Comparison of the multi-lineage differentiation ability of craniofacial and limb skeletal muscle derived progenitor cells

This thesis is submitted in part-fulfilment for the degree of Doctor of Philosophy at UCL

By Khalid Moaed Alqahtani, BDS, MSc

Division of Biomaterial and Tissue Engineering
UCL Eastman Dental Institute, UK
February 2012

Dedication

To my beloved family

You are the greatest treasure of my life and without your love, support and

prayers; I would have never produced this work

Declaration

I, Khalid M. Alqahtani confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Khalid Alqahtani

Acknowledgments

Abstract

Craniofacial skeletal muscles (CSkM) have a number of properties that distinguish them from other skeletal muscles. They have different embryological origin than limb muscles and are believed to have different regenerative capacity. In addition, they may offer an easy to access source of progenitor cells for tissue engineering applications.

The aim of this study was to isolate craniofacial muscle progenitor cells based on their adhesion properties to tissue culture plastic and investigate their differentiation ability compared to cells derived from limb muscles.

Mononuclear cells were isolated from mouse masseter and hind-limb muscles (LM) with a modified enzymatic digestion method. The mononuclear 'parental' population (PC) was serially plated through three different cultures to form pre-plate 1, 2 and 3 (PP1, PP2 and PP3) for correlating with adherent cells at 1 h, 48 h and 120 h of contact with tissue culture plastic. Part of the original isolate was kept unsorted for 5 days and named PC.

Early adhered cells from both muscle tissues appeared to be larger and grew faster than late adhered cells. They also showed higher stem cell gene expression compared to late adhered cells. Moreover, early adhered cells in CSkM showed higher expression of neural crest genes compared to all other isolated cells. The myogenic assessments indicated PP3 to be comparatively more capable of desmin, Myog and MyoD expression, as well as myotube formation in inductive media. In contrast, osteogenic differentiation indicated early adhered cells from both muscle groups to have higher ability compared to late adhered cells in expression of osteogenic genes, alkaline phosphatase protein and extracellular matrix calcium mineralisation.

A summary conclusion is that the cells that adhere early to tissue culture plastic seem skewed to osteogenic commitment compared to the late adherent population, which displays myogenic properties. Cells isolated from both muscle tissues seem to have similar differentiation abilities despite the documented developmental differences between those two sites though the early adhered cells isolated from CSkM have higher expression of neural crest genes than LM, and hence indicate a possible connective tissue origin.

Table of contents

List of figures

List of tables

Abbreviations

Alp	Alkaline phosphatase
AP-2c	Transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
AsAc	L-ascorbate 2-phosphate
Bcl-2	B-cell lymphocytic-leukaemia proto-oncogene
bHLH	Basic helix–loop–helix
BMP	Bone morphogenic protein
BMP2	Bone morphogenetic protein 2
BMP7	Bone morphogenetic protein 7
BSA	Bovine serum albumin
Bsp	Bone sialoprotein
C	Celsius
Ca	Calcium
CAMs	Cell Adhesion Molecules
CD34	Cluster of differentiation molecule cell surface antigen
cDNA	Complementary Deoxyribonucleic Acid
c-Met	Tyrosine kinase receptor
CPC	Craniofacial muscle parent population cells
CPP1	Craniofacial muscle preplate 1 cells
CPP2	Craniofacial muscle preplate 2 cells
CPP3	Craniofacial muscle preplate 3 cells
CSkM	Craniofacial Skeletal Muscle
CT	Crossing Threshold
d	Days
DAPI	4',6-diamidino-2-phenylindole
ddH$_2$O	Distilled water
DM	Dermomyotome
DMEM	Dulbecco's modified eagle medium high and low glucose
DNA	Deoxyribonucleic Acid
dNTP	DeoxyriboNucloside Triphosphate
ECM	Extracellular Matrix

ES	Embryonic stem cell
F	Forward primer
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
Flk-1	Foetal liver kinase-1
FOP	Fibrodysplasia ossificans progressiva
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GM	General growth medium
h	Hours
HCl	Hydrogen Chloride
HGF	Hepatocyte growth factor
HMM-MAA	High-molecular-weight melanoma-associated antibody
ICM	Inner cell mass
IGF-1	Insulin-like Growth Factor 1
IgG1	Immunoglobulin G 1
LM	Limb Muscle
LPC	Limb muscle parent population cells
LPP1	Limb muscle preplate 1 cells
LPP2	Limb muscle preplate 2 cells
LPP3	Limb muscle preplate 3 cells
MDCs	Muscle Derived Cells
MDSC	Muscle derived stem cells
Mg	Magnesium
min	Minutes
MM	Myogenic Medium
mm	Millimetre
mM	Milli Molar
MNF	Myocyte nuclear factor
MPCs	Muscle precursor cells
MPT	Modified preplate technique
MRF	Myogenic regulatory factors
MRF4	Myogenic regulatory factor 4
mRNA	Messenger Ribo-Nucleic Acid

MSC	Mesenchymal stem cell
Myf4	Myogenic factor 4
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation factor
Myog	Myogenin
N	Number of isolates
n	Number of experimental replicates
NaCl	Sodium chloride
NaN$_3$	Sodium azide
NCAMs	Neural cell adhesion molecules
NCC	Neural crest cell
Oct-04	Octamer-binding transcription factor 4
OD	Optical Density
OM	Osteogenic medium
P75	Nerve growth factor receptor
Pax3	Paired box gene 3
PBS	Calcium and magnesium free phosphate buffer saline
PC	Parent population cells
PCR	Polymerase Chain Reaction
PD	Population doubling
PHM	Paraxial head mesoderm
Pitx2	Paired-like homeodomain transcription factor 2
PP1	Preplate 1 cells
PP2	Preplate 2 cells
PP3	Preplate 3 cells
PT	Preplate technique
PTH	Parathyroid hormone
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse-Transcriptase Polymerase Chain Reaction
R	Reverse Primer
rhBMP4	Recombinant human bone morphogenic protein 4
RNA	Ribonucleic Acid
rpm	Revolutions per minute

RT	Reverse Transcription
Runx-2	Runt-related transcription factor 2
Sca-1	Stem cell antigen 1
SD	Standard Deviation
Shh	Sonic hedgehog
SMSP	Skeletal muscle side population cells
Sox10	SRY-box containing gene 10
Sox9	SRY-box containing gene 9
SP	Side population
T	Temperature
Tbx-1	T-box 1
TCF21	transcription factor 21 or Capsulin
TGF-β1	Transforming growth factor beta 1
um	Micrometre
uM	Micro Molar
VWF	Von Willebrand factor
W	Weeks
Wnt	Wingless gene
αSMA	Alpha sarcomeric actin
β-Gp	β-glycerophosphate

Chapter 1: Introduction

1.1.   Background

Tissues that are exposed to injury or in continuous turnover (e.g. muscle and blood cells) need an internal and immediate replacement and repair of damaged cells. This is accomplished in the mammalian body with an ability of tissue regeneration. The cells that are believed responsible for this regeneration and repair are commonly known as stem cells.

Skeletal muscle shows a remarkable ability to adapt to physiological demands such as growth, injury and stress endured in physical or athletic training. Satellite cells are believed to play a major role in these adaptive processes. Satellite cells were first discovered in 1960s (Mauro, 1961). Since the original description of the myogenic satellite cells, considerable interest and research efforts have focused on the identification of cells from muscle tissue with stem cell like biology and differentiation abilities.

A considerable amount of literature has been published on the differences between craniofacial skeletal muscles (CSkM) and other body muscles including limb muscle (LM). However, most of muscle derived stem cells studies have focussed on mononuclear cells derived from limb and trunk skeletal muscles (Katagiri *et al.,* 1994; Bosch *et al.,* 2000; Lee *et al.,* 2000; Li and Huard, 2002; Qu-Petersen *et al.,* 2002; Mastrogiacomo *et al.,* 2005); very little data is available regarding isolation and characterisation of craniofacial skeletal muscle stem cells (Sinanan *et al.,* 2004).

1.2. Muscle Tissue

1.2.1. Muscle

Muscle is a contractile tissue and is derived from the mesodermal layer of embryonic germ cells in mammals. Muscle cells contain contractile filaments that move past each other and change the size of the cell. They are classified as skeletal, cardiac, or smooth muscles. Their function is to produce force and cause motion. Muscles can cause either locomotion of the organism itself or movement of internal organs. Cardiac and smooth muscle contraction occurs without conscious thought and is necessary for survival. Examples are the contraction of the heart and peristalsis which pushes food through the digestive system. Voluntary contraction of the skeletal muscles is used to move the body and can be finely controlled. Examples are movements of the eye, or gross movements such as the quadriceps muscle of the thigh. There are two broad types of voluntary muscle fibres: slow twitch and fast twitch. Slow twitch fibres contract for long periods of time but with little force while fast twitch fibres contract quickly and powerfully but fatigue very rapidly.

1.2.2. Skeletal muscle

Skeletal muscle is attached to the skeleton through tendons and is found throughout the body forming an integrated network responsible for voluntary movements and coordination of human body. The skeletal muscles are adequately vascularised and innervated with nerves, which allow them to achieve their function accurately.

Skeletal muscle is composed of abundant muscle fibers closely integrated into a functional unit by connective tissue. The entire muscle is separated from surrounding tissues and organs by a dense layer of collagenous connective tissue called epimysium. The perimysium is a connective tissue that surrounds groups of muscle fibres called

fascicles. It contains blood vessels that maintain blood supply and nerves that innervate these fascicles. The endomysium is the connective tissue that envelopes each muscle fibre and contains a capillary network that provides the fibre with a ready blood supply, nerve fibers, and satellite cells responsible for repair of damaged muscle tissues. An over view of the structure of skeletal muscle segment is presented in figure 1.1.
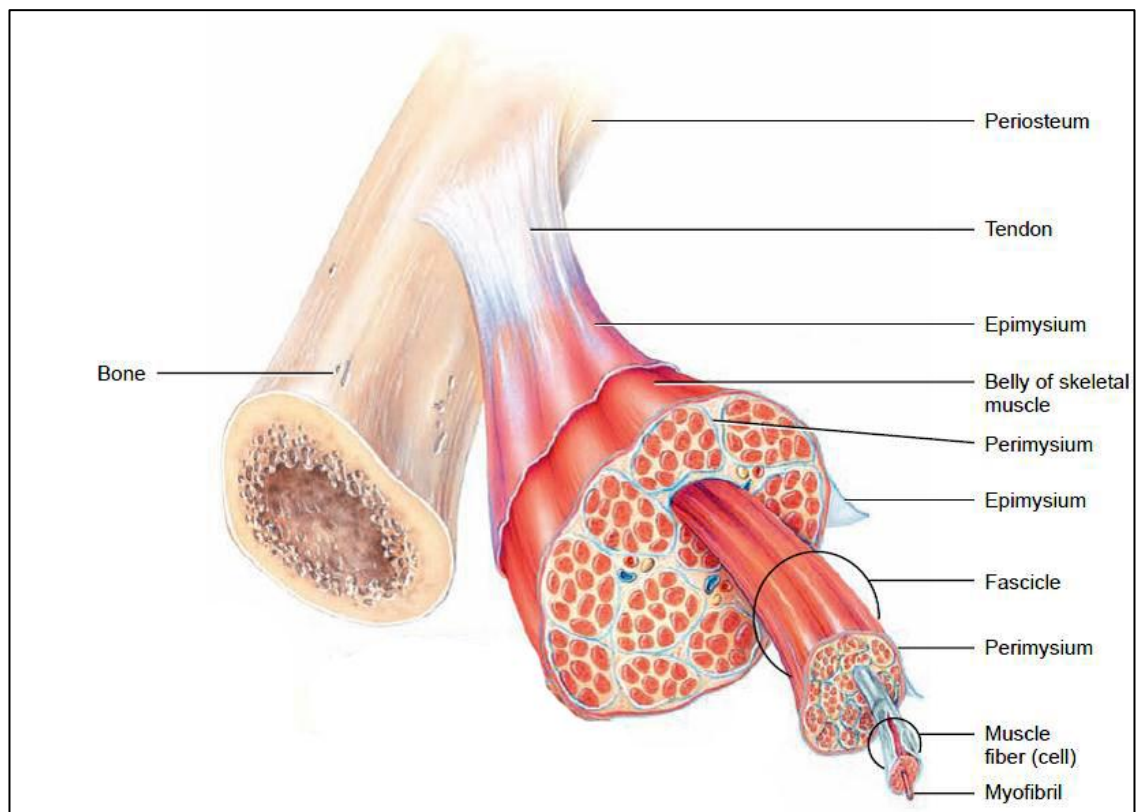
Figure 1.1 General structure of a muscle segment.
Figure shows a cross section of skeletal muscle attached via tendon to a bone. The substructures are highlighted. The epimysium is the outermost sheet that surrounds the whole muscle. The perimysium is the intermediate layer of the extracellular matrix (ECM) that covers each fascicle. The endomysium is the innermost layer of the ECM and surrounds each fibre (Figure taken with permission from Tortora & Derrickson 2009).

The functional unit of skeletal muscle is known as a muscle fibre. It is an elongated, cylindrical cell with multiple nuclei, ranging from 10 to 100 μm in width and from a few millimetres up to 30 centimetres in length. The cytoplasm of the fibre is called the sarcoplasm, which is encapsulated inside a cell membrane called the sarcolemma. It is made up of bundles of thick and thin protein filaments known as myofibrils. Theses filaments are organised in repeating functional units called sarcomeres (Martini and Ober, 2006). The structure of sarcomere unit is shown in figure 1.2.   Each sarcomere has dark bands called anisotropic (A bands), consisting of thicker myofilaments made of the protein myosin. The light bands are referred to as isotropic (I bands), and consist of thin myofilaments made of the protein actin as shown in figure 1.2.
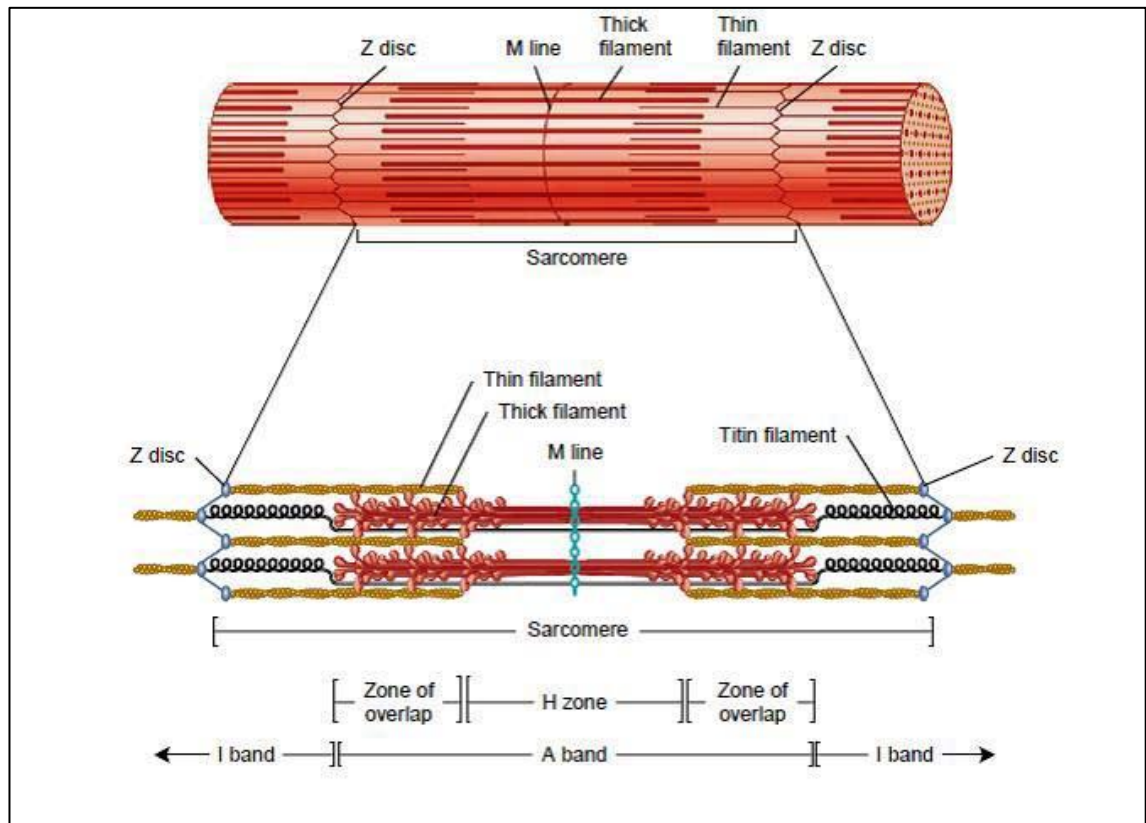
Figure 1.2 Structural aspects of muscle fibre.
Image shows a muscle fibre with constituent structural units: with mitochondria interspersed within a longitudinal aggregate of myofibrils enclosed by an outer membranous sarcolemma. Each myofibril is composed of repeating structural units called sarcomeres. These are separated from each other by Z discs that contain dense actin. The darker middle part of the sarcomere is composed of myosin and is called the A band that extends the entire length of the thick filament. Towards each end of the A band is a zone of overlap where thick and thin filaments lie side by side; this is the I band. A and Z disc passes through the centre of each I band. A narrow H zone presents in the centre of each A band and only contains thick filaments. The thick filaments at the centre of H zone are hold together by the M line. (Figure taken with permission from Tortora & Derrickson 2009).

Muscle proteins can be divided into three groups. These are described below:

1. Contractile proteins, such as myosin and actin, which generate force during contraction. Myosin functions as a motor protein, which push and pull various cellular structures to achieve movement by converting the chemical energy in ATP to mechanical energy. Myosin is shaped like two golf clubs twisted together where the tail points towards the M line. The myosin heads project outward from the shaft in a spiralling fashion, each extending towards one of the six thin filaments (actin) that surrounds each thick filaments (figure 1.2).

2. Regulatory proteins, such as tropomyosin and troponin that regulate the process of contraction.

3. Structural proteins, such as titin keep thick and thin filaments in proper alignment, confer the myofibril with elasticity, and help in linking the myofibril to the sarcolemma and extracellular matrix.

Muscle cells also contain several intermediate filament proteins. Desmin is one such example and is the major muscle-specific intermediate filament protein. It maintains muscle cytoarchitecture by forming a three-dimensional scaffold around the myofibrillar Z-disk and by joining the entire contractile apparatus to the subsarcolemmal cytoskeleton, the nuclei, and other organelles. (Paulin and Li, 2004).

1.2.3. Ontogeny of skeletal muscle

Skeletal muscle develops prenatally in a pre-programmed multistep process involving intrinsic and extrinsic cues that result in the generation of different precursor cell types. Nearly all skeletal muscles in body, except some craniofacial muscles, develop from the paraxial mesoderm (Hawke and Garry, 2001, Buckingham *et al.,* 2003, Grefte *et al.,* 2007, Sambasivan and Tajbakhsh, 2007). This process starts in the mouse from

embryonic day 8.5 ⁄ 9 and proceeds to 18 days with birth occurring at~19 days. Further maturation during the postnatal period takes about 2–3 weeks (Tajbakhsh, 2009). The differences exist in the development of skeletal muscle tissue between the trunk and limb section and craniofacial locations will be discussed below.

1.2.3.1. Development of Trunk and limb muscles

The first step of skeletal muscle development is the formation of cell clusters called somites which originate from paraxial mesoderm in an anterior-posterior direction. The ventral side of the somite forms the sclerotome, which gives rise to the axial skeleton whilst the dorsal somite, called the dermomyotome (DM) contains skeletal muscle progenitor cells of the trunk, limb and some head muscles and can be divided into epaxial and hypaxial parts (Christ and Ordahl, 1995). Figure 1.3 shows the developing origin of various body muscles.

The myotome is first formed underneath the epaxial DM and is responsible for the formation of trunk musculature (Tajbakhsh and Buckingham, 2000). Structures surrounding the DM produce signals that induce myogenic factor 5 (Myf 5) gene expression, which in turn activate myogenic differentiation factor (MyoD) gene. Cells then start to fuse at somite boundaries and form multinucleated myotubes. At the limb level, the muscle progenitor cells delaminate from the hypaxial DM and migrate into the limb buds (Buckingham *et al.,* 2003, Gros *et al.,* 2004).
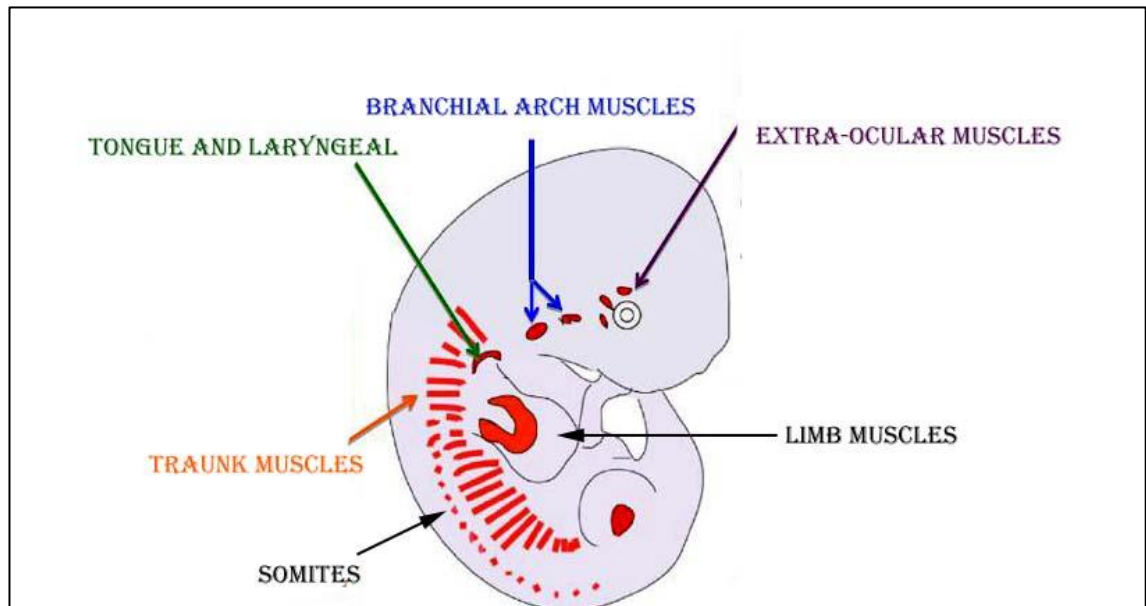
Figure 1.3 Diagram showing the developmental origin of various body muscles.
Figure showing a mouse embryo with the developmental origin of Branchial arch, Extra-ocular, limb, tongue, laryngeal and trunk muscle. It also show the location of developing somites (figure taken with permission from Rios and Marcelle, 2009)

This delamination and migration process has been described to be mediated by c-met, a tyrosine kinase receptor that binds hepatocyte growth factor (HGF), and the paired box gene 3 (Pax3) (Bober *et al.,* 1994, Tajbakhsh *et al.,* 1997). These migrated cells will then reach the limb where they will start activating myogenic regulatory factors (MRFs), a family of basic helix–loop–helix (bHLH) transcription factors that regulate myogenesis.

Myogenic cells forming back muscles are initially activated by Myf 5, which then activate the MyoD gene. In contrast, myogenic cells forming the limbs activate MyoD via pax3 and c-met dependent pathway (Pownall and Emerson, 1992; Braun and

Arnold, 1996; Cossu *et al.,* 1996). Both groups of cells then express myogenin (Myog) and myogenic regulatory factor 4 (MRF4) to produce their myotubes and myofibres (Braun and Arnold, 1996).

MyoD and Myf 5 belong to a family of transcription factors called the myogenic bHLH or MRFs. The proteins of this family all bind to similar sites on DNA and activate muscle specific genes. Even though limb and trunk require different genetic pathways to initiate the myogenic differentiation process, these myogenic transcription factors can compensate for the loss of one or the other. Rudnicki and colleagues (1992) showed that Myf 5 and MyoD could achieve the same functions. However, when mice lacking either MyoD or Myf 5 genes; the resulting mice have normal muscle development but may have breathing difficulties due to rib malformation (Braun *et al.,* 1992).

Soon after migration of cells to their target destination, they fuse to form multinucleated myotubes through the activation of MRFs. Fusion is initiated when the myoblasts leave the cell cycle, stop dividing and secrete fibronectin into their extracellular matrix, binding to it through their major fibronectin receptor, $\alpha5\beta1$ integrin (Menko and Boettiger, 1987). Cell membrane glycoproteins, including several cadherins and cell adhesion molecules (CAMs) will then direct cell alignment (Knudsen *et al.,* 1990). After alignment is accomplished cells will start fuse together by calcium ionosphere activation that carries calcium across cell membrane.

1.2.3.2.   Development of Craniofacial muscles

The genetic regulation of skeletal muscle development in the craniofacial region is still subject to study compared to the large amount of knowledge gained concerning the transcription factors and signalling molecules driving myogenesis in the limb and trunk.

The craniofacial muscles can be divided into three main groups based on their developmental origin:

1. Somite derived tongue and neck muscles

2. Muscles controlling eye movement or the extra ocular muscles

3. Branchomeric muscles

Developmentally, craniofacial muscles experience a unique environment compared to those in the trunk and limbs. Distinct to muscles of the trunk and limbs, which are all somitic in origin, craniofacial muscles developed from three different regions of the embryo; namely the occipital somites, precordal (cranial to the notochord) and paraxial head mesoderm (PHM).

Extraocular muscles, which control eye movements, are derived from precordal and paraxial head mesoderm (PHM). They have metabolic and fibre type compositions distinct from most of trunk muscles (Cheng *et al.,* 2004). On the other hand, the progenitors of the branchiomeric muscles originate from the PHM, and the first five somites (occipital somites) give rise to the muscles that elevate or rotate the skull in addition to the tongue and laryngeal muscles (Noden and Francis-West, 2006).

The PHM is unsegmented unlike its trunk counterpart (Kuratani, 2005). Consequently, the stem and progenitor cells of most of the head musculature reside in a uniquely organised embryonic tissue compared to that of the other muscle progenitors.

Muscles of the craniofacial region derive their connective tissue component from neural crest cells that provide the connective tissue of extrinsic ocular, glossal and all branchial arch skeletal muscles. The mesoderm derivatives provide connective tissues for other craniofacial muscles (Sperber, 1989). Neural crest may play a crucial role in migration,

patterning, proliferation, and differentiation of head skeletal muscle progenitors (Rinon *et al.,* 2007). The migration of neural crest cells and formation of branchiomeric muscles are explained in figure 1.4.
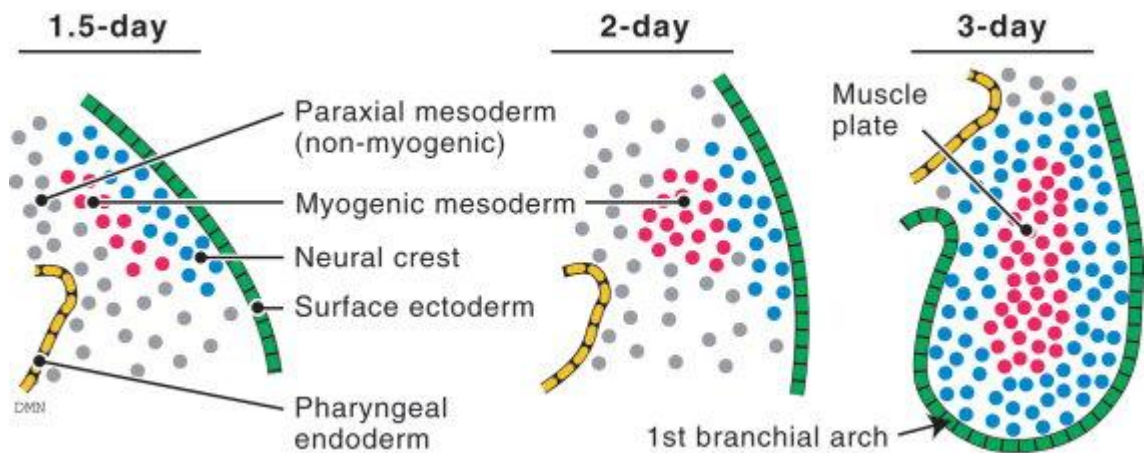
Figure 1.4 Neural crest cells migration and formation of branchial arch muscles.
Figure shows transverse views of three stages of avian head and the combined migration and expansion of surface ectoderm (green), neural crest (blue) and superficial myogenic paraxial mesoderm (red) populations. All population shift and expand in the same dorsal to ventral direction during these stages (Taken with permission from Noden and Francis-West 2005).

Craniofacial myogenesis has a distinct regulatory programme in the early stages of myogenesis, such as the specification and migration stages, compared to other muscles. The signals that involved in formation of craniofacial and limb muscles are highlighted in figure 1.5. While Wnt and sonic hedgehog (Shh) signals from the surrounding structures induce myogenesis in somites and bone morphogenetic protein (BMP) signals

from the lateral plate and dorsal neural tube inhibit myogenesis, the antagonist of Wnt (Frzb) and BMP (noggin and Gremlin) arising from the neural crest cells and other surrounding tissues encourages myogenesis in the somitomeres and branchial arches indirectly, by repressing the inhibitory actions of Wnts and BMPs (Munsterberg *et al*., 1995, Dietrich *et al*. 1998, Tajbakhsh *et al*. 1998, Cossu and Borello, 1999, Linker *et al*., 2003, Tzahor *et al*., 2003; Yamane, 2005).
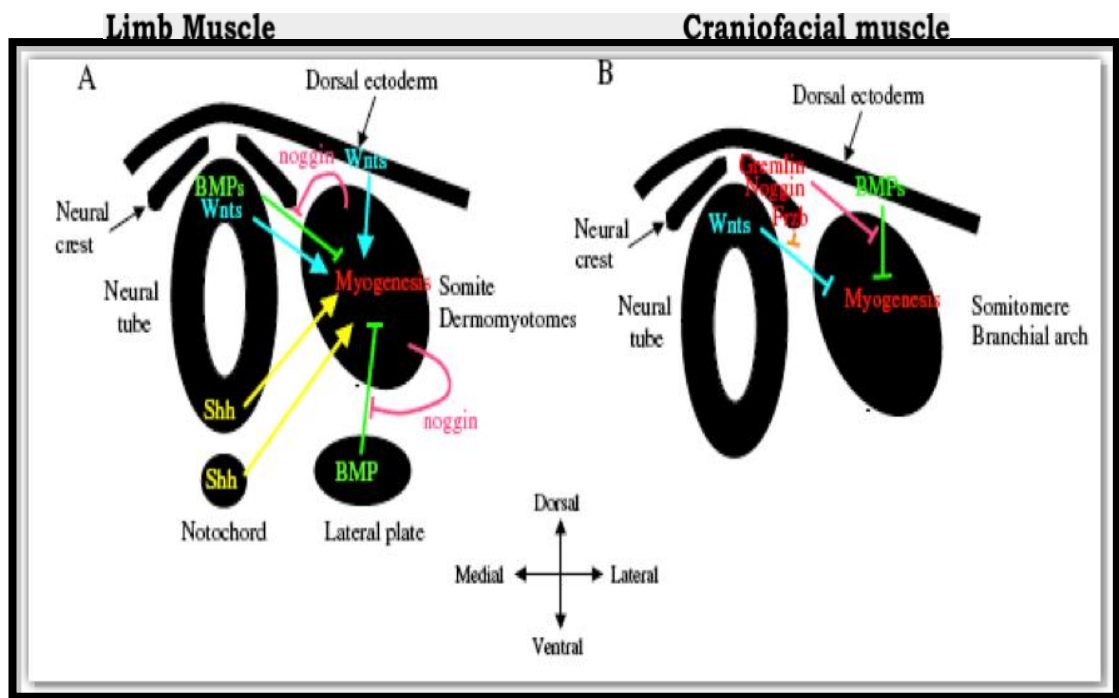
Figure 1.5 The differences in myogenesis pathways between CSkM and LM.
Figure of cross sections of mouse embryo showing regulatory molecules in (A) the somite (dermomyotomes, myotomes) and (B) somitomeres (branchial arch) during the early stages of myogenesis. Wnt and sonic hedgehog (Shh) signals from the surrounding structures induce myogenesis in somites. While antagonists of BMP (noggin and Gremlin) and Wnt (Frzb) promote head myogenesis. (Taken with permission from Yamane, 2005)

Although MRFs are required for craniofacial myogenesis, their upstream regulators appear to be distinct from those essential in the trunk and limb myogenesis. Knockouts of the transcription factors Tbx1, Pitx2, musculin and transcription factor 21 (TCF21 also known as capsulin); result in defects in the formation of distinct head muscles (Lu *et al.,* 2002, Kelly *et al.,* 2004). By contrast, Pax3 is neither expressed nor required during head muscle formation and, consequently, mice that lack both Pax3 and Myf5 develop craniofacial muscles normally, whereas trunk and limb muscles are largely absent (Tajbakhsh *et al.,* 1997, Mootoosamy and Dietrich, 2002).

In addition to the previously mentioned differences between craniofacial and non-craniofacial muscles, the craniofacial muscles appear to also have different regenerative potentials. Pavlath and colleagues found that there is a regenerative difference between masseter and limb muscle, which may be related to intrinsic differences between the muscle precursor cells in these two anatomically distinct muscle types (Pavlath *et al.,* 1998). Additionally, satellite cell isolated from masseter muscle are reported to proliferate more and differentiated rather later than those from limb muscle (Ono *et al.,* 2010). However, when transplanted in limb region, masseter-derived satellite cells regenerated limb muscles as efficiently as those from limb muscle (Ono *et al.,* 2010).

There are also some reports indicating that craniofacial muscles (masseter muscle) continue to express many molecules normally downregulated in adult limb skeletal muscle, such as developmental and neonatal myosin heavy chain (Butler-Browne *et al.,* 1988) and embryonic fibronectin splice variant EIIIA (Price *et al.,* 1998).

In addition to the differences in development between these muscle groups, bone development also differs between the craniofacial and limb region of a vertebrate. These differences are explained in the following section.

1.3. Osteogenesis

1.3.1. Bone

Bone is a strong anatomical structure that has the ability to provide protective covering of vulnerable structures in the body such as brain, rigid internal support for the body and the articulation and attachments of muscles. It provides a site of storage of minerals and lipids in addition to blood cell production (Martini and Ober, 2006).

Bone can be divided into two types; compact and spongy bone. These are depicted in figures 1.6 and 1.7.

Based on the microscopic features, osseous tissue is a collagen and calcified mineral based extracellular matrix (ECM). It is divided into two distinct types based on the architecture of the connective tissue ECM; cortical and cancellous (figure 1.6). The former is a relatively dense, compact type of osseous forming the outer part of a bone. The cancellous type occurs as spongy with a 'honey comb' of branching bars, plates and rods called 'trabeculae. This architecture accommodates a higher level of vascular tissue compared to cortical bone that nourishes bone marrow and its embedded populations of various stem cells (Tortora & Derrickson 2009a).

Cortical and cancellous bones constitute 80 % and 20 % of the adult skeleton, respectively. The predominant mineral salt is a combination of calcium phosphate and calcium hydroxide called hydroxyapatite, (Tortora & Derrickson 2009a).

Macroscopically, skeletal bones are divided into four types; long, short, flat and irregular. A prototypic long bone is composed of seven structural entities: diaphysis, epiphyses, metaphysis, articular cartilage, periosteum, medullary cavity and endosteum. The initial three constitute the larger bone structure; with the articular cartilage and

periosteum forming on the outside, and medullar cavity and endosteum within it. Other bones are composed of different combinations of these structural elements. The various macro structural attributes of a prototypic long bone are described in figure 1.7 (Tortora & Derrickson 2009a).

The vertebrate skeleton is the outcome of cells from three distinct embryonic lineages. Cranial neural crest cells are responsible for the formation of craniofacial skeleton, whereas the axial skeleton is derived from paraxial mesoderm (somites), and the limb skeleton is the product of lateral plate mesodermal cells.

There are three major bone cell types. Osteoblasts are responsible for building up bone in a process called osteogenesis. They secrete the collagenous and non-collagenous proteins of bone matrix. The osteocytes are mature bone cells that are contained in a lacunar space filled with bone fluid, mineralised collagen fibrils and proteoglycans. Osteoclasts have the function of bone resorption by degrading old mineral matrix to facilitate bone deposition by osteoblasts.
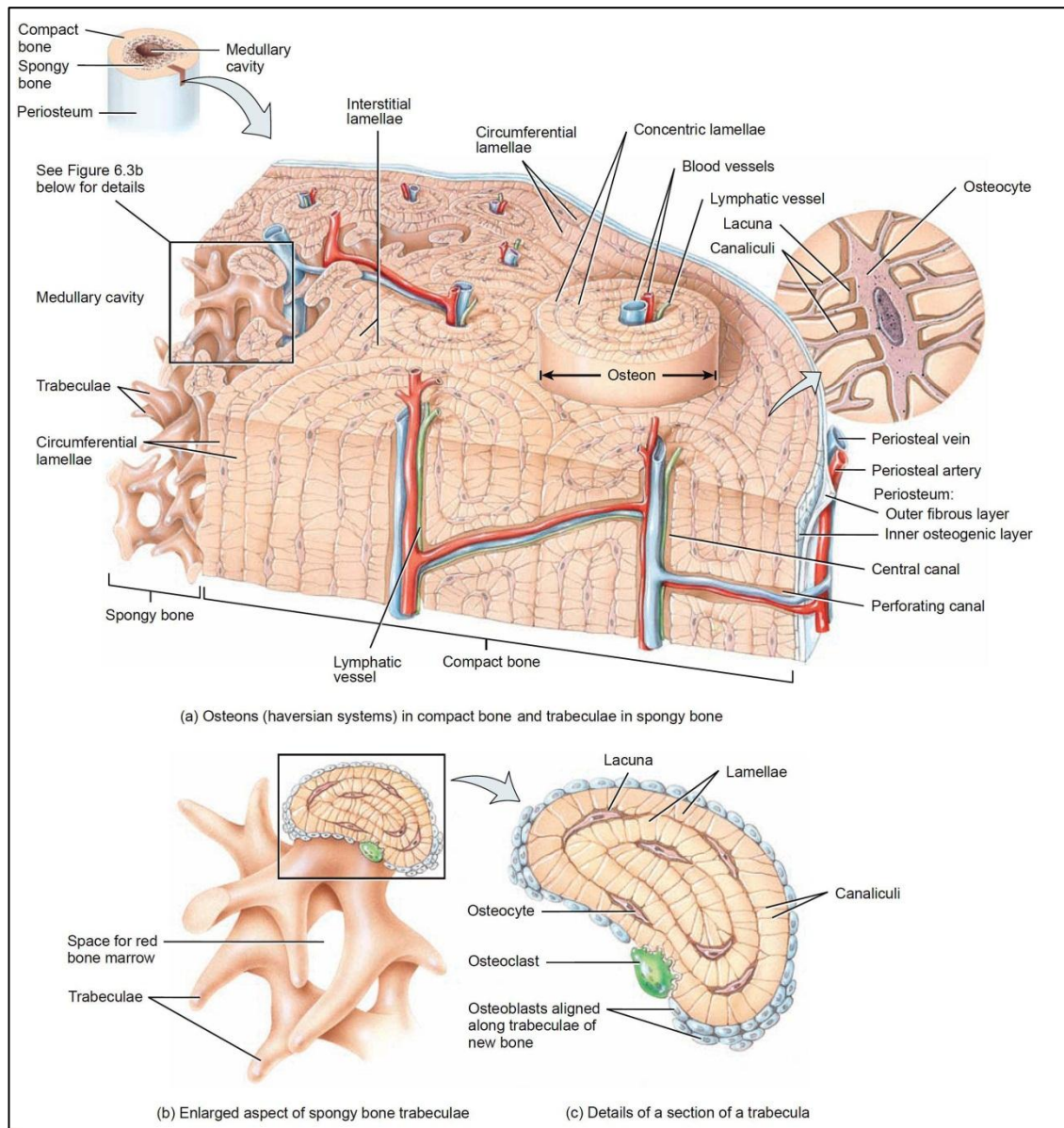
Figure 1.6 Microscopic structure of long bone
Figure shows a cross section along the transverse and sagittal planes of a hypothetical disaphyseal segment of bone. (a) The axis from right to left shows periosteum followed by compact cortical bone in the middle and spongy bone to the left. An osteon, haversian canal, resident blood and lymphatic vessels are surrounded by concentric rings of lamellae. Spaces between lamellae are either lacunae (osteocyte sites) or emanating canaliculi, indicated in the magnified representation on the upper right hand side are trabecular rods that lamellae are arranged into. (b & c) Magnified views of the cross section of a trabecula indicating the irregular arrangement of lamellae. Fig c also depicts osteoblastic and osteoclastic activity on the periphery. (Figure taken with permission from Tortora and Derrickson 2009)
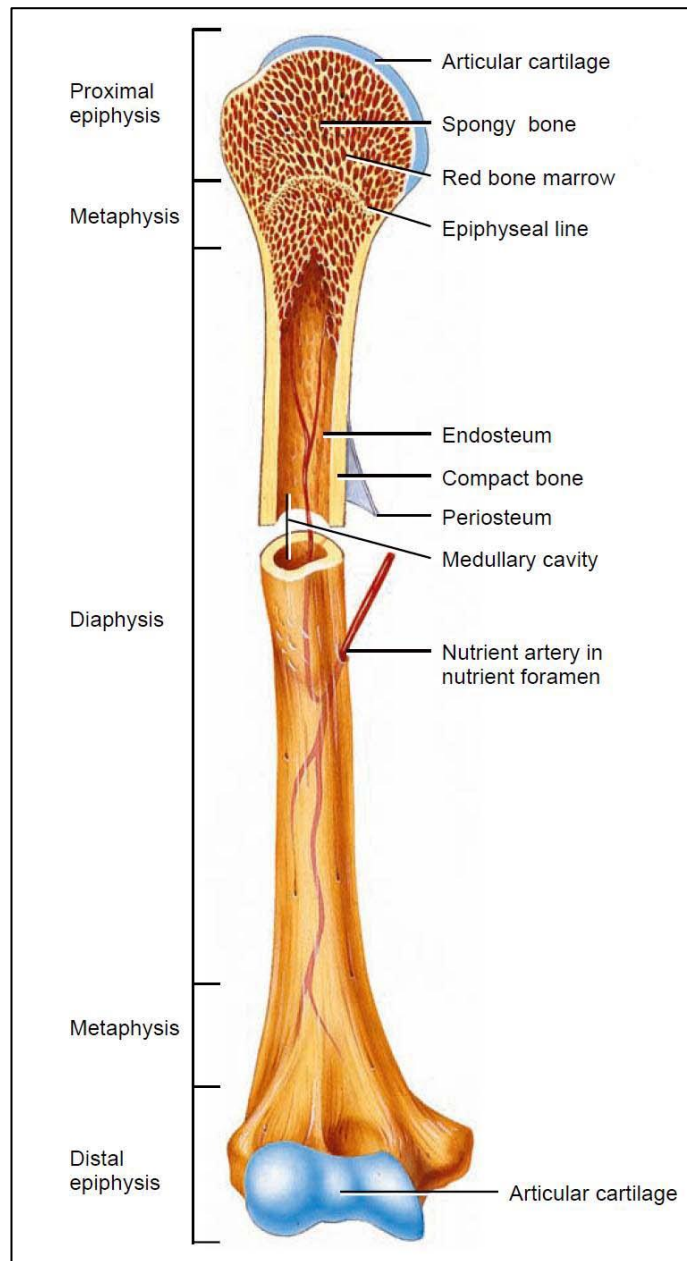
Figure 1.7 Macroscopic structure of a long bone
Image shows a long bone (humerus, arm) with structural segments highlighted along a descending vertical axis on the left hand side of image; and sub-cortical structures of a transverse section on the right hand side (Figure taken with permission from Tortora & Derrickson 2009).

An interplay between several molecules leads to the formation of bone. The main ones of high significance include bone morphogenic proteins (BMPs), Runx2, alkaline phosphatase (Alp) and bone sialoprotein (Bsp).

Several bone morphogenic proteins (BMPs) are capable of acting as potent bone inducer factors, including BMP-2, -4, and -7 (Canalis *et al.,* 2003; Ito and miyazono, 2003; Chen *et al.,* 2004; Farhadieh *et al.,* 2004). BMP-7 is considered a weaker bone inducer than BMP-2 and -4 (Chen *et al.,* 2004). The BMPs initiate their signalling pathway by binding to BMP receptors that activate the expression of target genes (Chen *et al.,* 2004). Runx2 is also involved in this signalling cascade mediating the osteogenic differentiation initiated by BMPs (Chen *et al.,* 2004). Other proteins and hormones involved in osteogenic regulation and differentiation include Parathyroid hormone (PTH), vitamin D and TGF-β1.

Runt-related transcription factor 2 (Runx2) (a member of a small transcription factor family that shares DNA-binding domains of homology with *Drosophila Runt*) is a key regulator of osteoblast differentiation and function (Ducy *et al.,* 1997, Komori *et al.,* 1997, Otto *et al.,* 1997, Jonason *et al.,* 2009). It also interacts with and controls the expression levels of several osteogenic genes, including osteocalcin (an osteoblast-specific marker), Bsp, Alp, and type I collagen (Ducy *et al.,* 1997).

Alkaline phosphatase (Alp) is a commonly used osteogenic marker; mature Alp is glycolysed and anchored to the cell plasma membrane. Alp is expressed at high levels in mineralised tissues and its importance for osteogenic differentiation is well established as an inorganic phosphate donor to growing calcium phosphate crystals. Alp is essential

for expression of necessary genes to produce osteogenic extracellular matrix (ECM) (Golub and Boesze-Battaglia, 2007)

Bone Sialoprotein (Bsp) is first expressed at the onset of bone, cementum, and dentin formation. Its expression is restricted to mineralised connective tissues (Chen *et al.,* 1992; Ogata, 2008). Bsp is believed to initiate calcium deposition by binding calcium ions. It is also involved in the regulation of cell attachment to bone matrix (Arnett and Henderson, 1998).

1.3.2. Ossification

Osteogenesis, or bone development takes place through two ways; these are intramembranous and endochondral ossification. Intramembranous ossification occurs in craniofacial region, in which cranial neural crest cells migrate to the location where the skeletal elements will develop and differentiate into osteoblast cells that start bone formation by replacement of sheet-like connective tissue membrane with bony tissue (Gilbert and Singer, 2006). The endochondral ossification, leading to development of limbs and trunk, involves the formation of cartilage tissue from aggregated mesenchymal cells, and subsequent replacement of cartilage tissue by bone (Gilbert and Singer, 2006). A depiction of this process is illustrated in figure 1.8 and 1.9.
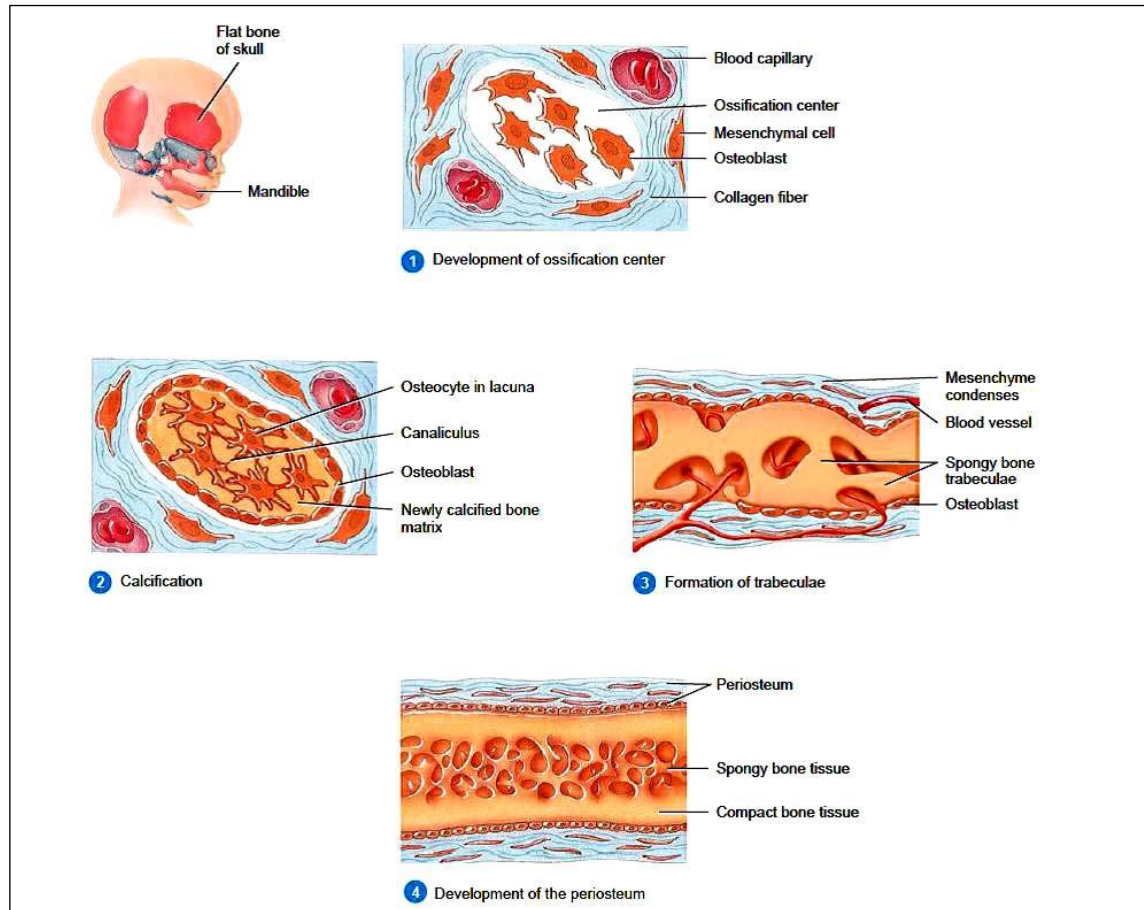
Figure 1.8 Schematic diagram of intramembranous ossification.
(1-2) Mesenchymal cells condense to produce osteoblasts, which deposit osteoid matrix. These osteoblasts become arrayed along the calcified region of the matrix. Osteoblasts that are trapped within the bone matrix become osteocytes (3) The extracellular matrix develops into trabeculae that fuse one to another to form spongy bone. (4) The mesenchyme condenses at the periphery of the bone and develops into the periosteom, a thin layer of compact bone will then replace the surface layers of the spongy bone, but spongy bone remains in the centre. (Taken with permission from Tortora and Derrickson, 2009).

The mechanism of intramembranous ossification involves bone morphogenetic proteins and the activation of Runx2. Bone morphogenetic proteins from the head epidermis are thought to instruct the neural crest-derived mesenchymal cells to become bone cells directly (Hall, 1988). The BMPs activate the Runx2 gene in the mesenchymal cells. The Runx2 transcription factor appears to be able to transform mesenchyme cells into osteoblasts.

22

The process of endochondral ossification starts by the commitment of the mesenchymal cells to become cartilage cells (chondrocytes), which proliferate rapidly to form a model for the bone. The chondrocytes then stop dividing and increase their volume (hypertrophic chondrocytes); they alter the matrix they produce to enable it to become mineralised. Finally the blood vessels invade the cartilage model formed.

The hypertrophic chondrocytes die by apoptosis. This space will become bone marrow. As the cartilage cells die, a group of cells that have surrounded the cartilage model differentiate into osteoblasts. The osteoblasts begin forming bone matrix on the partially degraded cartilage (Bruder and Caplan, 1989; Hatori *et al*., 1995). Eventually, all the cartilage is replaced by bone. Thus, the cartilage tissue serves as a model for the bone that follows. The skeletal components of the vertebral column, the pelvis, and the limbs are first formed of cartilage and later become bone. This process is depicted in figure 1.9.
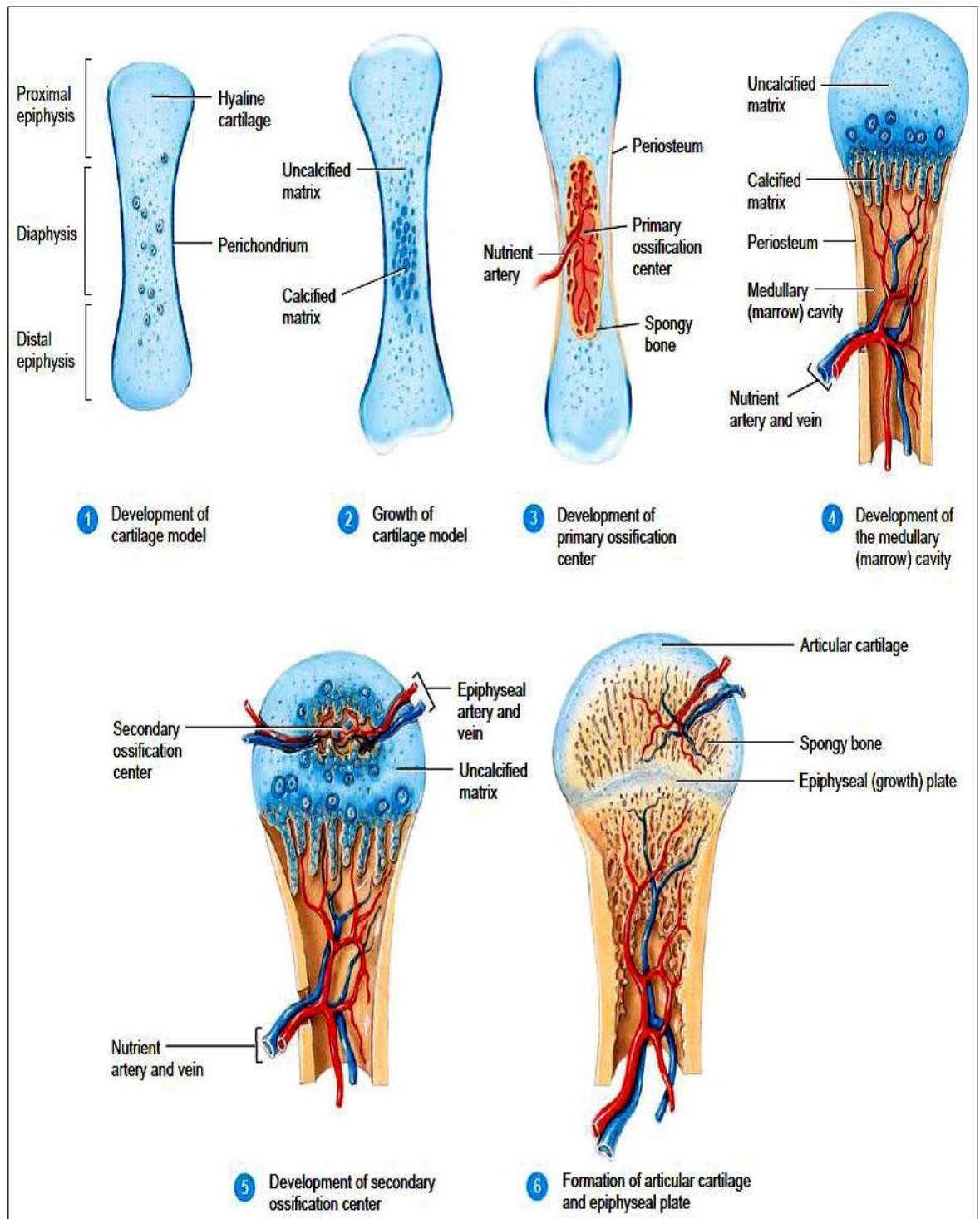
Figure 1.9 Schematic diagram of endochondral ossification.
(1-2) Mesenchymal cells condense and differentiate into chondrocytes to form the cartilaginous model of the bone. (3) Chondrocytes in the centre of the shaft undergo hypertrophy and apoptosis while they change and mineralise their extracellular matrix. Their deaths allow blood vessels to enter. (4) Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. (5-6) Bone formation and growth consist of ordered arrays of proliferating, hypertrophic, and mineralising chondrocytes. Secondary ossification centres also form as blood vessels enter near the tips of the bone. (Taken with permission from Tortora and Derrickson, 2009)

1.3.3. Heterotopic ossification

Ectopic bone in skeletal muscle has been observed in states of disease and experimental conditions. Heterotopic ossification, myositis ossificans and more aggressive diseases such as fibrodysplasia ossificans progressive (FOP) and musculoskeletal neoplasms exhibit unwanted bone formation in skeletal muscle (Sawyer *et al.,* 1991; Kaplan and Smith, 1997; Mahboubi *et al.,* 2001). Several experimental studies have shown that ectopic bone formation occurs as a result of microinjuries and subsequent muscle necrosis, which leads to macrophage invasion followed by proliferation of chondrocytes and osteoblasts. The bone formed in both humans and mice arises via the endochondral ossification route (Aro *et al.,* 1991; Kan *et al.,* 2004), but the origin of the chondrogenic cells is unknown. FOP, on the other hand, is believed to be a rare genetic disorder of connective tissues characterised by congenital malformation of the great toes and by a progressive heterotopic osteogenesis (Kaplan and Smith, 1997; Mahboubi *et al.,* 2001). These observations have led to the investigation for osteoprogenitor cells within skeletal muscle tissues.

1.3.4. Osteogenic potentials of muscle precursor cells

Despite the fact that mesenchymal stem cells are being widely recognised as a primary source of osteoprogenitor cells, non-bone-marrow sources are important to pursue as an alternative to bone marrow when patients do not wish to have a bone marrow aspirate or when the bone marrow is compromised, as can occur in the elderly or in those with bone marrow disease. Non-bone-marrow-derived cells might also be more easily obtained when tissues are accessible during a surgical procedure.

Most published work is on cells isolated from non-craniofacial muscle (Katagiri *et al.,* 1994; Gussoni *et al.,* 1999; Bosch *et al.,* 2000; Lee *et al.,* 2000; Asakura *et al.,* 2001; Li and Huard, 2002, Qu-Petersen *et al.,* 2002; Mastrogiacomo *et al.,* 2005); only a few have investigated the osteogenic efficiency of CSkM (Sinnan *et al.,* 2006).

There is considerable evidence supporting the skeletogenic ability of muscle-derived cells (MDCs) both from *in vivo* and *in vitro* experiments, as well as from pathological conditions. Van de Putte and Urist (1965) and Urist *et al*., (1970) originally showed that demineralised bone implanted intramuscularly could lead to the formation of new bone, and that this activity was mediated by BMP (Urist and Strates, 1971). Many researchers are challenged to identify and isolate multipotent stem cells from the skeletal muscle. This is due to the lack of specific markers to identify cells with osteogenic ability in skeletal muscles (Bosch *et al.,* 2000; Levy *et al.,* 2001).

Bosch *et al.,* in 2000 found that cells isolated from hind limb muscles of dystrophic neonatal mice (mdx), when treated with BMP2, produced positive Alp stain and participated in *in vivo* bone formation.

On the other hand, Lee *et al*., (2000) have gone further and isolated a clonal cell population from the muscle of mdx mice (mc13). They showed that these cells could be induced *in vitro* by BMP2 to express osteocalcin mRNA (Lee *et al.,* 2000). Furthermore they showed that transduced mc13 cells, constitutively expressing BMP2, formed bone when implanted subcutaneously into immunocompromised mice.

Satellite cells on the other hand also showed to have osteogenic potential when properly stimulated with rhBMP4 and/or BMP7. It can express Alp and up-regulated the expression of mRNA for osteocalcin and alkaline phosphatase (Asakura *et al*., 2001).

Based on their differential adherence of human muscle cells to tissue culture plastic; Levy and colleagues (2001) isolated two different cell populations from muscles tissue. According to them, the cells that adhered to the uncoated flasks form the connective tissue compartment of the isolate while suspended cells from their supernatant that adhered when transferred to collagen coated flasks, were deemed as satellite cells. They found that, alkaline phosphatase activity was detectable at early stages of isolated connective tissue cells (adhered to plastic). They concluded that, human skeletal muscle connective tissue contains osteo-progenitor cells that may be of pericytes or perivascular origin.

In vitro experiments on cells isolated from human masseter muscle have shown that a selected pre-myogenic population [NCAM$^+$ (neural cell-adhesion molecule), a satellite cell marker] can up-regulate osteogenic markers when stimulated with BMPs (Sinanan *et al.,* 2004).

In another human study, Mastrogiacomo *et al.,* (2005) found that plastic adhering human muscle cells (cells adhered to uncoated flask for 72 hours) showed positive staining for Alp, osteopontin and osteonectin in addition to the ability to form bone *in vivo*. They reported the isolation of an Alp$^+$/CD34$^-$ cell population from the human skeletal muscle, which forms both cartilage and bone *in vivo*. Isolated cells were 85% positive for Alp. At the beginning of the culture, skeletal muscle cells expressed chondro-osteogenic commitment markers such as Sox9 and Runx2. They demonstrated

that these cells isolated and expanded *in vitro* could undergo osteo-chondrogenic differentiation as evidenced by detection of calcium (osteogenic) and proteoglycan (chondrogenic) deposition as well as by the expression of master genes characteristic of both pathways.

In 2008, Hashimoto and colleagues, isolated human muscle progenitor cells on collagen coated flasks, and showed that isolated cells were spontaneously expressing osteoblast-specific proteins Alp and Runx2 and were able to undergo terminal osteogenic differentiation without exposure to an exogenous inductive agents.

These studies strongly suggested that skeletal muscle may contain osteoprogenitor cells that can differentiate into bone forming cells both *in vitro* and *in vivo.*

1.4. Skeletal Muscle Stem cells

1.4.1. Background

1.4.1.1. Stem cells

These are unspecialised cells that have two distinct properties; the ability for long term self-renewal and the capability to differentiate into one or more specialised cells. The differentiation ability or so called plasticity decreases with maturity. Cells taken from the early divisions of the zygote are totipotent, that is they are able to form any cell type in the embryo and the cells of the placenta (Alison et al., 2002). A few days later, the inner cell mass (ICM), a group of cells within the blastocyst, from which embryonic stem cells (ES) are derived, and they are capable of producing any cell product of the three germ layers but not the placenta. Hence, these cells are called pluripotent (Alison et al., 2002). Postnatally and during adult life tissue specific stem cells, adult stem cells, reside in most organs of the developing animal. The role of these cells is to act as a source of new cells for postnatal growth and repair or as a mechanism for tissue maintenance, growth and repair in later life (Blau et al., 2001).

They are present in various niches throughout the body, such as bone marrow, brain, liver and skin, as a mechanism for tissue maintenance, growth and repair in later life (Lavker and Sun 2000; Uchida *et al.* 2000; Vessey and de la Hall 2001; Wagers *et a,.* 2002). In addition to the above mentioned tissues adult stem cells were also reported to be present in craniofacial skin, palate, oral mucosa, periodontal ligaments surrounding the tooth and dental pulp etc. (Fernandes *et al.,* 2004; Widera *et al.,* 2009; Davies *et al.,* 2010; Seo *et al.,* 2004; Gronthos *et al.,* 2000).

In theory, adult stem cells could be harvested from a patient, differentiated in the laboratory and transplanted back into the same individual for tissue repair, thus bypassing the need for immunosuppression. However, for some stem cell types, low frequency, difficulties in accessing the niche and isolating the cells, restricted lineage potential and poor growth in cell culture may render their use impractical for tissue engineering purposes. In these cases, ES cells are likely to provide a more appropriate cell source (Vogel, 2001).

Until recently, adult stem cells were thought to be committed to regeneration of specific lineages associated with their niche, however a growing amount of data suggests these stem cells may actually be pluripotent (Alison et al., 2002). Plasticity of the adult stem cell was originally investigated by studies on stem cells derived from bone marrow (mesenchymal stem cell (MSC)). These studies found, experimentally, that MSCs have the ability to differentiate into other cell types including muscle, fat, liver, etc. (Caplan, 1991; Baddoo et al., 2003; Alhadlaq and Mao, 2004).

Human embryonic and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. One major difference between adult and embryonic stem cells is their different differentiation potentials compared to adult stem cells. Embryonic stem cells can become all cell types of the body while adult stem cells are thought to be limited to differentiating into different cell types of their tissue of origin (Strauer and Kornowski, 2003).

Finding an easy reproducible method for isolating and expanding adult stem cells is still subject to investigation. Embryonic stem cells, on the other hands, can also be relatively

easily grown in culture. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies.

Other main difference would be the immune rejection after transplantation. Adult stem cells, and tissues derived from them, are currently believed less likely to initiate immune rejection after transplantation. This is because a patient's own cells could be expanded in culture, properly treated by necessary agents, and then reintroduced into the patient. This represents a significant advantage, as immune rejection can be avoided only by continuous administration of immunosuppressive drugs (Strauer and Kornowski, 2003).

Biological difficulty is also a challenge for using bone marrow stem cells as patients may have compromised bone marrow or simply do not want to have bone marrow aspirate (Krebsbach and Robey, 2002). Therefore, work has focused on finding other sources of stem cells that may have the same abilities of the MSCs and might also be more easily obtained when tissues are exposed as part of surgical procedure. These include skeletal muscle which considered being a promising source of stem like cells as it is relatively easier to access especially craniofacial muscle (masseter), which can be accessed intra-orally under local anaesthesia with no scar formation.

These are unspecialised cells that have two distinct properties; the ability for long term self-renewal and the capability to differentiate into one or more specialised cells. The differentiation ability or so called plasticity decreases with maturity. Cells taken from the early divisions of the zygote are totipotent, that is they are able to form any cell type in the embryo and the cells of the placenta (Alison *et al.,* 2002). A few days later, the inner cell mass (ICM), a group of cells within the blastocyst, from which embryonic

stem cells (ES) are derived, and they are capable of producing any cell product of the three germ layers but not the placenta. Hence, these cells are called pluripotent (Alison *et al.,* 2002). Postnatally and during adult life tissue specific stem cells, adult stem cells, reside in most organs of the developing animal. The role of these cells is to act as a source of new cells for postnatal growth and repair or as a mechanism for tissue maintenance, growth and repair in later life (Blau *et al.,* 2001).

1.4.2. Muscle derived cells

1.4.2.1. Background

Several advances in 1961 provided a fundamental understanding of skeletal muscle cell biology. Firstly, the multinucleated skeletal myofibre was demonstrated to be formed by the fusion of many mononucleate myoblasts (Cooper and Konigsberg 1961). Later on the same year, Katz and Mauro described an apparently quiescent cell located between the sarcolemma and basement membrane of the muscle fibre. Mauro gave them the name satellite cells for their anatomical location on muscle fibre periphery (Katz 1961; Mauro 1961). The satellite cell occupies an identical anatomical position in the majority of vertebrates and is responsible for post-natal growth and regeneration of the muscles.

The remarkable capacity of muscle tissue to regenerate following injury highlights the potential of these quiescent cells. Satellite cells are activated upon damage to muscle, proliferate and become muscle precursor cells (MPCs) that fuse to make myofibres thereby regenerating the tissue (Cornelison and Wold, 1997).

MPCs express muscle specific markers (such as desmin) (Seale and Rudnicki, 2000) before they start to fuse to form myofibre. This process is mediated by several growth factors and genes such as insulin-like growth factor-I (IGF-I), which is synthesised and

secreted by muscle cells to enhance proliferation and differentiation (Stewart and Rittweger, 2006; Clemmons, 2009), Myof 5, MyoD, Myog, and MRF 4 genes (Stewart and Rittweger, 2006).

However, the ratio of satellite cells is different among different muscles. Renault *et al.,* (2002), found that the ratio of satellite cells was slightly higher in limb (biceps brachii) muscle than in masticatory (masseter) muscle. In addition, the satellite cells ratio in both muscle groups decreased with age. Even though the number of mean nuclei in masseter muscle is higher than limb muscle; it has been found that the regenerative capacity is reduced in masseter muscle compared to that of limb muscle after exogenous (freeze or crush injuries) traumas (Pavlath *et al.,* 1998). These differences might be explained by the differences in embryologic origins; myogenic pathways and gene expression pattern of craniofacial and limb muscle (Pavlath *et al.,* 1998; McLoon *et al.,* 2007).

1.4.2.2.   Types of muscle derived stem-like cells

In addition to the satellite cells, there are other stem cell-like populations within skeletal muscle that may be of interest in the field of skeletal muscle repair and regeneration. Potential sources of stem/precursor cells from various tissues in the skeletal muscle complex are shown in figure 1.10.
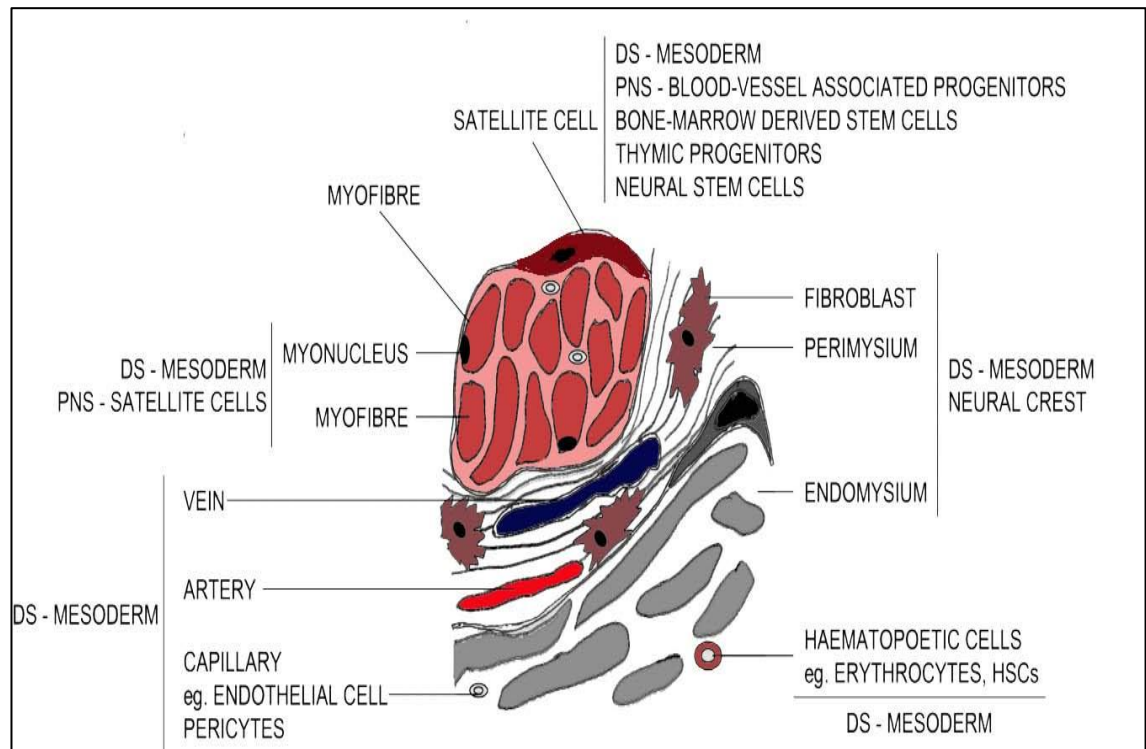
Figure 1.10 Potential sources of stem/precursor cells from various tissues in the skeletal muscle complex. Skeletal muscle has an intricate anatomy and is composed of multinucleate myofibres arranged into functional units called fascicles decorated with peripheral satellite cells and encompassed within three connective tissue sheaths, the endo-, peri- and epi-mysium. Each of the component layers contains a variety of different cells: Schwann cells, satellite cells, endothelial cells, haematopoietic cells and interstitial mesenchymal fibroblasts. Any or all of these cell types could give rise to multipotent cells. The developmental origin of the cells may also play a role in defining this multipotentiality. DS, developmental source of the tissue; PNS, postnatal source of the tissue. (Taken with permission from Sinanan *et al.,* 2006). "This figure was originally published in Biology of the cells. Sinanan and colleagues, muscling on stem cells. Biology of the cells; 2006; 98: 203-214 © Biology of the Cell"

A variety of methods for isolating MDCs (muscle-derived cells) have been employed, with the initial step usually involving the release of mononuclear cells from the host tissue, followed by fractionation of the population to isolate stem cell/precursor populations (Sinanan *et al.,* 2006).

Depending on the isolation method used or described features of the isolated cells, they are called either muscle-derived stem cells (MDSCs) (Lee *et al.,* 2000), skeletal muscle side population (SMSP) (Asakura *et al.,* 2001), or muscle derived hematopoietic stem cells (MD-HSCs) (McKinney-Freeman *et al.,* 2002). Whether these cells are distinct populations or the same population of cells at different stages of maturation is still a subject to study (Cao and Huard, 2004).

In recent years, several groups have attempted to characterise undifferentiated cells situated in skeletal muscles. The identification of muscle stem cells was based on the presence of certain surface markers such as: stem cell antigen-1 (sca-1) and CD34 (marker of hematopoietic and satellite cells), CD45 and c-kit (markers of hematopoietic cells) and desmin (a marker of myogenic cells), Oct4 (embryonic stem cell marker (Jiang *et al.,* 2002; Romero-Ramos *et al.,* 2002). However, the level of expression of these markers is not consistence between the different studies (Meeson *et al.,* 2004, Deasy *et al.,* 2005, Jankowski *et al.,* 2002, Lee *et al.,* 2000), which may suggest that several distinct populations of multipotential muscle derived stem cells may exist. Proposed markers distinguishing various stages along the myogenic differentiation pathway are shown in figure 1.11.
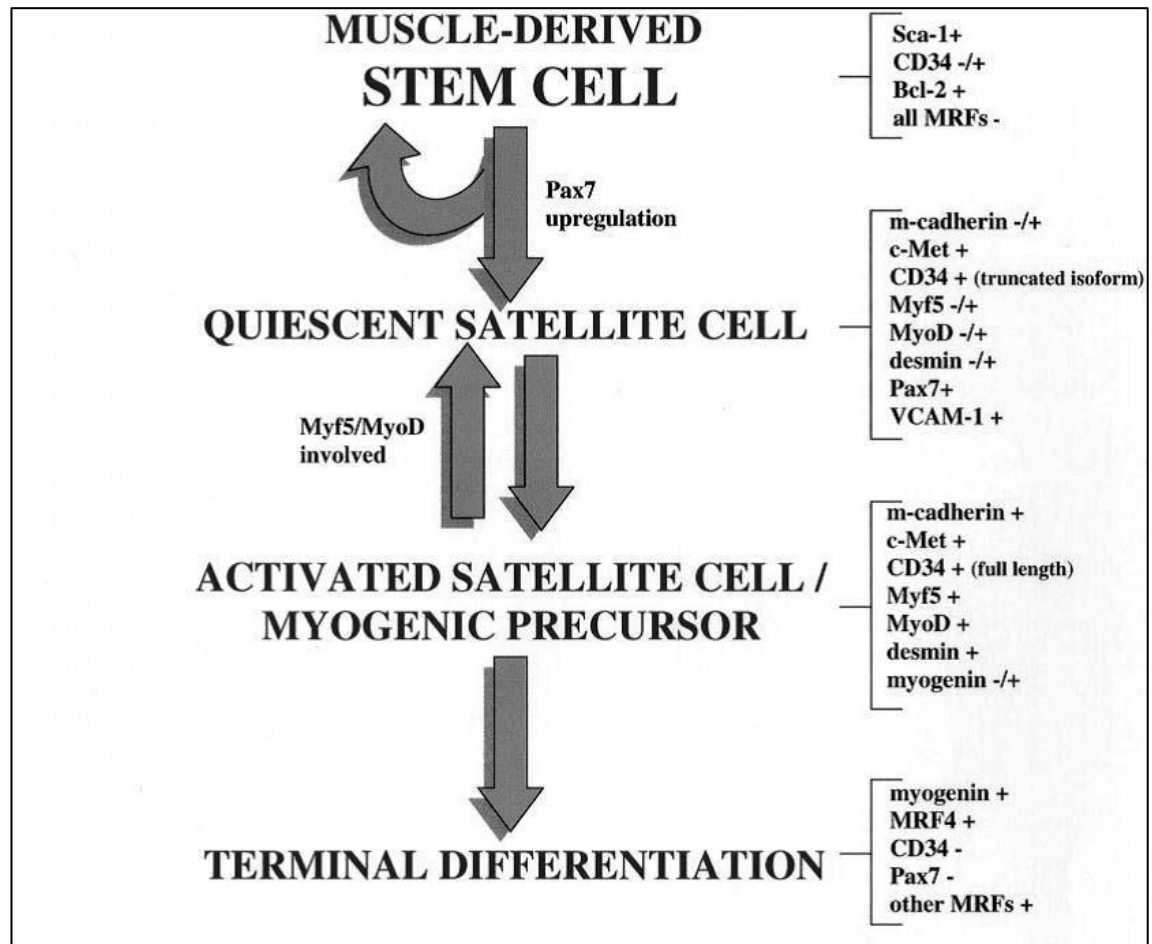
Figure 1.11 Markers distinguishing various stages of differentiating myogenic stem cell
Figure shows the potential markers that can be used to differentiate between muscle derived cells at different stages of differentiation. Discrepancies in expression are distinguished as -/+ and highlight the difficulties associated with describing distinct cellular phenotypes. Sca-1 and CD34 expressed in muscle derived stem cells while myogenic specific markers expressed later along myogenic lineages (Taken with permission from Deasy *et al.,* 2001)

The variability in expression profiles reported could be a result of either using different methods of protein analysis, the use of different isolation method or be dependent on the status of the cells at the time of analysis (for example whether they are quiescent, proliferating, differentiating, or apoptotic) (Beauchamp *et al.,* 2000, Royer *et al.,* 2002, Mitchell *et al.,* 2005).

MDSCs were isolated based on differences in adhesion properties of cells (Qu-Petersen *et al.,* 2002). Cells were differentially plated on pre-coated tissue culture flask in a systematic time course. Firstly, after enzymatic digestion of tissues, cells were seeded on a culture flask for one hour. The non-adherent cells were then transferred to a new flask. Non-adherent cells were then sequentially transferred every day to new flask up until reach plate 6 which believed to have the myogenic cell fraction. This method was called differential pre-plating method, which first described by Yaffe (1968) and Richler and Yaffe (1970) to isolate more purified myogenic cells. Recently this method has been modified and used to isolate MDSCs (Qu-Petersen *et al.,* 2002). MDSCs cultured *in vitro* differentiate spontaneously into myotubes, but also, when appropriately stimulated (by addition of growth factors to the medium or transfection with certain genes), these cells can also give rise to osteoblasts (Bosch *et al.,* 2000; Lee *et al.,* 2000), chondroblasts (Kuroda *et al.,* 2006), hematopoietic cells (Sherwood *et al.,* 2004) or endothelial cells (Arriero *et al.,* 2004).

Skeletal muscle side population (SMSP) cells on the other hand are isolated using flow cytometry sorting after staining them with Hoechst dye 33324 (Asakura *et al.,* 2002). The dye effluxing is a description of SMSP cells rather than defining certain features or functions of these cells. Even though initially associated with a subpopulation of hematopoietic stem cells (Kim *et al.,* 2002), side population cells have been found in other tissues, in addition to skeletal muscle, such as, mammary gland, lungs, small intestines, bone marrow, and cancer cells (Asakura and Rudnicki, 2002, Asakura *et al.,* 2002, Summer *et al.,* 2003, Jonker *et al.,* 2005, Ho *et al.,* 2007).

Therefore, side population phenotype is a rather ubiquitous across various tissues associated with low level of differentiation than certain features or origin of cells (Burdzińska *et al.,* 2008).

Skeletal muscle side population cells (show higher efflux of Hoechst dye 33324) are spherical, small, and poorly adhered to the tissue culture flask (Gussoni *et al.,* 1999). About 90% of them expressed sca-1 and CD34. They also express CD45 and c-kit, but not desmin (Asakura *et al.,* 2002, Sherwood *et al.,* 2004, Uezumi *et al.,* 2006). Assessment of the differentiation potential revealed that SMSP could give rise into all haematopoietic lines both *in vitro* and *in vivo* (Asakura *et al.,* 2002, Jackson *et al.,* 1999). However, their myogenic differentiation abilities are limited. Although SMSP cells in myogenic environment can undergo myogenic differentiation, they do so with low efficiency (Gussoni *et al.,* 1999, Asakura *et al.* 2002). Interestingly, only the CD45$^-$ subpopulation of SP cells possesses such a capability whereas CD45$^+$ SP cells do not undergo myogenic specification even when co-cultured with myoblasts (Asakura *et al.,* 2002) due to their commitment to haematopoietic lineage. When tested *in vivo*, side population cells contribute to formation of new fiber in damaged muscle and can be located in the position of satellite cells after transplantation. However, the CD45$^-$ subpopulation of cells differentiates into muscle fibers in at greater degree than CD45$^+$ cells, confirming *in vitro* studies (Asakura *et al.,* 2002, McKinney-Freeman *et al.,* 2002).

Myogenic specification of SMSP does not appear to be dependent on Pax7 as in Pax7$^{-/-}$ mice, the number and function of SMSP was not different to that of wild type (Asakura *et al.,* 2002, Seale *et al.,* 2004).

The origin of SMSP is not known however there are several suggestions that SMSP may be considered as a subset of endothelial cells present in capillaries or veins in undamaged skeletal muscle (McKinney-Freeman *et al.,* 2003, Peng and Huard, 2004, Uezumi *et al.,* 2006); other researchers have suggested a bone marrow origin at least for

CD45$^+$ subpopulation (Ojima *et al.,* 2004, Uezumi *et al.,* 2006). All this data may suggest that SMSP and satellite cells are distinct populations and probably have different origin. In each case, many points concerning their origin, potency and functions remain to be elucidated.

1.5. Statement of thesis

1.5.1. Observations

Muscle tissue has a remarkable ability to repair and regenerate implying the presence of stem like cells in this tissue. The occurrence of ectopic ossification suggests a subpopulation of these stem-like cells possess the ability to differentiate along the osteogenic lineage forming osteoblasts that can deposit mineralised osseous deposits in muscle. This situation is further complicated by the observed differences in the development of the limb and craniofacial muscle and bone tissues in vertebrates, with the former arising from mesoderm derived cells and the latter from neural crest derived cells

A variety of *in vitro* approaches have led to the isolation and partial identification of certain stem-like cells from muscle tissue. These studies have mainly focused on stem-like cells from non-craniofacial sources of vertebrate muscle tissue; ignoring the underlying embryonic developmental differences with the craniofacial region. The relative accessibility of craniofacial muscle and the possibility of isolating multipotent stem-like cells with osteogenic capacity present a potential source of cells for tissue engineering applications.

1.5.2. Hypothesis

Progenitor cells isolated by an approach of differential plating from the limb and craniofacial muscle possess different myogenic and osteogenic differentiation capacities.

1.5.3. Aims

The aims of the project were to isolate mononuclear cells from the craniofacial and limb muscles of neonatal mice; undertake their preliminary characterisation with phenotypic evaluations *in vitro*; and compare their myogenic and osteogenic potentials *in vitro*.

1.5.4. Objectives

The specific objectives were as follow:

1. Isolate mononuclear cells by differential tissue culture plastic adherence and assess growth characteristics, morphological differences and expression of muscle specific desmin protein.

2. Assess the expression of four sets of developmental genes in isolated mononuclear cells. The genes sets were stem cellness, myogenic, osteogenic and neural crest genes.

3. Compare the myogenic differentiation potentials of isolated mononuclear cells by exposing to myogenic inductant supplemented growth media *in vitro*, and assessing myotube formation and expression of myogenic genes.

4. Compare osteogenic differentiation potentials of isolated mononuclear cells by exposing to osteogenic inductants supplemented growth media and assessing alkaline phosphatase protein expression calcium mineralisation and gene expression.

A summary of analyses conducted in this study is presented in figure 1.12 in the form of flow chart that indicates the steps taken to address the project hypothesis.
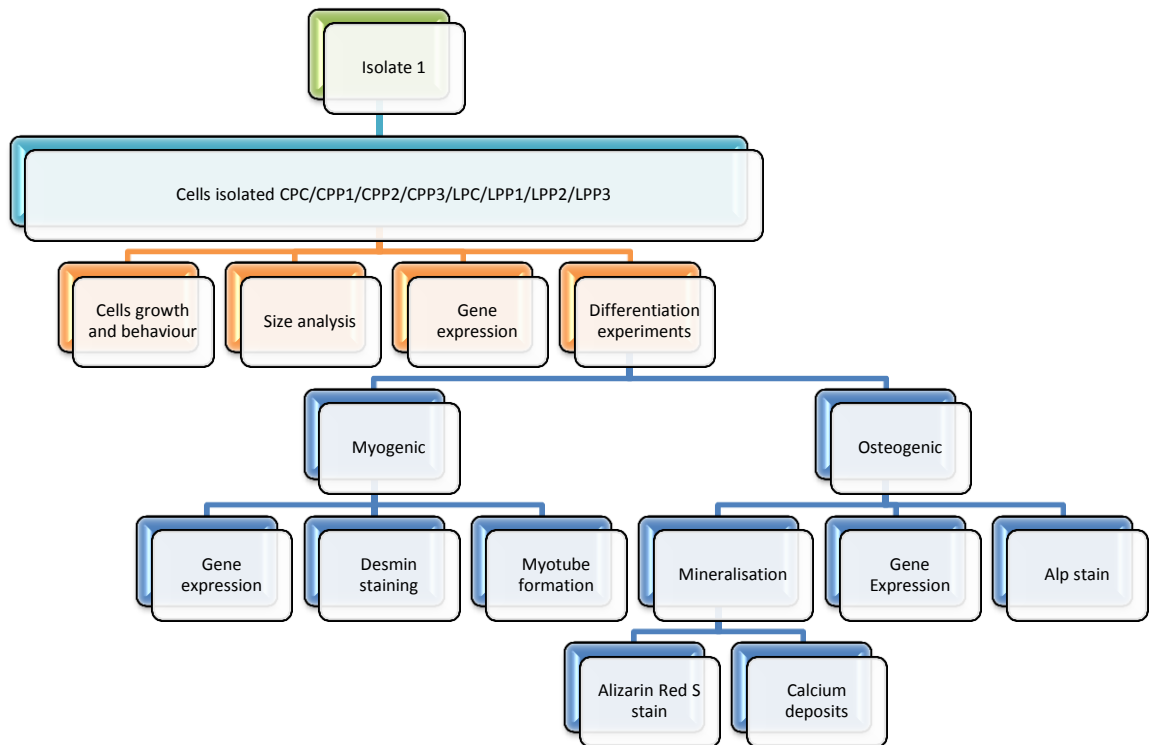
Figure 1.12 Outline of study.
The sequence of analyses conducted is presented. The experiments were conducted on three occasions of cell isolation. CPC, CPP1, CPP2 and CPP3 represent CSkM derived mononuclear cells; while LPC, LPP1, LPP2 and LPP3 indicate Limb derived mononuclear cells. Cells growth behaviour, size, gene expression profile were initially investigated. The myogenic and osteogenic differentiation abilities of the isolated cells were then investigated and analysed.

Chapter 2 Materials and Methods

2.1. Isolation of muscle precursor cells

2.1.1. Animal used

Normal (wild type) neonatal mice of the breed ICR/CD1 were used in this study. They were purchased from Harlan UK Limited laboratory. All procedures were carried out in accordance with the Animal (Scientific Procedures) ACT 1986. This strain of mice is inexpensive, robust and readily available outbred population. These strands of mice were available due to the work by departmental colleagues on bone tissue application (Buxton *et al.,* 2008).

2.1.2. Isolation of muscle tissues

The isolation procedure of muscle tissues was carried out on three occasions with 10 neonatal mice at each instance. Following sacrifice of mice by cervical dislocation and cleaning of skin over the head and back limbs with 70 % v / v ethanol in water, muscle tissues were carefully exposed by elevation of the skin with fine scissors. Masseter muscles and back limb muscles were carefully detached from underlying bone and transferred to a Petri dish containing calcium free Phosphate Buffer Saline (PBS); (PAA labs.) until muscles from all mice were isolated.

2.1.3. Isolation of Muscle cells

The method applied for mononuclear cells from extracted tissues was a modification of Richler and Yaffe, (1970) Rando and Blau, (1994) and Qu *et al.,* (1998). According to this approach a single cell suspension would be achieved by dissociating tissue fragments with enzymes. Extracted muscle tissues were minced in PBS using surgical blades and then homogenised into 2 ml of solution containing 1.2 U / ml dispase II (Roche), 0.75 U / ml Collagenase D (Roche) and 2.5 mM $CaCl_2$ at 37ºC with gentle mixing every 10 min to break up any large pieces of tissues. Following incubation,

equal volumes of foetal bovine serum (FBS) containing medium was added to each cell suspension and the mixture filtered through a piece of 70 µm nylon cell strainer (Falcon) to remove large pieces of tissue. The filtered tissues were centrifuged for 5 min at 1200 rpm forming a dense pellet at the base of a 15 ml tube (Nunc, Switzerland). This pellet was resuspended in 5 ml of general growth medium (GM) consisting of low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % heat-inactivated FBS (PAA Laboratories, Linz, Austria), 100 U / ml of penicillin (Gibco) and 100 µg / ml of streptomycin (Gibco). The suspended cells were transferred to a sterile T-75 (75 cm^2 surface area) polystyrene flask (Nunc, Switzerland) with added GM for cell culture.

2.2. Cell culture

2.2.1. Cell Culture environment

Cell culture was used for the maintenance, propagation, experimentation and storage of adherent primary cells in sterile conditions. This work was conducted in a fully equipped tissue culture laboratory. The macro equipment used for tissue culture is tabulated in table 2.1. Most tissue culture consumables were acquired sterile. Non-sterilised items, including micropipette tips, forceps etc. were autoclaved and oven dried at 60° C prior to use.

Table 2.1 Cell culture environment

Item No.	Equipment	Manufacturer	Application
1	Laminar Flow Cabinet	NUAIRE Class II with UV-tube	Confined clean work environment; pre-cleaned with 70% ethanol spray and propanol wipes prior to every use
2	Incubator	Nuaire NU-5500 DH AutoFlow Co_2 Incubator	Set at 5% CO_2 and 37^0C for cell incubation; cleaned every fortnight with bleaches.
3	Cryogenic cell storage	Thermo Scientific	Liquid nitrogen containing chamber with removable lid and six vertical racks, each capable of containing 5 x 96 sample cryo-boxes. Re-stocked with liquid nitrogen every fortnight.
4	Water bath	ETI 800 series	Weekly cleaned 50 L chamber with adjustable thermostat, set at 37^0C
5	Inverted microscope	Olympus CXK31	Standard device with fixed binocular observation tube and 4, 10, 40 and 100x magnification objective lenses.
6	Haemocytometer with trypan blue	Sigma	Standard 9x (1 x1 mm) grid with cover slip.
7	Centrifuge	Different	Tissue culture purposed large centrifuge. Microcentrifuge for smaller samples.
8	Pipettes	Qiagen	Calibrated pipette 'gun' and 1000, 200, 20 and 10 μl micropipettes.

The general consumables used for cell culture are detailed in table 2.2. The flasks and tubes were purchased sterile and were disposed following a single use.

Table 2.2 Cell culture consumables

Item No.	Equipment	Manufacturer	Applications
1	Flasks	Nunc	Enclosed polystyrene flasks with a negatively charged inner substratum for cell culture. Two sizes used in this study: 150 and 75 cm^2 flasks. Inner space accessed through an air-permeable screw-able cap.
2	Plates	Nunc	Treated polystyrene multi-well plates with removable lid. Three types used depending on individual well size: 9.6 cm^2 6-well; 1.9 cm^2 24-well; and 0.19 cm^2 96 well plates.
3	Tubes	Nunc	Sterile polystyrene tubes for various cell culture purposes. Sizes used: 50, 15, 2 and 1.5 ml.
4	Microtubes	Nunc	Sterile plastic tubes of 500 and 100 μl.
5	Cryopreservation tubes	Nunc	Male-screw sterile plastic vials of 2 ml volume for cryopreserving cells.
6	Tube Racks	Generic	Autoclavable plastic racks of various sizes.

The cell culture media and reagents used are tabulated in table 2.3.

Table 2.3 Cell culture reagents
Abbreviations of reagents used in text are provided in the corresponding row under the applications column.

Item No.	Reagent	Manufacturer	Applications
1	Dulbecco's modified eagle's medium	PAA Lab.	**DMEM**; L-Glutamine supplemented basal medium. Also used as basal medium for forming osteogenic inductive medium.
2	Dulbecco's modified eagle's medium high glucose	PAA Lab.	**DMEM**; L-Glutamine supplemental medium used as basal medium for forming myogenic inductive medium.
3	Trypsin/EDTA	Gibco.	A solution of 0.05 % / 0.002 % Trypsin/EDTA in calcium and magnesium free phosphate buffered saline.
4	Ca^{2+} Mg^{2+} free Phosphate Buffered Saline	PAA Lab.	**PBS**; an isotonic solution of NaCl in water used for cell washing, and dilutions.
5	Foetal Bovine Serum (FBS)	Gibco	**FBS**; heat inactivated serum sourced from South America, supplemented to basal medium to nourish cells whilst in culture.
6	Antibiotics	PAA Lab.	A mixture of Penicillin 10 Units / ml and Streptomycin 10 mg / ml in PBS. This was occasionally supplemented with the fungicide Amphotericin B at 0.2 % of culture medium.
7	DMSO	Sigma	Dimethyl sulfoxide; used as a cryoprotectant for cryopreserving cells.
8	IGF-1	Sigma	Growth factor used in myogenic differentiation medium.
9	β-glycerophosphate	Fischer	Water soluble phosphate donor in osteogenic differentiation.
10	L-Ascorbate-3-phsphate	Fluka	Metabolite used for collagen synthesis in osteogenic differentiation.

The instruments and consumables used for the sterilisation of equipment are tabulated in table 2.4.

Table 2.4 Sterilisation equipment

Item No.	Equipment	Manufacturer	Applications
1	Alcohols	Various	Industrial methylated spirit at 70 % in water and used as a sterilizing spray. Propanol wipes (Azowipes) were used also applied to clean large surfaces.
2	Autoclave		Pressurised steam sterilizer set at 126 ^{o} C for 20 min.

Spectrophotometric measurements of optical densities (OD) of samples were conducted in a Tecan M200 (Switzerland) multi-plate spectrophotometer. This was equipped with a monochromator capable of creating light of a wide range of wavelengths. It was also equipped with a NanoQuant (Tecan) plate for ultra violet spectral quantification of nucleic acid. Fluorometric measurements were conducted in a Fluoroskan (Fischer UK) multi-well fluorometer.

2.2.2. Isolation of different mononuclear cell sub-populations

The cell suspensions from the muscle tissue homogenates achieved from the previous step were divided in to two groups; the first group comprise mononuclear cells plated in tissue culture flask for about 5 d and called parent cells (PC) or unsorted cells. The second group consisted of suspension serially plated into three different plates; these were called pre-plate 1, 2 and 3 (PP1, PP2, and PP3 respectively), based on their adhesion differences. PP1 consisted of mononuclear cells that had adhered within 1 h of initial plating of suspension in tissue culture flask. The non-adherent cells from the first plating were transferred to a new flask for a period of 48 h. The cells that attached within 48 h period were called PP2. The non-adherent cells in suspension from PP2 were transferred to a new flask for 5 d and were designated PP3. The above described process is illustrated in figure 2.1.
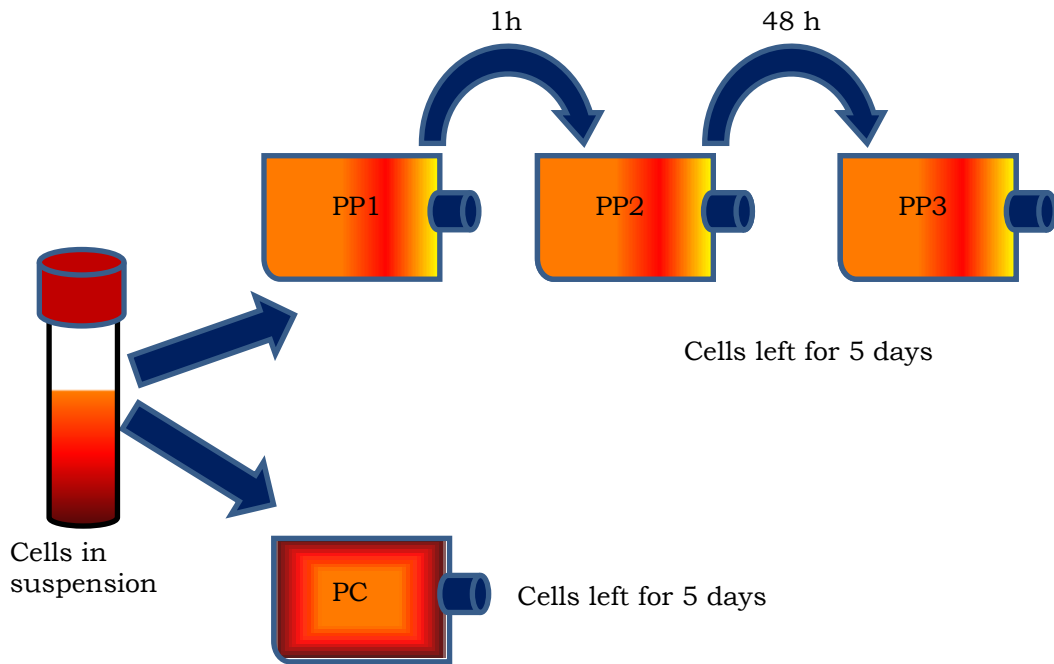
Figure 2.1 Diagrammatic representation of the isolation process of the different preplates used in this study.
This diagram shows muscle tissue derive cell suspension in a tube one third of this suspension would be plated in a tissue culture flask called PC. The other two thirds of the suspension would undergo a series of plating leading to the isolation of three separate populations of mononuclear cells. These cells are different based on their adhesion potentials and called PP1, PP2 and PP3.

The adherent cells in culture after a few days become closely associated with each other (confluent). The confluent adherent cells were detached from the surface by incubating with trypsin/ethylene diaminetetra acetic acid (EDTA) (0.25 % trypsin, 1 mM EDTA) (Gibco) for 5 min at 37ºC, after a gentle wash with PBS. Trypsin activity was neutralised by the addition of serum containing culture medium at ratio of 3:1 medium to original volume of trypsin used. The cell suspension was centrifuged at 1000 rpm, 4° C, for 5 min and supernatant discarded. The pellet was resuspended in an appropriate volume of GM for counting using haemocytometer. A description of counting method is provided with figure 2.2. Cells were plated into new tissue culture flask at a density of 1000 cells / cm$^{2.}$
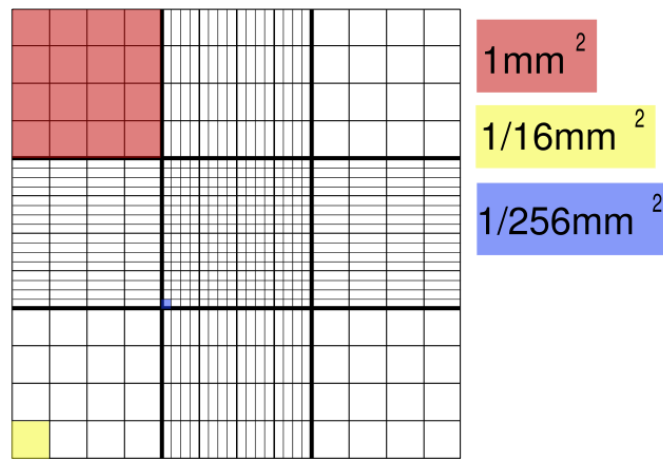
Figure 2.2 A haemocytometer counting grid.
The grid contains 9 large squares each with surface area of 1 mm^2. The depth of the chamber is 0.1 mm equivalent to 10 μl. the average cell count from four large corners squares was taken and multiplied by 10^4 to give the cell density in 1 ml of solution http://commons.wikimedia.org/wiki/Image:Haemocytometer_grid.svgg

2.2.3. Cryopreservation of primary cell cultures

The ability to store small aliquots of frozen cells in liquid nitrogen (-196 °C) without the loss of proliferative or differentiative capacity provides great advantage in allowing replication of experiments and comparative studies. However, the viability of cells can be reduced by mechanical damage caused by ice crystal formation. Freezing is therefore facilitated by the cryopreservative Dimethylsulphoxide (DMSO).

After trypsinisation and quantification of cells with a haemocytometer, the cells were then centrifuged and resuspended in freezing medium (90 % FBS and 10 % DMSO) at a density of 1 x 10^6 cells / ml. cells were then transferred to prelabled cryovials (1 ml) and placed in Styrofoam insulator container with isopropanol, which was stored in mechanical freezer overnight, to allow a cooling rate of -1° C / min down to - 70° C. The vials were then transferred to the liquid nitrogen cryopreservation tank the following day.

2.2.4. Retrieval and re-suspension of cryopreserved primary cell culture

Frozen vials of cells were retrieved and thawed in a 37° C water bath with constant agitation. The cell suspension was then transferred to a 15 ml centrifuge tube containing growth medium and centrifuged at 1000 rpm for 5 minutes. The supernatant was then discarded and the remaining pellet was resuspended in growth medium and seeded at a density of 1000 cells / cm^2.

2.3. Analysis of morphological characteristics

Cells were imaged under light microscopy using a standard camera type, with image size calibrated to the grid of a haemocytometer. The image analysis was performed on a PC computer using the public domain NIH Image program NIH ImageJ version 1.34e software. This software was developed at the U.S. National Institutes of Health (available on the Internet at http://rsb.info.nih.gov/nih-image/the NIH ImageJ version 1.34e software (http://rsbweb.nih.gov/ij/). The parameter measured was cell surface area. The outcomes were statistically analysed using ANOVA test to compare the surface area of different isolates.

2.4. Immunocytochemistry

2.4.1. Background

Immunocytochemistry is a valuable technique which used antibodies for identifying proteins and molecules in cells and tissues. The specific antibodies used possess highly specific binding to unique sequences of amino acids in proteins, thereby permitting visualisation and examination under microscope (Burry, 2010).

There are two main methods of immunofluorescent labelling. The direct method is where the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. The second method is the indirect immunofluorescence, where the specific antibody (called the primary antibody) is unlabelled, and a second anti-immunoglobulin antibody directed toward the constant portion of the first antibody (called the secondary antibody) and is tagged with the fluorescent dye.

Direct immunocytochemistry is simple and labelled primary antibodies from the same species can be used for multiple primary antibody experiments. On the other hand, indirect immunocytochemistry technique are less expensive more flexible than the direct technique. It also has the advantage of increased labeling because several secondary antibodies can bind a single primary antibody (Burry, 2010).

2.4.2. Cell fixation

The aim of cell fixation is to preserve cell structure and make target antigens available to the appropriate antibodies. In this study, a combination of paraformaldehyde and methanol was used for this process.

A solution of 4 % paraformaldehyde was prepared by diluting a stock solution of 10 % paraformaldehyde in PBS. Paraformaldehyde was added to each specimen-containing well by pipetting the solution down the side of each well for 10-20 minutes in order to lightly fix the cells. After the removal of the paraformaldehyde and transferring the solution to the waste solvent bottle, the cells were washed twice with 2 ml of freshly prepared PBS. Thereafter, 1.5 ml of cold methanol was added drop wise for further fixation for 10 min. The methanol was decanted and the cells were washed twice with PBS. The samples were covered in 2 ml of 0.0625 % sodium azide (NaN_3) in PBS and sealed with parafilm$^®$ for storage at 4°C, ready for antibody staining.

2.4.3. Desmin antibody staining

Desmin is a cytoplasmic intermediate filament protein that is expressed by muscle precursor cells (MPCs) and myotubes and therefore used as a myogenic marker for such cells.

Prior to application, the optimal dilution was determined for the primary antibody by a titration. The following dilutions with antibody diluent were tested: 1:20, 1:50, 1:100, 1:200 and 1:400. Clear desmin intermediate filament staining was seen in dilutions up to and including 1:200, and therefore the 1:200 dilution was used for all the staining procedures.

After fixation of cells, coverslips were removed from the wells and placed on custom-made pedestals. Cells were washed six times with freshly prepared PBS and permeabilised by incubation with 0.25 % Triton-X 100 / 0.0625 % NaN_3 in PBS for 10-15 min. A blocking step with 0.5 % bovine serum albumin (BSA) in PBS for 30 min was performed in order to prevent non-specific binding of the primary antibody.

Thereafter, the cells were washed six times with PBS. The mouse monoclonal anti-mouse desmin antibody (Isotype: IgG1, Abcam, UK) was diluted at the appropriate dilution in antibody diluent (PBS, 10 % heat inactivated FBS, 0.0625 % NaN3, 0.1 M lysine).

After overnight incubation, the samples were washed in freshly prepared PBS and incubated with secondary antibody for 30 min at room temperature. The anti-mouse IgG1 class specific FITC (Abcam, UK) was diluted 1:200 in antibody diluent. The nuclei were identified using the fluorescent DNA probe 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, U.K), which was diluted 1:10,000 in the antibody diluent.

The cells were washed for a final six times with PBS and coverslips then mounted on glass slides with anti-fade media (Citifluor Ltd, U.K) with the edges sealed with nail varnish. The samples were visualised with epifluorescence using the following filters to determine the expression of muscle specific antibody and the degree of cell differentiation: FITC (excitation at 485 nm, emission at 520 nm), and DAPI (UV excitation at 372 nm, emission at 456 nm).

2.5.   Phenotypic Differentiation

The types of phenotypic differentiation undertaking in this study were myogenic and osteogenic differentiation. For this purpose cells were plated in triplicate in 24-well plates at a density of 25×10^3 cells / cm^2. Cells were then left in GM until confluence was reached. At that point, the GM was removed and substituted with differentiation medium. All differentiation experiments included negative control, which are cells in GM at the same time points.

2.5.1. Myogenic differentiation

It was essential to establish the myogenicity of the isolated cells to see if there is any difference in myogenic differentiation abilities between cells from different preplates. *In vitro* proliferation is enhanced by high serum levels (10-20 %) whereas low serum levels (0-2 %) can promote myotube formation by depletion of growth factors (Ham *et al.,* 1988). Cells were plated at a density of 25×10^3 cells / cm^2 and maintained at 37°C in humidified atmosphere of 5 % CO_2 in air with GM until confluent reached.

Culture medium was then replaced with Myogenic medium (MM) containing high glucose DMEM supplemented with 2 % FBS and IGF-1 at a final concentration of 10 ng / ml (Sigma).

2.5.2. Osteogenic differentiation

This was carried out by culturing cells in osteogenic medium, which comprised low glucose DMEM supplanted with 10 % FBS, 50 μM L-ascorbate 2-phosphate (AsAc) (Fluka), 8 mM β-glycerophosphate (β-GP) and 100 ng / ml recombinant mouse BMP-2 (R&D systems).

As a positive control, mouse calvarial osteoblasts were used (MOB), which was kindly donated by the former second supervisor Dr Paul Buxton. MOB cells were expanded in

GM supplemented with ascorbate-2-phosphate (50 µg / ml; Fluka). Early passages were then used for osteogenic differentiation experiments in OS medium (Buxton *et al.,* 2008).

2.6.   Alkaline phosphatase protein expression

Alp activity was assessed with using Alp stain. The method consisted of fixing the cells with 10 % formalin for 15 min. Then the cells were washed three time with distilled water and then incubated for 45 min in Tris-HCl Buffer (0.2 M, PH 8.3) with AS-MX phosphate (Sigma Chemical Co., Poole, UK) and Fast Red violet (Sigma Chemical Co., Poole, UK).The Alp positive cells stained red to purple to the naked eye. Light microscope images of the samples were taken at eye piece objectives x 40.

2.7.   Calcium mineralisation

Mineralisation of osteogenic differentiated cells was assessed by measuring calcium deposition with Alizarin Red S stain and QuantiChrom assay kit (BioAssay Systems, USA).

2.7.1. Assessment of mineralisation with Alizarin red S stain

A working solution of a 2 % Alizarin Red S stain was prepared by dissolving 1 g Alizarin Red S dye in 45 ml ddH$_2$O at a final pH of 4.1-4.3 (adjusted with either 1 M HCl or 1 M NaOH). Volume was finalised to 50 ml upon pH stabilisation. Stain was prepared fresh each time. For the stain, medium was aspirated from cultures and cells washed 1x with PBS. Cells were fixed in 10 % formalin for 10-15 min. After aspirating fixative and washing samples with PBS, stain was performed by pouring Alizarin dye at 0.5 mL per well of a 24 well plate for 10 min. Samples were washed with PBS or

ddH$_2$O x4 and allowed to air dry. Alizarin Red positive cells stained red, and density was calcium mineral content dependent.

2.7.2. Assessment of mineralisation with QuantiChrom

Calcium was measured using QuantiChrom calcium Assay kit according to manufacturer's instructions. After washing cells three times with PBS, 500 µl of 1 M HCl was added to samples in each well for 45 min. After 45 min, 5 µl of the 1 M HCl solution was transferred to a 96 well plate. Equal amount of solution A and B (provided in the kit) were mixed and 200 µl of this solution was added to each well containing 5 µl of HCl cell lysate. Calcium concentration was measured using spectrophotometer at 610 nm wavelength and expressed as mg / dl.

2.8. Polymerase Chain Reaction (PCR)

2.8.1. RNA isolation

Total RNA was extracted from all cells using RNeasy$^{®}$ mini kit (Qiagen, UK). All procedures were conducted following the RNeasy$^{®}$ mini kit technical manual. All working surfaces and instruments (e.g. centrifuge, pipettes and bottles) were cleaned with special wipes (RNaseZap$^{®}$ wipes) which remove RNase enzymes. In addition, the tips, centrifuge tubes and water used were also RNase free.

Following the manufacturer's protocol, chemical disruption was performed using the RLT lysing buffer mixed with 2-Mercaptoethanol. Both materials are strong reagents that can dissolve proteins to release the RNA from the cells. Furthermore, the RLT® buffer included guanidiniumthiocyanate which inhibits RNase activity. The resulted solution was further disrupted by repeated pipetting to aid in freeing RNA from cells.

To initiate the selective binding of RNA to the silica membrane, the lysate solution was mixed with an equal amount of 70 % ethanol. The lysate solution then transferred to a clean silica membrane tube and passed through the column membrane by centrifugation. The RNA was bound to the filter membrane, while the flow-through supernatant containing the DNA and protein contaminants passed through the filter and collected in a tube which was then discarded. The filter membrane was then washed and centrifuged several times using different buffers to remove any remaining DNA or protein contaminants attached to the membrane. The RNA remained attached to the silica membrane. The final step was the elution of the isolated RNA, which was done by adding 30 µl of RNase free water to the membrane for five minutes at room temperature to dissolve the bound RNA. This was followed by centrifugation at maximum speed to elute the total RNA in a clean tube. The total RNA was then kept in RNease free tubes and stored at − 80 °C for further use. The steps and specific reagents used for this purpose are tabulated in table 2.5.

Table 2.5 Summary of RNA preparion

Laboratory steps	Materials	Volume	Settings	
i) Prior to start Clean the working bench, pipettes and all plastic ware used	-RNase*Zap*® wipes -RNase*Zap* spray			
iii) Disruption and homogenisation	RLT buffer	350µl		
	Repeated pipetting			
	Measure lysate			
	70% ethanol	350µl*		
Total		700µl		
	Spin column	~700 µl	**Centrifuge**	**15s 10,000 rpm**
iv) Purification** -Use the lysate from the previous step	RW1 buffer	350µl	**Centrifuge**	**15s 10,000 rpm**
	RW1 buffer	350µl	**Centrifuge**	**15s 10,000 rpm**
	RPE buffer	500 µl	**Centrifuge**	**15s 10,000 rpm**
	RPE buffer	500 µl	**Centrifuge**	**2m maximum speed**
	New tube open cap	-	**Centrifuge**	**1m maximum speed**
v) Elution**			**Keep**	**5 min at room T**
	RNase free water	**30 µl**	**Centrifuge**	**1 min maximum speed**

*Measure the remaining lysate after homogenisation and add equal volume of ethanol. ** Following the RNeasy® mini kit manual.

2.8.2. RNA quality control

The quantity of RNA was assessed using a spectrophotometer which measures the optical density (OD) of the RNA at a wavelength of 260 nm. The purity of RNA was routinely assessed by the A260 / A280 ratio that is derived from the spectrophotometer. The A260 / A280 ratio is generated from the light absorbance of the genetic material at a wavelength of 260 nm and the protein at 280nm. Acceptable RNA purity was represented by a ratio of 1.68-2.06. Samples having an A260 / A280 ratio lower than 1.68 (protein contamination) or higher than 2.06 (DNA contamination) were excluded from the experiments.

2.8.3. cDNA preparation

Total RNA was converted into single-strand cDNA via reverse transcriptase (RT) reaction. Two protocols were used, one for the qRT-PCR using high capacity cDNA reverse transcription kit (Applied Biosystems, Cheshire, U.K). The other protocol was used for the end point RT-PCR reaction following manufacturer's recommendations.

2.8.3.1. cDNA preparation for end point RT-PCR

cDNA synthesis was done using M-MuLV Reverse Transcriptase enzyme (Fermantas, UK). The table 2.6 summarises the amount used of each reagent for 20 µl reaction and thermal cycler program used.

Table 2.6 Component of cDNA synthesis for end point RT-PCR

Component	Volume (µl/20µl reaction
Oligo dt	1
RNA	X
10mM dNTP	1
RNase free dH2O	To 12 µl
Heat mixture to 65°C for 5 min and quick chill on ice	
5X First-Strand Buffer	4 µl
0.1M DTT	2 µl
RNaseOUT	1 µl
M-MLT RT	1 µl
Total	20 µl
cDNA cycle (One Cycle)	
37°C.	70°C	4°C
50 min	15 min	∞

The amount of cDNA needed was first calculated based on the number of reactions, and then the amount of reagents was calculated based on the amounts explained above in table. After the addition of the first set of reagents (Oligo (dT)18, RNA, 10mM dNTPs mix, and dH2O) into thin walled PCR tube, the mixture was heated to 65°C for 5 min and then chilled on ice. The second set of reagents was then added to the same tube according to the order explained in table 2.2. The tube was then loaded onto thermal cycler and run at 37 °C for 50 min, at 70°C for 15 min, and at 4°C until samples transferred to -20 °C for further use.

2.8.3.2. cDNA preparation for qRT-PCR

The cDNA was prepared so that 10 ng of RNA was used in every reaction in subsequent PCR. The amount of cDNA required was calculated for the polymerase chain reaction (PCR) in order to make up the correct amount of RT master mix. Table 2.7 outlines the amount of RT master mix components needed for 20 µl reaction

Table 2.7 Componant of RT master mix and their volumes used in this study

Component	Volume (µl/20µl reaction)
10x RT buffer	2.0
25x dNTP mix (100mM)	0.8
10x RT random primers	2.0
Multiscribe™ Reverse transcriptase	1.0
Nuclease-Free H$_2$O	4.2
Total	**10.0**

For the 20 µl reaction, 10 µl of RT master mix was placed into a nuclease-free microcentrifuge tube together with the 10 µl RNA water mixture. The tubes were then loaded into the thermal cycler (100™ PTC, MJ Research, Inc, U.S.A) and incubated at 25 °C for 10 min, 37°C for 120 min, 85 °C for 5 sec, and 4 °C. The samples were then collected and stored in -20 °C until further use.

2.8.4. End-point and quantitative RT-PCR

In q-PCR the level of expression of a particular gene is detected by incorporating a special fluorescent dye during the cDNA amplification. The gene expression intensity can be detected either at the end of the PCR procedure or during amplification; the higher the level of expression, the higher the fluorescent intensity. In the standard PCR technique, the level of expression is determined at the end of the reaction by running the PCR products on an agarose gel; a procedure called end-point measurement while, if the level of expression is measured during the amplification phase of the PCR, the procedure is said to be in real-time. There is a direct relationship between the level of expression of a particular gene and the amplification cycle at which the fluorescence will occur (i.e. the earlier the cycle at which the fluorescence will occur the higher the gene expression). This relationship cannot be detected by the end-point procedure. Therefore, real-time PCR has the advantage of being more sensitive in detecting minute variations in the level of expression (Schmittgen *et al.,* 2000). This is achieved by using a special thermocycler that is equipped with a sensitive camera and a monitor to display the accumulated fluorescence of the gene in each amplification cycle.

Real-time PCR provides a quantitative measurement of the expression of a particular gene and it has, therefore, been called the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Relative quantification is used to answer most clinical questions and provides a relative quantification of the gene expression of one sample compared to a calibrator or a control sample, as in time-point experiments or case-control studies. This procedure relies on the differences in the crossing threshold (Ct) cycle value between a sample and a control. The Ct value is the cycle at which a significant increase in the fluorescence of

63

a particular gene is detected. Therefore, the procedure is called the comparative Ct method (Cikos *et al.*, 2007). The relative qRT-PCR (comparative Ct method) was adopted for this research. Figure 2.3 shows the Ct relative quantification method used in this study.
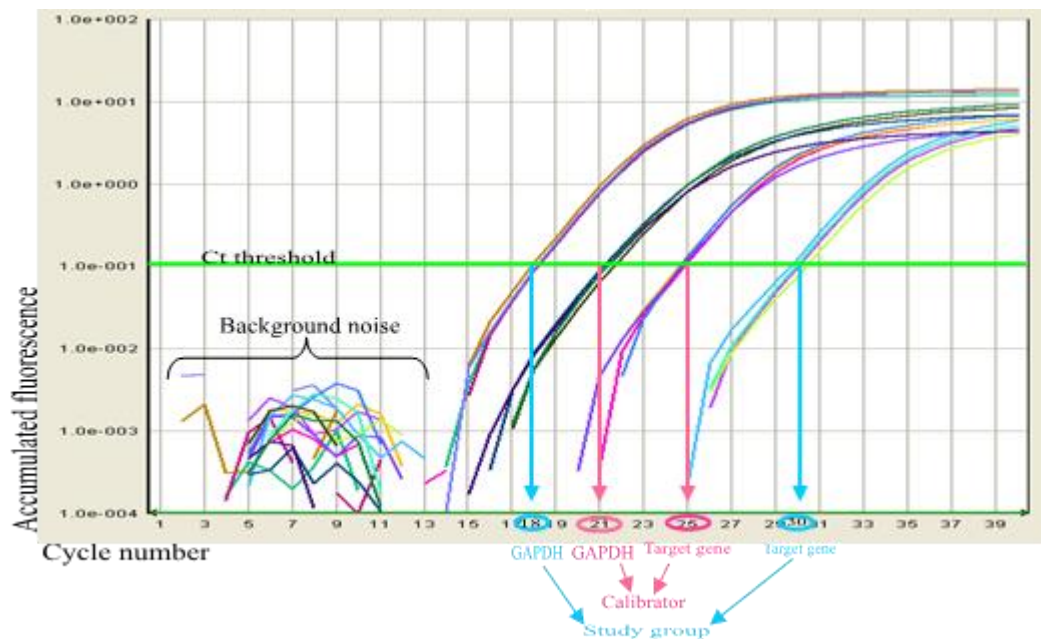
Figure 2.3 An example of calculating the relative gene intensity value using the 2-ΔΔCt equation
Both GAPDH and target gene for each sample have been tested in triplicate reactions. These are presented as 3 lines in close proximity. The background noise is the level where no fluorescence is seen; usually up to cycle 13.The Ct threshold is the cycle at which a detectable increase in the fluorescence is observed. The later the Ct value the lower the gene expression, as in the study group target gene, occurring at almost Ct 30. The Ct values obtained from the triplicate reactions are summated to produce the average Ct for each gene .For example, to calculate the gene intensity value: 1) Calculate the ΔCt (study group=30-18=12; calibrator=25-21=4); 2) Calculate ΔΔCt(study group=12-4=8; calibrator= 4-4=0); 3) Calculate 2-ΔΔCt(study group=2-8=0.004; calibrator=20 =1). Interpretation of the data indicates that the study group sample has a relatively lower gene expression (0.004 fold change) compared to the calibrator sample.

2.8.5. Protocol for end point PCR

End-point PCR reaction mixture was done following manufacturer's recommendation for 25 µl reaction volume. A master mix was first prepared and then transferred to each reaction tube. The specific primers and cDNA was then added to the prelabled PCR tubes. The tubes then loaded into the thermal cycler and PCR reaction started according to a preoptimised thermal cycles as shown in table 2.8.

PCR products were checked by 2% Agarose-TBE Ethidium bromide gels. To exclude genomic DNA contamination RT sample were loaded without reverse transcriptase enzyme and to monitor the quality of RNA, amplification of the house-keeping gene, glyceraldehyde- 3 phosphate dehydrogenase (GAPDH) was used.

Table 2.8 Reagents used for end point PCR and thermal cycles.

Reagents	Volume (µ/25µl)
10XPCR Buffer+15mM MgCl$_2$	5
10 mM dNTPs mix	0.5
Amplification primer (Forward)	0.5
Amplification primer (Reverse)	0.5
Tag DNA polymerase (5U/µl)	0.2
cDNA	1
Nuclease-free H$_2$O	17.3
Total	**25**

PCR reaction			
Pre-denaturation	**Denaturation**	**Annealing**	**Extension**
95°C	95°C	55-60°C	70°C
3 min	1 min	1 min	1 min

2.8.6. Protocol for qPCR

The following sections will describe the materials and methods used for the qRT-PCR experiment. The Applied Biosystems 7300 Real Time PCR System and related sequence detection software (Version 1.4) was used to carry out the real time quantitative polymerase chain reaction (qPCR). Each sample was examined in triplicate for expression of the targets genes and endogenous control (GAPDH). To ensure accuracy of the singleplex PCR, preparation was undertaken in a specified nuclease-free zone utilising nuclease-free equipment on ice. Additionally, high reliable TagMan® gene expression assays and PCR master mixes (Applied Biosystems, UK) were employed to reduce pipetting errors associated with the use of multiple reagents.

Each reaction was 25µl, which had been optimised previously. All reagents were thawed on ice and mixed gently. Reagents were added according to manufacturer protocol into nuclease free eppendorf tube and gently mixed (as shown in table 2.9). Then 25µl was transferred into each well of a 96 well PCR plate. The 96 well plate was then sealed with an optical adhesive film, briefly centrifuged and then place into PCR system for commencement of the reaction.

Table 2.9 Reagents used for q-PCR and their volume

Component	Volume (µl)
TagMan® Universal PCR master Mix	12.5
TagMan® probes	1.25
cDNA	2.5
Nuclease free water	8.75
Total	25

The normalisation process is required to remove any source of variation in RNA concentration and cDNA preparations between different samples. This procedure, and the calculation of the final gene expression value, is not a direct process and require the use of the Ct value in several equations. These equations have been described in detail by Livak and Schmittgen (2001) and will be discussed briefly in the following sections. All Ct values were exported from the qRT-PCR machine, and were transferred to an Excel spread sheet where the gene expression value was calculated using the final equation $2^{-\Delta\Delta Ct}$. Figure 2.3 shows an example of a qRT-PCR amplification plot by which the Ct values were derived.

The process of normalisation of q-PCR data was carried out along the following steps:

- Step 1 (repeatability)

  Objective: To ensure high precision, both the target and the reference genes were experimented in Triplicates. This produced several Ct values which were summated to produce an average Ct value obtained for both genes.

  Equation: = Average Ct of reference gene

  Average Ct of target gene

- Step 2 (normalisation)

  Objective: To normalise the average Ct of the target gene in relation to the average Ct of the reference gene of the same sample thereby producing the ΔCt

  Equation: = Average Ct target – Average Ct reference = ΔCt

- Step 3 (calibration)

Objective: The control sample was selected as the calibrator sample. This was to produce ΔΔCt for each sample. During calibration, the calibrator sample was also calibrated to itself to produce a value of zero. The reason for generating a zero value for the calibrator sample was to produce a value of one when used in the next equation, and one would act later as a baseline for relative gene expression comparison.

Equation: = ΔCt target – ΔCt calibrator = ΔΔCt

- Step 4 (gene expression value)

Objective: The fourth equation was derived from the belief that each gene was doubled during each amplification cycle, assuming 92-100% amplification efficiency (Livak and Schmittgen, 2001). The equation transferred the calibrator value into 1 which acted as a baseline for gene expression comparison. The generated gene expression values of the other samples which were higher or lower from the baseline were considered a fold change. The results were then used for further data analysis to assess the significance of the difference.

Equation: $= 2^{-\Delta\Delta Ct}$

2.9. Statistical analysis

The Graphpad Prism version 5.02 statistical package was used for mathematical calculation and statistical analyses of raw data. The calculations included (i) basic statistics (mean and standard deviation); (ii) Checking for the normality between data sets and (iii) evaluation of significance of the difference of means.

Statistical analyses were conducted after generating a data matrix consisting of readings from three different isolates with three replicates per isolate ($N = 3$; $n = 3$). Data was first tested for normality. This was followed by ANOVA measure (analysis of variance) with a 2-way or 1-way measures test utilising the Bonferonni post-test comparing all variables.

Chapter 3: Isolation and phenotypic comparison of CSkM and LM derived mononuclear cells

3.1. Introduction

3.1.1. Background

Skeletal muscle can adapt to physiological and reparative demands due to cells with stem-like characteristics that can differentiate and form muscle fibres. These cells are further suggested to have multipotential capacity (Lee *et al., 2000*, Asakura *et al., 2001, 2002*, Qu-Petersen *et al., 2002*) as well as, mainly deduced from the observation of heterotopic ossification in muscle tissue in certain disease conditions (Aho *et al., 1988*, Aro *et al., 1991*, Kaplan and Smith, 1997, Mahboubi *et al., 2001*).

As there are differences in the embryonic development of skeletal muscle tissue between the limb and craniofacial regions of a vertebrate body, it is suggested that stem-like cells isolated from these anatomical sites would have different differentiation capacities or degree of multipotency, and hence may offer various possibilities for the design of tissue engineering applications.

Several mononuclear cell types can be isolated from muscle tissue with various methods. These are described in section 1.3.2.2. One of the methods reported in (Yaffe, 1968; Richler and Yaffe, 1970, Rando and Blau, 1994, Qu *et al., 1998*) separates different mononuclear cells based on their adherence properties. This method was originally developed to increase the yield of myogenic cells and since its inception has been applied with varying modifications to isolate different mononuclear cells from skeletal muscle tissue.

3.1.2. Rationale

Much of the literature concerning skeletal muscle tissue mononuclear cells have focused on cells derived from limb and trunk skeletal muscles (Lee *et al*., 2000; Li and Huard,

2002; Mastrogiacomo *et al*., 2005). The data available of the isolation and characterisation of mononuclear cells from the craniofacial skeletal muscle are relatively less (Sinanan *et al.,* 2004). Distinct to muscles of the trunk and limbs that are somitic in origin, craniofacial muscles develop from three different regions of the embryo; namely the occipital somites, precordal (cranial to the notochord) and paraxial head mesoderm (PHM). Additionally, the connective tissues of CSkM muscles are derived from neural crest cells (Sperber, 1989). This implies that mononuclear cells isolated from skeletal muscle may possess varying differentiation potentials.

3.1.3. Aims and objectives

This initial section of the thesis hypothesised that mononuclear cells with varying phenotypic characteristics can be isolated from skeletal muscles of the cranium and limb of a vertebrate animal. The aim was to isolate various mononuclear cells based on the principle of adherence per time with a modification of the (Rando and Blau, 1994, Qu *et al.,* 1998) pre-plate technique; and evaluate differences between the cell sub-populations by assessing differences in (i) cellular proliferative potentials by comparing population doublings in culture; (ii) evaluate morphology and compare parameter of cell surface area, (iii) examine the expression of muscle-specific desmin protein; and (iv) evaluate the phenotype of the cells by analysing the expression of (a) potential stem cell markers Oct4 (Jiang *et al*., 2002), Sca-1 (Kafadar *et al*., 2009) and CD34 (Lee *et al.,* 2000, McKinney-Freeman *et al*., 2002, Majka *et al*., 2003); (b); myogenic markers MyoD and Desmin. (c) osteogenic markers Runx-2 and Alp; as well as the (d) neural crest genes Sox10 (Dupin *et al.,* 2007), P75 (Yan *et al.,* 2006, Dupin *et al.,* 2007) and AP2 (Mitchell *et al.,* 1991, Williams and Tjian, 1991). The details and significance of these genes are tabulated in table 3.1

Table 3.1 Developmental markers analysed in muscle derived mononuclear cells
a Gene annotations are derived from NCBI Gene. b annotations derived from (Kafadar *et al.* 2009).

Gene	Information
OCT-4	A transcription factor that forms a trimeric complex with SOX2 on DNA and controls the expression of embryonic pluripotency associated genes[a]
Sca-1	Cell surface protein that negatively regulates proliferation and differentiation of progenitor cells[b]
CD34	A possible cell surface adhesion molecule expressed selectively on hematopoietic stem cells but also myogenic satellite cells[a]
MyoD1	A transcription factor that regulates muscle cell differentiation by inducing cell cycle arrest, a prerequisite for myogenic initiation[a]
Desmin	A class of intermediate filaments found in muscle cells[a]
Runx-2	Master transcription factor regulating osteogenic differentiation[a]
Alp	An osteoblastic enzyme that provides inorganic phosphate to a developing calcium phosphate crystal[a]
P75	Transcriptional coactivator involved in neuroepithelial stem cell differentiation and neurogenesis[a]
AP-2c	AP2 isoform c; transcription factor involved in the activation of several genes important for eye, face and neural tube development[a]
Sox10	Transcription factor involved in the regulation of neural crest development and in the determination of the cell fate[a]

3.2.   Experimental protocols

3.2.1. Isolation of mononuclear cells

3.2.1.1.   Animals

Normal (wild type) neonatal mice of the breed ICR / CD1 were purchased from Harlan

UK Limited laboratory. All procedures were conducted in accordance with the Animal

(Scientific Procedures) ACT 1986. A description of the methods carried out is provided

in section 2.1.1. Isolation of muscle tissues from craniofacial and hind limb region was

conducted on three occasions with 10 neonatal mice in each instance.

3.2.1.2.   Isolation of muscle tissue

Required tissues were extracted with surgical scissors and transferred to petri-dishes for

enzymatic digestion, as described in section 2.1.2 and 2.1.3.

The extracted tissues were digested to a cell suspension that was subjected to the

modified pre-plating method described below.

3.2.1.3.   Adherence based fractionation of mononuclear cell isolate

The method used to separate the different sub-types of cells in suspension is described

in section 2.2.2. In brief, the cell suspension achieved from the digestion of muscle

tissue was divided in to two groups; group one was plated in a T75 tissue culture flask

for 5 days and designated as the parent or unsorted cells (PC). The second aliquot of

suspension was serially plated through three different plates; pre-plate 1, 2 and 3

represented by PP1, PP2, and PP3 respectively. Cells were fractioned based on their

adherence properties to tissue culture plastic per time. PP1 constituted cells that had

adhered within 1 h of contact with culture plastic. The non-adhered cells were

transferred to a clean culture flask and adherent cells by 48 h were called PP2. The non-

adherent cells in suspension from PP2 cultures were then cultured with a new flask for 5 d and the resulting adherent cell population was the PP3. Cells were sub-cultured and / or cryopreserved as described in sections 2.2.2 and 2.2.3, respectively. Three batches of each population were conducted in this study.

3.2.2. Assessment of proliferative differences between cell fractions

The growth behaviour of isolated cells was investigated by recording cell numbers at the start and end of a passage during routine serial sub-culture performed till senescence, which is the point of a lack of cell replication. Earliest passage cells would be trypsinised according to the method described in section 2.2.2. These detached cells were counted as described in section 2.2.2. They were seeded at a density of 1000 cells / cm^2 in T75 tissue culture flasks in GM and incubated in humidified conditions. Cell numbers were recorded at the end of culture. These were used to arithmetically determine the population doubling value. A population doubling is an increase of two-fold in the total number of cells during the exponential phase of growth in culture. The formula used to calculate the population doublings (PD) in a passage was: PD = Log (N - No) / log2, where: N is number of cells counted at the end of growth period, No is the original number of cells plated. Results were calculated as cumulative population doubling (PD) number against time.

3.2.3. Assessment of differences in morphological parameters

Light microscopic images of cell cultures in T75 flasks were used to assess morphological differences. Images were taken and analysed with Image J software, as described in section 2.3. In general, two images per flask were taken at x40 objective with a standard microscope-mounted camera. A total of 7 cells per image were selected

and analysed for surface area. This experiment was conducted on each of the three occasions of cell isolation and for each cell subpopulation.

3.2.4. Assessment of muscle specific desmin protein expression

An Immunocytochemistry analysis for the muscle-specific desmin protein was conducted on all isolated cells. For this purpose, 25×10^3 cells were seeded on sterile glass cover slips immobilised in a well of a 24-well plate in GM. Each sample was assessed in triplicate on each of the three occasions of cell isolation. The method applied for this experiment is described in section 2.4. Cells were first fixed and then stained for desmin using mouse monoclonal anti-mouse desmin antibody (Isotype: IgG1, Abcam, UK). Cells were then incubated with secondary antibody (anti-mouse IgG1 class specific FITC (Abcam, UK)) for 30 minutes at room temperature. The nuclei were identified using the fluorescent DNA probe DAPI (Invitrogen, U.K).

3.2.5. Polymerase Chain Reaction analysis of phenotypic genes

The genes tabulated in table 3.1 were examined due to their association with multipotent cell phenotypes.

Two variants of PCR were used for this experiment. End-point PCR was used to assess the presence or absence of gene expression; and qPCR was used to evaluate fold differences in the level of expression between samples. This experiment was conducted in triplicate (n = 3) on each of three occasions of cell isolation (N = 3). The methods for PCR applied are described in section 2.8. The different steps undertaken are highlighted below.

3.2.5.1. RNA isolation

This was conducted with Qiagen Total RNA kit as described in section 2.8.1. Cells were homogenised with buffer RLT. Homogenates were mixed 1:1 with 70 % v / v ethanol in water and passed through a silicon membrane-column. RNA was eluted in RNase free water.

3.2.5.2. RNA quantification

RNA was quantified by spectrophotometry according to the method described in section 2.8.2. A 2 ul aliquot of the RNA isolate was checked for concentration and integrity with spectrophotometer Tecan, M200 (A260 : A280).

3.2.5.3. Reverse transcription

This step was conducted according to the method described in section 2.8.3. A total of 100 ul cDNA was prepared with the equivalent of 10 ng RNA per 2.5 ul of reverse transcribed product.

3.2.5.4. End point PCR

This was performed by amplifying target genes in cDNA by conventional PCR and running the product on a 2 % agarose gel, according to the method described in section 2.8.5. The primers and the physical parameters for this are stated in table 3.2. Three replicates representing one of each of the three occasions of cell isolation were analysed simultaneously to omit any errors.

Table 3.2 Conditions for end-point PCR analysis
Table indicates the genes, primer sequences, and physical conditions used for end-point PCR analysis. (C= Celsius, min= Minutes, sec= Seconds, F= Forward, R= Reverse)

	Gene	Primer	Primer sequence	Primers and PCR conditions for End-point PCR					PCR product size (pb)
				PCR condition					
				Denature	Annealing	Extension	Final extension	No. of Cycles	
1	OCT-4	F	TGTGGACCTCAGGTTGGACT	94℃,2 sec	55℃,30 sec	72℃,1 min.	72℃,5 min.	35	201
		R	CTTCTGCAGGGCTTTCATGT						
2	Sca-1	F	TCTGAGGATGGACACTTCTC	94℃,2 sec	55℃,30 sec	72℃,1 min.	72℃,5 min.	35	384
		R	CTCAGGCTGAACAGAAGCAC						
3	CD34	F	TTGACTTCTGCAACCACGGA	94℃,2 sec	60℃,30 sec	72℃,1 min.	72℃,5 min.	35	300
		R	TAGATGGCAGGCTGGACTTC						
4	MyoD1	F	AGGACACGACTGCTTTCTTC	94℃,2 sec	55℃,30 sec	72℃,1 min.	72℃,5 min.	35	360
		R	GCACCGCAGTAGAGAAGTGT						
5	Desmin	F	TGATGAGGCAGATGAGGGAG	94℃,2 sec	55℃,30 sec	72℃,1 min.	72℃,5 min.	35	250
		R	TGAGAGCTGAGAAGGTCTGG						
6	Runx-2	F	GAACCAAGAAGGCACAGACA	94℃,30 sec.	55℃,10 sec	72℃,1 min.	72℃,5 min.	35	452
		R	AACTGCCTGGGGTCTGAAAA						
7	ALP	F	GCCCTCTCCAAGACATATA	94℃,30 sec.	58℃,10 sec	72℃,1 min.	72℃,5 min.	35	372
		R	CCATGATCACGTCGATATCC						
8	GAPDH	F	ACCACAGTCCATGCCATCAC	94℃,30 sec.	55℃,10 sec	72℃,1 min.	72℃,5 min.	30	451
		R	TCCACCACCCTGTTGCTGTA						
9	P75	F	GTGCGGGGTGGGCTCAGGACT	94℃,30 sec.	60℃,10 sec	72℃,1 min	72℃,5 min.	35	422
		R	CCACAAGGCCCACAACCACAGG						
10	AP-2c	F	CCTGGATTTAACTGGCGACT	94℃,30 sec.	55℃,10 sec	72℃,1 min.	72℃,5 min.	35	676
		R	CCTCCAGCCCTGAAATATGG						
11	Sox10	F	GGAGGTTGCTGAACGAAAGTG	94℃, 1min.	60℃,1min.	72℃,1 min.	72℃,5 min.	35	444
		R	TCCATGTTGGACATTACCTCG						

3.2.5.5. QPCR analysis for developmental genes

This was performed with $2^{-\Delta\Delta Ct}$ method according to the methods described in section 2.8.5. The Taqman probes used in this experiment are presented in table 3.3.

Table 3.3 TagMan probe catalogue number for q-PCR analysis

Gene	Probe ID
Sca-1	Mm00726565_s1
CD34	Mm00519283_m1
MyoD1	Mm00440387_m1
Desmin	Mm00802455_m1
Runx-2	Mm00501580_m1
ALP	Mm00475834_m1
GAPDH	Mm99999915_g1
P75	Mm01309635_m1
AP-2c	Mm00493473_m1
Sox10	Mm01300162_m1

3.2.6. Statistical analysis

Variations in gene expression and surface area comparisons were statistically assessed with a 1-way ANOVA using the Graphpad Prism v5.02. A p-value of ≤ 0.05 was considered to be statistically significant. The growth data were analysed using 2-way ANOVA and a p-value of ≤ 0.05 was considered to be statistically significant.

3.3. Results

3.3.1. Isolation of mononuclear cells from muscle tissue

Mononuclear cells were isolated with the absence of infection. The neonatal mice were de-skinned and target tissues homogenised with digestive enzymes. The resulting pellet of cells was re-suspended in GM and tissue fragments removed by passing through a sieve. The filtrate was collected. Cells were counted and then seeded to form the parental cells population and the first pre-plate (PP1), with non-adhered cells removed to achieve the PP2 and PP3 population of cells. Light microscopy revealed cells were mononuclear. Aseptic techniques ensured no infections developed in the extraction process. An average of 2,000,000 cells were isolated on each of the three occasions of isolation.

3.3.2. Assessment of proliferative differences between cell fractions

Expansion potentials of muscle-derived cells were determined by calculating the population doublings (PD) that the cells could achieve before senescence. Population doublings for all CSkM and LM cells (PC, PP1, PP2, and PP3) from three different isolates were measured. The mean of the PDs for the three isolations was plotted against the time. The results of this experiment are shown in figure 3.1 for CSkM cells and 3.2 for LM. Cells, from both CSkM and LM that adhered within the 1st hour of initial plating (PP1), cells that adhered within 48 h (PP2) and unsorted cells (PC), showed significant increases in growth rate for 5 w compared to late adhered cells (PP3), which displayed a relative lag in proliferation during this time. Moreover, late adhered cells (PP3) demonstrated an increase in growth rate after 5 w. From 7 w to 15 w, no significant differences in growth behaviour were found between cells from different pre-plate. A comparison between the muscle groups showed that the PC, PP1 and PP2 populations from CSkM differed significantly from their counterparts isolated from LM

at 3 to 9 week of culture. The PP3 populations did not differ significantly at any time points.

Mean population doublings for craniofacial skeletal muscle cells
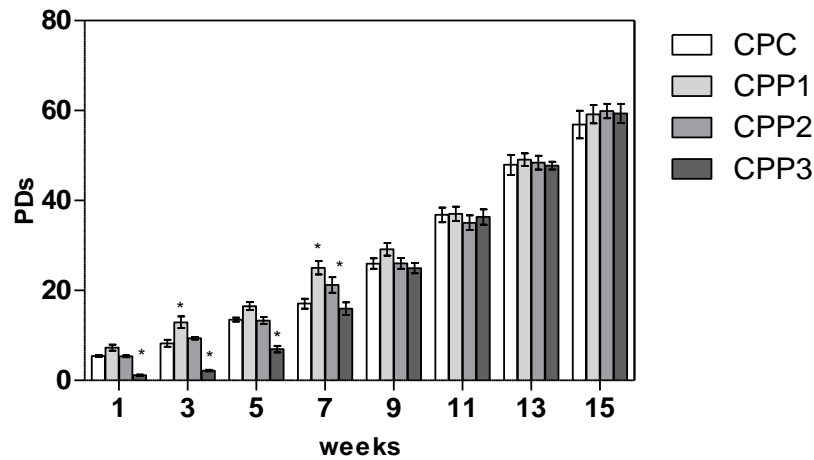
Figure 3.1 Mean population doublings for CSkM derived mononuclear cells.
Figure shows bar chart with the mean and SD of the population doublings (PDs) of cells plotted against time in culture (Weeks). Bars show mean with standard deviation, n= 3. * represent p < 0.05, PC vs. PP-type. Cell types displayed differences in the initial 7 w of culture. PP1 seemed highly replicative due to a higher cumulative PD value by 3 and 7 w. PP3 displayed a significantly lower PD rate compared to PC from 1 to 5 w in culture. All cultures displayed similar PD values from 9 to 15 w.

Mean population doublings for limb muscle cells
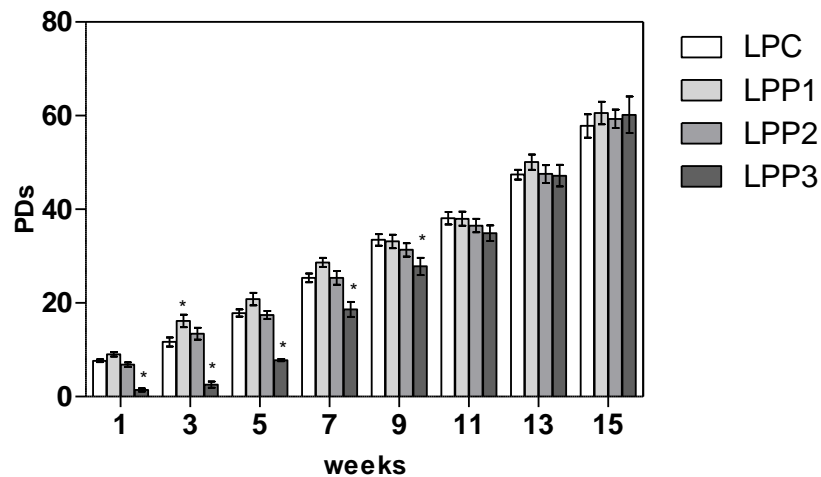
Figure 3.2 Mean population doublings for LM derived mononuclear cells.
Figure shows bar chart with the mean and SD of the population doublings (PDs) of cells plotted against time in culture (Weeks). Bars show mean with standard deviation, n= 3. * represent p < 0.05, PC vs. PP-type. PC, PP1 and PP2 proliferated at similar rates. PP3 displayed a significant lag in PD counts till 9 w in culture. All cultures displayed similar PD values from 11 to 15 w.

3.3.3. Assessment of differences in morphological parameters

Cells isolated from both CSkM and LM were analysed for morphological size differences. Images were taken from each pre-plate with light microscope mounted camera and images analysed using image J software for surface area of cells.

The analysis showed gradual decrease in cell size from the early adhered cells (PP1) toward the late adhered ones (PP3) in all samples. Examples of the images obtained in this experiment are shown in figures 3.3 for CSkM and 3.4 for LM. The mean value of surface area obtained for the different cultures are illustrated in a box-whisker plot in figure 3.5.

The CSkM derived PC cells seemed large and irregularly shaped. The CPP1 cells displayed similar characteristics. Their morphology differed from PP2 and PP3, which seemed to skew towards a more rounded shape. Similar was the case for LM derived cells. This trend was supported by the measurements of mean surface area, which indicated PC and PP1 to be significantly largely than PP2 and PP3.

A comparison between CSkM and LM derived cells indicated that although small, the differences between the surface area of the two types of muscle derived cells were significant.
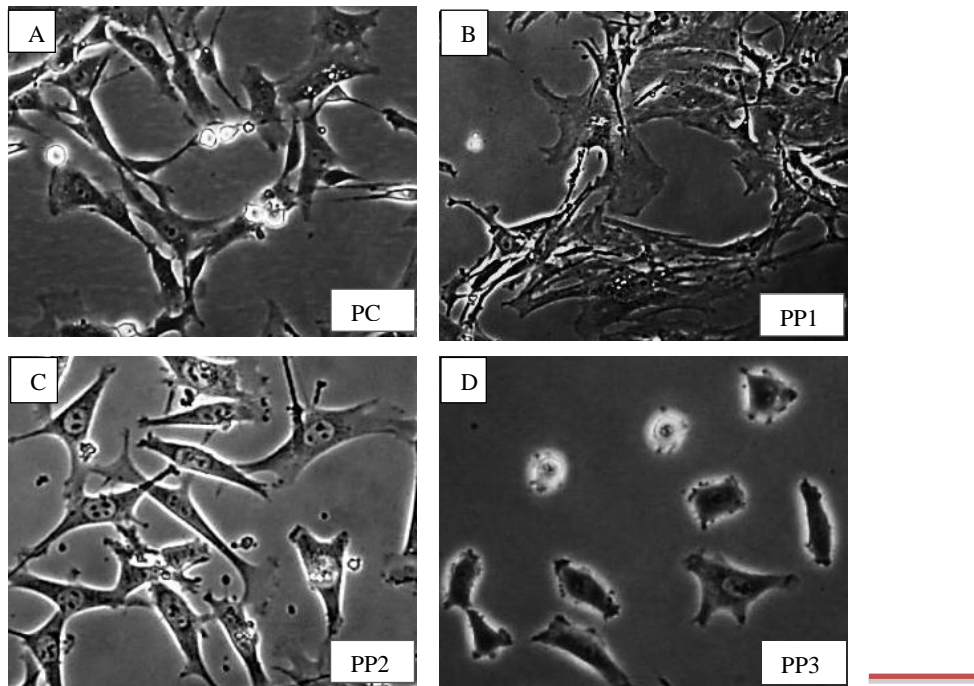
Figure 3.3 Images of CSkM derived mononuclear cells.
Figure shows grey-scaled images taken at x40 magnification of (A) PC, (B) PP1, (C) PP2, and (D) PP3 cells in tissue culture flasks at 1 d after cultures had settled. (A) PC cells appeared elongated in shape as were cells of (B) PP1. (C) The PP2 appeared elongated and flattened while (D) the PP3 appeared somewhat satellite or rounded. Bar equal 100 µm.
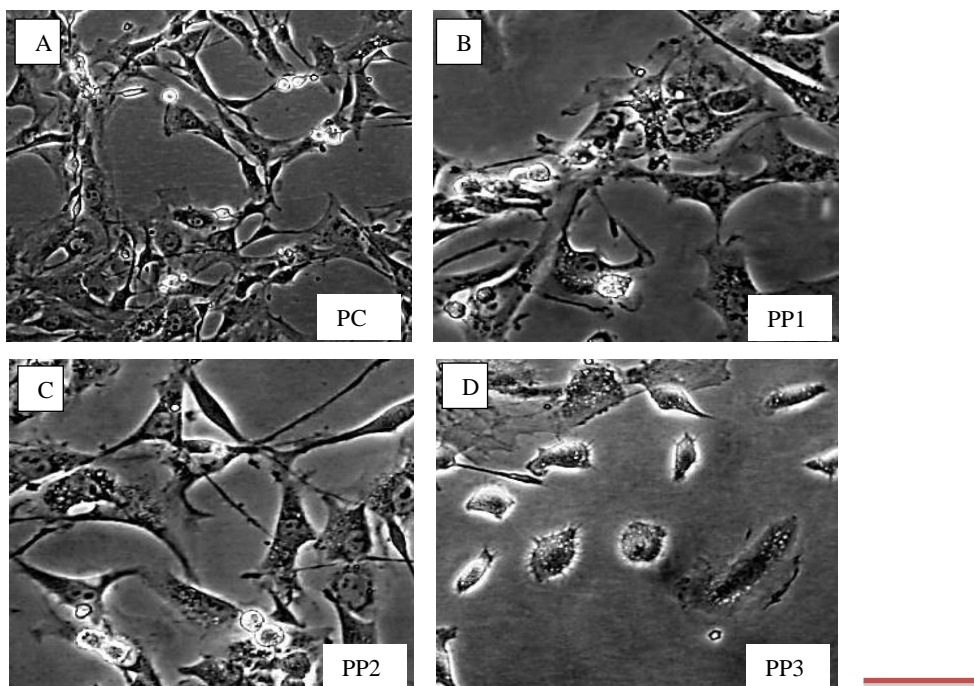
Figure 3.4 Images of LM derived mononuclear cells.
Figure shows grey-scaled images taken at x40 magnification of (A) PC, (B) PP1, (C) PP2, and (D) PP3 LM cells in tissue culture flasks at 1 d after cultures had settled. (a) PC and PP1 cells appeared similar as irregularly shaped cells. (c) The PP2 appeared elongated but flattened while (d) the PP3 appeared somewhat satellite or rounded. Bar equal 100 µm.

Mean surface area of cells isolated from CSkM and LM
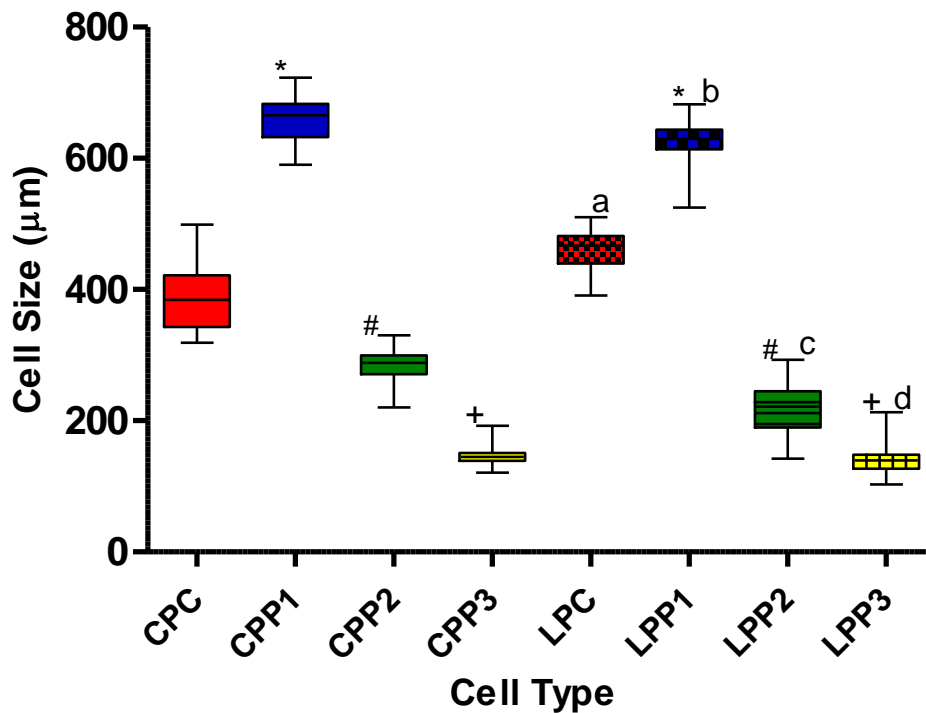
Figure 3.5 Mean surface area of cells isolated from CSkM and LM.
A box and whisker plot indicating readings (n = 42) obtained for surface area of CSkM and LM muscle derived cells with minimum to maximum range represented by vertical bars. *, #, + represent p < 0.05, PC vs. PP1, PP2 and PP3, respectively. Comparisons between the sub-types of both tissues are annotated by a, b, c, d, which represent p < 0.05, PC vs. PC, PP1 vs. PP1, PP2 vs. PP2 and PP3 vs. PP3, respectively. CPP1 and LPP1 had a larger surface area than PC while PP2 and PP3 had smaller surface areas. Comparisons between the muscle types indicated differences between respective pairs to be significant for all except PP3. LPC cells are significantly bigger than CPPC. LPP1 and LPP2 cells were significantly smaller than their counterpart cells from CSkM (CPP1 and CPP2). There is no significant difference in size between CPP3 and LPP3.

3.3.4. Assessment of muscle specific desmin protein expression

The different cultures were assessed for the expression of muscle specific desmin protein as a percentage of desmin positive cells in a culture of 25,000 cells on glass cover slips. It was found that cells displayed different degrees of desmin immunoreactivity. The images obtained of desmin stained cultures in this experiment are shown in figure 3.6 and 3.7 for CSkM and LM, respectively. The determined percentages of desmin positive cells from all samples are shown in figure 3.8.

The percentage of desmin positive cells was calculated by counting green fluorescence desmin positive cells against DAPI blue positive total cells. Desmin positive cells counting were performed in three random fields at objective x 40.

The images indicate a presence of desmin in all cultures. The relative intensity of the protein stain was least in PP1 and then PP2 compared to PC and PP3 for both muscle groups. Moreover, PP3 stain was highest in the case of either muscle type.

The analytical data supported these findings by clearly indicating a stark difference between PP1 and PP2 with PP3 in expression of desmin. There was no significant difference between the muscle types.
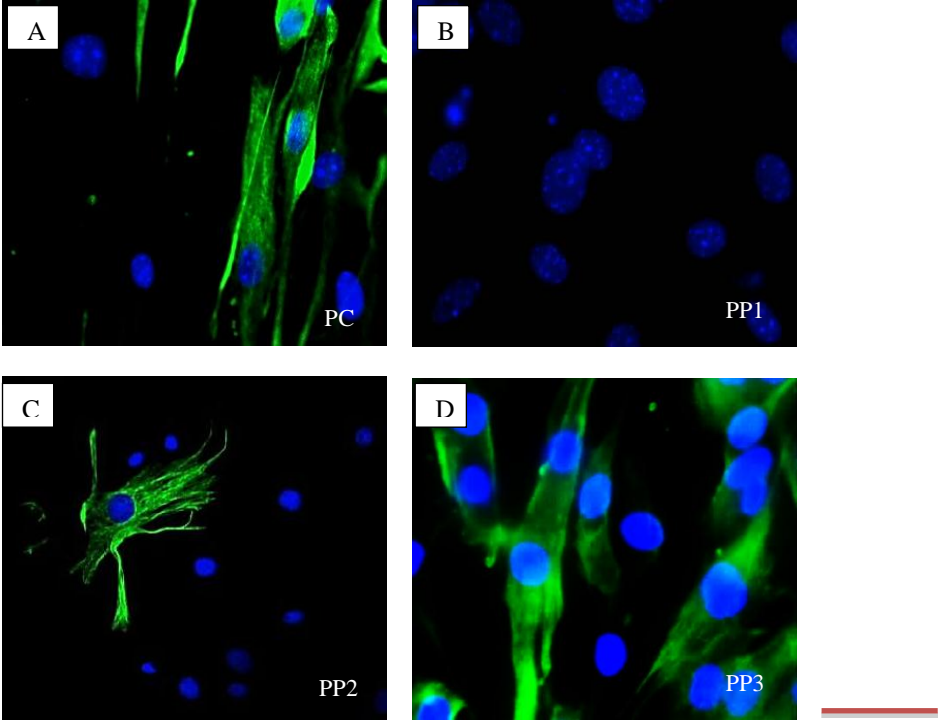
Figure 3.6 Confocal immuno-fluorescence based images of CSkM isolated cells.
Nuclei are stained blue and desmin green. PC (A) and PP3 (D) displayed high intensity of
desmin fluorescence. (B) PP1 and (C) PP2 displayed absence of any significant levels of the
protein. Bar equal 100 μm.
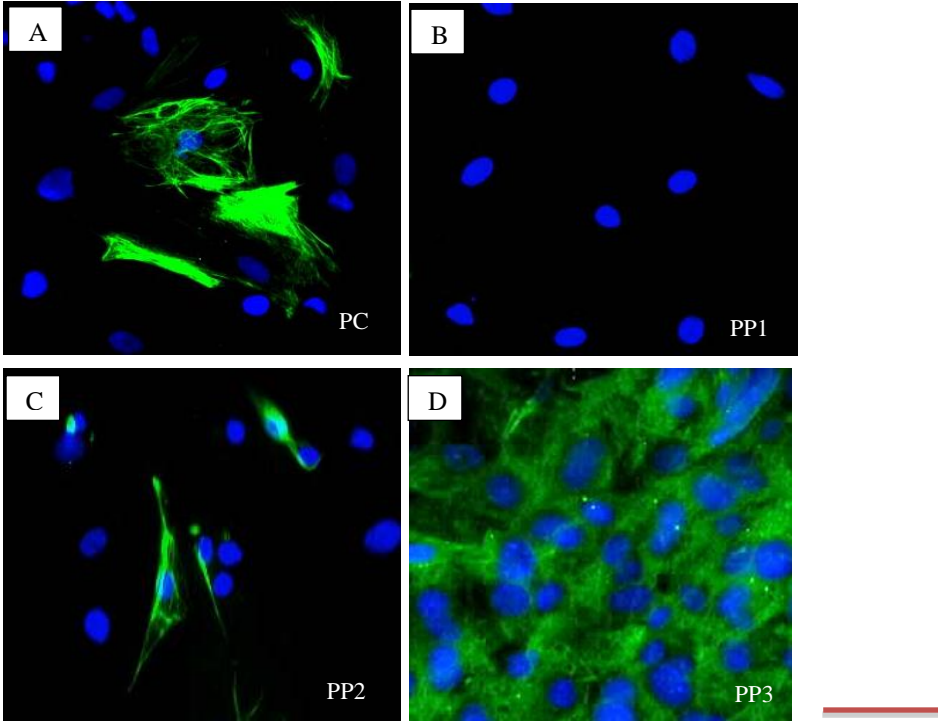
Figure 3.7 Confocal immuno-fluorescence based images of LM isolated cells.
Nuclei are stained blue and desmin green. PC (A) and PP3 (D) displayed high intensity
of desmin fluorescence. (B) PP1 and (C) PP2 displayed absence of any significant levels
of the protein. Bar equal 100 μm.

87

Mean percentage of desmin expression in muscle derived mononuclear cells isolated from CSkM and LM
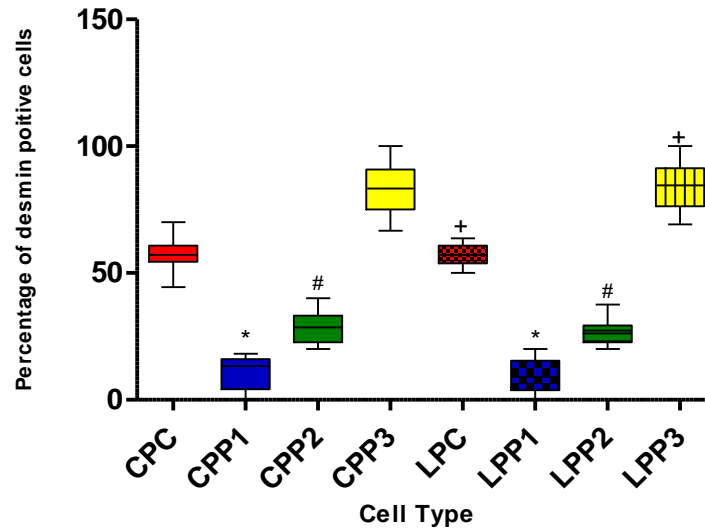
Figure 3.8 Mean percentage of desmin expression in muscle derived mononuclear cells isolated from CSkM and LM.

A box and whisker plot indicating readings (n = 9) obtained for the percentage of desmin expression in CSkM and LM muscle derived cells, with minimum to maximum range represented by vertical bars. *, #, + represent $p < 0.05$, PC vs. PP1, PP2 and PP3, respectively. The PP3 fractions displayed highest expression of desmin compared to the rest. The PP1 and PP2 fractions displayed the least positive cells for desmin. Comparisons between tissue types showed no significant differences between any of the tissues.

3.3.5. PCR analysis of phenotypic genes

The general trend seen was that early adhered cells PP1 and PP2 showed higher expression of stem cell, neural crest cell, and osteogenic markers compared to PP3 cells. In contrast, the PP3 expressed myogenic genes at significantly higher levels than the remainder. The results of this analysis are described in the following sub-sections.

3.3.5.1. End-point RT-PCR

The data from this analysis is shown in figure 3.9. It shows the bands obtained following agarose gel electrophoresis. The GAPDH house keeping gene was detected in all samples analysed. The Oct4 gene seemed non-transcribed in any of the samples. A band for Sca-1 was detected in all samples analysed. The CD34 band intensity was higher for PP1 and PP2 compared to PC and PP3 for both muscle types. The AP2c, Sox10 and P75 gene bands displayed high intensity for PP1 and PP2 of CSkM derived cells compared to others. The Runx2 band intensity was higher in PC, PP1 and PP2 cells from both muscle tissues while the gene seemed non-transcribed in PP3 cells. The Alp gene was found expressed in all samples. The MyoD gene seemed transcribed in all samples, while desmin gene was mainly expressed by PP3 compared to others.

Genes			Expression												Size (bp)
			PC	PC	PC	PP1	PP1	PP1	PP2	PP2	PP2	PP3	PP3	PP3	
Stem Cell genes	Oct4	CSkM													201
		LM													
	Sca1	CSkM													385
		LM													
	CD34	CSkM													300
		LM													
Myogenic genes	MyoD	CSkM													360
		LM													
	Desmin	CSkM													250
		LM													
Osteogenic genes	Runx2	CSkM													452
		LM													
	ALP	CSkM													372
		LM													
NCC gene	AP2c	CSkM													676
		LM													
	Sox10	CSkM													444
		LM													
	P75	CSkM													424
		LM													
Endo Ctrl	GAPDH	CSkM													451
		LM													

Figure 3.9 End-point gene expression of CSkM and LM derived cells.
Three isolates of each of the four mononuclear cell-fractions were analysed in parallel for the expression of developmental genes. Potential stem cell genes (Oct-4, Sca-1 and CD34); myogenic genes (MyoD and Desmin); osteogenic genes (Runx-2 and Alp); neural crest (NCC) genes (Ap2c, Sox 10 and P75). In brief, oct4 genes seemed non-transcribed in all samples. The early adhered cells expressed stem cell genes and osteogenic genes whereas the late adhered cells expressed Sca-1 gene and myogenic genes. Early adhered cells from CSkM expressed neural crest specific gene.

3.3.5.2.   QPCR analysis for developmental genes

The qPCR was conducted with $2^{-\Delta \Delta CT}$ method with the CT readings of CPC cells used as the calibrator.

The result for Sca-1 and CD34 gene expression are presented in figure 3.10. Sca-1 was expressed at a significantly higher fold value in PP1 and PP2 cells compared to their respective PC samples. It displayed a similar pattern of expression in CSkM and LM derived cells and sub-populations. CD34 was expressed at a significantly higher fold value by PP1 and PP2 cells and in both tissue types, compared to respective control. There were no significant differences between the two muscle groups.
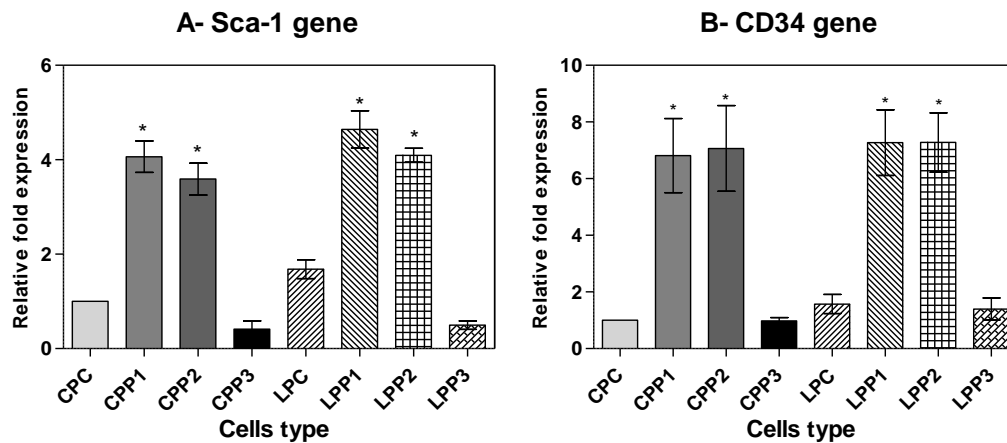
Figure 3.10 QPCR analysis of stem cellness markers
Figure shows fold values plotted against cell type for (A) Sca-1 and (B) CD34. The bars represent mean±1SD, N = isolation attempts = 3; n = replicates = 3. (A) PP1 and PP2 genes expressed significantly higher levels of Sca-1 compared to rest. (B) PP1 and PP2 genes expressed significantly higher levels of CD34 compared to the rest. *= p < 0.05, PC vs. PP-type.

The results of the myogenic genes MyoD and Desmin gene are shown in figure 3.11. The MyoD gene was expressed at significantly higher levels in PP3 cells of both isolates compared to PC or other sub-populations. The Desmin gene was similarly found expressed at significantly higher levels in PP3 cells compared to PC and other sub-populations. The PC cells expressed the gene at a relatively higher level than PP1 and PP2. No significance difference was found between the two muscle groups.
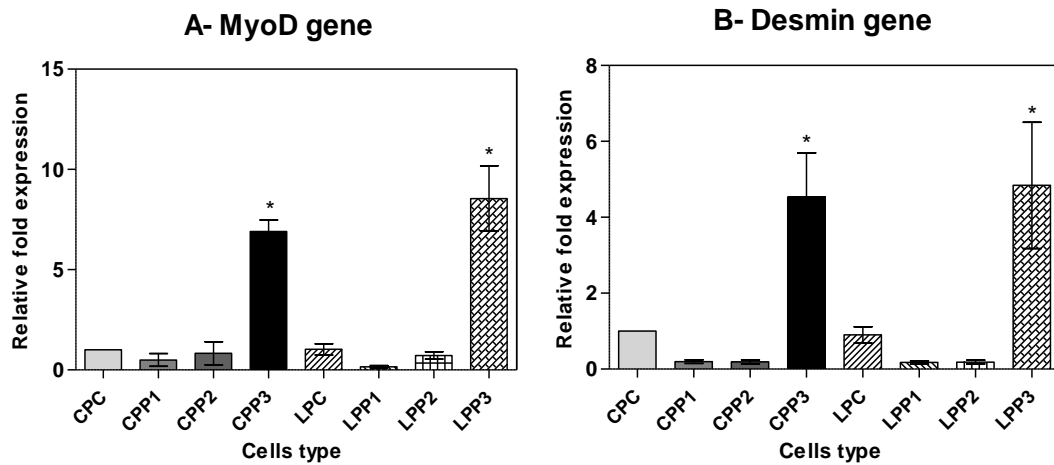
Figure 3.11 QPCR analysis of myogenic genes
Figure shows fold values plotted against cell type for (A) MyoD and (B) Desmin. The bars represent mean ±1SD, N = isolation attempts = 3; n = replicates = 3. (A) PP3 cells expressed the gene at significantly higher levels compared to rest. (B) PP3 cells expressed the gene at significantly higher levels compared to rest. There was no expression of the gene in the early adherent cells. * = p < 0.05, PC vs. PP-type.

The results for Runx2 and Alp are shown in figure 3.12. Runx2 was expressed at a significantly higher value in PP1 and PP2 from both muscle origins, compared to PC cells. The Alp gene was expressed significantly higher in PP1 and PP2 cells from both muscle isolates, compared to their respective PC samples. No significance difference was found when comparing CSkM cells with their equivalent isolates from LM tissue.
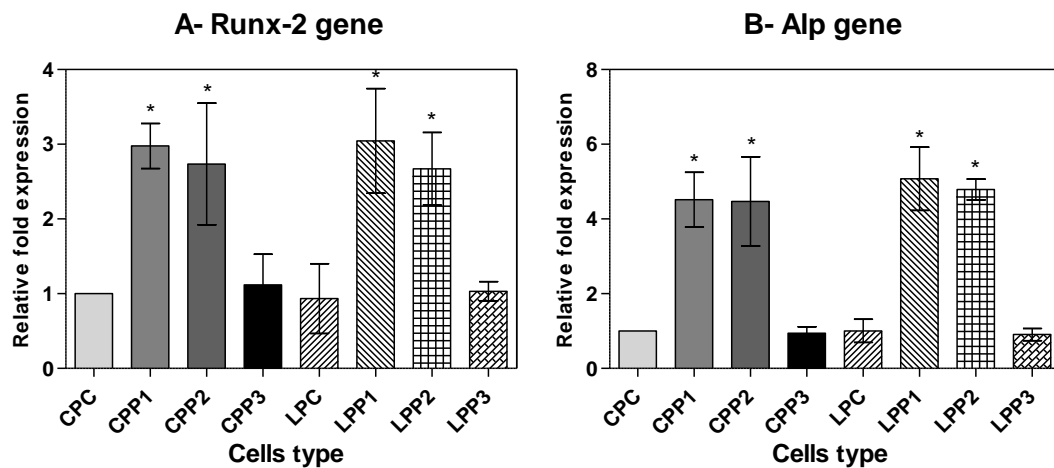
Figure 3.12 QPCR analysis of osteogenic genes
Figure shows fold values plotted against cell type for (A) Runx2 and (B) Alp. . The bars represent mean ±1SD, N = isolation attempts = 3; n = replicates = 3 (A) PP1 and PP2 expressed significantly higher levels of Runx2 compared to all other samples. Late adherent cells did not express the gene (B) PP1 and PP2 expressed significantly higher levels of Alp compared to rest. PP3 did not express the gene.* = p < 0.05, PC vs. PP-type.

The expression of AP-2 was found to be similar to P75 and Sox10. The results are presented in figure 3.13. AP-2 gene was expressed at significantly higher values by CSkM derived PP1 and PP2 cells only. The LPP1 and LPP2 displayed a difference with LPC but this was insignificant. The differences between CPP1 vs. LPP1 and CPP2 vs. LPP2 were found to be significant. The Sox10 gene was expressed at significantly higher values by CSkM derived PP1 and PP2 cells only. The differences between CPP1 vs. LPP1 and CPP2 vs. LPP2 were found to be significant ($p < 0.05$). The P75 gene was similarly expressed at significantly higher values by CSkM derived PP1 and PP2 cells only. The differences between CPP1 vs. LPP1 and CPP2 vs. LPP2 were found to be significant ($p < 0.05$).
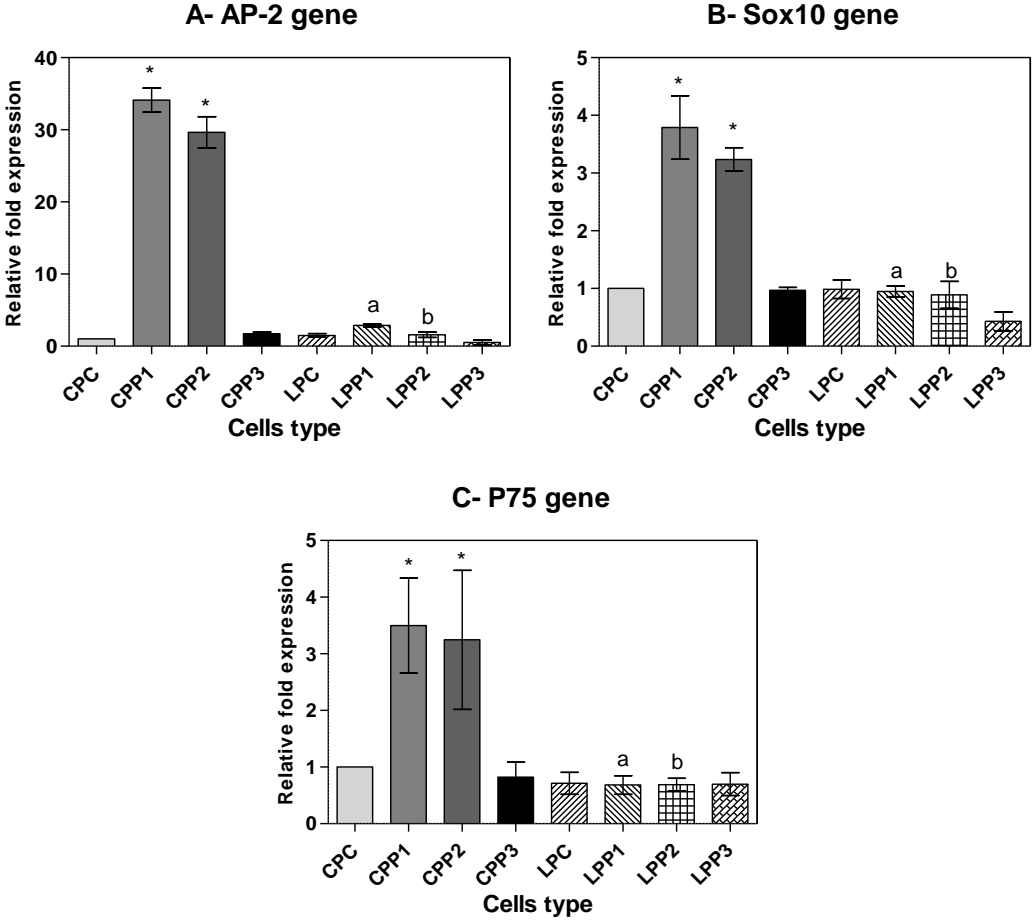
Figure 3.13 QPCR analysis of neural crest markers
Figure shows fold values plotted against cell type for (A) AP-2, (B) Sox10, and (C) P75. The bars represent mean ±1SD, N = isolation attempts = 3; n = replicates = 3 (A) The CSkM derived PP1 and PP2 expressed significantly higher levels of P75 compared to all samples. * (B) The CSkM derived PP1 and PP2 expressed significantly higher levels of Sox10 compared to all samples. (C)The CSkM derived PP1 and PP2 expressed the gene significantly higher levels compared to all samples. * = p < 0.05, PC vs. PP-type; a, b = p < 0.05, CPP1 vs. LPP1 and CPP2 vs. LPP2 respectively.

Table 3.4 summarises the gene expression profile of all isolated cells. It shows that early adhered cells from both muscle groups express stem cell (Sca-1 and CD34) and osteogenic (Runx-2 and Alp). The late adhered cells from both muscle groups only express the myogenic genes (MyoD and desmin) and to a lesser extent stem cell gene Sca-1. The early adhered cells from CSkM uniquely expressed the neural crest markers genes (Ap-2, P75 and Sox10).

Table 3.4 Summary of genes expressed by isolated cells

Cells	Sca-1	CD34	MyoD	Desmin	Runx-2	Alp	Ap-2	P75	Sox10
CPP1	+	+	-	-	+	+	+	+	+
CPP2	+	+	±	±	+	+	+	+	+
CPP3	±	-	+	+	-	-	-	-	-
LPP1	+	+	-	-	+	+	-	-	-
LPP2	+	+	±	±	+	+	-	-	-
LPP3	±	-	+	+	-	-	-	-	-

3.4. Discussion

3.4.1. Isolation of mononuclear cells from muscle tissue

A modification of the preplate method developed by Yaffe (1968), Rando and Blau (1994), Qu *et al.,* (1998) was applied here to isolate mononuclear cells from muscle tissue. The modifications included (i) the use of non-coated tissue culture plastic dishes for cell attachment in contrast to collagen coated substrates as used by others and (ii) alternate contact durations of 1 h, 48 h and 5 d compared to the day-wise pre-plating applied by Rando and Blau 1994 and Qu *et al.,* (1998). In addition, comparisons between the CSkM and LM derived mononuclear isolates have been conducted to a relatively lesser extent. As the developmental origins between these tissues differ, a requirement of a comparison between pre-plate isolates from both anatomical locations was deemed necessary. In this part of the study, mononuclear cells were successfully isolated from the two anatomically different skeletal muscle tissues. The isolated cells displayed differences in the various parameters assessed here.

3.4.2. Proliferative assessment

A comparison of the replicative potential of the cells indicated differences between the early and late adhered cells. The replicative potential of early adherent PP1 and PP2 sub-populations was significantly higher than the late adherent PP3 cells. The later adherent cells displayed a relative lag in proliferation in the initial period of culture, extending from 1 to 9 w.

This initial finding is suggestive of a difference in the isolated cells phenotypic capacities. Replication in cells is phenotype dependent; terminally differentiated cells tend to be post-mitotic whilst those that is of a less committed nature display a relatively greater capacity to multiply. This is in contrast to cancerous cells, which may be highly

replicative but whose aberrant phenotype would disqualify it from the context of normal developmental processes.

The comparative proliferation of the early adherent sub-populations compared to the slow proliferative rate displayed by later adherent PP3 substantiate, within limits, the phenotypic characteristics of the differentially plated cells. Accordingly, the replicative nature of the early adherent sub-populations could be indicative of their multipotentiality compared to the restricted nature of proliferation in PP3, which reflects it's somewhat committed, if not differentiated, nature. In agreement with Pavlath *et al.,* (1998) early adhered cells from CSkM showed slower growth rate compared to their equivalent cells from LM during the period from 3 to 9 weeks in culture. This different may be due to the developmental differences documented between those two muscle sites.

3.4.3. Morphological assessment

The differences between the sub-populations were further evaluated by examining morphology and comparing mean surface area of cells attached to tissue culture plastic. This analysis also showed differences between the isolates, with the early adherent PP1 and PP2 cells seemingly fibroblast like with a significantly larger surface area than the late adherent PP3, which appeared rounded in shape and of a very small surface area when attached to plastic.

The methodology applied to assess cellular morphological characteristics could have been elaborated to incorporate additional parameters, which in turn may have added contrast to the initial findings presented here. A method that may have served well to this purpose would have been flow cytometry, as it would have allowed the three

dimensional view of the cell; hence providing an assessment unadulterated by physical topography of tissue culture plastic. Additionally, flow cytometry would have provided a view of cellular granularity; an important factor that may reflect cytoplasmic differences. Such an assessment may have elaborated the morphological differences to a further extent.

However, the relatively larger fibroblastic morphology of the early adherent cells renders them comparable to multipotent cells isolated from other body segments, such as the bone and adipose tissue derived mesenchymal stem cell. In a similar manner, the restricted replicative potential and smaller size of the PP3 cells emphasises a difference in cellular function, if not multipotential abilities, of the sub-populations.

3.4.4. Muscle-specific desmin expression

This experiment further elaborated the phenotypic difference between the isolates. The expression of desmin in early adherent PP1 and PP2 was negligible. Though these cells were found to express the protein, the levels were several folds in magnitude lesser than the late adherent PP3, which were strongly desmin positive. This experimental finding strongly emphasises the phenotypic differences between cells by suggesting that the later adherent PP3 cells may be committed or differentiating myogenic precursors. This experimental finding is particularly important as it sums the preceding results by indicating that the phenotypic difference observed between the pre-plates.

3.4.5. Gene expression analysis with PCR

The phenotypes of isolated cells from an early passage were assessed initially with end-point PCR. The aim of this experimental approach was to validate the presence or

absence of certain genes that represent four possible facets of a stem-like cell, i.e. multipotentiality, myogenic, osteogenic capacities, and neural crest origin.

The first group of genes indicated that Oct4, a gene normally assigned to embryological pluripotency, was not expressed in these isolates. Sca-1, which is reported to be associated with several stem like cells in mice, was detected in all samples but its expression was relatively higher in the early adherent cells than late. Interestingly it was paralleled by CD34, yet another gene associated with multipotent cells from mice, but in the early adherent cells only. These suggest the multipotent nature of cells, compared to a pluripotent one that may be easily perceived due to the neonatal source of the mice; as well as that the early adherent cells may be more 'plastic' than the late adherent cells. This notion was supported by the findings of the myogenic and osteogenic genes. In brief, the early adherent cells displayed expression of osteogenic genes but lack myogenic gene expression. The converse was true for the late adherent PP3 cells, which expressed myogenic genes but lacked osteogenic genes. These findings indicate the early adherent cells were likely multipotent that expressed transcripts of genes, the lack of expression of these genes in the late adherent cells, complemented by the strong expression of myogenic genes, is highly indicative that PP3 were committed and/or differentiating myoprecursor cells.

The assessment of neural crest genes was conducted to determine if the different development origin of these muscles contributed to an altered phenotype, and also to attempt to distinguish the origin of early adherent cells between the muscle and connective tissues. This analysis indicated the expression of neural crest related markers in early adherent CSkM cells but not the remaining samples. The importance of this result is twofold; it clearly indicates the developmental and hence physiological

difference between muscle from these two anatomical regions, and it also shows that the early adherent cells may have a connective tissue origin. Though, comparable markers for connective tissue of the limb muscle region, or general connective tissue markers that may overlap both anatomical regions were not evaluated the positive expression of osteogenic with lack of myogenic gene expression support the notion of their neural crest derived connective tissue origin.

3.4.6. Significance of findings

The different pre-plate isolated cells displayed differences in their phenotype, which may possibly extend to their differentiation potentials. The early adherent PP1 and PP2 were found to be large, fibroblastic cells with a strong replicative potential that lacked desmin expression. These expressed the stem cell markers Sca-1 and CD34 at a higher level with Runx2 and Alp gene transcripts; and displayed a lack of myogenic gene expression. The findings clearly indicate that the early adherent cells may not have been committed muscle precursor-like cells. Instead, the proliferative and morphological assessments suggest that these were multipotential possibly capable of differentiating to osteogenic, adipogenic or chondrogenic cells.

The late adherent PP3, in contrast, displayed characteristics that suggest it may be a muscle-precursor or of least, a mononuclear cell committed to the myogenic lineage. This is deduced from their relative shape and size compared to the early adherent cells, but more so from the observation of desmin positive cells in this sub-population. The desmin molecule is a muscle specific cytoskeletal protein and can only be detected in cells that are committed myogenic precursor cells. The relatively reduced expression of Sca-1 and CD34 transcripts with the lack of expression of osteogenic genes but strong expression of myogenic MyoD and Desmin clearly support its suggested identity

as a committed myoprecursor cell (Rando and Blau, 1994; Lee *et al.,* 2000; Jankowski *et al.,* 2001; Qu-Petersen *et al.,* 2002; Deasy *et al.,* 2005; Rouger *et al.,* 2007).

A finding of interest is the expression of neural crest markers in the early adherent cells. It is shown here that cells originating from the neural crest retain the expression of certain genes within cells isolated from CSkM, which in turn implies that the origin of the early adherent cells may have been of a connective tissue type.

3.4.7. Conclusion

Based on the observations from the experiments conducted in this section, it is indicated that mononuclear cells can be isolated from muscle tissue. These cells, when separated based on duration of contact with tissue culture plastic, can be divided into at least three different sub-populations. The sub-populations that adhered very early to culture dishes were similar; but not exactly alike. These collectively differed from the later adherent PP3 sub-population in proliferative capacities, morphology and the expression of desmin. The differences were shown to extend to the expression of stem cellness, myogenic and osteogenic genes. The differences between the two muscle groups were evident in the expression of neural crest markers in early adherent cells of the CSkM, indicating their difference in developmental origin, and hence physiological properties with LM; which also shows that early adherent cells may have a connective tissue origin and the late adherent cells seem to be myogenic precursors. These differences were also seen as proliferative differences between the early adherent cells of the two muscle types at 3 to 9 wk.

.

3.5.   Summary of the results

- Mononuclear cells can be isolated from muscle tissue.

- The initially isolated suspension can be fractioned into sub-populations based on the factor of 'adherence per time'.

- Proliferative potential of early adherent cells is higher than the late adherent cells, which display an extended lag.

- There are some differences in growth behaviour between early adhered cells together with parent population isolated from CSkM and their equivalent cells isolated from LM from week 3 to week 9.

- There were no differences between late adhered cells from both muscle groups.

- Morphology and cell-surface area differ between the pre-plates, with early adherent cells appearing fibroblastic and larger in surface area than later adherent cells.

- Early adherent cells display minute levels of desmin expression but the later adherent cells display a strong desmin expression.

- Early adherent cells strongly express stem cell and osteogenic genes.

- Later adherent cells strongly express myogenic genes.

- Early adherent cells from CSkM, but not LM, express neural crest markers.

Chapter 4: Examination of the myogenic potential of muscle derived mononuclear cells

4.1. Introduction

4.1.1. Background

Skeletal muscle is an organ that has the ability to regenerate following trauma or physical activity. Myogenic differentiation is the default developmental pathway of skeletal muscle precursor cells, which enables the repair of damaged tissues, as well as adaptation to physical changes.

The process of myogenesis is orchestrated through a series of transcription factors controlled by the myogenic regulatory factors (MRFs). MyoD, a basic helix–loop–helix (bHLH) transcription factor is the founding member of the MRF family, which also includes the closely related Myf5, myogenin (Myog), and MRF4 proteins (Buckingham 2001). The activity of these factors leads to phenotypic change, characterised in part by the expression of muscle-specific desmin protein. Myogenic differentiation progresses through irreversible cell cycle arrest of muscle precursor cells (MPCs) that display a gradual increase in the expression of muscle specific genes, which in turn lead to the fusion of myoblasts into multinucleate myofibres. This conversion process can be recapitulated *in vitro* with high efficiency in well-established models.

Based upon the different adherence properties of cells obtained from freshly dissociated muscle, pre-plating has been used to obtain myogenic populations of cells that have slow adhesion characteristics (Rando and Blau, 1994). These cells have been shown to possess greater and promising efficiency to regenerate dystrophin-positive myofibres when injected intramuscularly in mice models (Jankowski *et al.,* 2002; Qu-Petersen *et al.,* 2002).

4.1.2. Rationale of study

As shown in the previous sections, the isolated cells from both muscles and their sub-populations may have different myogenic differentiation abilities. This is strongly evident in the differential expression of cytoplasmic desmin in PP3 cells compared to PC or PP1 or PP2 from either muscle type. Furthermore, it was shown that PP1 and PP2 express various developmental genes except for MyoD and Desmin. Incidentally, these genes are expressed in PP3 cells, which simultaneously show little or no expression of the remaining developmental markers. These findings strongly imply a difference in the potentials of the various cell isolates to differentiate along the myogenic lineage.

4.1.3. Aims and objectives

The aim of the present section of the thesis was to assess and compare the potential of CSkM and LM cells to undergo myogenic differentiation. Based on the observation presented in their preliminary characterisation, it is hypothesised that the late adherent PP3 sub-population of muscle derived mononuclear cells is relatively more myogenic than the early adherent fractions. The objectives were to induce myogenic differentiation in the different cell types to (i) conduct a light microscopic comparison for myotube formation; (ii) assess myotube formation and multi-nucleation by examining the expression of muscle-specific desmin at 14 d culture in differentiation medium; and additionally (iii) examine the expression of myogenic genes (MyoD, Desmin, Myog) in cultures at different time points over the course of 21 d.

4.2.   Experimental protocol

4.2.1. Cell culture

Mononuclear cells were isolated from CSkM and LM as described in sections 2.1 and 2.2. The stock suspensions were fractionated based on adherence to plastic per time to form the sub-populations PP1, PP2 and PP3 as described in section 2.2.2.

4.2.2. Myogenic differentiation

The differentiation experiments were carried out in 24-well plates as described in section 2.5.1. Cells were seeded at an initial seeding density of 25 x 10^3 cells / cm^2 in GM. At confluence, GM would be replaced by myogenic medium (MM). Myogenic differentiation was achieved by incubating a confluent monolayer of cells with MM. This medium comprised high glucose DMEM supplemented with 2 % FBS, 1 % antibiotics and 10 ng / ml IGF-1 (Sigma). Culture medium was replaced every three days.

4.2.3. Assessment of myogenic differentiation

4.2.3.1.   Bright field light microscopic evaluation for myotube formation

This was performed to visualise myotube formations in confluent CSkM and LM cells cultured in MM for 14 d. For this purpose, cultures were viewed at x40 magnification under bright field of a phase contrast light microscope. Images were taken as described in section 2.3.

4.2.3.2. Immunocytochemical examination of myotube formation using muscle-specific desmin protein expression

This was performed similar to the description in section 3.2.4 with a detailed methodology provided in section 2.4. Cells were grown in GM till confluence on sterile glass coverslips, which were immobilised in 24 well plates. GM was then replaced with MM. After 14 d in MM, cells were first fixed with 4 % paraformaldehyde and then stained for desmin with a mouse monoclonal anti-mouse desmin antibody (Isotype: IgG1, Abcam, UK). Cells were then incubated with secondary antibody (anti-mouse IgG1 class specific FITC (Abcam, UK) for 30 minutes at room temperature. The nuclei were stained with the fluorescent DNA probe DAPI (Invitrogen, U.K).

4.2.3.3. QPCR analysis for muscle-specific MyoD, Desmin and Myog genes

The expression of myogenic genes MyoD, Desmin and Myog were examined in cultures of confluent cells in MM over the course of 21 d. The specific time points examined were 1, 7, 14 and 21 d. The methods used for this experiment are described in section 2.8. Sample cultures comprising confluent monolayers were setup in GM and MM in 24-well plates. The two media were evaluated due to their differential effects on cell growth; as the GM supports replication but may not have the necessary differentiation queues of MM. At the required time, monolayers were washed x3 with PBS and lysed with buffer RLT. RNA was processed as described in section 2.8.1. Reverse transcription was performed as in section 2.8.3.2. The qPCR reactions were carried out as described in section 2.8.6. The table 4.1 shows the details of the genes and qPCR probes used.

Table 4.1 QPCR Gene probes for myogenic differentiation

Gene	Taqman Probe	Function
MyoD	Mm00440387_m1	Master transcription factor
Desmin	Mm00802455_m1	Muscle-specific cytoskeletal protein
Myog	Mm00446194_m1	Muscle-specific transcription factor

The CT values were normalised to the GAPDH gene, and resulting ΔCT values calibrated to CPC 1 d in GM.

4.3. Results

4.3.1. Bright field light microscopic evaluation for myotube formation

The confluent cultures were observed at 14 d with bright phase light microscopy for the formation of myotubes. The results of this experiment are shown in figures 4.1 for PP1 and 4.2 for PP3 from both muscle types. PP3 showed the highest ability for myotube formation. Moreover, cells in PP1 showed the least myotube formation.
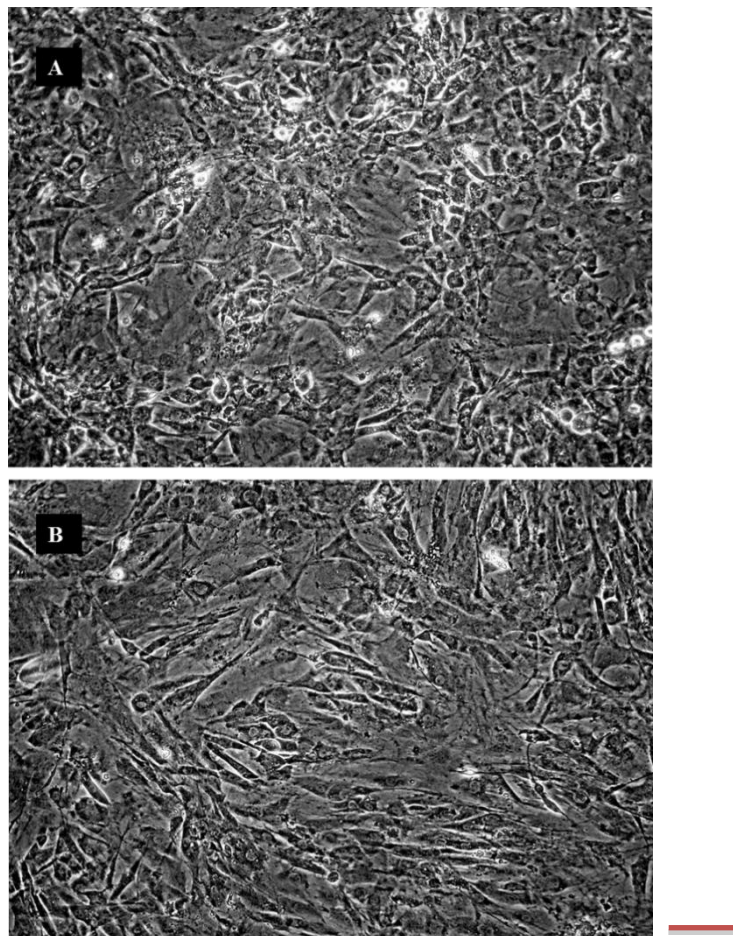
Figure 4.1 Observation for myotube formation in PP1 cells.
Images are x40 objective views of (A) CSkM and (B) LM derived PP1 cells 14 d in MM. These indicate a lack of myotube formations. Bar equal 100 μm.
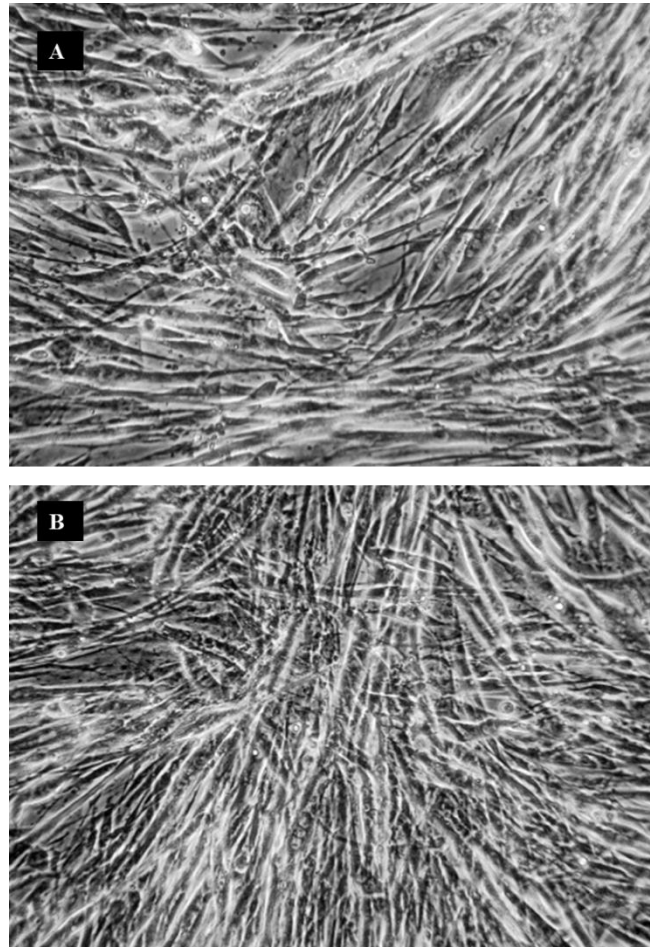
Figure 4.2 Observation for myotube formation in PP3 cells.
Images are x40 objective views of (A) CSkM and (B) LM derived PP3 cells 14 d in MM. They clearly show the formation of fibre-like myotubes. Bar equal 100μm.

4.3.1.1. Immunocytochemical examination of myotube formation using muscle-specific desmin protein expression

This analysis was used to seek the expression of desmin (fluorescent green) against nuclei (DAPI blue) in CSkM and LM derived cells cultured in MM for 14 d. The results of this experiment are presented in figure 4.3 and 4.4 for the early and late adherent sub-populations respectively.

The analysis revealed early adherent PP1 sub-population did not express any desmin by this time. The PP2 cells from both muscle types expressed very small quantity of desmin positive cells, as shown in figure 4.4. The late adherent PP3 cells from both muscle types displayed strong desmin expression. In addition, these cells isolated from both tissue types displayed multi-nucleation, as shown in the figure 4.4
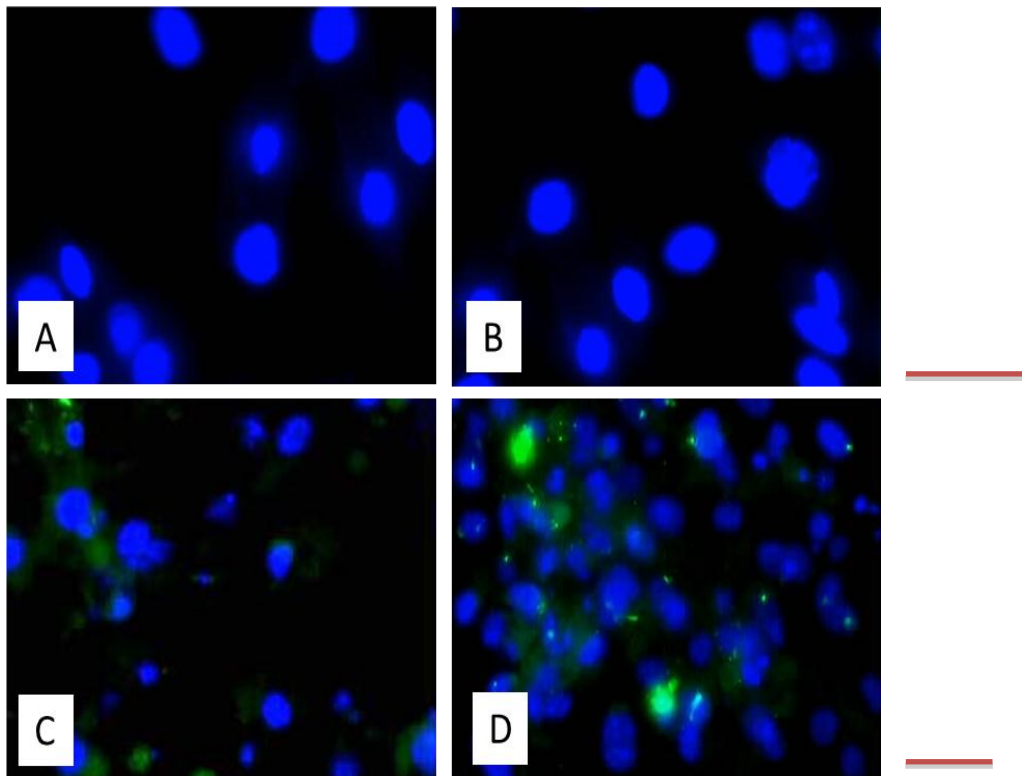
Figure.4.3 Desmin immunocytochemistry staining of early adherent cells
Images are views at objective x63 for (A) CSkM PP1 and (B) LM PP1; x40 for (C) CSkM PP2 and (D) LM PP2. The PP1 sub-population (A) and (B) did not express any desmin protein. The PP2 sub-population from (C) CSkM and (D) LM showed few desmin positive cells at 14 d in MM. Multi-nuclei due to myotube formation were not observed in any of these cells. Bar equal 100 μm.
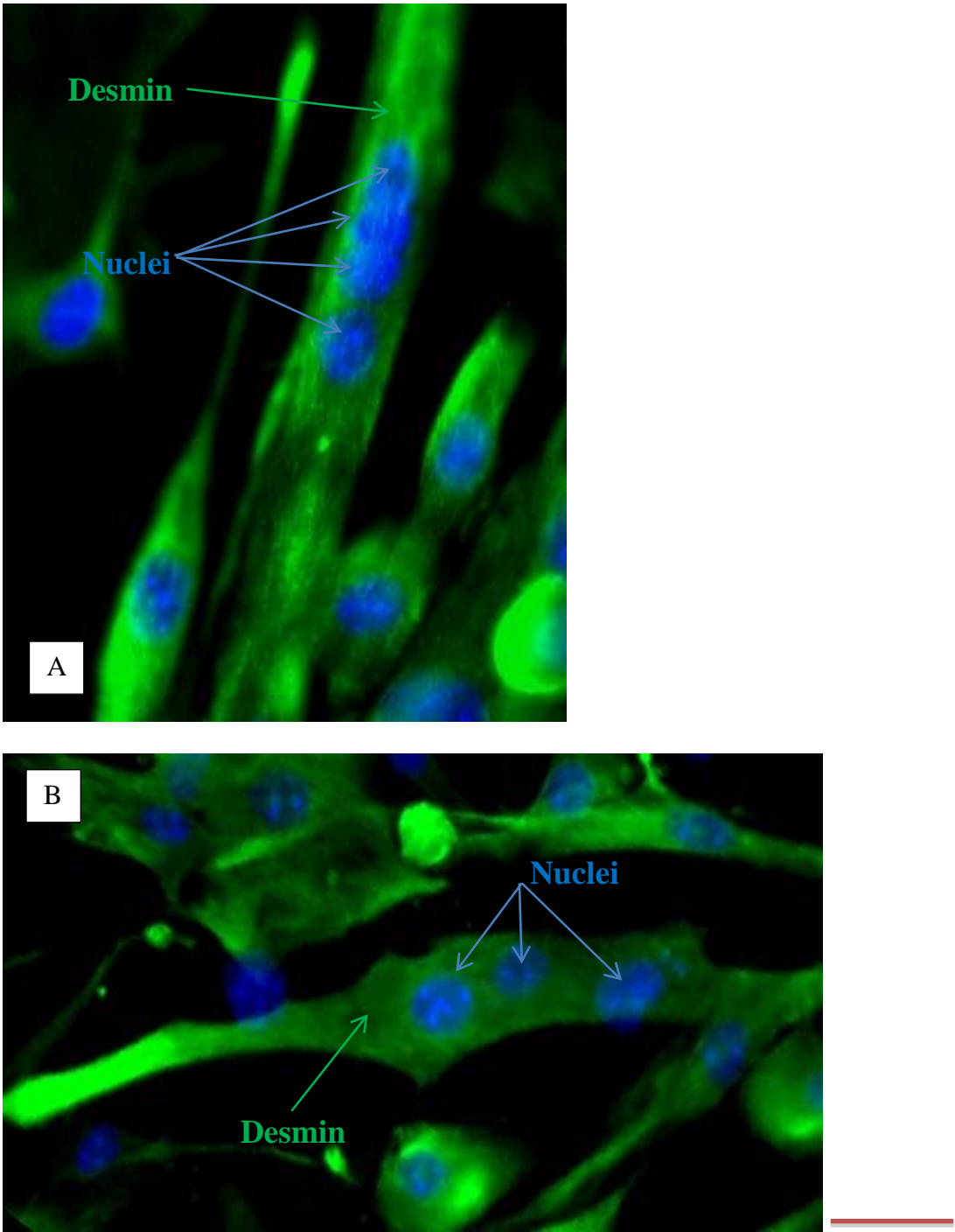
Figure 4.4 Desmin immunocytochemistry staining of late adherent cells
Images are confocal microscopic views at objective x63 of late adherent cells from (A) LM and (B) CSkM after 14 d in MM. The two PP3 populations displayed strong expression of desmin with multi-nuclei apparent. Bar equal 100 μm.

4.3.2. QPCR analysis for muscle-specific MyoD, Desmin and Myog genes

The muscle tissue specific genes MyoD, Desmin and Myog were examined in CSkM and LM derived cells cultured in GM and MM for 1, 7, 14 and 21 d. The results of this experiment are shown in figures 4.5, 4.6 and 4.7 for MyoD, Desmin and Myog, respectively.

The MyoD gene was detected in all samples at 1 d. In GM, the PC and PP3 cells from both muscle types displayed a progressive increase in the expression of this gene over the course of culture. The gene was found expressed significantly higher by PP3 than PC at all-time points analysed. The PP1 cells did not exhibit any change in expression over the course of culture. The PP2, however, did seem to increase expression of the gene by 14 d and 21 d.

In MM, MyoD expression was detected in all samples at 1 d but was significantly higher in PP3 than the rest. Its expression increased by 7 d in all samples except the PP1 fractions. The PP3 cells significantly increased expression of MyoD by 14 d compared to the other cell types. At 21 d, expression of the gene had increased in all samples. It was expressed at a higher fold value by PP3 followed by PP2; while PC and PP1 shared a small difference at a much lower fold. No significant difference was found when comparing cells isolated from CSkM and their equivalent cells from LM.

The results indicate the differential effects of media in the form of differences in the magnitude of relative fold values, which indicate MM caused a significantly higher upregulation of MyoD expression than GM. No significant difference between cells from the two muscle groups.

A- MyoD gene CSkM GM
B- MyoD gene LM GM
C- MyoD gene CSkM MM
D- MyoD gene LM MM
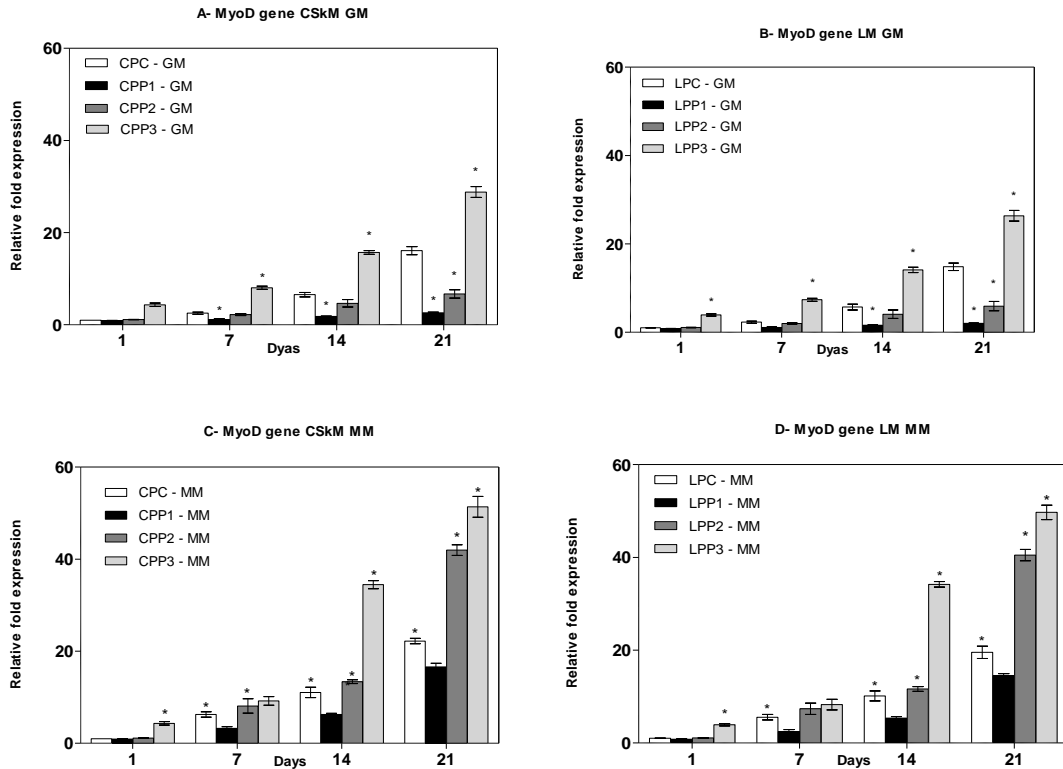
Figure 4.5 QPCR analysis for myogenic MyoD expression
Figure shows the relative fold values of expression of MyoD in CSkM and LM cells
Specimen were cultured in 24-well plates in GM or MM. (A and B) In GM, PP3 of both muscle types express significantly higher levels of MyoD followed by PC and then PP2, while PP1 do not express the gene. (C and D) In MM, expression of the gene increased in all samples through the course of culture. It was upregulated to a high level earlier by PP3 at 14 d. At 21 d, expression in PP3 and PP2 was many folds higher than PC and PP1. The expression of the genes was found stronger in MM compared to GM. Bars indicate mean±1SD, N = 3, n = 3. * = p < 0.05, PC vs. PP-type. No significant difference between cells from the two muscle groups.

116

The desmin gene was found to be almost-selectively expressed at significantly high relative fold values by PP3 from both muscle types and in both media. The remaining samples upregulated Desmin expression in MM; but this was very small compared to PP3. In MM, the PP2 cells displayed an upregulation at 14 d. This increased slightly in value along with PC cells by 21 d. The PP1 did not seem to have significant increase in the expression of desmin through the course of culture in either media. There were small but significant differences between PC and PP2 from CSkM and their equivalent from LM tissues.
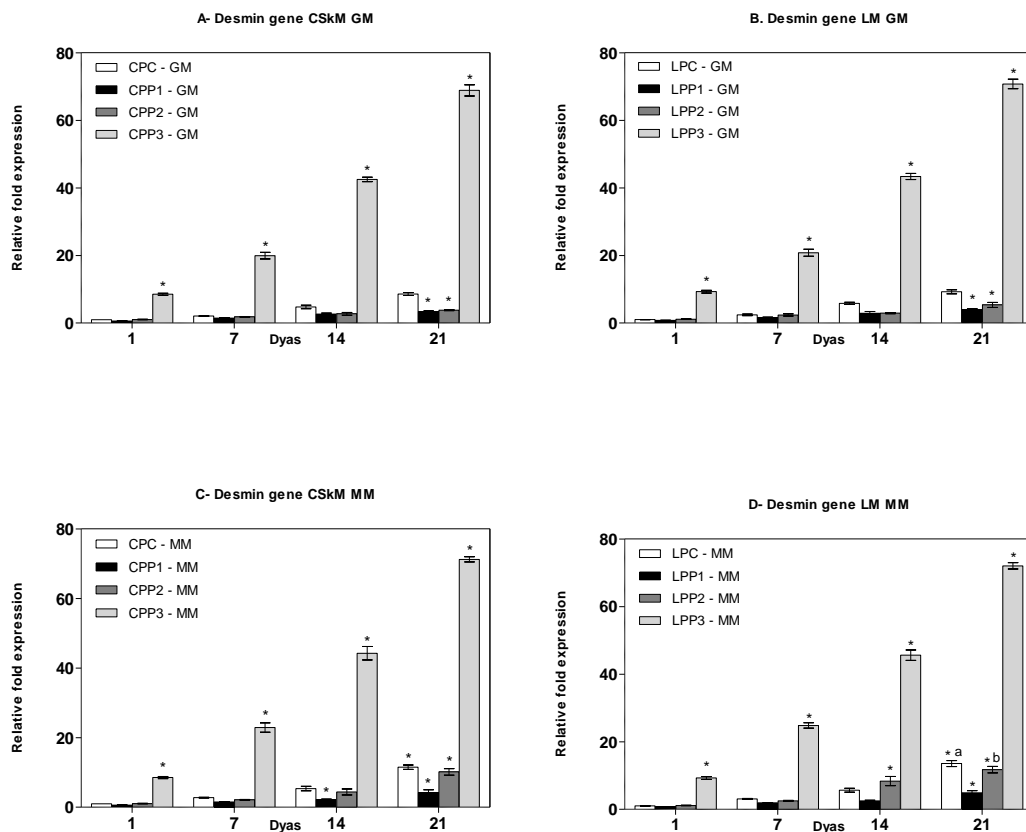
Figure 4.6 QPCR analysis for myogenic Desmin expression
Figure shows the relative fold values of expression of Desmin in CSkM and LM derived cells. Specimen were cultured in 24-well plates in GM or MM. (A and B) In GM, PP3 of both muscle types express significantly higher levels of Desmin. PC cells display a small fold upregulation of the gene by 21 d. (C and D) In MM, expression of the gene increased in all samples through the course of culture. It was upregulated relatively faster and to a high level earlier by PP3 from both muscle types. At 21 d, PP3 was many folds higher than PC, PP1 and PP2. PC and PP2 displayed a steady upregulation of Desmin but PP1 did not display any stark upregulation of the gene. The expression of the genes was found stronger in MM compared to GM. Bars indicate mean$\pm$1SD, N = 3, n = 3. * = p < 0.05, PC vs. PP-type. ^{a,b}p<0.05, CPC vs. LPC and CPP2 vs. LPP2.

The Myog gene displayed a similar pattern of expression to Desmin. It was detected at very low levels in all samples at 1 d. The PP3 cells from both muscles significantly expressed higher levels of the gene at all time points in the two media conditions. The other cells displayed a steady increase in Myog expression but were many fold lesser than PP3. The PC cells and PP2 cells expressed higher levels of the gene at 21 d compared to PP1, which displayed a comparative delay in upregulating the gene. A comparison between the media types indicates the expression of the gene to be highly increased by MM than GM. No significant difference between cells from both muscle groups.
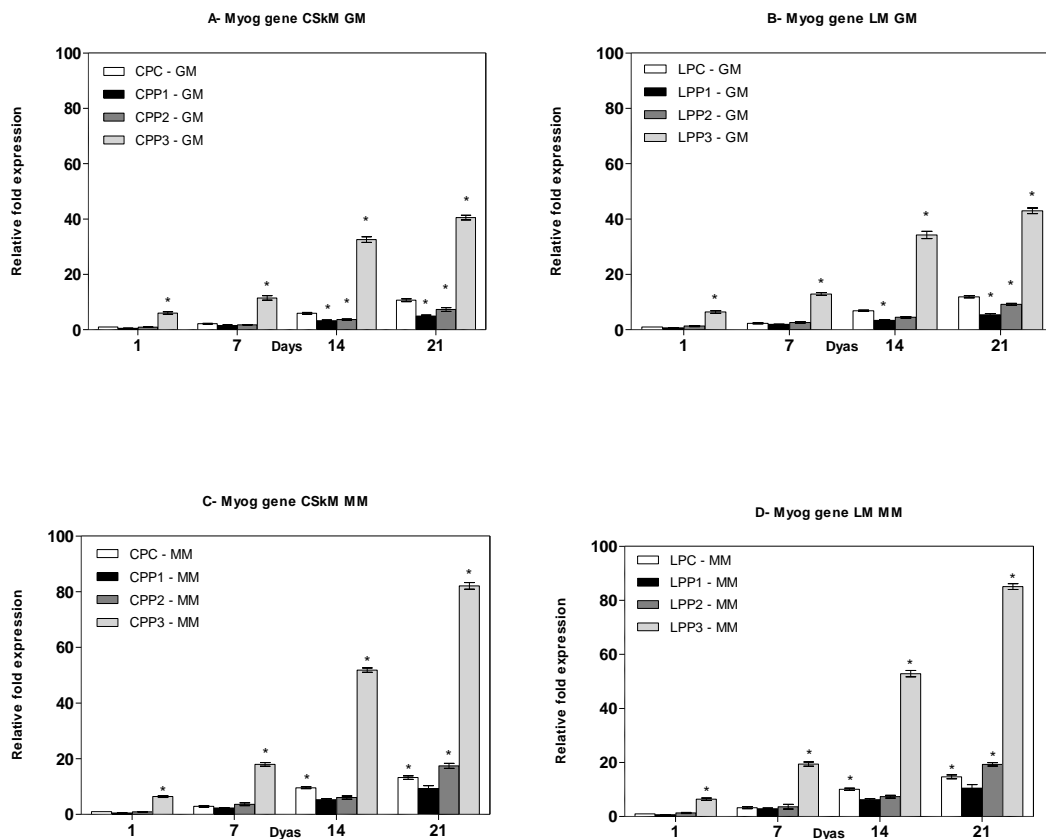
Figure 4.7 QPCR analysis for myogenic Myog expression
Figure shows the relative fold values of expression of Myog in CSkM and LM derived cells. Specimen were cultured in 24-well plates in GM or MM. (A and B) In GM, PP3 of both muscle types express significantly higher levels of Myog. PC and PP2 cells display a small fold upregulation of the gene by 21 d. PP1 display a delay in Myog upregulation. (C and D) In MM, expression of the gene increased in all samples through the course of culture. It was upregulated relatively faster and to a higher level earlier by PP3 from both muscle types. At 21 d, PP3 was many folds higher than PC, PP1 and PP2. PC and PP2 displayed a steady upregulation of Myog but PP1 displayed an obvious delay in upregulation of the gene. The expression of the genes was found stronger in MM compared to GM. Bars indicate mean±1SD, N = 3, n = 3. * = p < 0.05, PC vs. PP-type. No significant difference between cells from both muscle groups.

4.4. Discussion

4.4.1. Evaluation of myotube formations

This experiment showed the early adherent cells were not capable of forming myotubes, while the PP3 sub-populations from both muscle groups displayed positive myotube formation. The initial findings made with bright field microscopy were supported by immunocytochemical stains of fixed 14 d cultures of cells in MM, which clearly show the presence of multinuclear myotubes in late adherent cells. The comparison of the myogenic ability of the late adherent cells suggests these may be committed myoprecursor cells.

4.4.2. QPCR analysis of myogenic genes

The analysis of genetic change of MyoD, Desmin and Myog were evaluated over the course of 21 d. It positively supported the initial findings by indicating the late adherent PP3 to strongly express myogenic genes compared to other specimen (Rando and Blau, 1994; Lee *et al.,* 2000; Jankowski *et al.,* 2001; Qu-Petersen *et al.,* 2002; Deasy *et al.,* 2005). MyoD gene is essential for the acquisition of myogenic identity it also involved in the transcriptional activation of other muscle specific genes (Buckingham, 2001). Desmin was used because of its characteristic appearance in myoblasts as muscle-specific marker gene and its common use to detect cells with myogenic potential. The increased expression of desmin shows the myogenic potential of the late adhered cells. On the other hand Myog promotes conversion of myoblasts to myotubes and is expressed exclusively in myotubes (Blais *et al.,* 2005); therefore it is also used as a late marker of muscle differentiation.

The early adherent cell type PP2 displayed a small upregulation of myogenic genes towards the end of the experiment; but this significantly small in value. The expression

119

of stem cell genes by these cells may suggest their ability to differentiate towards myogenic differentiation. It might be also as a result of possible contamination of culture with some muscle committed cells.

4.4.3. Significance of findings

The results suggest that the late adherent cells isolated on plastic using differential adherence method from two different muscles are highly myogenic. The early adherent cells do display some myogenic tendencies but that is restricted to the PP2 sub-population, while the PP1 is apparently have very limited tendency. The observed differences in cell behaviour can be ascribed to the different origin of cells suggested by the positive expression of neural crest related markers in the early adherent cells of the CSkM suggesting a connective tissue origin of the early adherent cells. Though an overlapping set of genes representing connective tissue origins of both muscle groups may have been beneficial, the relative lack of any myogenic potential in the early adherent cells supports the notion of their non-myogenic nature.

The ability of the PP3 cells to differentiate is seemingly high. These cells were shown in the previous section to express myogenic genes and in particular, to express desmin gene right at the onset of isolation. The experimental findings in this section support the previous suggestion that these may represent committed and/or early differentiated muscle precursor cells (Lee *et al.,* 2000; Qu-Petersen *et al.,* 2002; Rouger *et al.,* 2007).

There were no differences observed between cells isolated from CSkM and their equivalent cells isolated from LM in term of myogenic differentiation abilities.

4.4.4. Conclusion

Based on the previous results; the late adhered cells from both muscle groups appeared to be more myogenic as evident by the formation of myotubes and the increased myogenic gene expression compared to late adhered cells. There were no significant differences between cells isolated from CSkM and their equivalent cells from LM in term of myogenic differentiation ability.

4.5. Summary of the results

- The later adherent PP3 sub-population of muscle derived mononuclear cells can form myotubes in myogenic medium. The early adherent PP1 and PP2 lack the ability to form myotubes.

- The myotubes formed by late adherent cells were multi-nucleated with a high intensity of desmin expression.

- The myogenic gene expression profile conducted on cells cultured in MM for up to 21 d showed the PP3 were expressing higher levels of myogenic genes at all times and both media conditions.

Chapter 5: Examination of the osteogenic potential of CSkM and LM muscle derived mononuclear cells

5.1. Introduction

5.1.1. Background

In addition to the differences in the embryonic origin and myogenic development, osteogenic development in the craniofacial region is different from that in long bones. Bone in CSkM develops through intramembranous ossification mediated by cranial neural crest cells, whereas long bone in limb region develops through the endochondral ossification.

Despite the fact that bone marrow stem cells have been generally recognised as the main source of osteoprogenitor cell, evidence exists that in a number of pathological conditions ectopic bone formation occurs in sites other than the anatomically correct ones (Smith and Triffitt, 1986; Sawyer *et al*., 1991). Heterotopic ossification, myositis ossificans and more aggressive diseases such as fibrodysplasia ossificans progressiva and musculoskeletal neoplasms exhibit unwanted bone formation in skeletal muscle (Kaplan and Smith, 1997, Mahboubi *et al*., 2001, Aho *et al*., 1988, Aro *et al*., 1991).

5.1.2. Rationale of study

Over the previous chapters, it has been shown that based on differential adherence to plastic, at-least three sub-populations of cells can be separated from a muscle tissue derived mononuclear cell isolates that have distinct properties. These are the PC, and sub-population PP1, PP2 and PP3 designated sub-populations of cells under study here. In experiments conducted, the early adherent PP1 and PP2 sub-populations displayed high proliferative capacities and fibroblastic morphologies compared to the less proliferative, rounded, late adherent PP3 cells. The early adherent cells were mainly desmin negative as opposed to PP3 and displayed a higher expression of stem cell markers and osteogenic gene transcripts. The late adherent population exhibited

myogenic characteristics, which were shown in the section 4 to be due to a relatively higher capability of myogenic gene expression and the formation of multi-nucleated myotubes compared to the early PP1 and PP2. These observations indicate the relative myogenic nature of PP3 cells; but also indicate a possible mesenchymal multipotentiality of the early adherent populations, particularly in regard to the higher expression of Runx2 and Alp transcripts found in chapter 3.

5.1.3. Aims and objectives

The aim of this section of thesis was to evaluate and compare the potential of isolated mononuclear cells from CSkM and LM to undergo osteogenic differentiation *in vitro*. This was assessed by culturing cells with osteoinductive medium and analyse (i) the genetic expression of osteogenic markers Runx 2, Alp and Bsp; (ii) compare the expression of alkaline phosphatase protein; and (iii) assess matrix calcification. The experiments were carried out in 24-well tissue culture plates and analyses conducted in the later phase of a 28 d culture period that parallels the process of osteogenic differentiation of a cell.

5.2.   Experimental protocols

5.2.1. Cell culture

Mononuclear cells had been isolated from CSkM and LM as described in sections 2.1 and 2.2. The stock suspensions were fractionated based on adherence to plastic per time to form the sub-populations PP1, PP2 and PP3 as described in section 2.2.2.

Mouse calavarial osteoblasts (MOB) were used as a positive control for osteogenic differentiation. These were isolated from calavarial bone of sacrificed mice that had been used for the extraction of muscle specimen. The osteoblasts were cultured as described in section 2.5.2. These were cultured in GM with ascorbic acid at a concentration of 50 µM (Sigma).

5.2.2.   Osteogenic differentiation

The differentiation experiments were carried out in 24-well plates as described in section 2.5. Cells of an early passage were seeded at an initial seeding density of 25 x 10^3 cells / cm^2 in GM. At confluence, GM would be replaced by osteogenic medium (OM). It comprised low glucose DMEM supplemented with 10 % FBS, 50 µM L-ascorbate 2-phosphate (Fluka), 8 mM β-Glycerophosphate (Fischer) and 100 ng / ml BMP-2 (R&D systems).

5.2.3. Assessment of osteogenic differentiation

This was carried out at various times during a 28 d culture period in cells cultured in OM. The experiments were conducted in triplicate on each of the three occasions of cell isolation and were performed on cells that were of an early passage.

126

5.2.3.1. QPCR gene expression analysis for osteogenic genes

This was carried out for Runx2, Alp and Bsp genes in cells cultured for 10, 21 and 28 d. The analysis was conducted in GM and OM due to the differential effects of the media on cell function; GM promotes replication whereas OM induces osteogenic commitment and differentiation. The methods used here are detailed in section 2.8. Cells monolayers were homogenised with buffer RLT. RNA was extracted as in 2.8.1 and quantified (2.8.2) and then reverse transcribed as in 2.8.3.

The qPCR was carried out as described in section 2.8.5. The CT values of target genes were normalised to GAPDH housekeeping gene and the ΔCT values calibrated to CSkM PC 1 d. The details of the probes used are in table 5.1.

Table 5.1 Taqman probes for osteogenic markers

Gene	Probe catalogue number	Function
Runx2	Mm00501580_m1	Master transcription factor
Alp	Mm00475834_m1	Inorganic phosphate donor
Bsp	Mm00492555_m1	Bone matrix protein

5.2.3.2. Assessment of Alp expression by staining

This was conducted according to the methods described in section 2.6. Samples consisted of confluent monolayers cultured in OM for 28 d. After fixing the cells with 10 % formalin for 15 min, the cells were washed three times with distilled water and then incubated for 45 min in Tris-HCl Buffer (0.2M, PH 8.3) with AS-MX phosphate (Sigma Chemical Co., Poole, UK) and Fast Red violet (Sigma Chemical Co., Poole,

UK). The Alp positive cells would be stained red/purple. This experiment was conducted on isolates from the three attempts of cell isolation.

5.2.3.3.   Semi-quantitative assessment of mineralisation

This was assessed in cells cultured in OM for 28 d with 2 % Alizarin Red S Staining. The method used for this experiment is described in section 2.7.1. For this analysis, samples were fixed and then stained with 2 % working solution of dye. Samples were washed several times and air dried. Images of samples were taken at objective x40 as described in section 2.3. This experiment was conducted on cells from each of the three occasions of cell isolation.

5.2.3.4.   Quantitative assessment of mineralisation

This was conducted with the aid of the QuantiChrom Calcium kit (BioAssay Systems, USA) according to the method described in section 2.5.2. Samples consisted of cells cultured in OM for 28 d. Samples were assayed by initially washing x3 with PBS and then homogenising monolayer with 500 µl of 1 M HCl. A 5 µl aliquot of homogenate would be transferred to a 96-well plate and to it added 200 ul of a 1:1 mixture of working reagents A and B. Coloration was measured with a spectrophotometer at 610 nm wavelengths. Absorbance values were interpolated from a standard curve and expressed as mg / dl. This experiment was conducted in cells isolated from the three attempts at cell isolation from tissue; and at three replicates per cell type per attempt.

5.3. Results

5.3.1. QPCR gene expression analysis for osteogenic genes

The expression of osteogenic master transcription factor Runx2 was investigated in cultures of cells in GM and OM at 10, 21 and 28 d. The results of this experiment are presented in figure 5.1. It shows that Runx2 was detected in all cells at 10 d in both media. Its expression was higher in PP1 and PP2 cells compared to PC and PP3. The fold expression of the gene remained at a higher level in the early adherent sub-populations compared to the remaining samples through the remainder of culture. The early adhered CSkM cells appeared to have significantly higher expression of the gene in 21 d compared to same cells isolated from LM.
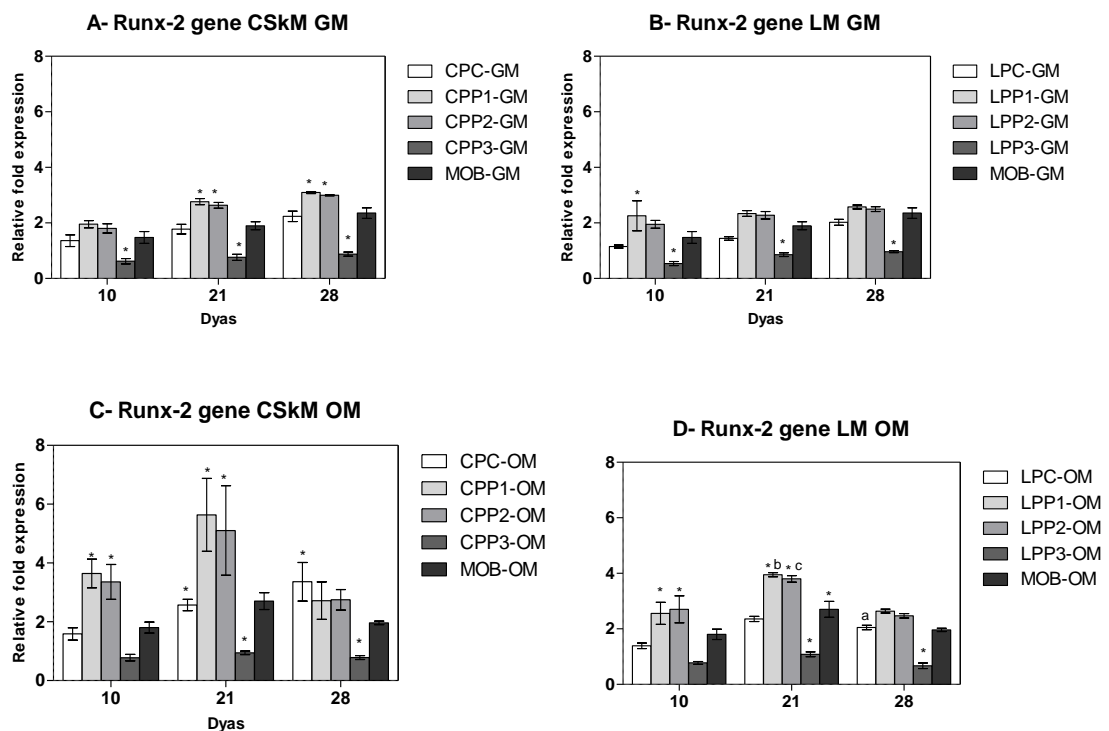
Figure 5.1 QPCR analysis of osteogenic Runx2
Figure shows the relative fold values of expression of Runx2 in CSkM and LM derived cells. Specimens were cultured in 24-well plates in GM or OM. (A and B) In GM, PP3 of both muscle types express significantly lower levels of Runx2. PP1 and PP2 cells displayed a small fold upregulation of the gene by 21 and 28 d. PP1 and PP2 display a level of expression comparable to the positive control MOB. (C and D) In OM, expression of the gene increased in PP1 and PP2 from 10 to 21 d and then decreased by 28 d. Runx2 was not modulated at any time in culture by PP3 cells. A comparison between the muscle groups indicated the CSkM early adherent sub-populations to express the gene significantly more than LM derived equivalents. The expression of the gene was found stronger in OM compared to GM. Bars indicate mean$\pm$1SD, N = 3, n = 3. * = $p < 0.05$, PC vs. PP-type. [a, b, c] = $p < 0.05$, CPC vs. LPC, CPP1 vs. LPP1, and CPP2 vs. LPP2 respectively.

The Alp gene was detected in all samples at 10 d in GM and OM. In GM, its expression increased in PP1 and PP2 by 21 d and further by 28 d. The difference was significantly higher for PP1 than PP2 by the final time point. PP3 displayed a small change in expression but its fold values of expression were very low than the early adherent sub-populations. A comparison with positive control MOB showed the early adherent sub-populations were expressing the gene at a significantly higher level. In OM, Alp displayed a similar trend to GM. The gene was upregulated several fold by early adherent cells than PC and late adherent PP3. This difference is obvious for both muscle types. At 21 d, the PP1 expressed Alp at a significantly higher level than PP2. The PP1 and PP2 levels of Alp expression decreased by 28 d. The MOB expressed Alp at a smaller fold value than the early adherent fractions. A comparison between the media types indicated similar range of expression values with the temporal difference in the upregulation of the genes by the early adherent cell fractions, which showed earlier modulation in OM than GM as shown in figure 5.2.
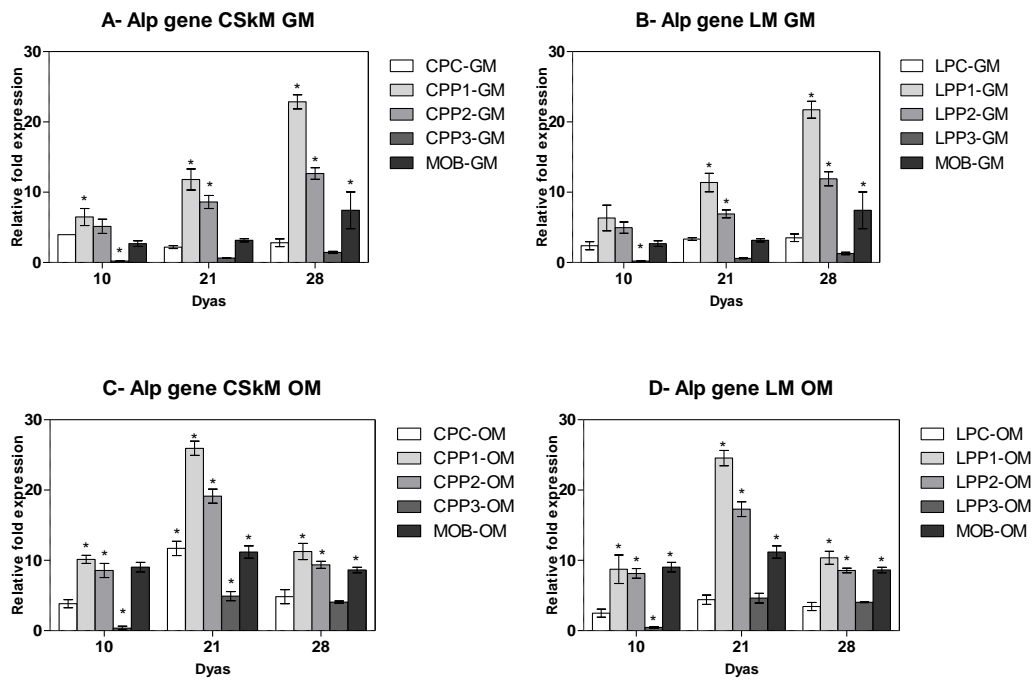
Figure 5.2 QPCR analysis of osteogenic Alp

Figure shows the relative fold values of expression of Alp in CSkM and LM derived cells. Specimens were cultured in 24-well plates in GM or OM. (A and B) In GM, PP1 and PP2 displayed a higher level of expression than PC, PP3 and the positive control cells (MOB) at 21 and 28 d. The PP3 did not modulate the gene to a significant level. (C and D) In OM, PP1 and PP2 displayed heightened expressed of Alp at 10 and 21 d. The expression of Alp decreased in the early adherent cell populations by 28 d. It was significantly lower in PP3 than the rest. The expression of the gene was found stronger in OM compared to GM. Bars indicate mean+1SD, N = 3, n = 3. * = p < 0.05, PC vs. PP-type.

The results for Bsp are shown in figure 5.3. Bsp gene was detected in all samples at 10 d. Its expression was many folds higher in OM than GM. In either medium, the gene was expressed at significantly higher levels by PP1 and PP2 cells compared to the rest. The PP3 cells did not express Bsp early but had upregulated it several fold by 28 d. There were no differences in the expression of Bsp between the early adherent cells. These differed from the positive control in GM, where the early adherent populations were significantly lower than the MOB. A comparison between the muscle groups indicated Bsp expression was significantly higher in CSkM derived PP1 and PP2 cells compared to their LM derived equivalents.
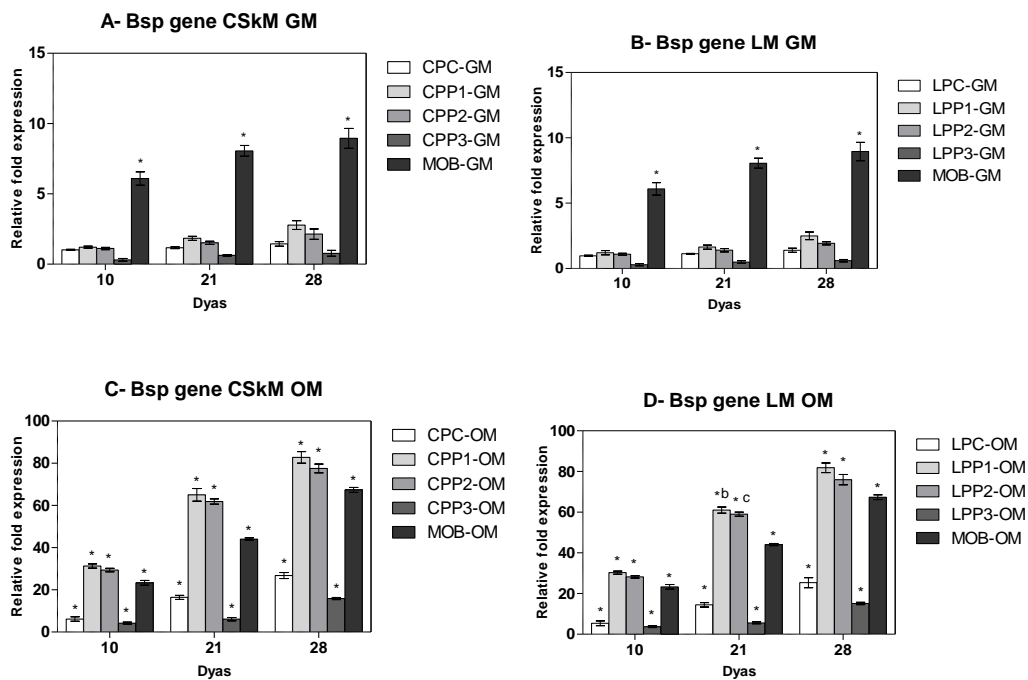
Figure 5.3 QPCR analysis of osteogenic Bsp
Figure shows the relative fold values of expression of Bsp in CSkM and LM derived cells. Specimens were cultured in 24-well plates in GM or OM. (A and B) In GM, PP1 and PP2 displayed a higher level of expression than PC and PP3 cells at all time points. The positive control (MOB) showed significantly higher expression than all other cells. (C and D) In OM, PP1 and PP2 displayed a progressive increase in the expression of Bsp over the course of culture. This was significantly higher than the positive control MOB cells. The PC and PP3 cells increased expression from 21 to 28 d. But PP3 expressed the least Bsp compared to all samples. A comparison between the muscle groups indicated the CSkM early adherent sub-populations to express the gene significantly more than LM derived equivalents. The expression of the gene was found stronger in OM compared to GM. Bars indicate mean$\pm$1SD, N = 3, n = 3. * = $p < 0.05$, PC vs. PP-type. [b, c] = $p < 0.05$, CPP1 vs. LPP1, and CPP2 vs. LPP2 respectively.

5.3.2. Alkaline phosphatase activity

The results of this analysis are shown in figures 5.4 and 5.5 for CSkM and LM derived cells. These show that osteogenic Alp was positively detected in all samples. The intensities of expression varied. The PP1 and PP2 cells from both muscle types displayed highest intensity of stain. These were followed by PC and MOB osteoblastic cells. The PP3 displayed least intensity of staining.
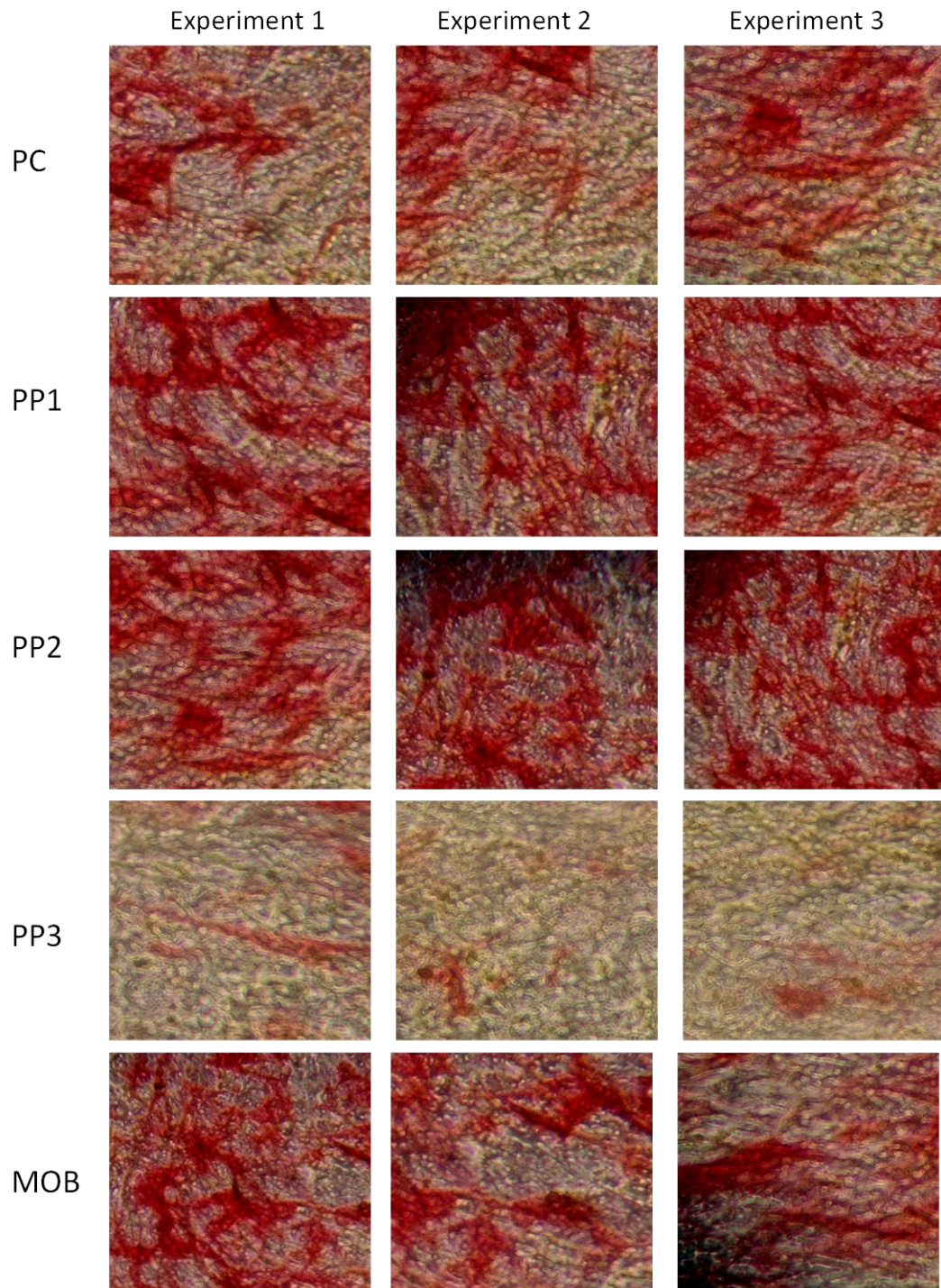
Figure 5.4 Alp staining for CSkM derived cells
Images were view and taken at objective x40 of Alp stained monolayers of different cell types of three different isolates at 28 d. The PP1 and PP2 cells displayed highest intensity of stain. PC and MOB displayed intermediate intensities of stain. The PP3 displayed a very small degree of staining.

	Experiment 1	Experiment 2	Experiment 3
PC			
PP1			
PP2			
PP3			
MOB			
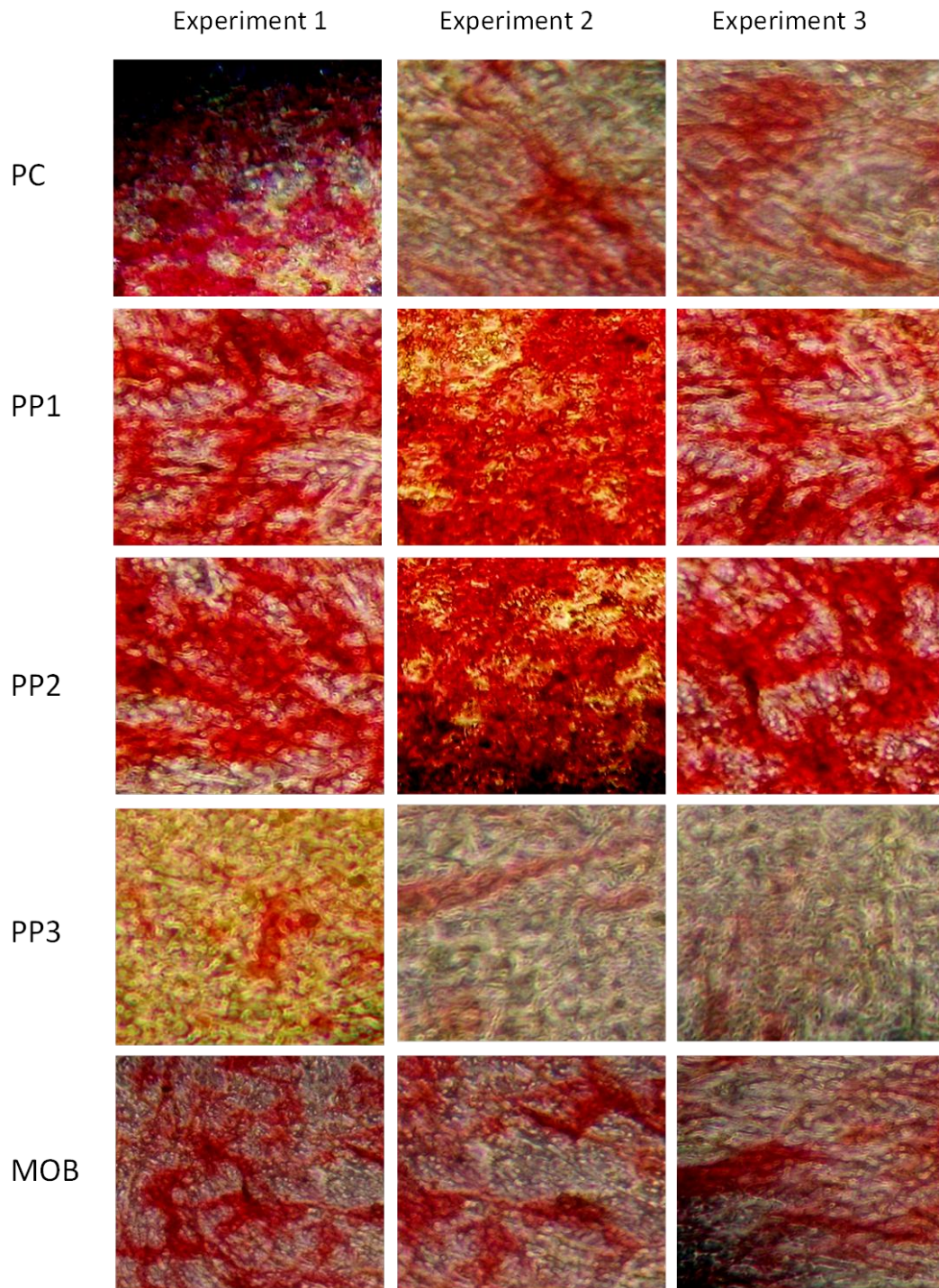
Figure 5.5 Alp staining for LM derived cells
Images were view and taken at objective x40 of Alp stained monolayers of different cell types of three different isolates at 28 d. The PP1 and PP2 cells displayed highest intensity of stain. PC and MOB displayed intermediate intensities of stain. The PP3 displayed a very small degree of staining.

5.3.3. Semi-quantitative assessment of mineralisation

The results of the Alizarin Red S staining of monolayers are shown in figures 5.6 and 5.7 for CSkM and LM cells. These indicate that PP1 and PP2 cells stained the highest and were followed by PC cells. The PP3 and MOB were similar and seemed much less intense compared to the rest. These differences were observed for cells isolated from the two muscle types.
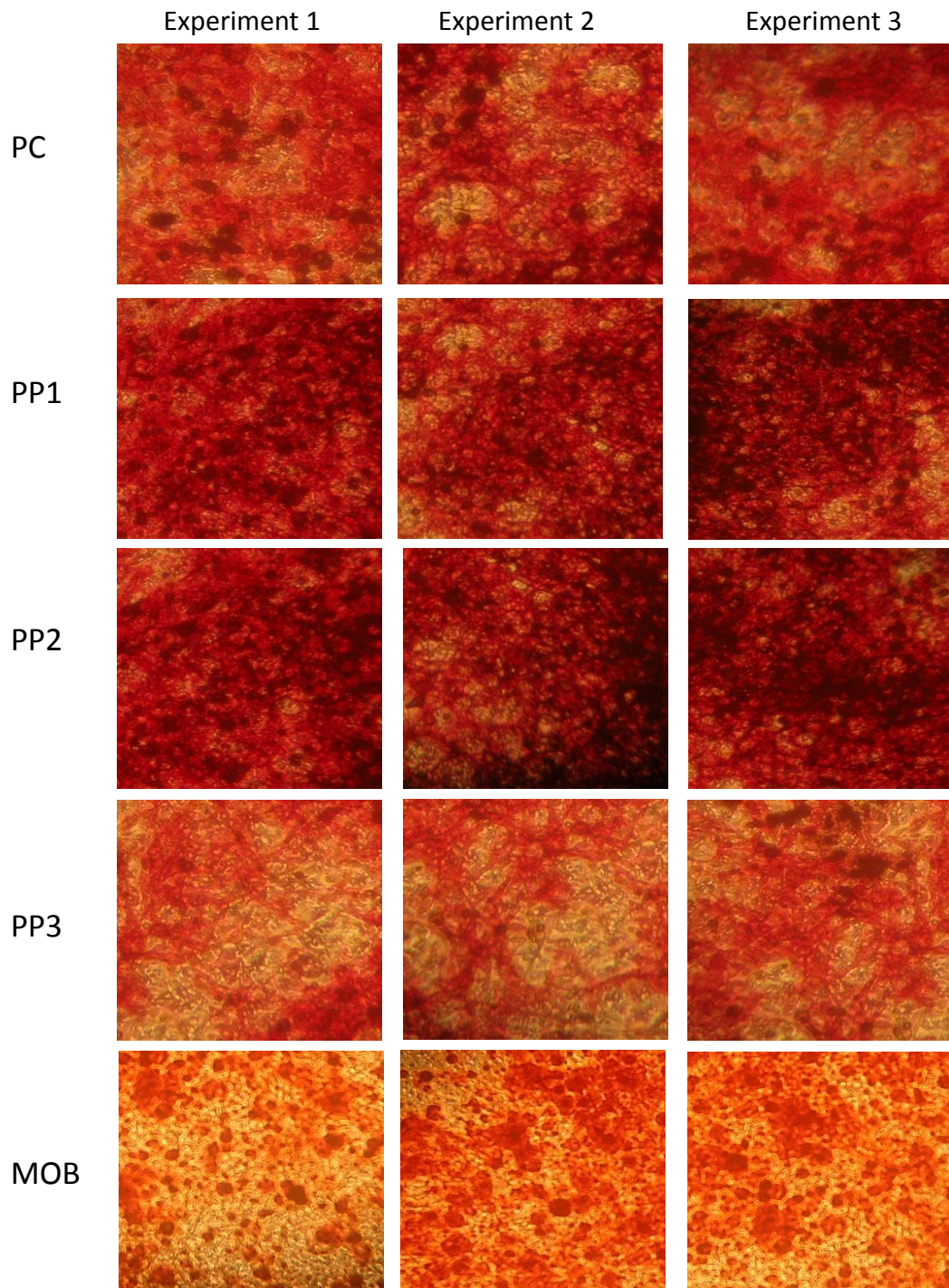
Figure 5.6 Alizarin Red S staining of CSkM derived cells
Images were viewed and taken at objective x40 views of stained cultures from three different isolates at 28 d in
OM. PP1 and PP2 stained the highest followed by PC. PP3 and MOB formed lesser stained monolayers.

Experiment 1 · Experiment 2 · Experiment 3

PC

PP1

PP2

PP3

MOB
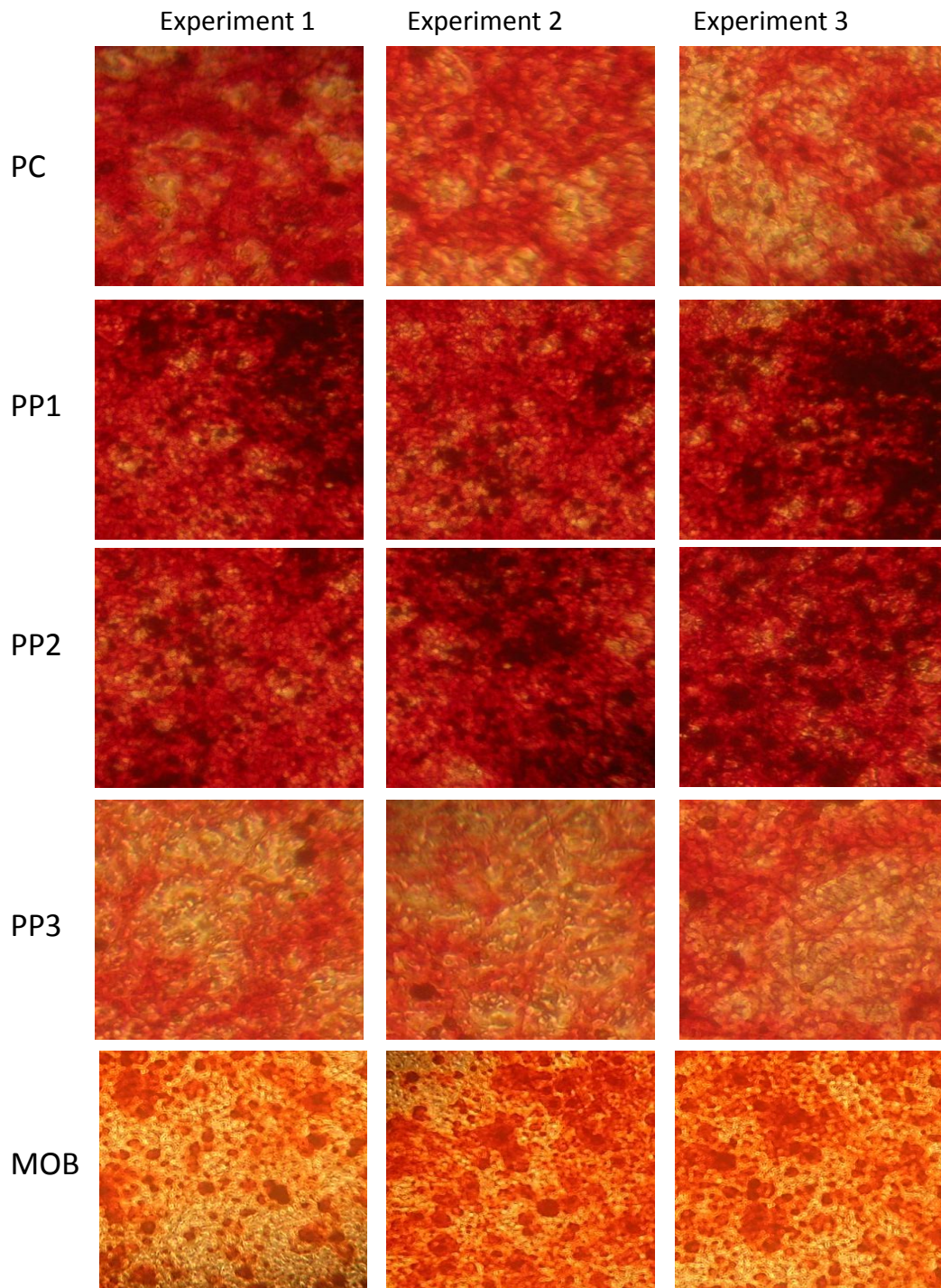
Figure 5.7 Alizarin Red S staining of LM derived cells
Images were viewed and taken at objective x40 views of stained cultures from three different isolates at 28 d in
OM. PP1 and PP2 stained the highest followed by PC. PP3 and MOB formed lesser stained monolayers.

5.3.4. Quantitative assessment of mineralisation

Cells from CSkM and LM were examined for their mineralisation capabilities after treatment with OM for 21 and 28 d. The results of this analysis are shown in figure 5.8. It shows that cells had deposited detectable levels of calcium mineral by 21 d in culture, which increased to significantly higher levels by 28 d. A comparison between the sub-types of cells indicated a similar level of calcium mineralisation in PC, PP1 and PP2 cultures by 21 d that was significantly higher than that detected for PP3 cells and MOB positive control cells. The 28 d readings indicate a higher deposition of calcium mineral by PP1 and PP2 compared to all others. PP3 displayed an increase in calcium mineral by this point but its final readings were significantly lower than the other cell types. A comparison between cells isolated from CSkM and their equivalent isolated from LM shows no significant difference in calcium mineralisation.

A. Calcium detection analysis in CSkM cells

B. Calcium detection analysis in LM cells
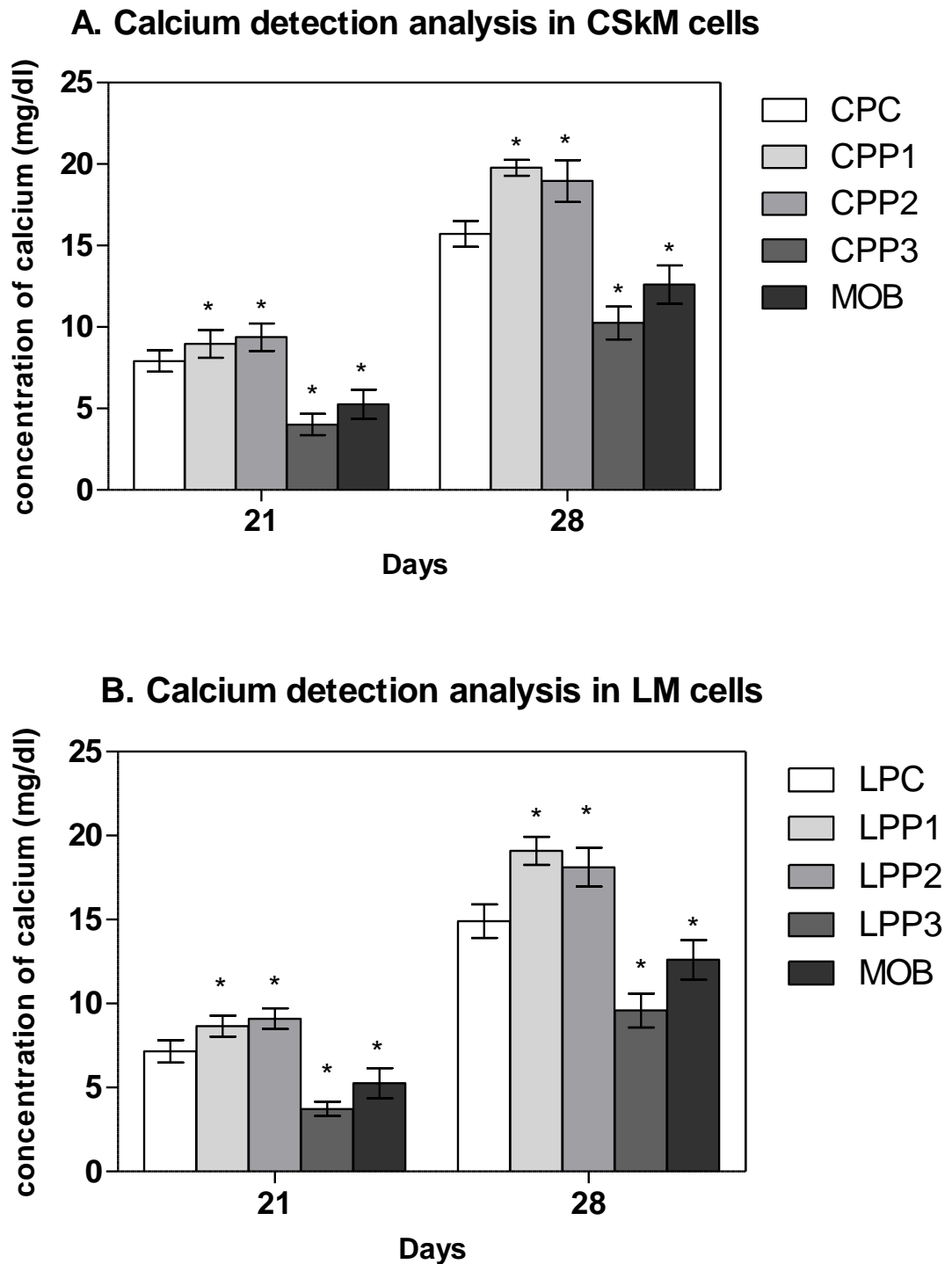
Figure 5.8 Calcium mineral detection in CSkM and LM cultures
Graph shows bar chart with mean±1SD, N=3, n=3 of calcium concentration in mg / dl. (A) = CSkM, (B) = LM.
Mineral was detected in cells from both muscle groups. It was detected lower in PP3 sub-populations from
both muscle types. PP1 and PP2 displayed highest concentration of calcium deposition. * = $p < 0.05$, PC vs.
PP-type.

5.4. Discussion

5.4.1. QPCR gene expression analysis for osteogenic genes

This analysis examined the genes Runx2, Alp, and Bsp in cells cultured in GM or OM for up to 28 d. The early adherent PP1 and PP2 expressed each of these genes at a significantly higher level than later adherent PP3. In the case of Alp, this relative high fold of expression was starkly evident in both media types. In comparison, the late adherent PP3 did not express these osteogenic genes at a higher level. Instead, the expression of these genes was found at a baseline that did increase by 28 compared to 10 d but this incremental raise was very small in value.

In addition, small yet significant differences were observed in the expression of these osteogenic genes over time between the CSkM and LM derived specimen. This is an indication of the differential influence that developmental origin may bear on adult stem cell characteristics. The phenotypic significance of these gene expression patterns are discussed below.

5.4.2. Assessment of Alp expression by staining

The staining indicated a higher expression of the alkaline phosphatase enzyme in the early adherent PP1 and PP2 cells compared to PP3 and even the MOB. This was particularly interesting as the last cell type is a dedicated mature osteoblast that would have been thought to differentiate and function at a relatively higher level. Interestingly, the PP3 cells displayed expression of the protein, albeit at significantly lower levels. These cells were considered as committed precursors due to their relative strong potential to differentiate to the myogenic lineage with the expression of myogenic genes and myotube formations. The observation that these cells express alkaline phosphatase after sufficient duration in inductive medium indicates its possible potential to deviate from the myogenic lineage.

5.4.3. Assessment of osteogenic mineralisation

The formation of a calcified extra-cellular matrix is the hallmark of osteogenic differentiation. The analyses conducted here indicated clearly that the early adherent cells were relatively osteogenic by forming more calcified matrices than the remaining specimens, including mature osteoblasts. In a manner similar to the osteogenic alkaline phosphatase expression, the late adherent PP3 cells displayed some mineralisation at 28 d. The increase observed from the initial to final time point indicates that these cells may have adopted an osteogenic profile. A comparison between the muscle groups indicated a slight difference in the concentration of calcium, which was insignificant.

5.4.4. Significance of findings

The findings indicate that the early adherent PP1 and PP2 sub-populations possessed a higher capacity to differentiate along the osteogenic lineage. These cells expressed the osteogenic genes at a higher level, expressed abundant levels of alkaline phosphatase and displayed higher matrix calcifications compared to PC, PP3 and MOBs. The findings support the presence of osteoprogenitor cells within skeletal muscle tissue as shown in previous work of Urist and colleagues (1970); Khouri *et al.,* 1991; Bosch *et al.,* 2000; Lee *et al.,* 2000; Levy *et al.* 2001; Mastrogiacomo *et al.,* 2005; Hashimoto *et al.,* 2008.

Interestingly, the PP3 cells displayed a degree of change with the presence of osteogenic inductive medium with an increase in gene expression, alkaline phosphatase expression and calcium mineralisation at 28 d compared to 10 d in culture. This is an important implication for the plasticity of PP3, as the findings of the previous sections suggested these were committed myogenic precursors, and hence were likely to not be capable of responding to inductants of a different lineage. The findings suggest that these cells

while being myogenic on the onset, my have the potential to 'trans-differentiate' from early myogenic commitment to that of an osteogenic phenotype. This can be seen in the long duration of culture with strong osteo-inductive medium; with time the defining factor that drives the change of fate in these cells. It may also be a suggestive of partial contamination of the late adhered cells with the more osteogenic cells from rely adhered ones (Bosch *et al.,* 2000).

5.4.5. Conclusion

The results presented here showed that early adhered cells from both muscle groups appeared to be more osteogenic than late adhered cells and the positive control MOB cells. This was evident by the higher expression of Alp and Alizarin Red stains. This was further confirmed by the higher expression of osteogenic genes (Runx2, Alp and Bsp) and detectable calcium depositions by early adhered cells from both muscle groups. Early adhered cells from CSkM showed slightly higher Runx2 and Bsp genes expression at 21 d. however, this change was faded by 28 d and cells from both muscle groups behave similarly again. Therefore, there were no significant differences in term of terminal osteogenic differentiation between cells isolated from CSkM and their equivalents cells isolated from LM.

5.5. Summary of the results

- The early adherent PP1 and PP2 express higher levels of Runx2, Alp and Bsp genes in OM compared to the late adherent PP3 cells at all times in a 28 d culture.

- PP3 display an increase in osteogenic gene expression at the end of 28 d in OM.

- PP1 and PP2 expressed alkaline phosphatase protein at a higher intensity than PP3.

- The PP3 cells express the protein at the end stages of a 28 d culture in OM.

- Calcium mineralisation assessed by Alizarin Red S stain followed the trend observed for alkaline phosphatase expression being higher in the early adherent sub-populations than late adherent PP3.

- This was confirmed by the quantitative analysis of calcium deposition.

Chapter 6: Discussion

6.1.   The hypothesis

The present study was based on the hypothesis that neonatal mouse muscle tissue from the head and limbs contains mononuclear cells that may, in turn, bear differences in their capacity to differentiate along the osteogenic and myogenic lineages.

Analytical methods have been developed to isolate mononuclear cells with stem cell like characteristics; these have been described in section 1.4.2.2. The pre-plate method that was originally developed by Yaffe (1968), Richler and Yaffe, (1970) and Rando and Blau, 1994, uses 'contact duration' as a method to fraction a total cell isolate into sub-populations, different in their adherent properties and hence possibly phenotypic characteristics. This method has been applied with varying modifications to isolate mononuclear cells with a capacity to differentiate to different lineages. Many such studies have mainly focused on late adherent cells and evaluated their multilineage potentials whilst the initial isolates have not been paid much attention.

Developmentally, CSkM and LM originate from two distinct parts of the embryo. Most of the head muscles arise from prechordal and paraxial head mesoderm (PHM) (Noden, 1983) but the connective tissues are neural crest derived (Noden and Trainor, 2005). The progenitors of tongue and posterior neck muscle arise from the occipital somites whereas branchiomeric muscles progenitors arise from the unsegmented PHM (Kuratani, 2005; Noden and Francis-West, 2006). On the other hand, trunk and limb musculature derive from the pre-somitic paraxial mesoderm, which runs parallel to the body axis flanking the neural tube. Accordingly, most of head muscle progenitor cells reside in a distinct embryonic tissue compared to limb and trunk muscle progenitors. The connective tissue compartment of CSkM develops from NCC in contrast to LM muscle tissues that are of mesodermal origin.

The signals that regulate myogenesis in the head vary to those that operate in trunk and limb regions. Early transcription factors that control early somitic muscle development are not expressed in the developing CSkM (Mootoosamy and Dietrich, 2002). All the somitic muscles, including limb muscle, that migrate from somites, are Pax3-positive and express homebox factor Lbx1 during early myogenesis, but craniofacial muscles are Pax3 and Lbx1 negative (Tajbakhsh *et al.,* 1997; Buckingham, 2001; Brown *et al.,* 2005; Mootoosamy and Dietrich, 2002). Postnatally there are differences in their regenerative potentials. Pavlath and colleagues found that there is a regenerative difference between masseter and limb muscle, which may be related to intrinsic differences between the muscle precursor cells in these two muscles (Pavlath *et al.,* 1998). There are also some report that indicate that craniofacial muscles (masseter muscle) continue to express many molecules normally downregulated in adult limb skeletal muscle, such as developmental and neonatal myosin heavy chain (Butler-Browne *et al.,* 1988) and embryonic fibronectin splicing variant EIIIA (Price *et al.,* 1998). Additionally, satellite cells isolated from masseter muscle reported to proliferate more and to differentiate rather later than those from limb muscle (Ono *et al.,* 2010). However, when masseter-derived satellite cells transplanted in limb region; the masseter-derived satellite cells regenerated limb muscles as efficiently as those from limb muscle (Ono *et al.,* 2010).

Furthermore, bone formation in craniofacial region and other parts of the body experience different pathways. Craniofacial ossification follows the intramembranous pathway which is differs from the endochondral pathway occurs in long bones.

The above-mentioned studies have documented differences between CSkM and LM development and regeneration. However, whether these differences positively or

negatively affect the proliferation and differentiation abilities of cell population isolated from these tissues remain to be evaluated.

6.2.   Overview of the thesis

The current study was divided into three main levels: firstly, to isolate and characterise muscle derived mononuclear cells from CSkM and LM tissues; secondly, compare the myogenic differentiation abilities of the isolated cells; and thirdly, examine differences in their osteogenic differentiation potentials.

6.2.1.   Isolation of mononuclear cells

In this study, mononuclear cells were isolated from both CSkM and LM tissue based on their adhesion properties to plastic. These populations were named, PP1, PP2 and PP3, which represented the adherent portion of a total cell isolate by 1 h, 48 h and 120 h on plastic.

A modification of the preplate method developed by Yaffe (1968) and Rando and Blau (1994), was applied here to isolate mononuclear cells from muscle tissue. The modifications included (i) the use of non-coated tissue culture plastic dishes for cell attachment in contrast to collagen coated substrates as used by others and (ii) various contact durations, comprising 1 h, 48 h and 5 d. The different substrates and contact intervals used by other researchers are tabulated in table 6.1. It shows the differences between method used in this study and same method used by most of the other studies. Most of work undertaken so far has been on non-craniofacial sources of muscle tissue, with the use an extracellular matrix protein as a substrate, and a main focus on the late adherent cells isolated with variable time points for cell attachment procedure. Hence

this was the first attempt to investigate and compare cells isolated from CSkM and LM based on their adhesion properties to tissue culture plastic.

Table 6.1 Various modes of muscle derived cells isolation by the pre-plate method
HMM-MAA (high-molecular-weight melanoma-associated antibody), VWF (Von Willebrand factor), MPT (Modified preplate technique), PT (preplate technique), αSMA (Alpha sarcomeric actin), h (Hour). MDSC (Muscle derived stem cell), c-Met (Tyrosine kinase receptor), MNF (Myocyte nuclear factor), Flk-1 (Foetal liver kinase-1), Bcl-2 (B-cell lymphocytic-leukaemia proto-oncogene), Sca-1 (Stem cell antigen 1), P75 (Nerve growth factor receptor), Sox10 (SRY-box containing gene), AP2c (Transcription factor AP2 gamma), Alp (Alkaline phosphatase)

Muscle origin	species	Isolation method	Cells investigated	Substrate	Lineage potential	Markers used	Main differences	Main similarities	References
Limb	Mice	MPT (1h, 2h) (PP1, PP2) PP1 discarded	Late adhered cells	Collagen	Hematopoietic	Hoechst 33342/ Ly-5.1/ c-Kit/ Sca-1	Method/ substrate/ Lineage potential/markers	N/A	Jackson *et al.*, 1999
Limb	Mice	PT (2h, 24h until PP6) PP1 discarded	EP (PP2-5), LP (PP6), MDSC subpopulation of PP6	collagen	Muscle/ neural/ endothelial	Desmin/ Sca-1/ CD34	Method/ substrate/ Lineage potential/markers/ cells used (MDSC) EP higher desmin than MDSC initially	Expanded MDSC are 95% desmin+	Qu- Petersen *et al.*, 2002
Limb	Mice	MPT (1h, 2h, 18h, 24h), PP1 to PP4-6.	All cells	Collagen	Myoblast	Desmin	Method/ substrate/ Lineage potential/markers	late adhered more myogenic	Qu *et al.*, 1998
Limb	Mice	MPT (1h, 2h, 18h, 24h), PP1 to PP4-6.	Late adhered cells	Collagen	Myoblast	Sca-1/ CD33/ desmin	Method/ substrate/ Lineage potential/markers/ cells investigated PP4-6 more Sca-1 and CD34	late adhered more myogenic	Jankowski *et al.*, 2001
Limb	Mice	MPT (1h, then when 30% to 40% of cells attached) PP1 to PP5-6.	Late adhered cells	Collagen	Myogenic/ Osteogenic	Sca-1/ desmin/ CD34/ Bcl-2/ Flk-1/ c-Met/ MNF	Method/ substrate/ Lineage potential/markers/ cells investigated no comparison made	Late adhered myogenic and osteogenic	Lee *et al.*, 2000
Limb	Mice	PT (2h, 24h until PP6) PP1 discarded	PP1, PP3 and late adhered MDSC	Collagen	Bone formation	Desmin	Method/ substrate/ Lineage potential/markers/ cells investigated Late adhered (MDSC) more osteogenic	MDSC higher desmin expression	Shen *et al.*, 2004
Limb	Mice	PT(selective pre-plating by trypsinisation)	Late adhered	Collagen	Myogenic	Desmin	Method/ substrate/ Lineage potential/markers/ cells investigated	Late adhered cells higher desmin expression	Rando and Blau, 1994
Limb	Mice	MPT (1h, 2h, 24h), PP1 to PP3-6.	All cells	No coating	Myogenic	Sca-1/ CD34/ Desmin	Method/ substrate/ Lineage potential/markers/ PP6 more Sca-1 and CD34.	PP4 and PP5 cells higher desmin expression	Torrente *et al.*, 2001
Limb	Mice	PT (2h, 24h until PP6) PP1 discarded	Late adhered cells	Collagen	Osteogenic	N/A	Method/ substrate/ Lineage potential/markers/ cells investigated/ no comparison	N/A	Usas *et al.*, 2009
Limb	Mice	PT (2h, 24h until PP6)	Late adhered cells	Collagen	Smooth muscle	αSMA/ CD34/ Sca-1	Method/ substrate/ Lineage potential/markers/ cells investigated/ no comparison	N/A	Nolazco *et al.*, 2008

Muscle origin	species	Isolation method	Cells investigated	Substrate	Lineage potential	Markers used	Main differences	Main similarities	References
Limb	Mice	MPT (1h, 2h, 18h, 24h), PP1 to PP4-6.	Late adhered cells and MC13 clone	Collagen	Muscle cell expansion	N/A	Method/ substrate/ cells investigated	N/A	Deasy *et al.*, 2002
Limb	Mice	PT (2h, 24h until PP6) PP1 discarded	Late adhered cells	Collagen	Muscle dystrophy	N/A	Method/ substrate/ Lineage potential/ cells investigated	N/A	Ambrosio *et al.*, 2009
Limb	Mice	PT (2h, 24h until PP6)	Late adhered cells	Collagen	Chondrogenic	N/A	Method/ substrate/ Lineage potential	N/A	Kubo *et al.*, 2009
Limb	Rat	MPT (1h, 2h, 3h, 1d, 2d, 3d).	Late adhered cells	Collagen	Osteogenic	CD44/ CD45	Method/ substrate/ Lineage/ species/ potential/markers/ cells investigated/ no comparison	N/A	Kim *et al.*, 2008
N/A	Human	PT (1h, 24h interval until PP5)	All cells	Collagen	Osteogenic	Desmin	Method/ substrate/ Lineage potential/markers/ PP3 more desmin expression/ PP3 higher Alp production/ PP1 higher BMP-2 secretion	Early adhered cells less desmin expression	Musgrave *et al.*, 2002
Head and neck	Human	Brief culture (1h (discarded), 24h used)	24h adherent cells	No coating	Hematopoietic	CD45/ CD31	Method/ substrate/ Lineage / potential/ species/ markers/ no comparison	N/A	Dell'Agnola *et al.*, 2002
N/A	Human	PT (2h, 24h until PP6)	CD34$^+$ cells	Collagen	Adipogenic, myogenic, osteogenic and chondrogenic	CD34	Method/ substrate/ Lineage potential/ species/ markers	N/A	Lu *et al.*, 2009
Limb	Mice	MPT (1h, then when 30% to 40% of cells attached) PP1 to PP6	Late adhered cells	Collagen	Osteogenic	Desmin	Method/ substrate/ Lineage potential/markers/ cells investigated/	Late adhered cells higher desmin expression	Bosch *et al.*, 2000
gracilis and semitendinosus	Human	Brief culture (1h uncoated then transferred supernatant to coated flask)		Uncoated Collagen	Osteogenic	HOP-26/ αSMA/ Desmin/ CD31/ VWF/ HMM-MAA/ β nonmuscle (NM) actin	Method/ substrate/ Lineage potential/ species/ markers/ cells investigated	Early adhered more osteogenic	Levy *et al.*, 2001
Limb and craniofacial	Mice	MPT (1h, 48h, 5d)		Uncoated	Myogenic and osteogenic	Sca-1/ CD34/ P75/ Sox10/ AP2c/ Alp/ Runx2/ MyoD/ Desmin			This study

Cells that adhered within the 1h of attachment, PP1, from both muscle tissue sites, showed higher growth rate initially compared to other sub-populations. These cells also appeared to be larger and with no desmin expression. The PP2 on the other hand, showed similar growth behaviour to PP1, however, their size was smaller and cells had minimal expression of desmin.

Late adhered cells, PP3, showed less growth rate compared to PP1 and PP2 and were significantly smaller in size. They were also highly positive for desmin expression. These observations were similar in both muscle tissues. Comparing isolated cells from CSkM to its counterpart equivalent from LM revealed that early adhered cells, PP1 and PP2, were slightly larger in size compared to PP1 and PP2 from LM. In contrast PP3 seemed to be similar from both muscles.

The multilineage potentiality of isolated cells was indicated by the expression of potential stem cells genes (Oct4, Sca-1 and CD34). The early adhered cells from both muscle tissue expressed both Sca-1 and CD34 in contrast to the late adhered cells which only expressed Sca-1.

The assessment of myogenic gene expression for MyoD and desmin indicated their selective expression in later adherence cells. This may suggest that those cells are residing in muscle tissue and therefore express muscle specific genes.

Heterotopic ossification in muscle tissue led to investigating the early osteogenic markers in isolated cells. These markers were osteogenic specific Runx2 and Alp that had the high chance of being detected, if expressed at all in the mononuclear cells

The expression of osteogenic markers in only early adhered cells, PP1 and PP2, from both muscle tissues indicates that they had the potential to differentiate to an osteogenic lineage. This may indicate their non-muscle origin, such as connective tissue origin and have multipotentiality towards osteogenic lineage.

As documented in developmental studies such as Noden and Francis-West, (2006), Noden and Schneider, (2006); Evans and Noden, (2006); Noden, (1983); Grenier *et al.,* (2009). CSkM connective tissue is believed to originate from neural crest cells whereas connective tissue of LM has a mesodermal origin. Therefore, the expression of three NCCs specific markers namely Sox10, P75 and AP-2 was assessed in these cells. The expression of those genes in only PP1 and PP2 of CSkM strongly support available evidence of NCC involvement in CSkM development. It also suggests that early adhered cells are present in the connective tissue of CSkM muscle. The use of connective tissue specific markers for other muscle origin would have provided a clearer picture of the origin of early adhered cells from LM.

6.2.2. Myogenic capacity of isolated mononuclear cells

An investigation of the myogenic differentiation abilities was carried out in order to further elaborate the phenotypic differences between the cells observed in the initial analysis.

In this study, early adhered cells, especially PP1, form both muscle tissues have a very limited ability to differentiate along myogenic lineage as clearly observed from bright field microscopy and desmin immune staining compared to late adhered cells.
PP2 cells shows relatively more myogenic gene expression than PP1 but lesser than PP3. This could be due to the stemness of those cells as they express stem cell specific

genes, Sca-1 and CD34. They also present in the muscle niche and may have the ability to differentiate to myogenic lineage, which in the *in vitro* method used may require longer time in culture. This is ascertained from the pattern of genetic expression, which indicates a possibility that early adhered cells especially PP2 may form myotubes if allowed further culture. On the other hand, PP3 showed increase in myotube formation and gene expression by 7 days.

The PP2 cells seemingly more myogenic than PP1 cells; this difference was small initially but presented as a progressive increase in transcription level of myogenic genes. However, absence of myotube formation may require further examination as this may suggest the ability of these cells to trans-differentiate from a seemingly non-myogenic phenotype to a myogenic one.

6.2.3. Osteogenic differentiation of isolated mononuclear cells

In this section we aimed to evaluate and compare the osteogenic potentials of isolated cells. Based on previous findings, cells showed different phenotypic and myogenic potentials. These could be complicated by the different origin of cells with and the difference in bone formation between craniofacial and long bones.

Early adhered cells, PP1 and PP2, from both muscles showed higher osteogenic potential compared to PP3 cells. This can be seen from Alp, Alizarin Red S stain, and calcium measurement assay presented in chapter 5.

Based on the findings PP1 cells from both muscle groups seemed to have a relatively higher osteogenic potential than PP2. The late adhered cells, PP3, from both muscle tissues showed gradual increase in osteogenic gene expression towards the end of 28 d of culture. They also show a minute amount of Alp stain. The Alizarin Red S staining

also confirmed the presence of few points of calcium deposition, which was further confirmed by the increase of calcium deposition from 21d to 28 d. This finding may be due to a sub-population of osteogenic cells present within the PP3 fraction of mononuclear cells. However, the early passaged cells used in this study and the time needed for the fibroblastic cells to expand and contribute to these readings may rule out the possibility of contamination. On the other hand, the expression of stem cells gene, Sca-1, by these cells and the gradual increase in osteogenic genes, Alp and Alizarin Red S stains and calcium deposition may indicate their ability to trans-differentiate from a myogenic cell to an osteogenic lineage. All these findings show that PP3 can differentiate towards osteogenic lineage but to an obviously lesser extent than PP1 and PP2.

Even though, differences were notices between CSkM PP1 and PP2 and their equivalent cells isolated from LM in term of osteogenic gene expression (Runx2 and Bsp) at 21 d; there were no significant differences at later time point (28 d). This transient different may be related to the developmental differences in muscle and bone formation between those two sites.

6.3.   Significance of findings and agreement

Cells isolated in this study based on their ability to adhere to uncoated tissue culture plastic. In addition, the time selected for cells to plastic contact was different among other published work in this field (Rando and Blau, 1994; Lee *et al.,* 2000; Jankowski *et al.,* 2001; Qu-Petersen *et al.,* 2002; Deasy *et al.,* 2005). Few studies have used the criteria of adherence to plastic in order to isolate osteogenic faction of muscle derived mononuclear cells (Levy *et al.,* 2001; Mastrogiacomo *et al.,* 2005, Gharaibeh *et al.,* 2008). A direct comparison of isolated cells in this study to same cells isolated in previous studies is not applicable. However, the data confirmed previous work that

mononuclear cells can be isolated from muscle tissue based on their adhesion characteristics. Also the late adhered cells appear to be more myogenic compared to early adhered cells. The cellular isolation process of preplating was originally developed to enrich cell culture preparations for myogenic cells used in muscle transplantation experiments (Rando and Blau, 1994; Qu *et al.,* 1998). Early adhered cells (PP1) on collagen had been reported by several researchers to be mainly fibroblast and discarded. However, Levy and colleagues (2001) have suggested that cells that adhered to plastic initially had more osteogenic potentials than late adhered cells or cells that adhered to collagen coated plastic. The findings support that early adhered cells to uncoated tissue culture flask appeared to have more growth potentials initially, low desmin expression and high osteogenic differentiation ability.

Sca-1 genes appeared to be present in all cells with higher expression in early adhered cells compared to late adhered ones. This supports the idea that all sub-population may have undifferentiated cells present in the culture with varying degrees of multipotency. The expression of CD34 mainly in early adhered cells from both muscles may suggest that those cells may have the ability to differentiate into non-myogenic cell types.

Taking into consideration the expression of all genes we can suggest that early adhered cells from both muscles share the same expression profile, which may indicate the multipotent nature of those cells. Expression of NCC genes by early adhered CSkM also suggests that those cells may originate from the connective tissue compartment of the muscle hence may have the ability to differentiate into non-myogenic lineages. However, further investigation of the other stem cell, connective tissue and multipotential genes may be of great help in understanding the identity of those cells.

Regarding osteogenic potential, the data in this study is in agreement with previous reports supporting the existence of osteoprogenitor cells within skeletal muscle. Prior work on this subject includes the diffusion chamber and muscle pouch implantation experiments of Urist *et al.,* (1970), muscle flap experiments by Khouri *et al.,* (1991), and numerous *in vitro* experiments using both muscle-derived cell lines (Katagiri *et al.,* 1994) and primary muscle cell preparations (Bosch *et al.,* 2000, Lee *et al.,* 2000; Levy *et al.,* 2001, Mastrogiacomo *et al.,* 2005; Hashimoto *et al.,* 2008). However, prior to this report, any efforts to isolate an enhanced population of osteoprogenitor cells from primary craniofacial skeletal muscle samples were not readily available; as was not a comparison between equivalent cells isolated from limb muscle. As researchers strive to improve fracture healing or bone formation at a cellular level, procurement of progenitor cells from a variety of sources may prove to be significant.

Recently, several groups of researchers were interested in identifying and isolating multipotent stem cells from the skeletal muscle but as of today, due to the lack of specific markers, the identity of the cells giving rise to osteoblasts within skeletal muscles has not been determined (Bosch *et al.,* 2000; Levy *et al.,* 2001). A number of candidates have been proposed: satellite cells, for example, have been found to be early progenitors of the muscle cell and also to retain the ability to undergo osteo-chondrogenic differentiation *in vitro*. Asakura *et al.,* (2001) have provided evidence *in vitro* that isolated mouse muscle satellite cells, in the presence of rhBMP4 and/or BMP7, can express alkaline phosphatase, an early marker of osteoblast differentiation and osteocalcin gene

Lee *et al.,* (2000) showed that muscle cells could be induced *in vitro* by BMP2 to express osteocalcin mRNA. Furthermore they showed that transduced cells,

constitutively expressing BMP2 formed bone, when implanted subcutaneously into immune-compromised mice. Levy *et al.,* (2001) also provided evidence of a cell population isolated from skeletal muscle expressing in culture high levels of osteocalcin mRNA after 24 h of induction with vitamin D.

Among potential contaminant cells from skeletal muscle, pericytes are particularly interesting as the possible source of osteoprogenitor cells for ectopic bone formation. Others have shown that pericytes isolated from other tissues are capable of mineralisation and behave phenotypically similarly to osteocytes in response to different oxygen tensions and growth factors *in vitro* (Reilly *et al.,* 1998; Schor *et al.,* 1995). These data support a widely believed connection between angiogenesis and osteogenesis (Reilly *et al.,* 1998; Schor *et al.,* 1995). Urist refers to pericytes as the cells induced by demineralised bone matrix or BMP to form bone within muscle tissue (Urist, 1965; Urist *et al.,* 1983, Nilsson *et al.,* 1986). Based on available data, the relative contribution of pericytes to the early adhered cells population is not clear. It is not mutually exclusive for osteoprogenitor cells within skeletal muscle to be associated with satellite cells and pericytes.

A stem cell population should fulfil several criteria in order to be used for tissue engineering applications. These criteria include:

• Should be found in abundance.

• Can be easily collected and harvested.

• Can be reproducibly differentiated along multiple cell lineage pathways.

• Can be safely and effectively transplanted to either an autologous or allogeneic host.

Several researchers have used, with some limitation, MDSC in the treatment of several diseases such heart failure, urological disordered, muscular dystrophies, orthopaedic

applications (Menasche, 2008; Huard *et al.,* 2002; Rando and Blau, 1994, Qu-Petersen *et al.,* 2002; Gussoni *et al.,* 1999; Bosch *et al.,* 2000; Lee *et al.,* 2002).

The cells isolated in this study have both the ability to differentiate in to myogenic and osteogenic lineages. After being investigated in 3D model; early adhered cells could be utilised in bone and/ or cartilage repair and regeneration. While the late adhered cells could be used in repair and regenerate damaged muscle tissues and other diseases such as DMD.

Two different strategies are pursued for application of stem cells in regenerative medicine. The one and easier way is simple injection of cell suspension into the blood circulation or the target tissue a procedure called "cell therapy," the second approach (tissue engineering); utilising the use of tissue-like construct consisting of a three-dimensional matrix colonised by cells is engineered in vitro and subsequently implanted into the tissue defect of the recipient. Mostly, addition of growth factors is necessary for proliferation and differentiation of stem cells in vitro (Ehnert *et al.,* 2009).

6.4.   Conclusions

The experimental findings presented in this thesis reveal that mononuclear cells can be isolated from muscle tissues of the mouse cranium and limbs. This initial isolate can be differentially fractioned into at least three populations based on their variable adherence to tissue culture plastic. Phenotypically, the early adherent cells seem to be relatively multipotent compared to the late adherent cells, which display characteristics of committed myogenic precursors. The osteogenic differentiation ability of the early adherent cells is higher than the late. Conversely, the ability of myogenic differentiation is higher in late adherent than early adherent cells. Interestingly, the different cells display varying degree of trans-differentiation. Accordingly, the early adherent cells display progressive increase in myogenic differentiation while PP3 exhibit an increase in osteogenic parameters after 21-28 day stimulation in inductive media. Cells isolated from both muscles displayed certain differences. These pertained mainly to cell size as surface area being larger in early adherent cells from CSkM than LM, but also due to the positive expression of neural crests specific genes in the former. No significant differences were observed in myogenic differentiation ability between CSkM and LM isolated cells. There was transient difference in osteogenic differentiation ability between early adhered cells from CSkM and their equivalent from LM by 21 d. However, this difference was small and faded by 28 d. Therefore, cells isolated from both muscle tissues seem to have similar differentiation abilities despite the documented developmental differences between those two sites.

6.5.   Future directions

Presented in this study are the preliminary findings of a promising shift in bone tissue engineering where muscle tissue, due to the presence of cells capable of osteogenic differentiation, may offer a suitable source of multipotent cells. Before moving to more advanced *in vivo* investigation of the ability of the cells isolated in this study, a full understanding of the biological behaviour and characteristics is required.

An initial examination would be to use a substrate other than plastic. This may include extracellular matrix molecules, such as collagen and fibronectin, as well as biomaterial substrates, such as modified titanium implant surfaces or polymeric substrates. The mechanism of cell adhesion depends on the type of cell surface molecules and the ability of these molecules to interact with extracellular matrix components. Tissue culture plastic is a widely used substrate but is non-native to the body and, while bearing the chance of fractionating a heterogeneous population as seen in the result provided here, it may fail to divide cells based on subtle molecular differences.

As it hasn't been done before, a comparison between cells isolated from both muscle groups adhered to plastic and cells adhered to other substrate such as collagen would give more insight into their biological characteristics and potential applications.

Multipotent cells can differentiate along several lineages. Some lineages tested successfully in cells that can undergo osteogenesis include, adipogenic differentiation, chondrogenic differentiation and neurological differentiation. These cells types form the organ and represent fates that local stem cells may adapt in cases of injury or physiological regeneration. Evidence exists that adult stem cells isolated from different anatomical sites exhibit multipotentiality. Hence, it may have been advantageous to assess the potential of isolated cells to differentiate and trans-differentiate along these lineages.

161

A method that is relatively accessible and can be conducted with ease is analysing the transcriptional activity of genes key to multipotentiality of cells. This could be achieved using mouse pluripotency stem cell arrays from Applied Biosystems. The gene content on these arrays is a result of the work of the I.S.C.I consortium: Characterization of human embryonic stem cell lines by the International Stem Cell Initiative (Adewumi et al., 2007). (https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd= catNavigate2&catID=604089&tab=DetailInfo). The genes can characterise cellular phenotype with respect to stem cellness, and with a careful experimental design, can allow the assessment of differentiation to different mesenchymal lineages, as well as trans-differentiation between these lineages. Furthermore, the cells isolated here, and their potential comparatives isolated from a substrate other than plastic, may be further evaluated with flow cytometry. This is a powerful method to delineate phenotypically different cells in a heterogeneous cell isolate. A point of interest is the possibility of assigning a biological identity to the different isolated cells using flow cytometry, a feat that has not been wholly achieved in the case of the human mesenchymal stromal cell, for example.

However, it is the possibility of the isolated cells to be used in treating some muscle diseases (such as Duchenne muscular dystrophy (DMD) and heterotopic ossification) or bone defects (tumour, large infections). An animal model with some of these diseases would give a more definitive answer of the cells abilities. This could be achieved by using the late adhered cells from both muscle groups in a DMD mouse model assessing the outcome in craniofacial and limb muscle areas. The osteogenic abilities would be investigated by creating a bone defect in craniofacial and long bone regions. Injecting the isolated cells from both groups into the same area where they were isolated from and to the opposite site. i.e. isolated CSkM cells will be injected into craniofacial bone

defect and long bone defect. Same will be adopted for the cells isolated from LM. This experiment would assess if CSKM and LM cells would lead to a beneficial treatment of muscle or bone loss. The outcomes of the above stated steps, if successful, will pave the way for human based experimentations targeted at correcting a particular tissue defect through the design of efficient therapeutic procedures that will isolate cells from muscle and apply them to the body.

References

Adewumi, O., Aflatoonian, B., Ahrlund-Richter, L., Amit, M., Andrews, P. W., Beighton, G., Bello, P. A., Benvenisty, N., Berry, L. S., Bevan S, Blum B, Brooking J, Chen KG, Choo AB, Churchill GA, Corbel M, Damjanov I, Draper JS, Dvorak P, Emanuelsson K, Fleck RA, Ford A, Gertow K, Gertsenstein M, Gokhale PJ, Hamilton RS, Hampl A, Healy LE, Hovatta O, Hyllner J, Imreh MP, Itskovitz-Eldor J, Jackson J, Johnson JL, Jones M, Kee K, King BL, Knowles BB, Lako M, Lebrin F, Mallon BS, Manning D, Mayshar Y, McKay RD, Michalska AE, Mikkola M, Mileikovsky M, Minger SL, Moore HD, Mummery CL, Nagy A, Nakatsuji N, O'Brien CM, Oh SK, Olsson C, Otonkoski T, Park KY, Passier R, Patel H, Patel M, Pedersen R, Pera MF, Piekarczyk MS, Pera RA, Reubinoff BE, Robins AJ, Rossant J, Rugg-Gunn P, Schulz TC, Semb H, Sherrer ES, Siemen H, Stacey GN, Stojkovic M, Suemori H, Szatkiewicz J, Turetsky T, Tuuri T, van den Brink S, Vintersten K, Vuoristo S, Ward D, Weaver TA, Young LA, Zhang W. (2007). Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. Nature Biotechnology, 25 (7), pp.803-816.

Aho, H.J., Aro, H., Juntunen, S., Strengell, L. & Michelsson, J.E. (1988) Bone formation in experimental myositis ossificans. Light and electron microscopy study. APMIS: Acta Pathologica, Microbiologica, Et Immunologica Scandinavica, 96 (10), pp.933-940.

Alhadlaq, A. & Mao, J.J. (2004) Mesenchymal stem cells: isolation and therapeutics. Stem Cells and Development, 13 (4), pp.436-448.

Alison, M.R., Poulsom, R., Forbes, S. & Wright, N.A. (2002) An introduction to stem cells. The Journal of Pathology, 197 (4), pp.419-423.

Ambrosio, F., Ferrari, R.J., Fitzgerald, G.K., Carvell, G., Boninger, M.L. & Huard, Johnny (2009) Functional overloading of dystrophic mice enhances muscle-derived stem cell contribution to muscle contractile capacity. Archives of Physical Medicine and Rehabilitation, 90 (1), pp.66-73.

Arnett, T.R. & Henderson, B. (1998) Methods in bone biology. 1st ed. London, Chapman and Hall.

Aro, H.T., Viljanto, J., Aho, H.J. & Michelsson, J.E. (1991) Macrophages in trauma-induced myositis ossificans. APMIS: Acta Pathologica, Microbiologica, Et Immunologica Scandinavica, 99 (5), pp.482-486.

Arriero, M., Brodsky, S.V., Gealekman, O., Lucas, P.A. & Goligorsky, M.S. (2004) Adult skeletal muscle stem cells differentiate into endothelial lineage and ameliorate renal dysfunction after acute ischemia. American Journal of Physiology. Renal Physiology, 287 (4), pp.F621-627.

Asakura, A, Komaki, M. & Rudnicki, M (2001) Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation; Research in Biological Diversity, 68 (4-5), pp.245-253.

Asakura, A & Rudnicki, M (2002) Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Experimental Hematology, 30 (11), pp.1339-1345.

Asakura, A, Seale, P, Girgis-Gabardo, A & Rudnicki, M A (2002) Myogenic specification of side population cells in skeletal muscle. The Journal of Cell Biology, 159 (1), pp.123-134.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G.C. & Phinney, D.G. (2003) Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. Journal of Cellular Biochemistry, 89 (6), pp.1235-1249.

Beauchamp, J.R., Heslop, L., Yu, D.S., Tajbakhsh, S, Kelly, R G, Wernig, A, Buckingham, M.E., Partridge, T.A. & Zammit, P S (2000) Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. The Journal of Cell Biology, 151 (6), pp.1221-1234.

Blais, A., Tsikitis, M., Acosta-Alvear, D., Sharan, R., Kluger, Y. & Dynlacht, B.D. (2005) An initial blueprint for myogenic differentiation. Genes & Development, 19 (5), pp.553-569.

Blau, H M, Brazelton, T.R. & Weimann, J.M. (2001) The evolving concept of a stem cell: entity or function? Cell, 105 (7), pp.829-841.

Bober, E., Brand-Saberi, B., Ebensperger, C., Wilting, J., Balling, R., Paterson, B.M., Arnold, H.H. & Christ, B. (1994) Initial steps of myogenesis in somites are independent of influence from axial structures. Development (Cambridge, England), 120 (11), pp.3073-3082.

Bosch, P., Musgrave, D.S., Lee, J Y, Cummins, J, Shuler, T., Ghivizzani, T.C., Evans, T., Robbins, T.D. & Huard (2000) Osteoprogenitor cells within skeletal muscle. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society, 18 (6), pp.933-944.

Braun, T. & Arnold, H.H. (1996) Myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. The EMBO Journal, 15 (2), pp.310-318.

Braun, T., Rudnicki, M A, Arnold, H.H. & Jaenisch, R. (1992) Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell, 71 (3), pp.369-382.

Brown, C.B., Engleka, K.A., Wenning, J., Min Lu, M. & Epstein, J.A. (2005) Identification of a hypaxial somite enhancer element regulating Pax3 expression in migrating myoblasts and characterization of hypaxial muscle Cre transgenic mice. Genesis (New York, N.Y.: 2000), 41 (4), pp.202-209.

Bruder, S.P. & Caplan, A.I. (1989) Cellular and molecular events during embryonic bone development. Connective Tissue Research, 20 (1-4), pp.65-71.

Buckingham, M (2001) Skeletal muscle formation in vertebrates. Current Opinion in Genetics & Development, 11 (4), pp.440-448.

Buckingham, Margaret, Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., Montarras, D., Rocancourt, Didier & Relaix, F. (2003) The formation of skeletal muscle: from somite to limb. Journal of Anatomy, 202 (1), pp.59-68.

Burdzińska, A., Gala, K. & Paczek, L. (2008) Myogenic stem cells. Folia Histochemica Et Cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society, 46 (4), pp.401-412.

Burry, R.W. (2010) Immunocytochemistry: A Practical Guide for Biomedical Research. 1st ed. Springer, New York.

Butler-Browne, G.S., Eriksson, P.O., Laurent, C. & Thornell, L.E. (1988) Adult human masseter muscle fibers express myosin isozymes characteristic of development. Muscle & Nerve, 11 (6), pp.610-620.

Buxton, P.G., Bitar, M., Gellynck, K., Parkar, M., Brown, R.A., Young, A.M., Knowles, J.C. & Nazhat, S.N. (2008) Dense collagen matrix accelerates osteogenic differentiation and rescues the apoptotic response to MMP inhibition. Bone, 43 (2), pp.377-385.

Canalis, E., Economides, A.N. & Gazzerro, E. (2003) Bone morphogenetic proteins, their antagonists, and the skeleton. Endocrine Reviews, 24 (2), pp.218-235.

Cao, B & Huard, J (2004) Muscle-derived stem cells. Cell Cycle (Georgetown, Tex.), 3 (2), pp.104-107.

Caplan, A.I. (1991) Mesenchymal stem cells. Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society, 9 (5), pp.641-650.

Chen, Di, Zhao, M. & Mundy, G.R. (2004) Bone morphogenetic proteins. Growth Factors (Chur, Switzerland), 22 (4), pp.233-241.

Chen, J., Shapiro, H.S. & Sodek, J. (1992) Development expression of bone sialoprotein mRNA in rat mineralized connective tissues. Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research, 7 (8), pp.987-997.

Cheng, G., Merriam, A.P., Gong, B., Leahy, P., Khanna, S. & Porter, J.D. (2004) Conserved and muscle-group-specific gene expression patterns shape postnatal development of the novel extraocular muscle phenotype. Physiological Genomics, 18 (2), pp.184-195.

Christ, B. & Ordahl, C.P. (1995) Early stages of chick somite development. Anatomy and Embryology, 191 (5), pp.381-396.

Cikos, S., Bukovská, A. & Koppel, J. (2007) Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Molecular Biology, 8, p.113.

Clemmons, D.R. (2009) Role of IGF-I in skeletal muscle mass maintenance. Trends in Endocrinology and Metabolism: TEM, 20 (7), pp.349-356.

Cooper, W.G. & Konigsberg, I.R. (1961) Dynamics of myogenesis in vitro. The Anatomical Record, 140, pp.195-205.

Cornelison, D.D. & Wold, B.J. (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Developmental Biology, 191 (2), pp.270-283.

Corsi, K.A., Pollett, Jonathan B, Phillippi, J.A., Usas, Arvydas, Li, G. & Huard, Johnny (2007) Osteogenic potential of postnatal skeletal muscle-derived stem cells is influenced by donor sex. Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research, 22 (10), pp.1592-1602.

Cossu, G & Borello, U. (1999) Wnt signaling and the activation of myogenesis in mammals. The EMBO Journal, 18 (24), pp.6867-6872.

Cossu, Giulio, Tajbakhsh, Shahragim & Buckingham, Margaret (1996) How is myogenesis initiated in the embryo? Trends in Genetics, 12 (6), pp.218-223.

Davies, L.C., Locke, M., Webb, R.D.J., Roberts, J.T., Langley, M., Thomas, D.W., Archer, C.W. & Stephens, P. (2010) A multipotent neural crest-derived progenitor cell population is resident within the oral mucosa lamina propria. Stem cells and development, 19 (6), pp.819–830.

Deasy, B M, Gharaibeh, B.M., Pollett, J B, Jones, M.M., Lucas, M.A., Kanda, Y. & Huard, J (2005) Long-term self-renewal of postnatal muscle-derived stem cells. Molecular Biology of the Cell, 16 (7), pp.3323-3333.

Deasy, B M, Qu-Peterson, Z., Greenberger, J.S. & Huard, J (2002) Mechanisms of muscle stem cell expansion with cytokines. Stem Cells (Dayton, Ohio), 20 (1), pp.50-60.

Deasy, B M., Jankowski, R J. & Huard, J (2001) Muscle-Derived Stem Cells: Characterization and Potential for Cell-Mediated Therapy. Blood Cells, Molecules, and Diseases, 27 (5), pp.924-933.

Dell'Agnola, C., Rabascio, C., Mancuso, P., Capillo, M., Pruneri, G., Gobbi, A., Minucci, S., Ronzoni, S., Volorio, S., Calabrese, L., Tradati, N., Martinelli, G., Shultz, L. & Bertolini, F. (2002) In vitro and in vivo hematopoietic potential of human stem cells residing in muscle tissue. Experimental Hematology, 30 (8), pp.905-914.

Dietrich, S, Schubert, F.R., Healy, C., Sharpe, P.T. & Lumsden, A. (1998) Specification of the hypaxial musculature. Development (Cambridge, England), 125 (12), pp.2235-2249.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L. & Karsenty, G. (1997) Osf2/Cbfa1: A Transcriptional Activator of Osteoblast Differentiation. Cell, 89 (5), pp.747-754.

Dupin, E., Calloni, G., Real, C., Gonçalves-Trentin, A. & Le Douarin, N.M. (2007) Neural crest progenitors and stem cells. Comptes Rendus Biologies, 330 (6-7), pp.521-529.

Ehnert, S., Glanemann, M., Schmitt, A., Vogt, S., Shanny, N., Nussler, N.C., Stöckle, U. & Nussler, A. (2009) The possible use of stem cells in regenerative medicine: dream or reality? Langenbeck's archives of surgery / Deutsche Gesellschaft für Chirurgie, 394 (6), pp.985–997.

Evans, D.J.R. & Noden, Drew M (2006) Spatial relations between avian craniofacial neural crest and paraxial mesoderm cells. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 235 (5), pp.1310-1325.

Farhadieh, R.D., Gianoutsos, M.P., Yu, Y. & Walsh, W.R. (2004) The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. The Journal of Craniofacial Surgery, 15 (5), pp.714-718.

Fernandes, K.J.L., McKenzie, I.A., Mill, P., Smith, K.M., Akhavan, M., Barnabé-Heider, F., Biernaskie, J., Junek, A., Kobayashi, N.R., Toma, J.G., Kaplan, D.R., Labosky, P.A., Rafuse, V., Hui, C.-C. & Miller, F.D. (2004) A dermal niche for multipotent adult skin-derived precursor cells. Nature cell biology, 6 (11), pp.1082–1093.

Gharaibeh, B., Lu, Aiping, Tebbets, J., Zheng, Bo, Feduska, J., Crisan, M., Peault, B., Cummins, James & Huard, Johnny (2008) Isolation of a slowly adhering cell fraction containing stem cells from murine skeletal muscle by the preplate technique. Nat. Protocols, 3 (9), pp.1501-1509.

Gilbert, S.F. & Singer, S.R. (2006) Developmental biology. Sinauer Associates, Inc. Sunderland, USA.

Golub, E.E. & Boesze-Battaglia, K. (2007) The role of alkaline phosphatase in mineralization. Current Opinion in Orthopaedics, 18, pp.444-448.

Grefte, S., Kuijpers-Jagtman, A.M., Torensma, R. & Von den Hoff, J.W. (2007) Skeletal muscle development and regeneration. Stem Cells and Development, 16 (5), pp.857-868.

Grenier, J., Teillet, M.-A., Grifone, R., Kelly, Robert G & Duprez, Delphine (2009) Relationship between neural crest cells and cranial mesoderm during head muscle development. PloS One, 4 (2), p.e4381.

Gronthos, S, Mankani, M., Brahim, J, Robey, P G & Shi, S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America, 97 (25), pp.13625–13630.

Gros, J., Scaal, M. & Marcelle, C. (2004) A two-step mechanism for myotome formation in chick. Developmental Cell, 6 (6), pp.875-882.

Gussoni, E, Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L M & Mulligan, R.C. (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature, 401 (6751), pp.390-394.

Hall, B.K. (1988) The Embryonic Development of Bone. American Scientist, 76 (2), pp.174-181.

Ham, R.G., St Clair, J.A., Webster, C. & Blau, H M (1988) Improved media for normal human muscle satellite cells: serum-free clonal growth and enhanced growth with low serum. In Vitro Cellular & Developmental Biology: Journal of the Tissue Culture Association, 24 (8), pp.833-844.

Hashimoto, N., Kiyono, T., Wada, M.R., Umeda, R., Goto, Y.-ichi, Nonaka, I., Shimizu, S., Yasumoto, S. & Inagawa-Ogashiwa, M. (2008) Osteogenic properties of human myogenic progenitor cells. Mechanisms of Development, 125 (3-4), pp.257-269.

Hatori, M., Klatte, K.J., Teixeira, C.C. & Shapiro, I.M. (1995) End labeling studies of fragmented DNA in the avian growth plate: evidence of apoptosis in terminally differentiated chondrocytes. Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research, 10 (12), pp.1960-1968.

Hawke, T.J. & Garry, D.J. (2001) Myogenic satellite cells: physiology to molecular biology. Journal of Applied Physiology, 91 (2), pp.534 -551.

Ho, M.M., Ng, A.V., Lam, S. & Hung, J.Y. (2007) Side Population in Human Lung Cancer Cell Lines and Tumors Is Enriched with Stem-like Cancer Cells. Cancer Research, 67 (10), pp.4827 -4833.

Huard, J, Yokoyama, T., Pruchnic, R., Qu, Z., Li, Y., Lee, J Y, Somogyi, G.T., de Groat, W.C. & Chancellor, M.B. (2002) Muscle-derived cell-mediated ex vivo gene therapy for urological dysfunction. Gene therapy, 9 (23), pp.1617–1626.

Ito, Y. & Miyazono, K. (2003) RUNX transcription factors as key targets of TGF-beta superfamily signaling. Current Opinion in Genetics & Development, 13 (1), pp.43-47.

Jackson, Kathyjo Ann, Mi, T. & Goodell, M.A. (1999) Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proceedings of the National Academy of Sciences, 96 (25), pp.14482 -14486.

Jankowski, R J, Deasy, B M & Huard, J (2002) Muscle-derived stem cells. Gene Therapy, 9 (10), pp.642-647.

Jankowski, R J, Haluszczak, C., Trucco, M. & Huard, J (2001) Flow cytometric characterization of myogenic cell populations obtained via the preplate technique: potential for rapid isolation of muscle-derived stem cells. Human Gene Therapy, 12 (6), pp.619-628.

Jiang, Y., Vaessen, B., Lenvik, T., Blackstad, M., Reyes, M. & Verfaillie, C.M. (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Experimental Hematology, 30 (8), pp.896-904.

Jonason, J.H., Xiao, G., Zhang, M., Xing, L. & Chen, D (2009) Post-translational Regulation of Runx2 in Bone and Cartilage. Journal of Dental Research, 88 (8), pp.693-703.

Jonker, J.W., Freeman, J., Bolscher, E., Musters, S., Alvi, A.J., Titley, I., Schinkel, A.H. & Dale, T.C. (2005) Contribution of the ABC transporters Bcrp1 and Mdr1a/1b to the side population phenotype in mammary gland and bone marrow of mice. Stem Cells (Dayton, Ohio), 23 (8), pp.1059-1065.

Kafadar, K.A., Yi, L., Ahmad, Y., So, L., Rossi, F. & Pavlath, Grace K (2009) Sca-1 expression is required for efficient remodeling of the extracellular matrix during skeletal muscle regeneration. Developmental Biology, 326 (1), pp.47-59.

Kan, L., Hu, M., Gomes, W.A. & Kessler, J.A. (2004) Transgenic mice overexpressing BMP4 develop a fibrodysplasia ossificans progressiva (FOP)-like phenotype. The American Journal of Pathology, 165 (4), pp.1107-1115.

Kaplan, F S & Smith, R.M. (1997) Fibrodysplasia ossificans progressiva (FOP). Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research, 12 (5), p.855.

Katagiri, T., Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J.M., Fujisawa-Sehara, A. & Suda, T. (1994) Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. The Journal of Cell Biology, 127 (6 Pt 1), pp.1755-1766.

Katz, B. (1961) The Terminations of the Afferent Nerve Fibre in the Muscle Spindle of the Frog. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 243 (703), pp.221 -240.

Kelly, Robert G, Jerome-Majewska, L.A. & Papaioannou, V.E. (2004) The del22q11.2 candidate gene Tbx1 regulates branchiomeric myogenesis. Human Molecular Genetics, 13 (22), pp.2829-2840.

Khouri, R.K., Koudsi, B. & Reddi, H. (1991) Tissue transformation into bone in vivo. A potential practical application. JAMA: The Journal of the American Medical Association, 266 (14), pp.1953-1955.

Kim, K.S., Lee, J.H., Ahn, H.H., Lee, Ju Young, Khang, G., Lee, B., Lee, H.B. & Kim, M.S. (2008) The osteogenic differentiation of rat muscle-derived stem cells in vivo within in situ-forming chitosan scaffolds. Biomaterials, 29 (33), pp.4420-4428.

Kim, M., Turnquist, H., Jackson, J., Sgagias, M., Yan, Y., Gong, M., Dean, M., Sharp, J.G. & Cowan, K. (2002) The Multidrug Resistance Transporter ABCG2 (Breast Cancer Resistance Protein 1) Effluxes Hoechst 33342 and Is Overexpressed in Hematopoietic Stem Cells. Clinical Cancer Research, 8 (1), pp.22 -28.

Knudsen, K.A., McElwee, S.A. & Myers, L. (1990) A role for the neural cell adhesion molecule, NCAM, in myoblast interaction during myogenesis. Developmental Biology, 138 (1), pp.159-168.

Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R.T., Gao, Y.H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. & Kishimoto, T. (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell, 89 (5), pp.755-764.

Krebsbach, P.H. & Robey, P.G. (2002) Dental and skeletal stem cells: potential cellular therapeutics for craniofacial regeneration. Journal of Dental Education, 66 (6), pp.766-773.

Kubo, S., Cooper, G.M., Matsumoto, T., Phillippi, J.A., Corsi, K.A., Usas, Arvydas, Li, G., Fu, Freddie H & Huard, Johnny (2009) Blocking vascular endothelial growth factor with soluble Flt-1 improves the chondrogenic potential of mouse skeletal muscle-derived stem cells. Arthritis and Rheumatism, 60 (1), pp.155-165.

Kuratani, S. (2005) Developmental studies on the vertebrate head evolution. Zoological Science, 22 (12), pp.1361-1366.

Kuroda, R., Usas, Arvydas, Kubo, S., Corsi, K., Peng, Hairong, Rose, T., Cummins, James, Fu, Freddie H & Huard, Johnny (2006) Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. Arthritis and Rheumatism, 54 (2), pp.433-442.

Lavker, R.M. & Sun, T.T. (2000) Epidermal stem cells: properties, markers, and location. Proceedings of the National Academy of Sciences of the United States of America, 97 (25), pp.13473–13475.

Lee, J Y, Peng, H., Usas, A., Musgrave, D., Cummins, J., Pelinkovic, D., Jankowski, R., Ziran, B., Robbins, P. & Huard, Johnny (2002) Enhancement of bone healing based on ex vivo gene therapy using human muscle-derived cells expressing bone morphogenetic protein 2. Human gene therapy, 13 (10), pp.1201–1211.

Lee, J Y, Qu-Petersen, Z, Cao, B, Kimura, S, Jankowski, R, Cummins, J, Usas, A, Gates, C, Robbins, P., Wernig, A & Huard, J (2000) Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. The Journal of Cell Biology, 150 (5), pp.1085-1100.

Levy, M.M., Joyner, C.J., Virdi, A.S., Reed, A., Triffitt, J.T., Simpson, A.H., Kenwright, J., Stein, H. & Francis, M.J. (2001) Osteoprogenitor cells of mature human skeletal muscle tissue: an in vitro study. Bone, 29 (4), pp.317-322.

Li, Y. & Huard, J (2002) Differentiation of muscle-derived cells into myofibroblasts in injured skeletal muscle. The American Journal of Pathology, 161 (3), pp.895-907.

Linker, C., Lesbros, C., Stark, M.R. & Marcelle, C. (2003) Intrinsic signals regulate the initial steps of myogenesis in vertebrates. Development (Cambridge, England), 130 (20), pp.4797-4807.

Liu, R., Schindeler, A. & Little, D.G. (2010) The potential role of muscle in bone repair. Journal of Musculoskeletal & Neuronal Interactions, 10 (1), pp.71-76.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.), 25 (4), pp.402-408.

Lu, J.-R., Bassel-Duby, R., Hawkins, A., Chang, P., Valdez, R., Wu, H., Gan, L., Shelton, J.M., Richardson, J.A. & Olson, E.N. (2002) Control of facial muscle development by MyoR and capsulin. Science (New York, N.Y.), 298 (5602), pp.2378-2381.

Lu, S.-H., Yang, A.-H., Wei, C.-F., Chiang, H.S. & Chancellor, M.B. (2009) Multi-potent differentiation of human purified muscle-derived cells: potential for tissue regeneration. BJU International. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19712114> [Accessed 8 January 2012].

Mahboubi, S., Glaser, D L, Shore, E M & Kaplan, F S (2001) Fibrodysplasia ossificans progressiva. Pediatric Radiology, 31 (5), pp.307-314.

Majka, S.M., Jackson, Kathyjo A, Kienstra, K.A., Majesky, M.W., Goodell, M.A. & Hirschi, K.K. (2003) Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. The Journal of Clinical Investigation, 111 (1), pp.71-79.

Martini, F. & Ober, W.C. (2006) Fundamentals of anatomy & physiology. 7th ed. Pearson Benjamin Cummings.

Mastrogiacomo, M., Derubeis, A.R. & Cancedda, R. (2005) Bone and cartilage formation by skeletal muscle derived cells. Journal of Cellular Physiology, 204 (2), pp.594-603.

Mauro, A. (1961) Satellite cell of skeletal muscle fibers. The Journal of Biophysical and Biochemical Cytology, 9, pp.493-495.

McKinney-Freeman, S.L., Jackson, Kathyjo A, Camargo, F.D., Ferrari, G., Mavilio, F. & Goodell, M.A. (2002) Muscle-derived hematopoietic stem cells are hematopoietic in origin. Proceedings of the National Academy of Sciences of the United States of America, 99 (3), pp.1341-1346.

McKinney-Freeman, S.L., Majka, S.M., Jackson, Kathyjo A, Norwood, K., Hirschi, K.K. & Goodell, M.A. (2003) Altered phenotype and reduced function of muscle-derived hematopoietic stem cells. Experimental Hematology, 31 (9), pp.806-814.

McLoon, L.K., Thorstenson, K.M., Solomon, A. & Lewis, M P (2007) Myogenic precursor cells in craniofacial muscles. Oral Diseases, 13 (2), pp.134-140.

Meeson, A.P., Hawke, T.J., Graham, S., Jiang, N., Elterman, J., Hutcheson, K., Dimaio, J.M., Gallardo, T.D. & Garry, D.J. (2004) Cellular and molecular regulation of skeletal muscle side population cells. Stem Cells (Dayton, Ohio), 22 (7), pp.1305-1320.

Menasché, P. (2008) Skeletal myoblasts and cardiac repair. Journal of molecular and cellular cardiology, 45 (4), pp.545–553.

Menetrey, J., Kasemkijwattana, C., Day, C.S., Bosch, P., Vogt, M., Fu, F H, Moreland, M.S. & Huard, J (2000) Growth factors improve muscle healing in vivo. The Journal of Bone and Joint Surgery. British Volume, 82 (1), pp.131-137.

Menko, A.S. & Boettiger, D. (1987) Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell, 51 (1), pp.51-57.

Mitchell, P.J., Timmons, P.M., Hébert, J.M., Rigby, P.W. & Tjian, R. (1991) Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Genes & Development, 5 (1), pp.105-119.

Mitchell, P.O., Mills, T., O'Connor, R.S., Kline, E.R., Graubert, T., Dzierzak, E. & Pavlath, Grace K (2005) Sca-1 negatively regulates proliferation and differentiation of muscle cells. Developmental Biology, 283 (1), pp.240-252.

Mootoosamy, R.C. & Dietrich, Susanne (2002) Distinct regulatory cascades for head and trunk myogenesis. Development (Cambridge, England), 129 (3), pp.573-583.

Münsterberg, A.E., Kitajewski, J., Bumcrot, D.A., McMahon, A.P. & Lassar, A B (1995) Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. Genes & Development, 9 (23), pp.2911-2922.

Musgrave, D.S., Pruchnic, R, Bosch, P., Ziran, B.H., Whalen, J. & Huard, J (2002) Human skeletal muscle cells in ex vivo gene therapy to deliver bone morphogenetic protein-2. The Journal of Bone and Joint Surgery. British Volume, 84 (1), pp.120-127.

Nilsson, O.S., Urist, M.R., Dawson, E.G., Schmalzried, T.P. & Finerman, G.A. (1986) Bone repair induced by bone morphogenetic protein in ulnar defects in dogs. The Journal of Bone and Joint Surgery. British Volume, 68 (4), pp.635-642.

Noden, D M (1983) The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. Developmental Biology, 96 (1), pp.144-165.

Noden, D M & Francis-West, P. (2006) The differentiation and morphogenesis of craniofacial muscles. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 235 (5), pp.1194-1218.

Noden, D M & Schneider, R.A. (2006) Neural crest cells and the community of plan for craniofacial development: historical debates and current perspectives. Advances in Experimental Medicine and Biology, 589, pp.1-23.

Noden, D M & Trainor, P.A. (2005) Relations and interactions between cranial mesoderm and neural crest populations. Journal of Anatomy, 207 (5), pp.575-601.

Nolazco, G., Kovanecz, I., Vernet, D., Gelfand, R.A., Tsao, J., Ferrini, M.G., Magee, T., Rajfer, J. & Gonzalez-Cadavid, N.F. (2008) Effect of muscle-derived stem cells on the restoration of corpora cavernosa smooth muscle and erectile function in the aged rat. BJU International, 101 (9), pp.1156-1164.

Ogata, Y. (2008) Bone sialoprotein and its transcriptional regulatory mechanism. Journal of Periodontal Research, 43 (2), pp.127-135.

Ojima, K., Uezumi, A., Miyoshi, H., Masuda, S., Morita, Y., Fukase, A., Hattori, A., Nakauchi, H., Miyagoe-Suzuki, Y. & Takeda, S. (2004) Mac-1(low) early myeloid cells in the bone marrow-derived SP fraction migrate into injured skeletal muscle and participate in muscle regeneration. Biochemical and Biophysical Research Communications, 321 (4), pp.1050-1061.

Ono, Y., Boldrin, L., Knopp, P., Morgan, J.E. & Zammit, Peter S (2010) Muscle satellite cells are a functionally heterogeneous population in both somite-derived and branchiomeric muscles. Developmental Biology, 337 (1), pp.29-41.

Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosewell, I.R., Stamp, G.W., Beddington, R.S., Mundlos, S., Olsen, B.R., Selby, P.B. & Owen, M.J. (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell, 89 (5), pp.765-771.

Paulin, D & Li, Z (2004) Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. Experimental Cell Research, 301 (1), pp.1-7.

Pavlath, G K, Thaloor, D., Rando, T A, Cheong, M., English, A.W. & Zheng, B (1998) Heterogeneity among muscle precursor cells in adult skeletal muscles with differing regenerative capacities. Developmental Dynamics: An Official Publication of the American Association of Anatomists, 212 (4), pp.495-508.

Peng, H & Huard, J (2004) Muscle-derived stem cells for musculoskeletal tissue regeneration and repair. Transplant Immunology, 12 (3-4), pp.311-319.

Pownall, M.E. & Emerson, C.P., Jr (1992) Sequential activation of three myogenic regulatory genes during somite morphogenesis in quail embryos. Developmental Biology, 151 (1), pp.67-79.

Price, N., Hunt, N P & Lewis, M P (1998) Expression of an embryonic fibronectin splicing variant in human masseter muscle. Archives of Oral Biology, 43 (11), pp.911-915.

Qu, Z., Balkir, L., van Deutekom, J.C., Robbins, P.D., Pruchnic, R & Huard, J (1998) Development of approaches to improve cell survival in myoblast transfer therapy. The Journal of Cell Biology, 142 (5), pp.1257-1267.

Qu-Petersen, Zhuqing, Deasy, B., Jankowski, Ron, Ikezawa, Makato, Cummins, James, Pruchnic, Ryan, Mytinger, J., Cao, Baohong, Gates, Charley, Wernig, Anton & Huard, Johnny (2002) Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. The Journal of Cell Biology, 157 (5), pp.851-864.

Rando, T A & Blau, H M (1994) Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. The Journal of Cell Biology, 125 (6), pp.1275-1287.

Reilly, T.M., Seldes, R., Luchetti, W. & Brighton, C.T. (1998) Similarities in the phenotypic expression of pericytes and bone cells. Clinical Orthopaedics and Related Research, (346), pp.95-103.

Renault, V., Rolland, E., Thornell, L.-E., Mouly, V. & Butler-Browne, G. (2002) Distribution of satellite cells in the human vastus lateralis muscle during aging. Experimental Gerontology, 37 (12), pp.1513-1514.

Richler, C. & Yaffe, D. (1970) The in vitro cultivation and differentiation capacities of myogenic cell lines. Developmental Biology, 23 (1), pp.1-22.

Rinon, A., Lazar, S., Marshall, H., Büchmann-Møller, S., Neufeld, A., Elhanany-Tamir, H., Taketo, M.M., Sommer, L., Krumlauf, R. & Tzahor, E. (2007) Cranial neural crest cells regulate head muscle patterning and differentiation during vertebrate embryogenesis. Development (Cambridge, England), 134 (17), pp.3065-3075.

Romero-Ramos, M., Vourc'h, P., Young, H.E., Lucas, P.A., Wu, Y., Chivatakarn, O., Zaman, R., Dunkelman, N., el-Kalay, M.A. & Chesselet, M.-F. (2002) Neuronal differentiation of stem cells isolated from adult muscle. Journal of Neuroscience Research, 69 (6), pp.894-907.

Rouger, K., Fornasari, B., Armengol, V., Jouvion, G., Leroux, I., Dubreil, L., Feron, M., Guevel, L. & Cherel, Y. (2007) Progenitor cell isolation from muscle-derived cells based on adhesion properties. The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society, 55 (6), pp.607-618.

Royer, C.L., Howell, J.C., Morrison, P.R., Srour, E.F. & Yoder, M.C. (2002) Muscle-derived CD45-SCA-1+c-kit- progenitor cells give rise to skeletal muscle myotubes in vitro. In Vitro Cellular & Developmental Biology. Animal, 38 (9), pp.512-517.

Rudnicki, M A, Braun, T., Hinuma, S. & Jaenisch, R. (1992) Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell, 71 (3), pp.383-390.

Sambasivan, R. & Tajbakhsh, S. (2007) Skeletal muscle stem cell birth and properties. Seminars in Cell & Developmental Biology, 18 (6), pp.870-882.

Sawyer, J.R., Myers, M.A., Rosier, R.N. & Puzas, J.E. (1991) Heterotopic ossification: clinical and cellular aspects. Calcified Tissue International, 49 (3), pp.208-215.

Schmittgen, T.D., Zakrajsek, B.A., Mills, A.G., Gorn, V., Singer, M.J. & Reed, M.W. (2000) Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. Analytical Biochemistry, 285 (2), pp.194-204.

Schor, A.M., Canfield, A.E., Sutton, A.B., Arciniegas, E. & Allen, T.D. (1995) Pericyte differentiation. Clinical Orthopaedics and Related Research, (313), pp.81-91.

Seale, P & Rudnicki, M A (2000) A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Developmental Biology, 218 (2), pp.115-124.

Seale, Patrick, Ishibashi, J., Holterman, C. & Rudnicki, Michael A (2004) Muscle satellite cell-specific genes identified by genetic profiling of MyoD-deficient myogenic cell. Developmental Biology, 275 (2), pp.287-300.

Seo, B.-M., Miura, M., Gronthos, Stan, Bartold, P.M., Batouli, S., Brahim, Jaime, Young, M., Robey, Pamela Gehron, Wang, C.-Y. & Shi, Songtao (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet, 364 (9429), pp.149–155.

Shen, H.-C., Peng, Hairong, Usas, Arvydas, Gearhart, B., Cummins, James, Fu, Freddie H & Huard, Johnny (2004) Ex vivo gene therapy-induced endochondral bone formation: comparison of muscle-derived stem cells and different subpopulations of primary muscle-derived cells. Bone, 34 (6), pp.982-992.

Sherwood, R.I., Christensen, J.L., Conboy, I.M., Conboy, M.J., Rando, Thomas A, Weissman, I.L. & Wagers, A.J. (2004) Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell, 119 (4), pp.543-554.

Sherwood, R.I., Christensen, J.L., Weissman, I.L. & Wagers, A.J. (2004) Determinants of skeletal muscle contributions from circulating cells, bone marrow cells, and hematopoietic stem cells. Stem Cells (Dayton, Ohio), 22 (7), pp.1292-1304.

Sinanan, A.C.M., Buxton, Paul G & Lewis, Mark P (2006) Muscling in on stem cells. Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 98 (4), pp.203-214.

Sinanan, A.C.M., Hunt, Nigel P & Lewis, Mark P (2004) Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. Biotechnology and Applied Biochemistry, 40 (Pt 1), pp.25-34.

Smith, R. & Triffitt, J.T. (1986) Bones in muscles: the problems of soft tissue ossification. The Quarterly Journal of Medicine, 61 (235), pp.985-990.

Sperber, G.H. (1989) Craniofacial Embryology. 4th ed. Oxford, Butterworth-Heinemann Ltd.

Stewart, C.E.H. & Rittweger, J. (2006) Adaptive processes in skeletal muscle: molecular regulators and genetic influences. Journal of Musculoskeletal & Neuronal Interactions, 6 (1), pp.73-86.

Strauer, B.E. and Kornowski, R. (2003) Stem Cell Therapy in Perspective. Circulation, 107 (7), pp.929–934.

Summer, R., Kotton, D.N., Sun, X., Ma, B., Fitzsimmons, K. & Fine, A. (2003) Side population cells and Bcrp1 expression in lung. American Journal of Physiology. Lung Cellular and Molecular Physiology, 285 (1), pp.L97-104.

Tajbakhsh, S (2009) Skeletal muscle stem cells in developmental versus regenerative myogenesis. Journal of Internal Medicine, 266 (4), pp.372-389.

Tajbakhsh, S & Buckingham, M (2000) The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. Current Topics in Developmental Biology, 48, pp.225-268.

Tajbakhsh, S, Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D, Buckingham, M & Cossu, G (1998) Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development (Cambridge, England), 125 (21), pp.4155-4162.

Tajbakhsh, S, Rocancourt, D, Cossu, G & Buckingham, M (1997) Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell, 89 (1), pp.127-138.

Torrente, Y, Tremblay, J P, Pisati, F., Belicchi, M., Rossi, B., Sironi, M., Fortunato, F., El Fahime, M., D'Angelo, M.G., Caron, N.J., Constantin, G., Paulin, D, Scarlato, G. & Bresolin, N. (2001) Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. The Journal of Cell Biology, 152 (2), pp.335-348.

Tortora, G.J. & Derrickson, B. (2009) Principles of anatomy and physiology. 12th ed. Hoboken, NJ, John Wiley & Sons, Inc.

Tzahor, E., Kempf, H., Mootoosamy, R.C., Poon, A.C., Abzhanov, A., Tabin, C.J., Dietrich, Susanne & Lassar, Andrew B (2003) Antagonists of Wnt and BMP signaling promote the formation of vertebrate head muscle. Genes & Development, 17 (24), pp.3087-3099.

Uchida, N., Buck, D.W., He, D., Reitsma, M.J., Masek, M., Phan, T.V., Tsukamoto, A.S., Gage, F.H. & Weissman, I.L. (2000) Direct isolation of human central nervous system stem cells. Proceedings of the National Academy of Sciences of the United States of America, 97 (26), pp.14720–14725.

Uezumi, A., Ojima, K., Fukada, S.-ichiro, Ikemoto, M., Masuda, S., Miyagoe-Suzuki, Y. & Takeda, S. (2006) Functional heterogeneity of side population cells in skeletal muscle. Biochemical and Biophysical Research Communications, 341 (3), pp.864-873.

Urist, M.R. (1965) Bone: formation by autoinduction. Science (New York, N.Y.), 150 (698), pp.893-899.

Urist, M.R. & Strates, B.S. (1971) Bone morphogenetic protein. Journal of Dental Research, 50 (6), pp.1392-1406.

Urist, M.R., DeLange, R.J. & Finerman, G.A. (1983) Bone cell differentiation and growth factors. Science (New York, N.Y.), 220 (4598), pp.680-686.

Urist, M.R., Jurist, J.M., Jr, Dubuc, F.L. & Strates, B.S. (1970) Quantitation of new bone formation in intramuscular implants of bone matrix in rabbits. Clinical Orthopaedics and Related Research, 68, pp.279-293.

Usas, Arvydas, Ho, A.M., Cooper, G.M., Olshanski, A., Peng, Hairong & Huard, Johnny (2009) Bone regeneration mediated by BMP4-expressing muscle-derived stem cells is affected by delivery system. Tissue Engineering. Part A, 15 (2), pp.285-293.

Van de Putte, K.A. & Urist, M.R. (1965) Osteogenesis in the interior of intramuscular implants of decalcified bone matrix. Clinical Orthopaedics and Related Research, 43, pp.257-270.

Vessey, C.J. & de la Hall, P.M. (2001) Hepatic stem cells: a review. Pathology, 33 (2), pp.130–141.

Vogel, G. (2001) Stem cell policy. Can adult stem cells suffice? Science, 292 (5523), pp.1820–1822.

Wagers, A.J., Christensen, J.L. & Weissman, I.L. (2002) Cell fate determination from stem cells. Gene therapy, 9 (10), pp.606–612.

Widera, D., Zander, C., Heidbreder, M., Kasperek, Y., Noll, T., Seitz, O., Saldamli, B., Sudhoff, H., Sader, R., Kaltschmidt, C. & Kaltschmidt, B. (2009) Adult palatum as a novel source of neural crest-related stem cells. Stem cells (Dayton, Ohio), 27 (8), pp.1899–1910.

Williams, T. & Tjian, R. (1991) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. Genes & Development, 5 (4), pp.670-682.

Yaffe, D. (1968) Retention of differentiation potentialities during prolonged cultivation of myogenic cells. Proceedings of the National Academy of Sciences of the United States of America, 61 (2), pp.477-483.

Yamane, A. (2005) Embryonic and postnatal development of masticatory and tongue muscles. Cell and Tissue Research, 322 (2), pp.183-189.

Yan, Z., Lin, Y., Jiao, X., Li, Zhiyong, Wu, L., Jing, W., Qiao, J., Liu, L., Tang, W., Zheng, X. & Tian, W. (2006) Characterization of ectomesenchymal cells isolated from the first branchial arch during multilineage differentiation. Cells, Tissues, Organs, 183 (3), pp.123-132.

Appendices

Appendix A. Materials

Materials used for RNA extraction and quality control

Experimental step	Materials	Supplying company
RNase inhibition -Clean surfaces -Tips -Centrifuge tubes -Water	-RNase*Zap*® wipes -RNase® Zap spray -Eppendorf® epT.I.P.S, -RNase/ DNase free centrifuge tubes -RNase free water	-Ambion (Europe) Ltd, Applied Biosystems, Warrington, UK -Sigma-Aldrich Ltd, Dorset, UK. -Qiagen, West Sussex, UK -Qiagen, West Sussex, UK
Purification and elution -selective binding -Elusion	-Ethanol -RNeasy® mini kit	-Fisher Scientific UK Ltd, Loughborough, UK -Qiagen, West Sussex, UK
RNA quantity and purity -Spectrophotometer	-Ultrospec 2000 machine - Tecan M200	-Amersham Pharmacia Biotech, GE Healthcare Ltd, Buckinghamshire, UK -TECAN group Ltd Switzerland

Materials used for quantitative RT-PCR (qRT-PCR)

Experimental step	Materials	Supplying company
<u>cDNA synthesis</u>	-High-capacity cDNA reverse transcription kit - PTC-100 Thermocycler machine,	* -MJ Research Inc, Genetic research instrumentation Ltd, Essex, UK
<u>qRT-PCR</u>	-File builder software (to design LOC gene assay) -TaqMan® universal PCR master mix -MicroAmp® optical 96-well reaction plates -MicroAmp® optical adhesive cover -7300 Real time PCR machine* -7300 system software* -Large centrifuge	* * * * * * -Jouan CR4.12, DJB labcare Ltd, Buckinghamshire, UK.
*Supplied by Applied Biosystems, Warrington, UK.		

Appendix B. Protocols

Isolation and differentiation of isolated muscle mononuclear cells protocol

Materials for muscle and cells isolation:

70% ethanol in a squirt bottle

Sterile phosphate buffered saline (PBS)

Collagenase/Dispase/CaCl$_2$ solution

DMEM medium

Neonatal mice, preferably 1-3 days old

Sharp curved surgical scissors

2 pairs of fine forceps

Low power stereo dissecting microscope

Sterile razor blade

70 μm nylon mesh

Small sterile funnel

Humidified 37°C, 5% CO$_2$ incubator

Inverted microscope

T75 flask

Solutions for muscle and cells isolation:

Collagenase D

(Roche #1 088 858) >0.15 U/mg

Reconstitute 100 mg in 10 ml D-PBS = 1.5 U/ml = 10 mg/ml

Filter sterilize. Store –20 C in 1 ml aliquots

Dispase II

(Roche #295 825) 2.4 U/ml

Liquid 1x (working conc. 0.6–2.4 U/ml)

Sterile. Store –20 C in aliquots

1. Dissolve 10 mg dispase in 1 ml PBS (without calcium and magnesium). This concentration is 10mg/ml. It is also 10 U/ml.

2. Dilute that stock 1:5 by adding 4 ml PBS to 1 ml stock (10 U/ml dispase). The concentration is now 2 U/ml.

100 mM CaCl2

1.47 g/100 ml

Filter sterilize

Collagenase/Dispase/CaCl2 solution

1 ml 1.5 U/ml collagenase (Final conc. 0.75 U/ml)

1 ml 2.4 U/ml dispase (Final conc. 1.2 U/ml)

50 µl 100 mM CaCl2 (Final conc. 2.5 mM)

DMEM medium

500 ml DMEM Low Glucose (1g/l) with L-Glutamine.

50 ml foetal bovine serum (10% final)

5 ml 100x penicillin/streptomycin (GIBCO BRL; final concentration is 100 U/ml and 100 µg/ml, respectively)

Store at 8°C

Phosphate buffered saline (PBS)

Store at room temperature.

Primary Culture preparation:

The primary culture will compose of Craniofacial and hind limb muscles of neonate mice.

All muscle samples will be isolated using enzymatic dissociation technique previously described by Rando and Blau, (1994).

Isolate craniofacial muscle

Sacrifice 10 neonatal mice by decapitation.

Rinse the head with 70% ethanol and remove the skin with sterile scissors. Dissect the muscle (Masseter) bilaterally away from the skin and bone with sterile forceps.

Dissection is easier if done under a stereo dissecting microscope. Store the muscle tissue in a culture dish on ice in a drop of sterile PBS as successive muscles are being processed, maintaining sterility in the accumulated tissue.

Dissociate muscle cells

All subsequent steps should be done in a sterile tissue culture hood

Add enough PBS to keep the tissue moist, and mince to slurry with razor blades in the culture dish.

Transfer to 50 ml tube with 10 ml PBS and let sit to allow fat to float in the buffer. Discard buffer carefully

Add approximately 2 ml of collagenase/dispase/CaCl$_2$ solution per gram or per 250mg of tissue and continue mincing for several minutes.

Transfer the minced tissue to a sterile tube and incubate at 37°C until the mixture is fine slurry, usually about 20-30 min. in shaking water bath. (Gently triturate with a plastic pipette to break up clumps 2-3 times while incubation with collagenase solution).

If desired, the slurry can be filtered through a piece of 70 μm nylon mesh in a sterile funnel to remove large pieces of tissue. (You may need to add equal amount of the growth medium to the solution)

Centrifuge the cells for 5 min at 1000 rpm. Re-suspend the pellet in 10 ml of DMEM medium supplemented with 10% FBS and 1% P/S, and plate in T75 culture flask.

It is common to see a great deal of debris and very few recognizable cells; however, after two days, many cells will be evident and the debris will be rinsed away during the first change of medium.

Incubate in a 37°C 5% CO_2 incubator for 1hr. transfer the supernatant from the original flask (Pre-plate 1 (PP1)) into new flask. The original flask will be replenished with a fresh medium.

The supernatant from flask 2 (Pre-plate 2 (PP2)) will be transferred to new flask (PP3) after incubation for 48h. The PP2 flask will be replenished with a fresh medium.

The cells in PP3 will be allowed to settle for about 5 d

Medium will be changed every 2-3 days for all pre-plates.

When the cells are ready to be split, remove the cells from the dish using Trypsin as Follows:

View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.

Remove spent medium.

Wash the cell monolayer with PBS without Ca_2^+/Mg_2^+ using a volume equivalent to half the volume of culture medium. Repeat this wash step if the cells are known to adhere strongly.

Pipette trypsin/EDTA onto the washed cell monolayer using 1ml per 25 cm^2 of surface area. Rotate flask to cover the monolayer with trypsin. Decant the excess trypsin.

Return flask to the incubator and leave for 2-10 min

Examine the cells using an inverted microscope to ensure that all/majority of the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.

Resuspended the cells in a small volume of fresh serum-containing medium (approx 3-4 x the volume of trypsin) to inactivate the trypsin. Centrifuge cells at 1000rpm for 3-5 minutes and resuspended before performing a cell count.

Transfer the required number of cells to a new labelled flask containing pre-warmed medium. Incubate in a 37ºC, 5% CO_2 incubator until confluent.

In vitro **cell differentiation:**

Media used:

Osteogenic: DMEM+ 10%FBS+ 1%P/S+ 100ng/ml BMP-2, 50ug/ml ascorbic acid (AsAc) and 8mM β-glycerophosphate.

Myogenic: DMEM high Glu+ 2%FBS+ 1%P/S+ 10ng/ml IGF-1.

GM: DMEM+ 10%FBS+ 1%P/S.

Cells from each plate were plated in 24-well plate. Three repeats for each differentiation method (Figure 1).

Cells will be stimulated to differentiate into osteogenic lineage by application of BMP-2 as follows:

Stimulate cells with 100 ng/ml of BMP-2, 50ug/ml ascorbic acid (AsAc) and 8mM β-glycerophosphate in general medium .

AsAc stock solution [1000X]:

1. Dissolve 50 mg ascorbic acid-2 phosphate in 10 ml DMEM/15 mM HEPES.

2. In the tissue culture hood, filter it through a 0.22 μm pore size membrane.

3. Store the solution in a closed flask at 4°C. It should be stable for several months.

β-glycerophosphate stock solution [10X]:

1. Dissolve 630 mg β-glycerophosphate in 20 ml DMEM.

2. In the tissue culture hood, filter it through a 0.22 μm pore size membrane.

3. Store the solution in a closed flask at 4°C. It should be stable for at least two months.

Osteogenic induction procedure:

• Reserve a confluent culture to perform the differentiation.

• The osteogenic induction consists of switching from complete culture medium to osteogenic induction medium, and performing medium changes twice a week for 4 weeks.

• Generally, mineralised extracellular matrix deposition can be observed after 2-3 weeks week after the onset of differentiation, and becomes more evident along time.

Myogenic Differentiation:

Stimulate wells with low serum containing medium supplemented with 10ng/ml IGF-1.

Medium changed every 2 days.

Analyse cells after 2-3 weeks.
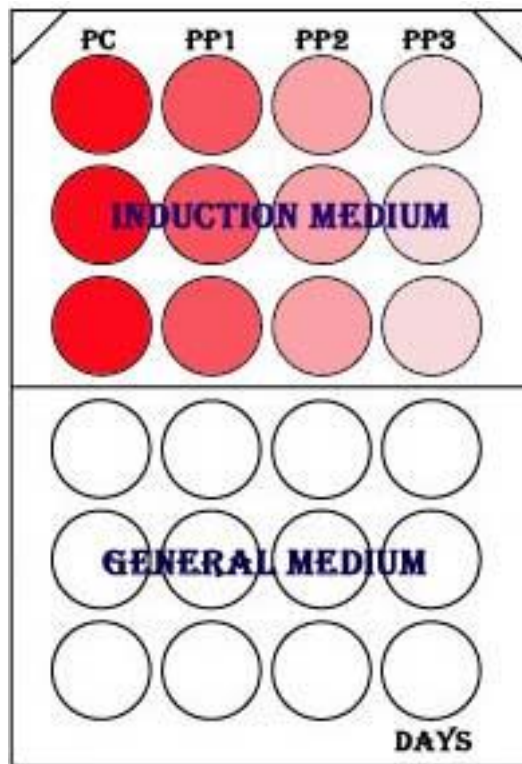
Figure 1: The differentiation set up for myogenic and osteogenic differentiation experiments. This setup was repeated for all isolates

Final RNA extraction protocol

Laboratory steps	Materials	Volume	Settings
i) Prior to start Clean the working bench, pipettes and all plastic ware used	-RNase*Zap*® wipes -RNase*Zap* spray		
ii) Handling the sample Transfer the cell lysate to the working bench			
iii) Disruption and homogenisation Total	RLT buffer Measure lysate 70% ethanol	~ ~350 µl ~350 µl * ~700 µl	
iv) Purification** -Use the lysate from the previous step	Spin column RW1 buffer RW1 buffer RPE buffer RPE buffer New tube open cap	~700 µl 350µl 350µl 500 µl 500 µl -	Centrifuge 15s 10,000 rpm*** Centrifuge 15s 10,000 rpm Centrifuge 15s 10,000 rpm Centrifuge 15s 10,000 rpm Centrifuge 2m maximum speed Centrifuge 1m maximum speed
v) Elution**	RNase free water	**30 µl**	Keep 5m at room T Centrifuge 1m maximum speed

*Measure the remaining lysate after homogenisation and add equal volume of ethanol. ** Following the RNeasy® mini kit manual. ***Capacity of the spin column is only 700 µl, hence the spinning can be conducted more than once. T, temperature.

qRT-PCR laboratory protocol

Laboratory steps	Materials	Volume	Settings
i) cDNA synthesis -Adjusting RNA concentration (equal volume to the cDNA MM) Total	RNA sample (total concentration **5ng/reaction**) RNase free water **Adjusted RNA sample**	Variable Variable **10µl**	
-Preparation of cDNA MM Total	10X RT Buffer 25X dNTP mix (100mM) 10X RT Random primers MultiScribe Reverse transcriptase enzyme RNase free water **cDNA MM/reaction**	2µl 0.8µl 2µl 1µl 4.2µl **10µl**	
-cDNA synthesis Total	cDNA MM Adjusted RNA sample **cDNA mixture**	10µl 10µl **20µl**	T/ cycle (35 cycles used) 25°C 10m 37°C 120m 85°C 5s 4°C ∞
ii) qRT-PCR -Preparation of qRT-PCR mix Total	TaqMan Universal PCR MM RNase free water cDNA mixture Gene of interest assay **qRT-PCR mixture/well** qRT-PCR mixture	12.5µl 8.75µl 2.5µl 1.25µl **25µl**	
-Loading each well and cover the whole plate -Applying the 96-well plate into the relative quantification real-time PCR machine		25µl	Centrifuge 15s maximum speed in a large centrifuge T/ cycle (40 cycles used) 50°C 2m 95°C 10m 95°C 15s 60°C 1m

MM: Master mix. T: Temperature. M: minutes. S: seconds

Appendix C. Publications

Publications:

Mulhall H, Patel M, Alqahtani K, Mason C, Lewis MP, Wall I. (2011) Effect of capillary shear stress on recovery and osteogenic differentiation of muscle-derived precursor cell populations. Journal of Tissue Engineering and Regenerative Medicine, **5**(8):629-35

Patel M, Mulhall H, Al-Qahtani K, Lewis M, Wall I. **(2011)** Muscle-derived precursor cells isolated on the basis of differential adhesion properties respond differently to capillary flow. Biotechnology Letters. **33**(7):1481-6.

Lewis MP, Smith A, Shah R, Al-Qahtani K, Carlqvist K, Sinanan AC, Mudera V, **(2009)** A little bit of this and a little bit of that! 3D tissue engineered skeletal muscle-Generating basic mechanisms in maintenance and turnover. Comparative Biochemistry and Physiology A-Molecular and Integrative Physiology **153** (2): 73.

Posters and Oral presentations:

Alqahtani, K., Buxton, P., Parkar, M., Lewis, M.P. Osteoprogenitor cells within skeletal muscle tissue. TCES, Glasgow, UK, 2009.

Alqahtani, K., Buxton, P., Parkar, M., Lewis, M.P. Isolation of Progenitor Cells from Craniofacial Skeletal Muscle based on Adhesion Properties. Presented at UCL stem cell day, December 2007.

Alqahtani, K., Buxton, P., Parkar, M., Lewis, M.P. Isolation of Progenitor Cells from Craniofacial Skeletal Muscle based on Adhesion Properties. Presented at 20th head group meeting held at ICH, January 2008.

Alqahtani, K., Buxton, P., Parkar, M., Lewis, M.P. Isolation of Progenitor Cells from Craniofacial Skeletal Muscle based on Adhesion Properties. Presented at Eastman 60th anniversary meeting, April 2008.

Alqahtani, K., Buxton, P., Parkar, M., Lewis, M.P. Isolation of Craniofacial Muscle Progenitor Cells Based on Adhesion Properties. Presented at PEF IDAR meeting, London 2008.

Carlqvist K, Alqahtani K, Sinanan ACM, Aggrewal S, Mudera V, Lewis M.P, 2008, craniofacial Muscle Derived Stem Cells. Presented at PEF IDAR meeting, London 2008.