Oral Progenitor Cells as a Cell-Based Treatment for Neural Damage

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Abstract

Over the past few decades stem cells have been extensively investigated due to their potentially invaluable therapeutic use. Embryonic stem cells (ESCs) have wide-ranging therapeutic applications in tissue repair and regeneration due to their pluripotent properties and their ability to self-renew indefinitely. However, ethical concerns surround their use and hence alternatives are sought. Adult stem cells (ASCs) have been isolated from various adult tissues including the oral mucosa lamina propria (OMLP). This study aims to isolate ASCs from the OMLP, reprogram these cells to induced pluripotent stem cells (iPSCs) and determine the potential for both to differentiate into functional neurons due to the limited regeneration of neurons in the central nervous system. Such investigations into strategies for the treatment of neural damage are invaluable and timely due to current limitations in the availability of human-derived cells for potential autologous or allogeneic tissue repair. OMLP-PCs represent an ideal cell source for use in regenerative medicine given their ease of isolation, proliferative potential, multipotent properties and immunosuppressive activities. Work in this Thesis has now demonstrated that these oral progenitors expressed numerous pluripotency markers and for the first time, that they could be reprogrammed to iPSCs utilising safer, non-integrating plasmids, thus increasing their potential for use in clinical applications. OMLP-iPSCs were positive for a number of pluripotent stem cell markers including SSEA-4, SSEA-5, TRA-1-60, TRA-1-81, Oct-4 and Sox-2. Moreover, their expression of early stage germ layer markers indicated their potential to differentiate into cell types of the mesoderm, endoderm and ectoderm. OMLP-PCs were also demonstrated within this Thesis to differentiate down an early neural lineage as evidenced by the presence of typical neural markers (Nestin, βIII tubulin, MAP-2 and NF-M). The presence of both ligand-gated and voltage-sensitive calcium channels indicated some limited potential functional phenotype. Unfortunately, utilising the same neural differentiation methodology, OMLP-iPSCs were not able to be similarly driven down a neural pathway. None-the-less this data suggests that OMLP-PCs and OMLP-iPSCs may hold great promise for a wide range of regenerative medicine applications.

Abbreviations

AMPA	-	2-Amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)Propanoic Acid
ANOVA	-	Analysis of Variance
ASC	-	Adult Stem Cell
ATP	-	Adenosine Triphosphate
BDNF	-	Brain-Derived Neurotrophic Factor
bFGF	-	basic Fibroblast Growth Factor
BMSC	-	Bone Marrow Stromal Cell
BMP	-	Bone Morphogenetic Protein
BSA	-	Bovine Serum Albumin
CFE	-	Colony Forming Efficiency
CNS	-	Central Nervous System
CNTF	-	Ciliary Neurotrophic Factor
DF	-	Dermal Fibroblast
DEPC	-	Diethylpyrocarbonate
DPPC	-	Dental Pulp Progenitor Cell
DMEM	-	Dulbecco’s Modified Eagle Medium
DMSO	-	Dimethyl Sulfoxide
eGFP	-	enhanced Green Fluorescent Protein

EB	-	Embryoid Body
ECM	-	Extra-Cellular Matrix
EDTA	-	Ethylenediaminetetraacetic Acid
EGF	-	Epidermal Growth Factor
ERK	-	Extracellular signal-Regulated Kinase
ESC	-	Embryonic Stem Cell
FACS	-	Fluorescence-Activated Cell Sorting
FBS/FCS	-	Foetal Bovine Serum / Foetal Calf Serum
FES	-	Functional Electrical Stimulation
FITC	-	Fluorescein I-sothiocyanate
F-SCM	-	Fibroblast-Serum Containing Medium
GABA	-	Gamma-Aminobutyric Acid
GAPDH	-	Glyceraldehyde 3-Phosphate Dehydrogenase
GDNF	-	Glial cell line-Derived Neurotrophic Factor
GMSC	-	Gingiva-derived Mesenchymal Stem Cell
GSK3	-	Glycogen Synthase Kinase-3
hESC	-	human Embryonic Stem Cell
hMSC	-	human Mesenchymal Stem Cell
hOMSC	-	human Oral Mucosa Stem Cell
HFF	-	Human Foreskin Fibroblast

HOCl	-	Hypochlorus acid
iFCF	-	inactivated Foreskin Fibroblast
iMEF	-	inactivated Mouse Embryonic Fibroblast
iPSC	-	induced Pluripotent Stem Cell
IGF-1	-	Insulin-Like Growth Factor-1
JAK	-	Janus Kinase
LIF	-	Leukaemia Inhibitory Factor
mESC	-	murine Embryonic Stem Cell
MAP-2	-	Microtubule-Associated Protein-2
MEF	-	Mouse Embryonic Fibroblast
MP	-	Merged Progenitor
MPO	-	Myeloperoxidase
MSC	-	Mesenchymal Stem Cell
NA	-	Non-Adherent
NADPH	-	Nicotinamide Adenine Dinucleotide Phosphate
NCAM	-	Neural Cell Adhesion Molecule
NEAA	-	Non-Essential Amino Acids
NF-M	-	Neurofilament-Medium
NGF	-	Nerve Growth Factor
Ngn-2	-	Neurogenin-2

NMDA	-	N-Methyl-D-Aspartate
NPC	-	Neural Progenitor Cell
NT-3	-	Neurotrophin-3
OEC	-	Olfactory Ensheathing Cell
OI	-	Osteogenesis Imperfect-
OMLP	-	Oral Mucosa Lamina Propria
OMLP-PC	-	Oral Mucosa Lamina Propria Progenitor Cell
O/N	-	Overnight
OP	-	Oral Progenitor
PB	-	PiggyBac
PBS	-	Phosphate Buffered Saline
PC	-	Progenitor Cell
PCR	-	Polymerase Chain Reaction
PD	-	Population Doubling
PDL	-	Population Doubling Level
PDLSC	-	Periodontal Ligament Stem Cell
PFA	-	Paraformaldehyde
PI3K	-	Phosphatidylinositide 3-Kinase
PNS	-	Peripheral Nervous System
qPCR	-	quantitative Polymerase Chain Reaction

RA	-	Retinoic Acid
SC	-	Schwann Cell
SCAP	-	Stem Cells from the Apical Papilla
SCI	-	Spinal Cord Injury
SD	-	Standard Deviation
SEM	-	Standard Error of the Mean
SFM	-	Serum Free Medium
SHED	-	Stem Cells from Human Exfoliated Deciduous teeth
SHH	-	Sonic Hedgehog
SOD	-	Superoxide Dismutase
STAT	-	Signal Transducer and Activator of Transcription
TAE	-	Tris base Acetic acid and EDTA buffer
TGFβ	-	Transforming Growth Factor β
TP	-	Total Population
TX	-	Triton X-100
VEGF	-	Vascular Endothelial Growth Factor

1 - General Introduction

1.1 Introduction

Stem cells have extensively been studied due to their invaluable potential use for therapeutic purposes. Although there is much yet to be learnt, it is an area that is fast moving and new discoveries are continually reported. It is also an area surrounded by much ethical debate which is mainly focused around the use of embryonic stem cells (ESCs) due to the way in which they are obtained.

ESCs are pluripotent cells, capable of forming cells from all 3 germ layers; the endoderm, ectoderm and the mesoderm (Figure: 1.1). They are derived from the inner cell mass of the blastocyst and have the capability to self-renew indefinitely (Thomson et al, 1998). As a result of these characteristics, it is clear to see why they are an attractive target for therapeutic purposes.

Adult stem cells (ASCs) have fewer ethical issues associated with them as they are isolated from adult tissues and, therefore, do not require the destruction of foetal tissue. These cells have the potential to be used as an autologous source of cells and as such, an immune response is also reduced. ASCs are however, sometimes partially committed to become cells of their germ layer of origin and so are not pluripotent but are considered to be multipotent, with some very limited evidence of pluripotency. (Reviewed by Young and Black, 2004)

Recently it has been demonstrated that somatic cells can be reverted back to an embryonic stem cell-like state, restoring pluripotent properties, by forcing the expression of factors known to keep cells in an undifferentiated state.

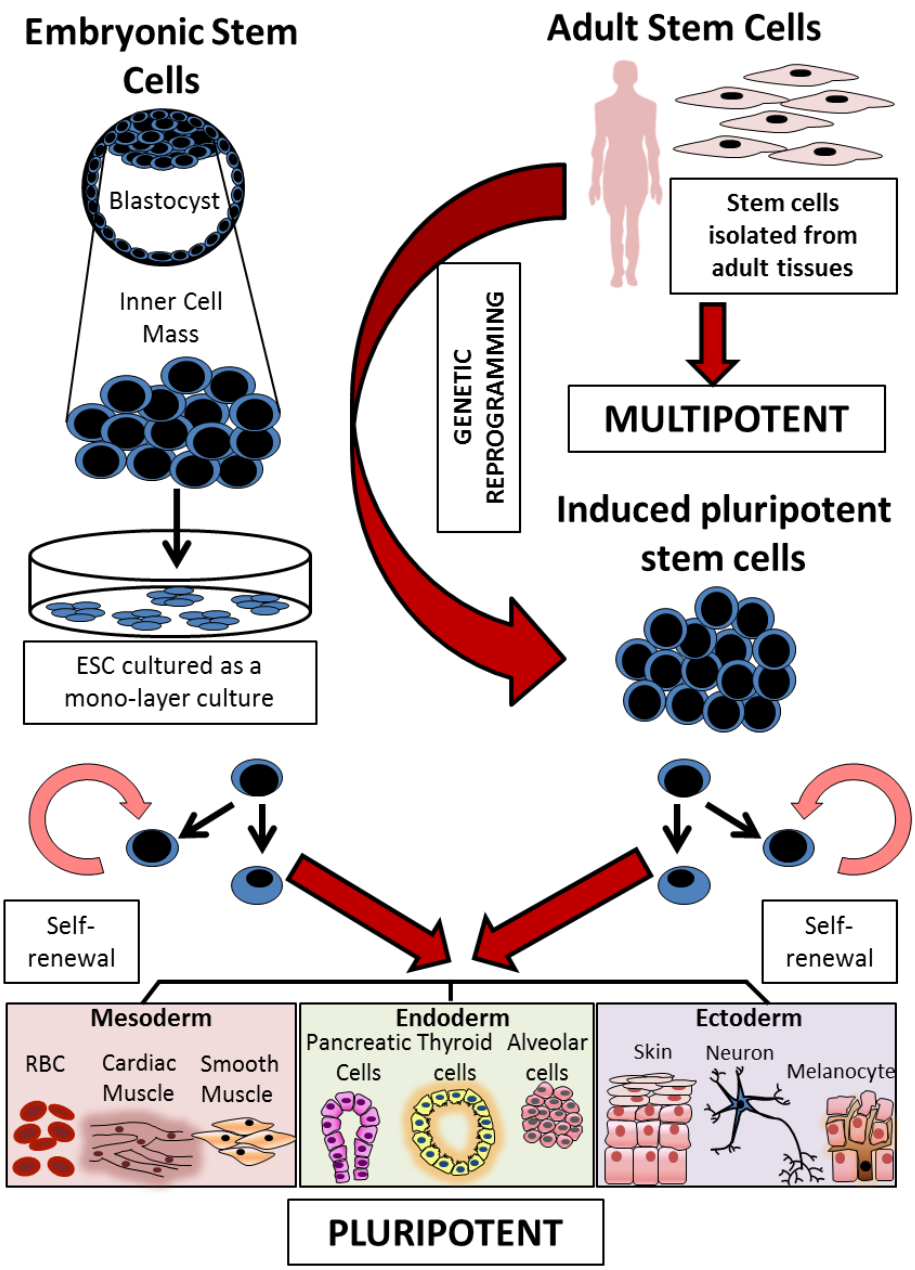

Figure 1.1*: Properties of ESC, ASC and iPSC.* Different fates of stem cells from different origins. Cells derived from the blastocyst are considered to be pluripotent as they can form cells from all three germ layers; ectoderm, endoderm and the mesoderm. Multipotent stem cells exist within different adult tissues with the ability to form cells from their germ layer of origin and there is some evidence for the potential to form cells from other cell lineages. iPSCs are cells that have been genetically reprogrammed back towards an embryonic stem cell state, restoring pluripotency.

These cells are known as induced pluripotent stem cells (iPSCs). This technique was first developed using mouse fibroblasts (Takahashi and Yamanaka, 2006) and then later successfully applied to human dermal fibroblasts (Takahashi et al, 2007) using four viral vectors containing Oct4, Sox2, c-Myc and Klf4. The efficiency of converting cells to iPSCs still remains relatively poor and studies are ongoing to improve this.

The purpose of this project is to isolate, expand and characterise a population of multipotent progenitor cells (PCs) that we know already resides within the oral mucosa lamina propria (OMLP) (Stephens and Genever, 2007; Davies et al, 2010; Marynka-Kalmani et al, 2010) and reprogram these to iPSCs, giving the cells pluripotent properties. The differentiation of these two cell populations (OMLP-PCs and iPSCs) to form functional neurons *in vitro* will be investigated to identify their potential for use as cell based treatments for neuronal damage.

1.2 The Nervous System

The adult nervous system is composed of two main components: (i) The Central Nervous System (CNS); consisting of the brain, spinal cord, optic, olfactory and auditory systems and (ii) The Peripheral Nervous System (PNS); consisting of cranial nerves, spinal nerves and sensory nerves. The purpose of the CNS is to receive and interoperate signals, providing the PNS with excitatory signals. The PNS responds to these signals and stimulates muscle tissue, whilst also relaying signals to and from the CNS.

Cells of the nervous system can be divided into two main cell types; neurons and glial cells. The neurons are the main functional cells of the CNS and consist of a cell body, axons and dendrites. Dendrites function to receive intercellular signals and relay these to the cell body. Axons are utilised to transmit electrical signals away from the neuron to downstream cells. In the PNS, neurons consist of a cell body, motor and sensory axons. Glial cells provide a support network: in the PNS these cells are known as Schwann cells and in the CNS the glial cells consist of oligodendrocytes, microglia, astrocytes. Together these cells support neuronal survival and help maintain the myelin sheath which acts as an insulating layer and functions to facilitate and increase conductivity in the axons.

1.2.1 Neural Damage

Studies into strategies for the treatment of neural damage to the adult CNS are invaluable due to the limited regeneration of neurons in the CNS. Unlike neurons of the adult PNS, axons in the CNS do not regenerate back into functional neurons once they have become damaged (Figure 1.2). This is largely known to be as a result of inhibition from the environment surrounding the damaged cells, rather than the properties of the cells themselves (Richardson et al, 1980). Molecules released by myelin sheath breakdown and the glial scar actively inhibit the re-growth of axons in the CNS (Mukhopadhyay et al, 1994; Chen et al, 2000), whereas, Schwann cells, macrophages and monocytes actively scavenge myelin at the site of injury in the PNS, allowing axonal re-growth (Stoll et al, 1989).

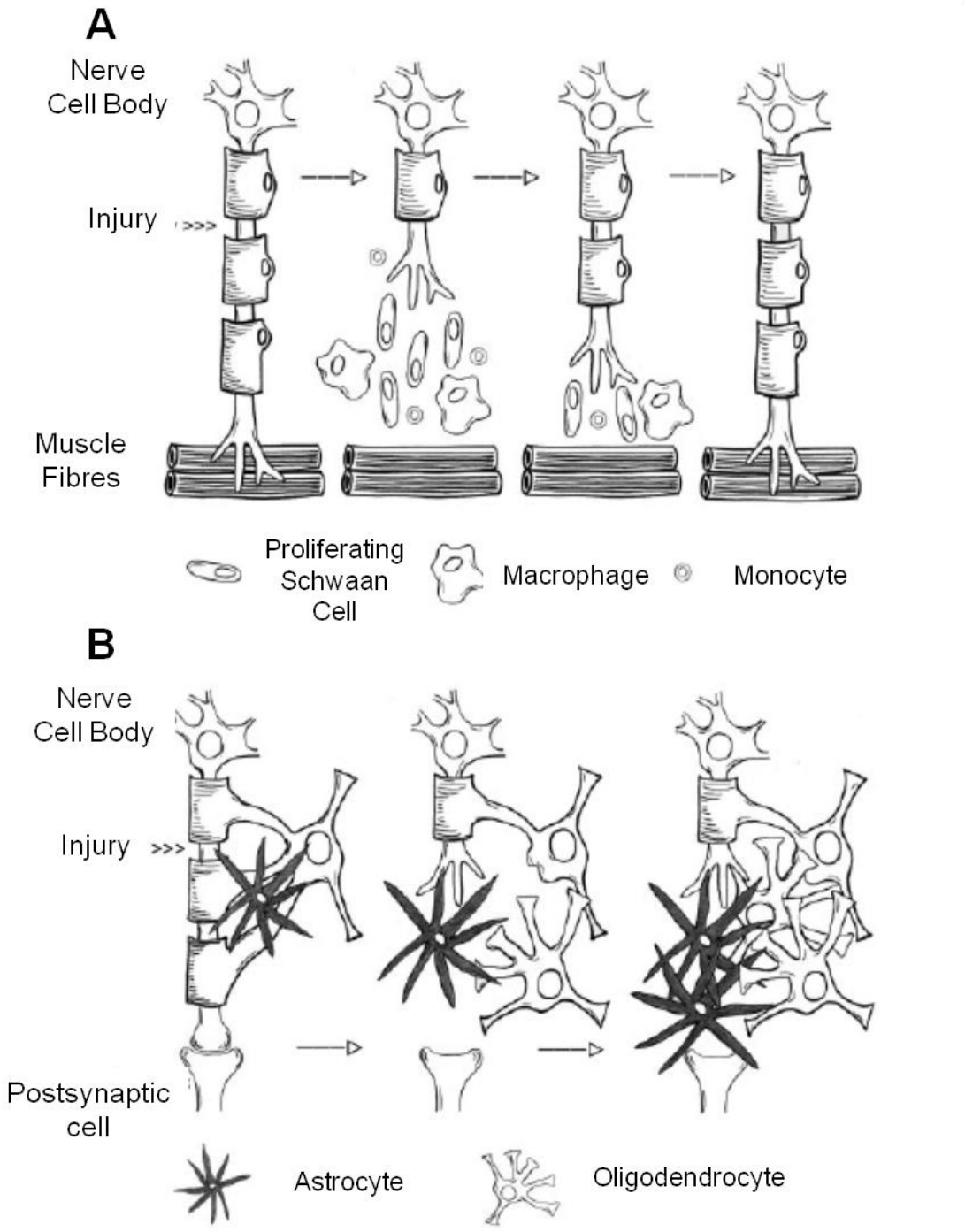

Figure 1.2*: Differences between neural repair in the PNS and CNS*. **(A)** Axon regeneration following injury of a neuron in the PNS. Myelin debris is removed by the collective work of the proliferating schwaan cells, macrophages and monocytes. **(B)** Inhibition of axon regeneration in the CNS. Glial scar formation following injury which consists of astrocytes, oligodendrocytes and cell debris. The glial scar blocks axon regeneration. (From Schmidt and Leach, 2003)

Damage to the nervous system can occur as a result of trauma to the body or disease. Whilst extensive damage to the PNS can be treated surgically by suturing severed nerves or using autologous nerve grafts (Sinis et al, 2009), damage to the CNS still remains a surgical challenge (Ruff et al, 2012). Neurodegenerative disorders, such as Alzheimer's and Parkinson's, present a stern challenge for regenerative medicine due to the continuous degeneration of axons and the resultant damage that is cumulative in nature. Spinal cord injury (SCI), as a result of trauma, lacks this progressive degradation.

Injury to the spinal cord can be devastating with patients encountering loss of movement of limbs and/or bodily functions depending on the site of injury. There are two stages following SCI; primary injury and secondary injury. Primary injury occurs as a direct result of trauma, damaging blood vessels, axons and neural cell membranes. This is followed by a secondary injury phase leading to loss of cells, swelling of the spinal cord to fill the spinal canal space, infiltration of inflammatory cells, activation of astrocytes and microglia, and the release of excitotoxic chemicals that damage the surrounding tissue (Balentine, 1978; Kwo et al, 1989; Macdonald and Sadowsky, 2002; Fleming et al, 2006).

Inflammation occurs during the acute stages of SCI and some of the inflammatory cells can persist for weeks and even months after the injury (Fleming et al, 2006). Secondary damage at the site of injury and to the surrounding tissue is caused by reactive oxygen species, proteolytic enzymes, pro-inflammatory cytokines and nitric oxide.

These are released by infiltrating neutrophils, macrophages, activated microglia and T-lymphocytes into the site of injury which is well documented in the acute stages of SCI in animal models (Banati et al, 1993; Taoka et al, 1997; Popovich et al, 1997; Carlson et al, 1998; Popovich et al, 2002).

Studying inflammation in the acute stages of human SCI does present a challenge due to limited availability of tissue. Nonetheless, there are some limited reports that demonstrate the production of pro-inflammatory cytokines from neurons and microglia following the primary injury (Yang et al, 2004) and infiltration of neutrophils, macrophages and T-lymphocytes to the site of injury in addition to microglia activation (Yang et al, 2004; Fleming et al, 2006). Neutrophils, activated microglia and macrophages demonstrate expression of Myeloperoxidase (MPO), and $gp91^{Phox}$ (a catalytic subunit of nicotinamide adenine dinucleotide phosphate (NADPH). MPO and NADPH are both oxidative enzymes and are involved in secondary damage to the surrounding healthy tissue (Flemming et al, 2006). As such, therapies to reduce the inflammatory response have been considered. Blocking the influx of neutrophils and macrophages to the site of injury has demonstrated reduced functional deficits following SCI in animal models (Taoka et al, 1997; Popovich et al, 1999) and as such these present an interesting target for human SCI.

During the acute stages of SCI excitotoxicity and oxidative damage contribute to secondary injury. Glutamate plays a crucial role in the destruction of healthy neurons during secondary injury as large quantities of it are released from damaged axons and astrocytes. This overexcites the

surrounding neurons, allowing masses of calcium ions to flow into the cell via activated N-methyl-D-aspartate (NMDA) receptors (Choi, 1992) resulting in the release of damaging free radicals, increasing oxidative stress which damages neurons and can induce apoptosis (Patel et al, 1996). Glutamate is also known to destroy oligodendrocytes (Macdonald et al, 1998) and has been suggested to be the reason behind the chronic de-myelination of healthy neurons following SCI (Macdonald and Sadowsky, 2002). A strategy to help regenerate and repair damaged nerves using cell based treatments is indeed an important area of research for SCI patients.

1.2.2 Strategies for Neural Repair and Regeneration in Spinal Cord Injuries

In the UK and Ireland, approximately 1000 people per year suffer a SCI and it is estimated that there are approximately 50,000 people living with such injury (Spinal Research, UK). There is much research being carried out in the area that has added to our knowledge of the extent of the injury. However there is currently no cure and, at present, treatments focus on symptom management as research has yet to find a strategy that permanently improves neuronal function for SCI patients (Kwon et al, 2010). Therapies that are currently involved in clinical trials include; pharmaceutical intervention, functional electrical stimulation (FES) and cell based therapies. At present there has been no lasting functional benefit reported.

1.2.2.1 Pharmaceutical Intervention

Methylprednisolone is a corticosteroid that initially demonstrated encouraging neurological benefits (Bracken et al, 1990). However, recent developments have since demonstrated no difference between the recovery of neurological function in treated and untreated patients (Pointillart et al, 2000). Furthermore, the rate of infection is high following the administration of methylprednisolone and as such its use is now in decline (Pointillart et al, 2000; Hurlbert and Hamilton, 2008).

Anti-Nogo-A is an antibody against one of the inhibitory molecules produced as a result of myelin breakdown in the CNS. Its use has promoted axon sprouting and some functional recovery in rat and primate SCI models (Freund et al, 2009; Leibsher et al, 2005). This antibody is currently undergoing clinical trials with the hope that blocking this inhibition will allow axonal re-growth following injury in humans.

Ion channel inhibitors have been developed with the hope that these will attenuate the damage cause by excitotoxic chemicals in the secondary injury stages of SCI. Magnesium was investigated as a potential antagonist of NMDA receptors to reduce calcium influx into neurons as a result of the increase in glutamate levels. Initial studies of magnesium administered with polyethylene glycol (PEG) have demonstrated positive results in rat SCI models, where a neurological recovery was observed (Kwon et al, 2009). Phase I clinical trials have since been completed demonstrating the safety of the therapy and phase II clinical trials are currently underway (Kwon et al, 2010).

1.2.2.2 Functional Electrical Stimulation

FES is a process that has previously been used to suppress plasticity and pain but has more recently been used to stimulate the spinal cord to restore some locomotive function to SCI patients (Gerasimenko et al, 2008). Studies have demonstrated that SCI patients treated with FES are able to bare their own weight and reactivation of remaining neural networks can restore some function. However this is largely lost following removal of the stimulus (Harkema et al, 2011). Nevertheless, these are promising results and as such there are several FES clinical trials currently in progress.

1.2.2.3 Cell-Based Therapies

Cellular loss and death as a result of injury to the spinal cord remains a challenge for the regeneration and repair of a functional spinal cord. This is largely due to the secondary injury cascade that occurs following the initial trauma. As such, cell-based therapies could play a number of important roles in restoring function to SCI patients. Not only could they directly replace lost cells but could also modify the environment, encourage endogenous repair and promote the re-myelination of axons that remain around the injury site to restore their ability to function.

Cells, such as Bone Marrow Stromal Cells (BMSCs), Schwann cells (SCs), Olfactory ensheathing cells (OECs), ESCs, iPSCs and neural progenitor cells (NPCs) have received much attention for their potential use in SCI (Reviewed by Ruff et al, 2012). Each of these cells have demonstrated promise and naturally, each have their own advantages and disadvantages.

1.2.2.3.1 Schwann Cells and Olfactory Ensheathing Cells

SCs are present in the PNS and aid recovery of damaged axons following injury. SC grafts have been used in CNS damage to promote and guide axon re-growth and re-myelination following SCI (Weidner et al, 1998; Pearse et al, 2004). However, axonal re-growth was only demonstrated when SC transplantation was performed immediately following SCI in rodent models (Martin et al, 1996) which limits their clinical application. OECs are similar to SCs of the PNS and astroglia of the CNS. They act in a similar fashion to SCs in neural repair of the CNS, promoting myelinated axonal regeneration across lesion sites in SCI (Sasaki et al, 2004). The ability of OECs to promote neural repair is variable and this has been linked to the different origins of these cells and also attributed to the *in vitro* handling of these cells (Richter et al, 2005).

1.2.2.3.2 Stem Cells for Neural Transplantation Therapies

BMSCs have received the most attention for their potential to be used as an autologous or allogeneic tissue source for tissue engineering applications. As such several groups have demonstrated that human mesenchymal stem cells (hMSCs) isolated from BMSC can be used to promote functional recovery in SCI rodent models (Kim et al, 2006, Cizkova et al, 2006; Sasaki et al, 2009). However, some reports only demonstrate functional recovery with the addition of basic fibroblast growth factor (FGF-2: Kim et al, 2006) or brain derived neurotrophic factor (BDNF: Sasaki et al, 2009).

Additionally improved locomotive function in rodent SCI models varies with different hMSC donors (Neuhuber et al, 2005) indicating that there are some inconsistencies with the use of hMSCs and further research is required.

NPCs are naturally found in adult neural tissues and present an attractive source of cells for use in neural repair. They have demonstrated the ability to activate re-myelination in myelin deficient mice (Eftekharpour et al, 2007) and promote functional recovery in SCI rat models (Moreno-Mansano et al, 2009). Despite these promises, NPCs exist in very small numbers in adult tissues and harvest of these cells represents a risk to patients which limits their clinical application, therefore safer and more clinically relevant options are being explored. These options include the use of ESCs and iPSCs.

ESCs have self-renewal properties and are pluripotent in nature giving these cells the ability to differentiate into many different cells types including neural and glial cells (Reviewed by Erceg et al, 2009). These properties make ESC a great source of cells for use in SCI repair and have demonstrated the ability to promote re-myelination and functional repair *in vivo* (Kierstead et al, 2005). However these positives are not without downsides. ESCs represent a population of cells that have the ability to differentiate into many different cell types, failure to differentiate all cells down a specific lineage before transplantation has clinical risks, including the formation of tumours (Matsuda et al, 2009). In addition to this, there are also potential problems with host rejection upon transplantation of the cells and the ethical issues that surround the derivation of ESCs. More recently iPSCs have been developed (Takahashi et al, 2007). These cells are pluripotent and, therefore, have a

similar differentiation potential to ESCs. However, unlike ESCs, these cells have the potential for use in autologous tissue engineering applications, representing an appealing target for SCI repair.

Several of these therapies have reached human clinical trial stage, but as yet, none have demonstrated convincing efficacy data (Kwon et al, 2010; Ruff et al, 2012).

It is clear that no single therapy alone will result in the complete functional recovery of SCI patients. Rather, potential treatment is likely to consist of a combination of these therapies given together at appropriate times to control the environment, encourage native cell re-growth and repair, as well as incorporating new cells, potentially derived from stem cells, to replace those lost in the initial injury.

1.3 Stem Cells

Stem cells can be defined as a cell with the ability to self-renew as well as the potential to differentiate into many other cell types (Figure 1.1). Stem cells can either divide to produce two identical daughter cells with the same self-renewal capacity or asymmetrically to produce one daughter cell identical to itself with self-renewal capacity and one with more limited capabilities. Stem cells can be considered totipotent, pluripotent or multipotent. Totipotent cells have the ability to form all cells of a given organism. Pluripotent cells have the ability to form cells from the 3 germ layers; mesoderm, endoderm and ectoderm, whilst multipotent stem cells have a more limited differentiation potential often forming cells of only a

single lineage. Stem cells can be divided into three broad categories: ESCs, iPSCs and ASCs.

1.3.1 Embryonic stem cells

The early development of a fertilised oocyte gives rise to many cell types making up all tissues of the entire organism. Cells in the fertilised oocyte remain totipotent for five cell divisions. After this, primary differentiation occurs forming the outer cells of the blastocyst, the trophoblast. The inner cell mass of the blastocyst remains undifferentiated and these cells maintain the ability to form cells from all three germ layers (Thomson et al, 1998). Much of the initial ESC work was carried out on mouse ESCs (mESC), which were first isolated and expanded in culture in the early 1980s. They were derived from the early embryonic blastocyst and characterised by their ability to proliferate whilst remaining undifferentiated (Evans and Kaufman, 1981). In the 1990s human ESCs (hESC) were successfully isolated and cultured from *in vitro* fertilisation of zygotes donated for research purposes (Thomson et al, 1998). mESCs were historically characterised by the expression of surface markers such as SSEA-1 (Solter and Knowles, 1978). More recently these undifferentiated mESCs have been characterised by the expression of certain transcription factors known to be involved in pluripotency of cells, such as Oct4 (Scholer et al, 1990) and Nanog (Chambers et al, 2003) and also by telomerase activity maintaining the proliferative potential of ESCs (Armstrong et al, 2000). Whilst expression of Oct4 and Nanog are also used to characterise hESCs, SSEA-1 is absent. Instead the presence of alkaline phosphatase, SSEA-4, TRA-1-60 and TRA-1-81 are commonly used to

characterise hESC (Andrews et al, 1984; Wenk et al, 1994; Thomson et al, 1995; Thomson et al, 1998) in addition to teratoma formation upon transplantation into immuno-deficient mice (Thomson et al, 1998). These tumours demonstrate the presence of tissues from all three germ layers, confirming pluripotency (Thomson et al, 1998).

1.3.1.1 ESC Culture

ESCs have successfully been cultured *in vitro* for a number of years however, studies concerning the optimum culture conditions are ongoing. It is common when growing hESCs to culture these using a feeder layer consisting of inactivated mouse embryonic fibroblasts (iMEFs). Initially it was thought that the iMEFs were required to supply hESCs with leukaemia inhibitory factor (LIF), a key component that maintains mESCs on gelatin-coated plates in an undifferentiated state. Whilst mESCs differentiate following removal of LIF (Williams et al, 1988), it has been found that in the absence of a feeder layer, hESCs differentiate regardless of whether LIF is present (Thomson et al, 1998). This therefore suggests that the iMEF feeder layer is providing other factors that are important for maintaining hESCs in an undifferentiated state.

mESCs and iMEFS are frequently cultured using foetal bovine serum (FBS) which has certain implications, specifically with culture of hESC, if they were ever to be used for clinical application. Alternative methods have recently been investigated for hESC culture, with potential use in human medicine in mind, as the use of animal products would need to be avoided. These methods include: (i) use of human derived feeder layers (Amit et al, 2003)

and (ii) use of extracellular matrix proteins (Xu et al, 2001). Both methods have been found to support the growth of hESC and maintain their undifferentiated properties.

1.3.1.2 Embryoid Body Formation

The potential for ESCs to be used in regenerative medicine is vast and many differentiation routes have been followed with mESCs and hESCs. *In vitro* differentiation often begins with the formation of a three-dimensional embryoid body and these are said to imitate post-implantation embryos (Desbaillets et al, 2000).

Embryoid bodies form spontaneously in the absence of anti-differentiation factors and importantly consist of mesoderm, ectoderm and endoderm tissues, allowing the subsequent differentiation into any cells from these germ layers. This has been demonstrated using hESC by expression of ζ-globin for presence of mesoderm tissue, neurofilament 68Kd for presence of ectoderm, and α-fetoprotein for endoderm (Itskovitz-Eldor et al, 2000). Formation of these bodies can be carried out in suspension culture on bacterial grade dishes. This technique was first carried out with mESCs whereby a non-treated hydrophobic polystyrene bacterial grade dish was used to culture ESCs (Doetschman et al, 1985). The basis of the technique is that ESCs do not adhere to the bacterial grade plate and instead adhere to each other forming aggregates. The formation of these aggregates occurs spontaneously but a disadvantage of this is that there is great variability in size and shape resulting in a population of heterogeneous aggregates, affecting differentiation.

As a result, this technique has been improved to generate a more uniform aggregate by rotating/stirring the cell suspension, resulting in a more uniform aggregate which is preferential for differentiation (Zweigerdt et al, 2003; Cameron et al, 2006). Methylcellulose cultures and hanging drop cultures have also been developed; however, these methods are more commonly used with mESC as they require the formation of a single cell suspension (Wiles and Keller, 1991; Kurosawa, 2007).

1.3.1.3 Differentiation of ESCs

mESC and hESC remain an area of extensive research and to date both have demonstrated the ability to successfully produce cells of a number of different lineages. Table 1.1 demonstrates some examples of these lineages that have been followed. Differentiation of ESC from embryoid bodies to cells of specific lineages requires the addition of defined factors to culture conditions. During embryoid body formation, cells randomly form cells of various lineages, with no further changes to the composition of the medium, myocardial cells will make up approximately one third of the cell types (Doetschman et al, 1985; He et al, 2003). In contrast, addition of certain factors, including growth factors and cytokines, is needed to direct cells preferentially down other lineages. For example, addition of bone morphogenetic protein-4 (BMP-4) to culture medium during embryoid body formation causes preferential differentiation of cells into haematopoietic cells (Chadwick et al, 2003). A further regulation of haematopoietic cells has been demonstrated with the addition of vascular endothelial growth factor (VEGF-A_{165}) which aids differentiation of erythrocytes (Cerdan et al, 2004)..

Table 1.1 ***Differentiation fates of human and murine embryonic stem cells.*** Details some of the common differentiation pathways that have been followed from all three germ layers: endoderm, mesoderm and ectoderm.

Germ Layer	Cell lineage	Differentiation of mESCs	Differentiation of hESCs
Endoderm	Hepatic	Hepatocytes (Lavon et al, 2004; Chinzei et al, 2002)	Hepatocytes (Rambhatla et al, 2003; Lavon et al, 2004)
	Pancreatic	Insulin secreting cells (Lumelsky et al, 2001; Blyszczuk et al, 2003)	Insulin-expressing endocrine cells (D'Amour et al, 2006; Kroon et al, 2008)
Mesoderm	Haematopoitic	Erythroid cells, macrophages and mast cells (Wiles and Keller, 1991)	$CD45^{+,}$ $CD34^{+,}$ VEGF and RUNX1 cells(Chadwick et al, 2003;Ng et al, 2005)
	Cardic	Myocardium (Doetchman et al, 1985) Cardiomyocytes identified using whole patch clamp (Matsev et al, 1994)	Directed differentiation into cardiomyocytes (Mummery et al, 2003; He et al, 2003; Passier et al, 2005)
	Bone	3D mineralised alginate hydrogels (Hwang et al, 2009). Osteogenic lineage differentiation (Woie et al, 2007)	Osteoblast differentiation and formation of mineralising nodules (Sottile et al, 2003; Ahn et al, 2006)
Ectoderm	Neural	Formation of neural precursors (Bibel et al, 2004). Motor neuron differentiation (Wu et al, 2012)	Formation of neural rossettes and differentiated neurons (Reubinoff et al, 2000; Zhang et al, 2001)
	Epidermal	Terminally differentiated epideral cells that produce a cornified layer (Troy and Turksen,2005)	Keratinocyte differentiation (Green et al, 2003)

1.3.1.3.1 Neural Differentiation of ESCs

Neural differentiation of hESCs is an area that has received much attention over the last decade due to the potential wide-ranging therapeutic applications of these differentiated cells in a number of clinical applications.

As with all differentiation of hESCs, neural lineage differentiation requires the addition of factors at specified time points determined by the specific cell type required. Several approaches have been utilised to differentiate hESCs towards neural cell types present in the CNS including; astrocytes and motor neurons. A key element to note is the level of variation between different protocols. Each protocol differs in the different factors used and the duration of exposure to these factors as well as the different cell starting points; confluent ESC colonies, single hESCs, EBs and neurally induced spheres (Table 1.2 and Table 1.3). However, there are several key factors that are required in neural differentiation protocols. The most commonly adopted method historically was to treat the cells with retinoic acid (RA). Although this method does result in a large proportion of the cells differentiating towards neural lineages, the population of cells remains very heterogeneous and requires further separation (Carpenter et al, 2001). More recently, methods utilising stromal feeder layers to form neural rosettes (Lee et al, 2007) and subsequent treatment of cells with RA and sonic hedgehog (SHH) demonstrated formation of neurons with morphological and physiological characteristics consistent with spinal motor neurons (Lee et al, 2007; Takazawa et al, 2012).

Table 1.2: .*Neural differentiation of hESC*. Different starting cell conditions and methods utilised for neural differentiation of HESC.

Reference	Starting Cells	Base medium	Supplements	Growth Factors	Coating	Duration	Outcome
(Reubinoff et al, 2000)	High density hESC	DMEM	0.1mM β-Mercaptoethanol, 1%NEAA, 50 units/mL penicillin, 50µg/mL streptomycin	none	Non-tissue culture coated plates	24hours	Formation of neurospheres, confirmed by the presence of N-CAM,, nestin and vimentin
	Nuerospheres	DMEM	As above with 1µM Retinoic acid	none	Tissue culture plastic	7-15 days	Cell outgrowth with long processes from the plated sphere positive for 200KDa neurofilament, 168KDa neurofilament, MAP-2, synaptophysin and βIII-tubulin. Also positive for $GABA_a$ and α2 neurotransmitter subunits.
(Zhang et al, 2001)	EBs	DMEM/F-12	N2	2µg/ML Heparin and 20ng/mL bFGF	Tissue culture plastic	8-10 days	Formation of neural rosettes (removed for subsequent culture by dispase)
	Neural Rossettes	DMEM-f12	N2	20ng.mL bFGF	Poly-(2hydroxyethyl-methacrylate)	cultured for a number of weeks	Neurospheres positive for nestin, musashi-1 and PSA-NCAM
	Neurospheres	DMEM/F-12	N2, cAMP (100ng.mL)	10ng/mL brain derived neurotrophic factor	Ornithine/laminin	14 days	Processes produces from the spheres, positive for MAP-2, βIII tubulin and majority of cells adopted a glutamateregic phenotype. Astrocytes present following continued culture beyond 2 weeks and in presence of platelet-derived growth factor a, oligodendrocytes were formed.
(Barhavard et al, 2007)	Undifferentiated hESCs	DMEM/F-12	20% knockout serum replacement, 2mM L-glutamine1%NEAA, 100 units/mL penicillin, 100µg/mL streptomycin, RA 4x 10^6 M	40ng.mL bFGF and 100ng/mL noggin	Matrigel	4-5 days	Neuroectodermal cells and formation of neural rossettes confirmed by presence of nestin staining. These were expanded for a further 6 days without tRA, then a further 6 days with 25ng/mL bFGF to form neural tubes
	Neural tubes	Neuro-basal	1% N2, 2% B27	none	5µg/mL Laminin/15µg/mL poly-l-ornithine	30 days	Cells extending from neural tubes were positive for βIII tubulin, MAP-2, synaptophysing and neurofilamentt protein.

Table 1.3: Different starting cell conditions and methods utilised for neural differentiation of HESC *continued.*

Reference	Starting cells	Base medium	Supplements	Growth Factors	Coating	Duration	Outcome
(Reubinoff et al, 2001)	High density hESC.	DMEM/F12	B27 (1:50), 2mM L-glutamine, , 50 units/mL penicillin, 50µg/mL streptomycin	20ng/mL EGF, 20ng/mL bFGF	Standard tissue culture plastic	7-21 days	Formation of neurospheres positive for N-CAM, nestin and Pax-6
	Neurospheres	DMEM/F-12	As above, with addition of RA after 6 days in culture	None	10µg/mLPoly-D-Lysine/ 4µg/mL Laminin	14-21 days	Resulting neural differentiated cells were positive for βIII tubulin, 70KDa neurofilament, 160KDa neurofilament, MAP-2, synaptophysin, glutamic acid decarboxylase, GABA, glutamate, serotonin, tyrosine hydroxylase, GFAP and oligodendrocyte marker ,O4
(Chambers et al, 2008)	Single hESCs	DMEM/F-12	20% knockout serum replacement, 0.1mM β-Mercaptoethanol, 10nM TGF-β inhibitor (removed after day 5) and increasing amounts of N2 added every 2 days (25%, 50%, 75%)	500ng/mL noggin	Matrigel	11 days	Greater the 80% positivity of Pax-6 in neurally inducted cells as well as increased expression of sox-1 confirming induction towards neuroectoderm lineage.
	Neurally inducted cells	DMEM/F-12	N2, (ascorbic acid, cAMP added at day 11)	sonic hedgehog added at day 5 and FGF-8 added at day 9;(both SHH & FGF-8 withdrawn at day) BDNF, GDNF and TGF-β3 added from day 11	Matrigel	19 days	Dopaminergic neurons present expressing βIII tubulin co-expressed with tyrosine hydroxylase. (presence of motor neuron markers; ISL1 and HB9 when threated with BDNF, SHH, ascorbic acid and retinoic acid for 19 days)

Brain derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and insulin-like growth factor-1 (IGF-1) have also demonstrated improved differentiation towards motor neurons *in vitro* (Zurn et al, 1996; Zhang et al, 2001). Despite the successful formation of differentiated neural cells from hESCs, these still exist within heterogeneous cell populations of undifferentiated cells, posing a risk for clinical application and further research is required to purify these cell populations for safe therapeutic use. Increased knowledge in this area has resulted in the development of different protocols that do not require the need for embryoid body formation or the co-culture with stromal cells. Instead, direct differentiation of hESCs in high density monolayer cultures have been developed, taking advantage of two inhibitors of SMAD signalling; Noggin and small molecule inhibitor SB431542 (Chambers et al, 2009). Treatment of high density hESC culture with these two inhibitors have resulted in complete conversion of cells to a neural phenotype which adds promise to this area for therapeutic potential for these cells.

1.3.2 Adult Stem Cells (ASCs)

ASCs have promised new hope for tissue repair and regeneration in recent years. Unlike hESCs, ASCs have fewer ethical issues surrounding their use due to the fact that they are isolated from adult tissues. Their ability to differentiate into a number of different tissues is beginning to demonstrate their usefulness for clinical application.

It was initially thought that these cells only existed in tissues that retain proliferative potential and maturation into specialised cells throughout adult life, such as bone marrow and skin (de Hann, 2002; Watt, 2001). However, in recent years it has become apparent that stem cells reside in many other adult tissues and have been isolated from a number of different sources to date. These stem cells are often referred to as progenitor cells or precursor cells, given their ability to differentiate into several different cell types that comprise their tissue of origin. ASCs have been found to exist in tissues that lack a continuous renewal mechanism, for example in liver, central nervous system tissue and skeletal muscle (Theise et al, 1999; Reynalds and Weiss, 1992; Beauchamp et al, 1999). It has been suggested that similar stem/progenitor cells potentially exist in many other adult tissues and will only proliferate and differentiate in response to signals produced from the organ they reside within (Jiang et al, 2002).

1.3.2.1 ASC Culture

ASCs are initially isolated from tissue samples either obtained by aspiration techniques (in the case of blood and bone marrow), biopsies, discarded tissue or from donated tissue postmortem. Unlike ESC culture, the expansion of some ASCs once isolated, has been found to be quite challenging. Haematopoietic stem cells, isolated from the bone marrow, are from a tissue where regeneration of new cells occurs quite readily. However, they have been difficult to culture *in vitro*, with little success until recent years following discovery of Wnt proteins acting as growth factors to successfully expand haematopoietic stem cells (Willert et al, 2003). A second population

of stem cells originating in the bone marrow have also been isolated, that unlike haematopoietic stem cells, proliferate readily in culture and these are termed bone marrow mesenchymal stem cells or bone marrow stromal cells (BMSCs) (Caplan, 1991). These cells are characterised by their ability to form colonies that can be isolated (Friedenstein et al, 1974) and undergo approximately 20 population doublings (PDs) before the onset of cellular senescence. Mixed clonal populations on the other hand can undergo more PDs but are limited to 30-40 PDs before growth arrest (Bruder et al, 1997). Although BMSCs are a potential preferential source of cell to utilise in clinical applications, there are some disadvantages to their use including the low cell numbers obtained on isolation and the invasiveness of obtaining human samples (Rickard et al, 1996; Bruder et al, 1997; Pittenger et al, 1999). Due to these shortcomings, MSC-like populations are continually being isolated and investigated from alternative tissue sources, with their potential usefulness in clinical application at the forefront of these investigations. The various conditions that cells require for expansion and differentiation *in vitro* is of great importance if these cells are to be used for clinical application.

1.3.2.2 Differentiation of ASCs

Much of the early work with ASCs was carried out using BMSCs. These cells demonstrate differentiation down adipogenic, chondrogenic and osteogenic lineages from clonally isolated cell populations (Pittenger et al, 1999). Non-bone marrow derived MSCs have received more attention over the last decade following reports that these cells were able to differentiate into epithelial cells, haematopoietic cells, and have also demonstrated the ability

to form cells with mesoderm, endoderm and neuroectoderm characteristics (Jiang et al, 2002). This suggests a potential for MSCs to be used in tissue regeneration and repair throughout the body. Progenitor cells have been isolated from several other human tissues and demonstrated differentiation down multiple lineages. Some of these are summarised in Table 1.4.

1.3.2.3 Stem cells in Dental Tissues

Dental tissues have demonstrated great promise as a potential source of MSC-like cells in recent years. These progenitor cells have been isolated from several different dental tissues to date including; dental pulp (Gronthos et al, 2000), exfoliated deciduous teeth, (Miura et al, 2003), apical papilla (Sonoyana et al, 2008), dental follicle (Morsczeck et al, 2005), periodontal ligament (Seo et al, 2004), oral mucosa (Davies et al, 2010; Marynka-Kalmani et al, 2010) and gingiva (Zhang et al, 2009).

These isolated cell populations share some distinct similarities in their MSC-like properties as well as characteristics that separate them. The relationship between the different stem cell populations that reside within the dental tissue is still unclear, however, investigations are ongoing.

Dental pulp progenitor cells (DPPCs) were the first progenitor cells to be isolated from dental tissues in 2000 (Gronthos et al, 2000). Like BMSCs, these cells can be clonally isolated but more interestingly, demonstrate a higher proliferative capacity compared to BMSCs (Gronthos et al, 2000).

Table 1.4: ***Adult stem cell differentiation*****.** Examples of stem cells isolated from different adult tissues and their differentiation capacity.

Tissue	Progenitor Cells	Differentiated Cell Types	References
CNS	Neural Stem Cells	Neurons, Astrocytes (limited oligodendrocytes)	Uchida et al, 2000; Palmer et al, 2001
Blood	Circulating Skeletal Stem Cells	Osteocytes, Adipocytes	Kuznetsov et al, 2001
	Umbilical cord blood stem cells	Osteocytes, Adipocytes and chondrocytes	Lee et al, 2003; Kern et al, 2006; Schuh et al, 2009
Adipose	Processed lipoaspirate cells (PLAs); Adipose Derived Multilineage Cells	Adipocytes, Chondrocytes, Myogenic Cells and Osteocytes	Zuk et al, 2001; De Ugarte et al, 2003; Kern et al, 2006
Pancreas	Nestin-Positive Islet-Derived Multipotential Stem Cells (NIP)	Islets of Langerhans, Hepatic cells, Pancreatic, Acinar Cells,	Ramiya et al, 2000; Zulewski et al, 2001
Craniofacial	Dental Pulp Progenitor Cells,	Dentine-Like Cells, Osteoblasts	Gronthos et al, 2000; Gronthos et al, 2002
	Oral Mucosa Lamina Propria Progenitor Cells	Osteogenic, Adipogenic, Chondrogenic & Neuroectoderm	Davies et al, 2010; Marynka-Kalmani et al, 2010

DPPCs also demonstrate multilineage differentiation potential, expressing typical markers and morphology of adipogenic and neurogenic cells (Gronthos et al, 2002) as well as the ability to differentiate down osteogenic, chondrogenic and myogenic lineages (Zhang et al, 2006). Shortly after the isolation of DPPCs, stem cells from human exfoliated deciduous teeth (SHEDs) were isolated. SHEDs represent a population of cells from a tissue source that would otherwise be discarded if not used and therefore represent a cell population with great potential. In addition to this, these cells demonstrate greater proliferative capacity compared to DPPC (Miura et al, 2003) and express numerous development markers including; Oct4, Sox2, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Kerkis et al, 2006).

The apical papilla, which develops to form the pulp structure of permanent teeth, has also demonstrated a resident stem cell population termed stem cells from the apical papilla (SCAP). These cells, like DPPCs, demonstrate multilineage differentiation down osteogenic, adipogenic and neural lineages (Abe et al, 2007; Sonoyama et al, 2008) and are thought to be of neural crest origin (Sonoyama et al, 2008). Similarly, stem cells have been isolated from the dental follicle of developing permanent teeth (Morsczeck et al, 2005). Although these cells demonstrate colony formation and osteogenic differentiation, the initial cell numbers obtained from a single isolation are low (Morsczeck et al, 2005) and therefore potentially have little use for clinical applications. Periodontal ligament stem cells (PDLSCs) have been isolated from extracted molars and give rise to a population of stem cells that are positive for typical MSC markers, including STRO-1 and, consistent with

characteristics of other MSC populations, demonstrate differentiation down osteogenic, adipogenic and chondrogenic lineages (Seo et al, 2004).

More recently investigations have been carried out on the tissue surrounding the teeth, including gingiva and the oral mucosa. These are easily accessible tissue sources applicable to all patients, regardless of age as it does not require presence of deciduous teeth, tooth extraction, pulp extraction, or the presence of impacted teeth.

The gingiva is a keratinized structure that, like the oral mucosa, bears a resemblance to the structure of the skin (Stephens and Genever, 2007). Gingiva-derived mesenchymal stem cells (GMSCs) have been isolated from the lamina propria of the gingiva (Zhang et al, 2009). These cells maintain a higher proliferative rate than BMSCs, express stem cell markers; Oct4, SSEA-4 and STRO-1, and possess multipotent properties, demonstrating positive expression of typical markers of adipocytes, osteoblasts, endothelial cells and neural cells (Zhang et al, 2009). In addition to this, these cells demonstrate immunosuppressive properties making them an attractive cell source for tissue engineering applications (Zhang et al, 2009; Zhang et al, 2012).

This thesis is based on a population of progenitor cells isolated from the lamina propria of the oral mucosa, which like gingiva, is a tissue that is easily isolated from patients undergoing routine dental treatment.

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1.3.2.3.1 Oral Mucosa

The oral mucosa is the term used to describe the soft tissue lining of the oral cavity. It has many different functions and consists of a distinct layered structure that is very similar to the structure and function of skin (Figure 1.3). The several discrete layers help to protect from bacterial infection and work to regulate temperature and water balance, much like the skin (Sloan et al, 1991). Three distinct layers make up the oral mucosa: the oral epithelium, lamina propria and sub mucosa.

The epithelium makes up the surface of the oral mucosa and consists largely of multiple layers of keratinocytes ranging in their degree of maturation; immature cells at the base and mature cells at the surface. The epithelial layer is supported by a connective tissue layer, the lamina propria, which gives integrity to the oral mucosa. This consists of an interwoven mesh of collagen (Type I and III), glycosaminoglycans, proteoglycans, glycoproteins, capillaries and elastic fibres which have been produced by the residing fibroblasts. Various other cells types exist in this layer including; endothelial cells, pericytes, smooth muscle cells, Schwann cells, lymphocytes and macrophages.

The sub-mucosa is the deepest layer of the oral mucosa and consists mainly of adipocytes. The sub mucosa acts to provide a network of blood vessels to the lamina propria in order to supply nutrients to the tissue and remove waste.

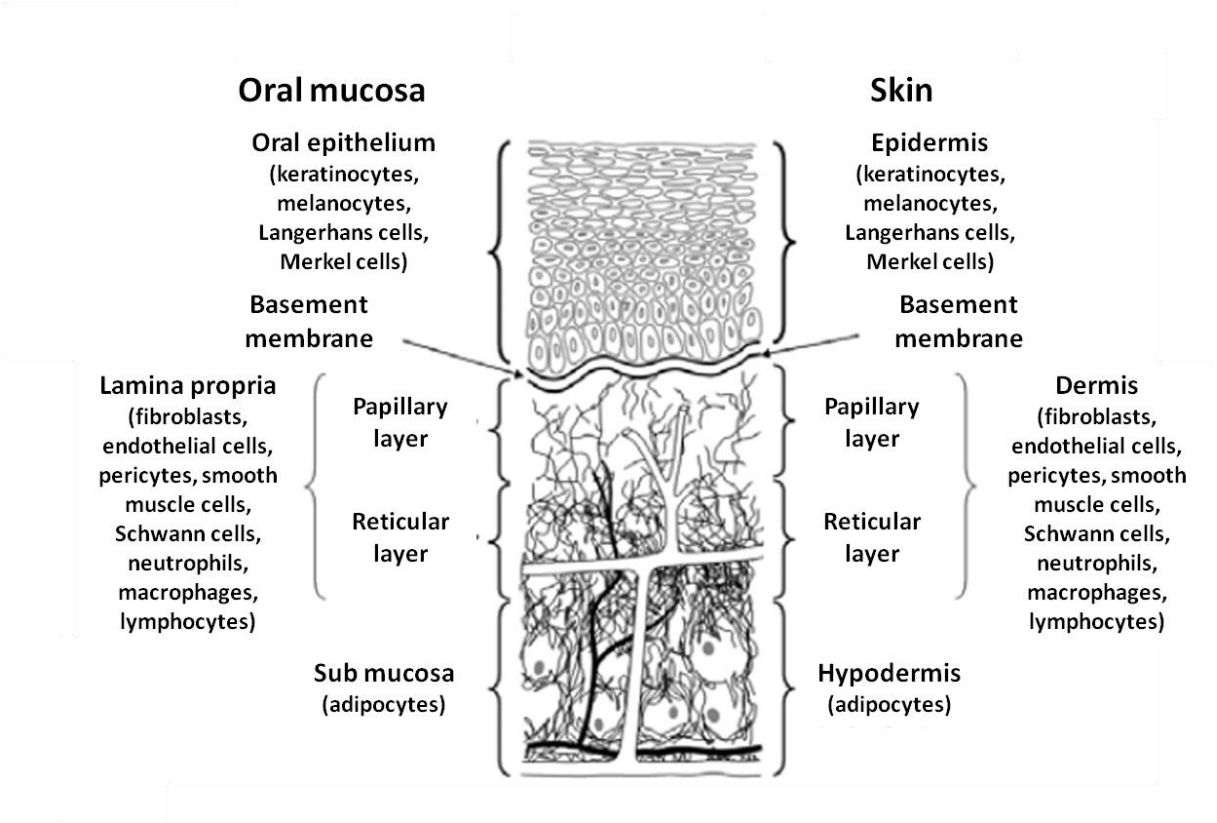

Figure 1.3: ***Similarities between the oral mucosa and the skin.*** The skin is a discrete layered structure consisting of the epidermis, dermis and the hypodermis which is similar in structure and function to the oral epithelium, lamina propria and the sub mucosa of the oral mucosa. (Image from Stephens and Genever, 2007).

The composition of the sub mucosa determines the flexibility of the tissue that lies above it and is only present in non-keratinised tissues such as the buccal mucosa and found to be absent in keratinised tissues, such as gingiva and hard palate, where the oral mucosa is attached directly to the periosteum of bone beneath (Squier and Kremer, 2001; Stephens and Genever 2007).

1.3.2.3.2 Wound Healing Properties of the Oral Mucosa

Wound healing responses consist of tightly regulated and overlapping phases of haemostasis, inflammation, proliferation and remodelling, a process that frequently leads to scarring of skin. Visualised healing of the oral mucosa differs from that of the skin due to the lack of scar formation (Stephens and Genever, 2007) and there are various reasons underlying this that can be used to support this difference. It has been demonstrated that oral mucosal wounds have a diminished inflammatory phase with significantly fewer T-cells, macrophages and neutrophils in comparison to dermal wound (Szpaderska et al, 2003). Another such potential reason is that oral mucosal fibroblasts have an increased ability over dermal fibroblasts to re-organise their ECM (Stephens et al, 1996). Recently a potential progenitor cell population residing within the lamina propria was suggested to be in part responsible for this wound healing response (Stephens and Genever, 2007). This population of progenitor cells has since been isolated (Davies et al, 2010; Marynka-Kalmani et al, 2010).

1.3.2.3.3 Oral Mucosa Lamina Propria Progenitor Cells (OMLP-PCs)

This progenitor cell population from the lamina propria has been isolated by two separate research groups and, as such, can be found in the literature under two different names; OMLP-PCs (Davies et al, 2010) or human oral mucosa stem cells (hOMSC; Marynka-Kalmani et al, 2010). Progenitor cells have been isolated from a heterogeneous cell population of the lamina propria by differential adhesion to fibronectin (Davies et al, 2010). This separation is based increased active functional $\alpha_5\beta_1$ integrins on the surface of progenitor cells compared to differentiated cells (Dowthwaite et al, 2003) and therefore progenitor cells adhere to a fibronectin coated surface in a shorter timeframe than more differentiated cell types. The cells of the OMLP demonstrate expression of numerous developmental markers including markers of the notch signalling pathway; Notch 1, 2 & 3, Delta 1 and Jagged 2 as well as neural crest markers; sox-10, snail, slug and twist. This confirms the neural crest origin of these isolated cells (Davies et al, 2010).

Similar to stem cells isolated from other dental tissues, these cells demonstrate a higher proliferative capacity compared to BMSCs and, in the case of GMSCs and OMLP-PCs, this is potentially due to the expression of hTERT (Zhang et al, 2009; Davies et al, 2010; Zhang et al, 2012). Like other MSC populations, OMLP-PCs also demonstrate multipotent properties, differentiating down adipogenic, osteogenic, chondrogenic and early neural lineages (Davies et al, 2010; Marynka-Kalmani et al, 2010). More recently, OMLP-PCs have demonstrated potent immunosuppressive properties, similar to GMSCs (Davies et al, 2012), making these cells interesting targets

for use in allogeneic tissue engineering applications as well as immune related disorders.

Additionally the tissue of the lamina propria has demonstrated expression of factors recently used for reprogramming of cells to iPSCs including Sox2, Oct4 and Klf4 (Davies et al, 2010; Marynka-Kalmani et al, 2010), making these cells an attractive and interesting target for reprogramming purposes.

1.3.2.3.4 Neural Differentiation of Stem/Progenitor Cells from Dental Tissues

Given the early association of the dental tissues with the neural crest, it is not surprising that many of the stem/progenitor cell populations isolated from dental tissues demonstrate neural crest marker expression in addition to some early stage neural markers including Notch-1 and Nestin (Reviewed by Huang et al, 2009). This is an encouraging early indication that these cells have the potential to differentiate towards neural lineages and, as such, have potential for use in neural repair. Early differentiation potential and expression of typical neural markers has been demonstrated for DPPCs (Arthur et al, 2008), SHEDs (Miura et al, 2003), SCAP (Sonoyama et al, 2008) Dental follicle stem cells (Morsczeck et al, 2009), PDLSCs (Kawanabe et al, 2010), GMSCs (Zhang et al, 2009) and OMLP-PC (Davies et al, 2010; Marynka-Kalmani et al, 2010).

Neural differentiation protocols differ for each dental tissue stem/progenitor cells. It is common to form a neurosphere-like structure prior to neural differentiation, similar to culturing of NPCs, which are grown as 3D bodies to help maintain their multipotency and their ability to differentiate into neuronal

cell types when plated in monolayer culture (Arsenijevic et al, 2001; Wachs et al, 2003). Neurosphere-like structures of dental tissue stem/progenitor is commonly achieved by culturing cells with FGF-2 and/or EGF (Widera et al, 2007; Morsczeck et al 2010, Davies et al, 2010). This is often followed by the addition of neural growth factors; BDNF, neurotrophin-3 (NT-3) and Nerve Growth Factor (NGF) once cells are disaggregated and plated in monolayer culture, to encourage terminal differentiation.(Marynka-Kalmani et al, 2010; Davies et al, 2010). Several groups have adopted an alternative method using direct differentiation of the cells in monolayer culture (Miura et al, 2003; Arthur et al, 2008; Sonoyama et al, 2008).

At present, characterisation of differentiated neural cells has demonstrated the presence of typical early and some more terminally differentiated neural markers (Huang et al, 2009). However, the ability of these cells to generate action potentials, and therefore function as a neuron, is yet to be reported.

1.3.3 Induced Pluripotent Stem cells (iPSC)

The reduced ethical issues that surround the use of ASCs over ESCs has led to further research into their use. The major difference between ESCs and ASCs lies with the capacity to form cells of different lineages. As already discussed, ESCs are considered to be pluripotent, with the ability to make cells types from all three germ layers, whereas ASCs are often only mulitpotent (Table 1.4) and therefore form a more limited number of cell types. This has led to research into the genetic reprogramming of adult somatic cells back to an ESC-like state using factors already known to

maintain cells in an undifferentiated state. Thus, the production of iPSCs creates a source of cells that are pluripotent in nature (Figure 1.4), but have fewer associated ethical issues with regard to their tissue of origin and have great potential for use in cell-base therapies as well as high throughput drug screening.

1.3.3.1 Important Factors in Pluripotency

Although the mechanisms that maintain cells in an undifferentiated state are still not yet fully understood, parts of the pathway involved with the process of self-renewal of mESC and hESC have been investigated. There are several genes known to be important in the maintenance of stem cell state, allowing self-renewal without terminally differentiating.

Oct4, Sox2 and Nanog are three transcription factors that have been found to be important in the regulation and maintenance of pluripotency in both mESCs (Nichols et al, 1998; Niwa et al, 2000; Avilion et al, 2003; Mitsui et al, 2003; Chambers et al, 2003) and hESC (Hyslop et al, 2005; Fong et al, 2008; Adachi et al, 2010; Wang et al, 2012). These three factors have played an important role in the reprogramming of both mouse and human somatic cells (Takahashi et al, 2007; Takahashi and Yamanaka, 2006) despite differences in the way the factors maintain pluripotency in the two species. Pluripotency in mESC is maintained by co-operative Oct4, Sox2 and Nanog regulation (Rodda et al, 2005), whereas in hESC, these transcription factors act independently to regulate different lineage specifications (Wang et al, 2012).

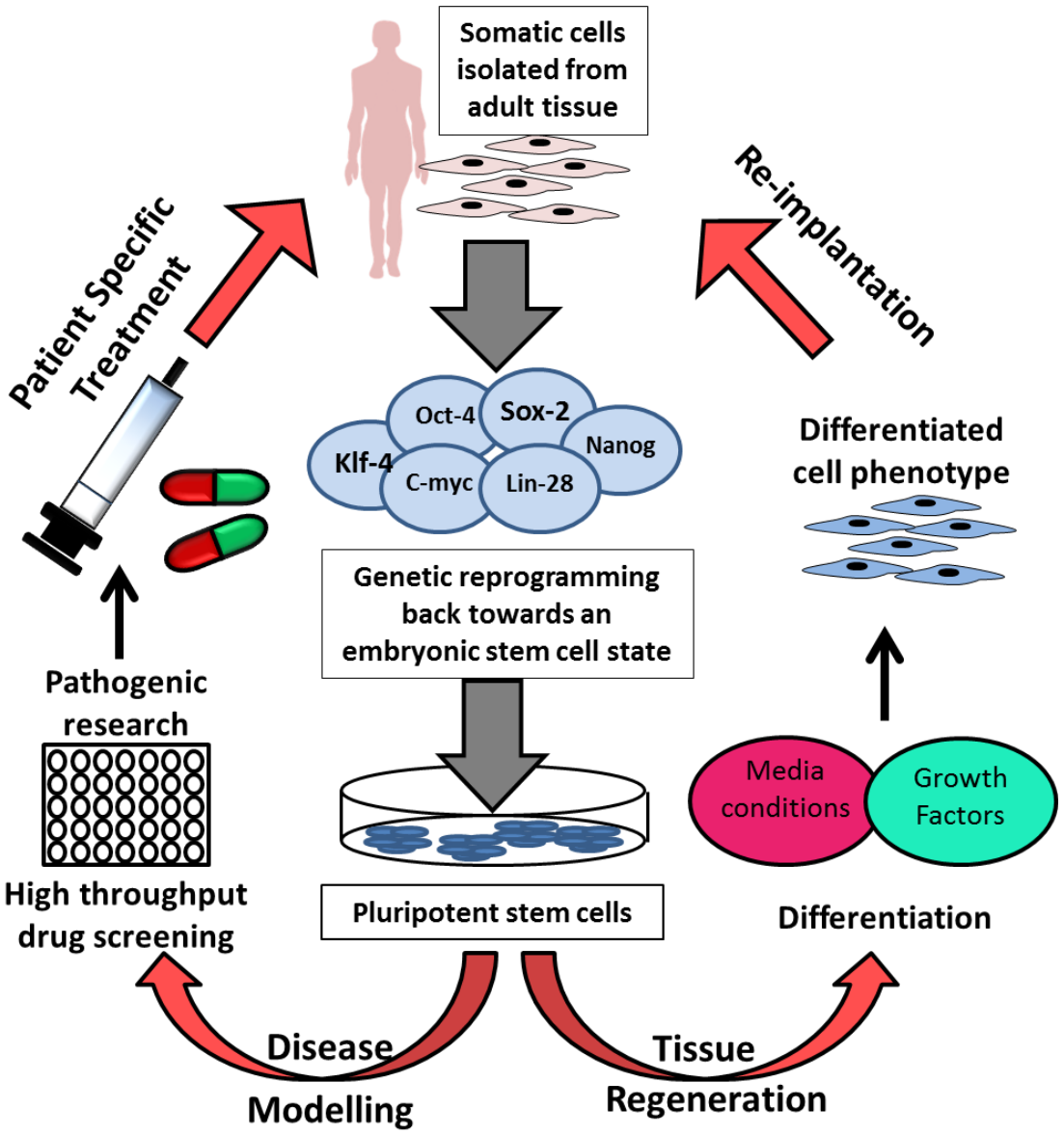

Figure 1.4*: Reprogramming of adult somatic cell to iPSC and potential therapeutic applications*. A number of methods have been utilised to reprogram cells back to an embryonic stem cell state.by introducing different combinations of factors; Oct4, Sox2, Nanog, Klf4, Lin28 and c-Myc, resulting in pluripotency. The resulting iPSCs are characterised as pluripotent cells by methods originally utilised by Thomson et al to characterise hESC (Thomson et al, 1998). These cells could potentially be utilised for development of patient specific drug therapies or could be used as cell based treatments following directed differentiation and re-implantation.

Following the successful genetic reprogramming of mouse dermal fibroblasts in 2006 and human dermal fibroblasts in 2007 (Takahashi and Yamanaka, 2006; Takahashi et al, 2007), much research has been carried out around the molecular basis of pluripotency and the factors involved. Myc has recently demonstrated a role in suppressing differentiation (Varlakhanova et al, 2011). Reports suggest that Klf4 acts directly to control Nanog expression (Chan et al, 2009, Zhang et al, 2010) and has more recently been linked with hTERT expression, implicating the importance of Klf4 as a factor in the maintenance of pluripotency (Wong et al, 2010). In addition to this, expression of Klf4 has been found to act directly with Oct4 and Sox2 to direct reprogramming of cells back to their ESC-like state (Wei et al, 2009). Similarly, Lin28 is implicated in the expression of Oct4 (Qui et al, 2009) highlighting its importance in the maintenance of pluripotency of hESCs. Research in this area is ongoing as there is still much to be discovered however, further research into the reprogramming of cells back to their ESC-like state is likely to lead to a greater insight into the molecular basis of pluripotency.

Signalling pathways play an important role in regulating these transcription factors to maintain the self-renewal properties of embryonic stem cells. Similar to the transcription factors, differences exist between the signalling pathways that maintain pluripotency of mESC and hESC, these pathways are summarised in Figure 1.5.

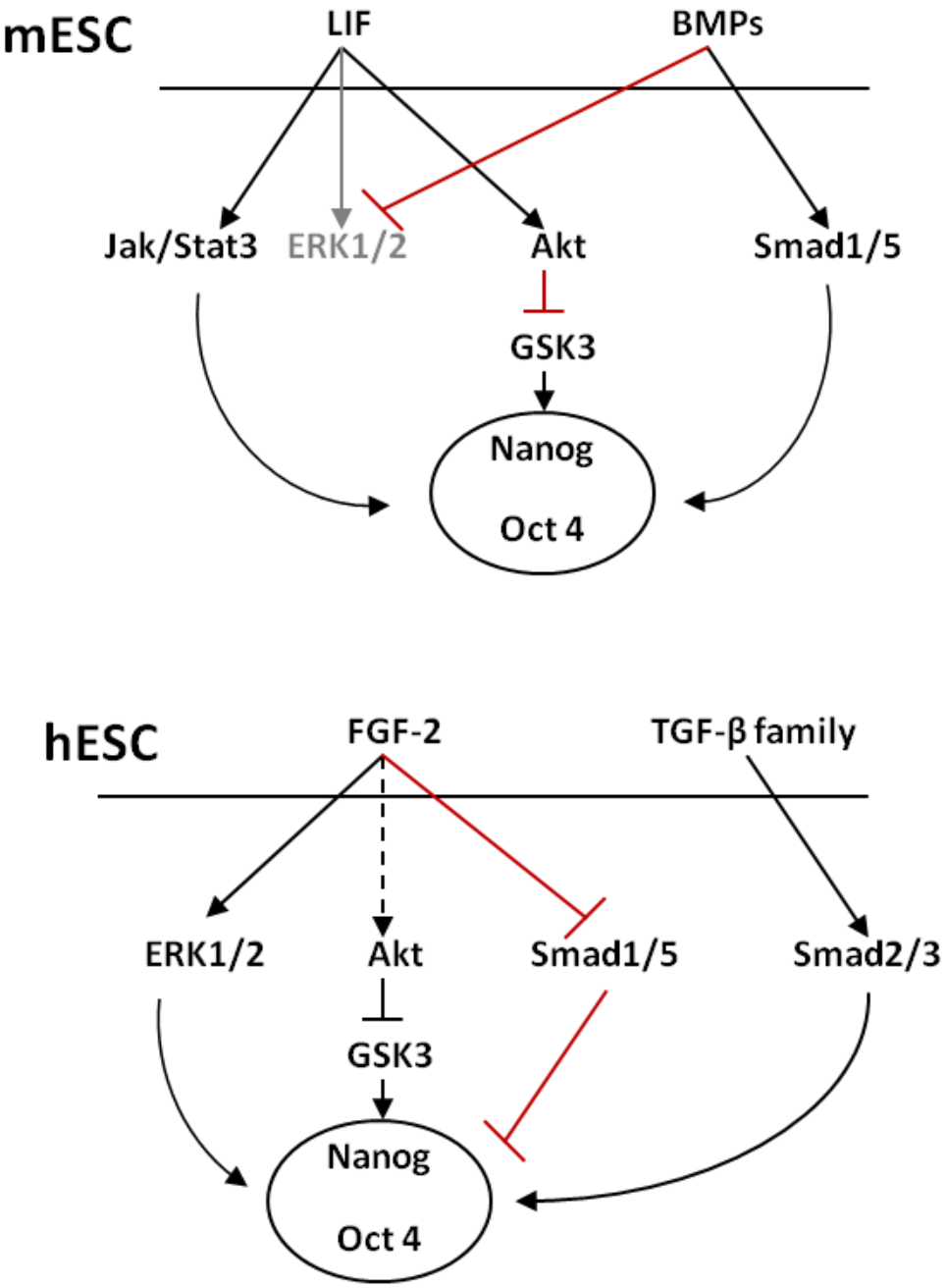

Figure 1.5: ***Signalling pathways involved in the maintenance of pluripotency.*** Summary of the different key signalling pathways; LIF, BMP's, FGF-2 and TGF-β involved in maintenance of pluripotency and self-renewal properties in mESC and hESC (adapted from Omelyanchuk et al, 2009).

LIF has been found to be an important factor in the maintenance of mESCs in an undifferentiated state. It activates the Janus kinase (JAK)/STAT3) signalling pathway to suppress differentiation (Burdon et al, 1999) and the PI3K/Akt signalling pathway to protect degradation of c-Myc by inhibition of GSK3 (Bechard and Dalton, 2009). LIF also activates the MEK/ERK1/2 signalling pathway, which results in differentiation of mESC. However, BMP acts against LIF to actively inhibit this pathway and stop differentiation, maintaining pluripotency (Lee et al, 2006; Ying et al, 2008). Self-renewal of hESCs is maintained independently of STAT3 (Humphrey et al, 2004; Sato et al, 2004) despite containing the elements of the JAK/STAT3 pathway (Humphrey et al, 2004). Instead, fibroblast growth factor- 2 (FGF-2) activates MEK/ERK1/2 pathway, which in contrast to mESC, maintains pluripotency of hESC (Dvorak et al, 2005; Li et al, 2007). FGF-2 also activates the PI3K/Akt pathway, which similarly to mESC, acts to maintain pluripotency (Li et al, 2007). Proteins of the TGFβ family are known to be involved in activation of SMAD 2/3 signalling, upregulating Nanog and Oct4 to maintain pluripotency in hESC (James et al, 2005; Vallier et al, 2005), again opposite from its role in mESCs. FGF-2 is known to inhibit SMAD 1/5/8 in the presence of TGFβ, suppressing differentiation of hESC (James et al, 2005).

Research in this field is very much ongoing and reprogramming produces not only a great source of cells for use in regenerative medicine, but also adds to our increasing knowledge of the molecular basis of pluripotency. This is an important factor when considering the use of these cells in transplant applications.

1.3.3.2 Reprogramming

iPSCs were first reported in 2006, when mouse fibroblasts were found to be reprogrammed back to an ESC-like state by the introduction of four factors; Oct4, Sox2, Klf4 and c-Myc in viral vectors (Takahashi and Yamanaka, 2006). It was later found that the same four factors could induce an ESC-like state in human skin fibroblasts (Takahashi et al, 2007). These key findings formed the basis of much research into the reprogramming of different cell populations back to their embryonic state. It was later discovered that retroviral induction of c-Myc may be of little use for any potential clinical application as chimeras formed using the genetically reprogrammed cells developed a large number of carcinomas (Okita et al, 2007; Yamanaka, 2007). This has led to the use of different factors and methods to enable reprogramming of cells that would be safer for potential therapeutic applications. These factors include the original factors used by Takahashi and Yamanaka, Oct4 and Sox2, with the addition of Nanog and Lin28 which is also known to be involved in the maintenance of pluripotency (Yu et al, 2007). These factors are sufficient for the reprogramming of human skin fibroblasts, with the resulting cells demonstrating ESC-like morphology, expression of classical pluripotency genes and cell surface markers such as SSEA-4. However, without the use of c-Myc, low reprogramming efficiencies were observed (Yu et al, 2007; Okita et al, 2007; Nakagawa et al, 2008). The development of innovative techniques is continually opening up new avenues for potential clinical application of these cells. Despite the development of such techniques, the use of viral vectors in the absence of c-Myc, to reprogram these cells still poses a risk to patients even though

chimeras produced from iPSCs without retroviral c-Myc integration demonstrate no tumour formation (Nakagawa et al, 2008), as the vectors still integrate and are potentially harmful. There are now a number of methods published for iPSC formation without the use of integrating viral vectors including: non-integrating adenoviral vectors (Stadtfeld et al, 2008), delivery of factors via PiggyBac (PB) transposition allowing the removal of the PB insertion (Woltjen et al, 2009), a minicircle DNA vector that does not contain any bacterial DNA (Jia et al, 2010) and vectors derived from the Epstein-barr virus (OriP/EBNA) with removal of the vector by culturing without drug selection (Yu et al, 2009). These techniques have used different combinations of the factors and, as they are potentially non-integrating, have also involved the use of c-Myc. In addition to this, it has been reported that iPSCs can be produced using significantly fewer reprogramming factors from human neural stem cells with the addition of Oct4 only (Kim et al, 2009). This is, of course, a step in the right direction for producing ESC-like cells suitable for clinical application.

The main disadvantage of producing iPSCs currently is the general low efficiency of the process. The removal of genes, such as the oncogene, c-Myc, and the use of non-integrating methods for reprogramming has resulted in the process becoming less efficient (Yu et al, 2007; Yamanaka, 2007; Yu et al, 2009). Several studies have concentrated on the efficiency of reprogramming, resulting in new ideas regarding the process including the addition of SV40 T antigen to vectors previously used (Mali et al, 2008) and the role of p53 in regulating the reprogramming of cells with short telomeres

(Marion et al, 2009). It has also been suggested that reducing the oxidative stress by addition of antioxidants such as vitamin C (Esteban et al, 2009) or culturing under low-oxygen conditions can increase the efficiency of reprogramming cells to iPSCs (Yoshida et al, 2009).

Reprogramming of terminally differentiated cells has been found to be an inefficient process which can possibly be attributed to telomere length as cells with short telomeres are difficult to reprogram (Marion et al, 2009; Davy et al, 2009). p53 is now known to have a role in the regulation of reprogramming of damaged cells, such as those with short telomeres and other DNA defects, thereby controlling the formation of iPSCs from these cells (Marion et al, 2009). It is interesting to note that there are differences emerging between the capabilities of different cells to be reprogrammed (Aasen et al, 2008), potentially not only due to their endogenous expression of genes of interest in reprogramming, but also as a result of different resident progenitor cell population numbers within the tissue used for reprogramming (Okita et al, 2007; Aasen et al, 2008).

1.3.3.3 iPSC Culture

Much of the initial work on cell reprogramming was performed by culturing cells with FGF-2 and on an iMEF feeder layers to provide the factors required to maintain emerging iPSCs in a pluripotent state (Takahashi et al, 2007; Okita et al, 2007; Yu et al, 2007; Stadtfeld et al, 2008; Nakagawa et al, 2008), mimicking the environment known to sustain hESCs cultures. In order for these cells to be considered for any medical applications in humans, they would first need to be confirmed as safe. As such, much work is being

carried out, similar to the work carried out on hESC, to establish and maintain them in an environment free of animal products i.e. iMEF feeder layers. iPSCS have been derived using feeder-free culture conditions on matrigel® (Chung et al, 2012; Chang et al, 2013) and demonstrated decreased variability amongst iPSC lines when in these conditions compared to feeder-dependent conditions (Chung et al, 2012). In addition to this, human neonatal foreskin fibroblasts have been used to derive and expand iPSCs (Warren et al, 2012). More recently, human MSCs have been suggested as potential feeder layers for iPSC maintenance and interestingly are able to support the growth and pluripotency of iPSCs (Havasi et al, 2013). Research in this area is ongoing and of great importance for these cells to have any clinical relevance.

1.3.3.4 Differentiation of iPSCs

Like ESCs and ASCs, differentiation of iPSCs is of great importance if these cells are to be used in tissue regeneration and repair. Reports have described varying degrees of success with differentiation of iPSCs (Hu et al, 2010; Kim et al, 2011). Research is ongoing despite the unanswered questions over the safety of these cells as differentiation of iPSCs will further our understanding of their potential. iPSCs demonstrate the ability to develop into cells of all three germ layers. This has typically been performed as part of the characterisation process for reprogrammed cells (Takahashi and Yamanaka, 2006; Takahashi et al, 2007; Yamanaka, 2007; Yu et al, 2007; Okita et al, 2007) and several studies have further investigated this promise. Similar to ESCs, differentiation of iPSCs often involves formation of an EB

(Sullivan et al, 2009; Zhang et al, 2009; Hu et al, 2010) followed by the directed differentiation which commonly includes; cardiomyocytes (Zhang et al, 2009), neuronal cells (Hu et al, 2010) and hepatocytes (Sullivan et al, 2010). Examples of the differentiation potential and the source of iPSCs are summarised in Table 1.5. Differentiation studies at present are largely focused on dermal fibroblasts as a cell source for iPSC formation as these were the first cell type to successfully be reprogrammed.

Differences still remain between the differentiation of iPSC and ESCs. This has been demonstrated in studies which indicate that iPSC derived from mouse fibroblasts differentiate slower compared to mESCs (Morizane et al, 2009). Similarly, neural differentiation of iPSCs has been found to be an inefficient process with an even higher degree of variability than that seen in hESCs (Hu et al, 2010). More recently, it was found that the donor cell type used for reprogramming was important as the resulting iPSCs demonstrated preferential differentiation towards cell types associated with the donor tissue (Kim et al, 2011; Bar-Nur et al, 2011).

This has been explained due to an epigenetic memory maintained by the iPSCs following reprogramming (Kim et al, 2010; Kim et al, 2011; Bar-Nur et al, 2011). These investigations outline the need for further research into the underlying differences that exist between ESCs and iPSCs before such cells can be used in clinical applications, to ensure that risk to the patient is limited.

Table 1.5*: iPSC reprogramming and differentiation potential.* Examples of different cell sources, their reprogramming method and their differentiation potential towards different cell types.

Reprogrammed Cell Source	Reprogramming Method	Differentiated Cell Type	References
Dermal Fibroblasts	Lentivirus, Episomal Vector, Retrovirus	Neural	Hu et al, (2010); Lohle et al, (2012)
	Lentivirus	Cardiac	Zhang et al, (2009)
	Lentivirus	Endothelial & Haematopoietic	Choi et al, (2009)
	Retrovirus Lentivirus	Hepatic	Sullivan et al, (2010), Ghodsizadeh et al, (2010)
Neural stem cells	Retroviral	Neural	Lohle et al, (2012)
Keratinocytes	Retroviral	Neural	Aasen et al, (2008)
	Retrovial and small molecule inhibitors	Cardiomyocytes	Zhu et al, (2010)
Mesenchymal Stem Cells	Lentivirus	Bone	Deyle et al, (2012)

1.3.3.5 Neural Differentiation of iPSCs

iPSCs have characteristics that are similar to ESCs and, like ESCs, require the administration of different factors at defined time points in order to successfully differentiate these cells down the desired lineages. The major challenge for iPSC differentiation is to gain comparable differentiation with hESC to confirm that these ethically obtained cells are a better potential candidate for tissue engineering applications. When considering motor neuron differentiation in particular, the reported differentiation potential of iPSCs is variable (Hu et al, 2010). As with hESC, each differentiation protocol varies but the important factors utilised for hESC differentiation have been utilised for iPSCs (Hu et al, 2010; Boulting et al, 2011).

iPSC demonstrate differentiation towards motor neurons but with reduced efficiency compared to hESCs when culturing EBs with FGF-2, RA and SHH followed by mono-layer culture on laminin with BDNF, GDNF and IGF-1 (Hu et al, 2010). In contrast, another group have differentiated iPSCs to motor neurons similar to those obtained from hESC (Boulting et al, 2011). However, different culture conditions were employed including culturing iPSCs as EBs with RA and a small molecule inhibitor of SHH, HAg, followed by culture with BDNF, GDNF and Ciliary Neurotrophic Factor (CNTF) prior to dissociation of EBs and plating as mono-layer cultures to induce motor neuron differentiation.

The differences observed between the two studies is potentially attributed to the different protocols used for differentiation but could also be due to the source of cells used in reprogramming to iPSCs as well as the number of factors used as previously discussed (Kim et al, 2010; Kim et al, 2011, Bar-Nur et al, 2011).

1.3.4 Stem cells and oxidative stress

Oxidative stress is a process that has long been known to affect different aspects of cell behaviour. In the presence of oxygen, reactive oxygen species such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and nitric oxide radical ($NO^{\cdot}$) can be generated and inflict cellular damage (Slater et al, 1995). The unpaired electrons in the outer orbitals of these free radicals make them highly reactive species. Non-radical derivatives of oxygen include hydrogen peroxide (H_2O_2) and hypochlorus acid (HOCl) (Halliwell, 1994). Cellular defences against free radicals exist and include superoxide dismutase (SOD) which is produced by the mitochonidria and in the cytosol. SOD acts to convert superoxide radicals to H_2O_2 (McCord and Fridovich, 1969a). This is produced by cells when the level of oxidative stress is relatively low, high levels of oxidative stress may result in irreversible damage to the cell and even cell death (McCord and Fridovich, 1969b; Halliwell,1994).

The culturing of hESC is very difficult due to the spontaneous differentiation that can occur. This is most likely as a result of the suboptimal culture conditions used to maintain them. It is common practice to culture these cells

at 21% O_2 *in vitro*, however, the *in vivo* conditions are around 3-5% O_2. When culturing hESC *in vitro* at low O_2 tension spontaneous differentiation is reduced (Ezashi et al, 2005; Ludwig et al, 2006), pluripotency is maintained and proliferation is increased (Forristal et al, 2010).

Alternative methods for reducing oxidative stress exist for hESC culture including the addition of antioxidants like β-Mercaptoethanol to the culture conditions. Ascorbic acid is an additional antioxidant that is often added to culture media. It has been well investigated and has long been known that addition of ascorbic acid to the culture medium of human dermal fibroblasts increases the transcription of genes encoding type I and III collagen (Tajima and Pinnell, 1996). Its presence also alters the growth rates of various cell cultures as well as stimulating differentiation of mesenchymal derived cells *in vitro* potentially by changing their gene expression patterns (Alcain and Buron, 1994).

The benefits of reducing oxidative stress for the purpose of increasing reprogramming efficiency have been investigated using hypoxic environmental conditions (Yoshida et al, 2009) and, more recently, by supplementing media with ascorbic acid (Esteban et al, 2010). The mechanisms that underlie this improved efficiency are largely unknown at present however, it has been suggested that ascorbic acid acts on demethylases that potently regulate reprogramming by suppressing cellular senescence and stimulating cell cycle progression (Wang et al, 2011).

1.4 Current Clinical Applications of Stem Cells

It is often forgotten that stem cells are already in use as clinical treatments and much can be learnt from the successes and failures of these treatments. Epithelial tissues have historically been cultured using autologous keratinocyte cells for use as skin grafts (Langdon et al, 1988), limbal stem cells have been transplanted to damaged corneal surfaces (Shortt et al, 2007) and more recently the use of oral mucosa epithelial stem cells for corneal transplants has been reported (Inatomi et al, 2006; Nakamura et al, 2013). A treatment that has been widely used for many years is that of haematopoietic cell transplants (Perry and Linch, 1996) and, although it was not known at the time, stem cells underlie the success of this treatment. Bone marrow transplants from donor patients have been used in the treatment of cancers such as leukaemia for a number of years. Advances in immunology have meant that donor patients can now be screened to find the best match to the patient that requires treatment, reducing immune rejection and, therefore, increasing the success of these transplants (Perry and Linch, 1996). Additionally, treatment of children with osteogenesis imperfect (OI) with BMSCs resulted in the formation of new dense bone (Horwitz et al, 1999) which is now attributed to the presence of MSCs in the BMSCs. Treatment with purified populations of MSCs derived from BMSCs has been suggested to boost clinical benefit to OI sufferers (Horwitz et al, 2001). Although the mechanisms by which these therapies work are not yet fully understood, these are invaluable to our increasing knowledge of stem cells and their actions *in vivo*.

1.5 Aims

The OMLP represents a tissue with a population of progenitor cells that already express numerous stem cell and developmental markers and can be acquired utilising a minimally invasive biopsy procedure. The biopsy site will heal rapidly with minimal scarring to the patient. These cells therefore represent an excellent and promising source of cells for use in clinical applications.

There are three main aims to this project:

1. Isolate, expand and characterise progenitor cell populations from the Oral (buccal) Mucosa Lamina Propria.
2. Reprogram progenitor cells from the OMLP to iPSC and characterise such cells.
3. Investigate the potential of oral progenitor cells and oral mucosal-iPSCs to differentiate down a functional neuronal pathway.

2 - General Materials & Methods

2.1 Materials

All materials were obtained from Invitrogen UK unless otherwise stated.

2.1.1 Cell Strains and Cell Lines

Primary oral mucosal fibroblasts were obtained from healthy oral (buccal) mucosa samples obtained from dental patients undergoing routine dental procedures at the School of Dentistry, Cardiff University, who had given informed consent and after full ethical approval (09/WSE03/18). A 6mm biopsy from the buccal mucosa was obtained from each patient and placed into 1x Phosphate buffered saline (PBS). Human Embryonic Stem Cells (H9) were obtained from Dr N Allen, School of Biosciences, Cardiff.

2.2 Methods

2.2.1 Cell Culture

All cell culture was carried out using sterile conditions in a Microflow Advanced bio-safety Class II cabinet and cultures maintained in a Binder series CB CO_2 incubator. All tissue culture plastic was obtained from Greiner bio-one unless otherwise stated.

2.2.1.1 Differential adhesion assay (Davies et al, 2010)

Differential adhesion to fibronectin was undertaken for 6 patients; XLVII, XLVIII, XLIX, L, LII and LIV (Appendix I for patient details).

2.2.1.1.1 Enzymatic Digestion of the Oral Buccal Mucosa Biopsies

Oral mucosa biopsies were washed in 70% (v/v) ethanol (40 seconds) then placed in a tissue culture dish containing Fibroblast-Serum Containing Medium (F-SCM): Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS), 2mM L-Glutamine, antibiotics and antimycotics (100U/mL Penicillin G, 100µg/mL Streptomycin Sulphate and 0.25µg/mL Amphotericin B). Any fat was then removed and the remaining biopsy was cut into 2mm squares and placed in a solution of 2mg/mL pronase (Sigma, UK) made up in F-SCM. Tissue samples were left at 4°C overnight.

Epithelium was then removed from tissue pieces using tweezers and the remaining tissue was placed in a tissue culture dish (BD Falcon) containing a 1mg/mL solution of collagenase A (Roche, UK) made up in F-SCM. Tissue pieces were cut as finely as possible using a scalpel and placed in an incubator at 37°C overnight.

2.2.1.1.2 Differential Adhesion to Fibronectin (Figure 2.1)

Fibronectin coated plates were prepared using a 10µg/mL solution of bovine plasma fibronectin (Sigma, UK) diluted in 1x PBS+ (1x PBS supplemented with 0.1mM $MgCl_2$ and 0.1mM $CaCl_2$). One mL of this solution was used to coat the bottom of each well of a 6-well plate. Plates were covered with Parafilm and left overnight at 4°C.

A 1mL pipette was then used to disaggregate the tissue by pipetting up and down, followed by successively smaller pipette sizes.

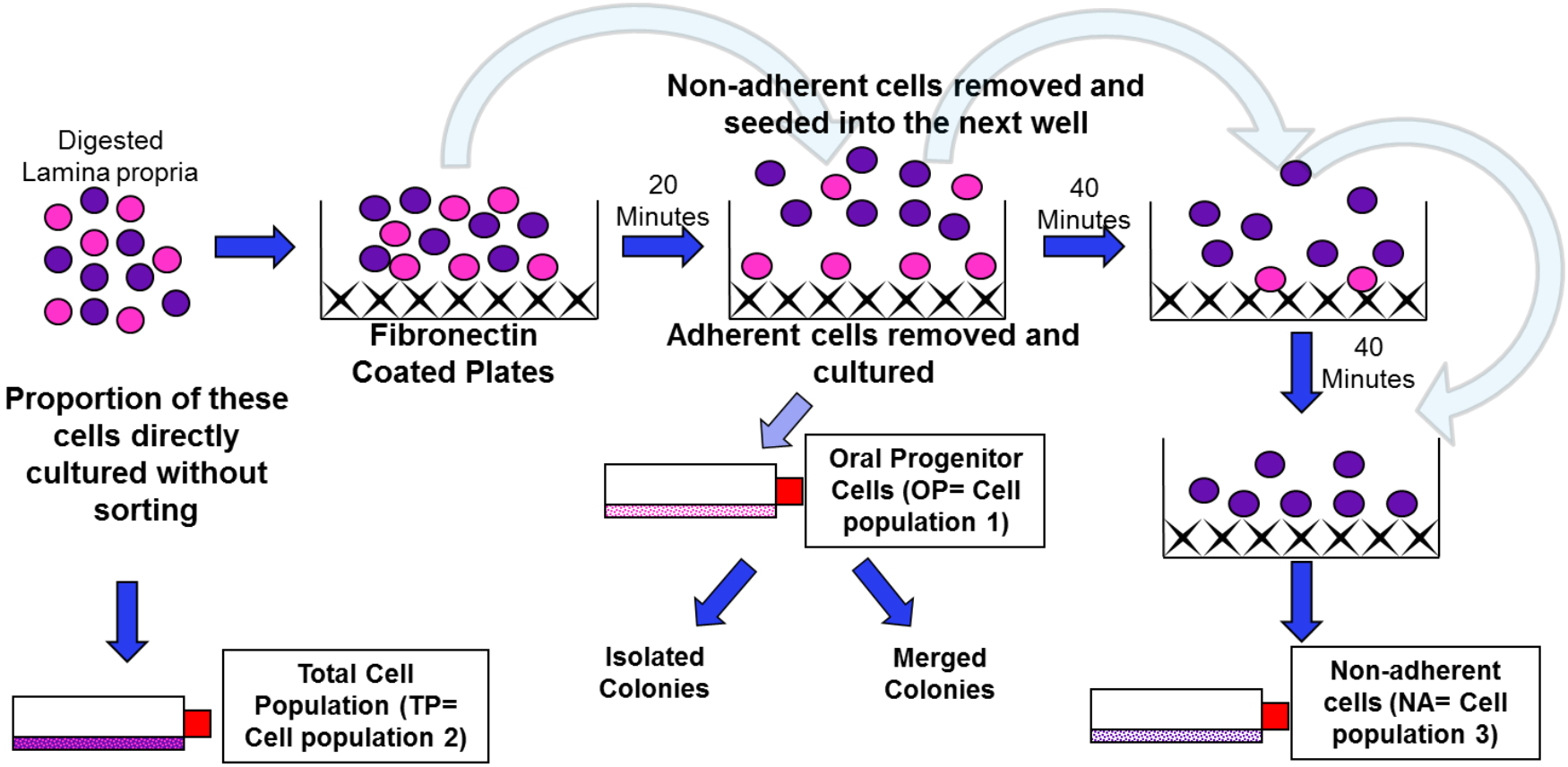

Figure 2.1 ***Differential adhesion of OMLP-PC to fibronectin***. Cells enzymatically digested from the OMLP seeded onto fibronectin coated plates to separate progenitor cell populations (OP) from a mixed cell population based on increased expression of functional $\alpha_5\beta_1$ integrins on their surface. Three time steps utilised to remove all progenitor cells from the population to culture non-progenitor cells (NA). Mixed population directly from the lamina propria digest cultured (TP).

Finally a 10mL syringe with a 0.8/21 gauge needle attached was used to remove cells from the dish after the addition of serum free medium (SFM) (DMEM supplemented with 2mM L-Glutamine, antibiotics and antimycotics (100U/mL Penicillin G 100µg/mL Streptomycin Sulphate and 0.25µg/mL Amphotericin B) and placed into a 15mL centrifuge tube. This was centrifuged at 400 x g for 5 minutes and the pellet re-suspended in 1mL SFM. The cells were counted by mixing 20µL of cell solution with 20µL trypan blue exclusion and counted using a Fastread 102 haemocytometer. A cell solution was made up to 1 x 10^4 viable cells/mL in SFM.

Cell populations were then selected on their bases to bind/not to bind to fibronectin (Figure 2.1). Fibronectin solution was aspirated off each well of the 6 well plate and 1mL of the cell solution was placed in each well (leaving two wells free for further incubation) and the plate incubated at 37°C for 20 minutes to isolate any potential oral progenitor cells (OP = Cell Population 1). Any remaining left over cell solution containing the total cell population (TP = Cell Population 2) was centrifuged at 400 x g, re-suspended in 1mL F-SCM and counted using trypan blue exclusion (1:1) and Fastread® 102 haemocytometer. Cells were seeded in a separate 6 well plate at a density of 2 x 10^4 cells/cm^2 in F-SCM. After the 20 minute incubation the medium in the primary 6-well plate (containing cells that had not adhered to fibronectin within the 20 min incubation period) was aspirated off the and 3mL of F-SCM placed into each of these wells to permit onward culture of the OPs. The 'non-adherent' cell medium was then centrifuged at 400 x g for 5 minutes, the medium aspirated off and the cells re-suspended in 3mL of serum free

medium. This was then added to another fibronectin coated well and incubated at 37°C for 40 minutes. This was then removed and transferred to another fibronectin coated well for a second 40 minute incubation at 37°C (F-SCM was added to the second population of adherent cells to permit their culture). Medium was aspirated off following the second 40 minute incubation and replaced with 3mL of F-SCM. Finally, the remaining (aspirated) cell solution was centrifuged at 400 x g and counted using a haemocytometer. These cells, the non-adherents (NA = Cell Population 3), were then re-seeded onto standard tissue culture plastic and left to adhere O/N. OP cells developed as single cell derived colonies and were cultured in two ways; isolated as single cell derived clonal populations and expanded or the OP cells were allowed to merge together to form a merged progenitor (MP) cell population.

2.2.1.2 Isolation of Colonies

Colonies were picked from the fibronectin coated plates when colony cell number was greater than 32 cells. Cell number for each colony was recorded. Each colony was marked on the plate, medium removed and cells washed once in PBS. A cloning ring was placed over the colony, using Vaseline® to make a seal. One hundred µL of accutase was pipetted into the cloning ring and the plate was placed into the incubator at 37°C for 5 minutes. The contents of the cloning ring were pipetted up and down to release the cells from the surface of the plate. Each colony was placed into 1 well of a 96 well plated containing 150µL of F-SCM. These were maintained in an incubator at 37°C and with 5% CO_2/95% air and fed every 2-3 days

until 80-90% confluency was reached. Medium was removed from the cells and cells were washed once with 200µL of PBS. This was removed and replaced with 100µL accutase and returned to the incubator for 5 minutes. Following incubation cells were mixed with 100µL F-SCM to inactivate the accutase and re-plated into a 24 well plate. Each passage following this, a cell count was performed as described below and cells were re-seeded as required.

2.2.1.3 Oral Mucosa Lamina Propria Cell Culture

Cell cultures were fed with F-SCM every 2-3 days. Cells were left until they were 85-95% confluent before passaging. Medium was aspirated off and the cell culture was washed once with PBS (2mL in 1 well of a 6 well plate, 5mL in a T25 and 10mL in a T75) which was then aspirated and replaced with accutase (500µL in 1 well of a 6 well plate, 1mL in a T25 and 3mL in a T75) to coat the plate/flask. This was left at 37°C for 5-10 minutes until the cells detached from the plastic. F-SCM (3mL in 1 well of a 6 well plate, 5mL in a T25 and 10mL in a T75) was added to the plate/flask to inactivate the accutase. Medium was removed and placed into a centrifuge tube, centrifuged at 400 x g for 5 minutes, supernatant was removed and pellet resuspended in 1mL of medium. Trypan blue (Sigma-Aldrich, UK) exclusion was used to calculate cell viability by mixing 20µL of Trypan blue with 20µL of cell suspension. Viable cells were counted using a Fastread® 102 haemocytometer Cell populations were re-seeded at the required density.

2.2.1.4 Cryopreservation and Re-establishment of Oral Mucosa Lamina Propria Cells

Cells were re-seeded as required. The remaining cells (between 3 x 10^5 and 5 x 10^5) were re-suspended in a 1mL cryopreservation solution containing: 10% (v/v) dimethyl sulfoxide (DMSO) and 90% (v/v) FCS and transferred to cryogenic vials and frozen in an iso-propanol freezing container at -80°C for 24 hours. Following this, cryovials were transferred to liquid nitrogen for long term storage. When required, these frozen cells were thawed rapidly at 37°C, added to 10mL of pre-warmed (37°C) F-SCM and centrifuged at 400 x g for 5 minutes to remove the DMSO and serum. The supernatant was removed and the pellet re-suspended in 1mL of F-SCM. Cells were counted using a haemocytometer and re-seeded as required.

2.2.1.5 Dermal Fibroblast Cell Culture

Human dermal fibroblast (DF) were previously isolated and expanded by members of the Wound Biology Group, (School of Dentistry, Cardiff University). DF were treated in the same way as oral mucosa lamina propria cells and re-established from cryopreservation as previously described in section 2.2.1.4 and cultured as described in section 2.2.1.3.

2.2.1.6 Mycoplasma Testing

All cell populations were mycoplasma tested at confluency. One hundred µL of conditioned medium was taken and tested using a Mycoplasma detection kit by PCR (Minerva Biolabs, Germany). All mycoplasma testing was carried out by Dr M Stack, (School of Dentistry, Cardiff University). Any contaminated cell populations were treated with F-SCM containing 10µg/mL

Ciprofloxacin for two weeks and then re-tested for mycoplasma. Subsequent mycoplasma test were carried out every 5 passages. Only mycoplasma negative cells were utilised in experiments.

2.2.1.7 Formation of Feeder Layers

Human embryonic stem cells require feeder layers in order to maintain their pluripotent state and mouse embryonic fibroblasts (MEFs) are often used to provide the required factors to maintain these stem cells (Thomson et al, 1998).

A pregnant mouse (CF1 strain) was sacrificed between days 13 and 14 post coitum by cervical dislocation. The abdomen was washed with 70% (v/v) ethanol and cut using a pair of scissors to reveal the uterine horns. These were dissected out and placed in a 50mL falcon containing 20mL PBS to wash them. This was repeated twice more with a fresh 50mL falcon containing 20mL PBS. The contents were placed in a 10cm tissue culture dish after the final wash and transferred to a sterile tissue culture hood. The placenta and amniotic membranes were removed from each embryo, the head and organs were removed and discarded. Each embryo was washed with PBS and transferred to a clean 10cm tissue culture dish. Embryos were minced using a sterile scalpel and then further disaggregated using a 1ml pipette before adding 2mL of Trypsin/EDTA per embryo and incubating the plate at 37ºC for 15 minutes. Following incubation the cell suspension was passed several times through a syringe fitted with an 18 G needle before adding an equal volume of fresh Trypsin/EDTA to the dish before returning to the incubator for a further 15 minutes. An equal volume of Mouse Embryonic

Fibroblast (MEF) medium: DMEM supplemented with 10% FCS, 2mM L-Glutamine, 100U/mL Penicillin, 100µg/mL Streptomycin Sulphate and Non-Essential Amino Acids (NEAA) (Glycine 7.5mg/L, L-alanine 8.9mg/L, L-asparagine 1.32mg/L, L-aspartic acid 1.33mg/L, L-glutamic acid 1.47mg/L, L-proline 1.15mg/L and L-serine 1.05mg/L) was added to the plate to neutralise the Trypsin/EDTA. A 1mL aliquot of this was counted using a haemocytometer. The cell suspension was spun at 400 x g for 5 minutes to pellet the cells and re-suspend in MEF medium to 1 x 10^6 cells/mL. These cells were plated at 10mL/10cm tissue culture dish and returned to the incubator. Medium was changed for 10mL of fresh MEF medium after 24 hours to remove debris and non-viable cells. Once cells reached high confluency after 2-3 days, medium from each 10cm dish was removed (1mL was kept for mycoplasma testing), washed once with 10mL PBS, replaced with 3mL of Trypsin/EDTA and returned to the incubator for 5 minutes. When cells had detached, 5mL of MEF medium was added to each plate and cells were washed from the surface and collected in a 50mL falcon. These cells were spun at 400 x g for 5 minutes, supernatant was removed, replaced with fresh MEF medium and cell suspension was re-plated into a 15cm tissue culture dish. These were termed passage 2 cells. Each 15cm tissue culture dish was split at a ratio of 1:5 when confluent to give passage 3 MEFs. Cells after passage 4 were not used as embryonic stem cell feeders.

2.2.1.8 Mitotic Inactivation of MEFS using γ-Irradiation

MEF cells that were free of mycoplasma contamination were mitotically inactivated by γ-irradiating in order to be used as a feeder layer (iMEFs).

Cells to be irradiated were split by removing MEF medium from the 15cm tissue culture dish, washing once with 15mL PBS and replacing with 5mL of Trypsin/EDTA and returning to the incubator for 5 minutes. Once the cells were detached an equal volume of fresh MEF medium was added to inactivate the Trypsin/EDTA and cell suspensions were collected in a 50mL falcon. An aliquot of this was used to determine the cell number with a haemocytometer as in section 2.2.1.3. The cell suspension was then spun at 400 x g for 5 minutes to pellet the cells. The supernatant was removed and the pellet resuspended to a cell concentration of 2 x 10^6 cells/mL before irradiating. The cells were irradiated at 3000 rads using a γ-irradiator. Following irradiation, the cells were once again spun at 400 x g for 5 minutes to pellet the cells. The supernatant was removed and the cells resuspended to 1 x 10^6 cells/mL with cryopreservation solution and transferred to cryogenic vials (1mL/vial). These were placed in an iso-propanol freezing container at -80°C for 24 hours. Following this, cryogenic vials were placed in liquid nitrogen for long term storage.

2.2.1.9 Human Embryonic Stem Cell Culture

2.2.1.9.1 Preparing Feeder Layers

MEFs were seeded at a density of 7.5 x 10^3 cells/cm^2, equivalent to 7.2 x 10^4 cells into each well of a 6 well plate. Plates were pre-coated with 0.1% (w/v) gelatin (Milipore, UK) for 1 hour at room temperature. Gelatin was removed and replaced with 2mL MEF medium containing the required number of MEFs. Plates were returned to the incubator to allow the MEFs to settle. Feeder layers were best used after 24 hours and within 2 days of plating.

When feeder layers were required for ESCs, MEF medium was removed, cells were washed twice with 2mL PBS to remove all serum and replaced with 2mL Embryonic Stem Cell (ESC) Medium: Knockout® DMEM supplemented with 20% (v/v) Knockout® Serum Replacer, 2mM L-Glutamine, 100U/mL Penicillin, 100µg/mL Streptomycin Sulphate, (NEAA) (Glycine 7.5mg/L, L-alanine 8.9mg/L, L-asparagine 1.32mg/L, L-aspartic acid 1.33mg/L, L-glutamic acid 1.47mg/L, L-proline 1.15mg/L and L-serine 1.05mg/L) and 10ng/mL basic Fibroblast Growth Factor (FGF-2, Peprotech, UK). Plates were returned to the incubator to allow medium to equilibrate.

2.2.1.9.2 Splitting and re-plating Human Embryonic Stem Cells

When Human ESC colonies were at an optimal size, determined by a flattened colony with a tight boundary that was not touching adjacent colonies and could be viewed completely using the size of 10 x objective lens, they were prepared for splitting.

Any differentiated colonies (distinguished by a change in morphology of the cells and colonies that lacked a tight colony boundary) were removed by scraping, using a flame pulled glass Pasteur pipette. ESC Medium was removed and colonies were washed once with PBS to remove differentiated colonies and medium. This was replaced with 2mL of 1mg/mL Collagenase IV in Knockout® DMEM and returned to the incubator for 40 minutes. When colonies began to curl up at the edges, plates were removed from the incubator and colonies were gently washed from the surface using a 5mL pipette, keeping the colonies as large as possible. The colonies were collected in a 15mL falcon and spun at 200 x g for 2 minutes to pellet them.

The supernatant was removed and the pellet was re-suspended with 1mL of pre-warmed ESC medium, pipetting up and down with a 1mL pipette tip (to break up the colonies) a maximum of 8 times depending on colony size. The colonies were split in a 1:3 ratio onto the prepared MEF feeder layers, returned to the incubator and left undisturbed for 48 hours to allow colonies to attach. After 48 hours, ESC medium was removed and replaced with 2mL of fresh pre-warmed ESC medium. This was repeated daily until colonies required splitting.

2.2.1.9.3 Cryopreservation and Re-establishment of Human Embryonic Stem Cells

Colonies were re-seeded as required and when ready to be split, differentiated colonies were removed from the plates using a flame pulled Pasteur pipette, ESC medium was removed and colonies washed with 2mL of PBS to remove debris. This was removed and replaced with 2mL 1mg/ml Collagenase IV solution and returned to the incubator for 40 minutes until colonies began to curl at the edges. Colonies were washed from the plate with a 5mL pipette, keeping the colonies as large as possible. The colonies were collected in a 15mL falcon tube and spun at 200 x g for 2 minutes to pellet the colonies, the supernatant was removed and colonies were gently re-suspended, avoiding breaking up the colonies, with 500µL of ESC medium and 500µL of 2 x Freeze mix: 20% (v/v) DMSO in 80% (v/v) FCS and transferred to cryogenic vials; colonies from one well of a 6 well plate were frozen into one vial. Vials were frozen in an iso-propanol freezing container at -80°C for 24 hours. Following this, cryovials were transferred to liquid

nitrogen for long term storage. When required, these frozen colonies were thawed rapidly at 37ºC, transferred to 10mL of pre-warmed ESC medium and spun at 200 x g for 2 minutes to pellet colonies. The supernatant was removed and colonies gently re-suspended (to avoid breaking the colonies) in 1mL of pre-warmed fresh ESC medium. These colonies were then gently re-seeded onto prepared MEF plates, returned to the incubator and left undisturbed for 48 hours to settle.

2.2.1.10 Imaging of Cells

Digital images of cells were obtained using a Nikon Eclipse TS100 microscope fitted with a Canon LA DC 58E digital camera (F stop 5.6) at 10x and 20x objective. Confocal laser scanning microscope images were obtained using a Leica TCS SP5 confocal laser scanning microscope (Leica, Milton Keynes, UK). Fluorescent images were obtained using an Olympus AX7 microscope fitted with a Nikon DXM-1000 camera (both Nikon, Japan) and images were acquired using ACT-1 imaging software.

2.2.2 Gene Expression Studies

2.2.2.1 Extraction of Total RNA

When cell cultures were 90-95% confluent, medium was removed from cells and cells were washed once with PBS. PBS was removed and replaced with 1ml of Trizol®. This was left to incubate at room temperature for 5 minutes. Cells were removed from the surface of the plastic with a cell scraper and placed into a 1.5mL eppendorf. 175µL of chloroform was added to each 1.5mL eppendorf, inverted and left to incubate at room temperature for 3

minutes. Heavy phase-lock tubes (5 Prime, UK) were centrifuged in an Eppendorf Centrifuge 5415R at 12000 x g for 1 minute to pellet the gel. Following the 3 minute incubation the whole RNA extraction mixture was transferred to the phase-lock tube and centrifuged at 13000 x g for 2 minutes to separate out the phenol layer. The upper aqueous layer was removed from the phase-lock tube and transferred to a new 1.5mL eppendorf and an equal volume of isopropanol was added to each tube, mixed and left overnight at -20°C to precipitate the RNA. The RNA pellet was isolated by centrifugation at 12000 x g for 10 minutes at 4ºC. The supernatant was removed and replaced with 1mL of 75% ethanol to wash the pellet. Following washing, the pellet was centrifuged at 7500 x g for 5 minutes at 4ºC, supernatant removed and the pellet allowed to air-dry for 10 minutes before resuspending in 43µL of RNase free water to give a final volume of 50µL following DNase Treatment.

2.2.2.2 DNase Treatment of Extracted RNA

The resuspended pellet of extracted RNA was treated with 1µL of DNase (Promega, UK), 5µL of 10x DNase buffer (Promega, UK) and 1µL of RNasin (Promega, UK) giving a final volume of 50µL which was then heated to 37ºC for 15 minutes and rapidly cooled on ice.

2.2.2.3 Purification of Treated RNA

RNA was purified using the RNeasy® MinElute™ protocol for RNA cleanup (Qiagen, UK). DNase treated RNA was made up to 100µL with RNase free water. To this, 350µL of buffer RLT containing 1% (v/v) β-mercaptoethanol was added and mixed by vortex. Following this, 250µL of 100% ice cold

ethanol was added and mixed by pipetting. The 700µL was added to the top of an RNeasy® spin column and centrifuged for 1 minute at 8000 x g. The elutant was disposed of and 500µL of buffer RPE was added to the column and centrifuged for a further minute at 8000 x g to wash the membrane. The membrane was then dried by adding 500µL of buffer RPE and centrifuging at 14000 x g for 2 minutes. The RNA was eluted by the addition of 50µL of RNase free water to the column and centrifuging at 8000 x g for 1 minute. The concentration of RNA was determined using Nanovue® spectrophotometer, aliquoted and stored at -80ºC until required.

2.2.2.4 Generation of cDNA by Reverse Transcription

Following concentration determination of RNA samples, 0.5µg of RNA was mixed with 0.5µL of random hexamer primers (Promega, UK) and made up to 6.5µL with RNase free water. This was mixed and heated to 70ºC for 5 minutes, then cooled on ice for 5 minutes. A master mix of 2.5µL mmLV 5x buffer, 0.5µL RNasin (20u), 2.5µL dNTPs (10mM) and 0.5µL mmLV RT (100u) (all Promega, UK) was added to each sample, mixed and incubated at 25ºC for 10 minutes, 42ºC for 1 hour and finally 95ºC for 5 minutes. Newly synthesised cDNA was stored at -20ºC until required.

2.2.2.5 Amplification of cDNA by Polymerase Chain Reaction (PCR)

A master mix of 12µL of Platinum Blue Super mix (containing 22U/ml complexed recombinant *Taq* DNA polymerase with Platinum® *Taq* antibody, 22mM Tris-HCl (pH 8.4), 55mM KCl, 1.65mM $MgCl_2$, 220µM dGTP, 220µM dATP, 220µM dTTP, 220µM dCTP, stabilizers, glycerol and blue tracking dye) was mixed with 0.5µL of 1µM forward primer specific to the gene of

interest and 0.5µL of 1µM reverse primer for the same gene (for details of primers see Chapter specific methods). One µL of sample cDNA was added to each reaction to make the total volume 13 µL. This was amplified in a thermal cycler (Software version 2.3.2, VWR, UK). There was an initial denaturation step at 95°C for 10 minutes before amplification for 40 cycles, each cycle consisting of a 95°C denaturation step, followed by a 30 second annealing (Ta) step at the optimal temperature for each primer and finally an elongation step at 74°C for 1 minute. Amplification was completed by a final elongation step at 74°C for 10 minutes. Samples were stored at -20°C until required.

2.2.2.6 Sequencing of PCR Products

PCR products were purified to remove primers using the MinElute® PCR Purification kit (Qiagen, UK). For each target gene, an extra 30µL reaction was run using the positive control (detailed in chapter specific methods) in order for each PCR product to be sequenced. Ten µL of this reaction was run out on an agarose gel (see below) to check that the product was the correct size. To the remaining 20µL reaction, 100µL of Buffer PB was added and mixed. This was added to a MinElute® spin column in a 2mL collection tube and spun at 17900 x g in a bench top microcentrifuge for 1 minute to bind the DNA. The flow-through was discarded from the collection tube and the MinElute® column placed back into the same collection tube. Bound DNA was washed by adding 750µL Buffer PE to the spin column and spinning for 1 minute at 17900 x g. Again, the flow-through was discarded and the MinElute® was placed back into the same tube and spun one final

time at 17900 x g for 1 minute to remove residual ethanol contained within Buffer PE. Bound DNA was eluted from the MinElute® spin column by placing the column in a fresh 1.5mL microcentrifuge tube. Ten µL of Buffer EB was added to the centre of the column membrane and this was left to stand for 1 minute followed by centrifugation at 17900 x g for 1 minute. The purified products were sequenced by Andrew Francis from Central Biotechnology Service (CBS), Cardiff University.

2.2.3 Agarose Gel Electrophoresis

Agarose gels containing 0.5% (v/v) ethidium bromide were made up in 1 x TAE (50x stock: 242g Tris Base, 57.1mL glacial acetic acid and 100ml 0.5M EDTA) buffer. The percentage gel was determined by product size; 1% gels were made up for PCR products above 500bp, 2% gels for products between 500bp and 300bp and 3% gels for products smaller than 300bp. The gel was buffered in 1 x TAE running buffer (as described previously), 5µL 100bp DNA ladder (Promega, UK) was loaded into the first well for estimating molecular weights followed by 10µL of each sample and run for 50 minutes at 80V. Products were visualised using a transilluminator and images were acquired using a Gel Doc-17 ™ (UVP doc, VWR, UK).

2.2.4 Statistical Analysis

All data is shown ± SD when applicable, where mean values from individual patient samples have been combined to give the mean. Alternatively, when applicable, data has been shown ± SEM, where experimental repeats have been used to calculate the mean. Data was analysed using GraphPad InStat

software and multiple groups were analysed using One-way Analysis of Variance (ANOVA) followed by post hoc Tukey-Kramer test and statistical significance was assumed when $P<0.05$.

3 - Characterisation of cell populations from the oral mucosa

3.1 Introduction

Embryonic stem cells are an invaluable source of cells for use in tissue engineering and repair due to their pluripotent properties however, there is much controversy surrounding their use (Wert and Mummery, 2003; McLaren, 2007; Bahadur et al, 2010). This is due to the fact that these cells are isolated from a fertilised egg. Their derivation involves destruction of the embryo and, thereby, initiates discussion regarding at what point an embryo becomes a life form and therefore gains human rights? For this reason, alternative cell sources are currently being sought and, as a result, stem cell research is exploring the idea of isolating and modifying adult stem cell populations from patients before re-implanting them back into the patient as a cell-based treatment for tissue regeneration and repair. Adult stem cell populations have been identified in various tissues within the human body including; skin (Jones & Watt, 1993), cornea (Barradon and Green, 1987) and bone marrow (Caplan, 1991; Colter et al, 2000) and much work has been carried out around their potential uses. Adult stem cells are thought to be a more acceptable cell source for use in tissue regeneration. However, in contrast to embryonic stem cells, these cells are only multipotent, often only differentiating into cells from their germ layer of origin and, as such, much work has been carried out to isolate progenitor cells from many different sources. Work within the Wound Biology Group (Cardiff University) has recently demonstrated that a progenitor cell population exists within the lamina propria of the oral mucosa (Davies et al, 2010). These cells were isolated utilising differential adhesion to fibronectin (Jones & Watt, 1993; Dowthwaite et al, 2004) based on the presence of higher number of

functionally active $\alpha_5\beta_1$ integrins on their surface compared to terminally differentiated cells. The isolated cells are characterised by their ability to form colonies from single cells seeded at low density (a typical characteristic of progenitor cell populations; Jones and Watt, 1993), express numerous development markers and are of neural crest origin. They have also recently been demonstrated to possess immunosuppressive properties (Davies et al, 2012) making these cells an attractive target for use in tissue regeneration and repair.

More recently it was reported that somatic cells (e.g. human dermal fibroblasts) could be reprogrammed back towards an embryonic stem cell-like state, producing what has been termed induced Pluripotent Stem Cells (iPSCs; Takashia et al, 2007). This procedure involves reprogramming adult cells to restore their pluripotent properties, making these cells an ideal alternative to embryonic stem cells. Interestingly, cells isolated from the lamina propria have already been reported to express several pluripotency markers including Oct-4, Nanog and Sox-2 (Davies et al, 2010) and, as such, these cells would make an interesting target for applications in cellular reprogramming since these are three of the factors that have been used during iPSC reprogramming (Yu et al, 2007). Much research has been carried out recently investigating the best cell type to derive iPSCs from with these factors and how to make the process more efficient (Yamanaka, 2012). Human dermal fibroblasts have been extensively used for reprogramming as these were the first cell type reported to be reprogrammed (Takashia et al, 2007). Although reprogramming efficiency remains relatively poor, work is

on-going to determine methods that safely (thinking of eventual translation of this technology into patients) improve this reprogramming efficiency. One method found to improve the efficiency of these reprogramming methods is to reduce the levels of oxidative stress the cells in culture experience.

The benefits of reducing oxidative stress for the purpose of increasing reprogramming efficiency have been investigated using hypoxic environmental conditions (Yoshida et al, 2009) and, more recently, by supplementing media with ascorbic acid (Esteban et al, 2010). The mechanisms that underlie this improved efficiency are largely unknown at present however, it has been suggested that ascorbic acid acts on demethylases that potently regulate reprogramming by suppressing cellular senescence and stimulating cell cycle progression (Wang et al, 2011).

The purpose of this chapter is to isolate a source of progenitor cells from the oral mucosa by differential adhesion to fibronectin and investigate the effects of ascorbic acid on these cells. This method has previously been optimised form cells from the oral mucosa lamina propria by members of the wound biology group, Cardiff University (Davies et al, 2010). As a comparison, total cell population of the lamina propria, merged progenitor cells and cells that do not adhere to fibronectin after three different adhesion time points will also be investigated with the aim of identifying the most appropriate cell population to target for reprogramming purposes. Each of these cell populations will be treated and untreated with 50µg/mL ascorbic acid, the optimum concentration that demonstrated improved reprogramming efficiency (Esteban et al, 2010).

3.1.1 Hypothesis

Reducing environmental oxidative stress will have a beneficial effect on oral mucosal lamina propria progenitor cells (and thereby provide a better candidate cell population for cellular reprogramming).

3.1.2 Aims

There are three main aims for the characterisation of the OMLP-PC cells during culture with/without the antioxidant ascorbic acid:

1. To establish the colony forming efficiency of these cells when treated/untreated
2. To determine the population doubling level of each of the isolated cell populations when treated/untreated
3. To determine the baseline expression of pluripotency markers in each of the treated/untreated cell populations

Together these data will be used to determine the best population of cells to target for reprogramming.

3.2 Methods

A number of general methods (Chapter 2) were used in the characterisation of cell populations of the oral mucosa: Differential adhesion assay, oral mucosa lamina propria (OMLP) cell culture, cryopreservation and re-establishment of cells from the OMLP, dermal fibroblast cell culture, formation of feeder layers, mitotic inactivation of MEFS using γ-irradiation, human embryonic stem cell (hESC) culture, cryopreservation and re-

establishment of hESCs, mycoplasma testing, imaging of cells, extraction of total RNA, generation of cDNA by reverse transcription, amplification of cDNA by PCR, agarose gel electrophoresis, sequencing of PCR products and statistical analysis.

Two OP cell populations were developed; (i) individual colonies were left to merge together and (ii) colonies were individually isolated and maintained in monolayer culture to give a population of colonies from a single OP cell (colonies). Colonies were indicated by a number before the patient number (e.g. 5 XLIX is colony 5 from patient XLIX). From here on MP refers to a cell population of colonies that have merged together.

Cell Population of the oral mucosa lamina propria	**Abbreviation**
Merged oral progenitor cells	MP
Total cell population	TP
Non-adherent cell population	NA
Colonies	e.g 5 XLIX

3.2.1 Treatment of Oral Mucosa Lamina Propria Cell Cultures with Ascorbic Acid

Cells were isolated from buccal mucosa biopsies as described in Chapter 2 (Section 2.2.1.1). At the point of isolation, duplicate cell cultures were set up for the three cell populations; OP, TP and NA. One was maintained in F-

SCM, the other in F-SCM +50µg/mL Ascorbic Acid (Sigma, UK) so that cells were cultured and expanded directly from the digested lamina propria either with or without treatment with ascorbic acid. Medium was replaced daily for each cell culture. Colonies were isolated and expanded from each of the treated and untreated OP cultures as previously described in Chapter 2 (Section 2.2.1.2) and maintained ± ascorbic acid. Alternatively, treated and untreated populations of OP cultures were allowed to merge (MP) and again, maintained ± ascorbic acid. In summary, this experiment consisted of four cell populations, MP, TP, NA and isolated colonies, each of these cultures were treated or untreated with ascorbic acid and set up in triplicate.

3.2.2 Colony Forming Efficiency

Cells that attached during the 20 minute adhesion step and both 40 minute adhesion steps of the differential adhesion assay (Section 2.2.1.1.2) were counted after 24 hours to determine the number of cells that adhered in each well (n=3 wells for each patient sample). This was carried out with cell cultures ± ascorbic acid. For all subsequent counts after day 1, the number of colonies formed in each well was determined whereby only colonies > 32 cells were counted to ensure exclusion of transit-amplifying cells that terminally differentiate after 5 population doublings (Jones & Watt, 1993). Colonies were counted in each well on days 3, 6, 9, 12 and 15 and the colony forming efficiency (CFE) was calculated using:

$$\text{CFE} = \frac{\text{Number of colonies formed >32 cells}}{\text{Initial number of adherent cells}}$$

3.2.3 Calculation of Population Doubling Levels

Population doubling levels (PDLs) were plotted against time to compare the proliferative potential of the different cell populations (MP, TP, NA and colonies) from the oral mucosa. At each passage, cell numbers were determined using a haemocytometer, the number of cells reseeded was also recorded. PDL was calculated using:

$$PDL = \frac{\log_{10}(\text{total cell count obtained}) - \log_{10}(\text{total cell count re-seeded})}{\log_{10}(2)}$$

3.2.4 End-point PCR Analysis of Pluripotency Markers

PCR was carried out as described in Chapter 2 (Section 2.2.2.6) using primers specific to pluripotency markers and a house keeping gene (Table 3.1). These were the 6 factors that had been used in various combinations for the purpose of reprogramming cells to iPSCs (Takahashi et al, 2007; Yu et al, 2007, Yu et al, 2009).

3.2.5 Quantitative analysis of Pluripotency Markers

cDNA (prepared as in Chapter 2: Section 2.2.2.5) from the cell populations isolated from patients XLIX, L, LII and dermal fibroblasts samples were used to establish the level of pluripotency gene expression in relation to hESCs (H9). Taqman® Gene Expression Assays for Oct-4, Sox-2, Nanog, Klf-4 and C-myc were utilised (Table 3.2). GAPDH was used as a house keeping gene. A standard curve method was employed to ensure linear reactions of the target genes of interest. H9 cDNA was used as a standard and ten-fold

serial dilutions were carried out (namely 1/10, 1/100, 1/1000, 1/10000 and 1/100000). Gene expression assays and cDNA samples were thawed on ice. TaqMan® Gene Expression Master Mix (containing: AmpliTaq Gold® DNA Polymerase (Ultra Pure), Uracil-DNA Glycosylase, dNTPs with dUTP, Passive Reference dye ROX and invitrogen optimized mix components) was swirled to mix the contents. The number of required reactions was calculated with each sample being assessed in triplicate. For each reaction the following was mixed in a sterile eppendorf: 10µL Expression Master mix, 8µL RNase free water and 1µL Taqman Gene Expression Assay. This was mixed thoroughly and 19µl was added to the relevant wells of a white qPCR 96 well plate (Primer Design, UK). One µl of serially diluted standard or sample cDNA (at 1/10 dilution used to ensure levels were within the linear range of the standard curve) was added to the relevant wells of the 96 well plate. The plate was sealed with an adhesive optical seal (PrimerDesign, UK) and spun at 800 x g for 5 minutes. The 96 well plate was then loaded into the qPCR machine (Applied Biosystems 7000). There was an initial denaturation step at 95°C for 10 minutes before amplification for 40 cycles, each cycle consisting of a 95°C denaturation step for 15 seconds then an annealing step at 60ºC for 30 seconds. When the reaction was complete, an appropriate threshold value was selected before analysis of cycle numbers (Ct) was made. The $2^{-\Delta\Delta Ct}$ method was adopted to analyse the relative quantities of gene expression (Livak and Schmittgen, 2001).

Table 3.1: ***Forward and reverse primers for target genes***; Oct-4, Sox-2, Nanog, Lin-28, Klf-4, C-myc and the house keeping gene β-actin. Annealing temperature and product length is demonstrated for each gene of interest.

Gene	Accession Number	Primer Sequence	Position (bp)	T anneal (°C)	Product Length (bp)
β-actin	NM_001101.3	F 5'-CCACACTGTGCCCATCTACGAGGGGT-3' R 5'-AGGGCAGTGATCTCCTTCTGCATCCT-3'	564 – 589 1043 - 1018	60	454
Oct-4	NM_002701.4	F 5'-AGGAGTCGGGGTGGAGAG-3' R 5'-CGTTTGGCTGAATACCTTCC-3'	351 – 368 600 - 581	54	249
Sox-2	NM_003106.2	F 5'-AACCCCAAGATGCACAACTC-3' R 5'CGGGGCCGGTATTTATAATC-3'	614 – 633 765 - 746	55	152
Nanog	NM_024865.2	F 5'-ATAACCTTGGCTGCCGTCTCTGG-3' R 5'-AAGCCTCCCAATCCCAAACAATACG-3'	1677-1699 1826-1802	44	150
Lin-28	NM_024674.4	F 5'-GAAGCGCAGATCAAAAGGAG-3' R 5'-GGGTAGGGCTGTGGATTTCT-3'	504 – 523 721 - 702	53	218
Klf-4	NM_004235.4	F 5'-ACCCACACAGGTGAGAAACC-3' R 5'ATGTGTAAGGCGAGGTGGTC-3'	1849 – 1868 2018 - 1999	55	149
C-myc	NM_002467.4	F 5'-CCCTTGCCGCATCCACGAAACT-3' R 5'-AAGGGAGAAGGGTGTGACCGCA-3'	341-362 816-795	50	476

Table 3.2: ***Invitrogen Taqman® Gene Expression Assays for target genes;*** Oct-4, Sox-2, Nanog, Klf-4, C-myc and GAPDH detailing accession number, primer location and product size.

Invitrogen ID	**Gene**	**Accession Number**	**Assay location (bp)**	**Size (bp)**
Hs00999634_gH	Oct-4	NM_002701.4	872	64
Hs01053049_s1	Sox-2	NM_003106.3	1541	91
Hs02387400_g1	Nanog	NM_024865.2	363	109
Hs00358836_m1	Klf-4	NM_004235.4	723	110
Hs00905030_m1	C-myc	NM_002467.4	561	87
Hs03929097_g1	GAPDH	NM_002046.4	1226	58

3.3 Results

3.3.1 Phenotypic Analysis of Cell Populations from the Oral Mucosa

OP cells from digested 6mm biopsies of the lamina propria were isolated by differential adhesion to fibronectin after a 20 minute incubation step and maintained in monolayer culture. A further two incubation steps (40 minutes each) followed this. Cells that failed to adhere to the fibronectin in these steps were maintained in a separate monolayer culture, the NA cells. A third monolayer (unselected) culture was maintained; the TP cells of the digested lamina propria. Figure 3.1 demonstrates the formation of a single colony following adhesion to fibronectin-coated plates.

3.3.1.1 The Effects of Vitamin C on Colony Forming Efficiency

Two sets of cultures were set up for each of the 3 differential adhesion steps (20 minute step and two 40 minutes steps) for cells from three patients (XLIX, LII and LIV) so that cell cultures could be maintained in F-SCM ± 50µg/mL ascorbic acid. Triplicate wells were prepared for each condition. Colony forming efficiency (CFE) and the effects of ascorbic acid on OP cell populations was determined over a 15 day period. Initial numbers of cells that adhered to the fibronectin coated plates for each time step (20 minute well, 40 minute well and second 40 minutes well) were determined on day 1 and colonies > 32 cells were counted for each time step on days 3, 6, 9, 12 and 15.

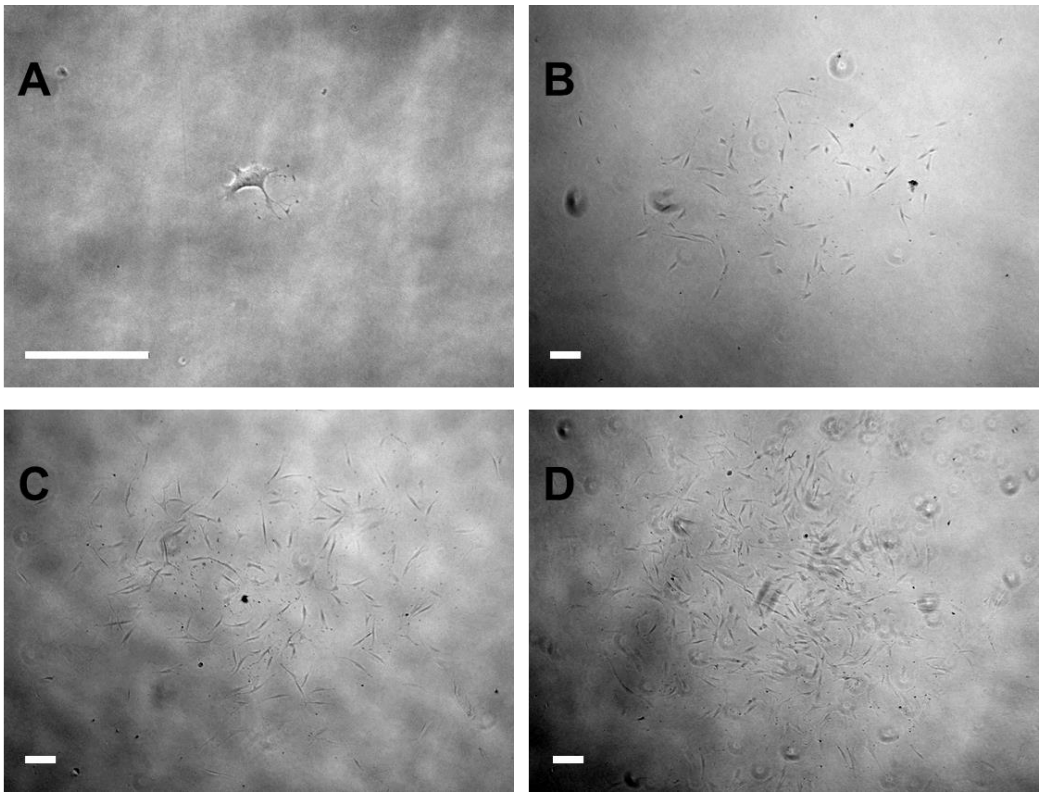

Figure 3.1: ***Example of colony formation in Patient LIV***. **(A)** Day 1 following adhesion to fibronectin-coated plates after the first 20 minute time point, **(B)** Day 7 post-differential adhesion, **(C)** Day 9 post-differential adhesion, **(D)** Day 13 post-differential adhesion. Scale bar = 100µm

Days 6, 9 and 12 were used to make comparisons between treated and untreated cultures (Figure 3.2) as colonies had not formed in either culture by day 3 and on day 15, colonies of untreated cells had merged and could no longer be distinguished and counted as separate colonies. Formation of colonies in cultures treated with ascorbic acid was much slower and significantly less (p<0.001) on days 9 and 12 compared to untreated cell cultures.

When comparing the difference in colony formation of each of the different time steps of the differential adhesion assay (20 minute and both 40 minute time steps), for treated and untreated cells, day 12 was used. After this time, colonies of untreated cells merged in the 20 minute wells. Comparisons between treated and untreated cells in the 20 minute adhesion wells on day 12 have already been described above and demonstrated a significant difference. However, differences between treated and untreated cultures in both 40 minute wells of the differential adhesion assay did not demonstrate a significant difference (Figure 3.3).

CFEs for both treated and untreated cultures were highest in the 20 minute well when compared to CFEs in both 40 minute wells of the differential adhesion assay (Figure 3.3). The differences in CFEs were only found to be significant in the untreated cell populations, with differences between the 20 minute time step and the first 40 minute step demonstrating a significant difference (p<0.05) and the difference between the 20 minute step and the second 40 minute step demonstrating a highly significant difference (p=<0.001).

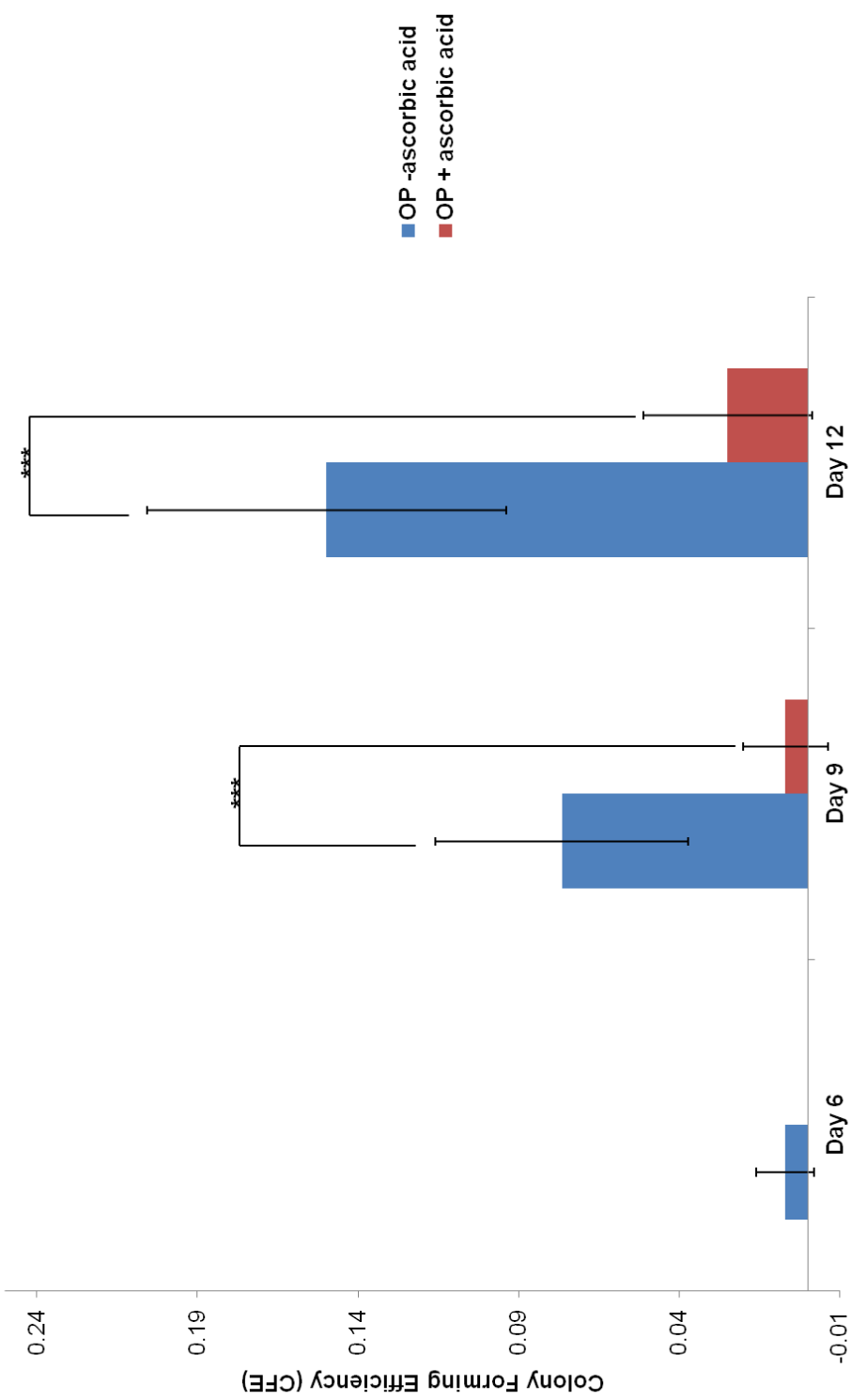

Figure 3.2: *CFE data for Patient XLIX, LII and LIV ± 50µg/mL ascorbic acid.* Cultures were fed daily ± ascorbic acid and colonies >32 cells were counted. Days 6, 9 and 12 are shown *n*=3±SEM *** =P<0.001. F value= 23.644. Degrees of freedom=89

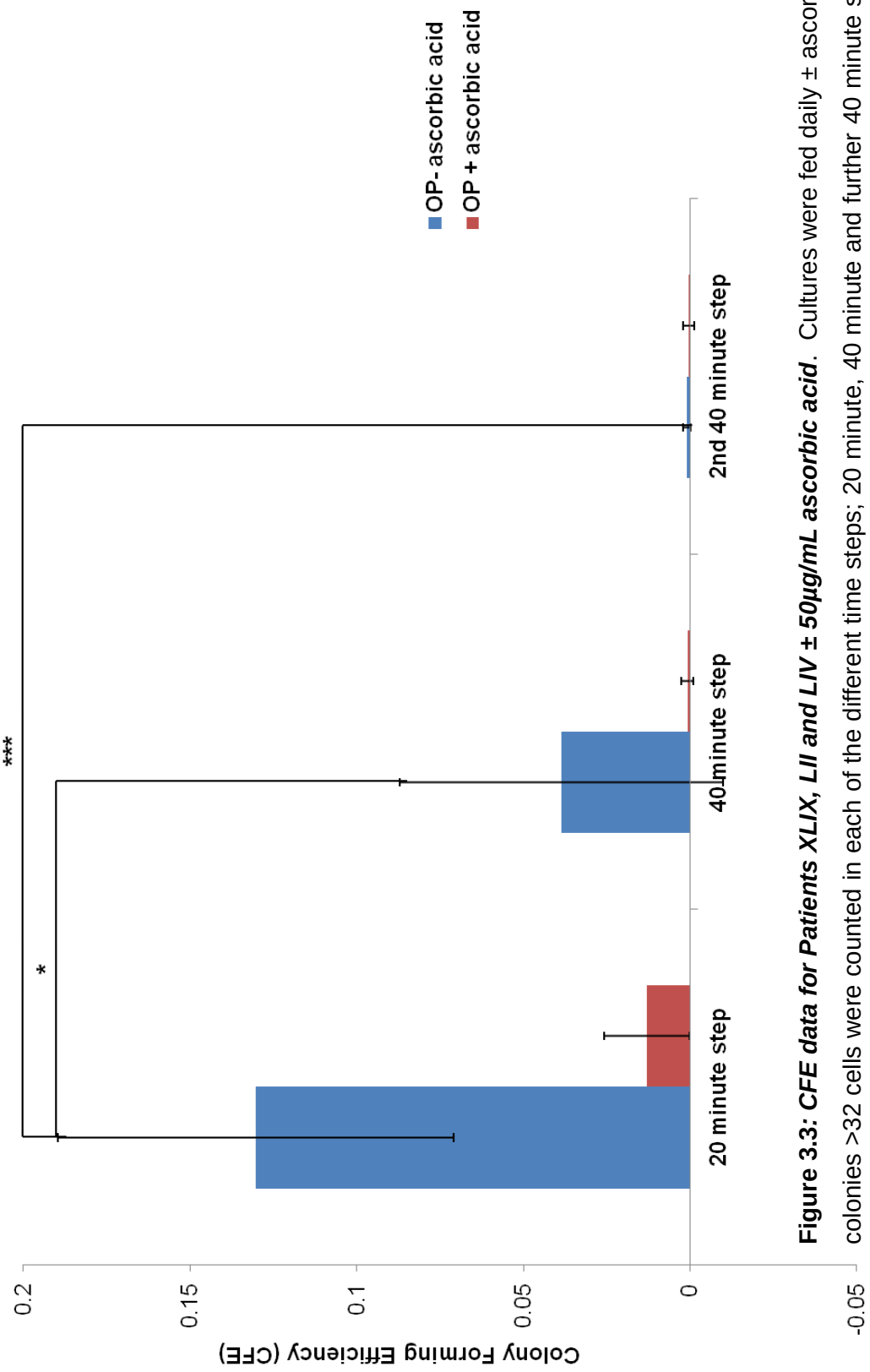

Figure 3.3: *CFE data for Patients XLIX, LII and LIV ± 50µg/mL ascorbic acid*. Cultures were fed daily ± ascorbic acid and colonies >32 cells were counted in each of the different time steps; 20 minute, 40 minute and further 40 minute step. Day12 is used to make comparisons. *n*=3 ± SEM * =P< 0.05 ***= P<0.001 F value = 23.644. Degrees of freedom=89

3.3.1.2 The Effects of Vitamin C on Cell Proliferation

Separate cultures were maintained for each cell population (MP, TP and NA), one culture fed with F-SCM and one with F-SCM + 50µg ascorbic acid. Each culture was fed daily due to the degradation of ascorbic acid when dissolved in medium. Cultures were established from which to pick colonies and were also maintained ± 50µg/mL ascorbic acid. Following isolation of the individual colonies, 3 colonies were maintained for longer periods of time in culture ± ascorbic acid.

Growth profiles were established for all cell populations (MP, TP, NA and colonies) isolated from the oral mucosa lamina propria for three patients; XLIX, L and LII. Growth profile of MP cells for patient XLIX demonstrated the fastest initial rate in comparison to TPs and NAs (Figure 3.4A). This was followed by TP cells which demonstrated a sharp increase in proliferative rate after about 20 days in culture and NA cells demonstrated the lowest proliferative rate. A similar growth profile was demonstrated with the different cell populations (MP, TP and NA) of patients L (Figure 3.5A) and LII (Figure 3.6A). When comparing the different growth profiles ofthe cell populations of XLIX treated with ascorbic acid, the same pattern of growth existed between MP, TP and NA cell populations of untreated cells (i.e. MP+ascorbic acid >TP+ascorbic acid >NA+ascorbic acid) and no difference between treated and untreated MP, TP and NA cell populations was demonstrated. Again, a similar pattern existed with respect to the treated cell populations of patient L and no difference was observed between the treated and untreated MP, TP and NA cell populations.

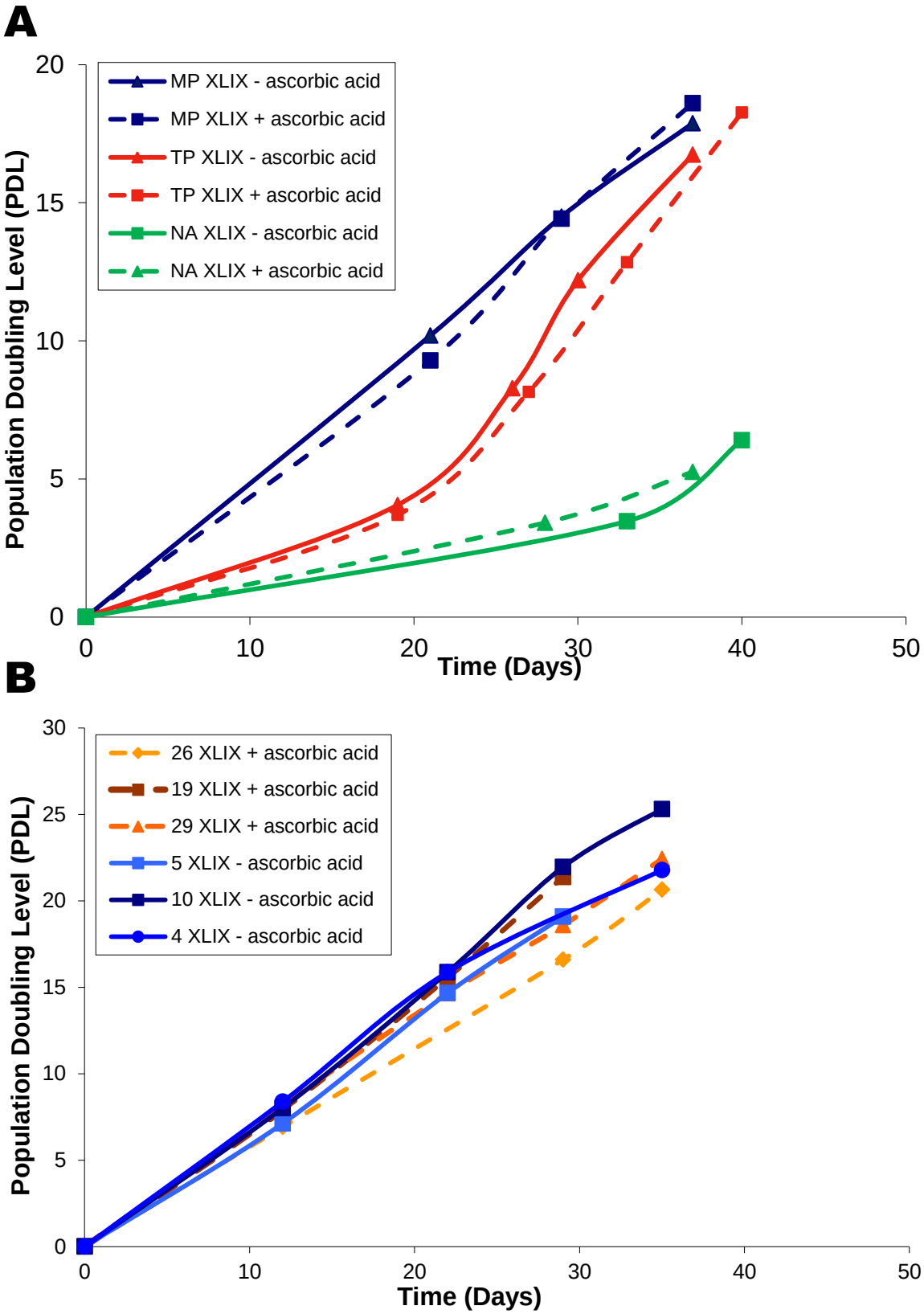

Figure 3.4: ***Growth profiles for patient XLIX ± 50µg ascorbic acid treatment.*** **(A)** Growth profiles of OP, TP and NA ± ascorbic acid. **(B)** Growth profiles of isolated colonies 19, 26 and 29 + ascorbic acid, 4, 5 and 10 – ascorbic acid. (*n=1*)

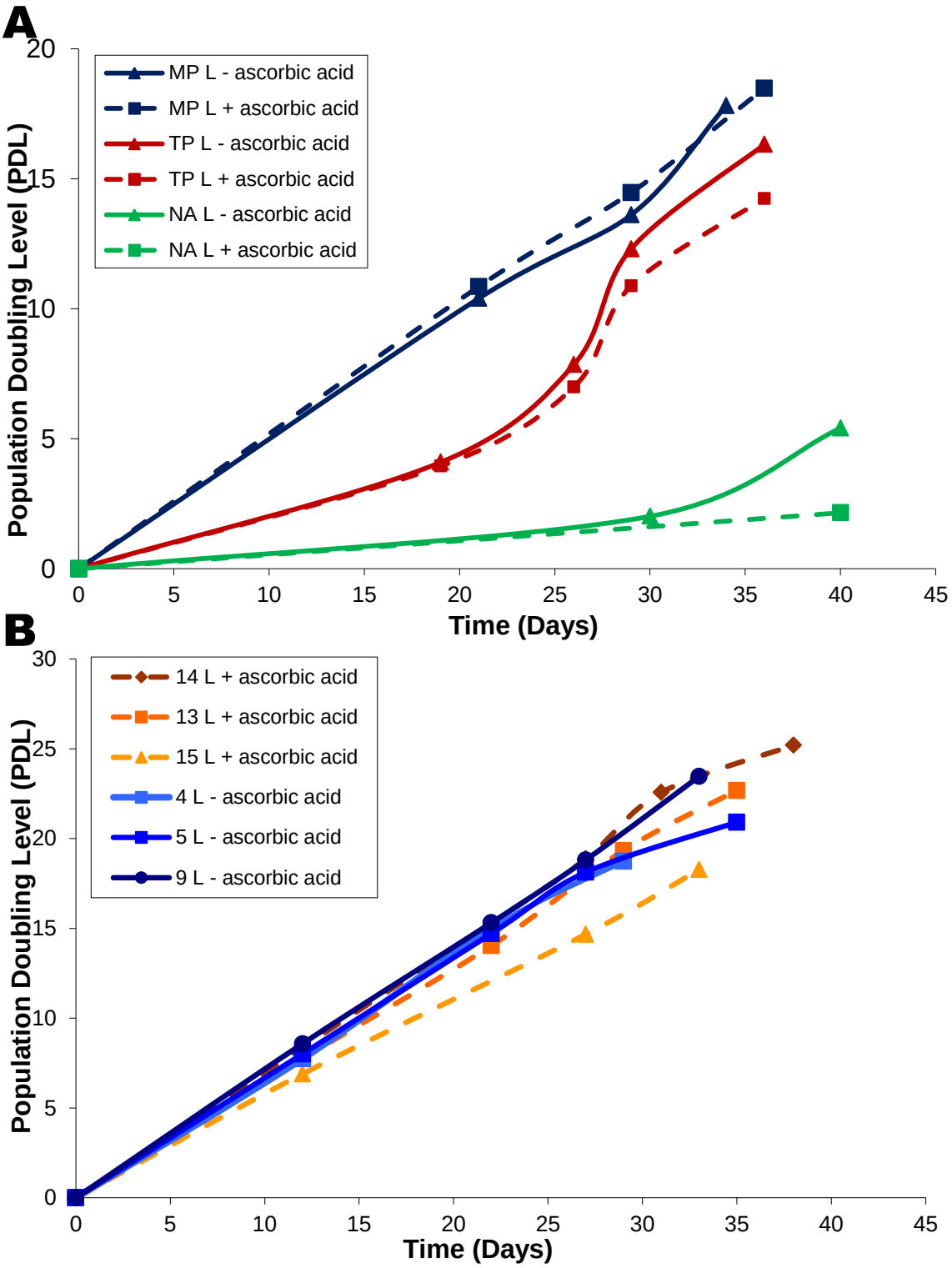

Figure 3.5: ***Growth profiles for patient L ± 50µg ascorbic acid treatment.*** **(A)** Growth profiles of OP, TP and NA ± ascorbic acid. **(B)** Growth profiles of isolated colonies; 13, 14 and 15 + ascorbic acid, 4, 5 and 9 – ascorbic acid. (*n=1*)

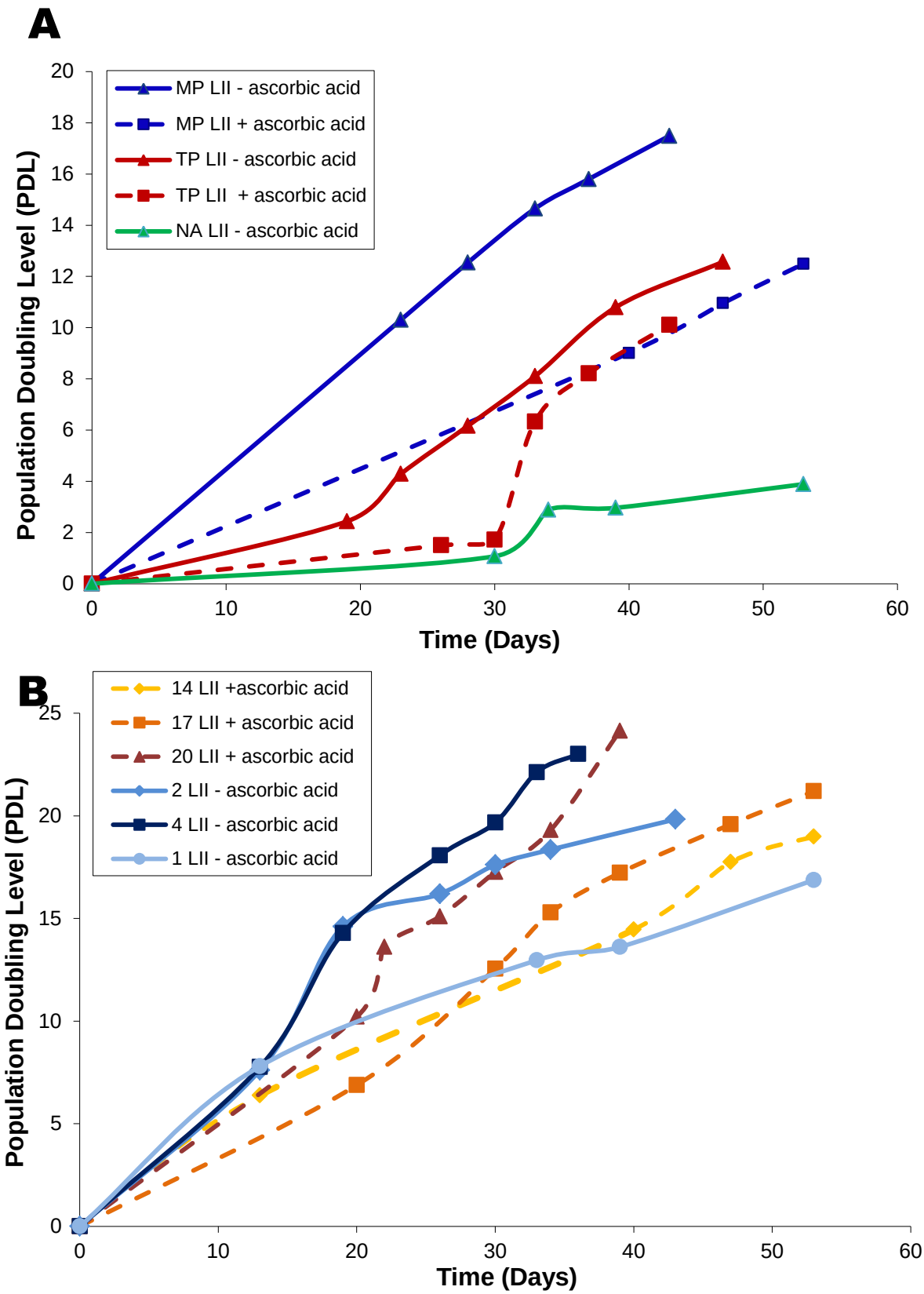

Figure 3.6: ***Growth profiles for patient LII ± 50µg ascorbic acid treatment.*** **(A)** Growth profiles of OP, TP and NA ± ascorbic acid. **(B)** Growth profiles of isolated colonies; 14, 17 and 20 + ascorbic acid, 1, 2 and 4 – ascorbic acid. (*n=1*)

Cell populations of patient LII treated with ascorbic acid demonstrated different growth profiles compared to the untreated cell populations. Treated cell populations typically demonstrated slower rates of proliferation when compared to untreated cell populations, with NA+ascorbic acid failing to grow. Treated TP cells demonstrated a sharp increase in growth after 30 days in culture with, at this point, them demonstrating a similar PDL as untreated TP populations. Over the time course studied, treated MP demonstrated a much slower initial growth rate compared to untreated MP. Overall, across the three patients there was no consistent alteration in the growth profiles of the treated cell populations compared to untreated cells populations.

Growth profiles of the colonies isolated from cultures ± ascorbic acid and then maintained ± ascorbic acid from patient XLIX all demonstrated similar growth patterns (Figure 3.4B). The isolated colonies from patient L, again ± ascorbic acid, and as with patient XLIX, all demonstrate similar growth patterns with no obvious differences between cells treated with ascorbic acid (Figure 3.5B). Colonies isolated and maintained ±ascorbic acid from patient LII demonstrate some variation in growth profiles but with no obvious difference between treated and untreated cell populations (Figure 3.6B).

3.3.1.3 Expression of Pluripotency Markers in Different Cell Populations Obtained from the Oral Mucosa

RNA was extracted from MP, TP and NA cell populations of three patients XLIX, L and LII, all ±ascorbic acid, at early time points (between PDL 10-15) to determine which pluripotency markers were being expressed by each cell

population and whether the addition of ascorbic acid to the cultures was affecting this expression. The pluripotency markers analysed were Oct-4, Sox-2, Nanog, Lin-28, Klf-4 and C-myc as these are the classical induced pluripotent stem cell (iPSC) factors used to reprogram cells back to their embryonic stem cell-like state (Takahashi et al, 2007; Yu et al 2007). For each gene, RNA extracted from skin fibroblasts (DF) was utilised as a comparative control as these cells have been classically used to reprogram back to an embryonic stem cell state. DFs demonstrated positive expression of all genes (Figures 3.7-3.9). The positive control, human embryonic stem cell (H9), as expected, demonstrated positive expression of all pluripotency markers. Two negatives were used, a reverse transcriptase negative and a DEPC water negative (DEPC).

All treated and untreated cell populations for all three patients were found to express Oct-4, Klf-4, C-myc and Nanog (Figures 3.7-3.9). However, expression of Lin-28 and Sox-2 demonstrated patient-specific differences. Untreated MP and TP cell populations express Sox-2 in all three patients. However, expression of Sox-2 was absent in the treated MP cell population of patient XLIX and NA of patient L and absent in both treated and untreated NA cell populations of patient XLIX. Expression of Lin-28 was absent in all treated and untreated cell populations of patient XLIX, patient L with the exception of treated MP and patient LII with exception of untreated NA. Authenticity of the PCR products was confirmed by DNA sequencing (Appendix II).

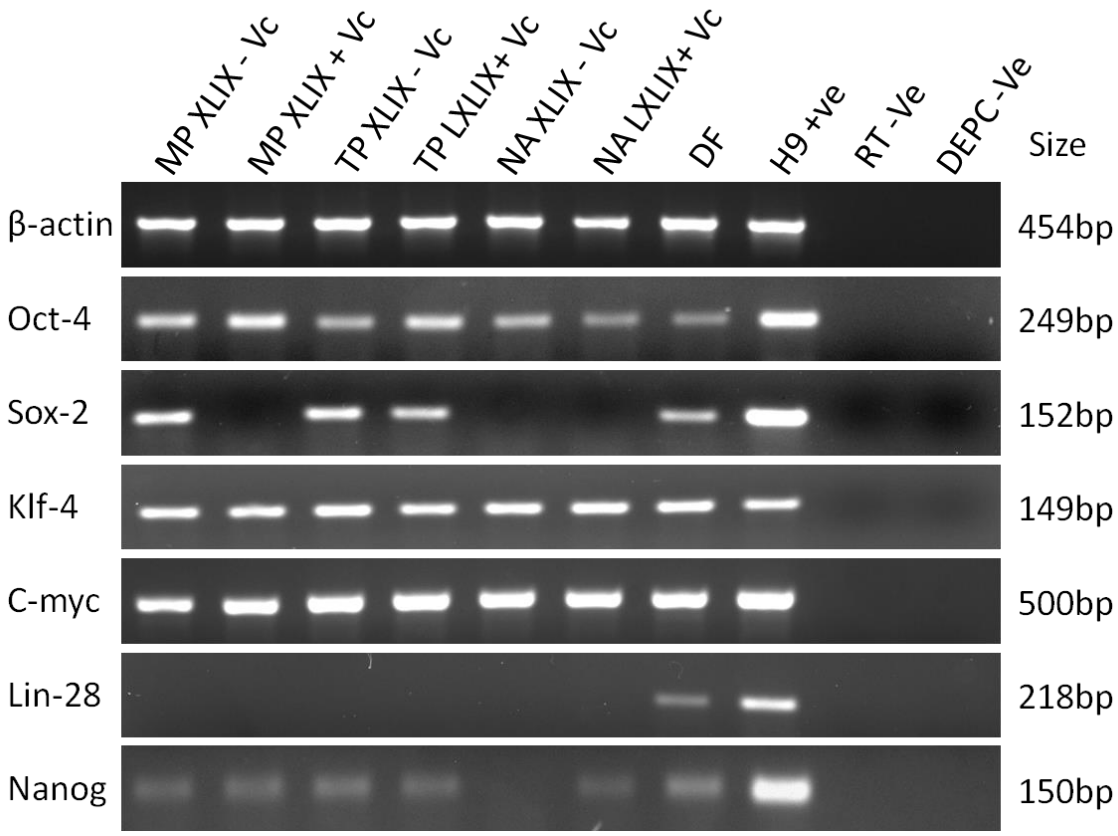

Figure 3.7: ***Pluripotency marker expression for different populations from the OMLP ± 50µg/mL ascorbic acid at early time points.*** Expression of pluripotency markers; Oct-4, Sox-2, Klf-4, C-myc and Lin-28 for Patient XLIX MP, TP ± ascorbic acid and NA+ ascorbic acid between PDL 10-15.

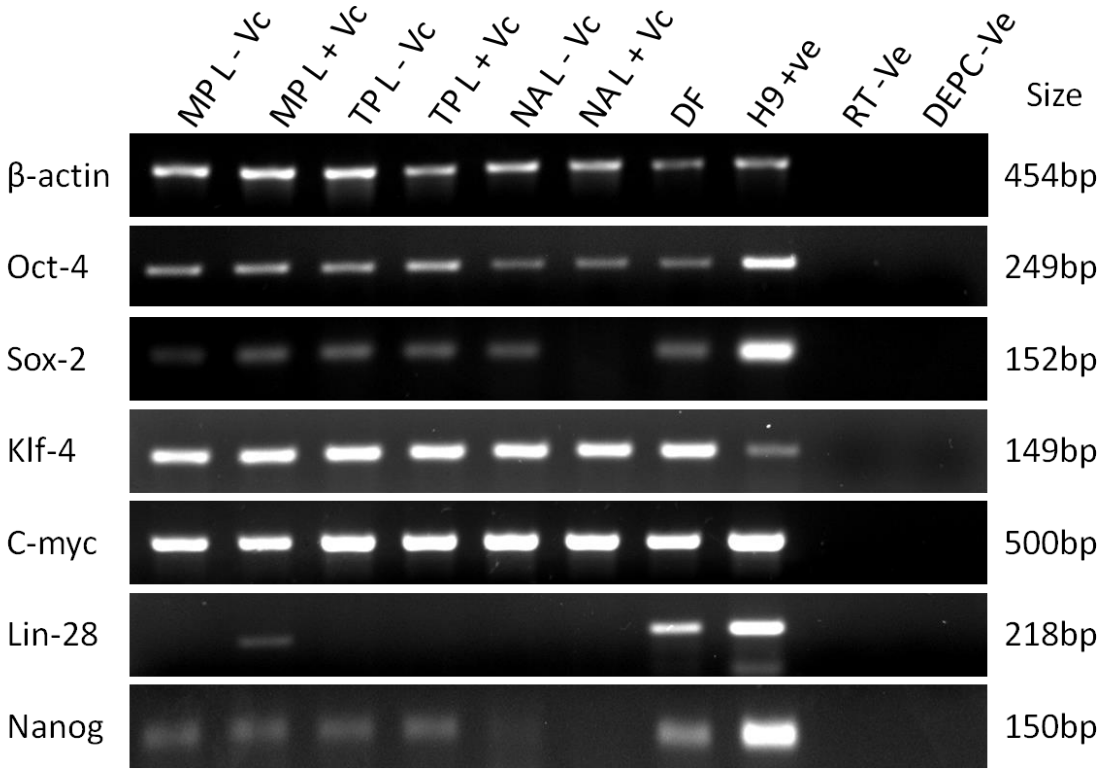

Figure 3.8: ***Pluripotency marker expression for different populations from the OMLP ± 50µg/mL ascorbic acid at early time points.*** Expression of pluripotency markers; Oct-4, Sox-2, Klf-4, C-myc and Lin-28 for Patient L MP, TP ± ascorbic acid and NA ± ascorbic acid between PDL 10-15.

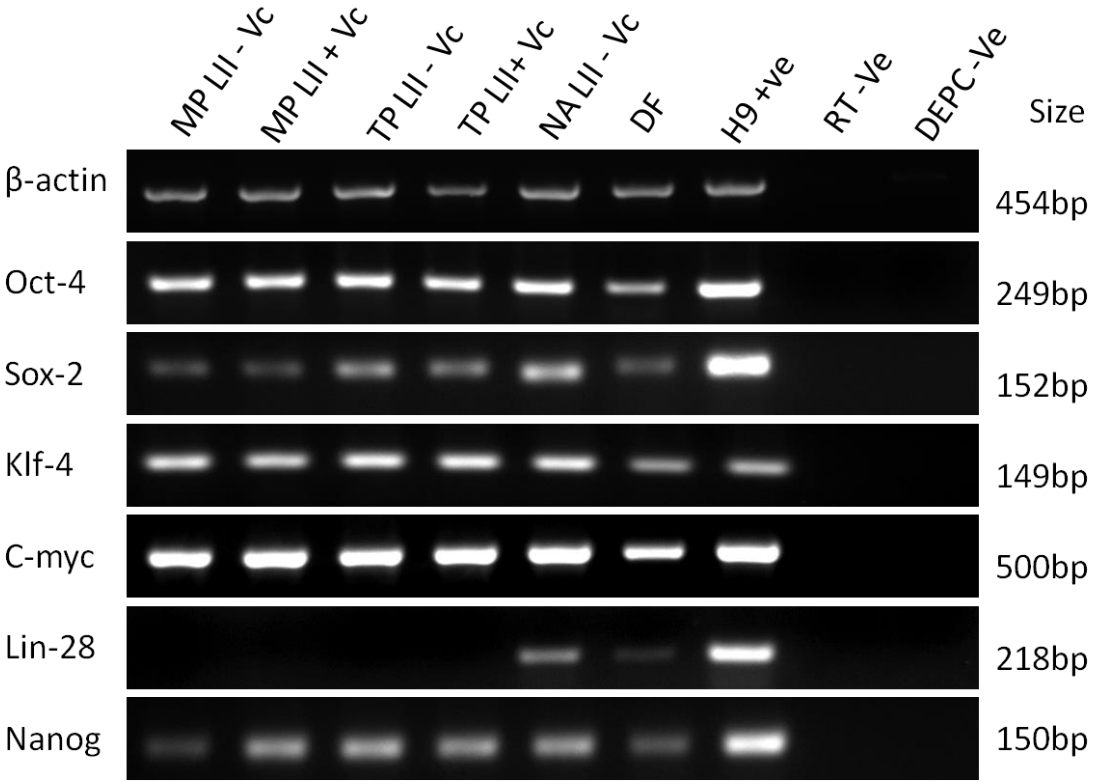

Figure 3.9: ***Pluripotency marker expression for different populations from the OMLP ± 50µg/mL ascorbic acid at early time points.*** Expression of pluripotency markers; Oct-4, Sox-2, Klf-4, C-myc and Lin-28 for Patient LII MP, TP ± ascorbic acid and NA ± ascorbic acid between PDL 10-15.

3.3.1.4 Quantitative analysis of Pluripotency Markers

Endpoint PCR analysis is useful as an indication of presence or absence of target genes however, it does not provide quantitative information. Quantitative PCR allows confirmation of expression levels of target genes compared to a calibrator known to express the genes of interest. H9 were utilised as a calibrator as they are known to express all the pluripotency genes of interest (see Figures 3.7-3.9). Untreated MP cell populations from patients XLIX, L and LII were selected for reprogramming and further analysis by qPCR as treatment of cell populations with ascorbic acid appeared to offer no benefit in terms of proliferative potential and expression of pluripotency markers. Before relative quantities of the pluripotency genes could be determined, a standard curve was established using serial dilutions of the calibrator to determine whether the reaction was linear or not. Each reaction was linear (Figure 3.10A) confirming the relative quantities could be determined between H9 and the patient samples. Expression of Oct-4, Sox-2 and Nanog was found to be significantly ($P<0.001$) lower in MP XLIX, MP L, MP LII and DF (Figures 3.10B, 3.11A and 3.12A). Expression of C-myc was also found to be significantly lower in MP XLIX, MP L and MP LII ($P<0.001$) however, expression levels were higher in DF and, therefore, the difference between C-myc expression in H9 and DF was less significant than in OP samples (Figure 3.11B, $P<0.01$). Expression of Klf-4 was the only gene that was found to be expressed at a higher level in MP samples and DF when compared to H9 (Figure 3.12B).

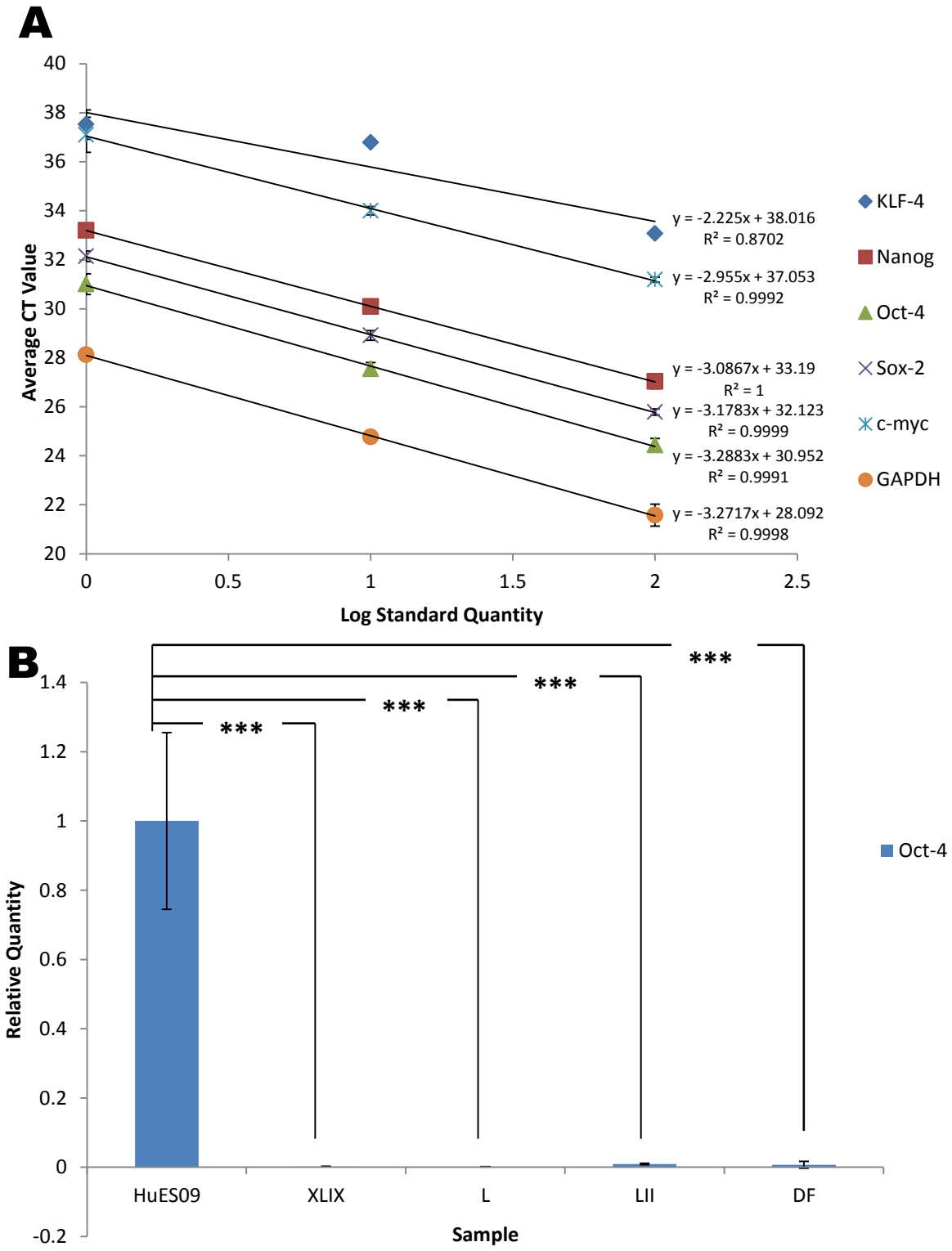

Figure 3.10: ***Quantitative PCR comparing pluripotency marker expression levels of OMLP and DF with hESCs.*** **(A)** Standard curve data for genes of interest; Oct-4, Sox-2, Nanog, Klf-4, C-myc and the house keeping gene GAPDH demonstrating linear reactions *n*=3 ± SEM. **(B)** Relative expression levels of Oct-4 in H9, MP XLIX, MP L, MP LII and DF *n*=3 ±SEM ***P<0.001. F value=47.512. Degrees of freedom 14

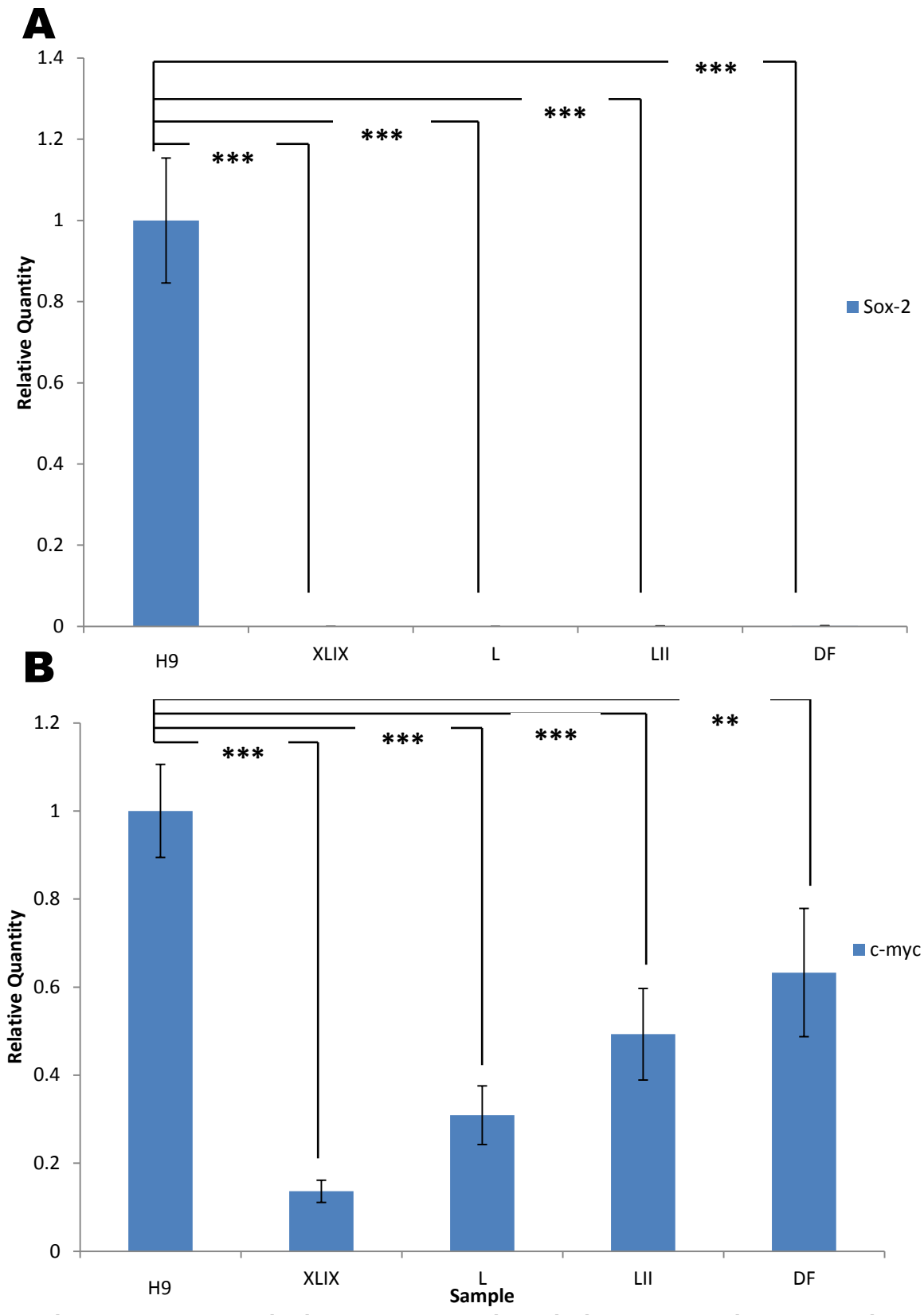

Figure 3.11: ***Quantitative PCR comparing pluripotency marker expression levels of OMLP and DF with hESCs.*** **(A)**. Relative expression levels of Sox-2 in H9, MP XLIX, MP L, MP LII and DF *n*=3 ±SEM ***P<0.001. F value=128.33. Degrees of freedom 14. **(B)** Relative expression levels of C-myc in H9, MP XLIX, MP L, MP LII and DF *n*=3 ±SEM ***P<0.001 **P<0.01. F value=34.069. Degrees of freedom 14

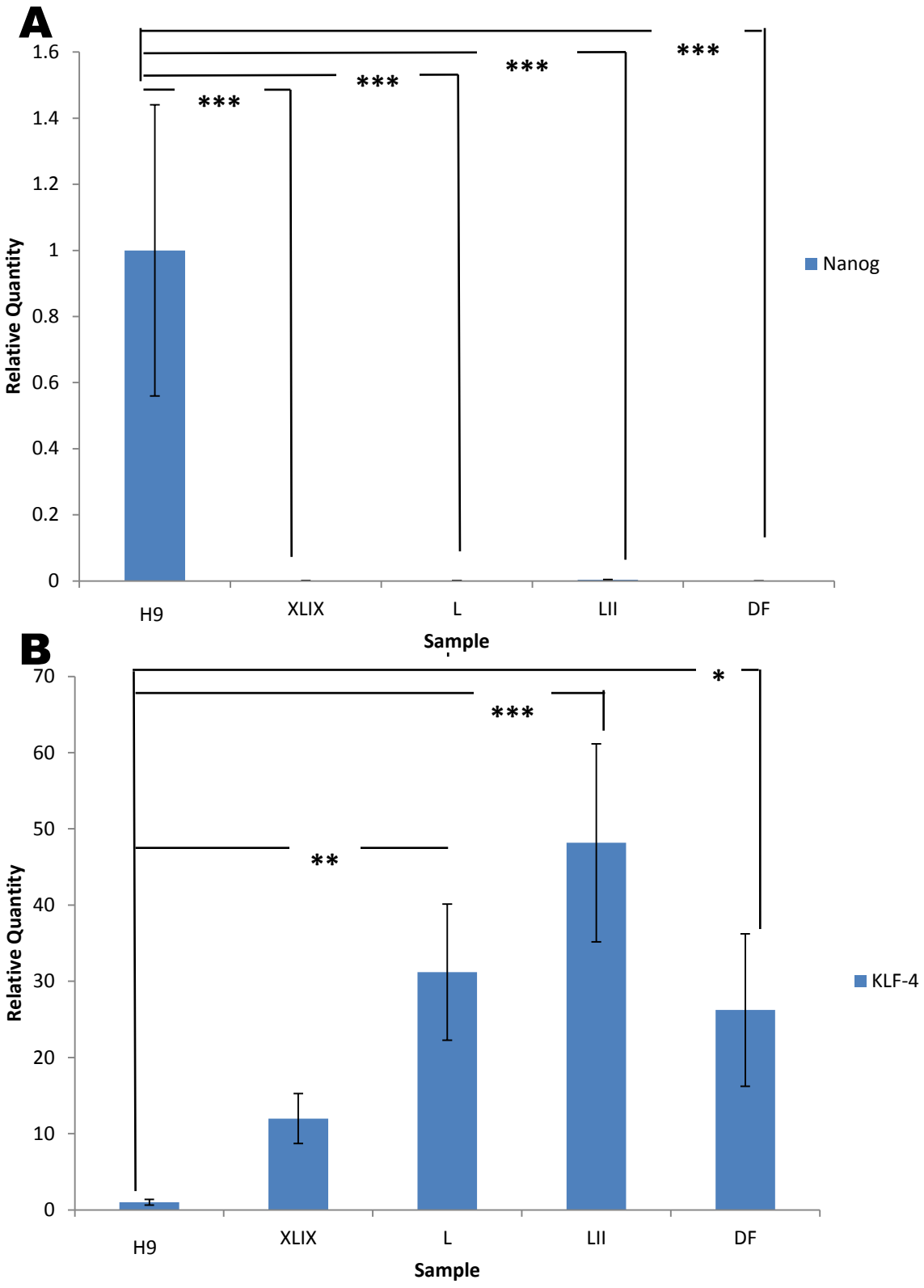

Figure 3.12: ***Quantitative PCR comparing pluripotency marker expression levels of OMLP and DF with hESCs.*** **(A)** Relative expression levels of Nanog in H9, MP XLIX, MP L, MP LII and DF *n*=3 ±SEM ***P<0.001. F value=17.347. Degrees of freedom 14. **(B)** Relative expression levels of Klf-4 in H9, MP XLIX, MP L, MP LII and DF *n*=3 ±SEM ***P<0.001 **P<0.01 *P<0.05. F value=14.414. Degrees of freedom 14

For Klf-4 the most significant difference was demonstrated between LII and H9 ($P<0.001$), the difference between patient L and H9 and DF and H9 was less significant ($P<0.01$ and $P<0.05$ respectively); the difference between patient XLIX and H9 was not significant. Quantitative PCR product sizes were confirmed utilising gel electrophoresis (Appendix III).

3.4 Summary

- Ascorbic acid reduces the CFE of OMLP-PCs
- Ascorbic acid has no effect on proliferation of cell populations of the OMLP
- Addition of ascorbic acid does not change expression of pluripotency markers at end point PCR level
- With the exception of Klf-4, pluripotency markers are expressed at lower levels compared to H9 cells

3.5 Discussion

Many different cell populations exist within each tissue of the body and progenitor cell populations have been successfully isolated from a variety of different tissues including the epidermal layer of the skin (Jones and Watt, 1993) and articular surface cartilage (Dowthwaite et al, 2004). Similarly, progenitor cells have been isolated from various dental tissues including; dental pulp, deciduous teeth, dental follicle, periodontal ligament, apical papilla and gingival (Gronthos et al, 2000; Miura et al, 2003; Seo et al, 2004; Morsczeck et al, 2005; Sonoyama et al, 2008; Zhang et al, 2009). However, oral progenitor cells isolated from the oral mucosa present an attractive source of progenitor cells due to the fact that they are easily accessible via a relatively non-invasive procedure and such biopsying of tissue generates wounds that will heal with little to no scarring (Stephens and Genever, 2007).

The investigations in this chapter have been carried out to establish the effects of ascorbic acid on cell populations isolated from the OMLP as one of the aims of this thesis is to generate oral iPSCs. Recent reports suggest that iPSC reprogramming efficiency is improved in cultures supplemented with ascorbic acid (which acts as an antioxidant, reducing levels of oxidative stress; Esteban et al, 2010). Reducing oxidative stress has been known for some time to support embryonic stem cell growth as culturing these cells in hypoxic conditions is thought to mimic the natural growth conditions that embryonic stem cells would be subjected to *in vivo* (Ezashi et al, 2005). The addition of antioxidants, such as ascorbic acid, is thought to work in a similar

way by reducing the oxidative stress that has been found to affect different aspects of cell behaviour (Reviewed by Halliwell, 2003).

3.5.1 The Effects of Vitamin C on Colony Forming Efficiency

Colony forming efficiency of cells has long been used as a way of discriminating between terminally differentiated transit-amplifying cells and populations of potential progenitor cells. This is due to the increased CFE of progenitor cells when compared to such transit-amplifying cells. These transit amplifying cells can only typically undergo 5 population doublings before becoming terminally differentiated (Jones and Watt, 1993). Cells with the highest level of CFEs have also been found to have the greatest number of functional β1-integrins (Jones and Watt, 1993) which underpins the rationale for the isolation of OP cells from the oral mucosa using differential adhesion to fibronectin (Davies et al, 2010). This assay is based on the fact that progenitor cells have been found to have increased levels of functional β1 integrins on their cell surface in comparison to terminally differentiated cells allowing these progenitors to adhere preferentially to fibronectin in a shorter time-frame. This supports the results demonstrated in this study due to the fact that the cells that adhere within the first 20 minute step of the differential adhesion assay have the highest CFE, similar to previous studies on the isolation of progenitor cells from skin and cartilage (Jones and Watt, 1993; Dowthwaite et al, 2004). The same result was also observed in OPs treated with ascorbic acid however, CFEs were overall greatly reduced and the differences found in CFEs between each time step for the treated cells was not found to be significantly different. The findings for untreated OP

cells adds weight to the notion that the oral cells used throughout this project are progenitor cells which is in line with the findings of Davies et al (2010).

Treatment of cells with ascorbic acid resulted in a lower CFE in comparison to untreated cells at the final time point where comparisons could be made. This could potentially be explained by an initial growth arrest in these progenitor cells causing an increased lag time before colony formation begins. However, it is also possible that treatment with this concentration of ascorbic acid (the concentration suggested by the work of others; Esteban et al, 2010) could have potentially been cytotoxic at this very early time point resulting in the growth arrest observed. Interestingly, proliferation of human dermal fibroblasts at low density is inhibited following treatment with ascorbic acid (Duarte et al, 2007), whilst higher density cultures are unaffected. This is thought to be as a result of potential pro-oxidant effect of ascorbic acid in cell culture models, resulting in the production of ascorbate free radicals and H_2O_2. Additional investigations into the effects of different concentrations of ascorbic acid, by establishing a dose responsive curve, on CFE and the analysis of media components following treatment would need to be conducted in order to determine this. It is possible that addition of ascorbic acid has changed the CFE properties of these progenitor cells, modifying their stem cell characteristics however, this is purely speculative and further investigation of these cells would need to be carried out, including differentiation capacity, in order to confirm this.

3.5.2 Cell Proliferation in Different Cell Populations of the Oral Mucosa and the Effects of Vitamin C on these Populations

Growth profiles from the first 40 days of culture of all three cell populations (MP, TP and NA) isolated from the oral mucosa have demonstrated potential differences between the populations. MP cell populations for three patient samples demonstrated the fastest initial cell proliferation which was similar to previous findings whereby oral progenitor cells were found to have a rapid initial proliferation (Davies et al, 2010). The complete proliferative potential of this cell population is yet to be determined but would be expected to have a finite lifespan as previously demonstrated for clonal populations isolated from the OMLP (Davies et al, 2010) as well as mixed cell populations of the oral mucosa (Enoch et al, 2009). The proliferation of MP cell populations was closely followed by TPs for all patients. TP populations for each patient demonstrated a slower initial proliferation in comparison to MP cells however, each patient also demonstrated an increase in proliferation after approximately 25 days in culture. This may be explained by the fact that the cells have been derived from the total cell population of the oral mucosa and oral progenitors will be present within this mixed heterogeneous population, eventually becoming the predominant cell populations within the mixed culture and leading to this sudden increase in proliferation. This is supported by the fact that mixed heterogeneous cell populations, without any further isolation, have been utilised by several groups to investigate potential

progenitor cell populations within the OMLP-PC and dental pulp (Gronthos et al, 2000: Marrynka-Kalmani et al, 2010).

NA cell populations from both patients demonstrate the slowest initial proliferation which is as expected given that this cell population is a potentially 'older' (non-progenitor) cell population than that of the oral progenitor cells. This is because potential progenitor cells are removed from the NA cell population by three separate fibronectin adhesion steps. Each step demonstrates reduced CFEs indicating that very few progenitor cells would remain in final NA cell population consistent with previous findings (Jones and Watt 1993; Dowthwaite et al, 2003; Davies et al, 2010). This cell population would therefore consist mainly of transit-amplifying cells which eventually terminally differentiate reducing their proliferative potential resulting in replicative senescence at an earlier time point in comparison to oral progenitor cells (Watt, 2001). Colonies isolated from each of the three patients demonstrated similar rapid initial proliferation as the MP cell populations of all three patients, which is as expected and again these isolated colonies would be expected to have a finite lifespan with continued expansion *in vitro* consistent with the work by Davies et al, (2010). In similar studies using differential adhesion to fibronectin, dental pulp stem cells have been isolated from rat pulp, these clonal progenitor cells had PDLs of 10 over 50 days in culture (Waddington et al, 2009). In contrast, colonies from the OMLP appear to have a faster initial proliferation, undergoing approximately double the amount of PDLs in the same period. The PDLs for these cells is important when considering their potential for use in clinical applications.

As culturing cells with ascorbic acid has been reported to improve reprogramming efficiency towards iPSCs (Esteban et al, 2010), each of these cell populations (MP, TP, NA and colonies) were maintained ±ascorbic acid to determine the effects of this anti-oxidant on cell proliferation. Treated and untreated MP, TP, NA and the colonies for patients XLIX and L did not demonstrate any notable changes in their proliferative profiles *in vitro.* This is consistent with similar studies on human dermal fibroblast, where ascorbic acid did not affect the proliferation of these cells when the cells were at a high density (Duarte et al, 2007). This study was primarily investigating whether or not ascorbic acid was acting as a pro-oxidant not an anti-oxidant and interestingly another study demonstrated no difference in proliferation of MSCs when treated with low levels of H_2O_2. (Brandl et al, 2011). These studies suggest that low levels of treatment with ascorbic acid would not make a difference to the proliferative potential of cells from the OMLP. Additional investigations could be carried out for each of the individual colonies isolated from the OMLP, by culturing each colony with and without ascorbic acid which would require expanding the colony first without ascorbic acid treatment until there were sufficient cells to form two cultures. Primarily this investigation was looking at the effects of ascorbic acid from the initial isolation.

Patient LII demonstrated a longer lag in the treated growth profiles when compared to untreated populations, with treated NA cells actually failing to grow. Colonies from patient LII demonstrated the most variation in growth potential between different colonies, with no obvious pattern between treated

and untreated colonies. The general finding was that treatment with ascorbic acid had no beneficial or detrimental effect on cell growth. Analysis of additional patient samples and extended growth profile analysis would further confirm these findings.

3.5.3 The Expression of Pluripotency Markers in Different Cell Populations of the Oral Mucosa

Induced pluripotent stem cell studies have highlighted specific factors that are required to reprogram cells back to their embryonic stem cell like state. These factors include Oct-4, Sox-2, Klf-4 and C-myc and were the first factors found to reprogram adult human skin fibroblasts back to their embryonic stem cell-like state (Takahashi et al, 2007). Later it was demonstrated that the oncogene C-myc was not required and iPSC could be generated using a lentiviral system containing the factors Oct-4, Sox-2, Nanog and Lin-28 however, the reprogramming efficiency was lower (Yu et al, 2007). Subsequently, various combinations of these factors have been used in cellular reprogramming, including all 6 factors (Yu et al, 2009). It has since been reported that some cell types require much fewer factors in order to be reprogrammed; for example neural stem cells have been demonstrated to require only one factor, Oct-4, to reprogram these cells back to their ESC-like state (Kim et al, 2009).

It has previously been reported that cells within the lamina propria of the oral mucosa express some of these previously used reprogramming factors (namely Oct-4, Sox-2, Nanog and Klf-4) and that progenitor cells can be

isolated from these tissues (Davies et al. 2010; Marynka-Kalmani et al, 2010). This therefore, makes cells isolated from this tissue an interesting target for iPSC studies to form an ESC-like state with a potentially reduced requirement for genetic manipulation.

Within this Chapter, studies into the endpoint expression of these six pluripotency markers demonstrated positive expression of 5 of these factors in MP cells and TP cells of three patient samples. Expression of Oct-4, Sox-2, Nanog and Klf-4 is consistent with previous findings for OMLP cells as well as progenitor cells from the gingiva (Zhang et al, 2009; Davies et al, 2010; Marynka-Kalmani et al, 2010). Lin-28 was not expressed in all populations of the oral mucosa but, interestingly, it was expressed in patient LII NA cell populations and following treatment of patient L MP cells with ascorbic acid suggesting that ascorbic acid can potentially influence some gene expression. Changes in gene expression have previously been demonstrated following treatment of human dermal fibroblasts with ascorbic acid (Tajima and Pinnell, 1996). Treatment with ascorbic acid appeared to have no obvious effect on the expression of the other 5 pluripotency markers.

As these cell populations already seemed to be expressing the majority of the factors required for reprogramming, it was an obvious step to compare these expression levels with the expression levels of hESCs. Following analysis of the results, it was found that expression levels of three factors; Oct-4, Sox-2 and Nanog were all expressed at significantly lower levels than hESCs, consistent with previous findings for keratinocytes (Aasen et al, 2008). Expression of C-myc was found to be lower than hESC, but not quite

as low as Oct-4, Sox-2 and Nanog and, interestingly, expression of Klf-4 was found to be higher in all MP cell populations compared to hESCs. The elevated levels of Klf-4 and C-myc are consistent with findings for keratinocytes (Aasen et al, 2008) and have also been demonstrated by several other groups using endpoint PCR analysis to be present in dermal fibroblasts (Takahashi et al, 2007; Nakawada et al, 2008). Kfl-4 has recently been linked to the self-renewal properties of stem cells and has, moreover, been linked with the activation of hTERT, a catalytic subunit of human telomerase in dermal fibroblasts (Wong et al, 2010). The elevated levels of Klf-4 demonstrated by MP cells is therefore consistent with the elevated levels of telomerase activity previously reported in cells isolated from the OMLP (Davies et al, 2010). Furthermore, dermal fibroblasts demonstrate shorter telomeres and a reduced proliferative potential compared to cells from the oral mucosa (Enoch et al, 2009). Expression of Klf-4 was lower in DF cells compared to two patient MP cell populations which could potentially account for the differences in telomere lengths. To confirm this, additional samples of dermal fibroblast cells would need to be analysed since this investigation was carried out with only one dermal fibroblast patient sample. The elevated levels of Klf-4 and C-myc in dermal fibroblasts could potentially be the reason underlying the reprogramming of dermal fibroblasts without requiring the use of C-myc or Klf-4 (Yu et al, 2007). Reprogramming without these factors could potentially apply to OP cells from the OMLP.

3.6 Conclusions

In conclusion, the purpose of this chapter was to determine which cell population would be the most suitable population to select for reprogramming. It was demonstrated that treatment with ascorbic acid significantly reduced CFE of cells isolated from the OMLP; high CFEs is a typical characteristic of progenitor cell populations. Exposure to ascorbic acid did not increase cell proliferation of the different populations isolated from the OMLP as previously reported for Mesenchymal Stem Cells (MSCs) (Choi et al, 2008). With the exception of a few anomalies, gene expression was found to be the same when cultures were treated with ascorbic acid. For these reasons, it was decided to continue all further experiments without ascorbic acid as treatment appeared to offer no benefit.

It is well known that long-term culture of cells *in vitro* can change gene expression and given that cells from the OMLP express numerous pluripotency markers at an early time point, it was decided that these cells would be utilised for future studies at this early time point. Reprogramming cells to iPSCs requires a large numbers of cells due to the low efficiency of the process, therefore, the best populations to select for this would be the MP cell populations as they have the highest initial growth rate when compared to TP and NA populations. Thus, they would achieve the required number of cells faster *in vitro* (with less time in culture). Furthermore, when deciding between the use of either clonal (single cell) populations or MP (Merged clonal) populations, it again became apparent that cell number could be a limiting factor due to the fact that clonal populations developing from a single

cell would take significantly longer to reach the required cell number and would be much further though their proliferative lifespan. Hence, utilising the MP cells from numerous colonies that had merged together represent a cell population that would be much 'younger' in terms of proliferative potential. This is important, since cellular senescence is known to reduce reprogramming efficiency (Li et al, 2009; Marion et al, 2009). Thus, in future chapters, merged clonal MP populations were utilised to generate oral iPS cells.

4 - Reprogramming of oral mucosa lamina propria progenitor cells

4.1 Introduction

Although the idea of reversing the effects of development on early gene expression is not a new phenomenon (Briggs and King, 1952; King and Briggs, 1955), reprogramming of cells has received much attention over the last 6 years since the reprogramming of mouse somatic cells back towards an embryonic stem cell–like state was first reported (Takahashi and Yamanaka, 2006). One year later the reprogramming of human somatic cells was further reported (Takahashi et al, 2007). During this time, many methods for the formation of iPSCs have been described including retroviral (Takahashi and Yamanaka, 2006; Okita et al, 2007; Maherali et al, 2007) and lentiviral (Brambrink et al, 2008; Maherali et al, 2008) transduction of genes as well as transient expression of genes using non-integrating systems (Okita et al, 2008; Yu et al, 2009). Retroviral systems reprogram somatic cells with considerably higher efficiency compared to non-integrating transient expression systems and as such are sometimes a good initial starting point for producing iPSCs utilising cells with unknown reprogramming ability. However, like many of the reprogramming systems, there are limitations to the retroviral system. Many iPSCs from this system are often only partially reprogrammed and continue to rely on the frequently poorly silenced exogenous expression of the pluripotency factors in place of activating their own endogenous pluripotency gene expression to maintain their pluripotent state (Takahashi and Yamanaka, 2006). This can lead to reactivation of oncogenic transgenes and potential formation of tumours in chimeras (Okita et al, 2007). It has also been reported that transgene expression in lentiviral systems is often silenced even less efficiently than retroviral transgene

expression, which in turn affects the differentiation of the resulting iPSCs (Brambrink et al, 2008).

Non-integrating systems for transient expression of pluripotency gene expression result in a better likelihood of fully reprogrammed iPSC populations however, this is at the cost of a much lower efficiency (Okita et al, 2008). Weighing up these two factors, efficiency and safety, will be important when considering the use of these cells for translational medicine. Alongside these considerations, the choice of pluripotency factors used will also be an important factor to consider. Since the discovery of iPSCs, many different combinations of the pluripotency factors; Oct-4, Sox-2, Nanog, Lin-28, Klf-4 and C-myc have been tested. The first four that were confirmed to produce iPSC by retroviral transduction were Oct-4, Sox-2, Klf-4 and C-myc (Takahashi and Yamanaka, 2006). These factors have already been used to successfully produce iPSCs from oral mucosal fibroblasts with a retroviral transduction system (Miyoshi et al, 2010). There has been a reduction in the use of retroviral transduction of the oncogene C-myc since studies indicated that iPSCs reprogrammed using this factor later developed adult chimaeras with tumours due to reactivation of C-myc thereby limiting the use of this gene in retroviral systems for therapeutic purposes.(Okita et al, 2007; Sumi et al, 2007). Lentiviral systems without C-myc have been described and have demonstrated that Oct-4, Sox-2, Nanog and Lin-28 are sufficient to reprogram fibroblasts to iPSCs however, in the absence of C-myc the efficiency is much lower (Yu et al, 2007). Ideally a non-integrating system would be a safer option for translational applications and many systems have

been tested. It was found that using all six factors: Oct-4, Sox-2, Nanog, Lin-28, Klf-4 and C-myc across two plasmids in a non-integrating system was the most efficient way to produce iPSCs that were free of any exogenous gene expression (Yu et al, 2009).

Given the aim of producing iPSCs from oral progenitor cells for their potential use in therapeutic applications, a lentiviral approach without the use of C-myc or a non-integrating system approach would appear to be the most appropriate systems to use. In the first instance, given the low efficiency associated with plasmid transfection, an efficient lentiviral system would be most appropriate to establish whether these cells can first be reprogrammed, followed by a plasmid system utilising all six pluripotency factors. With translational medicine in mind, the way in which these cells are cultured will also be important. Historically, human ESCs have been cultured on mouse embryonic fibroblasts as a feeder layer to provide the appropriate factors in order to maintain the cells in their pluripotent state (Thomson et al, 1998). However, this raises the concern of the safety of these cells for therapeutic applications as cells would need to be grown in an animal-free culture system. More recently, ESC have been cultured using feeder free systems by culturing the cells on Matrigel® coatings (Xu et al, 2001) however, some argue that matrigel is such a highly complex (and undefined) mixture of extracellular matrix components, growth factors and cytokines, with batch to batch variation that caution should be exercised with its use (Hughes et al, 2010). As an alternative to animal culture systems and in vitro growth matrices, growing ESCs on human foreskin fibroblast feeder layers has been

found to maintain the cells pluripotent properties and as such reduces the need for concern over therapeutic safety of using these cultures (Amit et al, 2003). For this reason, producing and maintaining iPSCs from oral progenitor cells on foreskin fibroblast feeders, as well as mouse embryonic feeder layers, is of great interest.

Once reprogrammed, the resulting iPSCs undergo some classical characterisation similar to that originally published for hESC (Thomson et al, 1998). The main purpose of this characterisation is to confirm that the cells have been reprogrammed and poses pluripotent properties. There are key cell surface markers and transcription factors that are commonly utilised for this purpose including; SSEA-4, SSEA-5, TRA-1-60, TRA-1-81, Oct-4 and Sox-2 (Thomson et al, 1998; Mali et al, 2008; Li et al, 2009; Tang et al, 2011). To confirm the pluripotent nature of these cells, demonstrating the ability to differentiate into cells/tissues from all three germs layers is necessary. This is often carried out by formation of teratomas following implantation into mice (Thomson et al, 1998; Takahashi et al, 2007). The resulting teratomas are then analysed for presence of tissues from all three germ layers by H& E staining. In addition to this, the cells can be differentiated towards cell types of the endoderm, mesoderm and ectoderm and stained with specific markers of the germ layers to confirm early differentiation potential (Maherali et al, 2008).

4.1.1 Hypothesis

Oral mucosa lamina propria progenitor cells can be reprogrammed back towards an embryonic stem cell-like state utilising iPSC technology.

4.1.2 Aims

There are three main aims for the reprogramming of oral progenitor cells from the oral mucosa lamina propria:

1. Reprogram the oral progenitor cells to iPSCs

2. Establish the presence of typical ESC markers and pluripotent properties of iPSCs

3. Carry out a comparison of on two different feeder layers; inactivated mouse embryonic fibroblasts (iMEFs) and inactivated foreskin fibroblasts (iFCFs) for formation and maintenance of iPSCs.

4.2 Materials and Methods

A number of general methods have been utilised in this Chapter which have been previously described (Chapter 2) including OMLP cell culture, formation of feeder layers, human embryonic stem cell culture, mycoplasma testing and imaging of cells.

4.2.1 Formation of Foreskin Fibroblast Feeder Layers

Foreskin fibroblasts are sometimes used as an alternative to MEFs when culturing hESCs. Foreskin fibroblast were previously isolated and cultured by members of the Wound Biology Group, Cardiff University. Early passage foreskin fibroblasts (FCF101) were thawed rapidly from cryopreservation and placed in 10mL of pre-warmed MEF media: DMEM supplemented with 10% FCS, 2mM L-Glutamine, 100U/mL Penicillin, 100µg/mL Streptomycin Sulphate and NEAA. Cells were spun at 400 x g for 5 minutes to pellet the cells. The supernatant was discarded and the pellet re-suspended in 1mL of MEF media. Cells were counted using a haemocytometer seeded at an initial high seeding density of 5 x 10^5 cells/ T75 flask to allow cells to settle following cryopreservation. Cells were fed every 2-3 days with MEF media until 90-95% confluent. One mL of media was removed from the confluent cells to test for mycoplasma contaminations.

Cells were passaged using Trypsin/EDTA (0.05%(v/v)/0.53mM). Media was removed from the flask and cells washed with 10mL of PBS. This was replaced with 1mL of Trypsin/EDTA, and flasks were returned to the incubator for 3-5 minutes until the cells detached. Ten mL of MEF media

was added to neutralise the Trypsin/EDTA. The resulting cell suspension was placed in a 15mL falcon and spun at 400x g for 5 minutes to pellet the cells. These were counted using a haemocytometer and re-seeded at a density of 3.5 $x10^5$ cells/T175. Subsequent passages were split at a 1:5 ratio and resulting confluent cultures were irradiated as previously described in Chapter 2. These irradiated cells were termed iFCF and treated as per iMEFs for use as feeder layers.

4.2.2 Derivation of Induced Pluripotent Stem Cells

4.2.2.1 Plasmid induction of Pluripotency Factors

Several methods have been described for the formation of iPSCs. Induction of pluripotency factors using plasmids have previously been described by Yu et al, 2009. Plasmids were commercially available from addgene, UK (Figure 4.1). These plasmids were chosen to allow transient expression of the 6 pluripotency factors; Oct-4, Sox-2, Nanog, Klf-4, Lin-28 and C-myc.

4.2.2.2 Making LB Agar Plates

Sixteen g of LB agar was mixed with 500mL of distilled water in a 1L autoclavable glass bottle. This was autoclaved for one hour (121ºC steam temperature) then placed in a water bath at 56ºC until required. When ready to pour the plates, LB agar was allowed to cool until the bottle was warm to touch before ampicillin was added at a final concentration of 20µg/mL.

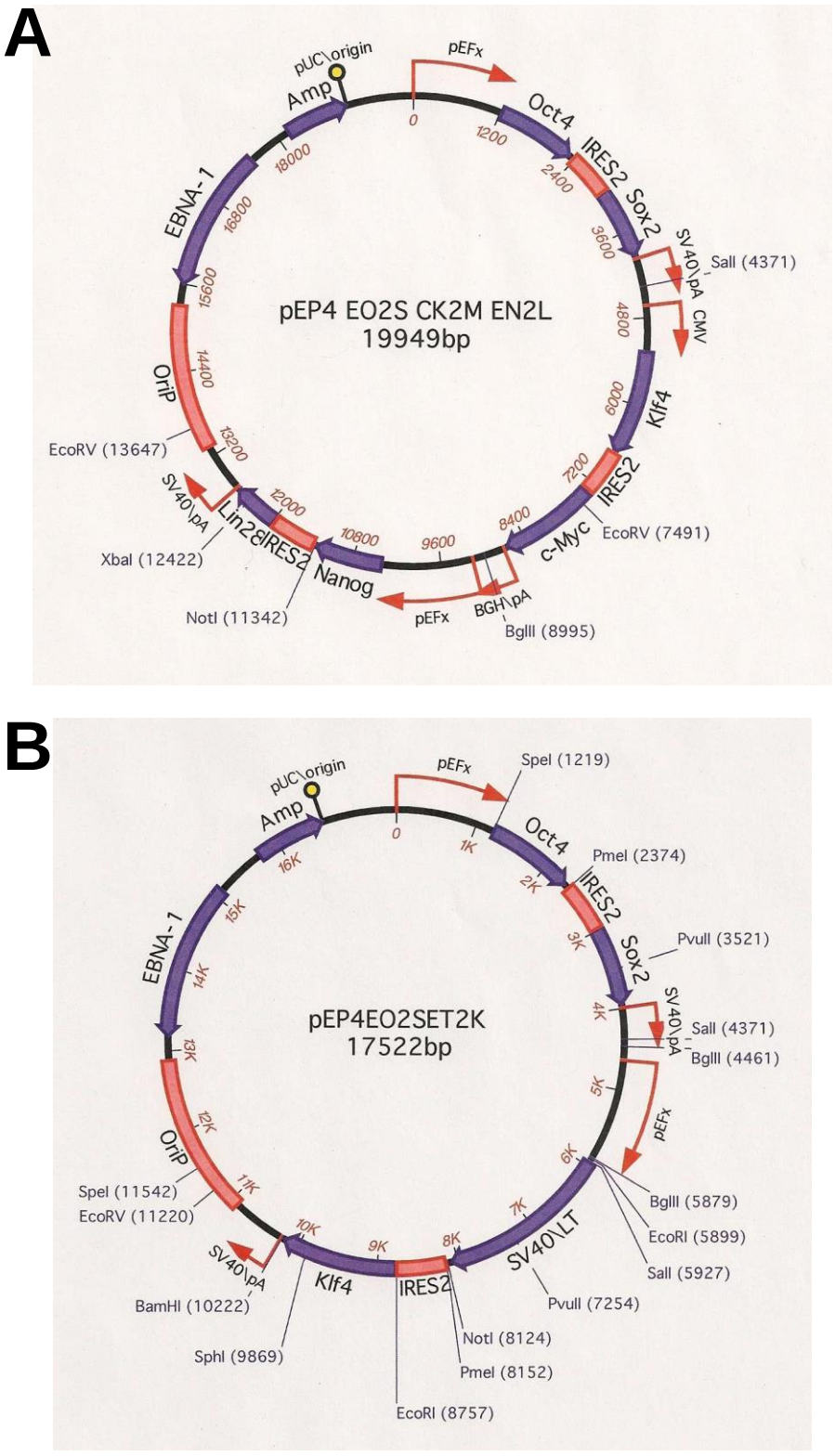

Figure 4.1: ***Plasmids utilised in the reprogramming of OMLP*** **(A)** AddGene Plasmid # 20924 containing all 6 pluripotency factors; Oct-4, Sox-2, Nanog, Klf-4, Lin-28 and C-myc. **(B)** AddGene Plasmid # 20927 containing Oct-4, Sox-2, SV40\LT and Klf-4 (images of plasmids from Addgene).

This was mixed thoroughly before carefully pouring the LB agar into a 10cm petri dish in a Microflow Advanced bio-safety Class II cabinet. LB agar was divided between petri dishes to a depth of 1cm, avoiding the formation of air bubbles and dishes left at the back of the cabinet to set. Once set, the plates were stored in a sealed container at 4ºC until required.

4.2.2.3 Growing TOP10 Containing Plasmids on LB Agar Plates

A vial of One Shot® TOP10 chemically competent E. coli was thawed on ice for each plasmid. The concentration of both plasmids was determined using a nanovue® spectrophotometer and 1µg of each plasmid was added to a separate thawed vial of TOP10 cells. These vials were very gently mixed before returning the vials to ice to incubate for 30 seconds. Following the short incubation period, cells were heat shock treated for a further 30 seconds in a water bath set to 42ºC. Two hundred and fifty µL of room temperature S.O.C medium (containing: 2% tryptone, 0.5%yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate and 20 mM glucose per litre.) was added to each vial, capped tightly and placed horizontally in a shaking incubator for 1 hour at 37ºC at 200 r.p.m. Two volumes, 100µl and 200µl, from each transformation were spread onto pre-warmed selective LB agar plates, to ensure well-spaced colonies.

These plates were then inverted and incubated O/N at 37ºC. Eight colonies were picked from each plasmid culture using a clean sterile 200µL pipette tip for each colony and plated onto a fresh selective LB agar plate and once again incubated O/N at 37ºC. The 200µL pipette tip was placed in 5µL of

DEPC water to dilute the remainder of the colony from the tip. PCR was carried out on this dilution for Oct-4 pluripotency gene (present on both plasmids) as previously described in Chapters 2 & 3 to check for plasmid expression.

4.2.2.4 Maxi Prep of Plasmids

As the plasmids have an extremely low copy number the Qiagen Endofree Maxi prep kit was used in order to obtain sufficient plasmid quantity for transfection. A colony from the fresh LB agar plates was picked to inoculate a starter culture of 5mL LB medium containing 20µg/mL ampicillin. This culture was incubated at 37ºC for 8 hours in a shaking incubator at 300 r.p.m. After 8 hours, 500µL of started culture was diluted in 250mL of LB medium containing 20µg/mL ampicillin in a 1L conical flask. This culture was grown at 37ºC for 12-16 hours at 300 r.p.m. in the shaking incubator. Following incubation, cells were pelleted by centrifugation at 6000 x g for 15 minutes at 4ºC. The supernatant was discarded and cells re-suspended with 10mL Buffer P1 until no clumps remained and were then transferred to a 50mL falcon. Ten mL of Buffer 2 was added to this and mixed by inverting the tube 4-6 times to lyse the cells. This solution was left to incubate at room temperature for 5 minutes. The QIAfilter Maxi Cartridge was fitted with a screw cap on the outlet nozzle and placed inside a 50mL falcon tube during the incubation step. Following incubation, 10mL of pre-chilled Buffer 3 was added to the lysate, mixed by inversion 4-6 times and added to the top of the Maxi Cartridge. This was left to incubate at room temperature for 10 minutes. The cap was removed after 10 minutes and a plunger inserted into

the top of the cartridge to filter the lysate. Two and a half mL of Buffer ER was added to the filtered lysate and mixed by inverting the tube 10 times before incubating on ice for 30 minutes. Following incubation, 10 mL of Buffer QBT was added to a QIAGEN-Tip 500 and allowed to flow through by gravity into a 50 mL Falcon tube. This was discarded and replaced with a fresh 50mL Falcon tube before the filtered lysate was added to the top of the QIAGEN-Tip 500 and, again, allowed to enter the Falcon tube by gravity flow. The flow-through was discarded and the bound DNA washed twice with 30mL Buffer QC before being eluted using 15mL of Buffer QN into a clean 50mL Falcon tube. DNA was precipitated by adding 10.5mL of isopropanol which was mixed and centrifuged at 15000 x g for 30 minutes at 4ºC. The supernatant was carefully removed before washing the DNA pellet with 5mL of endotoxin-free 70% ethanol and centrifuging at 15000 x g for 10 minutes. The supernatant was removed and the pellet left to air-dry for 5-10 minutes before re-suspension in 100µL of endotoxin-free Buffer TE. DNA concentration was determined using the nanovue®.

4.2.2.5 Nucleofection

Two different cell types were nucleofected; (i) DF, as a positive control as these had already been reported to be able to be reprogrammed using the two plasmids (Yu et al, 2009) and (ii) MPs. MP XLIX was selected for reprogramming due to available cell numbers at point of nucleofection. As part of these methodology two different feeder layers were tested; iMEFs and iFCFs. The nucleofection and subsequent culture of cells involved a number of different steps illustrated in Figure 4.2

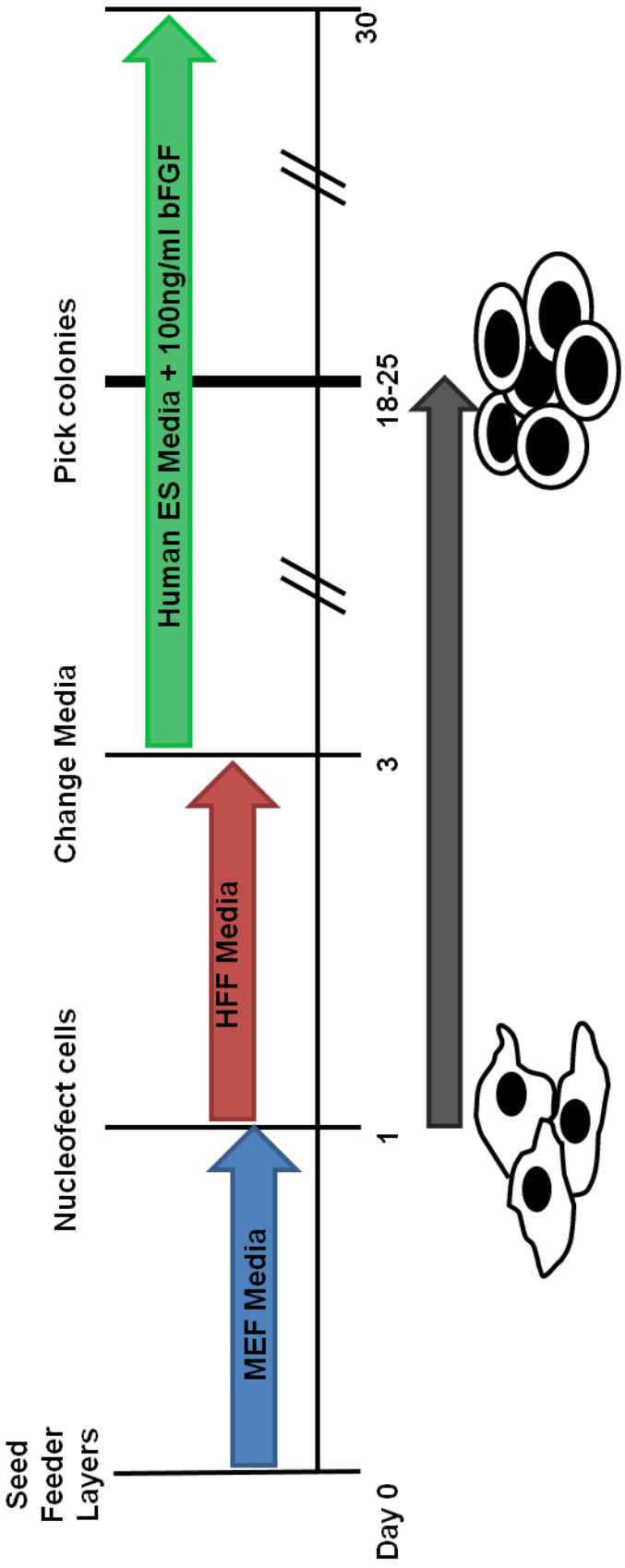

Figure 4.2: Reprogramming of cells with plasmids. Diagram illustrating the different stages involved in the reprogramming of MPs and DF to iPSC including the changes in media conditions.

Each condition (i.e. DF on iMEF or iFCFs and MP on iMEFs or iFCFs) was set up in triplicate. For each condition 3 x 10cm tissue culture dishes were coated for 1 hour with 6mL of 0.1% gelatin (Millipore, UK). The gelatin was removed and feeder cells were seeded at 8 x 10^5 cells per 10cm dish in 10mL of MEF media. The cells were allowed to attach for 24 hours. The following day, the MEF media was removed and replaced with 10mL of foreskin fibroblast media (HFF; Yu et al, 2009) containing: MEM (eagle) supplemented with 10% heat inactivated fetal bovine serum, 2.0mM Glutamax, 0.1mM β-mercaptoethanol, 100U/mL Penicillin, 100µg/mL Streptomycin Sulphate and 0.1mM NEAA.. This was returned to the incubator and allowed to equilibrate for at least 1 hour. Cells were seeded in to T175 flasks at a density of 2 x 10^4 cells/cm^2 as previously described. When cells reached 80-90% confluency, they were passaged. Culture media was removed and cells washed with 15mL HBSS. This was removed and replaced with 3mL Trypsin/EDTA. Cells were returned to the incubator for 1-3 minutes to allow the cells to detach. Once detached, Trypsin/EDTA was neutralised using 12mL DMEM supplemented with 2% FBS. The total number of cells was calculated by counting a 1mL aliquot. A volume of media containing 1 x10^6 cells was removed and placed in a 15mL falcon, this was spun at 200 x g for 10 minutes to pellet the cells. The supernatant was removed and cells were re-suspended in 100µL of room temperature nucleofector solution (NHDF - VPD-1001, Lonza, UK) in the 15mL falcon. To this, 7.3µg pEP4 E02S CK2M EN2L and 3.2µg pEP4 E02S ET2K plasmid DNA were added and mixed. The cell/DNA suspension was transferred to a certified cuvette (NHDF - VPD-1001, Lonza, UK) and placed into the

nucleofector (Amaxa, Lonza, UK). Program U-20 was selected. Following nucleofection, 500µL of pre-equilibrated HFF medium was added to the cuvette. The sample was removed from the cuvette very gently using a plastic pipette, avoiding repeated aspiration, and pipetted onto the prepared feeder dish. The plate was promptly returned to the incubator and allowed to settle for 24 hours. A transfection control was set up using the eGFP plasmid provided to calculate transfection efficiency. One µg of plasmid was added to 1 x 10^6 cells resuspended in 100µL of nucleofector solution. This was placed in the nucleofector and run on program U-20. The nucleofected cells were seeded into 10cm tissue culture dishes without feeder layers. The following day medium was removed and replaced with 10mL of fresh foreskin fibroblast media. The plates were returned to the incubator for a further 48 hours. The transfection control plates were removed and viewed using the Delta Vision in order for transfection efficiencies to be calculated (Detailed in section 4.2.1.4). On day 4 post-transfection, medium was removed and cells were washed with 10mL PBS and replaced with 10mL of fresh Human ES media containing DMEM/F-12 1:1 supplemented with 20% knockout serum replacement, 0.1mM NEAA, 1mM L-Glutamine, 0.1mM β-mercaptoethanol and 100ng/mL of basic fibroblast growth factor (bFGF). Cells were fed every 48 hours with fresh Human ES media. Conditioned medium was used from day 8 post-transfection due to deterioration of the feeder layers. Feeder layers for conditioned medium were seeded at a density of 5 x 10^4 cells/cm^2 in a pre-gelatin coated 6 well plate and allowed to settle for 24 hours in 2mL/well MEF medium. After 24 hours the feeder layers were washed twice with 2mL/well PBS and this was replaced with 2mL/ well Human ES media

without bFGF. Medium was removed and replaced with fresh human ES media daily. The conditioned medium was combined in 15mL Falcon tubes and stored at -20ºC until required. Cultures were fed with conditioned medium until colonies began to form on days 18-25.

4.2.2.6 Calculating Transfection Efficiency

Transfection control plates were viewed using an Olympus IX71 inverted microscope and camera (Applied Precision, USA). Imaging equipment was within an environmental chamber, maintaining the temperature at 37ºC. Images were analysed using Delta Vision imaging software (Applied Precision, USA). Images were acquired using a FITC filter (Excitation 461-489nm and emission 523-536nm), these were overlaid phase contrast images acquired from the same field of view and used to calculate transfection efficiency using the following equation:

$$\text{Transfection Efficiency} = \frac{\text{GFP positive cells}}{\text{Total number of cells}} \times 100$$

4.2.2.7 Alkaline Phosphatase Staining

A typical characteristic of hESC is presence of high levels of alkaline phosphatase when in an undifferentiated state (Thomson et al, 1998) therefore, to determine the presence or absence of iPSC colonies, one of the 10cm dishes was used to stain for alkaline phosphatase on day 20 post transfection. Medium was removed from the dish and cells were washed

twice with 10mL pre-warmed DMEM/F-12 1:1. Alkaline phosphatase stain was provided as 500x stock solution. Twelve µL was mixed with 6mL of pre-warmed DMEM/F-12 to make up a 1x working stock. Following washes, this working stock solution was added to the 10cm dish and incubated for 20-30 minutes. After this time, the solution was removed and the plate washed with 10mL DMEM/F-12 twice for 5 minutes each. The final wash was removed and replaced with 10mL of fresh DMEM/F-12 prior to visualisation. Cells were imaged using a FITC filter on an Olympus IX71 microscope as described in section 4.2.2.6 and alkaline phosphatase-positive colonies were marked.

4.2.2.8 iPSC Culture

4.2.2.8.1 First Passage Post-Nucleofection

During the initial stages of reprogramming when colonies first began to emerge, the colonies were very delicate and as such they were treated using mechanical passaging techniques. This was carried out using sterile flame pulled Pasteur pipettes. Feeder layers were set up as previously described in 24 well plates at a seeding density of 1.5 x 10^2 cells/mL. When required, MEF medium was removed and the cells washed twice with 500µL PBS. This was replaced with 500µL of Human ES medium supplemented with 100µg/mL bFGF. These cells were placed back in the incubator to allow the media to equilibrate at 37ºC and 5% CO_2. Each colony was cut into smaller pieces by scoring through the colony with the tip of a flame pulled Pasteur pipette and then around the borders of the colony to release the pieces. The colony pieces were removed using a 20µL pipette and seeded into one well

of the prepared feeder plates and returned to the incubator to settle for 48 hours. After the colonies had settled, the cultures were fed daily with fresh Human ES medium supplemented with 100ng/mL bFGF.

4.2.2.8.2 iPSC Colony Expansion

Not every colony picked from the initial transfection plate went on to form colonies that were similar in morphology to Human ES cells. The colonies that were similar to Human ES cells were selected for further expansion. The colonies were split from one well of the 24 well plate into one well of a 12 well plate prepared with feeder cells by mechanical passage, as previously described. These cultures were left to settle for 48 hours then fed daily with fresh Human ES medium supplemented with 100ng/mL bFGF. When the colonies were large enough to fill the x10 objective on the microscope they were split once more. The size of the plate used for expansion was determined by the number of colonies present. When the number of colonies in one well of a 12 well plate had reached 10-15 colonies, the culture was mechanically passaged to one well of a 6 well plate prepared with feeder cells. Once there were sufficient colonies to fill one well of a 6 well plate without the colonies merging, passaging was carried out enzymatically with collagenase IV.

4.2.2.8.3 Enzymatic Passaging of iPSCs.

When colonies were ready to be passaged, differentiated colonies were removed using the tip of a pipette, medium was removed and colonies were washed once with 2mL PBS to remove debris. This was removed and

replaced with 2mL of 1mg/mL Collagenase IV in DMEM/F-12 and returned to the incubator for 20-30 minutes until the colonies began to curl up at the edges. Once curled, the plates were removed from the incubator and colonies were gently washed from the surface using a 5mL pipette. Detached colonies were collected in a 15mL falcon and spun at 200 x g for 2 minutes to pellet the colonies. Supernatant was removed and colonies were resuspended 1mL pre-equilibrated medium from the prepared feeder plates. Colonies were broken by pipetting up and down using a 1mL pipette a maximum of 8 times. Colonies from one well of a 6 well plate were split at a ratio of between 1:4 and 1:6 depending on the number of undifferentiated colonies present. When plated, colonies were returned to the incubator and allowed to settle for 48 hours. Following this, cultures were fed daily with fresh Human ES media supplemented with 100ng/mL bFGF. Cultures were split typically every 4-5 days.

4.2.2.9 iPSC Characterisation

Oct-4, Sox-2, SSEA-4, SSEA-5, TRA-1-60 and TRA-1-81 are typical markers expressed by pluripotent stem cells (Thomson et al, 1998; Takahashi et al, 2007; Tang et al, 2011). Expression of these markers in MP iPSCs was determined by immunocytochemistry. H9 cells were used as a positive control.

4.2.2.9.1 Pluripotency Marker Expression

Chamber slides were prepared with feeder cells as previously described. Colonies were mechanically passaged onto chamber slides at a density of 4-6 colonies/well. Each well was allowed to settle for 48 hours before feeding

daily with fresh Human ES medium supplemented with 100ng/mL bFGF. When colonies were a sufficient size for passaging, they were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. MP XLIX cells were seeded onto chamber slides at a density of 2 x 10^3 cells/cm^2 and allowed to settle for 48 hours before fixing with 4% PFA. These control cells were used to determine presence of these markers prior to reprogramming. Each chamber was washed 3 times in PBS for 5 minutes, cells were then permeabilised with 0.1% (v/v) Triton X 100 (TX) for 20 minutes at room temperature. Non-specific binding of the secondary antibody was blocked using a solution of 2% (w/v) BSA (diluted in PBS) and left to incubate for 1 hour at room temperature. This was aspirated off and replaced with primary antibody (Rabbit polyclonal antibody against Oct-4, Mouse monoclonal antibodies against Sox-2, SSEA-4, TRA-1-60 and TRA-1-81 (All abcam, UK) and mouse monoclonal antibody against SSEA-5 (from Micha Drukker, Stanford University) were diluted to 1:200 (Sox-2 1.25µg/mL, SSEA-5 2.5µg/mL), 1:250 (Oct-4 2.8µg/mL) or 1:500 (SSEA-4 2µg/mL, TRA-1-60 4µg/mL and TRA-1-81 4µg/mL) in 2% BSA in PBS and incubated at 4°C overnight. Chamber slides were washed 3 times for 5 minutes with PBS. Secondary antibodies (Swine anti-rabbit (0.81g/L) and Rabbit anti-mouse (2.3g/L), both Dako, UK) were diluted 1:50 in 2% BSA in PBS and added to the cells and incubated for 1 hour at room temperature. Chamber slides were washed 3 times for 5 minutes with PBS before counterstaining the nuclei with DAPI (1.5µg/mL) in mounting media (Vector shield, UK). Appropriate IgG control and IgM control (Both Santa Cruz Biotechnology, UK) were used for each antibody to verify specificity by absence of staining.

4.2.2.9.2 Embryoid Body Formation

A key stage in the differentiation of Human ES cells is the formation of a 3D body termed an embryoid body. This 3D body is thought to mimic the environment of a stem cell in the embryo, aiding development into all three germ layers; Endoderm, Mesoderm and Ectoderm.

MP iPSC colonies were passaged enzymatically from 2 wells of a 6 well plate as previously described for splitting. Colonies were seeded into a 10cm non-tissue culture coated petri dish in 10mL fresh Human ES medium without bFGF. Cells were fed every second day. Again, as a comparative control, MP XLIX cells from 2 wells of a 6 well plate were enzymatically passage for splitting, as previously described, and seeded in a 10cm non-tissue culture coated petri dish in 10mL Human ES medium. Medium containing the cell bodies in suspension was removed and collected in a 15mL falcon. This was spun at 200 x g for 2 minutes to pellet the bodies. Supernatant was removed and replaced with 10mL fresh Human ES medium without bFGF. These embryoid bodies were grown for 8 days before differentiation was initiated.

4.2.2.9.3 Differentiation of Embryoid Bodies

Chamber slides and 24 well plates were pre-coated with 100µL and 250µL Matrigel® respectively at a concentration of 0.1% (v/v) in Human ES medium without bFGF for 1 hour at 37ºC and 5% CO_2. Plates were removed from the incubator and left at room temperature O/N. Media and 8 day old embryoid bodies were removed from the 10cm petri dishes and collected in a 15mL falcon. These were spun at 200 x g, supernatant was removed and embryoid bodies were re-suspended in 9mL Human ES medium. Three conditions

were set up to differentiate the embryoid bodies down the three lineages; Mesoderm, Endoderm and the Ectoderm. Medium alone was used to allow spontaneous differentiation, Human ES medium supplemented with 10µM retinoic acid (diluted in Dimethyl Sulfoxide (DMSO)) to promote ectoderm differentiation and Human ES medium supplemented with 1:1000 dilution of DMSO as a vehicle control for retinoic acid. Three 15mL falcons were set up containing 4mL of each medium condition. One mL of re-suspended EBs was taken from the main stock and added to each medium condition making a final volume of 5mL. When required, medium was removed from the Matrigel®-coated wells and replaced with 500µL of medium for one well of a 24 well plate and 250µL for one well of a chamber slide. These were returned to the incubator and fed with fresh medium (human ES medium, medium supplemented with retinoic acid and medium supplemented with DMSO) every other day.

4.2.2.9.4 Mesoderm, Endoderm and Ectoderm marker expression

Following 14 days of differentiation, chamber slides were fixed in 4% PFA for 15 minutes at room temperature. Each chamber was washed 3 times in PBS for 5 minutes, cells were then permeabilised with 0.1% (v/v) TX for 20 minutes at room temperature. Non-specific binding of the secondary antibody was blocked using a solution of 2% (w/v) BSA (diluted in PBS) and left to incubate for 1 hour at room temperature. This was aspirated off and replaced with primary antibody (Rabbit polyclonal antibody against Brachyury and Mouse monoclonal antibodies against α-1-fetoprotein and βIII tubulin (all abcam, UK) diluted 1:200 (Brachyury 5µg/mL, α-1-fetoprotein 5µg/mL) and

1:500 (βIII tubulin 2µg/mL) in 2% BSA in PBS and incubated at 4°C O/N. Chamber slides were washed 3 times for 5 minutes with PBS. Secondary antibodies (Swine anti-rabbit (0.81g/L) and Rabbit anti-mouse (2.3g/L), both Dako, UK) were diluted 1:50 in 2% BSA in PBS and added to the cells and incubated for 1 hour at room temperature. Chamber slides were washed 3 times for 5 minutes with PBS before counterstaining the nuclei with DAPI (1.5µg/mL) in mounting media (Vector shield, UK). Appropriate IgG controls (Santa Cruz Biotechnology, UK) were used for each antibody to verify specificity by absence of staining.

4.3 Results

The initial plan to produce iPSCs from OMLP-PCs involved a lentiviral system to transfect four pluripotency factors; Oct-4, Sox-2, Nanog and Lin-28 into the cells. This was performed as part of a collaboration with Dr James Uney, Bristol University. Three attempts using this system failed to produce any iPSC-like colonies and the plasmid based technology was selected in place of the lentiviral approach.

4.3.1 Reprogramming of Oral Mucosa Lamina Propria Progenitor Cells with Plasmid Vectors

4.3.1.1 Comparison of Reprogramming Dermal Fibroblasts versus Oral Mucosa Lamina Propria Progenitor Cells

DF were used as a positive control for reprogramming as they had already been successfully reprogrammed using these two plasmids (Yu et al, 2009). Each cell type; MP XLIX cells and DFs were cultured as previously described and prepared for nucleofection. MP XLIX cells were used at passage 5 and DF cells were used at passage 17. MP XLIX and DF were nucleofected with a GFP plasmid in order to calculate the transfection efficiencies of each cell type. Ten bright field images and corresponding GFP images were captured and analysed using image J software. The total number of cells and the number of GFP-positive cells were counted and used to calculate the transfection efficiency of DF (Figure 4.3 A) and MP XLIX (Figure 4.3 B). Transfection efficiency of DF was found to be 38.7% (± 2.290 SD) which was significantly lower ($p<0.001$) than MP XLIX which was 63.6% (±2.771 SD) (Figure 4.3 C).

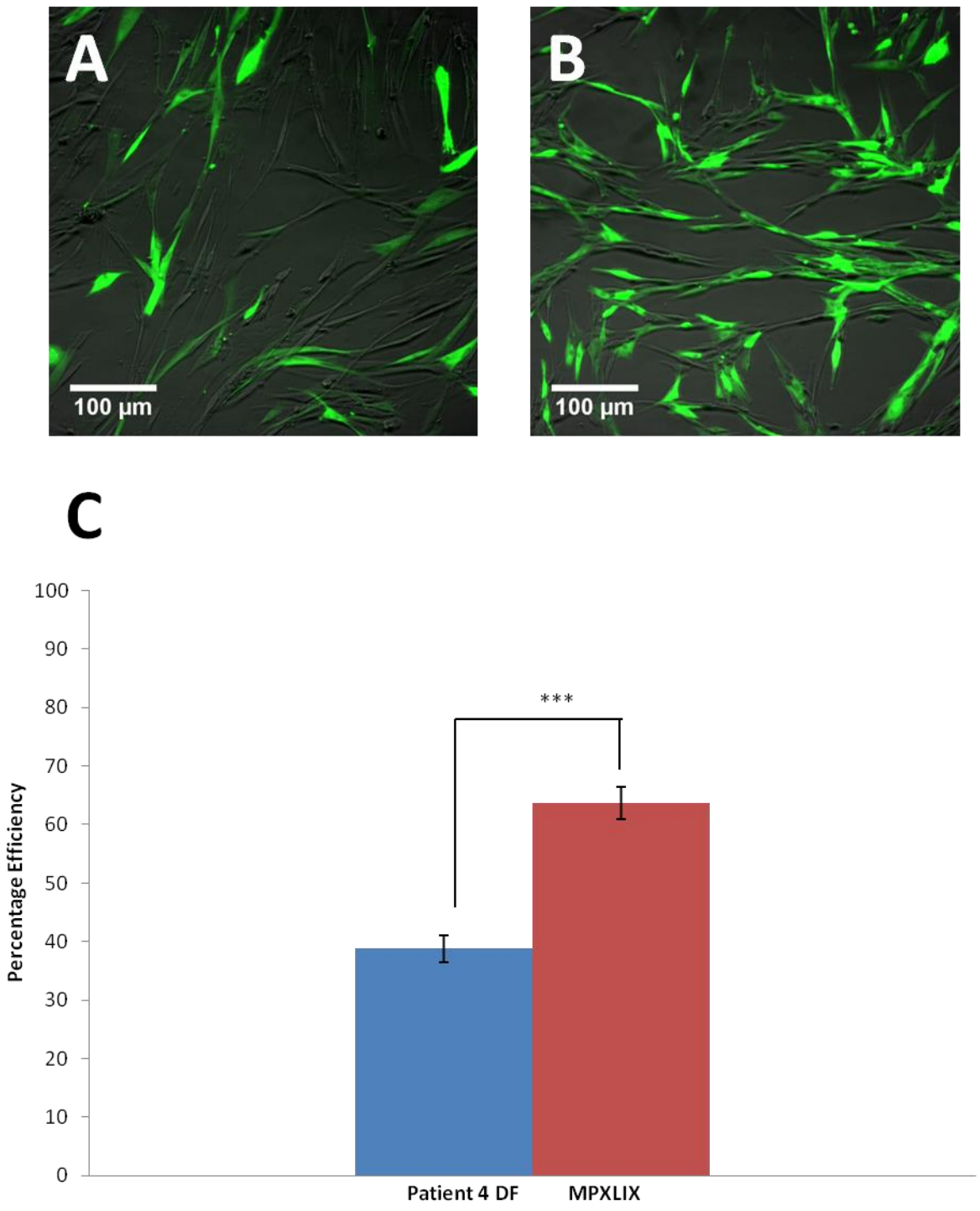

Figure 4.3: ***Transfection efficiencies of MP XLIX and DF.*** **(A)** Merged bright field and GFP images of transfected cells in Patient 4 DF. **(B)** Merged bright field and GFP images of transfected cells in MP XLIX. **(C)** Percentage transfection efficiency of both samples; Patient 4 DF and MP XLIX (*n=3* ±SD) (***= P< 0.001).

4.3.1.2 Comparison of Feeder Layers for Formation and Maintenance of iPSCs

Following nucleofection, each cell type, MP XLIX and DF, were seeded onto two different feeder layers; iMEFs and iFCFs, in order to carry out a comparison between colonies that developed on these plates. These four different culture conditions were set up in triplicate and followed for 30 days. Potential colonies developed on all MP XLIX iMEF plates and all MP XLIX iFCF plates after 18 days. No colonies were visible on DF plates (either feeder layer) after 30 days in culture. Live alkaline phosphatase stain was used to confirm the presence of potential iPSC colonies (Figure 4.4 B). Colonies were picked from MP XLIX iMEF and MP XLIX iFCF plates. Each colony was transferred into an individual well of a 24 well plate prepared with either iMEFs or iFCFs and left to settle for 48 hours. Colonies similar in morphology to human ES cells were present in the iMEF 24 well plate (Figure 4.4 C) and the iFCF 24 well plate (Figure 4.4.E) 5 days after mechanical passage from the original plate. These colonies consisted of small cells and demonstrated tight colony boundaries with a high nuclear to cytoplasmic ratio (Figure 4.4 D & F) that are normally observed in human ES cell culture. In total, six colonies displaying morphology similar to human ES cells were isolated and successfully cultured from the iMEF plates and one colony with this morphology was isolated from the iFCF plates.

4.3.2 Characterisation of MP iPSCs

Characterisation of iPSCs typically involves confirmation of the presence of pluripotency markers typically expressed by human ES cells.

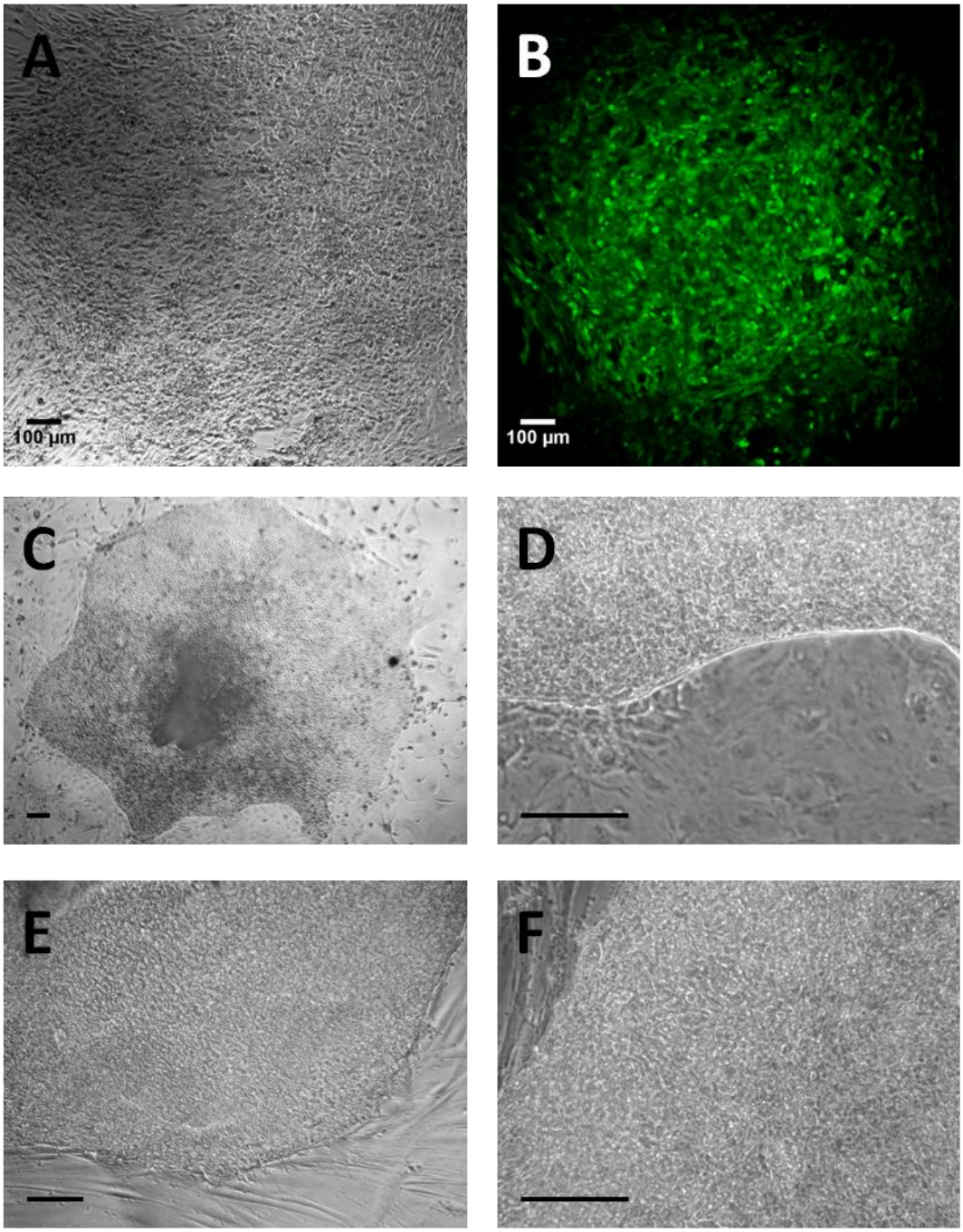

Figure 4.4: ***Alkaline phosphatase stain and first colony formation on iMEFs and iFCFs.*** **(A)** Bright field image of initial colony formation post-nucleofection. **(B)** Live Alkaline phosphatase staining of colony A. **(C)** First colony to form 5 days after first passage on iMEFs. **(D)** Higher magnification image of colony C demonstrating tight colony boundaries and cells with high nuclear to cytoplasmic ratio. **(E)** First colony to form 5 days after first passage on iFCF. **(F)** Higher magnification image of colony E demonstrating tight colony boundaries and cells with high nucler to cytoplasmic ratio. (Scale bar = 100µm)

Further characterisation can then be carried out to determine the potential of these cells to differentiate down the three germ layers of origin; mesoderm, ectoderm and endoderm, confirming pluripotent stem cell properties.

4.3.2.1 Expression of Pluripotency Markers

Each of the isolated colonies (iMEF (*n=6*) iFCF (*n=1*)) (n that were similar in morphology to human ES cells were expanded in culture, firstly by mechanical and then by enzymatic passaging. When a sufficient number of colonies were achieved, they were passaged onto culture slides prepared with feeder layers in order to carry out immunocytochemistry for the pluripotency markers. The colonies were stained for two transcription factors: Oct-4 and Sox-2 and four cell surface markers: SSEA-4, SSEA-5, TRA-1-60 and TRA-1-81. Human ES cells (H9) were cultured as previously described and used as a positive control for the markers. MP XLIX cells that had not been transfected with the plasmids were used as a comparative control. The transcription factors Oct-4 and Sox-2 were found to be produce by all 6 potential iPSC cultures on iMEFs (Figure 4.5 A, Figure 4.6 A and Table 4.1) and the potential iPSC culture on iFCF (Figure 4.5 B and Figure 4.6 B). H9 cells demonstrated positive production of Oct-4 and Sox-2 (Figure 4.5 C and Figure 4.6 C) and presence was also detected in MP XLIX cultures (Figure 4.5 D 4.6 D). Primary omission control, Swine anti Rabbit secondary omission control and IgG (Rabbit) control for Oct-4 were found to be negative (Figure 4.5 E, F, G).

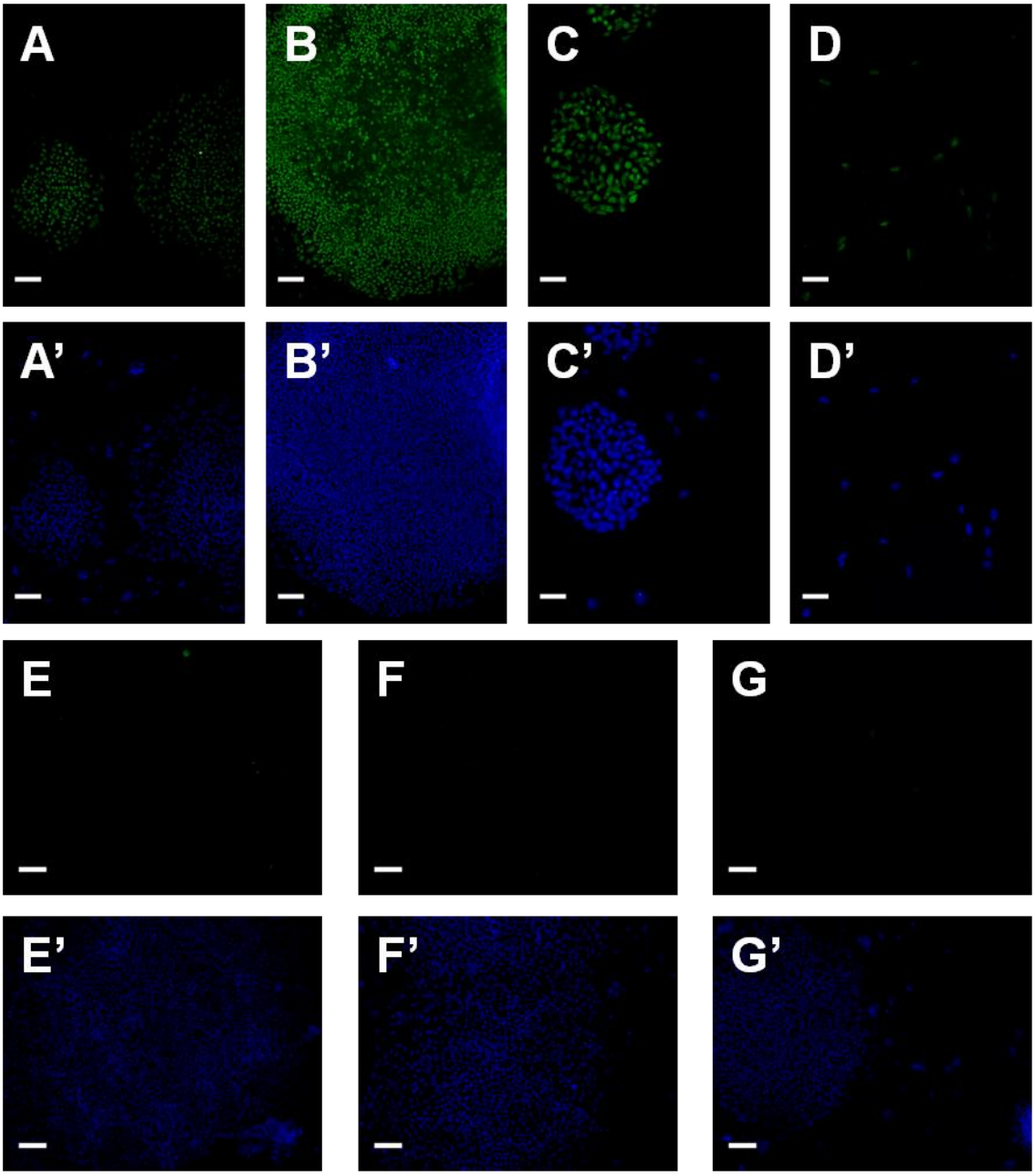

Figure 4.5: ***Immunocytochemistry for transcription factor Oct-4.*** **(A)** Oct-4 stain for iPSC on iMEF. **(A')** DAPI counterstain for iPSC on iMEF. **(B)** Oct-4 stain for iPSC on iFCF. **(B')** DAPI counterstain for iPSC on iFCF. **(C)** Positive control H9. **(C')** DAPI counterstain for H9. **(D)** Oct-4 stain for MP XLIX. **(D')** DAPI counterstain for MP XLIX. **(E)** Primary emission control for iPSC on iMEF. **(E')** DAPI counterstain for primary emission control. **(F)** Swine anti Rabbit secondary emission control for iPSC on iMEF. **(F')** DAPI counterstain for secondary emission control. **(G)** IgG (Rabbit) control for iPSC on iMEF. **(G')** DAPI counterstain fo IgG control(Controls for iPSC on iFCF, H9 and MP XLIX in appendix IV, V & VI (Scale bar = 100µm)).

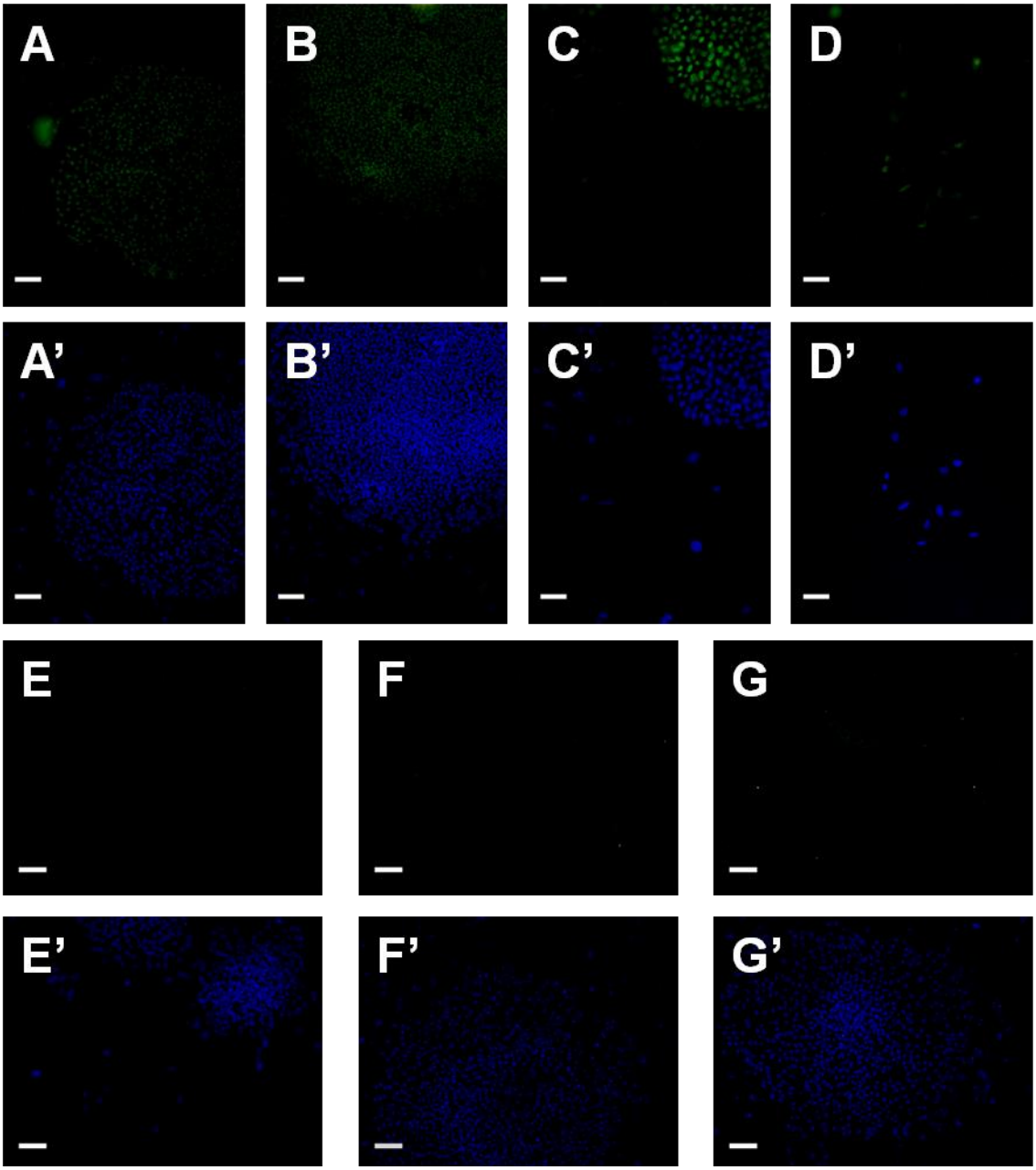

Figure 4.6: ***Immunocytochemistry for transcription factor Sox-2***. **(A)** Sox-2 stain for iPSC on iMEF. (**A')** DAPI counterstain for iPSC on iMEF. **(B)** Sox-2 stain for iPSC on iFCF. **(B')** DAPI counterstain for iPSC on iFCF. **(C)** Positive control H9. **(C')** DAPI counterstain for H9. **(D)** Sox-2 stain for MP XLIX. **(D')** DAPI counterstain for MP XLIX. **(E)** Primary emission control for iPSC on iMEF. **(E')** DAPI counterstain for primary emission control. **(F)** Rabbit anti mouse secondary emission control for iPSC on iMEF. **(F')** DAPI counterstain for secondary emission control. **(G)** IgG (Mouse) control for iPSC on iMEF. **(G')** DAPI counterstain for IgG control (Controls for iPSC on iFCF, H9 and MP XLIX in appendix IV, V & VI (Scale bar = 100µm))

Primary omission control, Rabbit anti Mouse secondary omission control and IgG (Mouse) control for Sox-2 were found to be negative (Figure 4.6 E, F, G). The presence of cell surface markers SSEA-4 and SSEA-5 were also found to be positively produced by all 6 potential iPSC culture on iMEFs (Figure 4.7 A, Figure 4.8 A and Table 4.1) and the potential iPSC culture on iFCF (Figure 4.7 B and Figure 4.8 B). H9 cells also demonstrated production of both SSEA-4 and SSEA-5 (Figure 4.7 C and Figure 4.8 C) however, MP XLIX cells were negative for these cell surface markers (Figure 4.7 D and Figure 4.8 D). Primary omission control, Rabbit anti Mouse secondary omission control and IgG (Mouse) control for SSEA-4 and SSEA-5 were found to be negative (Figure 4.7 &4.8 E, F, G). Presence of the final two cell surface markers; TRA-1-60 and TRA-1-81 were also found to be positively produced by all 6 potential iPSC colonies on iMEFs (Figure 4.9 A and Figure 4.10 A, Table 4.1) and the potential iPSC colony of iFCF (Figure 4.9 B and Figure 4.10 B). Protein production was detected in H9 cells for both TRA-1-60 and TRA-1-81 (Figure 4.9 C and Figure 4.10C) however, presences was not detected in MP XLIX cells for these markers (Figure 4.9 D and Figure 4.10 D). Primary omission controls, Rabbit anti Mouse secondary omission controls and IgM control for TRA-1-60 and TRA-1-81 were found to be negative (Figure 4.9 & 4.10 E, F, G).

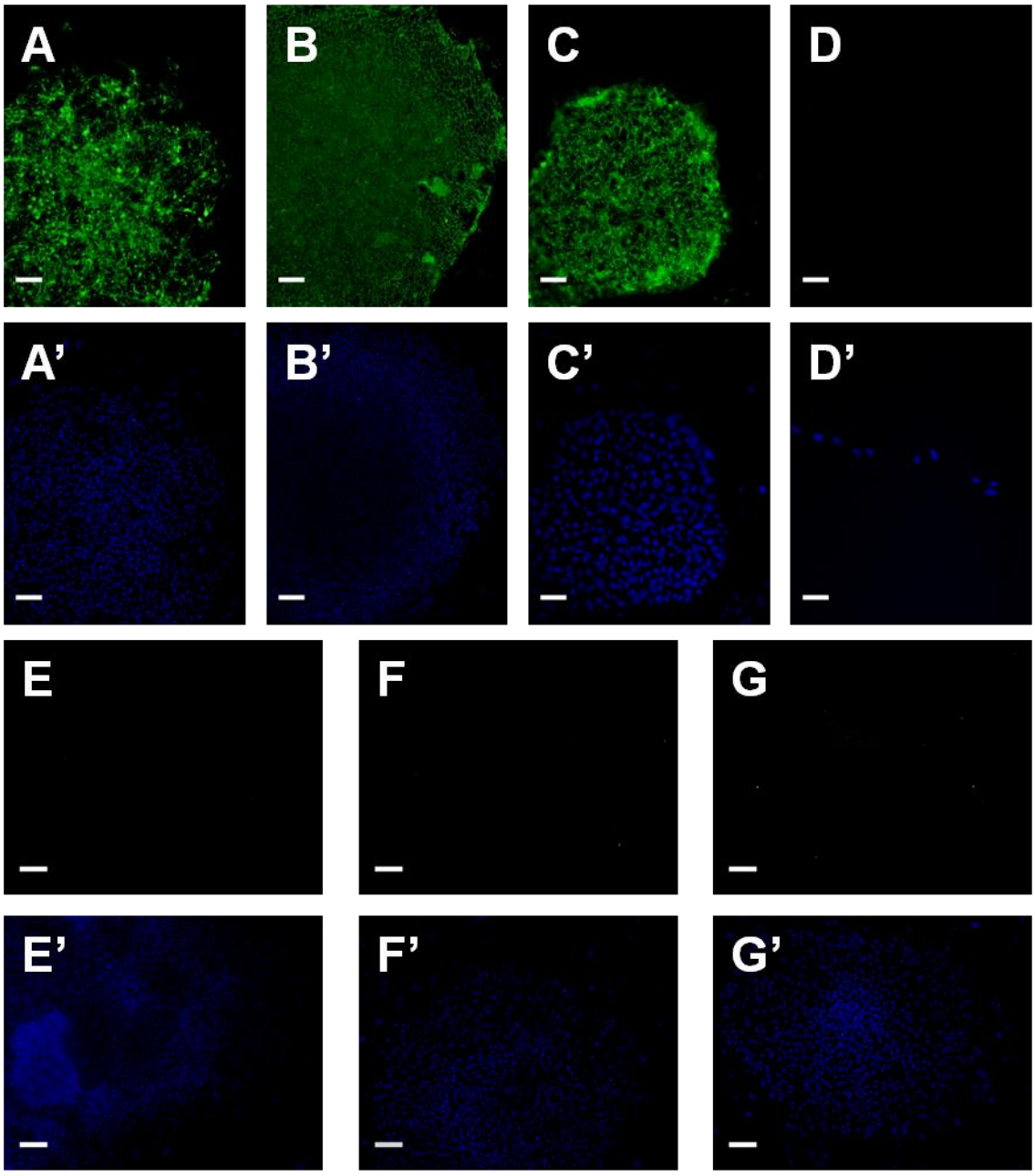

Figure 4.7: ***Immunocytochemistry for cell surface marker SSEA-4.*** **(A)** SSEA-4 stain for iPSC on iMEF. **(A')** DAPI counterstain for iPSC on iMEF. **(B)** SSEA-4 stain for iPSC on iFCF. **(B')** DAPI counterstain for iPSC on iFCF. **(C)** Positive control H9. **(C')** DAPI counterstain for H9. **(D)** SSEA-4 stain for MP XLIX. **(D')** DAPI counterstain for MP XLIX. **(E)** Primary emission control for iPSC on iMEF. **(E')** DAPI counterstain for primary emission control. **(F)** Rabbit anti mouse secondary emission control for iPSC on iMEF. **(F')** DAPI counterstain for secondary emission control. **(G)** IgG (Mouse) control for iPSC on iMEF. **(G')** DAPI counterstain for IgG control (Controls for iPSC on iFCF, H9 and MP XLIX in appendix IV, V & VI (Scale bar = 100µm)).

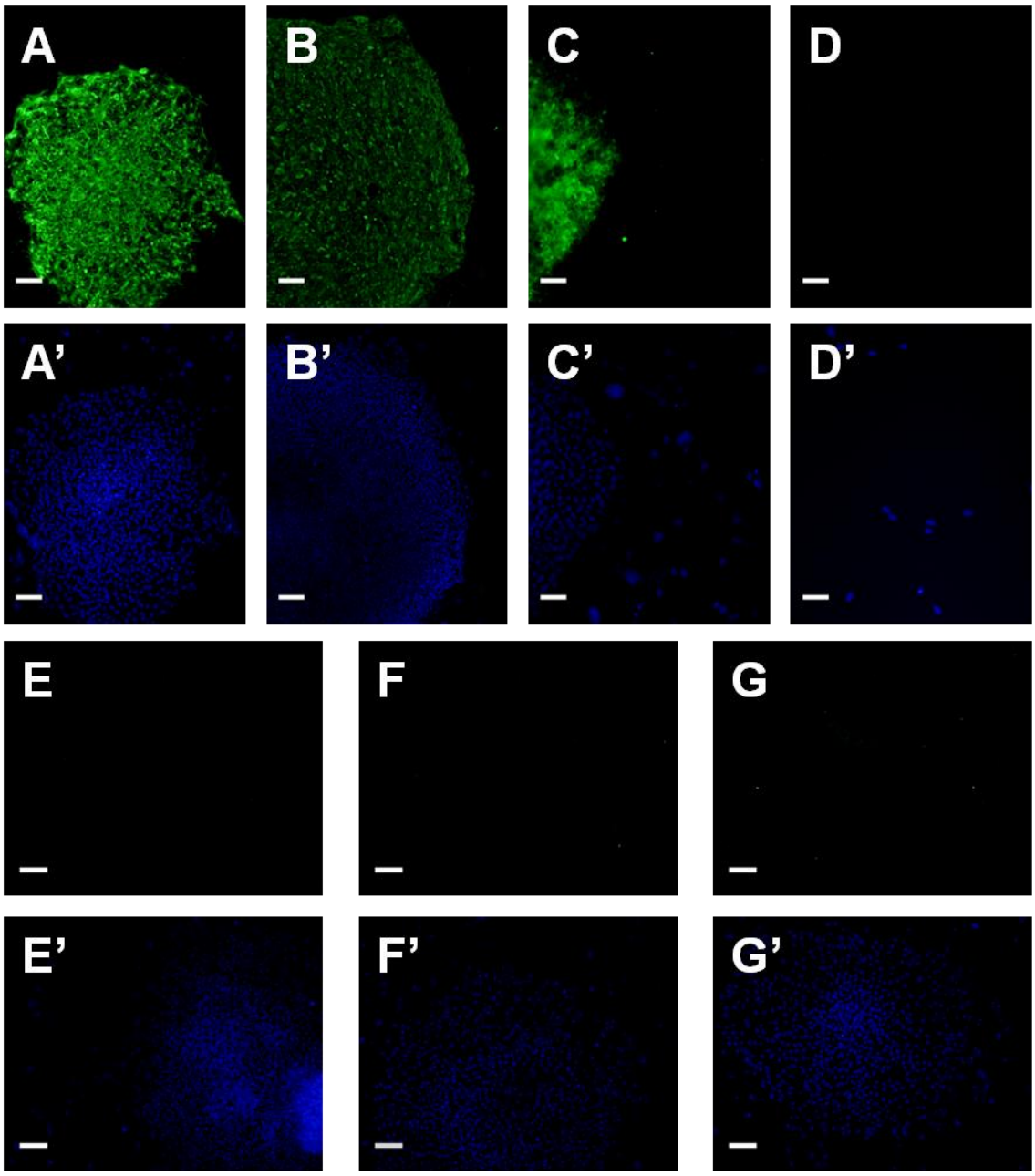

Figure 4.8: ***Immunocytochemistry for cell surface marker SSEA-5***. **(A)** SSEA-5 stain for iPSC on iMEF. **(A')** DAPI counterstain for iPSC on iMEF. **(B)** SSEA-5 stain for iPSC on iFCF. **(B')** DAPI counterstain for iPSC on iFCF. **(C)** Positive control H9. **(C')** DAPI counterstain for H9. **(D)** SSEA-5 stain for MP XLIX. **(D')** DAPI counterstain for MP XLIX. **(E)** Primary emission control for iPSC on iMEF. **(E')** DAPI counterstain for primary emission control. **(F)** Rabbit anti mouse secondary emission control for iPSC on iMEF. **(F')** DAPI counterstain for secondary emission control. **(G)** IgG (Mouse) control for iPSC on iMEF. **(G')** DAPI counterstain for IgG control (Controls for iPSC on iFCF, H9 and MP XLIX in appendix IV, V & VI)

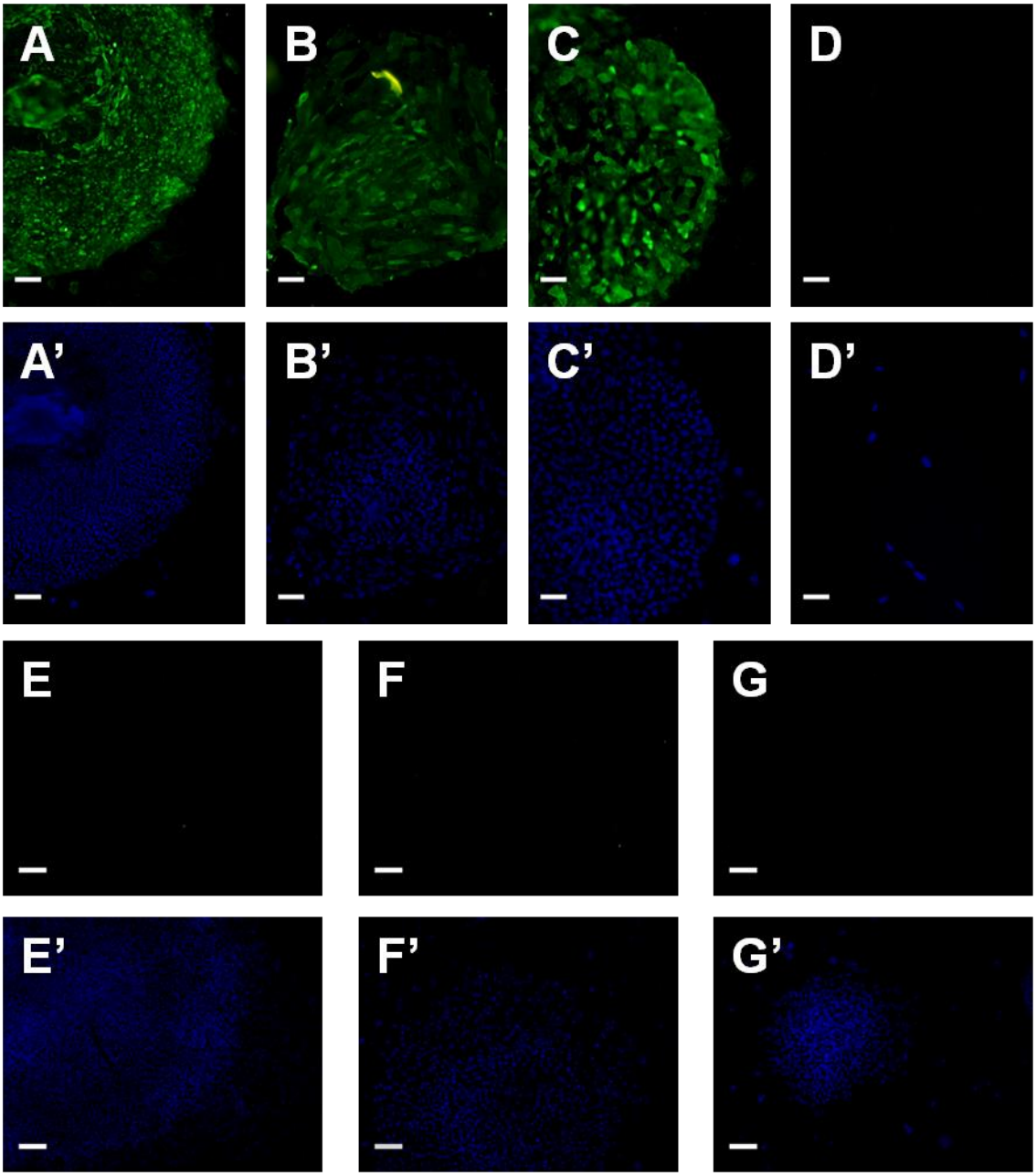

Figure 4.9: ***Immunocytochemistry for cell surface marker TRA-1-60.*** **(A)** TRA-1-60 stain for iPSC on iMEF. **(A')** DAPI counterstain for iPSC on iMEF. **(B)** TRA-1-60 stain for iPSC on iFCF. **(B')** DAPI counterstain for iPSC on iFCF. **(C)** Positive control H9. **(C')** DAPI counterstain for H9. **(D)** TRA-1-60 stain for MP XLIX. **(D')** DAPI counterstain for MP XLIX. **(E)** Primary emission control for iPSC on iMEF. **(E')** DAPI counterstain for primary emission control. **(F)** Rabbit anti mouse secondary emission control for iPSC on iMEF. **(F')** DAPI counterstain for secondary emission control. **(G)** IgM control for iPSC on iMEF. **(G')** DAPI counterstain for IgG control.(Controls for iPSC on iFCF, H9 and MP XLIX in appendix IV, V & VI (Scale bar = 100µm)

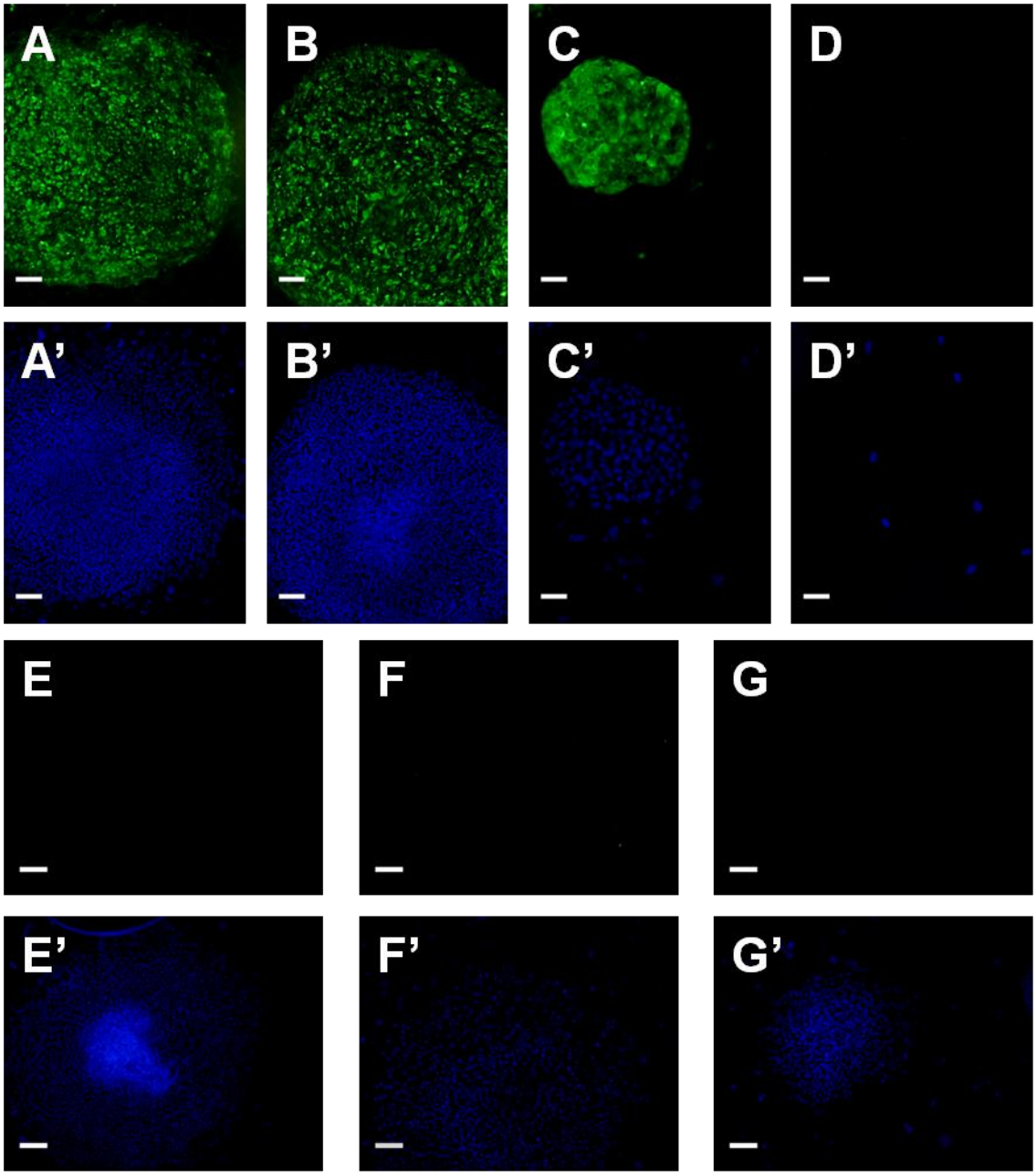

Figure 4.10: ***Immunocytochemistry for cell surface marker TRA-1-81.*** **(A)** TRA-1-81 stain for iPSC on iMEF. **(A')** DAPI counterstain for iPSC on iMEF. **(B)** TRA-1-81 stain for iPSC on iFCF. **(B')** DAPI counterstain for iPSC on iFCF. **(C)** Positive control H9. **(C')** DAPI counterstain for H9. **(D)** TRA-1-81 stain for MP XLIX. **(D')** DAPI counterstain for MP XLIX. **(E)** Primary emission control for iPSC on iMEF. **(E')** DAPI counterstain for primary emission control. **(F)** Rabbit anti mouse secondary emission control for iPSC on iMEF. **(F')** DAPI counterstain for secondary emission control. **(G)** IgM control for iPSC on iMEF. **(G')** DAPI counterstain for IgG control. (Controls for iPSC on iFCF, H9 and MP XLIX in appendix IV, V & VI (Scale bar = 100µm)).

Table 4.1: Pluripotency marker production of Oct-4, Sox-2, SSEA-4, SSEA-5, TRA-1-60 and TRA-1-81 for all isolated iPSC on both iMEFs and iFCFs, H9 and MP XLIX.

		Pluripotency Marker Expression					
Feeder Layer	**Sample Name**	**Oct-4**	**Sox-2**	**SSEA-4**	**SSEA-5**	**TRA-1-60**	**TRA-1-81**
iMEF	iP15	+	+	+	+	+	+
iMEF	iP14	+	+	+	+	+	+
iMEF	iP18	+	+	+	+	+	+
iMEF	iP1	+	+	+	+	+	+
iMEF	iP4	+	+	+	+	+	+
iMEF	iP40	+	+	+	+	+	+
iFCF	iP17	+	+	+	+	+	+
iMEF	H9	+	+	+	+	+	+
N/A	MP XLIX	+	+	-	-	-	-

4.3.2.2 Formation of Embryoid Bodies

Further characterisation of the potential iPSCs was carried out by determining the differentiation capacity of each culture. Numerous protocols exist for differentiation of human ES cells and iPSCs and many include an initial EB formation step before differentiation is commenced. The first potential iPSC culture isolated on iMEFs (iP14) was selected as a comparison with the potential iPSC culture on iFCF (iP17) for EB formation. MP XLIX cells that had not been transfected were again used as a comparative control. The simplest method for EB formation was selected to determine the potential for each culture to form EBs. Colonies were passaged, disaggregated and plated onto non-tissue culture coated plastic and cultured in iPSC media without bFGF allowing random formation of EBs. Following 48 hours in culture, iP17 readily formed small EB structures (Figure 4.11 A) and following 8 days in culture, EBs had increased slightly in size (Figure 4.11 B). Similarly, following 48 hours in culture iP14 readily formed small EB structures (Figure 4.11 C). However, following 8 days in culture EB size was considerably larger on average compared to EBs formed in iP17 cultures (Figure 4.11 D). When MP XLIX cells were cultured on non-tissue culture coated plastic, some cells were found to aggregate to form EB-like structures after 48 hours in culture however, despite the plate coating, some cells were found to have adhered to the bottom of the dish (Figure 4.11 E). Following 8 days in culture EB-like structures that were present were slightly larger in size compared to those after 48hours in culture and very regular in shape (Figure 4.11 F).

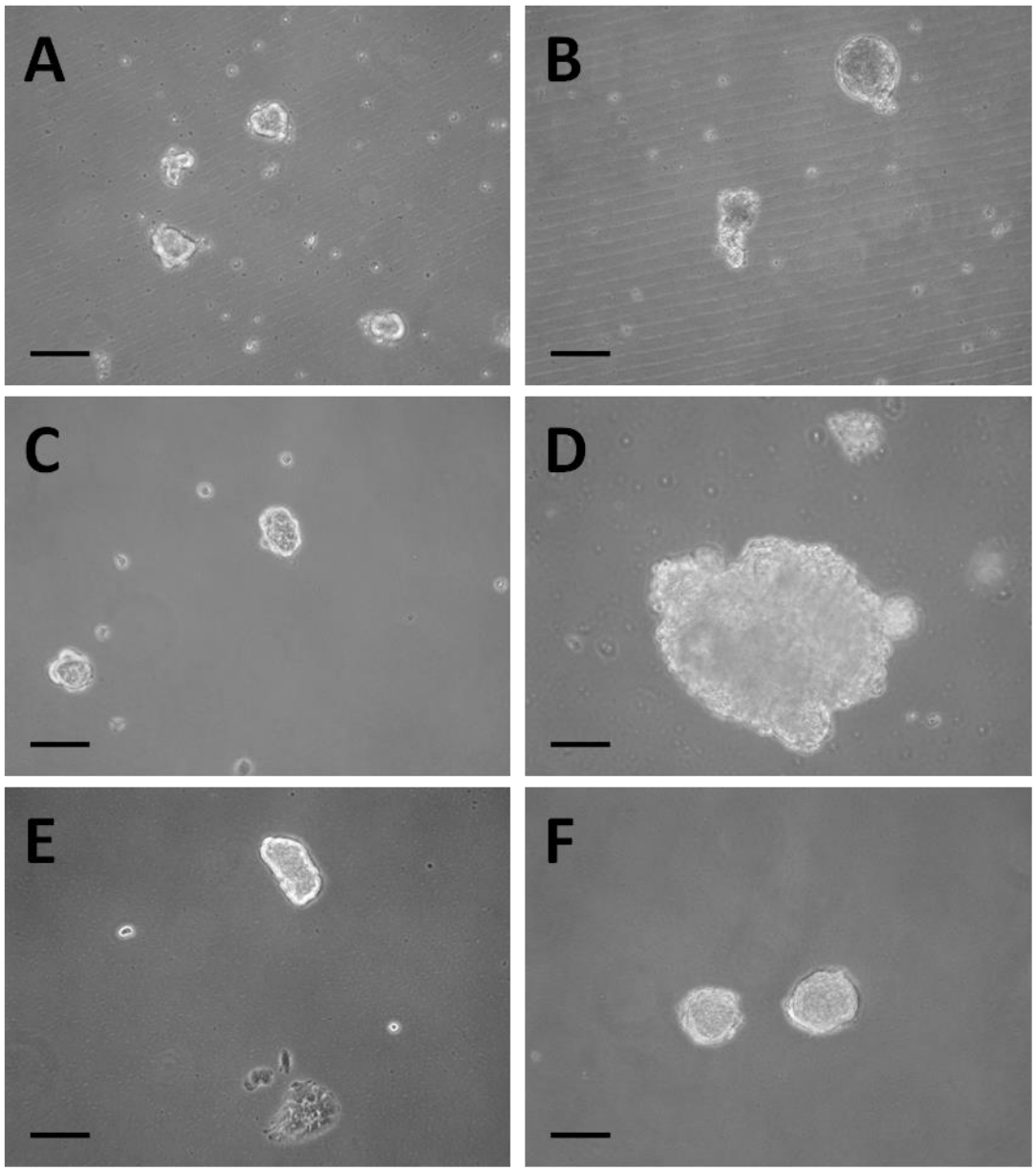

Figure 4.11: ***Embryoid body formation on non-tissue culture coated plastic cultured in Human ES medium without bFGF***. **(A)** EB formation from iP17 on iFCF 48 hours post passage. **(B)** EB formation from iP17 on iFCF after 8 days in culture. **(C)** EB formation from iP14 on iMEFs 48 hours post passage. **(D)** EB formation from iP14 on iMEFs after 8 days in culture. **(E)** EB formation in MP XLIX 48 hours after passage. **(F)** EB formation from MP XLIX after 8 days in culture. (Scale bar = 100µm)

4.3.2.3 Differentiation down Mesoderm, Endoderm and Ectoderm lineages

In order to determine whether these EBs cultured from iP17, iP14 and MP XLIX had the potential to form cells from all three germ layers, they were subjected to three different differentiation conditions. Each of the EBs were plated down onto dishes pre-coated with matrigel and cultured in (i) iPSC medium without bFGF to allow spontaneous differentiation of EBs (Figure 4.12 A, D & G), (ii) iPSC medium supplemented with retinoic acid to promote differentiation down a neuronal lineage to demonstrate ectoderm differentiation (Figure 4.12 B, E & H) and (iii) iPSC medium supplemented with DMSO as a vehicle control for retinoic acid (Figure 4.12 C, F & I). In all cases, EBs derived from each cell type demonstrated flattening out and outgrowth of cells from the original EB structure by day 14. Each of these cultures was stained for typical early stage markers of mesoderm, endoderm and ectoderm. iP17 EBs were found to differentiate down early endoderm lineages by demonstrating positive production of α-1-fetoprotein (Figure 4.13 A), early ectoderm lineages by presence of βIII tubulin (Figure 4.13 B) and early mesoderm lineage by some limited positive presence of Brachyury (Figure 4.13 C). All IgG controls and secondary omission controls were found to be negative (Figure 4.13 D, E, F & G). Cells cultured in all three media conditions were found to express the three lineage markers (Table 4.2). iP14 EBs demonstrated similar results to iP17, with positive production of α-1-fetoprotein (Figure 4.14 A), βIII tubulin (Figure 4.14 B) and Brachyury (Figure 4.14 C) with the three different culture conditions resulting in differentiation

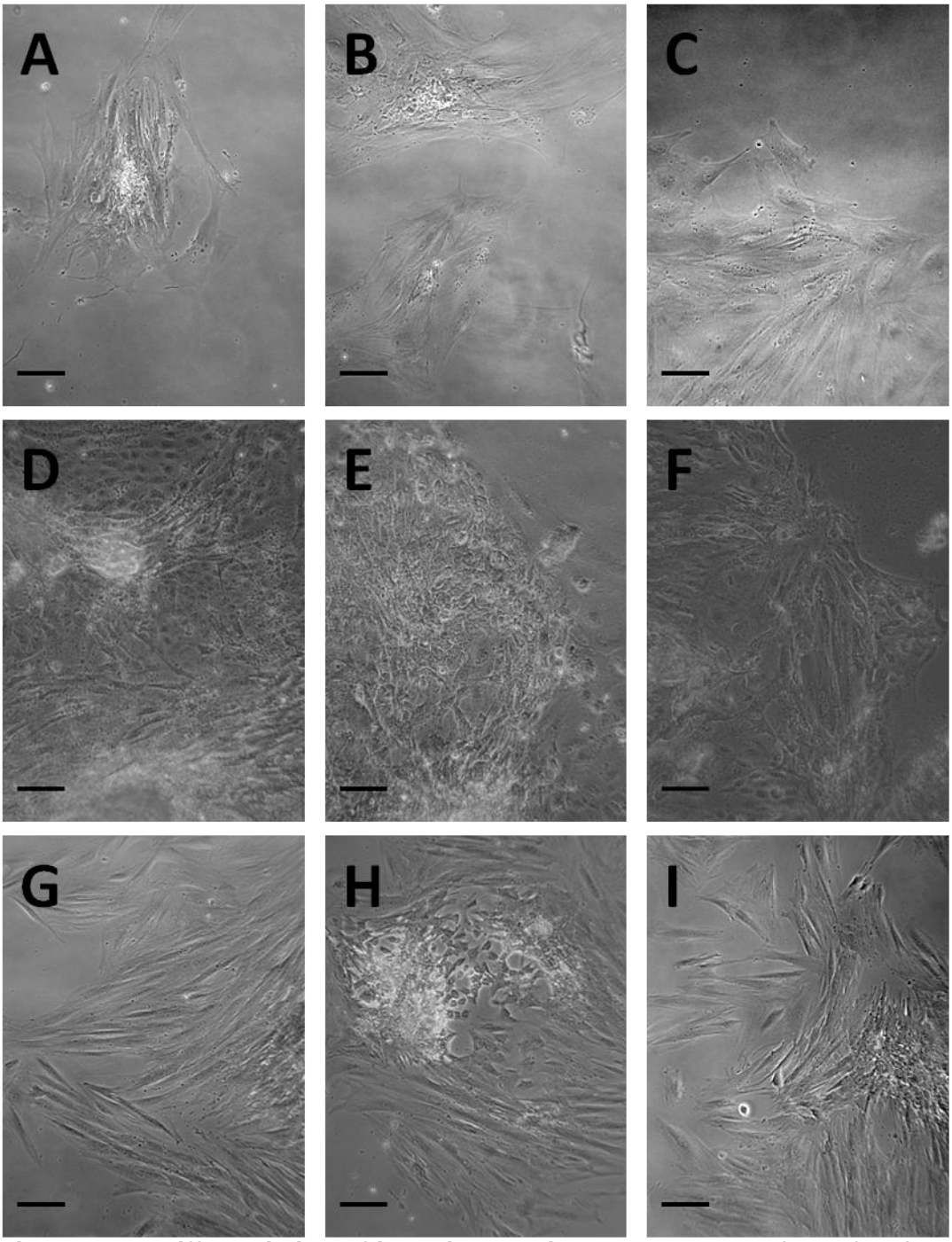

Figure 4.12: ***Differentiation of iP17, iP14 and MP XLIX EBs*** 14 days after they were plated down onto matrigel®-coated plastic in three different media conditions. **(A)** Differentiation of iP17 in iPSC medium. **(B)** Differentiation of iP17 in iPSC medium supplemented with retinoic acid. **(C)** Differentiation of iP17 in iPSC medium supplemented with DMSO. **D** Differentiation of iP14 in iPSC medium. **(E)** Differentiation of iP14 in iPSC medium supplemented with retinoic acid. **(F)** Differentiation of iP14 in iPSC medium supplemented with DMSO. **(G)** Differentiation of MP XLIX in iPSC medium. **(H)** Differentiation of MP XLIX in iPSC medium supplemented with retinoic acid. **(I)** Differentiation of MP XLIX in iPSC medium supplemented with DMSO. (Scale bar = 100µm)

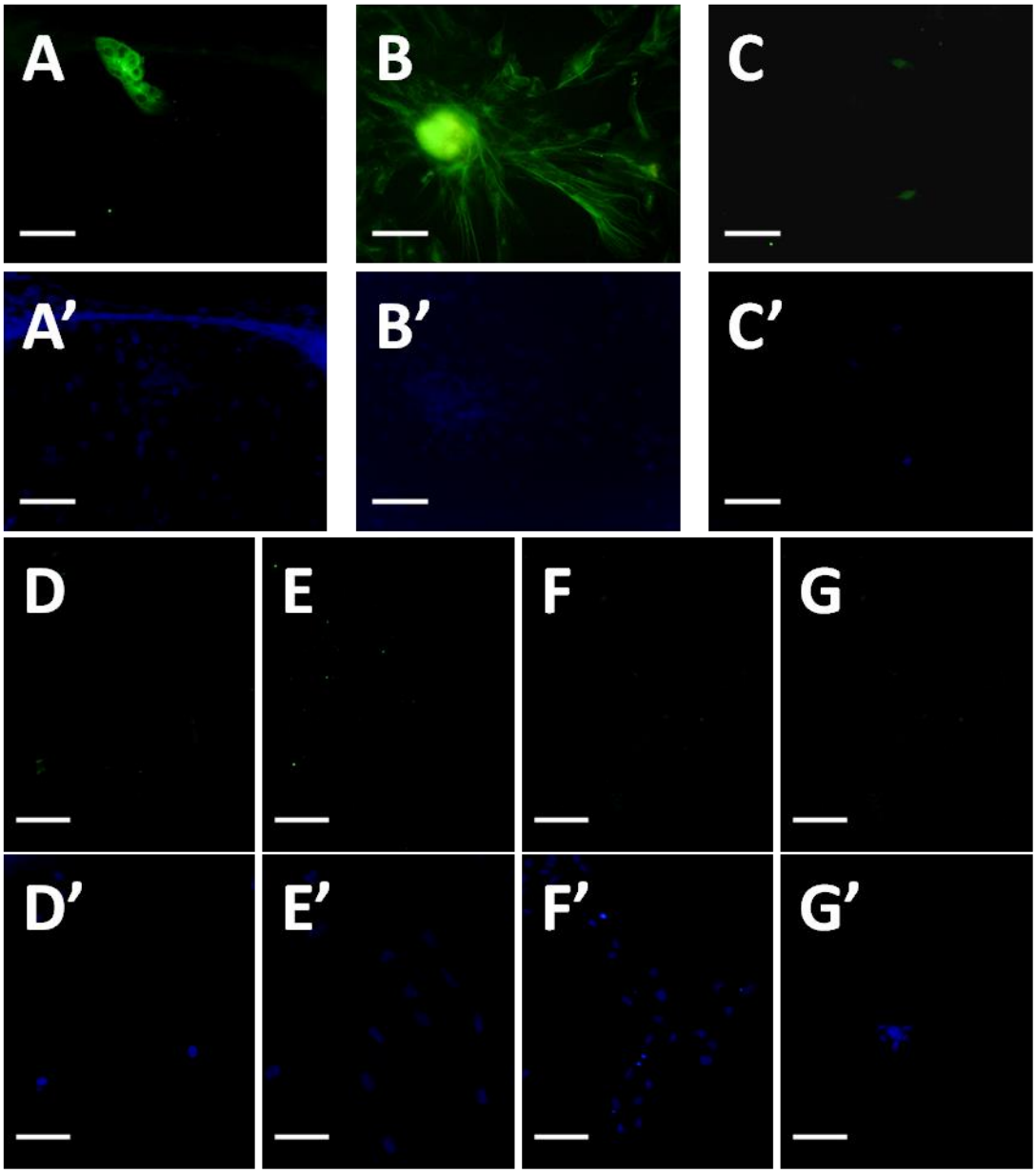

Figure 4.13: ***Immunocytochemistry for typical early stage endoderm, ectoderm and mesoderm differentiation for iP17.*** **(A)** Production of α-1-fetoprotein. **(A')** DAPI nuclear stain for A. **(B)** Production of βIII-tubulin. **(B')** DAPI nuclear stain for B. **(C)** Production of Brachyury. **(C')** DAPI nuclear stain for C. **(D)** IgG (Rabbit) control**.** **(D')** DAPI nuclear stain for D. **(E)** IgG (Mouse) control. **(E')** DAPI nuclear stain for E. **(F)** Swine anti Rabbit secondary emission control. **(F')** DAPI nuclear stain for F. **(G)** Rabbit anti Mouse secondary emission control. **(G')** DAPI nuclear stain for G. (Scale bar = 100μm).(*n=1*)

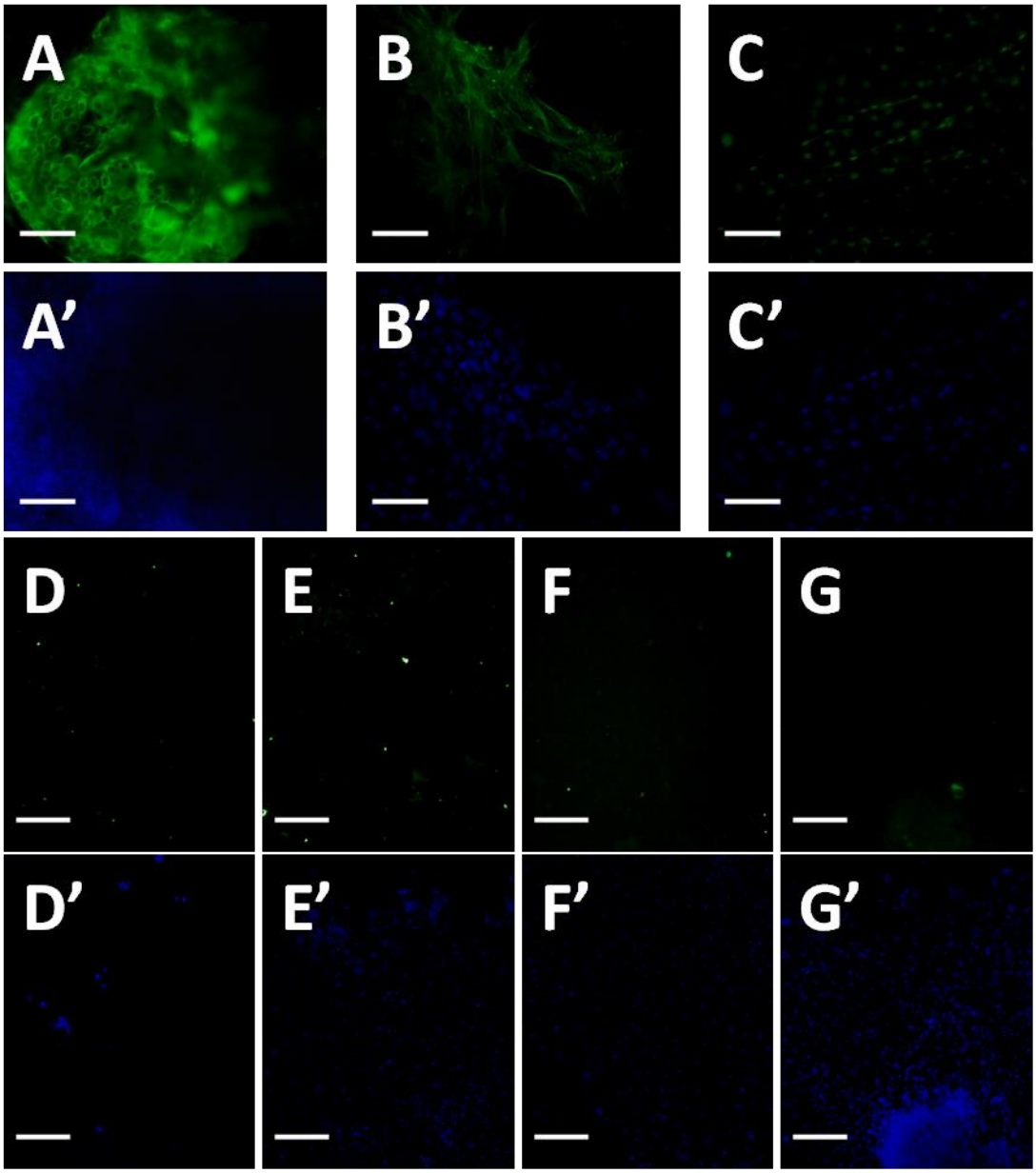

Figure 4.14: ***Immunocytochemistry for typical early stage endoderm, ectoderm and mesoderm differentiation for iP14.*** **(A)** Production of α-1-fetoprotein. **(A')** DAPI nuclear stain for A. **(B)** Production of βIII-tubulin. **(B')** DAPI nuclear stain for B. **(C)** Production of Brachyury. **(C')** DAPI nuclear stain for C. **(D)** IgG (Rabbit) control**. (D')** DAPI nuclear stain for D. **(E)** IgG (Mouse) control. **(E')** DAPI nuclear stain for E. **(F)** Swine anti Rabbit secondary emission control. **(F')** DAPI nuclear stain for F. **(G)** Rabbit anti Mouse secondary emission control. **(G')** DAPI nuclear stain for G. (Scale bar = 100μm)**.(*n=1*)**

down all three lineages (Table 4.2). Again, all controls were found to be negative (Figure 4.14 D, E, F & G). When subjected to identical differentiation conditions, MP XLIX EBs failed to demonstrate expression of any of the early lineage markers; α-1-fetoprotein (Figure 4.15 A), βIII tubulin (Figure 4.15 B) and Brachyury (Figure 4.15 C). Culturing in the different media conditions did not alter this (Table 4.2) and all controls were found to be negative (Figure 4.15 D, E, F & G).

4.4 Summary

- MPs can be reprogrammed to cells with morphology similar to hESCs
- The iPSC colonies produce cell surface markers and transcription factors typical of pluripotent stem cells
- iPSCs were able to differentiate into cells producing early stage markers of all three germ layers; mesoderm, endoderm and ectoderm, confirming their pluripotent properties

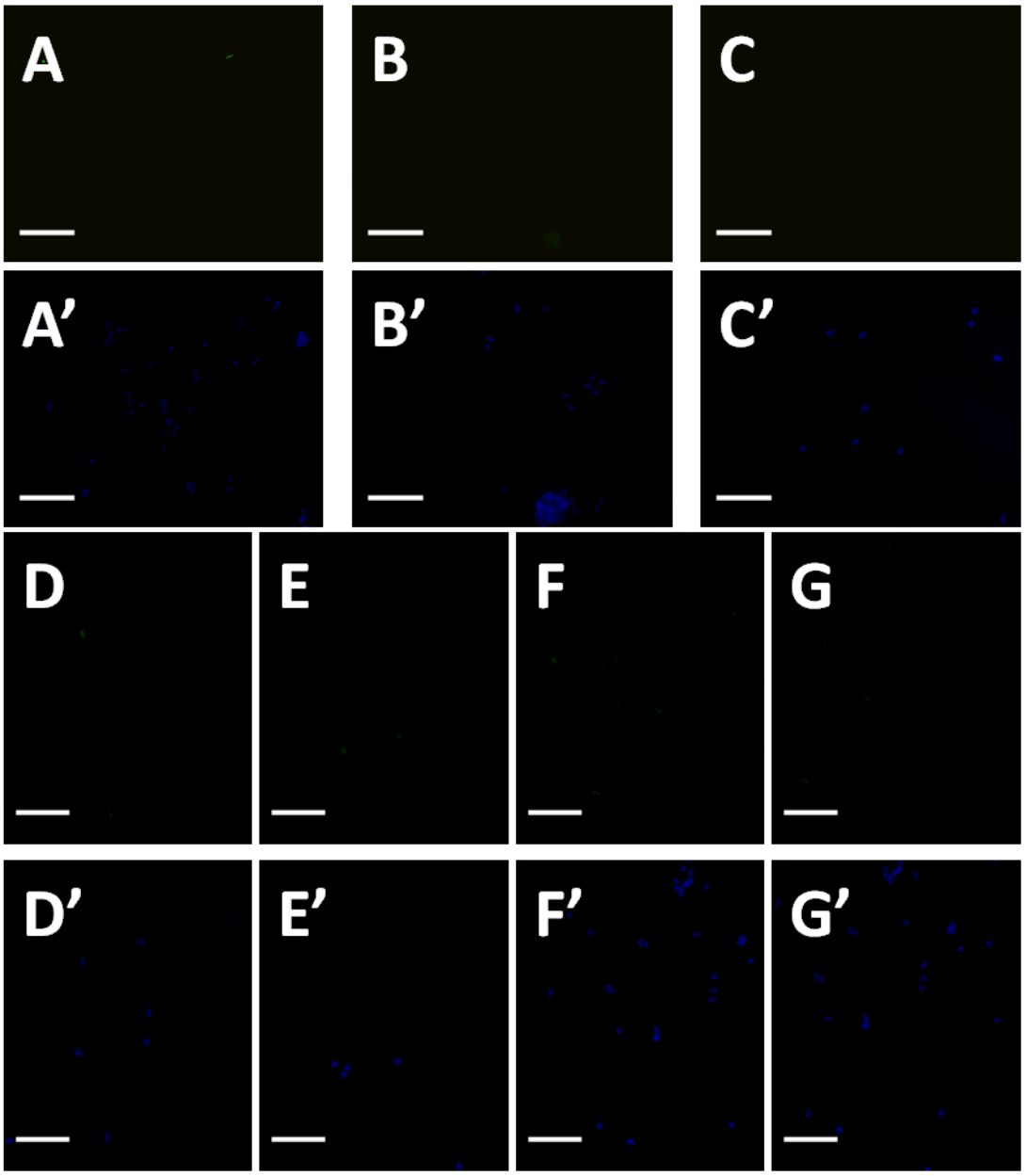

Figure 4.15: ***Immunocytochemistry for typical early stage endoderm, ectoderm and mesoderm differentiation for MP XLIX.*** **(A)** Production of α-1-fetoprotein. **(A')** DAPI nuclear stain for A. **(B)** Production of βIII-tubulin. **(B')** DAPI nuclear stain for B. **(C)** Production of Brachyury. **(C')** DAPI nuclear stain for C. **(D)** IgG (Rabbit) control**. (D')** DAPI nuclear stain for D. (**E)** IgG (Mouse) control. **(E')** DAPI nuclear stain for E. **(F)** Swine anti Rabbit secondary emission control. **(F')** DAPI nuclear stain for F. **(G)** Rabbit anti Mouse secondary emission control. **(G')** DAPI nuclear stain for G. (Scale bar = 100μm)**.(*n=1*)**

Table 4.2: Early stage endoderm (α-1-fetoprotein (AFP)), ectoderm (βIII tubulin) and mesoderm (Brachyury) marker production for iP17, iP14 and MP XLIX in iPSC medium, iPSC medium supplemented with 10μM retinoic acid and iPSC medium supplemented with DMSO.

Sample	**iPSC medium**			**iPSC medium + retinoic acid**			**iPSC medium + DMSO**		
	AFP	βIII tubulin	Brach-yury	AFP	βIII tubulin	Brach-yury	AFP	βIII tubulin	Brach-yury
iP17	+	+	+	+	+	+	+	+	+
iP14	+	+	+	+	+	+	+	+	+
MP XLIX	-	-	-	-	-	-	-	-	-

4.5 Discussion

4.5.1 Reprogramming with Plasmid Vectors

Two different cell types were used for reprogramming; oral progenitor cells and dermal fibroblast cells. Dermal fibroblasts were used as a positive control for the nucleofection of the plasmids since they have previously been used for this application. (Yu et al, 2009). However, no colonies formed in these transfected cells, which may be explained by the low transfection efficiency observed. Given that the efficiency of the non-integrating plasmid system is very low, high overall transfection efficiency would be required to result in colony formation. A potential explanation for this could be the age of the dermal fibroblasts used. These cells were isolated from adult tissue by a previous member of the Wound Biology Group (Cardiff University) and were used for this investigation at passage 17 as these were the lowest passage available in cryopreservation. Whilst still long before the onset of cellular senescence for this cell type, the manufacturer optimised protocol recommends that cells above passage 9 can reduce the transfection of plasmids and result in increased cell death when nucleofected with the amaxa® nucleofector®. In order to confirm this, additional experiments would need to be carried out by nucleofecting dermal fibroblasts at early, mid and late stages in their lifespan. In addition to this, human dermal fibroblasts utilised for reprogramming purposes are often isolated from neonatal foreskin tissue (Yu et al, 2007; Lowry et al, 2008; Yu et al, 2009), representing a much ‘younger’ population of cells, potentially better for reprogramming purposes.

MP cells from patient XLIX were successfully reprogrammed, using a non-integrating system of two plasmids containing all six factors, to hESC-like cells ; colonies were identified and isolated that demonstrated tight colony boundaries and cells with very high nuclear to cytoplasmic ratio, typical morphological characteristics of hESCs (Thomson et al, 1998). Although fibroblasts from the oral mucosa have already been successfully reprogrammed, it was carried out using retroviral transfer of the genes including the use of C-myc (Miyoshi et al, 2010), which has been linked to formation of carcinomas and poses a clinical risk (Okita et al, 2007; Yamanaka et al, 2007; Nakagawa et al, 2008). The work in this chapter suggests that reprogramming of oral progenitor cells with non-integrating plasmids for transient expression of the pluripotency factors is possible. The resulting iPSCs represent a potential source of cells that are clinically safer for translational research, however, this would need to be tested using numerous patient samples as definitive proof but these initial results are promising. In addition to this, to confirm that the plasmids have not integrated in to the genome, further investigations would need to be carried out comparing the episomal expression of the exogenous genes with the genomic expression of the endogenous genes in these isolated iPSC lines. These two plasmids have been utilised previously to successfully reprogram human dermal fibroblasts and demonstrated formation of iPSCs without integration of the plasmid (Yu et al, 2009). Furthermore, progenitor cells have been suggested as an underlying reason for reprogramming efficiencies of cells isolated from different tissues (Okita et al, 2007; Aasen et al, 2008), therefore, an isolated progenitor cell population may represent a preferential

source of cells for use in reprogramming. Moreover, studies have suggested that MSC-like cells isolated from DPPC and SHEDs are reprogrammed at a potentially higher efficiency (Yan et al, 2010).

Colonies were identified both on iMEFs and iFCFs, confirming that both cell types were able to support the growth of iPSCs from MPs. More colonies were identified and isolated from iMEF feeders than on iFCFs and this could potentially be as a result of the culture conditions which probably need optimising for use with iFCFs to improve iPSC production efficiency. In addition to this, iFCFs were prepared from foreskin fibroblasts previously isolated by members of the Wound Biology Group which had been in cryopreservation for a number of years. The resulting foreskin fibroblasts were irradiated for use as feeder layers at p15-18, corresponding to approximately 40-45 population doublings. This is relatively late when compared to MEFs, which are only used as feeders below p4 (approximately 10-15 population doublings) as they become less effective as feeder cells after this time (Jozefczuk et al, 2012). It has been demonstrated that foreskin fibroblast feeders can support growth of hESC when they are between 9-25 population doublings (Hovatta et al, 2003) so using cells as feeders beyond this time point may reduce their ability to support growth of hESCs cells. However, the fact that the foreskin fibroblasts utilised in this thesis were able to support the growth of the single iPSC line identified is encouraging for the future of these iPSCs for potential use in therapeutic applications and further investigations using foreskin fibroblasts at an earlier stage would be interesting to identify if this improves the reprogramming efficiency.

4.5.2 Characterisation of MP iPSCs

Characterisation of iPSCs formed on both types of feeder layers was next undertaken. Immunocytochemistry was employed as a simple and relatively quick method to screen these isolated lines and compare them to hESCs to confirm successful reprogramming. In addition to this, MP cells from the patient XLIX were used as a comparative control to determine if any of these markers were expressed prior to reprogramming. Oct-4 and Sox-2 are two transcription factors associated with pluripotent stem cells and it has already been demonstrated (Chapter 3) that MP cells express these transcription factors at the RNA level. MP XLIX were positive for these transcription factors, which is expected and confirms that MP XLIX are producing these factors at the protein level, validating the findings in Chapter 3 and is consistent with previous reports on OMLP-PCs (Marynka-Kalmani et al, 2010). All populations of iPSCs on iMEF, iPSC on iFCF and H9 were also positive for these transcription factors, which have previously been reported for both iPSCs and hESCs at the RNA and protein level (Bhattacharya et al, 2005; Lowry et al, 2008; Maherali et al, 2008; Mali et al, 2008; Li et al, 2009). On the other hand, the cell surface markers SSEA-4, SSEA-5, TRA-1-60 and TRA-1-18 (Thomson et al, 1998; Tang et al, 2011) were only positively produced by iPSCs and H9 cells but were not detected in MP XLIX cell population adding weight to the fact that these MP iPSC had possibly been reprogrammed. The presence of these typical stem cell markers, namely SSEA-4, TRA-1-60 and TRA-1-81, in the iPSCs is consistent with the original research carried out for hESCs (Thomson et al, 1998) and results demonstrated for iPSCs by several research groups (Takahashi et al, 2007;

Maherali, 2007; Miyoshi et al, 2010) confirming the presence of pluripotent stem cells. Moreover, the expression of SSEA-5 further confirms that these cells are in an undifferentiated state, given that expression of SSEA-5 is the first of the investigated markers known to be lost upon differentiation of the stem cells (Tang et al, 2011). The presence of pluripotency markers; Oct-4, SSEA-4, TRA-1-60 and TRA-1-81 observed for OMLP-iPSCs is consistent with those observed for iPSCs derivation from the OMLP utilising retroviral integration (Myioshi et al, 2010). The major difference demonstrated by the retroviral integration in comparison to plasmid transfection was formation of iPSC-like colonies after 13 days. The plasmid system resulted in a longer time period before colony formation was observed, suggesting better efficiency from retroviral systems (Myioshi et al, 2010).

In order to establish whether these iPSCs had pluripotent stem cell properties, their ability to differentiate down all three germ layers was investigated *in vitro*, a method commonly used as a test for pluripotency prior to carrying out a teratoma assay (Maherali et al, 2008). Before differentiation was initiated, the ability for iPSCs to form EBs was tested. EBs readily formed when populations of iPSCs, maintained both on iMEFs and iFCFs, were cultured on non-tissue culture coated plastic without growth factor, consistent with standard protocols for hESC EB culture (Bhattacharya et al, 2005) and EB formation observed with other iPSC lines (Zhang et al, 2009; Sullivan et al, 2010; Hu et al, 2010). Additionally, when MP XLIX cells were cultured on non-tissue culture coated plastic as a comparative control, they too formed EB-like structures. Although these EBs were observed to

increase in size in all tested cell populations, this was most apparent with iP14 where the EBs formed were larger, similar to previous findings with hESC (Itskovitz-eldor et al, 2000). When subjected to differentiation conditions only the differentiated EBs from iP14 positively produced early markers of mesoderm (Brachyury), endoderm (α-1-fetoprotein) and ectoderm (βIII tubulin), consistent with previous reports for iPSCs (Aasen et al, 2008; Maherali et al, 2008; Mali et al, 2008; Lin et al, 2009). Expression of both βIII tubulin and α-1-fetoprotein were detected in iP17 EBs but they had a very limited production of Brachyury, this indicates potentially a more limited differentiation capacity than iP14. The limited production of Brachyury further suggests that the foreskin fibroblasts used as feeder layers in this investigation potentially did not fully support the growth and maintenance of iPSCs. Staining with additional early stage markers of mesoderm, ectoderm and endoderm would provide further evidence of pluripotency and subsequent performance of an *in vivo* teratoma assay would provide definitive proof of the pluripotency of these cells. Presence of all three markers was not detected in differentiated EB-like structures of MP XLIX, suggesting that MP XLIX do not display these pluripotent properties prior to reprogramming. This provided further evidence that MP iPSCs are responding differently and have been reprogrammed.

It should be noted that iPSC colonies identified and isolated would need to be checked by karyotyping to ensure that the cells are karyotypically normal. This is routinely performed for hESCs (Narva et al, 2010) and importantly in iPSCs due to the genetic manipulation involved in reprogramming (Yu et al,

2007). In addition to this, the iPSCs still require testing for their ability to form teratomas in immune-compromised mice, which remains the ultimate test for confirmation of pluripotency (Thomson et al,1998; Takahashi et al, 2007).

4.6 Conclusion

The work in this Chapter has demonstrated that OP cells isolated from the oral mucosa lamina propria can be reprogrammed to cells demonstrating typical stem cell markers and pluripotent characteristics of hESCs using a non-integrating system with all 6 reprogramming factors; Oct-4, Sox-2, Nanog, Lin-28, Klf-4 and C-myc (Yu et al, 2009). The resulting iPSCs demonstrated expression of early stage markers of the mesoderm, endoderm and ectoderm, confirming the potential for these cells to differentiate into cell types from all three germ layers. Furthermore, the early indication that these MP iPSCs can be formed and maintained on foreskin fibroblast feeder layers, leading in colony formation with similar properties to those formed and maintained on mouse embryonic fibroblast feeder layers, adds more promise for the potential use of these cells in clinical applications making MP iPSCs a great potential source of cells for cell-based therapies.

5 - Neural differentiation of oral mucosa lamina propria progenitor cells versus oral mucosa derived iPSCs

5.1 Introduction

One area that has received much consideration recently is the possibility for ASC, hESC and iPSCs to differentiation into functional neurons. The resulting neurons would create an invaluable source of cells for use in neural damage. The limited regenerative capacity of neurons is well known and although some evidence does exist for neurogenesis in adult humans (Eriksson et al, 1998; Curtis et al, 2011), nerve regeneration following injury still remains a surgical challenge. This is largely due to the fact that once nerves are damaged, there are molecules present within the myelin sheath that inhibit axon re-growth, thereby limiting the regenerative capacity (Ng et al, 1996). This leads to devastating results, for example, paralysis following spinal cord injury, and therefore cell based therapies are being sought to overcome this problem.

The ability for pluripotent stem cells (ESCs and more lately iPSCs) to differentiate into neurons has received much attention in recent years including their differentiation towards neural precursors, the functional ability of these differentiated cells and promoted functional recovery of *in vivo* models (Dhara et al, 2008; Ladewig et al, 2008; Hu et al, 2010; Young et al, 2011; Haythornthwaite et al, 2012; Takazawa et al, 2012). Given the ability of ESCs and iPSCs to self-renew and the fact they possess pluripotent properties (Thomson et al, 1998; Takahashi et al, 2007), differentiation can be difficult to control and thus there are concerns about transplanting a population of cells that potentially still contain undifferentiated stem cells with self-renewal characteristics (Vats et al, 2005; Cunningham et al, 2012).

However, utilising the correct culture conditions, growth factors and signals to direct differentiation of pluripotent stem cells towards a pre-differentiated functional phenotype could make these an attractive cell source to target.

Adult stem cells have also demonstrated potential in this field and although adult stem cells have a more limited differentiation potential, they are an invaluable source of cells for differentiation. They have potential therapeutic purpose as an autologous, and potentially allogeneic, source of cells, with both MSCs and OMLP-PCs demonstrating immunosuppressive properties (Le Blanc et al, 2004, Davies et al, 2012). Progenitor cells isolated from the OMLP have demonstrated some limited potential to differentiate into cells with neural marker expression (Davies et al, 2010; Marynka-Kalmani et al, 2010). OMLP-PCs have been isolated from single cells forming a colonies of progenitor cells for use in differentiation studies. Clonally-derived cultures are advantageous as cells are, in theory identical and will therefore differentiate in a similar manner. Thus, these cells represent a more efficacious and controllable population for use in therapeutic applications. These protocols have involved the formation of neurpsphere-like structure followed by directed differentiation using defined media conditions (Davies et al, 2010). Additions of growth factors know to be involved with neural differentiation including; Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT3) and Nerve Growth Factor (NGR) have also been utilised (Davies et al, 2010, Marynka-Kalmani et al, 2010).

As yet however, the ability of OMLP-PCs to form a functional neural phenotype *in vitro* has not been investigated. Therefore, the purpose of this

chapter is to investigate and compare the abilities of multipotent OMLP-PCs verses pluripotent OMLP-iPSCs to differentiate into functional neural phenotypes to assess these different cell sources for use as potential avenues for therapeutic application. Not only will such cells have the potential for use in many regenerative medicine applications but they may also help further our understanding in this wide field.

5.1.1 Hypothesis

iPSCs are a better cell source for neural differentiation when compared to OMLP-PCs.

5.1.2 Aims

- To differentiate OMLP-PCs and OMLP- iPSCs down a neural lineage
- Carry out functional testing of these differentiated cell types
- Carry out a comparative analysis of the differentiation potential of OMLP-PCs and oral iPSCs

5.2 Materials and Methods

Methods describing specific cell culture protocols (oral mucosa lamina propria cells, human embryonic stem cells and oral iPSCs), imaging of cells and gene expression studies have been described elsewhere (Chapter 2).

5.2.1 End-point PCR Analysis of Stem Cell and Neural Crest Marker Expression

PCR was carried out as described in Chapter 2 using primers specific to stem cell markers, haemopoietic markers, neural crest markers and a house keeping gene (Table 5.1). These were used to confirm previous findings (Davies et al, 2010).

5.2.2 Neural Differentiation

In order to establish the differences in differentiation capacity, different colonies isolated from OMLP-PCs were examined for their ability to differentiate down a neural lineage. Colonies were isolated from several patients: XLVII, XLVIII, XLIX, L and LII. iPSCs derived from patient XLIX were also investigated.

5.2.2.1 Formation of neurospheres

Human laminin (Sigma-aldrich, UK) was diluted to 20µg/mL in cold Dulbecco's PBS. One well of a 6 well TC plate was coated with 1mL of the human laminin solution and left at room temperature overnight. The laminin solution was then removed and the well washed once with PBS before use. Each cell type was cultured and passaged when confluent as previously

described (Chapter 2, Section 2.2.1.3 and Chapter 4, Section 4.2.2.8.3). Cells (utilised between 20-25 PDL for colonies, MP and TP cells. iP14 were utilised at passage 28 and H9 cells at passage 92) were plated at a density of 5 x 10^5 cells/well of a 6 well plate. As passaging of H9 cells and iP14 cells leads to colony clumps, a parallel culture of cells was passaged using collagenase IV to lift the colonies which were then removed and spun at 200 x g for 2 minutes to pellet. The supernatant was removed and the pellet re-suspended in 1 ml of pre-warmed accutase and left for 5 minutes before being triturated, using a 200µL pipette, to form a single cell suspension. This was then used to perform a cell count to determine the approximate number of cells that would be obtained from one well of a 6 well plate of either iP14 of H9 cells. Cells were then maintained in 2mL X-vivo 10 medium (Lonza, UK) supplemented with 0.1mM β-Mercaptoethanol, 0.1mM NEAA and 80ng/mL bFGF. Medium was changed daily and neurosphere-like structures formed over 6-8 days.

Table 5.1: ***Forward and reverse primers of target genes***; housekeeping gene β-actin, P75, Twist, Slug, CD90, CD45 and CD34. Annealing temperature and product length is listed for each gene.

Gene	Accession Number	Primer Sequence	Position (bp)	T anneal (°C)	Product Length (bp)
β-actin	NM_001101.3	F 5'-CCACACTGTGCCCATCTACGAGGGGT-3' R 5'-AGGGCAGTGATCTCCTTCTGCATCCT-3'	564 – 589 1043 - 1018	60	454
P75	NM_002507.3	F 5'-CTGCAAGCAGAACAAGCAAG -3' R 5'-GGCCTCATGGGTAAAGGAGT -3'	956–975 1265-1246	58	310
Twist	NM_000474.3	F 5'-TCGAGAGATGATGCAGGACGT -3' R 5'-TCTGGCTCTTCCTCGCTGTT-3'	345-365 425-406	58	81
Slug	NM_003068.4	F 5'-CATACAGCCCCATCACTGTG -3' R 5'-CTTGGAGGAGGTGTCAGATG-3'	330-349 466-447	58	135
CD90	NM_006288.3	F 5'-ATGAACCTGGCCATCAGCATCG -3' R 5'-CACGAGGTGTTCTGAGCCAGCA -3'	449-470 873-852	55	425
CD45	NM_002838.4	F 5'-GTGACCCCTTACCTACTCACACCACTG -3' R 5'-TAAGGTAGGCATCTGAGGTGTTCGCTG -3'	353–379 808-782	65	456
CD34	NM_001025109.1	F 5'-ACAGGAGAAAGGCTGGGCGAAGACCCT -3' R 5'-TCCCCTGGGGGTTCCTGTATTGCGGCA-3'	1216–1242 1771-1745	65	556

5.2.2.2 Differentiation down a neural lineage using defined medium conditions

Matrigel® was thawed overnight at 4ºC as polymerisation at room temperature is rapid. An aliquot of Matrigel® was diluted on ice to 0.1% v/v in DMEM: F-12 (3:1) using cooled pipette tips. One well of a 6 well TC plate was coated with 1mL of this Matrigel® solution and 100µL of solution was used to coat one well of a chamber slide. These were placed in the incubator to polymerise at 37ºC for 1 hour and then left at room temperature overnight. When spheres were ready to be split, medium was removed from the adherent spheres and replaced with 1mL of 1mg/mL collagenase IV and the plate returned to the incubator and left for 5-10 minutes to digest the spheres. This resulted in cell clumping from the digested spheres, so a parallel culture of cells was set up to trypsinise for cell counting purposes as described above for iPSCs and H9 cells.

When matrigel-coated plates were required, the pre-incubated matrigel solution was removed and cells seeded onto the surface at a density of 5.6 x 10^3 cells/cm^2 in medium 1: DMEM-F12 supplemented with 10% FCS and 40ng/mL bFGF. Two mL of medium 1 was used per well of a 6 well plate and 200µL per well of a chamber slide. Medium changes were performed every 2-3 days for a period of 7 days. After this time medium 1 was exchanged for medium 2: DMEM-F12 supplemented with 10% FCS, a cocktail of neurotrophic factors; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3) (all at 10ng/mL). Cells were fed medium 2 every 2-3 days for a further period of 7 days.

5.2.3 Neural Marker Expression

5.2.3.1 End point PCR analysis of neural marker expression

End-point PCR was carried out as previously described in Chapter 2 (Section 2.2.2.6) for neural marker expression (see Table 5.2) of samples pre- and post-neural differentiation. Primers were designed using NCBI/Primer Blast and specificity was confirmed using Blast database and confirming product size following gel electrophoresis.

5.2.3.2 Immunocytochemistry for neural markers

Following 14 days of neural differentiation, chamber slides were fixed in ice cold 1:1 (v/v) acetone:methanol for 15 minutes 4ºC. Each chamber was then washed 3 times in PBS for 5 minutes. Non-specific binding of the secondary antibody was blocked using a solution of 2% (w/v) BSA (diluted in PBS) and left to incubate for 1 hour at room temperature. This was aspirated off and replaced with primary antibody (Rabbit polyclonal antibody against Neurofilament Medium (NF-M) diluted 1:500 (concentration not determined by manufacturer) and Mouse monoclonal antibodies against microtubule-associated protein 2 (MAP-2; 2.3mg/mL) diluted 1:500, Neuron-specific βIII tubulin, (βIII tubulin; 1mg/mL) diluted 1:1000 and glial fibrillary acidic protein (GFAP; 2mg/mL) diluted 1:200 in 2% BSA in PBS and incubated at 4°C overnight. Following this incubation the chamber slides were washed 3 times for 5 minutes with PBS. Secondary antibodies (Swine anti-rabbit (0.81g/L) and Rabbit anti-mouse (2.3g/L), both Dako, UK) were diluted 1:50 in 2%

Gene	Accession Number	Primer Sequence	Position (bp)	Tanneal (°C)	Product Length (bp)
β-actin	NM_001101.3	F 5’-CCACACTGTGCCCATCTACGAGGGGT-3’ R 5’-AGGGCAGTGATCTCCTTCTGCATCCT-3’	564 – 589 1043 - 1018	60	454
MAP-2	NM_001039538.1	F 5’-GTGACAAGGAGTTTCAAACAGGAA -3’ R 5’-CTGATGGATAACTCTGTGCGAGA -3’	2095–2118 2187-2165	54	93
NF-M	NM_005382.2	F 5’-GTCAAGATGGCTCTGGATATAGAAATC 3' R 5'-TACAGTGGCCCAGTGATGCTT -3'	1201-1227 1304-1284	54	104
Nestin	NM_006617.1	F 5’-CCCTGACCACTCCAGTTTAG -3’ R 5’-CCTCTATGGCTGTTTCTTTCTC -3’	1546–1565 1670-1649	56	125

Table 5.2: ***Forward and reverse primers of target genes***; housekeeping gene β-actin, MAP-2, NF-M and Nestin. Annealing temperature and product length is listed for each gene.

BSA in PBS and added to the cells and incubated for 1 hour at room temperature. Chamber slides were washed 3 times for 5 minutes with PBS before counterstaining the nuclei with DAPI in mounting medium (1.5µg/mL Vector shield, UK). Appropriate IgG controls (Santa Cruz Biotechnology, UK) were used for each antibody to verify specificity by absence of staining. Fluorescent images were acquired as described in Chapter 2 (Section 2.3.1.10).

5.2.4 Calcium imaging within differentiated cells

The presence of calcium channels was investigated using fura-2 acetoxymethyl ester (Molecular Probes, Eugene, USA), a calcium indicator dye that binds to intracellular free calcium and emits light at different wavelengths when bound and unbound to calcium. Cells were cultured on Matrigel®-coated 13mm glass cover slips and subjected to the 14 day neural differentiation protocol (see section 5.2.2). Following differentiation, cell were loaded with 6µM fura-2 in neural differentiation medium (DMEM:F12 supplemented with 10% FBS) and incubated for 30 minutes at 37ºC. Following incubation, cover slips were placed in a perfusion chamber sealed with petroleum jelly. Cells were visualised using an Olympus IX71 equipped with a monochromator-based fluorescence system (Cairn Instruments, Faversham, UK). Cover slips were continuously perfused with an Extracellular Solution (ECS) consisting of: 135mM NaCl, 5mM KCl, 5mM HEPES, 10mM Glucose, 1.2mM $MgCl_2$ and 1.25mM $CaCl_2$. Fura-2 was excited at 340 and 380nm and images were captured at 510nm by a slow-scan CCD camera (Kinetic Imaging Ltd, Nottingham, UK). Emission ratios

for 340/380nm were calculated following subtraction of background using Andor IQ 1.3 software (Andor Technology, Belfast, UK). Gamma-aminobutyric acid (GABA), L-Glutamic acid, N-methyl-D-aspartate (NMDA) (all at 100µM), kainite, 2-amino-3-(3-hydroxy-5-methyk-isoxazol-4-yl)propanoic acid (AMPA) and Adenosine triphosphate (ATP) (all at 50µM) (Sigma-aldrich, UK) were applied to the cells using a multi-barrel motorised solution exchanger (Bio Logic Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA) for 15 seconds each with 135 seconds perfused with ECS to depolarise the cells and determine neural excitability.

5.3 Results

5.3.1 Expression of Stem Cell and Neural Crest Markers in OMLP-PCs

Single cell-derived colonies were isolated from numerous patients and expanded as previously described in Chapter 2. To confirm these cells were of neural crest origin and expressed putative stem cell markers, RNA was extracted from each cell population prior to neural differentiation and PCR was performed for CD90 (a typical MSC marker), CD34, CD45, (Haematopoietic markers) and Twist, Slug and P75 (Neural crest markers) (Pittenger et al, 1999; Vernon and LaBonne, 2006; Lee et al, 2007; Davies et al, 2010). Total human RNA (Stratagene, UK) was used as a positive control for the markers and two negatives were used, a reverse transcription (RT) negative and a water negative (DEPC). All OMLP colonies were found to positively express CD90, Twist, Slug and P75 (Figure 5.1). Expression of both CD34 and CD45 were found to be absent in all colonies.

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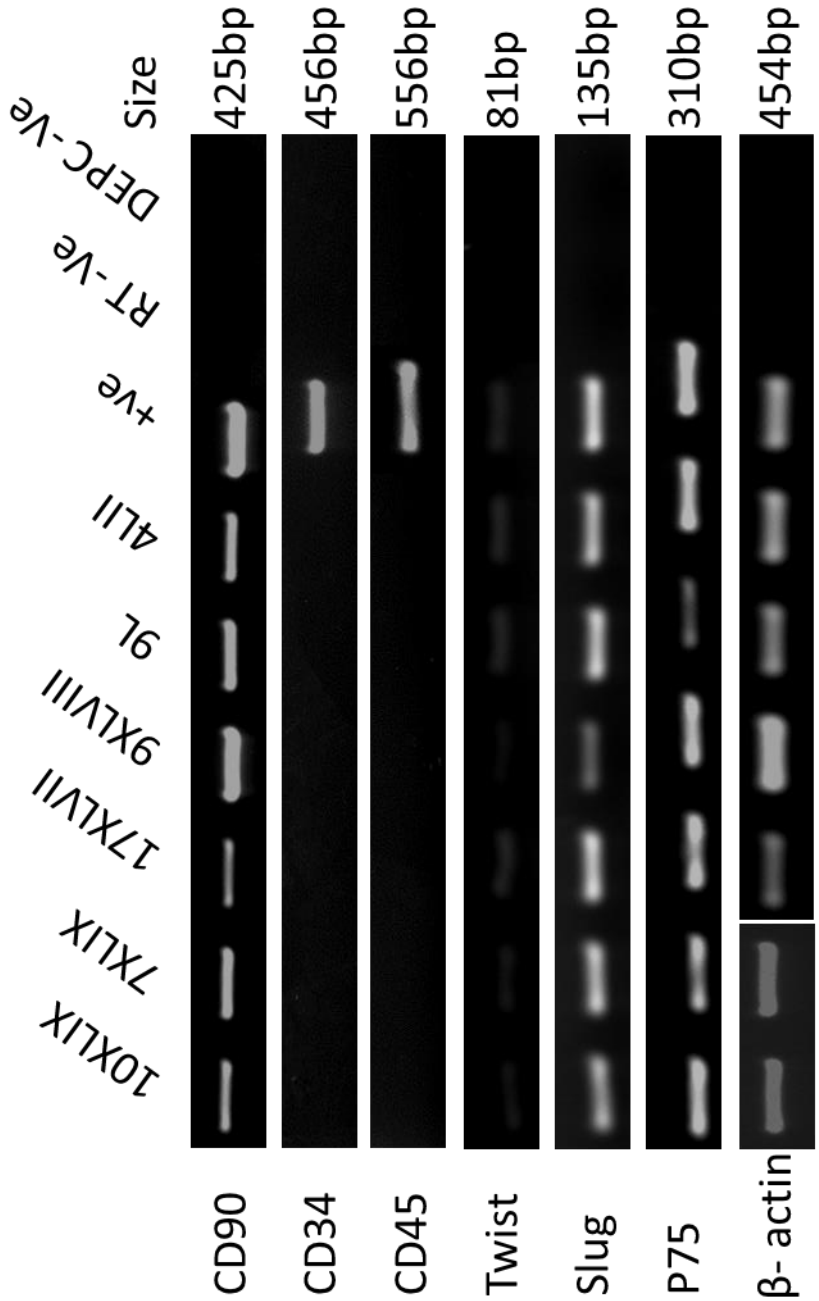

Figure 5.1:*Mesenchymal Stem Cell marker and Neural Crest marker expression.* Expression of CD90, CD34, CD45, Twist, Slug and P75 for colonies; 10XLIX, 7XLIX, 17XLVII, 9XLVIII, 9L and 4LII. (Irrelevant sample removed from lane 3 of β-actin gel)

5.3.2 Neural Differentiation of Cell Populations of the Oral Mucosa Lamina Propria Progenitor Cells and iPSCs

Single cell-derived colonies were isolated from five patients: XLVII, XLVIII, XLIX, L and LII and used for neural differentiation (10XLIX, 7XLIX, 17XLVII, 9XLVIII, 9L and 4LII). In addition to these colonies, iPSCs derived from patient XLIX (iP14), MP XLIX, TP XLIX and H9 were used to make comparisons with respect to differentiation potential

5.3.2.1 Formation of Neurosphere-like Structures Using Defined Culture Conditions

Each population of cells (colonies, iPSCs, MP XLIX, TP XLIX and H9) were cultured on laminin-coated plates in X-VIVO 10 with 80ng/mL of bFGF to induce neurosphere formation. During the first 24 hours of culture, three colonies; 7XLIX, 10XLIX & 9XLVIII demonstrated morphology typical of fibroblasts (Figures 5.2 A, B & D), two had cells that coalesced with a spherical morphology (Figures 5.2 C & E) and one colony was demonstrating both fibroblast-like morphology and some spherical cells (Figure 5.2 F). Following 7 days in culture, four colonies had successfully formed neurosphere-like structures (Figures 5.2 A', B', C' & E') whilst two colonies demonstrated a monolayer of confluent fibroblast-like cells (Figure 5.2 D' & F').

Twenty four hours following X-VIVO-10 treatment on laminin coated plates, TP XLIX and MP XLIX cell populations displayed a typical fibroblast-like morphology (5.3 A & B) whilst after the same duration in culture, iP14 and H9

cells demonstrated both cells with a spherical morphology that clumped together and flattened fibroblastic cells (Figures 5.3 C & D). Following 7 days in culture, TP XLIX failed to generate any neurosphere-like structures and had formed confluent monolayer of cells with a. fibroblast-like morphology (Figure 5.3 A'). MP XLIX formed neurosphere-like structures, however, there were still fibroblast-like cells populating the space around the neurosphere-like structure (Figure 5.3 B'). Large neurosphere-like structures were observed in iP14 cultures, again with some fibroblast-like cells in monolayer around the neurosphere-like structure (Figure 5.3 C'). H9 cells also demonstrated formation of neurosphere-like structures but again some fibroblast-like cells remained in monolayer (Figure 5.3 D'). The cell populations that formed neurosphere-like structures were used to determine the potential of these cells to differentiate down the neural lineage.

5.3.2.2 Neural Differentiation Using Defined Culture Conditions

7XLIX, 10XLIX, 17XLVII, 9L, MP XLIX, iP14 and H9 cell populations were used in neural differentiation experiments. Each cell population was seeded onto matrigel-coated plates and treated with two different neural differentiation conditions for a total of 14 days. Following 7 days of treatment with medium 1, OMLP colonies demonstrate a flattening out and the attainment of a bipolar conformation (Figures 5.4 A, B, C & D). After a further 7 days in medium 2, with a cocktail of neural growth factors, cells began to develop long neural-like processes.

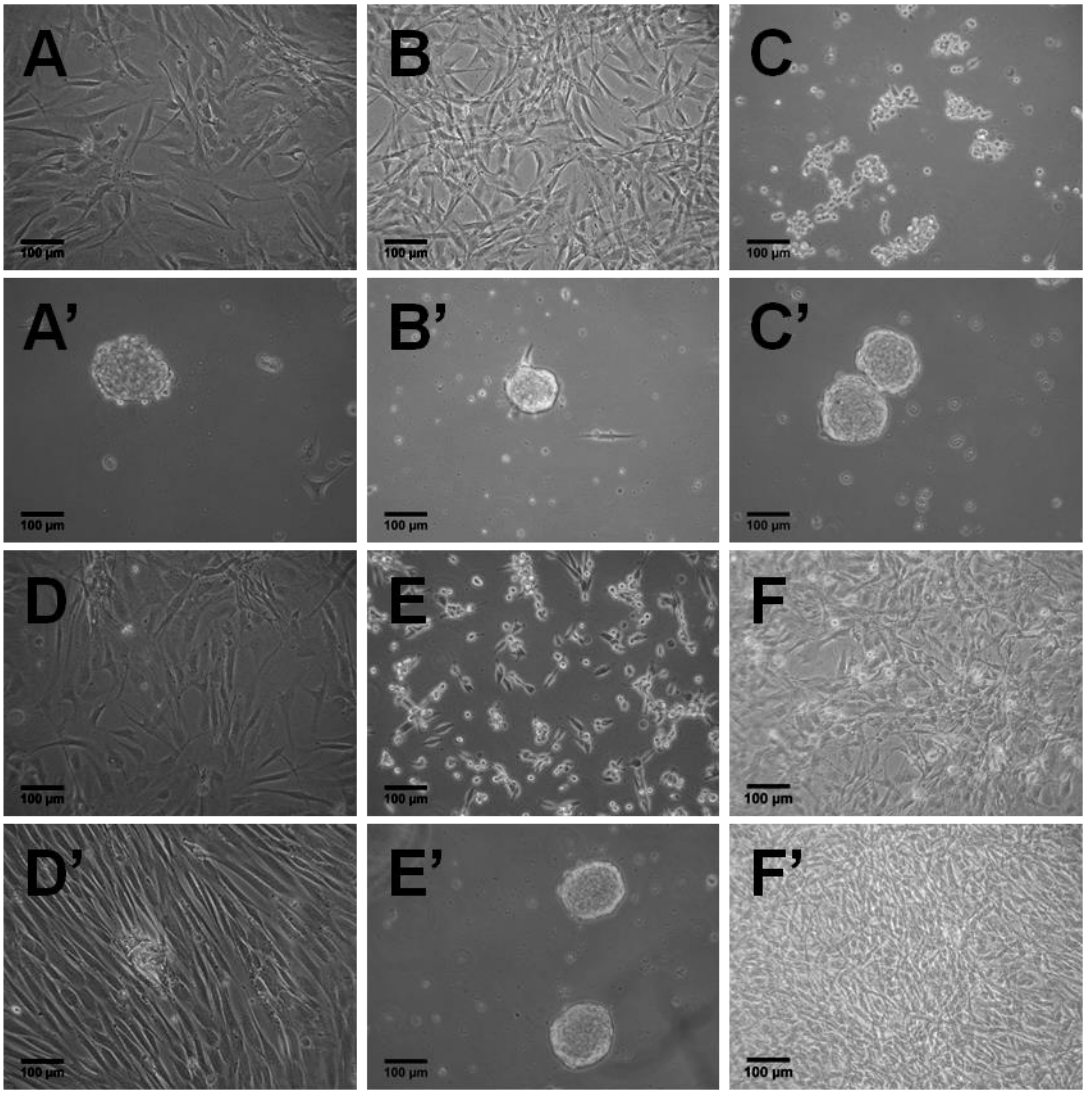

Figure 5.2*: Bright field images of Neurosphere formation for colonies cultured on laminin in X-VIVO 10 +80ng/mL bFGF.* **(A)** 7XLIX 24 hours following passage, **(A')** 7XLIX following 7 days of treatment. **(B)** 10XLIX 24 hours following passage, **(B')** 10XLIX following 7 days of treatment. **(C)** 17XLVII 24 hours following passage, **(C')** 17XLVII following 7 days of treatment. **(D)** 9XLVIII 24 hours following passage, **(D')** 9XLVIII following 7 days of treatment. **(E)** 9L 24 hours following passage, **(E')** 9L following 7 days of treatment. **(F)** 4LII 24 hours following passage, **(F')** 4LII following 7 days of treatment.

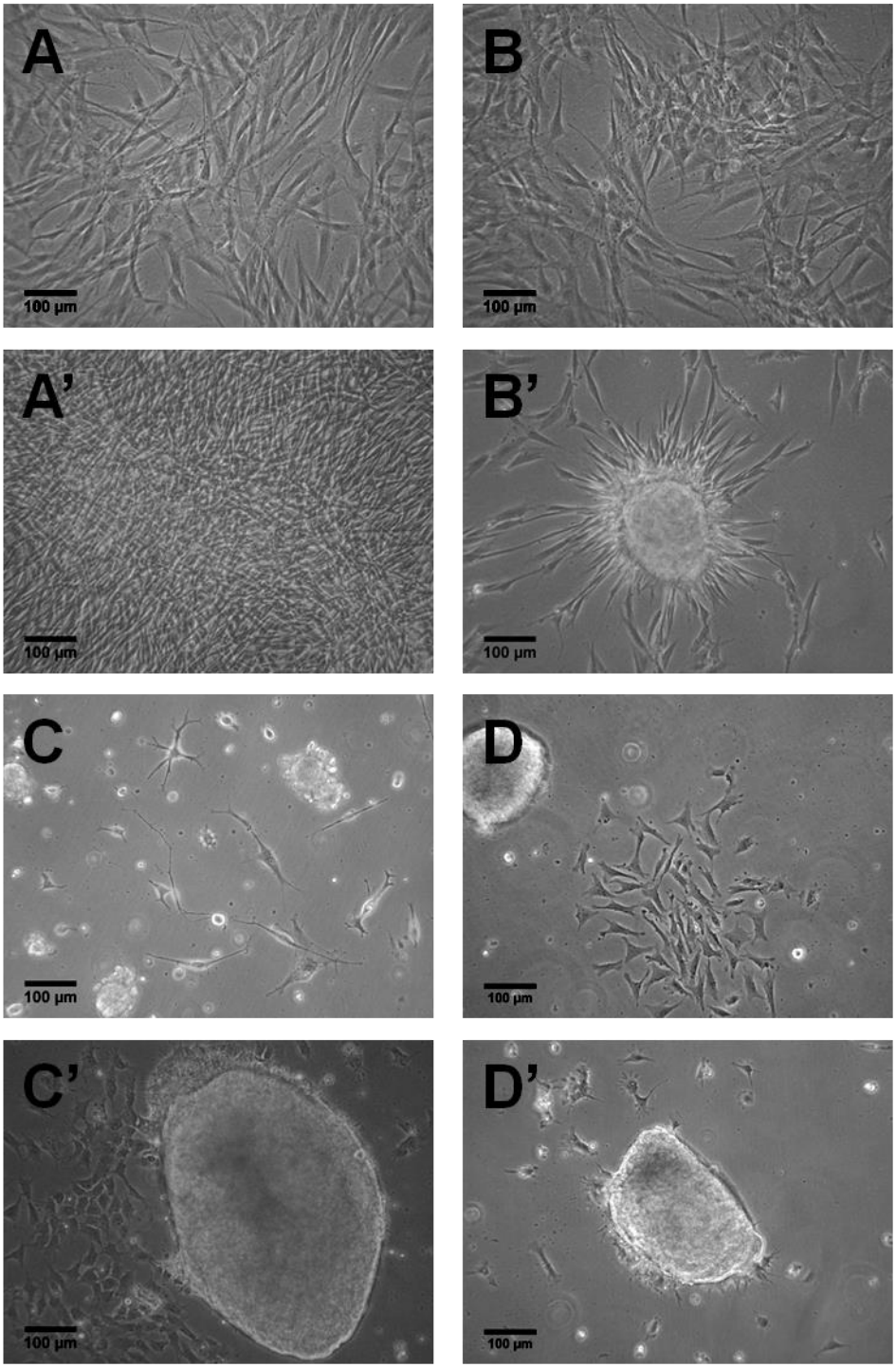

Figure 5.3: ***Bright field images of Neurosphere formation for TP XLIX, MP XLIX, iP14 and H9 cultured on laminin in X-VIVO 10 +80ng/mL bFGF.*** **(A)** TP XLIX 24 hours following passage, **(A')** TP XLIX following 7 days of treatment. **(B)** MP XLIX 2 4hours following passage, **(B')** MP XLIX following 7 days of treatment. **(C)** iP14 24 hours following passage, **(C')** iP14 following 7 days of treatment. **(D)** H9 24 hours following passage, **(D')** H9 following 7 days of treatment

These were most obvious in 7XLIX but were also evident in 10XLIX with 9L cultures demonstrating limited neural-like processes growing over the confluent monolayer (Figure 5.4 A', B' & D'). However, 17XLVII demonstrated a more confluent monolayer of cells and such neural processes were not evident (Figure 5.4 C').

After seven days of treatment MP XLIX cells, like the OMLP-PC colonies, flattened out and adopted a bipolar conformation (Figure 5.5 A). Unlike the colonies however, these cells were considerably more confluent. iP14 and H9 cells also demonstrated flattened cells after the first 7 days in medium 1 however, the morphology of these cells was different to MP XLIX and to the OMLP-PC colonies (Figures 5.5 B & C). Following a further 7 days in culture with medium 2, MP XLIX demonstrated a monolayer of confluent cells with no obvious neural-like processes (Figure 5.5 A'), iP14 demonstrated cells with a stressed appearance (large cells with many visible stress fibres) and some evidence of cell death (Figure 5.5 B') and H9 demonstrated some cells with long processes also with some evidence of cell death (Figure 5.5 C').

5.3.2.3 Expression of neural markers

Confirmation of neural differentiation was carried out utilising both, PCR analysis for typical early and late stage neural markers and immunocytochemistry to test for the presence of typical neural markers at the protein level.

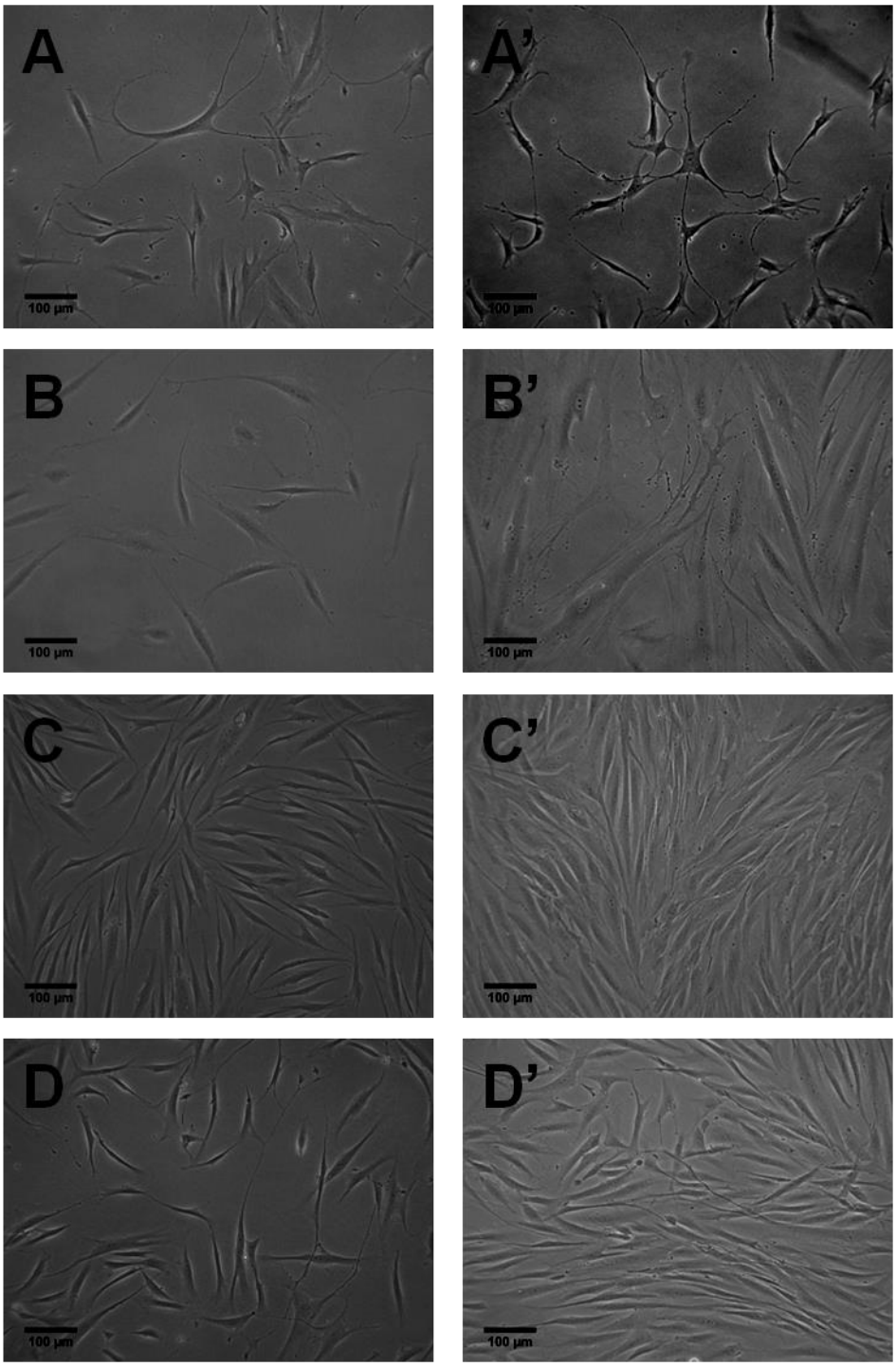

Figure 5.4*: Bright field images of neural differentiation of colonies*. **(A)** 7XLIX following 7 days on medium 1, **(A')** 7 XLIX following 7 days treatment on medium 2. **(B)** 10 XLIX following 7 days on medium 1, **(B')** 10 XLIX following 7 days treatment on medium 2. **(C)** 17 XLVII following 7 days treatment on medium 1, (**C')** 17 XLVII following 7 days treatment on medium 2. **(D)** 9L following 7 days treatment on medium 1, **(D')** 9L following 7 days treatment on medium 2.

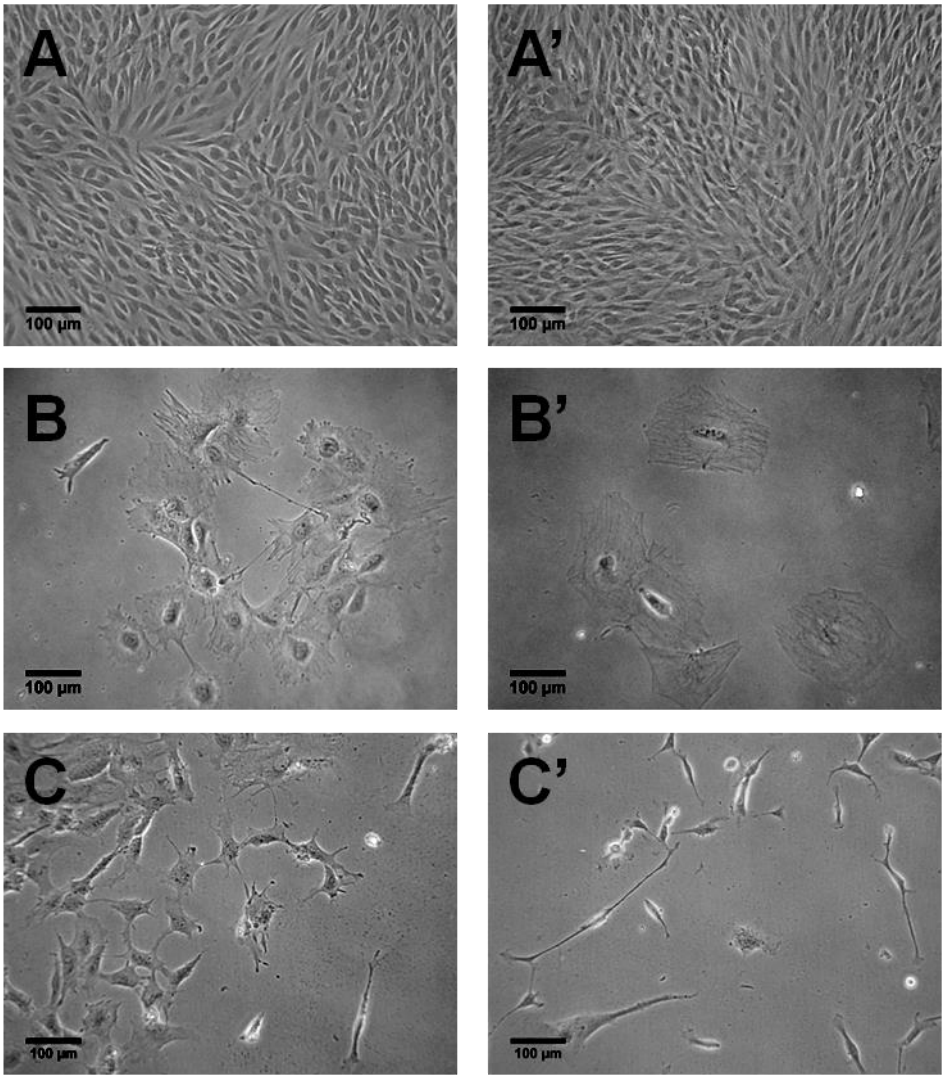

Figure 5.5: ***Bright field images of neural differentiation of MP XLIX, iP14 and H9.*** **(A)** MP XLIX following 7 days on medium 1, **(A')** MP XLIX following 7 days treatment on medium 2. **(B)** iP14 following 7 days on medium 1, (**B')** iP14 following 7 days treatment on medium 2. **(C)** H9 following 7 days treatment on medium 1, (**C')** H9 following 7 days treatment on medium 2.

5.3.2.3.1 PCR analysis

RNA was extracted from the cell populations before and after differentiation to carry out PCR analysis. Nestin is a neural precursor/progenitor cell marker expressed in early neural development and MAP-2 is an early stage marker of neural differentiation, whilst NF-M is a late stage marker of differentiation. Nestin was expressed in all OMLP-PC colonies pre- and post-differentiation (Figure 5.6 A). It was also positively expressed in MP XLIX and H9 pre- and post- differentiation, however, it was only expressed pre-differentiation in iP14 (Figure 5.6 B). Similarly MAP-2 was expressed in all OMLP-PC colonies, MP XLIX and H9 pre- and post-differentiation with the exception of 9L and 10XLIX (where MAP-2 was upregulated following differentiation) and iP14 (where expression was not detected after differentiation (Figure 5.6 A & B)). NF-M was found to be upregulated in MP XLIX and all OMLP colonies following differentiation (Figure 5.6 A & B). Expression of NF-M was detected pre-differentiation for both iP14 and H9, but only in H9 post-differentiation.

5.3.2.3.2 Immunocytochemistry analysis of neural differentiation markers

In addition to MAP-2, βIII tubulin is an additional early stage marker of neural differentiation and its expression at the protein level is commonly used as an indicator of neurons. βIII tubulin, MAP-2 and NF-M production was detected following neural differentiation of 7XLIX, 10XLIX, 17XLVII and 9L (Figures 5.7-5.10 A, B & C). All primary omission controls were negative (Figure 5.7-5.10 D, E & F) and all secondary and IgG controls were negative (Figures 5.7-5.10 G, H, I & J). Percentage positivity was found to be above 80% for

the neural markers in all colonies with the exception of NF-M which was found to be lower in 10XLIX (71.61%) and 9L (33.47%) (Table 5.3). A minimal level of βIII tubulin, MAP-2 and NF-M was detected in MP XLIX cell populations following differentiation (Figures 5.11 A, B & C and Table 5.3) but these neural markers were not detected in differentiated populations of iP14 & H9 (Figures 5.11 D, E, F, G, H &I). All primary omission, secondary omission and IgG controls were found to be negative (see appendix VII).

5.3.2.4 Functional Testing of Differentiated Neurons

Three OMLP-PC colonies were selected for calcium imaging; 10XLIX, 7XLIX and 9L. 17 XVII was not used at this stage as treated cells became too confluent after 14 days of differentiation making calcium imaging extremely difficult. MP XLIX, iP14 and H9 were also not selected for calcium imaging as they failed to form neurons as identified by immunocytochemistry (section 5.3.2.3). Each of the tested differentiated cell populations was treated with 6 neuron receptor agonists to evoke calcium influx. Following incubation with fura-2, 10XLIX cell populations failed to demonstrate calcium influx in response to treatment with KCl, NMDA, AMPA, Kainate or GABA however, calcium influx into the cell was evident following treatment with ATP (Figure 5.12). When 9L was treated with these neurotransmitters, an influx of calcium was evident following treatment with KCl and GABA (Figure 5.13). Following treatment 7XLIX cell populations demonstrate calcium influx following treatment with KCl, ATP and GABA (Figure 5.14).

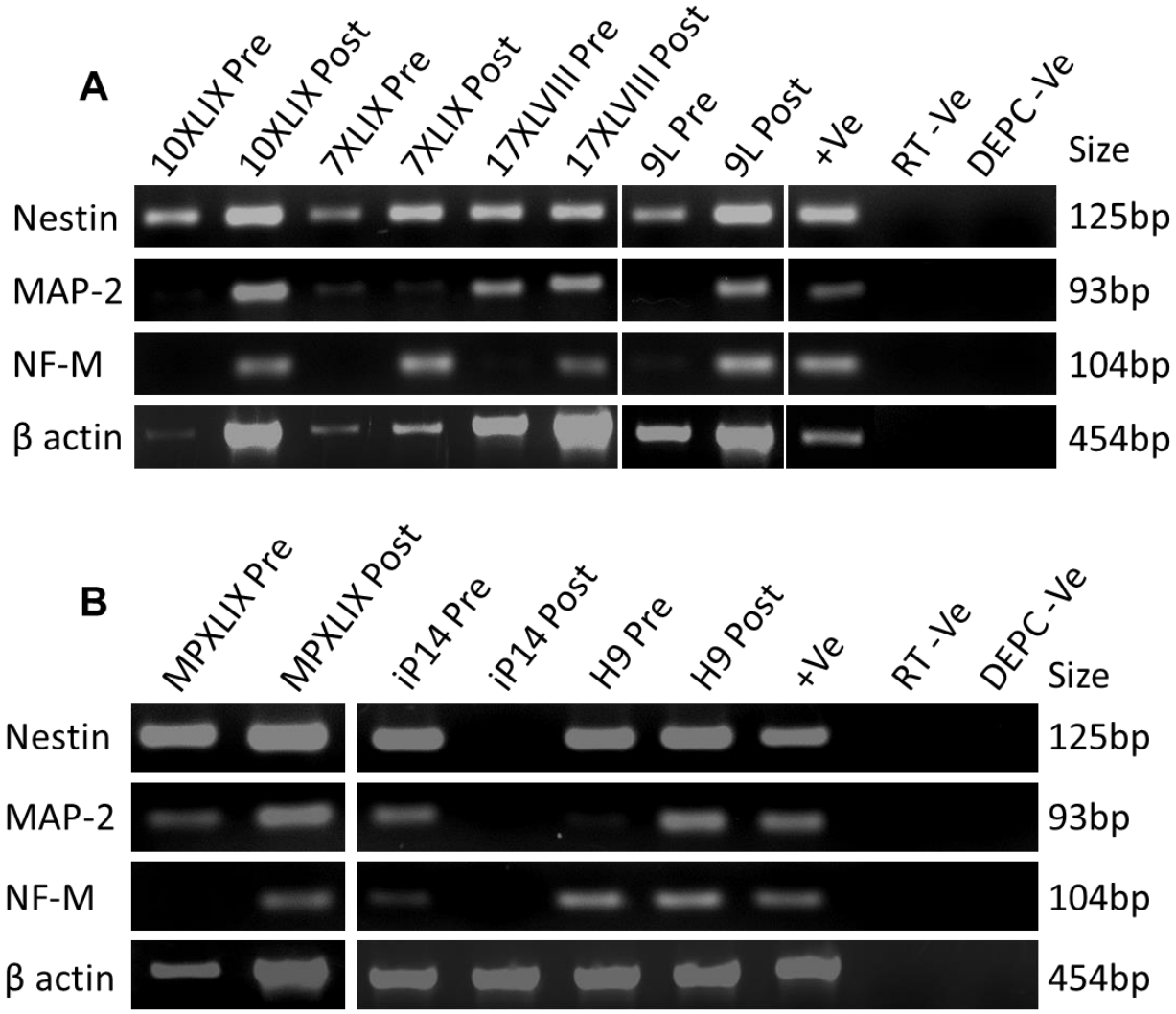

Figure 5.6: ***PCR analysis of early stage; nestin & MAP-2 and late stage; NF-M*. (A)** OMLP-PC colonies pre- and post-differentiation (Irrelevant samples removed from lanes 7 & 10). **(B)** MP XLIX, iP14 and H9 pre and post differentiation (irrelevant samples removed from lanes 3 & 4 of gel).

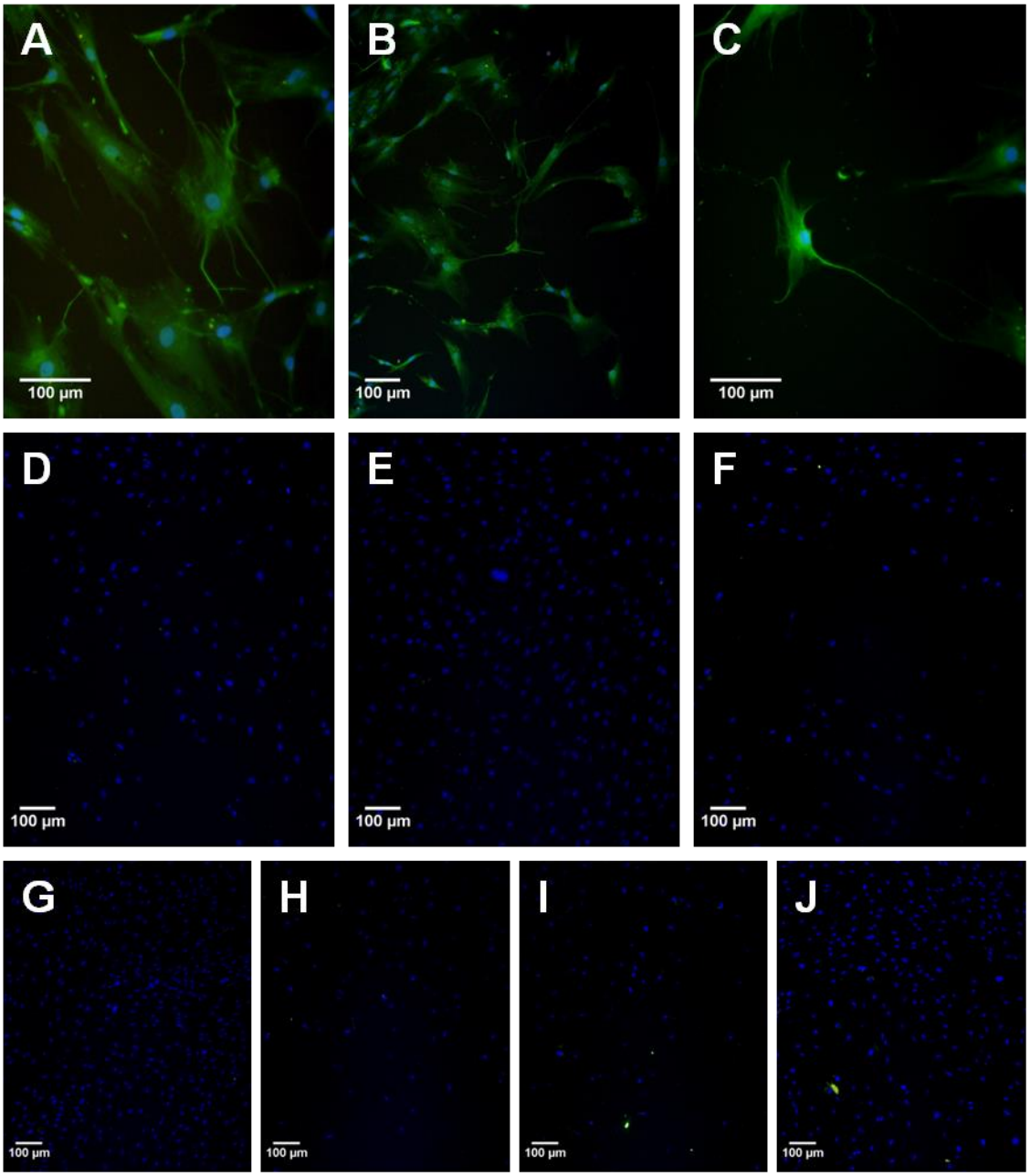

Figure 5.7: ***Immunocytochemistry analysis of early & late stage neural markers for 7XLIX:*** **(A**) βIII Tubulin with DAPI counterstain. **(B)** MAP-2 with DAPI counterstain. **(C)** NF-M with DAPI counterstain. **(D)** Primary omission control for βIII tubulin. **(E)** Primary omission control for MAP-2. **(F)** Primary omission control for NF-M. **(G)** Secondary omission control for swine anti-rabbit. **(H)** Secondary omission control for rabbit anti-mouse. **(I)** IgG (rabbit) control. **(J)** IgG (mouse) control.

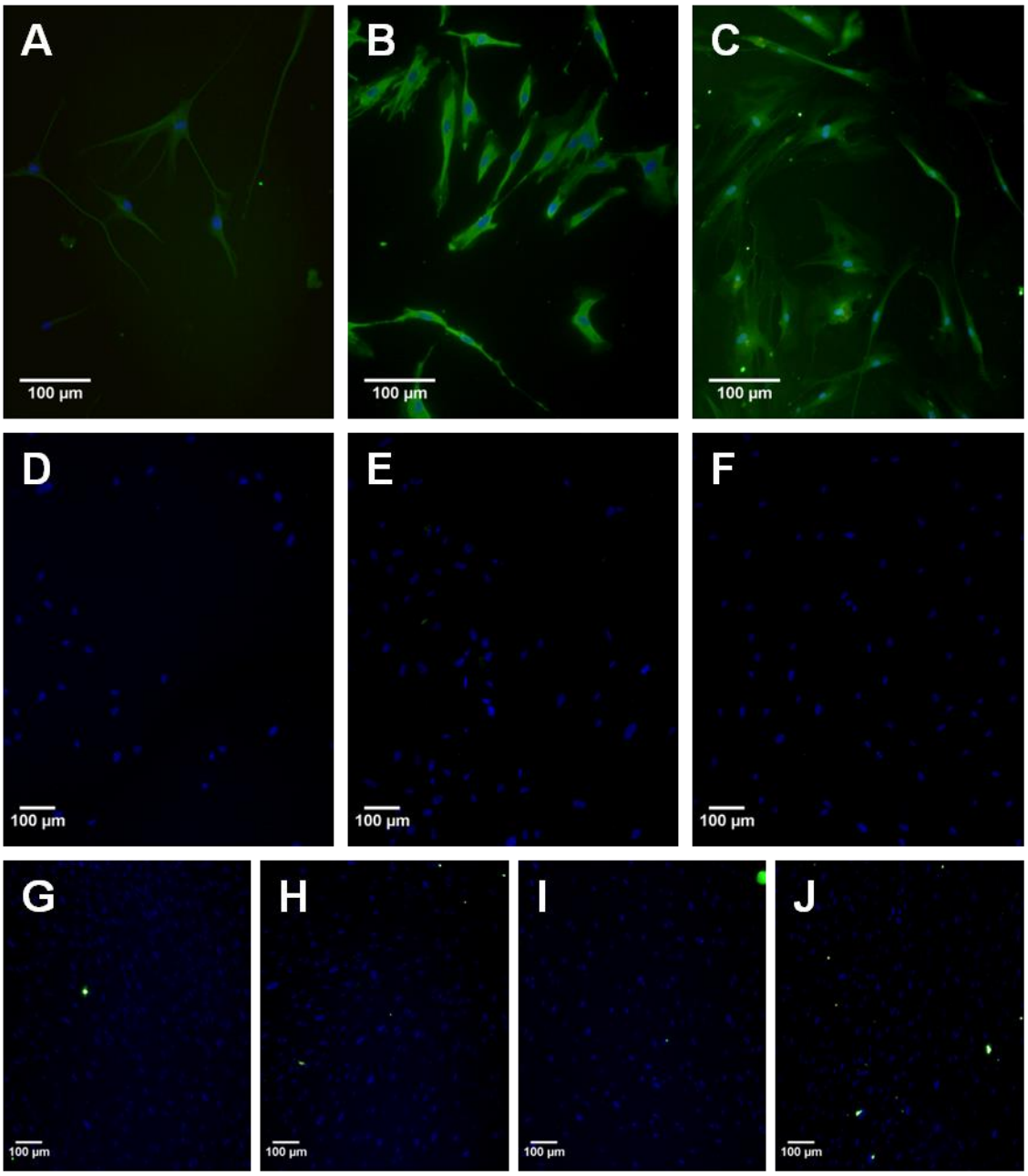

Figure 5.8*: Immunocytochemistry analysis of early & late stage neural markers for 10XLIX:* **(A**) βIII Tubulin with DAPI counterstain. **(B)** MAP-2 with DAPI counterstain. **(C)** NF-M with DAPI counterstain. **(D)** Primary omission control for βIII tubulin. **(E)** Primary omission control for MAP-2. **(F)** Primary omission control for NF-M. **(G)** Secondary omission control for swine anti-rabbit. **(H)** Secondary omission control for rabbit anti-mouse. **(I)** IgG (rabbit) control. **(J)** IgG (mouse) control.

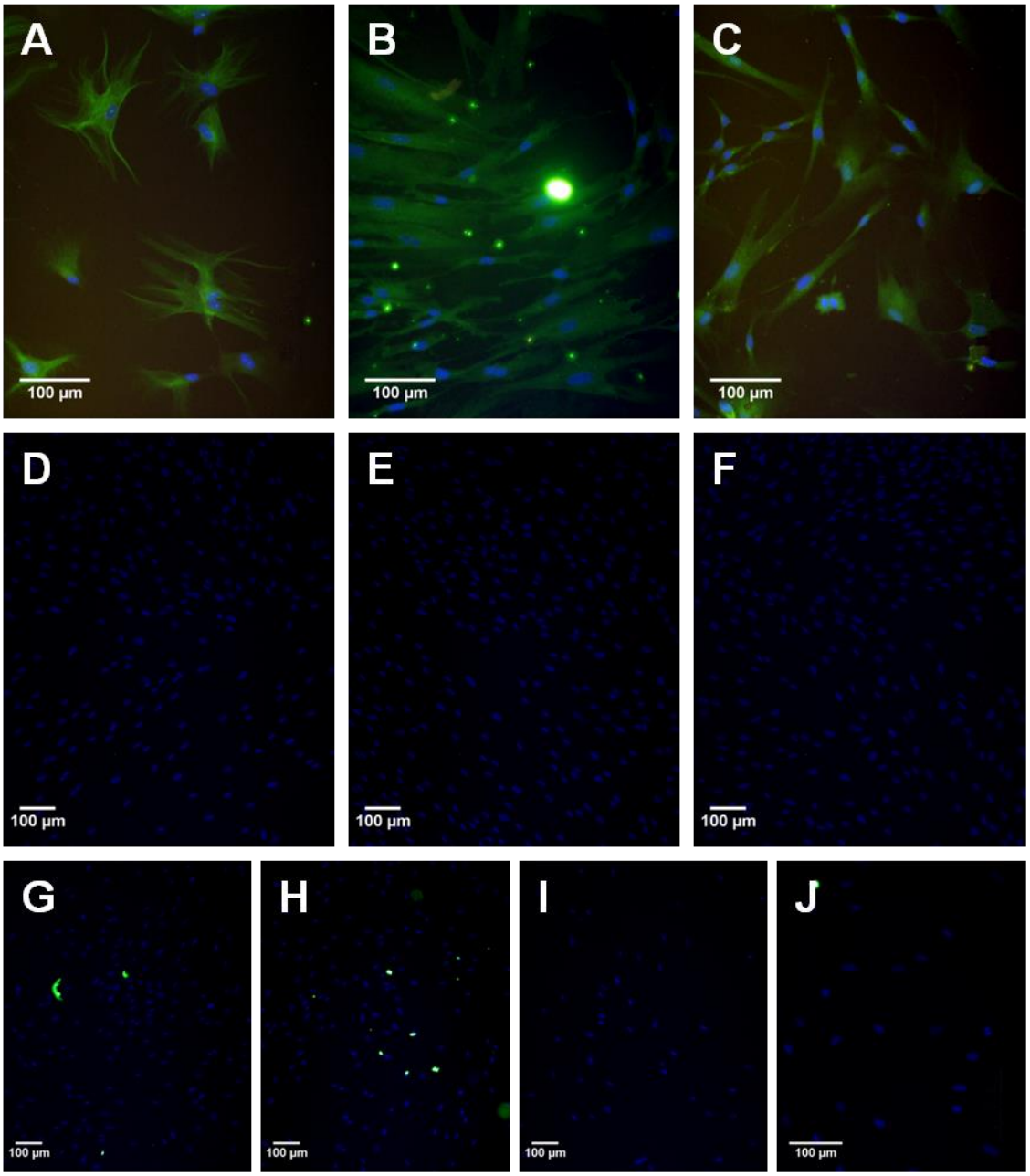

Figure 5.9*: **Immunocytochemistry analysis of early & late stage neural markers for 17XLVII:*** **(A**) βIII Tubulin with DAPI counterstain. **(B)** MAP-2 with DAPI counterstain. **(C)** NF-M with DAPI counterstain. **(D)** Primary omission control for βIII tubulin. **(E)** Primary omission control for MAP-2. **(F)** Primary omission control for NF-M. **(G)** Secondary omission control for swine anti-rabbit. **(H)** Secondary omission control for rabbit anti-mouse. **(I)** IgG (rabbit) control. **(J)** IgG (mouse) control.

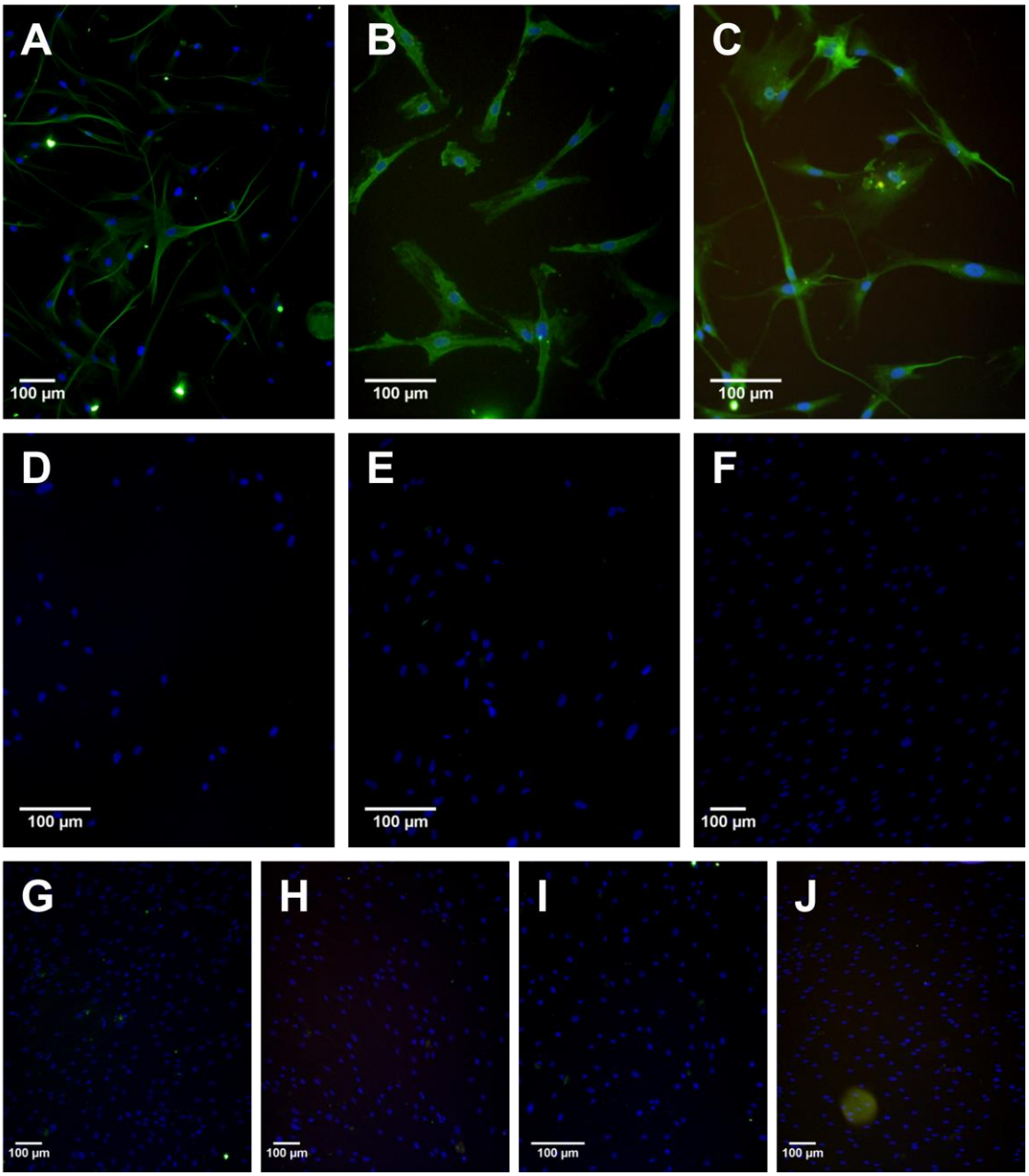

Figure 5.10: ***Immunocytochemistry analysis of early & late stage neural markers for 9L:*** **(A**) βIII Tubulin with DAPI counterstain. **(B)** MAP-2 with DAPI counterstain. **(C)** NF-M with DAPI counterstain. **(D)** Primary omission control for βIII tubulin. **(E)** Primary omission control for MAP-2. **(F)** Primary omission control for NF-M. **(G)** Secondary omission control for swine anti-rabbit. **(H)** Secondary omission control for rabbit anti-mouse. **(I)** IgG (rabbit) control. **(J)** IgG (mouse) control.

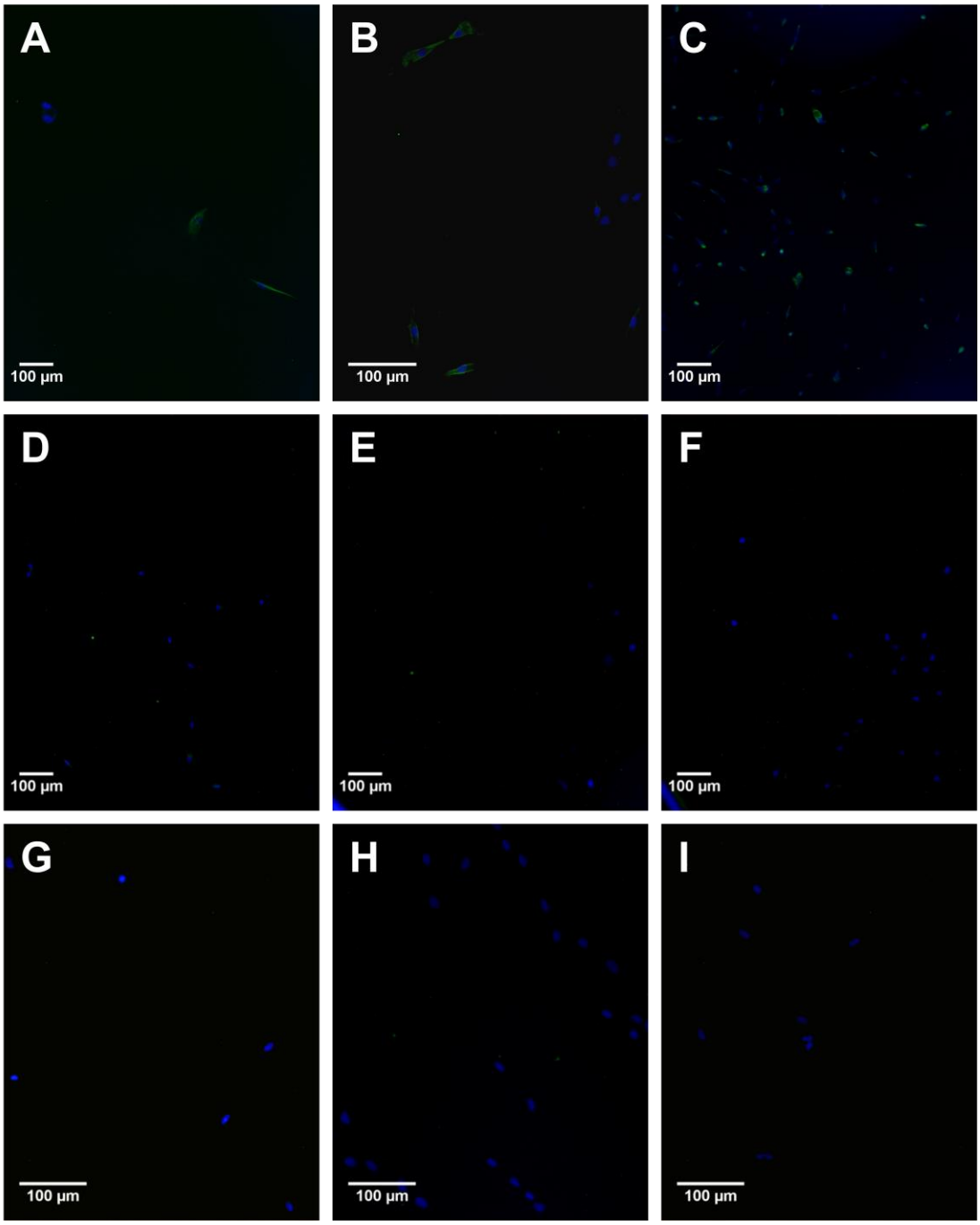

Figure 5.11*: **Immunocytochemistry analysis of early & late stage neural markers for MP XLIX, iP14 and H9:*** **(A)** βIII Tubulin with DAPI counterstain for MP XLIX. **(B)** Map-2 with DAPI counterstain for MPXLIX. **(C)** NF-M with DAPI counterstain for MP XLIX. **(D)** βIII Tubulin with DAPI counterstain for iP14. **(E)** MAP-2 with DAPI counterstain for iP14. **(F)** NF-M with DAPI counterstain for iP14. **(G)** βIII Tubulin with DAPI counterstain for H9. **(H)** Map-2 with DAPI counterstain for H9. **(I)** NF-M with DAPI counterstain for H9 (see appendix VII for controls)

Table 5.3: Percentage positivity of OMLP colonies, MPXLIX, iP14 and H9 stained with βIII tubulin, Map-2 and NF-M

	Neural Markers		
Cell Sample	βIII Tubulin	MAP-2	NF-M
10XLIX	82%	91%	72%
7XLIX	89%	95%	82%
17XLVII	90%	99%	82%
9L	93%	88%	33%
OPXLIX	<1.0%	<1.0%	<1.0%
iP14	0.0%	0.0%	0.0%
H9	0.0%	0.0%	0.0%

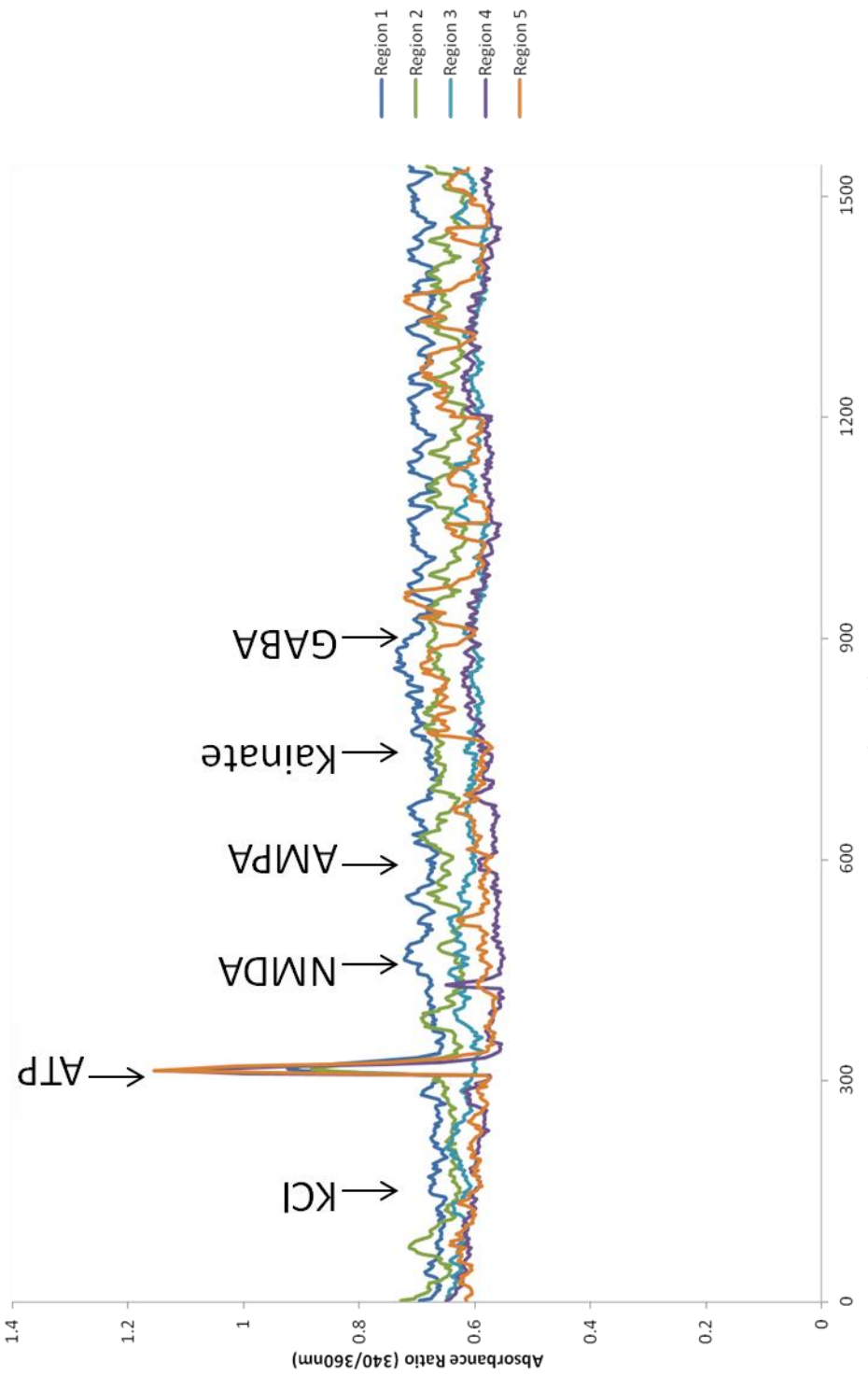

Figure 5.12: *Calcium Imaging.* Calcium imaging trace of absorbance ratio of Fura-2 calcium binding for 10XLIX differentiated cells treated with KCl, ATP, NMDA, AMPA, Kainate and GABA (*n*=5)

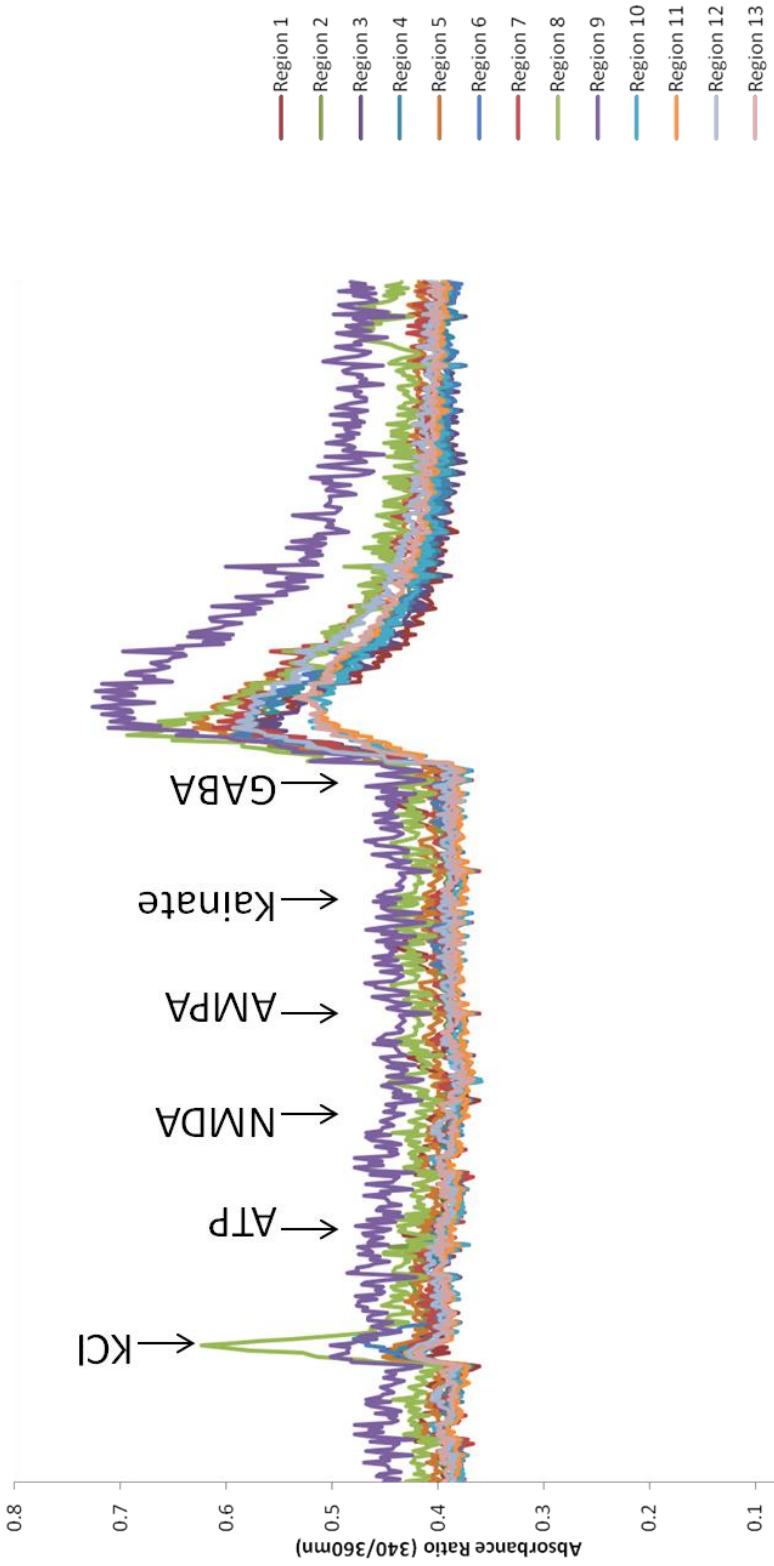

Figure 5.13: *Calcium Imaging.* Calcium imaging trace of absorbance ratio of Fura-2 calcium binding for 9L differentiated cells treated with KCl, ATP, NMDA, AMPA, Kainate and GABA (*n*=13)

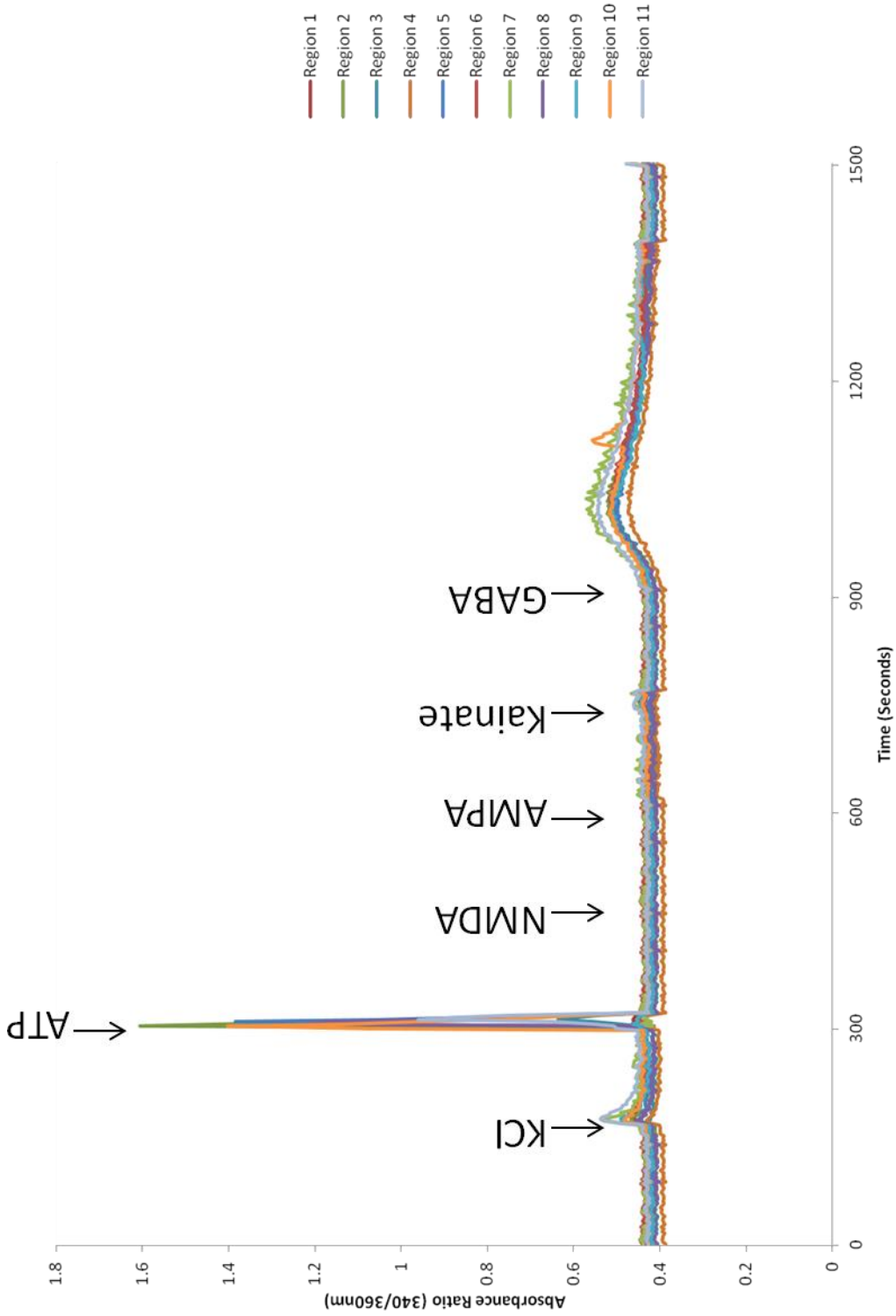

Figure 5.14: *Calcium Imaging.* Calcium imaging trace of absorbance ratio of Fura-2 calcium binding for 7XLIX differentiated cells treated with KCl, ATP, NMDA, AMPA, Kainate and GABA (*n*=11)

5.4 Summary

- OMLP-PC colonies form cells with morphology similar to neurons, positively express neural markers and demonstrate potential differentiation towards a functional neural phenotype
- Merged progenitors form partial neurospheres and appear to preferentially proliferate more than differentiate
- iPSC and H9 cells fail to differentiate into cells that demonstrate the presence of the neural markers at a protein level utilising the protocol investigated

5.5 Discussion

The main aim of this chapter was to carry out a comparative analysis of the potential for OMLP-PC colonies and iPSCs to differentiate down neural pathways. As the iPSC were derived from MP XLIX, this merged progenitor cell population was also used as a comparison in addition to TP XLIX. Given the similarities between iPSC and hESCs, the ability of human ES cells (H9) to differentiate down a neural lineage was also investigated. In this instance, one method for differentiation was used to compare differentiation potential between all populations.

Six colonies were selected from five patients to carry out neural differentiation due to their ability to grow in culture following isolation and expansion. Prior to differentiation, these cells were found to express CD90, Twist, Slug and p75 but were negative for CD34 and CD45, confirming that these cells are derived from the neural crest and consistent with previous findings of oral mucosal progenitor cells (Davies et al, 2010; Marynka-Kalmani et al, 2010). They express a typical MSC marker but were not of haematopoietic origin comparable to reports for progenitor cells isolated from other dental tissues including; SHEDs (Nourbakhsh et al, 2011) and gingival progenitor cells (Zhang et al, 2009) as well as MSCs isolated from BMSCs and other adult tissues including; fat, spleen and thymus (Pittenger et al, 1999; Krampera et al, 2007).

Varying degrees of differentiation potential between OMLP colonies was discovered. Here, four single cell derived colonies out of six demonstrated

the ability to form neurosphere-like structures similar to those described in previous reports for OMLP (Davies et al, 2010) and progenitor cells from other dental tissues including; dental follicle stem cells (Vollner et al, 2009) and PDL (Widera et al, 2007). Those colonies that failed to generate these structures continued to proliferate over the seven day protocol to confluence. Only the populations that formed neurosphere-like structures were taken through to the terminal neural differentiation protocol. Cells from these colonies extended long neural-like processes and were used for further analysis. The morphology observed was consistent with previous findings following neural differentiation of cells isolated from dental tissues such as OMLP, PDL, SHEDs and DPPCs (Arthur et al, 2008; Davies et al, 2010; Marynka-Kalmani et al, 2010; Nourbakhsh et al, 2011) as well as those observed from neural differentiated MSCs isolated from bone marrow, fat, spleen and thymus (Krampera et al, 2007). A varying degree of differentiation potential was also evident in the merged colonies, where, some cells coalesce to form neurosphere-like structures. However, there were other cells that continued to proliferate around the periphery of the sphere and when taken through the differentiation protocol. It was evident that these cells continue to proliferate to confluence and were potentially limiting the neural differentiation ability of the neurosphere-like structures. In contrast, TP XLIX failed to form any neurosphere-like structures and was therefore not taken through neural differentiation. It has been demonstrated that progenitor cells isolated from the OMLP can be differentiated to cells that express neural markers without the need for formation of a neurosphere-like structure however, this was achieved using much lower cell densities at the

onset of differentiation (Marynka-Kalmani et al, 2010). It is therefore possible that, if cells were seeded at a lower density, they may still possess the ability to differentiate down a neural lineage. In this case all cell seeding densities were kept the same in order to carry out a comparison.

Both iPSC and H9 cells formed neurosphere-like structures however, both cultures had numerous cells in monolayer culture that surrounded the sphere-like structures. When they were taken through neural differentiation, there was evidence of substantial cell death (as evidenced by a low cell density) in both cultures and the differentiated iPSCs that did survive the 14 days of culture, adopted a stressed appearance, typical of senescent cells. A limited number of surviving differentiated H9 cells did adopt cell morphology that was similar to that of neural cells. The ability for hESCs to differentiate down neural lineages has previously been reported (Lee et al, 2007; Dhara et al, 2008;) and similar studies have been carried out for iPSCs (Hu et al, 2010 Boulting et al, 2011). Moreover, a differentiated functional phenotype has been demonstrated by both hESCs and iPSCs (Lee et al, 2007; Young et al, 2011; Karumbayaram et al, 2009; Zeng et al, 2010; Haythornthwaite et al, 2012). However, differences between the neural differentiation potential of hESC and iPSCs have been reported, indicating that iPSCs do differentiate down a neural lineage with a much lower efficiency and more variability than hESCs (Hu et al, 2010).

The difference observed between the differentiation of OP cells compared to iPSCs and hESC is most likely due to the two different cell populations targeted for differentiation; adult stem cells (colonies and MP XLIX) and

pluripotent stem cells (hESC (H9) and iPSCs (iP14)). Adult stem cells are a population of cells that are already partially committed to become cells from their germ layer of origin, whereas hESC and iPSCs are at a much earlier developmental stage. Neural differentiation of iPSCs and hESCs commonly mimic neural stem cell culture and requires the formation of spherical aggregates known as embryoid bodies (EBs), formed by culturing the cells in suspension (Carpenter et al, 2001; Itsykson et al, 2005; Zhou et al, 2008). The downside of the formation of EBs from these pluripotent stem cells is that the process is variable and can largely depend upon the length of time in suspension culture and the culture conditions. Differentiation following plating can be spontaneous rather than lineage specific however, culturing EBs with Noggin has been found to increase the potency of neural differentiation due to the inhibition of BMP signalling pathways, suppressing non-neural differentiation (Itsykson et al, 2005). Similarly, the addition of Noggin following plating appears to increase neural differentiation efficiency (Zhou et al, 2008).

Only one protocol was utilised to compare the neural differentiation potential of the different cell types in this chapter and this protocol was initially developed for the differentiation of an adult stem cell population. Although the protocol includes the formation of a spherical body, these conditions were optimised for use with OP cells and it did not include the addition of inhibitors of BMP signalling pathways, which could potentially cause differentiation at a much lower efficiency with hESC and with great variability in iPSCs (Itsykson et al, 2005). This suggests that different culture conditions would be required

to optimise neural differentiation for each cell type. This would essentially require a greater understanding of the neural differentiation process of cells and the signals involved which is still not yet fully understood.

RNA was extracted pre- and post-differentiation to establish presence/absence of typical neural markers; nestin, MAP-2 and NF-M. Nestin is an early stage marker during neural development, often used as a neural precursor/progenitor cell marker and is down-regulated as cells become terminally differentiated, whilst NF-M (Steinert and Liem, 1990) and MAP-2 expression (Ferreira et al, 1990) increase as cells mature.

Expression of the neural markers by end-point PCR analysis was indicative of a neuronal-like phenotype and was evidenced by the OMLP-PC colonies and the merged colony population (MP XLIX). These cell populations demonstrated positive expression of the neural precursor marker, nestin both before and after differentiation which has previously been reported for oral progenitor cells (Marynka-Kalmani et al, 2010) in addition to progenitor cells isolated from other dental tissues including; SHEDs (Miura et al, 2003; Nourbakhsh et al, 2011) and DPPCs (Arthur et al, 2008). MAP-2 was also detected pre-differentiation in all OMLP colonies and MP XLIX with the exception of 9L and minimal detection in 10XLIX, where both colonies demonstrate up-regulation post-differentiation potentially confirming a more terminally differentiated phenotype. This is consistent with previous findings for oral mucosal progenitor cells (Marynka-Kalmani et al, 2010), whilst MAP-2 expression in other colonies and merged colony populations appeared unchanged following differentiation which is consistent with previous reports

for SHEDs (Nourbakhsh et al, 2011). NF-M was up-regulated following differentiation in colonies and MPXLIX which again is potentially indicative of a more terminally differentiated neural-like phenotype, similar to previous differentiation studies carried out on MSCs (Park et al 2011). Whilst iP14 and H9 both demonstrated expression of nestin, MAP-2 and NF-M pre-differentiation, these markers were not detected post differentiation in iP14 and, with the exception of MAP-2 which was up-regulated, appeared unchanged in differentiated H9 cells. These three markers have previously been reported following differentiation of hESC down neural lineages (Francis and Wei, 2010). Quantitative PCR analysis would be required to identify any changes in the levels of expression of these genes.

Immunocytochemistry for typical neural differentiation markers (βIII tubulin, MAP-2 and NF-M) was carried out to determine the presence/absence of these markers at the protein level and provide further evidence of a differentiated neural phenotype. Protein for all three markers was detected in all colonies of the oral progenitor cells which has previously been reported for progenitor cells derived from oral mucosa and gingival tissues (Zhang et al, 2009; Davies et al, 2010; Marynka-Kalmani et al, 2010), additionally the presence of βIII tubulin has been reported in neural differentiated SCAP cells (Sonoyama et al, 2008) and the presence of NF-M following neural differentiation of DPPCs has been reported (Arthur et al, 2008). Minimal detection was demonstrated in the merged colony populations, which is surprising as the merged population consists of many different colonies and several of these, like the colonies described in this chapter, will have the

potential ability to differentiate towards a neural-like phenotype. However, as already discussed, this may be as a result of the culture conditions including the incomplete formation of neurosphere-like structures as well as continued proliferation of the cell surrounding the partially formed spheres. There was no staining detected in iP14 or H9 differentiated cells, which is unsurprising given the amount of cell death observed in these cultures under these specified culture conditions.

Morphology and expression of neural markers simply provide further support of potential neural identity. However, in order to confirm potential ability to function as an excitable cell, these differentiated cells must be tested for critical characteristics that would allow the cell to function as a neuron. Based on morphology, end-point PCR analysis and presence of protein, three colonies (10XLIX, 7 XLIX and 9L) were selected to carry out functional testing by performing calcium imaging to detect the presence or absence of functional calcium channels. Calcium influx occurred in 10XLIX as a result of exposure to ATP. Whilst 9L did not react to ATP, an influx of calcium occurred following treatment with both KCl and GABA and interestingly 7XLIX had a calcium influx as a result of treatment with all three; ATP, KCl and GABA.

Calcium influx as a result of ATP suggests the presence of P2X purinoceptors which potentially indicates the presence of glial cells; astrocytes and oligodendrocytes, (James & Butt, 2001; James and Butt, 2002; Di Garbo et al, 2007), key cells in the functioning of the CNS. The response of the cells to both GABA and KCl caused membrane

depolarization by Ca^{2+} influx which occurs at presynaptic membranes. This provides evidence of neural-like activity, potentially via GABAa receptors, consistent with previous data (Stell et al, 2007; Stell, 2011). Development of GABAa receptors have recently been found to form before ionotropic glutamate receptors; NMDA & AMPA, during neural differentiation of mouse neural stem cells (Muth-Kohne et al, 2010). This would suggest that these cells here are potentially at an early stage of neural differentiation and are perhaps neural precursor-like cells, as they do not respond to NMDA and AMPA confirming that there were no active ionotropic glutamate receptors present. Further investigation would be required to confirm whether these cells will eventually form functional receptors by identifying the expression of the subunits for the NMDA and AMPA receptors. Additionally, expression of a limited number of neural markers was investigated utilising simple end point-PCR and therefore it may be beneficial to identify the expression level of these and additional neural markers using qPCR methods during the differentiation protocol. Investigations into the expression levels of genes known to be involved in neurogenesis by qPCR would provide further data on the potential neural development of these cells and provide potential reasons behind the lack of response to NMDA and AMPA.

The neural precursor-like cells that result from this protocol developed for differentiation of OMLP-PCs lack the ability for full neural functionality despite cells demonstrating morphology, marker expression and some calcium influx of neural cells. This could be as a result of a number of different reasons relating to the protocol. The protocol utilises a cocktail of growth factors;

BDNF, NGF & NT3 added to the final neural differentiation medium. These factors are known to induce process extensions and are potentially responsible for the neural-like morphology observed in the differentiated colonies. These process extensions would potentially result in upregulation of neural cell adhesion molecule (Ncam) via binding to P75 (Mirnics et al, 2005; Thornton et al, 2008). However, further investigation would be required to confirm expression levels of neural markers, such as Ncam, by qPCR analysis during the differentiation protocol to map any changes observed. It would also be interesting to determine the expression levels of factors actively known to inhibit neural differentiation and maturation to determine whether this protocol is limiting the differentiation potential by promoting expression of these factors. Factors such as Sox-2 are actively known to inhibit neural stem cell differentiation whilst maintaining cells in a pluripotent state (Graham et al, 2003). This would significantly impair the ability of these cells to form fully functional differentiated neurons as downregulation of Sox-2 is also known to be linked to additional markers upregulated during the onset of a functional neural phenotype; neurogenin-2 (Ngn-2) and Mash-1 (Bylund et al, 2003). These markers could be utilised to provide further evidence of level of neural differentiation/maturation.

5.6 Conclusion

The protocol utilised in these experiments allowed colonies from the OMLP to differentiate towards to neural-like phenotype. The differentiated phenotype was supported by morphology, expression of neural markers at the messenger RNA and protein level and their response to appropriate agonists

causing calcium influx. Together these data suggest that cells from OMLP-PCs can be differentiated towards neural precursor-like cells with the potential to form a functional neural phenotype following further differentiation. The merged colony populations and pluripotent cell populations did not respond to this protocol in the same way as OMLP-PCs colonies. This is not however, confirmation that these cells are less efficient at neural differentiation but more indicative that different optimised cell culture conditions are required to preferentially direct these cells down a functional neural lineage.

6 - General Discussion

Isolation of progenitor cells from the OMLP and their subsequent reprogramming provided two different stem cell populations from one tissue; OMLP-PC (multipotent ASCs) and OMLP-iPSC (pluripotent stem cells). The main purpose of this thesis was to take these two cell populations and determine the neural differentiation potential of each cell type. The objective was to establish whether the neural differentiation of reprogrammed OMLP-PCs was potentially superior when compared to direct neural differentiation of OMLP-PCs. The reality is however, that each route has its own distinct advantages and limitations and neural differentiation potential of the OMLP-iPSCs is yet to be determined.

Like many dental tissues, the buccal mucosa harbours a population of neural crest derived progenitor cells that exist within the lamina propria (Huang et al, 2009 Davies et al, 2010; Marynka-Kalmani et al, 2010). Typically, like other mesenchymally derived stem cells, they express MSC markers, demonstrate the ability to differentiate into chondrocytes, adipocytes, osteocytes (Davies et al, 2010; Marynka-Kalmani et al, 2010) and have potent immunomodulation properties (Davies et al, 2012). This means that they could be used in either autologous or allogeneic treatments without the need for immunosuppression. ASCs all tend to demonstrate many similar properties to one another but the question remains is one population of ASCs “better” than the other?

The OMLP represents an attractive source of cells for use in tissue engineering applications attributed to many features. Their proliferative potential is greater than bone marrow derived MSCs (Bruder et al, 1997;

Davies et al, 2010), making these cells potentially more useful when thinking about the scalability of clinically relevant cell numbers for patient treatments which may be more achievable with OMLP-PCs. More importantly, taking a biopsy from a patient is minimally invasive and can be obtained during routine dental procedures and would therefore, be available to majority of people. Furthermore, the wound site in the oral mucosa heals very rapidly with minimal scarring when compared to the skin leaving no lasting visible defect.

Despite these attractive properties, cells from the OMLP are developmentally more differentiated than hESC and would therefore be deemed to have a potentially more limited differentiation potential. For the first time, derivation of iPSCs has been demonstrated to be possible from the OMLP utilising non-integrating plasmid technology. Reprogramming these cells utilising iPSC technologies is thought to potentially counteract these limitations with reduced ethical complications compared to hESC use. At present the complete differentiation potential of each of these cell types; iPSC and ASCs, is not completely known or understood and therefore it is not known which cell type would offer the most beneficial starting point with respect to translational medicine.

Investigating the effects of oxidative stress on cells from the OMLP by treating with ascorbic acid was due to the reported benefit of an increased reprogramming efficiency of dermal fibroblasts to iPSCs following ascorbic acid treatment (Esteban et al, 2010). Having observed no benefit whilst culturing the OMLP-PCs with ascorbic acid, largely as a result of the ascorbic

acid potentially acting as a pro-oxidant in culture media at normal oxygen levels (Duarte et al, 2007), it was decided to discontinue with this approach and instead, untreated OMLP-PCs were utilised in reprogramming investigations. Although efficiency of the reprogramming technique is important when thinking about future clinical applications, reprogramming is a difficult technique that requires the process to work in a particular cell type before the efficiency can be improved.

It would nevertheless be interesting to investigate the effects of oxidative stress on stem cell properties of OMLP-PCs cultured in hypoxic and normoxic culture conditions. Minimising oxidative stress by reducing environmental oxygen levels would potentially more closely mimic the natural environment the cells exist within. In addition to this determining the potential effects of hypoxia on reprogramming efficiency of OMLP-PCs would also be interesting and studies have demonstrated increased efficiencies (Yoshida et al, 2009).

Reprogramming efficiencies of cells isolated from different tissues does vary and the presence of endogenous progenitor cells has been suggested to underlie these differences i.e better efficiencies are observed in host tissues where progenitor cells represent a larger proportion of the original population (Okita et al, 2007; Aasen et al, 2008). Cells isolated from dental tissues have demonstrated an increased reprogramming efficiency compared to dermal fibroblasts (Yan et al, 2010). Although differences were observed between the reprogramming of dermal fibroblasts and the OMLP-PCs in this thesis, this is potentially attributed to the differences in cell PDLs at the point of

nucleofection. Nevertheless, it would be interesting to test this hypothesis with the OMLP-PCs by utilising dermal fibroblasts at the same PDL.

The iPSCs derived from OMLP-PCs demonstrated some classical morphological and phenotypical characteristics typical of hESCs. This includes the production of stem cell surface markers; SSEA-4, SSEA-5, TRA-1-60, TRA-1-80 and transcription factors; Oct-4 and Sox-2 (Andrews et al, 1984; Wenk et al, 1994; Thomson et al, 1995; Thomson et al, 1998). Moreover, confirmation that the iPSCs had the potential ability to differentiate towards lineages of all three germ layers demonstrated by expression of early markers of the mesoderm, endoderm and ectoderm suggests that iPSCs from the oral mucosa may have pluripotent properties and therefore great potential for use in clinical applications.

In recent years, the epigenetic memory of reprogrammed cells has been investigated and as such this would form some interesting investigations to determine the level of memory these reprogrammed OMLP-iPSCs maintain and whether or not this would affect any resulting differentiation potential. Studies have demonstrated that the starting cell can affect reprogramming efficiency and also the differentiation potential of the resulting iPSCs. (Kim et al, 2010; Kim et al, 2010; Bar-Nur et al, 2011)

Future potential for reduction in the levels of genetic manipulation are promising for cells isolated from the OMLP given the expression levels detected for Klf-4 and C-Myc. It would be interesting to test the potential for iPSC derivation from OMLP-PCs utilising a reduced number of factors,

minimising genetic manipulation and hence improving the safety of these cells for potential therapeutic applications. Reprogramming of neural stem cells to iPSC has been demonstrated with retroviral induction of a single factor; Oct-4 (Kim et al, 2009) indicating that production of iPSCs with reduced genetic manipulation is possible. However, the use of retroviruses to introduce the factors, as previously discussed, limits the clinical usefulness of these cells. Therefore, developing a non-integrating method with fewer factors would be beneficial for future advances in this area.

The differentiation of colonies isolated from the OMLP demonstrated promise in their ability to differentiate down a neural lineage. The presence of typical neural marker expression at the RNA and protein level is encouraging. Moreover, their response to neural agonists adds weight to the fact that these cells have differentiated towards a more functional neural phenotype. However, their inability to respond to key agonists required for fully functional neurons (AMPA and NMDA) indicates that these cells are only partially committed to a differentiated functional neural phenotype.

The major difference in neural differentiation observed between OMLP-PCs and OMLP-iPSCs was due to the culture conditions utilised. The protocol utilised in this thesis had been optimised for OMLP-PCs (Davies et al, 2010), an ASC population that will be at a much later developmental stage than iPSCs. To truly determine the differentiation potential of the OMLP-iPSCs, a differentiation protocol optimised for hESCs would be required and will most likely involve the inhibition of BMP signalling in the early stages of differentiation which have shown some promising results (Itsykson et al,

2005; Zhou et al, 2008; Chambers et al, 2009). Once differentiated, these cells could then be tested for typical characteristics of neurons including; morphology, presence of neural markers and the potential to function like a neuron. One of the limitations of neural differentiation of iPSCs is the reported variability in differentiation potential (Hu et al, 2010). This may necessitate an additional cell selection step following differentiation due to potential safety concerns with regards to using undifferentiated iPSCs as cell transplants.

In addition to calcium imaging to determine the presence of calcium channels, differentiated cells for both OMLP-PCs and OMLP-iPSCs would ideally need to be tested for their ability to fire action potentials as definitive proof of the presence of functional neurons. This would be performed utilising whole cell patch clamp technique to record membrane potentials. Evidence of this action potential in neural differentiated iPSCs has previously been reported (Karumbayaram et al, 2009; Zeng et al, 2010; Haythornthwaite et al, 2012) however, this is yet to be reported for neural differentiated cells derived from OMLP and other progenitors isolated from dental tissues.

Only once OMLP-iPSCs demonstrate early neural differentiation, can a true comparison of the differentiation capabilities of OMLP-PCs and OMLP-iPSCs be made. This could potentially involve the use of *ex vivo* and potentially *in vivo* spinal cord injury models to test functional recovery following cell transplants.

Although evidence of the potential for hESCs, iPSCs or ASCs to terminally differentiate towards a neural phenotype is desirable, primarily to establish that the cells possess the ability to terminally differentiate *in vitro*, it may not be essential for clinical applications. It may be more realistic to produce a neural precursor cell for transplantation, rather than terminally differentiate the cells, and allow the cells to continue to differentiate *in vivo*. Stem cell transplants into spinal cord injury models have proved promising with some degree of functional recovery observed utilising ESCs and BMSCs (Kierstead et al, 2005; Kim et al, 2006, Cizkova et al, 2006; Sasaki et al, 2009), this provides hope for the use of the OMLP-PCs in such injuries. Furthermore, the ability of iPSCs to form functional neurons *in vitro* (Karumbayaram et al, 2009) adds promise for the future use of both OMLP-PCs and OMLP-iPSCs in spinal cord injury repair.

6.1 Conclusion

There were three main aims to this study; to isolate, expand and characterise cell populations from the OMLP, to reprogram OMLP-PCs to iPSCs and determine the potential of OMLP-PC and OMLP-iPSCs to differentiate down neuronal lineages.

Cells have been isolated and cultured as both single cell-derived colonies and merged progenitor populations. Isolated colonies have demonstrated differentiation towards a neural-like phenotype. Merged progenitor populations have been reprogrammed to iPSCs with promising pluripotent properties and the neural differentiation potential of these cells is yet to be

determined. However, the OMLP-iPSCs not only have the potential to differentiate towards a neural lineage, they may also demonstrate the ability to form cell types of all three germ layers bestowing wide-ranging therapeutic applications upon these cells.

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8 - Appendix

Appendix I: Table of patient information for samples utilised in this thesis

Patient Number	Gender	Age (At time of sample)
XLVII	Male	36
XLVIII	Male	30
XLIX	Female	38
L	Male	22
LII	Female	19
LIV	Male	22

Appendix II: DNA sequence data for pluripotency markers: C-myc, Klf-4, Sox-2, Lin-28 and Oct-4

Score	Expect	Identities	Gaps	Strand
455 bits(246)	3e-125	256/261(98%)	0/261(0%)	Plus/Plus

Features: myc proto-oncogene protein

```
Query  188       CCAGCAGCCTCCCGCGACGATGCCCCTCAACGTTAGCTTCACCAACAGGAACTATGACCT  247
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42194579  CCAGCAGCCTCCCGCGACGATGCCCCTCAACGTTAGCTTCACCAACAGGAACTATGACCT  42194638

Query  248       CGACTACGACTCGGTGCAGCCGTATTTCTACTGCGACGAGGAGGAGAACTTCTACCAGCA  307
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42194639  CGACTACGACTCGGTGCAGCCGTATTTCTACTGCGACGAGGAGGAGAACTTCTACCAGCA  42194698

Query  308       GCAGCAGCAGAGCGAGCTGccccccccGGCGCCCAGCGAGGATATCTGGAAAAAATTCGA  367
                 |||||||||||||||||||  |||||||||||||||||||||||||||| ||||||||
Sbjct  42194699  GCAGCAGCAGAGCGAGCTGCAGCCCCCGGCGCCCAGCGAGGATATCTGGAAGAAATTCGA  42194758

Query  368       GCTGCTGCCCACCCCGCCCCTGTCCCCTAGCCGCCGCTCCGGGCTCTGCTCGCCCTCCTA  427
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42194759  GCTGCTGCCCACCCCGCCCCTGTCCCCTAGCCGCCGCTCCGGGCTCTGCTCGCCCTCCTA  42194818

Query  428       CGTTGCGGTCACCCCTTTCTC  448
                 |||||||||||| || |||||
Sbjct  42194819  CGTTGCGGTCACACCCTTCTC  42194839
```

Score	Expect	Identities	Gaps	Strand
198 bits(107)	2e-48	124/132(94%)	2/132(1%)	Plus/Minus

Features: Krueppel-like factor 4

```
Query  20        CGGCTGTGGATGG-AATTCGCCCGCTCAGATG-ACTGACCAGGCACTACCGCAAACACAC  77
                 ||||||||||||| |||||||||||||||||| |||||||||||||||||| ||||||||
Sbjct  39514600  CGGCTGTGGATGGAAATTCGCCCGCTCAGATGAACTGACCAGGCACTACCGTAAACACAC  39514541

Query  78        AGGGCACCGGCCCTTCCAGTGCCAAAAATGCGACCGGGCCTTTTCCAGGTCGGACCACCT  137
                  |||||||| || |||||||||||||||||||| || ||||||||||||||||||||
Sbjct  39514540  GGGGCACCGCCCGTTCCAGTGCCAAAAATGCGACCGAGCATTTTCCAGGTCGGACCACCT  39514481

Query  138       CGCCTTACACAT  149
                 ||||||||||||
Sbjct  39514480  CGCCTTACACAT  39514469
```

Score	Expect	Identities	Gaps	Strand
189 bits(102)	8e-46	110/113(97%)	3/113(2%)	Plus/Plus

Features: transcription factor SOX-2

```
Query  20        GGCG-CGAGTGG--ACTTTTGTCGGAGACGGAGAAGCGGCCGTTCATCGACGAGGCTAAG  76
                 |||| |||||||  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87834929  GGCGCCGAGTGGAAACTTTTGTCGGAGACGGAGAAGCGGCCGTTCATCGACGAGGCTAAG  87834988

Query  77        CGGCTGCGAGCGCTGCACATGAAGGAGCACCCGGATTATAAATACCGGCCCCG  129
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87834989  CGGCTGCGAGCGCTGCACATGAAGGAGCACCCGGATTATAAATACCGGCCCCG  87835041
```

Score	Expect	Identities	Gaps	Strand
327 bits(177)	3e-87	177/177(100%)	0/177(0%)	Plus/Plus

Features: protein lin-28 homolog A

```
Query  18        GTCTAGATCATCATGCCAAGGAATGCAAGCTGCCACCCCAGCCCAAGAAGTGCCACTTCT  77
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13736514  GTCTAGATCATCATGCCAAGGAATGCAAGCTGCCACCCCAGCCCAAGAAGTGCCACTTCT  13736573

Query  78        GCCAGAGCATCAGCCATATGGTAGCCTCATGTCCGCTGAAGGCCCAGCAGGGCCCTAGTG  137
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13736574  GCCAGAGCATCAGCCATATGGTAGCCTCATGTCCGCTGAAGGCCCAGCAGGGCCCTAGTG  13736633

Query  138       CACAGGGAAAGCCAACCTACTTTCGAGAGGAAGAAGAAGAAATCCACAGCCCTACCC  194
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13736634  CACAGGGAAAGCCAACCTACTTTCGAGAGGAAGAAGAAGAAATCCACAGCCCTACCC  13736690
```

Score	Expect	Identities	Gaps	Strand
207 bits(112)	2e-51	119/122(98%)	2/122(1%)	Plus/Minus

Features: 540 bp at 5' side: homeobox protein NANOG
18559 bp at 3' side: solute carrier family 2, facilitated glucose transporter ...

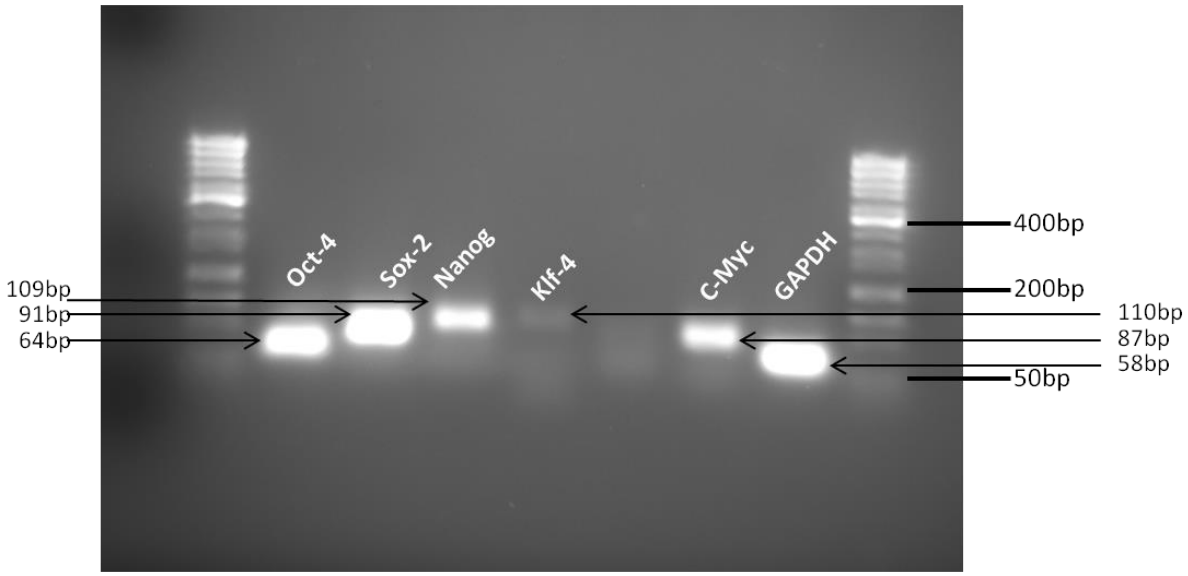
```
Query  11        ATCTTC-TCA-CAATTCGTACTTGTATTTCTTATTCTTGAGGTTAGATTCTAAACCCTAA  68
                 |||||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  7831514   ATCTTCATCACCAATTCGTACTTGTATTTCTTATTCTTGAGGTTAGATTCTAAACCCTAA  7831455

Query  69        AGATATCCAAACTAGTATTAGATCTACTTATCTATAGCCAGAGACGGCAGCCAAGGTTAT  128
                 |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  7831454   AGATATCCAAACTAGTATTAGATCTACTTATCTATAGCCAGAGACGGCAGGCAAGGTTAT  7831395

Query  129       TA  130
                 ||
Sbjct  7831394   TA  7831393
```

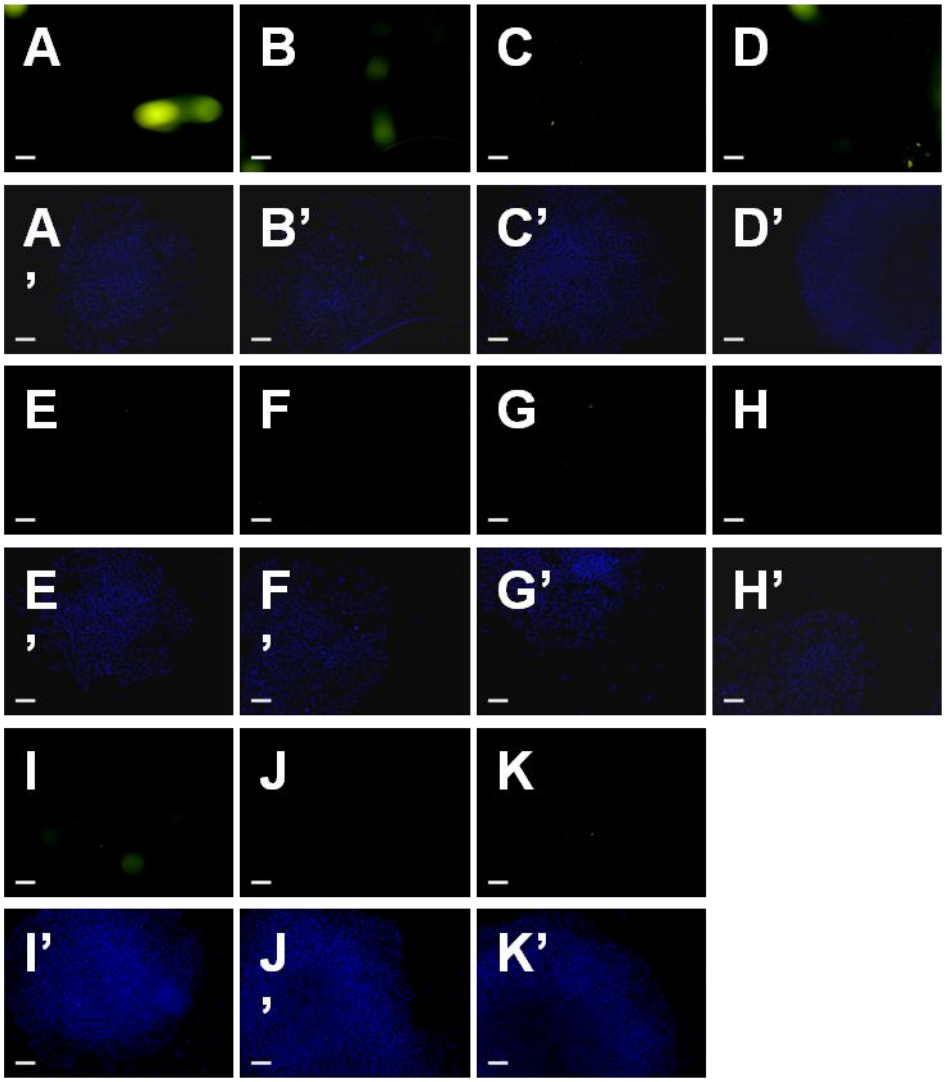
Score	Expect	Identities	Gaps	Strand
346 bits(187)	9e-93	200/206(97%)	1/206(0%)	Plus/Plus

Features: putative POU domain, class 5, transcription factor 1B

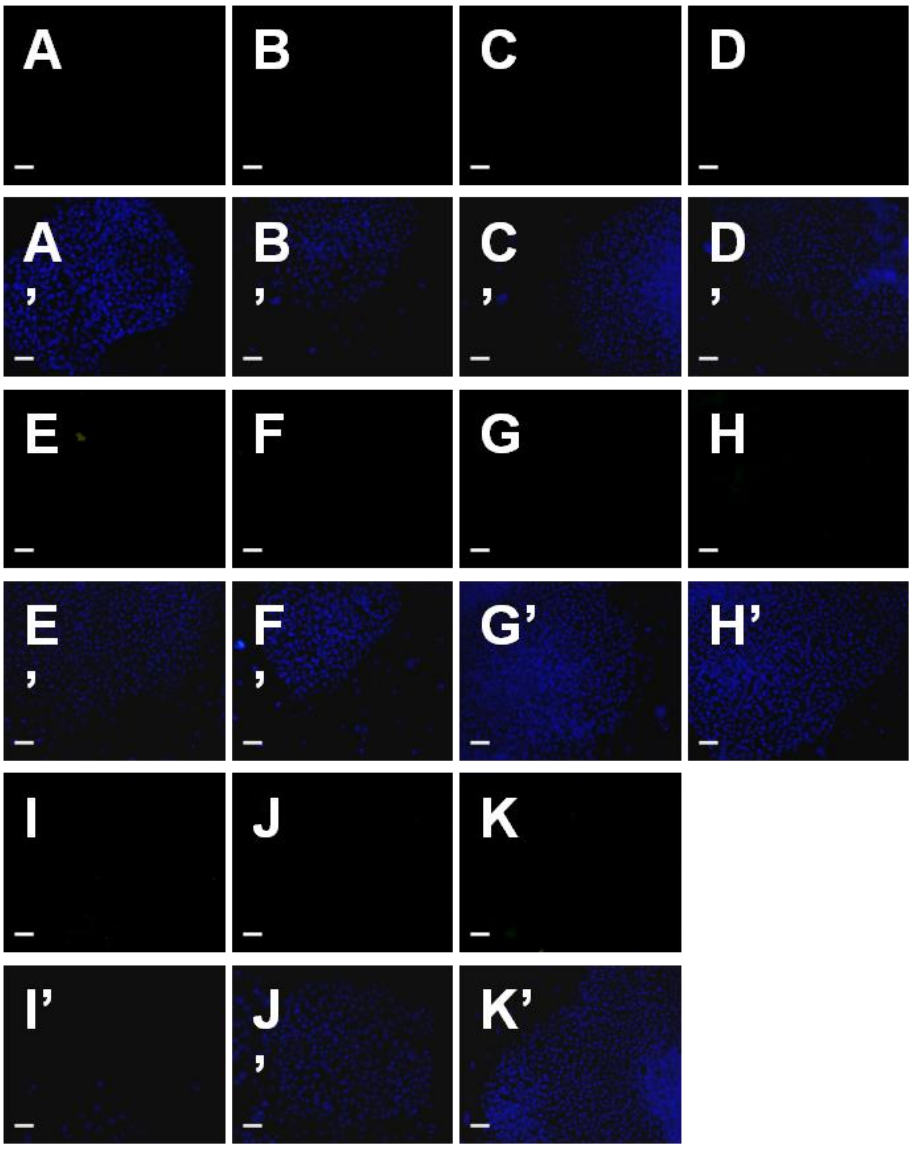
```
Query  27        CTGCA-CGTCACCCCTGGTGCCGTGAAGCTGGAGAAGGAGAAGCTGGAGCAAAACCCGGA  85
                 ||||| |||| ||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  41688133  CTGCACCGTCCCCCCTGGTGCCGTGAAGCTGGAGAAGGAGAAGCTAGAGCAAAACCCGGA  41688192

Query  86        GGAGTCCCAGGACATCAAAGCTCTGCAGAAAGAACTCGAGCAATTTGCCAAGCTCCTGAA  145
                 | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  41688193  GAAGTCCCAGGACATCAAAGCTCTGCAGAAAGAACTCGAGCAATTTGCCAAGCTCCTGAA  41688252

Query  146       GCAGAAGAGGATCACCCTGGGATATACACAGGCCGATGTGGGGCTCACCCTGGGGGTTCT  205
                 |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  41688253  GCAGAAGAGGATCACCCTGGGATATACACAGGCCGATGTGGGGCTCATCCTGGGGGTTCT  41688312

Query  206       ATTTGGGAAGGTATTCAGCCAAACGA  231
                 |||||||||||| ||||||||||||
Sbjct  41688313  ATTTGGGAAGGTGTTCAGCCAAACGA  41688338
```

Appendix III: Quantitative PCR product size confirmation for pluripotency markers and the house keeping gene: Oct-4, Sox-2, Nanog, Klf-4, C-myc and GAPDH

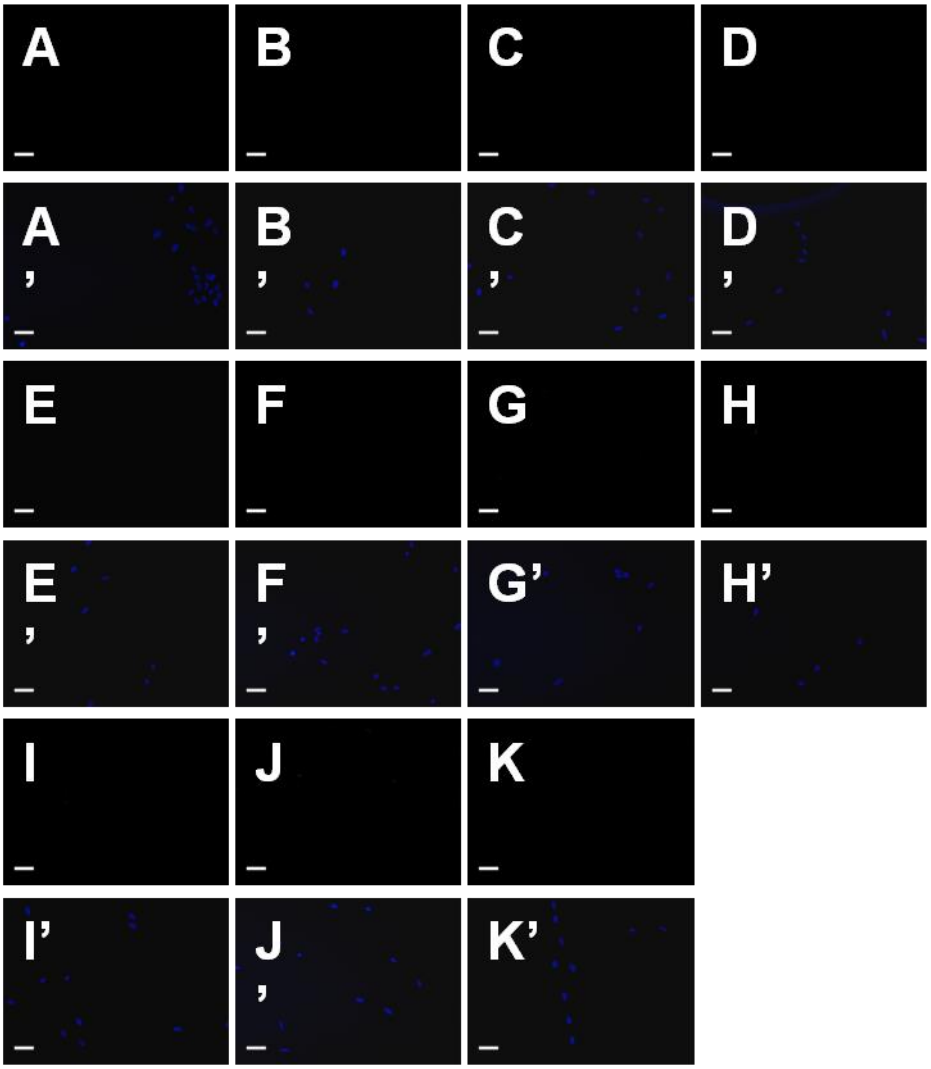




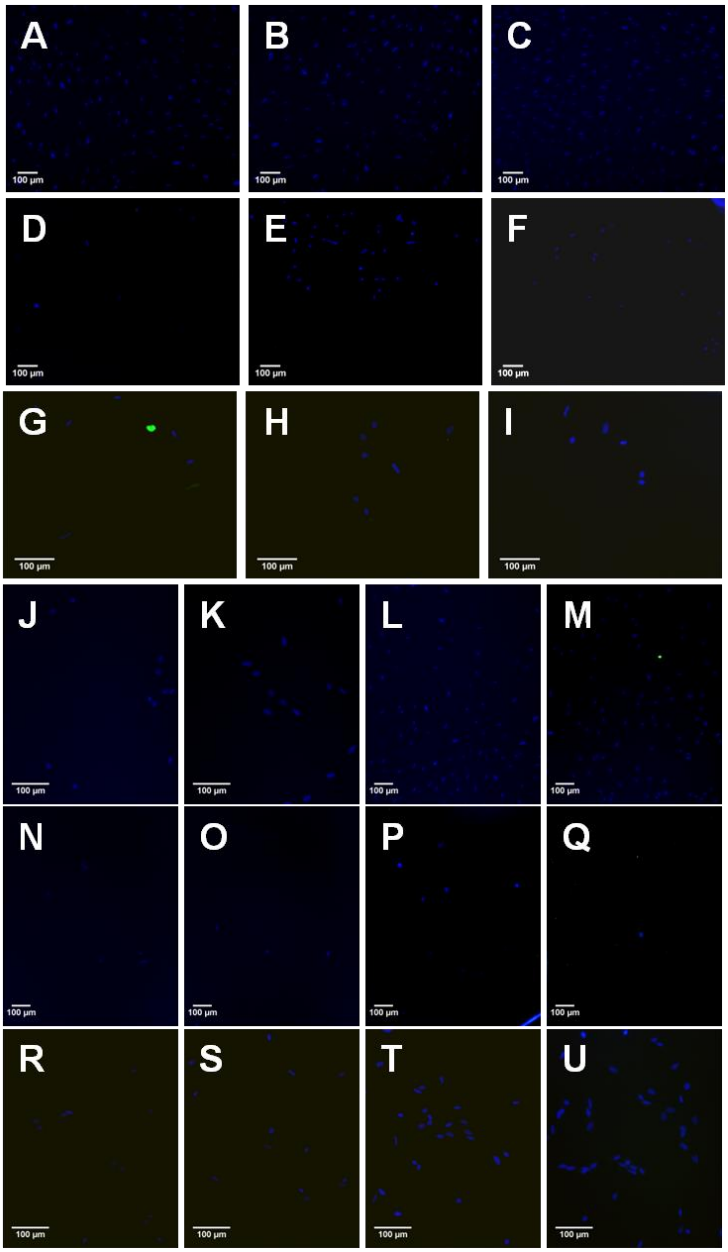
Appendix IV: iP17 controls for pluripotency markers. **(A)** Primary omission control for oct-4. **(A')** DAPI counterstain for oct-4 primary omission. **(B)** Primary omission control for sox-2. **(B')** DAPI counterstain for oct-4 primary omission. **(C)** Primary omission control for SSEA-4. **(C')** DAPI counterstain for SSEA-4 primary omission. **(D)** Primary omission control for SSEA-5. **(D')** DAPI counte stain for SSEA-5 primary omission. **(E)** Primary omission control for TRA-1-60. **(E')** DAPI counterstain for TRA-1-60 primary omission. **(F)** Primary omission control for TRA-1-81. **(F')** DAPI counterstain for TRA-1-81 primary omission. **(G)** IgG (Rabbit) control. **(G')** DAPI counterstain for IgG (Rabbit). **(H)** IgG (Mouse) control. **(H')** DAPI counterstain for IgG (Mouse). **(I)** IgM control. **(I')** DAPI Counterstain for IgM. **(J)** Secondary omission control for swine anti rabbit. **(J')** DAPI counterstain for secondary omission control. **(K)** Secondary omission control for rabbit anti mouse. **(K')** DAPI counterstain for secondary omission control. (Scale bar=100µm)



Appendix V: H9 controls for pluripotency markers. **(A)** Primary omission control for oct-4. **(A')** DAPI Counterstain for oct-4 primary omission. **(B)** Primary omission control for sox-2. **(B')** DAPI Counterstain for oct-4 primary omission. **(C)** Primary omission control for SSEA-4. **(C')** DAPI Counterstain for SSEA-4 primary omission. **(D)** Primary omission control for SSEA-5. **(D')** DAPI Counterstain for SSEA-5 primary omission. **(E)** Primary omission control for TRA-1-60. **(E')** DAPI Counterstain for TRA-1-60 primary omission. **(F)** Primary omission control for TRA-1-81. **(F')** DAPI Counterstain for TRA-1-81 primary omission. **(G)** IgG (Rabbit) control. **(G')** DAPI Counterstain for IgG (Rabbit). **(H)** IgG (Mouse) control. **(H')** DAPI Counterstain for IgG (Mouse). **(I)** IgM control. **(I')** DAPI Counterstain for IgM. **(J)** Secondary omission control for swine anti rabbit. **(J')** DAPI Counterstain for secondary omission control. **(K)** Secondary omission control for rabbit anti mouse. **(K')** DAPI Counterstain for secondary omission control. (Scale bar=100µm)



Appendix VI: MP XLIX controls for pluripotency markers. **(A)** Primary omission control for oct-4. **(A')** DAPI Counterstain for oct-4 primary omission. **(B)** Primary omission control for sox-2. **(B')** DAPI Counterstain for oct-4 primary omission. **(C)** Primary omission control for SSEA-4. **(C')** DAPI Counterstain for SSEA-4 primary omission. **(D)** Primary omission control for SSEA-5. **(D')** DAPI Counterstain for SSEA-5 primary omission. **(E)** Primary omission control for TRA-1-60. **(E')** DAPI Counterstain for TRA-1-60 primary omission. **(F)** Primary omission control for TRA-1-81. **(F')** DAPI Counterstain for TRA-1-81 primary omission. **(G)** IgG (Rabbit) control. **(G')** DAPI Counterstain for IgG (Rabbit). **(H)** IgG (Mouse) control. **(H')** DAPI Counterstain for IgG (Mouse). **(I)** IgM control. **(I')** DAPI Counterstain for IgM. **(J)** Secondary omission control for swine anti rabbit. **(J')** DAPI Counterstain for secondary omission control. **(K)** Secondary omission control for rabbit anti mouse. **(K')** DAPI Counterstain for secondary omission control. (Scale bar=100µm)



Appendix VII: Controls for neural markers. **(A)** Primary omission control of MP XLIX for βIII tubulin. **(B)** Primary omission control of MP XLIX for MAP-2. **(C)** Primary omission control of MP XLIX for NF-M. **(D)** Primary omission control of iP14 for βIII tubulin. **(E)** Primary omission control of iP14 for MAP-2. **(F)** Primary omission control of iP14 for NF-M. **(G)** Primary omission control of H9 for βIII tubulin. **(H)** Primary omission control of H9 for MAP-2. **(I)** Primary omission control of H9 forNF-M. **(J)** IgG (Rabbit) control for MP XLIX. **(K)** IgG (Mouse) control for MP XLIX. **(L)** Secondary omission control of MP XLIX for swine anti rabbit. **(M)** Secondary omission control of MP XLIX for rabbit anti mouse **(N)** IgG (Rabbit) control for iP14. **(O)** IgG (Mouse) control for iP14. **(P)** Secondary omission control of iP14 for swine anti rabbit. **(Q)** Secondary omission control of iP14 for rabbit anti mouse. **(R)** IgG (Rabbit) control for H9. **(S)** IgG (Mouse) control for H9. **(T)** Secondary omission control of H9 for swine anti rabbit. **(U)** Secondary omission control of H9 for rabbit anti. (Scale bar=100µm)