Characterisation of progenitor cell populations from within the Oral Mucosa Lamina Propria

Thesis submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy, Cardiff University

March 2011

James Thomas Roberts

School of Dentistry,

Cardiff University,

Cardiff.

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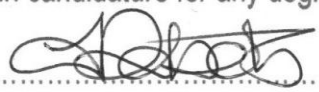
Signed  (candidate) Date 24/11/11

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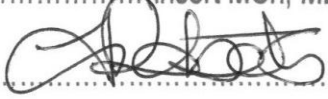
Signed  (candidate) Date 24/11/11

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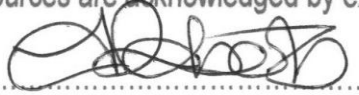
Signed  (candidate) Date 24/11/11

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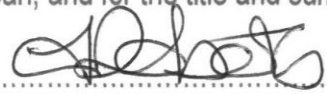
Signed  (candidate) Date 24/11/11

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List of Abbreviations

ALCAM	Activated Leukocyte Cell Adhesion Molecule
APC	Allophycocyanin
ASC	Adult Stem Cell
bFGF	Basic Fibroblast Growth Factor
BMSC	Bone Marrow Stromal Cell
cDNA	Complementary DNA
CD(x)	Cluster of Differentiation (x, e.g. CD34)
CFE	Colony Forming Efficiency
Clone (x)	Clonally Derived Cells (e.g. Clone 6)
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPSC	Dental Pulp Stem Cells
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ES/ESC	Embryonic Stem Cells
FACS	Fluorescence Activated Cell Sorting
FBS/FCS	Foetal Bovine/Calf Serum
FITC	Fluorescein Isothiocyanate
HCl	Hydrochloric Acid
HGF	Hepatocyte Growth/Scatter Factor
HSC	Haematopoietic Stem Cell
HSCT	Haematopoietic Stem Cell Transplant
hTERC	Human Telomerase RNA component
hTERT	Human Telomerase Reverse Transcriptase
K5-8	Keratin 5-8
K13-17	Keratin 13-17

LAM	Leukocyte Adhesion Molecule
LO	Whole Lamina Propria Cell Population
mL	Millilitre
MMP	Matrix Metalloproteinase
MPC	Mesenchymal Progenitor Cell
MSC	Mesenchymal Stem/Stomal Cell
NA	Non Adherent Cell Population
OMF	Oral Mucosal Fibroblast
PBS	Phosphate Buffered Saline
PBSC	Peripheral Blood Stem Cell
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDL	Periodontal Ligament
PDLSC	Periodontal Ligament Stem Cell
PE	Phycoerythrin
QRT-PCR	Quantitative Real-Time PCR
Rb	Retinoblastoma
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
SKP	Skin Derived Precursor
SRCR	Scavenger Receptor Cysteine Rich
TAE	Tris/Acetic Acid/EDTA Buffer
TGFα/β	Transforming Growth Factor α/β
TNF	Tumour Necrosis Factor
µL	Microlitre
µm	Micrometre
µM	Micromolar
xg	Gravitational Constant
XVII/XXVIII	Patient XVII/XXVIII

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Summary

Wound healing within the oral mucosa demonstrates “foetal-like” responses with minimal scarring. This preferential healing has been attributed to the phenotypic characteristics of Oral Mucosa Fibroblasts (OMF), which display enhanced replicative potential. Adult stem cell populations discovered throughout the body including the oral cavity led to the discovery of a novel stem cell population within the lamina propria.

This investigation aims to characterise this stem cell population in comparison with populations isolated from the lamina propria by different means, in an attempt to describe the preferential properties of a progenitor cell population within the tissue. Differential adhesion to fibronectin facilitated the isolation of clonally derived stem cell populations (clone x), a cell population representing the whole lamina propria, analogous to OMF (LO), and cells which do not adhere to fibronectin within the context of the differential adhesion assay (NA).

Population doublings indicated that all cell populations were capable of exceeding the growth potential of BMSCs, however populations demonstrated distinct growth characteristics during the early phase of growth. All cell populations demonstrated an increase in cell size over time in culture, associated with replicative senescence. Active telomerase was detected in all cell populations; however this was not sufficient to prevent cellular senescence in any cell population, with telomere degradation apparent in many cell populations.

Differentiation was achieved in all cell populations, with temporal differences in gene expression observed during adipogenesis. The success of the theoretically progenitor depleted NA population to successfully undergo differentiation suggests that the current isolation method, clonal expansion after fibronectin adherence, may not be the most practical means by which to enrich for a stem cell population from the Lamina Propria of the Oral Mucosa.

CHAPTER 1

INTRODUCTION

With recent advances in the field of stem cell research, the use of stem cells for therapeutic purposes has become the subject of much debate.

Embryonic stem (ES) cells derived from the inner cell mass of an embryo are capable of forming tissues from all embryonic germ layers, the endoderm, mesoderm and ectoderm (Fig. 1.1). With pluripotency and a limitless capacity for self-renewal *in vitro,* ES cells are the ideal choice for regenerative medicine. However, the use of ES cells and the manner in which they are obtained causes moral and ethical concerns for society and science alike.

Adult stem cells (ASCs) are a solution to the ethical concerns over ES cells, as they are isolated without the destruction of an embryo, depending on tissue of origin can be easily isolated, and represent an autologous source for tissue repair circumventing any tissue rejection concerns involved in allogeneic treatments. One of the main issues involved with the isolation of adult stem cells for clinical applications is the size of the biopsy required to isolate an adequate number of stem cells; as the stem cells found in adult tissues make up such a small proportion of the cells present. The oral mucosa however, has long been known to heal rapidly with minimal to no-scarring when compared to the skin, a tissue very similar in structure and function (Sloan et al., 1991, Stephens and Genever, 2007, Urmacher, 1990). This rapid healing has lead to the description of the oral mucosa as having a “foetal-like” phenotype (Irwin et al., 1994), and the hypothesis that a progenitor cell population plays a part in this effect.

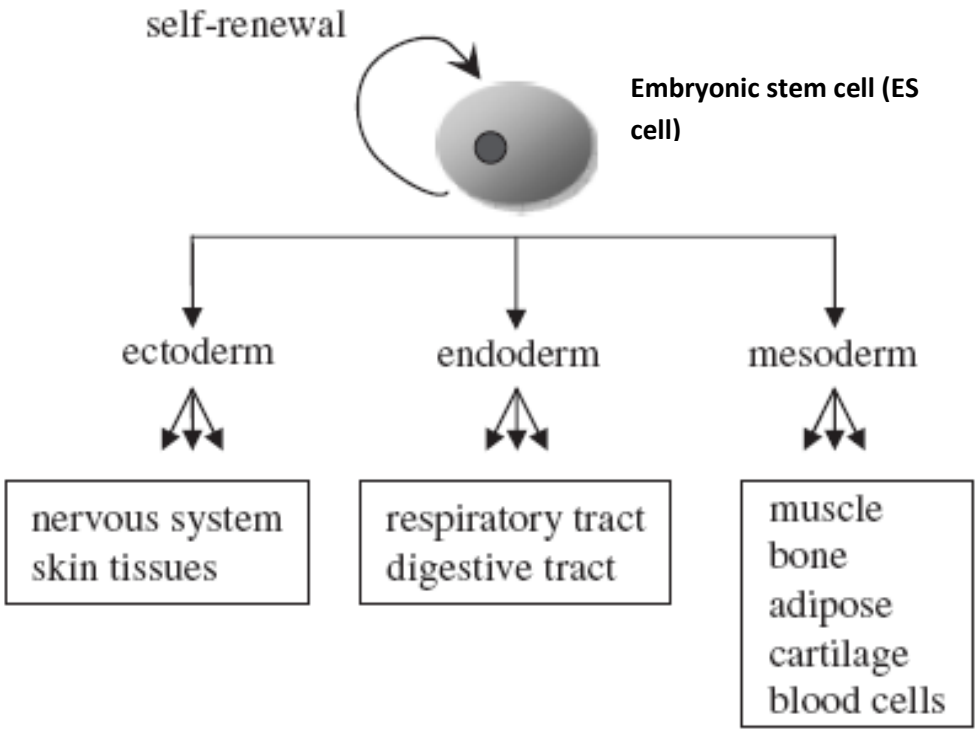

Fig 1.1: The principle definition of stem cells; self-renewal through replication, and differentiation into multiple lineages of all germ layers. (Fortier, 2005).

1.1 Developmental Origins of the Oral Mucosa

The mesenchymal of the head and neck is primarily constituted of neural crest cells, originating from the ectodermal germ layer that forms the nervous system. This is also known as the ectomesenchyme or neuroectoderm and gives rise to all of the connective tissues of the face, including dental structures. (James K. Avery, 2002). The oral mucosa is derived from this ectomesenchyme from neural crest cells which migrate from the midbrain to the relevant brachial arches and intersperse within the mesenchymal tissue already present. Once there these cells proliferate extensively and provide a great contribution to the development of oral cavity cultures including the oral mucosa and teeth (Winning and Townsend, 2000). Development of the craniofacial structures involves crucial epithelial-mesenchymal interactions, and these interactions are subsequently important in the maintenance of oral mucosa (Mackenzie and Hill, 1984). At 5-6 weeks development, two layers of epithelial cells line the oral cavity, by 10 weeks the multilayered epithelium is present and by 23 weeks *in utero*, an oral epithelium with adult characteristics is present.

1.2 Structure and Function of the Oral Mucosa

The oral mucosa, like the skin, is a continuous layer which plays a crucial role in the maintenance of an organism through a number of processes. These include regulation of water and electrolyte balance, thermoregulation

and as a barrier against factors such as micro-organisms (Sloan et al., 1991, Stephens and Genever, 2007, Urmacher, 1990).

Both tissues comprise a number of distinct layers; an epithelium principally composed of squamous stratified cells which vary in morphology from cuboidal at the basement membrane to flat squamous at the epithelial surface, an underlying layer of connective tissue cells containing dense vascular and neural networks, excretory and secretory glands and in the skin hair follicles, and a deeper layer of adipose tissue (Fig. 2 and Fig. 3) (Sloan et al., 1991, Stephens and Genever, 2007, Urmacher, 1990).

1.2.1 The epithelium

The epithelial layer in the oral mucosa (or epidermis in the skin) is composed principally of keratinocytes, which are named due to the production of filamentous keratins as the cells undergo terminal differentiation. Other cells are present in the epithelium of both the oral mucosa and the skin (Table 1). In the oral mucosa this layer can be keratinised (gingival, hard palate) or non-keratinised (soft palate, under-side of tongue, alveolar mucosa, labial mucosa, and buccal mucosa) (Sloan et al., 1991). Keratinised epithelium comprises four layers; the stratum (S.) basale, S. spinosum, S. granulosum and the S. corneum. The similarities observed in the keratinised epithelia of both tissues are due to similarities in function, such as the ability to withstand shear stress and friction. This is due to the cornified outer layers of the epithelia (Stephens and Genever, 2007). Cell division occurs within the S.

basale whereupon the cells can remain in the basal layer or migrate towards the S. corneum and terminally differentiate into a squamous stratified cell (Fig. 3b). The differentiation state of keratinocytes within the epithelium can be determined by the expression of cytokeratins, which changes as cells migrate and form the S. corneum, a layer of anucleated cells containing keratins (Fuchs and Green, 1980).

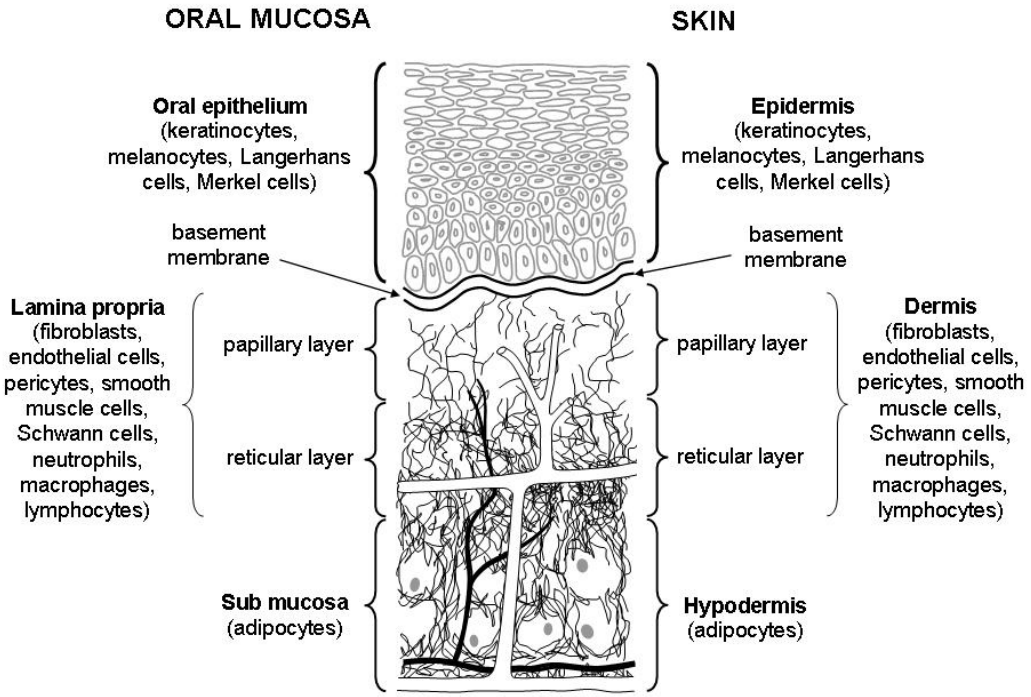

Fig 1.2: A schematic representation of the oral mucosa, highlighting the similarities to the skin (Stephens and Genever, 2007).

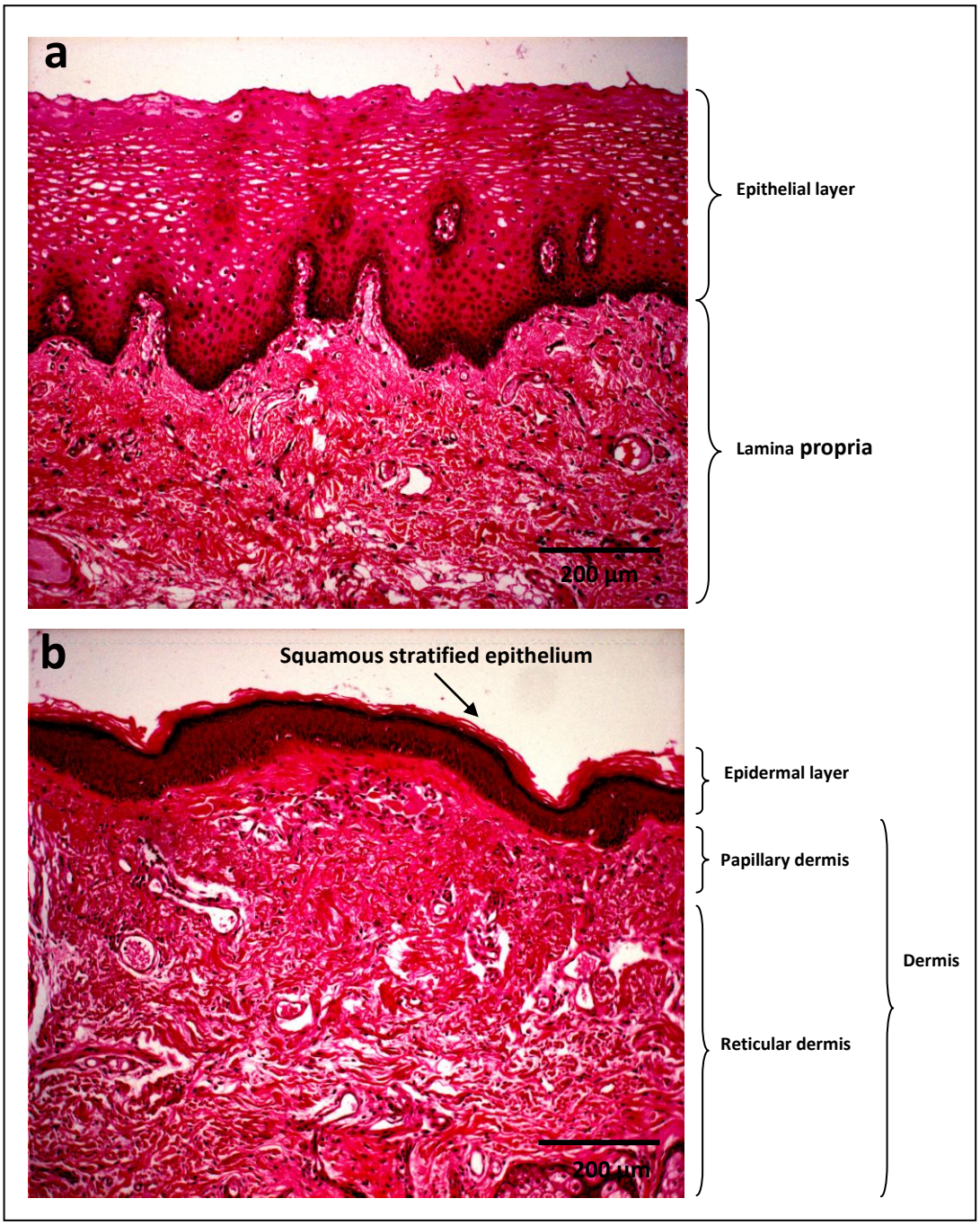

Fig 1.3: Histology of oral mucosa and skin. Haematoxylin and Eosin staining demonstrating the structure of non-keratinised oral buccal mucosa (Fig 3a) and (b) skin (Fig 3b). Oral mucosa contains a thicker epithelial layer than the skin with distinct protrusions into the lamina propria, which is thinner than observed in the skin. Note the lack of a keratinised layer in the oral mucosa, clearly visible in the skin (Stephens and Genever 2007).

Non-keratinised epithelium is structurally distinct from the epidermis in the skin and keratinised oral epithelia. It comprises three layers, the S. basale, S. intermedium and S. superficiale; lacking the cornified layer seen in the epidermis. It is less able to resist damage than keratinised epithelium. Keratin 14 is expressed highly in the S basale, and keratin 19 is heterogeneously expressed in all layers in contrast to keratinised epithelium where this is only found in Merkel cells (Table 1.1) (Su et al., 1996). In organotypic culture buccal mucosal fibroblasts have exhibited production of keratins K5-8 and K13-17 (Hansson et al., 2003). As cells migrate from the basal layer into the intermediate and superficial layers, the cells become flattened and show an increase in keratin filaments. Membrane thickening is also observed, as well as an increase permeability to water compared to the epidermis (Lesch et al., 1989).

Table 1.1: A table showing the cell types present in the oral and skin epithelia (Sloan et al., 1991).

Cell Type	**Characteristics**	**Function**
Keratinocyte	Population of rapidly renewing cells which undergo terminal differentiation and migration to the cornified layer	Resistant to compressive and sheer forces and abrasion. Barrier to the elements and infection by micro-organisms
Langerhans Cell	Bone marrow derived dentritic cells in the supra-basal region, often close to the papillary tips.	Immunity: presentation of antigens to regulatory T-lymphocytes
Granstein Cell	Similar to Langerhans Cell	Immunity: presentation of antigens to helper T-lymphocytes
Merkel Cell	Neural cell located in the basal layer	Epithelial sensory receptor
Melanocyte	Dendritic cell of neural crest origin forming a continuous network in the basal layer	Synthesis and transfer of melanin to adjacent cells

The epithelial layer is penetrated by undulating connective tissue protrusions known as papillae or Rete's ridges, which provide a complex epithelial-connective tissue junction with a large surface area for nutrition and attachment of the epithelia (Sloan et al., 1991).

The epithelial-connective tissue junction is known as the lamina in the oral mucosa and the basement membrane in the skin. This layer can be subdivided into two regions, the lamina lucida and the lamina densa (basal lamina). The lamina lucida is composed of laminin and decreases in density towards the epithelium, while the lamina densa contains laminin and type IV collagen, and is connected to the lamina propria. Basal epithelial cells are attached to the lamina lucida. Anchoring fibrils of type VII collagen are responsible for cell attachment to the lamina on the connective tissue side (Stephens and Genever, 2007). Other matrix components, such as fibronectin, tenascin, and entactin are present in the basal complex.

1.1.3 The lamina propria

The lamina propria in the oral mucosa and the dermis in the skin is the tissue underlying the epithelium (Fig. 1.2, Fig. 1.3). This can be divided into two regions, the papillary and the reticular lamina propria. The papillary layer contains the vasculature and neural components which provide the epidermis with nutrition and sensory information. The extracellular matrix (ECM) of the papillary layer is composed of a heterogeneous mixture of predominantly type I collagen, with type III collagen, elastic fibres, glycosaminoglycans,

proteoglycans and glycoproteins. The reticular lamina propria is thicker than the papillary layer, but similar in composition, and extends to the submucosa/hypodermis (Stephens and Genever, 2007).

Table 1.2: A table showing the cell types present in the lamina propria (Sloan et al., 1991).

Cell Type	**Characteristics**	**Function**
Fibroblasts	Heterogeneous cell population of spindle-like cells	Synthesis and turnover of extracellular matrix and growth factors. Interactions with epithelium and heavily involved in wound healing
Mast cells	Large polygonal cells containing metachromatic granules	Inflammatory and immune reactions. Interactions with blood vessels and the matrix
Immune cells	Macrophages, Lymphocytes etc…	Defence, scavenging, immune reactions, including inflammation
Vascular cells	Endothelial cells, lining of vessels or formation of capillaries	Nutrition, lymphatic drainage
Peripheral nerve cells	Schwann cells and specialised nerve endings	Transmission of sensory information

The principle cell type of the lamina propria is the fibroblast (Table 1.2), which produces the majority of the ECM, and plays a pivotal role in wound healing. Fibroblasts repopulate an area of lost tissue in a wound site and rapidly produce new provisional ECM. They also bring about contraction of the wound and remodel the ECM to form a fibrotic scar.

Although the ECM structure and composition is similar in both the lamina propria and the dermis, the non-keratinised oral mucosa shows differences related to function. Due to the requirement of a more flexible, recoiling tissue for mastication, speech and swallowing, the ECM is loose and has fewer, more slender papillae, with a higher elastic fibre composition. Also, the presence of other appendages varies with function; within the oral mucosa minor salivary and sebaceous glands interrupt the otherwise continuous structure; in the skin the salivary glands are absent, replaced by hair follicles.

The submucosa and the hypodermis lay beneath the lamina propria and the dermis, respectively. They are arranged into lobules, separated by thin bands of dermal connective tissue that make up the interlobular space. Within these lobules reside mature adipocytes, and an extensive network of nerves, blood and lymphatic vessels. The function of this layer is as a mechanical cushion and insulation. The presence of submucosa is dependent on function; keratinised oral mucosa has very little whereas non-keratinised has a significantly sized submucosa (Stephens and Genever, 2007)

1.3 Wound Responses in the Oral Mucosa

1.3.1 Wound Healing

In higher vertebrate animals, damage to tissues by wounding leads to a loss of haemostasis, triggering a fibroproliferative response resulting in wound closure and scar formation. The oral mucosa and skin follow a near-identical model of wound healing; this process can be divided into three well defined, overlapping stages; inflammation, proliferation and ECM formation and remodelling (Clark, 1996).

1.3.1.1 Haemostasis

Tissue injury causes microvascular injury and extravasation of blood into the wound space. Within minutes this stimulates platelet aggregation and activation of extrinsic and intrinsic coagulation cascades, ultimately facilitating the formation of a fibrin clot and prevention of further blood loss. This restores tissue haemostasis (Fig. 1.4). Platelet aggregation leads to the release of Hageman factor XII, which triggers the intrinsic pathway for coagulation through the conversion of a series of proenzymes to their active forms (Li et al., 2007). The extrinsic pathway is activated by the release of a lipoprotein named tissue factor from damaged tissues. This tissue factor is also present on the surface of activated monocytes and endothelial cells, which participate in the coagulation reactions. Platelets are also responsible for the release of other growth factors and cytokines which mediate the

recruitment of immune cells and tissue remodelling, such as transforming growth factors α and β (TGF-α/β) and platelet-derived growth factor (PDGF).

1.3.1.2 The inflammatory phase

After this restoration of haemostasis, the inflammatory phase sees the recruitment of monocytes and neutrophils from capillaries which migrate into the wounded tissue. In early inflammation (<12 hours) neutrophils are the predominant cell type, giving way to macrophages in late inflammation (>12 hours) (Li et al., 2007). Macrophages and neutrophils are recruited by the release of chemotactic factors released during haemostasis as well as mast cells, which secrete tumour necrosis factor (TNF), histamine, proteases and cytokines. Neutrophils destroy any bacteria which may have invaded the wound, as well as damaged matrix proteins. In late inflammation the macrophage is pivotal in the movement to the proliferative phase of wound healing, being responsible for the removal of any remaining pathogens, debris and neutrophils (Li et al., 2007, Clark, 1996).

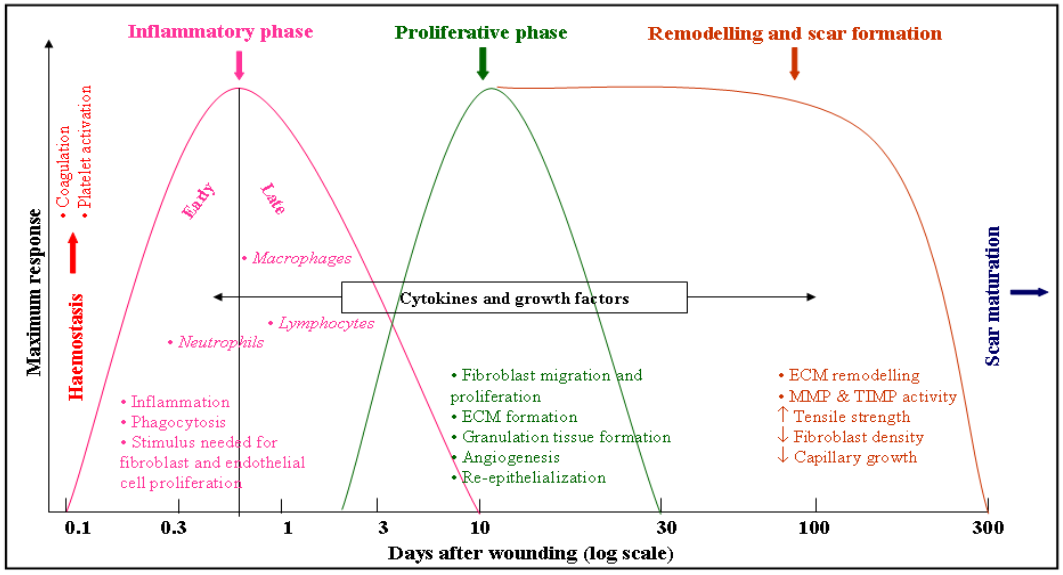

Fig 1.4. The main phases of wound healing, adapted from (Clark, 1996)

1.3.1.3 The proliferative phase

The proliferative phase involves the migration of cells into the wound space and the generation of granulation tissues and a new epithelium.

1.3.1.3.1 Re-epithelialisation

Re-epithelialisation begins with the migration of keratinocytes from the free edges of the wound within 24 hours. The keratinocytes at the wound edge lose the strong cell-cell and cell-matrix interactions found in normal tissue. Keratinocytes interact with the fibronectin rich provisional matrix formed during the clotting process; the migratory direction is regulated by the binding of integrin receptors to newly formed collagen in the wound bed (Li et al., 2007). The keratinocytes migrate by lamellipodial walking, moving in a “leap-frog” fashion. These migratory cells lack the associated keratins and filaggrin normally seen in terminally differentiated keratinocytes, yet remain phenotypically distinct from keratinocytes residing in the basal layer of the epidermis (Stephens and Thomas, 2002). Growth factors and matrix metalloproteinases (MMPs) have also been implicated, MMP-9 being involved in the degradation of type IV collagen and laminin, enabling detachment from the basement membrane and migration into the wound site. The formation of a new basement membrane completes the re-epithelialisation process.

1.3.1.3.2 Generation of ECM

Fibroblasts migrate into the wound space within 3-4 days of injury, using the provisional matrix as a substrate, and begin to provide scaffolding and contact guidance for cells to migrate and form granulation tissue. This process is known as fibroplasia. Fibroblasts proliferate in the wound space, producing a collagen rich matrix 2-7 days after injury (Li et al., 2007). This matrix is composed of collagen I and III, and contains fibronectin (Kurkinen et al., 1980). Angiogenesis also occurs, with the degradation of local basement membrane by endothelial cells, sprouting of new vasculature into the fibrin clot, reconstruction of basement membrane and maturation of the blood vessel.

1.3.1.4 Wound contraction

The final phase of wound healing involves wound contraction and the remodelling of the ECM produced by fibroblasts during the proliferative phase. The fibronectin produced in the formation of granulation tissue diminishes over time as the matrix is remodelled (Kurkinen et al., 1980) This process involves the induction of MMPs 1-3, enzymes which are each involved in the catabolism of different ECM components. These enzymes are controlled by natural tissue inhibitors of matrix metalloproteinases (TIMPS), and the balance between MMP and TIMP expression is crucial to normal matrix remodelling.

During wound contraction phenotypic changes to the fibroblasts populating the wound occur, with a switch to a pro-fibrotic myofibroblast phenotype, characterised by an increase in expression of α-smooth muscle actin. The attachment of these cells to each other and the surrounding matrix then facilitates wound contraction, with the myofibroblasts contracting pseudopodia attached to the ECM. This remodelling of the ECM in the skin leads to scar formation. However, the outcome observed in the oral mucosa has some marked differences, which may be due to intrinsic characteristics of the tissue (Szpaderska et al., 2003).

1.3.2 Differences in healing in the oral mucosa and the skin

Great differences have been observed in wound healing between the oral mucosa and the skin. Oral mucosa wound repair is characterised by rapid re-epithelialisation as well as enhanced wound repopulation and matrix reorganisation *in vitro*. Oral mucosal wounds were shown to contain significantly lower levels of macrophages, neutrophils and T-cells when compared with dermal wounds (Shannon et al., 2006, Szpaderska et al., 2003). Oral mucosal fibroblasts (OMFs) were shown to produce increased levels of epithelial mitogens keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), produce lower levels of α-smooth muscle actin, all factors implicated in fibrotic wound healing (Shannon et al., 2006, Stephens et al., 2001b). OMFs have also been shown to exhibit greater proliferation in the presence of basic fibroblast growth factor (bFGF) *in vitro* (Lee and Eun, 1999). It has been shown that OMFs exhibit an increased ability to remodel

ECM and contract a wound by use of fibroblast populated collagen lattices (Stephens et al., 1996, Lee and Eun, 1999, Okazaki et al., 2002). The matrix metalloproteinase activity of OMFs has also been shown to be superior to dermal fibroblasts, with increased levels of MMP-2 with a decreased production/activation of TIMPs (Stephens et al., 2001a). More recently, research had demonstrated that oral fibroblasts are phenotypically distinct, and are capable of achieving a higher number of cumulative population doublings *in vitro* when compared to patient matched skin fibroblasts (Enoch et al., 2010, Enoch et al., 2009), and it was hypothesised that the wound healing characteristics may be due to the presence of a progenitor/stem cell population within the lamina propria of the oral mucosa (Stephens and Genever, 2007).

1.4 Embryonic Stem Cells

Stem cells can be classified broadly into two groups, embryonic and adult. Embryonic stem (ES) cells are totipotent cells derived from the inner cell mass of a blastocyst, and are able to differentiate into cell lineages derived from the ectoderm, mesoderm and endoderm (Keller, 1995). ES cells have rapid growth rates, with cell division occurring every 36-48 hours, and can be cultured for prolonged lengths of time, up to 2 years (Fortier, 2005). This requires the correct conditions; either exogenous growth factors must be added to the culture media or the ES cells must be cultured on a feeder cell population to supply the necessary growth factors (Lee et al., 2005). ES cells have high levels of telomerase expression, which allows sustained renewal without compromising the chromosomal stability.

1.4.1 Embryonic Stem Cell Discovery

Embryonic stem cells were first isolated from early mouse embryos after the discovery that injection of these early embryos into histocompatible mice caused the formation of teratocarcinomas in the host (Stevens, 1970). These cells were discovered to have morphological and immunological properties in common with pluripotent embryonic cells, which had been studied extensively to understand embryonic development. In the 1980's the establishment of stable cell cultures from mouse embryos allowed investigation into all the mechanisms involved in embryonic differentiation and capacity for self renewal by the use of mitotically inactive embryonic

fibroblasts, known as “feeder cells” (Notarianni et al., 1990, Robertson et al., 1986, Kaufman et al., 1983, Evans and Kaufman, 1981). It was discovered that even after extended propagation *in vitro*, cells were still able to re-enter embryogenesis when injected into pre-implantation mouse embryos, giving rise to chimaeric mice (Bradley et al., 1984). Importantly, ESCs maintain a diploid karyotype, important in a cell capable of unrestricted self renewal.

Embryonic stem cells are described as totipotent, due to their ability to give rise to many cell types including germ cells however they cannot produce trophoectoderm due to the nature of ES cells, derived from the developing blastocyst (Smith, 2001). ES cells can be routinely expanded whilst remaining undifferentiated and large populations of cells (10^9-10^{10}) can be generated rapidly. However this required the necessary nutrients provided from a feeder cell population until the discovery that a single cytokine, LIF could sustain mouse ES cell self-renewal in the absence of feeder cells (Smith et al., 1988, Williams et al., 1988). Other factors were subsequently discovered to be essential for pluripotency, such as Oct-3/4 (Niwa, 2001).

1.4.2 Differentiation Potential of Human ES Cells

As previously mentioned, ES cells are isolated from the blastocyst of developing embryos, and as well as those discovered in mice, have also been characterised in humans (Thomson et al., 1998). The differentiation of ES cells typically allows the formation of free floating “embryonic bodies” in culture before a variety of differentiated cells are detected. In this manner

neurons, skeletal muscle and cardiomyocytes, liver and islet cells, capable of producing insulin, have been reported among many other cell types (Assady et al., 2001, Kaufman et al., 2001, Kehat et al., 2001, Itskovitz-Eldor et al., 2000, Odorico et al., 2001).

1.5 Adult Stem Cell Populations

Adult stem cells can be found in tissues of ectodermal, mesodermal and endodermal origin. These adult stem cells can be separated into two broad groups; the hematopoietic (HSC) and mesenchymal stem cells (MSC). HSCs are of mesodermal origin, and produce the cell types of the hematolymphatic system. More recently it has been observed that they can generate cell types from outside of this lineage (Fortier, 2005, Bellantuono, 2004).

1.5.1 Bone Marrow Stromal Cells

Mesenchymal stem cells (MSCs), also known as bone marrow stromal cells (BMSCs) or mesenchymal progenitor cells (MPCs) are derived from the mesoderm, and are the progenitors of all connective tissue cells such as fat, bone, tendon, muscle and cartilage. These cells have a fibroblastic morphology in culture and are capable of differentiating into multiple cell types, including those mentioned above. MSCs were first isolated from the bone marrow 40 years ago and where originally referred to as colony forming unit fibroblasts (Castro-Malaspina et al., 1984, Castro-Malaspina et al., 1982, Castro-Malaspina et al., 1980, Friedenstein et al., 1970). BMSCs represent an interesting target for cellular therapies due to their proliferation and differentiation characteristics (Johnstone et al., 1998). Bone marrow is a complex tissue comprising haematopoietic stem cells and their progeny and a connective tissue network, the stroma (Bruder et al., 1997). This stroma is comprised of adipocytes, reticulocytes, endothelial cells and fibroblastic cells,

and it has been noted that the adherent cells derived from bone marrow cultures are composed of different cell populations. The heterogeneous nature of BMSC cultures has led to the discovery that individual samples from within a culture of BMSCs can exhibit dramatically different expression of genes by microarray analysis (Xiao et al., 2010).

Single cell colonies of BMSC have been demonstrated to achieve 50 population doublings and are capable of differentiation into osteoblasts, adipocytes, chondrocytes, myocytes, astrocytes, oligodendrocytes and neurons (Kuznetsov et al., 1997b, Kuznetsov et al., 1997a, Aslan et al., 2006, Bosnakovski et al., 2004, Bosnakovski et al., 2006, Deliloglu-Gurhan et al., 2006, in 't Anker et al., 2003, Johnstone et al., 1998, Jones et al., 2002, Lecanda et al., 1997, Long et al., 1995, Majumdar et al., 2000, Mareddy et al., 2007) (Friedenstein et al., 1970, Bruder et al., 1997). This clonal culture system allows for the characterisation of a marrow stromal cell population in more detail. It has been determined that basic fibroblast growth factor (FGF-2) is a potent mitogen implicated in the maintenance of BMSC proliferation and osteogenic potential, able to extend the replicative potential of cultured cells to more than 70 population doublings (Yanada et al., 2006, Martin et al., 1997, Xiao et al., 2010).

1.5.2 Haematopoietic Stem Cells

Experiments on mice in 1940 showed that shielding the spleen during total body irradiation allowed the animal to survive previously lethal doses of radiation (Jansen et al., 2005). The discovery that haemopoietic stem cells (HSC's) were responsible for the animals' recovery then followed. HSC's are the progenitor cells of all lymphoid and myeloid cells within the blood and originate from the bone marrow (Janeway C.A, 2001). They have been categorised by the presence of CD34 on their surface, however this marker is not exclusive to stem cells and is also present on some mature lymphoid cells (Watt and Contreras, 2005). CD34 is a ligand for Leukocyte Adhesion Molecules (LAM) and helps with the adhesion of leukocytes to the endothelium. Shortly after HSC's discovery various research groups began working on the safe collection of these cells so they could be infused into patients undergoing chemo- and radiotherapy as a treatment.

Un-stimulated peripheral blood contains fewer HSC's naturally than in an equivalent volume of bone marrow (Jansen, J. et al, 2005) but the use of granulocyte colony stimulating factor (G-CSF) as a HSC liberating factor has increased stem cell yield so it is possible for PBSC's to be used in HSCT's (Basak et al., 2011, Grigg et al., 1995).

1.5.3 Adipose Derived Stem Cells

Human adipose tissue has been identified as a source of adult stem cells similar in phenotype and differentiation potential to BMSC, with some

differential expression of cell surface markers implicated in haematopoietic stem cell mobilisation (Zuk et al., 2002, Gabbay et al., 2006, Gimble and Guilak, 2003a, Gimble and Guilak, 2003b, Katz et al., 2005, Kern et al., 2006, Lee et al., 2004, De Ugarte et al., 2003). These cells are a heterogeneous population similar to BMSCs and successful clonal expansion from single progenitor cells has demonstrated differentiation along the standard lineages, including neurogenic differentiation (Guilak et al., 2006). It has also been observed that adipose derived stem cells are capable of forming insulin producing cells (Kim et al., 2010). The ease of access to obtain adipose-derived stem cells, from lipoaspirates, has generated interest in these cells as an alternative to BMSC for tissue regeneration and repair.

1.5.4 Skin Stem Cells

Within the skin, a tissue structurally and functionally similar to the oral mucosa, a number of stem cell populations have been identified, residing in discrete compartments of the tissue.

1.5.4.1 Epidermal Stem Cells

The epidermis, which is a highly proliferative layer of constant turnover has been shown to contain a stem cell population responsible for this high rate of self renewal (Jones and Watt, 1993). Recently it has been discerned that these epidermal stem cells can trans-differentiate into corneal epithelium-like cells (Gao et al., 2007).

1.5.4.2 Hair Follicle Stem Cells

The hair follicle has been shown to contain a stem cell population capable of differentiating into adipogenic and osteogenic lineages *in vitro* (Jahoda et al., 2003, Gharzi et al., 2003). More recently, evidence that these cells are capable of neuronal and glial potential has been demonstrated (Hunt et al., 2008).

1.5.4.3 Dermal Multipotent Stem Cells

Skin derived precursors (SKP's), cells isolated from the dermis of juvenile and adult rodents have been shown to differentiate into neurons, glia, smooth muscle cells and adipocytes (Toma et al., 2001, Fernandes et al., 2004). These cells were also identified in the human dermis of neonatal foreskin. These human SKPs have been maintained for long periods of time in culture, and are able to differentiate into neurons, glia, and smooth muscle cells, including cells with the phenotype of peripheral neurons and Schwann cells (Toma et al., 2005). More recently, these SKPs have been differentiated into functional melanocytes (Li et al., 2010). Dermal multipotent cells have also been shown to speed up skin wound healing and haematopoietic recovery in sub-lethally irradiated rats (Chunmeng et al., 2004)

1.5.6 Other Adult Stem cell populations

A number of other adult stem cell populations have been identified to date, in a variety of tissues and sites throughout the body. The renal papilla has been postulated to contain a kidney stem cell niche capable of deriving cells expressing neuronal markers and cells of uncharacterised phenotype (Oliver et al., 2004). Articular cartilage also contains a progenitor cell population capable of mesenchymal differentiation *in vitro* (Alsalameh et al., 2004, Dowthwaite et al., 2004, Fickert et al., 2004, Hiraoka et al., 2006, Williams et al., 2010).

1.6 Stem Cell Isolation Methods

The isolation of stem cells from adult tissues has lead to a number of techniques for the isolation of stem cells from the heterogeneous cell populations in which they reside.

1.6.1 Differential Adherence Properties

The adherence characteristics of stem cell populations can be exploited to isolate them from a heterogeneous cell population. In the case of muscle derived stem cells and blood derived MSC, this can be through their preferential adherence to tissue culture plastic (Sinanan et al., 2004, Wagner et al., 2005) or through differential adherence to extracellular matrix components such as collagen or fibronectin (Jones and Watt, 1993, Dowthwaite et al., 2004). The differential adhesion characteristic of stem cells allows for clonogenic expansion of single cells giving rise to a pure cell population, and in terms of fibronectin adherence can be explained by high levels of functional α5β1 integrin, the ‘classical’ fibronectin receptor (DeSimone et al., 1992)

1.6.2 Magnetic Separation/Fluorescence Activated Cell Sorting (FACS)

Markers present on the surface of stem cell populations can also be used to isolate these cells from a heterogeneous environment through magnetic separation, or through the detection of fluorescence conjugated antibodies to

markers of interest. Cardiac stem cells have been isolated using magnetic separation, staining with an anti stem cell antigen (sca-1) antibody conjugated to a magnetic microparticle allows for the enrichment of this cell population when cells are passed through a magnetic field (Oh et al., 2003). Similarly FACS has been used in Dental Pulp stem cells to isolate small cell populations using STRO-1 (Shi and Gronthos, 2003).

1.6.3 Hoechst Exclusion

Another method of isolating stem/progenitor cell populations involved the use of a fluorescent dye, Hoechst 33342 which is excluded by stem cells and provides a means of identification during FACS. This characteristic is due to the expression of ABCG2, a membrane transporter capable of rapid exclusion of the dye and resulting in a population with no fluorescence (Honda et al., 2007, Tsai et al., 2010).

As well as the isolation methods demonstrated above to physically acquire stem cell populations; a lot of research has been conducted into the phenotypic characteristics which differentiate stem cells from differentiated cells.

1.7 Stem Cell Characteristics used in Identification of Stem Cell Populations from Adult Tissues

1.7.1 Telomerase Activity and Replicative Senescence

In humans, individual cells can only undergo a predefined number of divisions. This phenomenon is seen in most somatic cells, and is referred to as Hayflick's limit (Hayflick, 1965). If cells are allowed to undergo unlimited replication, then over time mutations accumulated through erroneous DNA replication would cause damage to vital genetic information and could lead to carcinogenesis, by the inactivation of cell cycle checkpoints such as p21, and tumour suppressor genes, such as retinoblastoma (Rb) or p53. Limiting the number of population doublings a cell can achieve is a method of protecting against the development of cancer.

When cells have reached this defined number of population doublings they enter cell cycle arrest, and no longer divide. This is known as senescence, and a number of mechanisms can cause this (Fig 1.5). Replicative, or telomere-dependent senescence is caused by the dysregulation or shortening of telomeres, and will be the focus of this section

1.7.1.1 Telomeres

Telomeres are regions of repetitive, non-coding double stranded DNA found at the end of eukaryotic chromosomes. Human telomeres consist of 6 base-pair repeats of the sequence 5'- TTAGGG -3', with a 3' single stranded DNA overhang. Human telomeres are generally about 10,000 nucleotides in length, and capped with double-stranded and single-stranded DNA binding proteins. The overhanging strand of DNA folds back into the double stranded region of the telomere, forming a "T-loop" (Watson, 2003). This loop structure is designed to prevent the recognition of the telomere as a chromosomal break, which triggers cellular repair mechanisms involved in the maintenance of chromosomal integrity. Telomeres help to slow the end replication problem encountered during DNA synthesis (Shay and Wright, 2007).

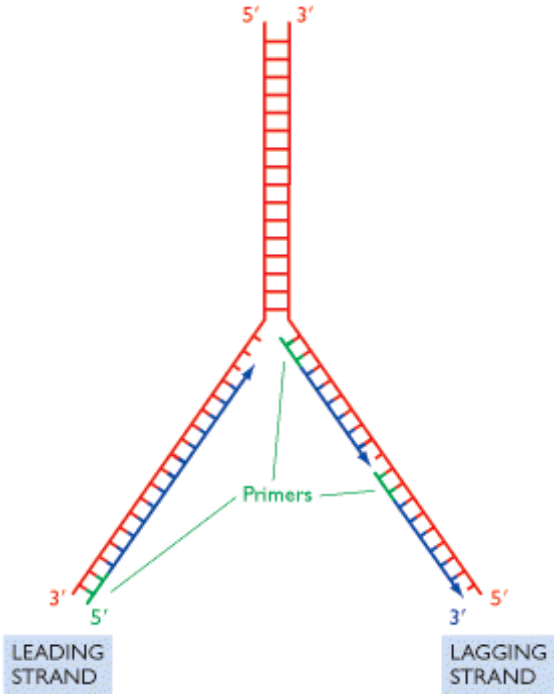

Fig 1.5: The end replication problem. Lagging strand synthesis involves the use of primers in a discontinuous manner, which poses a problem when the end of the lagging strand is reached (Brown, 2002). .

DNA polymerase only synthesises new DNA in a 5' to 3' orientation. This means that on the lagging strand of DNA synthesis occurs discontinuously, using RNA primers to facilitate the function of DNA polymerases (Fig.1.5). When the replication reaches the end of this lagging strand, an RNA primer cannot be inserted at the tip of the DNA molecule, meaning that with every replication a portion of genetic information is lost. Telomeres lose between 50-100 base-pairs with every cell division, and this loss acts as a record of the number of times a cell has undergone division (Alberts, 2002). When the telomere becomes too short then the cell cycle is arrested, to prevent the loss of critical genetic information contained in genes found near the ends of the chromosome, such as tumour suppressor genes of cell checkpoint genes. Loss of these genes can lead to a number of pathological processes such as cancers.

There are many diseases in which telomere shortening has been implicated (Wiemann et al., 2002, Plentz et al., 2004, Meeker et al., 2004, Benetos et al., 2004, Brouilette et al., 2003). In most human somatic cells, this degradation of telomeric DNA over numerous replications triggers replicative senescence. In highly proliferative somatic cells and germ line cells however, telomere lengths are maintained by a specialised reverse transcriptase known as telomerase (Wright et al., 1996).

1.7.1.2 Telomerase

The reverse transcriptase enzyme known as telomerase is responsible for the maintenance of telomere length, and is highly conserved in all eukaryotes (Autexier and Lue, 2006). It has been shown that in normal human fibroblasts, the reconstitution of telomerase activity by retroviral transfection can extend their replicative potential and extend the length of the telomeres (Vaziri and Benchimol, 1998) Telomerase is composed of two subunits, a catalytic subunit known as human telomerase reverse transcriptase (hTERT) in humans (Nakamura et al., 1997) and an RNA template known as telomerase RNA component (TERC) (Feng et al., 1995).

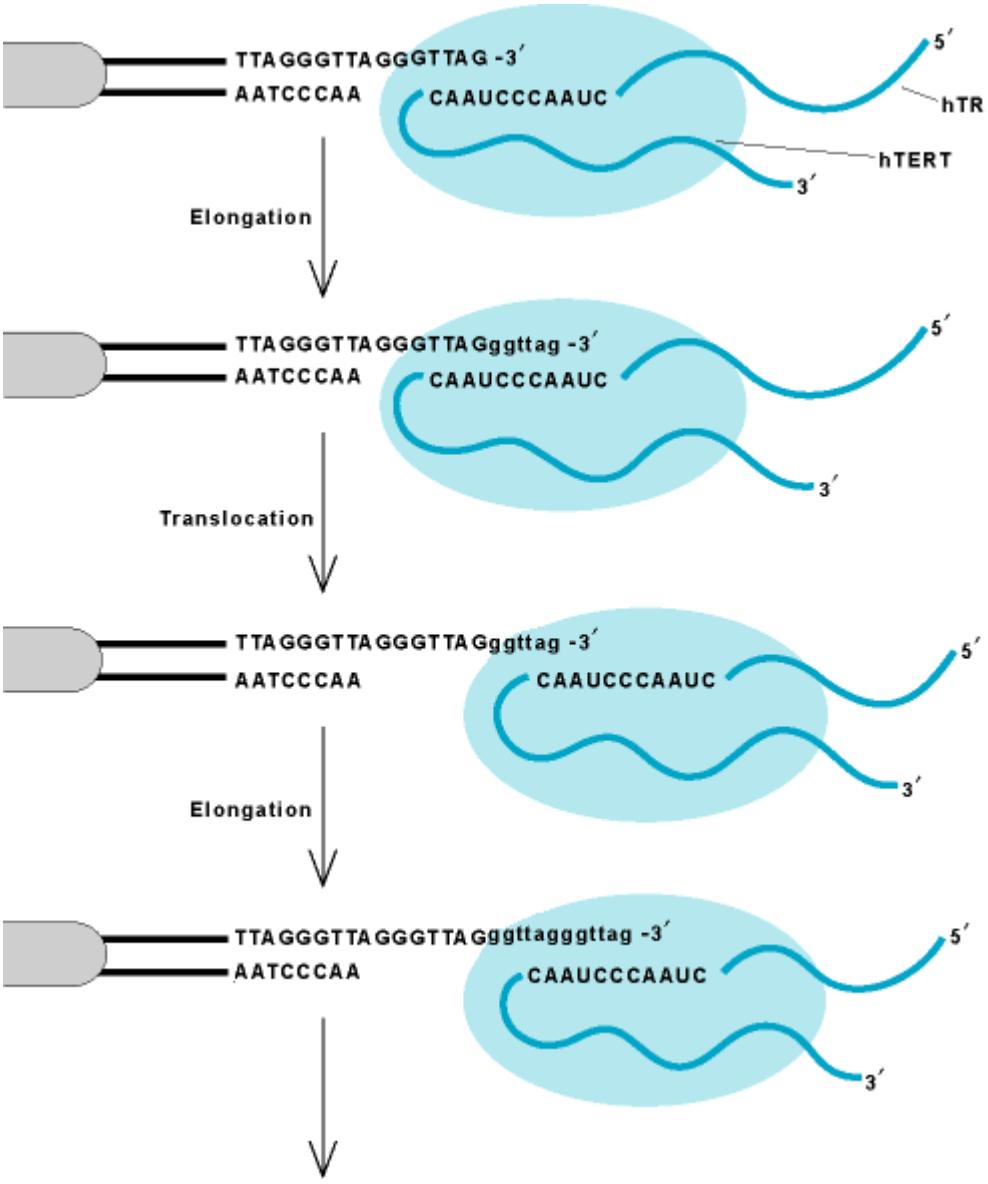

Fig 1.6: A schematic representing the elongation of telomeric DNA by the enzymatic activity of telomerase. (Image taken from http://accessscience.com/content/Cell-senescence-and-aging/YB000230)

The TERC subunit component contains 1.5 copies of the complement to the telomere sequence (5'-UAACCCUAA-3'), which anneals to the single stranded DNA at the 3' end of the telomere (Fig 1.6a) (Alberts, 2002). As well as this the telomerase binds to the DNA substrate and then the TERT reverse transcriptase activity sequentially adds new nucleotides to the DNA substrate until the end of the RNA template is reached (Fig 1.6b). At this point telomerase detaches and translocates and re-anneals to the end of the telomere and repeats the reverse transcription reaction (Fig 1.6d). This cycle is repeated a variable number of times, and leads to multiple copies of the 6 base-pair repeat being added to the DNA, and the maintenance of telomere length.

1.7.1.3 Telomerase in Stem Cells

Telomerase activity in ES is well characterised. It is involved in embryonic development and has been detected in cells of the inner cell mass of human blastocysts (Heins et al., 2004, Thomson et al., 1998). Embryonic stem cells and Embryonal Carcinoma cells demonstrate high levels of telomerase activity and hTERT expression, and during differentiation these are rapidly downregulated, suggesting that telomerase activity is a marker of undifferentiated stem cells (Armstrong et al., 2005).

The subject of adult stem cells and telomerase activity is at present unclear, with reports of telomerase activity in adult stem cells reported by groups being contradicted by the findings of others. Haematopoietic stem cells are

shown to possess telomerase activity (Hiyama et al., 1995, Morrison et al., 1996, Chiu et al., 1996). This activity is retained in some of the differentiated progeny. However, no telomerase activity has yet been found in MSCs from adult tissues and this lack of telomerase potential is believed to contribute to the limited replicative potential of MSC (Fehrer and Lepperdinger, 2005, Zimmermann et al., 2003). However, the addition of bFGF has been demonstrated to increase the replicative potential of MSC to over 100 population doublings, without the upregulation of telomerase (Yanada et al., 2006). Telomerase activity has been observed in human neural precursor cells, hepatic stem cells and cardiac stem cells (Ostenfeld et al., 2000, Schmelzer and Reid, 2009).

1.7.2 Cell Surface Marker Expression

In the process of isolating stem cells from adult tissues, a vast array of techniques are employed, and many groups have chosen to use flow cytometry to characterise these stem cells by examining a series of both cell surface and intracellular markers. This has led to a comprehensive list of markers which have been used as confirmation of mesenchymal stem cells within the subject tissue, which is summarised in table 1.3.

Table 1.3: Cell surface markers used in previous studies to identify stem cell populations residing within a variety of tissues

Tissue of origin	**Positive markers**	**Negative markers**	**Reference**
Periodontal Ligament	CD105, CD166, STRO-1	N/A	(Nagatomo et al., 2006)
Human trabecular bone	STRO-1, CD73, CD105	CD34, CD45, CD144	(Song et al., 2005, Tuli et al., 2003)
Osteoarthritic Cartilage	CD9, CD44, CD54, CD90, CD166	N/A	(Fickert et al., 2003)
Bone marrow	CD44, CD90, CD105, CD166, HLA-ABC	CD45, AC133 and HLA-DR	(Pasquinelli et al., 2007)
Osteoarthritic bone marrow	CD29, CD44, CD90, CD105, CD166	N/A	(Mareddy et al., 2007)
Adipose	CD34, CD90, CD105 CD166	CD31, CD45	(Oedayrajsingh-Varma et al., 2006)
Dental papilla	CD29, CD44, CD90, CD105, CD166	N/A	(Ikeda et al., 2006)
Bronchi	CD73, CD90, CD105, CD166, STRO-1	CD34, CD45	(Sabatini et al., 2005)
Articular cartilage	CD10, CD90, CD105, CD166	N/A	(Diaz-Romero et al., 2005)

1.7.2.1 Markers used for current stem cell identification

For the purpose of this investigation, the three markers CD90, CD105 and CD166 were chosen because of the frequency with which these markers appear in research into stem cells in other tissues, the intensity with which they are expressed and the wide ranging tissue in which they have been used for the identification of stem cells.

1.7.2.2 Structure and Function of selected cell surface markers

1.7.2.2.1 CD90: Thy-1

Thy-1, otherwise known as CD90, is a 25-37 kDa glycosylphosphoinositol (GPI)-anchored protein found on the external leaflet of the lipid bilayer. The gene for this protein is located on chromosome 11q22.3, and is expressed on human fibroblasts, neurons, blood stem cells and endothelial cells (Rege and Hagood, 2006a). Thy-1 has associations with the formation of focal adhesions, via modulation of p190 Rho GTPase-activating proteins, which results in RhoA activation (Barker et al., 2004). Thy-1 is also implicated in wound healing and fibrosis, with upregulation seen 3-6 days after injury (Saalbach et al., 1996). Interactions with a number of integrins has also been observed in endothelial cells and neurons (Rege and Hagood, 2006b). Thy-1 can act as both a receptor and a ligand dependent on the cell interactions involved.

1.7.2.2.2 CD105: Endoglin

CD105 is a disulphide-linked homodimeric cell membrane glycoprotein of 180kDa, which can exist as one of two isoforms, L and S (Gougos and Letarte, 1988). The gene for CD105 is located on chromosome 9q34, and arranged into 14 exons (Fernandez-Ruiz et al., 1993, McAllister et al., 1995). The L-CD105 variant is found predominantly on the surface of endothelial cells. Endoglin has been shown to be a receptor involved in transforming growth factor (TGF) β signalling, binding to TGF-β1 and TGFβ-3 with a high affinity resulting in down-regulation of phosphorylation, similar to the effect seen in the presence of protein kinase C inhibitors (Cheifetz et al., 1992, Lastres et al., 1994). Endoglin also has associations with angiogenesis with respect to tumour metastasis in a wide range of tumour endothelia (Duff et al., 2003). Evidence of the crucial involvement of Endoglin in angiogenesis can be seen in knockout mice, which have numerous cardiac and vascular defects (Li et al., 1999). It also binds growth factors such as bone morphogenetic proteins (BMPs) (Duff et al., 2003).

1.7.2.2.3 CD166: Activated leukocyte cell adhesion molecule

Activated leukocyte cell adhesion molecule (ALCAM) is a type I transmembrane protein and a member of the immunoglobulin superfamily (IgSF). It is a cell adhesion molecule comprised of five extracellular Ig domains, a single transmembrane domain and a short cytoplasmic tail (Masedunskas et al., 2006, Arai et al., 2002). First identified in Thymic cells and activated leukocytes it also displays significant sequence similarity to a

subset of the IgSF involved in neural adhesion (Bowen et al., 2000, Arai et al., 2002). ALCAM is a ligand of CD6, a scavenger receptor cysteine-rich (SRCR) protein belonging to the SRCR protein superfamily (Resnick et al., 1994). It is involved in the transendothelial migration of monocytes. ALCAM has also been implicated in invasion of endothelial cells into cartilage (Arai et al., 2002), as well as the implantation of blastocysts, neurite outgrowth and the invasion of melanoma cells.

1.8 Stem Cells from the Oral Cavity

Stem cells, as mentioned previously, have been isolated from a number of adult tissues. For the purpose of this thesis, this section will focus on the presence of stem cell populations in oral tissues.

1.8.1 Dental Pulp Stem Cells

Upon injury, reparative dentin is formed as a protective barrier for the pulp, suggesting that the dental pulp contains a population of progenitors to aid in this healing response (Mao et al., 2006). A stem cell population has been demonstrated in the dental pulp of impacted third molars from humans aged from 19-29 years of age (Gronthos et al., 2000). These cells were phenotypically similar to BMSCs with a higher colony forming efficiency, but exhibited a reduced capability for osteogenic differentiation *in vitro*, and a lack of adipogenic potential. When transplanted into immunocompromised mice, these cells were capable of forming a dentin like structure lined with human odontoblasts-like cells, and these cells surrounded a pulp-like tissue. In other experiments, the transplantation of dental pulp stem cells into immunocompromised mice demonstrated the ability to form bone (Otaki et al., 2007b). Since that initial discovery much work has been conducted to elucidate the full differentiation potential, with groups demonstrating successful adipogenesis and the generation of neural-like cells (Gronthos et al., 2002, Arthur et al., 2008). Dental Pulp Stem Cells (DPSCs) have now been isolated from permanent and deciduous teeth (Suchanek et al., 2007),

and culture conditions have been optimised for increased proliferative activity using additional supplements and low serum concentrations (Karbanova et al., 2010, Suchanek et al., 2009). An epithelial stem-cell like cell population has recently been discovered, within human deciduous dental pulp which may play a role as an epithelial component of repair and regeneration in teeth (Nam and Lee, 2009). Investigations into telomere erosion suggest that similar to MSCs, DPSCs do not express active telomerase (Mokry et al., 2010).

1.8.2 Periodontal Ligament Stem Cells

The periodontal ligament (PDL) connects the cementum to alveolar bone, and functions primarily to support the tooth in the alveolar socket (Mao et al., 2006).

Several groups have reported the isolation of multipotent periodontal ligament stem cells (PDLSCs). The expression of STRO-1 has been used to select for these stem cells, with evidence that these stem cells are comparable to BMSCs in terms of their differentiation potential along the common lineages (Gay et al., 2007). These cells have been successfully isolated from cryopreserved periodontal ligament. The preserved cells retained the ability to generate cementum/periodontal ligament tissues, as well as single cell colony generation and adipogenic, chondrogenic and osteogenic differentiation ability (Seo et al., 2004). The presence of embryonic pluripotency markers Oct-4, Nanog, SSEA-1 and SSEA-4 have suggested that PDLSCs are less differentiated than BMSCs.

1.8.3 Oral Keratinocyte Stem Cells

Within the Oral mucosa, a stem cell population has been identified and characterised. Using the expression of α6β4 integrin and CD71 by magnetic separation, three cell populations were isolated from keratinized oral mucosa, and the $\alpha6\beta4^{positive}$ $CD71^{negative}$ population where demonstrated to express Oct-4 and CD44 and where able to reconstitute an epithelial equivalent *in vitro* (Calenic et al., 2010). Another group used the high expression of p75 to isolate keratinocyte stem cells, whilst the presence of β1 integrin was used to characterise sorted oral keratinocytes by another group (Izumi et al., 2007, Nakamura et al., 2007).

1.8.4 Hard Palate Stem Cells

A stem cell population has been successfully isolated from adult palatum of Wistar rats. These cells were positive for Klf-4, Oct 4 and c-Myc, factors involved in the pluripotency of stem cells (Widera et al., 2009).

1.8.5 Oral Mucosa Lamina Propria Stem Cells

Within the context of oral stem cells, and of particular interest to this thesis is the recent discovery of multipotent stem cell populations within the lamina propria of the oral mucosa. These cells have been demonstrated to achieve higher numbers of population doublings compared to mesenchymal stem cells and other adult stem cell populations, are phenotypically similar in terms

of cell surface marker expression, and capable of differentiation along adipogenic, osteogenic, chondrogenic and neural lineages (Davies et al., 2010, Marynka-Kalmani et al., 2010). These cells express neural crest markers, as well as three of the four markers required for induced pluripotency (Nakagawa et al., 2008, Takahashi et al., 2007a, Takahashi et al., 2007b, Takahashi and Yamanaka, 2006).

1.8 Ethical Considerations of Embryonic Stem Cell Research

Whilst the potential of embryonic stem cells is clear, the ethical implications of use therapeutically remain restrictive to their clinical use, due to the nature of ESC isolation. This involves the destruction of an embryo, and in the case of human ES cells, this is deemed by many as an insurmountable problem. Adult stem cell populations are seen as an alternative that do not violate any ethical concerns, however limitation in replicative potential and lineage capability are still restricting the full potential of these cells for therapeutic benefit. The isolation of a pluripotent stem cell population with the benefits of embryonic stem cells would be of great scientific and therapeutic interest and would settle the debate over the use of embryonic stem cells for clinical uses.

1.9 Aims of this thesis

The overall aim of this thesis was to compare cell populations from the lamina propria of the oral mucosa, isolated by different means, in an attempt

to describe the preferential properties of a progenitor cell population within this tissue. In so doing the aim was to study these distinct cell populations with respect to their growth kinetics and cellular morphology throughout their proliferative lifespan until they reached senescence and thereby assess any cell-specific age related changes. Furthermore, to determine the expression of well known stem cell markers such as CD90, CD105, CD166 and active telomerase within these cell populations and relate this to *in vitro* ageing through a potential link to telomere length. Finally, to compare the potential of these populations to differentiate down classical cell lineages, namely undergo osteogenesis, chondrogenesis and adipogenesis.

It was hypothesized that:

- Clonally derived oral mucosal lamina propria cells, isolated by adherence to fibronectin, would exhibit a distinct cell phenotype when compared to whole cell populations (LO) or the residual cells that had not adhered to fibronectin (NA).
- The oral mucosal lamina propria clones would demonstrate stem/progenitor cell characteristics.
- Clonal populations would demonstrate a more stem/progenitor cell-like phenotype compared to the more heterogeneous LO and NA cell populations, as exhibited by their stem cell markers and superior differentiation capability *in vitro.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

All cell culture described in this thesis was carried out in sterile working conditions within an Astec Microflow 2 Cabinet. All flasks and plates were maintained within a Nuaire air-jacketed DH autoflow automatic CO_2 incubator at 37°C at 5% CO_2. All reagents used were purchased from Invitrogen, UK unless otherwise stated in the protocol

2.1.1 Isolation of cells from the lamina propria of the oral mucosa

Buccal mucosa tissue samples were collected from consenting patients undergoing routine dental procedures. Samples were sterilised by immersion in 70% ethanol for 40 seconds then dissected into 3mm long cubes and digested in culture media [Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS), 2mM Glutamine, antibiotics and antimycotics [(100 units ml^{-1} Penicillin G, 100µg ml^{-1} Streptomycin sulphate, 0.25µg ml^{-1} Amphotericin B)] containing 2mg ml^{-1} Pronase (Sigma®, UK) overnight at 4°C. Subsequent to pronase digestion the epithelium was removed using forceps, and the lamina propria minced and incubated in culture media containing 1mg ml^{-1} collagenase A (Sigma®, UK).

2.1.2 Isolation of progenitor cells from the oral mucosa using the differential adhesion assay

Progenitor cells were isolated from the lamina propria using the differential adhesion assay (Dowthwaite et al., 2004, Jones and Watt, 1993). The differential adhesion assay is based on the principle that progenitor cells express a higher level of functional β1 integrin receptors on their surface, which will allow adherence to fibronectin in a reduced timeframe compared to differentiated cells (Jones and Watt, 1993).

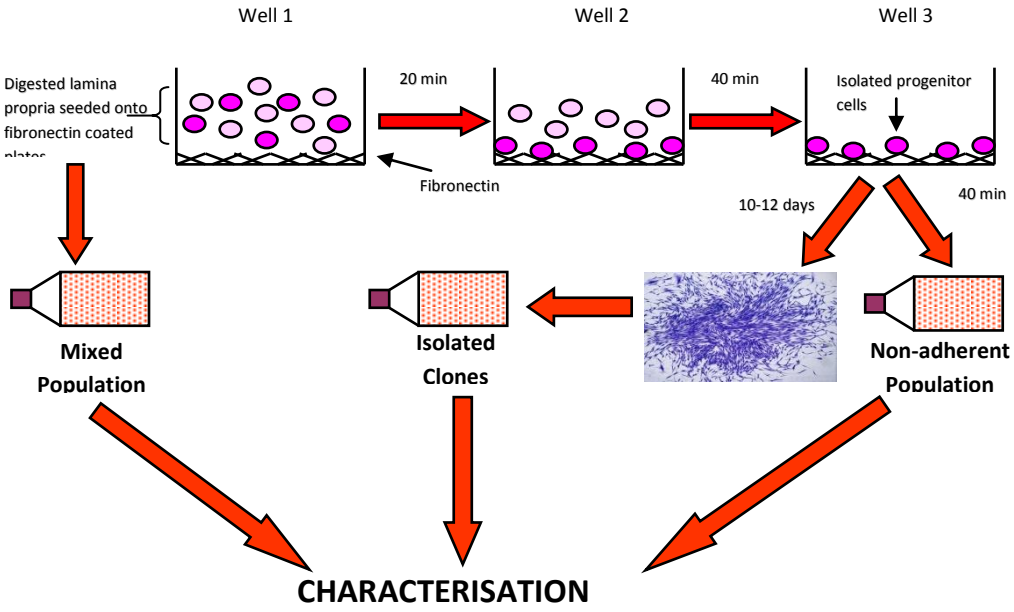

Fig 2.1 Schematic of the differential adhesion assay highlight the point in the protocol at which each cell population is isolated.

Six-well plates were coated with 10µg ml^{-1} bovine fibronectin (FN; Sigma, UK) in phosphate buffered saline (PBS pH 7.4) containing 1mM $MgCl_2$ and 1mM $CaCl_2$ (PBS+) overnight at 4°C. Control plates were treated with PBS+ containing 1% bovine serum albumin (BSA; Sigma®, UK). FN and BSA were aspirated before the addition of the cells, and the protocol was followed as demonstrated in Fig 2.1.

Isolated cell suspensions were adjusted to $8x10^4$ cells ml^{-1} in serum free media [DMEM media supplemented with 2mM glutamine and antibiotics/antimycotics (100 units ml^{-1} Penicillin G, 100µg ml^{-1} Streptomycin sulphate, 0.25µg ml^{-1} Amphotericin B)] seeded into a well and incubated at 37°C for 20 minutes (well 1) (Fig. 8). Media (and unattached cells) were removed and seeded into the next well for 40 minutes at 37°C (well 2). This was repeated and unattached cells were added to the final well for a further 40 minutes (well 3). Unattached cells removed after this final time point were centrifuged at 1500 RPM [Precision Durafuge 200, Roche, France] for 5 minutes. The pellet was resuspended in 1ml of culture media and the cells counted. These cells [from this point will be termed as the non-adherent population] were then seeded into the well of a separate uncoated 6-well plate and a further 1ml culture media added. After removal from 20 minute and both 40 minute time points (n=3), media was replaced with 2ml of culture media.

The remaining cells not used in the assay were also centrifuged (as above) and seeded into a well of a separate uncoated 6 well plate and termed the

"mixed" population, which reflects both the progenitor and fibroblast populations resident within the lamina propria.

2.1.3 Colony forming efficiency

To evaluate the colony forming efficiency (CFE) of this progenitor cell population, the initial number of adherent cells was counted in all 3 wells after 24 hours. On days 5, 9 and 14 the number of colonies visible exceeding both 4 cells and 32 cells were individually recorded. The CFEs were calculated using the formula:

$$\text{CFE} = \frac{\text{No. of colonies formed}}{\text{No. of adherent cells}}$$

2.1.4 Isolation of colonies

At day 14 individual colonies in excess of 32 cells originating from a single cell were isolated and removed from well 1 using trypsin [0.25% with EDTA] digestion. Media was aspirated from the plates and the cells washed with sterile PBS. After removing the PBS, cloning rings were placed over the colonies selected for study. Trypsin (200µl) was added to the cloning ring, incubated for 5 minutes at 37°C and removed and placed into a falcon centrifuge tube with an equal volume of culture media. Then culture media was added to cloning ring to remove any cells which had not been removed previously. The cell suspension was then centrifuged at 1500 RPM for 5 minutes. The supernatant was then aspirated and the pellet resuspended and seeded into a well of a 12 well plate and grown until confluent, and

passaged until senescence. At every passage cell numbers were recorded for both seeding and splitting of flasks to determine population doublings.

2.1.5 Cryopreservation and retrieval of cells

Cryopreservation was conducted at every passage to ensure adequate stocks were available for subsequent experiments. For cryopreservation, cells were resuspended in a solution of 10% (v/v) dimethyl sulfoxide (DMSO – Sigma®, UK), and 90% FBS. This cell suspension was placed into cryogenic vials (Greiner,UK) and stored at -80°C in an isopropanol cryopreservation container. After 24 hours cells were retrieved from this container and placed into long term liquid nitrogen storage. Frozen cells were retrieved from storage by rapid thawing at 37°C, washed in DMEM+10% FBS and following centrifugation (1500 RPM, 5 minutes) were resuspended in DMEM+10% FBS for cell counting.

2.1.6 Calculation of Population Doubling Levels

To allow comparisons between cell populations in terms of cellular proliferation, cumulative population doublings were plotted against time in culture. At every passage cells were counted, using Trypan blue exclusion to determine the number of viable cells, on a Neubauer haemocytometer (Sigma®, UK). The number of cells reseeded into a fresh flask was also recorded. Population doublings were then calculated using the formula

$PDL = [\log_{10}(\text{total cell number at passage}) - \log_{10}(\text{reseeded cell count})] / \log_{10}(2)$

Cells were considered to be senescent when their population doublings/week fell below 0.5 for three consecutive passages, at which point cells were cryopreserved awaiting subsequent analysis.

2.1.7 Routine testing for Mycoplasma contamination of cell cultures

Cell lines were routinely screened for presence of Mycoplasma contamination, every 2-4 passages. Cells were cultured overnight at $1x10^4$ cells per well in a 6 well plate containing 25mmx25mm coverslips, then washed with 1mL of PBS. Hoechst 33258 stain (bizbenzimide; Aventis, Germany) was applied to the coverslips at 1µg/ml in methanol (Sigma®, UK), and incubated for 15 minutes. After this time, the coverslips were removed from the plate wells using forceps, dabbed on tissue to ensure excess moisture was removed, and mounted on microscope slides for fluorescence imaging. Images were acquired on an Olympus PROVIS AX470 (TRF) Microscope using a Nikon DXM1200 Digital Camera and Nikon ACT-1 software. The images were reviewed in Adobe Photoshop Elements.

2.1.8 Morphological Analysis

Images of cells were taken at every passage, 24 hours after seeding (x10 and x20 magnification, F3.5, Nikon Coolpix 4500 Camera). Images taken at x10 magnification were then subjected to morphology analysis using the Java based software ImageJ (Version 1.37, obtained from the National Institutes of Health website http://rsb.info.nih.gov/ij/, USA. Programmer: Wayne Rasband). The scale was set using a haemocytometer image at x10 magnification, and cell area was traced and recorded (n=50). Average cell areas were then plotted for early and late passages. These timepoints were determined by the individual cell population, with early passages representing around 10 population doublings, and late timepoints determined by the total population doublings of each individual cell population.

2.2 Stem Cell Phenotypic Analysis by Flow Cytometry

2.2.1 Flow Cytometric analysis of stem cell associated cell surface marker

The cell surface markers, CD90, CD105 and CD166 were selected from the literature as a positive marker triplicate for mesenchymal stem cells. The antibodies CD34 and CD45 were also used as negative controls for the exclusion of haematopoietic and fibrocytic cell lineages. Isotype controls for each antibody were also used as negative controls.

Table 2.1: Antibodies raised against the CD markers CD34, CD45, CD90, 105 and 166 and corresponding isotype controls used in the flow Cytometric analysis of isolated clone. All antibodies were uses at a 1:50 dilution for staining of $5x10^5$ cells.

Antibody	Conjugate	Supplier
CD90	Allophycocyanin (APC)	RnD Systems
IgG_{2A}	Allophycocyanin (APC)	RnD Systems
CD105	Phycoerythrin (PE)	Ancell
IgG1	Phycoerythrin (PE)	Ancell
CD166	Fluorescein Isothiocyanate (FITC)	Ancell
IgG1	Fluorescein Isothiocyanate (FITC)	Ancell
CD34	Fluorescein Isothiocyanate (FITC)	Ancell
CD45	Phycoerythrin (PE)	Ancell

Table 2.2. Showing the key for the labelling of cell samples with respective antibodies

Tube Number	Antibody added
1	Anti-CD90, APC Conjugated
2	
3	Anti-CD105, PE Conjugated
4	IgG1, PE Conjugated
5	Anti-CD166, FITC Conjugated
6	IgG1, FITC Conjugated
7	Anti-CD90, Anti-CD105, Anti-CD166 triplicate
8	Anti-CD34 FITC Conjugated
9	Anti-CD45 PE Conjugated
10	Anti CD-90, Anti-CD34, Anti CD45 triplicate

After trypsinisation of flasks, half a million cells were added to each of 7 numbered microcentrifuge tubes, which were then centrifuged (Eppendorf Centrifuge 5415R, Eppendorf, UK) for 5 minutes at 2000 revolutions per minute (RPM) at 4°C and the supernatant aspirated. The antibodies (Table 2.1) were diluted 1:50 in PBS and added to the relevant tube (Table 2.2). The cells were incubated with the antibody for 45 minutes at 4°C, and then centrifuged at 2000 RPM for 5 minutes at 4°C. After aspirating the supernatant the pellets were washed in 1ml PBS and centrifuged again as above. This was repeated, and after aspiration of the supernatant the cells were fixed in 100µl 2% fridge-cold paraformaldehyde (Sigma®, UK) for 2 minutes on ice, and then centrifuged at 2000 RPM for 5 minutes at 4°C. The cells were then resuspended in 1ml PBS ready for analysis on the FACSCanto™ flow cytometer (Becton Dickinson, USA). Data was then analysed using Flowjo 7.2.1 (Treestar Inc, USA)

2.3 Quantification of active telomerase in cell lysates using the quantitative telomeric repeat amplification protocol (QTRAP)

The presence of active telomerase was investigated using a quantitative real time polymerase chain reaction assay which exploits the telomeric extension property of telomerase.

2.3.1 Extraction of active telomerase from cell pellets

After trypsinisation, pellets of $5x10^5$ cells were resuspended in PBS and transferred to a 1.5ml microcentrifuge tube for centrifugation at 15,000xg for 2 minutes at 4°C. The supernatant was removed and the pellet resuspended in $TRAP_{EZE}$ CHAPS lysis buffer (Millipore, UK). The amount of lysis buffer is proportional to the cell count, added at a quantity of 1µl per $5x10^3$ cells. Cells were then incubated on ice for 30 minutes before centrifuging at 20,000xg (Sigma® 3k15, Sigma®, UK) for 30 minutes at 4°C. The supernatants containing telomerase were snap-frozen in 10µl aliquots using dry ice.

2.3.2 Quantification of telomerase activity in frozen cell lysates

Table 2.3 Primer Sequences for QTRAP Protocol

TS Primer	5'-AATCCGTCGAGCAGAGTT-3'
ACX Primer	5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'

For each sample assayed, reactions were made in triplicate. A mastermix was created calculated from the number of wells required plus additional wells to account for pipetting error (n+3). The mastermix comprised (per RXN) 12.5µl Sybr Green PCR Mix (Applied Biosystems, UK) 1µl Primer Mix (100ng TS primer and 50ng ACX Primer per µl [Table 2.3]) and 6.5µl sterile H_2O giving a reaction volume of 20µl. Added to the wells of a 96 well clear plate (Abgene, UK) was 5µl of 1:5 diluted cell lysate. This gave a final reaction volume of 25µl per well.

To generate a standard curve of telomerase activity, lysates from the telomerase positive cell line 501mel were used. These standards represented 10,000, 1,000, 100, 10, 1 and 0.1 cell/µl lysates, and were prediluted prior to the beginning of the assay. Test lysates were used at a concentration of 10,000 cells/µl lysates, which was demonstrated to be the best concentration for this assay. As a negative control, heat inactivated cell lysates were also plated. These were achieved by pre-incubation of cell lysates at 95°C for 10 minutes, during which time the telomerase enzyme becomes denatured and telomeric extension fails to occur.

Plates were then sealed with Absolute™ QPCR seals, and run on the ABI PRISM 7000 (Applied Biosystems, Foster City, USA) with the following cycling parameters:

1. 25°C, 25 minutes
2. 95°C, 10 minutes

Followed by 40 cycles of:

1. 95°C, 15 seconds
2. 60°C, 90 seconds

.

Melting curve analysis was conducted for all reactions at 60°C.

2.3.3 Analysis of Telomerase Activity

Telomerase activity was quantified using the standard curve method. ABI PRISM 7000 SDS Software version 1.1 was used to determine the standard curve for the 501mel positive control. This curve was used to determine the relative telomerase activity of each sample as a percentage of the cell number matched 501mel lysate (10,000 cells).

2.4 Quantification of telomere length using the terminal restriction fragment (TRF) assay

2.4.1 Extraction of genomic DNA

At passaging, cells from early, mid and late time points in their replicative lifespan were pelleted in a 1.5mL RNase/DNase free microcentrifuge tube (Starlab, UK), at $5x10^5$ cells per tube. Cells were washed in PBS to remove any residual media. Samples were kept on ice and resuspended in 300µl lysis buffer [200mM Tris-HCl pH7.5, 250mM EDTA, 0.5% Sodium Dodecyl Sulphate (SDS) in sterile H_2O). To this was added 3µl ribonuclease A (RNase A, Sigma®, UK), and 6µl proteinase K (Sigma®, UK) and the solution was incubated overnight at 45°C. The next morning 300µl of phenol: choloroform:isoamyl:alcohol (25:24:1, Sigma®, UK) was added to each tube and the tubes mixed well by inversion periodically over 30 minutes at room temperature. The solution was subsequently centrifuged at 13000 RPM for five minutes, and the upper aqueous layer transferred to a fresh tube. DNA was precipitated using 300µl Sodium Acetate (3M, pH5.2, Sigma®, UK) and 900µl ice-cold 100% ethanol for 20 minutes at -20°C. DNA was centrifuged at 13000 RPM for 2 minutes, the supernatant decanted and the pellet washed in 500µl ice cold 70% ethanol and centrifuged for 2 minutes at 13000 RPM. The supernatant was decanted and the pellet air dried at room temperature for 2 hours. The DNA was then resuspended in 20µl RNase/DNase free

water and stored at -20°C until required. DNA Quantification was conducted using the Nanovue spectrophotometer (GE Healthcare, UK) using the DNA quantification program.

2.4.2 Digestion of Genomic DNA

Genomic DNA was digested according to the TeloTAGGG telomere length assay manufacturer's instructions (Roche, France). All reagents, unless otherwise stated, are available from Roche.

2.4.2.1 Agarose Gel Electrophoresis of genomic DNA.

For each sample to be tested, 1µg of genomic DNA was added to 1:5 dilutions of 5X loading buffer (Promega, UK) in a total volume of 20µl in a 0.2mL microcentrifuge tube. In addition, a tube containing 4µl DIG molecular weight marker 12µl nuclease-free water and 4µl of loading buffer was pulsed on a centrifuge along with the tubes containing genomic DNA, to ensure all liquid was deposited in the bottom of the tube. These samples were loaded onto a 0.8% Agarose gel in 1X TAE buffer with markers at each end of the gel, and one marker lane in the middle to ensure accurate measurements. The gel was then run at 100V for 2h in 1X TAE buffer until the bromophenol blue band had travelled at least 10cm from the loading wells.

2.4.2.2 Southern Blotting of Agarose Gel

The Agarose gel was submerged in 0.25M Hydrochloric Acid (HCl, Sigma Aldrich, UK) solution subjected to gentle agitation for 5–10 min at room temperature until the bromophenol blue stain changes color to yellow. The gel was then washed twice with H_2O and submerged in denaturing solution twice for 15 minutes at room temperature. The gel was then washed twice with H_2O before being submerged in neutralisation solution twice for 15 minutes at room temperature. The gel was then placed into a tray large enough to hold 20X SSC solution at a level lower than the Agarose gel being blotted (use of an internal mount to raise the gel above the liquid level achieved this). Onto the mount was placed 3mm filter paper, so that the filter paper ends were in contact with the 20X SSC solution. The Agarose gel was inverted and place upon the filter paper, and onto the gel was place a nylon membrane (Roche, UK). On top of this were placed four pieces of filter paper pre soaked in 2X SSC buffer. At this stage the tray and all of the contents were sealed with Saran wrap, and a weight placed over the tray to ensure compression of all the layers. This was left at room temperature for 6 hours, to ensure the DNA blotted to the nylon membrane by capillary action. After the southern transfer, the DNA was cross linked to the membrane using UV-crosslinking (120mJ).

2.4.2.3 Hybridisation and Chemiluminescence Detection of Telomere length

Prewarming of 25 ml of DIG Easy Hyb (Solution 7, Roche, France) was conducted to bring it to 42°C. The blotted membrane was submerged in 18ml of DIG Easy Hyb solution and incubated at 42°C with gentle agitation for 60 minutes. Meanwhile the hybridisation solution was prepared by adding 1µl telomere probe per 5mL fresh prewarmed DIG Easy Hyb solution with thorough mixing. At least 6.5mL of this solution is required per 200cm^2 blotting membrane. After 60 minutes the prehybridisation solution was removed and the hybridisation solution containing the telomere probe was immediately added and the membrane incubated for 3 hours at 42°C with gentle agitation. After 3 hours the hybridisation solution was discarded and the membrane washed twice with stringent wash buffer for 5 minutes at room temperature, with gentle agitation. The membrane was then washed twice with stringent wash buffer II in a heater water bath, for 15-20 minutes at 50°C. The membrane was then rinsed in at least 100mL 1X washing buffer for 5 minutes at room temperature with gentle agitation. The membrane was then washed in 1X blocking solution for 30 minutes at room temperature. Meanwhile, the Anti-DIG-AP working solution was prepared as per the manufacturer's instructions. After 30 minutes the membrane was washed twice with 1X washing buffer for 15 minutes at room temperature, before incubation with 100ml of 1X detection buffer for 5 minutes at room temperature with gentle agitation. After 5 minutes the buffer was discarded and the residual liquid removed by placing the membrane DNA side up on a

sheet of absorbent paper, however it was important not to let the membrane dry. The membrane was then placed in a hybridisation bag, DNA side facing upward and 40 drops (approximately 3mL) substrate solution was added to the membrane, with careful attention paid to the hetereogeneous distribution of the substrate buffer throughout the membrane. After sealing the edges of the hybridisation bag, the membrane was developed on the Typhoon Imager (GE Healthcare, UK).

2.4.2.4 Analysis of Telomere Length

The telomere lengths of individual samples were determined by calculating the median telomere length. Measurements were taken at the top and bottom of the visible bands of DNA, and the median calculated. This gives a numerical representation of telomere length within experimental samples, to allow comparisons between individual samples.

2.5 Adipogenic Differentiation

To investigate the lineage potential of isolated cell populations, cells were induced along the adipogenic lineage by supplementation of culture media for 28 days using a previously established differentiation cocktail (Choi et al., 2006). RNA was extracted at 7 day intervals until 28 days, and 28 day cultures were kept for histochemical staining for lipid deposition using Oil Red-O staining.

2.5.1 Adipogenic induction of cell cultures

Cells were seeded in 6 well cultures dishes (Greiner, UK) at $3x10^3$ cells/cm2. Cells were cultured in DMEM+10%FBS until confluency. At this point the media was replaced with adipogenic induction media (DMEM+10%FBS supplemented with 10µg/ml insulin, 1µM dexamethasone, 100µM indomethacin, 100µM 3-isobutyl-1-methyl-xanthine [IBMX] (All Sigma, UK). Cells were also culture in standard DMEM+10% FBS media as a differentiation control. In total, enough cultures for RNA extractions in triplicate at day 7, 14, 21 and 28 for both adipogenic and control cultures were used. In addition, cells were seeded into three 8-well chamberslides (BD Falcon, UK) for adipogenic induction and three for control cultures for Oil Red-O staining.

2.5.2 Quantitative Real-Time (PCR QRT-PCR) detection of adipogenic mRNA from adipogenic cell cultures

2.5.2.1 RNA Extractions of Adipogenic Cultures

Differentiation cultures were stimulated for the required number of days (7-28 days at 7 day intervals). Wells were washed with 1mL PBS and 1mL TRIzol (Invitrogen, UK) added for 5 minutes at room temperature. The wells were then transferred to 1.5mL microcentrifuge tubes (Starlab, UK) and 175µl Chloroform was added (Sigma®, UK), and the samples mixed well through gentle pipetting. The chloroform/TRIzol mix was transferred to a 1.5mL heavy phase lock tube (Fisher Scientific, UK) and centrifuged for 2 minutes at 13000*xg*. The upper aqueous layer was transferred to an RNase free 1.5mL microcentrifuge tube and the RNA was precipitated by adding 500µl Isopropanol (Sigma®, UK), and placing at -20°C overnight. The resulting precipitate was centrifuged for 10 minutes at 12000*xg* at 4°C using an Eppendorf Bench top Durafuge (Eppendorf, Germany). The supernatant was removed; the pellet washed with 75% Ethanol and the centrifuged for a further 5 minutes at 7500*xg.* The supernatant was removed and the pellet air dried, then resuspended in 50µL sterile water. RNA concentration was quantified using the Nanovue spectrophotometer (GE Healthcare, UK).

2.5.2.2 cDNA Generation

For the QRT-PCR, cDNA was generated from the total RNA isolated in section 2.5.2.1. For each reaction 1µg of total RNA was added to 0.5µg random hexamer primers (Promega, UK) in an RNase free 0.2ml microcentrifuge tube, with 13µl of sterile water. Reactions were incubated at 70°*C* for 5 minutes followed by 5 minutes on ice. To each reaction 1µl RNasin, 5µl MMLV 5X Buffer, 5µl dNTPs (dCTP, dGTP, dATP, dTTP) and 1µl MMLV Reverse Transcriptase were added (All Promega, UK). After thorough mixing, reactions were incubated for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 95°C. The resulting cDNA was stored at -80°C until required.

2.5.2.3 QRT-PCR detection of lineage associated markers

2.5.2.3.1 Primer Sequences

Primer sequences were designed using Primer3, a web based software for the generation of primer sequences with strict parameters (i.e. %GC content, T_a/T_m, http://frodo.wi.mit.edu/primer3/input.htm). Primers were designed to span exon-exon boundaries, to ensure amplification of mRNA generated cDNA only, not genomic DNA. Parameters were set for 50% GC content and T_a 0f 60°C to ensure that all primers could be used under the same conditions on the ABI PRISM 7000 lightcycler. Sequences for the primers were as follows:

β-Actin (Forward) 5’-AGGGCAGTGATCTCCTTCTGCATCCT-3’

β-Actin (Reverse) 5’- CACACTGTGCCCATCTACGAGGGGT -3’

Product Length – 500 base pairs (bp)

LPL (Forward) 5’- CCTGCTCGTGCTGACTCTGG-3’

LPL (Reverse) 5’-CATCCTGTCCCACCAGTTTGG-3’

Product Length – 389bp

PPARγ (Forward) 5’- CCACAGGCCGAGAAGGAG AA-3’

PPARγ (Reverse) 5’- CTGGCAGCCCTGAAAGATGC-3’

Product length – 302bp

C/EBPα (Forward) 5’- CCTTGTGCAATGTGAATGTGC-3’

C/EBPα (Reverse) 5’- CGGAGAGTCTCATTTTGGCAA-3’

Product Length – 150bp

2.5.2.3.2 Reference Control

To ensure the accuracy of comparisons between plates, a reference control was used. Stratagene QPCR Human Reference Total RNA (Stratagene, USA) was purchased at a concentration of 1µg/mL. cDNA controls were generated for the generation of standards at using 5µg RNA per reaction, giving 100µl of 200ng/µl total cDNA. This was used to generate standards curves for each of the PCR primer pairs.

2.5.2.3.3 Reference Gene

To ensure the differences in gene expression observed within the QRT-PCR reactions were a function of the biological processes involved, the used of β-actin as a reference gene was employed. This allowed the generation of normalized expression of all other genes of interest back to the expression of the housekeeping gene β-actin

2.5.2.3.4 QRT-PCR

Reactions were conducted in 96 well clear PCR plates (Abgene, UK). A standard curve of the reference control cDNA was generated representing 100ng, 10ng, 1ng, 0.1ng and 0.01ng cDNA to allow for comparisons between plates. Reactions were setup in triplicate for each sample with all timepoints on one plate, and the clonal populations on a plate together, and the NA/LO populations on another plate. Reactions were in a 25µl volume, comprising 12.5µl SybrGreen PCR mix (Applied Biosystems), 1µl of 10µM primer mix and 6.5µl water. Into the wells of the 96 well plate, 100ng of sample cDNA was diluted into 5µl in triplicate, and the 20µl reaction mix was added to the 5µl sample cDNA prior to running on the ABI PRISM 7000 (Applied Biosystems). Reactions were run under the following conditions: 2 minutes 50°C, 2 minutes 95°C followed by 50 cycles of 15 seconds 95°C, 60 seconds 60°C. Melting curve analysis was conducted at 60°C.

2.5.2.3.5 Analysis of Gene Expression

Gene expression was quantified using the standard curve method. ABI PRISM 7000 SDS Software version 1.1 was used to determine the standard curve for the reference control cDNA. This curve was used to determine the relative expression of the target gene, and normalisation to β-actin expression accounted for any plate to plate or well to well variation.

2.5.3 Histological investigation of adipogenic differentiation using Oil red-O staining

After 28 days in either differentiation media or control media, chamber slides were washed with PBS and cells fixed with 70% ice cold ethanol for 15 minutes at -20°C. Cells were washed again with 60% (v/v) Isopropanol (Sigma®, UK), for 5 minutes at room temperature before staining of cells with Oil Red-O (0.25% (w/v) Oil Red-O (Sigma®,UK) in Isopropanol) at room temperature with gentle agitation. Cells were rinsed with 60% isopropanol before washing with water. Chamberslide culture chambers were removed to reveal the underlying microscope slide, which was then in turn mounted with DPX (Sigma®,UK) and a coverslip was placed over the slide. After the DPX mount was dry, images were acquired using an Olympus PROVIS AX470 (TRF) Microscope using a Nikon DXM1200 Digital Camera and Nikon ACT-1 software. The images were reviewed in Adobe Photoshop Elements.

2.6 Chondrogenic Differentiation

To investigate the lineage potential of isolated cell populations, cells were induced along the chondrogenic lineage by supplementation of culture media for 28 days in a pellet culture system using an established differentiation cocktail (Lee et al., 2004). After 28 days, pellets were kindly paraffin embedded and 10μm sections cut on a microtome by School of Dentistry Pathology MLSO Kath Allsop.

2.6.1 Chondrogenic induction of cell cultures using the pellet culture system

Cell pellets were generated using in 1.5mL sterile microcentrifuge tubes (Starlab, UK). Briefly, $5xx10^5$ cells were centrifuges at 1500 RPM in 1mL of DMEM+10%FBS. Pellets were allowed to form overnight. DMEM+10%FBS was replaced with chondrogenic induction media (DMEM+10%FBS supplemented with 1x insulin, transferrin and selenium (ITS) supplement (Invitrogen, UK), 50μg/ml L-ascorbate (Sigma, UK) and 5ng/ml transforming growth factor beta 1 (TGF-β1) (Peprotech EC Ltd., UK)). Control pellets were incubated in the DMEM+10%FBS media alone. Media was change every two days, with care taken not to disrupt the pellet at the bottom of the tube, and after every media change pellets were centrifuged at 1500 RPM for 5 minutes and replaced back into the incubator.

2.6.2 Evaluation of chondrogenic differentiation using general histology

As mentioned in section 2.6, sections were kindly paraffin embedded and 10μm sections cut on a microtome by School of Dentistry Pathology MLSO Kath Allsop.

2.6.2.1 Haemotoxylin and Eosin (H&E) staining

Sections were washed in Xylene (Fisher Scientific, UK) twice for 2 minutes, followed by 2 minute washes in decreasing alcohol concentrations (100%, 90%, 75%). Cells were then washed in running tap water for 2 minutes, submerged in Mayer's Haemotoxylin for 2 minutes and washed in running water until the sections appear blue. Slides were then submerged in 1% Eosin solution for 2 minutes before washing in running water for 20 seconds. Sections were then dehydrated through increasing concentrations of alcohol as previously described, until two final 2 minute washes with Xylene. Sections were then mounted in DPX mounting medium (Sigma, UK) and coverslips placed on top, before images were acquired using an Olympus PROVIS AX470 (TRF) Microscope using a Nikon DXM1200 Digital Camera and Nikon ACT-1 software. The images were reviewed in Adobe Photoshop Elements.

2.6.2.2 Van Gieson's staining

Sections were rehydrated as described in section 2.6.2.1. Sections were stained with Celestine Blue [5% ammonium ferric sulphate (iron alum) with 5g/mL Celestin Blue] for 5 minutes, and then rinsed in distilled water. Cells were stained with Haemotoxylin for 5 minutes, washed well with tap water for 5 minutes and submerged in Curtis stain for 5 minutes. Sections were then blotted to remove excess liquid, rapidly dehydrated through alcohol to Xylene and mounted with DPX and coverslips. Images were acquired using an Olympus PROVIS AX470 (TRF) Microscope using the Nikon DXM1200 Digital Camera and Nikon ACT-1 software. The images were reviewed in Adobe Photoshop Elements.

2.6.3 Immunohistochemical evaluation of chondrogenic differentiation by aggrecan expression

Pellets after 28 days differentiation were placed into a 30% sucrose solution (Sigma®, UK) overnight at 4°C. After pellets had fallen to the bottom of the sucrose solution, they were removed from the sucrose solution using forceps, and placed onto a cryotome chuck in cryosection embedding media, and cooled until set in dry ice. 10µm sections were then cut on the cryotome (Cryotome Electronic 77210163, Shandon, UK) and placed on Poly L-lysine coated microscope slides (Fisher Scientific, UK). These pellet sections were carefully washed with PBS before blocking in a 1:20 dliution of goat serum (Santa Cruz, USA) in PBS+0.05% $Tween_{20}$ (Sigma®, UK), for 30 minutes at

room temperature. Sections were incubated in the presence of 1:50 dilution of 6B4 mouse anti-aggrecan antibody (kindly donated by Prof. Bruce Caterson, Cardiff University School of Biosciences) overnight at 4°C in a humidified chamber. The next day slides were washed three times with PBS+0.05% $Tween_{20}$ for 5 minutes, before incubation with an anti-mouse secondary antibody (1:50, Dako, UK) for 1 hour at room temperature. Sections were washed again with PBS prior to mounting under coverslips with Dako fluorescent mounting medium. Images were acquired using an Olympus PROVIS AX470 (TRF) Microscope using the Nikon DXM1200 Digital Camera and Nikon ACT-1 software. The images were reviewed in Adobe Photoshop Elements

2.7 Osteogenic Differentiation

To investigate the lineage potential of isolated cell populations, cells were induced along the osteogenic lineage by supplementation of culture media for 28 days using an established differentiation cocktail (Coelho and Fernandes, 2000, Coelho et al., 2000). Cultures were then assessed by histochemical staining for calcium deposition using Alizarin red staining.

2.7.1 Osteogenic induction of cell cultures

Cells were seeded into three 8-well chamberslides (BD Falcon, UK) for osteogenic induction and three for control cultures at $3x10^3$ cell/cm^2. Cells were cultured in DMEM+10%FBS until confluency. At this point the media was replaced with osteogenic induction media (Alpha modified Eagles medium (α-MEM, Invitrogen, UK) supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin G, 100µg/ml streptomycin sulphate, 0.25µg/ml amphotericin B, 50µg/ml L-ascorbate, 10µM dexamethasone and 10µM beta-glycerophosphate (Sigma, UK)). Cells were also culture in standard DMEM+10% FBS media as a differentiation control. Cells were cultured for 28 days before staining for calcium deposition using Alizarin red.

2.7.2 Evaluation of Osteogenic differentiation using Alizarin red staining

After 28 days in either differentiation media or control media, chamber slides were washed with PBS and cells fixed with 70% ice cold ethanol for 15 minutes at -20°C.

Cells were washed with PBS before the addition of Alizarin Red Solution [Alizarin Red 20g/L in distilled H_2O] at room temperature for 2 minutes. Chamberslide culture chambers were removed to reveal the underlying microscope slide, which was then in turn mounted with DPX (Sigma®, UK) and a coverslip was placed over the slide. After the DPX mount was dry, images were acquired using an Olympus PROVIS AX470 (TRF) Microscope using a Nikon DXM1200 Digital Camera and Nikon ACT-1 software. The images were reviewed in Adobe Photoshop Elements.

CHAPTER 3

The isolation and long term culture of oral mucosal progenitor cell populations from the lamina propria

3.1 Introduction

Fibroblasts derived from the oral mucosa have been known to display distinct phenotypic characteristics when compared to fibroblasts obtained from human skin particularly in the wound healing process, including lower levels of tissue infiltrating immune cells and increased levels of growth factors (Szpaderska et al., 2003, Smith et al., 2010, Shannon et al., 2006, Stephens et al., 2001b). In addition, oral fibroblasts demonstrate increased MMP activity and an increased ability to remodel extracellular matrix and close a wound *in vitro* (Lee and Eun, 1999, Stephens et al., 2001a). Ultimately these phenotypic characteristics play a role in the normally non-scarring wound healing process observed within the oral mucosa. It has been postulated that these differences in phenotype and the non-scarring wound healing process may be in part due to a previously unknown stem cell population resident within the oral mucosa (Stephens and Genever, 2007).

Embryonic stem cells are totipotent cells capable of generating cells from all developmental lineages (Keller, 1995). ES cells demonstrate rapid growth rates and can be cultured for up to 2 years without exhibiting signs of cellular senescence (Fortier, 2005). Whilst the potential for these cells to provide a therapeutic benefit for a number of disease conditions is vast, the ethical implications of their use, means that embryonic stem cell therapy may never become a standard practice. This has lead to research around the world into

alternative sources of multi-potential stem cells of non-embryonic origin, and the discovery of a number of adult stem cell populations.

Adult stem cells have been isolated from a number of tissues of different developmental origin since the discovery of mesenchymal stem cells in peripheral blood by Friedenstein et al in 1970. *In vitro* characterisation of MSCs determined that these cells were able to undergo 40 population doublings before exhibiting signs of replicative senescence (Friedenstein et al., 1970, Bruder et al., 1997). Other tissues shown to possess adult stem cell populations include adipose tissue and cartilage (Alsalameh et al., 2004, Hiraoka et al., 2006, Miyazaki et al., 2005, Dowthwaite et al., 2004, Fickert et al., 2004). The skin, functionally a similar tissue to the oral mucosa, also contains adult stem cell populations (Jones and Watt, 1993, Jahoda et al., 2003, Toma et al., 2005, Toma et al., 2001).

In the oral cavity, adult stem cell populations have been found in a number of tissues. The dental pulp contains a cell population with the capacity for self renewal and the ability to generate dentin-pulp like tissue when implanted in mice, and generating adipocytes and neural-like cells *in vitro* (Gronthos et al., 2002). Mesenchymal cells isolated from the dental papilla of impacted wisdom teeth have also exhibited multilineage potential and the presence of stem cell associated markers (Ikeda et al., 2006). Postnatal deciduous teeth have also been shown to contain a multipotent stem cell population capable of forming neural cells, adipocytes and odontoblasts (Miura et al., 2003). The periodontal ligament has also been shown to contain stem cells which have an extended capacity for self renewal and positive for stem cell markers

(Trubiani et al., 2005). The lamina propria also contains a keratinocyte stem cell population (Izumi et al., 2007, Calenic et al., 2010). All of this data suggests that a stem cell population may reside within the lamina propria (Davies et al., 2010, Marynka-Kalmani et al., 2010, Zhang et al., 2009, Zhang et al., 2010)

The isolation of adult stem cell populations can be undertaken utilising a number of methods, including adherence to plastic in the case of muscle derived stem cells (Sinanan et al., 2004) and blood derived MSC (Wagner et al., 2005) or through differential adherence to extracellular matrix components such as collagen or fibronectin (Jones and Watt, 1993, Dowthwaite et al., 2004). This differential adhesion characteristic of stem cells allows for clonogenic expansion of single cells giving rise to pure cell populations. As previously discussed stem cells demonstrate an increased replicative potential *in vitro*, with MSCs able to undergo at least 50 population doublings (Stenderup et al., 2003, Bonab et al., 2006). In the case of MSCs when this replicative potential is exhausted and cells exhibit signs of cellular senescence, they become larger with an irregular, flat shape, increasingly granular cytoplasm and circumcised nuclei when observed using phase contrast microscopy (Wagner et al., 2008).

The aim of this chapter was to successfully isolate a progenitor cell population from the Lamina Propria of the Oral Mucosa by the rapid adherence of this cell population to fibronectin, and observe the colony forming efficiency of those isolated cells. Once isolated these populations were compared with both heterogeneous, unseparated cells representing

whole lamina propria cultures (LO), and progenitor-depleted cells which did not adhere to fibronectin during differential adhesion (NA). These comparisons were based on the replicative potential of these cell populations during long-term culture, as well as associated changes in cell size as the cells reached senescence.

3.2 Results

3.2.1 Isolation of cells from the lamina propria of the oral mucosa

Cells were obtained from patients undergoing wisdom tooth extraction after ethical approval (LREC 05/WSE02/124) and informed patient consent (Chapter 2.2.1). During the extraction procedure, a 6mm section of buccal mucosa was excised before proteinase/collagenase digestion and subsequent differential adhesion and cell population isolation. In total 5 patients were consented for the study, shown in table 3.1. Only 2 patients were successfully taken from the point of isolation to senescence with 5 cell populations, patient XVII and patient XXVIII, male and female respectively. The other three patients were unsuccessful for a number of reasons, including failure of all cell populations to grow from point of isolation, to cultures succumbing to microbial infections.

The five populations from patient XVII and patient XXVIII represent three clonally derived populations obtained by adherence to fibronectin (clone 6, 7, 10 XVII and clone 3, 9, 10 XXVIII), a population representative of the digested lamina propria prior to the differential adhesion assay (LO) and those cells which did not adhere during the sequential adherences

incubations (NA). All subsequent experiments concern the comparative properties of these 5 populations from patient XVII and XXVIII.

Patient No	D.o.B	Gender	Successfully established?	Reason for failure
XVII	13/12/1972	M	YES	-
XXIII	30/09/1975	M	NO	Infection
XXVIII	07/09/1983	F	YES	-
XXXVI	09/06/1985	F	NO	No NA population isolated
XXXVIII	27/10/1985	F	NO	No NA population isolated

Table 3.1: Anonymised patient information for all oral mucosal biopsies obtained, and information regarding the success of colony isolation and subsequent cultures. Patient samples were each given an identifier in roman numerals (e.g. XVII = Patient 17) to differentiate between each sample. Although five samples were obtained, only two provided the necessary populations (three clones, whole lamina propria [LO] and non fibronectin adherent [NA]) to continue culture. Patient numbers began at XVII due to previous studies carried out within the wound biology group.

3.2.2 Differential Adhesion and Colony Forming Efficiency

After collagenase digestion of the mucosal biopsy, cells were used in a differential adhesion assay (See Chapter 2.2.2). Initial adhesion to fibronectin was recorded after 24h and compared with BSA controls (Fig 3.1A). This data represents the combined data from patients XXVIII, XXXVI and XXXVIII. The adherence to fibronectin was measure over 1 hour and 40 minutes, at three timepoints: 20 minutes (FN20), 40 minutes (FN40 [1]) and a further second 40 minutes (FN40 [2]). At 20 minutes, fibronectin demonstrated a significantly higher level of adherence than BSA controls when analysed using a one-way ANOVA with a Bonferroni multiple comparisons post test ($P<0.001$). In addition, the 20 minute incubation also demonstrated a significantly higher adherence when compared to the other fibronectin timepoints ($P<0.001$ in both instances). After the initial adherence, cells were cultured for 12 days and colonies in excess of 32 cells (Fig 3.2B) were counted at days 5, 9 and 12 to determine colony forming efficiency (CFE - by dividing number of colonies by initial adherence). Comparisons were made between fibronectin at 20, 40[1] and 40[2] timepoints at these three intervals (Fig 3.2). At Day 5 only the FN20 timepoint demonstrated any colony formation. By day 9 however, all three timepoints exhibit colony formation. By day 12 the difference in CFE was significant between FN20 and FN40 [2], with the FN20 timepoint giving rise to a CFE at 0.042 ($p<0.01$). Colonies were removed from the culture plates after the day 12 measurement and

cultured as clonally derived cell populations originating from a single seeded cell.

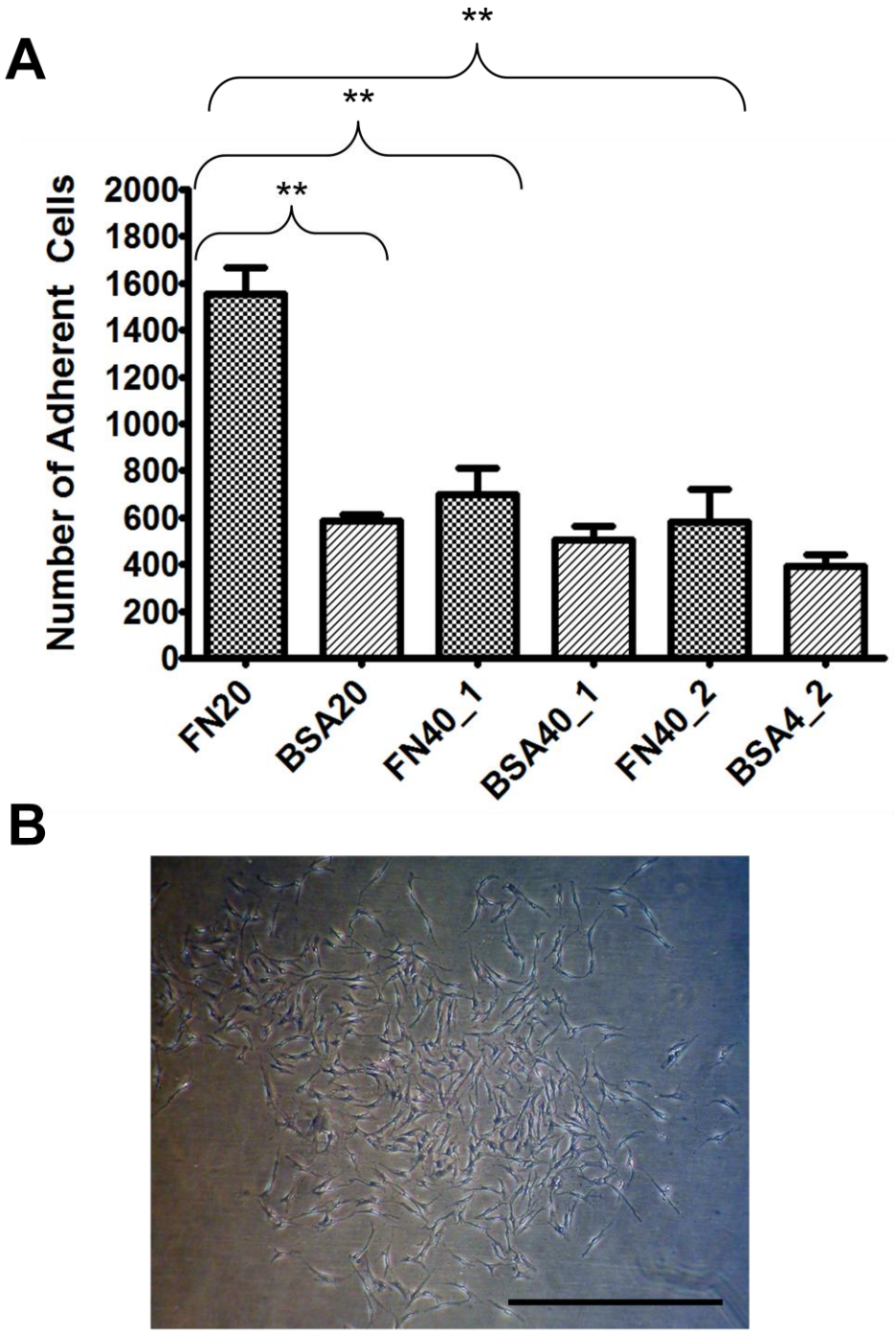

Fig 3.1: (A) Combined data from three patients demonstrating the initial adherence of cells isolated from the oral mucosa lamina propria during the differential adhesion assay. Cells were sequentially incubated at three timepoints of 20 minutes, 40 minutes (40[1]) and a final 40 minutes (40[2]) on fibronectin coated plates with BSA coated plates for control. After 12 days in culture any colonies in excess of 32 cells (B) were identified and isolated by trypsin digestion and reseeded into 24 well plates ready for ongoing culture. Data combined from three separate patient experiments. Adherence data analysed for significance by one way ANOVA with a bonferroni post test. *n* = 3 ± SD, ** P<0.001. Scale bar = 200 μm.

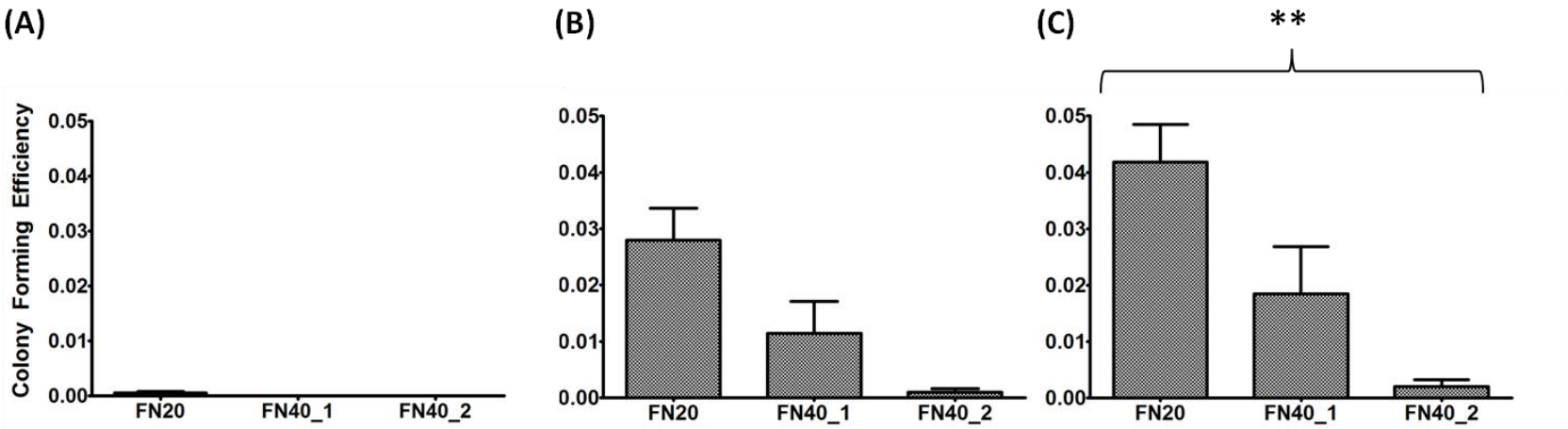

Fig 3.2: Colony forming efficiency calculated from the combined data from three patients. After initial adhesion of isolated oral mucosa lamina propria cells to fibronectin (FN) or BSA, colonies derived from a single cell were counted when exceeding 32 cells per colony. Counts were conducted at day 5 (A), day 9 (B) and day 12 (C). The number of colonies in combination with the initial adherence data was used to calculate the colony forming efficiency (CFE) of the cells at different time points. At day 5 only FN20 cells demonstrated colony formation. By day 12 all cell populations exhibited the formation of colonies, with significantly higher CFE in FN20 when compared to FN40_2 $n = 3 \pm SD$, ** $P<0.01$.

In summary, the adherence to fibronectin was significantly higher than BSA controls, and fibronectin adherence decreased across the three sequential timepoints significantly. Differences in CFE do not become highly significant during the first 9 days of the assay, but by day 12 the FN20 timepoint demonstrates a significantly higher level of colony formation when compared to FN40 [1] and FN40 [2].

3.2.3 Growth Kinetics of Isolated cell populations

After isolation of clonally derived cell populations (clone x) and alongside the other populations isolated as mentioned previously, cells were cultured long-term as described in materials and methods (Chapter 2.2.3). Three clones which demonstrated the highest growth rate during the intial passage were selected for culturing until senescence, with the remainder frozen for future use. Growth kinetics for all cell populations were plotted for both patient XVII (Fig 3.3) and XXVIII (Fig 3.4) from isolation until senescence, a pre-determined threshold of three repeated passages at a rate of less than 0.5 population doublings per week was used for this point. In patient XVII (Fig 3.3), all clonally derived populations demonstrated a rapid initial phase of growth after isolation, XVII clone 6 underwent 76 population doublings (PD) over 597 days, the highest number of population doublings for a clone from this patient. All three clones exhibited similar growth kinetics over the first 20 PD; however, after this time clone 7 demonstrated a diminished growth rate for 100 days before an increase in growth rate back to similar levels to those observed in clone 6. Clone 10 however, exhibited signs of senescence after

37 population doublings. The LO and NA populations differ in growth kinetics to the clonally derived populations, initially demonstrating a low rate of growth before an increase in growth kinetics was observed around 100 days. The LO population achieve the highest number of population doublings, with a growth rate consistently higher than the NA population. Also of note was the “stepped” growth rate with many increases and decreases in growth rate in the LO population. The LO and NA populations achieved 91 and 73 PD over 650 and 633 days respectively. When comparing average growth rates over the entire replicative lifespan of the cell populations (Table 3.2), it is apparent that the LO and NA populations demonstrate a higher average growth rate, and over their entire lifespan, the LO population were the cells with the highest replicative potential.

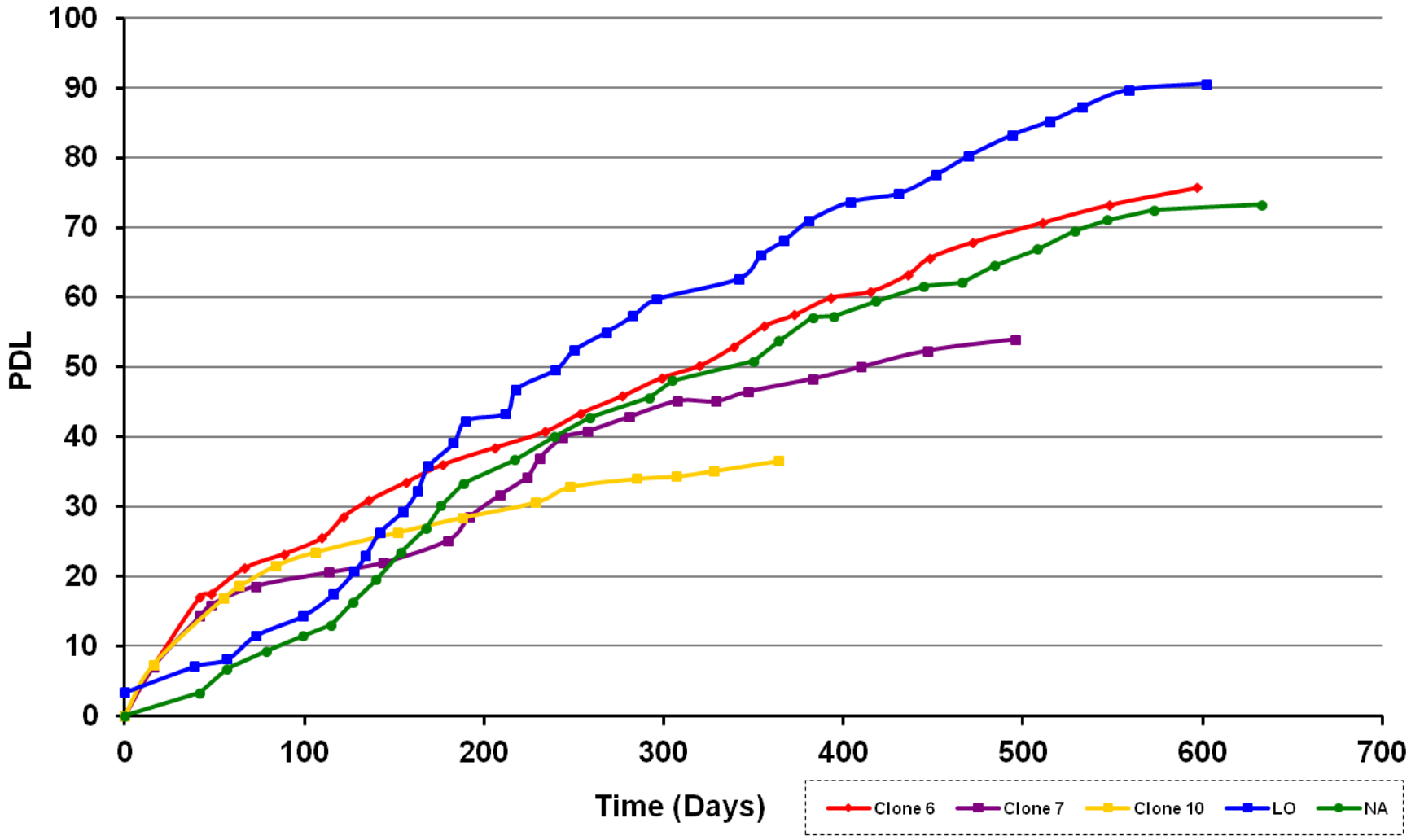

Fig 3.3: Relative population doubling level (PDL) data for patient XVII. Oral mucosa lamina propria (LO) cells and non-adherent (NA) cells, and clones 6 and 7 all achieve in excess of 50 population doublings before senescence, with LO cells showing the highest replicative potential (senescence was determined as when population doubling dropped below 0.5 per week).

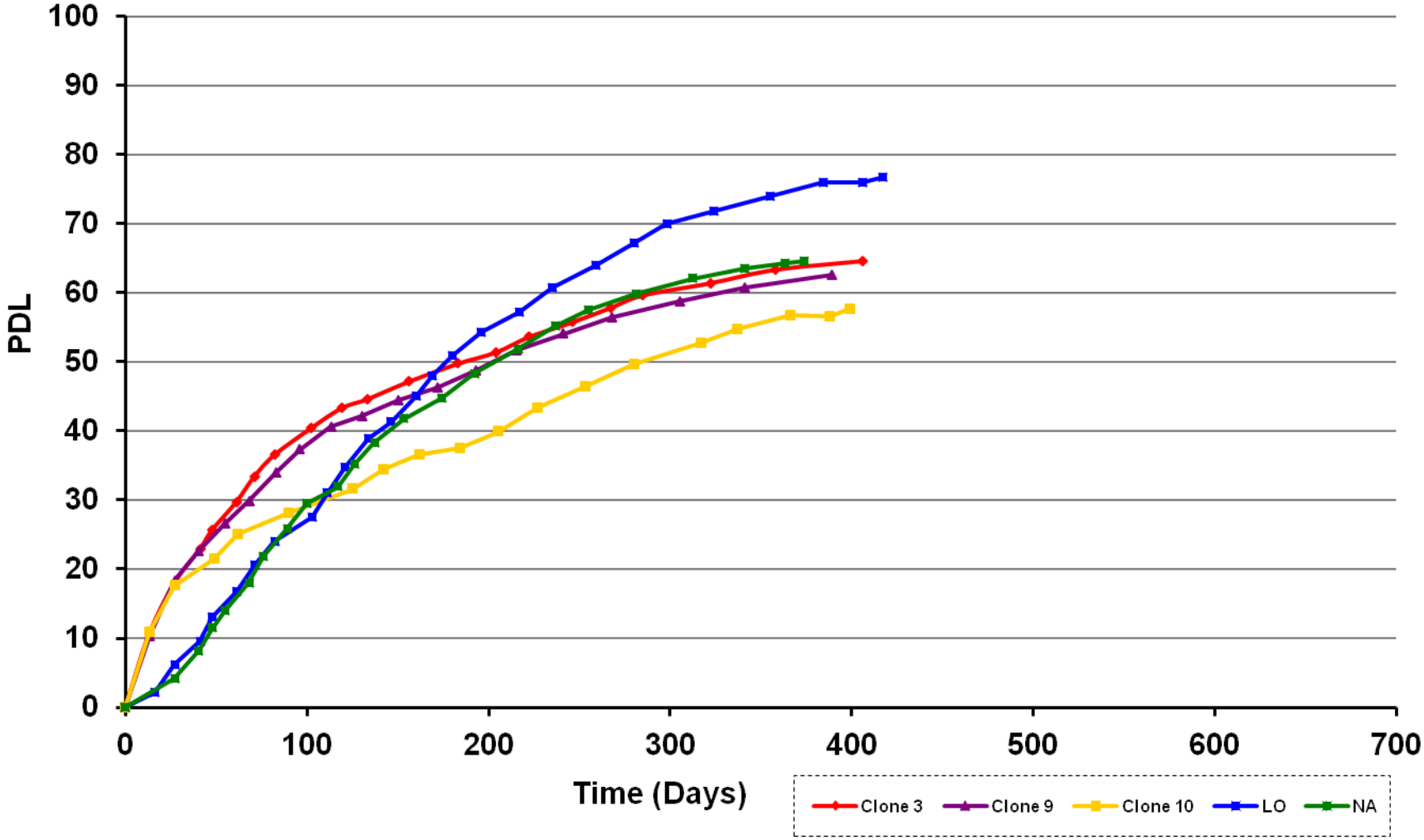

Fig 3.4: Relative population doubling level (PDL) data for patient XXVIII. All cell populations achieved in excess of 50 population doublings before senescence, with LO cells showing the highest replicative potential (senescence was determined as when population doubling dropped below 0.5 per week for consecutive passages), with the LO population achieving the highest number of population doublings.

Patient	Population	Total Population Doublings	Total Time in Culture (Days)	Average Growth Rate
XVII	6	76	597	0.888692
XVII	7	54	496	0.913513
XVII	10	37	364	0.632324
XVII	LO	91	650	1.338273
XVII	NA	73	633	1.041652
XXVIII	3	64	406	1.301062
XXVIII	9	57	389	1.24451
XXVIII	10	62	399	0.95248
XXVIII	LO	76	417	1.574508
XXVIII	NA	64	374	1.503925

Table 3.2: Table showing the average growth rates of cell populations from patients XVII and XXVII, excluding the first passage, as the clonally isolated cells underwent a rapid expansion during this first passage which could not be compared to the LO and NA populations. Population doubling per week from each passage were used to generate an average growth rate over the replicative lifespan of the cells. Note that the LO and NA populations show a higher average growth rate compared to the clonal populations when considered in terms of the entire time in culture.

Patient XXVIII population doublings follow a similar trend to those seen in patient XVII however, the cell populations did not achieve the same levels, with the highest PD being observed in LO XXVIII, however (Fig. 3.4, Table 3.2) only achieving 76 PD over 417 days. In terms of the growth kinetics however, the pattern of the clonally derived populations rapidly achieving 20 PD before slowing in rate are maintained across the two patients, as was the lag in the growth of both the LO and NA populations before an increase in proliferation rate. The NA population also demonstrated a reduction in proliferation in line with the clonally derived population toward the end of their replicative lifespan.

The average growth rate of the XXVIII populations when compared to those of patient XVII were consistently higher, with the clonal growth rates comparable to the LO and NA rates from XVII (1.3, 1.2 and 0.9 PD/week respectively), however, the LO and NA rates were again higher than those observed in the clones for patient XXVIII, with the LO XXVIII population exhibiting the highest average growth rate of 1.57 PD per week. As with patient XVII, one of the clones did not grow in the same way as the others, Clone 10 XXVIII. This clone showed a marked decrease in proliferation after 20 PD however, it did overall achieve 57 population doublings before entering senescence, similar to that observed in clones 3 and 9, but with a lower average growth rate. In summary, whilst the final number of divisions between patients differs, the growth kinetics of the different cell populations seem to exhibit distinct characteristic maintained between patients, with the

heterogeneous populations (LO and NA) demonstrating higher growth rates and a higher number of population doublings over the lifetime in culture.

3.2.4 Maintenance of Long Term Cultures

Over the replicative lifespan of all the cell populations, routine testing for Mycoplasma was conducted using Hoechst staining as described in the materials and methods (Chapter 2.2.4) at every other passage. Briefly, cells were stained with a DNA intercalating dye and visualised under fluorescence microscopy. Any cells contaminated with Mycoplasma exhibited a ‘speckled’ staining throughout the cytoplasm. Cells which were negative for Mycoplasma infection demonstrate nuclear-only staining. When conducting this routine Mycoplasma staining (Fig 3.5), samples from XVII were found to be positive for Mycoplasma contamination (Fig 3.5a). These cells were then treated with alternating tetracycline derived antibiotics (BM Cyclin 1 &2) for three weeks as recommended by the manufacturer, after which time the cells were retested and found to be negative (Fig 3.5b) for non-nuclear staining and were then cultured as normal. Samples from Patient XXVIII were found to be routinely negative for Mycoplasma testing throughout their replicative lifespan.

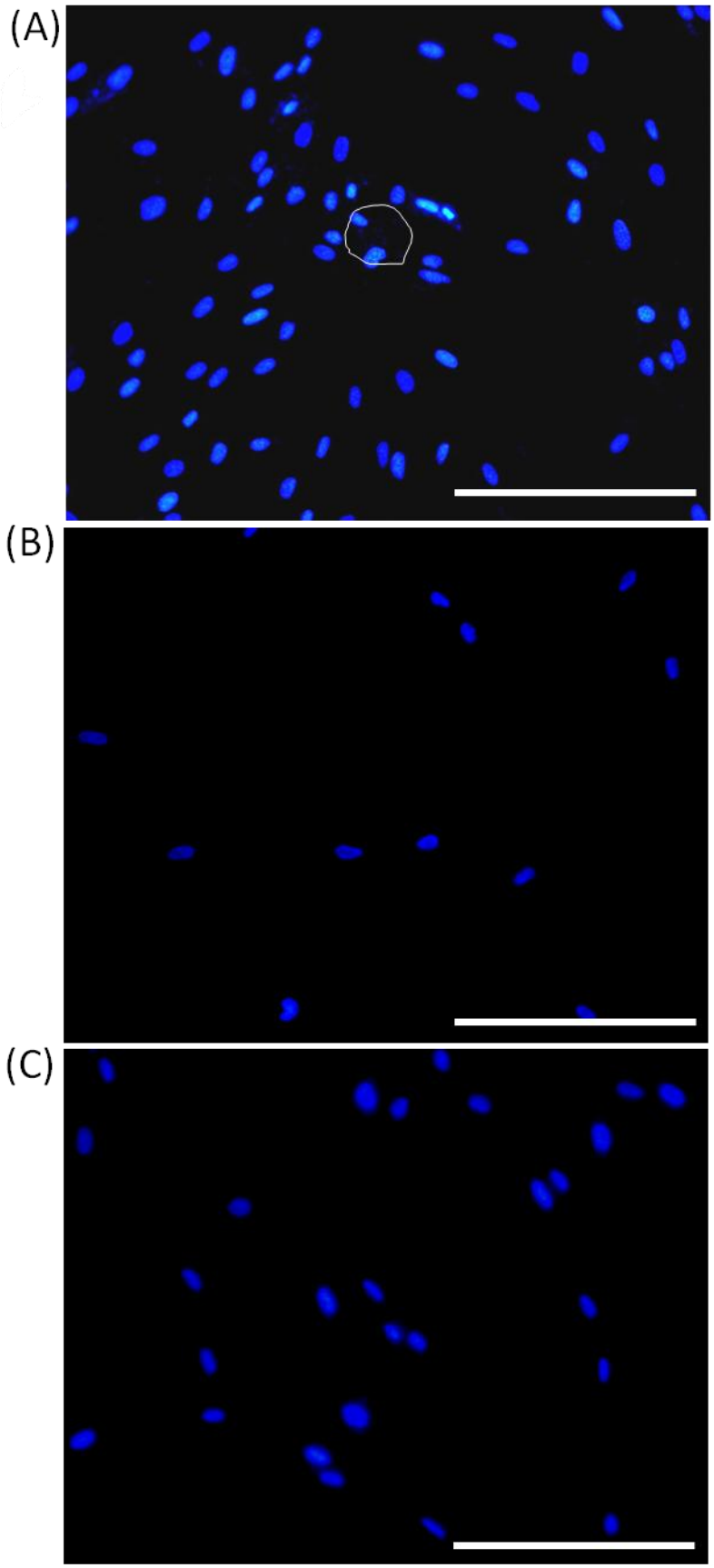

Fig 3.5 Mycoplasma testing using Hoechst staining protocol. (A) At an early passage cell populations from patient XVII demonstrated positive staining for Mycoplasma, as seen by the presence of non-nuclear, cytoplasm localised staining (circled in white). (B) After treatment with tetracycline antibiotics, this Mycoplasma staining was abolished. (C) Cell populations from XXVIII remained Mycoplasma free. Scale bar = 200um

3.2.5 Morphological changes in isolated cell populations during long-term culture

To determine how long-term culture may affect the morphology of these cell populations, images were captured at every passage as described in the materials and methods (Chapter 2.2.5). The cell images at both early and late passages were used for analysis of morphological changes in terms of an increase of cell size using ImageJ, using a haemocytometer image at the correct magnification to set the scale. Once the correct scale was set, 50 cells were traced by hand to calculate an average cell area in μm^2 as demonstrated in Fig 3.6. Images of each cell population at early and late passages are shown in Fig 3.7 and 3.8 for patient XVII and XXVIII respectively. A visual analysis of the images for each cell population demonstrated that all cell populations appear initially like small bi-polar 'fibroblast-like' cells with minimal cellular processes. However, by the late passages these cells are visually larger, exhibiting roughened edges with more branched cellular processes, as observed for example in clone 10 XXVIII (Fig 3.8).

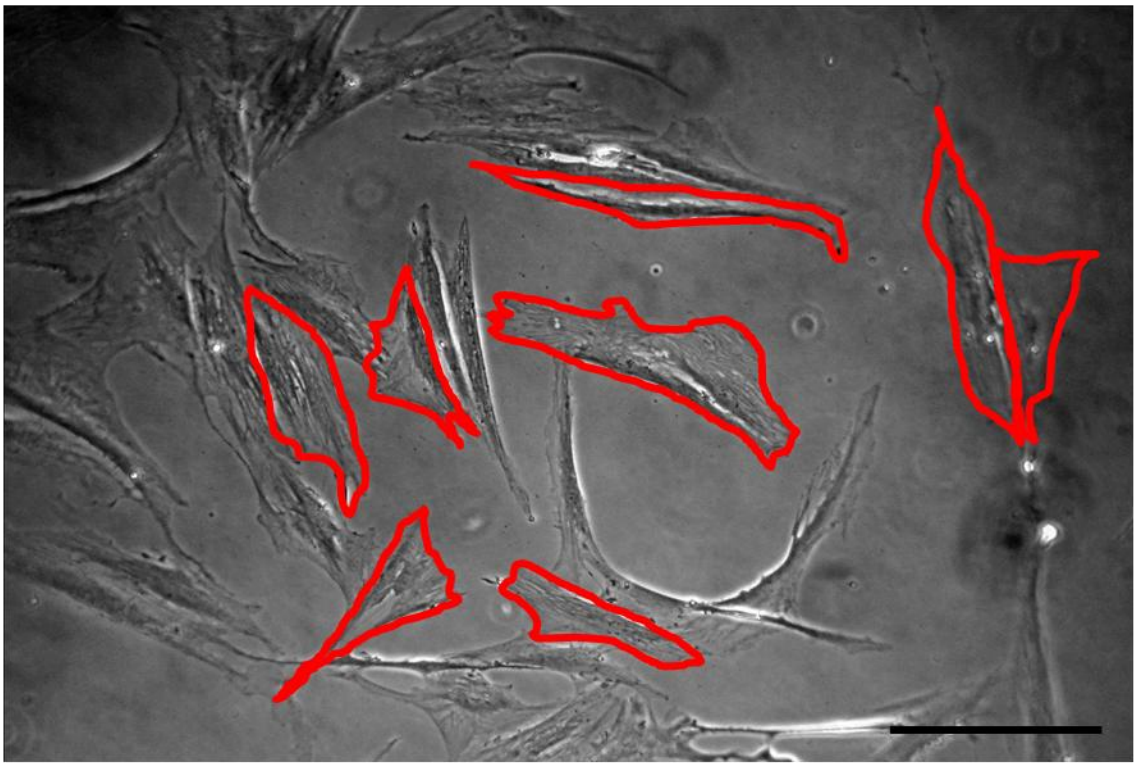

Fig 3.6: Cells size analysis was carried out using the ImageJ software package. Tracing of the cell perimeter and calibration of the software to a haemocytometer grid allowed the calculation of cell area. The perimeter of 50 cells were traced (ImageJ) for analysis using the graphad prism statistical analysis software package. Scale bar = 200µm.

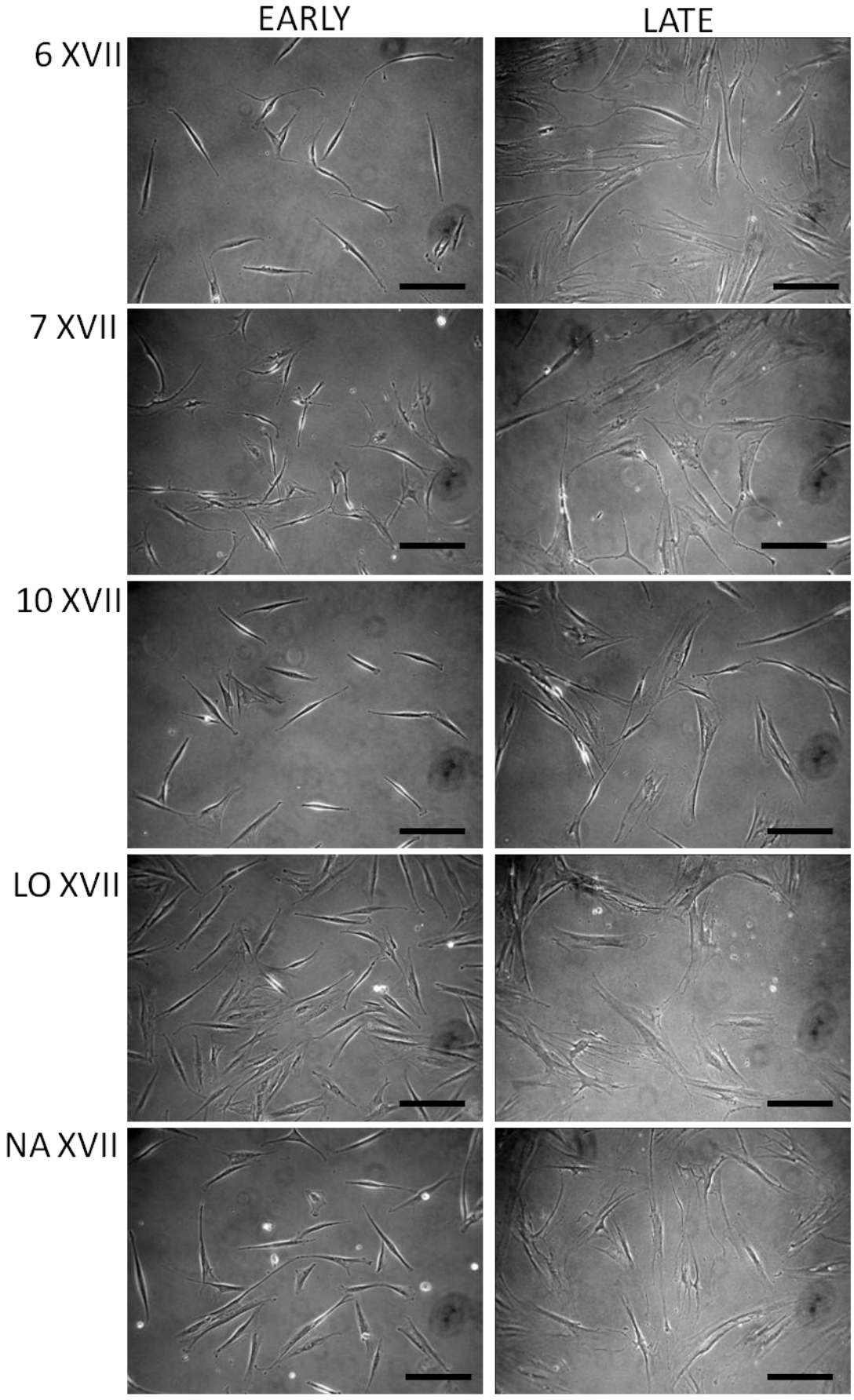

Fig 3.7: Cellular morphology of cell populations from patient XVII at early and late timepoints in culture, to show the differences in morphology associated with *in vitro* ageing. Scale bar = 200µm.

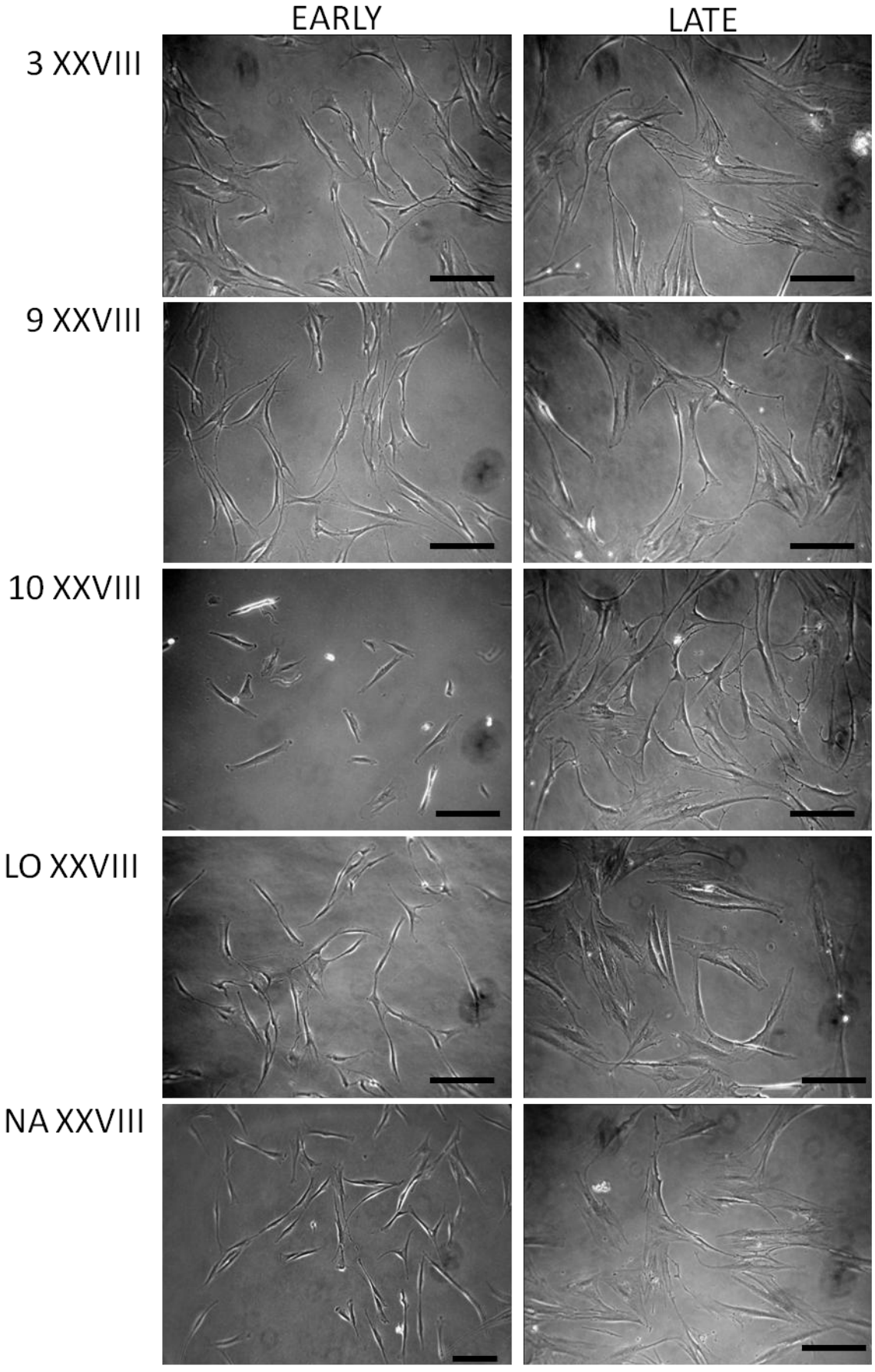

Fig 3.8: Cellular morphology of cell populations from patient XXVIII at early and late timepoints in culture, to show the differences in morphology associated with *in vitro* ageing. Scale bar = 200µm.

The cell size data was analysed for any statistical significance by Kruskal-Wallis non-parametric One-way Analysis of Variance (ANOVA) with a Dunn's post test conducted where $P<0.05$, as data was not normally distributed and therefore failed the assumptions necessary for a parametric statistical test. Graphs were plotted showing the average cell size for both early and late passages of all cell populations from patients XVII and XXVIII (Fig 3.9 and Fig 3.10). All cell populations from patient XVII demonstrated a significant increase in cell size over their replicative lifespan, with the exception of clone 10 XVII which saw no significant increase in cell size during time in culture before undergoing cellular senescence. All these results were highly significant ($P<0.001$), and confirm the visual analysis of morphological changes observed in the cell images. When comparing cell populations from patient XVII at the early passage, there were no significant differences in cell populations however, there were significant differences between cell populations at the late passage, with Clone 6 XVII cells exhibiting a larger cell area than Clones 7 and 10 respectively ($P<0.05$ and $P<0.001$), but not the LO or NA populations.

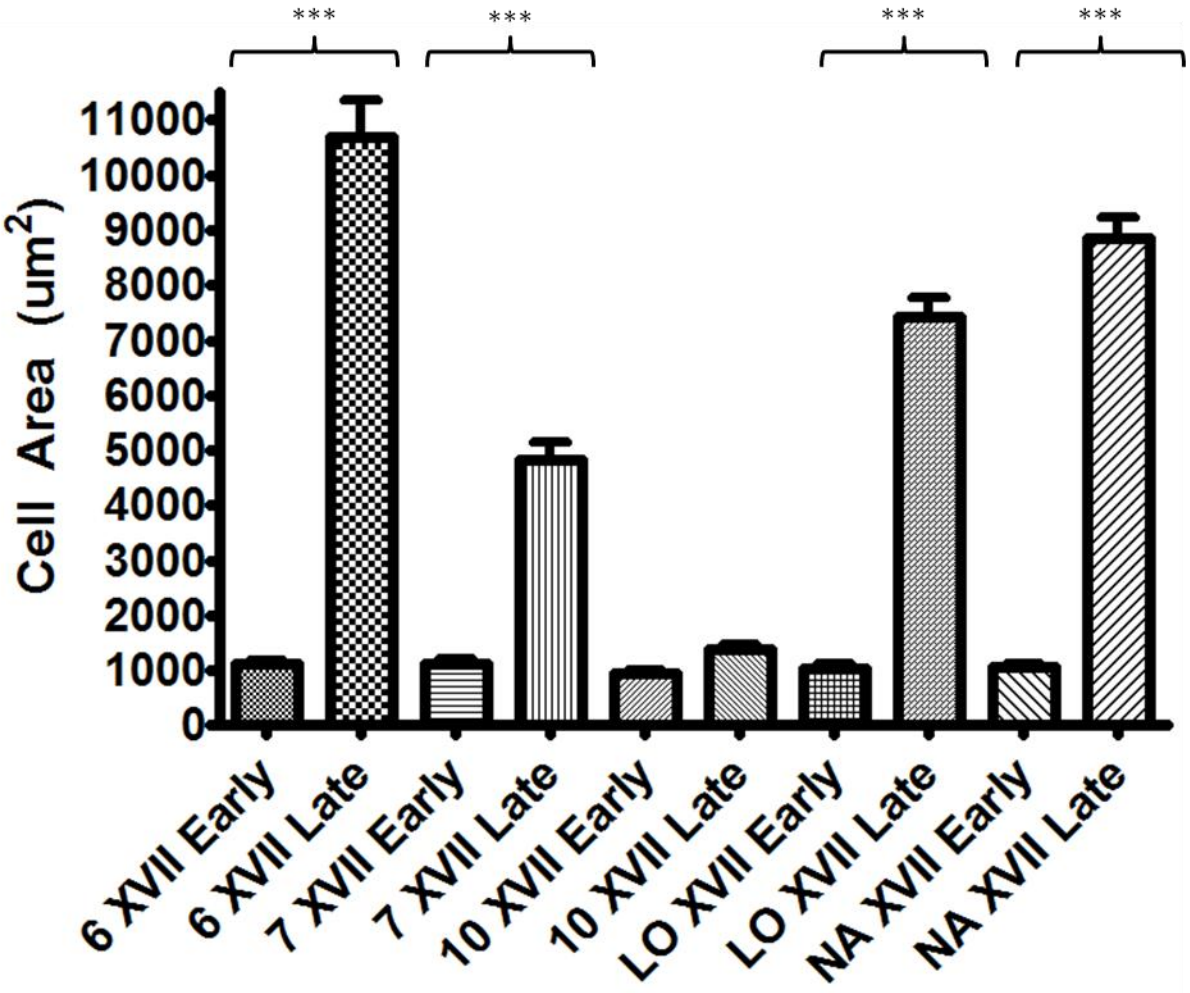

Fig 3.9: Cell morphology analysis data from patient XVII at early and late timepoints, to determine if cell size has any correlation with cellular senescence. Cell area was calculated using ImageJ and the data imported into Graphpad Prism for analysis. n=50 ± SD?. *** = P<0.001

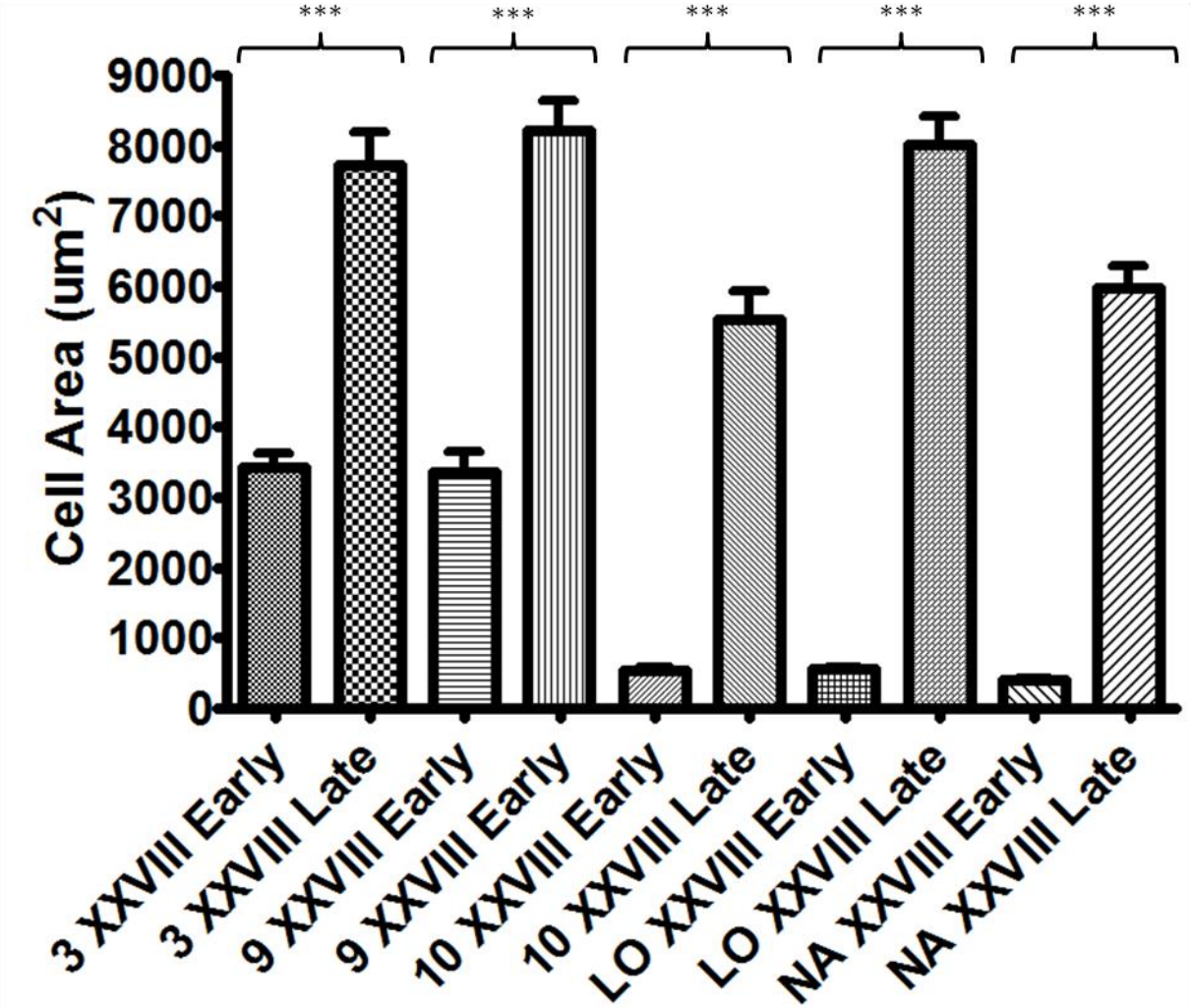

Fig 3.10: Cell morphology analysis data from patient XXVIII at early and late timepoints, to determine if cell size has any correlation with cellular senescence. Cell area was calculated using ImageJ and the data imported into Graphpad Prism for analysis. *n=50* ± SD *** = P<0.001

3.3 Discussion

The aims of this chapter were to isolate multiple cell populations from the Lamina Propria of the Oral Mucosa by differential adhesion to fibronectin, and investigate if any differences in growth kinetics and cellular morphology exist between these different cell populations during long term culture. Previous work within the Wound Biology Group, School of Dentistry, Cardiff University demonstrated the increased replicative potential of oral mucosal fibroblasts *in vitro* (Enoch et al., 2009), a heterogeneous cell population with which no selection by differential adherence prior to culture was conducted. The question of whether clonally expanded cells still exhibit an increased replicative potential is important in understanding the mechanism behind the oral fibroblast's longevity *in vitro*. Changes in cellular morphology with *in vitro* ageing are also important to address the question of whether these isolated cells are stem cells, as changes in cell size have already been observed in MSC populations (Wagner et al., 2008).

Successful isolation of all required cell populations for comparison proved problematic in three of five patients for a number of reasons. Infection of initial cultures from patient XXII lead to the loss of those cells cultured from this patient. The oral cavity has a diverse collection of microflora and while attempts were made to ensure any microbes were sterilised from the lamina propria biopsies prior to tissue disaggregation. However, in this instance it

became apparent that an opportunistic pathogen was able to colonise the culture media preventing growth of any cells for comparison. Patients XXXVI and XXXVIII also failed to produce NA populations and where therefore not

suitable for comparisons between cell populations. The reasons for this observed difference in the ability to form non-adherent cell populations between patients are unclear. Age does not appear to be a factor as patient XXVIII, which successfully produced a NA population and patients XXXVI and XXXVIII, which did not were all born within 2 years, whilst the other successful patient (XVII) was at least 10 years older. Patient to patient variability may be responsible however, proving this would require a much larger data set. Attempts to get another biopsy to bring the number of samples to 3 after the unsuccessful biopsies were not possible in part due to lack of eligible patients attending clinic, and time constraints of obtaining a sample and culturing to senescence meant that it was not possible in the time remaining.

Using the differential adhesion to fibronectin demonstrated a significant number of cells were able to adhere during the first 20 minute incubation compared to the corresponding BSA coated control, and compared to the subsequent 40 minute incubations on both fibronectin and BSA. This can be explained by high levels of functional α5β1 integrin expressed on the surface of those cells which adhere to the fibronectin coated wells after 20 minutes, as the ‘classical’ fibronectin receptor (DeSimone et al., 1992). In the BSA coated controls, adherence occurs via non-specific interactions and will not select for a cell with higher levels of integrin expression, explaining the consistent levels of adherence observed at all timepoints to BSA .

This increased integrin expression has been observed in progenitor cells isolated from articular cartilage (Dowthwaite et al., 2004) however, it is

suggested that higher integrin expression does not provide an indicator of colony forming efficiency of adherent cells. In this study however, those cells which adhere rapidly, perhaps due to higher levels of integrin expression, demonstrated a higher colony forming efficiency. Quantification of integrin expression would be a useful addition to the current data to determine if the integrin expression is truly responsible for the adherence characteristics, and a potential marker of those cells with a high replicative potential.

The growth kinetics of both patients observed demonstrated similar kinetic profiles. In both patients the clonally derived cell populations underwent a brief phase of high proliferation, achieving 7/10 population doublings in 16/13 days for patient XVII and patient XXVIII respectively. This rapid burst in proliferation is probably due to the clonal nature of these cell populations and the high number of cell divisions to achieve a cell population similar in size to those observed in the NA/LO populations. Interestingly in both patients clone 10 did not follow the same kinetic profile as the other clones, in the case of patient XVII only reaching 35 population doublings before entering cellular senescence. These clones were both initially frozen after two passages, as each patient generated more clonally derived cell populations than required and only three clones were selected to be culture until senescence. However, when one of the clones selected in each patient senesced early, suggesting they were transit amplifying cells (Bluteau et al., 2008), clones 10 XVII and XXVIII were thawed out and cultured as replacements for these senescent cell populations, and this may be responsible for the retarded growth kinetics of these cell populations. In the instance of clone 10 XXVIII however, it was

able to still achieve in excess of 50 population doublings whereas clone 10 XVII was only able of achieving 35 population doublings. Notably this is still higher than reported for some MSC populations where only 27 population doublings were achieved (Bieback et al., 2004). With the exception of clones 10 XVII and XXVIII, this differential adhesion and clonal selection assay demonstrated a method by which higher proliferative cells with replicative potential similar or greater than MSC populations can be successfully isolated. This clonal selection allows for a pure cell population uncontaminated with transit amplifying and terminally differentiated cells, and this has been shown to increase the differentiation potential (Kotobuki et al., 2004). After this initial phase the clonal population growth rates decreased until reaching a rate similar to that observed in the NA population. The LO population however, maintained consistent growth kinetics throughout their replicative lifespan, similar to those observed previously within the Wound Biology Group's reports on oral fibroblast replicative potential (Enoch et al., 2009). The heterogeneous nature of this cell population may enhance the replicative potential of the resident stem cell population by allowing them to remain quiescent and only dividing when those terminally differentiated cells become senescent and effectively 'drop out' of the proliferating population. This may explain the higher number of population doublings achieved before senescence was observed within the LO populations of both patients. Comparisons of the average growth rates for cell populations show an interesting phenomenon. In both patients the average growth rate of the clonal populations was lower than the NA populations, which in turn were lower than the LO population. This is interesting given the high growth rates

in the clones during early passages, meaning the LO/NA populations are consistently achieving a higher growth rate over the lifespan of these cultures, compared to the clonal populations.

During the culture life of these cells routine Mycoplasma testing was conducted to detect and subsequently eradicate any infections. Mycoplasma has been shown to alter the behaviour of cell cultures (Hendershot and Levitt, 1985, Levine et al., 1968) and MSC have been found to be efficient vectors of Mycoplasma infection (Zinocker et al., 2011). During this routine screening all cell populations from patient XVII were found to be positive for Mycoplasma as determined by the non-nuclear localised staining with Hoechst fluorescence dye. Cells were immediately quarantined from all Mycoplasma negative cells and treated with tetracycline derivative antibodies as recommended by the manufacturer until testing demonstrated nuclear only staining, and therefore Mycoplasma contamination was eradicated. This allows the conclusion that all of the subsequent analysis of these cell populations is a result of the behaviour of the cells in question and not an artefact of Mycoplasma contamination.

The final analysis undertaken on cell populations was to determine the effect of long term culture and ensuing senescence on the cell morphology/cell area. When cells undergo replicative senescence, their morphology changes as reported for skin fibroblasts and MSC (Gorbunova et al., 2003, Wagner et al., 2008, Stephens et al., 2003, Baxter et al., 2004). With the exception of Clone 10 XVII, all cell populations demonstrated a significant increase in cell area over time in culture, with cell morphology changing from a classical

fibroblastic, spindle bi-polar morphology to a larger, more flattened morphology with an increase in cellular processes. The replicative potential of the cell populations appears to have no bearing on the cell size at senescence, but with data for only two patients any conclusions drawn are limited; the analysis of populations from a larger number of patients may highlight the importance of patient variability or identify a trend within the data which cannot be elucidated at present in this thesis.

The data presented in this chapter demonstrates that the differential adhesion assay allows for the selection of clonally derived cell populations which have a high replicative potential, and demonstrate distinct growth characteristics when compared to more heterogeneous unselected cell populations. This selection may be due to the higher level of α5β1 integrin expression on the surface of cells allowing for more rapid adherence during this assay (Jones and Watt, 1993, DeSimone et al., 1992). Without further analysis of integrin expression to determine if these cells do show differential expression of integrins, it is not possible to draw conclusions on this. With some exceptions, all cell populations were able to exceed 50 population doublings *in vitro*, higher than those observed in normal somatic cells and in particular MSCs cells (Baxter et al., 2004, Banfi et al., 2000, Bruder et al., 1997, Hayflick, 1965). This suggest that all cell populations have a high capacity for self renewal, and that in the instance of the lamina propria this may be a characteristic of all cells resident within the tissue, not just of stem cells. Changes to cell morphology and the associated increase in cell area follow the characteristics of both fibroblasts and stem cell populations and

suggest that the cell populations isolated from the oral mucosa behave in a traditional manner. At this stage without further characterisation, the nature of these populations and the presence of stem cells are uncertain, and hence the characterisation and differentiation studies within the next chapters will address these issues.

CHAPTER 4

The characterisation of cell populations isolated from the oral mucosa lamina propria

4.1 Introduction

The ethical and moral issues surrounding the use of pluripotent embryonic stem cells (ESCs) have led to an increased level of interest in adult stem cell populations (ASCs). To date, these adult stem cells have been identified from multiple sites within the body however, those cells which have been most characterised remain those first identified, namely bone marrow stromal cells (BMSCs) (Yoo et al., 2005, Wagner et al., 2005, Quirici et al., 2002, Oliveira et al., 2009, Mareschi et al., 2006, Mareddy et al., 2007, Ishii et al., 2005, Friedenstein et al., 1970). Adult stem cells have been successfully isolated from a number of other tissues including oral tissues (Otaki et al., 2007a, Ikeda et al., 2006, Gronthos et al., 2002, Webb et al., 2004, Toma et al., 2005, Toma et al., 2001, Fernandes et al., 2004, Chunmeng et al., 2004, Nakamura et al., 2007, Izumi et al., 2007, Calenic et al., 2010). Characterisation of these isolated stem cell populations often includes immunophenotyping of cell populations for the presence of specific cell surface markers such as CD90, CD105, CD166 and STRO-1 (Table 1.3, Chapter 1.7.2) and the absence of markers such as CD34 and CD45 to ensure that those cells are not of haematopoietic or fibrocyte origin (Nagatomo et al., 2006, Pasquinelli et al., 2007, Song et al., 2005, Diaz-Romero et al., 2005).

The expression of active telomerase is also suggested as a stem cell marker, with telomerase expression well characterised in embryonic stem cells (Heins et al., 2004, Thomson et al., 1998). It is also implicated in a large number of cancers (Herbert et al., 2001, Kishimoto et al., 1998, Poremba et al., 2000, Tatsumoto et al., 2000). The subject of adult stem cells and telomerase activity is at present unclear, with reports of telomerase activity in adult stem cells reported by groups contradicted by the findings of others. A sub-population of haematopoietic progenitors from adult human bone marrow has been shown to possess telomerase activity *in vitro* (Chiu et al., 1996). Telomerase activity has also been observed in human neural precursor cells (Ostenfeld et al., 2000). However, no telomerase activity has yet been found in bone marrow stromal cells (Fehrer and Lepperdinger, 2005, Zimmermann et al., 2003).

Telomerase is an enzyme responsible for the maintenance of telomeres, the large regions of repetitive DNA at the ends of chromosomes. The lack of telomerase is associated with premature ageing; the erosion of telomeres *in vitro* is one mechanism responsible for replicative senescence (von Zglinicki, 2002, Harley et al., 1990, Baxter et al., 2004, Shay and Wright, 2007). Work carried out within the Wound Biology Group has previously shown that oral fibroblast lifespan is telomerase independent (Enoch et al., 2009). Current work into the isolation of stem cells from the Lamina Propria has demonstrated that colonies formed from single cells using differential adhesion express telomerase by immunocytochemistry and this in part explains the ability of these cells to rapidly proliferate and form colonies *in vitro* (Davies et al., 2010).

The aim of this chapter is to confirm the presence of stem cell associated cell surface markers by flow cytometry, to confirm the phenotype of these isolated cell populations conform to that observed in other adult stem cell populations within the literature. Secondly, the quantification of telomerase activity will allow comparisons between cell populations and determine if clonal expansion enriches a cell population for increased telomerase activity. The final analysis of this chapter will be to determine the level of telomere erosion occurring with *in vitro* ageing within the different cell populations. All of this analysis will be conducted to determine if differential adhesion enriches the clonal cell populations for these stem cell associated markers, or whether an unselected, heterogeneous cell population is phenotypically similar in terms of telomerase, protein expression and telomere maintenance.

4.2 Results

4.2.1 Flow cytometry detection of stem cell associated markers

Cells were analysed for expression of stem cell markers CD90, CD105 and CD166 and negative markers CD34 and CD45 as described in the materials and methods (Chapter 2.2) at the earliest passage possible to generate a cell number sufficient for the experiment. In the Clone populations, the cells were used at passage 4 (~20 PD), with population doubling matched LO/NA cells. These markers were selected due to their consistency among the scientific literature, where an abundant number of markers are used to determine the presence of a stem cell population. Fig 4.1-Fig 4.10 demonstrates the expression of these markers in each cell population by flow cytometry, where the red histogram peaks represent mean fluorescence intensity (MFI) of the isotype control antibody, and the blue histogram represents the mean fluorescence of the corresponding cell surface marker. In each figure, graph F represents the morphological characteristics defined by the forward and side scatter (FSC and SSC). This graph determined the gating strategy for subsequent analysis of marker expression. Typically, the mean fluorescence of CD34 and CD45 after isotype control subtraction was negative, indicating that those cells were negative for those markers. However in three instances (10 XXVIII, LO XXVIII and 6 XVII) demonstrated a very small shift in mean fluorescence even after isotype subtraction in both CD34 and CD45, with 6

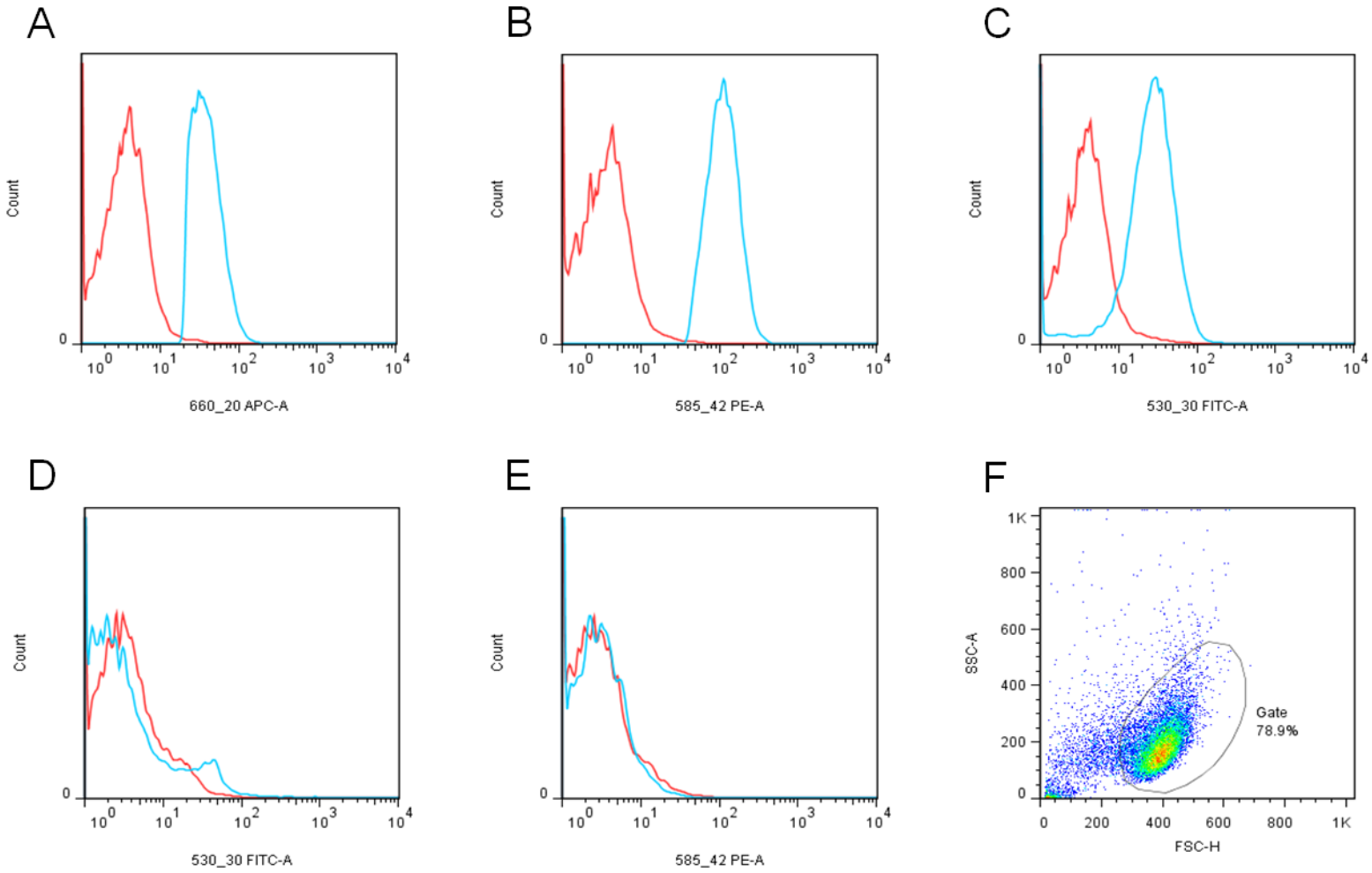

Fig 4.1: Flow Cytometry data from patient XVII, Clone 6. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

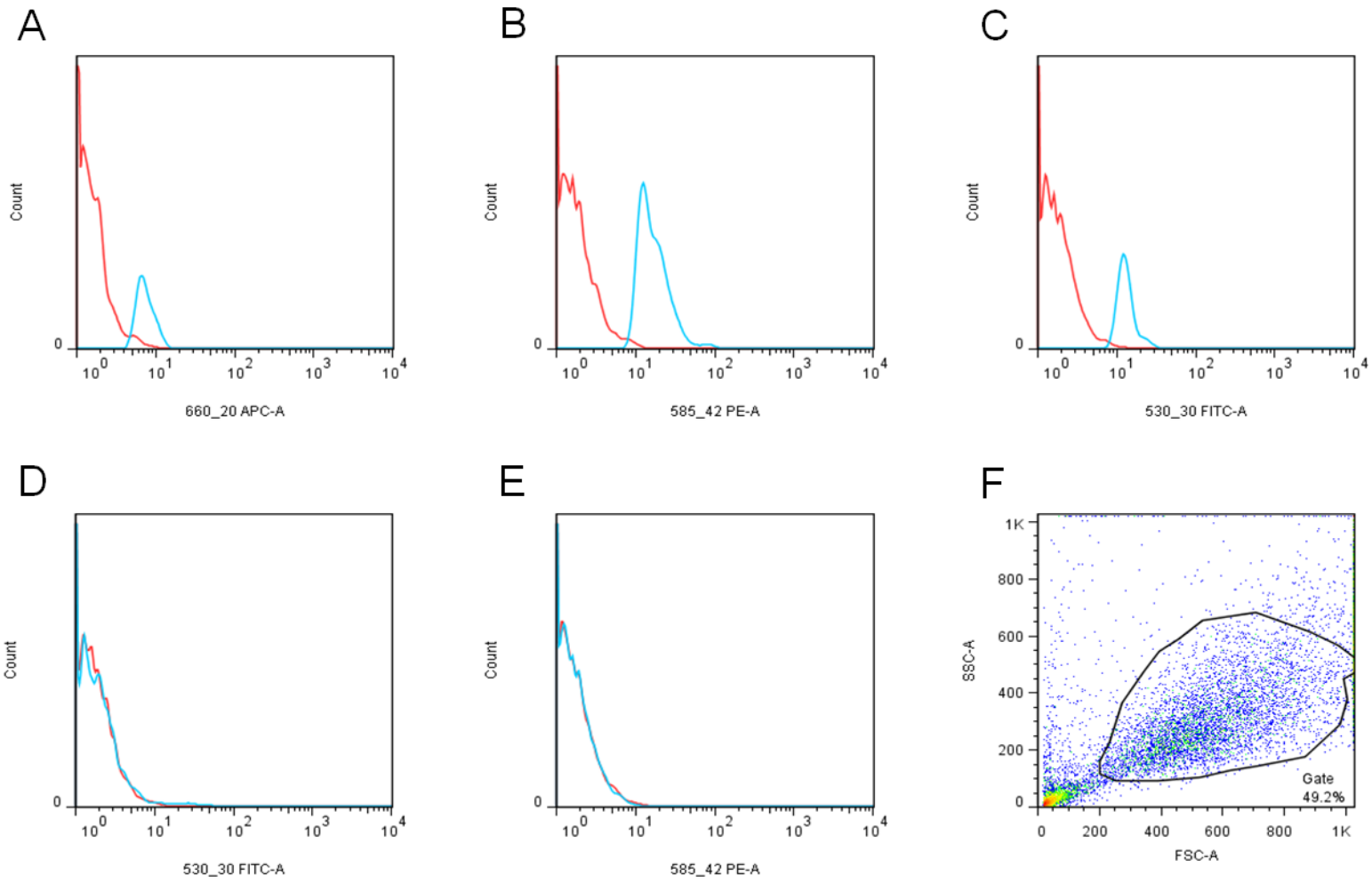

Fig 4.2: Flow Cytometry data from patient XVII, Clone 7. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

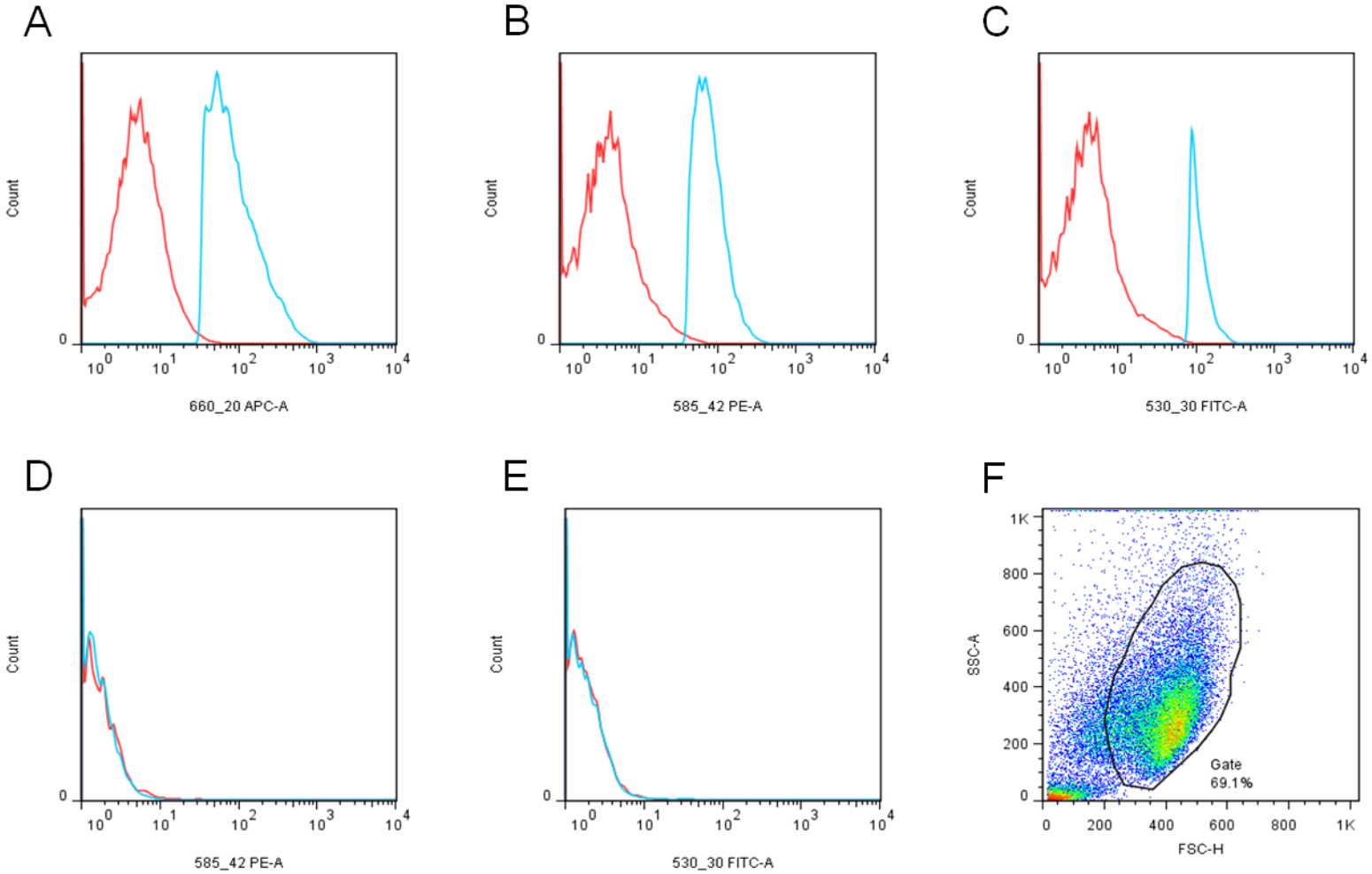

Fig 4.3: Flow Cytometry data from patient XVII, Clone 10. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

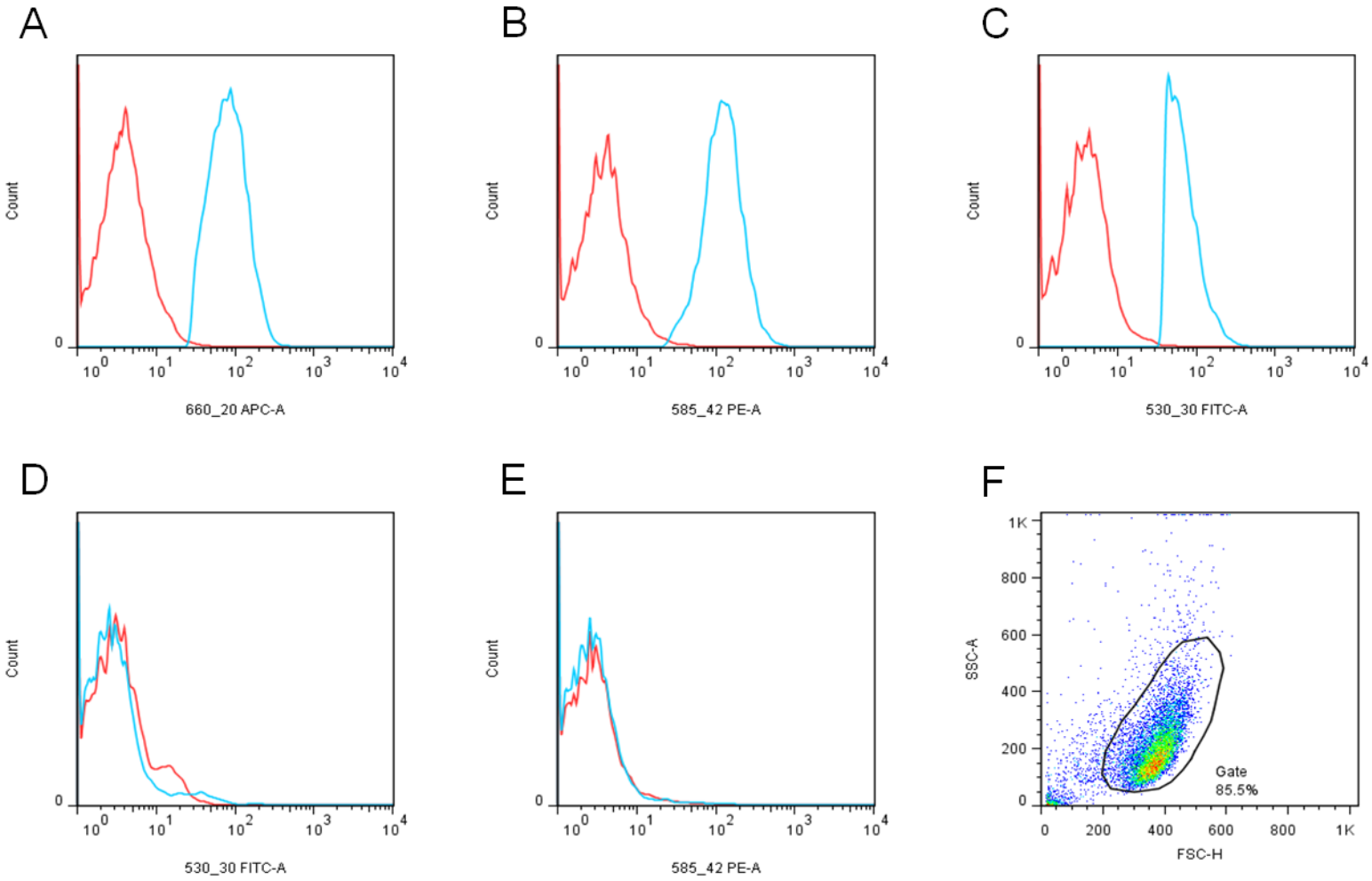

Fig 4.4: Flow Cytometry data from patient XVII, LO. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

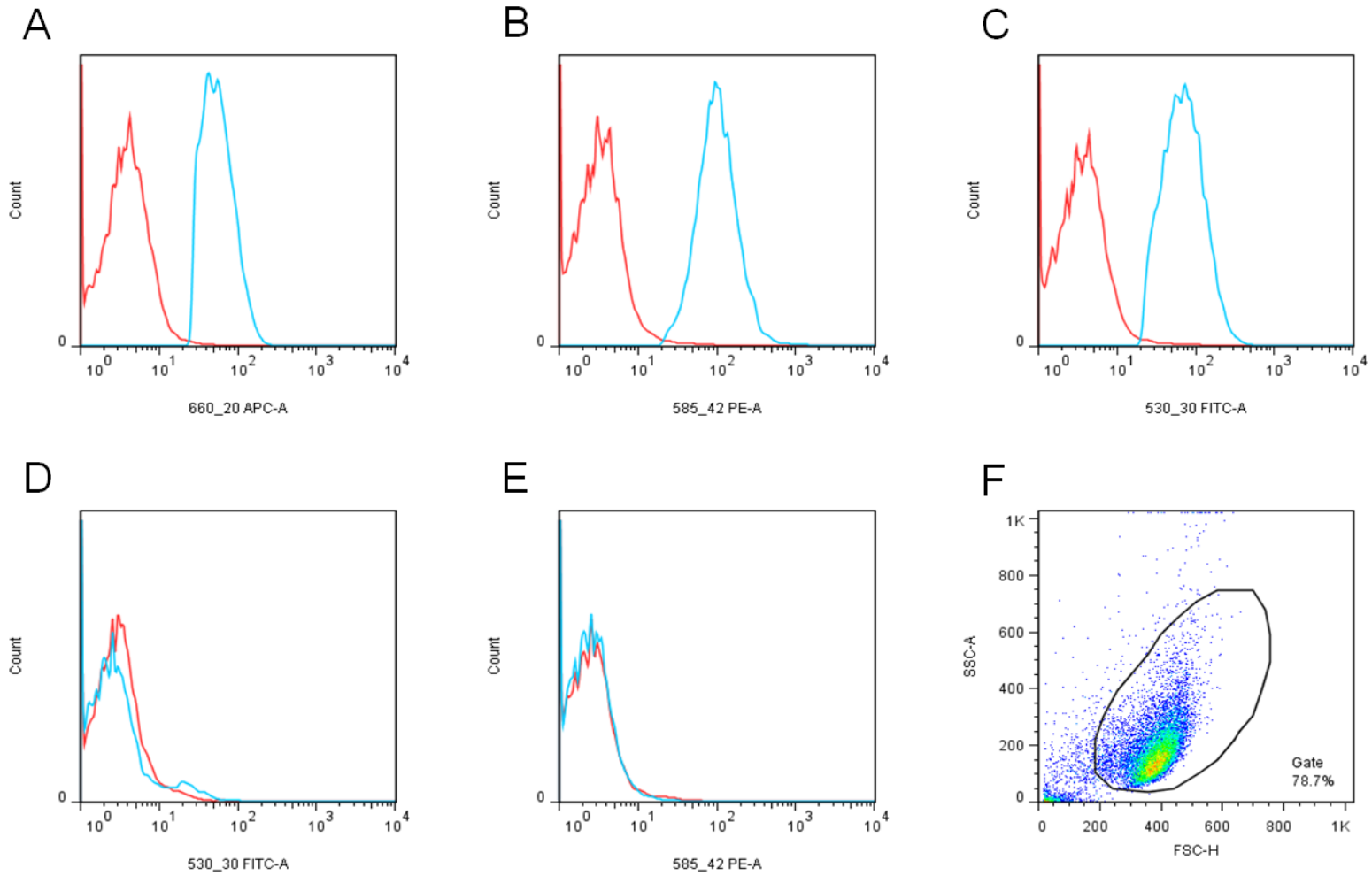

Fig 4.5: Flow Cytometry data from patient XVII, NA. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

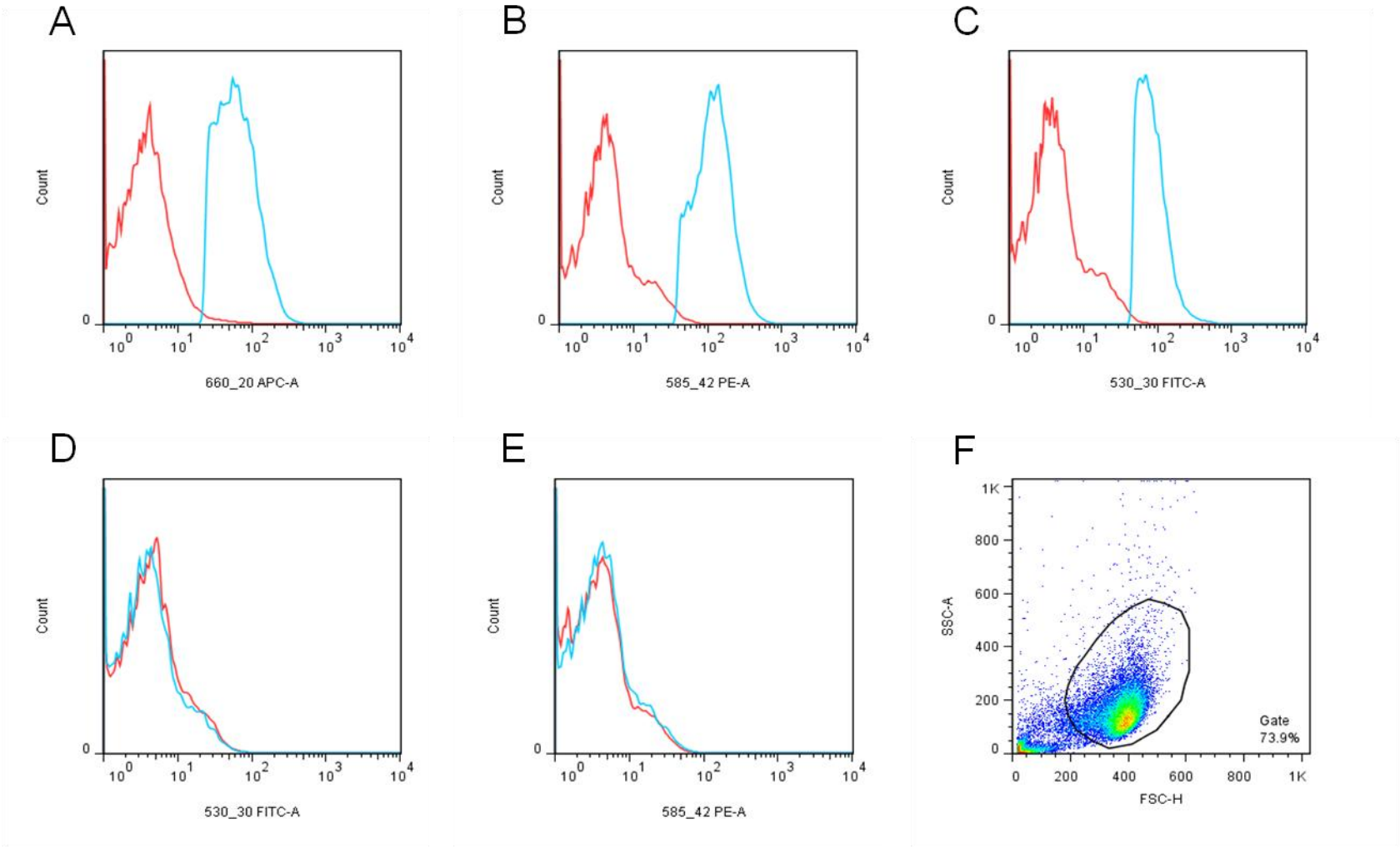

Fig 4.6: Flow Cytometry data from patient XXVIII, Clone 3. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

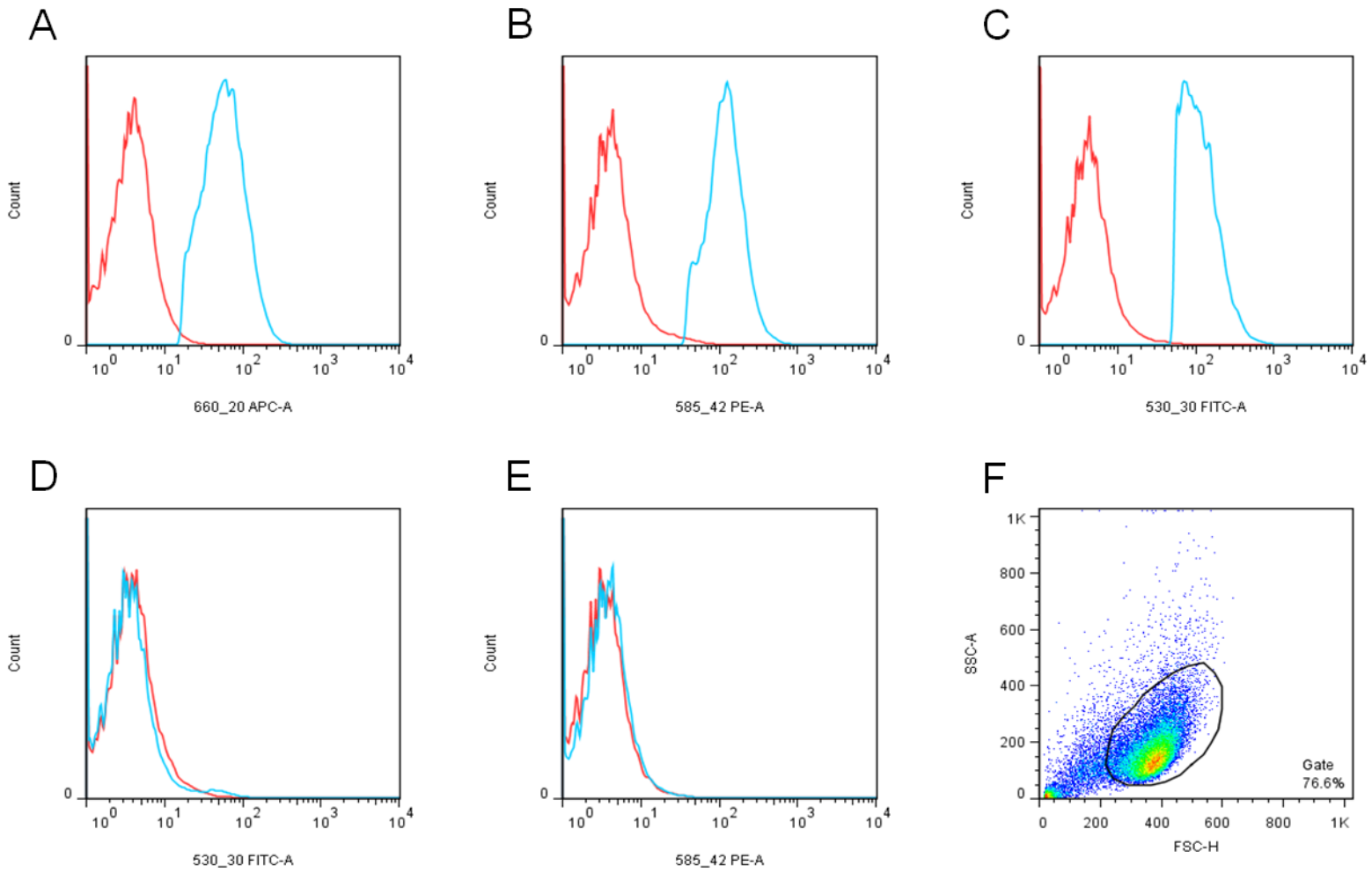

Fig 4.7: Flow Cytometry data from patient XXVIII, Clone 9. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

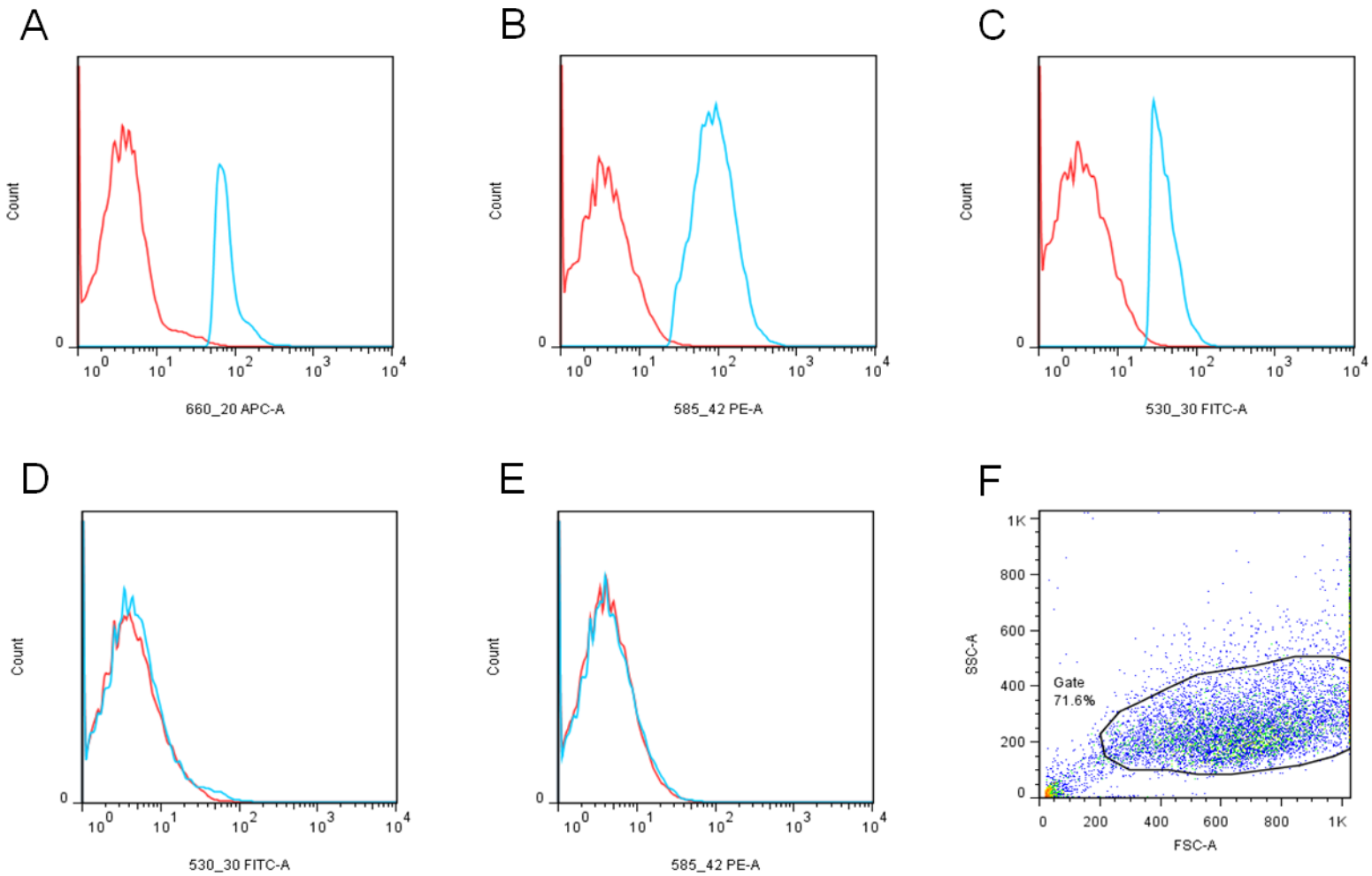

Fig 4.8: Flow Cytometry data from patient XXVIII, Clone 10. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

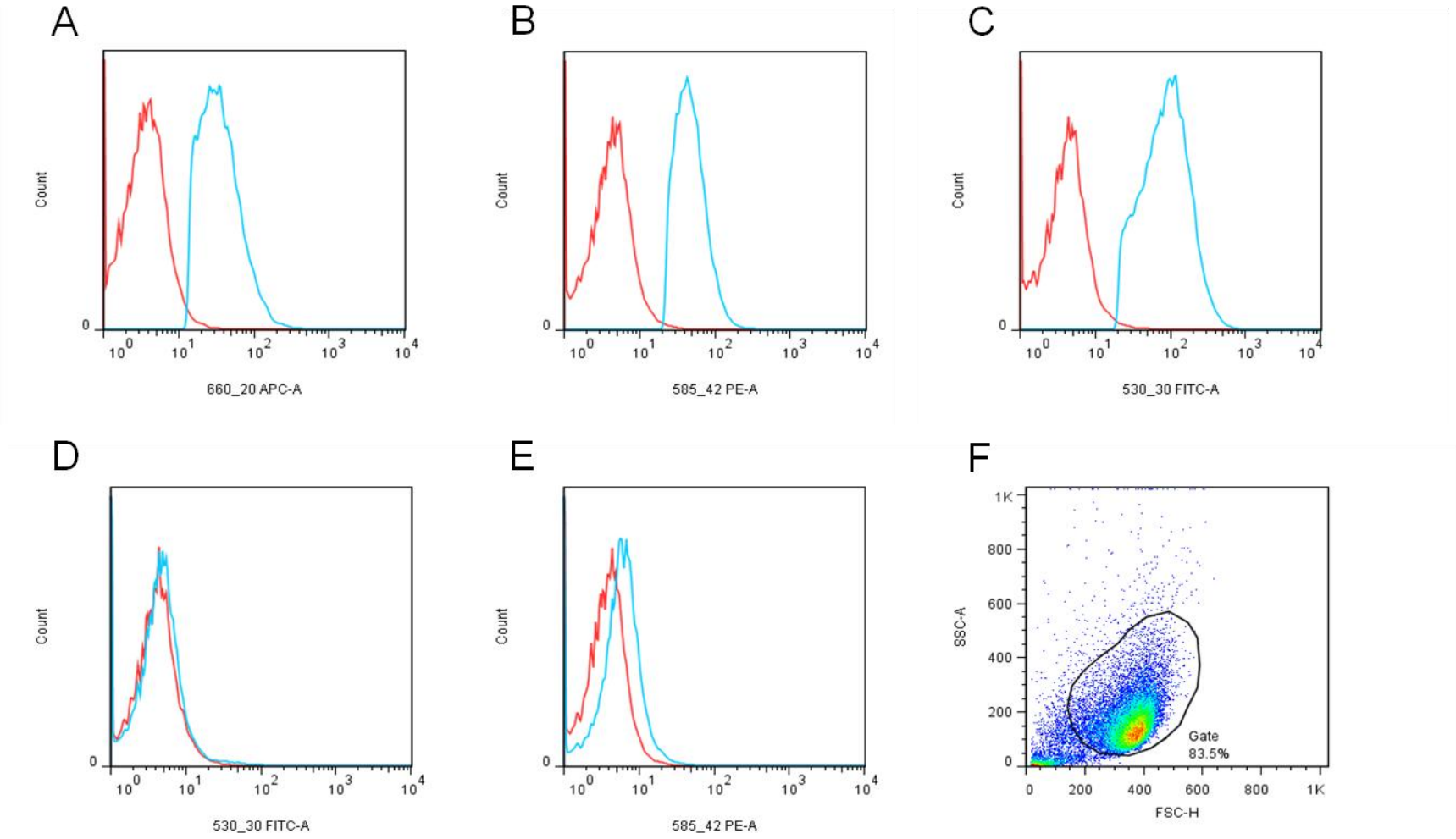

Fig 4.9: Flow Cytometry data from patient XXVIII, LO. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

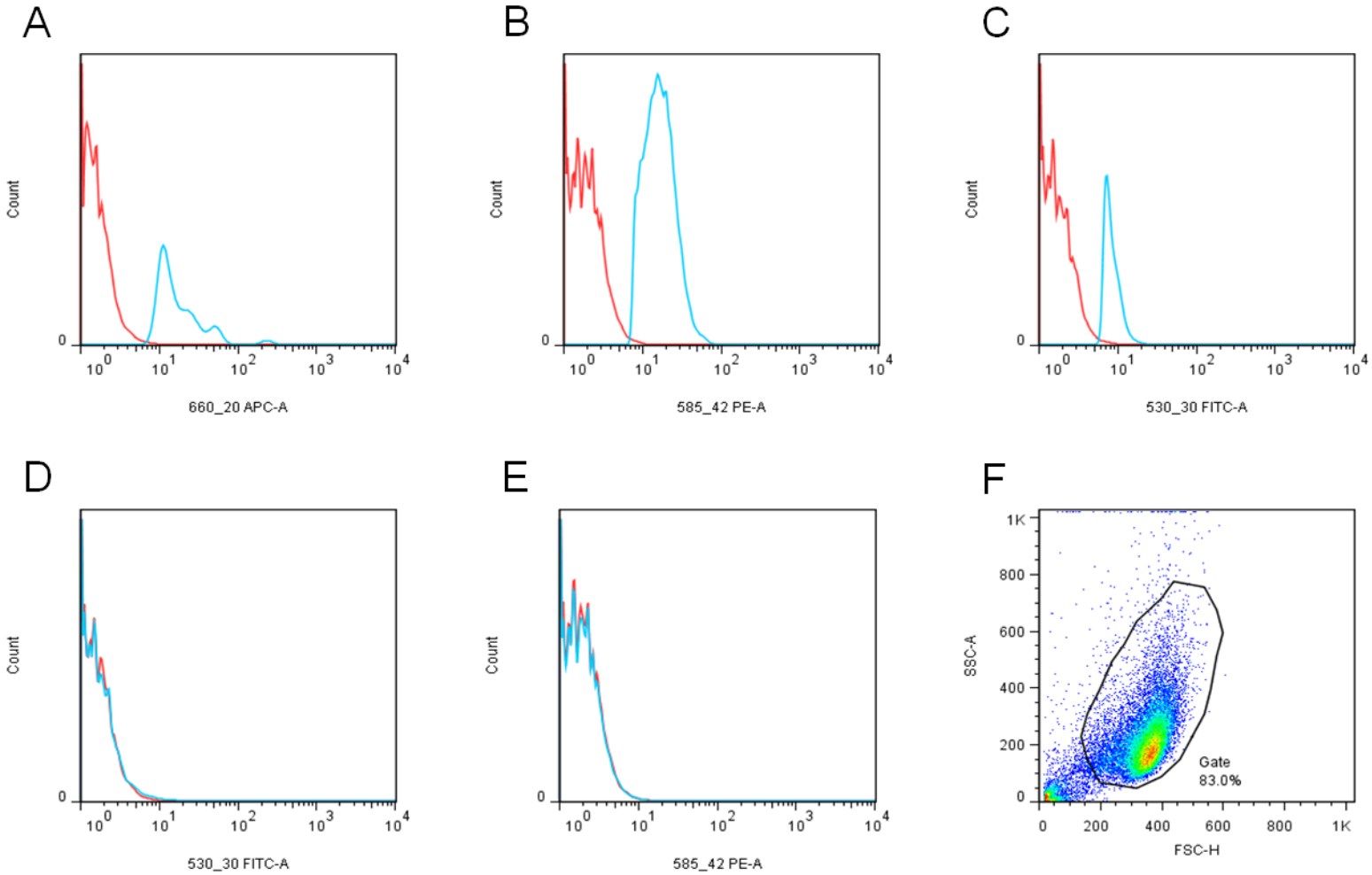

Fig 4.10: Flow Cytometry data from patient XXVIII, NA. Cells were analysed for the presence of (A) CD90, (B) CD105, (C) CD166, (D) CD34, (E) CD45. Morphology characteristics (Forward scatter vs Side scatter) demonstrating the gating strategy employed for subsequent analysis.

	CD34	CD45	CD90	CD105	CD166
3 XXVIII	-0.11	-0.18	51.08	141.7	75.92
9 XXVIII	-0.44	-0.78	45.94	84.54	98.21
10 XXVIII	1.58	0.76	68.37	100.12	37.83
LO XXVIII	0.9	7.86	25.37	58.91	96.36
NA XXVIII	-0.01	-0.03	4.13	3.82	6.54
6 XVII	3.72	1.34	37.9	114.67	63.04
7 XVII	0.03	-0.02	6.26	16.77	11.8
10 XVII	-0.13	-0.15	60.91	76.88	69.56
LO XVII	-0.01	0.06	79.5	108.8	68.21
NA XVII	0.17	-1.08	75.52	124.46	93.3

Table 4.1: Mean fluorescence intensity of cell populations from patients XVII and XXVIII. Positive markers CD90, CD105 and CD166 were determined to represent a stem cell phenotype, while CD34 and CD45 were used for the elimination of haematopoietic and fibrocytic phenotypes. These values represent isotype subtracted values from the tested antibodies. All cell populations were positive for all three positive markers, however there were differences between patients, and between cell populations.

XVII showing the highest shift in CD34 (3.72) and LO XXVIII recording a fluorescence shift of 7.86 (CD45). The analysis of CD90, CD105 and CD166 demonstrate different expression profiles between patient XVII and XXVIII. Patient XVII demonstrated higher MFIs for CD90 in the LO and NA populations (79.5 and 75.5 respectively) than observed in the clonal populations, with clones 6, 7 and 10 giving MFIs of 37.9, 6.26 and 60.91 respectively. Interestingly, the corresponding MFIs in patient XXVIII were the inverse, with the clonal populations having MFIs of 51.08, 45.4 and 68.37 for clones 3, 9 and 10 XXVIII respectively; which were higher than LO and NA populations for CD90, at 25.37 and 4.13 respectively. The MFI for CD105 from patient XVII again demonstrated higher MFIs in the LO and NA populations than the clonal populations with MFIs of 108.8 and 124.46, with the exception of 6 XVII which had an MFI similar to the LO and NA populations, at 114.67. In patient XXVIII the MFI for CD105 were higher in the clonal populations than in the LO and NA populations. The final CD marker, CD166 demonstrated variable expression in both patients, with the highest MFI in patient XVII observed in the NA population at 93.3, and the lowest at 11.8 in 7 XVII. The MFI for 6, 10 and LO populations from patient XVII were all similar, at 63.04, 69.56 and 68.21 respectively. In patient XXVIII, the highest MFIs were in 9 XXVIII and LO XXVIII (98.21 and 96.36) with the lowest MFI observed in the NA population at 6.54. It is worth noting that the MFI in the NA XXVIII population for all positive markers is very low by comparison to all other populations (Table 4.1).

4.2.2 Quantification of telomerase activity in cell lysates using the QTRAP assay

To determine the presence of telomerase within cell lysates, the telomeric repeat amplification protocol was initially utilised. However the technique lacked the sensitivity to detect low levels of telomerase activity which may be present in heterogeneous cell populations such as the LO and NA populations, and did not detect telomerase in all clonally derived cell populations (see Appendix Fig 8.1 and 8.2). Instead, a quantitative PCR based detection method based on the SybrGreen reagents was implemented, known as the QTRAP assay (Wege et al., 2003). The method is described in full in the materials and methods (Chapter 2.2.6). Cell lysates were analysed in triplicate at early, mid-stage and late passages to elucidate and changes in telomerase activity during the replicative lifespan of the cell populations in vitro. Data was analysed using one way ANOVA with a Tukey's *ad-hoc* test where $p<0.05$.

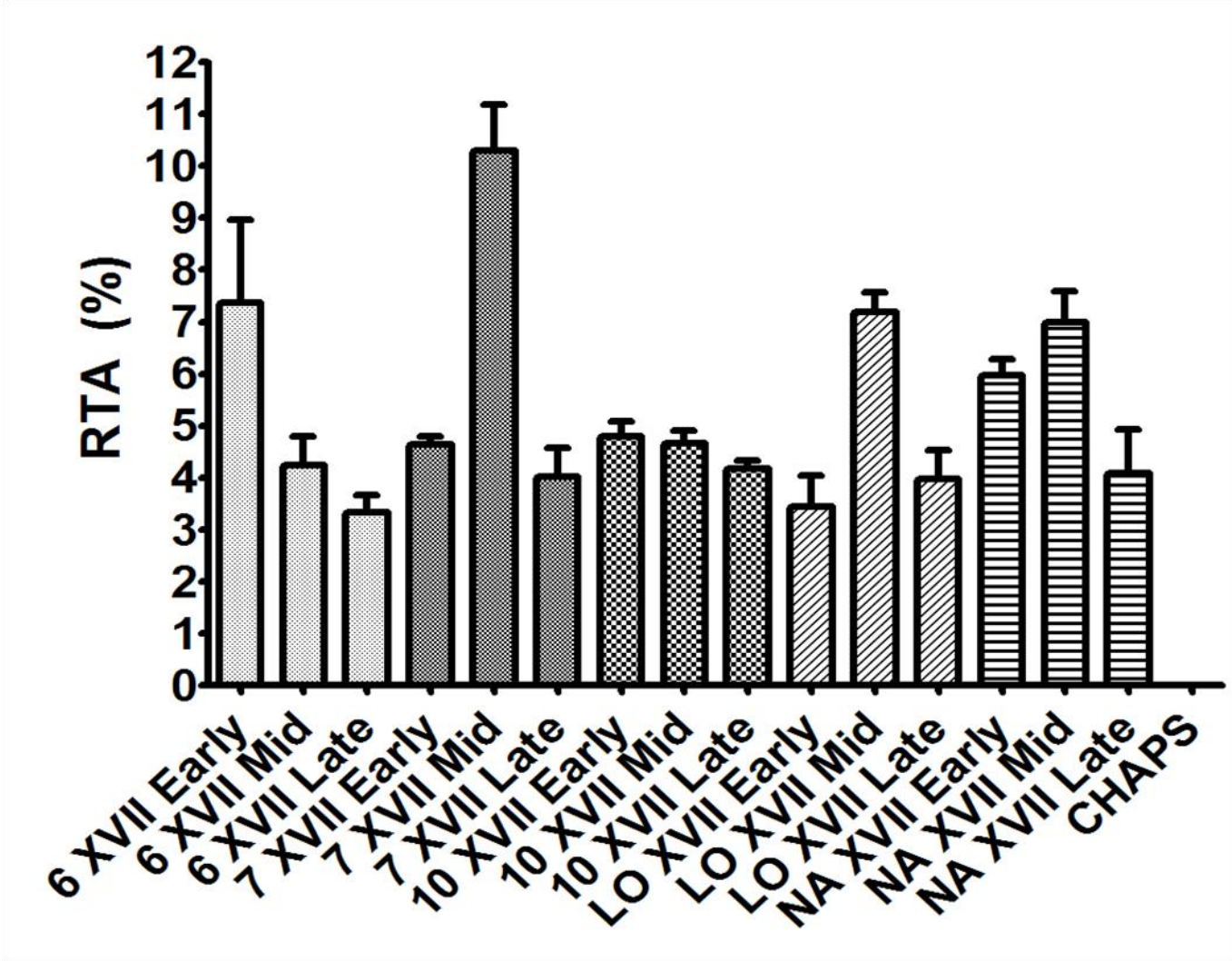

Fig 4.11: Relative telomerase activity (%RTA) compared to a hTERT positive, 501 melanoma cell line for cell populations from patient XVII. Cell lysates from early, mid, and late timepoints were taken from cell populations from patient XVII and assayed for telomerase activity. N=3 ± SD

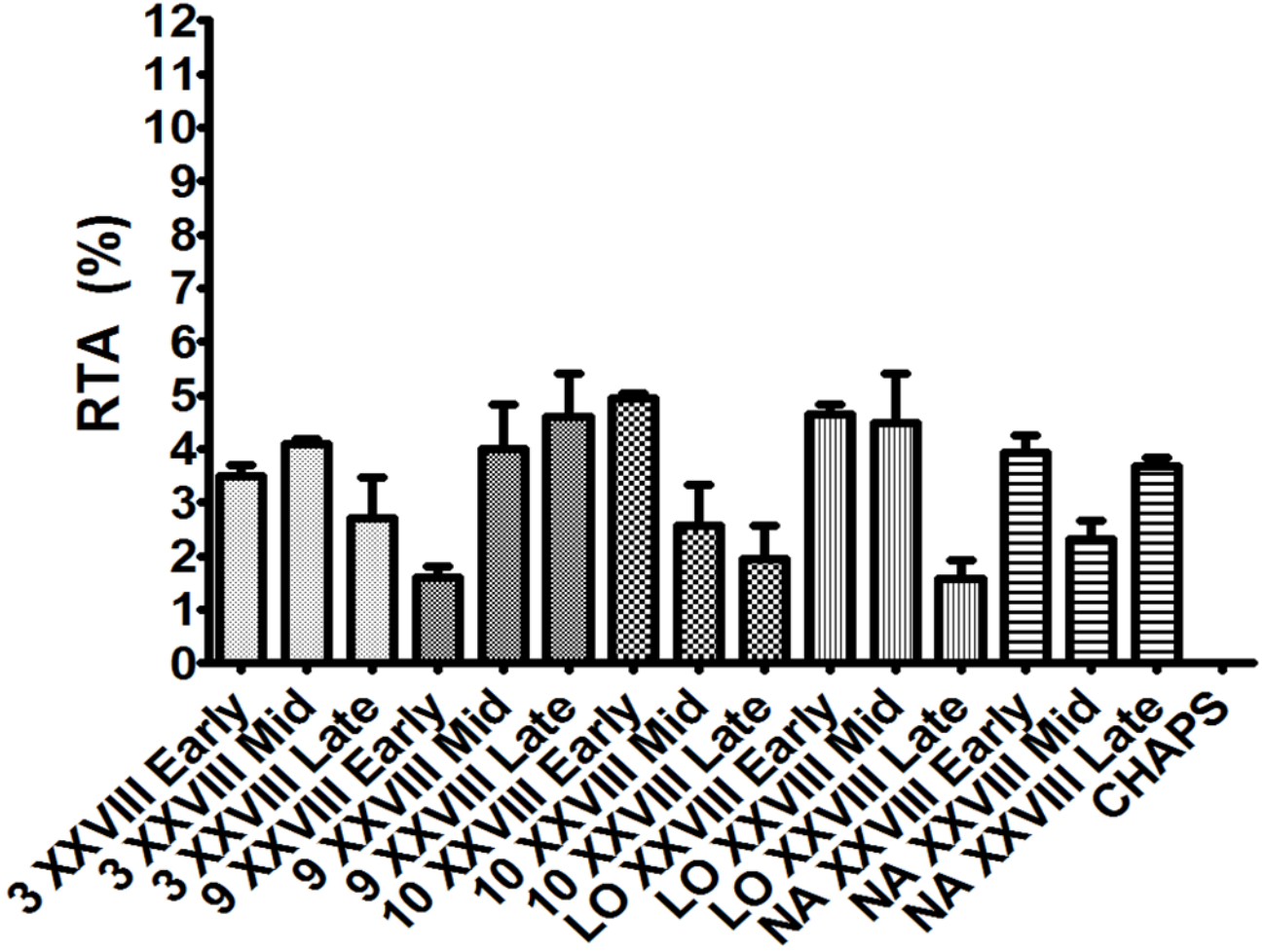

Fig 4.12: Relative telomerase activity (%RTA) compared to a hTERT positive, 501 melanoma cell line for cell populations from patient XXVIII. Cell lysates from early, mid, and late timepoints were taken from cell populations from patient XVII and assayed for telomerase activity. N=3 ± SD

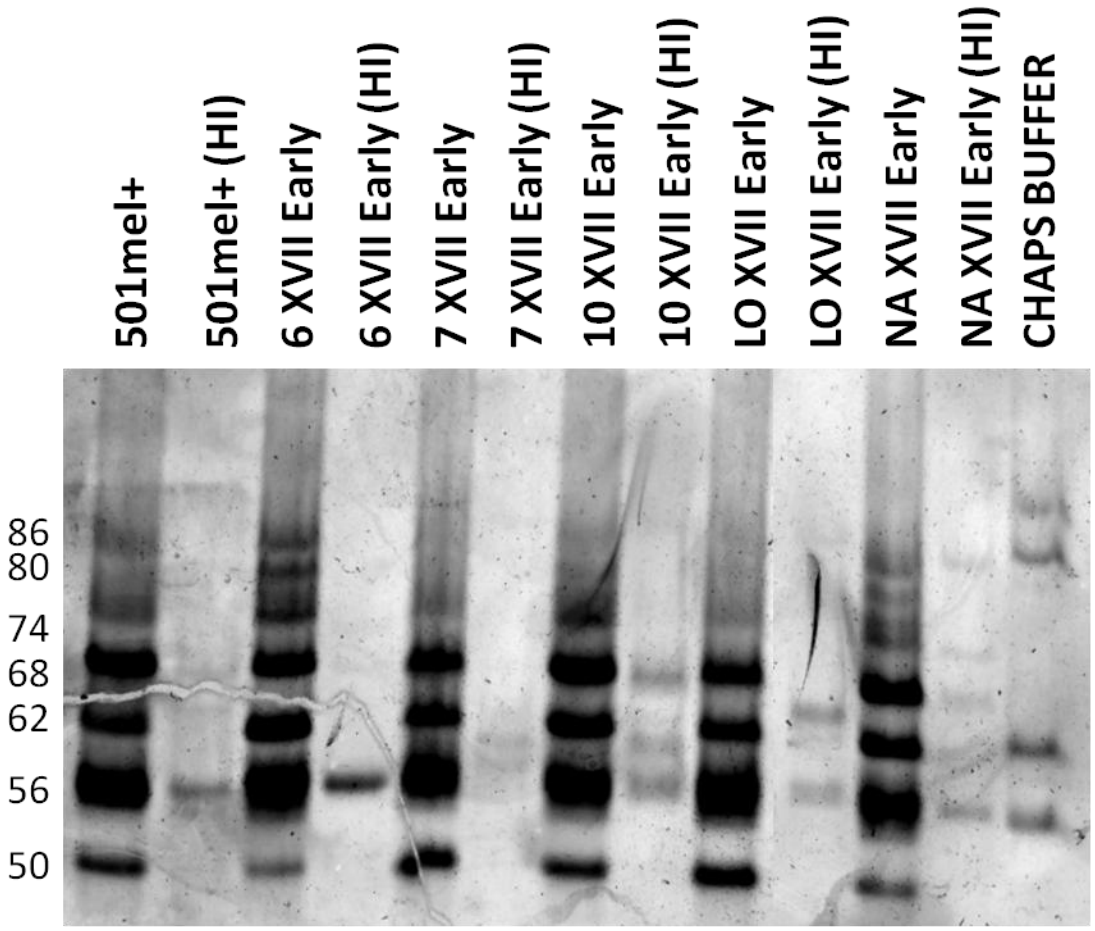

Fig 4.13 Polyacrylamide gel showing the results of the a QTRAP assay in patient XVII, showing the functionality of the assay. Products for the early timepoints of patient XVII cell populations were resolved on a 4% polyacrylamide gel for 2 hours and stained with SybrGold before acquiring an image on the Typhoon Imager. The strong banding corresponds to the positive signals observed in the data, however some faint banding is still observed in heat inactivated and CHAPS buffer only controls. This banding does not correspond to a detectable signal during the assay. Bands represent repeating 6bp extension of telomeric substrate primer from 50bp as shown on left of gel.

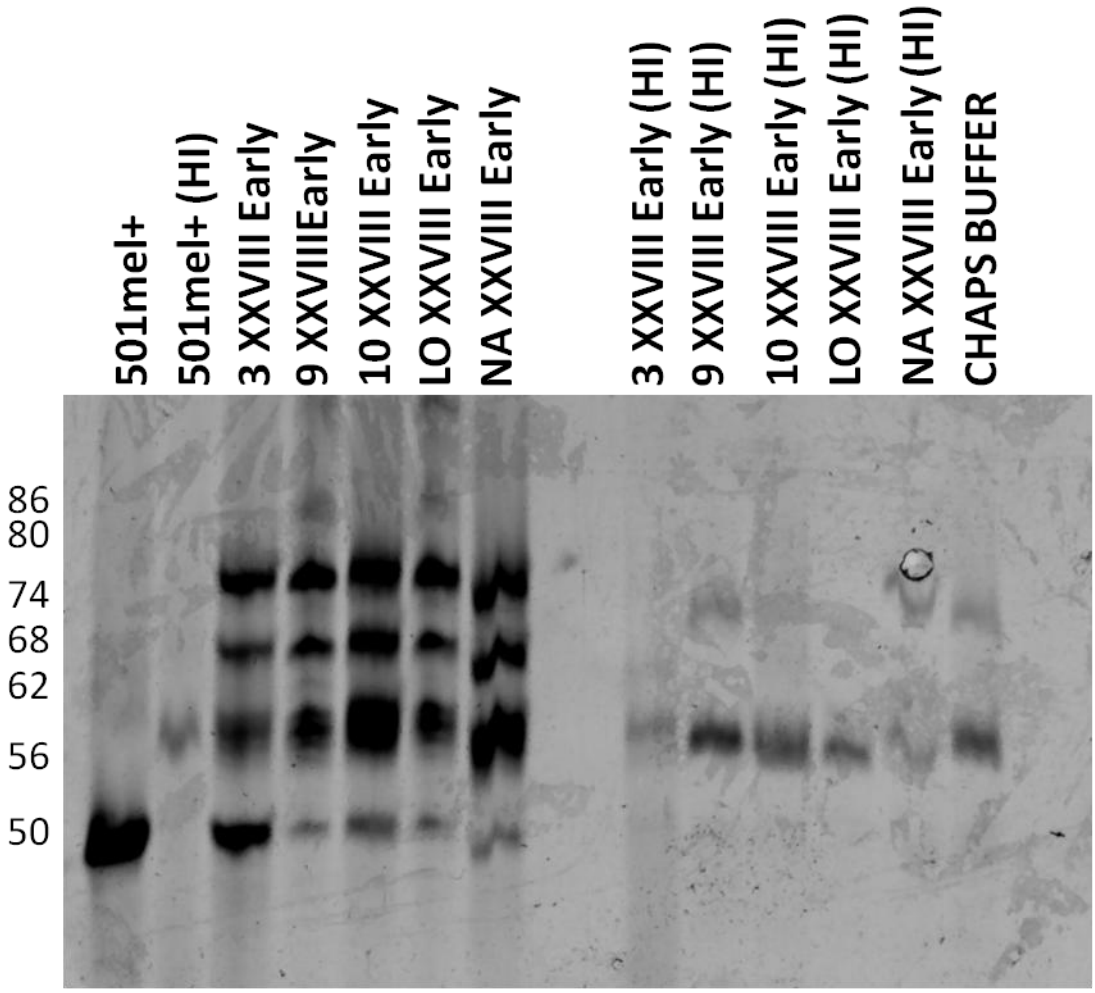

Fig 4.14 Polyacrylamide gel showing the results of the a QTRAP assay, showing the functionality of the assay. Products for the early timepoints of patient XXVIII cell populations were resolved on a 4% polyacrylamide gel for 2 hours and stained with SybrGold before acquiring an image on the Typhoon Imager. The strong banding corresponds to the positive signals observed in the data, however some faint banding is still observed in heat inactivated and CHAPS buffer only controls. This banding does not correspond to a detectable signal during the assay. Bands represent repeating 6bp extension of telomeric substrate primer from 50bp as shown on left of gel.

The level of telomerase activity in individual cell populations was investigated first (Fig 4.11). Clone 6 XVII does not show a significant change during time in culture ($P>0.05$). Clone 7 XVVI exhibits a significant increase in telomerase activity at the mid-stage of its replicative lifespan ($p<0.01$), before significantly decreasing back to similar levels as observed in the early timepoint by the late time point ($p<0.001$). Clone 10 XVII shows no change in telomerase expression ($P>0.05$). LO XVII cells demonstrated telomerase activity similar to 7 XVII with a peak at the mid time point ($p<0.01$) before falling back to a similar level at the late time point ($p<0.05$), however the peak telomerase activity is still lower than recorded in 7 XVII ($p<0.05$). The NA XVII population maintains similar levels at early and mid time points before a significant reduction in telomerase activity by the late timepoint ($p<0.05$). Comparisons between the cell populations at early time points demonstrated that only 6 XVII expressed higher levels of telomerase ($p<0.05$), At the mid-stage in patient XVII cell populations *in vitro* lifespan, 7 XVII expressed higher levels of telomerase than all other cell populations (all $p<0.05$, $p<0.001$ for 6 XVII), whilst LO XVII expresses a higher level of telomerase than 6 XVII ($p<0.05$). By the late time point, levels of telomerase are similar across all cell populations ($p>0.05$).

In patient XXVIII, Clone 3 demonstrates no change in telomerase activity across the three timepoints (Fig 4.12, $p>0.05$). Clone 9 XXVIII demonstrates a significant increase between early and late time points ($p<0.05$), whilst 10 and LO XXVIII demonstrated a significant decrease ($p<0.05$). NA XXVIII demonstrate a drop in telomerase activity at the mid time point, which then

returns to early levels by the late time point ($p<0.05$). Comparisons between cell populations demonstrated that at the early time point, 3 XXVIII expressed higher levels of telomerase than 9, 10 and LO XXVIII respectively ($p<0.001$, $p<0.01$, $p<0.05$, respectively), but not NA XXVIII ($p>0.05$). At the late time point, 9 XXVIII expresses higher levels of telomerase than 10 XXVIII and LO XXVIII ($p<0.05$).

To confirm the results of the QTRAP assay were due to the amplification of the telomeric substrate, the products of the reaction were resolved on an acrylamide DNA gel. The results shown in Fig 4.13 and Fig 4.14 samples from patient XVII and XXVIII at early time points, with heat-inactivated controls to demonstrate that dentaturation prior to the reaction abolished telomerase activity. The laddered banding is distinctive of telomerase activity, representing a 6bp extension of the original 50bp TS primer substrate. This occurs multiple times giving rise to bands increasing incrementally by 6bp. Faint banding is observed however even in the heat inactivated controls and the buffer control (CHAPS), as a small amount of primer dimerisation is unavoidable in this assay. The strong, dark banding observed in the 501mel positive control and in the XVII lysates is typical of those seen using the QTRAP and TRAP assay.

4.2.3 Quantification of telomere length by terminal restriction fragment (TRF) assay

The analysis of telomere length was conducted using the terminal restriction fragment (TRF) using the TeloTAGGG assay, to determine if the presence of active telomerase as determined using QTRAP was capable of telomere maintenance in culture. This assay gives an estimate of telomere length through the visual representation of a smeared band of DNA on a hybridisation membrane (Chapter 2.4). By measuring the top and bottom of each band and determining the median telomere length, comparisons can be drawn between telomere length at early, mid-stage and late time points corresponding to the replicative lifespan of the cell population, and between cell populations from both patients (Fig 4.15, Table 4.2)

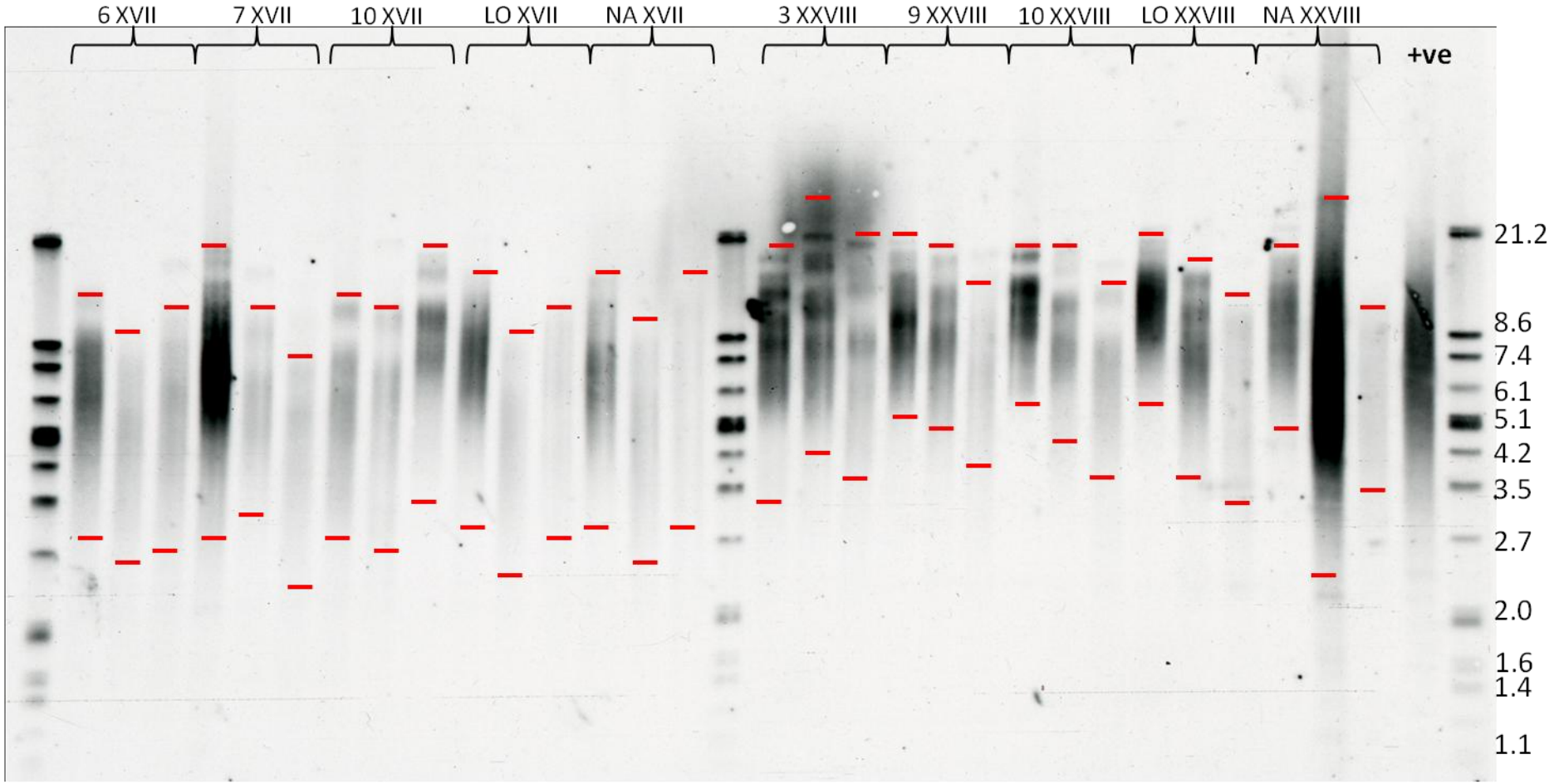

Fig 4.15 Telomere length analysis using the terminal restriction fragment assay. DNA was extracted from cell pellets containing $1x10^6$ cells from early, mid and late passages (from left to right) of all cell populations cultured. As a positive control DNA provided with the assay was used. By southern blotting using telomere specific oligonucleotides, differences were observed in the banding patterns between different populations and different timepoints. Markers represent multiple kilobase products as shown (right). Red markers represent the upper and lower limits of the bands used for the measurement of mean telomere length.

Sample	MEDIAN TERMINAL RESTRICTION FRAGMENT (base pairs)		
	EARLY	MID	LATE
6 XVII	8.80	5.65	10.38
7 XVII	11.35	8.80	5.05
10 XVII	7.75	7.35	11.55
LO XVII	10.25	5.75	7.60
NA XVII	10.00	5.50	7.60
3 XXVIII	11.70	12.95	12.55
9 XXVIII	11.90	11.90	11.30
10 XXVIII	12.80	11.45	10.98
LO XXVIII	13.10	10.25	7.63
NA XXVIII	12.70	11.65	6.75

Table 4.2: Median telomere length determined from the upper and lower boundaries of the bands from the terminal restriction fragment (TRF) assay. At three timepoints (early, mid and late) from the replicative lifespan of the isolated cell populations, genomic DNA was extracted and the length of the telomere assayed using a southern blot based technique. The bands resolved using a polyacrylamide gel

Clone 6 XVII shows an increase in median telomere length by the late time point from 8.8 kilobases (kb) to 10.38kb however, at the mid-point demonstrated a reduction in telomere length to 5.66kb. Clone 7 XVII exhibited erosion of telomere length from 11.35kb at the early time point to 5.05kb by the late time point. Clone 10 XVII demonstrated an increase in median telomere length. Both LO and NA populations exhibited similar early and late telomere lengths of 10.25kb and 10kb at the early and 7.60kb at the late timepoint respectively. In patient XXVIII all cell populations begin with similar telomere lengths of 11.70, 11.90, 12.80, 13.10 and 12.70 for 3, 9, 10, LO and NA XXVIII respectively. Clone 3 XXVIII demonstrated an increase in telomere length overall, peaking at the midpoint of 12.95kb before decreasing to 12.55kb by the late time point. Clone 9 XXVIII maintained the telomere length until the late timepoint where it decreased to 11.3kb. Clone 10 XXVII exhibited a decrease in telomere length over the three time points, reaching 10.98kb by the late time point. The LO and NA populations however, demonstrated a dramatic reduction in telomere length reaching 7.63kb and 6.75kb respectively.

4.3 Discussion

The aims of this chapter were to compare the isolated cell populations in terms of the presence of stem cell associated markers CD90, CD105 and CD166 and the presence of active telomerase. In addition, investigations into the levels of telomere erosion with *in vitro* ageing were conducted to determine whether senescence was telomere-dependent in the different cell populations, and if the effects of telomere erosion were linked to levels of active telomerase.

Flow cytometry data demonstrated varied levels of expression of CD90, CD105 and CD166 in the cell populations from both patients, with the patterns observed between patients XVII and XXVIII in terms of MFI. However, all cell populations proved positive for the markers CD90, CD105 and CD166 in line with the classical definition of mesenchymal stem cells (Dominici et al., 2006). These cell surface markers have been found in stem cells from multiple origins including bone marrow, periodontal ligament, dental pulp, cartilage, synovial fluid and adipose tissue (Trubiani et al., 2010, Wada et al., 2009, Tomokiyo et al., 2008, Gay et al., 2007, Nagatomo et al., 2006, Hiraoka et al., 2006, Fickert et al., 2004, Dowthwaite et al., 2004, Alsalameh et al., 2004, Zannettino et al., 2008, Ikeda et al., 2006, Morito et al., 2008). Interestingly the presence of these markers had been previously discussed in regard to buccal mucosal fibroblasts (Lindroos et al., 2008). In this instance they were considered to be a control population, with the exception of the CD marker expression, a non-stem cell population. In terms

of presentation of this data in the scientific literature, the normal format of flow cytometry data is to show a positive shift on histograms as seen in Fig 4.1 In this manner, the important factor to observe here is that all cell populations are positive, and those observed variations in MFI may not be important in terms of their stem cell phenotype. This suggests that the differential adhesion of fibronectin may not be sufficient to completely remove progenitors from within the NA population, and that subsequent cultures will enrich for this cell population over time as senescent cells are removed from the replicating cell “pool”. This evidence supports data published on oral mucosa stem cells cultured by explantation in SCID mice, and work carried out within the Wound Biology Group (Davies et al., 2010, Marynka-Kalmani et al., 2010). An interesting finding was the presence of a shift in MFI in CD34 and CD45 in three cell populations (6 XVII, 10 XXVIII and LO XXVIII). These cell populations were not expected to express these markers, as the presence of a haematopoietic cell lineage should be prevented by the culture conditions, which are not optimised for this cell type. However, these values were extremely low and may just represent experimental error/variation. Analysis of the telomerase activity of cell lysates using the QTRAP assay allowed for quantification of telomerase activity relative to 501mel, a cancer cell line with high levels of telomerase activity. All cell populations expressed active telomerase, with distinct differences arising between cell populations. Of particular interest was the presence of telomerase within LO and NA populations. In patient XVII, the mid time points of both LO and NA populations demonstrated increased telomerase activity suggesting that by the mid stage of culture an increase in the proportion of progenitor cells

within these populations, as a possible explanation for this increase. Clone 10 XVII, cells which senesced early at only 35 population doublings; do not appear to demonstrate any changes in telomerase activity, suggesting their senescence was telomerase independent. This has been reported previously in oral fibroblasts and may be responsible in this instance, however in that study telomerase activity could not be detected in oral fibroblasts, an analogous population to the LO population characterised in this thesis (Enoch et al., 2009). The telomerase activity detected in patient XXVIII did not reflect those difference of patient XVII, differences in telomerase activity between cell populations limited to a higher level of telomerase in Clones 3 and 9 at the early timepoint, an increase which by the late timepoint was only apparent between Clone 9 and LO populations. A larger investigation involving more patient samples is required to determine the presence of a characteristic telomerase “signature” within the stem cell populations. The presence of telomerase within these cell populations is another indicator of stem cell phenotype. Mesenchymal stem cells have not demonstrated active telomerase (Zimmermann et al., 2003), however, this has been observed in other lineages, such as a subpopulation of haematopoietic progenitors and neural precursors (Chiu et al., 1996, Ostenfeld et al., 2000, Haik et al., 2000, Wang et al., 2010). Most importantly, telomerase activity has been identified in cells of the inner cell mass of human blastocysts, the area from which ESCs are isolated (Heins et al., 2004, Thomson et al., 1998). Here the maintenance of telomere length is responsible in part for the unlimited potential for self renewal, one of the defining characteristics of totipotent stem cells such as ESCs (Amit et al., 2000). This data suggests that the cell

populations from the oral mucosa demonstrate a more pluripotent phenotype that MSC phenotype, and further investigation into the differentiation potential and plasticity of these cell populations would be crucial to fully understand the use of these cells in a therapeutic context for tissue engineering purposes.

The final analysis conducted in this chapter was of telomere erosion/maintenance of telomere length using the terminal restriction fragment assay (Chapter 2.2.7). This assay gives a representation of the telomere length of DNA extracted from cell lysates by the production of a smeared band on a membrane after DNA hybridisation. To calculate the median telomere length, measurements were taken of the upper and lower limits of the observed bands, and the median taken from this value. The results showed variation between the telomere lengths at all time points, with no discernable pattern of telomere erosion detected, and even in the instance of 6 XVII, 10 XVII and 3 XXVIII an observed increase in median telomere length. Clone 9 XXVIII, expressing a stable level of telomerase expression throughout the replicative lifespan of the cell population, demonstrated maintenance of telomere length. When taken in context of the telomerase activity of these cell populations, upregulation of telomerase expression in LO and NA populations from patient XVII at the mid time point suggest that this allows the recovery of telomeres, but ultimately not escape from replicative senescence. In Clone 7 XVII, a large increase in telomerase activity has no effect on telomere erosion. Clone 10 XVII, senescent after only 35 population doublings demonstrated a large increase in telomere length by the late time

point, when the cells were actually senescent, however this was not enough to escape replicative senescence. The observed increase in telomere length and indeed telomerase activity within the samples may be due to culture selection of progenitors with longer telomeres, or by the loss of cells with smaller telomeres causing an increase to the median, rather than reflecting a real upregulation of telomerase within the whole cell population. The detection of telomere shortening has been linked with rapid *in vitro* ageing in MSC and is one of the mechanisms responsible for replicative senescence (Baxter et al., 2004, Rubin, 2002, Wagner et al., 2008, Hayflick, 1965). However in this study it is clear that particularly in the case of 6 XVII, 10 XVII, and 3XXVIII, another mechanism of senescence was employed.

The TRF assay, a crude method to determine telomere length has been surpassed by sensitive techniques which allow a higher level of quantification. Single telomere length analysis (STELA) is a method by which accurate measurements of the full spectrum of telomere lengths from individual chromosomes (Baird et al., 2003). This technique has previously been utilised for investigation of *in vitro* ageing in oral fibroblasts (Enoch et al., 2009). However for this investigation it was not used due to practical difficulties experienced by other researchers in the Wound Biology Group with respect to consistent experimental repetition. In future studies it would benefit the research if a more exact quantification of telomere erosion could be conducted.

The aims of this chapter were to investigate the presence by flow cytometry of CD90, CD105 and CD166, commonly used stem cell surface markers

used in the identification of MSCs and other adult stem cell populations. The comparison of relative telomerase activity within the different cell populations was conducted to determine if clonal expansion of fibronectin adherent cells were enriched for this stem cell associated marker. The final analysis was to determine the levels of telomeric degradation occurring with *in vitro* ageing to determine if the telomerase activity detected affected the maintenance of telomere during long term culture. All cell populations were found to be positive for markers CD90, CD105 and CD166, and with the exception of three cell populations negative for CD34 and CD45, negating the presence of fibrocytic or haematopoietic cells within these cell populations. All populations also exhibited active telomerase within cell lysates, suggesting these cells are phenotypically distinct from MSCs and characteristically similar to embryonic stem cells. The presence of telomerase did not appear to prevent the erosion of telomeres in the different cell populations; however a more accurate method of telomere length analysis, such as STELA, may highlight smaller changes in telomere length with more accuracy that this technique could provide. All the data suggests that not only the clonally selected cell populations, but the heterogeneous cell populations contain stem cells which are capable of self-renewal and as previously shown in chapter 3 can exceed the population doublings observed in MSCs. To further elucidate the therapeutic potential of these isolated cell populations, it is crucial that the multi-lineage potential of these cells is fully investigated to determine how effectively the different cell populations form tissues *in vitro.*

CHAPTER 5

Investigating the chondrogenic, osteogenic and adipogenic differentiation potential of isolated oral mucosal cell populations

5.1 Introduction

As mentioned in previous Chapters, adult stem cells have been isolated from multiple sites in the body, with the most characterised site being the bone which were the first to be identified (Yoo et al., 2005, Wagner et al., 2005, Quirici et al., 2002, Oliveira et al., 2009, Mareschi et al., 2006, Mareddy et al., 2007, Ishii et al., 2005, Friedenstein et al., 1970). Adult stem cells have been successfully isolated from a number of other tissues including synovial fluid, articular cartilage and skin (Chunmeng et al., 2004, Fernandes et al., 2004, Toma et al., 2001, Toma et al., 2005, Webb et al., 2004, Alsalameh et al., 2004, Dowthwaite et al., 2004, Fickert et al., 2004, Hiraoka et al., 2006, Williams et al., 2010). With the focus of this project being the lamina propria of the oral mucosa, a number of other oral tissues have demonstrated the presence of a stem cell population including the oral mucosa epithelium, dental papilla and periodontal ligament (Gay et al., 2007, Ivanovski et al., 2006, Nagatomo et al., 2006, Techawattanawisal et al., 2007, Tomokiyo et al., 2008, Trubiani et al., 2010, Wada et al., 2009, Ikeda et al., 2006, Calenic et al., 2010, Izumi et al., 2007, Nakamura et al., 2007).

The characterisation of stem cell populations requires the elucidation of differentiation capacity of those cells along a number of potential lineages. Stem cells may be multipotent, restricted to differentiate only into the lineages of the tissue from which they are isolated such as cortex stem cells or haematopoietic stem cells (Wright et al., 2006, Izumi et al., 2007), or totipotent, able to develop into any lineage such as in embryonic stem cells (Keller, 1995, Thomson et al., 1998, Heins et al., 2004).

The lineage potential of BMSCs is well characterised with differentiation along chondrogenic, adipogenic and osteogenic differentiation observed in these cells (Romanov et al., 2005, Kolf et al., 2007).(Kotobuki et al., 2008, in 't Anker et al., 2003). These differentiation characteristics have also been documented in the stem cell populations isolated from oral tissues including the periodontal ligament, dental pulp and oral mucosa (Gay et al., 2007, Nagatomo et al., 2006, Davies et al., 2010, Marynka-Kalmani et al., 2010, Gronthos et al., 2002, Otaki et al., 2007a). Clonal expansion can affect the differentiation potential of the cells as documented by the increased osteogenic potential of osteoblast like cells after selection of cells exhibiting high alkaline phosphatise activity (Kotobuki et al., 2004).

The aim of this chapter was to determine the potential of the isolated cell populations to differentiate along adipogenic, osteogenic, and chondrogenic lineages, to determine if this potential is similar to the well characterised adult stem cell populations. As well as determining the differentiation potential of the isolated populations, the comparisons between cell populations to determine if clonal selection increased the differentiation potential or if using a heterogeneous cell population from the oral mucosa would be just as successful in a therapeutic setting for tissue engineering purposes.

5.2 Results

5.2.1 Adipogenic Differentiation

5.2.1.1 Quantitative PCR detection of Adipogenic mRNA from patient XXVIII cell populations

To determine the adipogenic potential of cell cultures mRNA was isolated from adipogenic cultures induced (A = adipogenic induced)) for 7, 14, 21 and 28 days, with non induced cultures used as controls (AC). The expression of three markers Lipoprotein Lipase (LPL), PPARγ and C/EBP-α, was quantified and normalised to the expression of the housekeeper gene β-actin. Due to time limitations only patient XXVIII samples were analysed for quantitative expression of these markers - 2 clones (3 XXVIII and 9 XXVIII) along with the corresponding patient LO and NA populations. Data was analysed for statistical significance using one-way ANOVA with a bonferroni post-test where $p<0.05$.

5.2.1.1.1 Peroxisome Proliferator Activated Receptor-gamma (PPARγ) expression

Fig 5.1 demonstrates the PPARγ expression of patient XXVIII cell populations over a 28 day differentiation study. Expression of PPARγ in Clone 3 XXVIII demonstrated a significant increase at day 14 ($p<0.001$), before reducing significantly at day 21 and remaining at a low level until day 28 ($p<0.001$, Fig 5.1A). Whilst at 14 days the control expressed significantly lower levels of PPARγ than adipogenic cultures, at all other timepoints the difference between adipogenic and control cultures demonstrated no difference ($p>0.05$). Similarly 9 XXVIII saw a significant increase at 14 days ($p<0.001$), before a significant reduction in expression by day 21, at which level the adipogenic cultures remained at day 28 (Fig 5.1B). As with Clone 3 XXVIII, the day 14 control expression remained significantly lower ($p<0.001$), after which time the difference between control and adipogenic cultures was no longer significant ($p>0.05$). In LO XXVIII cultures, PPARγ expression remained stable up to 21 days without significant changes in expression before decreasing at day 28 ($p<0.001$, Fig 5.1C). For LO cultures all control cultures demonstrated significantly lower levels of expression compared to adipogenic cultures ($p<0.001$). NA XXVIII cultures saw expression increase at day 14 ($p<0.001$), remain stable at day 21 before expression deceased significantly at day 28 ($p<0.001$). Expression in control cultures remained significantly lower up to day 21 ($p<0.001$), however at day 28 no significant difference between control cultures or adipogenic cultures was observed (Fig 5.1D). Comparisons between cell populations at each time point determined

that LO and NA populations had higher levels of PPARγ expression at day 7, compared to Clone 3 XXVIII and Clone 9 XXVIII (all $p<0.001$).By day 14 the differences had reversed with 9 XXVIII demonstrating higher levels of expression than LO XXVIII ($p<0.001$), with Clone 3 XXVIII and NA XXVIII exhibiting a similar level of expression to Clone 9 XXVII. At day 21, PPARγ expression within Clone 3 XXVIII cultures is significantly lower than all other cultures ($p<0.001$), with no difference in expression detected between the LO, NA and Clone 9 XXVIII cultures ($p>0.05$). At the final time point (day 28), the expression in LO XXVIII has decreased to a level comparable to Clone 3 XXVIII, whilst the only population expressing significant levels of PPARγ in relation to the other cell populations is Clone 9 XXVIII.

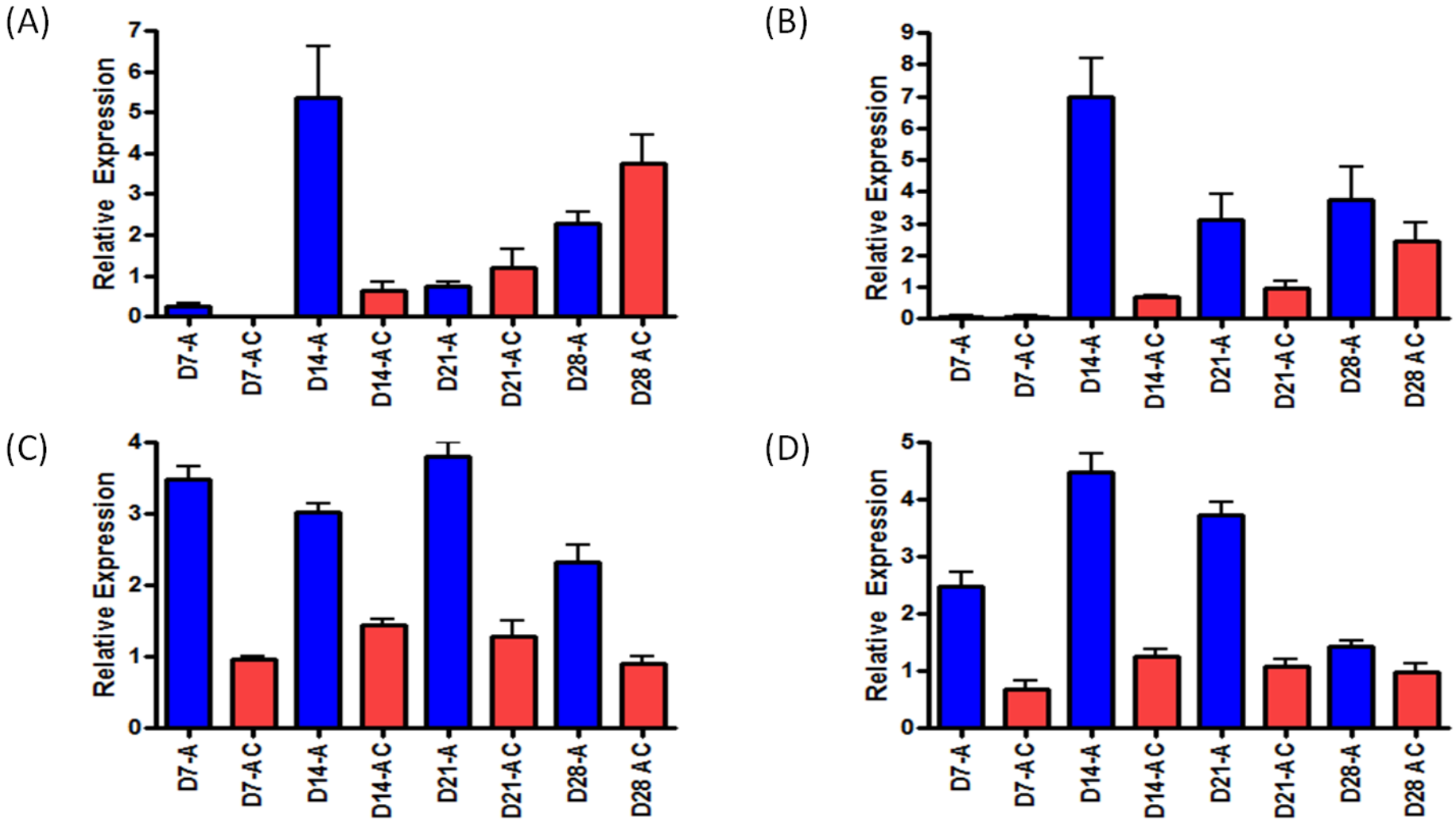

Fig 5.1 Quantitative-PCR analysis of RNA for the adipogenic marker PPARγ in cell populations (A) Clone 3, (B) Clone 9, (C) LO and (D) NA from Patient XXVIII. RNA Samples for both Adipogenic (D^x-A) and control cultures (D^x-AC) were taken from cultures at 4 timepoints, day (D) 7, 14, 21 and 28, and the results normalised to the expression of beta-actin. n=3 ± SD

5.2.1.1.2 CCAAT/Enhanced Binder Protein-alpha (C/EBPα) expression

Expression of C/EBPα mRNA was low by day 7, increasing significantly at day 14 ($p<0.001$) before immediately decreasing back to low levels at day 21 ($p<0.001$), where expression remained until day 28 (Fig 5.2A). Whilst expression in control cultures at day 14 was significantly lower than the adipogenic cultures ($p<0.001$), difference in adipogenic and control C/EBPα expression at all other timepoints were not significant ($p>0.05$). Clone 9 XXVIII also demonstrated low levels of expression at day 7, before a significant increase in expression at day 14 ($p<0.01$, Fig 5.2B). Expression did not change significantly from day 14 onwards. Differences between expression in control cultures and adipogenic cultures were not significantly different at day 7 or day 21 ($p>0.05$), but expression was significantly higher in adipogenic cultures when compared to controls at day 14 ($p<0.05$) and day 28 ($p<0.001$) respectively. LO XXVIII demonstrated low levels of expression until day 21, when a significant increase in expression was detected ($p<0.001$). This increase continued until day 28 ($p<0.001$, Fig 5.2C). Whilst differences between adipogenic cultures at day 7 and day 14 were not significant ($p>0.05$), the expression of C/EBPα in adipogenic cultures was significantly higher at day 21 ($p<0.001$) and day 28 ($p<0.001$). NA XXVIII cultures demonstrated a similar pattern of expression to LO XXVII, with no significant increase observed until day 21 ($p<0.001$, Fig 5.2D), after which time expression remained at a high level. As with LO XXVIII, adipogenic culture expression was significantly higher than controls at day 21 ($p<0.0010$ and day 28 ($p<0.001$), however no differences between adipogenic or control

C/EBPα expression were observed at day 7 or day 14 (Fig 5.2). Comparisons between cell populations demonstrated that at day 7, LO XXVIII expressed the highest levels of C/EBPα compared to NA ($p<0.05$), Clone 3 XXVIII ($p<0.001$) and 9 XXVIII ($p<0.001$). The levels of expression of the other three cell populations were not significantly different. By day 14, Clone 3 XXVII has significantly higher levels of expression than LO ($p<0.01$) and NA ($p<0.001$) and Clone 9 XXVIII ($p<0.05$) populations. Clone 9 XXVIII however expresses lower levels of C/EBPα than LO ($p<0.05$) and NA populations ($p<0.05$) at day 14. By day 21, LO XXVIII expresses higher levels of C/EBPα expression when compared to NA ($p<0.0010$. Clone 9 ($p<0.001$) and Clone 3 ($p<0.001$). NA XXVIII expression is significantly higher than Clone 3 XXVIII ($p<0.05$) at day 21. Finally at day 28 expression of C/EBPα is highest in LO XXVIII when compared to Clone 3 ($p<0.001$), Clone 9 ($p<0.001$) and NA XXVIII ($p<0.001$). Expression in 9 XXVIII is significantly higher than Clone 3 ($p<0.001$) and NA XXVIII ($p<0.001$) populations at day 28.

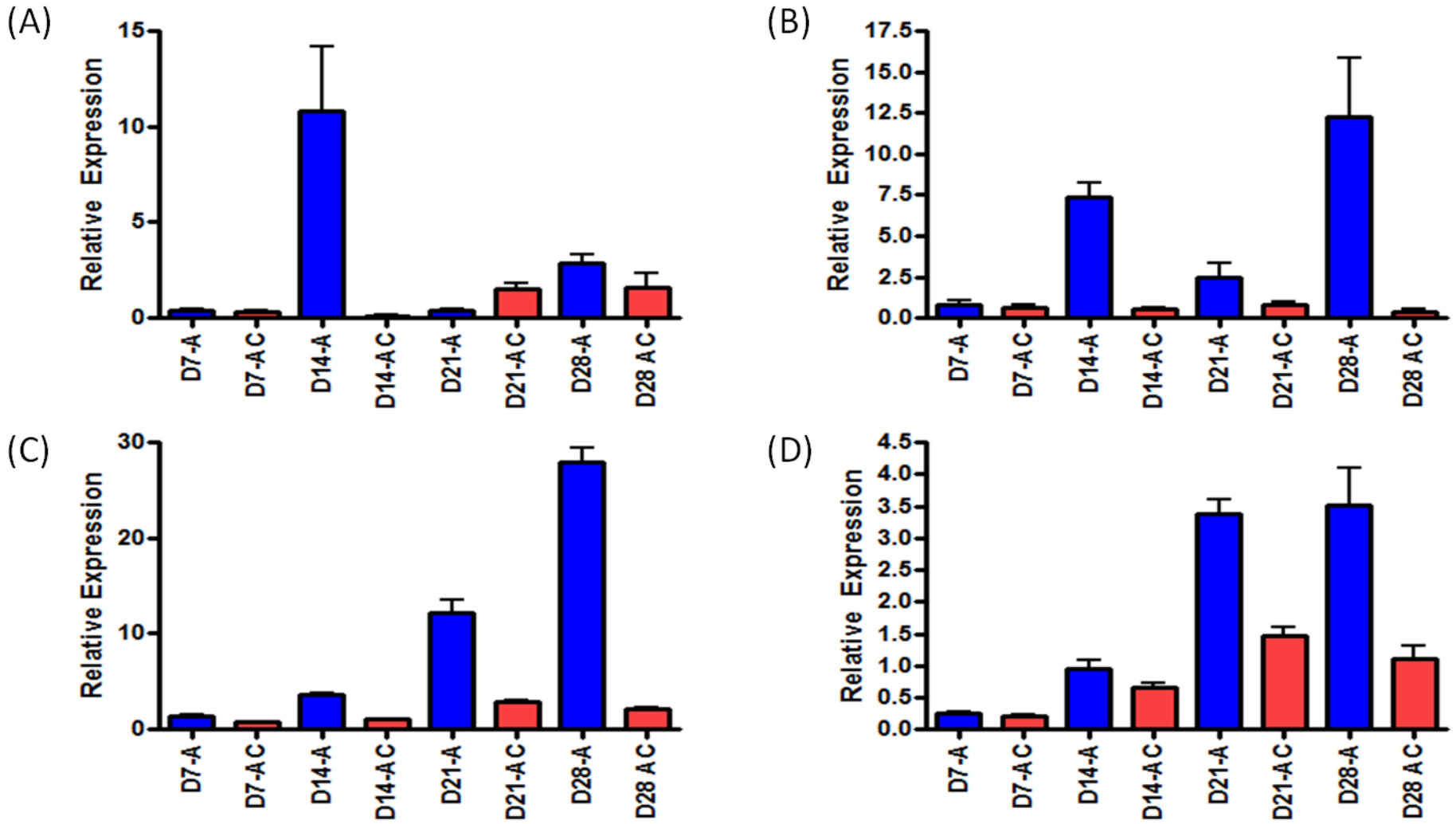

Fig 5.2 Quantitative-PCR analysis of RNA for the adipogenic marker C/EBP-alpha in cell populations (A) Clone 3, (B) Clone 9, (C) LO and (D) NA from Patient XXVIII. RNA Samples were taken from cultures at 4 timepoints, day (D) 7, 14, 21 and 28, and the results normalised to the expression of beta-actin. n=3 ± SD

5.2.1.1.3 Lipoprotein Lipase (LPL) expression

Expression of LPL within Clone 3 XXVII demonstrated no significant difference in LPL expression at all timepoints, in either adipogenic or control cultures (Fig 5.3A). The same result was observed in Clone 9 XXVIII (Fig 5.3B). LO XXVIII cultures demonstrated no significant change in expression from day 7 to day 14. At day 21 a significant increase in expression was observed ($p<0.05$), and expression remained at this level at day 28 (Fig 5.3C). Adipogenic cultures expressed significantly higher levels of LPL expression compared to controls at day 7 ($p<0.01$), day 21 ($p<0.01$) and day 28 ($p<0.001$). In the NA cultures, expression of LPL increases significantly at day 21 ($p<0.01$) and remains at this level at day 28 (Fig 5.3D). Levels of expression in adipogenic and control cultures are not statistically different ($p>0.05$) at any timepoint in the NA XXVIII cultures. Comparisons of cell populations at each timepoint demonstrated that LO XXVIII expressed higher levels of LPL at day 7 than Clone 3, Clone 9 and NA populations ($p<0.001$). NA XXVIII also expresses higher levels of LPL than Clone 3 ($p<0.001$) and Clone 9 ($p<0.01$) at day 7. At day 14, there is no significant difference in expression in any cell populations ($p>0.05$). At both day 21 and day 28, Clone 9 expresses higher levels of LPL than Clone 3 ($p<0.05$), LO XXVIII expresses higher levels of LPL when compared to Clone 3 at day 21 and day 28 ($p<0.01$ and $p<0.001$, respectively).

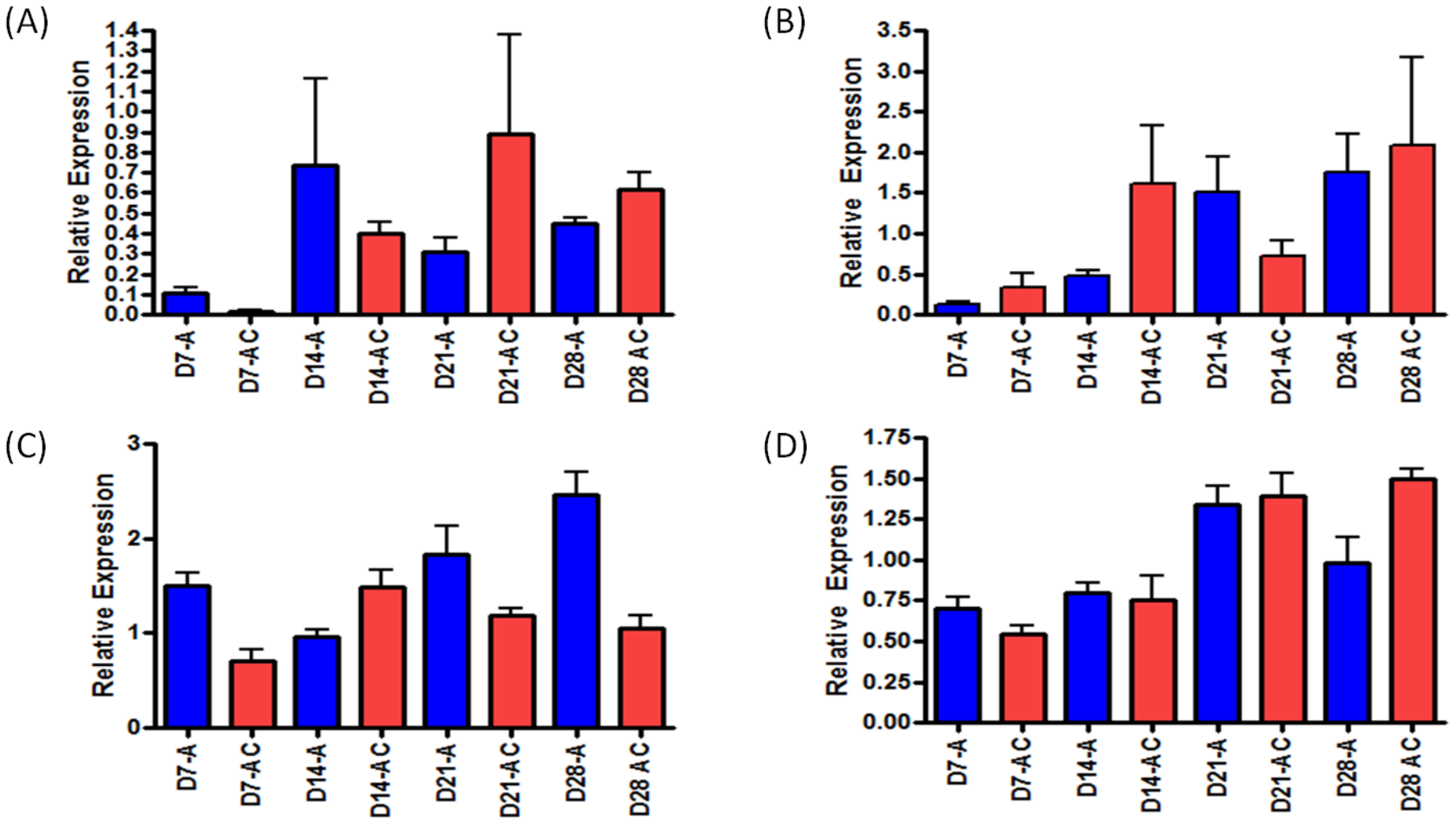

Fig 5.3 Quantitative-PCR analysis of RNA for the adipogenic marker LPL in cell populations (A) Clone 3, (B) Clone 9, (C) LO and (D) NA from Patient XXVIII. RNA Samples were taken from cultures at 4 timepoints, day (D) 7, 14, 21 and 28, and the results normalised to the expression of beta-actin. n=3 ± SD

5.2.1.2 Oil Red-O Staining of Adipogenic Cultures

To determine the effectiveness of adipogenic differentiation (i.e. would the attempts to drive cell populations down an adipogenic lineage prove successful as evidenced through the production of lipid deposits), cells cultured for 28 days in adipogenic induction media or control media were fixed and stained with Oil Red-O, which allows for the visualisation of fat droplets via bright field microscopy. As a control, 3T3-L1 cells were also incubated for 28 days in adipogenic media for a positive Oil Red-O staining.

In patient XVII all cell populations demonstrated the presence of lipid droplets after 28 days adipogenic induction, with the most intense staining observed in 7 XVII (Fig 5.4). In the LO and NA populations, staining was widespread. Clone 6 XVII however showed a very low level of Oil Red-O staining. Interestingly in the control cultures, LO XVII also demonstrated positive staining for lipid droplets; however this was not as distinct or widespread as the staining observed in the adipogenic cultures. However, it was notable that all of the cell populations demonstrated dramatically lower level of staining when compared to the 3T3-L1 positive control.

Patient XXVIII demonstrated more concentrated staining compared to patient XVII, with no staining present in controls of 3 XXVIII and 9 XXVIII and NA XXVIII (Fig 5.5). As already observed in patient XVII, the control LO population demonstrated some limited lipid deposition. Clone 3 XXVIII demonstrated quite extensive lipid deposition, although this was not as extensive as LO XXVIII. Clone 9 XXVIII showed minimal staining with Oil Red-O, and NA XXVIII also exhibited a minimal level of staining.

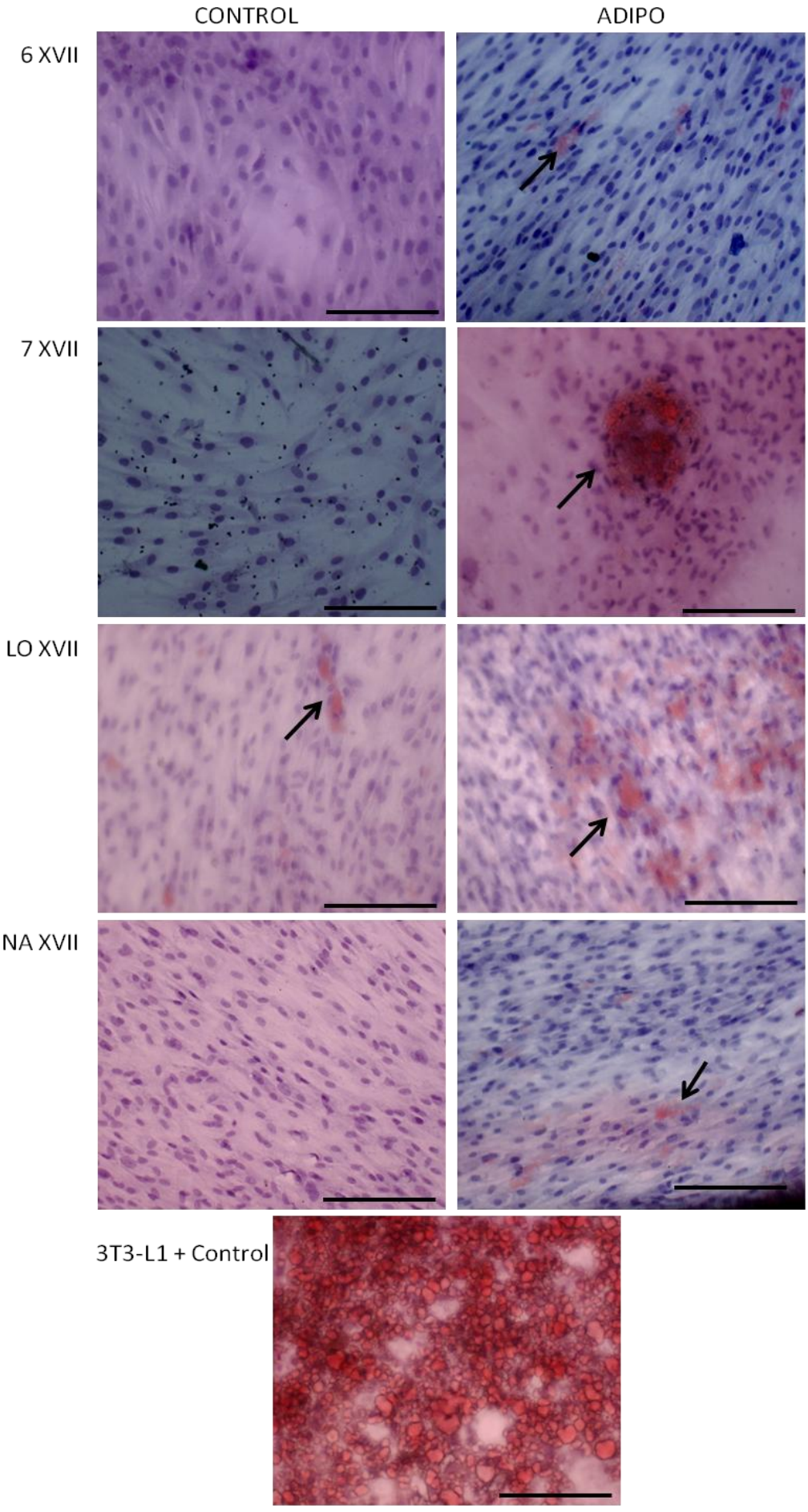

Fig 5.4: Oil Red-O Staining of Adipogenic induced and control cultures from patient XVII. Evidence of lipid deposition within cultures marked with arrows to highlight red staining. Scale = 200um

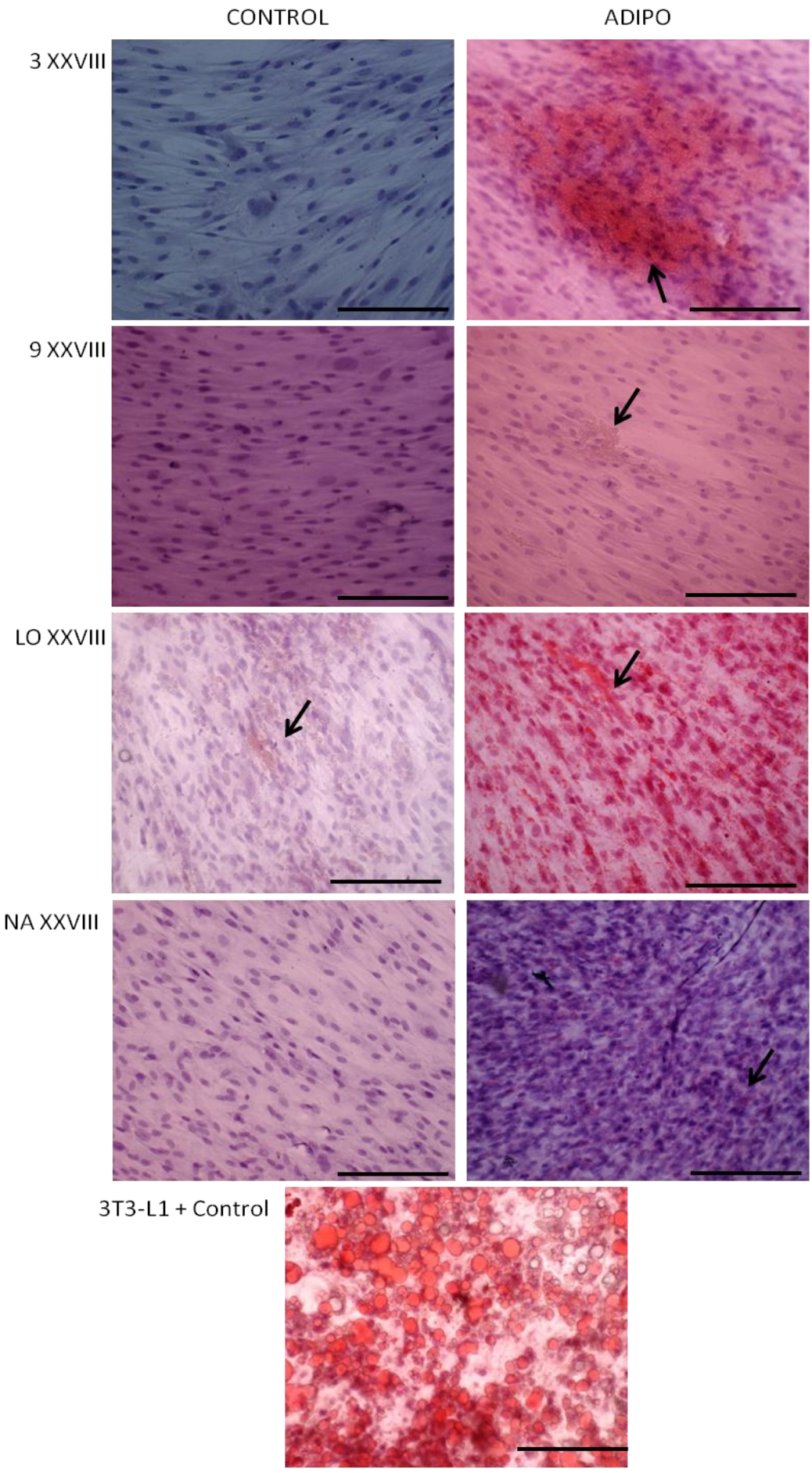

Fig 5.5: Oil Red-O Staining of Adipogenic induced and control cultures from patient XXVIII. Evidence of lipid deposition within cultures marked with arrows to highlight red staining. Scale = 200um

5.2.2 Chondrogenic Differentiation

5.2.2.1 Histological Analysis of Chondrogenic Cultures

After induction of chondrogenic differentiation for 28 days in a pellet culture system as described (Chapter 2.6.1), pellets were kindly paraffin embedded and 10μm sections cut on a microtome by School of Dentistry Pathology MLSO Kath Allsop attached. Sections were staining using Haemotoxylin and Eosin (H&E) staining, and for more specific collagen staining using Van Gieson's stain. It was notable that after 28 days in culture only chondrogenic-induced pellets remained intact with the controls disintegrating (therefore there was no data for control pellets).

5.2.2.1.1 Haemotoxylin and Eosin staining

The result of H&E staining of the chondropellets for patient XVII demonstrated that all cell nuclei are distributed throughout the pellet (Fig 5.7). The general structure of the pellets appeared more compact in the LO and NA pellets with fewer breakages and voids within the pellet than Clones 3 and 9 XXVIII, which contained many voids around the perimeter of the cell pellets. These may be artifacts from sectioning which however, may in turn suggest an underlying weakness in such chondropellets formulated form the clonal cells.

The sections for patient XXVIII exhibit similar characteristics to those observed for patient XVII, with the exception of Clone 9 XXVIII. The sections

of 9 XXVIII were not able to remain intact during processing and sectioning however, the remainder of the section was able to demonstrate similar staining to that observed within the other cell populations. Clone 3 XXVIII demonstrated similar morphology to the clones from patient XVII with voids within the sections however, in this instance spread throughout the section not just at the periphery. As observed in patient XVII, the LO and NA section of patient XXVIII exhibit a more compacted morphology with fewer voids present throughout the sections. The nuclei were again distributed throughout the cell pellets, again suggesting that the entire pellet remained viable for the entire 28 day differentiation culture.

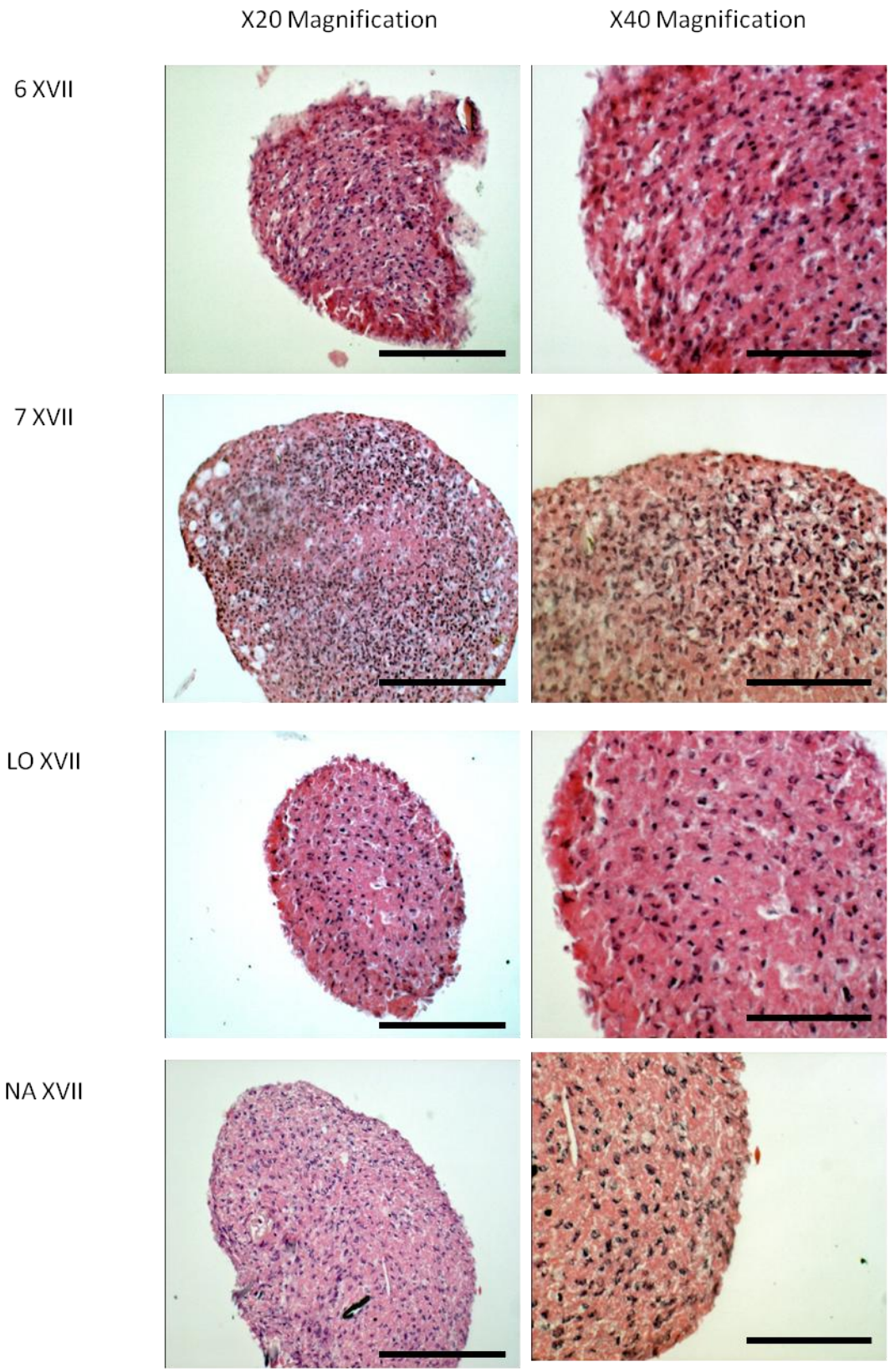

Fig 5.6: H&E Staining of 10µm paraffin embedded sections for the detection of general matrix components. Sections from Clones 6 and 7, LO and NA XVII were stained with Haemotoxylin and Eosin and images acquired at x20 and x40 magnification Scale bar (x20) = 200um, (x40) = 100um

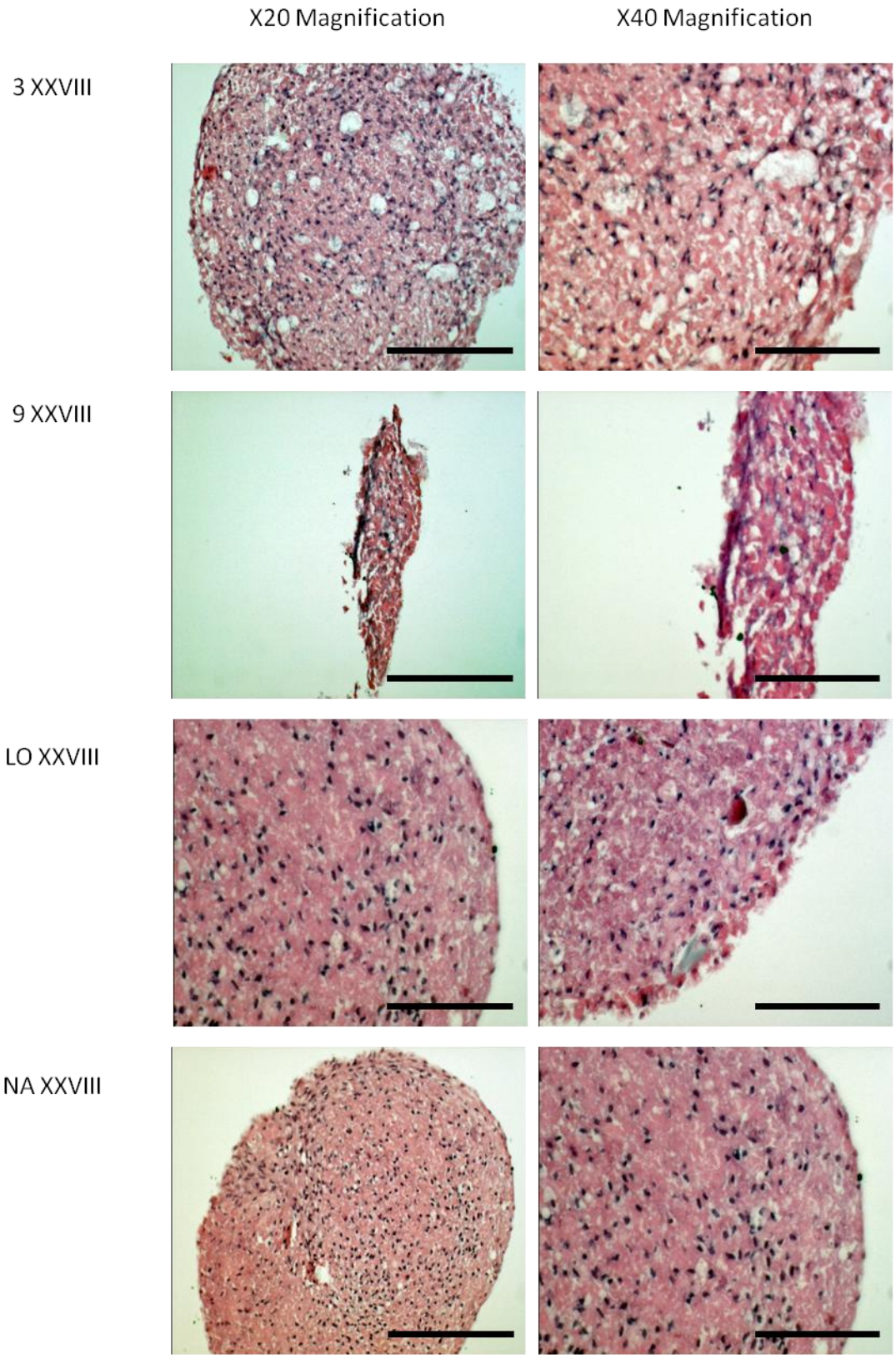

Fig 5.7: H&E Staining of 10µm paraffin embedded sections for the detection of general matrix components. Sections from Clones 3 and 9, LO and NA XXVIII were stained with Haemotoxylin and Eosin and images acquired at x20 and x40 magnification. Scale bar (x20) = 200um, (x40) = 100um

5.2.2.1.2 Van Gieson's

In addition to the general histology staining, Van Gieson's staining was used for investigating the presence of collagen within the chondrogenic pellets; an indication of successful differentiation along the chondrogenic lineage. Collagen will stain red, whilst cellular cytoplasm and muscle stains yellow/brown. Sections from patient XVII demonstrated staining for collagen throughout the sections, with the exception of 7 XVII which exhibited an unusual pattern of staining. The presence of collagen with 6 XVII was prevalent throughout the pellet, although staining was not as strong as observed within the pellets from LO and NA XVII (Fig 5.9), where the most distinct staining was observed at the periphery of the pellet sections. Patient XXVIII samples also contained collagen, and this was concentrated at the periphery of NA XXVIII sections, but more widespread in Clone 3, 9 and LO XXVIII sections. Sections from 9 XXVIII were again unable to remain intact during the sectioning process; however, the partial sections still demonstrated the presence of collagen through red staining.

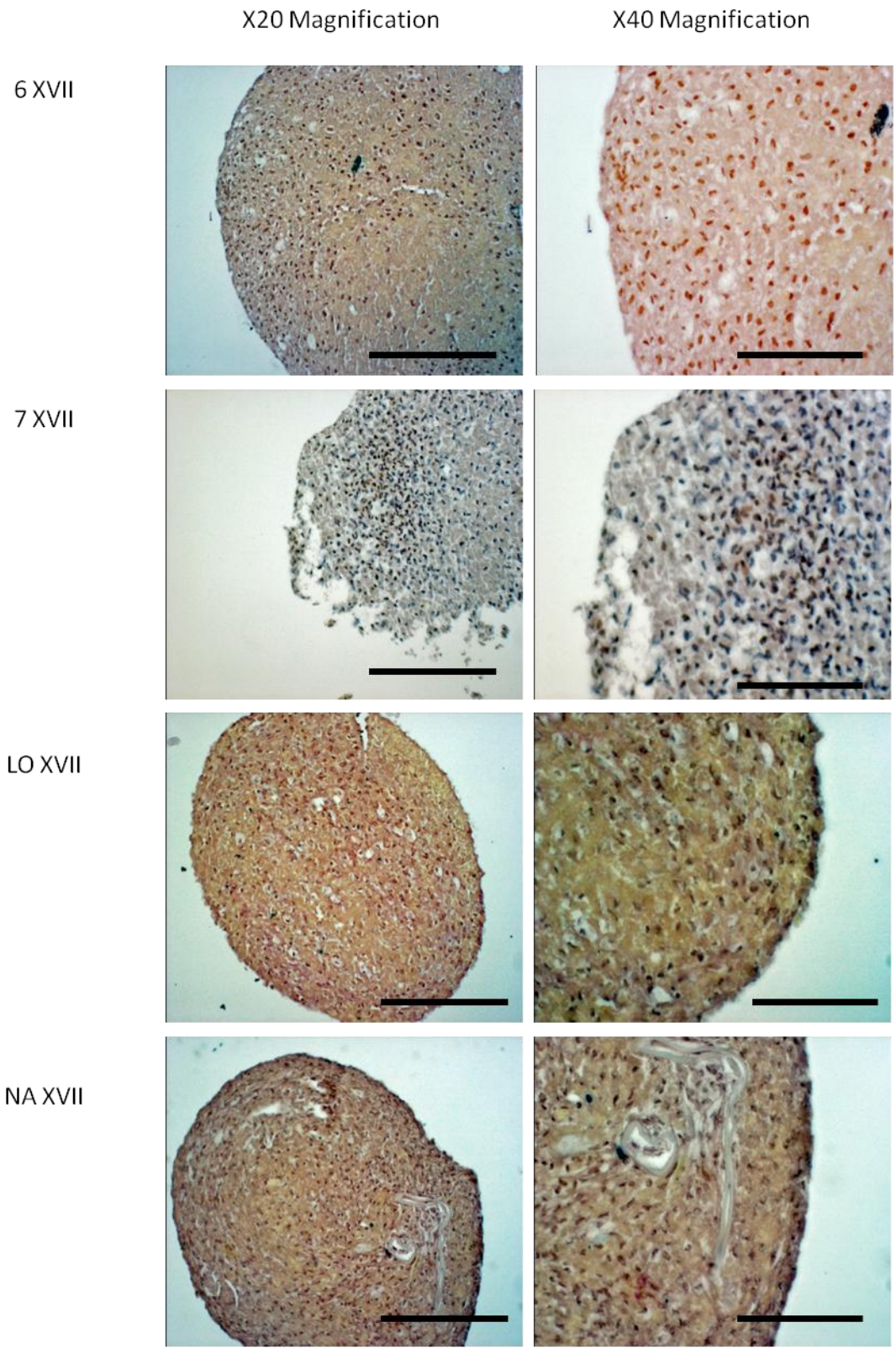

Fig 5.8: Van Gieson's stain of 10µm paraffin embedded sections for the detection of general matrix components. Sections from Clones 6 and 7, LO and NA XVII were stained with and images acquired at x20 and x40 magnification. Scale bar (x20) = 200um, (x40) = 100um

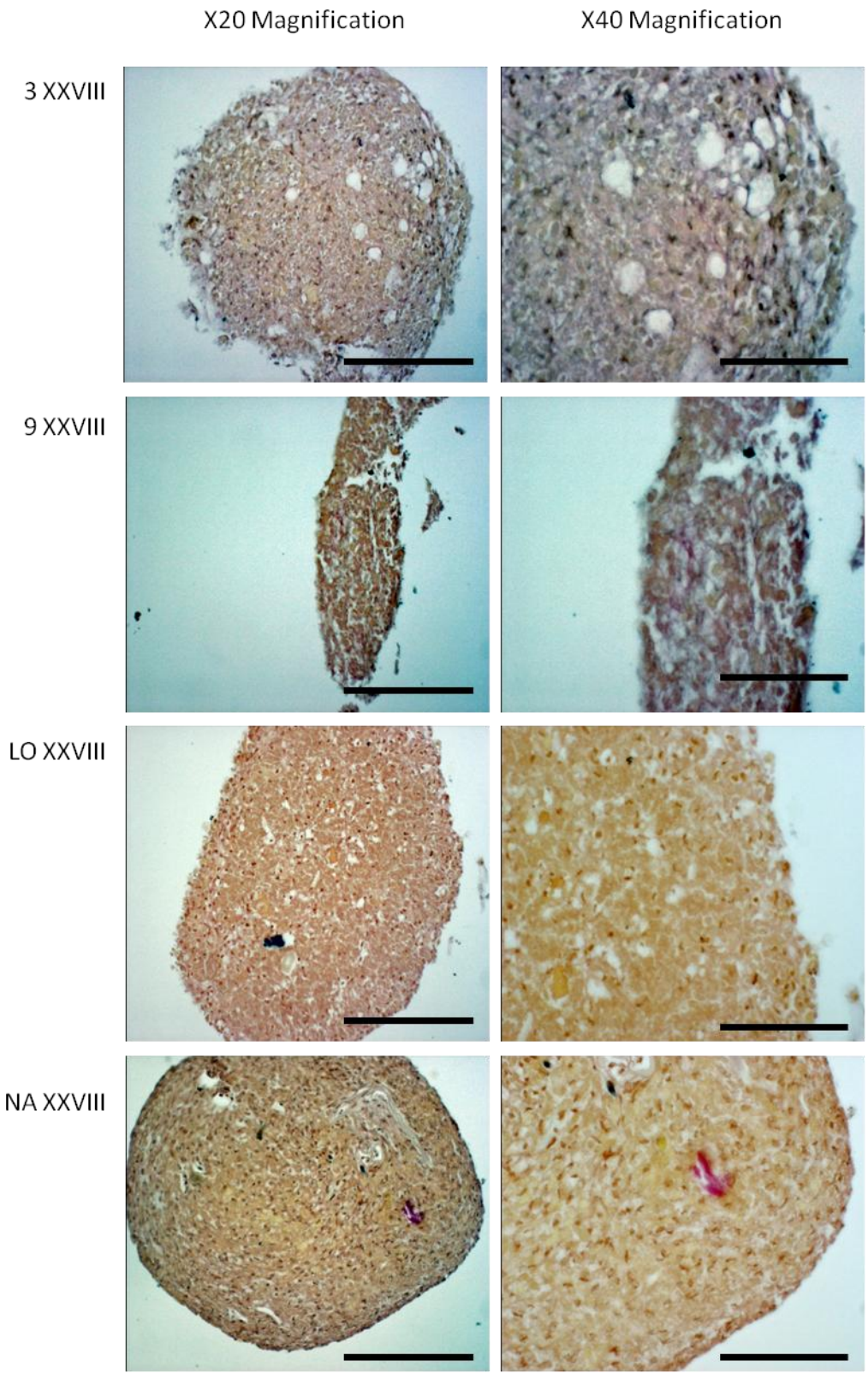

Fig 5.9: Van Gieson's stain of 10µm paraffin embedded sections for the detection of general matrix components. Sections from Clones 6 and 7, LO and NA XVII were stained with and images acquired at x20 and x40 magnification. Scale bar (x20) = 200um, (x40) = 100um

5.2.3 Immunhistochemical Analysis of Chondrogenic Cultures

To determine if the results of the general histology staining represented true chondrogenic differentiation, cryopreserved sections were investigated for the presence of aggrecan, a proteoglycan which together with type II collagen comprises one of the main structural components of (Bayliss et al., 2000). All staining was conducted using the 6B4 aggrecan antibody kindly donated by Prof. Bruce Caterson School of Biosciences, Cardiff University. Primary negative and secondary negative controls were used to ensure non-specific binding was not responsible for the staining.

Clone 6 XVII demonstrated positive staining with aggrecan (Fig 5.11A) and corresponding DAPI staining indicates this is most prominent at the periphery of the pellet sections. Closer microscopic examination of the pellet confirms that the expression of aggrecan is highest at the edge of the section (Fig 5.11D). Clone 7 XVII demonstrated faint aggrecan staining compared to 6 XVII, and lacked the peripheral staining mentioned in 6 XVII pellets (Fig 5.12). LO XXVIII pellets demonstrated very strong staining at the periphery of the sections, and the same pattern of expression was also observed in the NA XXVIII population, with a strong band of staining around the section edge (Fig 5.13, Fig 5.14). In all sections, both controls remained negative for any staining.

Clone 3 XXVIII stained for aggrecan, located primarily at the periphery of the section. However some unbound secondary antibody remained bound after washes and can be observed as bright spots located all over the section (Fig 5.15A). Clone 9 XXVIII exhibited widespread staining for aggrecan

throughout the section without extensive staining at the periphery as observed in 3 XXVIII (Fig 5.16A). As observed with the LO and NA populations from patient XVII, staining for aggrecan was most prominent at the edge of the sections from LO and NA XXVIII but staining was present throughout the pellet sections.

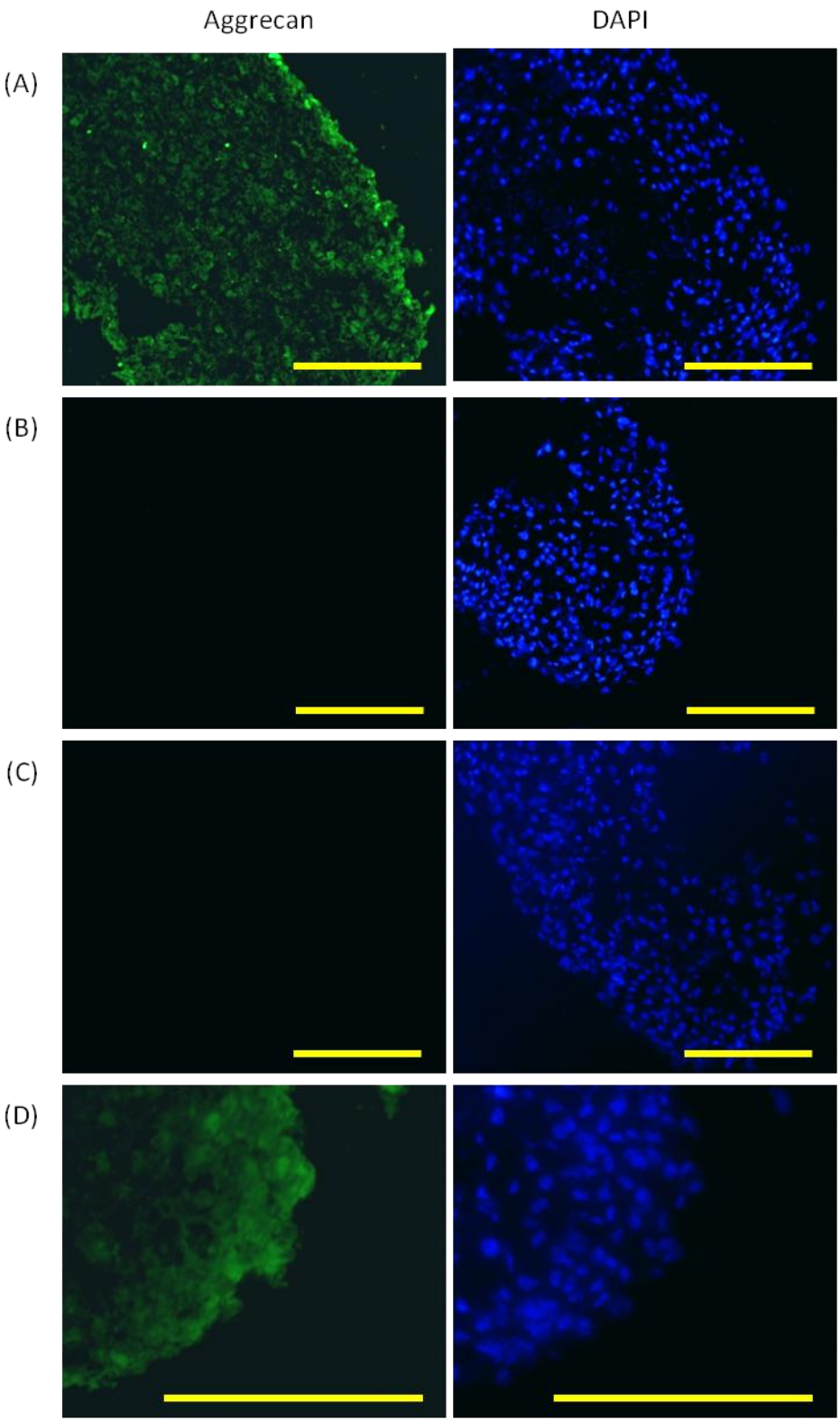

Fig 5.10: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) 6 XVII x20 magnification (B) 6 XVII Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) 6 XVII Aggrecan x40 magnification. Scale bar 200um

FIG 5.10

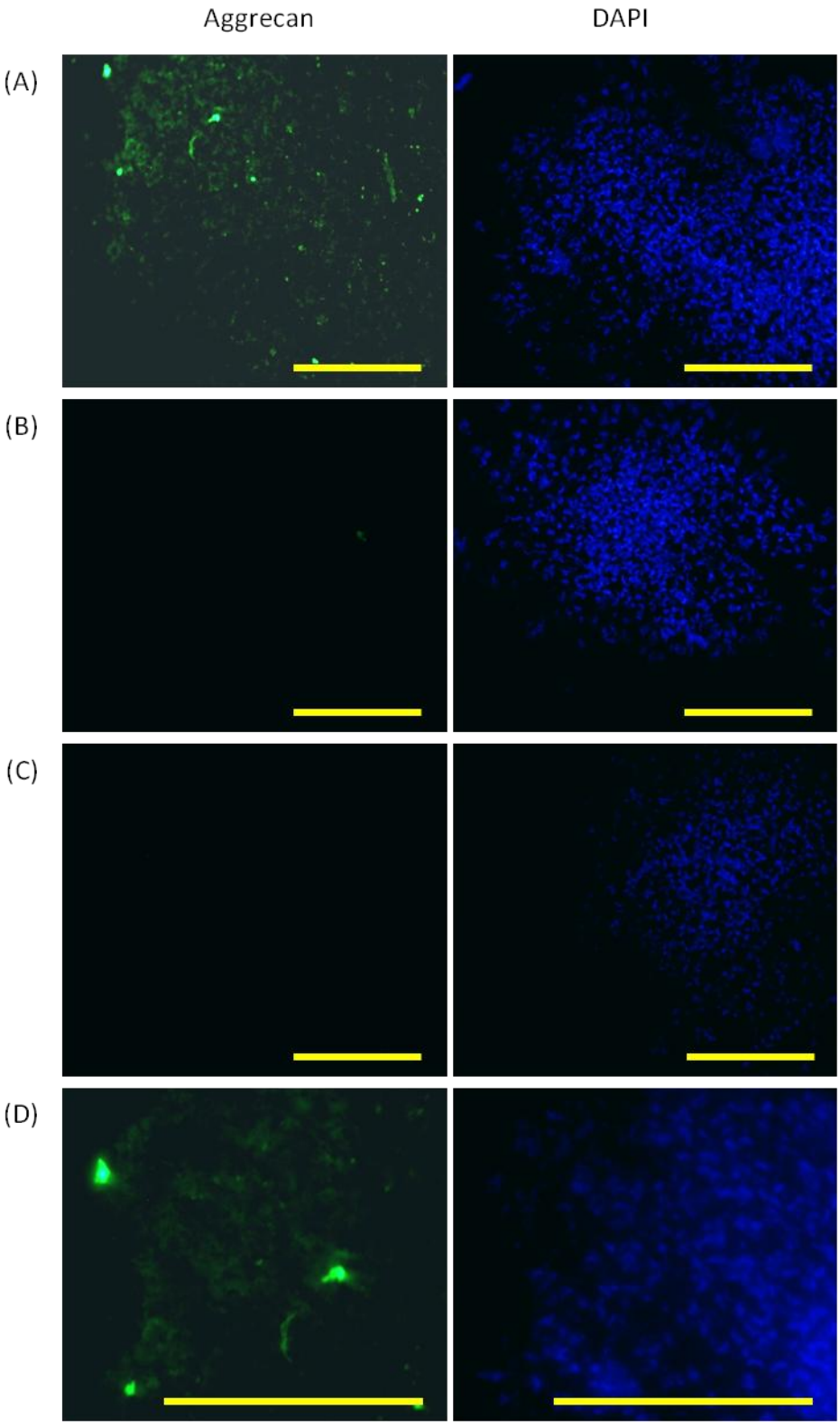

Fig 5.11: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) 7 XVII x20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar 200um

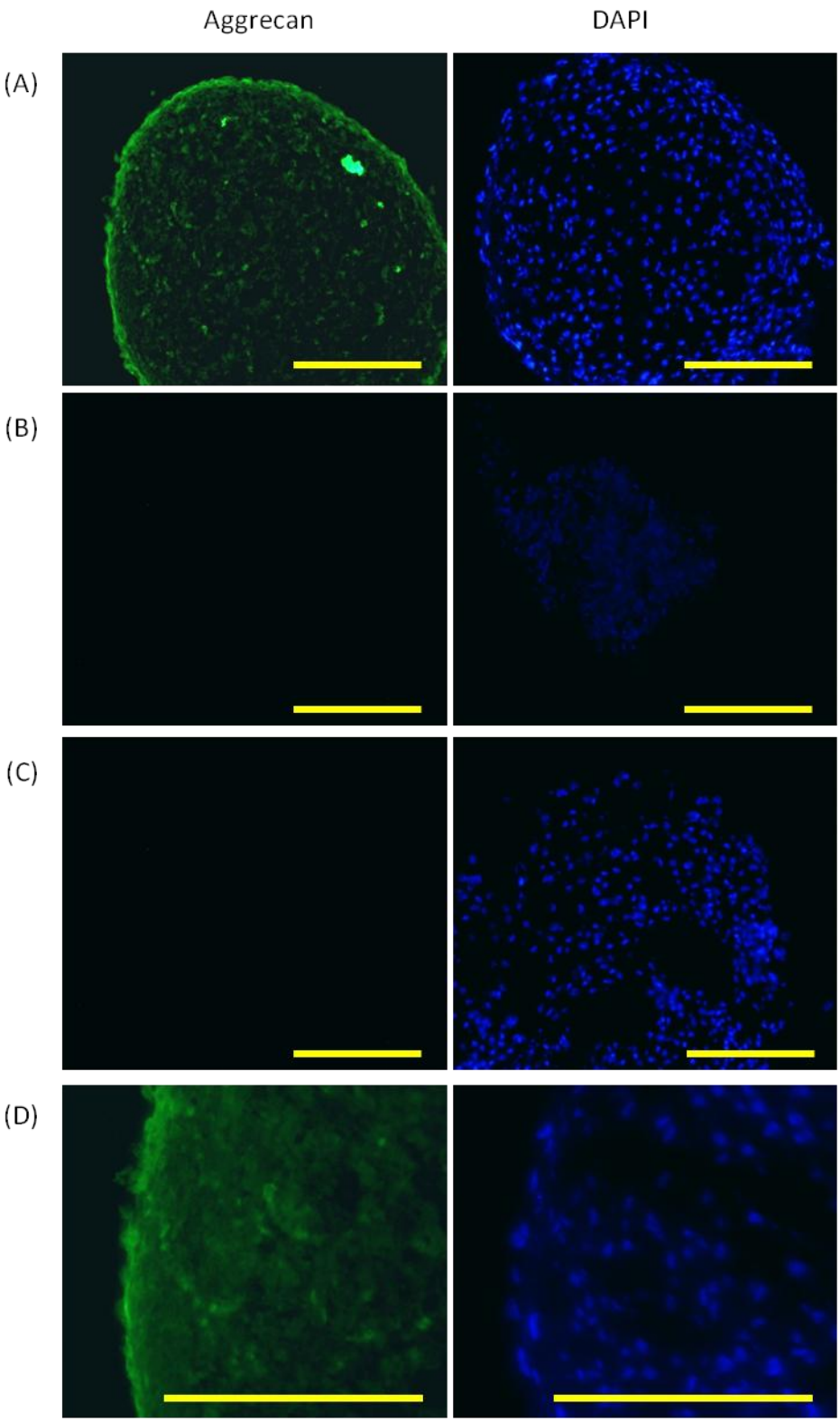

Fig 5.12: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) LO XVII x20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar = 200um

FIG 5.11

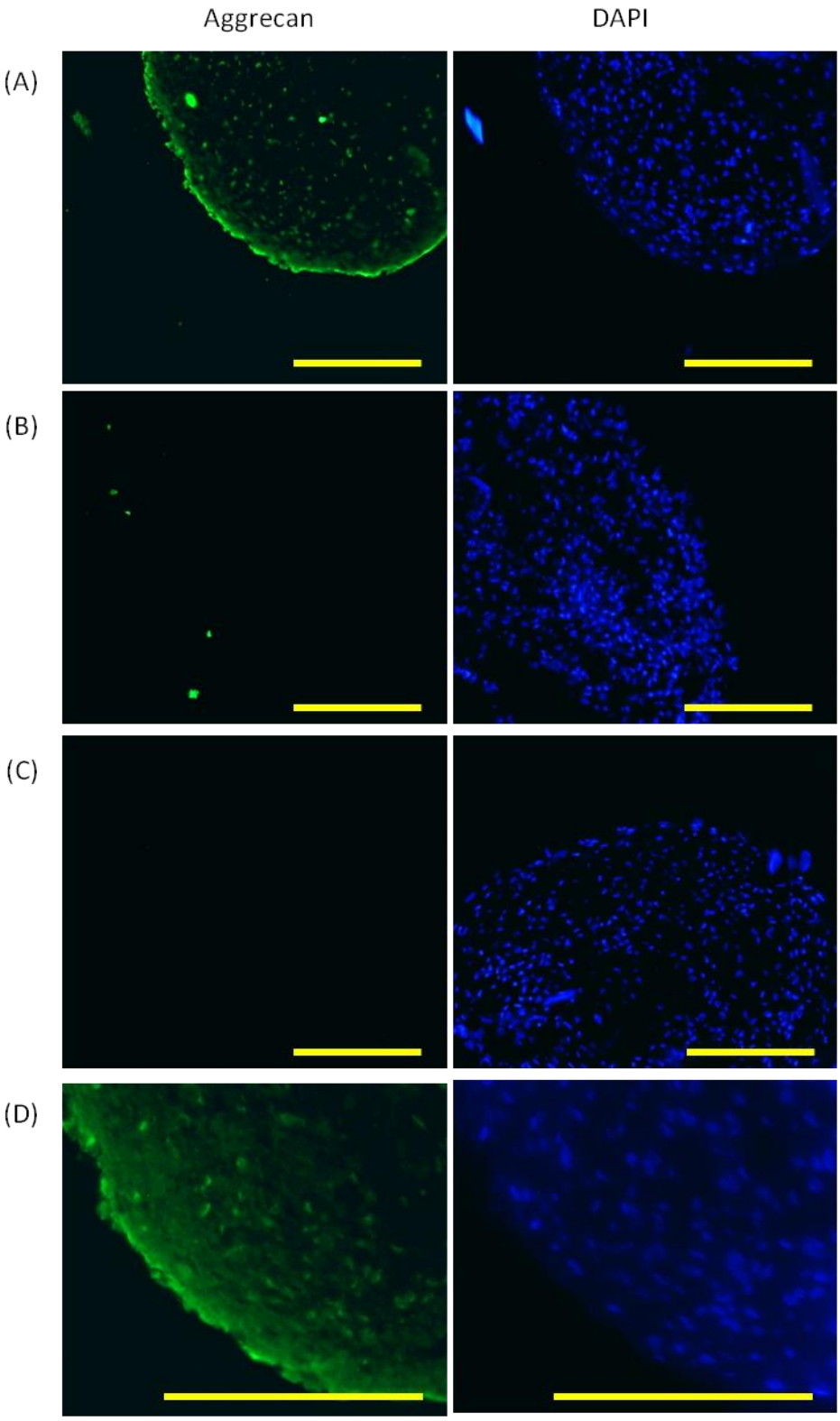

Fig 5.13: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) NA XVII x20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar = 200um

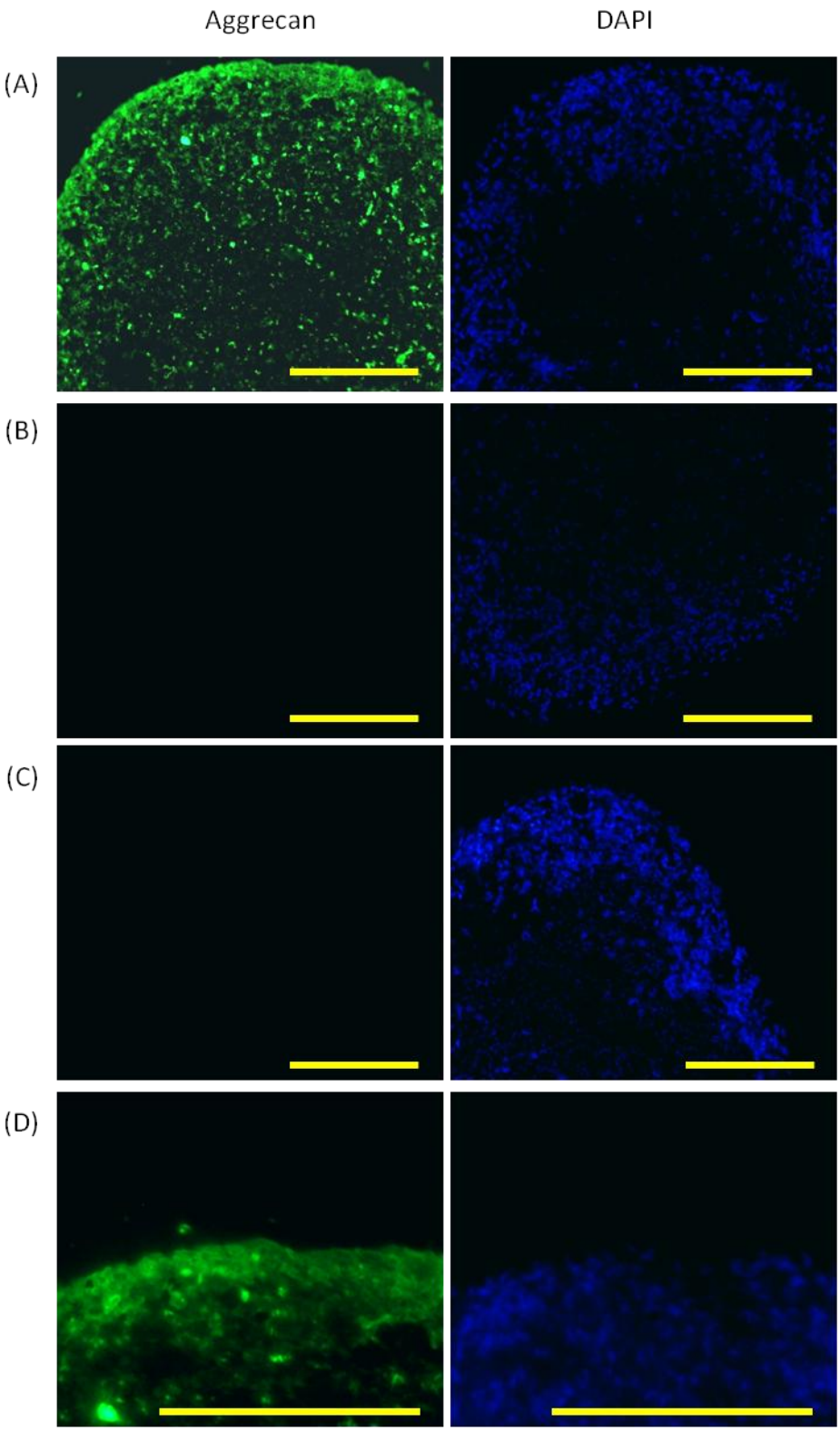

Fig 5.14: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) 3 XXVIII x20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar = 200um

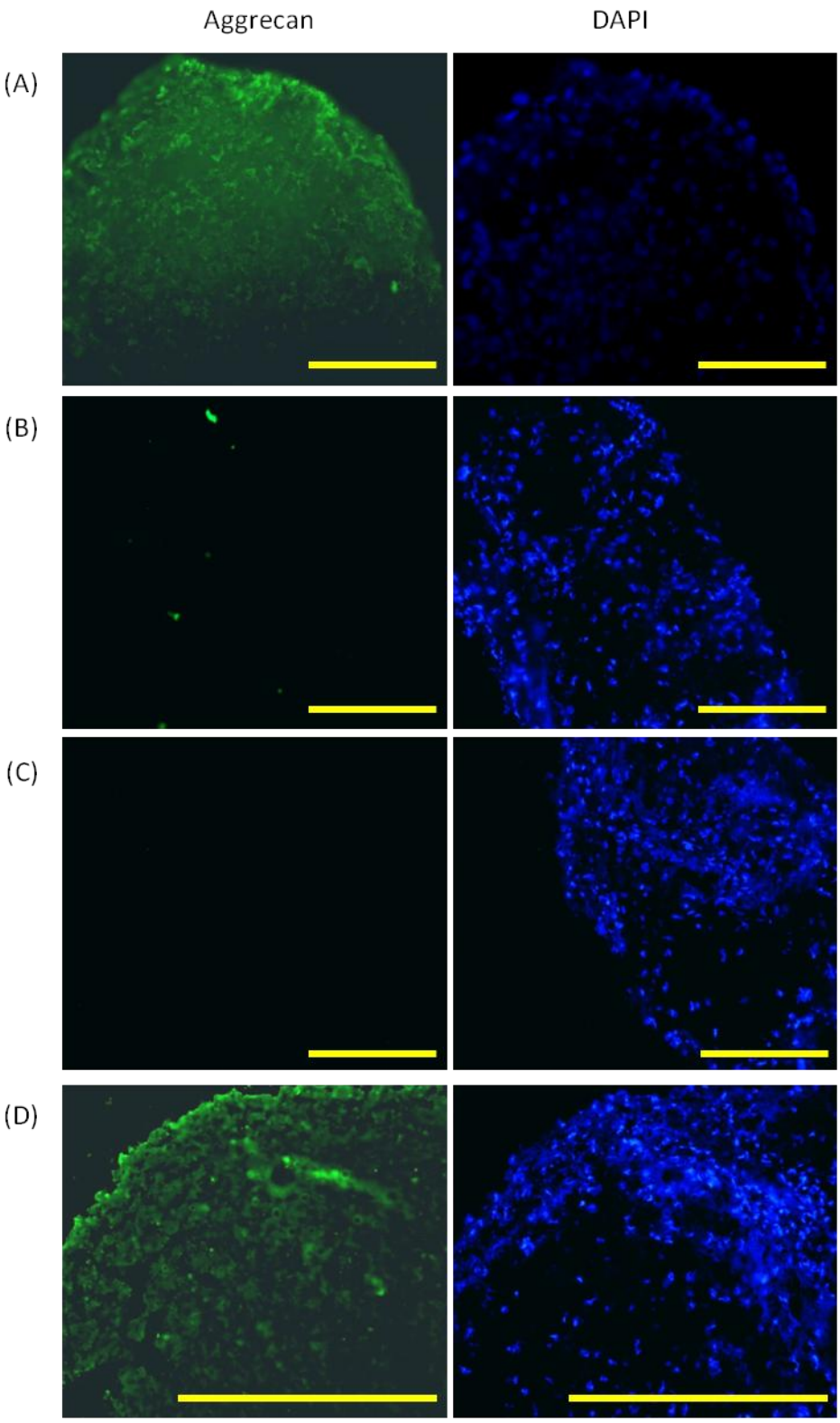

Fig 5.15: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) 9 XXVIII x20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar = 200um

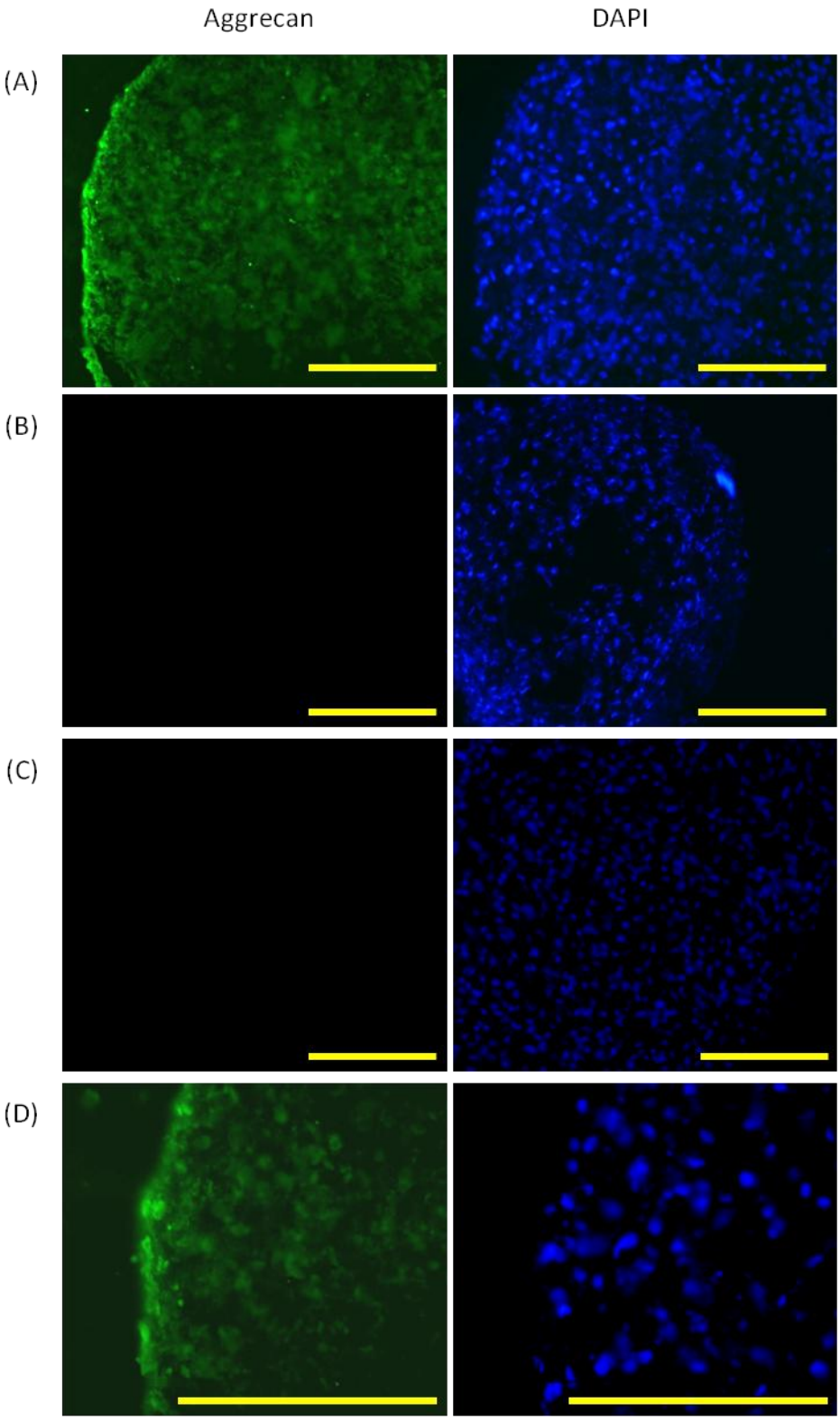

Fig 5.16: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) LO XXVIII x20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar = 200um

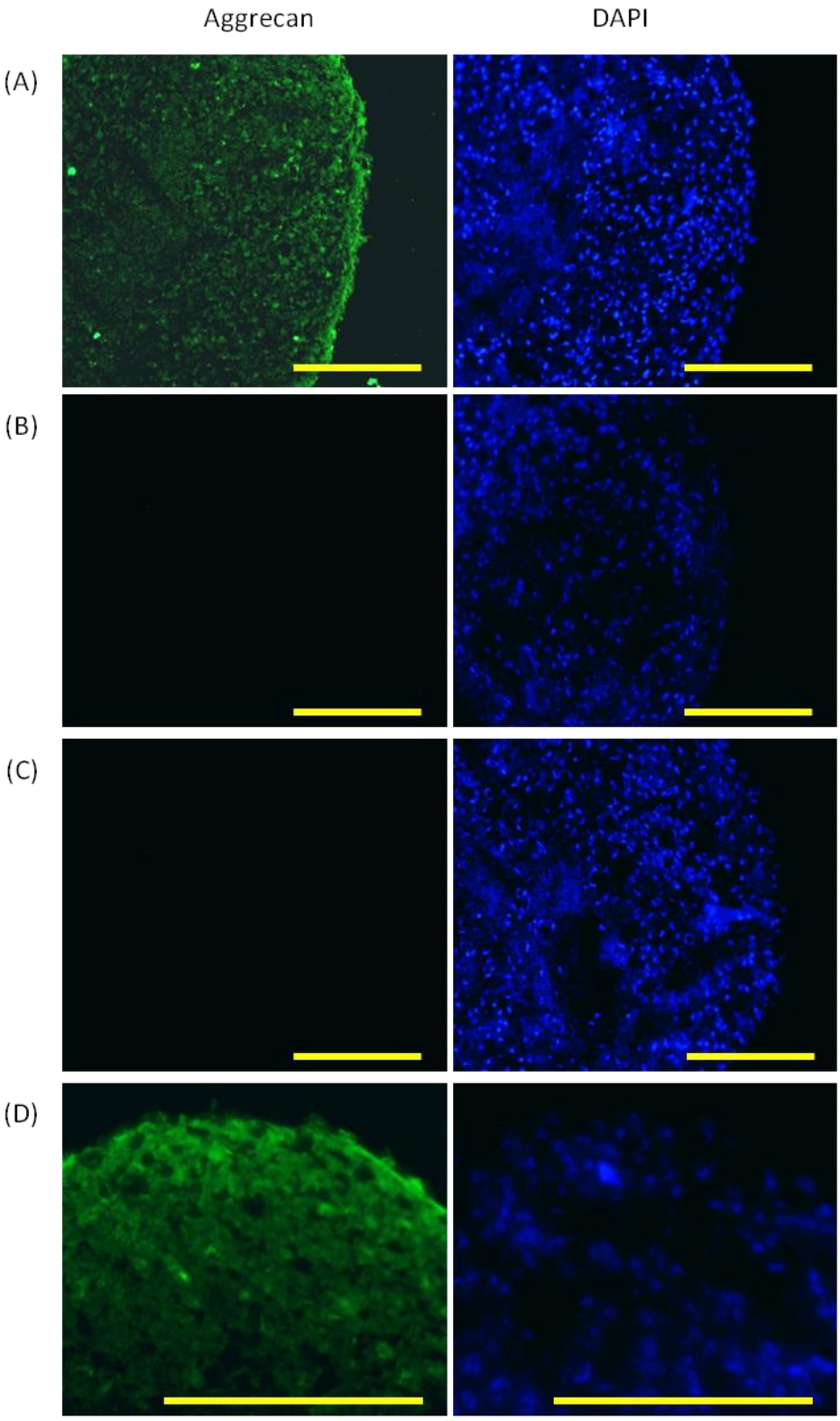

Fig 5.17: Aggrecan staining of cryopreserved chondrogenic pellets, showing the presence of aggrecan (green) and dapi stained nuclei (blue) within 10um sections. (A) NA XXVIIIx20 magnification (B) Primary Negative x20 magnification (C) Secondary Negative x20 magnification(D) Aggrecan x40 magnification. Scale bar = 200um

5.2.3 Osteogenic Differentiation

5.3.1 Alizarin Red Staining of Osteogenic Cultures

To determine if the isolated cell populations were capable of osteogenic differentiation *in vitro* under induction conditions, cells were cultured for 28 days and stained with alizarin red, a histological stain which binds to calcium deposits and produces a red colour. In patient XVII all cell populations were capable of osteogenic differentiation as observed by strong staining of calcium deposits, with no apparent differences in intensity of staining between cell populations (Fig 5.18). Similarly all populations of patient XXVIII are capable of osteogenic differentiation however; both NA XXVIII and 9 XXVIII demonstrate lower levels of staining, with small localised deposits compared to the more widespread staining observed in 3 XXVIII and LO XXVIII (Fig 5.19). In both patients, control populations appear to have no calcium deposits, suggesting that the effects of the osteogenic media are responsible for the presence of staining in osteogenic cultures.

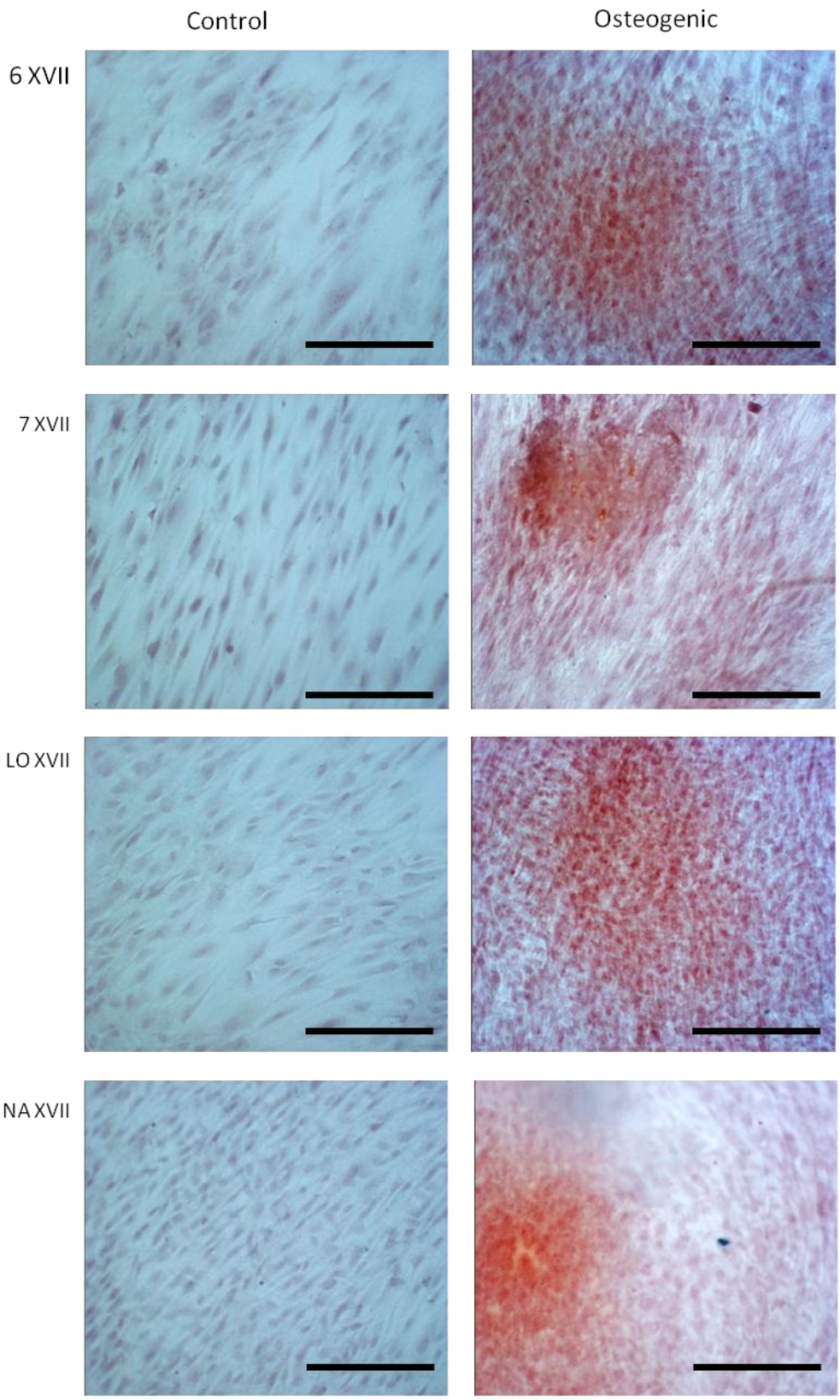

Fig 5.18: The detection of osteogenic differentiation by calcium deposition in osteogenic and control cultures from patient XVII. Cells were cultured for 28 days in the presence or absence of osteogenic factors, after which time cells were fixed and stained with Alizarin red solution. If calcium deposition had occurred the presence of red staining was detectable under bright field microscopy. Scale bar = 200μm

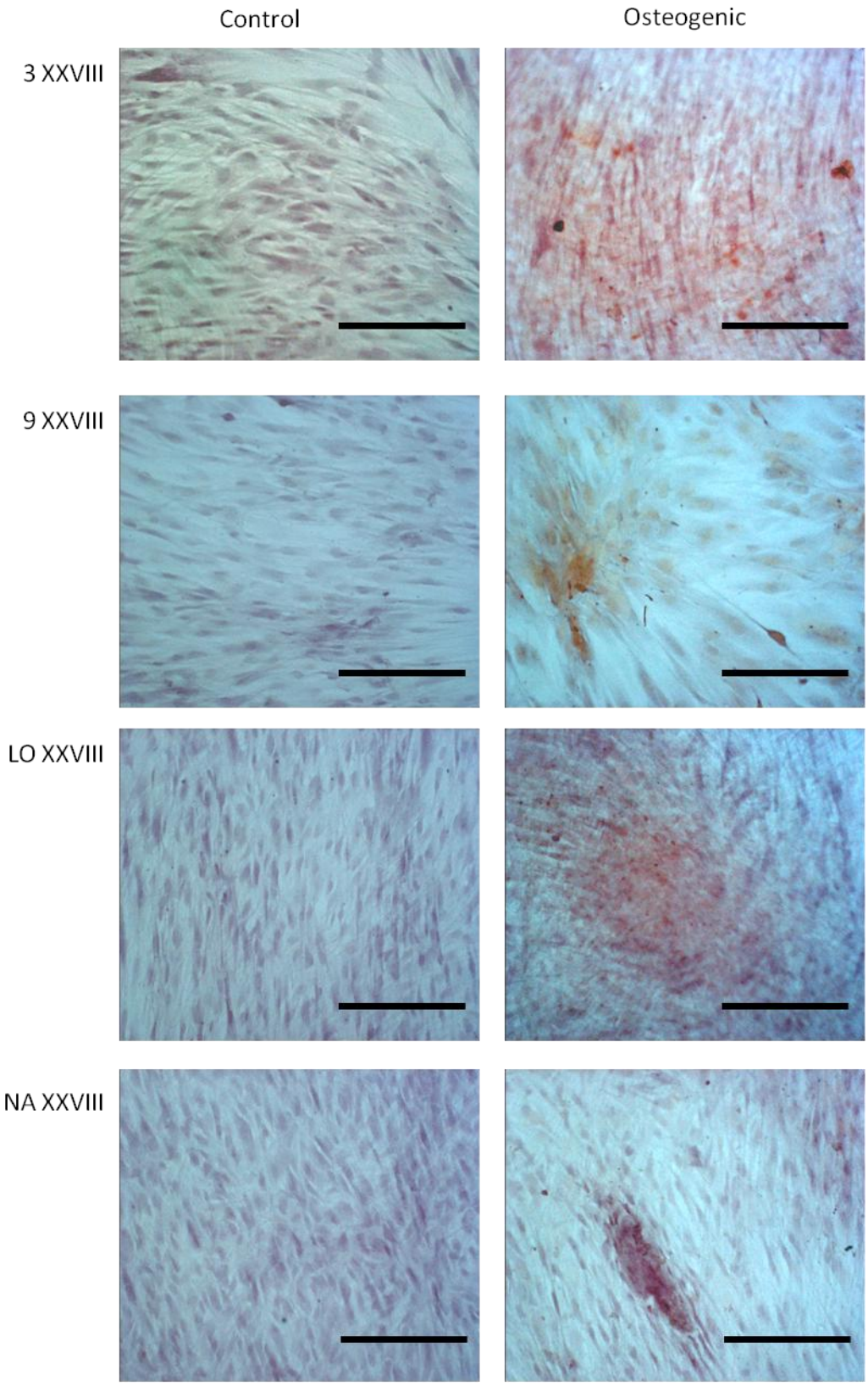

Fig 5.19: The detection of osteogenic differentiation by calcium deposition in osteogenic and control cultures from patient XXVIII. Cells were cultured for 28 days in the presence or absence of osteogenic factors, after which time cells were fixed and stained with Alizarin red solution. If calcium deposition had occurred the presence of red staining was detectable under bright field microscopy. . Scale bar = 200um

5.3 Discussion

The aims of this chapter were to determine the differentiation potential of the isolated oral mucosal cell populations and to determine if their lineage potential is similar to that observed within other adult stem cell populations. To this end the potential toward osteogenic, adipogenic and chondrogenic differentiation were probed.

Adipogenic differentiation was determined through mRNA expression of lineage specific markers CCAAT/enhancer binding protein-alpha (C/EBPα), peroxisome proliferator activated receptor (PPARγ) and Lipoprotein lipase (LPL) using QRT-PCR. C/EBPα and PPARγ are both early stage indicators of Adipogenesis whilst LPL is classed as a late stage marker of adipogenic differentiation (Morrison and Farmer, 2000).. In terms of PPARγ expression, LO and NA populations appear to respond to adipogenic induction earlier than Clonal populations, with higher levels of expression at day 7, but by day 14 the Clonal populations demonstrated a stronger response with higher expression. C/EBPα expression was delayed in LO and NA populations compared to the Clones which increased expression at day 14 compared to day 21/28 seen in the LO/NA XXVIII populations. Expression of LPL within the LO/NA populations was higher than the level recorded in the other cell populations at day 7, whilst at day 14 there was no significant difference in expression. Clone 3 did not appear to express LPL at levels near those observed in the cell populations, suggesting that whilst the early markers of adipogenesis are upregulated, the differentiation of these cells requires additional stimulation for full adipogenesis. The variation in LPL expression

suggests that this data is not conclusive, as levels of LPL appear to increase in control cultures at levels comparable to the adipogenic induction cultures. Further experiments are required to determine the true role of LPL within the cell populations during adipogenic differentiation. Confirmation of differentiation Oil Red-O staining to confirm the presence of intracellular lipid droplets, suggests that whilst temporal differences are apparent in the different cell populations in terms of the expression of the early markers of adipogenesis, this has little bearing on the level of lipid deposition. In both patients LO populations appear to show the most widespread lipid staining, suggesting that some aspect of this cell population precludes it to undergo adipogenic differentiation. An interesting result was the presence of lipid staining within the control cultures of LO for both patients. This may be an artefact of culturing the cells at confluence for 28 days, which may induce a, adipogenic-like state. The inclusion of a day 0 timepoint or from cells which were nearing senescence may highlight genetic changes that may be distinct from adipogenesis, but still lead to lipid staining. It may suggest a level of contamination of the heterogeneous cell population during the isolation process with adipocytes from the lamina propria, and could explain why the other clonally selected and NA populations did not appear to display lipid accumulation in the control cultures. This phenomenon has been observed previously in bronchial lung fibroblasts which exhibited a mesenchymal stem cell phenotype, so is perhaps not unsurprising (Sabatini et al., 2005). In other tissues, spontaneous differentiation has been documented, adipose stromal cells have been reported to differentiate into cardiomyocytes (Planat-Benard et al., 2004). Utlimately the differentiation along the adipogenic lineage was

achieved in all cell populations, with the lipid staining suggesting that the heterogeneous LO cell population was more capable of adipogenic differentiation *in vitro* due to higher levels of observable lipid accumulation however, without a validated method of quantification then conclusions cannot be drawn at this time. In the future use of a technique incorporating the use of DAPI counterstaining for quantification of lipid production in relation to cell number may provide a better level of differentiation (Gorjup et al., 2009). This data contrasts with the use of buccal mucosa fibroblasts as a negative control for dental pulp stem cell experiments, where it was reported that buccal mucosal fibroblasts, which are effectively the LO population in these experiments, were unable to undergo adipogenic differentiation (Lindroos et al., 2008). The ability of these cells to successfully undergo differentiation along the adipogenic lineage conforms to the data from other adult stem cell populations both BMSC and cell populations derived from oral tissues including the lamina propria of the oral mucosa (Seo et al., 2004, Pittenger et al., 1999, Romanov et al., 2005, Sabatini et al., 2005, Schilling et al., 2007, Davies et al., 2010, Marynka-Kalmani et al., 2010).

Chondrogenic differentiation was successful in all cell populations, with the expression of aggrecan confirmed by immunohistochemistry and the presence of collagen detected by Van Gieson's staining. The pellet culture system was chosen for chondrogenic differentiation as it provides a three-dimensional culture system exposed to mechanical stresses through regular centrifugation simulating load bearing as it would be experienced in cartilage (Yang et al., 2004). Differences were observed in structural integrity of the

pellets, with the LO and NA populations in both patients appearing to remaining intact during sectioning, suggesting the underlying tissue produced during differentiation was more developed that that observed in the clonally derived cell populations. The failure of control pellets to survive the culture period suggests that cells from the oral mucosa are not able to differentiate spontaneously just from the pellet culture condition as reported in bovine mesenchymal stem cells (Bosnakovski et al., 2004, Bosnakovski et al., 2006), and that the presence of induction factors is crucial. To draw comparisons of the levels of differentiation beyond the structural differences observed in the histology staining would require additional analysis. Attempts were made to isolate RNA from chondrogenic pellets for RT-PCR analysis however; these were unsuccessful due to the low concentrations of RNA isolated. If this had been successful, temporal changes in chondrogenic gene expression may have been detected which would allow differences between cell populations to be observed. Chondrogenic differentiation has been demonstrated in adult stem cell populations from multiple sites including BMSC and oral tissues (Davies et al., 2010, Marynka-Kalmani et al., 2010, Bernstein et al., 2009, Bosnakovski et al., 2004, Lin et al., 2005, Majumdar et al., 2000, Yanada et al., 2006, Yoo et al., 1998, Hiraoka et al., 2006).

Osteogenic differentiation was confirmed using Alizarin red staining for the presence of calcium deposits after 28 days culture in the presence of osteogenic induction medium. The control cultures did not exhibit any staining. In patient XVII the level of calcium deposition was comparable in all cell populations, with large areas of staining observed. In patient XXVIII the

NA population appeared to show a reduced capacity for osteogenic differentiation. The same was apparent from the levels of staining within 9 XXVIII cultures. Further characterisation of the differentiation is needed, in terms of the expression of bone specific differentiation markers during differentiation. RNA was extracted from osteogenic cultures to determine whether the cell populations did express the common markers of osteogenic differentiation however, technical issues and time constraints prevented further analysis. In the future this is a key analysis for the better understanding of any differences which may occur in differentiation between the cell populations. Successful differentiation along the osteogenic lineage, as with the chondrogenic and adipogenic lineages, supports the findings in other stem cell populations from BMSCs to dental pulp stem cells, and supports the work carried out within the Wound Biology Group and elsewhere into lamina propria stem cell populations (Aslan et al., 2006, Bruder et al., 1997, Gabbay et al., 2006, Ikeda et al., 2006, Im et al., 2005, Jahoda et al., 2003, Kotobuki et al., 2005, Kotobuki et al., 2008, Davies et al., 2010, Marynka-Kalmani et al., 2010).

The aims of this chapter were to investigate the differentiation potential of the isolated cell populations and determine whether the differentiation capacity of these cell populations was affected by the stage of the differential adhesion assay from which the population derived. All cell populations successfully demonstrated the ability to differentiate along the common mesenchymal lineages, which supports the findings in other stem cell populations from adult tissues populations (Aslan et al., 2006, Bruder et al., 1997, Gabbay et

al., 2006, Ikeda et al., 2006, Im et al., 2005, Jahoda et al., 2003, Kotobuki et al., 2005, Kotobuki et al., 2008, Davies et al., 2010, Marynka-Kalmani et al., 2010, Bernstein et al., 2009, Bosnakovski et al., 2004, Lin et al., 2005, Majumdar et al., 2000, Yanada et al., 2006, Yoo et al., 1998, Hiraoka et al., 2006, Seo et al., 2004, Pittenger et al., 1999, Romanov et al., 2005, Schilling et al., 2007, Sabatini et al., 2005). Interestingly, the heterogeneous LO and NA populations appear to demonstrate the ability to differentiate as well as the clonally derived cell populations, and whilst there appear to be temporal differences in expression of markers at mRNA level, as observed in the adipogenic differentiation studies, the end point of successful differentiation is still achieved.

Cells of the lamina propria are developmentally derived from the neural crest (Winning and Townsend, 2000, Davies et al., 2010). Neural crest stem cells have been identified in a number of tissues, including oral tissues such as the adult palate (Widera et al., 2009). Work within the wound biology group has demonstrated the ability of clonally derived lamina propria stem cells to form neural lineages *in vitro* (Davies et al., 2010), suggesting that the lineage potential of these cells is greater than the potential reported in mesenchymal stem cells and would therefore make lamina propria progenitor cells a useful resource for tissue engineering and stem cell therapies. The work presented in this Chapter suggests that clonally selecting cells using their adherence properties may not be as important as first hypothesised, as the heterogeneous LO population, and even the NA population which should be depleted of stem/progenitor cells based on the principle of the differential

adhesion assay are capable of mesenchymal differentiation *in vitro.* Investigation into whether the neural differentiation observed in the clonal populations could be replicated in the LO and NA populations would be a useful addition to the research already conducted on lamina propria stem cells.

CHAPTER 6

General Discussion

6.1 General Discussion

In this thesis, the growth kinetics, telomerase activity and differentiation potential of multiple cell populations isolated from the lamina propria of the oral mucosa were described. The oral mucosa is well documented for superior healing compared to the skin, with increased replicative potential when compared to skin fibroblasts *in vitro* (Enoch et al., 2010, Enoch et al., 2009, Stephens et al., 1996, Stephens et al., 2001a, Lee and Eun, 1999). It has been hypothesised that these characteristics are due to a progenitor/stem cell population within the lamina propria which contribute to this preferential healing response (Stephens and Genever, 2007). Recent discoveries of stem cell populations within other craniofacial tissues have given more weight to the argument for the presence of a stem cell population within the oral mucosa (Calenic et al., 2010, Gagari et al., 2006, Huang et al., 2009, Izumi et al., 2007, Nakamura et al., 2007). Ultimately this hypothesis has been demonstrated by work conducted within the Wound Biology Group, and another research group based in Israel into stem cells within the buccal and gingival mucosa (Davies et al., 2010, Marynka-Kalmani et al., 2010, Zhang et al., 2010, Zhang et al., 2009). In order to further explore the characteristics of these cells, clonally derived cell populations derived from the buccal mucosa, along with those cells unable to adhere during the adhesion assay and heterogeneous cell populations representing the whole lamina propria were grown until senescence and the presence of stem cell associated markers and characteristics investigated. Therefore the first aim of this thesis was to successfully isolate cell populations using the differential

adhesion assay, and investigate their growth characteristics in regard to the wider field of adult stem cell biology.

In Chapter 3 the colony forming efficiency, growth potential and morphological changes with *in vitro* ageing were studied to determine if the clonally derived cell populations differed *in vitro* to the other cell populations derived from the lamina propria during the differential adhesion assay. This assay has been used for the isolation of stem cells within the epidermis of the skin, and articular cartilage, exploiting the increased expression of α5b1 integrin on the surface of progenitor/stem cells to ensure those cells are able to adhere rapidly and (Dowthwaite et al., 2004, Williams et al., 2010, Jones, 1996, Jones and Watt, 1993). As well as demonstrating an increased adherence to fibronectin, there was evidence of an increased colony forming efficiency within those rapidly adhering cells as determined by the ability to form colonies from a single adherent cell, and this effect was seen in all patients used in this assay. In this respect these cells share similarities with mesenchymal stem cells, which have long been isolated using the colony forming unit fibroblast (CFU-F) assay, another method of calculating the ability to derive from a single cell (Castro-Malaspina et al., 1984, Castro-Malaspina et al., 1982, Castro-Malaspina et al., 1980, Deans and Moseley, 2000). After isolation of clonally expanded cell populations, along with the LO and NA populations previously described, cells were cultured through to cellular senescence, and at each passage cells were counted and the cell number along with the seeded cell number were used to calculate total

population doublings. Over the lifetime of the cell populations, an average growth rate was calculated to determine if within the context of total average growth rate, there were any differences within the different cell populations. The results demonstrated the ability of all cell populations to exceed 50 population doublings *in vitro* with the exception of one clonal cell population (10XVII). This growth potential is comparable, if not greater than the replicative potential observed in MSCs isolated from bone marrow or cord blood (Bieback et al., 2004, Colter et al., 2000, Banfi et al., 2000, Baxter et al., 2004, Bruder et al., 1997). With respect to adult stem cell populations derived from other tissues, stem cells isolated from the dental pulp have been reported to in excess of 20-40 population doublings (Gronthos et al., 2002, Suchanek et al., 2009, Suchanek et al., 2007). Articular cartilage progenitor cells have been reported reaching 60 population doublings (Dowthwaite et al., 2004, Williams et al., 2010). Other groups have reported findings in terms of population doubling time (Widera et al., 2009). This does not however provide any information about the proliferative capacity of these cell populations however and ultimately cannot be used in the same way to determine the replicative potential of isolated stem cell populations. The results found with the lamina propria derived cell populations suggest that, irrespective of clonal selection of cells based on adherence characteristics, these cells are capable of a vastly increased replicative potential, as already characterised from work on oral fibroblasts (Stephens et al., 1996, Stephens et al., 2001a, Enoch et al., 2010, Enoch et al., 2009). The final analysis of Chapter 3 was to determine any morphological changes associated with *in vitro* ageing in the isolated cell populations. The age related changes

considered markers of senescence, observed in both MSC and fibroblast populations include an increase in cell size with an associated change in cell morphology (Baxter et al., 2004, Banfi et al., 2000, Bruder et al., 1997, Hayflick, 1965). In this manner, the isolated cell populations demonstrated the same age related changes, with an increase in cell size observed in all cell populations with the exception of clone 10 XVII, which underwent early senescence. Along with change in cell size, a visible flattening of cells and an increase in the number of cellular processes occurred. The differences in cell size between cell populations varied between patients, and with no distinct difference between clonal populations and LO populations. The lack of a third patient for analysis excludes the presentation of a common trend within the cell populations and is crucial for any future work into these cell populations.

Overall the analysis presented in Chapter 3 indicates that differential adhesion does allow for the selection of a highly clonogenic cell population which exhibits distinct growth kinetics when compared to whole lamina propria (LO) digests, and that those cells which do not adhere during the assay (NA) behave in a similar manner to those LO cells. Interestingly the cell population with the highest cumulative population doublings remains the LO population, suggesting that this heterogeneous population allows for the maintenance of the resident stem cell population over time in culture. The clonal populations undergo many population doublings before senescence, but the heterogeneous cell population may enhance the replicative potential of the resident stem cell population, remaining quiescent and only dividing when those terminally differentiated cells become senescent and effectively

‘drop out’ of the proliferating population. This may explain the higher number of population doublings achieved before senescence was observed within the LO populations of both patients. Finally changes in cell morphology appear to occur as expected from both MSC and fibroblast characterisation as mentioned previously.

The second aim of this thesis was the characterisation of the isolated cell populations with regard to the presence of stem cell associated markers. These included the presence of stem cell surface markers as identified from the literature; CD90, CD105 and CD166. The inclusion within this panel of markers of CD34 and CD45 allowed for the confirmation that these cells were not of fibrocyte or haematopoietic origin (Pasquinelli et al., 2007, Nagatomo et al., 2006, Song et al., 2005, Diaz-Romero et al., 2005). All cell populations proved to be positive for all three markers as observed in stem cell populations isolated from a number of tissues including bone marrow, periodontal ligament, dental pulp, cartilage, synovial fluid and adipose tissue (Trubiani et al., 2010, Wada et al., 2009, Tomokiyo et al., 2008, Gay et al., 2007, Nagatomo et al., 2006, Hiraoka et al., 2006, Fickert et al., 2004, Dowthwaite et al., 2004, Alsalameh et al., 2004, Zannettino et al., 2008, Ikeda et al., 2006, Morito et al., 2008). It also confirms the findings already observed in the buccal and gingival mucosa (Davies et al., 2010, Marynka-Kalmani et al., 2010). Slight levels of staining were present in three of the cell populations analysed by flow cytometry for CD34 and CD45, but this was attributed to non-specific binding due to out of date antibodies and not due to

the phenotype of the cell population in question. The next analysis was to determine the telomerase potential of isolated cell populations to understand the replicative potential observed within the cell populations, and to determine if they are superior to MSCs by the presence of active telomerase, as this has yet to be identified with MSCs (Zimmermann et al., 2003, Fehrer and Lepperdinger, 2005). Telomerase has been identified in haematopoietic cells and neural precursor cells and in chondrogenic progenitors from articular cartilage (Chiu et al., 1996, Ostenfeld et al., 2000, Williams et al., 2010). The presence of active telomerase suggests that these isolated cell populations are superior to MSCs in confirmation of their superior replicative potential, and present a more promising therapeutic choice for tissue repair. The stem cells which demonstrate well documented telomerase activity are embryonic stem cells (Heins et al., 2004, Thomson et al., 1998). The expression of telomerase within the isolated cell populations suggests a “younger” phenotype more akin to ESCs than MSC. This correlates with the phenotypic analysis of oral mucosal fibroblasts and may contribute to the preferential healing *in vitro*. Interestingly the presence of active telomerase in the LO and NA populations suggests that even after differential adhesion, a progenitor cell population with telomerase potential and expressing stem cell surface markers persist within that NA population.

The final analysis was to determine the telomere lengths within isolated cell populations at early, mid-stage and late time points to observe any age-related changes in telomere length which may indicate the levels of telomerase detected within the samples are responsible for the high

replicative potential of these cell populations. The erosion of telomeres has been demonstrated to contribute to replicative senescence in somatic cells, and telomere shortening has been linked with rapid *in vitro* ageing of MSCs (Baxter et al., 2004, Rubin, 2002, Wagner et al., 2008, Hayflick, 1965). The presence of telomerase within the samples did not appear to prevent the shortening of telomeres within cell populations, indeed the cells which were able to demonstrate longer telomeres at the late time point were the cell population which underwent senescence at a much earlier PD, suggesting that senescence in these cell populations was not caused by a telomerase-dependent mechanism. This supports the findings reported in oral fibroblasts, that senescence occurs by telomerase independent mechanism. (Enoch et al., 2009).

The overall aim of Chapter 4 was to determine if the cell populations isolated demonstrated the characteristics attributed to stem cell populations. Investigations of marker expression and telomerase activity demonstrate that these cells are superior to BMSCs and other adult stem cell populations, and the presence of telomerase would allow for a more plentiful source of stem cells for tissue regeneration and repair. The resulting telomerase activity reported in the LO/NA population, along with the presence of stem cell associated marker suggests that the lamina propria of the oral mucosa could be used without clonal selection of progenitor cells within the population, as even the fibroblastic NA population exhibits stem cell characteristics.

The aims of Chapter 5 were to determine if the isolated cell populations, already identified as stem cell like by the data of the previous two chapters

were able to differentiate along a number of mesenchymal lineages. The understanding of the lineage potential of these cells is crucial for tissue engineering and repair applications, to determine if they represent a more suitable choice of adult stem cell than other, better characterised cells such as BMSCs. The lineage potential of BMSCs is well characterised with differentiation along chondrogenic, adipogenic and osteogenic differentiation observed in these cells (Romanov et al., 2005, Kolf et al., 2007).(Kotobuki et al., 2008, in 't Anker et al., 2003). Differentiation potential of stem cell populations isolated from the periodontal ligament, dental pulp and oral mucosa suggest they would all be useful replacements for BMSC to this end (Gay et al., 2007, Nagatomo et al., 2006, Davies et al., 2010, Marynka-Kalmani et al., 2010, Gronthos et al., 2002, Otaki et al., 2007a). Clonal expansion can affect the differentiation potential of the cells (Kotobuki et al., 2004), and therefore the comparison between cell populations to determine if differential adhesion selected for cells with an increased differentiation capacity would be important to determine if this isolation method is necessary, or if simple heterogeneous cultures are equally capable of repairing tissue *in vivo*. The three “classical” lineages were chosen for this thesis, adipogenic, chondrogenic and osteogenic differentiation. Recent findings suggested buccal mucosal fibroblasts were incapable of differentiation and were included in a paper on the stem cell properties of other dental stem cell populations, however in this thesis the successful differentiation of all cell populations, including the LO population which was comparable to the buccal mucosal fibroblasts along adipogenic, osteogenic and chondrogenic lineages (Lindroos et al., 2008) in confirmation of the

research already conducted into the differentiation potential of lamina propria stem cell populations (Davies et al., 2010, Marynka-Kalmani et al., 2010). Adipogenic differentiation was demonstrated through the presence of lineage specific mRNA using both quantitative PCR. Differences in expression and time of response observed by did not relate to differences in levels of lipid accumulation observed in the adipogenic cultures, and the LO populations appeared to demonstrate a higher level of differentiation, at least at a histological level observed by Oil Red-O staining. Chondrogenic and Osteogenic differentiation were confirmed at a histological level through the staining of collagen and aggrecan within pellet culture sections and alizarin red staining for the presence of calcium respectively. In both lineages the use of more quantitative methods of assaying successful differentiation would be crucial to a better understanding of the differences between cell populations during differentiation, however it would appear that the whole cell population is able to undergo differentiation. This may be due to the increase of stem cells within the heterogeneous cell populations in culture. It has been demonstrated through the rapid intial proliferation during differential adhesion that those cells are capable of clonally expanding *in vitro*. These cells may be increasing within the heterogeneous population, and perhaps even within the NA populations a small sub population of multipotent stem cells remains which is responsible for the observed differentiation capability of these cells. Ultimately these *in vitro* differentiation experiments are not representative of the three-dimensional environment for which tissue repair therapies will be designed, and therefore more work is required to identify if these cell

populations are capable of generating multiple tissues in an *in vivo* environment.

The overall aim of this thesis was to compare the clonally expanded oral mucosa lamina propria stem cells (OMLPSCs) with cell populations isolated by different means, in the context of the tissue from which they derive. This was to determine if any phenotypic characteristics differentiated them from normal cells resident within the tissues. However through comparisons of the cell populations for their replicative potential and other stem cell associated markers it became apparent that the LO and NA populations, both heterogeneous populations derived from the lamina propria, are potentially equal to the clonally derived cells in terms of stem cell markers and lineage potential. Further investigation into the differentiation mechanisms of those lineages investigated in this thesis and indeed of further lineages to probe the full lineage potential of these cells is crucial to the success of the lamina propria as the superior source of multipotent stem cells. The ease of access and minimally invasive procedure involved in the isolation of cells from the lamina propria, along with the minimal scarring characteristics of oral tissues, make it an exploitable option for tissue engineering and repair. The initial hypothesis of this thesis, that the clonally derived cells would exhibit a distinct phenotype was found to be incorrect. The presence of stem cell characteristics and the capability of differentiation along adipogenic, osteogenic and chondrogenic lineages suggest that contrary to the initial hypothesis, LO and NA populations appear to be enriched for stem/progenitor cell populations, and this is an interesting finding in the wider

context of adult stem cell populations, which typically represent a small niche within the host tissue.

6.2 Future Work

The potential of cells isolated from the oral mucosa lamina propria for tissue engineering purposes appears promising on the basis of the work contained within this thesis and work completed within the Wound Biology Group. However with the study of only two patient samples it is problematic to draw solid conclusions from the data presented. If continuing this work in future the first point to address would be the addition of at least one extra patient to the study, preferably a larger number of patients than three to ensure the statistical power of any observations and ensure that variation due to patient heterogeneity is controlled for within the experimental setup. Analysis of telomere lengths was conducted using the TRF assay, which is only a crude method of monitoring changes to telomere length. In the future use of the STELA technique, to observe telomere erosion at a very high resolution would be more informative to elucidate any telomere-dependent changes to the different cell populations. The differentiation of the cell populations along the chondrogenic, osteogenic and adipogenic differentiation was demonstrated, however the ability of Oral Mucosal cell populations compared to progenitors from each of these development origins would help determine if the Oral Mucosa is the best source of cells for regenerative medicine, and

indeed if any differences between cell populations are present in terms of differentiation potential.

If continuing this study, direct comparison of differentiation ability with populations such as chondroprogenitors for chondrogenic differentiation (Williams et al., 2010), may identify where oral mucosal progenitor cells reside within the field of stem cell biology. As well as an extension of the differentiation potential already observed within this thesis, in the future it would be prudent to investigate other potential lineages which may be possible with lamina propria cell populations. Work already conducted has demonstrated that adult stem cell populations from other tissues are not limited to the adipogenic, chondrogenic and osteogenic lineages. Adipose derived stem cells have been demonstrated to be capable of expressing neuronal lineage markers when subjected to the correct growth conditions (Guilak et al., 2006, Gimble and Guilak, 2003b, Gimble and Guilak, 2003a, Safford et al., 2002). In addition the presence of a neurogenic cell population within the dermis of mammalian skin, dental pulp has been demonstrated (Toma et al., 2001, Arthur et al., 2008). Work within the Wound Biology Group and elsewhere has demonstrated that clonally derived stem cells from the lamina propria are also capable of differentiating along the neuronal lineage *in vitro* (Davies et al., 2010, Marynka-Kalmani et al., 2010). Recently, the potential for *in vitro* oogenesis was reported in cells derived from porcine skin (Song et al., 2011). A potential avenue of future research would be to replicate the neuronal differentiation demonstrated in the clonally derived cells, and expand this work to include the LO and NA populations studied in

this thesis, to determine if clonal expansion does indeed isolate cells with a greater differentiation repertoire.

Another recent development in stem cell biology was the discovery of induced pluripotency of reprogrammed skin fibroblasts – iPS cells (Takahashi and Yamanaka, 2006, Takahashi et al., 2007b). This was achieved through the introduction of four factors required for embryonic stem cell maintenance and self-renewal. These factors are Oct-3/4, Sox-2, Klf-4 and c-Myc, and reverts skin fibroblasts back to an embryonic stem cell state, from which point it is possible to drive differentiation along any endo-, ecto- or mesodermal lineage. Recent advancements have led to the refinement of this method, the removal of c-Myc, a potent oncogene, and in the case of neural stem cells, which endogenously express three of the four factors, only oct-4 is required to induce pluripotency (Wernig et al., 2008, Kim et al., 2009, Kim et al., 2008). Work within the Wound Biology Group has demonstrated that whole oral mucosa lamina propria digests are positive for expression of Oct-3/4, Sox2, and Klf-4, suggesting that this cell population is already in a pluripotent state without induction, and may be capable of unrestricted differentiation as observed in iPS cells. An intriguing avenue of research would be to investigate the induction of pluripotency within the oral mucosal cell populations, to determine if (a) it is possible to achieve this using the introduction of the final required factor via genetic modification, in this case c-myc and (b) if successful in reprogramming all of the cell population studied in this thesis, would any differences in phenotype arise. Additionally, investigating if the presence of the iPS markers as reported by Davies et al

within the lamina propria can be exploited to alter the phenotype through culture conditions alone. A similar technique was employed using recombinant proteins in murine fibroblasts, eliminating the need for genetic modification (Zhou et al., 2009).

In terms of future work to be conducted on the basis of the findings presented in this thesis, I believe a variety of experiments could be conducted to further characterise the different cell populations described, and to determine if the Oral Mucosa does represent a good source of stem cells for the purposes of regenerative and tissue engineering.

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CHAPTER 8

Appendix

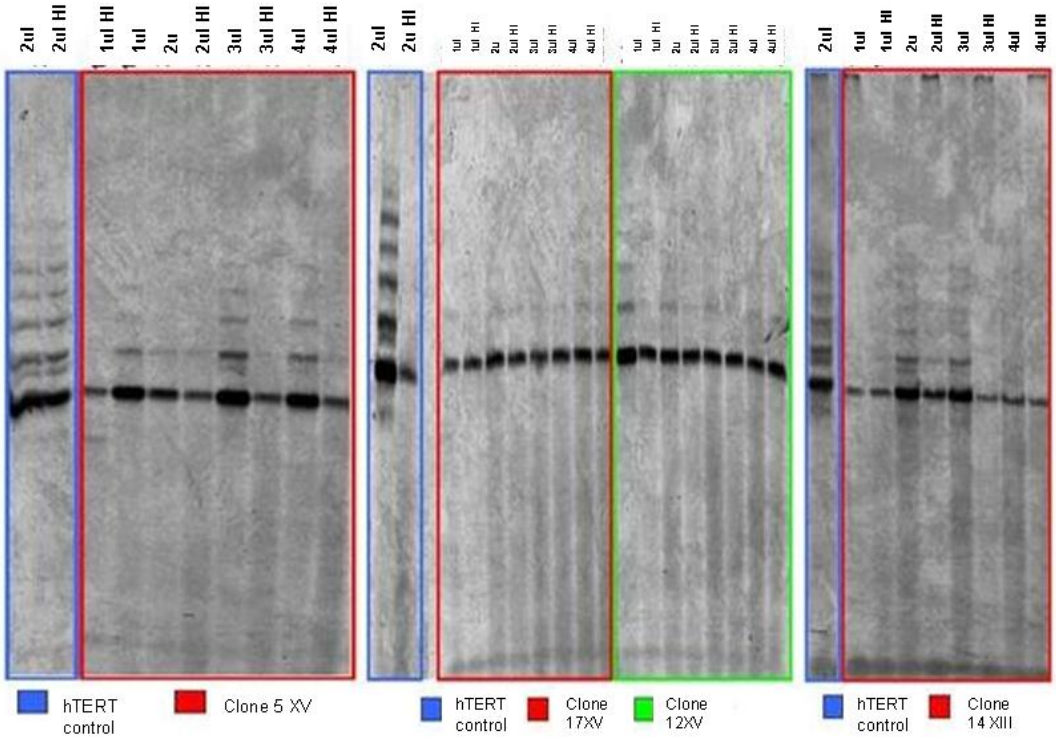

Fig 8.1. The Telomeric Repeat Amplification Protocol (TRAP) assay results for clones 5, 12 and 17 XV and clone 14 XIII using varying amounts of cell lysate. hTERT transfected cells were used as a positive control (+ve) and heat inactivated cell lysate was used as a negative control (HI)

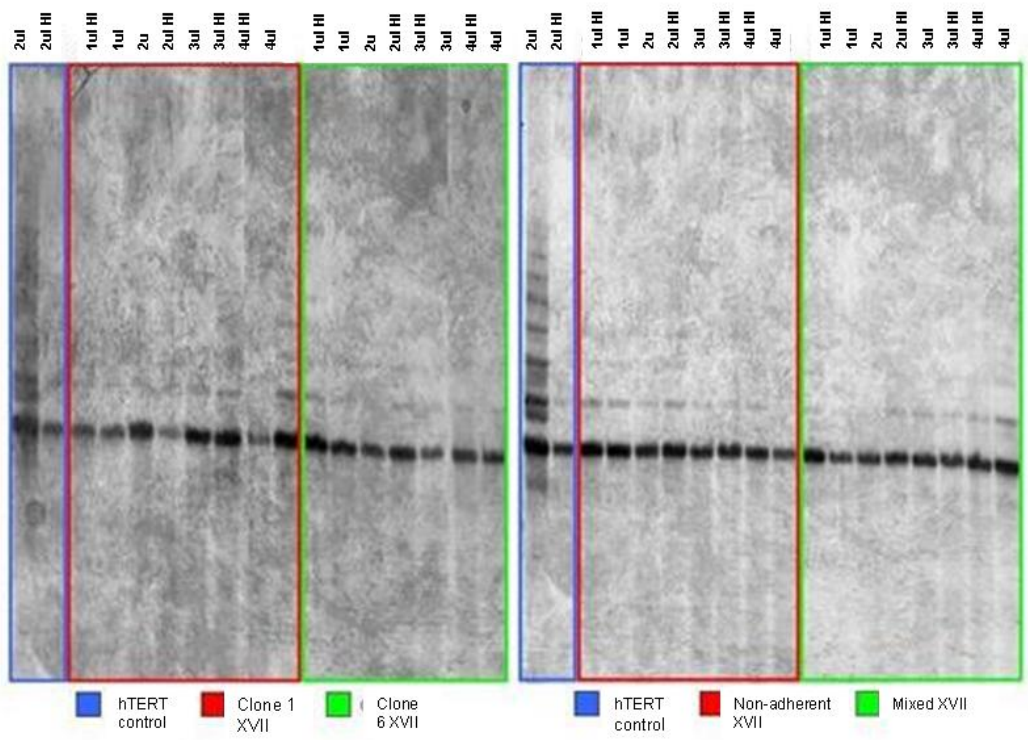

Fig 22. The Telomeric Repeat Amplification Protocol (TRAP) assay results for clones 6 and 10 XVII, and NA XVII and LO XVII using varying amounts of cell lysate. hTERT transfected cells were used as a positive control (+ve) and heat inactivated cell lysate was used as a negative control (HI)