Molecular Control of Tooth Mesenchymal Stem Cell Activation

By

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Dedication

There are so many people, without whom I would not have made it to this point. As there's a size limit on this thesis I can't list them all. But please know, that I am so very grateful to all my family, friends and colleagues for your unceasing support. I would like to take the time to personally acknowledge a few however, who have been especially instrumental in getting me through what has been a rewarding yet challenging journey.

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For my family

Abstract

Miss Jemma Victoria Walker

MOLECULAR CONTROL OF TOOTH MESENCHYMAL STEM CELL ACTIVATION

Background

The continuously growing mouse incisor provides a robust model for studying molecular mechanisms of stem cell (SC) fate determination. While the epithelial SCs are well studied within this model, the identification and characterisation of a mesenchymal SC (MSC) population has yet to be defined.

Aims

This study aims to identify the molecular signatures of a novel MSC pool within the lower murine incisor. This work aims to investigate the molecular mechanisms governing the maintenance and transition of MSC cells and their progeny, particularly the role of Notch signalling in this process.

Methods

Isolation of proposed putative MSC containing and known mesenchymal transit amplifying cell (MTAC) containing regions were undertaken. Laser capture microdissection and subsequent comparative analysis of MSC marker expression between populations was performed. Investigation of the molecular

mechanisms governing MSC activation and maintenance was undertaken. Specifically, the role of Notch signalling was investigated, through analysis of Notch pathway transgenic mouse models including tissue specific RBP-Jκ knock out, Dlk1 null, and tissue specific Dlk1 overexpressing strains. *In vitro* models were developed to validate findings.

Results

MTAC and MSC containing regions exhibited distinct expression signatures of quiescent MSC marker genes. Notch pathway ligands, receptors and downstream effectors were differentially expressed between these populations. Modulation of Notch signalling *in vivo* impacted the behaviour of incisor MSCs. Manipulation of Dlk1 *in vitro* identified it as a potent regulator of MTAC maintenance.

Conclusions

The novel endogenous MSC population exists within the mouse incisor mesenchyme. The MSCs give rise to cells of the MTAC region, which in turn express Notch ligand Dlk1. Dlk1 is pivotal in balancing the lineage differentiation and maintenance of the incisor MSCs. Thus, Notch signalling plays a key role in the molecular regulation of the activation of tooth mesenchymal stem cells.

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Table of Abbreviations

Abbreviation	Unabbreviated form
2D	Two-Dimensional(ly)
3D	Three-Dimensional(ly)
ASPA	UK Animals Scientific Procedures Act 1986
BMC(s)	Bone Marrow Cell(s)
BMSC(s)	Bone Marrow Stem Cell(s)
BSA	Bovine Serum Albumin
CD1	ICR (CD-1®) Mice
ChIP	Chromatin Immunoprecipitation
cKO	Conditional Knock Out
CL(s)	Cervical Loop(s)
CL-MSC(s)	Cervical Loop Mesenchymal Stem Cell(s)
Co-A	Co-activators
Co-R	Co-repressors
Conc^N	Concentration
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate treated distilled water
DMEM	Dulbecco modified Eagle's medium
DMEM/F12	Dulbecco modified Eagle's medium/F12
DMP	Dentine matrix protein
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DP	Dental Pulp
DPSC(s)	Dental Pulp Stem Cell(s)
DSPP	Dentine sialophosphoprotein
EDTA	Ethylenediaminetetraacetic acid
ESC(s)	Embryonic Stem Cell(s)
EpSC(s)	Epithelial Stem Cell(s)
ETAC(s)	Epithelial Transit Amplifying Cell(s)
ETDC(s)	Epithelial Terminally Differentiated Cell(s)
FBS	Fetal Bovine Serum
FFPE	Formalin Fixed Paraffin Embedded
FUCCI	Fluorescent Ubiquitination-based Cell Cycle Indicator
gDNA	Genomic Deoxyribonucleic Acid
HBSS	Hank's balanced salt solution
HERS	Hertwig's Epithelial Root Sheath
HFE	Human Fertilisation & Embryology

HFEA	Human Fertilisation & Embryology Authority
hESC(s)	Human Embryonic Stem Cell(s)
HSC(s)	Haematopoietic Stem Cell(s)
ICM	Inner Cell Mass
IDE	Inner Dental Epithelium
IF	Immunofluorescence
IMS	Industrial Methylated Spirit
iPSC(s)	Induced Pluripotent Stem Cell(s)
IVF	*In Vitro* Fertilisation
KO	Knock Out
LCM	Laser Capture Microdissection
M1, M2, M3	1st Molar, 2nd Molar, 3rd Molar
MTDC(s)	Mesenchymal Terminally Differentiated Cell(s)
MEF(s)	Mouse Embryonic Fibroblast(s)
mRNA	Messenger Ribonucleic Acid
MSC(s)	Mesenchymal Stem Cell(s)
MTAC(s)	Mesenchymal Transit Amplifying Cell(s)
NICD	Notch Intracellular Domain
NRES	National Research Ethics Service
NVB(s)	Neurovascular Bundle(s)
NVB-MSC(s)	Neurovascular Bundle Mesenchymal Stem Cell(s)
Od(s)	Odontoblast(s)
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Triton
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PIC	Protease Inhibitor Cocktail
PMSF	Phenylmethylsulphonyl Fluoride
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SC(s)	Stem Cell(s)
Shh	Sonic Hedgehog
TAC(s)	Transit Amplifying Cell(s)
TDC(s)	Terminally Differentiated Cell(s)
Wnt	Wingless
WT	Wildtype
w/w	Weight by weight

Acknowledgements

Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.
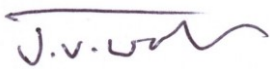
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Presentation at Conferences:

Conference Name	Presentation Level	Title of Presentation (if given)
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British Society for Oral and Dental Research (Cardiff – 2015)	Oral Presenter	Molecular Control of Dental Mesenchymal Cell Development & Regeneration (Senior Colgate Prize Commendation Winner)

Plymouth University Peninsula Schools of Medicine & Dentistry Annual Research Event (Plymouth – 2016)	Oral and Poster Presenter	Molecular Control of Dental Mesenchymal Tissue Development & Regeneration
Plymouth University Peninsula Schools of Medicine & Dentistry Annual Research Event (Plymouth – 2017)	Oral and Poster Presenter	Molecular Control of Dental Mesenchymal Tissue Development & Regeneration (Best oral presentation prize winner)
British Society for Oral and Dental Research (Plymouth - 2017)	Oral Presenter	Notch Signalling Controls Tooth Mesenchymal Stem Cell Activation (Senior Colgate Prize Finalist)

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Signed………. J.v.uᴑᴧ …….

Date……………20/11/2018………….

1 Background

1.1 Stem cells

Stem cells (SCs) are undifferentiated cells which can both self-renew and give rise to progeny of specific fates (Bolontrade and García, 2016). When discussing SCs it is important to note that SCs can be categorised by the tissue from which they originate and also by their potential to differentiate.

Figure 1. Differentiation potential decreases as cells differentiate
Schematic of mammalian cell potency restriction through development.
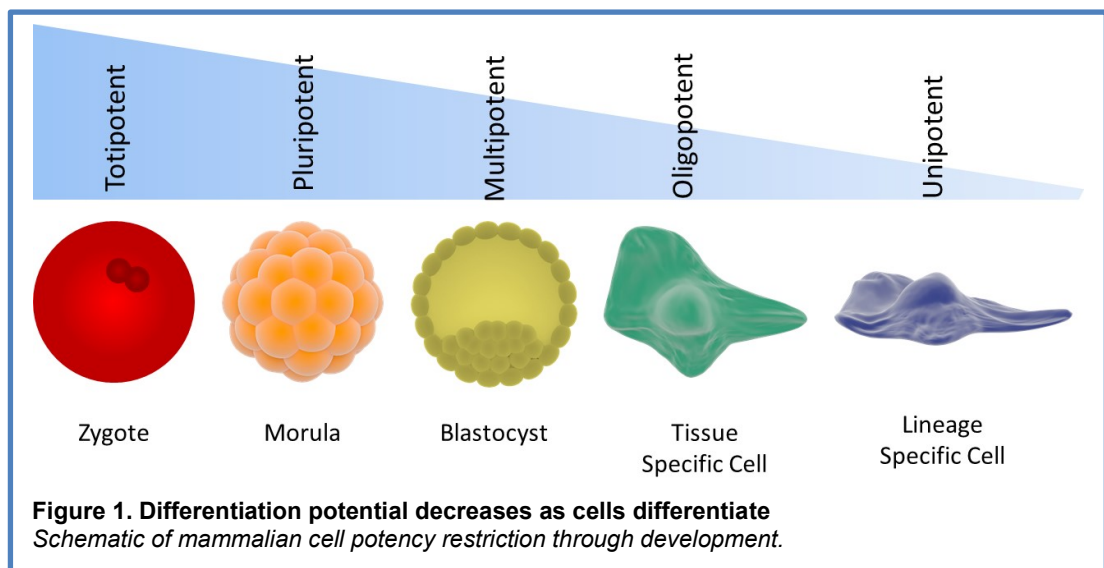
Within the embryo until the completion of the first few cellular divisions, cells are described as totipotent as they can give rise to all the extraembryonic material (Baker and Pera, 2018). As the embryo develops these cells begin to commit to develop along specific lineages. Through this process of commitment, cells lose their potential to differentiate along other lineages (Figure 1). In mammals, at conception the newly formed zygote has potential to differentiate into any cell type which will make up the embryo and the extraembryonic tissue (Condic, 2014). Once the early embryo has formed the multicellular morula, the potential of the cells is moderately restricted. By blastocyst stage, the Inner Cell Mass (ICM) cells are multipotent as they can give rise to the multiple lineages within the embryo

proper, however they can no longer give rise *in vivo* to the extra embryonic material. Within the foetal and adult tissues, cells are generally committed to give rise to only the cells of a specific tissue type. Generally, cells which can give rise to a specific subset of differentiated cell types are described as pluripotent. Further differentiation gives rise to lineage specific cells, which are only able to differentiate further into one mature cell type. Once the cells have reached their fate, many differentiate to a point whereby they can no longer enter the cell cycle and are described as terminally differentiated cells (TDCs) (Serrano, 2010, Hsu et al., 2014).

The regulation of SCs is a vital process in both homeostasis and development (Simons and Clevers, 2011, Lane et al., 2014). Careful coordination of the cell cycle is necessary to balance the often opposing needs for growth and differentiation (Ruijtenberg and van den Heuvel, 2016). If this regulation is lost or disturbed, tissue loss and aberrant growth can result (Simons and Clevers, 2011). While aberrant growth can cause excessive tissue growth, it has also been observed that over proliferation of SCs can result in the depletion of SC pools, which can lead to tissue loss and ultimately failure (Singh and Hansen, 2017). Similar loss of tissue has also been observed when premature differentiation of SCs occurs due to dysregulation of SC maintenance, such as in aging (Choi and Artandi, 2009).

The molecular mechanisms which underpin the maintenance and regulation of SCs have been widely investigated. The findings of this large body of work have shown a great deal of tissue and temporal specificity (Jung and Brack, 2014).

1.1.1 Embryonic stem cells

It was long suspected that pluripotent SCs existed within the developing embryo, however it was not until 1981 that these cells were first successfully isolated and cultured *in vitro* (Evans and Kaufman, 1981). These pluripotent cells were defined as embryonic stem cells (ESCs) and characterised for their ability to proliferate continuously in culture, as well as their ability to differentiate when cued to do so. Traditionally ESCs are defined as cells from the ICM of a blastocyst stage embryo, which have the capacity to divide without differentiating spontaneously in culture (NIH, 2009). The technique used to isolate these ESCs involved the destruction of the blastocyst stage embryo. This technique was adapted and later used to isolate the first SCs from the ICM of a human embryo, as outlined in Figure 2 (Thomson et al., 1998).

Figure 2. The isolation and propagation of human embryonic stem cells traditionally requires the co-culture of cells with growth factor producing fibroblasts
A Initially mesenchymal cells are cultured adherently as "feeder" cells. These cells are often Mouse Embryonic Fibroblasts (MEFs). B A biopsy of the Inner Cell Mass of a blastocyst is then performed, extracting one or more of these cells. C The biopsied cells are then plated on top of the MEFs, which function to provide necessary molecular signals to encourage the embryonic cells to grow and maintain pluripotency. D The culture is then allowed to expand, thus increasing the number of embryonic cells. This schematic is adapted from (Gepstein, 2002).

Concerns over the ethical implications of destroying human embryos for the derivation of these cells, drove the development of embryo preserving techniques of cell isolation (Ilic and Ogilvie, 2017). Ultimately single blastomeres were able to be biopsied from cleavage stage embryos (which themselves were shown to be pluripotent cells) while the remaining embryo was still able to continue to develop (Chung et al., 2008, Klimanskaya et al., 2006). While this circumnavigated some of the ethical debate that had arisen, as these cells are not derived from the blastocyst stage embryo, there were new concerns that these cells may not fall under the guidelines and regulations that govern human embryonic stem cell (hESC) work.

The potential uses of hESCs clinically and in research are wide ranging. Perhaps the most widely publicised potential application for hESCs is in SC transplantation, as a therapeutic intervention to encourage regeneration and repair (Keller and Snodgrass, 1999). In order to develop clinical grade hESCs, which could be used in this manner, there is a drive towards animal product free hESC propagation (Stephenson et al., 2012, Lukovic et al., 2014).

SC therapy employs the ability of hESCs to differentiate to produce new tissues within a damaged or deficient host. Currently, studies are underway to investigate the therapeutic potential of SC therapy in the treatment of macular degeneration (Ouyang et al., 2016), diabetes (Lysy et al., 2016) and spinal cord injuries (Manley et al., 2017). In addition to exploiting the differentiation potential of hESCs to produce new tissues, researchers have been able to produce potentially viable natural killer cells which could go on to help fight cancer *in vivo* (Woll et al., 2005). Recent studies have gone on to suggest that even non-somatic cell types, such

as gametes could be made from hESCs as a therapeutic alternative for infertile couples (Bhartiya et al., 2017).

Due to the proliferative properties and differentiation potential of hESCs, such cells are also valuable tools for academic researchers. Whether using them in the naïve SC state or through first differentiating them, hESCs provide insight into the mechanisms that govern embryogenesis, cell cycle regulation and cell fate determination (De Jaime-Soguero et al., 2017, Dvash et al., 2006, Chen et al., 2017b).

One interesting application for hESC is the production of cell lines which contain disease-specific mutations. Human ESCs can be derived from embryos carrying these specific mutations, which is confirmed by preimplantation genetic diagnosis (Ilic and Ogilvie, 2017). By using such cell lines researchers are able to investigate the specific effects of these natural mutations on the cells' behaviour, thus creating a better understanding of the disease and the molecular mechanisms which underpin it.

The limitations of the applications of hESCs in research and in clinic, are both ethical and scientific (Lo and Parham, 2009). One of the major concerns about *in vitro* cell culture is that spontaneous mutations have been observed within the genome of the cells being cultured (Rebuzzini et al., 2016). While these mutations can be beneficial for the proliferation and survival of these cells (Amir et al., 2017), they pose a hazard to the reproducibility of research conducted using these cell lines.

When considering the use of hESCs in SC therapies, their proliferative potential is both their strength and their weakness. The characteristics of hESCs are

similar in nature to those of cancer cells, their persistence in an undifferentiated state and long proliferative life span along with reduced levels of apoptosis, in particular (Werbowetski-Ogilvie et al., 2009). These features, among others, indicate a potential for hESCs to form teratomas if they are administered into a human as part of a therapeutic intervention (Herberts et al., 2011, Hentze et al., 2009). Furthermore SCs have been shown to affect the growth of existing tumour cells (Prockop and Olson, 2007), suggesting that the administration of hESCs to patients with underlying tumours could cause further tumour cell proliferation. The innate properties of hESCs, coupled with the spontaneous acquisition of genetic alterations in culture make the use of hESCs in SC therapies a potential tumour formation risk.

As with all cellular transplantation, SC therapy carries with it a risk of the graft rejection by the host. Current data suggests that while SCs can elicit an immune response (Nussbaum et al., 2007, Swijnenburg et al., 2007) hESCs more often have a low immunogenic potential (Li et al., 2004) and even immune regulatory activity (Mohib et al., 2010). However, while the administered cells begin as pluripotent hESCs they are able to differentiate within the host and may acquire greater immunogenic properties (Herberts et al., 2011).

Once hESCs are used as a therapeutic treatment their specificity and efficacy are yet to be proven (Shroff and Barthakur, 2015). SCs have been shown to be able to migrate away from the location of administration (Imitola et al., 2004), raising concerns that both the therapeutic effects and negative side effects of SC therapies could act on unpredictable sites.

Arguments against the use of hESCs for ethical reasons have been long debated and brought more publicly than the scientific limitations. Particularly with the

effectiveness of hESCs, as research and therapeutic tools, yet to be proven and the possibility of pluripotent cells being obtained by other means (Takahashi and Yamanaka, 2006). The primary concern of many scientists and lay persons alike, remains the destruction of the human embryo for the derivation of hESCs (Sherley, 2004). Techniques have improved that allow the biopsy of cells from an embryo without causing it to arrest, both for use in the production of hESCs and also for preimplantation genetic diagnosis (Chen et al., 2017a, Pickering et al., 2003). However the manipulation of human embryos is a tightly regulated process, in the UK this falls under the 1990 Human Fertilisation and Embryology (HFE) Act (Lovell-Badge, 2008). As part of this act, it is stated that embryos cannot be kept in culture for more than 14 days, as after this time the developing nervous system begins to form and the embryo loses its ability to form into twins (Cavaliere, 2017, Hyun et al., 2016). As hESCs are taken from the early blastocyst, this does not involve using embryos past this cut off and so the process is governed under many of the same regulations as other embryonic interventions and research. In order to be granted permission and funding to derive hESCs in the UK a licence must be granted by the HFE Authority (HFEA). Often the embryos used are created by *in vitro* fertilisation (IVF) and are surplus to the requirements of the patients receiving treatment, however embryos can be produced solely for the purpose of deriving hESCs (Lovell-Badge, 2008). In addition to HFEA licences permission from the National Research Ethics Service (NRES) is also necessary. Once derived, hESCs are no longer considered under the HFEA's jurisdiction. The process of using IVF to make embryos specifically for hESC derivation is in itself another moral grey area due to the physical burden that oocyte recovery places on the donor.

Although hESCs have great potential in clinical and research fields, the many ethical and scientific limitations of these cells have made the rate of advancement in these areas slow. Consequently, alternative sources of pluripotent SC have been greatly investigated.

1.1.2 Postnatal stem cells

Within the developing embryo, SCs must proliferate and differentiate in order to pattern and grow into the appropriate tissues. Postnatally , while less patterning and development is observed, tissue homeostasis and response to injury require a great deal of cellular plasticity (Jessen et al., 2015) (Ge and Fuchs, 2018). In postnatal tissues, TDCs make up the majority of cells. TDCs are often damaged or lost due to injury, ageing and disease. The inability of most TDCs to re-enter the cell cycle (Myster and Duronio, 2000) limits the regenerative capability of postnatal tissues however multipotent cells persist within many regenerative postnatal tissues in order to maintain tissue homeostasis.

Multipotent postnatal SCs were first identified within the bone marrow (Till and Mc, 1961) and have since been identified in other tissues including neural, epithelial & mesenchymal derived organs (reviewed in (Gonzalez-Perez, 2012, Bianco et al., 2008, Blanpain et al., 2007)). The field of postnatal SC research has grown rapidly over the last two decades, leading to the discovery of postnatal SCs in many different organs (Figure 3). It is believed that most identified postnatal SCs play important roles in regeneration and repair of the tissue and so are intriguing research targets which may potentially be important in the development of regenerative therapies (Barry and Murphy, 2004).

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Figure 3. Adult stem cells have been identified and isolated from many human tissues
Since their initial discovery within the bone marrow, adult SCs have since been identified within all of the tissues depicted here (non-exhaustive list) (Diaz-Flores et al., 2006, Gonzalez-Perez, 2012, Stange, 2013, Naveau et al., 2014, Hugnot and Franzen, 2011, Yu et al., 2015).
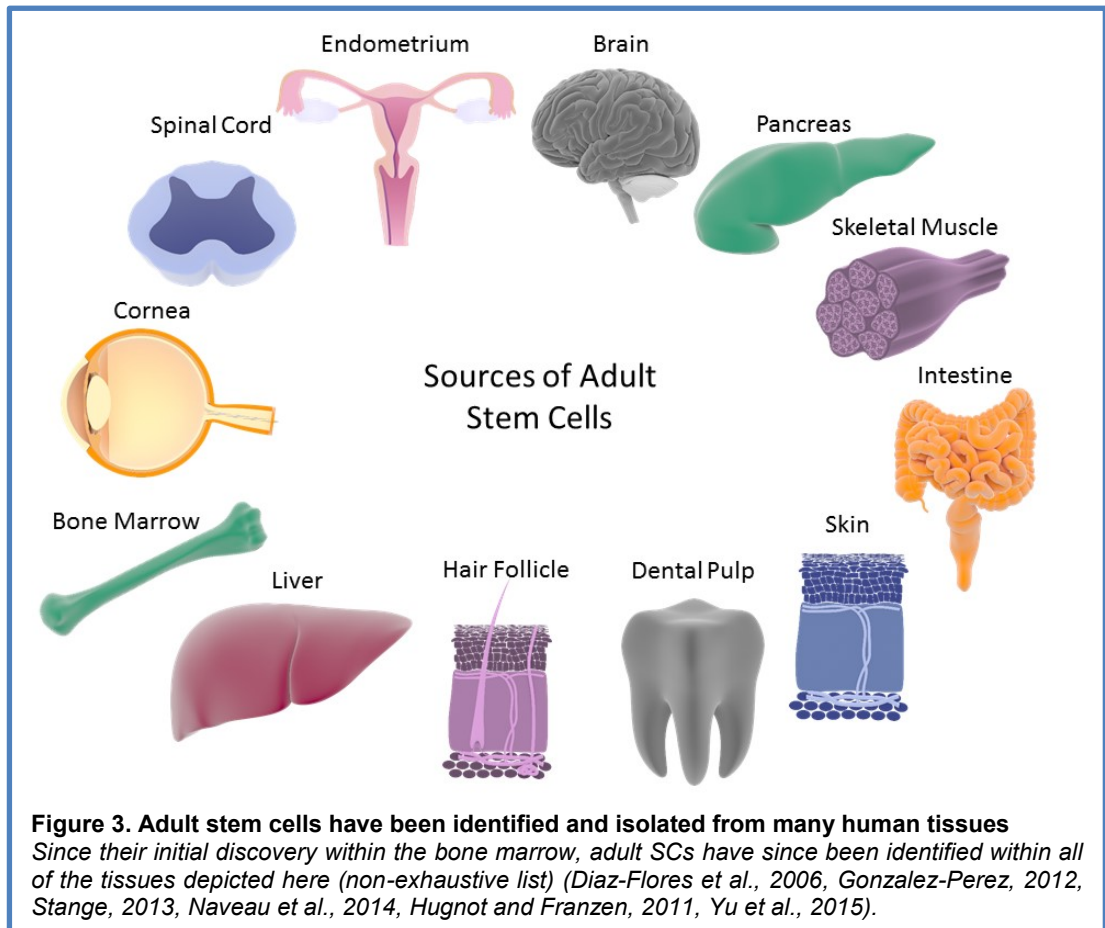
As with hESCs, postnatal SCs have been used in research and clinically. Postnatal SCs are often committed to a specific germ layer or tissue lineage, as such they can be very useful research tools for the investigation of tissue specific mechanisms and behaviours. In addition to their uses in molecular research, postnatal SCs have been used therapeutically. Since the first successful allogenic transplantation of SCs in the treatment of cancer (Thomas et al., 1971) interest in the therapeutic applications has increased, however the successful utilisation of such therapies have been limited (Watt and Driskell, 2010). Questions about the limited efficacy of existing therapies have led to vast debate about the ethical implications of providing these therapies (Poulos, 2018). In addition to the direct utilisation of SCs in therapeutic procedures, recent studies have begun investigating the possibility of using the exosomes and secreted factors. For example the exosomes of postnatal SCs have been collected and

applied successfully to promote regeneration of the myocardium after ischemic heart attack (Safari et al., 2016).

Despite these successes in the field, the isolation and propagation of postnatal SCs has proven to be a substantial research challenge. The relative low abundance of SCs within postnatal tissues makes isolation of SCs particularly difficult. Once isolated, postnatal SCs can often be difficult to culture two-dimensionally (2D) due to the loss of the tissue architecture and supporting cells from the niche. In order to circumnavigate the issues of 2D culture, postnatal SCs are often grown more successfully in a three-dimensional (3D) organoid culture (Drost and Clevers, 2017). As with 2D cultures, postnatal SCs grown into organoids have been used in the study of disease progression, drug action and for therapeutic transplantation. Organoid culture can be performed without the need for SC isolation from the original tissue (Sato et al., 2009) by using small pieces of material. Organoid expansion of postnatal SCs has been successfully employed in the modelling of drug actions in specific genetic disorders such as cystic fibrosis (Dekkers et al., 2013). These 3D cultures have also shown great promise in understanding host pathogen interactions (Leslie and Young, 2016). Within the field of infection and immunity alone, the clinical promise of 3D cultured postnatal SCs is wide ranging. Already, postnatal SC organoids have been used to culture immune cells which could ultimately be reintroduced therapeutically into patients (Nozaki et al., 2016). These early studies into the clinical applications of 3D cultured tissues have shown promising results across many tissue types. It has been the retention of characteristics from the tissue of origin, by organoid cultured SCs has also been a key factor in the successful engrafting of organoids back into a donor following *in vitro* expansion (Yui et al., 2012).

1.1.3 Mesenchymal stem cells

Mesenchymal tissue is derived from the embryonic mesoderm (and neural Crest in some tissues (Noden, 1988)) and develops into blood, connective tissues and skeletal tissues (Figure 4).

Figure 4. The three germ layers and their derivatives
Within the early developing embryo three predominant germ layers develop (in addition to the germ cell precursors). From these three germ layers all adult tissues are derived (Kiecker et al., 2016). The neural crest is a specialised group of cells found in certain vertebrates. Cells derived from the neural crest include those which make up many mesenchymal like tissues such as those of the craniofacial bone, cartilage, neural tissue and muscle (Hall, 2018).

Mesenchyme is characterised by its loosely organised stromal cell structure surrounded by abundant extracellular matrix. Within many mesenchyme derived tissues, mesenchymal stem cells (MSCs) have been discovered and isolated. The first such tissue identified as containing MSCs was the bone marrow (Caplan, 1991). Haematopoietic stem cells (HSCs) had previously been identified in the

bone marrow and found to give rise to the cells of the blood. For many years previously, experiments using bone marrow had shown a propensity for osteogenesis within the cells of the marrow (Tavassoli and Crosby, 1968). The stromal cells from within the bone marrow were later identified as being the causative cells and were also shown to be able to differentiate into bone, cartilage and adipose (Friedenstein et al., 1987) (Figure 5). The isolation of these stromal bone marrow cells (BMCs) lead to the resultant derived cells being termed as MSCs when referring to them *ex vivo* (Bianco et al., 2008).

Figure 5. Within the bone marrow, two well described stem cells pools have been identified
Within the bone marrow, BMSCs are a form of MSCs which are capable of giving rise to the skeletal tissues including (but not limited to) adipocytes, chondrocytes and oligodendrocytes. The hematopoietic stem cells are also found within the bone marrow and are able to differentiate into the cell types which make up the blood, including the red and white blood cells. Figure adapted from (Yin and Li, 2006).

The term "mesenchymal stem cell" has caused much controversy (Bianco et al., 2008). One of the most largely debated issues with the nomenclature is that not all mesenchymal like tissues are in fact derivatives of the early mesoderm, for

example the bones of the craniofacial region are of a neuroectodermal origin (Olsen et al., 2000).

Since the discovery of bone marrow mesenchymal stem cells (BMSCs) , pools of mesenchyme derived SCs have been identified and characterised in many tissues including, bone, muscle and fat (Ba et al., 2012, Caplan, 1991, Gopinath et al., 2014, Beltrami et al., 2007, Jiang et al., 2002). Although these cells are committed to differentiate into tissue specific lineages *in vivo*, they have been shown to have the potential to differentiate along many mesenchymal lineages when given appropriate cues to do so experimentally.

In order to identify isolated cells as true MSCs, their stemness, multipotency and self-renewal properties must be assessed (Bianco et al., 2008). Isolating and expanding MSCs has posed a technical issue to researchers. In part due to the fact that they often behave similarly to non-SCs within the same tissues, when cultured. For example, BMSCs have been shown to always be clonogenic, frustratingly many other non-stem cell cells within the bone marrow are also capable of forming colonies. However, when these cells are transplanted into a host, only the true BMSCs are multipotent (Gronthos et al., 2003). Following the discovery of MSCs, the signature of expression of cell surface antigens has been widely used as a method of distinguishing BMSCs from their haematopoietic neighbours (Simmons and Torok-Storb, 1991, Boxall and Jones, 2012, Ramos et al., 2016). Primarily the expression of CD44, CD73 and CD90 identified BMSCs from their CD45 expressing HSC counterparts within the marrow. In addition to these marker proteins being expressed within MSCs, they also play an important role in maintaining the pluripotent state of the cells and regulating differentiation (Moraes et al., 2016).

Within solid tissues, many of these antigens are harder to identify and so other marker profiles have been investigated to aid in the identification of MSCs within these tissues. An effort to consolidate the vast array of work done to identify MSC specific marker genes has been undertaken. It was found that across different MSC models, similar signalling pathways are at work, including those involved in coordination of the cell cycle, mitochondrial function and transcriptional regulation (Cheung and Rando, 2013). Interestingly many of the genes identified in this study were linked to cell cycle regulation, with those involved in promoting progression being downregulated in quiescent MSCs, while genes known to negatively regulate the cell cycle were upregulated.

1.2 Stem cells and the cell cycle

Postnatal SCs often exist in a state of quiescence, where the cells still have the potential to divide but are not in an active state of mitosis (Figure 6) (Oki et al., 2014). It is believed that by maintaining mitotic quiescence, SCs are protected from deoxyribonucleic acid (DNA) damage that is likely to occur through the cell cycle process. Therefore, allowing the cells to persist within the tissue until they are required to activate to participate in growth and repair.

The quiescent SCs differ from other non-dividing cells, such as TDCs, which do not readily re-enter the cell cycle (Serrano, 2010). Quiescent cells are slow cycling or resting in a G0 state, which then can be activated to exit mitosis, given the correct cues and favourable biological conditions (Larsson et al., 1985, Cheung and Rando, 2013).
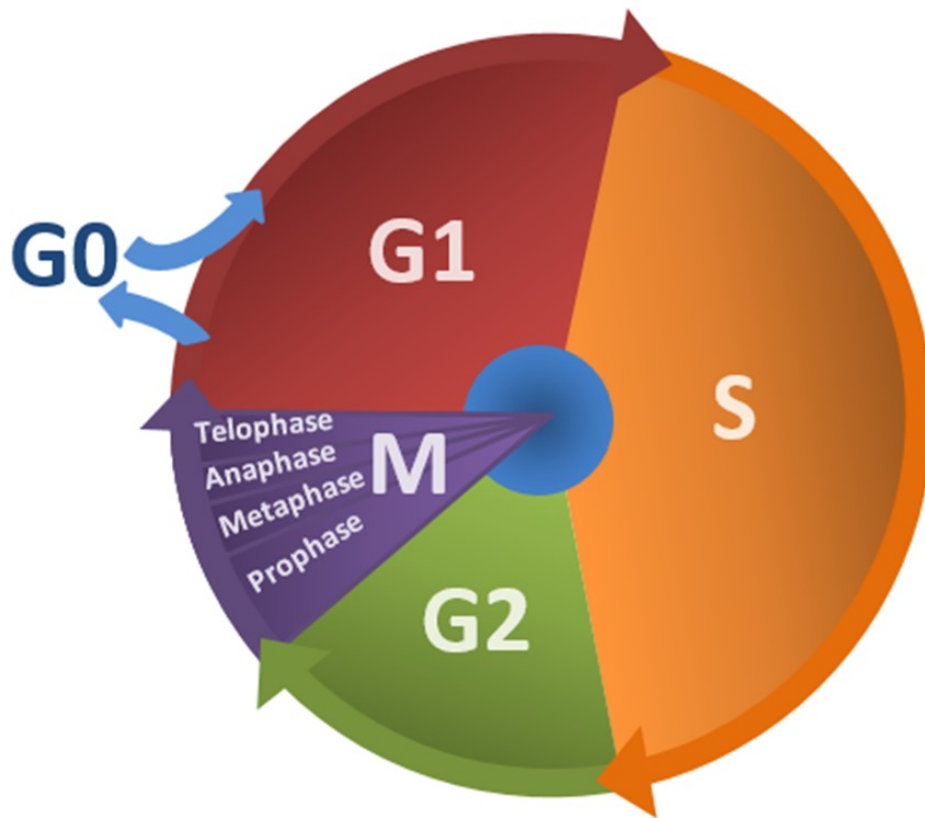
Figure 6. The cell cycle consists of phases of growth, synthesis, mitosis and rest
Stages G1, S and G2 are defined as interphase. G1 represents the initial growth phase during which time the cell prepares for DNA synthesis. S represents the phase of DNA replication, G2 represents the second growth phase where the cell prepares for mitosis. M represents mitosis, this phase is made up of 4 mitotic phases, prophase, metaphase, anaphase and telophase. G0 represent the cells exit from the cell cycle and a subsequent rest period. During G0 a cell may be able to re-enter the cell cycle again at G1 phase if the biological conditions are favourable and the cell is capable. Not all cells pass through a G0 stage before continuing onto G1 following mitosis.

Once quiescent SCs re-enter the cell cycle, the daughter cells can be quiescent themselves or may be mitotically active Transit Amplifying Cells (TACs) (Watt, 2001, Clayton et al., 2007). The process of SCs, TACs and TDCs becoming activated and fated is illustrated in Figure 7., which follows the mostly accepted SC concept (Bachor and Suburo, 2012, Alison et al., 2010). Quiescent MSCs have distinct signatures as they express specific cell cycle genes such as Cdkn1a, p21 & Ccnd3 and transcription regulators Zbtb20 & Thra (Cheung and Rando, 2013). TACs self-renew to increase cell number within the tissue and also have the potential to give rise to lineage differentiated cells (Fuchs et al., 2004). Some TACs are multipotent and can give rise to a number of differentiated cell types,

while others can only give rise to one cell type in addition to self-renewing (Slack, 2000).

Normally TACs are transiently present within a tissue, often they are lost after development has been completed (Zhang and Hsu, 2017) but can be replenished via the activation of SCs within certain tissues following wounding (Xin et al., 2016). In tissues where constant replenishment of TDCs is required, such as in the skin and intestinal epithelium, TACs persist and reside as neighbours to the SCs within the niche (Clevers, 2013, Kretzschmar and Watt, 2014).

Figure 7. Stem cell differentiation occurs in stages, distinguishable by differences in the rate of mitosis
SCs are defined by their ability to self-renew. When conditions are appropriate the SC is cued to begin the process of differentiation. Initially SCs often transition into a TAC type. TACs can also self-renew, but are more mitotically active, thus increasing the pool of available cells within the tissue. Once again, when cued to do so, the TACs can further differentiate and become TDCs. TDCs generally make up the majority of the cells within the adult tissue. They are mitotically inactive as they have acquired characteristics which allow them to fulfil their function, but which also prohibit mitosis.

In hESCs, cell division is required in order to produce sufficient cell numbers for development, this is achieved by having mitotically active SCs with a shortened G1 phase (Becker et al., 2006). However, in postnatal tissues cell division is required for homeostasis rather than tissue genesis. Consequently postnatal SCs are less mitotically active than their embryonic counterparts (Orford and Scadden, 2008). Studies of the HSC system, have shown that up to 75% of these cells

reside in a G0 or G1 state *in vivo* (Cheshier et al., 1999). Further work in this SC model has found that mice deficient of important cell cycle inhibitors such as p21, exhaust their HSC pools through over proliferation (Cheng et al., 2000, Hock et al., 2004).

In addition to controlling SC number, the regulation of the cell cycle also provides a platform for fate determination to be regulated. During the early G1 phase, cells are particularly sensitive to the cues which determine commitment and survival decisions (Massague, 2004). The expression levels of genes which regulate cell cycle progression may therefore act both as markers of SC quiescence, and also as regulators of maintenance and differentiation in these cells.

1.3   Commitment of stem cells

While a SC is defined in part by its multipotent state and ability to self-renew, it is the cell's capacity for differentiation which allows it to become a TDC in the postnatal or regenerated tissue. The process of a SC differentiating along a specific lineage is a tightly regulated and dynamic process (Zhang and Wang, 2008). Initial cell fate determination is multifactorial, with influences from genetic, epigenetic and environmental factors all contributing to fate determination (Vincent and Van Seuningen, 2009, Wagers et al., 2002). Commitment of cells to a specific fate had long been believed to be an irreversible process, however it has since been shown that cells can be manipulated to return to a more potent state *in vivo* (Tata et al., 2013) and *in vitro* through the invention of induced pluripotent stem cells (iPSCs)(Takahashi and Yamanaka, 2006).

Some of the most famous work in the field of differentiation was published by Conrad Hal Waddington in the 1940's. His theoretical frameworks outlined the concepts of genes and the environment organising the development of an embryo (Waddington, 1940). A major concept from this work, which has become a foundation stone to many theories on commitment, is that of a marble rolling down a hill (Figure 8) (Waddington, 1957). As the marble rolls down the hill, it is directed down valleys and ultimately will reach the bottom of the hill. The final resting point of the marble is resultant from the path which it travelled; a path determined by the environment that it encounters as it rolls. Likewise, a cell will follow a path of commitment based on the spatial and temporal cues which it receives from its environment.
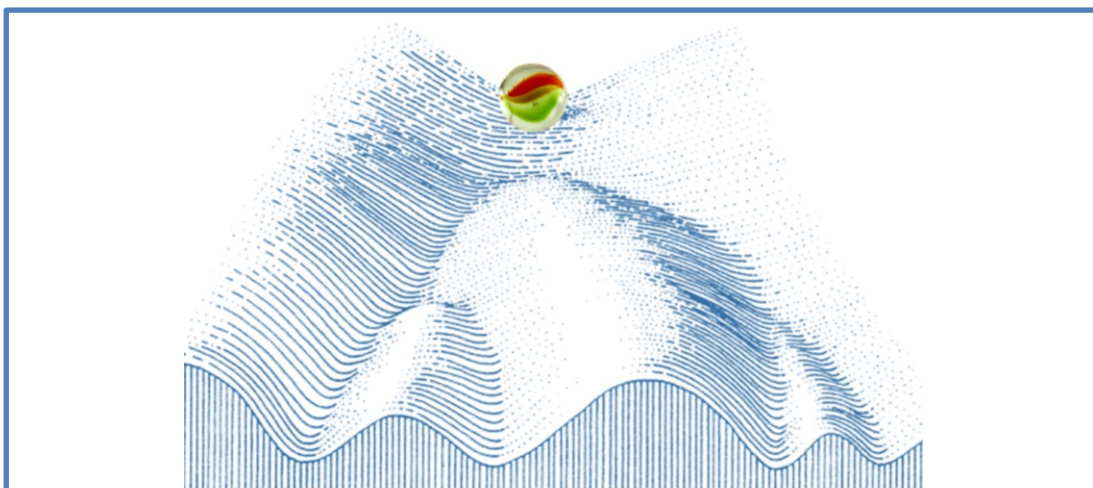
Figure 8. The process of stem cell fate determination is likened to a marble rolling through a landscape
The analogy of an object rolling down a hill is commonly used to depict the concept of cell fating. As the marble rolls down it is funnelled along certain valleys, which restrict its ability to travel down another path (Waddington, 1940). This metaphor mirrors the way in which a SC is committed to a lineage based on the cues it has received from the local environment as it differentiates.

The signals which cue cells to begin the process of lineage commitment include chemical and physical signals. It has been shown that the stiffness of the substrate on which cells are grown can influence differentiation along specific skeletal tissue lineages (Engler et al., 2006). Further research in this field has also shown that the shape of the cell, the area of the cell in contact with the

substrate and the components of the extracellular matrix, all influence the fate determination of cultured SCs (Trappmann et al., 2012, McBeath et al., 2004).

The specific molecular mechanisms which underpin the process of SC fate determination are largely tissue specific, and so are not well defined as a broad universal mechanism. One common element to all SCs as they differentiate is that they must overcome their propensity to maintain self-renewal. In order to maintain an appropriate pool of SCs whilst also allowing differentiation for development and repair, a fine balance between maintaining cell cycle arrest and lineage commitment must be achieved (Zhang and Wang, 2008).

1.4   Maintenance of self-renewal in stem cells

Homeostasis of postnatal tissues requires regulated mechanisms of cell proliferation and differentiation in order to govern repair and regeneration (Simons and Clevers, 2011). This regulation is important to maintain tissue integrity and avoid aberrant growth or loss of cells. To ensure that SCs are not depleted with time it is vital that a pool of SCs remain within the tissue. This is achieved through either population asymmetry or by a process of singe cell asymmetric division (Watt and Hogan, 2000). In population asymmetry strategies, proliferation occurs and while some SCs produce two SC daughters others will produce two cells which will both differentiate, these two divisions occur at an equal ratio in order to maintain the SC pool. The method of asymmetric cell division is perhaps more commonly described, whereby a SC creates two daughters, one a SC itself and the other committed to differentiate (Figure 9). These two asymmetric SC proliferation strategies both work to maintain SC pools while still contributing to the cells available for repair and regeneration of the

tissue. The mechanisms which determine which of these strategies are employed are unclear. However, links to cell polarity, intrinsic cues and signals from the surrounding tissue have all been identified (Morrison and Kimble, 2006, Yamashita et al., 2010).

Figure 9. Stem cells employ asymmetric division strategies in order to maintain the stem cell pool while also producing more differentiated progeny
Two different asymmetric division strategies have been shown to be employed by stem cells. The process which decides the fate of the daughters is often linked to the orientation of the SC mother and the cues received from the surrounding microenvironment.
A *Single cell asymmetric division (as shown in the top part of this figure) is a process whereby each individual SC divides to give rise to one TAC daughter and one SC daughter.*
B *Population asymmetry (outlined in the lower portion of this figure) describes the process whereby the SC pool is maintained through some SCs producing only SC daughters, while other SCs produce all of the TACs (Watt and Hogan, 2000).*

In recent years the concept of microenvironmental influences on SC behaviour has come in vogue (Scadden, 2006). With regards to the maintenance of SCs, it has been proposed that when daughter cells are physically removed from the

anatomical region of the niche, they may then lose their self-renewal properties (Simons and Clevers, 2011). This is due to the loss of contact with the surrounding cells which may provide contact activation/inhibition, regulating the cell's behaviour. Much of this work has been undertaken through the study of gametogenesis, where the niche architecture is well defined and self-contained, however similar mechanisms have been observed in other tissues (Simons and Clevers, 2011). In the gonads, SCs leave the microenvironment as they divide and transform through a process of TAC divisions. A similar process has been observed in the skin and intestines (Diaz-Flores et al., 2006). The specific mechanisms regulating SC self-renewal in different postnatal tissues are largely tissue specific, however there are many common molecular signals which have been discovered which control self-renewal, the cell cycle and fate determination.

1.5   Signalling pathways in mesenchymal stem cells

SCs receive a range of cues and signals from their micro-environment and the other cells within the niche. These signals often coordinate in a complex feedback mechanism with the SCs themselves to drive SC maintenance or differentiation (Morrison and Spradling, 2008). In postnatal tissues, many developmentally active signalling pathways have been found to be reutilised in the regulation of postnatal SCs, such as the Sonic Hedgehog (Shh), Wingless (Wnt) and Notch pathways (Ahn and Joyner, 2005, Reya and Clevers, 2005, Perdigoto and Bardin, 2013, Lim et al., 2013). The Notch pathway specifically has been known to be involved in a wide array of developmental and SC processes (Artavanis-Tsakonas et al., 1999) from organ patterning to cell fate determination. Such signalling pathways often rely upon a crosstalk between the mesenchyme and

epithelial structures during development and repair (Blanpain et al., 2007). In addition to signalling between different embryonic lineages within the niche, intra-lineage signalling, from TACs for example, also plays an important role in SC regulation (Hsu et al., 2014). Efficient signalling is required to maintain correct SC function and disruption can cause aberrant growth or faulty repair.

Wnt signalling is a highly conserved pathway which is utilised throughout development in a range of processes, including cell fate determination and differentiation (Logan and Nusse, 2004). The Wnt proteins themselves are a large group of molecules which can act as secreted hydrophobic ligands, these ligands can often interact with the plasma membrane to maintain prolonged signalling within the SC niche (Nusse, 2008). Within MSCs, Wnt signalling has been shown to be a regulator of proliferation and differentiation in a number of MSC containing tissues, including adipose, bone, cartilage and muscle (Van Camp et al., 2014).

As with many signalling pathways there is a great deal of cross talk. Wnt signalling has been linked to Shh signalling in the regulation of SC fate determination (Ouspenskaia et al., 2016). Shh itself is an evolutionarily conserved pathway which plays a vital role in embryonic development and patterning (Wu et al., 2010). Hedgehog proteins are powerful morphogens which can act both locally and distally to create patterning gradients. In hESCs, Shh signalling is particularly important in the regulation of lineage specific differentiation (Wu et al., 2010). In postnatal SCs the role of Shh is widely contested and appears to be strongly tissue specific. In dental SCs, Shh has been suggested as an important regulator of SC quiescence in both epithelial and mesenchymal SCs (Ishikawa et al., 2017, Zhao et al., 2014). However in the

postnatal pituitary, Shh induces SC proliferation within the gland (Pyczek et al., 2016).

A third highly conserved developmental signalling pathway, which has a strong regulatory role within SCs is the Notch pathway. Of course there are many other signalling pathways at play in SCs which act independently and in conjunction with these pathways (Bhaskar et al., 2014, Tanavde V.M., 2009).

1.6   The Notch pathway

The canonical Notch pathway is an important signalling pathway found in many, if not all, metazoans as homologues between vertebrates are highly conserved (Gazave et al., 2009). Notch signalling has a range of functions in development and regeneration including a key role in cell fate determination. Central to this pathway is the presentation of Notch receptors on the plasma membrane, these receptors can be activated canonically by ligands or independent of traditional ligand binding, through non-canonical activation (Kopan and Ilagan, 2009).

Canonical Notch signalling was first described in the development of the Drosophila nervous system as a mechanism of lateral inhibition (Simpson, 1990). This method of activation of the Notch receptor involves binding of a complementary ligand (D'Souza et al., 2008). The first of these ligands were identified as being bound to adjacent cells and so Notch signalling was believed to be a juxtracrine pathway, however diffusible ligands have since been discovered (Kopan and Ilagan, 2009). The binding of ligand to receptor triggers a multi-step cleavage of the intracellular domain of the Notch receptor (NICD). The NICD then translocates to the nucleus, where it interacts with the RBP-

Jκ/CSL complex, causing a reversal of transcriptional repression and so transcriptional activation occurs. This process is summarised in Figure 10.

Figure 10. The canonical Notch receptor activation pathway involves the use of proteolytic enzymes and intranuclear mediators
Upon activation by a ligand the Notch receptor undergoes a second cleavage (S2) (after S1 which occurs during synthesis). A further 2 cleavage steps (S3 & S4) release the NICD into the cytoplasm. From the cytoplasm NICD translocates to the nucleus. In the nucleus RBP-Jκ (aka. CSL in humans) is ordinarily part of a complex with co-repressors (Co-R). Once the NICD interacts with the Rbp-Jκ complex a conformational change occurs and with the co-activators (Co-A) transcription is activated.
Adapted from (Kopan and Ilagan, 2009, Yu et al., 2015).

In mammals there are four known Notch receptors and an array of canonical ligands (Figure 11). The expression patterns of these receptors and ligands differ between tissues; as such the function of each receptor is largely context dependent. These canonical ligands can be divided into distinct groups based on their homology to the serrate and delta ligands of the drosophila and the conserved domains they possess (Kopan and Ilagan, 2009, D'Souza et al., 2010).

The downstream effectors of canonnical Notch activation have important roles in regulation of proliferation, differentiation and cell fating. Hes1, for example controls proliferation through the repression of cell cycle inhibitor Cdkn1b (Murata

et al., 2005). While Notch effectors Hey1 and Hey2 have been linked to cell fate determination in arterial development (Fischer et al., 2004).

Figure 11. Mammalian Notch receptors and ligands show high levels of inter and intra species conservation

The Notch receptor family of proteins are identified as containing multiple extracellular EGF-like repeats. The number of repeats differ between the four mammalian Notch paralogues, some of which are known to regulate ligand binding. Within the extracellular domain of the receptor there is also a cysteine rich negative regulatory domain. The transmembrane and intracellular domains (NICD) of the different mammalian Notch receptors are more highly conserved in their gross structure, each containing an RBP-Jκ association domain and nuclear localisation sequences, which are important for canonical Notch function after ligand binding and NICD cleavage.
Notch ligands are grouped based on the domains which they contain. Classical ligands contain both a Delta/Serrate/Lag_2 (DSL) and a Delta & OSM-11 like (DOS) domain. Other classes of ligands contain only one of these two motif types. The DSL ligands have been shown to be able to function alone or in conjunction with the DOS ligands. Adapted from (Kopan and Ilagan, 2009)

Non-canonical Notch signalling was identified when differentiation of myoblasts was inhibited by Notch, even when the NICD was unable to interact with the RBP-Jκ complex (Shawber et al., 1996). Further work went to on to show that modulation of Notch receptors could cause an effect even in the absence of RBP-Jκ (Rusconi and Corbin, 1998). These findings, which predominantly focussed on progenitor and SC populations (Andersen et al., 2012), highlighted the potential for Notch receptors to have a function independent of canonical Notch signalling. The mechanisms of non-canonical Notch signalling have yet to be fully explained,

however one interesting action which has been identified is the antagonistic effect of ligand independent Notch signalling on the Wnt pathway (Andersen et al., 2012). Work into the interplay between Wnt and Notch signalling has revealed a Notch-ligand independent role of the Wnt-Notch axis in the regulation of SC lineage progression (Chavali et al., 2018), while canonical Notch signalling has been shown to crosstalk with Wnt to promote expansion of SCs (Hayward et al., 2008).

1.6.1 Notch function in stem cells

There is a great deal of debate over the specific role of Notch in different SC groups, however it is often the case that Notch signalling (whether canonical or ligand independent) is an important regulator of SC maintenance and differentiation (Liu et al., 2010a).

In BMSCs, data suggests that Notch activity has a role in promoting differentiation along an osteogenic lineage through inhibiting cells from residing in a G0 state (Tian et al., 2017, Cao et al., 2017). A similar differentiation promoting action of Notch has been observed in SCs of the hair follicle and inter-follicular epidermis (Williams et al., 2011, Estrach et al., 2008). However in muscle SCs, genetic ablation of RBP-Jκ causes premature differentiation of the cells, suggesting a role in SC maintenance for canonical Notch signalling (Mourikis et al., 2012, Luo et al., 2005). Likewise, in neural SCs the Notch pathway is a key regulator of the maintenance of quiescence and also in fate determination (Chapouton et al., 2010). Furthermore, once SCs have begun to differentiate into TACs, Notch is still an important player in the cell fate progression of these cells (Artavanis-Tsakonas et al., 1999). Part of the reason for these discrepancies may lie in the

method of Notch signalling activation utilised in these different cell types. Due to the RBP-Jκ knock out phenotype observed, it appears that it is canonical Notch signalling which is maintaining the SC pool in such neural tissues (Chapouton et al., 2010). The role of the non-canonical Notch pathway is yet to be elucidated, however work on adipose derived MSCs may elude to a role for this type of signalling in these cells.

In adipose MSCs the SC pool is maintained by Notch dependent regulation of cell cycle progression (Perdigoto and Bardin, 2013, Moriyama et al., 2014, Osathanon et al., 2012). Through the inhibition of differentiation the SCs are kept from beginning their lineage differentiation towards adipogenesis. A key regulator of adipogenesis is Delta-like Homologue 1 (Dlk1), also known as Preadipocyte Factor 1 (Pref1). This molecule is long since been known to promote adipogenesis in BMSCs (Sul, 2009, Wang et al., 2010). In BMSCs, Dental Pulp Stem Cells (DPSCs) and even cancer SCs elevated Dlk1 expression has been linked to the highly proliferative cells within the lineages, sugegsting that this molecule may also have a role in TACs maintenance (Xu et al., 2012, Qi et al., 2017, Kluth et al., 2010). Dlk1 functions both as a membrane bound ligand and can be cleaved to act as a diffusible signalling molecule. The role of Dlk1 in its diffusible and membrane bound forms in SC regulation have been found to exert different effects (Mirshekar-Syahkal et al., 2013). Of particucular interest is the fact that Dlk1 is a well known Notch ligand, which has been identified as a negative regulator of Notch1 possibly through competition with the classical ligands (Baladron et al., 2005, Nueda et al., 2007, Falix et al., 2012). Therefore it is possible that while canonical Notch signalling maintains the SC pool, the action of Dlk1 may be to promote the progression of SCs towards differentiation.

1.7 Epigenetic regulation of stem cells & the cell cycle

The term epigenetics was first coined in the 1940's by C. Waddington, to refer to the field of biology investigating how a phenotype is brought about by the causal interactions between genes and their products (Waddington, 1968). Since this definition was first introduced, the boom in molecular biological research has allowed many fields to fall under this banner. Consequently this definition has since been refined to refer only to the way in which gene function is heritably changed without a DNA sequence alteration (Wu and Morris, 2001). At present the description of epigenetic modifications is used to refer to histone modifications and covalent alterations of the DNA itself (Figure 12) (Dupont et al., 2009, Bernstein et al., 2007).

Histone modifications have been strongly implicated in the epigenetic regulation of chromatin structure, thus altering the accessibility of the DNA for transcription. The amino acid tails of histones are vulnerable to histone modifying enzymes, which can cause posttranslational modifications on selected arginine, lysine and serine residues. The wide array of possible histone modifications and phenotypic outcomes have been extensively investigated in order to determine patterns in expression changes, giving rise to the histone code hypothesis (Strahl and Allis, 2000). The acetylation of many histone 3 and 4 lysine residues, have been associated with transcriptional activation of local genes (e.g. *H3K9* & *H3K27* (Goda et al., 2013, Marinho et al., 2017)). While methylation of the same (and other) lysine residues are associated with transcriptional repression (Dupont et al., 2009). The targeted modification of specific histone amino acids is believed to be regulated by both the recognition of specific DNA sequences and by the

interaction of intermediate proteins, such as the polycomb group proteins (Kohler and Villar, 2008). While histone modification is a widely studied part of epigenetic research, the presence and mechanism of heritability of such alterations to histones in not clear. Some have argued that during cellular division histones are semi-conservatively distributed during duplication (Tagami et al., 2004). However this is a widely contested mechanism, and consensus on an alternative method is yet to be reached (Dupont et al., 2009).

Figure 12. Potential sites of epigenetic modification occur through interaction with the DNA, histones or assembled chromatin
Epigenetic regulation of transcription can occur at different levels within the chromatin, as indicated by the yellow shapes. Covalent modification of the DNA bases (often through the methylation of cytosine residues) alters the accessibility of the DNA to transcription factors and other DNA binding molecules, thus regulating transcription levels. or through mRNA interactions with the chromatin (Deaton and Bird, 2011). The modification of histone structure, again through the covalent addition or removal of methyl moieties, alters the structure of chromatin thus making the DNA more or less accessible to transcriptional machinery (Wu and Morris, 2001). More recently, the role of mRNAs in modulating these other forms of epigenetic marks, has led to some including mRNA mediation as a further form of epigenetic regulation (Holoch and Moazed, 2015).

The other focus of epigenetic study is that of covalent modification of the DNA itself. DNA bases were first observed to be covalently modifiable in the 1940's (Hotchkiss, 1948). The majority of these modifications were found to be methylation events of cytosine, adenine and to some extent guanine bases (Hotchkiss, 1948). 20 years later it was found that such modifications can regulate gene expression levels (Griffith and Mahler, 1969). The inheritance of DNA methylation through mitotic events is regulated by DNA methyltransferase DNMT1, which stabilises and replicates the modifications during replication

(Dupont et al., 2009, Reik et al., 2001). Key regulators of *de novo* methylation include DNA methyltransferases DNMT3A & DNMT3B. The targeted binding of these enzymes is site and developmental stage specific, but can involve the action of RNA interference and recognition of specific DNA sequences (Dupont et al., 2009).

While methylation of cytosine can occur throughout the genome (Woodcock et al., 1987), one well documented epigenetic regulation process is the methylation of cytosine at CpG dinucleotides (Deaton and Bird, 2011). CpG islands are a common feature of the promotor sequences of many transcribed genes. Current findings in the field of DNA epigenetic modification have shown that CpG methylation in promotor sequences causes transcriptional repression (Deaton and Bird, 2011). This repression occurs due to two effects of the DNA modification. Firstly, the binding site of transcription factors can be blocked by the covalent modification of the bases within the promotor sequence. The second repressive event which can occur, is that the methylation of the DNA can attract chromatin and histone remodelling proteins which in turn repress transcription.

When considering the role of epigenetic regulation in SCs, it is important to take note of the array of cellular processes within SCs which can be modulated. For example, the necessity of SCs and TACs to undergo proliferation, and ultimately differentiation, requires robust genomic stability. The loss of DNA methylation has been shown to allow inappropriate recombination and mutagenesis, which can lead to immortalisation and thus inhibited differentiation (Dupont et al., 2009, Dodge et al., 2005).

One such important cellular process which SCs utilise is the maintenance of cellular quiescence. H3K9 methylation has been shown to be important in the

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regulation of quiescence in muscle precursor cells (Cheedipudi et al., 2015). Specifically, PRDM2 associates with promoters in quiescent myoblasts and colocalises with methylation at H3K9. This molecule has also been found to be able to further modulate quiescence by regulating the transcription of TAC marker genes such as CCNA2 (Cheedipudi et al., 2015). DNA methylation has also been shown to be an important regulator of the G0 state, through the modification of promoter sequences for genes which modulate quiescence (Gu et al., 2011).

In postnatal SCs, self-renewal is a vital cellular process, which is tightly regulated. The loss of specific chromatin modifiers has been shown to impair the ability of cells to self-renew (Xi and Xie, 2005, Rinaldi and Benitah, 2015). For example, the loss of Ring1A in intestine has been found to cause tissue failure through impaired self-renewal. Histone modifiers have also been found to have a profound impact on self-renewal. Over expression or knock out of EZH1, a H3K27 methyltransferase, has been shown enhance or impair mammary gland cell self-renewal respectively (Avgustinova and Benitah, 2016). In addition to chromatin and histone modifications affecting the ability of SCs to self-renew, DNA methylation is also an important regulator. In HSCs, knock out of DNMT1 inhibits the mitotic inheritance of DNA modifications and results in impaired self-renewal. Meanwhile, ablation of DNMT3A and DNMT3B in the same cell type has been shown to enhance self-renewal (Challen et al., 2014).

With changes in self-renewal being observed when epigenetic modifications are externally manipulated, changes in lineage commitment have also been observed (Avgustinova and Benitah, 2016, Srinageshwar et al., 2016). Changes in the methylation of DNA in HSCs have given rise to impaired lymphoid differentiation, indicating a role for covalent DNA regulation in determining the

fate of these cells (Broske et al., 2009). Similarly, loss of histone deacetylation modifiers such as HDAC1 in the epidermis cause a loss of lineage specificity (Winter et al., 2013).

Further to lineage determination being affected by epigenetic modification, regulation of differentiation is also impacted. Knock out of other H3K27 methyltransferases (e.g. JARID2) have been shown to affect the timing of differentiation of epidermal SCs (Mejetta et al., 2011). Meanwhile, overexpression of another H3K27 methyltransferase, EZH2, has been found to cause premature differentiation of mammary gland SCs (Li et al., 2009). Mammary gland SCs have also been shown to have limited differentiation capability, leading to impaired terminal end bud development, when *de novo* DNA methylation is inhibited (Pathania et al., 2015).

The role of different epigenetic modifications in different SC types are largely cell type specific. As with most cellular events and regulations there is no one-size-fits-all hypothesis which would determine the effect of a specific regulatory event on the phenotype of all SCs. However, it is clear that both histone and DNA epigenetic modifications play an important role in the regulation of SC quiescence, self-renewal and differentiation.

1.7.1 Epigenetic regulation of Notch signalling

When considering the way in which epigenetic regulations affect cellular phenotypes, it is important to bear in mind the specific molecular pathways that are being modulated. The epigenetic modulation of the Notch pathway has been studied in terms of the synergistic function of epigenetics on Notch target gene

expression, and in terms of the expression of Notch pathway components themselves. The downstream effect of the Notch pathway is the expression of target genes, which is often epigenetically modulated (Schwanbeck, 2015).

A number of the key interaction partners of RBP-Jκ are histone acetyl transferases, which are vital for appropriate targeting of the binding complex (Wallberg et al., 2002). Furthermore, research into the binding partners of the NICD have identified other known epigenetic regulators, including ATP-dependent chromatin remodellers (such as SmarcA2 of the SWI/SNF complex) and polycomb repressing complexes (Schwanbeck, 2015). The effect of chromatin remodelling and Notch target gene promoter occupation, on Notch target gene expression is an area of much study within epigenetics. Specifically, in ESCs, some 95% of studied Notch target genes were identified as having histone methylation events in the promoter region (Meier-Stiegen et al., 2010).

Chromatin modifying proteins are important epigenetic regulators. KDM5A, a lysine demethylase, has been identified as a highly conserved, essential component of the RBP-Jκ repressor complex (Liefke et al., 2010). In cancer cells, the polycomb repressor complex and KDM5A interact to remove histone methylations in order to regulate Notch target genes including Hes1 (Liefke et al., 2010). The regulation of Notch target genes by polycomb group proteins is further supported following the identification of polycomb repressor complex proteins at Notch target gene promoter regions (Schwanbeck et al., 2011). Specifically, Ring1B and EZH2 have been shown to be enriched at the promoter regions of Notch target genes, suggesting a role for polycomb repressive complexes as antagonists to Notch signalling (Yatim et al., 2012).

In addition to potentially regulating levels of downstream Notch effects, a role for polycomb group proteins has also been investigated in "reading" epigenetic histone marks and thus targeting or preventing intracellular Notch pathway components from binding to specific chromatin regions (Kutateladze, 2011).

Notch signalling pathway components have also been shown to interact with chromatin remodelling factors, including those of the SWI/SNF complex (Schwanbeck, 2015). This complex is an ATP-dependent chromatin remodelling complex, which allows DNA to be more accessible to transcription, replication and repair machinery, by restructuring the nucleosome (Tang et al., 2010). Chromatin remodelling complexes have also been identified as having histone acetyl transferase interaction capabilities, thus allowing the acetylation of histones (Schwanbeck, 2015). Components of chromatin remodelling complexes have been identified as being able to interact with Notch receptors and act as vital mediators of NICD – RBP-Jκ binding (Yatim et al., 2012, Takeuchi et al., 2007). Of particular note is the findings that ATPases of the SWI/SNF complex, SmarcA2 and SmarcA4, can interact strongly with RBP-Jκ and NICD (Kadam and Emerson, 2003, Yatim et al., 2012). SmarcA2 and SmarcA4 are exclusively and interchangeably expressed within the SWI/SNF complex, and the role of each complex type is believed to be distinct. In cancer cells the interaction of SmarcA4 with the core Notch effector complex is necessary for endogenous Notch effector gene expression (Takeuchi et al., 2007, Yatim et al., 2012). However, in retinal SCs, SmarcA2 interactions have been shown to have inhibitory effects on Hes1 and Hes5 expression (Das et al., 2007).

As with all cellular pathways, the expression of the ligands, receptors and effectors of the Notch pathway can be epigenetically regulated. Epigenetic DNA

modification has been shown to both regulate and be regulated by the Notch Pathway. Methylation of cytosine, 5mC, is a well characterised inhibitory epigenetic change which occurs often within the DNA in regulatory elements, gene bodies and CpG islands. RBP-Jκ has been found to bind strongly to 5mC motifs, suggesting a role for DNA methylation in increasing RBP-Jκ binding to certain areas of chromatin (Bartels et al., 2011). Notch target genes themselves have been shown to have different levels of DNA methylation in their promoter regions in progenitor and differentiated cells (Reister et al., 2011, Martini et al., 2013). Further suggesting that the effect of Notch signalling is modulated epigenetically throughout a cell's life. In addition to methylation changes in Notch target genes, the components of the Notch pathway themselves are also upregulated by hypomethylation (Terragni et al., 2014).

A further important epigenetic event when considering the regulation of expression of Notch pathway components through DNA methylation is genomic imprinting. Since the 1980's it has been known that mammalian embryogenesis requires both paternal and maternal genetic contributions (McGrath and Solter, 1984). The epigenetic silencing of specific parental alleles occurs through the modification of the DNA in the oocyte or spermatozoon. Whether a gene is madumnally or padumnally expressed conveys different heritable fitness to the offspring (Wilkins and Haig, 2003). Some genes remain imprinted throughout an organism's life, while others are temporally and spatially transient. Similar to the way in which X chromosome inactivation occurs at some loci during gametogenesis, autosomal genomic imprinting can occur during germ cell development (Dupont et al., 2009).

The gene of Notch ligand Dlk1 lies within a region of the genome which is well documented as paternally imprinted in mammals (Wallace et al., 2010). Consequently, Dlk1 is expressed from the maternal allele in early development (Schmidt et al., 2000). This imprinting occurs through the methylation of DNA in a series of CpG rich regions within the loci. The Dlk1 gene sits within a well characterised, epigenetically regulated locus. In the region of the genome containing the Dlk1 gene, there are two areas of CpG islands. One sits upstream and the other downstream of the gene. These islands are referred to as the 5' and 3' islands respectively (Figure 16 B). Methylation of these sites has been linked to transcriptional repression of Dlk1. Targeting of DNA methylation has been linked to the modification of histone proteins in the region (Rose and Klose, 2014). The imprinting is maintained and inherited through the function of DNTPs which can be used to modify levels of repression of the gene (Schmidt et al., 2000). The modification of levels of epigenetic imprinting of Dlk1 have been linked to levels of potency in embryonic and iPSCs (Liu et al., 2010b). Thus, suggesting an important role for the levels of epigenetic regulation of this Notch ligand in SC maintenance and differentiation.

1.8   The tooth as a model for mesenchymal stem cell research

Since the first description of vertebrate tooth development (Owen, 1840), the processes and mechanisms at work have been studied in depth. Research has been conducted using animal models including reptiles, fish and mammals (Jernvall and Thesleff, 2012), with many commonalities and conserved pathways being identified.

1.8.1 Development of the tooth

The morphological stages of tooth development are defined as initiation, bud, cap, bell and mineralisation (as shown in Figure 13). The stages are assigned predominantly by the morphology of the tissues and not by the age of the animal, as the teeth of different species develop at different rates and in different formula (Cobourne and Sharpe, 2010).

The gross stages of tooth development are common to many mammals and to some animals of other classes. Rodents are often used as mammalian models of tooth development, as although their postnatal dentition is markedly different from our own, many of the developmental processes and gross morphology throughout development have been shown to often be conserved (Hu et al., 2013).

Figure 13. **The stages of tooth development are described primarily based on the morphology of the molar tooth**
The stages of tooth development are characterised morphologically. This schematic outlines the process in the molar teeth. During initiation the dental epithelium thickens. This thickening begins to invaginate into the mesenchyme as the mesenchymal cells condense around it in the bud stage. The cap stage begins as the early cusps are patterned with the interaction of the condensed mesenchyme and the epithelium above. During bell stage odontoblasts and ameloblasts differentiate from the mesenchyme and epithelium respectively. Following this, mineralisation and eruption begins, whereby the hard tissues of the tooth are deposited, and the tooth migrates into the oral cavity. Adapted from (Tucker and Sharpe, 2004, Hugnot and Franzen, 2011)

1.8.1.1 Murine molar development

The general process of molar development in mice is conserved between strains, however the precise timing of these events can differ (Pugach and Gibson, 2014). Within the field of research into dental development, and in the research presented here, strains with a background of ICR (CD-1®) and BL6 are commonly used (Caton and Tucker, 2009). The combined work on multiple mouse strains has allowed for a broad understanding of the overall process of dental formation, patterning and development.

1.8.1.1.1 Initiation

During the initiation phase (generally E11.5 in mice 1st molar (M1)) there are two tissue layers, the mesenchymal tissue and the epithelial tissue. While the epithelial tissue is derived from the ectoderm and endoderm as expected, the mesenchymal tissue is not mesodermal in origin, but is derived from the neural crest (Soukup et al., 2008).

The dental epithelium proliferates to a two-layer thickness during initiation. The layer of cells contacting the mesenchymal tissue then begins to polarise and press towards the mesenchyme. The mesenchyme then condenses around this newly formed placode. Once initiated, early dental development is controlled by the interaction of the epithelial and mesenchymal tissues (Balic and Thesleff, 2015).

In many developing organs within vertebrates, the mesenchyme initiates developmental signalling though cross-talk with the epithelium. However in the tooth it has been shown that the epithelium is the instigator of signalling which acts upon the mesenchyme causing condensation (Moss, 1969, Thesleff et al., 1995). This may suggest that the different origin of the tooth mesenchymal tissue

may lead to different functionality. These recombination experiments also identified a range of transcription factors and growth factors which are key players in the development of mammalian teeth, including BMPs, TGFβs and FGFs (Thesleff et al., 1995).

1.8.1.1.2 Bud

The bud stage (generally E12.5-E13.5 in mice M1) is identified by the invasion of the epithelial tissue into the mesenchyme. By this stage there is increased cross talk between the epithelium and the underlying mesenchymal tissue. Effective budding requires BMP4 signalling from the epithelium to act though Msx1 to induce BMP4 expression in the mesenchyme (Saadi et al., 2013). It is at this stage that development is often halted in the absence of the necessary signalling molecules. In the diastema region of the mandibles of the mouse, the reduction of Shh signalling inhibits the further development of early tooth buds in this area (Kangas et al., 2004). This effect has been shown to be rescued by mutations which cause elevated Wnt signalling (Ahn et al., 2010). These findings suggest a complementary role whereby reduction of Wnt and restriction of Shh results in a reduced postnatal murine dentition, while vestigial teeth remain undeveloped in the diastema of these adults.

1.8.1.1.3 Cap

By the cap stage (generally E14.5-E15.5 in mice M1) the epithelium begins to differentiate into two distinct layers, the inner enamel epithelium (at the convexity of the cap) and outer enamel epithelium (at the concavity). These layers are separated from the underlying mesenchymal tissue by a basement membrane, which has also been implicated in the crosstalk between the epithelium and mesenchyme (Thesleff and Hurmerinta, 1981). At the cervical loop (CL) the cells

between these two epithelia begin to take on a star-shaped phenotype, while still maintaining contact to one another. The dental epithelial stem cells (EpSCs) are located in the stellate reticulum and surrounding outer enamel epithelium (Li et al., 2012).

During the cap stage, the enamel knot is first visible. The enamel knot is an epithelia derived structure which contains densely packed cells (Jernvall and Thesleff, 2012), In multi-cuspid teeth a secondary enamel knot later forms from non-proliferative cells of the primary knot (Coin et al., 1999), thus allowing for an increase in cusp complexity in these teeth. The enamel knots are transient developmental structures which are later apoptosed once the enamel knot has performed its function of establishing the future cusp pattern (Vaahtokari et al., 1996). In addition to the dense clustering of epithelial cells at this time, the underlying mesenchymal tissue can also be observed condensing. In the mouse incisor capillaries are seen to begin forming within the mesenchyme (Kerley, 1975).

By late cap stage one side of the forming bell elongates before the other follows suit. This elongation is marked by increased Shh expression in the Inner Dental Epithelium (IDE) (Hu et al., 2013). The expression pattern of Shh has also been observed in the developing human dentition, showing the inter species conservation of signalling mechanisms in ontogenesis (Hu et al., 2013).

1.8.1.1.4 Bell

During the bell (generally E16.5-E18.5 in mice M1), the tissue is said to be undergoing histodifferentiation (Phinney and Halstead, 2003). The cells of the inner enamel epithelium begin to differentiate into preameloblasts, while the mesenchymal cells contacting the basement membrane become preodontoblasts.

As the cells differentiate and undergo different rates of mitosis, the epithelium folds into a bell shape. By the late bell stage (E19) the developing dental organ loses its attachment to the ectoderm (Kerley, 1975).

1.8.1.1.5 Differentiation

During the process of differentiation (generally perinatally in mice M1) the TACs of the teeth begin to differentiate into the terminally differentiated odontoblasts and ameloblasts (Yildirim, 2013). During this process the cells polarise and align along the basement membrane between the mesenchyme and epithelium. This process is tightly regulated though reciprocal signalling between these two tissues and through interaction with the extracellular matrix (He et al., 2010).

1.8.1.1.6 Mineralisation & Eruption

During the process of eruption (generally postnatal day 0-2 in mice M1) crown formation is finalised. The now mature ameloblasts secrete enamel while the odontoblasts secrete dentine. The ameloblasts on the cusps of the tooth begin to deposit enamel, starting at the tips and slowly developing down the sides of the tooth. The last remnant of the attachment between the surface epithelium and the enamel organ is degraded concurrently with increased proliferation of the enamel organ itself (Lungova et al., 2011). The dental mesenchyme is also active during this stage of development, displaying increased proliferation and remodelling. By postnatal day 2, ameloblasts of the crown are no longer proliferating but rather are utilised in the production of enamel. The ameloblasts of the rest of the tooth slowly begin to reduce their proliferation and increase secretion in a wave towards the CLs, until all of the dental epithelium is no longer mitotically active.

Alongside the mineralisation of the crown, the root is beginning to form and remodel (Lungova et al., 2011). This involves not only dental, but bone

remodelling. By postnatal day 4, bone turnover is increased along the base of the tooth crypt (Villarino et al., 2005). In rooted teeth, the epithelial cells of the CL begin to differentiate into the Hertwig's Epithelial Root Sheath (HERS) which then secretes cementum (Jernvall and Thesleff, 2012). After the formation of the root, the HERS loses its ability to continue to grow and the SC containing Stellate Reticulum is lost (Baratella et al., 1999). Molar teeth erupt into the oral cavity around postnatal day 14 (Lungova et al., 2011). By this stage the teeth are beginning to locate into their functional position and can be seen within the cavity fully by postnatal day 16.

1.8.1.2 Murine incisor development

The development of the mouse incisors follows a generally similar process to that of their molar counterparts. The key differences are observed at the later stages, as the continuously growing mouse incisor maintains its proliferative potential and does not develop a classical root (Juuri et al., 2013).

In the development of the murine dentition, the initiation phase of the incisor is morphologically very similar to that of the molar teeth (Harada et al., 2002). However the molecular cues which determine the fate of the developing tooth are already being put into place at this early stage (Caton and Tucker, 2009).

By bud stage early differences between molars and incisors can be observed. Within the incisors the condensation of the mesenchyme begins to cause asymmetry in the epithelium, causing the bud to begin to tilt away from being entirely perpendicular to the ectoderm above (Harada et al., 2002).

This asymmetry continues into the cap stage. By the end of the cap stage the morphological features of the developing incisor are distinct from the molar teeth.

It has been observed that the time taken to reach this stage of development may be slightly swifter in incisors than the molar teeth, further differentiating this tooth from the molars. The tilting of the dental epithelium and the beginnings of the bell shape form so that the region which will ultimately go on to become the dental pulp forms a chamber parallel to the developing oral cavity (Harada et al., 2002). Enamel knot, which is a prerequisite structure of cusp formation is notably absent from the rodent incisor.

Throughout the bell stage the patterning of the incisor teeth clearly demarks this tooth as being different from the non-continuously growing molars. The tooth lies almost horizontal within the mandible with the crown at the distal tip of the jaw. Within the epithelium of the incisor the two CLs which have formed are also beginning to be distinct from one another (Suomalainen and Thesleff, 2010). The lingual CL is notably smaller than the larger labial CL below (Harada et al., 2002). Both CLs begin to grow deeper into the mesenchyme, thus elongating the tooth and ultimately coming to rest closer to the angle of the mandible (Kerley, 1975).

While differentiation and polarisation of the cells within the tooth still occur within the incisor, it is notable that many of the cells of the CL maintain their proliferative capability. As no traditional root is forming it is ameloblasts rather than cementoblasts which differentiate from the epithelial tissue (Kawano et al., 2004). Similarly, to molars the odontoblasts which have differentiated from the mesenchymal cells go on to produce the dentine during mineralisation, while the ameloblasts secrete enamel.

In the mouse incisor and other continuously growing teeth, the process of eruption is associated with increased vascularisation (Balic and Mina, 2010), this is vital to provide an adequate oxygen and nutrient supply to the tooth which will

grow throughout life. As the tooth elongates the incisor tooth passes through the external epithelium of the oral cavity and erupts early after birth (Lungova et al., 2011).

1.8.2 Stem cells in the non-continuously growing teeth

In humans the deciduous teeth are typically lost before adolescence and replaced by the permanent dentition (Cobourne and Sharpe, 2010). After this time the teeth are increasingly limited in their ability to repair after injury (Volponi et al., 2010). However a population of reactive mesenchymal DPSCs has been identified, these cells can be activated upon injury to produce a reparative dentine that strengthens the damaged hard tissue (Gronthos et al., 2002, Huang et al., 2009).

In teeth which do not continuously grow, including all human teeth and rodent molars, the epithelium is lost by the time of eruption. Not only does this limit the regenerative capacity of the enamel, it also has a profound impact on the mesenchymal cell lineages and dentine production, due to the loss of epithelial-mesenchymal communication. However the mesenchymal dental pulp is a dynamic tissue which can still contribute to the reparation of dentine in these teeth (Sloan and Smith, 2007, Yu et al., 2015).

In teeth which do not continuously grow there is no CL, as the corresponding structure during development differentiated into the HERS (Jernvall and Thesleff, 2012) and the remaining epithelium is differentiated into ameloblasts by adulthood. The apical papilla of the postnatal tooth has been shown to contain an EpSC niche (Morotomi et al., 2005), while the dental pulp within the postnatal

tooth has been shown to contain a non-terminally differentiated cell population which can be activated upon injury (Gronthos et al., 2000).

Dental SCs were first isolated from the dental pulp (Gronthos et al., 2000) in 2000. Since then, dental SCs have also been harvested from exfoliated deciduous teeth after they have been shed (Bluteau et al., 2008). The characterisation of true dental pulp SCs (DPSCs) has been much debated. Currently the most accurate method of characterisation is though the presence or absence of cell surface antigens. DPSCs have been found to express a number of markers that are common to many MSCs (i.e. CD44, CD29, CD105 & Stro-1) (Kawashima, 2012), while being low for haematopoietic antigens (i.e.. CD45 & CD34) (Lindroos et al., 2008, Mitsiadis et al., 2011). Dental SCs have been shown to be multipotent and can be cultured to differentiate into cardiomyocytes (Gandia et al., 2008), chondrocytes and adipocytes (Patil et al., 2013). Induced pluripotency assays have also shown that DPSCs can be reverted to a pluripotent state (Yan et al., 2010). However, unlike embryonic SCs, the dental SCs have a finite mitotic potential and with each division the risk of genetic alterations within the cells increases (Gronthos et al., 2002).

1.8.3 Stem cells in the continuously growing teeth

Unlike human teeth, the incisors of rodents and lagomorphs, continue to grow throughout adulthood and are worn down by gnawing (Derrell-Clark, 2013). The murine incisor is an elegant model for the study of SCs. By nature, it grows continuously throughout the lifetime of the animal and the tooth is worn down through friction. If this process does not occur, aberrant overgrowth can occur which carries with it high levels of morbidity and mortality.

The incisor tooth of rodents is by far the largest of its teeth (Figure 14). The exposed tip, which is visible, is only a fraction of the entire organ. Within the mandible the incisor takes up large proportion of the jaw.

Figure 14. The sagittal view & sectioning of the mouse mandible shows the extent of the incisor
The top image is an Illustration of the mouse mandible after sagittal sectioning has exposed the tooth chambers. M1, M2 & M3 denote the 1st, 2nd and 3rd molars respectively. "In" denotes the incisor. Cut out shows cervical loop region of the incisor tooth as seen in a histological sagittal sectioned preparation. The epithelial tissue is marked in blue, containing regions of EpSCs, ETACs and ETDCs. Mesenchymal tissue is coloured pink, containing the MTAC and MTDC pools. Neurovascular bundles (NVBs) are also depicted.

The hard, exposed portion of the incisor is comprised of two hard tissues, dentine and enamel. The epithelium and mesenchyme at the apical end of the incisor provide precursors, these precursors then go on to become the terminally differentiated enamel secreting ameloblasts, and dentine producing odontoblasts respectively (Seidel et al., 2010). Enamel is produced by TDCs specific to the tooth, known as ameloblasts. Meanwhile dentine is produced by mesenchymal odontoblasts (Smith, 1980).

It is known that ameloblasts originate from an epithelial structure located at the apical end of the incisor called the CL (Baker et al., 2009). Within the CL a pool of well characterised EpSCs has been documented. The CL epithelium expresses a range of growth factors, including FGFs 3, 7 & 10, alongside a range of SC markers such as Sox2, BMI1 & LGR5 (Kettunen et al., 2000, Biehs et al., 2013, Juuri et al., 2012, Suomalainen and Thesleff, 2010). These SCs persist within the adult, and commit to differentiate through a TAC state before terminally differentiating (Smith and Warshawsky, 1975, Harada et al., 1999). The origins of odontoblasts however are less well characterised.

1.8.4 Mesenchymal stem cells in the mouse incisor tooth

In the incisor mesenchyme there is a highly proliferative region, adjacent to the TAC region of the epithelium (Lapthanasupkul et al., 2012). This region of mesenchyme highly expresses a number of polycomb genes including transcriptional regulator Ring1b and cell cycle controller Skp1 (Lapthanasupkul et al., 2012). The cells from this population have since been shown to be capable of migrating to the mesenchymal odontoblast layer within the incisor (Feng et al., 2011, Zhao et al., 2014).

Contrary to the mesenchymal transit amplifying cells (MTACs), the origin of MSCs in the mouse incisor model has yet to be fully confirmed. Recently published findings indicate a glial origin for dental MSCs, having identified a population of label retaining cells around the neurovascular bundles (NVB) of the mouse incisor (Kaukua et al., 2014a). A periarterial slow cycling cell population, which is Gli1 and Thy1 positive has also recently been identified (Zhao et al., 2014, An et al., 2018). Both of these NVB associated populations of MSCs (NVB-MSCs) have

been shown to be capable of migrating to areas of the odontoblast layer and participating in repair of the tooth's hard tissues after damage. However, lineage tracing data was not able to link either of these cell populations to the native MTACs of the incisor. Additionally, these cells also did not express known MSC markers. Therefore, the endogenous incisor MSCs have not been identified so far.

At least in the epithelium, the SCs, TACs and TDCs of the epithelium can all be visualised on the same sagittal plane, alongside their mesenchymal counterparts (Naveau et al., 2014), (Figure 14), this is observed in relatively few tissue niches (Li and Clevers, 2010) making the murine incisor an exciting model for studying the interactions and transitions between each of the cell groups.

The continuously growing mouse incisor provides a potential model for better understanding the regulation of SC maintenance and renewal. It is hoped that by providing further insight into the regulation of mesenchymal cells within this tissue, further advances in the MSC field could be made. In order to utilise the mouse incisor for this purpose, a series of research questions were posed and addressed. The question of whether the mesenchyme contains a similar SC, TAC, TDC axis to the epithelial cells remains. An equivalent SC population within the mesenchyme which gives rise to the odontoblasts has yet to be identified. If such cells are present, novel methods of identifying and distinguishing them from the other cells of the tissue will need to be employed. Further research questions about these cells will also need to be addressed, including the molecular control of these cells in both development and regeneration.

1.8.5 Dentine regeneration

In humans, tooth damage is most commonly caused by erosion of the enamel through physical, chemical or bacterial assault. Dental caries is the most common dental pathology among humans and is caused by bacterial infection of compromised teeth (Bjorndal, 2008). The infection causes demineralisation and damage to all the hard tissues of the dentition. As caries is such a common complaint, a wealth of research has been conducted into preventative and reparative strategies.

Upon dental injury there is initial apoptosis of odontoblasts. The surviving odontoblasts begin to secrete a reactive dentine to counteract the damage. The apoptosis of neighbouring odontoblasts causes an activation of the DPSCs, which proliferate, migrate and differentiate into odontoblasts-like cells and begin producing a reparative dentine. Ordinarily this repair response occurs so rapidly that there is little recruitment of inflammatory markers to the site of injury. Part of the reason why the repair mechanisms are so quick to initiate is that during physiological conditions, signalling molecules and growth factors (such as TGF-beta (Baker et al., 2009)) are secreted by pulp cells and sequestered by the dentine (Silva et al., 2004). Upon injury these molecules are released from the dentine and are free to induce wound healing, differentiation and proliferation responses in the surrounding tissue.

In order for efficient regeneration (and continued tooth growth) a pool of stem like cells is required. In the tooth there are two broad classes of dental SCs, those of epithelial origin and those which originate from the mesenchymal tissue.

While the SC niches of developing teeth (and continuously growing teeth) have a great potential to differentiate and proliferate, the fully formed dentine and

cementum have very little regenerative ability and the enamel has not been observed to have any. However the presence of postnatal SC niches in the dental pulp and the CL of the murine incisor, have led many to believe that reactivating these SCs could provide a regenerative therapy to counteract demineralisation and possibly whole tooth loss (Bluteau et al., 2008). In addition to advances in clinical dentistry, dental SCs have been suggested as a potential pool of postnatal SCs that could be utilised in regenerative medicine systemically (Huang et al., 2009).

1.8.6 Notch function in the tooth

In the tooth, Notch signalling has a number of regulatory roles (Mitsiadis et al., 2005, Campa et al., 2008). Notch signalling has been shown to be indispensable for tooth development (Mitsiadis et al., 2010). Furthermore, the Notch pathway has been shown to be important in tooth pulp reparation (Mitsiadis et al., 2011, Mitsiadis and Rahiotis, 2004). However, how the Notch pathway is activated and how the level of Notch activation contributes to tooth SC behaviour is not yet understood.

Throughout murine dental development, the expression pattern of Notch receptors and ligands are temporally and spatially regulated (Cai et al., 2011). The epithelial cells of the developing tooth broadly express the Notch receptor proteins. However, within the dental mesenchyme Notch transcription has been identified within the pre-odontoblast regions but is absent from the odontoblasts (Mitsiadis et al., 1995), suggesting a potential role for Notch signalling in maintaining the pool of odontoblast precursors.

During early murine molar development, the Notch receptors 1, 2 and 3 are expressed broadly within the dental epithelial tissue. As development progresses through to the bell stage, the localisation of each receptor is restricted into distinct compartments as the fate of each of the epithelial cell types are adopted (Cai et al., 2011). Initially Notch receptor mRNA (messenger ribonucleic acid) is not detected within the developing molar tooth mesenchyme, however by late bell stage Notch receptor genes 2 and 3 are being transcribed in this tissue. By eruption, each of the three major Notch receptors are expressed transiently within the dental mesenchyme, however the differentiated mesenchymal cells remain absent of Notch expression (Mitsiadis et al., 1995). Classical ligands of Notch signalling, Jag1 and Jag2 are also expressed within the developing mouse molar. During early development, Jag1 is highly expressed within the condensing mesenchyme and epithelial stellate reticulum (Mustonen et al., 2002). This expression pattern is strongly correlated with the expression of Notch receptors. As the molar develops, the expression of Jag1 in the mesenchyme becomes more restricted to the regions of mesenchyme which contain the developmental dental pulp SCs. Jag2 conversely is most highly expressed within the epithelial tissues throughout development of the mouse molar (Mitsiadis et al., 2005). This initially broad expression is restricted as development progresses until by the time of eruption, Jag2 is expressed almost exclusively in the IDE and ameloblasts (Harada et al., 2006).

During early incisor development in the mouse, the Notch receptor gene expression patterns have been shown to be similar to that of their molar counterparts (Mitsiadis et al., 1995). Of note is the differential expression of Notch2, which has been shown to be expressed in the condensing mesenchyme of incisor teeth during the bud/cap stage (Mucchielli and Mitsiadis, 2000). By bell

stage, the rotation of the incisor has begun and so the pattern of Notch receptor gene expression is no longer symmetrical within the tooth, thus highlighting the difference in expression to molar teeth. However, the majority of the expression of Notch1, 2 and 3 at this stage, is restricted to the epithelial tissue. By the time that the incisor tooth is erupting, Notch1 mRNA is once again detectable in the region of mesenchyme containing odontoblast precursors (Harada et al., 2006). Much of this work has been carried out using *In-situ* hybridisation techniques some 20 years ago. With the development of modern techniques such as laser capture microdissection, more accurate assessment of the expression patterns of these genes may show more accurately the precise regions of expression.

The expression of classical Notch ligands within the developing incisor is almost entirely restricted to the epithelial compartment throughout. However Dll1 has been shown to be expressed in the mesenchymal tissue around the labial CL by late bell stage (Mitsiadis et al., 1998). The apparent lack of Notch ligands within the mesenchyme has led to the suggestion that cross talk between the epithelium and mesenchyme, and the interaction with other molecular pathways, may play an important role in incisor development (Harada et al., 1999). Together this dynamic spatiotemporal Notch receptor and ligand expression pattern shows the potential for Notch signalling to be utilised by the tooth in order to direct cell fate determination, and the maintenance of precursor cell pools within the developing tooth.

Within the adult dentition, Notch pathway gene expression is largely absent from the differentiated cells which make up the teeth. Primarily the expression of these proteins are restricted to the SC containing CL region of the incisors (About and Mitsiadis, 2001). However, following injury, the adult teeth require the reactivation

of SCs in order to undertake repair and regeneration. Work on molar teeth has shown that this reactivation correlates with the triggering of Notch signalling within the dental mesenchyme and preodontoblasts adjacent to the injury (About et al., 2000, Mitsiadis et al., 1999). Notch receptor activity is greatly upregulated in the reactive dental pulp, specifically Notch2 protein is strongly expressed (Mitsiadis et al., 1999). While Notch1 is upregulated in the mesenchymal tissue local to the injury, and adjacent to the surrounding NVBs, Notch2 has also been shown to be upregulated more distally, in the odontoblast precursor pool (Mitsiadis et al., 1999). This Notch2 upregulation correlates with increased expression of ligand protein Dll1, and a drive towards differentiation of these precursor cells (About and Mitsiadis, 2001). It is believed that Notch signalling in the injured tooth mesenchyme may indicate pools of reactive dental pulp SC. Notch signalling has already been shown to be required for the survival of dental epithelial SCs, and so it is possible that the same signalling pathway plays a similarly vital role in the mesenchymal SCs of the teeth (Felszeghy et al., 2010).

Postnatal dental SCs, including DPSCs, Notch signalling has been shown to regulate differentiation and maintenance of the SC pool (Bray, 1998, Carlesso et al., 1999). The presence of Notch signalling has also been used to identify the presence of undifferentiated dental cells in organ culture conditions (Morsczeck et al., 2005). Once identified the fate determination of these DPSCs *in vitro* is modifiable through the manipulation of Notch signalling, suggesting a role for Notch in the differentiation of reactive dental SCs *in vivo* (Grottkau et al., 2010).

The perivascular cells of the dental pulp have been found to contain a population of DPSCs (Machado et al., 2016, Shi and Gronthos, 2003). Notch 3 is an established marker for the identification of these cells and the upregulation of this

protein following injury indicated a further role for the Notch pathway in fate determination of reactive SCs (Lovschall et al., 2005).

As Notch signalling is an important and well documented regulator of developmental and reparative SCs within many mesenchymal tissues, the question of whether Notch activity may indicate pools of SCs within the mouse incisor, and how the same pathway may function in these cells, is an interesting basis for further study.

2 Aims & Objectives

The primary hypothesis of this research is that within the mouse incisor mesenchyme, there is a novel endogenous MSC population which gives rise to the MTAC and ultimately odontoblast cells. It is also hypothesised that Dlk1 will have a regulatory role on the maintenance and transition of MSC and MTACs.

This work therefore aims to investigate the molecular mechanisms underpinning the behaviour of these mesenchymal cells, specifically to determine the role of Notch signalling in this process.

Specific objectives are therefore as follows:

2.1 Classification of an endogenous mesenchymal stem cell pool

To identify the location of novel MSCs in the incisor, regions will be examined for MSC marker gene expression, at a protein and mRNA transcription level. It is expected that the MSC and MTAC regions will display distinct molecular expression signatures.

Qualification that the identified cells of the endogenous MSC pool are capable of differentiating into MTACs and ultimately odontoblasts will also be determined. This will initially be investigated though microinjection and lineage tracing methodologies. Secondly, an *ex vivo* model will be developed. This will involve the resection and culture of MSC containing incisor tissue with mature devitalised dentine, in order to display the ability of these cells to reproduce a mature odontoblast like phenotype and functionality.

2.2 Determination of the role of Notch signalling in the maintenance and transition of mesenchymal cells of the incisor

To investigate the role of Notch signalling in the behaviour of MSC and MTAC cells *in vivo* Dlk1 conditional overexpression transgenic mouse line, Collagen 1 a1 Dlk1 overexpression, and Dlk1 knock out mice will be characterised. The expression of MSC marker proteins in the potential cervical loop MSC (CL-MSC) region of these animals will be determined, to define the effect of mediation of the Notch signalling pathway through Dlk1 modulation.

To further investigate the role of Notch signalling pathway members (with a focus on Dlk1) on MSC behaviour, an *in vitro* model of MSCs will be developed. Both primary cells and established cell lines will be utilised. Cell cycle synchronisation of the cultured cells will be performed to model quiescent MSC and MTAC cells. The effect of the addition or silencing of Dlk1 and MSC markers on the cell cycle status and expression profiles of the cells will be investigated.

2.3 Investigation into the translational potential of findings to clinical applications

Finally, to investigate the translational potential of this study, in terms of regenerating a non-continuously growing teeth, a dental injury model will be employed. This will be used to investigate the mouse incisor MSC/MTAC regulating proteins at sites of dental injury in a rat model.

3 Materials & Methods

3.1 Animal models

Mouse strains used included ICR (CD-1®) Mice (CD1) mice from The University of Plymouth, UK; Collagen 1 α2 Cre x Rosa26 mice and Collagen 1 α2 Cre x Rbp-Jκ flox/flox and Transgenic Notch Reporter mice both obtained from The University of Lausanne, Switzerland; Collagen 1 α1 Dlk1 overexpression mice from The University of Southern Denmark, Denmark; and Dlk1 -/- mice from the University of Castilla-La Mancha, Ciudad Real, Spain.

All *in vivo* and *in vitro* experiments used mice at postnatal ages of day 0, day 7, day 14 and day 30.

Six female Wistar rats were used at age 30-45 days, which were raised at Peking University, China.

All animals used in this work were humanely euthanized using cervical dislocation in accordance to Home Office regulation under the UK Animals Scientific Procedures Act 1986 (ASPA). Plymouth University Animal Welfare and Ethical Review Board granted ethical approval for all mice experiments. Experiments using rats were conducted at Peking University under local ethical approval.

3.2 Rat molar capping animal experiment

Wistar rats aged 30 days were used. Bilateral 0.5mm wounds were created in the first molar to open up the pulp chamber. One wound was capped with a control calcium hydroxide substance (Dycal®), while the contralateral wound was treated by being capped with the same substance containing Dlk1 protein. Rats were

sacrificed 14 days after the procedure. Mandibles were fixed in 10% Formalin (Sigma, HT50-128) for 24 hours before being embedded as formalin fixed paraffin embedded (FFPE) blocks (see section 3.3 Fixation & embedding of formalin fixed paraffin embedded animal tissues). FFPE blocks were sectioned at 10μm thickness and mounted on Superfrost slides (Sigma Aldrich, Z692255). Slides were processed for immunofluorescent staining (see section 3.22 Immunofluorescent staining of frozen tissue, formalin fixed paraffin embedded tissue & cultured cells), or Haematoxylin & Eosin staining (see section 3.23 Haematoxylin & eosin staining of formalin fixed paraffin embedded tissue).

3.3 Fixation & embedding of formalin fixed paraffin embedded animal tissues

FFPE tissue blocks were prepared by initially fixing the tissue in formalin (Sigma, HT50-128). Where appropriate, tissue was decalcified in 14% Ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher, BP2482-100), for 30 days at 4°C prior to processing. Samples were then processed using a Leica HistoCore Pearl through the following program of solutions (Table 1);

Table 1. **Reagents used for formalin fixation of tissue**

Reagent	Company	Catalogue Number	Time in solution	Temperature
Ethanol 70%	VWR Chemicals	20821.321	90 seconds	Room Temperature
Ethanol 70%	VWR Chemicals	20821.321	45 seconds	37°C
Ethanol 80%	VWR Chemicals	20821.321	45 seconds	37°C
Ethanol 95%	VWR Chemicals	20821.321	45 seconds	37°C
Ethanol 100%	VWR Chemicals	20821.321	135 seconds	37°C
Xylenes	Sigma Aldrich	534056	120 seconds	37°C
Paraffin wax	PFM Medical	9000R2010	180 seconds	62°C

After processing tissue was formed into blocks using Leica EG1150H and Leica EG1150C embedding instruments. Blocks were then stored for processing as

outlined in section 3.22 Immunofluorescent staining of frozen tissue, formalin fixed paraffin embedded tissue & cultured cells.

3.4 Freeze embedding of fresh and cultured animal tissues

Fresh tissue was immediately submerged in dry ice cooled 2-methylbutane (Sigma Aldrich, M32631) for 10 minutes. Frozen tissue was then removed and stored at -80°C.

For incisor samples from p30 mice, CL regions were dissected first under a Leica M80 stereo microscope then embedded in Tissue-Tek® O.C.T.™ (Sakura, 4583) and stored at -80°C before sectioning.

Cultured tissues were initially fixed in 10% Formalin (Sigma, HT50-128) for 20 minutes before being embedded in Tissue-Tek® O.C.T.™ and stored at -80°C, as fully described in section 3.22 Immunofluorescent staining of frozen tissue, formalin fixed paraffin embedded tissue & cultured cells.

3.5 Organ culture of murine tissues

Incisors of postnatal day 0-7 mice were extracted by microdissection under a Leica M80 stereo microscope. The dentine of the incisor was isolated, and the dentine washed and stored in Hank's balanced salt solution (HBSS) (Gibco, 14175-053) for 24 hours. From further mice, the incisors were again extracted and the CL region isolated. The tissue was placed into dispase (Roche, 4942078001) for 1 hour at 37°C. After which the dispase was neutralised by 10ml of Dulbecco modified Eagle's medium (DMEM) (Gibco, 31966021) containing 10% foetal bovine serum (FBS) (Sigma, F7524) and 1% penicillin-streptomycin

(Hyclone, SV30079.01). The epithelial tissue was then removed mechanically by microdissection under a Leica M80 stereo microscope.

The dentine slides were placed on sterile cell strainers (Corning 431750) in a 6 well plate (Fisher Scientific, 140685) containing Dulbecco modified Eagle's medium/F12 (DMEM/F12) (Gibco, 31331-028) with 20% FBS, 1% penicillin-streptomycin and 1% L-Ascorbic acid (w/w) (Sigma, A4403). The extracted incisor mesenchyme was then placed on top of the dentine and cultured for 4-7 days.

The dentine-mesenchyme samples were removed from culture and placed directly into 4% paraformaldehyde (PFA) (Sigma Aldrich, 158127) solution in 10mM phosphate buffered saline (PBS) (Sigma, 4417) for 20 minutes at room temperature. After fixation the samples were washed twice in PBS. Samples were then passed through serial solutions of 10%, 20% and 30% sucrose (Sigma, S0389) in PBS for 20 minutes each, before being embedded in Tissue-Tek® O.C.T.™ (Sakura, 4583) and snap frozen by plunging into liquid nitrogen. Once frozen samples were stored at -80 before being sectioned at 15μm and stained for immunofluorescent analysis (see section 3.22 Immunofluorescent staining of frozen tissue, formalin fixed paraffin embedded tissue & cultured cells).

Alternatively, sections were used to visualise the actin cytoskeleton. Samples were cryosectioned at 15μm thickness on a Leica CM1850 cryostat onto Polysine™ Microscope Adhesion Slides (Thermo Scientific, J2800AMNZ). The tissue was fixed in ice cold Acetone (Sigma Aldrich, 34850) for 30 minutes. Slides were washed three times in PBST (PBS containing 0.1% Triton-X100, (Sigma, X100)) for 5 minutes per wash, and then incubated for 30 minutes in 0.1μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich, D9542) with 1:200 DyLight

554 Phalloidin (Cell Signalling, 13054) in PBS. Stained slides were processed as outlined in section 3.24 Three-dimensional reconstruction of the mouse incisor and cultured tissues following immunofluorescent staining.

3.6 Cell culture from established cell lines & primary culture of human and mouse cells

Immortalised odontoblast cell lines MO6-G3 cells (MacDougall et al., 1995) were obtained from Professor Shou Chen, at UT Health San Antonio. Cells were cultured in DMEM (Gibco, 31966021) containing 10% foetal bovine serum (FBS) (Sigma, F7524), 1% penicillin-streptomycin (Hyclone, SV30079.01) and 300μg/ml Geneticin (Gibco, 10131019).

Human BMC's were extracted and cultured in DMEM/F12 (Gibco, 31331-028) containing 20% FBS and 1% penicillin-streptomycin.

CL mesenchyme cells were isolated as follows; Incisors were dissected from postnatal day 30 CD1 mice shortly after death. The CL region was dissected under a Leica M80 stereomicroscope. The explants were incubated for 60 minutes in 1% Collagenase I (Sigma, C0130) in HBSS (Gibco, 14175-053) with 1% penicillin-streptomycin and 1% Fungizone® Antimycotic (Gibco, 15290-018). Following incubation collagenase was neutralised with DMEM/F12 containing 10% FBS and 1% penicillin-streptomycin. The resulting cell suspension was centrifuged at 1000 RCF, at room temperature for 5 minutes. Supernatant was discarded, and the pellet resuspended in complete AmnioMax Media, which combines AmnioMAX™ C-100 Basal Medium (Gibco, 17001-07) and

AmnioMAX™ C-100 Supplement (Gibco, 12556-023) before being cultured on Poly-D-Lysine CELLCOAT® dishes (Greiner Bio One, 628940).

3.7 Synchronisation of cell cycle of cultured cells

Adapting the protocol from(Chen et al., 2012a), cultured cells UT Health San Antonio were grown under different culture conditions in order to induce cell cycle synchronisation. Twenty-four hours after initial seeding and culture under normal cell culture conditions, cells were grown in basal or fully supplemented media as described in Figure 15. After the defined amount of time under this treatment, cells were replenished with new media, either maintaining the current supplement positive or negative condition or returning the appropriate supplements to the culture (Figure 15).

Figure 15. A quiescent mesenchymal stem cell in vitro model system utilises serum starvation to model quiescence
Cultured incisor mesenchyme, when grown under mesenchymal selective conditions displays heterogeneity in cell cycle state. Cells are deprived of serum to induce starvation conditions and so promote cell cycle arrest, producing a largely quiescent cell population. When serum is returned to the culture conditions, cells are encouraged to re-enter the cell cycle, thus producing a more TAC-like population.

Cells were cultured in their standard conditions and classified as "fed" cells, within this population the mixed expression of quiescent MSC and MTAC marker genes was observed. By removing serum supplementation from the culture media cells were considered "starved". The third culture regimen was also undertaken whereby the cells were starved initially before having serum replenished before analysis, thus encouraging the cells to re-enter the cell cycle and mimic TAC-like cells, termed "recovered" cells.

3.8 Cell culture confluency assay

Cells were seeded at equal densities of $1x10^5$ into a 6 well plate (Fisher Scientific, 140685). Wells were synchronised as described in section 3.7 Synchronisation of cell cycle of cultured cells. In certain conditions siRNA was added to the culture at the point of replenishing the supplements to the media (see section 3.11 Transfection of cultured cells with short interfering RNA).

Cells were fixed in 10% Formalin (Sigma, HT50-128) for 30 minutes at room temperature before being washed 3 times for 5 minutes each in HBSS (Gibco, 14175-053). Cells were visualised using 0.01% Crystal Violet solution (Sigma, V5265). Images were taken of four areas of each well on a Leica DM1000 LED microscope. Confluency was measured using FIJI (Image J 1.51n).

3.9 Infection of cultured cells with Ki67-FUCCI cell cycle indicator

Ki67-mCherry-BSD and Ki67-mAG-NEO plasmids were gifted by Alexander Zambon. Human BMCs were incubated with the plasmid containing viral containing supernatant with 10µg/ml polybrene (Millipore, TR-1003-G) for 2 hours.

Following the incubation, the supernatant was replaced with normal growth media. Infected cells were definitively selected using 10µg/ml blasticidin (Sigma Aldrich, 15205) and 250µg/ml Geneticin (Gibco, 10131019).

3.10 Flow cytometric analysis of cultured cells

FUCCI infected BMCs (see section 3.9 Infection of cultured cells with Ki67-FUCCI cell cycle indicator) were harvested (see section 3.7 Synchronisation of cell cycle of cultured cells), fixed in 4% PFA (Sigma Aldrich, 158127) solution in 10mM PBS (Sigma, P4417) for 30 minutes at room temperature, and analysed using the BD FACSCanto II SOR (Beckman Coulter). Data was acquired using blue laser (488 nm) for mAzamiGreen signal and yellow-green laser (561 nm) for mCherry. Results were analysed using the FlowJo software v10.3 (Tree Star Inc.).

For flow cytometry analysis of stained cultured cells, the cells were collected and stained using the Mouse Mesenchymal Stem Cell Marker Antibody Panel (R&D, SC018). Cells were harvested and fixed in flow staining buffer (R&D, FC001) for 15 minutes at room temperature. After 30 minutes of incubation with the primary antibodies provided in the kit, the cells were washed and incubated for 30 minutes with secondary antibodies appropriate to the primary antibodies used (Alexa 488 donkey anti-mouse IgG (Life Technologies, A21202) or Alexa 488 donkey anti-rat IgG (Life Technologies, A21208).

Cells were analysed on a BD Accuri C6 (Beckman Coulter) Data was acquired using the blue laser (488nm) to detect the Alexa 488 signal from the bound secondary antibodies. Analysis was performed in the BD Accuri C6 Software (Accuri Cytometers Inc, version 1.0.264.21)

3.11 Transfection of cultured cells with short interfering RNA

Short interfering RNA was added to cells in accordance with the protocol as outlined in the information sheet provided by polyplus INTERFERin kit (Polyplus, 409-10).

Table 2. **siRNA constructs used for cell culture transfection**

Target	Conc^N	Company	siRNA Code
Mm_SmarcA2_4	10µM	Qiagen	2182015
Mm_SmarcA2_3	10µM	Qiagen	2182014
mDlk1	10µM	Invitrogen	39
mDlk1	10µM	Invitrogen	38

In brief, siRNA was complexed with interferin reagent in DMEM (Gibco, 31966021) containing 1% penicillin-streptomycin (Hyclone, SV30079.01) for 15 minutes. The media on the cells is then replaced with fresh complete media and 100µl of each siRNA complex was added per well of a 24 well plate. Cells were collected or fixed after 48 or 72 hours as appropriate. siRNAs used are listed in Table 2.

3.12 Treatment of cultured cells with recombinant proteins

Cells were treated with recombinant proteins either through direct addition of the protein to fresh media (without the use of carriers) or via an indirect coating method.

Direct protein treatment involved the addition of the protein into fresh un-supplemented or fully supplemented media as appropriate to the cell line. This fresh media was then placed onto the cells which had been cultured previously for 24 hours. Media containing the protein was replaced every 48 hours.

The indirect binding method involved passaging cells onto pre-prepared dishes which had already been coated with protein. Dish preparation was performed as follows; 6 well plate (Fisher Scientific, 140685) plates were coated by placing 10μg/ml of Goat anti-human IgG (Sigma, I1886) or Goat anti-Mouse IgG (Sigma, M8642) in HBSS (Gibco, 14175-053) into the dish. The dish was then incubated for 30 minutes at 37°C. Dishes were quickly washed five times in HBSS and then blocked by incubating with a 2% solution of bovine serum albumin (BSA) (Sigma, A2153) in HBSS over night at 4°C. Following blocking 10μg/ml of the recombinant Human Dlk1 protein (R&D, 144-PR/CF, lot HNE041302) was added to the blocking solution and incubated for 2 hours at 37°C. Following preparation of the dishes they were washed quickly in HBSS 5 times and immediately cells are plated into them.

3.13 Treatment of cultured cells with synthetic probe, PFI3

Cells were treated with PFI-3 (Sigma, SML 0939) by adding PFI-3 directly to fresh cell culture media. As PFI-3 was dissolved in Dimethyl sulphoxide (DMSO) Hybri-Max (Sigma, D2650) as stock, the 50mM stock was diluted 1:1000 in media before being used at final concentrations of 5μM 2μM or 1μM.

After culture cells were collected for crystal violet staining and RNA as described in section 3.8 Cell culture confluency assay.

3.14 Chromatin immunoprecipitation of cultured cells

ChIP was performed on cells using the ChIP-IT high sensitivity kit (Active Motif, 53040) in accordance with the provided protocol. In summary the procedure was as follows;

Cells were fixed using Complete Cell Fixation Solution added directly to the culture media for 15 minutes at room temperature. Fixation was quenched using Stop Solution. Cells were scraped and collected by centrifugation for 3 minutes at 1250 RCF at 4°C. The pellet was resuspended and washed in ice-cold PBS Wash Buffer twice via centrifugation. The resultant pellet was resuspended in Chromatin Prep Buffer supplemented with protease inhibitor cocktail (PIC) and phenylmethylsulphonyl fluoride (PMSF) and incubated for 10 minutes on ice. Using a Dounce homogeniser the cells were homogenised, collected by centrifugation and resuspended in fresh ChIP Buffer supplemented with PIC and PMSF.

Chromatin was sheared using a Diagenode Bioruptor Pico (Diagenode) by sonicating for 15 seconds followed by 30 seconds of rest for four cycles. Cell debris was removed by pelleting this out by centrifugation.

Antibodies or control IgG was mixed with Blocker as outlined in Table 3;

Table 3. Antibodies used in ChIP assays

Antibody	Company	Catalogue Number	Amount	IgG	Company	Catalogue Number
h,mRBPSUH(D10A4)	Cell Signalling	5313	100ng	rabbit IgG from serum	Sigma-Aldrich	I5006
H3K27me3	Diagenode	pAb-069-050	1µg	rabbit IgG from serum	Sigma-Aldrich	I5006
H3K9me3	Diagenode	pAb-056-050	1µg	rabbit IgG from serum	Sigma-Aldrich	I5006

This antibody mix was mixed with ChIP Buffer, PIC and sonicated chromatin and incubated over night at 4°C.

Immunoprecipitation was performed by washing Protein G agarose beads in TE pH 8.8 and then mixing the washed beads to the antibody/chromatin mixture and incubating for 3 hours at 4°C. Following incubation with the beads the solution was passed through a ChIP filtration column to collect the bound chromatin. The bound chromatin was washed and eluted using the provided solutions. The cross-links within the eluted sample was reversed and the DNA purified by overnight incubation at 37°C in Proteinase K. The DNA was collected by heating the sample to 95°C for 8 minutes and then plunging into ice for 1 minute to deactivate the Proteinase K function. DNA Purification Binding Buffer and Sodium Acetate was added to the sample which was then passed through a DNA Purification Column using centrifugation. The column was washed in DNA Purification Wash Buffer and finally eluted from the column using DNA Purification Elution Buffer.

Figure 16. The regions of the genome targeted by ChIP assay primers
A A schematic showing the location of RBP-Jκ binding sites within 10000 base pairs of the SmarcA2 transcript variant 1 start codon (numbered 1-20) with the target region of primers designed to cover these (marked a-k & z-v) B Illustration of the Dlk1 loci with CpG islands as presented on USCS genome browser (2011).

Input DNA was prepared in the same manner, however rather than column-based purification. Following proteinase K treatment, the DNA was immunoprecipitated in 2-Propanol (Fluka Analytical, 34965) with 1ul GlycoBlue (Ambion, AM9515), before washing in 70% ethanol (VWR Chemicals, 20821.321) and resuspended in 0.1% diethylpyrocarbonate (Sigma Aldrich, D5758) treated water (DEPC).

Regions of the genome targeted by primers designed for ChIP as described in 3.19 Real-time PCR & data analysis, to target the regions of CpG islands associated with the Dlk1 gene loci, and the Promotor region of the SmarcA2 gene containing Rbp-Jκ binding sites (Figure 16).

3.15 Laser capture microdissection of frozen & formalin fixed paraffin embedded tissues

Frozen tissue was prepared in OCT then frozen at -80°C before being sectioned using a Leica CM1850 cryostat at a thickness of 20μm. Sections were mounted onto PEN Membrane Glass Slides (Applied Biosystems, LCM0522).

FFPE blocks were sectioned on a Microm HM320 microtome at a thickness of 10μm and placed onto PEN Membrane Glass Slides. After drying overnight, deparaffinisation was performed. Slides were heated to 55°C for 20 minutes before being twice washed in xylenes (Sigma Aldrich 534056) for 10 minutes. Slides were then washed in 100% industrial methylated spirits (IMS) (VWR, 23684.360) for 5 minutes, before being washed for 2 minutes in 95% IMS, then 70% IMS and finally in distilled water.

All prepared slides were stained using 1% Methyl Green (Fluka Analytical, 67060) in 0.1% DEPC (Sigma Aldrich, D5758), then washed three times for 30 seconds

in 0.1% DEPC before being allowed to dry for 5 minutes in order to make visualisation of the structures clear. An ArcturusXT™ LCM instrument was used to perform LCM onto CapSure® Macro LCM Caps (Applied Biosystems, LCM0211). Capture was performed utilising the ultraviolet laser to cut around regions of interest, particularly the CL-MSC, MTAC, Odontoblast and NVB containing dental pulp. The infrared laser was then deployed to fix the cap material to the section allowing the region to be lifted from the slide.

Captured tissue to be used for RNA analysis was stored in Tri-Reagent (Sigma, T9424) at -80°C within 30 minutes of initial sectioning. Captured tissue to be used for gDNA analysis was processed immediately (see section 3.18 Extraction of genomic DNA from laser capture microdissected tissue).

3.16 Extraction of mRNA from cultured cells & LCM captured tissue

Samples were collected from tissue using LCM (see section 3.15 Laser capture microdissection of frozen & formalin fixed paraffin embedded tissues). Alternatively, from cultured cells by washing the cells in HBSS (Gibco, 14175-053) briefly before covering the cells in Tri Reagent (Sigma, T9424) for 5 minutes at room temperature, before scraping the cells, collecting 500ul of cell suspension.

RNA was extracted and purified using an acid guanidinium thiocyanate-phenol-chloroform extraction protocol. Briefly, the total RNA was extracted using Tri-Reagent. The samples were vortexed and incubated for 5 minutes at room temperature before addition of Chloroform (Sigma Aldrich, C2432) at a ratio of 5:1 (lysate: chloroform). The samples were then incubated for a further 5 minutes before centrifugation for 10 minutes at 13000rpm. The aqueous phase was then

collected and added 1:1 with 2-Propanol (Fluka Analytical, 34965) with 1ul GlycoBlue (Ambion, AM9515). The sample was incubated at -20°C overnight before centrifugation at 13000rpm for 45 minutes at 4°C. The subsequent pellet was then washed in 1ml 70% Ethanol (VWR Chemicals, 20821.321) and centrifuged for 10 minutes at room temperature at 9000rpm. The resulting pellet was resuspended in 10ul of 0.1% DEPC (Sigma Aldrich, D5758).

Purified RNA was quantified on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). Quality control was assessed by analysis of the 260/230 ratio, which highlights phenol contamination and 260/280 ratio more than 1.8 which can be used as an indicator of DNA contamination.

3.17 Reverse transcription of mRNA extracted from cultured cells & laser capture microdissected tissue

Reverse transcription polymerase chain reaction (RT-PCR) was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) in accordance with the manufacturers protocol on a Veriti™ Thermal Cycler 96 well (Applied Biosystems) using manufacturers program of 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C indefinitely thereafter. Samples were diluted in 180µl of 0.1% DEPC (Sigma Aldrich, D5758) and stored at -20°C.

3.18 Extraction of genomic DNA from laser capture microdissected tissue

LCM was used to capture regions of tissue of interest (see section 3.15 Laser capture microdissection of frozen & formalin fixed paraffin embedded tissues). CapSure® Macro LCM Caps (Applied Biosystems, LCM0211) with captured

tissue attached, were placed into 30µl of DNA lysis buffer. Lysis buffer contained 10mM TrisCL ph8 (Sigma Aldrich, T1503), 1mM EDTA (Thermo Fisher, BP2482-100), 1% Tween-20 (Sigma Aldrich, P9416), 0.04% Proteinase K (RPROTK-RO ROCHE, 03115836001). Samples were inverted and incubated at 58°C overnight. Caps were inspected visually to confirm that the tissue had been removed into the reagents. The sample was heated to 95°C for 8 minutes, before being plunged into ice for 1 minute. DNA concentration of the sample was measured on a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). Analysis was performed by real-time PCR (see section 3.19 Real-time PCR & data analysis) followed by gel electroporation (see section 3.21 Electroporation of PCR produced amplicons).

3.19 Real-time PCR & data analysis

Real-time RT-PCR analysis was performed on a LightCycler 480 Real-Time system (Roche) for 45 cycles, using a SYBR Green I MasterMix (Roche) and primers listed below. Internal control primers were used to determine relative quantification of gene expression using the 2-ΔΔCt method. Analyses were performed using three technical replicates.

Real Time PCR was performed in triplicate. Samples were combined with LightCycler 480 SYBR Green I master kit (Roche Life Science, 4887352001) (diluted 1:1 with the water provided in accordance with the manufacturer's instructions) and appropriate primers in a ratio of 1:8:1 totalling 10µl. This equates to a final primer concentration of 1µM. The samples were analysed with each primer in triplicate as a technical control.

Figure 17. Molecular markers, differentially expressed in quiescent mesenchymal stem cells and mesenchymal transit amplifying cells, are involved in diverse cellular processes
Schematic representation of a panel of genes identified as being up or down regulated in quiescent MSCs adapted from (Cheung and Rando, 2013). Genes are grouped by cellular function/role. Green arrows indicate genes which are commonly upregulated in quiescent MSCs. Red arrows denote downregulation is commonly observed in quiescent MSCs, thus genes which are more commonly associated with mitotically active MTAC-like cells.

A panel of genes known to be upregulated or down regulated within quiescent MSCs in a number of tissues was identified as potential targets for analysis using real time polymerase chain reaction (PCR). This panel was made up of a selection of genes identified in Cheung & Rando's 2013 paper (Cheung and Rando, 2013). These genes were identified as being involved in many cellular processes which govern self-renewal, cell cycle arrest/progression and differentiation (Figure 17).

Primers for mRNA transcription analysis were designed against the CDS sequence for each gene, provided by NCBI gene (http://www.ncbi.nlm.nih.gov/gene/). Primers for the assessment of genomic DNA (gDNA) were designed against the gDNA sequence. All primers were generated using Primer 3 website (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and validated on UCSC In-SilicoPCR (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) assembly Dec11(GRCm38/mm10).

Table 4. Primers used for mRNA assessment

Gene for mRNA Analysis		Product Size	Forward Primer / Reverse Primer
Anln	anillin, actin binding protein	153	Anln-F, 5'-ATTTTGTGCAAGACGCAACA-3'; Anln-R, 5'-ATCCAGAGATCCCGCTCTTC-3';
Ccna2	cyclin A2	164	Ccna2-F, 5'-CATTGGCACAACAGACTGGA-3'; Ccna2-R, 5'-TGTCTCTGGTGGGTTGAGAA-3';
Ccnb1	cyclin B1	*159*	*Ccnb1-F, 5'-TTCCTGTTATGCAGCACCTG-3'; Ccnb1-R, 5'-CGGCCTTAGACAAATTCTGA-3';*
Ccnd3	cyclin D3	*164*	*Ccnd3-F, 5'-GCGATGTATCCTCCATCCAT-3'; Ccnd3-R, 5'-GCAGCTTCGATCTGTTCCTG-3';*
Cdh1	cadherin 1	*245*	*Cdh1-F, 5'-CAGCTGCCCCGAAAATGAAAAGG-3';* Cdh1-R, 5'-TCCACCGCTTCCCCATTTGATG-3';
Cdkn1A	cyclin-dependent kinase inhibitor 1A (P21)	142	Cdkn1A-F, 5'-CTTGTCGCTGTCTTGCACTC-3'; Cdkn1A-R, 5'-TCTCTTGCAGAAGACCAATCTG-3';
Cdkn1B	cyclin-dependent kinase inhibitor 1B	152	Cdkn1B-F, 5'-TTGGGTCTCAGGCAAACTCT-3'; Cdkn1B-R, 5'-TCTGTTGGCCCTTTTGTTTT-3';
Cdkn1C	cyclin-dependent kinase inhibitor 1C (P57)	146	Cdkn1C-F, 5'-TCTAGGGGAATGGTTGTTGA-3'; Cdkn1C-R, 5'-GATTTTTGTTGGGCCTCTTT-3';
Ctdsp1	carboxy-terminal domain, RNA polymerase II, polypeptide A, small phosphatase 1	149	Ctdsp1-F, 5'-CGCCTCCTATGTCTTCCATC-3'; Ctdsp1-R, 5'-GCCTAGGCTGTCTGAGCACT-3';
Cycs	cytochrome c, somatic	159	Cycs-F, 5'-AAATCTCCACGGTCTGTTCG-3'; Cycs-R, 5'-GCGAAGATCATTTTTGTTCCA-3';
Dlk1	delta like non-canonical Notch ligand 1	276	Dlk1-F, 5'-GCGTGGACCTGGAGAAAG-3'; Dlk1-R, 5'-GGAAGTCACCCCCGATGT-3';
Ezh1	enhancer of zeste 1 polycomb repressive complex 2 subunit	149	Ezh1-F, 5'-ACCCCAACTGTTATGCCAAA-3'; Ezh1-R, 5'-CCTCTCGATGCCCACATACT-3';
Foxo3	forkhead box O3	130	Foxo3-F, 5'-ACAAACGGCTCACTTTGTCC-3'; Foxo3-R, 5'-CTGTGCAGGGACAGGTTGT-3';
GapDH	glyceraldehyde-3-phosphate dehydrogenase	148	GapDH-F, 5'-ATCACTGCCACCCAGAAGAC-3'; GapDH-R, 5'-CAGTGAGCTTCCCGTTCAG-3';
Hat1	histone aminotransferase 1	164	Hat1-F, 5'-TTCGACTGCTGGTGACTGAC-3'; Hat1-R, 5'-TGGTCTCAGGCATTTCTTCA-3';
Hes1	hes family bHLH transcription factor 1	148	Hes1-F, 5'-CCAAGCTAGAGAAGGCAGACA-3'; Hes1-R, 5'-GTCACCTCGTTCATGCACTC-3';
Hes5	hes family bHLH transcription factor 5	164	Hes5-F, 5'-AGGGTAGCAGCTTTCAGGAT-3'; Hes5-R, 5'-AGCCTCTGGGATCTCCTCTA-3';
Heyl	hairy/enhancer-of-split related with YRPW motif-like	141	Heyl-F, 5'-TTTGAGAAACAGGGCTCCTC-3'; Heyl-R, 5'-CCCAATACTCCGGAAGTCAA-3';
K14	keratin 14	190	K14-F, 5'-CCTGCTGGATGTGAAGACAA-3'; K14-R, 5'-ATCGTGCACATCCATGACCT-3';
Ki67	antigen identified by monoclonal antibody Ki 67	194	Ki67-F, 5'-AGCAAACCAGCTGCAGAAAT-3'; Ki67-R, 5'-TTCTTGGTGCATACAATGTCTT-3';
Notch1	notch 1	150	Notch1-F, 5'-TGTTGTGCTCCTGAAGAACG-3'; Notch1-R, 5'-TCCATGTGATCCGTGATGTC-3';
Notch2	notch 2	157	Notch2-F, 5'-GAGGCGACTCTTCTGCTGTT-3'; Notch2-R, 5'-CCATGTGGTCAGTGATGTCC-3';
Pcna	proliferating cell nuclear antigen	167	Pcna-F, 5'-GAAGAGGAGGCGGTAACCAT-3'; Pcna-R, 5'-TGTCCCATGTCAGCAATTTT-3';
PdgfrB	platelet derived growth factor receptor, beta polypeptide	210	PdgfrB-F, 5'-CAGAAATGCTGGGAAGAAAA-3'; PdgfrB-R, 5'-AACAGAGCTGGTGTCCAGAG-3';
Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	153	Pdk1-F, 5'-TACGGGACAGATGCGGTTAT-3'; Pdk1-R, 5'-GGTCATGTCTTTCGGCTCTC-3';
Prdm5	PR domain containing 5	166	Prdm5-F, 5'-AGTGCTCAGAGTGCAGCAAG-3'; Prdm5-R, 5'-ATCGGACGGTTAGGATTGTG-3';
Ptov1	prostate tumour over expressed gene 1	145	Ptov1-F, 5'-CGCCTGGTACAGTTCCACTT-3'; Ptov1-R, 5'-TCCGAGGAGTACAGGAGCAT-3';
Rbp-Jк	recombination signal binding protein for immunoglobulin kappa J region	166	Rbp-Jк-F, 5'-GACAAGGCCGAGTACACGTT-3'; Rbp-Jк-R, 5'-CATCCCCAAACCACACTCTT-3';
Sgol1	shugoshin 1	145	Sgol1-F, 5'-CCAGCAGTGGCTCTGACTAA-3'; Sgol1-R, 5'-TCATACCCTTTTCTGCTTGA-3';
SmarcA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	172	SmarcA2-F, 5'-AGGCGAAATCTGTGAAGGTG-3'; SmarcA2-R, 5'-TCAGTTCCACTTGCTTCTGACT-3';
Tef	thyrotroph embryonic factor	147	Tef-F, 5'-AGCTCTTCAACCCTCGGAAG-3'; Tef-R, 5'-GAGCGTTTAGCTGCCACATT-3';
Thra	thyroid hormone receptor alpha	155	Thra-F, 5'-GGCTGTGCTGCTAATGTCAA-3'; Thra-R, 5'-TCACCTTCATCAGCAGCTTG-3';
Top2a	topoisomerase (DNA) II alpha	147	Top2a-F, 5'-GAACAGTCGCAAAAGGAAGC-3'; Top2a-R, 5'-GCTCGAGGAGCTATCGTGTC-3';
Vimentin	vimentin	148	Vim-F, 5'-CCAACCTTTTCTTCCCTGAA-3'; Vim-R, 5'-GGTCATCGTGATGCTGAGAA-3';
Zbtb20	zinc finger and BTB domain containing 20	169	Zbtb20-F, 5'-CAAGGCGACAAAAAGCCTTA-3'; Zbtb20-R, 5'-GCGTCACCATGTGCTTGATA-3';
Zfp30	zinc finger protein 30	154	Zfp30-F, 5'-GGCTGAGAAGAGAAGGTGGA-3'; Zfp30-R, 5'-TTTCCCCAAAAGGGAGTCTT-3';

Primers were designed to give an amplicon length that reduces the risk of dimers and hairpins. Primers for the assessment of mRNA levels were also designed to cross an exon to negate the problems of gDNA contaminants being amplified. To reduce the risks posed by RNA degradation leading to non-binding of primers, primers were designed to bind towards the 3' end of the cDNA. Primers were validated against cDNA extracted from mouse embryonic fibroblasts, to ensure that they detect cDNA and the amplicons produced are of the anticipated length. Mouse and human primers used in this study are shown in Table 5.

Table 5. **Primers used for gDNA assessment**

Experiment	Product Size	Forward Primer 5'-3'	Reverse Primer 5'-3'
Dlk1 methylation ChIP	5' CpG island site 1	CTTTTCGTGGTGGTTTTCGT	GCAAGTCTCAGGAACCAAGC
Dlk1 methylation ChIP	5' CpG island site 2	GTGCAACCCTGGCTTTCTT	AAGAAAGCCAGGGTTGCAC
Dlk1 methylation ChIP	5' CpG island site 3	GTGGTTTTCGTGTGTGCATC	GCAAGTCTCAGGAACCAAGC
Dlk1 methylation ChIP	5' CpG island site 4	ACCCGTCTAGCCAAAGAGTG	AAGAAAGCCAGGGTTGCAC
Dlk1 methylation ChIP	5' CpG island site 5	GTGCAACCCTGGCTTTCTT	GGCTCACCATAGGTGCTGTG
Dlk1 methylation ChIP	3' CpG island site 1	CTAACCCATGCGAGAACGAT	GCTTGCACAGACACTCGAAG
Dlk1 methylation ChIP	3' CpG island site 2	GGATTCGTCGACAAGACCTG	TCGGTGAGGAGAGGGGTACT
Dlk1 methylation ChIP	Non-CpG island control site 1	AAACACAAGCTCACAGCCTCT	TGCAGTTTCAAGAGCACCAG
Dlk1 methylation ChIP	Non-CpG island control site 2	CCCATCTAAAAATCCCAACATC	AAAACCCCAGGTTCAGTTCC
Dlk1 methylation ChIP	Non-CpG island control site 3	GGAACTGAACCTGGGGTTTT	CCCCACACGGTAGAAGAAGA
Rbp binding SmarcA2 ChIP	Non-Rbp-Jκ binding control Site	GACACATGGTTCACATTCTCC	AGTCACTTGATGGCACCTGT
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 3	CCACCAATGGCATCCTTAAC	CAGGGAACATTGGGCATATT
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 3	GCACACTGGAAAAGGACCTC	TTCTGGGGATTCCATTCATC
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 4 & 5	CTGAGCCCTGTGACAGATCA	GCACACTGGAAAAGGACCTC
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 6 & 7	CGCATGGAAGCCTAAATGTT	TTAGGCTGCTGGCCATAAAT
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 8	TCACCTTTGCAGGTCCCTAT	CACTCCCACAGAACCCTGAG
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 9 & 10	CAAGGCTTATCGCTCTCCTG	ACCTCCAAAACTGCCCTTCT
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 11	GAGGCTTAGGCTGACTGTGG	TCCCTGCCACATTACTCTCC
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 12 & 13	TGGAGGGGGTCTTTGTGATA	TCTGGGTCATGTCGATAGGG
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 14	TTGGAGATCACCCATCATCA	GGAAATGGGAAGTGATGGAA
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 15	GGCTGGCTGGTCTAATTTCA	TTCCGGCAGAAATCAAAGAT
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 16	GACCAATTCTGGGGCCTAGT	ATGCCTGAGAAGACGGGTAA
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 17	GGGCTGGTCTTTCCTCATTT	TGGTTAAAGTGGCACATGGA
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 18	TCCATGTGCCACTTTAACCA	CTCCAGCAAACAAACAAGCA
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 19	CCCCACTTTTGCTTGTTTGT	TGTCAAGGTGTCACCCCATA
Rbp binding SmarcA2 ChIP	Rbp-Jκ binding site 20	GAGGGAAAATGTGGAGGACA	GGAAGCACACAAGCTCAAAA

Real-time PCR was performed using a Roche Lightcycler 480 Instrument II 384-well block real-time PCR machine (Roche), in accordance with the manufacturer's instructions. Program details are outlined in Table 6.

Table 6. **Program used for Real-Time PCR**

Program Step	Temperature	Time (hh:mm:ss)	Repeats
Pre-Incubation	95°C	00:05:00	1
Amplification	95°C	00:00:10	45 (from cultured cell sample)
	60°C	00:00:20	55 (from LCM sample)
	72°C	00:00:10	
Melting Curves	95°C	00:00:05	1
	65°C	00:01:00	
	97°C	Until reached	
Cooling	95°C	00:00:01	1

Results were analysed using comparative Ct methods. PCR amplification was checked to ensure appropriate amplification curves and annealing temperature was obtained. Results were exported into Microsoft Excel.

3.20 Statistical analysis of real-time PCR data

Experimental data was analysed using PRISM 5 software (Graph Pad Software). For Real-Time PCR analysis data is shown as mean with standard error of mean, a Two-way ANOVA followed by Bonferroni correction was performed. Statistical significance was set at * $p < 0.05$, ** $p<0.01$ & *** $p<0.001$. Experimental n numbers are shown in figure legends.

3.21 Electroporation of PCR produced amplicons

Amplicons were run on an agarose gel (2% Agarose (Thermo Fisher BP160-100) in TAE buffer (Life Technologies, 15558-042)). Once microwaved and cooled 20µl of Sybr Safe DNA gel stain (Invitrogen, PIN533102) was added to the molten

gel before being cast. Following pouring the gel was allowed to set for 30 minutes at room temperature.

The gel was submerged in TAE buffer and the comb removed. 20µl of PCR product was mixed with 4µl of loading dye (Thermo Scientific, R0611) and loaded into each well. The gel was electroporated for 15 minutes at 125 volts at room temperature. The gel was then imaged using a UV light chamber.

3.22 Immunofluorescent staining of frozen tissue, formalin fixed paraffin embedded tissue & cultured cells

Preparation and fixation of cells to be used for immunofluorescent (IF) analysis was performed. Cells were washed in HBSS (Gibco, 14175-053). Then fixed in ice cooled 4% PFA (Sigma Aldrich, 158127) solution in 10mM PBS (Sigma, P4417) for 30 minutes.

Preparation and fixation of frozen tissue is as follows; Frozen tissue was cryosectioned at 15µm thickness on a Leica CM1850 cryostat. Sections were mounted onto Polysine™ Microscope Adhesion Slides (Thermo Scientific, J2800AMNZ) and allowed to air dry for 30 minutes before were fixed in ice cold Acetone (Sigma Aldrich, 34850) or freshly made ice cooled 4% PFA (Sigma Aldrich, 158127) solution in 10mM PBS (Sigma, P4417) for 30 minutes.

Preparation of FFPE samples is performed as described here; FFPE samples were sectioned on a manual microtome at a thickness of 10µm, and placed onto Superfrost slides (Sigma Aldrich, Z692255). After drying overnight, deparaffinisation was performed. Slides were heated to 55°C for 20 minutes before being twice washed in xylenes (Sigma Aldrich 534056) for 10 minutes.

Slides were then washed in 100% IMS (VWR, 23684.360) for 5 minutes, before being washed for 2 minutes in 95% IMS and then 70% IMS. Antigen retrieval was performed by microwaving the slides in a 0.01M citrate buffer solution (citric acid (Sigma Aldrich, C2404) & 0.05% Tween (Sigma Aldrich, P9416)) for 1 minute.

Table 7. Antibodies used in immunofluorescence assays

Primary Antibody	Company	Catalogue number	Secondary antibody (catalogue number, company)
Dlk1	Abcam	21682	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Dmp1	R&D Systems	AF4386	Alexa 488 donkey anti-sheep IgG (A11015, Life Technologies)
Dsp	Gifted by Dr Larry Fisher (NIH)		Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
GFP	Abcam	ab6673	Alexa 488 donkey anti-goat IgG (A11055, Life Technologies)
Gli1	Novus	NBP1-78259	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
H3k9me3	Diagenode	pAb-056-050	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
H3k27me3	Diagenode	pAb-069-051	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Hes1	Cell Signalling	11988s	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Jag1	Cell Signalling	2620	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Jag2	R&D Systems	MAB4748	Alexa 488 donkey anti-rat IgG (A21208, Life Technologies)
K14	Covance	PRB-155P-100	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Ki67	R&D Systems	AF7649	Alexa 488 donkey anti-sheep IgG (A11015, Life Technologies)
Notch1	eBioscience	14-5785	Alexa 488 donkey anti-mouse IgG (A21202, Life Technologies)
Notch2 NICD	Sigma	410353	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Notch2	Cell Signalling	5732	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Notch3	R&D Systems	AF1308	Alexa 488 donkey anti-goat IgG (A11055, Life Technologies)
Pdgfrβ	eBioscience	16-1401	Alexa 488 donkey anti-rat IgG (A21208, Life Technologies)
Smarca2	AbCam	15597	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)
Thy-1	eBioscience	11-0903-82	Alexa 488 donkey anti-rat IgG (A21208, Life Technologies)
Zbtb20	Sigma	HPA016815	Alexa 568 donkey anti-rabbit IgG (A10042, Life Technologies)

Once prepared all sample types were washed three times in PBST (PBS containing 0.1% Triton-X100, (Sigma, X100)) for 5 minutes per wash. Non-Specific binding was blocked by incubation for 60 minutes with PBST containing 5% Donkey Serum (Sigma, D9663), 0.25% cold water fish gelatine (Sigma, G7765) and 0.25% BSA (Sigma, A2153).

Primary antibodies were incubated overnight. Slides were washed three times in PBST at room temperature before incubation with secondary antibodies for 2hrs at room temperature. Secondary antibodies were incubated for 2 hours. Nuclei were counterstained with 2µg/ml DAPI (Sigma Aldrich, D9542) for 10 minutes.

An overview of antibody concentrations and secondary pairings is summarised in Table 7.

IF images were captured using a Leica DMI6000 confocal microscope with a Leica TCS SP8 attachment at a scanning thickness of 1µm per section. The microscope is running LAS AF software from Leica. Images for comparison were taken using the same settings and post imaging processing was conducted using Adobe Photoshop CC also in parallel between comparable samples.

3.23 Haematoxylin & eosin staining of formalin fixed paraffin embedded tissue

Slides of FFPE sectioned tissue are stained using Haematoxylin and Eosin histological dyes according to the following protocol. FFPE samples were sectioned on a manual microtome at a thickness of 10µm, and placed onto Superfrost slides (Sigma Aldrich, Z692255). After drying overnight, deparaffinisation was performed. Slides were heated to 55°C for 20 minutes. Subsequently slides were washed twice in xylenes (Sigma Aldrich, 534056) for 5 minutes. The tissue is then rehydrated through 2 changes of 100% alcohol, followed by a wash in 95% alcohol and then 70% alcohol (VWR, 23684.360), each for 2 minutes. The slides were briefly washed in distilled water before being stained for 8 minutes in Harris haematoxylin (Sigma, HHS16). After staining was

sufficient the slides were washed in tap water for 5 minutes. Differentiation of the stain was achieved by placing the slides in 1% Acid alcohol (Sigma Aldrich, 56694) for 30 seconds, before a further 5-minute tap water wash. Subsequent counterstaining was performed by washing the slides briefly in 95% alcohol, followed by a 1-minute incubation in 0.25% eosin Y solution (Sigma, 230251). After staining was complete, the tissue was dehydrated by passing the slides through two 5-minute washes in 95% alcohol, then 100% alcohol. In order to clear the sample, the slides were washed twice more in xylenes for 5 minutes before being mounted using Eukit, xylenes based mounting medium (Fluka, 03989).

3.24 Three-dimensional reconstruction of the mouse incisor and cultured tissues following immunofluorescent staining

For dentine culture assay, IF images were captured using A Leica DMI6000 confocal microscope with a Leica TCS SP8 attachment at a scanning thickness of 1μm per section. The microscope is running LAS AF software from Leica. These images were used to reconstruct the growth of the cells into the dentine tubules using Imaris 9 (Bitplane).

The 3D reconstruction of the CL and surrounding transit amplifying mesenchyme region was performed by taking serial immunofluorescently labelled cryosections. The epithelium was outlined from approximately 20 consecutive 20-μm-thick sagittal sections of the apical end of the mouse incisor. The corresponding TAC region was defined by the location of ki67 positive nuclei in the mesenchyme adjacent to the basement membrane. Computational reconstruction using BioVis software (version: 3.1.1.11) (http://www.biovis3d.com) was used to calculate the volume of the TAC region.

4 Results

4.1 There is a novel endogenous pool of mesenchymal stem cells within the murine incisor

In order for the mouse incisor to continuously grow throughout an animal's life, it is necessary for a pool of SCs to reside within the tissue.

Within the epithelium there is a pool of well characterised quiescent epithelial SCs in a structure called the CL at the apical end of the mouse incisor. The epithelial CL is surrounded by mesenchymal tissue. Within the dental mesenchyme of the incisor there is a known region of MTACs. These cells can differentiate to populate the odontoblast layer and participate in the production of dentine. The hypothesis of this body of work is that within the mesenchyme of the continuously growing mouse incisor there is a quiescent, cervical loop adjacent, MSC population which provide the pool of cells which go on to populate the MTAC region.

4.1.1 The region of incisor mesenchyme adjacent to the epithelial stem cell region displays a quiescent mesenchymal stem cell marker signature

The mesenchyme contacting the EpSC region of the CL has been highlighted by Gli1 reporter studies (Seidel et al., 2010, Zhao et al., 2014). This region's proximity to the MTAC region of the incisor tooth, adjacent position to the EpSCs of the CL, indicated that this may be a region of interest in the identification of MSCs within the incisor tooth. Using immunofluorescent analysis, the expression of Thy1 and Gli1 was indeed expressed within the region of interest in addition to being expressed within the NVB-MSCs (Figure 18).

Figure 18. Confirmation of stem cell marker gene expression in the region of mesenchyme adjacent to the cervical loop
*Postnatal day 7 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained with antibody specific to **A** Gli1 & **B** Thy1, counterstained with DAPI. Representative images, n=2*
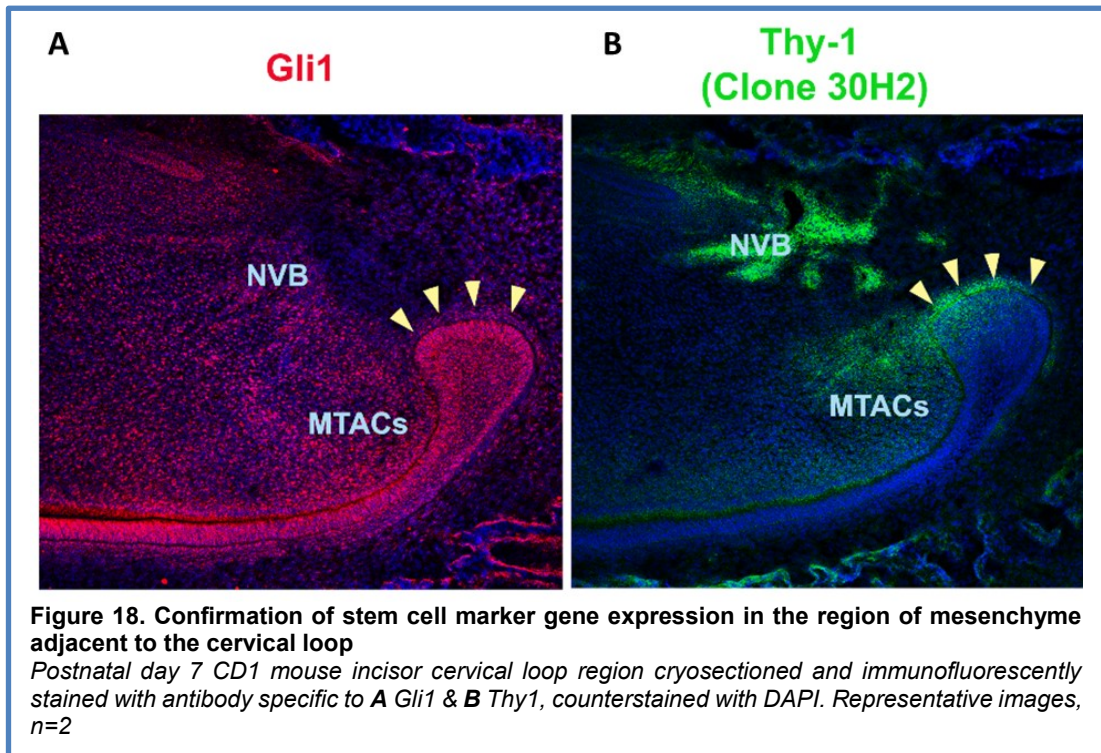
Laser capture microdissection (LCM) followed by mRNA extraction and analysis was conducted in order to confirm if the region of interest expresses known quiescent MSC markers. Regions of tissue captured included the known MTAC region, the proposed CL adjacent MSC region and the region containing the NVBs (Figure 19).

Figure 19. Regions of incisor mesenchyme can be located and captured by Laser Capture Microdissection
A Representative postnatal day 7 CD1 mouse incisor sectioned onto Laser Capture Microdissection (LCM) membrane slide & stained with methyl green. (n=11) B The same preparation of tissue as shown in panel A, following LCM to capture the region of mesenchyme proposed to contain a Cervical Loop associated Mesenchymal Stem Cells (CL-MSC) region, Neurovascular Bundle (NVB) region, Odontoblast (Ods) region and the region of known Mesenchymal Transit Amplifying Cells.(MTACs) C Captured tissue sections taken from the section shown in panel A

The CL-MSC region displayed high expression of transcripts of quiescent marker genes compared to the expression of these genes in the neighbouring MTACs (Figure 20 A). The pattern of increased expression was not limited to genes which regulate only one cellular pathway, but included genes involved in chromatin assembly, cell cycle arrest and transcriptional control.

In addition, this region also had reduced expression of genes expected to be down regulated in quiescent MSCs (Figure 20 B). Most notably a reduction in proliferation and cell cycle progression markers, was observed in the MSC region.

Figure 20. The markers gene expression profile of known quiescent mesenchymal stem cells is observed within the proposed mesenchymal stem cell region of the mouse incisor
*Real Time PCR analysis of the mRNA expression levels of quiescent MSC marker genes in tissue captured by LCM from the MTAC region & the proposed MSC region of the p7 CD1 incisor tooth. **A** Genes with upregulation of expression in other known quiescent MSC pools are more highly expressed in the proposed incisor MSC region than in the MTAC region. **B** Genes which are downregulated in quiescent MSC populations are less highly expressed in the Notch active region than in the MTAC region. * p<0.05, ** p<0.01, *** p<0.001 Representative data, n=4*

Of this panel of markers, two genes that were significantly upregulated in the CL-MSC region, SmarcA2 and Zbtb20, were selected for additional investigation.

Figure 21. Quiescent mesenchymal stem cell marker proteins are expressed in the mesenchymal stem cell region of the mouse incisor
*Postnatal day 7 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibody specific to **A** SmarcA2, **B** Zbtb20, **C** SmarcA2 with Ki67 & counterstained with DAPI. Representative images, A n=14, B n=14, C n=6*

These markers displayed high levels of consistency between experimental data sets, the antibodies were readily available, and the functions of these genes are not fully understood, making them interesting and accessible research targets. Immunofluorescent analysis of these two marker genes supported the real time PCR data and showed stronger immunoreactivity within the MCS region than in

the MTAC region (Figure 21). The expression pattern of SmarcA2 is distinct from that of Ki67, with a transitional zone of dual expressing cells at the boundary of the MTAC region (Figure 21 C).

Further analysis of the erupted and functional postnatal tooth is hampered by the tooth's hard tissue structure. However, immunofluorescent analysis was undertaken at postnatal day 30. It is clear that once the incisor tooth has erupted and become functional, the profile of protein expression within the incisor mesenchyme persists (Figure 22). Immunoreactivity of Ki67 and Zbtb20 antibodies were distinctly localised, with Zbtb20 expression restricted within the mesenchyme to the CL-MSC region. SmarcA2 immunoreactivity was observed within the MTAC region at this time, however the expression remained highest in the Ki67 negative, CL-MSC region.

Figure 22. Quiescent mesenchymal stem cell marker expression patterns persist within the functional murine incisor
*Postnatal day 30 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibodies specific to **A** Zbtb20 & Ki67 and **B** SmarcA2 & Ki67, counterstained with DAPI. Representative images, n=8*
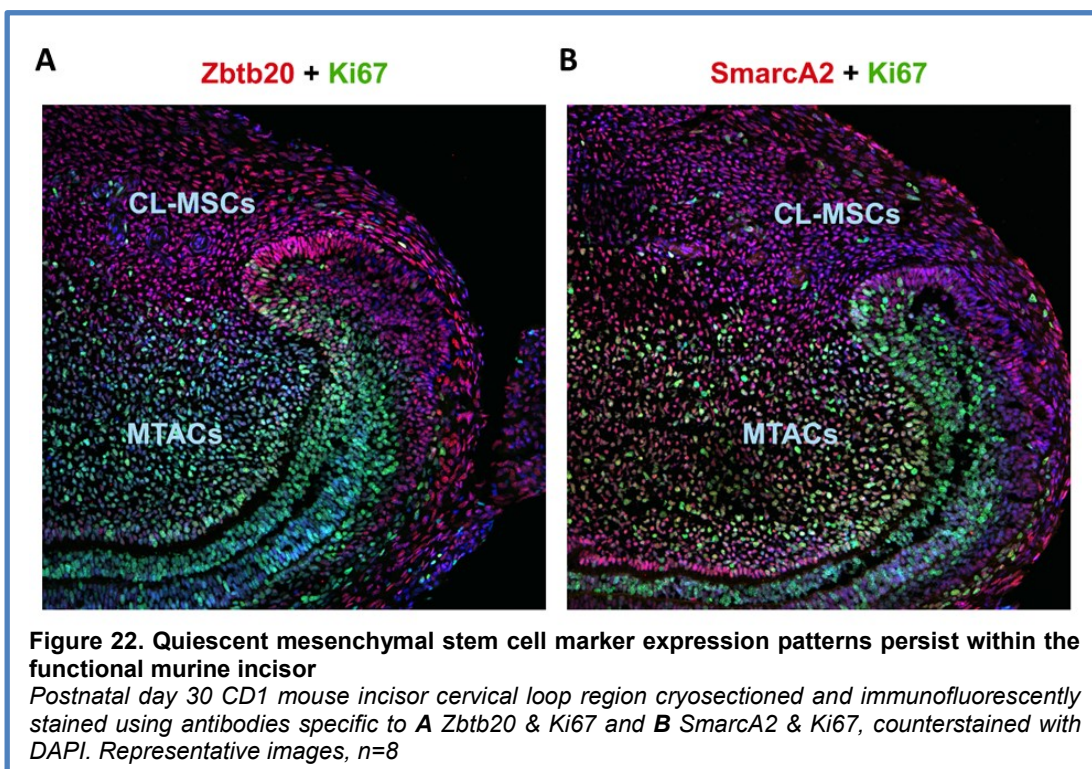
Together this data shows that the region of incisor mesenchyme adjacent to the CL epithelial SCs displays a quiescence MSC marker gene signature which is

maintained into adulthood. Consequently, this region is a likely location of an endogenous MSC pool.

4.1.2 The endogenous mesenchymal stem cells of the incisor mesenchyme are progenitors of odontoblasts and differentiate through a transit amplifying lineage

In order to prove that the novel pool of endogenous quiescent CL-MSCs identified within the mouse incisor are the progenitors of those within the odontoblast layer, lineage tracing is required.

To circumnavigate the issues associated with *in vivo* and *in vitro* lineage tracing, examination of transgenic mice post mortem was undertaken. In previous work carried out within the research group of Dr Bing Hu, LacZ staining was undertaken on Colagen1a2 Cre x Rosa26 mice, which were therefore identified as having the Cre recombinase expressed in some of cells within the proposed CL-MSC region. However, no such expression was observed in the MTAC or odontoblast areas. The unpublished data here in (Figure 23 A) was kindly given by Dr Bing Hu (The University of Plymouth) and is printed here with his permission.

Cre recombinase was found to be expressed primarily in the bone, but also at low levels in the MSC region, at embryonic, postnatal and adult aged mice (Figure 23 A). By crossing this mouse line with RBP-Jκ flox/flox mice, their offspring could be assessed to determine the location of the progeny of cells where Cre recombinase has acted to excise RBP-Jκ. Assessment of the MTAC and odontoblast regions of these mice by LCM and genomic DNA analysis was undertaken.
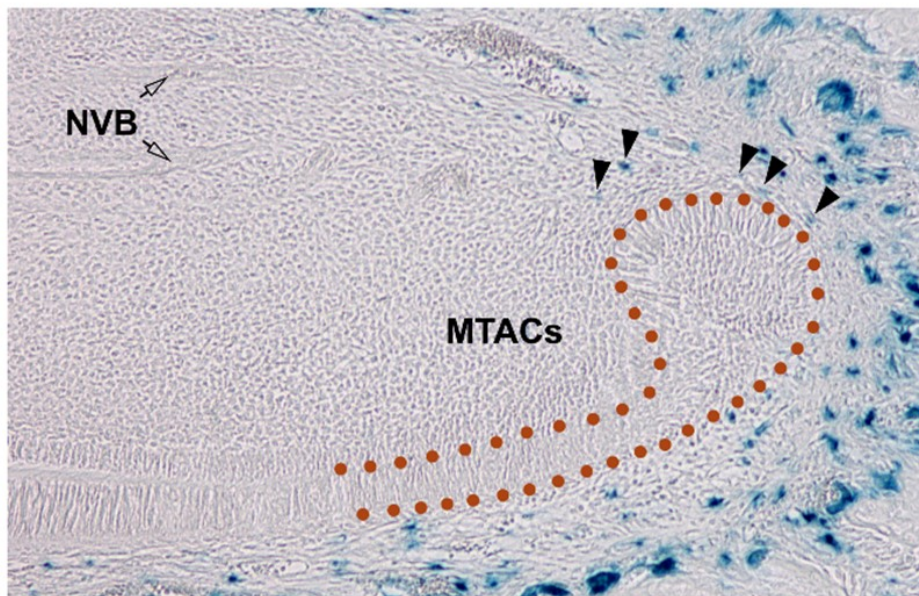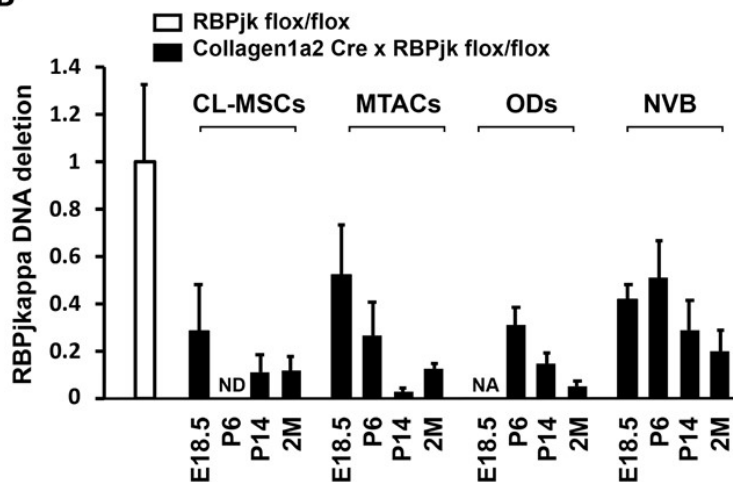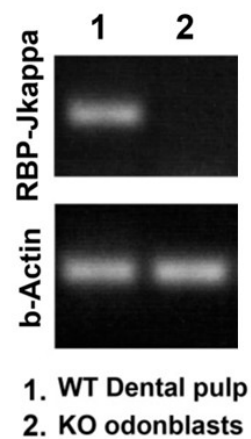
Figure 23. Transgenic mouse line Collagen 1a2Cre x RBP-Jκ flox/flox shows that cells of the MSC region go on to populate the MTAC and odontoblast regions
A Collagen 1a2Cre x Rosa26 postnatal day 1 mouse mandible, lac z stained. Representative image from Dr Bing Hu, unpublished data. Within the mesenchyme, the MSC region shows some cells of positive lac z staining. (n=2) B Real time PCR undertaken on LCM samples from Collagen 1a2Cre x RBP-Jκ flox/flox mice of a range of ages (embryonic day 18.5 (E18.5), postnatal day 6 (p6), postnatal day 14 (p14) & 2 months (2M)), and from the pooled mesenchymal LCM sample from a control mouse of age p6. (ND denoted not detected, NA denotes non-capturable region) (n=1 for each age)) C Gel image of real time PCR showing RBP-Jκ and βActin (control) gDNA within the dental pulp of WT mouse LCM dental pulp sample and Collagen 1a2Cre x RBP-Jκ flox/flox odontoblast sample. RBP-Jκ is not present within the odontoblasts of the KO mouse(n=3).

Primers were designed against the second exon of the RBP-Jκ gene which is unaffected by the action of Cre recombinase on the DNA, and also against exon

6/7 which is cleaved from the DNA. Comparison between the levels of exon 2 and exon 6/7 in each LCM gDNA sample was performed.

RBP-Jκ exon 6/7 was removed from the gDNA of the cells within the CL-MSC region of the postnatal day 6 transgenic mouse. There was also a reduction in the presence of this gDNA within the MTAC and odontoblast samples. By postnatal day 14, the levels were still reduced within the CL-MSC region and are more strongly reduced in the MTAC and odontoblast samples. In the 6-month-old mouse, levels of the floxed gDNA were reduced in all three LCM samples (Figure 23 B). Confirmation using traditional PCR showed that RBP-Jκ was not present within the odontoblasts of the Collagen 1a2Cre x RBP-Jκ flox/flox adult mouse (Figure 23 C).

In this work there appears to have been Cre-recombinase activity in the cells which gave rise to the odontoblasts at all ages, suggesting that the CL-MSC region continues to give rise to odontoblast layer cells throughout the animals' life.

The ability of the CL mesenchyme to give rise to dentine producing cells was further investigated to support the hypothesis that the cells of this region contain SCs which are capable of differentiating into odontoblasts. Microdissection of the CL region was undertaken. The resected tissue was enzymatically and mechanically dissociated to allow discard of the epithelial tissue. The isolated mesenchyme was placed on devitalised dentine and cultured in organ culture conditions. Histological analysis showed that the cells of the mesenchyme were able to align with the dentine slice and begin to project into the existing dentine tubules. These projections showed a typical odontoblastic branching phenotype (Figure 24 A). Continuing culture for a further 3 days showed that the projections

were able to produce new DSPP and DMP1 protein, indicating that the cells are beginning to become functional odontoblast like cells (Figure 24 B-D).

Figure 24. Dental mesenchyme from the cervical loop mesenchymal stem cell region of the mouse incisor can form odontoblast like cells when cultured on devitalised dentine slices *in vitro*

*Postnatal day 7 CD1 mouse incisor CL-MSC tissue, cultured on devitalised p30 CD1 incisor dentine slides. **A** Tissue cultured on dentine slice for 4 days, frozen, cryosectioned, stained with phalloidin and counterstained with DAPI. Image shows actin cytoskeleton of cultured cells protruding into the dentine tubules, 3D rendered on IMARIS software. **B-C** Tissue cultured on dentine slice for 7 days, frozen, cryosectioned, stained with (B) DMP1 & (C) DSP antibodies and counterstained with DAPI. **D** Merged image of B&C with phase contrast image overlaid (n=2).*

Together this data has shown that cells from within the CL-MSC region are capable of populating the MTAC and odontoblast cell regions. In addition, these cells have the capacity to differentiate into odontoblast like cells when in a permissive environment.

4.2 Development of an *in vitro* model to use in investigating the molecular mechanisms of dental mesenchymal stem cell maintenance

In order to further investigate the molecular mechanisms underpinning SC maintenance and transition, accurate *in vitro* cell culture model systems were employed. Initial attempts at producing a primary cell-line based approach were moderately successful. However, the cells showed limited propensity to survive after passage 7 and were not sufficiently robust to survive transfection and infection manipulations. Consequently, established cell lines were also used in order to better understand the molecular mechanisms governing quiescence, self-renewal and differentiation is dental mesenchymal cells.

4.2.1 Primary mesenchymal cell culture can be utilised to propagate dental mesenchymal cells from the mouse incisor

Development of an adherent primary-cell system in order to better understand the nature of the mesenchymal cells of the mouse incisor was undertaken.

Figure 25. Mouse incisor mesenchymal stem cell region tissue can be cultured *in vitro*
A Microdissected cervical loop tissue following enzyme digestion and mechanical dissociation of the surrounding mesenchyme from the epithelial tissue. Illustrates the accuracy of microdissected area and the completeness of dissociation of epithelium and mesenchyme possible by this method. scale bar 200nm. B Phase contrast image of the mesenchymal cells at passage 2 following extraction from the mouse incisor and culture under mesenchymal permissive conditions.

Microdissection of the CL of the mandibular incisors was undertaken. The apical region containing the CL and surrounding mesenchyme was collected and enzymatically digested (Figure 25 A). The resultant single cell suspension was then applied to precoated culture dishes and grown under mesenchymal or epithelial permissive conditions. In poly-d-lysine coated dishes and with culture conditions utilising a SC permissive media, AmnioMax, the cells which grew were able to survive and expand in culture and exhibited a mesenchymal like morphology (Figure 25 B).

In order to determine if cells grown in these permissive conditions were exhibiting mesenchymal and epithelial specific behaviours, the mRNA expression of known mesenchymal and epithelial specific markers were assessed. K14 and CDH1 are well documented epithelial markers, with K14 expression being entirely epithelial specific in the incisor tooth (Figure 26 A).

Meanwhile PDGFRβ and Vimentin are common mesenchymal markers, the former being almost exclusively expressed in the mesenchymal compartment of the mouse incisor (Figure 26 B). Analysis of the mRNA expression of these cultured cells, showed that when cultured in mesenchymal culture conditions, the cells exhibited high expression of known mesenchymal marker genes, and negligible expression of epithelial markers (Figure 26C&D). Suggesting that the culture system is capable of selectively culturing mesenchymal cells.
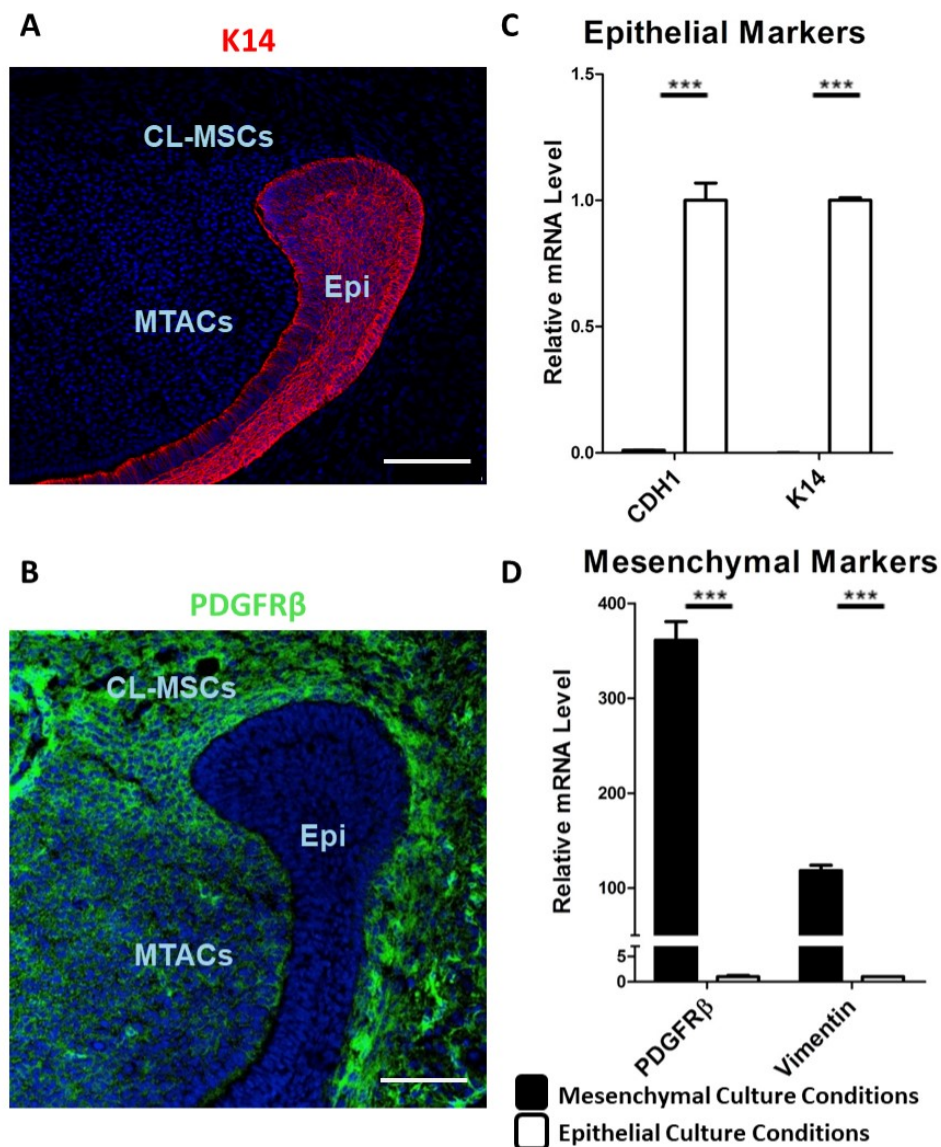
Figure 26. Cultured incisor mesenchymal cells have a mesenchymal expression signature
A & B Representative Postnatal day 7 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibodies specific to A K14 (n=2) and B PDGFRβ (n=4), counterstained with DAPI. Scale bars 100nm. C & D Representative Real Time PCR analysis of the mRNA expression of dental epithelial specific markers (C) and dental mesenchymal specific markers (D) in the cultured mesenchymal and epithelial cells grown from the mouse incisor. * p<0.05, ** p<0.01, *** p<0.001 n=3

Further validation of the mesenchymal cell culture model was undertaken using flow cytometry. In BMSCs a number of cell surface antigens have been shown to be routinely expressed. These markers include Sca1, CD29, CD73, CD105 and CD106. Antibody kits for flow cytometric analysis of MSCs are commercially available.

Figure 27. Flow cytometric analysis suggests that cultured incisor mesenchyme cells contain cells expressing some known mesenchymal stem cell marker genes
*Flow cytometric analysis using a commercially available panel of MSC marker genes. **A-E** Representative histogram data of the flow cytometry analysis. Black lines indicate the background fluorescence of cells having been incubated with IgG protein. Coloured lines indicate the distribution of fluorescent cells within the sample following incubation with the antibody indicated. **F** For all MSC markers tested observable peak shift in fluorescence was noted (n=2).*

Preliminary work found that when the cells of the incisor were cultured under mesenchyme permissive conditions, the population contained cells which displayed many of these antigens on their surface (Figure 27). Indicating that the cultured cells may contain MSC-like cells.

The mesenchymal cells cultured from the resected tissues expressed many genes which are known to be both up regulated and down regulated when MSC are in a state of quiescence (Figure 28). The heterogeneity of the cultured cells may be owing to the imprecise nature of the initial tissue extraction, capturing both MTAC and quiescent MSCs.

Figure 28. Heterogeneity within the cultured incisor mesenchymal cells indicates a mixed population of quiescent mesenchymal stem cell like and proliferative transit amplifying cell like cells

A Representative Real time PCR analysis indicating raw data (CT values) of MSC and MTAC marker gene expression from two batches of cultured incisor mesenchymal cells. Both MSC and MTAC marker gene transcripts are detectable within the cells (n=2). B Cultured incisor mesenchymal cells fixed and immunofluorescently stained using antibodies specific to MSC marker Zbtb20, counterstained with DAPI. Representative image, n=2. White arrow indicates two adjacent cells each with markedly different levels of immunoreactivity to the MSC marker antibody.

In addition, the culture conditions are high in nutrients which may have encouraged formerly quiescent cells to begin cycling again once they were placed into culture. In order to better model the MSC and MTAC populations synchronisation of the cell cycle within these cells was required.

4.2.2 The cell cycle status of cultured cells can be synchronised through adjusting culture conditions

Using an established cellular synchronisation protocol, serum deprivation was used to encourage the cells to enter into quiescence (Figure 15) (Chen et al., 2012a).

Initial analysis of cells under these three regimens was undertaken through an immunofluorescence (IF) based approach. Fed cultures showed a great deal of heterogeneity with populations of cells which were positive for known quiescent MSC marker genes, Zbtb20 and SmarcA2, while other cells within the same field

were negative (Figure 29 A&D). Cells which were recovered from starvation displayed fewer cells positive for these markers (Figure 29 B&E) and an overall more homogenous pattern of expression. While starved cells maintained the expression of Zbtb20 and SmarcA2 in some cells possibly with a modest increase in expression of these markers although this was not able to be quantified through IF (Figure 29 C&F). Synchronisation does not produce entirely positively or negatively immunoreactive, homogenous populations. However starved culture conditions appear to encourage more cells to adopt a quiescent MSC-like expression pattern.

Figure 29. Synchronisation of the cell cycle through serum starvation produces a population of cells which express more quiescent mesenchymal stem cell marker proteins
Cultured incisor mesenchymal cells, grown under fed, recovered or starved conditions, fixed and immunofluorescently stained using antibodies specific to MSC markers Zbtb20 & SmarcA2, counterstained with DAPI. yellow arrows indicate cells with nuclear localisation n=1

Following from the immunofluorescent analysis, a molecular transcript expression approach was undertaken in order to allow for accurate quantification. Molecular profiling of cells cultured under each regimen showed that mRNA expression of the cells under each condition was quite different (Figure 30).

Figure 30. Synchronisation of cultured incisor mesenchyme cells produces populations of cells which exhibit more quiescent stem cell like transcription signatures
Representative Real Time PCR analysis of the mRNA expression levels of quiescent MSC marker genes in cultured incisor mesenchymal cells which have been subjected to fed, starved and recovered conditions. *** p<0.001 n=3

When compared to the expression profile of fed cells, starved cells upregulated quiescent SC marker genes and downregulated MTAC markers. Conversely recovered cells downregulated quiescent MSC markers and upregulated MTAC markers respectively.

4.2.3 Established dental mesenchymal cell lines are capable of being synchronised into a state of quiescence

While incisor culture cells were capable of being manipulated into quiescent MSC-Like and MTAC-like behaviours, these cells are limited by their nature. Being primary cells, they are not capable of being passaged repeatedly, nor are they robust enough to withstand manipulations such as infection or transfection. Consequently, and alternative model was sought.

Figure 31. Established dental pulp cell lines display a similar morphology, expression profile and response to synchronisation as cultured incisor mesenchyme cells
A&B Representative phase contrast image of the established dental pulp cell line (MO6 G3). C Gel image of PCR products in mouse embryonic fibroblasts (MEF) and MO6 G3 cell sample, against a selection of quiescent MSC and MTAC marker genes. (n=1) D Representative Real time PCR analysis of MO6 G3 cells following synchronisation (n=3) data not significant.

In the 1990's MacDougall et al, established a pre-odontoblast like cell line (MacDougall et al., 1995). These cells, MO6 G3, are an immortalised cell line which can be grown *in vitro* and are sufficiently robust (Figure 31 A&B). PCR analysis revealed that these cells expressed many of the quiescent MSC and MTAC marker genes which were identified in the mouse incisor mesenchyme (Figure 31 C). Both MSC and MTAC marker transcripts were detectable in MO6 G3 cells, suggesting a similar heterogeneity to fed cultured incisor mesenchyme cells. Furthermore, when exposed to the synchronisation regimens used on the incisor culture primary cells, starved cells showed an upregulation of MSC marker genes and a downregulation of TAC gene expression. Conversely, recovered cells displayed upregulation of TAC genes and a downregulation of MSC genes (Figure 31 D).

4.3 CL-MSCs & MTACs display distinct patterns of Notch pathway utilisation

4.3.1 Notch signalling activity in the incisor tooth mesenchyme is spatially restricted

Notch signalling has long been connected to quiescent SC maintenance and activation. It is well documented that Notch activity can indicate the presence of quiescent SCs in mesenchymal tissues.

Transgenic Notch Reporter (TNR) mice were employed to determine if Notch activity is present within the CL-MSC region. Indeed, at postnatal day 0 and persisting at postnatal day 14, the CL-MSC region is strongly immunoreactive

when anti-GFP is applied (Figure 32 A&B). This indicates that RBP-Jκ binding sites are occupied in this region (Figure 32 C), which in turn is indicative of Notch pathway regulation occurring here.

Figure 32. Transgenic Notch Reporter mice indicate the presence of Notch signalling regulation within the cells of the cervical loop mesenchymal stem cell region
A Representative postnatal day 0 TNR mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibodies specific to GFP, counterstained with DAPI. (n=2) B Representative postnatal day 14 TNR mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibodies specific to GFP, counterstained with DAPI. Yellow arrows indicate the positive staining regions. (n=2) C Schematic of the TNR construct, showing the expression of GFP under the control of RBPj binding sites.

Immunofluorescent analysis of the localisation of Notch receptors within the mouse incisor was undertaken, to confirm previous reports. Within the mouse incisor mesenchyme, Notch receptors 1 and 2 were widely expressed throughout the tissue, while Notch receptor 3 was largely undetectable by IF analysis (Figure 33 A-C). With Notch2 displaying enhanced immunoreactivity in the known MTAC region of the mesenchyme (Figure 33 B). The Notch1 antibody shown here (Figure 33 A) is specific to the intra cellular domain and therefore indicates not

only ubiquitous expression but also the presence of the cleaved ICD, suggestive

of active Notch signalling.

Figure 33. Notch1 & Notch2 receptors are expressed within the incisor mesenchyme
A-C Representative postnatal day 7 CD1 mouse cervical loop region of the incisor, cryosectioned and immunofluorescently stained using antibodies specific to A) Notch1, n=15 (ICD), B) Notch2 n=14 & C) Notch3 n=5, counterstained with DAPI. Yellow arrows indicate the immune-unreactive CL-MSC region. D Representative Real Time PCR analysis of the mRNA expression levels of Notch receptor genes in LCM captured populations of p7 CD1 mice cells. *** p<0.001 n=4

Using LCM as previously described, the expression of *Notch1* and *Notch2*

indicated a greater expression of both receptors in the CL-MSC region (Figure 33

D). The presence of Notch receptors indicates a potential for active Notch

signalling to take place, however assessment of the prevalence of active Notch signalling can be undertaken though identification of cleaved Notch receptor intracellular domains.

Immunofluorescent detection of cleaved Notch1 and Notch2 ICDs is another powerful tool used to determine the regions of Notch receptor activation. As the intracellular domain is only cleaved once the Notch receptor is activated, presence of the cleaved domain is indicative of canonical Notch signalling. Within the mouse incisor mesenchyme, Notch1 ICD antibodies showed very low levels of immunoreactivity throughout the mesenchyme (Figure 33 A). Notch2 intracellular domain antibodies displayed elevated immunoreactivity in the mesenchyme known to contain MTACs (Figure 34). Thus, supporting the suggestion that Notch signalling is an important regulator of SC activation and maintenance.

Figure 34. Cleaved Notch2 intracellular domains localise to the nuclei of cells within the mesenchymal transit amplifying region
*Representative postnatal day 7 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibody specific to cleaved Notch2 intracellular domain (Notch2 ICD) & counterstained with DAPI. Dotted line marks the basement membrane, separating the epithelial CL from the mesenchymal tissue. Boxes **B** & **C** depict area expanded and shown in panels to the right, CL-MSC and MTAC regions respectively. yellow arrow heads indicate nuclei with nuclear Notch2ICD staining n=3*

Analysis of nuclear localisation of cleaved Notch2 intracellular domains showed that Notch2 ICD was expressed within the CL-MSC and MTAC regions, it was in the MTAC region where the localisation was most strongly within the nucleus (Figure 34). Thus, indicating a contrasting role for Notch2 receptor within the incisor mesenchyme in these two areas.

The combination of Notch2 expression and activation being highest in the MTAC region, coupled with enhanced TNR activity in these cells, suggest that Notch signalling is active in this region. In order to confirm this, immunostaining of the mouse incisor for down-stream Notch target Hes1 was carried out. While Hes1 expression was most striking in the epithelial tissue of the incisor tooth, there was a modest upregulation of this protein in the MTAC region over that of the CL-MSC area (Figure 35 A). Suggesting that Notch signalling is truly most abundant in the MTAC region. Furthermore, mRNA expression analysis of LCM captured CL-MSC and MTAC tissue, found that Notch effector genes were more highly upregulated in the CL-MSC region.

Figure 35. Notch downstream target protein (Hes1) are highly immunoreactive in the mesenchymal transit amplifying cell region
*A Representative postnatal day 7 CD1 mouse cervical loop region of the incisor, cryosectioned and immunofluorescently stained using specific antibodies for Hes1, counterstained with DAPI. Arrows indicate low reactivity in the CL-MSC region. n=13 B Real Time PCR analysis of the mRNA expression levels of Notch effector genes in LCM captured populations of p7 CD1 mice cells. **p<0.01, *** p<0.001*

Canonical Notch signalling is ordinarily activated by Notch ligands binding to the receptors. In order to determine if this is likely to be the case in this system, immunofluorescent analysis was undertaken to investigate the location of expression of Notch ligands Jag1, Jag2 and Dlk1.

Figure 36. Expression of Notch ligands occurs in distinct locations within the mouse incisor *Representative postnatal day 7 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibody specific to **A** Jag1 n=11, **B** Jag2 n=8 and **C** Dlk1 n=14 & counterstained with DAPI. Arrows mark the CL-MSCs. **D** Real Time PCR analysis of the mRNA expression levels of Notch effector genes in LCM captured populations of p7 CD1 mice cells. (N.D. denotes not detected, Ods = Odontoblasts, DPs = Dental Pulp cells) n=1 *** p<0.001*

In the case of classical ligands Jag1 and Jag2 immunoreactivity was detected throughout the incisor mesenchyme at relatively homogeneous levels however there was a clear increase in expression within the incisor epithelium (Figure 36

A&B). DSL ligand Dlk1 however is most highly expressed within the MTAC region (Figure 36 C). Dlk1 mRNA expression was also found to be upregulated in the MTAC region (Figure 36 D).

4.4 Notch signalling modulation affects the maintenance of the mesenchymal transit amplifying cells and the transition of the endogenous mesenchymal stem cell pool

Notch pathway components are spatially restricted within the mouse incisor indicating that they may have a role in maintaining and regulating this tissue. Notch signalling is known to be important in the maintenance and transition of SCs. As Notch receptor, Notch2, and ligand, Dlk1, are each most highly expressed and possibly functional in the MTAC region, the modulation of Notch signalling could have profound effects on the mitotic potential of the MTACs and the endogenous CL-MSC pool.

4.4.1 *In vivo* modulation of Notch signalling affects the terminally differentiated progeny of the mesenchymal transit amplifying cell pool

The effect of removing canonical Notch signalling from the incisor tooth was investigated using mice which are conditionally deficient of RBP-Jκ within the mesenchymal compartment of the incisor. These Collagen 1 α2 Cre x RBP-Jκ flox/flox mice display a significant dentine disorganisation phenotype (Figure 37).

Histologically the odontoblast layer is disorganised, and the dentine produced is similarly disorganised (Figure 37 C&D). In the Collagen 1 α2 Cre x Rbp-Jκ flox/flox the odontoblast layer is disrupted to the point of almost entire loss. The

dentine is disorganised, and dentine tubule orientation is eccentric. Similarly, scanning electron microscopy, (performed by Dr Bing Hu (The University of Plymouth) and printed here with his permission) reveals that the hydroxyapatite crystals of the dentine have lost their characteristic organisation and density pattern (Figure 37 E&F).

Figure 37. *In vivo,* **dental mesenchymal specific knock out of RBP-Jκ gives rise to disruption of normal dentine organisation and maturation**
A&B Representative Collagen 1 α2 Cre x Rbp-Jκ flox/flox mouse incisor dentine*, frozen, cryosectioned, stained with DMP1 & DSPP specific antibodies and counterstained with DAPI (n=2)* **C&D** *Representative Haematoxylin and eosin staining of sectioned wildtype and Collagen 1 α2 Cre x Rbp-Jκ flox/flox incisor dentine (n=4)* **E&F** *Scanning electron microscopy images of wildtype and Collagen 1 α2 Cre x Rbp-Jκ flox/flox mouse incisor dentine (n=1).*

Fluorescent immunohistochemical analysis of the RBP-Jκ conditional knock out mouse incisor highlights that the dental mesenchyme displayed more immunoreactivity for markers of terminally differentiated odontoblasts, DSP and DMP1 (Figure 37 A&B). This phenotype suggests that in the absence of Notch

signalling, the lineage of cells responsible for the production of odontoblasts, and thus dentine, is dysregulated. The presence of increased differentiation markers indicates that premature differentiation has occurred, potentially due to inability to maintain the self-renewal of the progenitor cells.

4.4.2 *In vivo* modulation of Notch ligand, Dlk1, affects mesenchymal transit amplifying cell maintenance and mesenchymal stem cell transition

Notch ligand, Dlk1 was identified as being highly upregulated in the MTAC region of the incisor. In order to investigate the function of Dlk1 in these cells, microscopic phenotypic analysis of Dlk1 null mice (Raghunandan et al., 2008) and of mice overexpressing Dlk1 in a tissue specific manner (Abdallah et al., 2011) was undertaken.

Figure 38. Incisors of mice deficient of Dlk1 display a pattern of expression of MTAC and MSC marker genes consistent with an increase in quiescent cells and a decrease in proliferating cells
Representative Postnatal day 7 Dlk1 KO and wildtype mouse incisor cervical loop regions cryosectioned and immunofluorescently stained using antibodies specific to Ki67 & SmarcA2, n=5

Initial immunofluorescent analysis of the CL region of Dlk1 knock out mice was undertaken. The expression of MTAC and MSC marker genes, Ki67 and SmarcA2, were similar in pattern in the knock out and wild type mice. SmarcA2 immunoreactivity is observed in the CL-MSCs region in both wildtype and knock out mice, while Ki67 immunoreactivity is most prevalent in the MTACs region within the mesenchyme (Figure 38). However there appeared to be a greater level of immunoreactivity for the MSC marker, SmarcA2, within the MTAC region of the knock out incisor (Figure 38). This was coupled with a reduced level of Ki67 immunoreactivity throughout, indicating a reduction in proliferative cells (Figure 38 A&D).

In order to quantify if there is a reduction in the MTAC marker genes in the MTAC region of the knock out incisor compared to the wildtype, LCM and PCR was undertaken. The expression pattern of upregulation of quiescent MSC marker genes in the CL-MSC region was observed in both the wildtype and knock out (Figure 39). There is no statistically significant difference between the expression of the quiescent MSC and MTAC marker genes in the knock out, the two mesenchymal cell populations are more similar to one another than in the wildtype.

In order to determine if the MTAC region had indeed been diminished by the loss of Dlk1, 3D remodelling was undertaken. By digitally analysing serial sections of the incisor, having been cryosectioned and immunofluorescently stained, a 3D model of the tissue has been reproduced (Figure 40 A&B). The CL was defined using the basement membrane as a boundary, while the MTAC region was determined through the location of Ki67 positive cells adjacent to the basement membrane. The volume of the MTAC region was calculated using Biovis3D

software, which showed that the relative volume of the MTAC region was reduced by 22.5% (Figure 40 D). In addition, the number of Ki67 positive cells within the region was also reduced (Figure 40 C). Further suggesting the Dlk1 is necessary for MTAC maintenance.

Figure 39. The mesenchymal stem cell and transit amplifying regions of Dlk1 null mice display more similar expression of mesenchymal stem cell marker genes than in wildtype incisors *Representative Real Time PCR analysis of the mRNA expression levels of quiescent MSC marker genes in samples of incisor CL-MSC and MTAC regions collected by LCM from **A** Dlk1 WT mice (n=3) and **B** Dlk1 KO mice (n=2). *** p<0.001*

Similar immunofluorescent and 3D modelling analysis of Collagen 1 α1 Dlk1 overexpression mice was also conducted. Preliminary work was undertaken to microscopically analyse the incisor phenotype of these mice which overexpress Dlk1 under the promotor for rat collagen 1. Interestingly the expression of quiescent MSC marker gene, SmarcA2, was found to be widespread throughout the mesenchyme of the incisor (Figure 41 A). This quiescent marker was observed within the region usually described as the MTAC location, which itself displayed a modest decrease in Ki67 immunoreactivity (Figure 41 B).

Figure 40. The mesenchymal transit amplifying cell region of incisors of mice deficient in Dlk1 are reduced in volume
A&B Representative 3D reconstruction of immunofluorescently stained serial cryosections form Dlk1 WT and Dlk1 KO incisors, stained with antibodies specific to Ki67 and counter stained with DAPI. Computational reconstruction produces models as show, with volume calculations for the MTAC regions in apple-blue-sea-green. C Number of Ki67 positive cells observed within the mesenchyme of the CL structure, based on quantification of cells from serial immunofluorescently stained cryosections. D Relative volume of WT and KO MTAC regions following computational analysis of 3D models. WT; n=3, KO; n=3 data not found to be significant

Due to the limited availability of these mice, this work is only preliminary and requires more investigation before these findings can be proven. It is unclear however, if the depletion of the MTAC is primarily due to a loss of maintenance of self-renewal in these cells, or due to enhanced transition of MTACs to odontoblasts.

Figure 41. Incisors of mice overexpressing Dlk1 within the incisor mesenchyme show an apparent reduction of the MTAC region and increased quiescent MSC marker gene expression
*Postnatal day 7 Collagen 1 α1 Dlk1 overexpression mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibody specific to **A** SmarcA2 (n=1) and **B** Ki67 (n=1) & counterstained with DAPI*

Additional work has been undertaken within the research group of Dr Bing Hu, which indicates that these mice have increased dentine deposition (Walker et al, *in review*). Together this indicates a potential mechanism whereby constitutive expression of Dlk1 in the incisor enhances MTAC proliferation and subsequent differentiation to the point of exhausting the MTAC pool.

4.4.3 *In vitro* manipulation of Dlk1 reveals that Dlk1 is necessary for mesenchymal transit amplifying cell maintenance

In order to further consolidate the *in vivo* findings of Dlk1 modulation in the mouse models, *in vitro* analysis was also carried out.

Figure 42. *In vitro* reduction of Dlk1 reduces expression of genes commonly downregulated in quiescent mesenchymal stem cells in cultured incisor mesenchyme cells
Representative Real Time PCR analysis of the mRNA expression levels of genes which are downregulated in MSCs in cultured incisor mesenchyme cells transfected with siRNA, before mRNA samples were collected 24hours later. Scrambled siRNA used as control. siDlk1 treatment successfully reduced Dlk1 expression levels within the sample. In this sample all four tested MTAC marker genes also displayed reduced transcription. *p<0.05, *** p<0.001 n=2

Following the finding that Dlk1 deficient mice appear to have a reduction in the MTAC phenotype within the mouse incisor, the effect of interfering with Dlk1 *in vitro* was assessed. Mesenchymal cells cultured from the mouse incisor were transfected with siDlk1 constructs. The level of Dlk1 reduction was modest. However, it was clear that with the reduction of Dlk1, other MTAC marker genes were also downregulated (Figure 42).

Using the MO6 G3 cell line model, the effect of removing endogenous Dlk1 through the use of siRNA transfection was more pronounced. Using two different siRNA probes, Dlk1 transcription could be downregulated in these cells (Figure 43 A). Simultaneously, other MTAC region marker genes, Ki67 and Top2A

indicate a trend of downregulation (Figure 43 A). The growth of the cells was reduced in the transfected cells, with a modest reduction in the area covered by the cells in sparse cell culture conditions (Figure 43 B).

Figure 43. *In vitro* reduction of Dlk1 reduces expression of genes commonly downregulated in quiescent mesenchymal stem cells and reduces cell culture coverage in immortalised dental pulp cell line
*Immortalised dental pulp cells were transfected with siRNAs, before mRNA samples were collected 24hours later. Scrambled siRNA used as control, and 2 different siDlk1 probes. **A** Representative Real Time PCR analysis of the mRNA expression levels of genes which are downregulated in quiescent MSCs siDlk1 A & B treatment successfully reduced Dlk1 expression levels within the sample .n=2 *** p<0.001 **B** Relative area covered by cell growth compared to growth within the control treated cells, following crystal violet staining n=2 data not significant.*

When considered together, it appears that dental mesenchymal cells in culture reduce their MTAC-like phenotype in the absence of Dlk1. Thus, highlighting a potential role for Dlk1 as a necessary factor in the maintenance of MTACs.

4.4.4 *In vitro,* Dlk1 is sufficient for quiescent mesenchymal stem cells to transition to a transit amplifying like state and functions to maintain this status

In addition to Dlk1's role as a maintainer of MTACs, the *in vivo* data also indicates that the overexpression of Dlk1 may encourage differentiation throughout the lineage. To investigate this further, Dlk1 protein was added to cultured dental mesenchymal cells.

Figure 44. *In vitro* treatment of dental pulp cells with Dlk1 protein encourages a more transit amplifying transcription profile and greater coverage of cell culture surface area
*A Immortalised dental pulp cells cultured on culture surfaces pre-treated with bound Dlk1 protein. Representative Real-Time PCR analysis of mRNA expression levels of quiescent MSC marker genes, analysis undertaken by real time PCR. In the presence of Dlk1 the cultured cells downregulate the expression of MSC marker genes. (n=2) *p<0.05, **p<0.005, *** p<0.001 B Relative cell culture surface coverage of immortalised dental pulp cells, synchronised to quiescence and treated with soluble Dlk1 protein compared to coverage of control starved cells, following crystal violet staining. In the presence of Dlk1 cells grow to cover a greater area of the cell culture surface. (n=2) data not significant C Representative Real time PCR analysis of the mRNA expression levels of genes upregulated and downregulated in quiescent MSCs in immortalised dental pulp cells, synchronised to quiescent and treated with soluble Dlk1 protein (n=3). *p<0.05, **p<0.005, *** p<0.001*

Initial experiments in this study investigated the local effects of Dlk1 protein on MTACs utilising a bound ligand culture method. As the protein was adhered to the cell culture surface before the cells were seeded, I the cells cannot be synchronised as they must be passaged into the prepared dish. However, within

the heterogenous "fed" cell population that was used, there are cells which exhibit MTAC-like properties and others which are more MSC-like (Figure 26).

The addition of bound Dlk1 protein into this culture system provoked a downregulation in the expression of an array of quiescent MSC marker genes (Figure 44 A). Indicating the ability of Dlk1 to promote a loss of quiescent phenotype and the adoption of a more MTAC-like phenotype within the heterogenous population.

This data supports the findings previously discussed in section 4.4.3 *In vitro manipulation of Dlk1 reveals that Dlk1 is necessary for mesenchymal transit amplifying cell maintenance*, that this effect may be due to the preservation and maintenance of the MTACs already within the heterogenous culture.

In order to discern if diffusible Dlk1 protein can encourage the quiescent MSCs to transition into MTAC-like cells, soluble Dlk1 protein was added to synchronised cells. Cell cycle synchronisation was performed in order to produce quiescence within the culture, following this Dlk1 protein was added. A dose dependent upregulation of marker genes commonly downregulated in quiescent MSCs was observed (Figure 44 C). This indicates an apparent loss of quiescence by these cells. Coupled with the increased expression of MTAC marker Ki67 (Figure 44 C), it is clear that the addition of Dlk1 has encouraged these MSC-like cells to adopt a more MTAC-like phenotype. This push towards an MTAC-like phenotype is supported by the finding that the cells grew to cover a greater area of the cell culture container when Dlk1 was added (Figure 44 B).

The addition of Dlk1 protein to cultured quiescent dental mesenchymal cells, is sufficient to encourage cells to adopt an MTAC-like phenotype. Indicating that Dlk1 has an important role as a regulator of quiescent to TAC state.

4.5 Mesenchymal stem cells and transit amplifying cells are epigenetically regulated within the mouse incisor

4.5.1 Epigenetic regulation of Dlk1 is utilised differently in mesenchymal stem cells and mesenchymal transit amplifying cells

Immunofluorescent analysis of the mouse incisor mesenchyme revealed different levels of common histone modifications, H3K9me3 and H3k27me3, between the MTAC and CL-MSC regions (Figure 45 B&C). This data indicates that there is a greater level of histone modification within the CL-MSC region.

In order to determine if the Dlk1 gene itself is subjected to such epigenetic regulation, Chromatin Immunoprecipitation (ChIP) assays were conducted. Briefly, chromatin was extracted from dental mesenchymal cells grown in standard or starved culture conditions, to simulate MTAC-like and MSC-like cells respectively. This chromatin was pulled down using antibodies specific to methylated histone proteins. The resultant DNA was analysed by PCR using probes specific to the 5' and 3' CpG islands of the Dlk1 gene (Figure 45 A).

When pulling down based on two common histone modifications, both 5' and 3' CpG islands of Dlk1 were more highly associated with the epigenetic regulatory marks in the starved cells than in the growing cells (Figure 45 B&C).

In the MSC-like cells, histone modification marks are a feature of the chromatin at the site of epigenetic DNA regulation regions. Indicating that Dlk1 expression may be inhibited in MSCs through an epigenetic mechanism.

Figure 45. Cultured dental pulp cells display altered levels of epigenetic marks at key regulatory sequences around the Dlk1 gene when synchronised into quiescent or transit amplifying like status
*A Illustration of the Dlk1 loci with CpG islands as presented on USCS genome browser (2011). **B&C** Representative postnatal day 7 CD1 mouse incisor cervical loop region cryosectioned and immunofluorescently stained using antibody specific to B) H3k9me3 and C) H3k27me3 & counterstained with DAPI. Arrows mark positive immunoreactivity in the CL-MSCs.(**n=2**) **D&E** Real time PCR analysis of samples from fed and starved dental pulp cells assayed by ChIP, using antibodies specific to histone modification marks H3K9me3 & H3K27me3, probed for gDNA using primers specific to the region of gDNA containing CpG islands at the D) 5' & E) 3' regulatory regions of the Dlk1 gene. n=1 ** p<0.005*

4.5.2 Chromatin remodelling factor SmarcA2 is necessary for the maintenance of mesenchymal stem cell like cell state
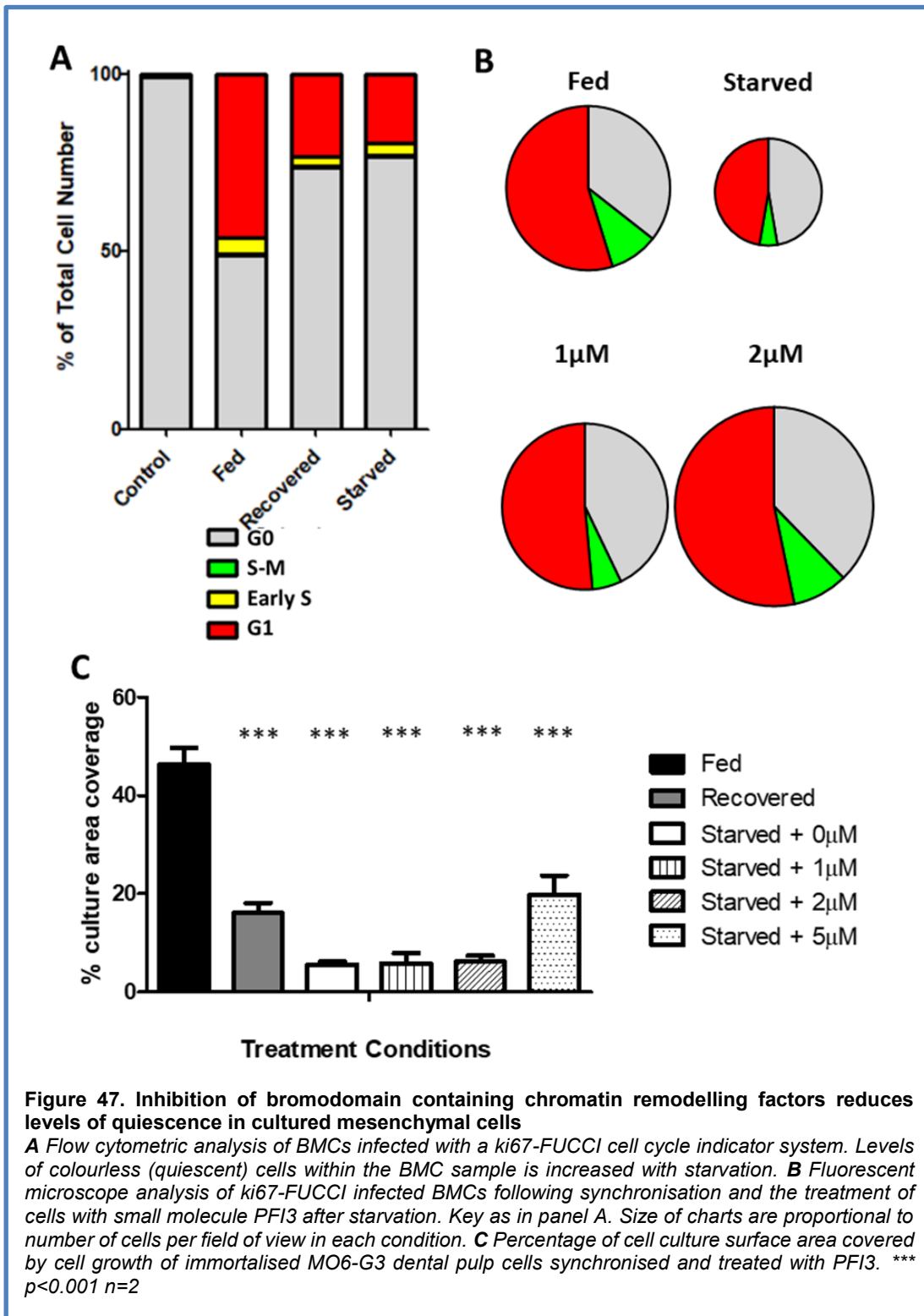
SmarcA2 is a well characterised chromatin remodelling factor, which is also a known quiescent MSC marker. In order to determine the effect of losing SmarcA2 function in quiescent cells, siSmarcA2 was transfected into synchronised dental mesenchymal cells. Although downregulation of SmarcA2 was only modest, MTAC marker genes Ki67 and Top2A showed a trend of upregulation (Figure 46 A). This correlated with an increase in the relative amount of cell culture surface area covered by the cells, when treated with siSmarcA2 (Figure 46 B). The loss of SmarcA2 from this cell model appears to have prevented the MSC-like cells form maintaining their quiescent MSC-like state.

Figure 46. *In vitro* reduction of quiescent mesenchymal stem cell marker, SmarcA2, increases expression of genes commonly downregulated in quiescent mesenchymal stem cells and increases cell culture coverage in immortalised dental pulp cell line
*Immortalised dental pulp cells were transfected with siRNAs, before mRNA samples were collected 24hours later. Scrambled siRNA used as control, against 2 different siSmarcA2 probes. **A** Representative Real Time PCR analysis of the mRNA expression levels of genes which are downregulated or upregulated in quiescent MSCs. siSmarcA2 A & B treatment modestly reduced SmarcA2 expression levels within the sample. n=3 data not significant **B** Relative area covered by cell growth compared to growth within the control treated cells, following crystal violet staining. In the presence of siSmarcA2, cells grow to cover more of the cell culture surface. ** p<0.005 n=2*

To further validate the effect of inhibiting SmarcA2 on MSC state, a fluorescent cell cycle indicator was utilised. The Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) system allows for the infection of cells with two fluorescently active constructs. Briefly, following infection and selection, cells within the mitotic phases exhibit green fluorescence (in S-M phases) and red fluorescence (in G1 to early S phases). When the cells are quiescent, they are colourless. As the infection system requires the use of antibiotics for selection, the cell models used previously in this study were unsuitable for this assay. However human bone-marrow mesenchymal cells (BMCs) were obtained for this purpose. Having subjected the cells to the synchronisation regime as described in section 4.2.2 The cell cycle status of cultured cells can be synchronised through adjusting culture conditions. Synchronised infected cells were subjected to flow cytometric analysis. In comparison to the infected control cells, all three conditions displayed some cells which were mitotically active and therefore coloured. The proportion of colourless cells was highest in the starved condition, indicating that in this condition many of the cells were able to enter quiescence (Figure 47 A). In this cell type, 24 hours of treatment to recover the cells produced only a slight decrease in the number of quiescent cells. Perhaps indicating that these cells require longer exposure to nutrients before they re-enter the cell cycle following starvation.

SmarcA2 was inhibited in these cells using small molecule inhibitor PFI3. This bromodomain specific probe sequesters SmarcA2 (and other bromodomain containing chromatin remodelling factors) thus preventing it from performing its endogenous function. Fluorescent microscopic analysis of FUCCI infected BMSCs revealed that with increasing doses of PFI3, the proportion of colourless

cells within the culture decreased (Figure 47 B). Furthermore, the number of cells

within each field of view in each culture condition also increased (Figure 47 B).

Figure 47. Inhibition of bromodomain containing chromatin remodelling factors reduces levels of quiescence in cultured mesenchymal cells
A Flow cytometric analysis of BMCs infected with a ki67-FUCCI cell cycle indicator system. Levels of colourless (quiescent) cells within the BMC sample is increased with starvation. B Fluorescent microscope analysis of ki67-FUCCI infected BMCs following synchronisation and the treatment of cells with small molecule PFI3 after starvation. Key as in panel A. Size of charts are proportional to number of cells per field of view in each condition. C Percentage of cell culture surface area covered by cell growth of immortalised MO6-G3 dental pulp cells synchronised and treated with PFI3. *** p<0.001 n=2

When the same small molecule inhibitor was added to starved dental mesenchymal cells, they too began to show a dose dependent increase in the amount of area the cells grew to cover in culture (Figure 47 C).

Together, this data suggests that, the inhibition of SmarcA2 encourages quiescent cells to lose their quiescent state and begin to proliferate. Indicating the SmarcA2 action is necessary for the maintenance of quiescence.

4.5.3 Quiescent mesenchymal stem cell marker gene SmarcA2 expression may be regulated by Notch signalling components

In addition to being an epigenetic regulator itself, SmarcA2 is also a marker of quiescent MSCs. The transcriptional regulation of this gene has been widely investigated. However, there are numerous RBP-Jκ binding sequence motifs in the DNA around the promoter region and first exon of SmarcA2 (Figure 16 A). Having designed primers to target these RBP-Jκ binding motif regions, ChIP was undertaken to determine if RBP-Jκ does indeed bind to these sites in dental mesenchymal cells.

The preliminary data shows that some background pull down was occurring at many of these sites. However, the DNA containing RBP-Jκ binding sites, 9 &10, 12 & 13 and 16, was exclusively pulled down with RBP-Jκ protein (Figure 48 B). Indicating that these sites are occupied by RBP-Jκ in these cells. The cells used in this preliminary work, were not synchronised, but rather represent a heterogenous population of fed dental mesenchymal cells.
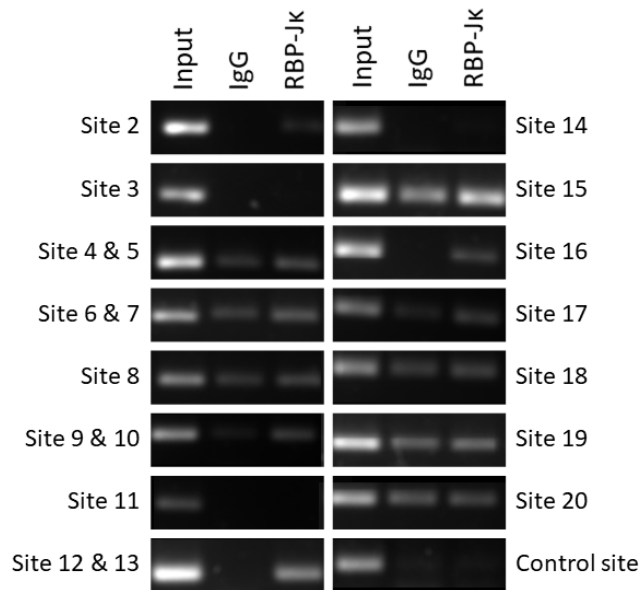
Figure 48. The region of the genome adjacent to the start codon of the SmarcA2 gene contains many potential RBP-Jκ binding sites, some of which are occupied by RBP-Jκ in dental pulp cells in vitro
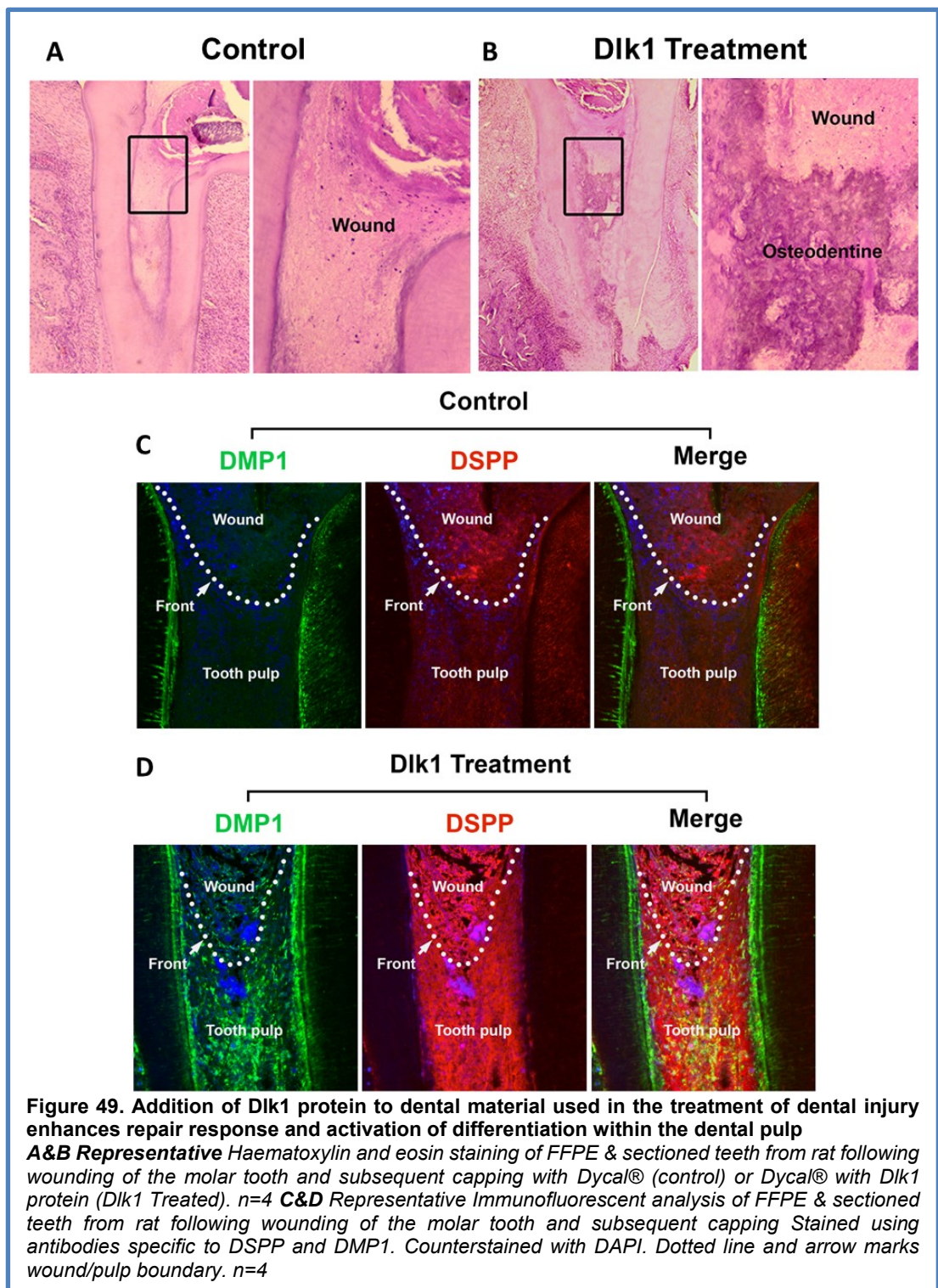*Gel image of PCR products following ChIP of MO6 G3 cells using RBP-Jκ specific antibodies, followed by PCR using probes designed to cover RBP-Jκ binding sites, as indicated in panel A. (n=1)*

Consequently, this work shows that SmarcA2 has the potential to be regulated by RBP-Jκ and thus Notch signalling. However, further assays are required to determine if this binding is differentially observed in cells exhibiting MSC-like and MTAC-like properties.

4.6 Dlk1 protein can be used to enhance dentine regeneration after dental injury

This study has discussed the role of Dlk1 in regulating the endogenous MSCs, and their progeny, in the incisor. Understanding these mechanisms may be important in the advancement of clinical SC applications generally. However, the human dentition does not contain continuously growing teeth, and so the translatability of this work into a clinical setting is limited.

Within the non-continuously growing dental pulp, reactive SCs have been identified, which can respond to injury to promote repair and regeneration. As Dlk1 has been shown to be a powerful regulator of endogenous MSCs in the mouse incisor, the effect of this molecule on molar dental mesenchyme following injury was investigated.

Figure 49. Addition of Dlk1 protein to dental material used in the treatment of dental injury enhances repair response and activation of differentiation within the dental pulp
***A&B** Representative Haematoxylin and eosin staining of FFPE & sectioned teeth from rat following wounding of the molar tooth and subsequent capping with Dycal® (control) or Dycal® with Dlk1 protein (Dlk1 Treated). n=4 **C&D** Representative Immunofluorescent analysis of FFPE & sectioned teeth from rat following wounding of the molar tooth and subsequent capping Stained using antibodies specific to DSPP and DMP1. Counterstained with DAPI. Dotted line and arrow marks wound/pulp boundary. n=4*

In order to investigate tooth injury and clinical treatment, the rat M1 was used as a model. This animal work was undertaken by Dr Heng Zhuang, who kindly provided the samples from this experiment. Briefly, bilateral wounds were made to open the pulp chamber in the mandibular 1st molars. The wounds were then capped with calcium hydroxide based filler. In the experimental tooth, Dlk1 protein was added to the capping material. The rats were later sacrificed and the effect of Dlk1 on the wound healing response was assessed.

In the control teeth, the wound edge abutted the dental pulp with some remodelling of the mesenchymal tissue (Figure 49 A). The contralateral, Dlk1 treated teeth, displayed the deposition of a substance resembling osteodentine at the pulp-wound boundary (Figure 49 B). Furthermore, while the dental pulp of control teeth displayed modest immunoreactivity to differentiation marker gene antibodies, the Dlk1 treated teeth showed a vast upregulation (Figure 49 C&D).

5 Discussion

5.1 The identification and classification of endogenous mesenchymal stem cells within the murine incisor

The cells of the CL-MSC region have here been shown to express a range of quiescent MSC marker genes. Comparisons to the established MTAC region within the incisor and the region containing the recently identified NVB-MSC, revealed the CL-MSCs to be a distinct population.

Technically, the experiments required to determine the existence of the CL-MSC region required careful consideration. During this assessment it was important that LCM of the regions be confined to the most strongly suggestive region of MSCs, as the true size of the population was unknown and capturing non-MSC tissue within the sample would dilute the concentration of MSC cell mRNA. While it would be preferable to investigate each of the MSC marker genes in turn, limitations on time and resources made this impossible. Consequently, two genes which were consistently upregulated in the CL-MSC region were taken forward for further analysis, SmarcA2 and Zbtb20. The protein expression analysis confirmed that the proposed MSC region was presenting proteins which are expected to be produced in quiescent MSCs. This work also showed that the region of expression was larger than the region captured by LCM for mRNA assessment, however the region of Ki67 positive MTACs is distinct from the quiescent MSC marker expressing region. These studies were undertaken on immature mice, at a stage when the incisor tooth is only partially in use. Therefore, it was necessary to determine if the same patterns of cell population expression were observed in the mature incisor. Due to the density of the mandibular tissue after postnatal day 7, assessment was particularly difficult. Consequently,

dissection was undertaken prior to sample processing. This brought its own issues, chiefly that the morphology of the incisor tissue can be affected. However, it was still demonstrated that the expression levels of quiescent MSC marker genes remained higher in the MSC region of the adult incisor. This finding indicates that the MSCs identified persist in the adult tooth.

The identification of a novel MSC population within the mouse incisor mesenchyme in this study was a primary aim of this work. Following the discovery of NVB-MSCs within the mouse incisor (An et al., 2018, Zhao et al., 2014, Kaukua et al., 2014b), it appears that this work has further identified the incisor mesenchyme as having two distinct MSC niches within the same tissue. Generally it is believed that single SC niches are present within tissues, so this is a particularly exciting discovery (Morrison and Spradling, 2008). The CL-MSC region is distinct both in its location, size (approximately 50µm in diameter) and expression profile.

It is likely that the NVB-MSCs and CL-MSCs receive distinct signals from the NVB and the epithelium, being geographically closer to one or other. And possibly that these two populations are from subtly different lineages developmentally. In order to fully investigate the differences, transgenic *in vivo* labelling would be required, which was not possible under the scope of this study. However, it is interesting that the CL-MSCs and NVB-MSCs appear to be utilised and activated differently within the tissue.

Specifically, the way in which the two MSC populations are utilised in physiological growth and in response to injury appears to be very different. NVB-MSCs have been shown to activate upon injury to populate the odontoblast region and assist in the production of reparative dentine (Zhao et al., 2014, Kaukua et

al., 2014a). However, the CL-MSCs appear to be activated also under normal physiological conditions. This difference is particularly interesting when considering the MSCs of the molar teeth. In molars, there is no CL and thus no location for a CL-MSC niche and no epithelial signal input. However, the NVBs are present within the molar (Mitsiadis et al., 2017). In molar teeth, which do not continuously grow, the regeneration observed following injury may be more like that of the repair induced by NVB-MSCs in the incisor, although this is not definitive from this investigation. If this indication is found to be true this finding would further support the notion that the NVB-MSCs play a role only in regeneration, while CL-MSCs are the endogenous SCs required for physiological lineage differentiation of the continuously growing tooth.

5.2 The determination of mesenchymal stem cell to odontoblast lineage

Validation that the identified endogenous quiescent MSC cell population is made up of progenitor cells of the odontoblasts was attempted.

Initially attempts were made to identify a transgenic mouse line with the option of applying an inducible label to the cells specifically expressing quiescent SC markers. Unfortunately, there are no existing transgenic mouse strains which allow for sensitive and specific tracing of the CL-MSC cells, and so alternative options have been investigated.

Traditional *in vivo* methods of lineage tracing utilising injected dyes which can be visualised after the animals are sacrificed at different time points, was not possible due to limitations in licencing, funding and time available for animal work.

However, utilising an organ culture model, mouse incisors can be extracted and maintained *in vitro*. At pre-eruption and post-eruption aged mice, microinjection of explanted incisors was attempted. CM-DiI injections into the CL region were performed to label the mesenchyme in this area. After 7 days the location of the dyed cells was analysed through fixation, sectioning and fluorescent imaging of the samples. Upon analysis, no tracing was able to be visualised. The fluorescent signal retention of cells stained with this dye is reported by the manufacturer as ">72 hours", while it may remain stable longer, it is likely that by 7 days this dye was no longer detectible in this preliminary experiment. The lack of visualisation is likely to also be compounded by the fact that maintaining the sample in organ culture for this length of time may have had a negative impact on the integrity of the tissues. Due to the unstable and toxic nature of the dyes, the inaccessibility of the tissue, and the issues with maintaining the integrity of this tissue in culture, these experiments were largely unsuccessful.

Figure 50. Transgenic mice can be used to trace the lineage of incisor mesenchymal stem cells to the odontoblast layer
Schematic explanation of conclusions based upon lineage tracing of endogenous CL-MSCs through the use of transgenic mice line
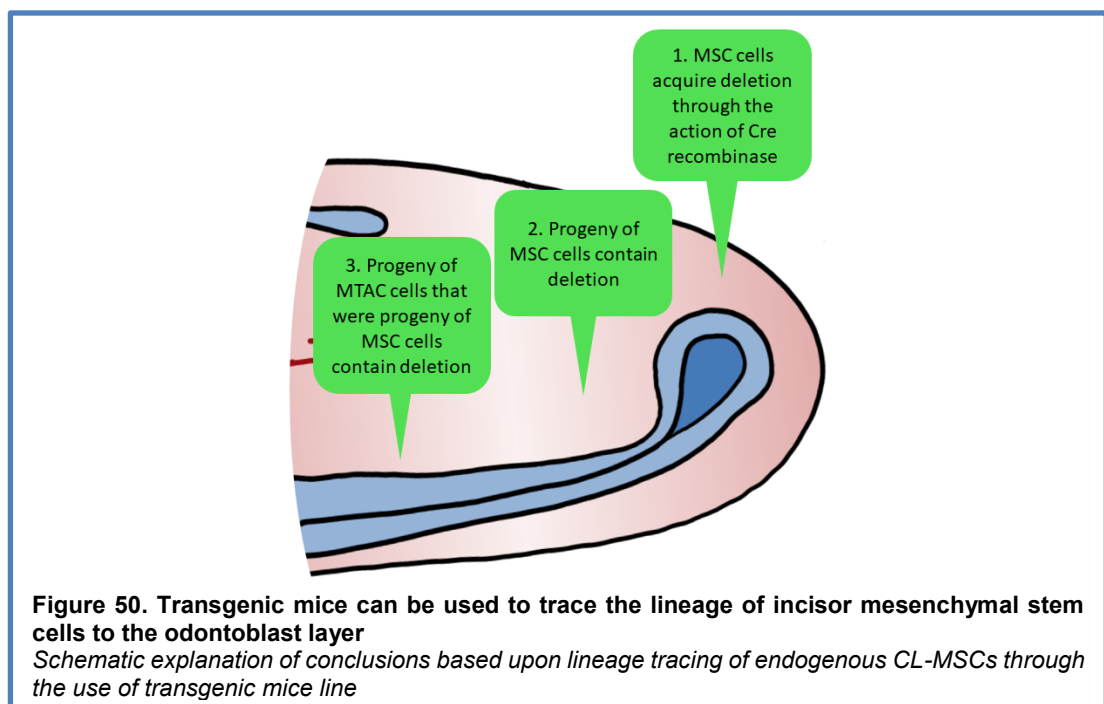
In an attempt to circumnavigate these problems transgenic mice were utilised. The expression of Cre recombinase under the promotion of Collagen 1 α2 in these mice was identified within the CL-MSC region.

The MTAC region and odontoblast layer both showed no recombinase expression. Assessment of the action of Cre was undertaken through the use of mice bred from this strain and another strain which carried a region of floxed DNA within its genome. By assessing the location of cells with the deletion of this DNA, the location of cells which were the progeny of the CL-MSCs (at the time of Cre recombinase action) were traced. This data showed that although the deletion was most prominent within the MSC region in young mice, as the mice aged a greater proportion of the MTAC, and ultimately odontoblasts, carried the deletion. This indicates that the CL-MSCs which first acquired the deletion, go on to populate the MTAC and later odontoblast regions (Figure 50).

While this system has been a useful tool to indicate the migration of the CL-MSC progeny, it would be preferable to validate this work using inducible transgenic mice lines, which could allow direct visualisation of the cells in real time. In addition, if this could be better targeted to the CL-MSCs specifically, this would allow for more robust assessment of the specific progeny of these cells. Unfortunately, neither of these options were available during the time of this research. However, the findings of this study do strongly indicate that the CL-MSC's progeny go on to populate the MTAC and odontoblast regions.

While CL-MSCs of the mouse incisor produce progeny, which populate the odontoblast region, it is not clear from the lineage tracing if these cells are functional odontoblasts. The ability of CL-MSCs to produce mature odontoblasts was assessed through the utilisation of an *in vitro* culture model. By resecting the

CL-MSC containing mesenchyme and culturing it on devitalised dentine, explanted cells were able to adopt an odontoblast like morphology, sending protrusions into the existing dentine tubules. Furthermore, the cells were able to produce mature odontoblast marker proteins, DMP1 and DSP. This together indicates that the CL-MSC containing incisor mesenchyme has the ability to differentiate into mature odontoblasts.

In order to be certain that it is the CL-MSCs themselves, and not other mesenchymal cells from within the resected tissue, that are producing these odontoblast-like cells, further work may be required. Work undertaken in the research group of Dr Bing Hu at the University of Plymouth, by other researchers in the group, has shown that extracted CL-MSCs, cultured from the resected tissue are also able to produce the morphology and expression pattern of odontoblast-like cells when grown on devitalised dentine (Walker et al., in review). Therefore, it is strongly suggestive that the CL-MSCs are the cells responsible for the observed effect in this study.

5.3 The development of *in vitro* model systems to investigate the molecular mechanisms which mediate mesenchymal stem cell and transit amplifying cell maintenance and transition

Further investigation into the role of Dlk1 in regulating dental mesenchymal cells required the development of appropriate *in vitro* systems. Primary cells can be a valuable tool when understanding the behaviours of cells. The fact that the cells are not artificially immortalised makes modelling their cell cycle potentially more relevant to the *in vivo* situation (Kaur and Dufour, 2012). However, this advantage has a corresponding disadvantage, the cells are not immortalised and therefore

cannot be passaged indefinitely. A similar boon/burden situation arises when considering the purity of the cells. In primary cell cultures, a mixed population of all cell types can potentially grow from the tissue (Stockholm et al., 2007). This can be a benefit, as it more closely resembles the *in vivo* environment and could provide additional growth factors, which the cells of interest require. The downside of this, is that any molecular samples collected from the cultured cells will be the combined molecules from all cell types, which may mask the molecular situation of the cells of interest.

In this study, an incisor mesenchymal cell primary culture was undertaken. In order to address the issue of mixed population growth, cells were cultured under permissive conditions to encourage the cell type of interest to thrive. Through a combined approach of coating the cell culture surface with a molecule known to encourage mesenchymal cell adherence and providing a culture medium which favours non-epithelial cell growth, mesenchymal cells were selected for (Somaiah et al., 2015). Analysis of the molecular signature of the cells within this system showed that the cells were indeed mesenchymal in their phenotype. The longevity of these cells was minimal, with growth stalling profoundly after passage 6. It was also noted that the cells did not readily survive the freezing and thawing process. Consequently, cells were used at low passages and always from fresh batches of cells. This has an inherent limitation, that each batch of cells was produced from a different litter of mice and therefore had a fair degree of genetic variation. However, repeated experiments did show similar results and reproducibility. This cell culture model was synchronised into a quiescent MSC-like state through serum starvation. Replenishment of the serum was also sufficient to encourage the cells to adopt a more MTAC-like phenotype and expression profile. Unfortunately, these cells were not very robust. Manipulation

of the cells through the use of recombinant proteins, small molecular inhibitors and plasmids, caused the cells to lose integrity and degenerate. An alternative model was required to investigate the molecular mechanisms which underpin the regulation of these cells *in vivo*.

Established cell lines are able to circumvent many of the pitfalls which can occur when using primary cells (Geraghty et al., 2014). However, they are not without their own issues. Chiefly, cell lines tend to be robust and have almost limitless mitotic potential. While they are often not as phenotypically similar to their original source tissue (and may acquire genetic alterations with time), established cell lines can be made from many organs and so can model tissue specific events. In this study, immortalised dental pulp cells were utilised. These cells were able to be synchronised into a state of quiescence in order to mimic quiescent MSC-like dental cells. This model allowed for the investigation of molecular mechanisms as the cells were robust and able to withstand an array of manipulation techniques.

5.4 The maintenance and transition of mesenchymal stem cell and transit amplifying cells, the role of Notch signalling

Having identified and characterised an endogenous quiescent CL-MSC pool within the murine incisor, understanding the molecular mechanisms which regulate the ability of MSCs to self-renew and to differentiate was a key aim of this work.

The number of active TACs directly influence and define the number of TDCs within a tissue. Insufficient activation of SCs results in insufficient TAC

populations and ultimately a failure of tissue replenishment (Chen et al., 2012b). However, overactivation of SCs can result in an exhaustion of the pool of available cells (Scheller et al., 2006). Therefore, fine regulation of this activation process throughout the lineage is imperative for proper functioning and maintenance of the tissue.

The identification of Notch receptors being utilised differentially between the CL-MSC and MTAC regions led to the investigation of Notch ligand expression pattern differences within the mouse incisor. Through the use of transgenic mouse lines with altered Notch pathway gene expression within the incisor mesenchyme, an important role for Notch signalling was reinforced.

In mice which are conditionally deficient in RBP-Jκ, the dentine displays an abnormal phenotype. Further investigation of this mouse line revealed that the odontoblasts were disorganised and appeared to be prematurely differentiated. Clearly canonical Notch signalling is an important signalling pathway in regulating the lineage and differentiation of the CL-MSCs of the incisor mesenchyme. A similar dentine phenotype has been observed in the Dlk1 KO mice. Whereby apparent precocious differentiation occurred, resulting in an aberrant dentine structure being seen.

Within mice deficient of Dlk1, the MTAC region was reduced in volume. Therefore, it is likely that the LCM regions captured were more greatly diluted by cells from outside of the targeted population. This is a probable reason for the apparent similarity between the expression profiles of the MTAC and CL-MSC sample. Nonetheless, it was clear that the MTAC region is diminished in the absence of Dlk1, thus indicating a potential loss of SC to TAC transition, a loss of MTAC maintenance or a combination of both of these factors.

The preliminary investigation of the Dlk1 Tg mice, in which Dlk1 was over expressed within the incisor mesenchyme, showed signs of MTAC pool exhaustion. This suggests that while some Dlk1 is required for MTAC maintenance, too much can cause over proliferation of these cells and subsequent reduction of MTACs from the tissue. Further investigation of these mice at a range of ages may illuminate how this phenomenon develops within these mice. However, during the time of this study such mice were unavailable.

This study has found that Dlk1 is expressed most strongly within the MTAC region. However, it's function on MSC-like and MTAC-like cells in culture indicate that it may have different modes of action on these cell types. *In vitro* bound Dlk1 appears to encourage mesenchymal cells to lose their MSC-like properties, indicating a role for bound Dlk1 in encouraging MSC to MTAC transition. Meanwhile, soluble Dlk1 protein added to the cells seems to encourage a greater adoption of MTAC-like marker expression. Together this data suggests that Dlk1, whether bound or soluble, is sufficient to encourage the shift of mesenchymal cells to a more MTAC-like state and a maintenance of this new state. Conversely, the inhibition of Dlk1 through the use of siRNAs in cultures containing proliferative cells, revealed that Dlk1 is necessary for the maintenance of the MTAC-like state of these cells. *In vivo* Dlk1 is likely to function differently both due to differences in presence of the soluble or membrane-bound form of the protein, but also in terms of different receptor availability.

Although Dlk1 has been long identified as having a role in BMSC differentiation in adipogenesis (Sul, 2009), no established specific Notch receptor for Dlk1 has been identified. In this study, Notch2 was found to be expressed in a similar pattern throughout the incisor mesenchyme to Dlk1, indicating a potential link

between them. Furthermore, a striking increase in Notch effector Hes5 has been observed in the MTAC region. If a Dlk1>Notch2>Hes5 axis is present within this tissue, it may indicate a potential pathway which could be explored further.

It is clear from this study together that Notch signalling plays an important function in MSC and MTAC lineage differentiation, activation and maintenance. Furthermore, physiological levels of Dlk1 are inextricably involved in the maintenance of MTACs and in prevention of precocious differentiation of this lineage.

Additional Notch pathway findings identified in this study, which have not been fully investigated here, include the clear spatial restriction of Jag1 and Jag2 expression within the CL epithelium. Both the MTAC and CL-MSC regions directly contact the CL epithelium. Many signalling pathways have been implicated in the epithelial-mesenchymal crosstalk of the incisor tooth (Kettunen et al., 2000, Balic and Thesleff, 2015). The clear localisation of Notch ligands within the CL epithelium, and the observed differences in Notch signalling between the MTAC and CL-MSC regions would make further investigation of this potential epithelial-mesenchymal interaction an attractive and, obvious, next step following this work.

5.5 The epigenetic regulation of markers and modifiers of mesenchymal stem cell and transit amplifying cell maintenance
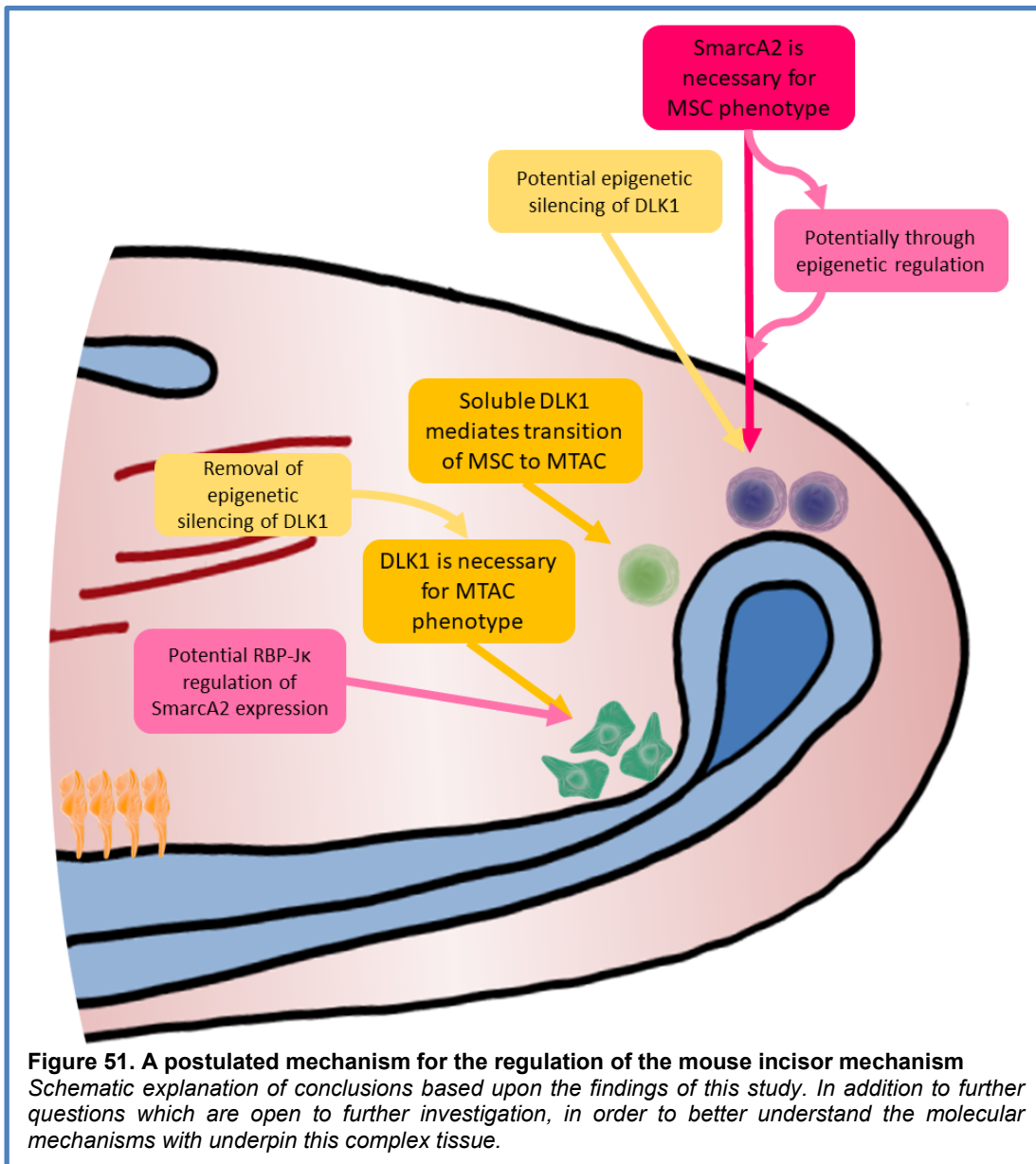
In addition to the main aims set out prior to undertaking this study, an interesting avenue of investigation into epigenetic regulation within the incisor tooth mesenchyme arose during the course of the project. Two molecules which

showed promise as important regulators of maintenance and transition within the cell lineage of interest, have strong links to epigenetics.

Dlk1 is a gene which has long been known to be heavily epigenetically regulated. Having been identified as residing within a maternally imprinted locus, the CpG islands around the Dlk1 gene are a prime target for epigenetic regulation throughout the life of a cell. Histone methylation marks are often used to identify regions of chromatin which are repressed. In this study, such marks were identified adjacent to the CpG islands of Dlk1 in starved (but not proliferative) cultured dental pulp cells. This highlights that epigenetic marks are in place in these MSC-like cells, indicating that Dlk1 expression may be influenced epigenetically.

The notion that CL-MSC and MTACs may be utilising epigenetic regulation to modulate the phenotype of the two populations is also supported by the differential expression of known epigenetic regulatory protein SmarcA2. SmarcA2, a quiescent MSC marker gene, displayed strong expression within the CL-MSC region of the murine incisor. This molecule is an important chromatin remodeller (Tang et al., 2010). The expression of SmarcA2 within the CL-MSCs indicates a potential role for this form of epigenetic modulation to be regulating the behaviour of the cells. Indeed, when SmarcA2 was inhibited in MSC-like cells in culture, using siRNAs, the cells upregulated the expression of a number of genes which were formerly downregulated. These treated cells also grow to cover a greater proportion of the culture surface. Together indicating that SmarcA2 may have been necessary in the repression of MTAC-like phenotype in the CL-MSCs, thus ensuring the maintenance of the MSCs in a quiescent state.

The transcription of SmarcA2 itself is regulated between the CL-MSC and MTAC region.

Figure 51. A postulated mechanism for the regulation of the mouse incisor mechanism
Schematic explanation of conclusions based upon the findings of this study. In addition to further questions which are open to further investigation, in order to better understand the molecular mechanisms with underpin this complex tissue.

The lack of expression within the MTAC region indicates a strong inhibition of transcription here. In order to begin to address which molecules and pathways may be responsible for the alternate expression patterns within the CL-MSC and MTAC regions, the promoter region of SmarcA2 was investigated. Interestingly, there are many RBP-Jκ potential binding sites within the promoter region of SmarcA2. Preliminary ChIP experiments revealed that the RBP-Jκ protein does

bind to many of these sites within cultured dental pulp cells. This combined with the knowledge that RBP-Jκ has been shown to interact with SmarcA2 protein itself (Schwanbeck, 2015), indicates the potential for a feedback loop whereby RBP-Jκ regulates SmarcA2 expression levels, and also acts in conjunction with the SmarcA2 as an epigenetic regulator itself. Moving forward from here, the effect of synchronising these cells on the binding state of these sites is now being investigated by colleagues at The University of Plymouth.

The postulated mechanism or regulation within the mouse incisor mesenchyme (Figure 51) requires further investigation in order to determine if, and how RBP-Jκ regulates cell behaviours within this model. The high expression of other Notch ligands within the epithelial tissue of this organ may also have a bearing on the function of RBP-Jκ, thus creating a complex inter tissue regulatory network.

5.6 The potential clinical impact of the molecular mechanisms which underpin mesenchymal stem cell regulation being utilised therapeutically

This study has investigated the role of Dlk1 in regulating the endogenous MSCs, and their progeny, in the incisor. While this may add to the body of knowledge regarding the maintenance and activation of MSCs generally, the translational applications would require a great deal more study. Within the human dentition there are no continuously growing teeth similar to the mouse incisor. However, there are known pools of reactive DPSCs. This study has shown that Dlk1 is a potent regulator of murine incisor MSCs and their progeny, the effect of Dlk1 on DPSCs in non-continuously growing teeth, however, is largely unknown. In order to model human dental injury and repair under the influence of Dlk1, a rat model

was utilised. Wounds were made in the rat molar teeth which were either treated with a hydroxyapatite based capping substance, or with the same substance impregnated with Dlk1. In the Dlk1 treated teeth, there was a greater level or osteodentine deposition, indicative of enhanced repair mechanisms. It is presumed that the Dlk1 protein is acting upon reactive DPSCs within the tooth to create this enhanced repair. While further investigation is required, it is encouraging that Dlk1 may be a simple additive which could be used in conjunction with traditional dental materials to enhance dental repair. Dental injury and its treatment is a costly and laborious affair, which when handled inappropriately can lead to tooth loss which impacts quality of life for the patient. If the process of repair and healing following dental injury can be accelerated, it would be a great boon for patients and health care providers alike.

6 Conclusions

Within the CL-MSC region identified by this work, Notch signalling plays a role in the molecular regulation of the cells. As outlined in the initial aims, a novel CL-MSC population has been found to exist within the mouse incisor mesenchyme. The CL-MSC and MTAC regions exhibit different quiescent MSC marker gene expression profiles. Strikingly, the Notch effector Dk1 is also differentially expressed, with an increased level of Dlk1 protein within the MTAC region. *In vivo* modulation of the Notch pathway results in aberrant incisor development, indicating the important role of this pathway in dental mesenchymal cell function. *In vitro* manipulation of this same pathway further supports the role of Notch signalling in maintenance and transition of the cells of the CL-MSC and MTAC regions. Although investigation of the translational applications of these findings is still at a preliminary stage, it appears that Notch effector Dlk1 may exert an effect on the cellular behaviour of wounded dental pulp during regeneration.

Specific conclusions are as follows:

6.1 There is a novel endogenous mesenchymal stem cell region in the incisor tooth

Within the murine incisor a distinct region of MSC containing cells has been identified. This region presents a quiescent MSC-like marker signature, both in terms of mRNA and protein expression. This region is geographically and phenotypically distinct from the known region of MTACs and the region of NVB-MSCs within the incisor mesenchyme.

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6.2 Notch signalling molecules are expressed at different levels between the distinct mesenchymal regions

The MTAC region highly expresses Notch2 and Notch ligand, Dlk1. These markers are expressed much less within the MSC region. Furthermore, the MTAC region displays a greater level of Notch2 activation. The differences in Notch pathway expression and activation between the CL-MSC and MTAC regions, indicate alternate utilisation of this pathway between the two cell populations.

6.3 The endogenous mesenchymal stem cell region can give rise to odontoblasts

Through *in vivo* lineage tracing of cells from the CL-MSC region, their progeny has been traced to the MTAC and odontoblast regions within the mouse incisor. Further *in vitro* assays revealed that tissue containing the CL-MSC region is capable of forming odontoblast like cells (both in terms of their morphology and their expression profile) when in contact with dentine. This together indicates the potential ability of MSCs to give rise to odontoblasts.

6.4 Mesenchymal stem cell to transit amplifying cell transition can be modelled *in vitro*

Utilising both primary cell culture and established cell lines, the MSC and MTACs of the incisor mesenchyme have been successfully modelled. These cells can be manipulated into a quiescent MSC-like state or encouraged to proliferate and take

on an MTAC-like phenotype. This cell model has then been successfully employed to investigate the role of specific molecules in regulating the behaviour of MSC-like and MTAC-like cells.

6.5 Modulation of Notch pathway activity regulates the maintenance and transition of mesenchymal stem cells and transit amplifying cells

Phenotypic analysis of RBP-Jκ conditional knock out mice, revealed that canonical Notch signalling is an important regulator of the dental mesenchymal cell lineage which gives rise to the odontoblasts. Analysis of Dlk1 overexpressing mice and Dlk1 knock out mice, revealed that Dlk1 is a vital molecule which regulates the MTACs maintenance and transition.

In vitro investigation went on to further identify that Dlk1 is necessary for the maintenance of an MTAC-like phenotype. Furthermore, Dlk1 is sufficient to induce an MSC to MTAC-like transition in cultured dental mesenchymal cells.

6.6 Notch ligand, Dlk1, may be epigenetically regulated within the incisor mesenchyme

The Dlk1 gene is located within a region of chromatin which is highly epigenetically regulated. Between MSC-like and MTAC-like cells there is a significant difference in the levels of epigenetic markers around the Dlk1 locus. Thus, indicating an epigenetic regulation of the expression level of Dlk1 difference between CL-MSC and MTACs in the incisor tooth.

6.7 Notch pathway ligand, Dlk1, can promote dentine reparation following injury

The addition of Dlk1 to the dental material used to cap wounded teeth, enhanced the levels of osteodentine deposition and repair in damaged rat molar teeth. The enhanced osteodentine deposition indicates an activation of DPSCs within the tooth, further supporting a regulatory role for Dlk1 in dental stem cells generally.

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