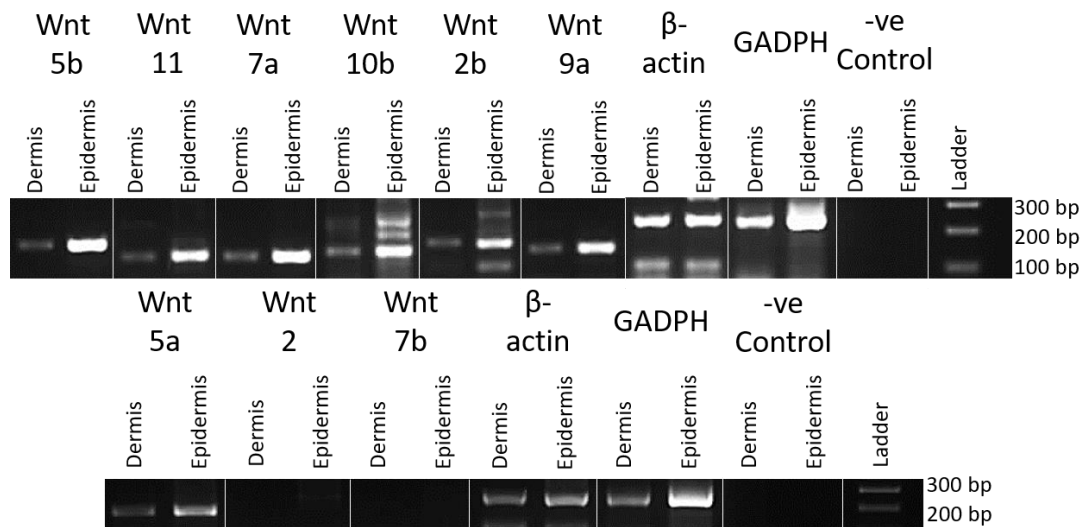


Figure 3.4: RT-PCR results for E13.5 dermis and epidermis (respective order of loading from left to right) for a variety of Wnt isoforms. 100 ng/ μ l cDNA of dermis; 50 ng/ μ l cDNA of epidermis. Wnt 5a, Wnt 2 and Wnt 7b repeated once; all others tested only once. β -actin and GADPH were used as positive controls and water as the negative (-ve) control.

E13.5 dorsolateral skin was sectioned and immunofluorescently stained for a number of Wnt family members that were of interest, and for which antibodies were available. Wnt 2 was shown to mark the epidermis but there was no specific staining of the dermis or developing panniculus carnosus (Figure 3.5.a). Similarly, Wnt 11 was shown to label the epidermis the most strongly, particularly basally (indicated by upper dashed lines). However, in the dermis, the region corresponding to the developing panniculus carnosus (indicated by lower dashed lines) was also labelled (Figure 3.5.d). Wnt 4 and Wnt 5a antibodies showed no specific staining of the skin (Figure 3.5.b and c). Nestin labelled cells throughout the dermis and developing panniculus carnosus (indicated by dashed lines) but no positive labelling was apparent in the epidermis (Figure 3.5.e).

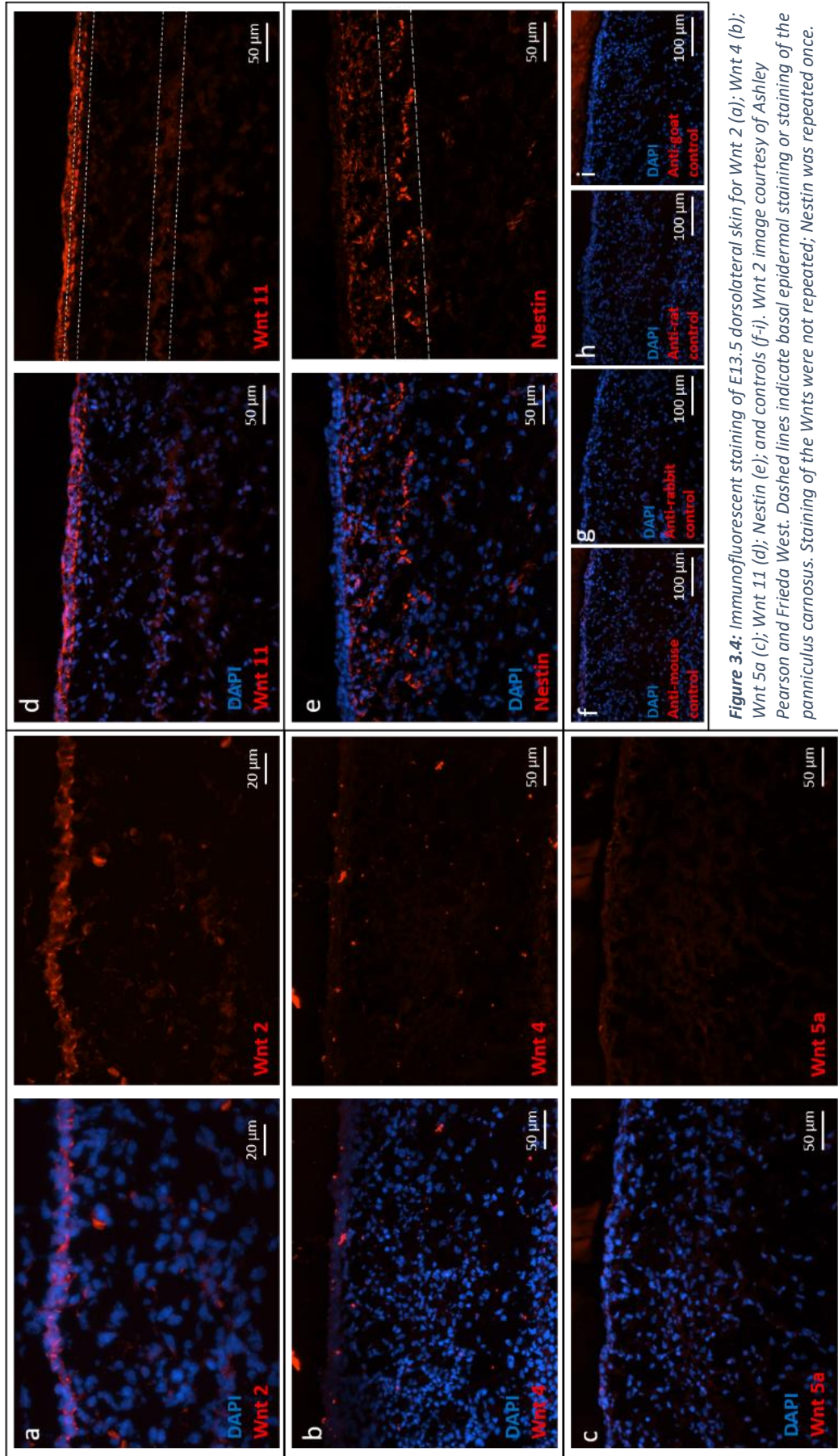


Figure 3.4: Immunofluorescent staining of E13.5 dorsolateral skin for Wnt 2 (a); Wnt 4 (b); Wnt 5a (c); Wnt 11 (d); Nestin (e); and controls (f-i). Wnt 2 image courtesy of Ashley Pearson and Frieda West. Dashed lines indicate basal epidermal staining or staining of the panniculus carnosus. Staining of the Wnts were not repeated; Nestin was repeated once.

3.4 Discussion

Skin dramatically changes its structure and composition during development in mice, starting with a thin epidermis and morphologically uniform dermis at E12.5. By E18.5, the epidermis has become multi-layered, incorporates multiple types of hair follicles and possesses a full barrier, while the dermis has also stratified (Zhang et al. 2008, Driskell et al. 2013, Driskell et al. 2014, Driskell and Watt 2015). The progressive cellular and molecular changes during epidermal and hair follicle initiation and maturation have been intensively studied (Fu and Hsu 2013, Liu, Zhang, and Duan 2013, Rishikaysh et al. 2014). However, the intricacies of how the dermis compartmentalises have only been addressed more recently (Driskell et al. 2013, Driskell et al. 2014, Driskell and Watt 2015). In particular, lineage tracing experiments have shown, in more detail, how the composition of the dermis changes over time, resulting in the papillary dermis, reticular dermis and DWAT (Driskell and Watt 2015). Wnt/ β -catenin signalling has long been known to play a role in skin development, particularly hair follicle formation. However, more recently, it has been associated with determining the lineage commitment of DCs (Fu and Hsu 2013, Donati et al. 2014, Driskell and Watt 2015, Mastrogiannaki et al. 2016, Plikus et al. 2017). Thus, Wnt signalling appears to have roles in multiple developmental pathways. Consequently, one of the challenges associated with this pathway is to distinguish between these roles. In this context, the upper dermis would be an obvious recipient of epidermal Wnt signalling from the adjacent epidermis, and also likely reciprocate in the other direction. Less clear is whether there is intradermal signalling involved in the delineation between the papillary and reticular dermis and/or the reticular dermis and DWAT. This study set out to explore these questions via investigation of the spatial and temporal expression of individual Wnt family members.

3.4.1 Mouse Skin Development – Wnt/ β -catenin Signalling and Progenitor Labelling

In this chapter, Wnt signalling was examined in developing mouse skin at the RNA and protein levels. Tracking Wnt/ β -catenin signalling via immunofluorescent labelling of LEF-1 (a transcription factor downstream of Wnt) revealed the progressive restriction of labelling from a general basal epidermal expression to intermittent epidermal basal expression and in the dermis, from cells at almost all depths to just the upper dermis and then, predominantly to the dermal condensations that subsequently become the follicle DP (Driskell and Watt 2015). At the level of transcription, RT-PCR showed that multiple Wnts were expressed in both the epidermis and dermis at E13.5, but immunofluorescent staining revealed strong epidermal labelling of some Wnts and little evidence of any dermal Wnt expression, except the region corresponding to the developing panniculus carnosus. *Dlk1* (a non-canonical Notch ligand associated with progenitor cells and the negative regulation of adipogenesis) was found, perhaps unexpectedly, to be limited to this lower dermal region of the developing panniculus carnosus, while Nestin (a neuroepithelial stem cell

marker) was more widely expressed dermally in early skin development (Smas and Sul 1993, Sellheyer and Krahl 2010, Vorotnikova et al. 2010, Falix et al. 2012, Traustadottir et al. 2016).

3.4.2 Wnt/ β -catenin Signalling and Lineage Commitment

The restriction of Wnt activity (shown by LEF-1 expression) to the papillary dermis at E13.5 is reflected in adult mouse skin as well. Mastrogiannaki et al. (2016) showed distinct gene expression profiles of papillary and reticular dermal lineages in postnatal day 2 dermis, in relation to regulators of the Wnt signalling pathway. For example, high levels of nuclear β -catenin were shown in the cells of the papillary dermis and low levels were observed in the reticular dermis (Mastrogiannaki et al. 2016). So how does the papillary dermis acquire this Wnt activity when Wnts are not shown to be expressed at the protein level?

Crosstalk between the epidermis and dermis is widely acknowledged so it would not be surprising if the expression of epidermal Wnts has a role in specifying the distinct gene expression profile of the papillary dermis, which resides directly below it (Chen et al. 2012, Fu and Hsu 2013). Wnt 2 and Wnt 11 showed bright epidermal immunofluorescent staining at E13.5 (the first stage LEF-1 is no longer expressed evenly throughout the dermis) and the Wnt 11 expression was particularly notable in the basal layer of the epidermis (Figure 3.4.d; indicated by the upper dashed lines). The fact that basal epidermal expression was so bright and that LEF-1 was restricted to the papillary dermis at E13.5 suggests support of the previous hypothesis (of the Jahoda lab) that the presence of epidermal Wnts directly adjacent to the papillary dermis help to specify its distinct Wnt-related expression profile. Chen et al. (2012) have shown that epidermal Wnts are needed for Wnt activity in dermal fibroblast progenitors. They showed this by knocking out Wntless (a gene involved in Wnt secretion) exclusively in the epidermis using a K14Cre/+;R26R/+;Wls^{fl/fl} strain. These mutant embryos had no hair follicles, a lack of Wnt11 at E14.5 in the dermis, a lack of LEF-1 in the dermis at E16.5, and the number of dermal fibroblasts between E16.5 and E18.5 was less than controls. In addition, Chen et al. (2012) knocked out Wntless exclusively in the dermis using a En1Cre/+;R26R/+;Wls^{fl/fl} strain. The mutant embryos showed very little difference with the controls regarding the histology of the dermis at E14.5 and E17.5, the expression of Twist2 and LEF-1 in the dermis, and epidermal differentiation. These findings therefore show the need for epidermal Wnts rather than dermal Wnts for dermal responsiveness to Wnt signalling and thus, hair follicle initiation (Chen et al. 2012). To further highlight the role of epidermal Wnts on the dermis, Driskell et al. (2014) have shown that sustained epidermal Wnt/ β -catenin signalling in adults induced hair follicle growth, the formation of new follicles, fibroblast proliferation and a remodelling of the dermal ECM (Driskell et al. 2014). One discrepancy with the Wnt 2 and Wnt 11 immunofluorescent staining was that, according to the microarray and RT-PCR, Wnt 11 should also be dermally expressed. This inconsistency may be due to the limitations of immunofluorescent staining with regards to the levels of expression it can detect compared to RT-

PCR and microarrays, or it may reflect the location of Wnt protein activity differing to its transcriptional activity. Regardless of this discrepancy, we know at least the downstream effects of Wnt/ β -catenin signalling are present in the papillary dermis (LEF-1 and β -catenin expression) and, thus, it is highly likely there is Wnt expression in this region. However, β -catenin can be activated by alternative ligands to Wnt which makes interpretation of these results harder (McNeill and Woodgett 2010).

Wnt/ β -catenin signalling has also been shown to have a role in specifying lineages not present in the skin and, thus, the regulation of this pathway is essential for correct development to take place. For example, when β -catenin activity was conditionally knocked out in the face and ventral trunk dermis (using En1Cre; R26R; β -catenin^{flox/null} mouse strain), the dermis was replaced with cells expressing Sox9 (a marker of cartilage cell fate), displaying cartilage morphology and were positive for Alcian Blue staining (Tran et al. 2010).

Mastrogiannaki et al. (2016) went on to investigate the influence of ectopic Wnt/ β -catenin signalling in the dermis and how it affected cell lineage. Conditional stabilisation of β -catenin throughout the dermis led to a reduction in DWAT size and disruption of the hair follicle cycle. In contrast, when β -catenin was expressed in only the lower DCs (Dlk1+ population at E16.5, Figure 3.2.I), DWAT was replaced by fibrotic dermis but the hair follicle cycle remained unaltered (Mastrogiannaki et al. 2016). This supports the idea that the presence of Wnt/ β -catenin signalling in the dermis promotes fibroblast formation and the absence of Wnt/ β -catenin signalling, adipocyte formation. In addition, Chen et al. (2012) showed sustained β -catenin expression in the dermis led to a thicker layer of dermal fibroblasts, as well as faster differentiation of hair follicles, larger placodes and larger dermal condensates (Chen et al. 2012). This, therefore, indicates that Wnt/ β -catenin signalling is involved in this lineage divergence between fibroblasts and adipocytes, as well as hair follicle initiation and cycle (Chen et al. 2012, Mastrogiannaki et al. 2016). These findings are also supported by Wei et al. (2011) who showed the replacement of the DWAT layer by a fibrotic dermis when a FABP4-Wnt10b transgenic mouse was used (Wei et al. 2011). This fits with the restriction of LEF-1 and β -catenin expression to the papillary dermis (the fibroblastic region of the dermis) shown in both this project and that of Mastrogiannaki et al. (2016).

In support of this, Plikus et al. (2017) showed that overexpression of Wnt/ β -catenin signalling in the epidermis (using K14-Wnt 7a mice) led to an increased number of follicles and no adipocyte regeneration (in contrast to the control) in wounded skin. Similarly, when β -catenin was constitutively expressed in the epidermis (using KRT14-Cre;ROSA26R mice), placodes had developed by E12.5 and were present in the footpad at E12 (an ordinarily glabrous (hairless) region). However, the placodes produced were unable to advance into follicles, indicating the

need for a removal of β -catenin expression at certain points in the process of follicular induction (Zhang et al. 2008). In addition, when epidermal β -catenin was deleted in adult mouse skin or *Dkk1* (a Wnt signalling inhibitor) was ectopically expressed in the epidermis, hair follicles were forced into catagen early (Choi et al. 2013). This highlights the divergent role Wnt signalling has in promoting hair follicle formation and inhibiting adipogenesis (Plikus et al. 2017).

Donati et al. (2014) contrast Plikus et al. (2017) when they showed that activation of Wnt/ β -catenin signalling in keratinocytes led to adipocyte differentiation. This was confirmed when ectopic expression of Wnt/ β -catenin signalling resulted in an expanded adipocyte layer (Donati et al. 2014). One might see these two papers as contradictory: one shows that Wnt signalling inhibits adipocyte differentiation, the other that Wnt signalling promotes adipocyte differentiation. However, it highlights the importance of the location, timing and type of signal (Jahoda and Gilmore 2016). This is what this chapter has aimed to uncover with regards to E13.5 skin.

The literature discussed in this section has provided evidence in support of Wnt signalling playing a role in DC fate specification. Therefore, the lack of dermal Wnt expression (at the protein level) found in this project is surprising. Obviously, more Wnt ligands need to be explored to draw concrete conclusions regarding Wnt signalling in the skin at E13.5 but the data here raises the question of the 'default' cell fate of DCs. The lack of dermal Wnt protein expression means only the papillary dermis, due to its adjacent location to the epidermis, will potentially be influenced by the epidermal Wnts shown to be expressed in this project (Wnt 2 and Wnt 11). In contrast, the reticular dermis (according to the lack of Wnt protein expression in the dermis) remains unaffected by the epidermal Wnts. Therefore, the fact that later in development the reticular dermis gives rise to adipocytes may imply adipocytes are the 'default' cell fate of DCs (Driskell et al. 2013). This concept will be discussed further in Chapter 4. On the other hand, the lack of dermal Wnt protein expression may indicate E13.5 is too early or too late a time point to detect the Wnt ligands tested in the dermis. Considering lineage divergence of the dermis has been shown to take place at E16.5, it would be entirely plausible that the priming of these events take place after E13.5 (Driskell et al. 2013). Alternatively, is the Wnt protein expression detected in the epidermis secreted from the papillary dermis (discussed later in this chapter)? This is particularly pertinent for Wnt 11 due to its basal epidermal expression. Perhaps this expression of Wnt protein in the papillary but not the reticular dermis is involved in the lineage divergence of the dermis. Further research into the timing and spatial localisation of the different Wnt family members will help provide answers to these questions.

3.4.3 Wnt/ β -catenin Signalling and Hair Follicle Induction

As mentioned above, Wnt/ β -catenin signalling and hair follicles are closely associated. At E13.5, there are no signs of hair follicles (placodes and dermal condensates are present at E14.5)

(Sennett and Rendl 2012, Fu and Hsu 2013). Therefore, the epidermal and dermal Wnts observed at E13.5 are likely to be involved in priming hair follicle formation.

Research into the role of Wnt/ β -catenin signalling in hair follicle induction has shown that epidermal Wnt signalling (thought to be Wnt 3, 4 and 6) communicates to the papillary dermis to trigger a dermal signal. However, this is unable to act in the epidermis due to an inhibitor. The dermal signal that subsequently occurs represses the activity of this inhibitor, activating epidermal Wnt signalling in the epidermis (thought to be Wnt 2, 7b, 10a and 10b). This allows placode formation in the epidermis (thought to be marked by Wnt 10b), which leads to the placode signalling to the dermis below, resulting in the formation of the dermal condensate and papilla. Finally, dermal Wnts signal to the placode to promote further induction (Zhang et al. 2008, Chen et al. 2012, Fu and Hsu 2013, Rishikaysh et al. 2014).

The RT-PCR and immunofluorescent staining have both indicated that the dermis and epidermis expressed a variety of Wnt family members. However, the key difference (shown more clearly in the RT-PCR data) was that, despite the lower concentration of epidermal cDNA (50 ng/ μ l), the epidermis had a higher transcriptional expression of these Wnts than the dermis (which had a concentration of 100 ng/ μ l). This, therefore, indicates that the skin was at the stage where the epidermis promotes Wnt signalling in the dermis and begins to express dermal Wnts. This fits with the knowledge that placodes form at E14.5, which is the next step in the process that Fu and Hsu (2013) have elucidated. However, the lack of Wnt 4 immunofluorescent staining throughout the skin does not match up with this mechanism or the microarray data. Wnt 5a (a Wnt known to be involved in hair follicle formation) also showed no immunofluorescent staining, despite being present in the epidermis and dermis in both the microarray data and RT-PCR (Reddy et al. 2001). Both these discrepancies may be due to a need for better antibodies.

Another caveat is that Wnt 2 was only epidermally expressed and Wnt 7b was not expressed at all in the RT-PCR, which contradicts the microarray data where they were expressed in both the epidermis and dermis. Their lack of expression may simply be due to non-optimal primers or experimental error. In partial support of the microarray data, the immunofluorescent staining of Wnt 2 shows clear expression in the epidermis but not in the dermis. This begs the question of whether Wnts are secreted. Just because the microarray data shows Wnt 2 was expressed on a transcriptional level in the dermis does not necessarily mean the protein remains there. A possible explanation is that Wnt 2 is secreted to the epidermis where it promotes placode formation in addition to epidermal Wnt 2.

All Wnt family members are secreted (indicated by a signal sequence for secretion) and Wnts have been shown to have short- and long-range signalling, despite being lipid-modified glycoproteins (Mikels and Nusse 2006, Port and Basler 2010, Solis, Luchtenborg, and Katanaev

2013). Their hydrophobic nature has led to questions of how Wnts are able to diffuse. One explanation is that they form complexes with secreted Frizzled-related proteins, which have been shown to increase the diffusion range of Wnt 8 and Wnt 11 in a concentration-dependent manner in *Xenopus* (Mii and Taira 2009). This role of Frizzled-related proteins has been shown to apply to *Drosophila* and mice, indicating conservation (Solis, Lüchtenborg, and Katanaev 2013). Microarray data (from the Jahoda and Christiano labs, data not shown) has shown that between E13.5 and E15.5 secreted Frizzled-related protein 1 was downregulated but secreted Frizzled-related protein 5 was upregulated. This adds an additional level of complexity, with regards to Wnt regulation, as Frizzled-related proteins have their own family members with differential expression. In addition GAG-modified proteins and lipoproteins have also been shown to aid the diffusion of Wnts (Mikels and Nusse 2006). However, whether Frizzled-related proteins, GAG-modified proteins and/or lipoproteins form complexes with all Wnt family members or just some; or whether the diffusion range varies for different Wnt family members needs further investigation. Secretion of Wnts has been known to play a key role in the hair follicle cycle. Myung et al. (2013) showed that the hair cycle was arrested at telogen/early anagen and that there was a lack of β -catenin activity in the DP and secondary hair germ when Wntless was knocked out of the basal layer of the epidermis and hair follicle cells (Myung et al. 2013). Therefore, research on the diffusion range of individual Wnt family members and the mechanisms that influence this range would aid the understanding and manipulation of this pathway and, by extension, its role in dermal development.

3.4.4 Nestin: A Stem Cell Marker that Persists in Adult Hair Follicles

Nestin is a neuroepithelial stem cell marker and was shown to be expressed throughout the dermis and panniculus carnosus in E13.5 skin, here and in previous reports (Sellheyer and Krahl 2010). However, the microarray data shows it should also be expressed in the epidermis but perhaps this expression is at much lower levels than immunofluorescent staining can detect. The literature also shows that Nestin is expressed in the full dermis of the mouse at E12.5 but this expression is subsequently restricted, resulting in expression only in the DSs in older embryos and then hair follicles in the adult (Falodah and Al-Karim 2016). A similar expression pattern is found in human scalp embryonic skin, where Nestin is restricted to the DS when the hair follicle is at the bulbous peg stage. In contrast to dorsolateral skin, Nestin is still expressed in the papillary dermis at this stage. However, this also eventually depletes so that, in the adult, Nestin is only expressed in the DS, DP and blood vessels. According to the hair follicle cycle, Nestin expression fluctuates in the DS and DP, with the highest level of Nestin expression being at early anagen and a reduced expression at catagen and telogen. In addition, Nestin expression increases in the DS of follicles close to a wound and the interfollicular fibroblasts within the wound overexpress Nestin. This indicates that Nestin-positive cells mark a stem cell niche in the follicles, which are a source of

cells needed to increase the hair follicle size during anagen and to replenish the lost cells in wound healing (Sellheyer and Krahl 2010). In addition, adult mouse whisker follicles express Nestin in the bulge area and staining has shown Nestin-positive cells migrate from the bulge area to the epidermis (in the context of wound healing) or down the hair shaft to the DP. This confirms Nestin-positive cells play a role in wound healing and also shows there may be more than one source of Nestin-positive cells in the hair follicle (Uchugonova et al. 2011).

Coming back to E13.5 skin, Nestin expression in the dermis and panniculus carnosus area indicates a primitive, undifferentiated nature of the E13.5 DCs, including those that will give rise to the panniculus carnosus. Further to this project, it would be interesting to confirm whether Nestin is marking where the mature panniculus carnosus will form, using double staining with panniculus carnosus muscle precursor markers, such as Myf5, Pax3 or Pax7 (Naldaiz-Gastesi et al. 2016).

3.5 Conclusions

The skin was shown to change dramatically during development: from a 1-2 cell thick epidermis, morphologically uniform and primitive dermis at E12.5 to a fully stratified epidermis with hair follicles and a subdivided dermis at E18.5.

This chapter aimed to answer two questions: firstly, regarding the spatial and temporal activity of particular Wnt family members in early skin development and secondly, regarding the location of progenitor cells in E13.5 skin-particularly to see if the dermis is beginning to subdivide at this stage.

Multiple Wnts were shown to be expressed at the transcriptional level in both the epidermis and dermis throughout early skin development but this was not reflected at the protein level, where only Wnt 2 was expressed in the epidermis and Wnt 11 was expressed in both the epidermis and the region of the developing panniculus carnosus. Wnt signalling has been discussed to have a role in lineage commitment in the dermis and hair follicle induction and, due to the arrival of placodes at E14.5, the Wnts expressed at E13.5 have been postulated to be involved in priming these hair follicle induction events. However, the divergent roles of Wnt/ β -catenin signalling still leaves the question of which family members, if not all, are involved in hair follicle induction and which in lineage commitment.

Nestin and Dlk1, both associated with marking precursors, showed differing immunofluorescent expression in E13.5 skin (Sellheyer and Krahl 2010, Falix et al. 2012). Nestin was expressed throughout the dermis and in the region of the developing panniculus carnosus, whereas Dlk1 was only expressed in the latter. Nestin expression is particularly associated with SKPs, even in adults (Toma et al. 2001). The high proportion of Nestin positive cells in the E13.5 dermis leads to

further questions concerning the stem cell properties of the dermal population at E13.5 that are addressed in Chapters 4 and 5.

4. Investigation of Embryonic Dermal Precursor Cell Properties via 2D Cell Culture

4.1 Introduction

Lineage tracing experiments in the developing mouse dermis have shown that, at E12.5, there is a homogeneous population of common 'fibroblast' progenitor cells. By E16.5, this population was shown to diverge into papillary and reticular fibroblast progenitors, indicated by the restriction of *Dlk1* expression (an early marker of adipogenesis) to the reticular dermis only, which further differentiate to the various cell types found in the adult dermis (Driskell et al. 2013, Driskell and Watt 2015). Therefore, this project has hypothesised that this common precursor population is responsible for producing all cell types of the dermis. However, in contrast to the Watt group, Rinkevich et al. (2015) have shown that *Engrailed-1* positive cells (CD26+) migrate from the papillary dermis to the reticular dermis at E16.5, implying that cell populations are not static and the mechanism of dermal development is still up for discussion (Rinkevich et al. 2015).

E13.5 dermis is the stage this project has chosen to focus on due to its cleaner microdissection from the underlying muscle and overlying epidermis, as well as being the stage prior to hair follicle development, which is visible morphologically at E14.5 (Fu and Hsu 2013). In addition, E13.5 is thought to still be a relatively primitive stage of the dermis (Driskell and Watt 2015).

There are two essential properties of stem cells: the ability to self-renew and to differentiate. Maintenance of a stem cell population usually involves asymmetric division, which results in a stem cell (remains undifferentiated) and a daughter cell (goes on to differentiate). The environment of stem cells, termed a niche, is also key to their maintenance. Often differentiation can result when a stem cell leaves its niche (He, Nakada, and Morrison 2009, Chen, Ye, and Ying 2015).

In adult skin, the dermis is known to contain cells that are MSCs or are MSC-like, indicated by their similar expression profile and functional properties to bone marrow MSCs, for example (Riekstina et al. 2009, Vaculik et al. 2012). The literature shows that embryonic and adult DCs can differentiate to a wide range of mesodermal lineages, such as adipocytes, chondrocytes and muscle, with some suggesting that cells can become non-mesodermal derivatives, namely neurons (Goldring, Jones, and Watt 2000, Goldring, Jones, Sewry, et al. 2002, Goldring, Jones, Thiagarajah, et al. 2002, Fernandes et al. 2004, Chen et al. 2007, Ambasadhan et al. 2011). Considering muscle and adipocytes are mesenchymal in origin and these layers appear to derive from the same precursors as the dermal fibroblasts, a reasonable hypothesis might be that the embryonic dermis, when put into culture, should contain at least some MSC-like cells, or even a full population of MSC-like cells, if taken at an early enough stage (Driskell et al. 2014, Driskell and Watt 2015)

One way in which to test MSC-like functionality is to push this E13.5 DC population to differentiate to various lineages. In this chapter, E13.5 DCs were cultured in 2D in adipogenic and osteogenic media thus, allowing the multipotency of this population to be tested, particularly, with regards to its ability to turn into bone which is not present in the skin.

Previous data from the Jahoda lab has highlighted the important difference between culturing embryonic DCs in 2D (where a fibroblastic population resides) vs 3D culture (where a spheroid, consisting of mostly adipocytes, forms) shown by Oil Red O staining and immunofluorescent labelling of markers associated with various stages of adipogenesis such as, C/EBP α , Dlk1 and FABP4 (Stephens 2012, Driskell et al. 2013, Moseti, Regassa, and Kim 2016). This led to the idea that embryonic DCs are primed to produce adipocytes, almost as their 'default' cell fate, and reiterated the importance of environment (Baker and Chen 2012). Therefore, when approaching the differentiation capabilities of the E13.5 DC population, one would predict they would have little problem in being pushed to differentiate to adipocytes given that this is what a high proportion of them become *in vivo* (Driskell et al. 2014). This echoes the question of whether the early dermal population is more restricted to cell types of the skin or is more like an MSC. It also raises the question of whether 3D culture is more representative of *in vivo* than 2D culture.

This chapter, therefore, aims to investigate the plasticity of this population and to see if a large proportion of the population, or just a small subpopulation, display stem-like properties as well as, determining if there are stem cell niches in the dermis at this early stage of skin development.

4.2 Materials and Methods

Figure 4.1 summarises the methodical approaches taken for this chapter of work (See Chapter 2 for detailed methods). This chapter focused on investigating the plasticity of E13.5 DCs *in vitro* by pushing the cells to differentiate in 2D to either adipocytes or osteoblasts.

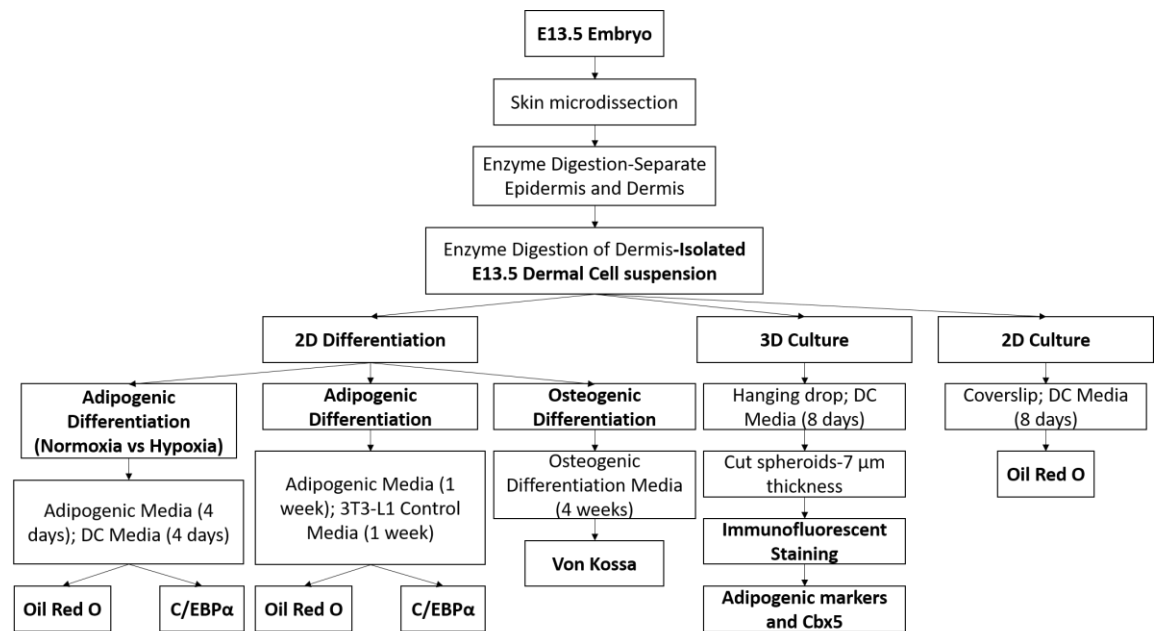


Figure 4.1: Schematic summary of the approaches used to investigate whether E13.5 DCs are capable of adipogenic and osteogenic differentiation. Oil Red O and immunofluorescent labelling of C/EBP α were the techniques used to analyse adipogenic differentiation and Von Kossa staining for osteogenic differentiation. Immunofluorescent staining was also used to characterise a 3D culture model and Oil Red O staining for 2D culture of E13.5 DCs. See Chapter 2 for more detailed methods.

4.3 Results

4.3.1 3D Culture

Figure 4.2.a shows the formation of E13.5 DC spheroids which are 3D spheres that formed when E13.5 DCs were isolated from the epidermis by enzymatic digestion, dissociated and cultured for 8 days using the hanging drop method. After 3 days, a sphere formed and this compacted over the next 5 days. Figure 4.2.b-e show the immunofluorescent staining of sectioned spheroids conducted to characterise the extent of adipogenic differentiation. Cbx5 (a protein involved in heterochromatin dynamics) and C/EBP α (a late marker of adipogenesis) were expressed in the nuclei of almost all cells (indicated by arrow heads) (Stephens 2012, Hinde, Cardarelli, and Gratton 2015). Dlk1 (an early marker of adipogenesis) was expressed in the nuclei of some cells (indicated by arrow head), otherwise was expressed in the cytoplasm (Driskell et al. 2013). FABP4 (a mature adipocyte marker) was expressed in the cytoplasm but the sections were more damaged than we would have liked (Moseti, Regassa, and Kim 2016).

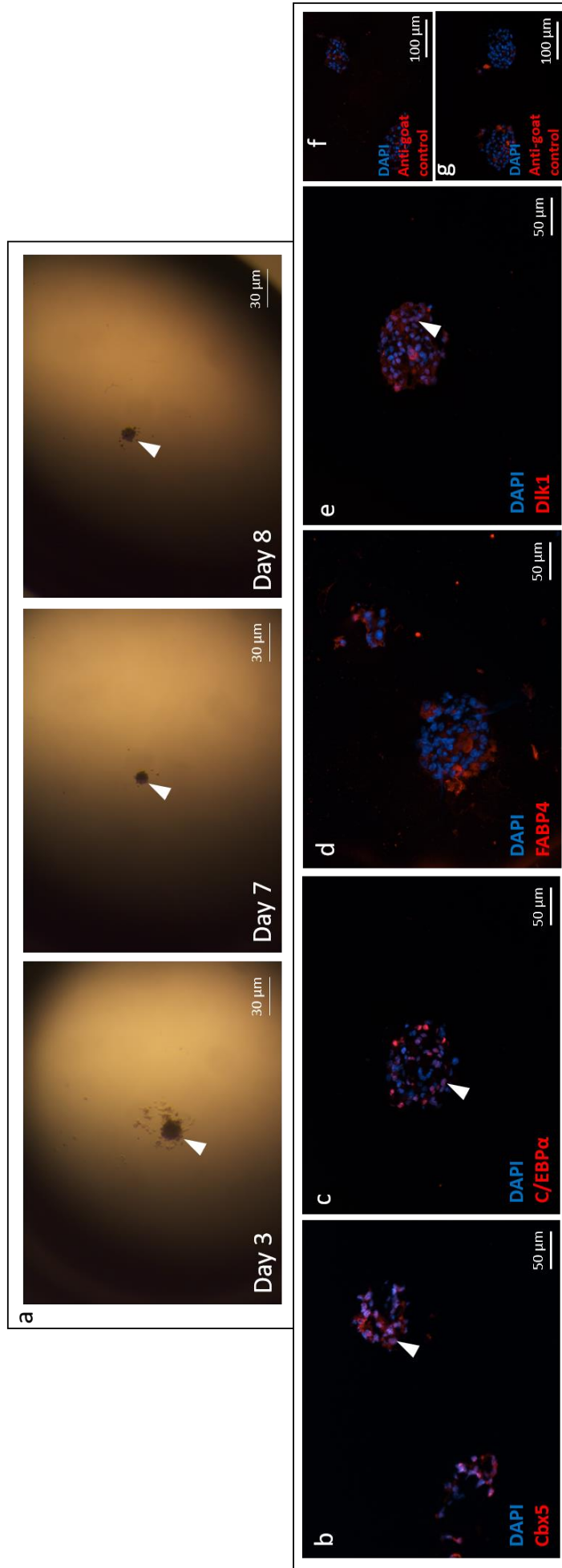


Figure 4.2: E13.5 DC spheroids made by culturing E13.5 DCs in 3D using the hanging drop method. Shown is their formation during cell culture for 8 days (a); and immunofluorescent staining for Cbx5 (b); C/EBPα (c); FABP4 (d); Dlk1 (e); and controls (f and g). Arrow heads indicate spheroids or nuclear staining. Repeated once.

4.3.2 2D Culture

Figure 4.3 shows that no Oil Red O staining was observed when E13.5 DCs were cultured in DC media for 8 days in 2D.

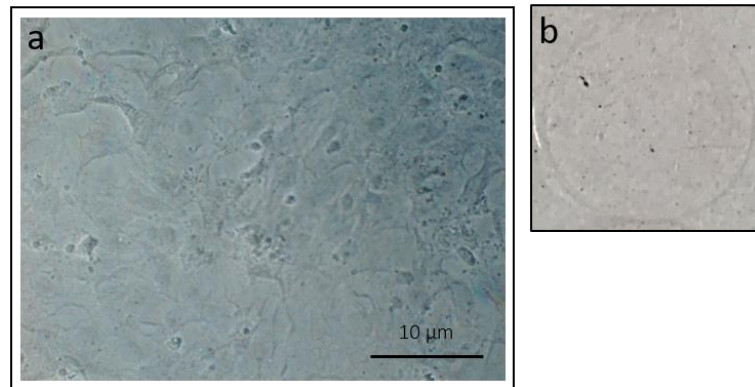


Figure 4.3: 2D culture of E13.5 DCs in DC media. The presence of adipocytes was characterised by Oil Red O Staining (a; and macroscopic image: b). Done in collaboration with Adam Gilmore. Repeated once but unsuccessfully due to fungal infection. However, consistent with multiple (>6) repeats by Adam Gilmore.

4.3.3 Adipogenic Differentiation

Oil Red O and C/EBP α immunofluorescent staining were used to characterise the adipogenic differentiation of the E13.5 DCs after 14 days in culture. Less than 10-15% of the experimental E13.5 DCs (in adipogenic media) differentiated to adipocytes, indicated by red staining of lipid droplets (indicated by arrow heads; Figure 4.4.a and e), and no significant staining was shown in the E13.5 DC control (in DC control media) (Figure 4.4.b and f). 3T3-L1 cells were used as a positive control however, less than 30% of the 3T3-L1 cells differentiated when cultured in adipogenic media (arrow heads indicate adipocytes; Figure 4.4.c and g). Figure 4.4.q shows the 3T3-L1 cells in adipogenic media on Day 14 (prior to fixation) and more mature adipocytes were visible compared to the Oil Red O and C/EBP α immunofluorescent staining for the same conditions (indicated by the arrow head). The control 3T3-L1 cells showed no significant differentiation (Figure 4.4.d and h). In addition, no nuclear C/EBP α staining was observed for any of the conditions but there was some cytoplasmic staining under all conditions (Figure 4.4.i-p).

Oil Red O and C/EBP α immunofluorescent staining were also used to characterise the adipogenic differentiation of E13.5 DCs but this time, in normoxic (20% oxygen) or hypoxic (5% oxygen) conditions and when cultured for 8 days. Oil Red O staining showed that no specific staining was observed for the hypoxia E13.5 DCs in adipogenic media (Figure 4.5.a and y) or control (Figure 4.5.b and z). Less than 1% of the normoxia E13.5 DCs in adipogenic media differentiated to adipocytes (Figure 4.4.c and c1) and no significant staining was indicated in the E13.5 DC normoxia control (Figure 4.4.d and d1). 3T3-L1 cells were, again, used as a positive control. However, less than 1% had large distinct vacuoles stained but small, grainy vacuoles were stained throughout (when in adipogenic media) in either hypoxia or normoxia (indicated by arrow heads; Figure 4.5.e and a1; and Figure 4.5.g and e1, respectively). The control 3T3-L1 cells showed

staining of small, grainy vacuoles throughout for both hypoxia and normoxia, but hypoxia cultures also had less than 1% staining of larger vacuoles (indicated by arrow heads; Figure 4.5.f and b1; and Figure 4.5.h and f1, respectively). In addition, no nuclear C/EBP α staining was observed in any of the conditions (Figure 4.5.i-x).

Supplementary Figure S6 shows cell culture images of the progression of adipogenic differentiation in E13.5 DCs when in adipogenic media in normoxic conditions.

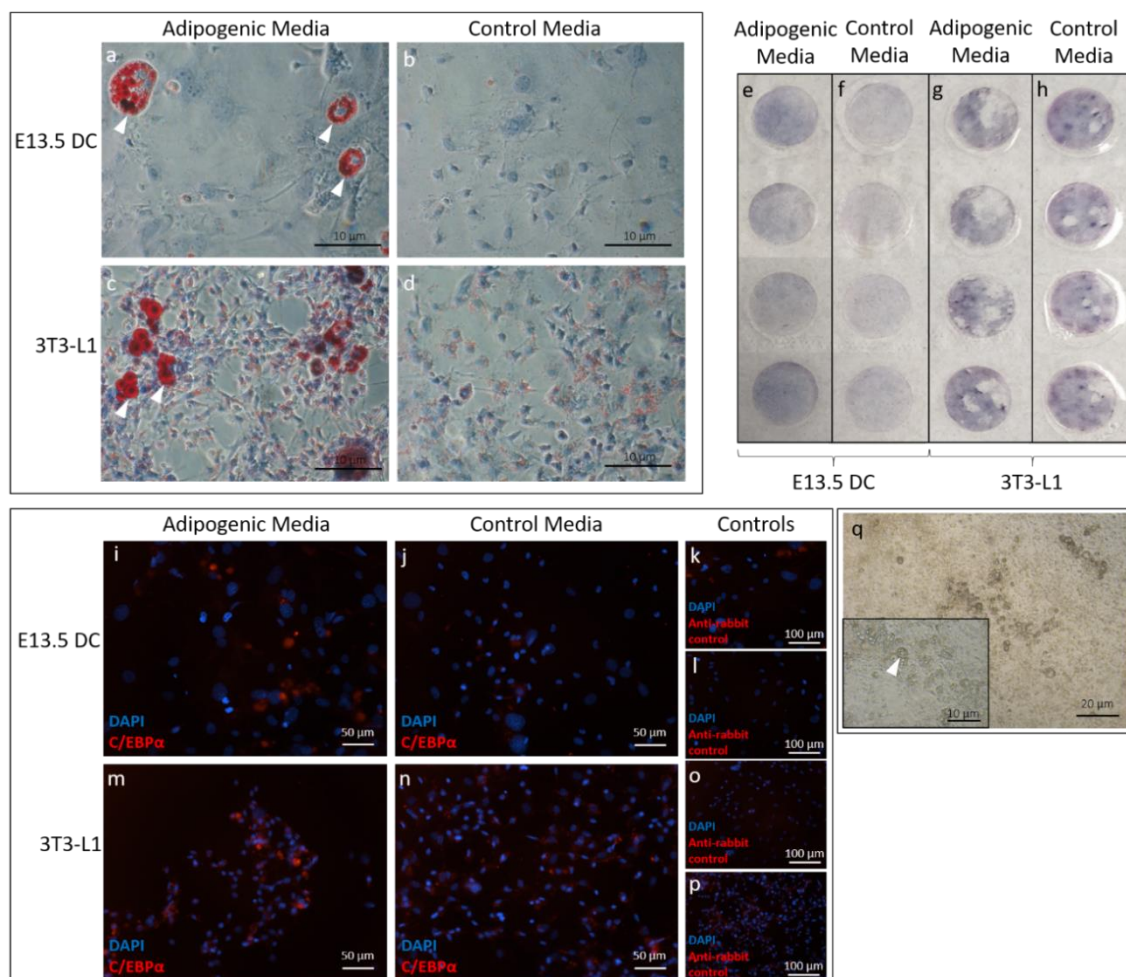


Figure 4.4: Adipogenic differentiation of E13.5 DCs and 3T3-L1 (P11) after 14 days in culture (7 days with adipogenic media and 7 days with 3T3-L1 control media). The presence of adipocytes was characterised by Oil Red O staining (a-d; macroscopic images: e-h) and C/EBP α immunofluorescent staining (i-n). Immunofluorescence controls are also shown: E13.5 DC in adipogenic media (k) or in DC control media (l) and 3T3-L1 in adipogenic media (o) or in 3T3-L1 control media (p). Also shown is a cell culture image of 3T3-L1 cells in adipogenic media on Day 14 prior to fixation (q). Arrow heads indicate adipocytes. Repeated once.

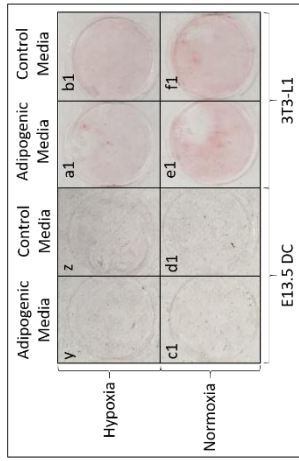
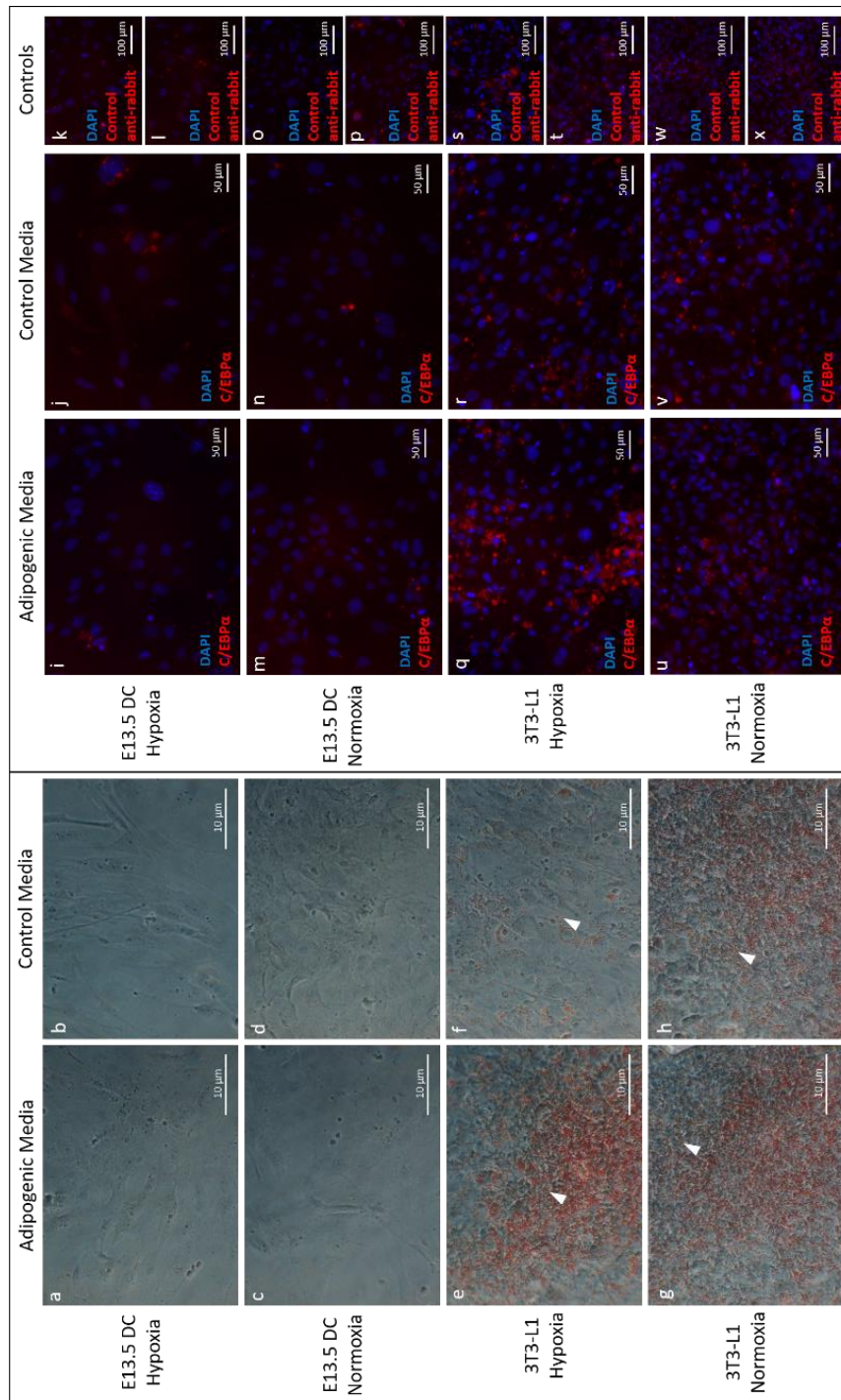


Figure 4.5: Adipogenic differentiation of E13.5 DCs and 3T3-L1 (P12) after 8 days (4 days adipogenic media and then 4 days with DC media or 3T3-L1 control media, respectively) under hypoxic or normoxic conditions. The presence of adipocytes was characterised by Oil Red O staining (a-h; macroscopic images: y-f1) and C/EBP α immunofluorescent staining (i-x). Immunofluorescence controls are also shown. For hypoxic conditions: E13.5 DC in adipogenic media (k) or in control media (DC media) (l) and 3T3-L1 in adipogenic media (s) or in 3T3-L1 control media (t). For normoxic conditions: E13.5 DC in adipogenic media (o), and in control media (DC media) (p), 3T3-L1 in adipogenic media (w) and in 3T3-L1 control media (x). Arrow heads indicate adipocytes. Work done in collaboration with Adam Gilmore. Repeated once but unsuccessfully due to fungal infection.



4.3.5 Osteogenic Differentiation

Von Kossa staining was used to characterise the osteogenic differentiation of E13.5 DCs and rMSCs after 28 and 21 days, respectively. The experimental E13.5 DCs differentiated minimally (less than 2%), shown by the brown calcium deposits (indicated by arrow heads; Figure 4.6.a and e), and less than 1% staining was observed for the control E13.5 DCs (Figure 4.6.b and f). rMSCs were used as a positive control and extensive differentiation (60-70%) was observed when cultured in the osteogenic media and only required 21 days to reach this stage (indicated by arrow heads; Figure 4.6.c and g). No differentiation was observed for the control rMSCs (Figure 4.6.d and h).

Supplementary Figure S7 shows cell culture images of the progression of osteogenic differentiation in E13.5 DCs when in osteogenic media.

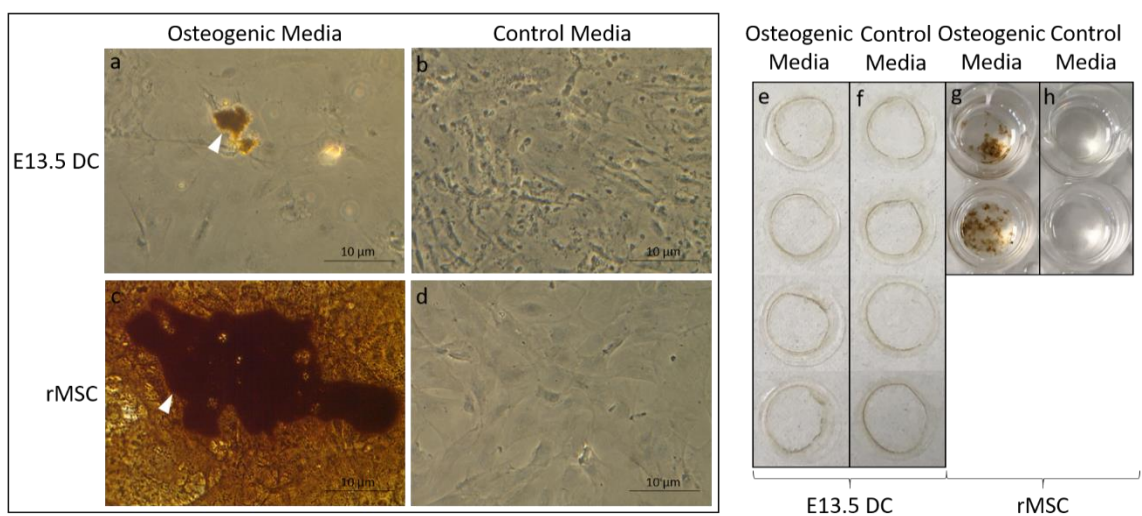


Figure 4.6: Von Kossa staining to characterise the osteogenic differentiation of E13.5 DCs when cultured in osteogenic (a) or DC control media (b) for 28 days, and rMSCs (P4) when cultured in osteogenic (c) or rMSC control media (d) for 21 days. Macroscopic images are also shown (e-h). Arrow heads indicate calcium deposits. E13.5 DCs were repeated once but unsuccessfully due to fungal infection. rMSCs were repeated twice but one was unsuccessful due to fungal infection.

4.4 Discussion

4.4.1 E13.5 DC Differentiation Potential in 2D and 3D

In this chapter, the differentiation potential of E13.5 DCs was investigated in 2D and 3D, with and without differentiation media. 3D culture work, here and in the Jahoda lab, indicated the 'default' cell fate of the E13.5 DCs may be adipocytes due to their adipogenic differentiation in the absence of the ECM, epidermis and differentiation media. This was characterised by immunofluorescent labelling of C/EBP α , FABP4 and Dlk1. In contrast, 2D culture of this dermal population (without differentiation media) resulted in a fibroblastic population with no adipocyte differentiation, shown by Oil Red O staining. The 2D culture of E13.5 DCs in differentiation media indicated that a small subpopulation of E13.5 DCs was capable of adipocyte differentiation (characterised by Oil Red O and immunofluorescent labelling of C/EBP α) and an even smaller subpopulation, osteogenic differentiation (characterised by Von Kossa staining). This surprising lack of adipogenic

differentiation, in both types of 2D culture experiments, was postulated to be related to oxygen levels thus, was repeated under hypoxic conditions. However, no difference was observed between normoxic and hypoxic conditions.

Therefore, this data has raised the following questions: is 3D culture more representative of *in vivo* than 2D culture; how do 2D and 3D culture influence differentiation; is this subpopulation restricted to only differentiate to cell types of the skin; and how does this embryonic DC differentiation potential compare to adult DCs?

4.4.2 The Role of the Culture Environment

When approaching the question of what lineages the E13.5 DC population is capable of differentiating into, it was strongly anticipated that some adipogenic differentiation was a given. This was due to the previous work carried out in the Jahoda lab (Gilmore, unpublished) where 3D culture of isolated DCs of the same age has been shown to produce spheroids consisting of almost all adipocytes. Figure 4.4 reproduced these findings and confirmed these 3D spheroids were mostly undergoing adipogenic differentiation by expressing nuclear C/EBP α (a late marker of adipogenesis), FABP4 (a mature adipocyte marker) and Dlk1 (an early marker of adipogenesis) (Stephens 2012, Driskell et al. 2013, Moseti, Regassa, and Kim 2016). Hence the idea that the 'default' state of embryonic DCs could be to produce adipocytes as culturing them without the influence of the ECM, epidermis and differentiation media resulted in adipocytes. In addition to adipocyte markers, Cbx5 expression was studied and nuclear expression in almost all cells was observed. Cbx5 is a protein involved in the dynamics of heterochromatin structure thus, indicates the changes on a genetic level that are involved in differentiation (Hinde, Cardarelli, and Gratton 2015).

In contrast to these 3D findings, culturing the same cells in the same conditions, except they were now in 2D, led to no such adipogenic differentiation. In addition, less than 10% of these cells produced adipocytes when cultured in adipogenic media in 2D. It is fascinating that the same DCs have such varying cell fates when cultured in 2D or 3D, indicating that the environment is important for differentiation. One explanation for this is thought to be because gene expression profiles of cells dramatically change when cultured in 2D due to a number of reasons. For example, adhesions are restricted to one plane as opposed to 3D; this leads to forced apical-basal polarity, reduced cell:cell interactions, reduced intercellular signalling and reduced cell:ECM interactions; there are a lack of soluble gradients and the stiffness of the cell increases (Baker and Chen 2012, Shen et al. 2013). Cells, for example chondrocytes, which have been shown to dedifferentiate in 2D culture have had their original differentiated state restored when returned to a 3D environment (Benya and Shaffer 1982). This shows how these morphological changes have links to differentiation and, thus, gene expression. Shen et al. (2013) have compared 2D

culture with the 3D hanging drop culture method using adipose-derived stromal cells to investigate osteogenic differentiation. They showed that 3D culture with osteogenic media resulted in increased osteogenic differentiation compared to 2D, as well as successful formation of bone when transplanted *in vivo*, unlike the transplantation of monolayer cells (Shen et al. 2013). It would be interesting to try this comparison of differentiation in 2D and 3D culture with E13.5 DCs to see how much the environment plays a role in the restricted phenotype they display. In addition to 2D vs 3D arguments, the observations in this study raise some interesting questions with regards to the status of DCs before and after they enter culture. It might be expected that culture would alter cells from a differentiated state to a less differentiated state. For example, DP cells become MSC-like (differentiate to adipogenic, osteogenic and myogenic lineages) and express Pax3 (associated with an undifferentiated state in cells of dermomyotome origin) when grown in 2D culture (Jahoda et al. 2003, Ruffaut et al. 2006). Therefore, it is interesting that mouse E13.5 DCs, which aren't fully committed to a cell type yet and, therefore, might be expected to be more 'plastic' originally, show less MSC-like activity in culture. However, this increased plasticity of DP cells in 2D culture may be due to the SKP niche (multipotent stem cell population derived from the skin) that the DP contains, rather than the effect of the culture environment. In addition, the DP cells were often clonally expanded prior to 2D culture, unlike the E13.5 DCs in this project, so this technique may be the reason for the difference in plasticity (Toma et al. 2001, Jahoda et al. 2003, Ruffaut et al. 2006, Biernaskie et al. 2009, Kellner and Coulombe 2009, Driskell et al. 2013, Driskell and Watt 2015). Therefore, to properly rule out this idea, the E13.5 DCs need to be clonally expanded prior to differentiation.

4.4.3 E13.5 DC Differentiation

When E13.5 DCs were pushed to differentiate to adipogenic and osteogenic lineages in 2D culture, a subpopulation of less than 10% seemed to be capable of producing adipocytes and less than 2% seemed to be capable of osteogenic differentiation. It could be argued that E13.5 is too late in development to find a more homogeneous, stem cell dermal population. However, Supplementary Figure S1 indicates that even at E12.5 less than 1% osteogenic differentiation took place. E12.5 DCs were not tested for adipogenic differentiation unfortunately, due to technical difficulties with the adipogenic media and time restraints.

With regards to adipogenic differentiation, it was especially odd that there seemed to be a lack of C/EBP α nuclear expression despite 10% of cells staining with Oil Red O. C/EBP α is a late marker of adipogenesis so perhaps the adipocytes that were present were too mature but this is quite unlikely (Stephens 2012). It was particularly strange that the positive control (3T3-L1 cells) did not appear to show as extensive differentiation as you would expect. Comparing the Oil Red O and C/EBP α immunofluorescent staining to the cell culture image taken prior to fixation indicated a

decrease in the number of adipocytes after staining. This may be because mature adipocytes more readily lift and so there is a higher tendency for their loss during the staining process.

Consequently, oxygen levels were considered as a potential factor. In the 3D spheroid, the oxygen levels would be lower for those cells in the centre compared to the levels cells in 2D culture would be exposed to. Therefore, we compared 2D differentiation in hypoxic (5% oxygen) and normoxic (20% oxygen) conditions. There was no significant difference apparent between these conditions so oxygen levels suggest no obvious role in adipogenesis. However, repeats are needed to confirm these findings. The literature has shown that hypoxic conditions between 0.1 and 2% prevent adipogenesis in 3T3-L1 preadipocytes so perhaps E13.5 DCs act in a similar way hence, lower oxygen levels did not enhance adipogenesis. Hypoxia has also been shown to have a role in maintaining pluripotency of mouse and human embryonic stem cells as demonstrated by the increased expression of OCT4, Sox2 and Nanog (pluripotency-associated genes) and increased proliferation rate of human embryonic stem cells cultured in 5% oxygen. In contrast, hypoxia has also been known to promote neural differentiation of embryonic stem cells. Therefore, oxygen levels should be considered when optimising conditions for differentiation assays (Hawkins, Sharp, and McKay 2013). However, in this case, oxygen conditions appear not to be the reason for minimal adipogenic differentiation of E13.5 DCs.

Although testing differentiation of only two different lineages is limiting, this data can provide some answers. Firstly, they suggest that the E13.5 DC population is more restricted in its differentiation potential rather than being overwhelmingly a plastic, MSC-like population. Secondly, they indicate that even at this relatively early stage of skin development there is only a very small subpopulation of cells with a more stem-like profile, despite being lower in number than one might have predicted.

As discussed earlier, it is important to remember the limitations 2D culture comes with, and so the differentiation experiments beg the question of whether they are representative of *in vivo*. Is the restrictive differentiation of E13.5 DCs simply a product of 2D culture not providing the optimal environment or is this restriction present *in vivo* to ensure the development of the dermis occurs correctly? Carrying out the differentiation experiments in 3D would help to answer these questions.

4.4.4 Differentiation of DCs in Adults and to Alternative Lineages

The presence of a subpopulation containing stem cells is known in the adult dermis as well, for example, the DP and DS of adult rat vibrissae have been shown to differentiate to both adipogenic and osteogenic cell fates (Jahoda et al. 2003). This is interesting due to the DP, as previously mentioned, being immunofluorescently labelled with Nestin (a neuroepithelial stem cell marker associated with SKPs) in human adult scalp skin (Sellheyer and Krahl 2010). In addition, Chapter 3

indicated the E13.5 dermis was labelled throughout by Nestin. Therefore, the subpopulation of embryonic DCs that were able to differentiate in this chapter are possible candidates for the precursors of the DP and, thus, SKPs (DP is a source of SKPs) and so would, by this logic, retain this differentiation potential and Nestin expression in adulthood (Biernaskie et al. 2009, Kellner and Coulombe 2009). One way to determine this would be to isolate DP precursor cells at E13.5 and compare their differentiation ability to the rest of the E13.5 DC population. Or alternatively, use mouse strains with specific genetic markers of papillary or reticular DC populations in order to isolate different parts of the dermis and compare their differentiation potential to narrow down the location of this differentiating subpopulation.

Another example of adult DC differentiation is shown when neonatal mouse DCs were cultured with galectin-1 and resulted in 30% of the population expressing desmin (a muscle-specific marker) (Goldring, Jones, and Watt 2000, Goldring, Jones, Thiagarajah, et al. 2002). Therefore, showing this stem-like population has the capability to differentiate to muscle. In a similar study, human foetal DCs showed comparable results of 17.7% of the population expressing desmin when cultured with galectin-1 (Goldring, Jones, Sewry, et al. 2002).

The minimal osteogenic differentiation in embryonic DCs, compared to adipogenic and myogenic, warrants the question of whether the resident stem cell subpopulation in the dermis is only capable of differentiating to lineages required in the mouse skin (dermal, adipogenic and panniculus carnosus). However, the literature suggests that this is not the case. For example, Fernandes et al. (2004) showed some E18 and adult skin cells were able to differentiate to neurons (a cell type not found in skin) when cultured in differentiation media. This was indicated by the expression of β III-tubulin, and p75NTR (Fernandes et al. 2004). In addition, adult human foreskin fibroblasts have been pushed to differentiate to neurons when transduced with miR-124, BRN2 and MYT1L. After 3 days, there were signs of neuronal differentiation such as, weak β III-tubulin expression and monopolar or bipolar projections from the cell bodies. After another 15 days, a high number of cells expressed the mature neuronal markers MAP2 (55%) and NeuN (46%) and there were more extensions and branching (Ambasudhan et al. 2011). This relatively high level of differentiation could perhaps be linked to a unique Nestin expression in the adult dermis of foreskin. The glabrous (hairless) nature of foreskin may lead to differential expression of Nestin compared to non-glabrous regions (Toma et al. 2005). Therefore, it would be interesting to compare the expression profile of Nestin in the foreskin dermis with non-glabrous adult dermis, as well as the E13.5 dorsolateral dermis (Chapter 3).

Additional evidence that DCs aren't limited to differentiate only to cell types of the skin, comes from Chen et al. (2007) who showed that dermal fibroblasts from foreskin were capable of adipogenic (20% of population), osteogenic (30%) and chondrogenic (20%) differentiation (the

latter two both being cell types not found in skin) when cultured in differentiation media. Clonal analysis of this population revealed that 6.4% of the clones were tripotent, 19.1% bipotent and 10.6% unipotent. Of the three tripotent clones only one underwent neurogenic and hepatogenic differentiation, indicating the presence of a pluripotent subpopulation of dermal fibroblasts (Chen et al. 2007). However, clonal expansion involves the isolation of cells. This has been shown to be a common culture condition when pushing cells to become more plastic, for example, multipotent adult progenitor cells are grown in sparse conditions and are diluted often to reduce cell:cell contacts; marrow-isolated adult multilineage inducible cells are grown in isolation; as are SKPs (when testing they are multipotent) (Shoshani and Zipori 2015). This indicates a lack of neighbouring cells (a stressful environment) induces cells to dedifferentiate. It has been postulated that stressful environments influence gene expression via epigenetics thus, leading to reprogramming events (Shoshani and Zipori 2011). Therefore, how representative of *in vivo* are these processes of dedifferentiation? Shoshani and Zipori (2015) investigated this question by inducing different types of stress *in vivo* but found that only stress involving cell loss led to dedifferentiation of cells. Therefore, perhaps this subpopulation of stem cells, observed in embryonic and adult dermis, does represent a resident population *in vivo* that is only revealed under stress conditions requiring replenishment of cells/tissue (for example injury or isolated cell culture conditions) via transdifferentiation or dedifferentiation of committed cells (Wagers and Weissman 2004, Shoshani and Zipori 2015).

When one compares the limited differentiation potential observed in the E13.5 DCs to the literature discussed here, it seems likely that the early embryonic DCs have a restricted potential that is broadened with age in some of the subpopulations that make up the later dermis. The literature discussed covers a range of ages, organisms and anatomical skin sites, as well as differences in technique (the use of clonal expansion as opposed to direct differentiation). Therefore, despite the need for further research, it could be interpreted that developmental changes lead to cells displaying more stem-like properties when in 2D culture. Other interpretations are that a) some cells in the dermis go from being in a more restricted to a less restricted state over time in development, perhaps due to some interactive event (for example hair follicle DCs being induced) or b) other cells migrate to the dermis later in development and are responsible for this more plastic nature in 2D culture. These concepts will be explored more in Chapter 5 in the context of SKPs.

4.5 Conclusions

To conclude, this data has suggested that the E13.5 DC population is not full of stem-like, primitive cells, as might be hypothesised, but there appears to be a subpopulation capable of producing adipocytes and very few osteoblasts. However, more types of differentiation need to be explored to fully answer the hypothesis and to determine whether the E13.5 DCs are restricted

to differentiate only to the cell types found in the skin. This proposed subpopulation continues to be present in the adult dermis and appears to become less restricted and more multipotent in the lineages it can produce, according to the literature (Goldring, Jones, and Watt 2000, Goldring, Jones, Thiagarajah, et al. 2002, Chen et al. 2007, Ambasadhan et al. 2011). This highlights the fact that changes occur during embryogenesis that result in the adult dermis expanding its differentiation abilities and results in a larger subpopulation displaying this stem-like property.

In addition, the 2D differentiation experiments have indicated that the concentration of oxygen did not appear to make a significant difference in the extent of adipogenic differentiation and there was an increased ability of embryonic DCs to produce adipocytes compared to osteoblasts. It could be hypothesised that this is due to the 'default' cell type of embryonic DCs, postulated by the 3D culture work, being adipocytes. Alternatively, it could be due to a more restrictive stem-like profile than predicted or 2D culture isn't reflective of the true properties of these cells.

Substantial research has been done to isolate these stem-like dermal subpopulations in order to fully elucidate their differentiation potential and to investigate how they can be used in clinical applications (Kwon et al. 2017). One identified isolated multipotent population, termed SKPs, characteristically expresses Nestin, Fibronectin and Vimentin (Fernandes et al. 2004, Toma et al. 2005). This population will be discussed more in Chapter 5 which will investigate SKP formation in the context of the embryonic dermis and its development.

5. Investigation of Embryonic Dermal Precursor Cell Properties Via the Formation and Characterisation of dSKPs

5.1 Introduction

The second approach taken to investigate the question of the plasticity of cells in the E13.5 dermis was to push these common 'fibroblast' precursor cells to an even more primitive state. This was achieved by investigating the production of SKPs from the E13.5 DC population.

SKPs are a multipotent precursor population derived from the skin and capable of differentiating into mesodermal and neuronal lineages (Toma et al. 2001, Fernandes et al. 2004). The origin of SKPs is a topic of some controversy and still remains unclear. However, similarities have been drawn between neural crest stem cells and SKPs. For example, Fernandes et al. (2004) showed SKPs had a similar differentiation potential to neural crest stem cells (Fernandes et al. 2004).

Another point of contention in this field are the questions of whether SKPs are skin-resident multipotent stem cells, whether they are a result of transdifferentiation of a particular cell type or whether they are a phenomenon seen only *in vitro* (Hunt, Jahoda, and Chandran 2009)? The DP of hair follicles has been hypothesised as a possible niche for SKPs and has provided some evidence in support of SKPs representing an endogenous dermal precursor population (Biernaskie et al. 2009, Kellner and Coulombe 2009). Despite evidence in favour of this, the production of SKPs from glabrous regions of skin (for example, foreskin) has proved the DP cannot be the only origin (Toma et al. 2005, Ruetze et al. 2013). Consequently, a variety of skin regions, including both glabrous and nonglabrous regions, have been used in the literature to produce SKPs, as well as a variety of mammalian species, such as mouse, human, pig and rat (Toma et al. 2001, Fernandes et al. 2004, Toma et al. 2005, Zhao et al. 2009, Ruetze et al. 2013).

The literature has mainly focused on using adult skin or older embryonic skin to produce these SKPs. Fernandes et al. (2004) found that they could not be produced from embryonic skin younger than 14 days (Toma et al. 2001, Fernandes et al. 2004, Toma et al. 2005). This chapter revisited the question of whether SKPs could be created from embryonic DCs (between E12.5 and E15.5). These SKPs have therefore been termed dSKPs to highlight their dermal origin as opposed to the whole skin origin often used in the literature (Fernandes et al. 2004, Toma et al. 2005).

In this study, characterisation and comparison of different aged dSKPs was achieved via immunofluorescent staining of Fibronectin, Vimentin, Nestin (all commonly accepted markers of SKPs), Sox2, OCT4 (both stem cell related markers) and Wnt 5a (associated with the DP) (Fernandes et al. 2004, Toma et al. 2005, Kellner and Coulombe 2009, Liu et al. 2013, Li et al. 2016). In addition, RT-PCR was conducted for genes known to be expressed in SKPs (Fernandes et al. 2004). However, the most stringent test for SKPs is their ability to differentiate so, in this

chapter, the dSKPs between E13.5 and E15.5 were pushed to differentiate to adipocytes and osteoblasts using differentiation media (Toma et al. 2001, Fernandes et al. 2004).

dSKPs that were produced from E13.5 dermis were the material which most of this chapter focused on. Again, due to the ease of dermal separation from the epidermis and muscle as well as their predicted primitive nature (Driskell and Watt 2015). E15.5 skin had been shown by Fernandes et al. (2004) to produce SKPs so was used as a positive control. In addition, E14.5 dSKPs were produced in order to investigate the effect of hair follicle initiation (which begins at E14.5) on SKPs and to provide an intermediate stage between the positive control and experimental dSKPs (Sennett and Rendl 2012, Fu and Hsu 2013).

Chapter 4 investigated the questions of whether the E13.5 DCs contained any cells with a more stem-like profile and showed that small subpopulations were able to differentiate into adipocytes and osteoblasts. However, further work is needed to determine if this population is restricted to only differentiate to lineages of the skin. This chapter will aim to further investigate the question of early embryonic DC plasticity by evaluating if E13.5 DCs are able to produce dSKPs and show the same multipotency as SKPs from older skin in the literature. Consequently, this is testing whether the hypothesis (that the common 'fibroblast' precursor in the early dermis is responsible for producing all cell types of the dermis) is true.

5.2 Materials and Methods

Figure 5.1 summarises the methods and approaches for this chapter (See Chapter 2 for detailed methods). This group of experiments focused on looking at the plasticity of E13.5 DCs by determining their capacity to create dSKPs and involved comparisons with older embryonic dSKPs.

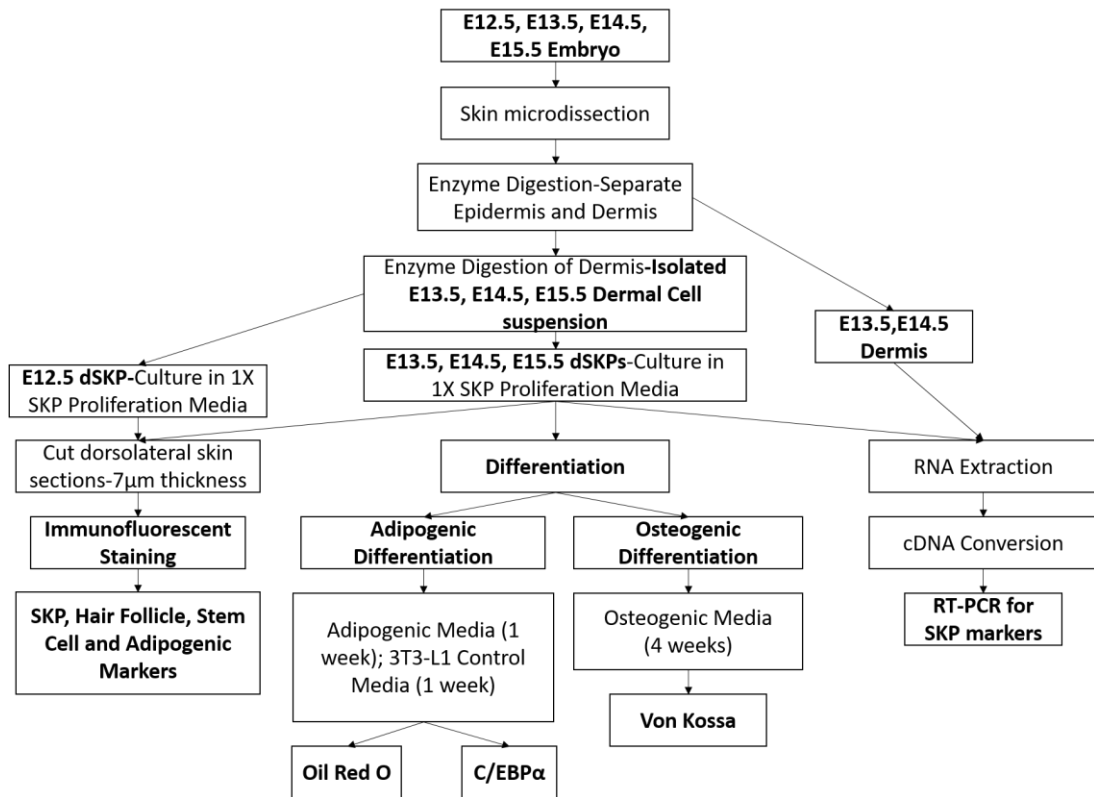


Figure 5.1: A schematic summary of the approaches used to characterise the expression and differentiation of E13.5-E15.5 dSKPs; and the expression profiles of E12.5 dSKPs, E13.5 and E14.5 dermis. See Chapter 2 for more detailed methods.

5.3 Results

5.3.1 Formation of dSKPs

When introduced into culture in 1X SKP proliferation media, freshly isolated cells from the dermis of all the ages tested (E12.5-E15.5) formed spheres, provisionally termed dSKPs because, at least morphologically, they all resembled SKPs. These spheres formed after only 2 days and increased progressively in size and number over the course of 8 days (Figure 5.2). A key difference between early (E12.5 and E13.5) and late (E14.5 and E15.5) dSKPs was that only a few cells were still adhered after 8 days in the early dSKP cultures, whereas a larger number were still adhered in the cultures containing later-age dSKPs.

One difference between E12.5 and E13.5 DCs (in their ability to make these dSKPs) was that E12.5 DCs appeared to produce a greater number of dSKPs than E13.5 DCs (data not shown). However, further quantification of this is required for conclusive findings. Quantification of E13.5-E15.5 dSKPs was conducted and indicated there was a small decrease in the efficiency of dSKP formation as the DCs increased in age (Figure 5.3).

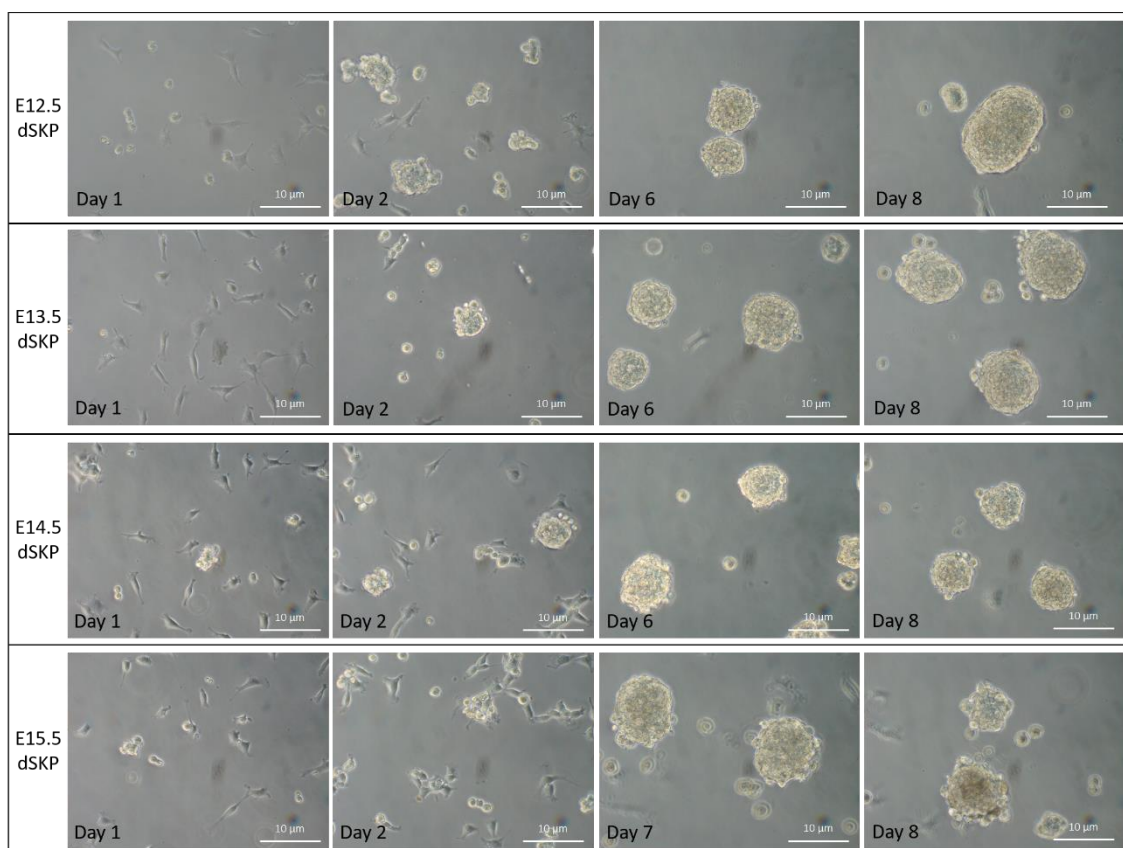


Figure 5.2: The formation of E12.5, E13.5, E14.5 and E15.5 dSKPs when cultured in 1X SKP proliferation media for 8 days. See Supplementary Table S5 for repeats.

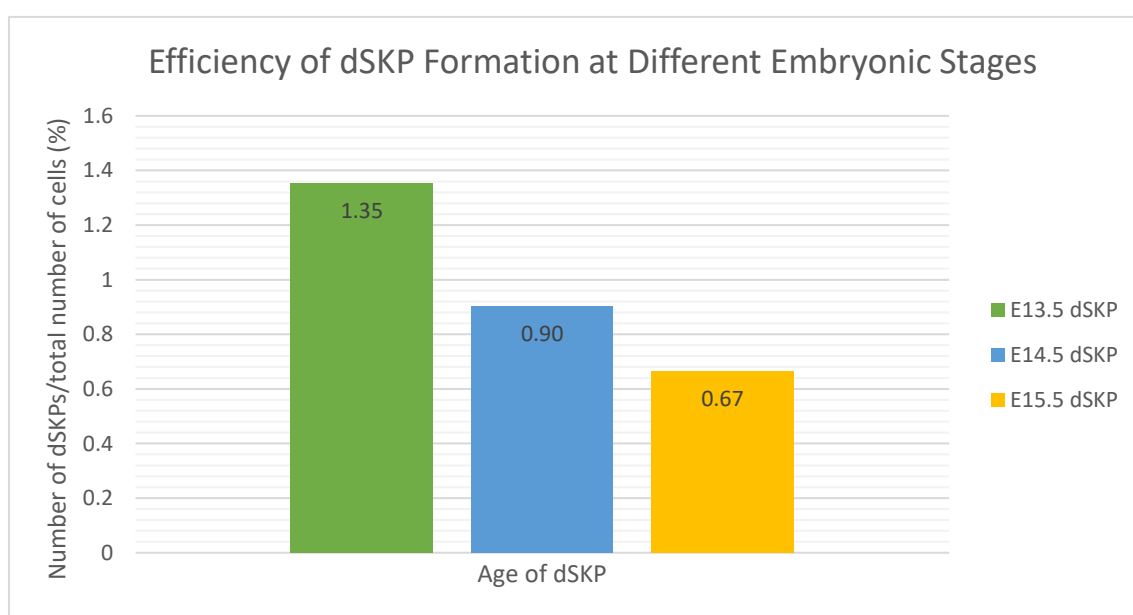


Figure 5.3: Comparison of the efficiency of DCs from different embryonic ages in the production of dSKPs: E13.5 dSKPs=3 samples counted; E14.5 dSKPs=3 samples counted; E15.5 dSKPs=3 samples counted.

5.3.2 Characterisation of dSKPs

5.3.2.1 Immunofluorescent Staining

dSKPs ranging from E12.5 to E15.5 were cultured and cryosectioned for immunofluorescent staining of the characteristic SKP markers: Nestin, Fibronectin and Vimentin (Figure 5.3) (Fernandes et al. 2004, Toma et al. 2005). Each age was represented by at least 10 dSKPs and all ages expressed Fibronectin and Vimentin consistently throughout the sections (as shown by repeats), even when there was variation in the size of dSKPs. Fibronectin was expressed extracellularly and Vimentin was expressed in the cytoskeleton. However, Nestin staining was less consistent (with no expression present on some occasions) but overall showed cytoskeletal staining in all ages of the dSKPs. In addition, there was some tendency for Nestin to be expressed in the outer regions of the dSKPs (as opposed to the centre) and this sometimes correlated to the dSKPs being larger. Also, E14.5 dSKPs and E15.5 dSKPs appeared to have slightly increased Nestin expression compared to E12.5 dSKPs and E13.5 dSKPs.

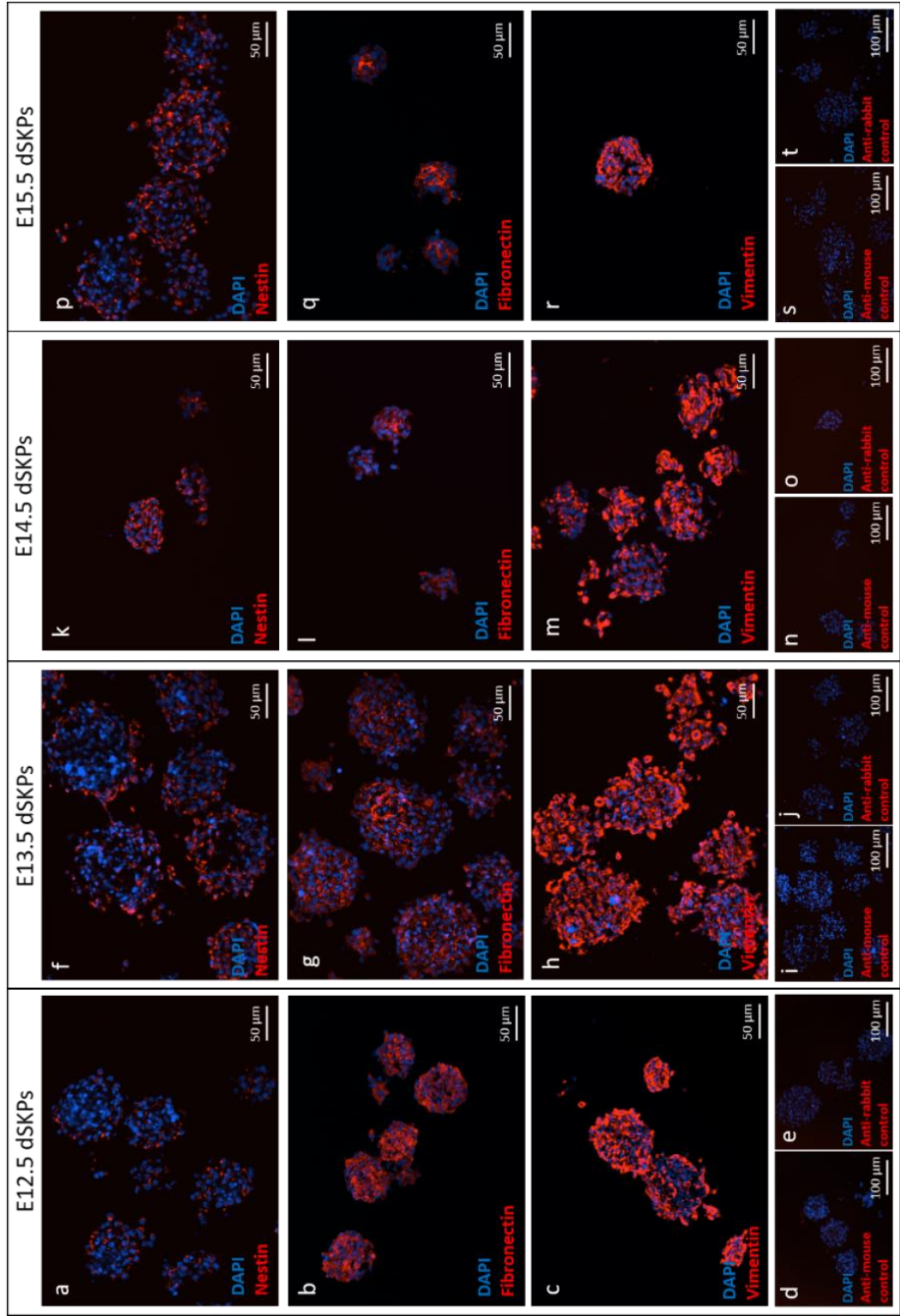


Figure 5.4: Immunofluorescent staining of E12.5 dSKPs (a-e); E13.5 dSKPs (f-j); E14.5 dSKPs (k-o); E15.5 dSKPs (p-t) for the characteristic SKP markers: Nestin (a, f, k, p); Fibronectin (b, g, l, q); Vimentin (c, h, m, r); and controls (d, e, i, j, n, o, s, t). E12.5 dSKPs repeated once; E13.5 dSKPs repeated twice; E14.5 dSKPs repeated once; E15.5 dSKPs only repeated once for Nestin. The dSKPs were cultured for the following number of days before being frozen down for staining: E12.5 dSKPs=9 days; E13.5 dSKPs=8 days; E14.5=7 days; E15.5=7 days.

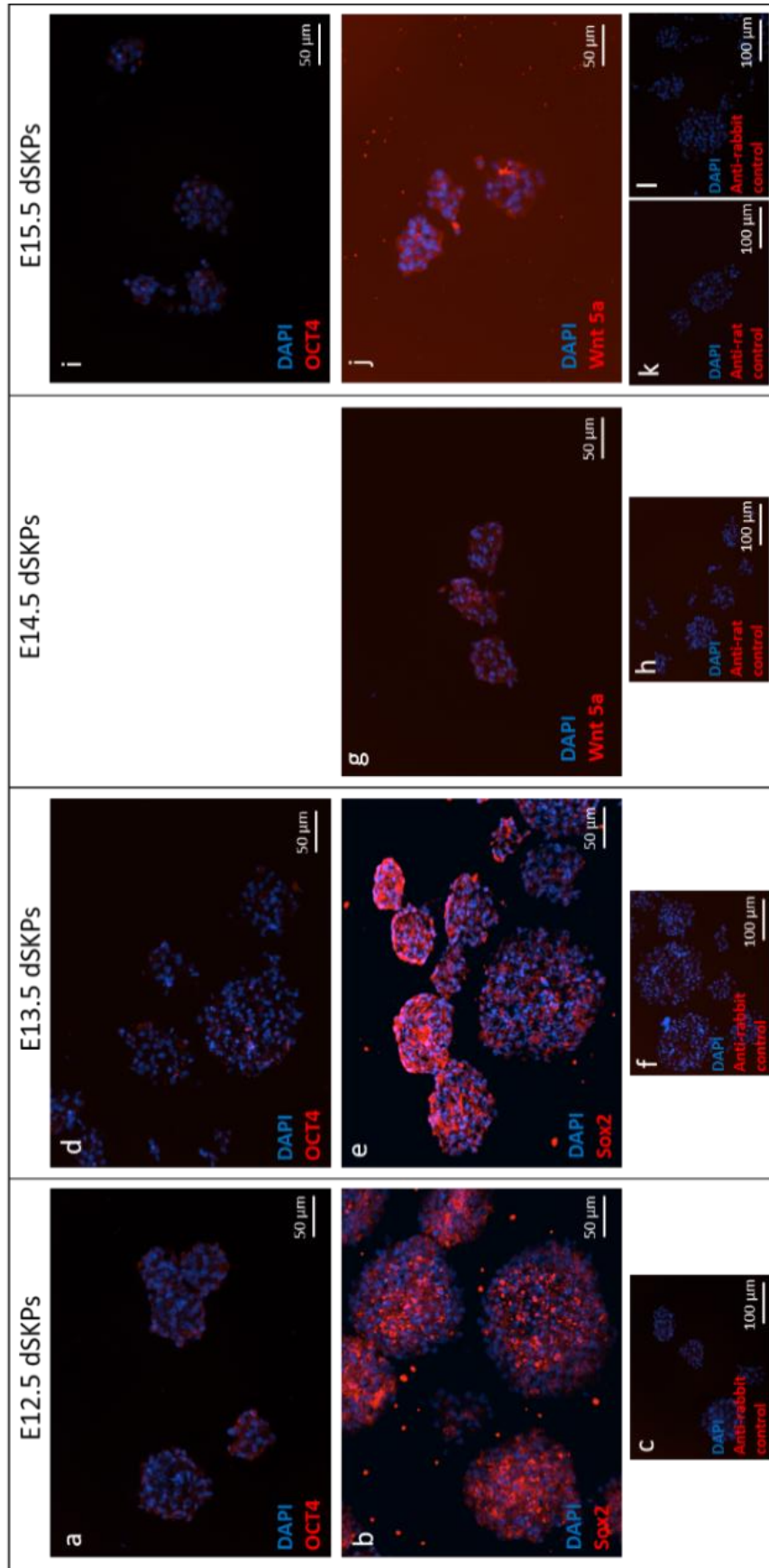


Figure 5.5: Immunofluorescent staining of E12.5 dSKPs (a-c); E13.5 dSKPs (d-f); E14.5 dSKPs (g-h); E15.5 dSKPs (i-l) for characteristic DP markers: Sox2 (b,e); Wnt 5a (g, j); and the stem cell marker: OCT4 (a, d, i); and controls (c, f, h, k, l). E12.5 dSKP no repeats; E13.5 dSKPs repeated once for OCT4 and no repeats for Sox2; E14.5 dSKPs no repeats; E15.5 dSKPs no repeats. The dSKPs were cultured for the following number of days before being frozen down for staining: E12.5 dSKPs=7 or 9 days; E13.5 dSKPs=8 or 12 days; E14.5=7 days; E15.5=7 days.

Further immunofluorescent staining was done to investigate the link SKPs have with stem cells (OCT4 and Sox2) and the DP (Wnt 5a) (Fernandes et al. 2004, Kellner and Coulombe 2009, Liu et al. 2013, Li et al. 2016). OCT4 was not expressed in the nucleus in all ages tested (E14.5 dSKPs were not tested), Sox2 was not expressed in the nucleus of E12.5 and E13.5 dSKPs (E14.5 and E15.5 dSKPs were not tested) and no specific Wnt 5a staining was shown in E14.5 and E15.5 dSKPs (E12.5 and E13.5 dSKPs were not tested) (Figure 5.5). OCT4 and Sox2 antibodies have been shown to be expressed in the nucleus of TERA2.cl.sp12 cells and DP cells of Lister Hooded rat follicles, respectively (Supplementary Figure S9).

Facial dermis is known to have a neural crest origin in contrast to dorsolateral dermis (the skin region of choice for this project) which has a somite origin (Jinno et al. 2010). Therefore, dSKPs were produced from E14.5 whisker pad (a region of the facial skin) DCs, as a comparison of skin regions, and they showed similar immunofluorescent staining for Nestin, Vimentin, Fibronectin and OCT4 (Supplementary Figure S4).

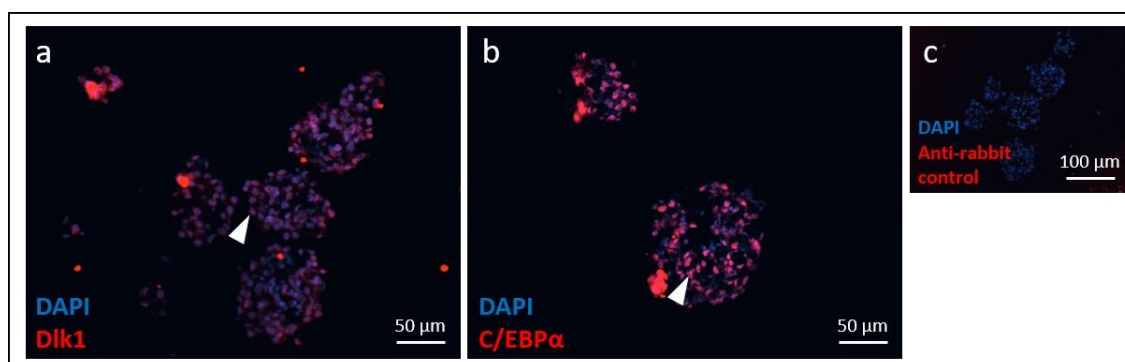


Figure 5.6: Immunofluorescent staining of E13.5 dSKPs for characteristic adipocyte markers: Dlk1 (a); C/EBP α (b); and control (c). Arrow heads indicate nuclear staining. Repeated once for Dlk1 but no repeats for C/EBP α . The E13.5 dSKPs were cultured for 12 days before being frozen down for staining.

Finally, E13.5 dSKPs were further stained for the adipocyte markers Dlk1 and C/EBP α and both appear to be present throughout the dSKPs (Stephens 2012, Driskell et al. 2013, Falix et al. 2013). C/EBP α was expressed in the nuclei (Figure 5.6; arrow head indicates nuclear staining), while Dlk1 was expressed in the nuclei, perinuclear regions and sometimes in the cytoplasm.

5.3.2.2 RT-PCR

Further characterisation of the E13.5, E14.5 and E15.5 dSKPs was carried out using RT-PCR to analyse the expression levels of genes linked with embryonic neural crest determination and migration (Slug, Twist, Snail, Sox9, P75, Pax3), dermal development (SHOX2, Dermo1), the DP (Nexin, Versican, Wnt 5a), stem cell maintenance (Sox2) and neuroepithelial stem cells and SKPs (Nestin) (Fernandes et al. 2004, Toma et al. 2005, Sellheyer and Krahl 2010, Liu et al. 2013). E15.5 dSKPs were used as a positive control. In addition, E14.5 dSKPs were investigated to look at the intermediate changes that may be taking place between E13.5 and E15.5. The whole E13.5 dermis and whole E14.5 dermis were also analysed to investigate the changes that may have taken place

during dSKP formation (Fernandes et al. 2004). Product size was used to confirm the PCR products were specific for the gene in question.

Firstly, a progressive increase in the number of genes expressed between E13.5 dSKPs and E15.5 dSKPs was shown by the RT-PCR, as well as a distinct difference between the gene expression of E13.5 dSKPs and E15.5 dSKPs (Figure 5.7). E13.5 dSKPs expressed Slug, Twist, Sox9, SHOX2, Dermo1, Nexin and Nestin and did not express Snail, P75, Versican, Wnt 5a, Sox2 or Pax3. The presence of Sox9 and Dermo1 were confirmed (as shown in Figure 5.7.b). E14.5 dSKPs expressed Slug, Twist, Sox9, P75 (faintly), SHOX2, Dermo1, Nexin, Nestin, Sox2 but did not express Snail, Versican, Wnt 5a or Pax3. Therefore, the differences between E13.5 dSKPs and E14.5 dSKPs were the expression of P75 and Sox2 in E14.5 dSKPs. E15.5 dSKPs expressed all the genes, except Pax3. Therefore, the differences between E15.5 dSKPs and E13.5 dSKPs were the expression of the neural crest-related markers Snail and P75, the expression of the DP markers Versican and Wnt 5a and the expression of the stem cell maintenance marker Sox2 in E15.5 dSKPs (Fernandes et al. 2004). The differences between E15.5 dSKPs and E14.5 dSKPs were very similar to E13.5 dSKPs, except P75 and Sox2 had been switched on in E14.5 dSKPs.

Secondly, there were changes in gene expression that occurred as a result of dSKP formation (Figure 5.7). The whole E13.5 dermis expressed Slug, Twist, Sox9, SHOX2, Dermo1, Nexin and Nestin but, like E13.5 dSKPs, did not express Snail, Versican, Wnt 5a or Pax3. However, in contrast to E13.5 dSKPs, it expressed P75 (faintly) and Sox2. Therefore, E13.5 dSKP formation resulted in genes associated with neural crest stem cells (P75) and stem cell maintenance (Sox2) being switched off (Toma et al. 2005, Liu et al. 2013). The whole E14.5 dermis expressed all the genes. Like E14.5 dSKPs, it expressed Slug, Twist, Sox9, P75, SHOX2, Dermo1, Nexin, Nestin and Sox2 but, in contrast to E14.5 dSKPs, also expressed Snail, Versican, Wnt 5a and Pax3. Therefore, E14.5 dSKP formation resulted in genes associated with neural crest determination and migration (Snail, Pax3) and the DP (Versican, Wnt 5a) being switched off (Fernandes et al. 2004, Toma et al. 2005).

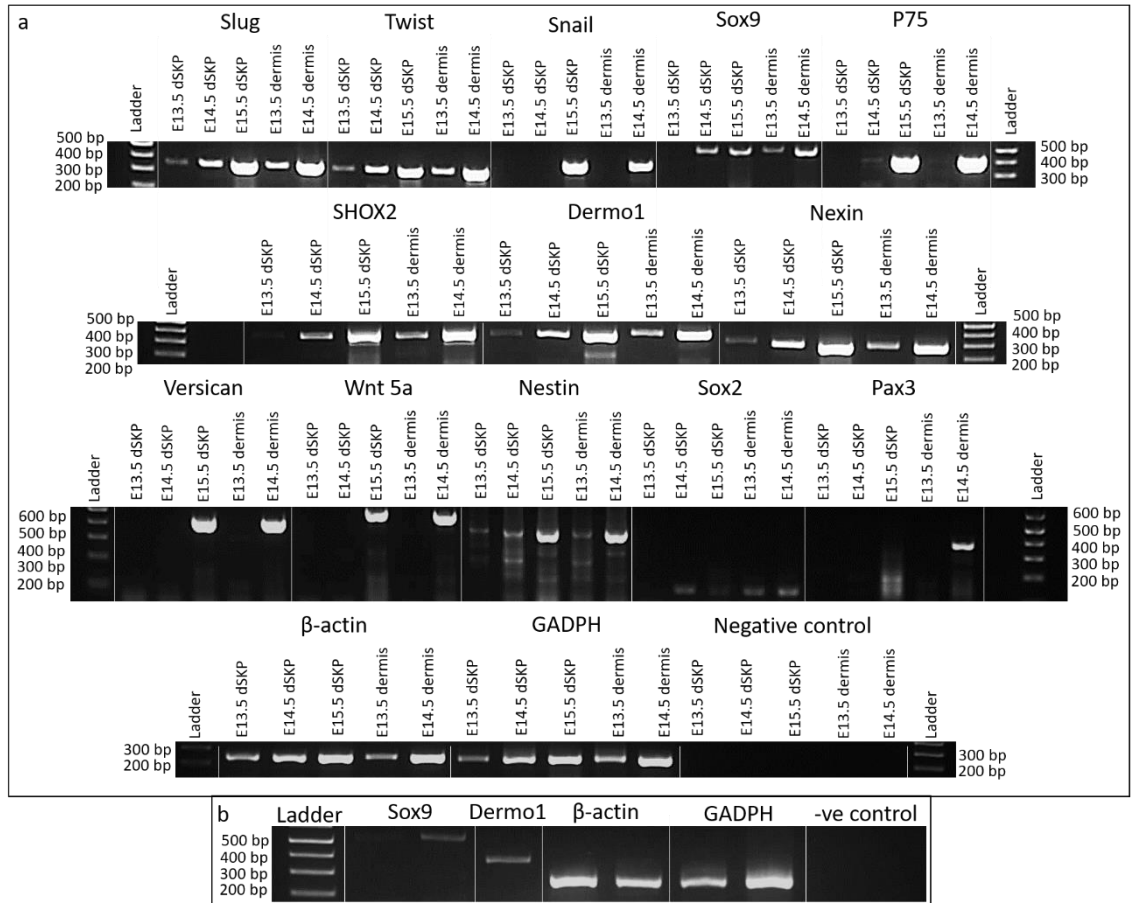


Figure 5.7: RT-PCR data for E13.5 dSKPs, E14.5 dSKPs, E15.5 dSKPs, E13.5 dermis and E14.5 dermis (respective order of loading from left to right) comparing the gene expression of characteristic SKP, skin, DP, neural crest and stem cell markers (a). Repeat RT-PCR for Sox9 in E13.5 dSKP and E13.5 dermis, and Dermo1 for E13.5 dSKP (respective order of loading from left to right) (b). In addition to the repeats shown in (b), all genes were repeated once for E13.5 dSKP, E15.5 dSKP and E13.5 dermis but not for E14.5 dSKP and E14.5 dermis. β -actin and GADPH were used as positive controls and water as the negative control. The dSKPs were cultured for the following number of days before RNA extraction was performed: E13.5 dSKPs=26 days; E14.5=8 days; E15.5=7 days.

5.3.3 Differentiation of dSKPs

5.3.3.1 Adipogenic Differentiation

E13.5, E14.5 and E15.5 dSKPs were cultured for 7 days in adipogenic media and the level of adipogenic differentiation was characterised using Oil Red O staining and C/EBP α (late marker of adipogenesis) immunofluorescent staining (Stephens 2012). Around 50% of E13.5 dSKPs in adipogenic media differentiated to adipocytes, indicated by the red staining of lipid droplets and nuclear expression of C/EBP α (Figure 5.8.a, i and y, arrow heads indicate adipocytes/nuclear staining). No significant staining was apparent in the E13.5 dSKP control (Figure 5.8.b, j and z). Less than 15-20% of E14.5 dSKPs in adipogenic media differentiated to adipocytes. This was again indicated by Oil Red O staining and nuclear expression of C/EBP α (Figure 5.8.c, m and a1; arrow heads indicate adipocytes/nuclear staining). No significant staining was apparent in the E14.5 dSKP control (Figure 5.8.d, n and b1). Around 50% of E15.5 dSKPs in adipogenic media differentiated to adipocytes, indicated by Oil Red O and nuclear C/EBP α expression (Figure 5.8.e, q and c1; arrow heads indicate adipocytes/nuclear staining). Less than 1% staining was apparent in the E15.5 dSKP control (Figure 5.8.f, r and d1). 3T3-L1 cells were used as a positive control and, surprisingly, appeared to undergo less than 1% differentiation to adipocytes when in adipogenic media (only shown by Oil Red O staining as there was no nuclear C/EBP α staining) but repeats are needed to confirm this negative result (Figure 5.8.g, u and e1; arrow heads indicate adipocytes). No significant differentiation was apparent in the 3T3-L1 control (Figure 5.8.h, v and f1).

Supplementary Figure S7 shows cell culture images of the progression of adipogenic differentiation in E13.5, E14.5 and E15.5 dSKPs when in adipogenic media.

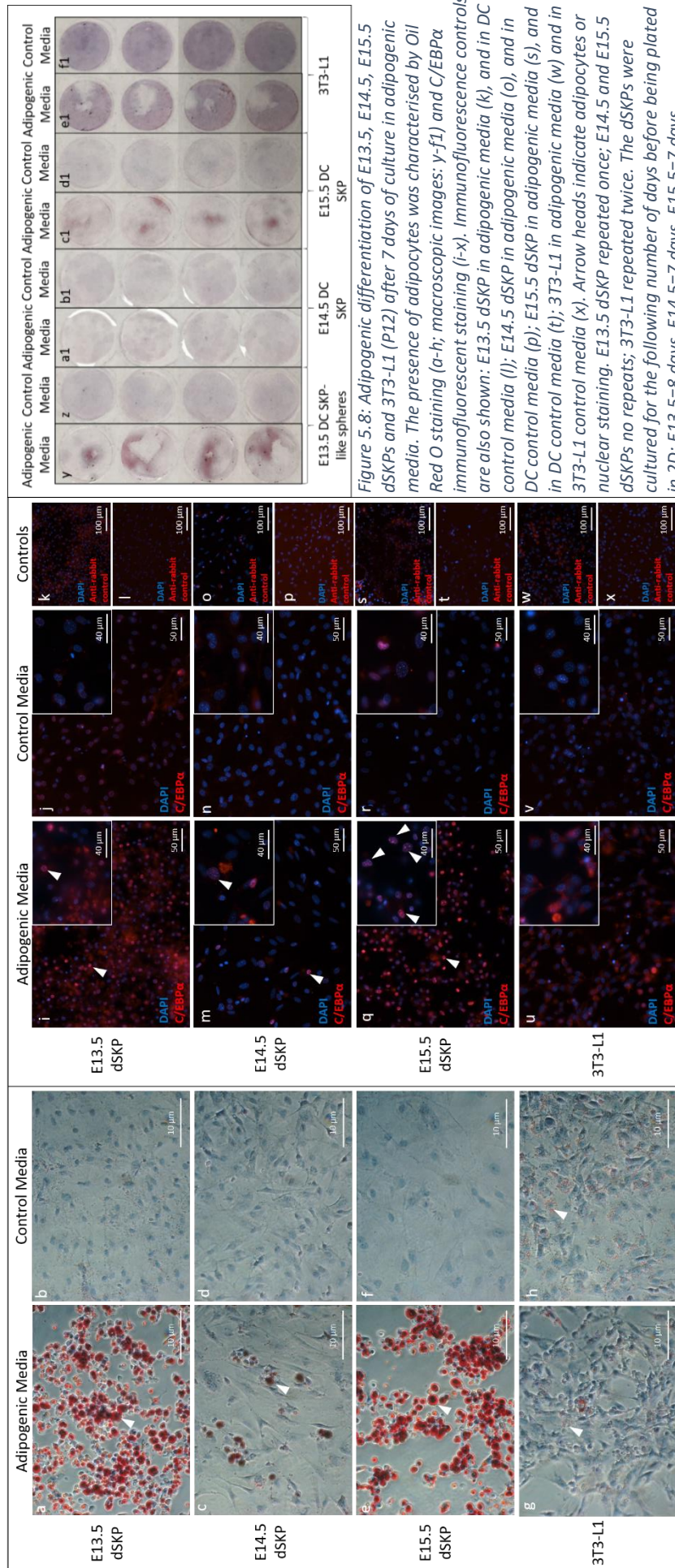


Figure 5.8: Adipogenic differentiation of E13.5, E14.5, E15.5 dSKPs and 3T3-L1 (P12) after 7 days of culture in adipogenic media. The presence of adipocytes was characterised by Oil Red O staining (a-h; macroscopic images: y-f1) and C/EBPα immunofluorescent staining (i-x). Immunofluorescence controls are also shown: E13.5 dSKP in adipogenic media (k), and in DC control media (l); E14.5 dSKP in adipogenic media (o), and in DC control media (p); E15.5 dSKP in adipogenic media (s), and in DC control media (t); 3T3-L1 in adipogenic media (w) and in 3T3-L1 control media (x). Arrow heads indicate adipocytes or nuclear staining. E13.5 dSKP repeated once; E14.5 and E15.5 dSKPs no repeats; 3T3-L1 repeated twice. The dSKPs were cultured for the following number of days before being plated in 2D: E13.5=8 days, E14.5=7 days, E15.5=7 days.

5.5.3.2 Osteogenic Differentiation

E14.5 dSKPs were cultured in osteogenic media for the desired 28 days but E13.5 dSKPs, E15.5 dSKPs and rMSCs were cultured in osteogenic media for only 20 days due to a fungal infection. Von Kossa staining was used to characterise the osteogenic differentiation. Less than 2% of the E13.5 dSKPs in osteogenic media appeared to undergo osteogenic differentiation, shown by the dark calcium deposits (Figure 5.9.a and l; arrow heads indicate calcium deposits), and no significant staining was indicated in the E13.5 dSKP control (Figure 5.9.b and j). Less than 1% of the E14.5 dSKPs in osteogenic media appeared to undergo osteogenic differentiation, shown again by calcium deposits (Figure 5.9.c and k; arrow heads indicate calcium deposits). No significant staining was indicated in the E14.5 dSKP control (Figure 5.9.d and l). Less than 5% of E15.5 dSKPs in osteogenic media appeared to undergo osteogenic differentiation (Figure 5.9.e and m; arrow heads indicate calcium deposits) and no significant staining was indicated in the E15.5 dSKP control (Figure 5.9.f and n). rMSCs were used as a positive control and less than 10% staining was apparent when in osteogenic media (Figure 5.9.g and o; arrow heads indicate calcium deposits) and no significant staining was indicated in the rMSC control (Figure 5.9.h and p).

Supplementary Figure S8 shows cell culture images of the progression of osteogenic differentiation in E13.5, E14.5 and E15.5 dSKPs when in osteogenic media.

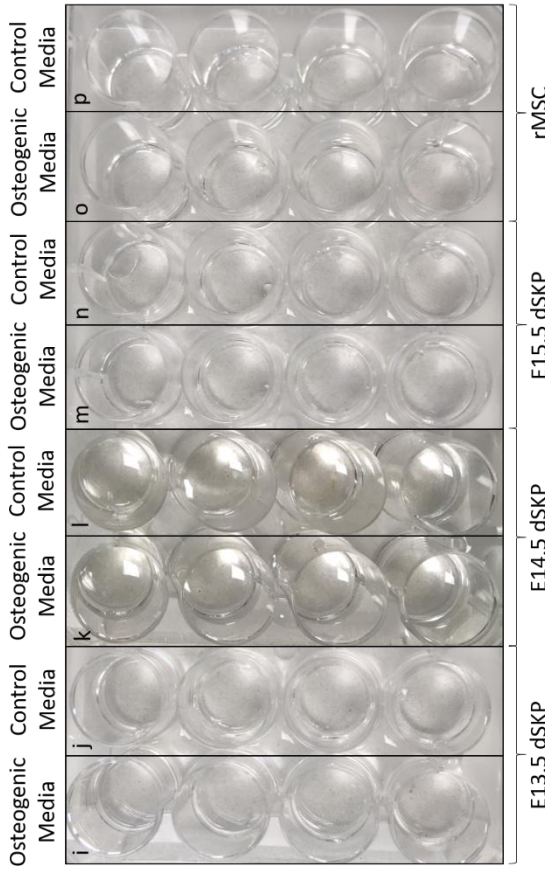
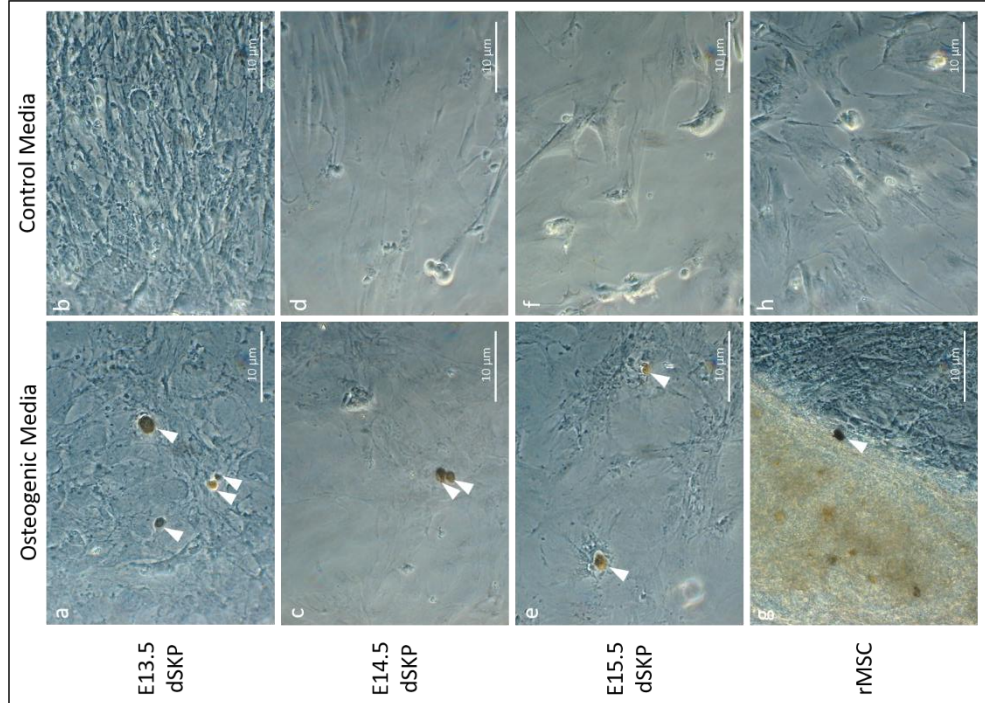


Figure 5.9: Von Kossa staining to characterise the osteogenic differentiation of E13.5, E15.5 dSKPs and rMSCs (P3) when cultured in osteogenic (a, e, g) or DC control media (b, f, h) for 20 days; and E14.5 dSKPs when cultured in osteogenic (c) or DC control media (d) for 28 days. Macroscopic images are also shown (i-p). Arrow heads indicate calcium deposits. (E13.5, E15.5 dSKPs and rMSCs only cultured for 20 days due to fungal infection). E13.5 dSKP repeated once; E14.5 and E15.5 dSKPs no repeats; rMSC were repeated three times. The dSKPs were cultured for the following number of days before being plated in 2D: E13.5=8 days, E14.5=7 days, E15.5=7 days.



5.4 Discussion

5.4.1 Embryonic dSKPs - Characterisation and Comparison

For this chapter, dSKPs were produced from dorsolateral skin aged E12.5-E15.5. The dSKPs from E13.5-E15.5 dermis were chosen as the focus and their expression profile and differentiation potential were characterised. Immunofluorescent labelling showed that Fibronectin, Vimentin and Nestin (characteristic SKP markers) were expressed in all ages. However, Sox2, OCT4 (stem cell markers) and Wnt 5a (DP marker) did not appear to be expressed (at least not in the nucleus for Sox2 and OCT4) in the ages tested (Fernandes et al. 2004, Toma et al. 2005, Kellner and Coulombe 2009, Liu et al. 2013, Li et al. 2016). By contrast, the RT-PCR revealed a drastic difference in gene expression between E13.5 dSKPs and E15.5 dSKPs as well as, E14.5 dSKPs and E15.5 dSKPs. This may be indicative of changes in the DC population at these stages of development - the reasons for which will be discussed in this chapter.

The dSKP differentiation assays showed more cells differentiated to adipocytes than osteoblasts. In addition, the level of osteogenic differentiation was very minimal, which raises questions about the plasticity of these cells and whether they are restricted only to the cell types of the dermis. These are the questions this chapter aims to explore.

5.4.2 E13.5 dSKPs

Most of the work done on SKPs has focused on the use of adult skin or older embryonic skin as the literature has stated that SKPs cannot be made from embryonic dermis younger than 14 days (Toma et al. 2001, Fernandes et al. 2004). This finding, as well as the limited evidence of multi-differentiation potential found in the previous chapter, sparked the investigation of the plasticity of pre-E14 DCs and began with an attempt to produce SKPs from E12.5 and E13.5 dermis. The results illustrated in Figure 5.2 appeared to contradict the literature. Both E12.5 and E13.5 DCs have produced floating spheres after only 2 days in culture and these spheres go on to increase in size and number so that, after about a week, the majority of cells are these floating spheres. This was a promising finding as not every cell type is able to form spheres in this way. For example, E12.5 epidermal cells in 1X SKP proliferation media did not make spheres even after 20 days in culture (Supplementary Figure S2). Toma et al. (2001) had a similar finding with epidermal cells (Toma et al. 2001).

The next step in trying to determine whether these dSKPs were truly SKPs was to characterise their expression. The literature has named Nestin, Vimentin and Fibronectin as commonly accepted markers of SKPs, while Sca-1, Versican and Wnt 5a are less characterised but have also been shown to be expressed in SKPs (Fernandes et al. 2004, Toma et al. 2005). Therefore, this project investigated the expression of Nestin, Vimentin, Fibronectin and Wnt 5a (discussed later in this chapter) at the protein level, due to available antibodies. Unfortunately, we did not have an

antibody for Sca-1 and we were not successful in optimising the Versican antibody we had. Immunofluorescent staining showed the expression of Nestin, Fibronectin and Vimentin for both E12.5 and E13.5 dSKPs and was, thus, reflective of a characteristic SKP profile (Fernandes et al. 2004, Toma et al. 2005).

There has been less investigation in the SKP literature of markers usually associated with stem cells: for example, Sox2 and OCT4 (both transcription factors involved in the maintenance and self-renewal of embryonic stem cells) (Kellner and Coulombe 2009, Liu et al. 2013, Li et al. 2016). In this project, we also wanted to look more generally at the potential multipotency of the dSKPs, so immunofluorescent staining of Sox2 and OCT4 was investigated. Neither showed nuclear staining for E12.5 or E13.5 dSKPs. The lack of Sox2 expression in E13.5 dSKPs was supported by the RT-PCR data. This is surprising, as you would expect the expression of some stem cell markers in SKPs. In contrast to these findings, Biernaskie et al. (2009) showed Sox2 was expressed in SKPs (derived from neonatal mouse back skin) at both the protein and transcript level (Biernaskie et al. 2009). In addition, Hill et al. (2012) showed the expression of both OCT4 and Sox2 in an RT-PCR of adult SKPs derived from P3 and P12 fibroblast monolayers. However, this is a less traditional method of producing SKPs and so may not be comparable (Hill et al. 2012).

The majority of studies would agree that the most important characteristic of a SKP is its ability to differentiate to neural and mesodermal lineages as, by definition, SKPs are a multipotent precursor population and, consequently, must have the ability to differentiate to several lineages (Toma et al. 2001, Fernandes et al. 2004). In order to explore this crucial characteristic, this study pushed E13.5 dSKPs to adipogenic and osteogenic lineages via the use of differentiation media.

The observation that around 50% of E13.5 dSKPs underwent adipogenic differentiation but less than 2% underwent osteogenic differentiation (when in differentiation media) was an unexpected finding and implied that the E13.5 dSKPs lacked the MSC-like potential of SKPs. This posed the question of why this was lacking. In my hands, E12.5 dSKPs also underwent less than 1-2% osteogenic differentiation (Supplementary Figure S5) so it is unlikely that this is related to E13.5 dSKPs deriving from cells that are further committed. This led me to question why, in this case, would a population that is less developmentally advanced in its progression of differentiation prove to be less able to become more SKP-like than older embryonic and adult cells? The literature has shown that SKPs made from adult skin and older embryonic SKPs (E15-E19) are capable of differentiating to both neural and mesodermal lineages, such as neurons, glial cells, smooth muscle cells, osteoblasts and adipocytes (the latter two only shown in adult SKPs) (Toma et al. 2001, Fernandes et al. 2004, Kang et al. 2011). It would seem counter-intuitive that these early embryonic dSKPs do not show the same level of potency. This is suggestive that either DCs (or even just a subpopulation of DCs) are altered during development (for example, as a result of

further differentiation) so that they are more able to produce SKPs. Alternatively, perhaps SKPs produced from older skin are derived from a population that is only present in the dermis later in development. These concepts will be discussed in more detail later in this chapter.

The differentiation assays in this project involved plating the SKPs in 2D. Therefore, changes in gene expression during the adherence of SKPs was tested. Supplementary Figure S5 shows immunofluorescent staining of E12.5 and E13.5 dSKPs cultured in 1X SKP adherence media after 48 hours (in 2D). Fibronectin and Vimentin expression still seem to be retained and H3K9me3 (a histone modification associated with silenced genes) appeared to display a gradient of expression, indicating that cells migrating at the edge of the explant are undergoing more gene expression changes than those in the centre (Krishnan, Horowitz, and Trievel 2011). Therefore, 2D culture appears to induce some changes in gene expression but the characteristic SKP markers seem to be maintained. Consequently, the lack of osteogenic differentiation of E12.5 and E13.5 dSKPs does not seem to be the result of the effects of 2D culture. However, changes in the expression of other SKP markers should be tested to confirm this.

In light of E13.5 dSKPs not acting like 'true' SKPs, E14.5 dSKPs and E15.5 dSKPs were produced as a comparison. Aside from Fernandes et al. (2004) showing SKPs older than E14 produced 'true' SKPs, these time points were especially of interest due to the significant changes that occur in the skin, such as hair follicle formation and the speculated neural crest migration around E14.5 (Mort, Hay, and Jackson 2010, Sennett and Rendl 2012). Formation of these late embryonic dSKPs in cell culture was very similar to E12.5 and E13.5, with the exception of more adhered cells in E14.5 and E15.5 dSKPs. Immunofluorescent characterisation was indicative of the same expression profile of Nestin, Fibronectin and Vimentin staining for both E14.5 and E15.5 dSKPs and no nuclear OCT4 was expressed by E15.5 dSKPs.

However, when E13.5, E14.5 and E15.5 dSKPs were quantified, a small reduction in dSKP-forming ability was directly correlated with the increasing age of the dermis. An interesting finding from this data was that regardless of the number of cells originally seeded (ranging between 100,000 and 500,000) between 3,000 and 4,000 dSKPs appeared to form irrespective of age (data not shown). Perhaps this is a flaw of the quantification technique used, which was quite rudimentary and requires more repeats (a more accurate technique would be to use fluorescent activated cell sorting (FACS)), or it is indicative of dSKPs representing a subpopulation present even at the more homogeneous stage of E13.5. This may be true of E14.5 and E15.5 dSKPs, where a greater number of cells remained adhered, but very few adhered cells were observed in E13.5 dSKPs after 8 days. This would imply the full population of cells became SKPs and yet the dSKP counts did not reflect this. One possible explanation is that the aggregation of cells has produced these dSKPs rather than single cell proliferation. Kawase et al. (2004) showed this to be a real possibility when

chimeric spheres formed from a culture of an equal number of wildtype and GFP ear skin cells (Kawase et al. 2004). However, other groups have established the clonality of SKPs either via clonal expansion or BrdU (Fernandes et al. 2004, Joannides et al. 2004). Unfortunately, due to time constraints, this was not tested in this project.

Returning to the characterisation of these dSKPs: so far, we have seen very little difference between the expression profiles of the early (E12.5, E13.5) and late (E14.5, E15.5) embryonic dSKPs. This led to a broader investigation of dSKP gene expression (via RT-PCR) for genes associated with embryonic neural crest determination and migration (Slug, Twist, Snail, Sox9, P75, Pax3), dermal development (SHOX2, Dermo1), the DP (Nexin, Versican, Wnt 5a), stem cell maintenance (Sox2) and neuroepithelial stem cells and SKPs (Nestin) (Fernandes et al. 2004, Toma et al. 2005, Sellheyer and Krahl 2010, Liu et al. 2013). There were three key findings from the RT-PCR.

Firstly, and perhaps unsurprisingly, changes seemed to take place between the whole E13.5 dermis to E13.5 dSKP and the whole E14.5 dermis to E14.5 dSKP. However, genes (for example, neural crest, stem cell and DP markers) were switched off in this process of whole dermis to dSKP formation which may be more surprising (Figure 5.7). This could be a sign of dedifferentiation but this contradicts the literature which discusses a neural crest and/or DP origin for SKPs (Fernandes et al. 2004, Kellner and Coulombe 2009). Consequently, one would expect these markers to be retained. Alternatively, it could mean that dSKPs were derived from a dermal population not expressing these distinctive markers but, again, this goes against the literature. This is a limiting factor of performing RT-PCR on the full dermis: it is hard to know if the full population shares the expression of the gene in question or if it is only a small number of cells. Lineage tracing techniques would be more informative regarding the origin of SKPs which will be further discussed later in this chapter.

Secondly, there appeared to be a distinct difference in gene expression between E13.5 and E15.5 dSKPs: most clearly shown by the absence of Snail, P75, Versican, Wnt 5a and Sox2 in E13.5 dSKPs. These genes are associated with the neural crest, DP and stem cells (Fernandes et al. 2004, Liu et al. 2013). Considering the whole E13.5 dermis does not express most of these genes (due to the lack of hair follicles and neural crest migration at this stage), it is not that surprising the E13.5 dSKPs reflect this. Nonetheless, this lack of expression is significant if it means E13.5 dSKPs are unable to differentiate like a 'true' SKP. It is particularly significant that the neural crest markers, Snail and P75, were not expressed in E13.5 dSKPs but were in E15.5 dSKPs. This implies that the neural crest migration (speculated to occur around E14.5) has influenced the dermis at E15.5 (Mort, Hay, and Jackson 2010). This potentially means E15.5 DCs have a broader differentiation capacity, as a result of the external influence of the neural crest migration. Thus, the original

hypothesis - that the dermis contains a common precursor responsible for producing all cell types of the dermis - may not be entirely true and perhaps should be changed to the following: the dermal embryonic common precursor population is more limited in the lineages it can produce and requires the influence of other cells not present at this early stage of development. This influence from other cells could be the neural crest. In a similar line of thinking, the lack of the DP markers in E13.5 dSKPs and dermis (naturally, due to lack of hair follicles at this stage) could mean the epidermal involvement in hair follicle formation is the influencing cell population in this new hypothesis. These two possibilities will be further explored later in this chapter.

Finally, there seemed to be a progressive acquisition of this characteristic SKP gene expression profile from E13.5 to E15.5 dSKPs (as shown by the intermediate profile of E14.5 dSKPs), implying that the emergence of dSKPs with 'true' SKP characteristics is a gradual process rather than a binary one. One of the reasons E14.5 dSKPs were investigated in addition to E15.5 dSKPs was to look at the influence the emergence of hair follicles had on SKP formation (Sennett and Rendl 2012, Fu and Hsu 2013). Apparently, this significant event in development had minimal impact on dSKPs as only P75 expression was switched on at this stage. However, it may have a role in priming the changes that result in the distinct expression profile of E15.5 dSKPs.

Overall, the RT-PCR data confirmed what the differentiation assays had led us to believe: the E13.5 dSKPs are not 'true' SKPs. However, it has raised some issues with the literature that states embryonic skin younger than 14 days cannot produce spheres, when they did so in this project. In addition, these findings have raised questions regarding the original hypothesis: the common dermal precursor is capable of producing all cell types of the dermis and warrants further discussion.

5.4.3 The New Hypotheses

In response to E13.5 dSKPs being shown to not be a 'true' SKP, the hypothesis (that the dermis contains a common precursor responsible for producing all cell types of the adult dermis) has come in to question. This leads us to discuss three new hypotheses that may better explain these findings. Firstly, that the dermal embryonic common precursor population is more limited in the lineages it can produce and requires the influence of other cells not present at this early stage of development and this influencing population may be the epidermis (particularly with regards to its role in hair follicle formation) (Sennett and Rendl 2012, Fu and Hsu 2013). Secondly, this influencing population could also be the neural crest migration which is speculated to occur around E14.5 (Mort, Hay, and Jackson 2010). Finally, the third hypothesis relates again to the population being more restricted but that this restriction is 'lifted' as the cells progress further down the path of differentiation.

5.4.3.1 The New Hypothesis: Epidermal Influence

The first morphological sign of pelage hair follicle formation is at E14.5 in mice when epidermal placodes become visible in response to a dermal signal (Sennett and Rendl 2012, Fu and Hsu 2013). Therefore, E14.5 is a significant time point in skin development and hair follicle induction is an obvious explanation of why dSKPs differ between E13.5 and E15.5 stages.

Hair follicle cells (more specifically, the DP cells) have been shown to provide a niche for SKPs. SKPs produced from the DP not only express Sox2 (in accordance with the DP and lower DS in anagen) but are capable of initiating hair follicle formation, dermal differentiation and migrate to the DP and DS area *in vivo*. They therefore display similar expression profiles and functional properties to the Sox2 positive cells of the DP. This is indicative of SKPs being 'real' *in vivo* which is a point of much debate (Biernaskie et al. 2009, Hunt, Jahoda, and Chandran 2009, Kellner and Coulombe 2009, Ruetze et al. 2013). Biernaskie et al. (2009) discussed the idea that the adult dermal precursors that SKPs may derive from (for example, Sox2 expressing cells of the DP) were themselves derived from embryonic mesenchymal precursors that acquired the expression of Sox2 as a result of interaction with the epidermal precursors. These epidermal precursors may in fact be the Nestin expressing cells shown to migrate from the bulge area (an epidermal stem cell niche) to the DP (Fernandes et al. 2004, Uchugonova et al. 2011). This concept also fits with the finding that the formation of the epidermal placode (observed at E14.5) subsequently leads to the formation of the dermal condensate that later forms the DP. Therefore, this reinforces the idea of an epidermal influence and crosstalk with the DCs, particularly those that eventually become the DP (Sennett and Rendl 2012, Fu and Hsu 2013). In addition, Hunt et al. (2008) showed that the vibrissa DP was a site of 1,000-fold enrichment for papillaspheres (which have very similar characteristics to SKPs) compared to whole facial skin of rats (Hunt et al. 2008). This indeed supports the new hypothesis that the influence of the epidermis is important in dermal development and by extension SKP formation (Biernaskie et al. 2009). In keeping with the idea that hair follicles provide the niche for 'true' SKPs, Figure 5.5 unsurprisingly indicated no nuclear Sox2 staining in the early dSKPs and the RT-PCR data supported this. However, the immunofluorescent data also indicated that no Wnt 5a (another DP marker) was labelled in the later dSKPs. This may be due to the antibody used, especially as the RT-PCR data showed Wnt 5a was expressed in E15.5 dSKPs but was not expressed in the earlier dSKPs. Similar findings were also shown for Versican (another DP marker) in the RT-PCR (Fernandes et al. 2004).

Unfortunately, there is one great flaw in this hypothesis: glabrous (hairless) skin is capable of producing SKPs (Toma et al. 2005, Ruetze et al. 2013). This does not mean the epidermis and dermis do not influence each other but it cannot have as significant a role on the common dermal precursor as previously thought. Ruetze et al. (2013) compared the quantities of SKPs produced from anatomical locations with differing hair follicle densities-scalp, breast, abdomen and foreskin

(glabrous). They found no link between the numbers of SKPs and density and also found Sox2 negative cells were capable of producing spheres. Therefore, alternative sources of SKPs to the DP must exist (Ruetze et al. 2013). This brings in the idea that SKP populations are heterogeneous as multiple sources within the dermis will most likely result in SKPs with varying characteristics. Agabalyan et al. (2017) have discussed this heterogeneity in the context of Sox2 positive cells from the hair follicle displaying unique qualities of induction not shared by dermal progenitor cells outside of the hair follicle (Agabalyan et al. 2017).

This calls into question the location of this other SKP niche. Perivascular cells have been their speculated location due to the papillary dermis showing a 5-fold increase in its ability to produce SKPs than the reticular dermis, the fact that the papillary dermis is more vascularised than the reticular dermis and because the SKPs expressed CD146 (a vascular endothelium marker) (Li et al. 2003, Ruetze et al. 2013). However, further work is needed to confirm this.

Overall, despite the evidence supporting epidermal influence on the dermis leading to 'true' SKPs, it cannot be the reason, or at least not the sole reason, for the differences seen in DCs and dSKPs at different embryonic stages. Experiments using glabrous skin have negated the epidermal influence hypothesis and, with this, we turn to the neural crest migration.

5.4.3.2 The New Hypothesis: Neural Crest Influence

The neural crest (a transient, stem-like population) is speculated to migrate to the dorsolateral skin at around E14.5 and so, yet again, E14.5 shows itself to be a crucial time point in embryonic skin development (Mort, Hay, and Jackson 2010). Previously, migrating neural crest cells were thought to terminally differentiate once they had reached their peripheral destination. However, it has been postulated that some of these multipotent neural crest precursor cells are maintained (possibly as SKPs) in some tissues into adulthood: for example, in the bulge area of the whisker follicle (Sieber-Blum et al. 2004, Fernandes, Toma, and Miller 2008, Liu and Cheung 2016). In addition to this, the similarities of the SKP gene expression profile to embryonic neural crest cells and the gradual acquisition of neural crest markers with increasing age of embryonic dSKPs (P75 switched on in E14.5 dSKPs, and Snail in E15.5 dSKPs) were the reasons behind this new hypothesis (Hagner and Biernaskie 2013).

As previously mentioned, Fernandes et al. (2004) were not able to produce SKPs with embryonic skin younger than E14 and found an increase in the ability to produce SKPs between E15 and E19 (Fernandes et al. 2004). This goes against the trend observed in Figure 5.3 of a small decline in SKP yield correlating with increased age but this was only looking between E13.5 and E15.5 dSKPs. This increase in SKP yield with age (from E15) has been linked to SKPs having a neural crest origin on the basis that they have similar characteristics to neural crest stem cells: for example, the ability to migrate along the neural crest migratory pathways when transplanted *in ovo* (Fernandes

et al. 2004, Toma et al. 2005). Therefore, it has been hypothesised that SKPs will only form after neural crest migration has taken place.

5.4.3.2.1 Exploring the Origin of SKPs

The high levels of similarities between SKPs and neural crest stem cells has led to the investigation of whether SKPs are neural crest-derived (Toma et al. 2005).

Fernandes et al. 2004, have used Wnt1-Cre/R26R neural crest reporter mice (cells expressing Wnt 1 will express β -galactosidase, allowing visualisation) to track the location of neural crest-derived cells in the skin. All SKPs produced from neonatal whisker pads of these reporter mice showed β -galactosidase expression. This, therefore, indicated neural crest-derived cells were present. This is not surprising considering facial skin is well-known to be neural crest-derived, confirmed by Fernandes et al. (2004); in which vibrissae DP expressed β -galactosidase in E18.5 facial skin (Fernandes et al. 2004). This project used dorsolateral skin, the mesenchyme of which is not neural crest-derived like the facial skin (Wong et al. 2006, Jinno et al. 2010). Therefore, this finding does not tell us much about the origin of the SKPs used in this project. However, Wong et al. (2006) investigated adult dorsal skin also using Wnt1-Cre/R26R mice to track neural crest-derived cells. They showed that β -galactosidase was expressed in the bulge area, the pigmented melanocytes and the nerves of adult back skin but not in the DP, DS or outer and inner root sheaths. In addition, all the SKPs produced from the back skin of these adult mice expressed β -galactosidase and this expression was maintained even after passaging (Wong et al. 2006). The data discussed here supports the hypothesis that SKPs are neural crest-derived and the DP is one niche for them. However, one drawback of this technique is that the expression of neural crest markers (for example, Wnt 1) may be acquired as a result of the changes to cell properties and gene expression that can occur in culture (Shoshani and Zipori 2015). Therefore, expression of neural crest markers may not necessarily mean these SKPs are neural crest-derived. This is a strong possibility as dorsal trunk skin has been shown to be somite-derived using a Myf5-Cre line and, when SKPs produced from the same region of skin were tested, they also showed a somite origin (Jinno et al. 2010). Perhaps, this reflects SKPs produced from an additional dermal source that is not neural crest-derived.

Despite the differences in origin, Liu and Cheung (2016) showed that facial and trunk SKPs have similar transcriptomes, differentiation capabilities and functions (Liu and Cheung 2016). This would imply the neural crest migration has minimal impact on SKP formation, which questions the seeming importance of neural crest markers' expression. Jinno et al. (2010) postulated that neural crest-like cells may arise from alternative origins to the neural crest. Many cells and tissues (for example, the dermis) are derived from both neural crest and mesodermal origins, depending on their anatomical location (Fernandes, Toma, and Miller 2008, Jinno et al. 2010). This could be one

solution to the conundrum of SKP origin. Alternatively, these dorsal SKPs may be derived from neural crest stem cells that do not express Wnt 1 (Fernandes, Toma, and Miller 2008).

Even though the origin of SKPs is yet to be fully ascertained, the concept of the neural crest migration raises an important point that the cell populations in the skin are not static during development and this is something to consider when researching this area.

Overall, SKPs from the dorsal skin appear to have neural crest and somite origins. It is unknown whether this represents two separate SKP niches in the dermis of differing origins, whether this is a result of changes in cell properties and gene expression (due to the culture environment) or because of converging developmental pathways (Shoshani and Zipori 2015). All that can be drawn from this project's findings is that the lack of neural crest and DP markers in SKPs is significant for its ability to function. However, more work is needed to elucidate the possible role of the neural crest migration.

5.4.3.3 The New Hypothesis: Differentiation of Early Embryonic DCs Eliminates the Restricted dSKP Phenotype

The advancement in differentiation of the DCs between E13.5 and E15.5 is obviously another key reason for differences between these cells. It could, therefore, be postulated that this is simply the reason for the differences in the dSKPs they produce. However, this way of thinking would imply that cells further down a pathway of differentiation are able to produce 'true' dSKPs, in contrast to more primitive, less differentiated cells, which are not. This defies the longstanding idea of younger cells being more plastic and stem-like than older cells. However, this suggests embryonic cells may be subjected to a greater level of regulation early on in development, such as the regulation of transcription factors (Frum and Ralston 2015). The correct development is crucial to life and survival so it would not be surprising if more regulatory mechanisms were in place during these critical stages of cell fate decisions.

This still leaves the question of how SKPs express neural crest markers if the neural crest migration is not involved in SKP formation. The expression of these genes could be a result of the changes in cell properties and gene expression that can occur during cell culture. According to this theory, older embryonic cells have less regulatory mechanisms in place to prevent these changes and are thus able to express markers not observed in early embryonic dSKPs (Shoshani and Zipori 2015).

Another question this hypothesis raises is why these regulatory mechanisms would be lifted later on in development. Surely unwanted differentiation is something the body still wants to prevent, even in adults. One explanation for this is the need for the body to regenerate and replenish cells that are lost through injury or cell turnover (Dahl 2012). Thus, there is a need for resident somatic stem cell populations or a need for transdifferentiation or dedifferentiation of committed cells to

provide the correct cell types to replenish the lost cells (Fernandes et al. 2004). Hence, why regulatory mechanisms may be lifted in later development.

5.4.4 What are E13.5 dSKPs?

Now that it has been established that E13.5 dSKPs are not 'true' SKPs, we needed to address what they actually are. The differentiation assays showed an increased disposition towards the adipogenic cell fate compared to osteogenic. Therefore, E13.5 dSKPs were stained for the adipogenic markers Dlk1 and C/EBP α as a comparison to the E13.5 DC spheroid 3D culture model discussed in Chapter 4 (Figure 4.2) (Stephens 2012, Driskell et al. 2013). E13.5 dSKPs appeared to express Dlk1 and nuclear C/EBP α throughout (like the E13.5 DC spheroids), which strongly implies the presence of preadipocytes. This further confirmed the findings that E13.5 dSKPs are not 'true' SKPs, as a multipotent stem cell population should not be expressing preadipocyte markers without differentiation media. In addition, it implies a predisposition of these DCs to an adipogenic cell fate, perhaps as the 'default' cell type. However, this does not agree with the findings of Chapter 4, where freshly collected E13.5 DCs did not show extensive differentiation when cultured in adipogenic media. Therefore, even though E13.5 dSKPs are not 'true' SKPs, the production of dSKPs in culture appears to have led to changes in the properties of the DCs (as displayed by their different differentiation potentials).

Interestingly, this favouring of adipogenic over osteogenic differentiation in the E13.5 dSKPs was also shown by the E13.5 DC population (Chapter 4). Therefore, one could make a connection between this subpopulation of E13.5 DCs (that were capable of differentiation) and the E13.5 dSKPs. Perhaps this subpopulation of E13.5 DCs were the cells the E13.5 dSKPs derived from in culture.

5.4.5 Differentiation of 'true' SKPs?

The RT-PCR data strongly implied that the E15.5 dSKPs were 'true' SKPs due to their distinct gene expression profile which corresponded with the literature (Fernandes et al. 2004). On the other hand, E14.5 dSKPs had a less SKP-like profile. Based on this, they are, therefore, unlikely to be 'true' SKPs (Figure 5.7). In order to confirm this hypothesis, these older embryonic dSKPs were pushed to differentiate to adipogenic and osteogenic lineages. Unfortunately, though, the data does not show what one would have expected.

The E14.5 dSKPs appeared to undergo less than 15-20% differentiation to adipocytes and less than 1% to osteoblasts. This low level of differentiation is not too surprising since there was very little difference in the gene expression with E13.5 dSKPs. However, the reduction in adipogenic differentiation compared to the E13.5 dSKPs is odd (50% adipogenic differentiation in E13.5 dSKPs). This may be simply due to experimental error (these differentiation assays were not repeated as many times as we would have liked due to time constraints) or perhaps this is a

significant finding of the changes that occur at E14.5, such as the emergence of hair follicles and concomitant arrival of Wnt/ β -catenin signalling as a key communicator between the epidermis and dermis (as discussed previously in this chapter and Chapter 3) (Sennett and Rendl 2012, Fu and Hsu 2013). Wnt/ β -catenin signalling has an interesting role in relation to adipocyte development. It has been shown to have a role in promoting and repressing adipogenesis, depending on the location of its source and its family member (Donati et al. 2014, Mastrogiannaki et al. 2016). It could therefore be postulated that a possible reason for this decline in adipogenic differentiation is the arrival of Wnt/ β -catenin signalling between the epidermis and dermis. In order to investigate this further, the levels of LEF-1 (transcription factor downstream of Wnt/ β -catenin signalling) in the different aged dSKPs could be monitored, for example, using qRT-PCR (Driskell and Watt 2015).

For E15.5 dSKPs, around 50% of cells appeared to differentiate to adipocytes and 5% to osteoblasts. This is comparable to E13.5 dSKPs, although there is a minimal increase in osteogenic differentiation. However, it is still not what you would expect from a 'true' SKP. A 'true' SKP should consist of a stem cell population capable of differentiating to mesodermal and neuronal lineages (Toma et al. 2001, Fernandes et al. 2004). Consequently, *all* cells should differentiate when exposed to environmental cues and, thus, despite the gene expression profile being so convincingly a SKP, it cannot be said with certainty that these E15.5 dSKPs are 'true' SKPs. Their behaviour (shown in this project) is different to that described in the literature and there are a number of possible explanations for this (Toma et al. 2001, Fernandes et al. 2004).

Firstly, the differentiation media used in this project may not be optimal. There were a number of issues getting the positive control cells to differentiate to adipocytes and this unpredictability continued, even when a more successful media and alternative positive control cell line were used. Similarly, the osteogenic media used did not consistently push rMSCs (positive control cell line) to differentiate, despite having done so originally.

Secondly, the osteogenic assays were subject to fungal infections which cut the culture time short for E13.5 dSKPs, E15.5 dSKPs and rMSCs. Perhaps another week would have shown a significant increase in osteogenesis but it would unlikely be enough to warrant 'true' SKP status. In addition, the presence of fungus in wells neighbouring those that were stained may have impacted the cells' ability to differentiate. Obviously, repeats of all the differentiation assays are essential to properly draw conclusions but time constraints did not allow this.

Thirdly, passaged SKPs are commonly used in the literature for differentiation assays (Fernandes et al. 2004, Toma et al. 2005). This project successfully passaged E12.5 dSKPs twice (Supplementary Figure S3) but passaging slowed down the process of SKP formation and more cells were adhered to the flask than at P0. This may lead to a refinement of the population,

resulting in a population consisting only of cells capable of differentiation. Toma et al. (2005) compared P16 SKPs (maintained in culture for 1 year) with lower passage SKPs and showed they expressed the same markers (Nestin, Fibronectin and Vimentin) and were capable of differentiating to neurons, glial cells, smooth muscle cells. In addition, P0 SKPs were pushed to differentiate and showed very similar results to passaged SKPs (Toma et al. 2005). In contrast, Wang et al. (2014) showed SKPs lost their sphere-forming abilities after 3 or 4 passages (Wang et al. 2014). Thus, it would be interesting to compare passaged and P0 embryonic dSKPs in differentiation assays to elucidate if there is a difference between the differentiation abilities of these particular embryonic dSKPs.

Another difference is the location of the skin used. This project has used dorsolateral skin, whereas Fernandes et al. (2004) used facial and dorsal skin, Toma et al. (2005) used foreskin and Toma et al. (2001) used abdomen and back skin. Therefore, comparisons drawn from the literature need to take this into account but it should not be the reason for this lack of differentiation.

Finally, clonal expansion was often used to culture SKPs in addition to the ordinary culture technique and these clonally expanded SKPs were then used in differentiation assays. For example, when Toma et al. (2005) differentiated ordinary SKPs between P3 and P9 to neuronal lineages, only $9.4\% \pm 0.2\%$ of the cells differentiated. Whereas, when clonally expanded SKPs were pushed to differentiate, all the clones were able to do so (Toma et al. 2005). Fernandes et al. (2004), among others, showed similar findings of only subpopulations of ordinary SKPs differentiating to neuronal lineages compared to clonally expanded SKPs, where all clones differentiated to neuronal and mesodermal lineages (Fernandes et al. 2004). As discussed in Chapter 4, clonal expansion creates a stressful environment, inducing changes in cell properties, gene expression and refinement of the SKP population (Shoshani and Zipori 2015). This may result in an increased ability to differentiate. Toma et al. (2005) demonstrated this as, when SKPs (between P3 and P9) were dissociated and clonally expanded, only 40% of these isolated SKP cells proliferated (Toma et al. 2005). This highlights a major refinement of an already selectively cultured population when clonal expansion is used.

Nonetheless, the literature is unclear as to the percentage of cells within these clonally expanded SKPs that are able to differentiate. It only references 'subpopulations', which may mean a majority or minority of cells, with no lower magnification images included for clarification (Toma et al. 2001, Fernandes et al. 2004, Toma et al. 2005). Therefore, while this project has deemed E13.5 dSKPs, E14.5 dSKPs and E15.5 dSKPs as falling short of a 'true' SKP's definition, it may not have held them to the same standard as the literature.

5.5 Conclusions

To conclude, this chapter has shown that E13.5 dSKPs have a distinct gene expression profile that differs to the characteristic SKP profile shown in E15.5 dSKPs and in the literature (Fernandes et al. 2004). This difference in gene expression, lack of differentiation to adipogenic and osteogenic lineages and expression of adipogenic markers has led to the conclusion that E13.5 dSKPs are not 'true' SKPs. These findings indicate that this population is more restricted in the lineages it can differentiate to (than was previously thought) and that it may rely on the influence of external populations not yet present at E13.5. The epidermis (in relation to hair follicle formation) and neural crest migration were both discussed as possible candidates for this influential population but the epidermis was ruled out by experiments using glabrous skin. The neural crest, however, is still a possibility that requires further investigation. In addition, the changes differentiation leads to in the embryonic DCs was discussed as a possible explanation for the restricted differentiation profile. However, the fact that this E13.5 DC population was unable to produce a 'true' SKP *in vitro* may not necessarily mean this population has the same level of lineage restriction *in vivo*. This is the flaw of using *in vitro* conditions, particularly in 2D.

Finally, E14.5 and E15.5 dSKPs were shown *not* to be 'true' SKPs due to their lack of MSC-like potential. However, this raised questions over the extent of differentiation required to class as a 'true' SKP.

6. Final Discussion, Conclusions and Future Directions

The first project aim was to elucidate the different Wnt family members expressed in E13.5 skin and their localisation. The findings of this project and expression analysis data from our laboratory suggest that skin development involves different Wnt family members, each with different roles. This is highlighted by their different spatial and temporal expression. The literature has shown Wnts are secreted but has not yet elucidated the diffusion range and mechanism for individual Wnt family members (Mikels and Nusse 2006, Port and Basler 2010, Solis, Lüchtenborg, and Katanaev 2013). Consequently, research on the diffusion range of individual Wnt family members and the mechanisms that influence this range would aid the understanding and manipulation of this pathway and, by extension, its role in dermal development. In addition, looking more specifically at the distinct papillary and reticular dermal Wnt expression profiles in embryonic dermis, using flow cytometry, would complement and further the work done in adult skin by Mastrogiannaki et al. (2016). The findings of this project did show some of the Wnt family members tested were expressed in the epidermis (Wnt 2 and Wnt 11), in agreement with the microarray data, but none showed dermal expression and especially no differential expression in the papillary and reticular dermis. More work needs to be done in this area, so future investigation should explore all the Wnt family members and look at their expression over a range of time points (particularly those around E13.5 due to their role in hair follicle formation at these stages) (Fu and Hsu 2013). To further explore the roles of Wnt family members, the use of knockout and overexpression mouse strains for different Wnt family members would be a helpful tool in deciphering the function of the individual family members, particularly during development. For example, a Wnt 5a^{-/-} mouse strain was used to elucidate Wnt 5a's essential role in anorectal development and a mouse strain that overexpressed Wnt 5a in the epidermis only (using a K14 promoter) was used to investigate Wnt 5a's role in psoriasis (Tai et al. 2009, Zhu et al. 2014). A similar approach can be used in the context of skin development. In addition, it would be worth exploring whether, at the other time points not studied here, Nestin (which was later shown to be expressed in the dSKPs) shows the same expression profile in embryonic dorsolateral skin as in the embryonic scalp (Sellheyer and Krahl 2010).

The second aim of this project was to investigate the plasticity of the embryonic dermis at E13.5. This was approached by looking at their differentiation potential when cultured immediately after dissection and when pushed to become a SKP. The E13.5 DC differentiation experiments indicated that this population is less plastic than might have been anticipated but there is a small subpopulation that is more MSC-like and capable of adipogenic and/or osteogenic differentiation. When in ordinary media, the 3D and 2D culture showed contrasting results, which calls into question which type of cell culture, 2D or 3D, is more representative of *in vivo*. In future, it would be interesting to compare the 2D differentiation experiments shown here (freshly dissected DCs

and dSKPs) with 3D versions in order to elucidate how much of an influence the culture environment has on the stem cell properties of the embryonic DCs. In addition, investigating different embryonic time points and different lineages, such as chondrogenic, myogenic and neuronal, would help to further the understanding of the plasticity of embryonic DCs. To further analyse the E13.5 DC population, a TCF/LEF-1 reporter mouse strain could be used in conjunction with flow cytometry to isolate the papillary and reticular DC populations and, thus, allow the comparison of the differentiation potential and SKP forming ability of these separate populations.

Disappointingly, the dSKPs produced in this project did not match up with the definition of a 'true' SKP due to their lack of differentiation to multiple lineages. Passaging and clonal expansion of SKPs prior to differentiation were popular methods in the literature and have been postulated as reasons for the discrepancy with this project's findings. It could, therefore, be interesting to try these techniques on the embryonic dSKPs. However, to be confident in these differentiation assay conclusions, they need optimising, repeating and expanding to alternative lineages. Optimisation could involve 3D culture, as mentioned above, which may prove to be a more representative technique. Moreover, in the literature, adult SKPs have been tested for their functionality *in vivo* so this could be a more representative approach to investigate the capabilities of these embryonic dSKPs than 2D differentiation (Fernandes, Toma, and Miller 2008).

The findings of this project question the hypothesis (that the dermis contains a common precursor responsible for producing all cell types of the adult dermis), contradict the idea that the embryonic dermis contains a large MSC-like progenitor population and, thus, queries the capabilities of this common dermal precursor. This led to the postulation that the epidermis and/or neural crest influence the DCs, enabling a wider differentiation potential. The neural crest's role in the embryonic dermis, particularly in the dorsolateral dermis, warrants further investigation. This could be achieved by performing similar lineage tracing experiments with Wnt1-Cre/R26R mouse strains but looking at embryonic time points (E12.5-E15.5) rather than adult (Fernandes et al. 2004, Wong et al. 2006). Additionally, Nestin (a marker associated with SKPs) has been shown to be expressed in the DP (among other locations) and the DP has been postulated as one source of SKPs (Toma et al. 2001, Sellheyer and Krahl 2010). On this basis, it would be interesting to see if those cells expressing Nestin in the E13.5 dermis are those going on to produce dSKPs. This could be investigated using lineage tracing, as previously discussed.

7. Supplementary Information

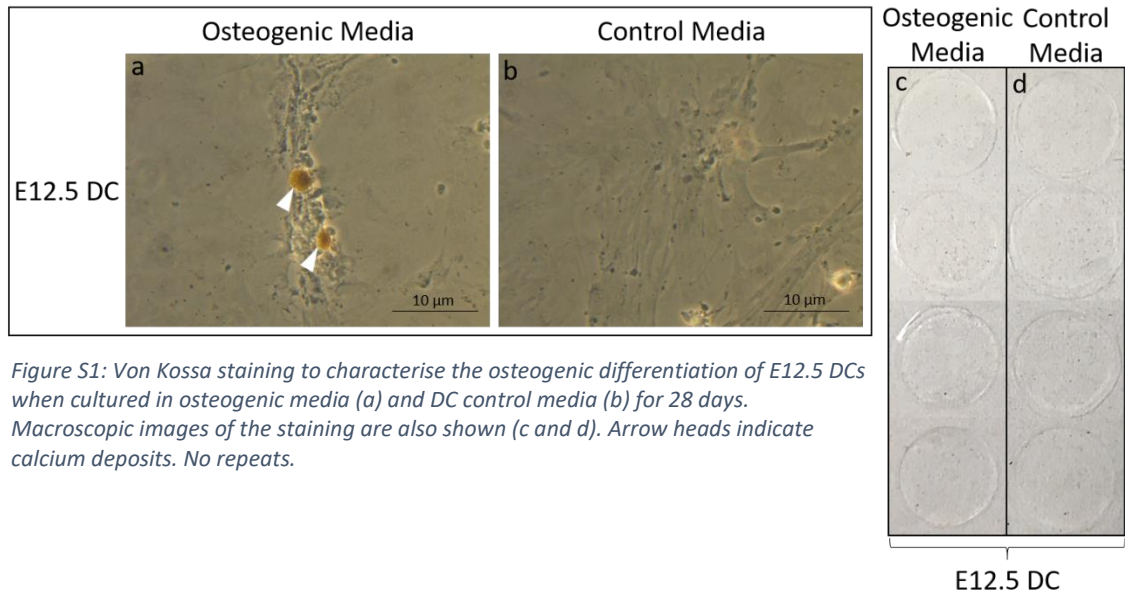


Figure S1: Von Kossa staining to characterise the osteogenic differentiation of E12.5 DCs when cultured in osteogenic media (a) and DC control media (b) for 28 days. Macroscopic images of the staining are also shown (c and d). Arrow heads indicate calcium deposits. No repeats.

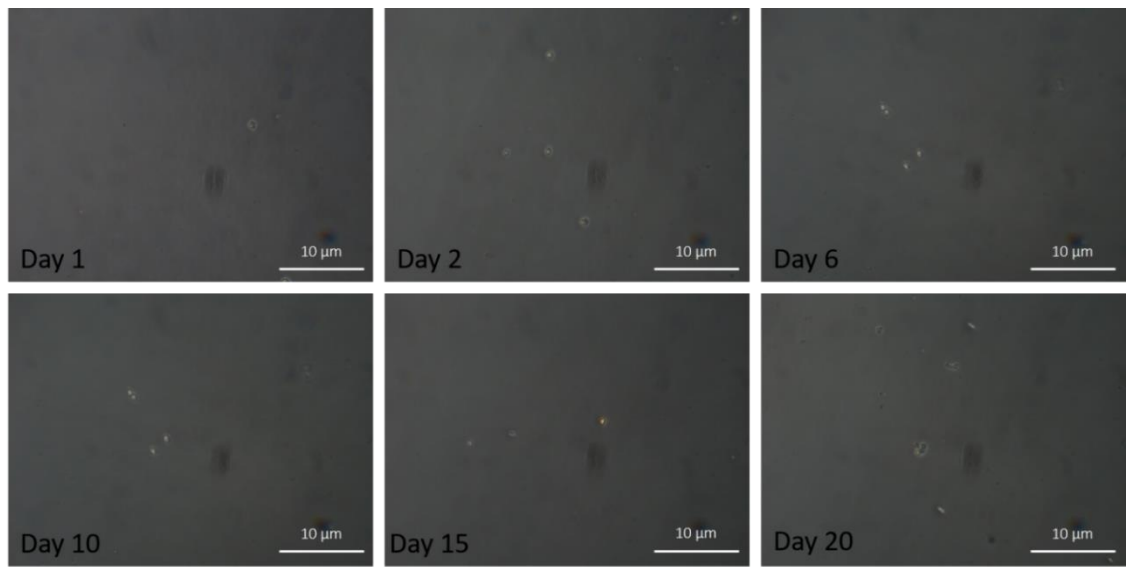


Figure S2: E12.5 epidermal cells cultured in 1X SKP proliferation media. No repeats.

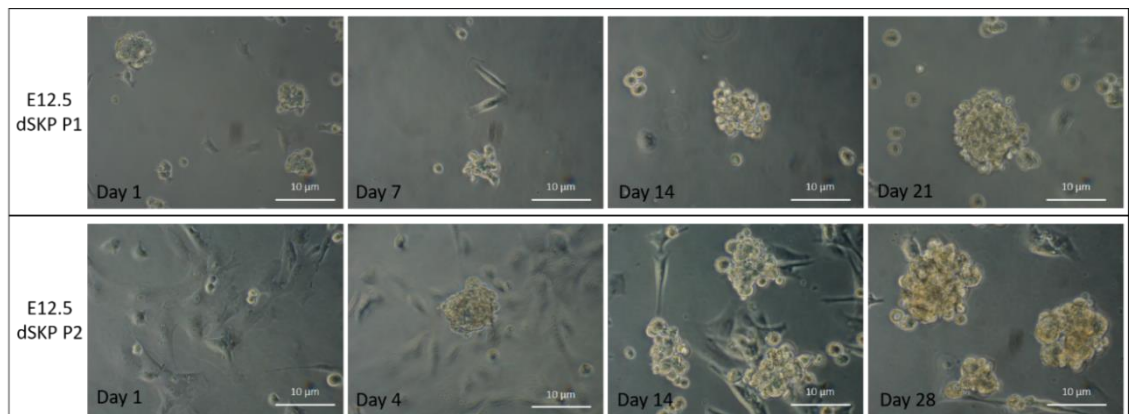


Figure S3: E12.5 dSKPs in culture after one and two passages (in 1X SKP proliferation media). No repeats.

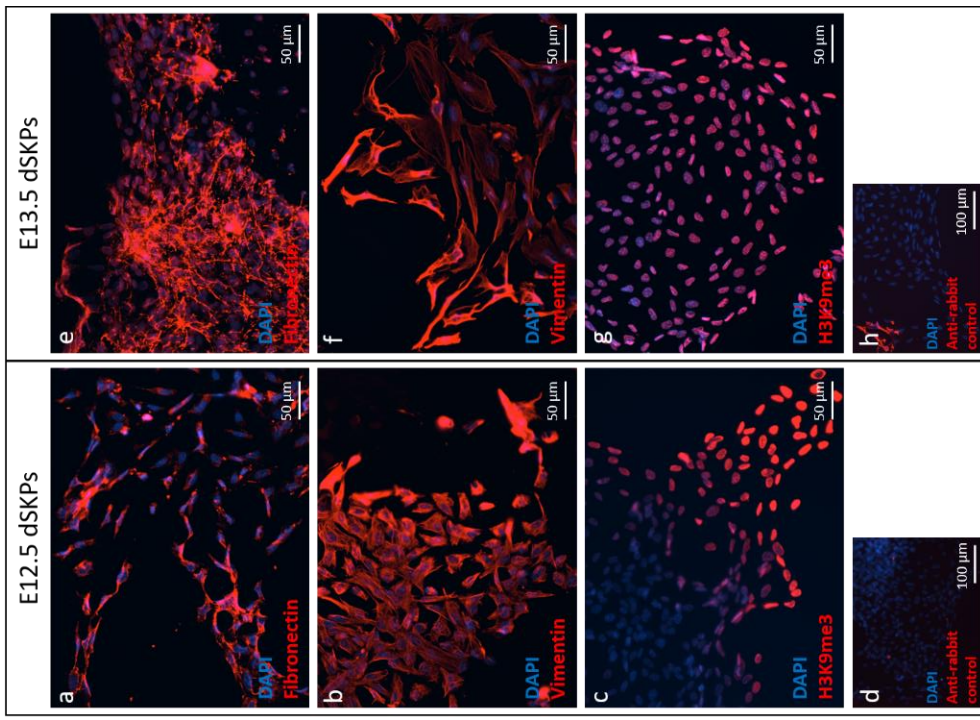


Figure S5: Immunofluorescent staining of 12.5 dSKP and 13.5 dSKP, when cultured in 1X SKP adherence media for 48 hours, for Fibronectin (a,e), Vimentin (b,f) and H3K9me3 (c,g). Arrow heads indicate nuclear staining. No repeats. E12.5 dSKPs were cultured for 7 days and E13.5 dSKPs 12 days before culture in 1X SKP adherence media.

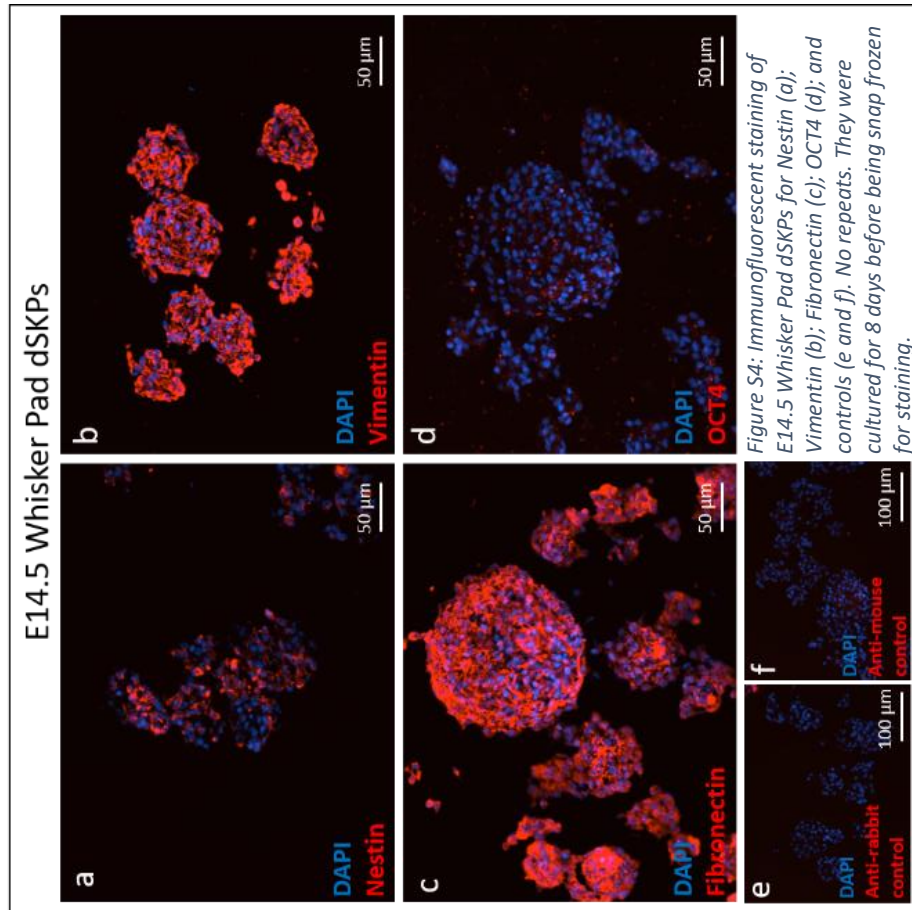


Figure S4: Immunofluorescent staining of E14.5 Whisker Pad dSKPs for Nestin (a); Vimentin (b); Fibronectin (c); OCT4 (d); and controls (e and f). No repeats. They were cultured for 8 days before being snap frozen for staining.

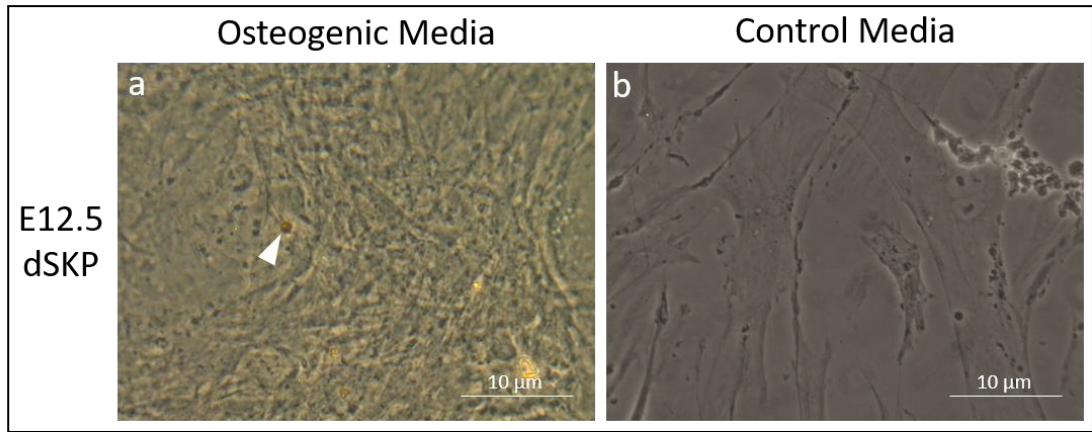


Figure S6: Von Kossa staining to characterise the osteogenic differentiation of E12.5 dSKPs when cultured in osteogenic media (a) and DC control media (b) for 28 days. Arrow heads indicate calcium deposits. No repeats. E12.5 dSKPs were cultured for 9 days prior to culture in osteogenic media.

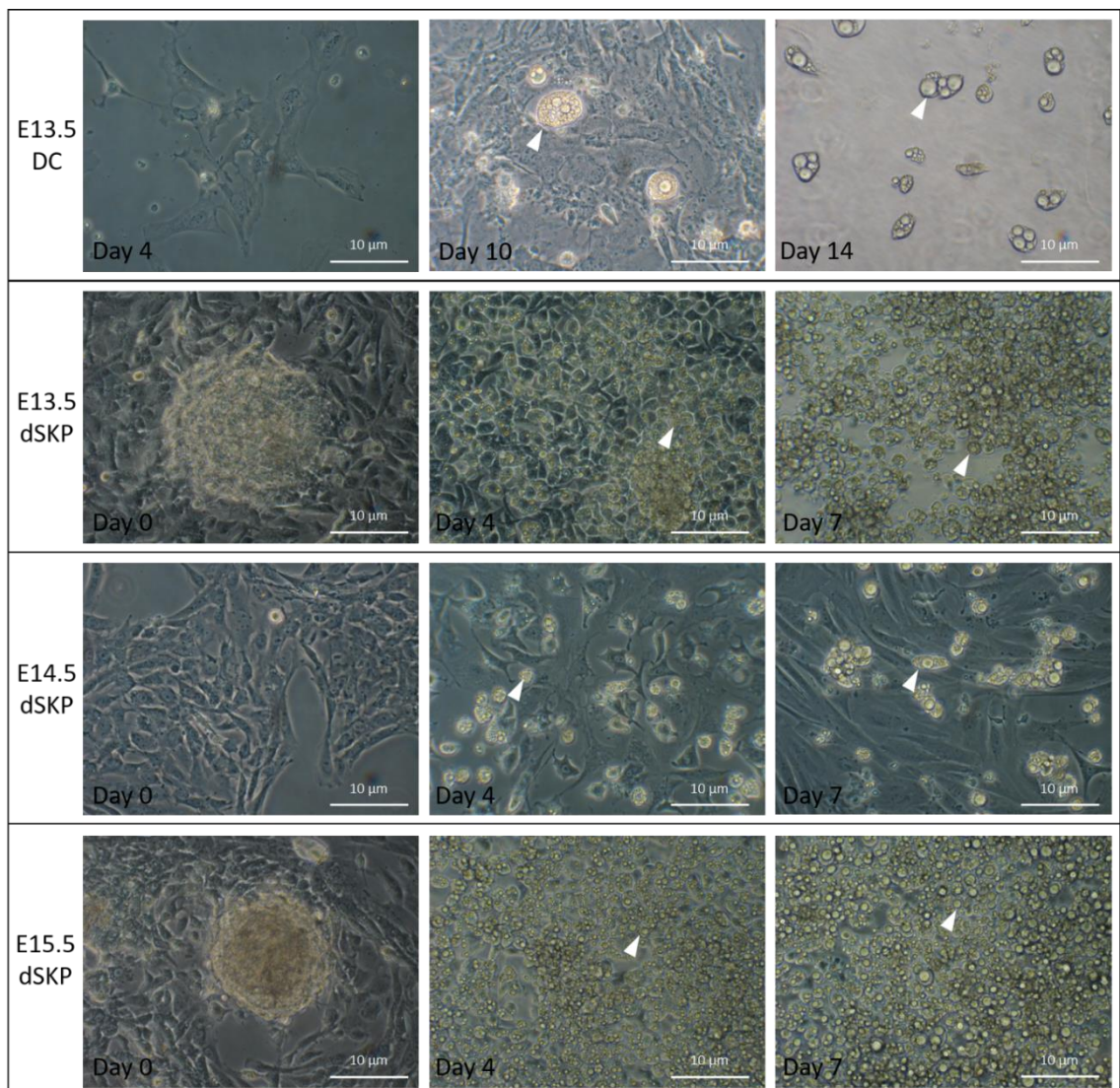


Figure S7: Cell culture images of the process of adipogenic differentiation of E13.5 DCs, E13.5, E14.5 and E15.5 dSKPs when cultured in adipogenic media. Arrow heads indicate adipocytes. E13.5 DC and E13.5 dSKP repeated once; E14.5 and E15.5 dSKP no repeats. The dSKPs were cultured for the following number of days before being plated in 2D: E13.5=8 days, E14.5=7 days, E15.5=7 days.

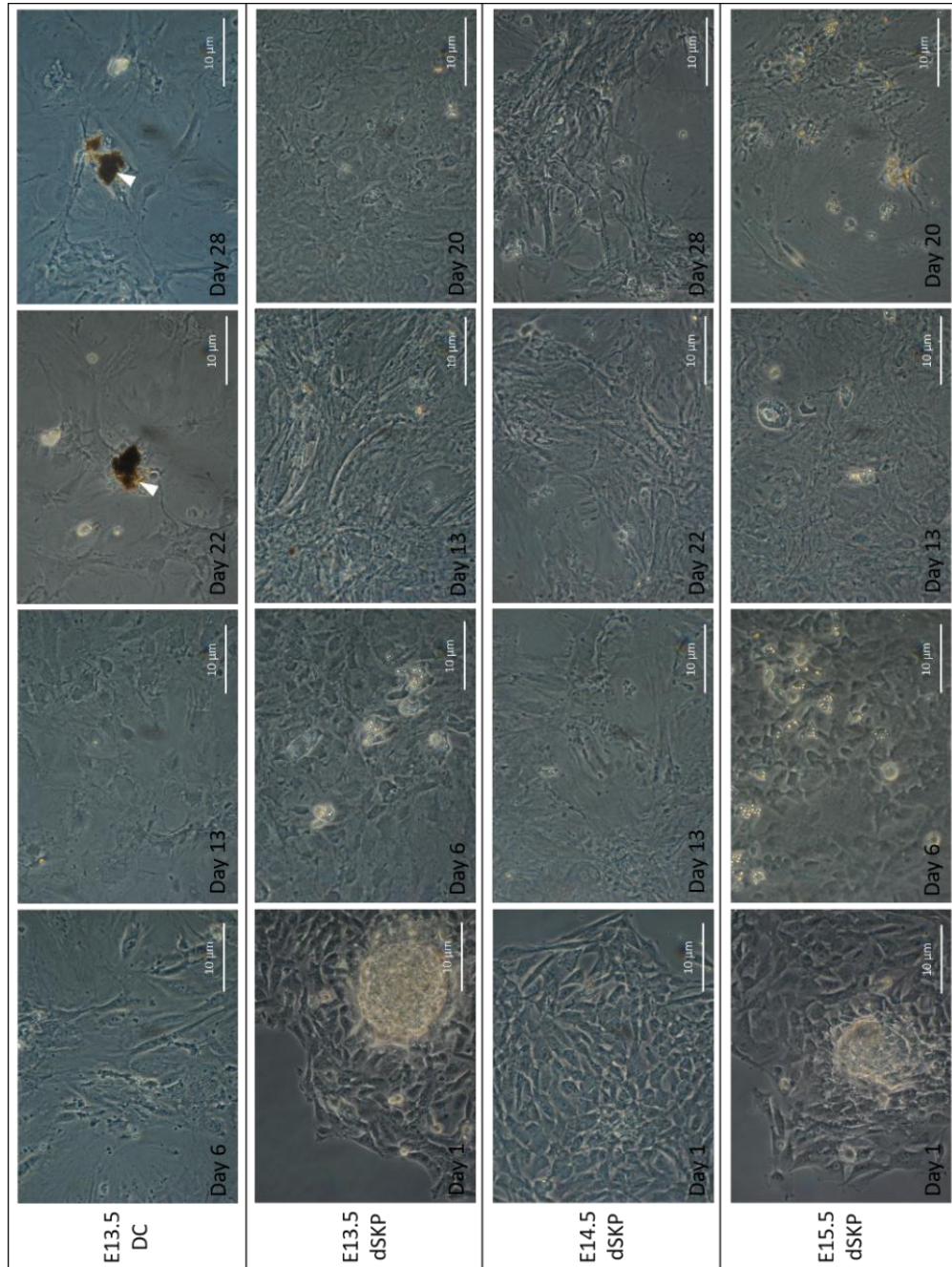


Figure S8: Cell culture images of the process of osteogenic differentiation of E13.5 DCs, E13.5, E14.5 and E15.5 dSKPs when cultured in osteogenic media. Arrow heads indicate calcium deposits. E13.5 DC and E13.5 dSKP repeated once (E13.5 DC unsuccessful repeat due to fungal infection); E14.5 and E15.5 dSKPs no repeats. The dSKPs were cultured for the following number of days before being plated in 2D: E13.5=8 days, E14.5=7 days, E15.5=7 days.

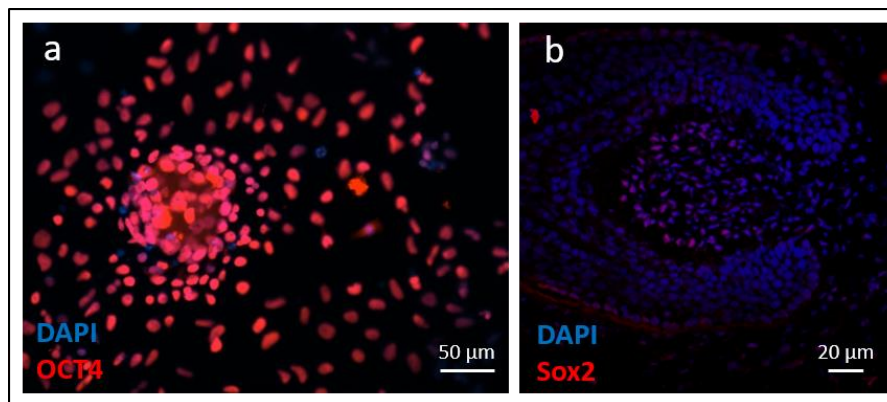


Figure S9: (a) OCT4 antibody staining of TERA.cl.sp12 cells courtesy of Hannah Broderick; (b) Sox2 antibody staining of a Lister Hooded rat hair follicle courtesy of Dr Elisa Carrasco.

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