Satellite Cell Subpopulations and Environmental Mediators of their Function: Implications for Stem Cell Therapy in Skeletal Muscle

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This thesis is submitted for the degree of Doctor of Philosophy

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Declaration

I, Alice Neal, confirm that the work presented in this thesis is my own. Where information has been derived from other sources this has been clearly indicated in the thesis.
Abstract

Satellite cells are myogenic cells found between the basal lamina and the sarcolemma of the muscle fibre (myofibre). Satellite cells are the source of new myofibres; as such, satellite cell transplantation holds promise as a treatment for muscular dystrophies. There is a need to investigate factors that enable satellite cell survival and/or proliferation post engraftment in order to obtain the optimal donor cell and host environment for efficient satellite cell transplantation. I have investigated sex differences in mouse satellite cell populations across the lifespan in vitro and in vivo. I show that satellite cell number and myogenic regulator factor expressions differ according to sex and developmental stage. Despite this, I show that engraftment efficiency is not mediated by the age or sex of the host or the donor. I hypothesise that there are two distinct satellite cell populations: one for muscle growth and maintenance and one for muscle regeneration. I have used high doses of ionising radiation to separate radio-resistant from radio-sensitive satellite cells. I demonstrate that radio resistant satellite cells do not contribute to growth, but are able to contribute to host muscle regeneration post transplantation and have compared their expression profiles using microarray. I hypothesise that satellite cells able to survive high dose ionizing radiation are the same population of satellite cells that are able to survive transplantation. Engraftment efficiency is greatly improved if host muscle is exposed to ionizing radiation prior to engraftment. I demonstrate that elimination of the host satellite cell pool is not sufficient to account for the improved
engraftment efficiency with radiation and I have therefore investigated the role of the vasculature as a mediator of radiation induced improvement in engraftment efficiency.

**Keywords.** Satellite cells, stem cells, regeneration
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# List of Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APES</td>
<td>aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>C57BL</td>
<td>C57 Black</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complimentary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome C Oxidase</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DMD</td>
<td>Duchenne Muscular Dystrophy</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DPX</td>
<td>Di-n-butyl Phthalate</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>DTA</td>
<td>Diptheria Toxin A</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
</tr>
<tr>
<td>EOM</td>
<td>Extra-Ocular Muscles</td>
</tr>
<tr>
<td>FACs</td>
<td>Fluorescent Activated Cell Sorting</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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</table>
GTP       Guanosine Triphosphate
H&E       Hematoxylin and Eosin
HGF       Hepatocyte Growth Factor
HIF1      Hypoxia Inducible Factor 1
HS        Horse Serum
HSP       Heat Shock Protein
IGF1      Insulin-like Growth Factor
MHC       Myosin Heavy Chain
MicroRNAs Micro Ribonucleic Acid
MLC       Myosin Light Chain
MMP       Matrix Metalloprotease
MRF       Myogenic Regulatory Factor
mRNA      Messenger Ribonucleic Acid
NAD       Nicotinamide Adenine Dinucleotide
OCT       Optimal Cutting Temperature
OH        Hydroxyl
PAs       Pharyngeal Arches
PBS       Phosphate Buffered Saline
PCR       Polymerase Chain Reaction
PFA       Paraformaldehyde
PTPRC     Protein Tyrosine Phosphatase Receptor Type C
qPCR      Quantitative Polymerase Chain Reaction
RMA       Robust Multichip Average
RNA       Ribonucleic Acid
ROS       Reactive Oxygen Species
RT-PCR     Real Time Polymerase Chain Reaction
SNF       Sucrose NonFermentable
SNP       Single Nucleotide Polymorphism
SRY       Sex Determining Region Y
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TA</td>
<td>Tibialis Anterior</td>
</tr>
<tr>
<td>TAP</td>
<td>Antigen Peptide Transporter</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TO</td>
<td>0 hours in culture</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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Chapter 1

Introduction

1.1 Skeletal Muscle

Skeletal muscle comprises 30-40% of a human’s total body mass and is responsible for all movement of the skeleton. Skeletal muscle is made up of bundles of long cylindrical multinucleated myofibres themselves comprised of myofibrils, repetitions of actin and myosin filaments which together form the sarcomere, the basic functional unit of skeletal muscle contraction (Jones & Round, 1990) (see figure 1.1 and figure 1.2). Fibroblasts found in and around the muscle fibres secrete the extracellular matrix to form layers of connective tissue that bring individual fibres into bundles and those bundles into large functional muscles. The endomysium, a layer of connective tissue containing capillaries, nerves and lymphatics, overlies the muscle cell membrane, the sarcolemma, and separates one fibre from the next. Groups of muscle fibres are surrounded by the perimysium which collects the fibres into bundles known as fascicles. Each fascicle is connected to the next via the epimysium, a dense layer of connective tissue continuous with the tendons (see figure 1.1). These fascicles are collectively known as skeletal muscle.

Skeletal muscle achieves its contractility via the action of the actin and myosin filaments of the sarcomere (see figure 1.2). Upon excitation via the
calcium-dependent release of acetylcholine at the motor neuron terminal of the neuromuscular junction, the nicotinic receptor of the muscle is activated allowing an action potential to propagate. Via deep invaginations of the muscle sarcolemma (T tubules), the action potential results in the release of calcium into the myofibre from the sarcoplasmic reticulum. Calcium binds tropinin C of the troponin complex which is found associated with actin filaments of the sarcomere. The calcium unlocks tropomyosin from actin which unobscures the actin binding site. This enables the myosin cross bridge, which is kept in an ever ready state due to the presence of adenosine diphosphate (ADP) and inorganic phosphate bound to its nucleotide binding pocket, to bind the actin. Adenosine triphosphate (ATP) binds myosin, causing the myosin to release ADP and phosphate and the crossbridge to assume the cocked back formation. The repetition of these steps causes the myosin to 'walk' along the actin filaments. This walk causes the actin filaments to be pulled towards each other, and the muscle to contract. The
process of actin binding and release will continue as long as the calcium, which is being actively pumped back into the sarcoplasmic reticulum, is available to bind the troponin complex and uncover the actin binding site. When tropomyosin once again covers the actin binding site, the filaments slide back to their original location and the muscle relaxes.

All skeletal muscles are comprised of bundles of fibres and have an actin myosin mediated mechanism of contraction as described. However, skeletal muscle is a heterogenous tissue. Fibres can be sub-categorised based on functional, molecular and metabolic properties. Myofibres can be classified as extrafusal or intrafusal. Extrafusal fibres are innervated by motor neurons and are responsible for the maintenance of posture and for all voluntary movement. Intrafusal fibres are responsible for monitoring changes in muscle length for proprioception via sensory neurons (reviewed Walro & Kucera...
Extrafusal fibres can be broadly subdivided into type I (slow) and type II (fast) muscle fibres depending on ATPase activity and the isoform of myosin heavy chain that they express (reviewed Schiaffino & Reggiani (1994)). Type I muscle fibres use aerobic metabolism and are thus highly resistant to fatigue, whilst type II fibres produce a higher power, have a faster contraction time but fatigue quickly due to their reliance on anaerobic glycolytic metabolism. Muscles are described as fast or slow depending on the fibre type from which they are predominantly comprised. Slow muscles are more red in appearance than the paler fast muscles due to their higher density of mitochondria (Eisenberg, 1983).

Skeletal muscle is a plastic tissue that frequently undergoes minor damage and repair processes and changes in fibre type, size and metabolic activity. Cytoplasmic volume can change in size, myonuclei can change in number and focal plasma membrane and myofibrillar disruptions can lead to myofibrillar remodelling and the formation of new sarcomeres. Fibre size, morphology and contractile characteristics are greatly influenced by extrinsic factors like usage electrical stimulation and hormones (reviewed Pette & Staron (1997)).

Occasionally, skeletal muscle is subject to more traumatic injuries: sport injuries, dytrophies or genetic diseases which cause inflammatory processes, histological changes and cell death. Fortunately, skeletal muscle is well equipped to deal with most eventualities. Upon injury, a rapid inflammatory response is followed by the creation of new myofibres and the remodelling of newly regenerated tissue. Early murine muscle graft experiments demonstrated that in mice, transplanted minced muscle can, within 30 days of grafting, go from a ‘porridge like’ state to a functional muscle able to respond appropriately to nerve input (Studitsky, 1964, Carlson, 1986). In humans, it is now known that a young skeletal muscle can be entirely regenerated within just 14 days, with the expression of neonatal myosin and
Figure 1.3: Muscle regeneration following Notexin Injury (Image courtesy of Rowan Ashfahani, Dubowitz Neuromuscular Centre 2012).

myofibre central nucleation the only remaining evidence of the initial injury (reviewed Ciciliot & Schiaffino (2010)) (see section 1.3). Identifying, understanding and harnessing the power of the cells responsible for this remarkable regenerative capacity has been the life’s work of many: it is a challenge still far from complete, and is the subject of the rest of this thesis.

1.2 Skeletal Muscle Regeneration and the Satellite Cell

It was postulated as early as the 1830’s that multinucleated myofibres may be formed by the fusion of multiple mononucleated cells. Experimental demonstration of this did not arrive until some 130 years later. Investigation of skeletal muscle injury in the mouse hind limb revealed that shortly following muscle damage, a pool of mononucleated cells assemble at the site of injury
and these fuse together to form myofibres. Tritiated thymidine is a specific radioactive DNA precursor that, if present during DNA replication, results in the synthesis of radioactive DNA and thus radioactive daughter nuclei. By pulsing injured muscle with tritiated thymidine it was demonstrated that up to 4 days after injury, only mononucleated cells had undergone cell division. However, at 4 days post injury, some myonuclei were observed to be radioactive. As nuclei can only incorporate the radioactive label if they are dividing at the time of exposure and only mononucleated cells were seen to be doing so, it follows that the labelled myonuclei must have been formed from those mononucleated cells. Crucially, if muscle was pulsed a longer time period after injury, myonuclei were not labelled (Bintliff & Walker, 1960). Thus, it was elegantly demonstrated that myonuclei themselves are post-mitotic, arising from mononucleated cells that gather at the site of injury: myoblasts.

In 1961, based on observations of frog muscles under the electron microscope, Alexander Mauro provided the first description of the satellite cell (Mauro, 1961). He described a cell that, due to its small cytoplasmic volume, appeared indistinguishable from myonuclei except for the unique position that it occupied, ‘wedged’ between the plasma membrane and the basement membrane of the muscle fibre. These findings were verified by another paper of the same year that observed such a cell within muscle spindles (Katz, 1961). In his original description, Mauro showed considerable foresight as to the myogenic relevance of the cell he had discovered, postulating that these satellite cells were ‘dormant myoblasts’. However, as the origin of the myoblast was very much open to debate, with hypotheses identifying precursors from the connective tissue, circulating leukocytes or from within the muscle itself (reviewed (Scharner & Zammit, 2011)), it was not until 1970 that the proliferative potential of the satellite cell was convincingly demonstrated.
Similar to the radiolabelling experiments described above that demonstrated the existence of myoblasts, radio labelling was used again, to demonstrate the myogenic potential of the satellite cell. In growing rats, 24 hours post injury and exposure to tritiated thymidine, it was again observed that only a pool of mononucleated cells incorporated the radioactive label. This time however, these mononucleated cells were identified under the electron microscope and defined by their position between the basal lamina and the sarcolemma, as satellite cells. 48 hours post injury, a number of myonuclei were radioactive and this number continually increased up to 72 hours after injury. Satellite cells were therefore shown to be the source of new myonuclei (Moss & Leblond, 1970).

The development of techniques that enabled the extraction and culture of segments of (Konigsberg et al., 1975) and entire (Bischoff, 1975) single fibres, free from contaminating cells and with their basal lamina intact (Rosenblatt et al., 1995), provided good support for these conclusions. After 24 to 48 hours in culture, mononucleated cells emanated from fibres and, due to the purity of the fibre preparation, were presumed to be satellite cells. These cells were seen to enlarge and proliferate to form colonies of cells which fused together to form myotubes (Bischoff, 1975). By 1975 it was therefore understood that satellite cells could form myoblasts and that these myoblasts form new multinucleated myofibres.

1.3 The Satellite Cell: The Principal Skeletal Muscle Stem Cell

To attain the status of stem cell a cell must meet two criteria: 1) the cell must produce progeny that will differentiate into at least one specialised cell type and 2) the cell must maintain its own numbers by self-renewal. Self-renewal requires that at least one daughter cell be specified to avoid differentiation
and retain the initial capacity of the parent cell. Thus, stem cell numbers are not diminished by proliferation. The research detailed thus far establishes satellite cells as a source of myoblasts, but this does not answer to whether satellite cells are the sole source, or just one of the many sources of muscle myoblasts. Furthermore, the source of the satellite cells themselves, was until relatively recently largely a mystery. There exists a body of evidence to suggest that satellite cells are not in fact bona fide stem cells but rather muscle precursor cells themselves renewed from another stem cell source.

Much research has described other cell types that are able to contribute to muscle regeneration. Some evidence suggests that bone marrow derived and skeletal muscle stem cells are of the same lineage. Bone marrow transplantation using ubiquitously expressing green fluorescent protein (GFP) expressing donors, showed GFP positive cells in regenerated tibialis anterior muscles (TA) 5 weeks post transplantation suggesting that bone marrow derived cells can contribute to skeletal muscle regeneration (Ferrari et al., 1998). Furthermore, muscle derived stem cells, after culture with bone marrow derived cells and transplantation into whole body irradiated hosts, have been shown to contribute to reconstitution of the bone marrow (Jackson et al., 1999). Similarly, side population cells, defined by their ability to expel the fluorescent dye Hoescht 33342, are located both within the bone marrow and in skeletal muscle. Side population cells have been shown to contribute to muscle regeneration in the mdx mouse and reconstitute the bone marrow of lethally irradiated mice (Gussoni et al., 1999).

The investigation of other stem cell types that may contribute to skeletal muscle regeneration has not been restricted to those that are bone marrow derived. Cells of various origins - dermal fibroblasts, mesangioblasts, neural tube derived cells - have been shown to convert to the myogenic lineage after a period in culture and can contribute to some muscle regeneration in the dystrophic mouse (Gibson et al., 1995, Saito et al., 1995, Tajbakhsh et al., 1995).
More recently it has been shown that a variety of circulating cells, including AC133 expressing and blood vessel derived pericytes, when transplanted can contribute to muscle regeneration in dystrophic mice (Torrente et al., 2004, Sampaolesi et al., 2003, Dellavalle et al., 2007).

However, when interpreting such data it is important to consider that the method of cell extraction is of great importance. Any contaminating cells in the transplanted cell population preparation would render data inconclusive. For example, Jackson et al. 1999 study muscle derived cells obtained by enzymatic disaggregation and draw conclusions as to their haematopoietic potential. However, enzymatic disaggregation does not permit the isolation of a pure population and it is likely to have contained many contaminating cell types, including haematopoietic progenitors. Culturing stem cells before transplantation also adds another layer of complexity to data interpretation. With any stem cell type, a period of cell culture will select for the fastest dividing sub population. This can result in mutated, tumorogenic and physiologically irrelevant cell populations. Furthermore, many studies use the mdx nude mouse as a host model system. The mdx nude mouse is the genetic homologue of Duchenne Muscular Dystrophy (see section 1.9.1), and lacks the dystrophin protein beneath the sarcolemma of muscle fibres (see section 1.9.2). There is some evidence to suggest that the incorporation of circulating cells is particular to dystrophic muscle and cannot be generalized to non pathological states (Bittner et al., 1999). It is entirely conceivable that circulating cells would require a disrupted myofibre membrane in order to pass into the muscle fibre.

With the description of other stem cell types that could contribute to myogenesis, the importance of the satellite cell for skeletal muscle regeneration was unclear. Cultured and radiolabelled myoblasts when re-implanted into their muscle of origin give rise to radiolabelled myonuclei even after multiple rounds of muscle regeneration, suggesting that some of those labeled
cells undergo self renewal and contribute to the maintenance of a progenitor cell pool (Lipton & Schultz, 1979, Snow, 1978). However, as the satellite cell is defined by its location, the available methods of myoblast extraction (analysis of cells emanating from fibres, enzymatic disaggregation or percoll gradient separation) meant that the identification of the starting population in these studies could only be guessed at.

The elucidation of transcription factors expressed exclusively by satellite cells was somewhat of a revolution. With transcription factor specificity came the opportunity to analyse the proliferation and commitment of the satellite cell population once outside the niche. Quiescent satellite cells express both the myogenic regulatory factor (MRF) Myf5 and a member of the paired box family of transcription factors Pax7 (Seale et al., 2000). Pax genes are defined by the presence of a DNA binding domain of 128 amino acids and are known to be crucial regulators of development in other systems (reviewed Hastie (1991)). Pax7 is expressed in skeletal muscle satellite cells, the mesencephalon, hindbrain, neural tube and adult brain (Jostes et al., 1990, Stoykova & Gruss, 1994). Although Pax7 null mice do not show an obvious muscle defect during development, by P11 their muscles are considerably reduced in size and postnatally, satellite cells are continually lost due to apoptosis (Relaix et al., 2004, Mansouri et al., 1996). Thus, Pax7 is not required for satellite cell specification but is necessary for maintenance of the satellite cell pool. Pax7 null mice die 3-4 weeks after birth due to the importance of Pax7 in development of the neural crest, a migratory cell population that gives rise to many cell lineages (Mansouri et al., 1996). Although Pax7 null mice die in gestation they do not show severe skeletal muscle pathology, this is likely due to the functional redundancy that Pax7 shares with its paralogue Pax3 (Relaix et al., 2004). Pax3 is expressed in the embryonic precursors of satellite cells and by satellite cells of many skeletal muscles post natally (see section 1.4). The Pax3/Pax7 double mutant mouse
The MRFs are basic helix loop helix transcription factors. Myf5 is the first MRF expressed in the embryo and is critical for muscle determination (Ott et al., 1991, Buckingham, 1992). Upon entrance into the cell cycle adult satellite cells express a second MRF, MyoD (Beauchamp et al., 2000). MyoD is expressed throughout the proliferative phase before being down regulated upon differentiation (Tajbakhsh & Buckingham, 2000). Although Myf5 acts upstream of MyoD they share a degree of redundancy (Kassar-Duchossoy et al., 2004, Rudnicki et al., 1993). Myf5/MyoD double knockout mice die soon after birth with no detectable skeletal muscle mRNAs (Rudnicki et al., 1993). Myf5 and MyoD are critical determinants of the myogenic lineage and are indispensable for satellite cell specification and differentiation. MyoD null mice show severe regeneration deficiency due to an inability of the satellite cells to differentiate and a failure of these cells to exit the cell cycle (Megeney et al., 1996, Sabourin et al., 1999, Yablonka-Reuveni et al., 1999).

Armed with tools to assess satellite cell progression along the myogenic lineage, in vitro experiments began to demonstrate a satellite cell capacity for self renewal. The immortalised myogenic cell line C2C12 (Yaffe & Saxel, 1977, Blau et al., 1983a), if allowed to differentiate, shows that a minority of cells retain a quiescent phenotype, that is they remain small and round, are MyoD negative and Myf5 positive. This suggests that C2C12 cultures maintain a reserve myogenic population (Yoshida et al., 1998). Intact single fibre extraction and suspension culture (For methods see section 2.4) enables the analysis of myofibre associated satellite cells as they undergo proliferation and a degree of differentiation (Zammit et al., 2004, Collins et al., 2005). In these conditions, by 24 hours post isolation all satellite cells become activated (MyoD positive). However, after multiple rounds of division, and the appearance of fibre associated satellite cell derived colonies, mononucleated
cells are observed to undergo one of two cell fates. The majority of satellite cells down regulate MyoD and up-regulate the muscle specific transcription factor, and marker of differentiation Myogenin. Crucially however, a small minority of cells down-regulate MyoD, thus exit from the cell cycle, but re-express Pax7 returning to a quiescent, undifferentiated state. This is good evidence to suggest that satellite cells are able to undergo self renewal and maintain their own numbers (Zammit et al., 2004).

Despite these converging lines of evidence, the stem cell status of the satellite cell remained controversial due to studies suggesting that satellite cells could be derived from other stem cell sources. Side population cells of the mouse, upon transplantation, were shown to give rise not only to myofibres, but also to satellite cells of donor origin (Asakura et al., 2002). After the transplantation of mouse bone marrow with donor GFP expressing cells, GFP positive cells were reported in the satellite cell position and were seen to give rise to small numbers of GFP positive myofibres (LaBarge & Blau, 2002). However, this research is directly contradicted by evidence showing that isolation of populations of bone marrow derived cells, previously described as having myogenic cell potential, were able to take up the satellite cell position upon engraftment but could not subsequently contribute to muscle regeneration. This suggests that bone marrow derived cells display no intrinsic myogenicity and that functional satellite cells can not be derived from circulating cells (Sherwood et al., 2004).

The most convincing body of evidence to demonstrate the satellite cell capacity for self renewal comes from transplantation studies using transgenic mice. The Myf5<sup>nlacZ</sup> mouse has nuclear lacz (nlacz) under the Myf5 promoter and therefore expresses nlacz exclusively in embryonic muscle precursors and postnatally in satellite cells (Tajbakhsh et al., 1996). βgal positive cells isolated from a Myf5<sup>nlacZ</sup>/+ adult mouse and injected into a wild type host show that cells of donor origin (βgal positive) can take up the
satellite cell position beneath the basal lamina (Heslop et al., 2001, Collins et al., 2005, 2007, Boldrin et al., 2009, 2012). Furthermore, after isolation of intact single fibres from mice engrafted with β gal positive cells, satellite cells of donor origin are able to dissociate from the fibre and proliferate in vitro (Heslop et al., 2001). This suggests that Myf5 positive cells (satellite cells) are able to both contribute to host muscle regeneration and give rise to functional satellite cells.

The isolation and engraftment of small and very pure populations of satellite cells strongly suggests that satellite cells are at the top of the skeletal muscle stem cell hierarchy. The engraftment of individual myofibres demonstrated that just the small number of cells associated with an individual fibre can give rise to thousands of myofibres within a host muscle (Collins et al., 2005). Using the Myf5\textsuperscript{\textsubscript{lacz}} mouse as a donor, and the mdx nude as a host allowed for the assessment of donor cell contribution both to reconstitution of the host satellite cell compartment and to regenerated myofibres (Collins et al., 2005). Results show that not only did the few transplanted myofibre associated satellite cells proliferate and differentiate to form donor derived muscle, importantly they also underwent self renewal to form donor derived satellite cells in the host satellite cell niche. Furthermore, donor derived satellite cells were shown to be functional, as they were able to give rise to large clusters of donor derived fibres with subsequent rounds of injury (Collins et al., 2005, 2007, Boldrin et al., 2009, 2012).

The self renewal capacity of satellite cells has been convincingly demonstrated again, by the transplantation of just a single luciferase expressing satellite cell. \textit{in vivo} luciferase imaging has shown that that not only could transplanted cells undergo multiple rounds of rapid proliferation, but that occasionally, a single cell can give rise to a population of reserve cells that can contribute to subsequent rounds of muscle regeneration (Sacco et al., 2008). These studies are a clear \textit{in vivo} demonstration that the satellite
cell possess all the necessary properties to be considered a true stem cell. Upon transplantation satellite cells undergo proliferation and differentiation to form myofibres and undergo self renewal to maintain and if necessary expand their own numbers.

It was thus established that the satellite cell is a skeletal muscle stem cell, yet the relative importance of the satellite cell with regard to other myogenic cell sources remained unclear, i.e. is the satellite cell one of many skeletal muscle stem cells, or is it one of a number of stem cell populations that collectively contribute to muscle regeneration. This question has recently been approached in an elegant study that utilises cre-recombinase transgenic mouse strains to conditionally ablate Pax7 expressing cells. The R26\textsuperscript{GFP-DTA} mouse has a floxed GFP tagged diptheria toxin A (DTA) fragment which is prevented from expression due to an upstream stop codon. Upon Cre activity the floxed GFP and stop region is inactivated and expression of DTA is allowed. The Pax7-Cre mouse expresses tamoxifen inducible Cre in Pax7 positive cells. By crossing the Pax7-Cre mouse with the R26\textsuperscript{GFP-DTA}, a mouse is generated in which, upon tamoxifen injection, Pax7 positive cells express Cre and therefore DTA. This results in Pax7 specific cell death upon tamoxifen injection (Lepper et al., 2011). This ingenious mouse model has shown that, when functional satellite cells are ablated, adult skeletal muscle cannot undergo any regenerative response to cardiotoxin injury. Intriguingly, mice did not survive more than 5 days post tamoxifen-induced Pax7 specific cell death, perhaps indicating an important role for Pax7 in other cell types. In order to assess the longer term affects of satellite cell ablation, whole muscle transplants were performed. Pax7 ablated muscles were considerably smaller than control muscles and showed no regeneration after cardiotoxin injection, even 68 days post injury. Furthermore, no Pax7 expressing cells were seen to reappear in the transplanted muscle, strongly suggesting that satellite cells cannot be replaced from other
Research described here provides ample evidence to conclude that satellite cells are the principle skeletal muscle stem cells, capable of self renewal and absolutely essential for skeletal muscle regeneration. It is likely that other stem cells are able to contribute to skeletal muscle regeneration in some circumstances, but such cells do not have a considerable impact during normal physiological conditions. It is now understood and widely accepted that skeletal muscle regeneration relies upon activation, proliferation and differentiation of myofibre-associated satellite cells (see figure 1.4). Satellite cells, via expression of a known set of transcription factors (see figure 1.4), form a pool of mononucleated cells which fuse either to each other, to form new myofibres, or to an existing damaged fibre for myofibre repair (see figure 1.4).
Figure 1.4: Satellite cell activation differentiation and myogenic regulatory factor expression (Image from Boldrin et al. (2010)).
1.4 Skeletal Muscle Development

Skeletal muscles of the body have their origin in the mesoderm-derived somites. Somites bud from the unsegmented paraxial mesoderm and develop in a rostral to caudal direction (see figure 1.5A.) using a ‘clock and wave’ mechanism. The clock is formed by pulses of alternate Notch and Wnt signalling. Notch are a family of transmembrane receptors that effect transcription when bound by specific transmembrane protiens of other cells (reviewed (Bray, 2006)). Wnts are a family of protein ligands commonly associated with the extracellular matrix (reviewed (Nusse, 2008)). The ‘wave’ is formed by a fibroblast growth factor (FGF) protein gradient in a rostral to caudal direction (reviewed (Christ & Ordahl, 1995, Pourqui, 2003)). Somites are comprised of a basement membrane which surrounds epithelial cells which themselves enclose a lumen filled with mesenchymal cells. The ventral portion of the somite undergoes de-epithelialisation to form the sclerotome (see figure 1.5B.) whilst the dorsal epithelium stretches to form the dermomyotome (see figure 1.5B) (reviewed (Scaal & Christ, 2004)). Myogenesis begins when cells delaminate from the dermomyotome elongate and migrate to form the somitic myotome (see figure 1.5B.).

The early myotome is made up of post mitotic mononucleated myotubes which span the length of the somite in a cranial to caudal direction. The mechanism of myotome growth has been elucidated with spatially accurate electroporated GFP labelling of dermomyotome regions in the chick embryo. Electroporation enables the labelling of all dividing cells at a known time point and, due to the long half life of GFP, visualisation of the destination of labelled cells. Such techniques demonstrate that during this period of myotome formation, proliferative progenitor cells remain within the dermomyotome (Gros et al., 2004). Daughter cells elongate and then migrate only once they are post mitotic (Gros et al., 2004). The initial myotome is therefore comprised of elongated post mitotic mononucleated cells formed.
from the proliferation of cells within the dermomyotome (reviewed Scaal & Christ (2004)).

Pax genes, a set of highly conserved homeobox transcription factors, are important for cell lineage specification in the somite (reviewed (Christ & Ordahl, 1995)). As described (see section 1.3) Pax3 and Pax7 are known to be critical regulators of skeletal muscle development and are both expressed within the dermomyotome (Tajbakhsh et al., 1997, Bajard et al., 2006, Relaix et al., 2005). At this stage in development Pax3 is thought to be the critical regulator of myogenesis. The homozygous Pax3 mouse mutant (splotch) (Auerbach, 1954, Franz et al., 1993, Goulding et al., 1994) dies at E15 with a highly disorganised dermomyotome and an absence of properly elongated myotomal cells (Tremblay et al., 1998, Borycki et al., 1999). Using GFP electroporation combined with immunohistochemical analysis of Pax3/Pax7/Myf5 and MyoD, it has been demonstrated that cells within the dermomyotome never co-express Pax3 and the myogenic markers of the myotome Myf5 or MyoD. This, together with investigations showing that Pax3 acts upstream of Myf5 and MyoD (Tajbakhsh et al., 1997, Bajard et al., 2006), suggests that Pax3 marks an undifferentiated progenitor cell type whose daughter cells give rise to the myoblasts that form the myotome (Relaix et al., 2005).

Proliferative cells within the dermomyotome (see figure 1.5B.) can be categorised according to their Pax3/Pax7 expression profiles. The majority of cells co-express Pax7 and Pax3. A small minority are Pax3 positive only and an even smaller fraction expresses only Pax7 (Relaix et al., 2005). When assessed for expression of Cyclin A, a protein found in cells at the S and G2 phases of the cell cycle, Pax3/Pax7 positive cells were shown to represent the main proliferative cell population of the dermomyotome. By contrast, MyoD expression occurs once cells have migrated into the myotome and is concomitant with exit from the cell cycle. Accordingly, the Pax3/Pax7 dou-
ble mutant develops very little dermomyotome, shows no myotome or limb muscle development and dies at mid gestation (Relaix et al., 2005). It is possible that the differential expression of the Pax genes specifies different cell fates. Cells from the dermomyotome give rise not only to skeletal muscle but also to smooth muscle (Pouget et al., 2006), dermal cells and endothelial cells of the limb. It has been suggested that whilst Pax3/Pax7 co-expressing cells form skeletal muscle, the Pax3 population of the dermomyotome are multi potent progenitor cells which form other somite derived tissue (Buckingham et al., 2006).

Whilst limb muscles are derived from the somitic dermomyotome (see figure 1.5B.), muscles of the head and neck have a different origin. Muscles of the face, jaw, and neck develop from cells that migrate from the cranial mesoderm (a region of seven pairs of contiguous somitomeres) to the pharyngeal arches (PAs) (Trainor et al., 1994, Noden & Francis-West, 2006). Evidence suggests dissociation between the genetic cascades controlling cell fate of the head and limb muscles (Hacker & Guthrie, 1998, Mootosamy & Dietrich, 2002, Tzahor et al., 2003). In particular, progenitors of the head muscles do not rely on the expression of Pax3 for their fate specification as the Pax3/Pax7 mutant mouse is able to develop head muscles but do not develop limb muscles (Tajbakhsh et al., 1997). In the pharyngeal arch Pax3 is thought to be replaced by Tbx1 and Pitx2 which, as with their limb muscle counterpart Pax3, are critical for myogenesis due to their regulation of Myf5 and MyoD (Sambasivan et al., 2009). In the absence of Tbx1 or Pitx2, myoblasts of the head are lost due to apoptosis (Sambasivan et al., 2009).
Figure 1.5: The somites and somite differentiation in the human embryo A. Drawing of a longitudinal section of an human embryo showing the somites allinged in a rostral to caudal direction along the neural tube. B. Drawing of a transverse section of an human embryo shows the differentiation of the somites, the dermomyotome and primitive myotome (original image a lithograph plate from Gray’s Anatomy, accessed and adapted from http://www.wikipedia.org/wiki/File:Gray20.png and File:Gray64.png under the creative commons licence).
1.5 The Origin of the Satellite Cell

The dermomyotome, which gives rise to the first skeletal muscle cells of the somitic myotome, is a transient structure (see figure 1.5B.). In the chick, 2 days after myotome formation, migration of myoblasts from the dermomyotome stops. The dermomyotome undergoes an epithelial-mesenchymal transition and forms the sclerotome, from which the ribs, neural arches, spinal nerve and later the vertebral bodies and invertebral discs develop (reviewed (Christ & Ordahl, 1995)). Skeletal muscle development must therefore proceed from a different source.

Mononucleated muscle fibres of the myotome become multinucleated fibres through fusion with mononucleated myoblasts in much the same way that muscle growth and repair occurs in the adult. In the adult, the source of such myoblasts is, as discussed (see section 1.2), the satellite cell. Elucidating the origin of the satellite cell itself and understanding the mechanism by which embryonic muscle grows has been the subject of much investigation.

Early quail-chick chimera studies suggested a somitic origin of the satellite cell (Armand et al., 1983) but these studies did not identify a somitic satellite cell progenitor population or describe how it developed, so the satellite cells somitic origin remained largely conjecture. Cells isolated from the mouse embryonic dorsal aorta were reported as having a similar morphology to satellite cells and were shown to give rise to skeletal myogenic cells in culture and to skeletal muscle post natal development after transplantation (De Angelis et al., 1999). This suggested that a subset of satellite cells derive from the embryonic vasculature. Studies of the Pax7 null mouse show that satellite cells are present at birth but that numbers diminish steadily during the mouse’s brief post natal life. Thus Pax7 is not necessary for satellite cell specification but is necessary for maintenance of the postnatal satellite cell pool (Oustanina et al., 2004). Such data was influential in the development of hypotheses that myotome progenitor cells were derived
from distinct multi-potent stem cells that could migrate into the somite and differentiate into satellite cells. Prior to a clear demonstration of satellite cell self-renewal, this mechanism was also proposed to explain how adult satellite cells were maintained (see section 1.3).

Analysis of Pax3/Pax7 expressing cells within the somitic dermomyotome of the mouse told a different story. At the time of dermomyotome disintegration, Pax3/Pax7 co-expressing cells can be found within the myotome. Unlike the majority of cells in the myotome, Pax3/Pax7 expressing cells are not committed to the myogenic programme as they do not express MRFs. These Pax3/Pax7 positive cells are found within the myotome at all embryonic stages (Relaix et al., 2005). Between E16 and E18 a basal lamina forms around the outside of muscle fibres. GFP marked Pax3 positive cells were seen to become trapped beneath this developing basal lamina in a position closely associated with the muscle fibre (the satellite cell position). These GFP positive cells were still detectable during postnatal growth (Relaix et al., 2005). This study strongly suggests that adult satellite cells are specified at the same time as the first myofibres.

This is supported by an elegant study in the chick embryo which used Brd-U labelling to show that the epithelial to mesenchymal transition in the dermomyotome is associated with an influx of Brd-U positive cells in the myotome (Gros et al., 2005). Electroporation of the central dermomyotome with GFP established that these cells migrated from the dermomyotome. This GFP population was seen to continually contribute to the differentiated muscle mass, importantly, not all cells adopted a differentiated phenotype, with 25% of the GFP positive population maintaining Pax7 expression. Quail-chick dermomyotome grafting experiments demonstrate that these Pax7/GFP positive cells are present in the early myotome, during late fetal development and post hatching (Gros et al., 2005). Together, these data convincingly demonstrate that satellite cells of the limb muscles are
specified in embryo from undifferentiated progenitors of the dermomyotome.

1.6 The Satellite Cell in Postnatal Growth and Muscle Maintenance

It is established that satellite cells have their origin in the somitic dermomyotome and are principally responsible for muscle regeneration in adulthood. However, as the source of myofibres, satellite cells also have a role in muscle development, growth and maintenance (Buckingham & Relaix, 2007, Gros et al., 2005, White et al., 2010). Both muscle regeneration and muscle growth require the fusion of satellite cells or satellite cell-derived myoblasts. In growth this requires myoblasts to fuse to an existing myofibre (Blaveri et al., 1999). After traumatic injury, regeneration requires that myoblasts fuse with each other to form entirely new myofibres. Regeneration is associated with a marked inflammatory response, necrosis, clearing of damaged tissue, and the transition of satellite cells from quiescence to activation (see section 1.2). However, muscle growth has different initiating signals, with some evidence to suggest that it is regulated by the fibre itself (Horsley et al., 2001, Jansen & Pavlath, 2006) and it is highly likely that muscle growth and muscle regeneration have differing regulatory molecular mechanisms (Lepper et al., 2011, Horsley et al., 2001, Lepper et al., 2009). The role of the satellite cell in normal postnatal growth and its role in the maintenance of adult muscle is, perhaps surprisingly, not very well understood.

Satellite cells undergo extensive proliferation during the first few weeks after birth. In rat muscles, the majority of these satellite cell derived myoblasts are incorporated into growing myofibres (Moss & Leblond, 1971). In the developing muscle of the mouse, two distinct stages of post natal muscle development have been delineated (White et al., 2010): Before post natal day (P) 21 satellite cells undergo extensive proliferation, contributing
to an increase in the number of myonuclei as the cytoplasm increases, the fibre expands and the muscle grows. However, post P21 the cytoplasm of the myofibre is seen to expand without an associated increase in myonuclei number and the satellite cells move into quiescence (White et al., 2010).

It seems that P21 also marks a change in the molecular requirements of the satellite cell population. Satellite cell expression of Pax7 is critical for satellite cell specification and muscle regeneration before P21. The Pax7 null mouse continually loses satellite cells due to apoptosis during postnatal life and is unable to mount an effective regenerative response (Oustanina et al., 2004, Kuang et al., 2006). Pax7 expression is therefore essential for maintenance of the satellite cell pool in neonatal mice. Until recently this evidence was taken to mean that Pax7 was essential for satellite cell pool maintenance and therefore muscle regeneration throughout life. Using tamoxifen inducible Cre to initiate the recombination of Pax7 and thus its conditional inactivation, it has been possible to bypass the early requirement of Pax7 and investigate its role in the adult. Surprisingly, if Pax7 is knocked down after P21, then satellite cells are able to mount an effective regenerative response and are still able to maintain their own numbers (Lepper et al., 2009). This demonstrates that expression of Pax7 is dispensable for muscle regeneration in the adult but not in actively growing mice. Here, it is important to distinguish between the importance of Pax7 expressing cells and the importance of expression of Pax7. Cells identified by the expression of Pax7 are, as discussed (see section 1.3) indispensable to muscle regeneration, however post P21 these Pax7 expressing cells do not require Pax7 in order to function.

The role of the satellite cell in adult muscle hypertrophy and the maintenance of muscle size is the source of much debate. It is estimated that human adult myofibres have a myonuclear turnover of 1 - 2 nuclei per week (Schmalbruch & Lewis, 2000), but how this changes in response to extrin-
suc factors e.g. endurance exercise or strength training is not clear. Until recently the pervading consensus has been that each myonucleus has a restricted area of cytoplasm over which it presides and that this area remains constant, such that any increase in cytoplasmic volume must be achieved by an increase in myonuclei number (Cheek et al., 1965). As satellite cells are the source of new myonuclei, it therefore follows that satellite cell activation, proliferation and fusion with existing fibres is the mechanism by which a muscle increases in mass (Roy et al., 1999, O’Connor & Pavlath, 2007). Accordingly, hypertrophy has been shown to be associated with an increase in myonuclear number (Bruusgaard et al., 2010).

Muscle hypertrophy is prevented after the ablation of satellite cell activity by exposure to high doses of gamma radiation (Mitchell & Pavlath, 2001, Rosenblatt & Parry, 1992, Rosenblatt et al., 1994), suggesting that satellite cell activity is required for fibre size increase. However, the analyses of these results is confounded by the fact that irradiation may have unknown effects on support cells, the vasculature and protein synthesis within the muscle itself. In both human and murine muscle, satellite cells do seem to be involved in the increase in muscle size in response to exercise (Kadi et al., 2004, Li et al., 2006). But interpretation of these data is complicated by recognition of the fact that exercise can also induce muscle damage: many protocols use damage causing maximal eccentric exercise and are arguably studying regeneration rather than hypertrophy. Nevertheless, it has been shown that satellite cell number increases in response to eccentric exercise even without muscle damage or inflammatory cell infiltrate (Mikkelsen et al., 2009).

The myonuclear domain hypothesis is challenged by research that shows changes in muscle fibre size that are not accompanied by changes in myonuclei number (Aravamudan et al., 2006, Rehfeldt et al., 1997, Verheul et al., 2004). Blockade of myostatin, a member of the TGF-β family of signalling molecules, by pharmacological agents causes a dramatic increase in body
weight and muscle mass, and as such has been proposed as a therapeutic strategy for muscle loss (Wagner et al., 2002, Bogdanovich et al., 2002). It has been shown that hypertrophy due to myostatin blockade is not associated with satellite cell activity and does not cause an increase in myonuclear number (Amthor et al., 2009). Similarly, activation of the IGF1/AKT signalling pathway or inducing activation of the transcription factor JunB, is associated with muscle hypertrophy but does not induce satellite cell activity (Schiaffino & Mammucari, 2011, Blaauw et al., 2009, Raffaello et al., 2010).

Recently, using the Pax7 Cre mouse (see section 1.3), McCarthy et al. show that conditional ablation of Pax7 expressing cells does not prevent muscle hypertrophy in response to synergist ablation (McCarthy et al., 2011). However, it is important to mention that, if satellite cells are conditionally ablated (McCarthy et al., 2011) the TA muscle undergoes a significant reduction in mass within 2 weeks. This atrophy is further exacerbated at 8 weeks post satellite cell ablation. Thus, it seems that satellite cell activity is important in routine muscle maintenance. Furthermore, in more physiologically relevant models, when satellite cells are functional, synergist ablation and consequent muscle hypertrophy is accompanied by the addition of new myonuclei and new myofibres (Ishido et al., 2009, Westerkamp & Gordon, 2005).

The role of the satellite cell in healthy adult skeletal muscle remains ambiguous. Either satellite cells are required for muscle growth and regulation of myofibre size and thus constitute a population of cells that regularly enter the proliferative state to respond to growth signals, or satellite cells are a population of cells that are in deep quiescence for the majority of their existence, becoming activated only upon muscle injury. In chapter 3 of this thesis, I address this question and my data suggest that there are distinct satellite cell subpopulations: one that is regularly activated to maintain
myonuclear numbers and one that is activated only upon extreme muscle injury.

1.7 Ageing Muscle and its Satellite Cells

With an ageing population and the ever growing demand that age-associated illness is placing on the world’s health services, the science of ageing has never been more relevant. One of the most influential and overarching explanations of the ageing phenotype states that ageing is the consequence of a tissue’s inability to maintain mass and function due to a decline in function of its tissue specific stem cells. Compromised stem cell function with increasing age has been observed across tissues. Hematopoietic stem cells are five times more frequent in old compared to young mice but are dramatically less efficient at homing and engrafting to host bone marrow (Morrison et al., 1996), liver tumours arise from transformation of resident stem cells and increase in occurrence (reviewed (Sigal et al., 1992)), and neuronal progenitors of the dentate gyrus decrease in proliferative capacity with increasing age (Kuhn et al., 1996).

A substantial loss of skeletal muscle is a defining characteristic of the ageing phenotype. Aged muscle shows a decrease in muscle mass, maximal force and regenerative capacity (Conboy et al., 2003, 2005). It has been proposed that aged associated changes in muscle structure and function are due to a reduced capacity of the myofibre associated satellite cells to maintain muscle mass. Analysis of Pax7 on single myofibres shows that the number of satellite cells per myofibre decreases in aged compared to young mice (Collins et al., 2007). Furthermore, when isolated, aged single fibre-associated satellite cells produce significantly smaller colonies of which a smaller percentage return to the quiescent phenotype than satellite cells of young mice (Collins et al., 2007).

There is some evidence to suggest that a stem cell is only capable of a
finite number of divisions before it moves into permanent senescence. Studies of satellite cells in muscles from Duchenne muscular dystrophy (DMD) patients suggest that this is applicable to human satellite cells. DMD is a severe muscle wasting disorder caused by a mutation in the dystrophin gene (see section 1.9.1). It is hypothesised that the replicative ageing of satellite cells is a major mediator of the accumulation of muscle damage with increasing age in DMD boys. The continuous cycles of degeneration and regeneration caused by an absence of a functional dystrophin protein beneath the sarcolemma of the muscle fibre (see section 1.9.1) eventually exhausts the satellite cell pool. Satellite cell exhaustion leads to the loss of skeletal muscle and its replacement with fibrotic tissue (Blau et al., 1983b, Webster & Blau, 1990). Satellite cell senescence has also been implicated in other myopathies e.g. sporadic inclusion-body myositis (Morosetti et al., 2010) and the mouse model of limb-girdle muscular dystrophy (Kudryashova et al., 2012).

Telomeres are simple tandem repeat sequences which cap the end of chromosomes to allow DNA to be accurately replicated without the loss of base pairs (reviewed (Blackburn, 1991)). Although the length of a cell’s telomere is a complex balance between processes that shorten and elongate them, on average telomeres shorten with each round of cell division as base pairs are missed at the 5’ end (reviewed (Blackburn, 1991)). Telomere length can therefore be used as a proxy for replicative age. A loss of chromosomal telomeres leads to chromosome instability and cell senescence (Allsopp et al., 1992). It follows then, that myoblasts from DMD patients are seen to have far shorter telomeres than non affected individuals. It is suggested that this replicative age is detrimental to satellite cell function even in non dystrophic environments as myoblasts isolated from human DMD patients show a very limited capacity to regenerate host mouse muscle (Decary et al., 2000, Mouly et al., 2005). It may therefore be more relevant to consider satellite cell
ageing in terms of replicative rather than chronological age.

The *mdx* mouse is the genetic homologue of DMD. Due to a naturally occurring nonsense mutation in the dystrophin gene (see section 1.9.2) *mdx* mice lack a functional dystrophin protein. However, *mdx* mouse muscle regenerates well compared to DMD muscle and so, although enormously useful to the field, the *mdx* mouse is not considered a good model for DMD. Mouse telomeres are 5-10 times longer than human telomeres and unlike human tissue, telomerase activity is detectable in a subset of mouse tissues (Hastie et al., 1990, Kim et al., 1994, Allsopp & Weissman, 2002). Telomerase is a ribonucleoprotein enzyme that synthesis and elongates telomeres (Blackburn, 1991). Over-expression of telomerase in the mouse has been shown to prevent telomere attrition, delay cell senescence and the ageing phenotype. Telomerase activity is detected in 80-90% of human tumours (reviewed Autexier & Greider (1996)) and in primary cell cultures after cell immortalisation protocols (reviewed (Harley & Kim, 1996)). Telomerase therefore plays a crucial role in regulating cell division. A possible explanation as to the lack of muscle pathology observed in the mouse is that the initial length of the satellite cell telomere, together with the action of telomerase, prevents telomere attrition and thereby satellite cell senescence. This allows the *mdx* mouse to successfully regenerate muscle beyond what is possible for a DMD boy. Indeed, *mdx* mice with a non functional telomerase enzyme show a muscular dystrophy that is considerably more severe and steadily worsens with age (Sacco et al., 2010).

Evidence is convincing that satellite cells can undergo only a finite number of cell divisions before they enter senescence. However, although this is likely to play a role in muscular dystrophies, there is little evidence to suggest that this mechanism is relevant to normal muscle ageing. Human somitic cells do not express detectable levels of telomerase and therefore satellite cell telomeres shorten with age. Experiments in fibroblasts have
shown that cells senesce with telomeres approximately 6kb in length (Allsopp et al., 1992). However, in culture, satellite cells enter senescence long before their telomeres would limit their capacity for cell division (Mamchaoui et al., 2011). Satellite cells isolated from aged humans were still capable of undergoing 15 rounds of cell division before senescence. This is comparable to the number of divisions observed in myoblasts from some 25 to 30 year olds (Renault et al., 2000). The number of cell divisions that can be undergone before senescence decreases significantly in human muscle biopsy derived myoblasts between birth and the age of 20. After this period there is no consistent decrease in the proliferative capacity of myoblasts and there exists a wide inter-subject variability (Renault et al., 2000). It seems then, that the satellite cell intrinsic mitotic clock is unlikely to be the limiting factor that causes the reduced regenerative capacity and a failure of muscle maintenance with age.

The decline in the regenerative ability of skeletal muscle with increasing age may be environmentally, rather than satellite cell intrinsically, regulated. Parabiotic pairing studies, in which two animals are joined together such that they share a circulatory system, have shown that, if aged skeletal muscles are exposed to a young systemic environment their regenerative capacity is comparable to young adult mice (Conboy et al., 2005). Furthermore if aged satellite cells are removed from their aged host and engrafted into young muscle, they are equally regeneration competent as young adult satellite cells (Collins et al., 2007). Therefore, although they are reduced in number, those satellite cells that remain in aged muscle have retained their regenerative capacity.

There are an infinite number of possible environmental regulators that could play a role in age-related changes in satellite cell function. Among those that have received the most attention are the Wnt and Notch signalling pathways (Hidestrand et al., 2008, Brack et al., 2008). Notch, is a
transmembrane protein that, when bound by its ligands, themselves transmembrane proteins on other cells, translocates to the nucleus and effects transcription. Notch is a known regulator of myogenic cell fate decisions in the embryo (Rios et al., 2011). In adult muscle, Notch has been shown to be essential for satellite cell activation and proliferation (Mourikis et al., 2012, Conboy & Rando, 2002). Wnts are a family of protein ligands found associated with cell membranes and the extracellular matrix defined by a common amino acid sequence (reviewed (Nusse, 2008)). Like Notch, the Wnt signalling pathway has been identified as an important regulator of embryonic myogenesis (Cossu & Borello, 1999). Wnt has been shown as essential in the satellite cell transition from proliferation to differentiation (Anakwe et al., 2003, Petropoulos & Skerjanc, 2002, Brack et al., 2007). Activation and inhibition of Wnt and Notch pathways in isolated satellite cell cultures suggest that changes in signalling from Notch to Wnt determine the balance between cell proliferation and differentiation in normal adult skeletal muscle regeneration (Brack et al., 2008).

Quiescent cells of aged muscle show an increase in Wnt signalling, and lineage tracing suggests that this acts to send satellite cells towards a fibrogenic lineage. Accordingly, systemic inhibition of Wnt in aged mouse muscle has been shown to improve its regenerative capacity following injury (Brack et al., 2007). Conversely, Notch signalling is seen to be reduced in aged muscle and activation of Notch 3 in aged muscles of the mouse improves their regenerative capacity (Pisconti et al., 2010, Conboy et al., 2003). Parabiotic pairing experiments show that the improved regenerative capacity of heterochronically paired aged muscle was concomitant with an increase in expression of the Notch ligand delta (Conboy et al., 2003).

An increase in Wnt and decrease in Notch signalling may offer an explanation as to the increase in fibrosis and decrease in satellite cell proliferation observed in aged muscles (Brack et al., 2007). However, it is unclear if these
changes in signalling pathways observed in the mouse can be generalised to human muscle. Human biopsy derived myoblasts cultured in the presence of serum derived from old or young humans showed that the age of the donor from which the serum was derived had no affect on satellite cell proliferation, fusion index, myogenic regulatory factor expression or myosin heavy chain expression (George et al., 2010). This suggests that, in the human, circulatory factors may not be critical mediators of the decline in skeletal muscle regenerative capacity with age.

As well as a loss of satellite cells and a change in circulating factors, the ageing muscle is characterised by a two fold increase in reactive oxygen species (ROS), DNA fragmentation and loss of myonuclei (Leeuwenburgh et al., 2005). The free radical theory of ageing states that oxygen free radicals, produced as by-products of normal metabolism, cause DNA damage which accumulates over the lifespan. This DNA damage eventually leads to mutations, cell damage, senescence and hence the ageing phenotype (Harman, 1956). There is good supporting evidence for this from caloric restriction experiments: Caloric restriction is known to increase lifespan and is associated with a reduction in ROS production and DNA damage (reviewed Heilbronn & Ravussin (2003)).

ROS are by-products of oxidative respiration and thus the principal source of ROS are the mitochondria. Thus, mitochondria are at high risk of suffering ROS mediated damage, this damage can cause mitochondrial dysfunction, leading to further ROS production in a positive feedback loop. Accumulation of ROS can lead to apoptosis of the mitochondria and, when enough mitochondria are lost, apoptosis of the cell (Harman, 1972). Mitochondrial ROS production and mitochondrial DNA damage increase with age in both mice and humans (reviewed Wei et al. (1998)). In culture, senescing cells show mitochondrial dysfunction. Delaying this dysfunction postpones senescence and, interestingly, slows telomere shortening, suggest-
ing that telomere shortening can be triggered by mitochondrial dysfunction (Passos et al., 2007).

Cytochrome C Oxidase (COX), an enzyme located at the mitochondrial membrane essential for ATP synthesis, is reduced in activity by 30% in aged compared to adult rat muscle (Chabi et al., 2008). Analysis of aged rat muscle sections suggests that ageing is associated with a specific loss in inter-myofibrilar mitochondria, whilst sub-sarcolemmal mitochondria are relatively preserved (Leeuwenburgh et al., 2005). In rhesus monkeys skeletal muscle atrophy has been associated with electron transport chain abnormalities and deletions in the mitochondrial genome (Lee et al., 1998) and in humans, mitochondrial DNA mutations have been shown to accumulate within skeletal muscle with increasing age (Bua et al., 2006). Evidence is consistent with the theory that skeletal muscle ROS accumulation leads to a loss of mitochondria, which may lead to a loss of myonuclei and satellite cells and thereby contribute to muscle atrophy and a reduction in regenerative capacity with age.

There is good evidence to suggest that instead, or perhaps as well, as an increase in ROS production, the ageing phenotype is caused by a failure of aged muscle to activate stress response pathways in response to ROS. Heat shock proteins (HSPs) are molecular chaperones that are normally rapidly upregulated in response to cellular stress. In both mouse and human muscle it has been shown that the up-regulation of HSPs in response to an increase in ROS production is significantly attenuated with increasing age (reviewed (McArdle et al., 2002)). Lifelong over-expression of HSP10 or HSP70 in mice prevents the age related decline in maximal tetanic force and protects against exercise-induced damage (Kayani et al., 2010, Vasilaki et al., 2010). This suggests that it is not the ROS itself that is detrimental, but rather age related changes in the cellular response to normal ROS production.

It is clear that skeletal muscle ageing is a complex combination of changes
in the satellite cell population and its surrounding environment. Data in chapter 3 of this thesis supports previous data (Collins et al., 2005) that show that satellite cell numbers are significantly reduced in aged muscles, but that a sub population of satellite cells survive skeletal muscle regeneration and remain regeneration competent. With increasing age, increased ROS production, decreased stress response pathways and changes in systemic factors will create an environment that impedes satellite cell function, even if this subpopulation of satellite cells are themselves relatively immune to age-related damage.

### 1.8 Satellite Cell Heterogeneity

Skeletal muscle is a heterogeneous tissue in terms of fibre type, myosin heavy chain expression, metabolic function, structure and embryological origin (see sections 1.1 and 1.4). Furthermore, the satellite cells associated with this heterogeneous tissue are also heterogeneous with regards to their gene expression profiles, proliferative, self renewal and regenerative capacities.

Whilst the majority of skeletal muscle derives from the somitic dermomyotome, satellite cells of the head and extra-ocular muscles (EOM) are derived from the pharyngeal arches. Satellite cells associated with these fibres of different embryological origins have been shown to have distinct regulatory cascades in both the embryo (see section 1.4) and the adult. All satellite cells in the adult EOM, head and body express Pax7. However head muscles show an up-regulation of Pitx1 and Pitx2 compared to satellite cells of the limb muscle and do not express Pax3 (Sambasivan et al., 2009). Interestingly, it seems this molecular memory can be overridden by environmental cues. When satellite cells isolated from EOMs are transplanted into the TAs of adult mice they produce large amounts of muscle regeneration and donor derived satellite cells. These donor derived satellite cells upon subsequent isolation, are found to have adopted the molecular signature of the
host TA (do not express Pitx1 or Pitx2) rather than their location of origin (Sambasivan et al., 2009).

The different embryological origin and gene expression profiles of satellite cells of the head and limb are reflected in their functional properties. In the rat, muscles of the head show less age-related changes in fibre size, fibre typing or contraction times (Norton et al., 2001). Head and limb muscles also have different regenerative capacities. In situ, head muscles do not regenerate after injury as efficiently as do limb muscles (Pavlath et al., 1998). In culture, satellite cells of the masseter are on average more proliferative and expressed differentiation or self renewing profiles earlier than satellite cells derived from the hind limb EDL muscle (Ono et al., 2010). However, after transplantation into the TA of the mdx nude mouse, masseter-derived satellite cells showed similar contribution to host muscle regeneration as EDL-derived satellite cells (Ono et al., 2010). Thus, there are clearly environmental factors that are critical regulators of satellite cell activity.

Although there are differences observed on average between populations of different muscles, satellite cells derived from both the head and the hind limb show considerable variance in the size of satellite cell-derived clones. In both muscles, there are some satellite cells that produce large colonies with cells that differentiate quickly, however, there are also satellite cells that show very limited proliferation. It is not just within whole muscles that such heterogeneity becomes apparent, even if isolated from the same fibre, two satellite cells can show huge differences in the size of the clusters they give rise to and their capacity for clonal expansion (Ono et al., 2010). This inter-cell variability between satellite cells of the same fibre is common to all skeletal muscles yet its underlying mechanism remains mysterious.

Satellite cells and their progeny are, like all stem cells, required to make cell fate decisions. A satellite cell may either die, differentiate to form new
myofibres or it must self-renew and contribute to the maintenance of the satellite cell pool. Studies of clones of satellite cells on single isolated myofibres show that a subset of satellite cell daughter cells re-express Pax7 and self renew (see section 1.3). However, the vast majority of satellite cell daughter cells exit the cell cycle and undergo differentiation (Zammit et al., 2004)(see section 1.3). The cell intrinsic genetic and molecular cascades that govern this crucial cell fate decision are unclear. It is an open question as to whether, prior to activation and proliferation, all satellite cells have the same capacity to self renew, or alternatively, whether self renewal is the responsibility of a sub-set of satellite cells that have distinct more stem cell-like characteristics.

Convincing evidence for a stem-like subset of satellite cells comes from satellite cell transplantation studies. Upon transplantation, donor satellite cells are able to contribute to host muscle regeneration, producing clusters of donor derived myofibres. Importantly, donor-derived satellite cells refill the host muscle satellite cell niche and can contribute to subsequent rounds of regeneration (see section 1.3). However, not all satellite cells have this same post engraftment regenerative capacity. The transplantation of radio-labelled male myoblasts into female mouse muscles shows that the majority of transplanted satellite cells die immediately following transplantation (Beauchamp et al., 1999). It is only the remaining few satellite cells that proliferate extensively to produce donor derived fibres. This same experiment observed that in culture, a subset of myoblasts are not labelled by the radioactive $^3$Hthymidine after a 16 hour exposure, demonstrating that they had not undergone cell division during this time. These slowly dividing cells, when injected into host muscle, proliferate extensively and give rise to donor derived muscle (Beauchamp et al., 1999). Cells that proliferate extensively in vivo and give rise to donor derived muscle are therefore those cell that divide slowly in culture.
These studies used satellite cells obtained through enzymatic digestion, known to be a crude and impure method of cell isolation. Furthermore, the use of radiolabelling requires a period of culture which will affect fundamental properties of the population. Subsequent transplantation studies have overcome these limitations, using small and very pure populations of satellite cells, to support the conclusion that not all satellite cells can regenerate muscle post transplantation. After transplantation of individual muscle fibres with their associated satellite cells (mean 7 satellite cells per fibre) only 1 in 8 engraftments successfully result in donor derived muscle 3 weeks later (Collins et al., 2005). Similarly, engraftment of isolated single muscle progenitor cells resulted in successful engraftment in just 2% of cases (Sacco et al., 2008). This is a remarkable accordance, in both cases it is observed that just 1 in 40 satellite cells can contribute to host muscle regeneration.

Many studies have suggested that a more stem-like satellite cell subpopulation can be identified by differing expression profiles e.g. Myf5, CD34, M-Cadherin, CXCR4 (Beauchamp et al., 2000, Kuang et al., 2007, Cerletti et al., 2008). However, much of this evidence has been difficult to interpret and findings are contradictory. For example, Kuang et al. (Kuang et al., 2007) used a transgenic mouse in which all cells that have expressed Myf5 will express yellow fluorescent protein (YFP) irreversibly, to demonstrate that 10% of satellite cells have never expressed Myf5. Upon transplantation, YFP negative (Myf5 negative) cells were shown to be more regeneration competent than YFP positive cells. Thus Myf5 has been postulated as an exclusion criterion for the isolation of a more stem cell like subpopulation of satellite cells. However, using the Cre-loxP recombination system to irreversibly label MyoD expressing cells, it has been demonstrated that 98% of all adult satellite cells have previously expressed MyoD (Kanisicak et al., 2009). As MyoD acts genetically downstream of Myf5 (Tajbakhsh
et al. (1997)) this should suggest that all satellite cells have also expressed Myf5.

Differences in methodologies employed in many of these studies may go some way to explaining the contradictory findings. It must be considered that to attribute functional relevance to a population based on a lack of expression, one must assume 100% efficacy of a system. It is possible that the YFP population observed by Kuang et al is the result of a loxP Cre recombination failure. Furthermore, markers can be expressed at a low level and therefore may be missed by immunostaining but detected by the more sensitive fluorescent activated cell sorting (FACs) or RNA sequencing techniques. Adding to the confusion, many studies fail to control for potential engraftment artefacts. Type IIa fibres show a green autofluorescence related to NADH hydroxylase activity. If not properly controlled for, autofluorescence can easily be mistaken for GFP expression (Jackson et al., 2004). Analysis of donor derived fibres within the mdx mouse is complicated by the presence of clusters of revertant fibres that arise due to naturally occurring splicing variations at the mRNA level (Fanin et al., 1992, Nicholson et al., 1989, Hoffman et al., 1990) (see section 1.9). Experiments that rely on dystrophin to assert donor origin of a myofibre are therefore unreliable. Furthermore, cytoplasmic markers such as GFP can overestimate the number of donor derived fibres due to cytoplasmic diffusion and a long half life, whilst nuclear markers will tend towards underestimation as they are easily missed on muscle cross sections. Thus separating satellite cell subpopulations based on marker expressions and assessing their subsequent regenerative capacity is fraught with methodological pitfalls.

In other systems, stem cell subpopulations have been delineated based on chromosomal segregation. Observations in the embryonic fibroblast and interstitial cells of the epithelium have led to the ‘immortal strand hypothesis’. This states that a stem cell selectively segregates template and newly
synthesised DNA strands such that template DNA strands are passed to the
daughter cell that will remain as an undifferentiated progenitor, whilst the
newly synthesised strands are passed to the daughter cell that will differ-
entiate and become post mitotic cells. Asymmetric segregation of template
and newly synthesised DNA strands is thought to be a mechanism by which
some stem cells avoid the accumulation of replication errors (Cairns, 1975,
Lark et al., 1966, Potten et al., 1978). This has relevance to the loss of tis-
sue specific stem cells with age. While template strand segregation protects
against replicative errors, in the long run it would contribute to the stem
cell accrual of replication independent damage (e.g ROS damage) and thus
may play a role in stem cell senescence (Charville & Rando, 2011).

Studies suggest that a rare sub population of satellite cells can undergo
template strand segregation. Using BrdU label retention, it was demon-
strated that between 7% and 50% (depending on experimental conditions)
of satellite cells asymmetrically segregate template from newly synthesised
stands. In accordance with the template strand hypothesis, daughter satel-
tite cells seen to inherit the template strand retained the undifferentiated
satellite cell state, whilst those that inherited the newly synthesised strands
went on to differentiate (Shinin et al., 2006, Conboy et al., 2007). It has been
convincingly demonstrated that haematopoietic stem cells do not asymmet-
rically segregate DNA (Kiel et al., 2007) and template strand segregation
cannot therefore be considered a general stem cell property. However, it
is possible that template strand segregation may occur in a sub popula-
tion of satellite cells, and that this property may define a stem satellite cell
population.

It is possible to define stem cell subpopulations based on radio-resistance
(Bao et al., 2006, Meijne et al., 1991, Heslop et al., 2000). When mouse
hindlimbs are treated with ionizing radiation, very little regeneration is ob-
served (Wakeford et al., 1991, Pagel & Partridge, 1999). However, when
irradiated muscle is subsequently treated with notexin, muscle is capable of regeneration. Single fibres isolated from irradiated fibres do not produce any emanating satellite cells, however single fibres isolated from irradiated and notexin treated muscles show a population of highly proliferative cells that emanate from the fibre (Heslop et al., 2000). Interestingly, these cells are observed in wild type, but are absent in mdx mice (Heslop et al., 2000). This demonstrates that there are a number of functionally distinct satellite cell populations, one of which can be defined by its resistance to high dose radiation and activation by extreme muscle injury (see chapter 4).

Ageing also appears to select for a specific subpopulation of satellite cells. Although aged muscles have significantly fewer satellite cells per fibre, they are able to regenerate host muscle equally as well as satellite cells isolated from young adults (Collins et al., 2007) (see section 1.7). This suggests that those satellite cells that are present on aged fibres are the subpopulation of satellite cells that divide slowly in culture and can engraft efficiently (Beauchamp et al., 1999). This is consistent with the smaller colony size observed from aged satellite cells using methods that only allow 96 hours of culture (Collins et al., 2007) (section 3 figure 3.4). It is still unclear if survival after transplantation is due to true heterogeneity or rather is the characteristic of a stochastic process, reflecting differing transient states within the satellite cell population (e.g. a cells position in the cell cycle). Nevertheless, it is tempting to speculate that those cells resistant to ionizing radiation, and those that survive skeletal muscle ageing, are the same population of satellite cells capable of muscle regeneration post engraftment in wild type adult mice (see chapters 4 and 5).
1.9 Satellite Cell Transplantation: A Therapeutic Tool?

1.9.1 Duchenne Muscular Dystrophy

DMD is an x-linked recessive severe muscle wasting disease (reviewed Sussman (2002)) caused by an absence of the functional dystrophin protein due to a mutation in the dystrophin gene (Hoffman et al., 1987). Dystrophin is normally located under the sarcolemma of individual muscle fibres (Zubrzycka-Gaarn et al., 1988), where it provides a flexible mechanical link between the internal cytoskeleton of the muscle and the extracellular matrix (reviewed Blake et al. (2002), Campbell (1995)). In the absence of dystrophin, muscles are damaged with use and are forced to undergo continual cycles of repair (Petrof et al., 1993). Due to the constant muscle degeneration, eventually regeneration can not keep up, satellite cells become exhausted and skeletal muscle is lost, to be replaced by fibrotic and fatty tissue. (Blau et al., 1983b, Webster & Blau, 1990).

The absence of dystrophin causes muscle degeneration when that muscle is mechanically stressed (reviewed (Campbell, 1995)). Therefore, the pathology of DMD is not evident until between the ages of 2 to 5 (Jennekens et al., 1991). Patients present with a typically waddling gait, difficulty climbing stairs (Jennekens et al., 1991) and Gower’s sign (the use of the arms to assist in going from a sitting to a standing position) (Gowers, 1879). Children show a delay in running, unsteadiness when walking, hypertrophy of the calf muscles and proximal limb muscle weakness. This weakness becomes steadily more severe and typically patients are wheel chair bound by the age of 12. Wheel chair confinement is followed by progressive weakness of the arms and neck, and scoliosis of the spine. Patients typically die of respiratory failure in their mid 20s (reviewed (Blake et al., 2002).

Becker Muscular Dystrophy (BMD) is a milder form of DMD. BMD
pathogenesis is similar to DMD but much less severe, i.e. proximal limb muscle weakness occurs but patients typically retain the ability to walk well into adulthood. Cardiac and respiratory problems are less severe in BMD compared to DMD and most patients live into their 40s with some cases reporting a near normal lifespan (Ringel et al., 1977, Beggs et al., 1991).

As with DMD, Becker patients have mutations in the dystrophin gene. However, BMD mutations occur at locations that maintain the reading frame and allow effective mRNA splicing such that either a shorter but functional dystrophin protein is produced and/or dystrophin is present but less abundant than in normal muscle (Monaco et al., 1988). At the muscle membrane, this shortened dystrophin protein is sufficient to prevent the dramatic muscle wasting observed in DMD (Koenig et al., 1989, Hoffman et al., 1988). The milder phenotype of BMD is encouraging from a therapeutic perspective, as it suggests that insertion of a smaller dystrophin transcript more amenable to viral packaging (reviewed (Chamberlain & Rando, 2006) or restoring only some dystrophin expression in DMD muscles would provide considerable therapeutic gain.

1.9.2 The \( \textit{mdx} \) Mouse

The \( \textit{mdx} \) mouse is the most widely used animal model of DMD and its use has been fundamental to our understanding of the mechanisms of DMD and the investigation of potential therapeutic strategies. The \( \textit{mdx} \) mouse was first reported as a result of a screening programme on C57BL/10 mice designed to uncover mutations that effect enzyme activity in glycolysis. During this screen some mice were noticed to have elevated creatine kinase and pyruvate kinase levels. Histological examination of the muscles of these mice revealed evidence of muscle degeneration and regeneration (electron-dense mitochondria and increased central nucleation) and a muscle specific pathology that worsened with age. Linkage analysis revealed this was due to a recessive X-
chromosome linked mutation and inbreeding created the C57BL/10sn.\textit{mdx} mouse (the \textit{mdx} mouse) (Bulfield et al., 1984). Due to its muscle pathology and the X-linked nature of inheritance, the \textit{mdx} mouse was proposed as a model for the human DMD (see section 1.7, and 1.9). In 1987 a human cDNA clone of a portion of the DMD transcript was isolated based on its conservation between mouse and man (Monaco et al., 1986). The protein product of the DMD locus was targeted using polyclonal antibodies and shown to be absent in both DMD boys and the \textit{mdx} mouse. This protein was therefore named dystrophin (Hoffman et al., 1987). Cloning and sequence analysis later revealed the causative mutation of the \textit{mdx} mouse to be a point mutation in exon 23 resulting in a premature stop codon (Sicinski et al., 1989).

As with DMD patients, the \textit{mdx} mouse lacks a functional dystrophin protein. The \textit{mdx} mouse undergoes a marked muscle degeneration and regeneration beginning at 3 weeks (Anderson et al., 1987, Coulton et al., 1988). This peaks between 4 and 8 weeks (McGeachie et al., 1993) and is associated with necrosis (Grounds & Torrisi, 2004) and an increase in the number of newly regenerated myofibres. Muscle turnover continues in the \textit{mdx} mouse throughout life, but at lower levels than those observed between 4 and 8 weeks (McGeachie et al., 1993, Lefaucheur et al., 1995, Pastoret & Sebille, 1995, Pagel & Partridge, 1999). However, limb muscles are able to regenerate well so muscle weakness and fibrosis is not observed until later life (Lefaucheur et al., 1995).

Occasionally muscle cross sections from the \textit{mdx} mouse, as with DMD patient muscle, will reveal small clusters of dystrophin-positive fibres (Wilton et al., 1997). Revertant fibres make the assessment of donor derived dystrophin complex (Arechavala-Gomeza et al., 2010) and arise due to random splicing variations of mRNA (see section 1.9.3). Splicing changes can result in the ‘skipping’ of mutated regions during translation. Dystrophin
expression occurs when this skipping takes place at a point that, when tran-
scription is resumed, the missing portion leaves two ends that can be joined
together. The result is a shorter dystrophin transcript, that lacks the mu-
tated site and thus produces a truncated but functional dystrophin protein
(Klein et al., 1992, Wallgren-Pettersson et al., 1993).

There exist other mdx mouse models with differing mutations of the
dystrophin gene. The mdx2cv and mdx3cv the mdx4cv were created by
screening chemical mutagen-treated mice from a C3H/HeHa strain crossed
with the C57BL/10Sn mdx mouse for X-linked muscle pathology (Chapman
et al., 1989). The mdx2cv has a 14 base pair insertion at base 9762 (Cox
et al., 1993), mdx3cv has a point mutation in intron 65 (Cox et al., 1993),
and mdx4cv a nonsense mutation in exon 53 (Im et al., 1996). Usefully, these
mice have fewer revertant fibres than the mdx mouse (Danko et al., 1992)
but mdx2cv and mdx3cv show disruption of both muscle and non muscle
dystrophin (Cox et al., 1993).

The mdx52 is another mouse model of DMD on a C57BL/6J background
created with a targeting vector to produce a large deletion in exon 52 of the
dystrophin gene which recapitulates mutations frequently found in DMD pa-
tients (Araki et al., 1997). The pathologies of these mice differ only slightly
from the originally reported mdx mouse (Bulfield et al., 1984).

1.9.3 Stem Cell Transplantation for Muscular Dystrophy

Intra muscular injection of donor skeletal muscle myoblasts can successfully
restore some dystrophin expression in the mdx mouse hind limb (Morgan
et al., 1988, Partridge et al., 1989, Morgan et al., 1990). Although the
first demonstrations of this came in the late 1980’s (Morgan et al., 1988,
Partridge et al., 1989) myoblast transplantation was, and still remains, a
highly inefficient process. The amount of donor derived muscle fibres rarely
exceeds the number of cells initially injected suggesting very limited prolifer-
ation and/or death of the majority of transplanted cells (reviewed Partridge (2003)).

On the strength of the initial encouraging reports in animal models, myoblast transplantation therapy was rapidly taken to human clinical trial. This was despite the objections raised by some of the leading scientists of the field concerned about the preliminary nature of the supporting evidence, particularly with regards to immune rejection, and myoblast transformation after engraftment (reviewed (Partridge, 2002)). Nevertheless, a number of clinical trials proceeded and, as foreseen by many, met with very limited success. Allogenic transplantation of myoblasts via multiple intramuscular injections resulted in no functional or biochemical improvement to patient muscles (Karpati et al., 1993, Mendell et al., 1995, Gussoni et al., 1992, 1997). One trial reported only a small increase in dystrophin positive fibres but a significant increase in maximal force in a subset of muscles (Tremblay et al., 1993). However this could be accounted for by the administration of the immuno suppressant cyclosporin alone (reviewed Grounds & Davies (2007)), which has been shown to improve muscle strength in DMD boys (Sharma et al., 1993, Miller et al., 1997) and the mdx mouse (De Luca et al., 2005). One clinical trial did claim success in restoring dystrophin expression and improving patient phenotype (Law et al., 1997), however, amid much controversy, accusations of scientific misconduct, legal proceedings and an investigation by the FDA this was found not to stand up against independent analysis. Initial clinical trials of myoblast transplantation therapy were on the whole, entirely unsuccessful.

Many of the initial murine myoblast transplants were performed with immuno-compromised hosts, such as the mdx nude (Partridge et al., 1989) or severe combined immuno deficient mice (Huard et al., 1994). Myoblast transplantation into immuno-competent mice results in a large immune reaction, infiltration of lymphocytes, macrophages and granulocytes resulting
in donor cell death and host muscle damage (Irintchev et al., 1995, Wernig & Irintchev, 1995, Fan et al., 1996). Immune rejection is observed even when donors are matched for the major histocompatibility complex (MHC) with host muscles developing a significant immune response against minor antigens (Roy et al., 1993). Complicating matters still further, hosts have also been observed to develop antibodies to the dystrophin protein itself (Huard et al., 1992). Clinical trials used the immuno-suppressive drug cyclosporin A, however this was clearly not sufficient to overcome this dramatic immune response and it is likely that most patient engraftments were immuno-rejected. Moreover, subsequent studies have shown that cyclosporin itself induces apoptosis of myoblasts at the point of differentiation (Hardiman et al., 1993, Hong et al., 2002). Other trials have used the immuno-suppressant cyclophosphamide (Karpati et al., 1993). However cyclophosphamide was later shown to kill transplanted myoblasts along with other rapidly proliferating cells (Vilquin et al., 1995).

Immune-rejection of transplanted myogenic cells presents a significant but not insurmountable obstacle to successful stem cell therapy for muscle. Clinical trials show improved results with more powerful immuno-suppressants and increased cell numbers. In order to correct for the dramatic cell loss due to immune rejection, more recent clinical trials have used high density injections, just 1mm apart in the biceps brachii and the gastrocnemius with increased cell numbers and continuous immune suppression. Such protocols have met with some success with patients showing a significant increase in the number of dystrophin positive fibres in the injected compared to control muscle (Skuk et al., 2004). At 18 months 35% of patient myofibres were dystrophin positive, however it is not clear how many fibres this relates to and most of the muscle, as in the control, was seen to be replaced by fat and fibrotic tissue (Skuk et al., 2007). Although more encouraging than previous trials, high density injection resulted in a poor survival rate
and continuous immuno-suppression is clearly far from an ideal therapeutic strategy.

In addition to immune rejection, initial myoblast clinical trials were limited by a protocol that required a period of in vitro expansion. It is now understood that expanding satellite cell numbers in culture is detrimental to the satellite cell population and significantly reduces their subsequent in vivo capacity. The average human myoblast is capable of 20-25 cell divisions before reaching quiescence. Of relevance to the development of autologous transplantation techniques, in myoblasts isolated from DMD patients this is reduced to just 5 divisions (Decary et al., 1997). As discussed, (see section 1.7) due to replicative senescence, achieving the required amount of in vitro amplification will therefore limit the degree of proliferation engrafted cells can undergo in vivo (Cooper et al., 2003). Yet, even if these issues could be overcome (e.g. by the addition of telomerase), there remains a more fundamental problem: The satellite cell population is heterogeneous in terms of engraftment capacity (see section 1.8). Those satellite cells that engraft well, divide slowly in vitro, whilst those that die shortly following injection proliferate quickly. It follows therefore that, not only does in vitro expansion of satellite cells run the risk, as with any stem cell expansion, of reducing their proliferative capacity, selecting for cells with faster replication times due to mutations, but also with the satellite cell population, in vitro expansion will specifically select for cells that are ineffective in vivo.

These concerns have been borne out experimentally: single isolated satellite cells engraft rapidly and undergo multiple rounds of cell division before tissue homeostasis is met. Cultured myoblasts undergo cell death immediately following engraftment and those that survive do not undergo proliferation (Sacco et al., 2008).

Autologous transplantation protocols have been proposed as an alternative strategy to overcome immune rejection of donor cells (reviewed Pault
et al. (2007)). Autologous transplantation of stem cells would require the in vitro manipulation of these cells so that they express the desired corrected gene, e.g. for DMD a corrected version of the dystrophin gene. As stem cell and particularly satellite cell culture presents many problems of its own, a better understanding of the donor cell population is required before such methods can be clinically useful.

In murine myoblast transplantation models it has been shown that the majority of transplanted myoblasts die even in immuno-compromised mice (Fan et al., 1996, Hodgetts et al., 2006, Beauchamp et al., 1999). The speed of this initial cell death is too rapid to be accounted for by immunological rejection (Beauchamp et al., 1999). Only a small sub population of myoblasts are able to survive transplantation and there is good evidence to suggest that subsequent donor derived fibres are oligoclonal (Fan et al., 1996, Beauchamp et al., 1999, Cousins et al., 2004, Beauchamp et al., 1997, Collins et al., 2005) (see section 1.8). The formation of donor derived fibres therefore depends upon the proliferation of just a few surviving cells (Beauchamp et al., 1997, 1999). In the mouse, muscle pre treatments are necessary in order for this surviving population to proliferate (Boldrin et al., 2012). Pre treatments include damage with myotoxins (Huard et al., 1994) cryoinjury (Morgan et al., 1987, Wernig et al., 1991, Irintchev et al., 1997) or irradiation (Morgan et al., 1990, 1993, 1996, Wakeford et al., 1991, Collins et al., 2005, Boldrin et al., 2009) (see section 5.1). High dose ionizing irradiation is the most effective muscle pre treatment in the mdx nude mouse for subsequent donor derived dystrophin expression (Boldrin et al., 2012) (see section 5.1). However, this is not appropriate for use in humans, and it is necessary to understand the mechanism of action of irradiation before this knowledge can be translated into a therapeutically viable solution (see chapter 5).

A further problem with the transplantation of myoblasts to treat body
wide diseases such as Duchenne is the cells lack of mobility. Although some
myogenic cells have been reported integrate into muscle after intra arterial
injection (Dellavalle et al., 2007), this has not been verified by other in-
vestigations (Meng et al., 2011) and the very limited migration of donor
intra muscularly injected muscle derived myoblasts (Moens et al., 1996) un-
less these muscle are injured (Morgan et al., 1987), suggests these are not
able to cross the vasculature walls. Some research has elucidated potential
strategies to improve myoblast migration: muscle pre treatments with FGF,
a known mediator of cell migration in embryo (Pourqui, 2003), suppression
of MyoD expression (Smythe & Grounds, 2001) or activation of matrix met-
alloproteinases (El Fahime et al., 2000) that are meeting with some success.
Although the migratory capacity of satellite cells currently presents a signif-
icant limitation to muscle stem cell therapy, it is entirely conceivable that
local injection of satellite cells will be able to maintain function of individual
muscles. Whilst this is perhaps not the revolutionary cure that stem cell
therapy was once believed to promise, the value of the use of a single muscle
to a patients quality of life (for example maintenance of finger movement)
ought not be underestimated.

The most successful clinical restoration of dystrophin in DMD patients
has been through the use of oligonucleotide mediated exon skipping. Exon
skipping capitalises on the naturally occurring pheneomenon of mRNA splic-
ing variations that are known to cause revertant fibres in both the mdx
mouse and DMD patients. Antisense oligonucleotides are single stranded
oligodeoxynucleotides that hybridize to mRNA or pre-mRNA thereby af-
fecting splicing. Antisenese oligonucleotides can be used to specifically mod-
ulate splicing of the dystrophin gene so that the deleted region is skipped,
the protein is put back ‘in-frame’ and dystrophin expression is observed in
myofibres (Muntoni & Wood, 2011).

Exon skipping by the administration of targeted anti-sense oligonucleotides
has produced encouraging results at initial clinical trials (Kinali et al., 2009, van Deutekom et al., 2007, Goemans et al., 2011, Cirak et al., 2011). Much work now focuses on improving the chemistry, efficiency and applicability of this approach (reviewed (Lu et al., 2011)). One might imagine the ideal therapy for DMD as a combination of stem cell and exon skipping technologies. If exon skipping could be induced in isolated DMD patient satellite cells and these autologously transplanted, then, due to their capacity for extensive proliferation and self renewal, they could provide a source of dystrophin positive fibres for a life time.

The failure of the initial clinical trials and the challenges presented by myoblast and satellite cell migratory capacity have led to the investigation of other cell sources as therapeutic agents. There are a number of cell types reported as having myogenic capacity. Although in normal physiological conditions they do not contribute to muscle regeneration (Lepper et al., 2011) (see section 1.2), research suggests they have myogenic capacity in some circumstances. Side population cells, defined by their expulsion of the dye Hoescht 33342, have been shown to engraft into mouse skeletal muscle after intra arterial injection (Bachrach et al., 2006). Circulating cells expressing the marker AC133 have been isolated from human blood and shown to produce dystrophin expression in skeletal muscle of mdx immuno compromised mice (Torrente et al., 2004). Mesoangioblasts, a type of mesodermal stem cell isolated from vessels have, after transplantation, been shown to give rise to dystrophin expression in skeletal muscle of dystrophic dogs (Sampaolesi et al., 2006). The crucial advantage of these cells is their potential for systemic delivery, which would allow them to reach all muscles of the body and preclude the necessity of multiple painful injections.

Despite the rise of other stem cell populations, promising in terms of their potential methods of delivery, satellite cells remain an optimal choice for skeletal muscle stem cell therapy. Satellite cells have a far superior
regenerative efficacy upon engraftment compared to any other stem cell yet described. Mesoangioblasts transplantation protocols in dogs use $5 \times 10^7$ cells to show dystrophin restoration (Sampalesi et al., 2006). AC133 mediated dystrophin restoration uses injection protocols that require $20 \times 10^4$ cells (Torrente et al., 2004). Numbers of donor derived fibres are unfortunately not reported in these papers, making direct comparisons difficult, however it is clear that intra muscular injection of satellite cells can restore dystrophin expression with considerably smaller starting populations (Collins et al., 2005, 2007, Sacco et al., 2008, Boldrin et al., 2009, 2012).

The methods used for satellite cell/myoblast isolation are of critical importance for their subsequent in vivo efficiency. In the mouse, cells obtained from enzymatic disaggregation or using a pre-plating technique are typically injected at high numbers for successful engraftment. For example, Montarras et al. inject $2 \times 10^4$ myoblasts per host mouse muscle and observed a mean of 500-600 donor derived fibres (Montarras et al., 2005). Non-cultured populations of satellite cells, isolated in such a way that ensures their purity, are considerably more efficient than these cruder preparations. Collins et al. report up to 200 donor derived fibres from just 6-7 EDL myofibre associated satellite cells (Collins et al., 2005). Satellite cells isolated using the single fibre method followed by mechanical stripping of the associated satellite cells and immediate injection show up to 800 donor derived fibres from 400 injected cells (Boldrin et al., 2012). However, such experiments show considerable inter-transplant variability, likely due to the heterogeneous nature of the small starting population (see section 1.8).

The capacity of the satellite cell to self renew, refill the stem cell niche and contribute to subsequent rounds of regeneration, along with evidence that satellite cells are an irreplaceable cell population that are absolutely essential for effective muscle regeneration (Lepper et al., 2011), places the satellite cell as the most promising cell type for the development of stem
cell therapies. Moreover, freshly isolated satellite cells are by far the most efficient cell type for transplantation in terms of dystrophin positive fibres produced per cell injected. In order for satellite cells to begin to reach some of their potential as effective therapeutic agents, it is necessary to gain a better understanding of their basic biology. Specifically, a better understanding of satellite cell heterogeneity and properties of the environment that mediate satellite cell proliferation and self-renewal may provide new strategies of cell selection and expansion for more effective transplantation protocols.

1.10 Thesis Aims

In general this thesis aims to improve satellite cell engraftment efficiencies. To this end, this thesis explores satellite cell heterogeneity with a focus on how this heterogeneity may effect donor satellite cell contribution to host muscle regeneration post engraftment. Chapter 3 investigates changes in satellite cell numbers and their proliferative capacity in mice between the ages of 2.5 weeks and 2.5 years. Here, potential sex differences are also explored and different combinations of pre-weaned and adult male and female host and donors are tested for efficiency. Chapter 4 explores a satellite cell sub population that is able to survive high dose radiation and asks whether these radio resistant cells play a distinct role in muscle maintenance or in muscle regeneration after transplantation compared to radio sensitive satellite cells. Satellite cell transplantation efficiency is greatly increased when the host muscle is treated with high dose ionising radiation prior to engraftment. Chapter 5 investigates the effects of different doses of host muscle radiation on donor satellite cell engraftment and explores potential mechanisms by which exposure to radiation may affect the donor satellite cell population.
Chapter 2

Materials and Methods

Throughout this thesis wild type refers to a C57BL/6 mouse (see section 2.1) that is negative for any transgene expression. All washes and incubations were performed with phosphate buffered saline (PBS) at room temperature unless otherwise specified.

2.1 Mice

Mice were bred and experimental procedures were carried out in the Biological Services Unit, of University College London, Institute of Child Health, in accordance with the Animals (Scientific Procedures) Act 1986. Experiments were carried out under Home Office licence.

All mice were on the C57BL/6 (Labs, 2012a) or C57BL/10 (Labs, 2012b) genetic background, unless otherwise stated. C57s are a general purpose in-bred mouse strain and are the most commonly used for experimental investigation. They are easy to breed and detailed knowledge of their physiology and genetics is readily available. The C57BL/6 mouse was initially developed by C.C. Little in 1921 and the BL/10 derives from the same stock separated before 1937 (reviewed (Beck et al., 2000). C57BLs have dark coats and generally speaking, bad temperaments: grouped females show barbering
behaviour (the dominant mouse removes hair of others in the group), whilst males are prone to fighting, neither males nor females are easily handled due to a tendency to bite (Labs, 2012a,b).

2.1.1 Transgenic Models

Myosin 3F-nlacZ-2E

Myosin 3F-nlacZ-2E mice are a C57BL/6J x SJL that express nuclear lacZ (nlacZ) driven by the myosin light chain 3F (MLC3F) promoter with a 3’ enhancer element that enables strong muscle specific expression (Kelly et al., 1995). LacZ is a gene from the bacterium Escherichia coli which encodes β-galactosidase (βgal). In its natural environment, β-galactosidase is part of a lac operon which facilitates lactose digestion. X-gal, an analogue of lactose is used to assess enzyme expression. X-gal, when cleaved by βgalactosidase, produces a blue insoluble product that is easily visualised in muscle fibres and sections under the light microscope. As MLC3F is expressed only in differentiated muscle (reviewed Barton & Buckingham (1985)), lacZ can be seen only in myonuclei and is absent in satellite cells (Beauchamp et al., 2000). MLC3F is a myosin isoform found predominantly in type II muscle fibres. Accordingly, nlacZ expression is reduced in muscles that contain fewer numbers of type IIB fibres e.g. the differentiated soleus, the diaphragm, and the heart (Kelly et al., 1995).

Myf5nlacZ/+ 

The Myf5nlacZ/+ mouse has an nlacZ gene inserted downstream of the MRF Myf5 (Tajbakhsh et al., 1994). Myf5, and therefore nlacZ is widely expressed in the developing embryo but is seen exclusively in quiescent satellite cells or activated myoblasts in adult muscle (Beauchamp et al., 2000). The homozygous Myf5nlacZ/+ mouse is embryonic lethal (Tajbakhsh et al., 1994). Mice are bred using one wild type parent and one heterozygous Myf5nlacZ/+
parent generating offspring that have a 25% chance of being heterozygous for the Myf5^{lacZ} allele.

**Md x nude**

This thesis uses the *mdx* mouse, originally described by Bulfield et al. 1984 (Bulfield et al., 1984) that has a premature stop codon in exon 23 of the dystrophin gene (Sicinski et al., 1989) and therefore has an absence of dystrophin beneath the sarcolemma of the muscle fibres (see section 1.9.2). This *mdx* model remains the best characterised and most widely used *mdx* model.

Due to a spontaneous mutation in the FOXN1 gene nude mice do not undergo proper thymus development and therefore cannot generate mature T lymphocytes (Flanagan, 1966). The nude mouse is therefore immunocompromised. The nude mouse derives its name from its lack of fur growth. This is not associated with the role of FOXN1 in the thymus but is due to a role for FOXN1 as a critical regulator of the acidic hair keratin gene (reviewed (Mecklenburg et al., 2001)).

To avoid immune rejection after transplantation (see section 1.9.3), these experiments use the *mdx* nude mouse originally derived from an *mdx* x 129/ReJ cross, in order to distinguish between host and donor isoenzymes, back crossed to the outbred nu/nu strain (Partridge et al., 1989). The *mdx* nu/nu mouse has considerably smaller limb muscles and higher muscle collagen levels than heterozygous or control mice, collagen levels are comparable to controls at 12 weeks (Morrison et al., 2005) but muscles display and ageing phenotype earlier than wild type mice (Boldrin et al., 2009). Nude female mice have underdeveloped mammary glands: mice must therefore be bred using an *mdx* nu/nu male with an *mdx* heterozygous (nu/+) female.
Genotyping

Myosin 3F-\textit{nlacZ}-2E and \textit{Myf5}^{\text{nLacZ}/+} mice were genotyped between the ages of 4 and 7 weeks of age. The tip of the tail was sprayed with ethyl chloride local anesthetic (cryogesic) and a piece no greater than 1 inch in length was removed using a sterilized disposable round blade scalpel (Swann-morton Ltd. Surgical Scalpel Blade no.22). Avoca 95% silver nitrate sticks were used on the end of the tail to stem bleeding. Mice were given numbers, with identifying ear punches. Tail tips were placed in a 10mm dish and the skin removed under the light microscope using sprung dissection scissors (curved 8mm blade spring scissor, World Precision Instruments Ltd.). The remaining tail muscle was placed in a flat bottom 24 well tissue culture plate (BD Falcon, BD Biosciences) and analysed for X-gal expression using 1 mg/ml X-gal diluted in X-gal solution (0.01% sodium deoxycholate, 0.02% nonidet P40, 2 mmol/L MgCl2, 5 mmol/L potassium ferricyanide, and 5 mmol/L potassium ferrocyanide). Tails were incubated in 500\,\mu l of X-gal at 37\,\degree C overnight in plates protected from light with tin foil. Tails from myosin 3F-\textit{nLacZ}-2E mice were assessed for diffuse X-gal staining and tails for \textit{Myf5}^{\text{nLacZ}/+} mice for X-gal positive nuclei under the light microscope.

2.2 Dissection

2.2.1 Dissection of Mouse EDL

Mice were sacrificed by cervical dislocation and immediately pinned to a cork board for dissection. Mice were pinned face up through both fore limb paws and the hind limb to be dissected whilst the contra lateral limb was kept free for ease of movement. Hind limbs were sprayed with 70% ethanol and dried to prevent infection. Skin was removed from the foot and hind limb under a dissection microscope to expose the TA muscle. Using fine sprung scissors and forceps the thin fascia surrounding the hind limb muscles was
completely removed with care taken not to damage the underlying muscles.

The EDL tendon is easily located in the foot due to its multiple branching (see figure 2.1). The EDL tendon was cut just before branching to ensure no attachment remained. The TA tendon was localised (see figure 2.1) and cut as low down as possible. Once cut, the TA and EDL tendons can be pulled through the ankle joint and are easily separated. Holding the muscles by the tendon only, and taking care to exert only the minimum required force, the EDL and TA were pulled upwards towards the proximal end. Before reaching the knee level, the EDL was separated from the TA by pulling it back towards the foot. The TA was then removed by pulling further towards the proximal end. The TA could then be mounted and prepared for sectioning (see section 2.3). Removal of the TA allows the EDL tendon to be exposed at knee level. This tendon was cut and the EDL gently removed and transferred to 2ml of collagenase solution for single fibre isolation (see section 2.4).
Figure 2.1: Anatomy of the mouse hind limb A. Mouse hindlimb ventral aspect showing the TA (yellow) and the EDL (red) from knee joint to foot. B. Mouse hindlimb dorsal aspect shows the gastrocnemius (green) and the soleus (purple).
2.3 Isolation and analysis of Mouse Muscle Cross Sections

Transverse sections of mouse hind limb muscles were used to analyse gross morphology, muscle myofibre size, protein and gene expression.

2.3.1 Muscle Freezing

Gum tragacanth (Sigma-Alldrich) was dissolved 6g per 100ml in H₂O. This was kept at 50°C overnight to produce a thick, gelatinous substance. Aliquots were kept at 4°C, as they are prone to fungal and bacterial contamination. For muscle freezing, a small amount of gum was transferred with a spatula to labelled 20mm diameter round corks (Harris Raymond A Lamb Ltd.) prior to muscle dissection. Dissected muscles were placed on a 10mm Petri dish and bisected using a flat bladed scalpel (Swann Morton Ltd. Blade 11). Muscles were then mounted on the prepared cork in a transverse orientation, such that the centre of the muscle faced upwards. Muscles were placed in isopentane cooled in liquid nitrogen for 60 seconds, then removed and placed immediately in liquid nitrogen. Muscles were transferred to a −80°C freezer for long term storage.

2.3.2 Cryosectioning

Cryosections were cut using a Leica CM1850W cryostat. Muscles were placed in the cryostat freezing block at -40C for at least 10 minutes. Corks were attached to cryostat chucks using OCT embedding matrix (Harris Raymond A Lamb Ltd.). 7µm transverse serial sections were collected in rows, using 6 aminopropyltriethoxysilane (APES)-coated glass microscope slides sequentially, such that the same column and row of each slide would represent a section of muscle no greater than 28µm from the other. Rows were separated at 100µm intervals. This was repeated until the entire muscle had
been sectioned. Sections were air-dried for at least 20 minutes before being stored at $-80^\circ\text{C}$ until use.

2.3.3 Hematoxylin and Eosin Staining of Muscle Sections

Thawed sections were submerged in Haematoxylin (Harris Raymond A Lamb Ltd.) diluted 1:1 in milliQ water for 2 minutes. They were then washed under running tap water for 2 minutes. Sections were placed in 1% Eosin (VWR International) in water milliQ for 20 seconds and washed under running tap water for 5 minutes, dipped quickly in 70% ethanol, followed by 100% ethanol and placed immediately in Histo-Clear (National Diagnostics.). Round glass cover slips thickness 1 (VWR International) were applied to slides with DPX mounting medium (VWR International).

2.3.4 X-gal Staining of Muscle Sections

Sections were allowed to thaw before being re-hydrated in PBS. Sections were fixed for 10 minutes on ice, in cold 0.5% gluteraldehyde (Sigma-Aldrich) and washed in PBS for 10 minutes. In order for the X-gal to penetrate the cell membrane sections were permeabilised with 0.01% sodium deoxycholate, 0.02% nonidet P40 and 2 mmol/L MgCl2 for 10 minutes. Sections were then incubated overnight at 37°C in 1 mg/ml X-gal diluted in X-gal solution (0.01% sodium deoxycholate, 0.02% nonidet P40, 2 mmol/L MgCl2, 5 mmol/L potassium ferricyanide, and 5 mmol/L potassium ferrocyanide). Sections were washed 3 times for 10 minutes in PBS. 8µl of fluorescent mounting medium (Dako) containing 1.5µg/ml DAPI was placed on the slide and sections were covered with a 25 x 60 rectangular glass cover-slip thickness 1 (VWR International).
2.3.5 Dystrophin Staining of Mouse Muscle Sections

Thawed and dried sections were circled with a liquid blocker PAP pen (Ted Pella inc.) before re-hydration with PBS. Sections were blocked with 10% goat serum (Sigma-Aldrich) in PBS and washed for 3 x 10 minutes. Sections were then incubated for 1 hour at room temperature with primary rabbit anti-dystrophin (P7) (Lu et al., 2005) diluted 1:1000 in 10% goat serum, PBS. Sections together with a secondary only control were washed for 3 x 10 minutes then incubated for 1 hour with Alexa Fluor 594-conjugated goat anti-rabbit Ig secondary antibody (Molecular Probes). Sections were washed a further 3 times and mounted with fluorescent mounting medium (Dako) containing 1.5µg/ml DAPI.

2.3.6 CD31/45 Staining of Mouse Muscle

Sections were thawed then re-hydrated with PBS. Sections were outlined with a liquid blocker PAP pen and fixed in 4% paraform-aldehyde for 10 minutes. After 3 x 10 min washes, sections were incubated overnight at room temperature with primary antibodies, polyclonal rabbit anti mouse CD31 (Abcam) and monoclonal rat anti mouse CD45 (Serotec) diluted 1:50 (see table 2.1). Stained sections, along with a secondary only control were washed for 3 x 10 minutes then incubated with secondary antibodies Alexa Fluor 594 goat anti rat, and Alexa Fluor 488 goat anti rabbit (Molecular probes). Sections were washed a further 3 times and mounted with fluorescent mounting medium (DAKO) containing 1.5µg/ml DAPI.

2.4 Single Fibre Analysis

Single fibre isolation is the process of isolating individual muscle fibres in a manner that avoids their contraction and keeps the basal lamina intact. For successful single fibre isolation, it is essential that the muscle is carefully
dissected from tendon to tendon, with special care taken not to pull the muscle during extraction as described in section 2.2.1. Mechanical stress on the fibre during dissection results in hyper contracted non-viable fibres. Due to the method of extraction and the multiple wash protocol, isolated single fibres are free from contaminating cells from outside the basal lamina (Bischoff, 1975, Rosenblatt et al., 1995).

Once isolated, single fibres can be plated and their associated satellite cells will activate, proliferate and emanate from the fibre. The substrate that single fibres and satellite cells are cultured on has been shown to change their activation, proliferation and self renewal properties (Engler et al., 2004) and their subsequent ability to regenerate muscle in vivo (Gilbert et al., 2010). Furthermore, the physical structure of the satellite cell niche plays an important role in regulating satellite cell properties ((Boldrin et al., 2012) and reviewed (Boonen & Post, 2008)). As an alternative to plating, single fibres can be kept in suspension culture where satellite cells remain within the basal lamina and form myofibre associated colonies within 72 hours (Zammit et al., 2004, Collins et al., 2007, Gnocchi et al., 2009). Single fibre suspension culture provides an important tool to study satellite cell activation and proliferation whilst maintaining the satellite cell interaction with its native matrix and its niche signalling (Zammit et al., 2004).

Satellite cells can be isolated from single fibres by physically stripping them from the basal lamina and removing myonuclei and debris based on size (Collins et al., 2009, Boldrin et al., 2009, Shefer et al., 2010). This is the purest method available of isolating satellite cells and does not require harmful chemical or mechanical dissociation.

### 2.4.1 Single Fibre Isolation

Prior to EDL dissection, 2% collagenase type I (Sigma-Aldrich) was prepared in Dulbecco’s modified eagles medium (DMEM; Gibco) and filtered
for sterilisation with a 0.22µm filter. Dissected EDL muscles (see section 2.2.1) were placed in collagenase 1ml/muscle and incubated in a water bath at 35°C for 60 (2 week old mice), 70 (3 month/1 year old mice) or 90 (2.5 year old mice) minutes.

After digestion, each muscle was placed in a 50mm horse serum-coated bacteriological Petri dish (Sterilin Ltd.) containing 8ml of warmed DMEM. Muscles were gently triturated using a horse serum coated modified short glass Pasteur pipette (230mm Corning Inc.) under microscopic observation. Pipettes were modified using a diamond tip pencil. For the short pipette the thin end of the Pasteur pipette was removed and the edge rounded with a flame to eliminate any edges that may damage the basal lamina. For the long pipette, Pasteur pipettes were bent at the thin end and rounded with a flame. As individual myofibres began to separate from the muscle mass they were selected with the long modified pipette and placed into a second horse serum-coated dish. In the second dish fibres were triturated once again and moved to a third dish. This process was repeated in the third and fourth dishes to ensure that all contaminants and debris had been removed from the outside of the fibre. After four washes, the final dish is then full of intact single myofibres.

After digestion of the muscle, single fibres were isolated as quickly as possible so as to avoid satellite cell activation. Analysis of single fibres isolated from adult male wild type mouse EDL muscles (N=2), with antibodies against Pax7 and MyoD shows that the trituration process activates satellite cells (see figure 2.2). As the length of trituration after digestion increases, so the percentage of satellite cells that are activated also increases. 50% of satellite cells are activated before fixation if this process takes longer than 1.5 hours. Therefore, in the experiments reported here, it was ensured that trituration took no longer than 20 minutes.
Figure 2.2: Satellite cells activated by trituration a) Graph shows the percentage of Pax7\(^+\) cells that were also MyoD\(^+\) (i.e. the activated proportion of the satellite cell population) on single fibres fixed after 20 minutes, 30 minutes and every 30 minutes thereafter up to 3 hours of trituration. Results show that as trituration time increases the percentage of the satellite cell population that is activated also increases. b) Example images of single fibres showing a Pax7\(^+\)/MyoD\(^-\) quiescent satellite cell on a fibre fixed after 20 minutes of trituration and a Pax7\(^+\)/MyoD\(^+\) activated satellite cell on a fibre fixed after 3 hours of trituration.
2.4.2 Single Fibre Culture

For single fibre suspension culture, isolated fibres were placed in horse serum coated 50mm bacteriological Petri dishes (Sterilin Ltd.). Fibres were individually selected and cultured at no more than 30 fibres per dish in 4ml of plating medium (10% horse serum (Gibco), DMEM, 0.005% chick embryo extract (Sera Laboratories), 4mM L-glutamine (Sigma-Aldrich), 1% penicillin and streptomycin antibiotics) and incubated at 37°C for up to 96 hours.

2.4.3 Single Fibre Immunostaining

Single fibres were removed from plating media using the long modified Pasteur pipette, and placed in a 2ml tube (Eppendorf). The tube was then filled with 4% paraformaldehyde (PFA), shaken and left for 10 minutes. After 3 x 10 minute PBS washes, fibres were permeabilized with 0.5% Triton X-100 (Sigma) for 6 minutes, and blocked with 10% goat serum (Sigma-Aldrich) for 30 minutes. Fibres were incubated overnight at 4°C with primary antibodies (see table 2.1). A secondary only control was incubated in PBS. The primary antibody was removed with 3 x 10 minutes PBS washes, and together with secondary only control, fibres were then incubated for 1 hour with the appropriate Alexa-Fluor secondary antibody (Invitrogen, Molecular Probes). Fibres were individually placed onto a glass polysine microscope slide (VWR International) using a modified glass Pasteur pipette under microscopic observation (see section 2.4.1). Taking care to remove any excess liquid, 6µl fluorescent mounting media (DAKO) containing DAPI was placed over the fibres and this covered with a round 22mm cover-slip (VWR International). Slides were allowed to dry before analysis under the fluorescent microscope.
2.4.4 X-gal Staining of Single Fibres

X-gal staining was performed on single fibres in conjunction with immunostaining. After gathering fibres in 2ml tubes (Eppendorf) (see section 2.4.3) fibres were incubated for 30 minutes in 1 mg/ml X-gal diluted in Xgal solution (0.01% sodium deoxycholate, 0.02% nonidet P40, 2 mmol/L MgCl2, 5 mmol/L potassium ferricyanide, and 5 mmol/L potassium ferrocyanide). Fibres were washed for 3 x 10 minutes in PBS and then fixed for 10 minutes with 4% PFA. Immunostaining could then proceed as described above (see section 2.4.3)

2.4.5 Single Fibre TUNEL Assay

Fixed single fibres were first immunostained as described above, (section 2.4.3) using only a monoclonal antibody. Taking care to protect fibres from the light, fibres were permeabilised with 70% ice-cold ethanol for 45 minutes at -20°C. Fibres were washed twice with PBS and once with wash buffer from the Merck Chemicals, Apo-BrdU kit. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining continued according to the kit protocol. Fibres were incubated for 60 minutes in DNA labelling solution at 37°C, washed as per manufacturers instructions and mounted on glass microscope slide as described (section 2.4.3).

2.4.6 Satellite Cell Isolation from Single Fibres

Following the final wash of the single fibre isolation protocol (see section 2.4.1), Single fibres were pooled in a horse serum coated dish containing 1ml of plating medium (10% Gibco horse serum DMEM, .005% chick embryo extract, 4mM L-glutamine (Sigma-Aldrich) and 1% penicillin and streptomycin antibiotics). To remove satellite cells from the myofibre, fibres were triturated continuously for 10 minutes with a 19-gauge needle (Terumo) attached to a 1ml syringe (BD Plastipak) after which all fibres could be seen
to be contracted. Media was then passed through a 40µm filter to remove myofibre sheaths and debris and cell suspension collected in a 50ml tube (Falcon). Cell suspension could then be centrifuged for satellite cell transplantation (see section 2.6), or cells placed immediately in flasks/chamber slides for cell culture (see section 2.4.7). Satellite cell number was estimated by counting the number of single fibres prior to stripping and multiplying this by the appropriate mean number of satellite cells per fibre (see figure 3.2)

2.4.7 Satellite Cell Culture

8 well permanox chamber slides (Nunclon;Nalgene Nunc International) were coated with Matrigel (0.1 mg/ml; BD Biosciences) and incubated for 30 minutes before the Matrigel was removed taking care to eliminate any excess from the corners. Satellite cells were isolated as described above (section 2.4.6) and cell suspension was plated in coated 8 well chamber slides at 250µl per well. Satellite cells were kept in plating media, 50% volume was changed 24 hours after plating and 100% changed every 3 days thereafter.

2.4.8 Satellite Cell Clonal Culture

For clonal culture, satellite cells were diluted to the appropriate volume based on myofibre number multiplied by the average number of satellite cells per fibre according to the age and sex of the mouse (see figure 3.2). 96 well flat bottom modified polystyrene plates (Falcon) were coated with 30µl matrigel (0.1 mg/ml; BD Biosciences) diluted 1:10 DMEM. Cells were plated in 20% Gibco horse serum, 2% chick embryo extract in DMEM at 2 satellite cells per well (volume 100µl per well). Plates were incubated at 37°C for 7 days. 50% of medium was changed after 24 hours of culture and every second day thereafter.
<table>
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**Table 2.1:** Primary Antibodies
2.5 Irradiation of Mouse Hindlimbs

For radiation, mice were anesthetised with 25% Hypnorm (Janssen: fentanyl citrate, final concentration 0.79 mg/mL: fluanisone, final concentration 2.5 mg/mL), and 25% Hypnovel (Roche: midazolam, 1.25mg) in distilled H₂O administered by subcutaneous injection. For transplantation mdx mice were irradiated at 3-4 weeks of age, WT mice were irradiated between 4 weeks and 3 months of age. Mice hind limbs were irradiated in a Gamma service Medical GmbH Cs-137 irradiator at a dose rate of 0.73Gy per minute as this is optimal for subsequent donor cell engraftment (Gross et al., 1999). Cs-137 is a non naturally occurring radioactive isotope formed by nuclear fission, which undergoes beta decay to become barium-137 and emits gamma radiation, with an estimated half life of 31 years. The body and head of the mouse was protected with 4cm of lead. For consistency with previous literature host mdx nu/nu and irradiated donors were irradiated 3 days prior to engraftment (Gross et al., 1999, Gross & Morgan, 1999, Morgan et al., 2002, Collins et al., 2005, 2007, Boldrin et al., 2009).

2.6 Satellite Cell Transplantation

Due to random splicing variations of the dystrophin mRNA transcript, mdx mouse muscle contains rare clusters of revertant fibres (Wilton et al., 1997) (see section 1.9). In order to demonstrate the presence of donor-derived dystrophin positive fibres it is therefore not sufficient simply to show the presence of dystrophin (reviewed Partridge et al. (1998)). To control for this, we use myosin 3F-nlacZ-2E donors. As myonuclei derived from myosin 3F-nLacZ-2E donor satellite cells express βgal and dystrophin this allows for a double marker of donor derived fibres. At analysis, only those sections that were first seen to be positive for X-gal staining were analysed for dystrophin expression (see section 2.6.3). Although this system is reliable
for the identification of donor derived fibres it has a number of limitations. 
\(\beta\)gal expression cannot be used to quantify donor derived fibre number due to promotor silencing and diffusion of \(\beta\)gal across muscle sections (for an example see figure 3.10). Thus \(\beta\)gal is used to verify donor origin of a 
dystrophin cluster but is not used for quantification. For quantification, the 
number of dystrophin positive fibres is counted (see section 2.6.3). However, 
this also has its limitations: it is unknown how many donor derived nuclei 
are required in order for a myofibre to become dystrophin positive, nor is it 
clear the extent to which dystrophin can spread across a myofibre mem-
brane. Although these limitations must be borne in mind when interpreting 
data, this system is preferred to alternative nuclear markers of donor origin 
as quantifying nuclei on cross sections will have a very high false negative 
rate, a nucleus of donor origin could only be counted if the cross section cut 
precisely through it.

2.6.1 Intra-Muscular Satellite Cell Injections

Donor EDL satellite cells were isolated using the single fibre method (see 
section 2.4.6) from myosin 3F-\(nlacZ\)-2E mice. Myofibres were counted prior 
to satellite cell removal and this used to estimate satellite cell number (see 
section 2.4.6). Satellite cells were centrifuged at 240g for 15 minutes at room 
temperature, the pellet was collected in as small a volume as possible and 
placed immediately on ice. The supernatant was centrifuged at 600g for a 
进一步 20 minutes at 4°C. This pellet was re-suspended, transferred to the 
Ependorff tube along with the first pellet and placed on ice.

Immediately after donor cell isolation, host \(mdx\)-nude mice were anes-
thesised with isoflourane. The TA was located and a small incision was 
made through the skin at the bottom of the TA nearest the foot with a ster-
ile flat bladed scalpel (Swann Morton blade 11). Satellite cells were drawn 
into a sterilised fine glass needle and this pushed through the incision and
upwards into the TA under microscopic observation. Satellite cells were thus injected into the middle of the TA muscle in a volume no greater than 10µl/muscle. Mice were kept warm on a heat mat until they were recovered from anesthesia and were provided with wet food for 24 hours post surgery. Host *mdx*-nude mice were all between 3 and 4 weeks old at the time of engraftment.

### 2.6.2 Devascularisation of Mouse Hind Limb

Donor EDL satellite cells were prepared as above (2.6.1). Host *mdx*-nude mice were anesthetised with isoflurane. Using a flat bladed scalpel the skin and surrounding thin fascia were cut through to expose the length of the TA muscle. A sterile 8mm round bodied silk suture suture (Johnson & Johnson int.) was passed underneath the TA tendon at the knee joint, by applying considerable force but being careful not to rip the tendons the suture was passed underneath the TA muscle for the entire length of the TA. The TA was seen to lose its red colour and turn white, thus it was clear that the connecting vascular network had been severed. The skin was sutured closed. Mice were kept warm on a heat mat until they were recovered from anaesthesia and were provided with wet food for 24 hours post surgery. Sutures were checked 24 and 48 hours post surgery and skin was re-sutured under isoflurane anesthesia where necessary.

### 2.6.3 Histological Analysis of Grafted Muscle

4 weeks post engraftment, host mice were killed by cervical dislocation and the TA muscles removed. TA muscles were frozen in isopentane cooled in liquid nitrogen (see section 2.3.1). Muscles were kept at -80 until cryosectioning (see section 2.3.2). 7µm transverse sections were collected at 100m intervals from the whole muscle. Cryosections were rehydrated with PBS and X-gal stained as described previously (see section 2.3.4). Sections se-
rial to those with X-gal positive donor-derived fibres were immunostained for dystrophin and the number of dystrophin positive myofibres in a single section counted. This figure was used for comparative statistics.

2.7 Gene Expression Analysis

Microarrays enable the analysis of the expression level of a large numbers of genes simultaneously (reviewed (Duggan et al., 1999) and (Stears et al., 2003)). The two-channel microarray requires the isolation of RNA from a control and an experimental group. This RNA is converted to a cDNA library via reverse transcriptase. Each library is then labelled with fluorescent fluorophores of differing emission wavelengths. The labelled cDNA libraries are then mixed and passed over a solid chip (Affymetrix array) or micro beads (Illumina) containing a set of oligonucleotide probes. Exon arrays contain approximately 1.2 million probe sets that can be grouped into over 1 million exon clusters and over 80,000 transcript clusters (groups of putative alternatively spliced transcripts from the same gene). cDNA binds to the probes and the amount of binding is measured by fluorescence intensity. The amount of binding is a proxy for how much starting material was present in the cDNA library and therefore how much of that specific RNA transcript there was present in the initial sample. Unlike next generation sequencing techniques, such as DNAseq or RNAseq, which give sequence data for all exons, microarrays give relative expression values of probed genes. Microarray results are therefore expressed either on a scale normalised to positive and negative control probes or a ratio or fold change between experimental and control samples.
2.7.1 RNA Extraction

Satellite cells were isolated from single fibres as described previously (see section 2.4.6). Satellite cells were pelleted by centrifugation at 240g for 15 minutes at room temperature, the supernatant was discarded and the pellet placed immediately on ice. Cells were spun for 2 minutes at 10000g and any remaining media carefully removed. RNA was purified using QIAGEN RNAeasy Micro Purification kit containing β mercaptoethanol. RNA was kept on ice throughout the procedure. RNA concentration was measured using thermo scientific NanoDrop and was stored at −80°C until use.

2.7.2 Microarray

Microarrays were performed by UCL genomics. Due to the rarity of radio resistant satellite cells RNA concentration from satellite cells isolated from irradiated muscle was very low. RNA was amplified using the NuGen Ovation Pico V2 Kit Protocol accordin to manufacturers instructions. Fragmentation and labelling of cDNA was performed using the NuGen Encore Biotin Module. Samples were then run on an Affymetrix GeneChip array chip type MoEx-1-0-st-v1. The hybridisation protocols were performed on GeneChip Fluidics Station 450, and scanned using the Affymetrix GeneChip Scanner.

2.7.3 Microarray Data Analysis

Array quality controls were created by UCL genomics using Expression Console followed by Bioconductor. Data were normalised using the robust multichip average (RMA) normalisation algorithm in Expression Console 1.2 and imported into Partek to calculate a transcript signal value from probes. Array data was checked for outliers and grouped using Principal Component analysis. Between groups, analysis took place on two levels, gene and exon. At the gene level, exon data was summarized by mean value to obtain one
expression value per gene. These values were used in ANOVA to determine significance in gene expression for each gene between wild-type and mutant groups and significance lists were created. To reduce false positives due to multiple testing the Benjamini and Hochberg algorithm was applied to ANOVA p values. This was carried out at UCL genomics.

I analysed gene and exon expression values using Excel and Matlab software and UCL genomics provided full ANOVA gene lists which I filtered with Excel software to create tables 4.1, 4.2.4.3 and 4.4. I analysed raw microarray chip data in Matlab and used GIMP image software for figure 4.7.

2.8 Data Analysis

2.8.1 Images

Fluorescence and brightfield images were taken using a Zeiss Axiophoto microscope (Carl Zeiss, UK) and Metamorph image capture software (Metamorph production, UK). Further image processing was achieved with ImageJ (rsbweb.nih.gov/ij) and photoshop. On all graphs * indicates significance p <0.05, ** p<0.01 and *** p<0.001.

Myofibre length and width were ascertained by capturing serial photos at 10X magnification along the entire length of the fibre. Ensuring that there was a degree of overlap at the left edge of each photograph enabled these images to be stitched together automatically using Photoshop stitch tool. Length was measured using metamorph and image J measure tools. As the width of the fibre can change along its length, width was measured at 6 locations and the mean value used to calculate volume.
2.8.2 Statistics

Preliminary data analysis was carried out in Excel. Data was collated, analysed and graphs created using Graphpad Prism software.

Means were compared for significance using either the student T test to compare the means between 2 groups, or ANOVA on occasions when the means of more than two groups required comparison. Both tests used a confidence interval of 95%. If ANOVA revealed a significant overall main effect or an interaction, post hoc T tests were performed in order to reveal the direction of change between selected means.

Myonuclear shape was analysed using ImageJ software. Circularity $C$ can be calculated as

$$C = \frac{4\pi \times \text{area}}{\text{parameter}}$$

Volume was calculated assuming that the myofibre is cylindrical in shape using

$$volume = \pi \times length \times r^2$$
Chapter 3

The Satellite Cell in Male and Female Developing and Adult Mice

3.1 Introduction

There is considerable evidence to show that muscle mass is in part mediated by exposure to circulating androgens. Muscle mass is decreased in androgen receptor knock out male mice, but not affected in knock out females (MacLean et al., 2008). Furthermore, androgens have been shown to directly regulate protein synthesis: gonadal steroid suppression in healthy men decreases whole body protein synthesis (Mauras et al., 1998), whilst testosterone administration increases net muscle protein synthesis in young (Sheffield-Moore et al., 1999) and ageing men (Urban et al., 1995, Ferrando et al., 2002).

Whether this androgen-mediated increase in muscle size involves satellite cell activity is unclear. There are some data to suggest that androgens have direct influence on satellite cell properties. Testosterone treatment has been
associated with an increase in myonuclei and satellite cell number in hypo
gonadal men and with the induction of DNA synthesis in the levator ani
Castration reduces satellite cell proliferation in pigs, with partial rescue
achieved by testosterone implantation (Mulvaney et al., 1988). Androgen
receptor knockout mice show altered expression of the MRFs, an increase
in differentiation and a decrease in proliferation markers within the satellite
cell population (MacLean et al., 2008).

Although research has demonstrated the importance of androgens in
skeletal muscle and satellite cell regulation, it remains possible that there
are other, androgen independent regulators of skeletal muscle size and its
sexual dimorphism. Male and female satellite cells may be intrinsically dis-

In vitro studies of EDL and soleus derived satellite cells of the mouse
have shown that male satellite cells have a higher proliferative rate and an
increased expression of proliferative markers compared to females (Manzano
et al., 2011). However, to properly assess differences between satellite cell
populations, it is necessary to consider that the satellite cell is responsible
for two largely distinct processes: muscle growth and muscle regeneration
(see section 1.6), that it is possible to have differences in one without dif-
ferences in the other, and that the relative efficiency of these processes will
be considerably confounded by the developmental stage of the muscle from
which these satellite cells originate.

The relative efficiency of male and female satellite cells to regenerate
dystrophic muscle remains unexplored. Considering the pro proliferation
effects of circulating androgens, it is likely that the host environment will
mediate donor cell engraftment efficiency such that more fibres from cells of
donor origin will occur in male as compared to female host muscle. If there
are indeed intrinsic differences in male and female satellite cell populations,
then it may also be observed that the sex from which the donor satellite
cells are derived will mediate donor cell contribution to regeneration.

The efficiency of skeletal muscle regeneration is influenced by the developmental stage of the muscle. Aged muscles show a decrease in regenerative capacity compared to adult, which is widely considered to be environmentally regulated (see section 1.7) (Boldrin et al., 2009, Roy et al., 1999, Mitchell & Pavlath, 2001). In the mouse, satellite cells from growing muscles have been shown to have distinct genetic requirements compared to those from the adult (see section 1.6) (Lepper et al., 2009). Muscle precursor cells derived from muscle of young and newborn mice are able to regenerate adult host muscle post transplantation (Morgan et al., 1988, Partridge et al., 1989, Morgan et al., 1990, Heslop et al., 2001), but whether these cells, derived from enzymatic disaggregation of muscle, are actually satellite cells or another cell type (e.g. PICS, present in large numbers in newborn mouse muscle (Mitchell et al., 2010) (see section 1.3) was not ascertained. The post transplantation regenerative capacity of a pure satellite cell population, derived from fibres of young actively growing mice, is unknown.

3.1.1 Aims

- To quantify satellite cell and myonuclei numbers in male and female mouse EDL muscle from 2 weeks to 2.5 years of age.

- To investigate potential cell intrinsic differences in MRF expression, self renewal and colony forming capacity between satellite cells isolated from male and female mice.

- To investigate potential cell intrinsic differences in MRF expression, self renewal and colony forming capacity between satellite cells isolated from actively growing adult and aged mice.

- To investigate differences in engraftment efficiency between satellite cells isolated from male or female donors and engrafted into male and
female host muscle.

- To investigate differences in engraftment efficiency between donor satellite cells isolated from actively growing and adult mice.

### 3.2 Satellite Cell and Myonuclear Numbers in Adult Male Compared to Female Mice

To begin my investigation of age and sex differences in satellite cell populations, I quantified satellite cell numbers on freshly-isolated myofibres of the EDL (see section 2.4) from 3 month, 1 year and 2.5 year old male and female C57BL/6 mice. Satellite cells were quantified using immunostaining for Pax7 and co-localisation with DAPI on fixed single fibres (see figure 3.1). Results show that the number of Pax7 expressing cells was at its highest at 2 weeks of age (8.5 ± 0.9, 6.5 ± 1.0 satellite cells/fibre respectively in males and females) and declined steadily thereafter, reaching a low of just 1.4 ± 0.1 satellite cells per fibre at 2.5 years of age in males (see figure 3.1 and 3.2.B). Each data point represents the mean of 60 fibres sampled from 3 mice. Males had significantly more satellite cells per fibre than females during the adult years (p = 0.02 at 3 months, p = 0.03 at 1 year).

To investigate the nature of the relationship between satellite cell and myonuclear number, I quantified the number of myonuclei per fibre on fibres isolated from male and female mice of different ages (see figures 3.1 and 3.2.C). Two week old mice had significantly less myonuclei per fibre than any other age group studied (173 ± 30, 182 ± 42 nuclei/fibre respectively in males and females). The decrease in satellite cell number observed between 3 month old and 2 week old mice coincided with an increase in myonuclear number (p < 0.0001). From this it may be inferred that many of the Pax7 expressing cells observed at 2 weeks of age undergo terminal differentiation without self-renewal and that the proliferation and differenti-
Figure 3.1: Satellite cells and Myonuclei on EDL myofibres from mice of 2 weeks to 2 years of age. Isolated single fibres immunostained with Pax7 and DAPI satellite cells and myonuclei respectively. Scale bar = 20 µm
Figure 3.2: Satellite cell and myofibre number per EDL myofibre in male and female mice across the lifespan (A) Satellite cells per fibre on single fibres isolated from male and female mice of 2 weeks, 3 months 1 year and 2.5 years of age. C) Myonuclei per fibre on single fibres isolated from male and female mice of 2 weeks, 3 months 1 year and 2.5 years of age.
nation of these cells is responsible for growth and the increase in myonuclear number. Mirroring the drop in satellite cell numbers, myonuclear number was significantly reduced in 2.5 year old compared to 1 year old mice (p < 0.001). Although these data do not demonstrate a causative relationship, it is possible that the decrease in number of myonuclei is due to a functional deficit in the ability of the diminished satellite cell population to maintain numbers of myonuclei in adult fibres.

3.3 Myogenic Regulatory Factor Expression Profiles in Growing, Adult and Aged Satellite Cell Populations

Satellite cells express different combinations of the myogenic regulatory factors Pax7, MyoD, and Myogenin according to their activation, proliferation and differentiation status (see section 1.3). MyoD is expressed by satellite cells in the cell cycle, that can either differentiate, in which case they will down regulate MyoD and upregulate Myogenin, or these cells can return to quiescence: down regulate MyoD and maintain Pax7 expression, thus maintaining a reserve cell population (Zammit et al., 2002). The number of cells positive for Pax7 and negative for MyoD after 72 hours in culture is therefore a measure of satellite cell self-renewal (Zammit et al., 2002) (see section 1.3).

To assess the functionality of satellite cells from the different populations, single fibres were assessed for satellite cell numbers and expression of Pax7, MyoD and myogenin (see methods 2.4.1 and 2.4.3) immediately after isolation or after 24, 48 and 72 hours in suspension culture (see figure 3.3.B). Twenty fibres were analysed per mouse from at least three mice per condition. After 72 hours in culture, fibres from 3 month old mice had significantly more satellite cells per fibre than fibres from 1 year and 2.5 year
Figure 3.3: Single Fibre Cultures after 48 and 72 hours A) Representative images of single fibres from a 3 month old mouse fixed after 48 or 72 hours in suspension and immunostained for Pax7 and MyoD. B) Representative image of a single fibre fixed after 72 hours in culture and immunostained for MyoD and Myogenin expression. Scale bar 50µm
Figure 3.4: Satellite Cell Numbers and Myogenic Regulatory Factor Expression on Cultured Single Fibres (Caption on page 87)
Figure 3.5: Quiescent satellite cells on single myofibres from 2 wk old male and female mice T0 (Caption on page 87)
Figure 3.6: Activated satellite cells on single myofibres from 2 wk old mice T0 (Caption on page 87)
Figure 3.4: Satellite Cell Numbers and Myogenic Regulatory Factor Expression in Cultured Single Fibres

A) Number of satellite cells/ fibre after 24, 48 and 72 hours in suspension culture. B) The percentage of myoblasts either Pax7+ (blue) only, MyoD+ (green) only, or co-expressing Pax7 and MyoD (red) on single fibres fixed immediately after isolation (i) and after 72 hours in culture (ii).

Figure 3.5: Quiescent satellite cells on single myofibres from 2 wk old male and female mice

Pax7+/ MyoD⁻ nuclei (DAPI+) representing quiescent satellite cell on single fibres fixed at T0 from 2 week old male and female mice. Scale bar 20µm

Figure 3.6: Activated satellite cells on single myofibres from 2 wk old mice

Pax7+/ MyoD⁺ nuclei (DAPI+) on EDL fibres fixed at T0 from 2 week old male and female mice. Scale bar 20µm
old mice (see figure 3.4.A). However this can be accounted for by the greater starting number of satellite cells/fibre. It is therefore more meaningful to analyse the number of population doublings. Satellite cells from 3 month old mice undergo 2 population doublings between 24 and 72 hours in culture. Aged satellite cells have a slower doubling time, as they have undergone only 1 population doubling within the same time period (see figure 3.4.A).

To observe satellite cell self-renewal, fibres were fixed after 72 hours in culture and analysed for the expression of Pax7 and MyoD (see figure 3.4.Bii). In accordance with previous data (Collins et al., 2007) it was observed that self-renewing satellite cells represent a smaller fraction of the satellite cell population in aged compared to adult mice.

In single fibres from 2 week old mice analysed immediately after isolation, the majority (91% in males, 92% in females) of satellite cells were seen to co-express Pax7 and MyoD (see figure 3.6 and 3.4.Bi) and are thus actively proliferating. This is to be expected from a fibre that, within the next 10 weeks, will almost double its number of myonuclei (see figure 3.2.C). Intriguingly, however, not all satellite cells are recruited into this growth program. A rare few satellite cells were in quiescence, MyoD negative Pax7 positive (see figure 3.5, 3.4.Bi, and 3.3.A). It was not possible to assess the fate of these cells on a single fibre in culture, as fibres from 2 week old mice could rarely be cultured successfully for any length of time. Fibres that could be analysed had no associated satellite cells by 48 hours. Possibly, this is due to changes in the basal lamina between 2 week and 3 month old mice. Fibres of 2 week old mice are more fragile and the basal lamina more easily damaged, such that the satellite cells may not remain associated with the fibre and/or the fibre contracts.

Satellite cells have been shown to be more abundant at the ends of isolated muscle fibres than in the middle, and it is suggested that fibres grow by adding nuclei to the ends rather than the middle of myofibres (Allouh
et al., 2008). It may therefore be expected to find quiescent satellite cells towards the middle of the fibre and proliferating satellite cells at the ends in growing mice. Although due to their rarity it is difficult to assess significance it appeared that on fibres from 2 week old mice quiescent cells were randomly distributed around the fibre (see figure 3.5).

3.4 Myonuclei to Cytoplasm Ratio is a Function of Developmental Stage

Next, I set out to investigate if the observed changes in myonuclear number are related to changes in myofibre size. The myonuclear domain hypothesis of muscle fibre growth states that the nucleus to cytoplasm ratio remains constant, hence any increase in muscle size must occur via the addition of new myonuclei from the proliferation of satellite cells (see section 1.6). Surprisingly, there was no difference in the volume of EDL fibres between males and females within any age group. Myofibre volume increased between 2 weeks and 3 months of age ($p < 0.001$) (see figure 3.7.A) and decreased between 1 year and 2.5 years ($p < 0.001$). These significant changes were concomitant with the changes in myonuclear number (see figure 3.2) that are predicted by the myonuclear domain hypothesis.

If fibre growth is inextricably linked to myonuclear addition, and thus satellite cell activity, then this relationship ought to be observed between fibres of different sizes within age groups. Pearson correlation coefficients between myonuclei per fibre and fibre volume within the four age group categories (see figure 3.7) reveal an overall positive correlation between myonuclear number and myofibre volume. However, this relationship did not exist within all age groups. Results reveal a significant positive correlation between myonuclear number and fibre size in adult mice (3 months $r = .77 \ p = 0.013$, 1 year $r = 0.58$, $p = 0.045$ respectively). However, at 2 weeks
Figure 3.7: Myonuclei and myofibre volume of isolated EDL single fibres A) Shows changes in myofibre volume across age groups. B) The relationship between the number of myonuclei per fibre and fibre volume. C) Representative images of EDL myofibres stained with DAPI at 2 weeks and 3 months of age. Each fibre is 6 stitched images at 10x.
Figure 3.8: Circularity of myonuclei on male and female fibres across the lifespan. Affects of sex and age on circularity of myonuclei. Myonuclei on fibres isolated from 2 week and 3 month old EDL fibres were significantly more circular than myonuclei from fibres from 1 year and 2.5 year old mice.
and 2.5 years of age this relationship was not observed. Results show that myonuclear domain is established between 2 weeks and 3 months of age and remains fairly constant for adult life. Myofibres from aged mice show an overall decrease in myonuclear number (see figure 3.2.C) and fibre volume (see figure 3.7.A) and a breakdown in the relationship between these two measures (see figure 3.7.B).

Whilst quantifying changes in myonuclear numbers, it was observed that myonuclei have distinctive morphologies according to age (figure 3.8). At 2 weeks of age, myonuclei are round in shape and appear to have tightly compact chromatin, whilst at 2.5 years nuclei are greatly elongated and chromatin appears less dense. A two way ANOVA showed a significant effect of age on circularity (F= 40.79, p < 0.0001). Post hoc T-tests demonstrate that myonuclei from 2 week and 3 month old mice were significantly more circular than 1 year or 2.5 year old mice (p < 0.001) (see figure 3.8).

### 3.5 Satellite Cell Transplantation efficiency with Male and Female Hosts and Donors

Thus far, single fibre analyses represent myofibre and satellite cell dynamics within a sedentary model where satellite cells are not challenged beyond the requirements of normal growth. It is possible that male and female satellite cells have functional differences with regards to their ability to survive transplantation and regenerate muscle after severe injury. The significantly greater number of satellite cells found in male compared to female mice may also suggest that male donor mice provide a greater pool of satellite cells and will therefore produce greater numbers of donor derived fibres, making them a preferable choice of donors in current models of satellite cell transplantation.

Satellite cells were harvested from freshly isolated male or female 3F-
Figure 3.9: Satellite cells from male and female 3F-nLacZ-2E mice show similar contribution to host muscle regeneration. A) Representative image of donor derived fibres in mdx-nude TA muscle sections. Scale bar 20µm. B) shows the number of donor derived fibres produced per injected cell when grafted with male and female donors. C) Number of donor derived fibres per injected cell from male donors in male and female hosts. D & E as in B & C but shows absolute number of dystrophin positive fibres. Green: male, blue: female.
*nlacZ-2E* EDL single fibres (see section 2.6). Satellite cells, stripped from approximately 100 male or female fibres were injected into contra lateral male and female *mdx*-nude TA muscles that had been irradiated 3 days before grafting (see methods 2.6) (Collins et al., 2005, Sacco et al., 2008, Collins et al., 2007, Morgan et al., 2002). Muscles were analysed four weeks later for donor contribution to muscle regeneration (see figure 3.9). Despite the differences in the number of satellite cells injected between male and female donors (5.5 and 3.9 satellite cells per male and female fibre respectively (see figure 3.9.B), there was no difference in the number of donor derived fibres in host muscles injected with satellite cells derived from male or female donors (see figure 3.9.D). This was the case for absolute number of dystrophin positive fibres (figure 3.9.D), and for number of donor fibres per injected cell (see figure 3.9.B). The number of donor derived fibres formed after transplantation therefore does not correspond in a linear fashion to the number of cells injected.

These results suggest that there is no intrinsic difference between male and female satellite cells in their ability to regenerate host muscle after transplantation. However, it is possible that sex differences in muscle regeneration may occur, and are governed by environmental, rather than via satellite cell intrinsic mediators e.g. circulating androgens. To investigate this, satellite cells were isolated from 3 month old male 3F-*nLacZ-2E* mice and injected into the TA muscles of male and female *mdx*-nude hosts. There was no difference between the number of donor-derived fibres per donor cell (see figure 3.9.C) or absolute number of dystrophin positive fibres (see figure 3.9.E) at 4 weeks post injection between male and female hosts (see figure 3.9.C). Together these data demonstrate that in this model of satellite cell transplantation, donor derived muscle formation is not altered either by the sex of the host or the sex of the donor.
3.6 Satellite Cell Transplantation Efficiency with Donors from Growing compared to Adult Mice

In vitro analysis of single fibres showed that the age at which the fibre is isolated has the greatest effect on all variables measured (sections 3.2, 3.4, and 3.7). However, previous research has shown that aged and adult donor satellite cells regenerate host muscle equally well (Collins et al., 2007) (see section 1.7). The regenerative capacity of satellite cells isolated from fibres derived from muscles that are actively growing is unknown.

Satellite cells were isolated from the EDL muscle fibres of male 2 week and 3 month old 3F-\textit{nLacZ}-2E mice (see methods 2.1.1 and 2.4.6). Satellite cells stripped from 100 donor fibres were injected into each host \textit{mdx} nude TA muscle (see methods 2.6). This equates to approximately 850 satellite cells from 2 week old donors and 550 satellite cells from 3 month old donors injected into host muscles (see figure 3.2). Despite the differences in injected cell numbers, differences in proliferative state and mitotic age of the cells, there was no difference in the number of donor derived fibres in TA muscles grafted with 3 month old or 2 week old satellite cells (see figure 3.10.A and B). These data strengthen the conclusion that donor derived muscle formation post transplantation is not the cumulative product of all cells transplanted. At best it seems there is a loose relationship between the number of satellite cells transplanted and the subsequent amount of donor derived muscle observed.
Figure 3.10: 3 month and 2 week old donor mice show similar contribution to host muscle regeneration A) Representative sections of *mdx*-nude host TAs shows co localisation of β-gal and dystrophin. 20x magnification scale bar 20µm B) The number of donor-derived fibres per injected cell between muscles grafted with cells from 3 month old or 2 week old donors in male and female hosts
3.7 Discussion

In this chapter I have demonstrated that myonuclear number is related to myofibre size, indicating that satellite cells are involved in muscle maintenance throughout life (figure 3.7). The satellite cell population changes in abundance as a function of age and sex with male adults having significantly more satellite cells per fibre than female adults and young mice having significantly more satellite cells per fibre than aged mice (see figure 3.2). However, engraftment experiments have shown that neither donor or host sex nor donor age effects transplantation efficiency (see figures 3.9 and 3.10). From these results I hypothesise that the satellite cells that are responsible for host muscle regeneration after transplantation are a distinct population from the more numerous satellite cells responsible for muscle growth and maintenance in situ.

In the In vitro studies of mouse EDL single fibres reported here, show that adult males have significantly more satellite cells per fibre than adult females (see figure 3.2). This is in accordance with previous data showing males have more satellite cells per fibre in mice ranging from 3 to 8 months of age (Day et al., 2010). Here it is shown that sex differences in satellite cell number can only be observed in adult mice (see figure 3.2). The time course of satellite cell population divergence between males and females suggests that androgens may be significant mediators of satellite cell number. Androgen levels peak during puberty, between days 30 and 35 in C57BL/6 mice (MacLennan et al., 2011) and decrease with old age (Nelson et al., 1975). This is parallel to the changes observed in satellite cell numbers in mice with increasing age. Previous data have shown that testosterone can increase satellite cell number and satellite cell proliferation (Mulvaney et al., 1988, Joubert & Tobin, 1989, 1995). In this model of satellite transplantation there was no difference in donor contribution to host muscle regeneration after in male and female mdx-nude host mice. From this, it is concluded
that, although androgens may mediate satellite cell numbers, exposure to a male or female host environment and thus circulating androgens does not mediate satellite cell survival or proliferation after transplantation.

There is research to suggest that male satellite cells have a greater proliferative rate and express higher levels of MRFs ex-vivo than female satellite cells (Manzano et al., 2011). Here satellite cells were studied in an in vitro system that maintains satellite cell myofibre interactions (see section 2.4), thus keeping satellite cells within an environment that mimics that seen in vivo. This system also allows the analysis of satellite cells at the individual cell level, rather than averaging expression profiles across the population. Data shows a comparable rate of self-renewal, and similar activation and proliferation profiles, in male and female satellite cell populations. There were greater numbers of myoblasts in male mice after 72 hours in culture, (see figure 3.4), however this is accounted for by the larger starting number and no difference was observed in population doubling times.

Despite differences in number, proliferative state, mitotic age and genetic requirements between adult and p14 mice, there is no difference in regenerative efficiency post transplantation. When engrafted into mdx-nude hosts, the absolute number of satellite cells does not have a linear relationship with the amount of muscle that can be regenerated from these cells. The number of dystrophin positive fibres obtained four weeks post transplantation was independent of the sex or the age of the donor mice. Together these data suggest that, despite considerable variance in the absolute number of satellite cells, the number of cells that can contribute to muscle regeneration post engraftment is small and remains constant. It is tempting to speculate that those cells observed to be quiescent on single fibres of p14 mice are the same small population of satellite cells that are able to contribute to muscle regeneration post transplantation.

In accordance with previous studies (Collins et al., 2005, 2007, Arava-
mudan et al., 2006, Conboy et al., 2003), our results show that satellite cell number declines steadily with increasing age (see figure 3.2). This decline in satellite cell number is accompanied by a decrease in myonuclear number, myofibre atrophy and a failure to maintain a myonucleus to cytoplasm ratio (see figures 3.2 and 3.7). Together these findings are indicative of a role for satellite cells in muscle maintenance throughout life and that the impairment in satellite cell function with old age contributes to sarcopenia. This is supported by analyses of myoblasts on isolated single fibres. After 72 hours in culture there were fewer fibre-associated myoblasts, and perhaps more interestingly, a smaller percentage of these myoblasts returning to quiescence, in aged compared to adult fibres (see figure 3.4).

It is possible that a diminished amount of self-renewal is the cause of the reduced number of satellite cells observed in freshly isolated aged fibres. Despite a diminished ability to maintain muscle mass and satellite cell numbers in situ, previous work has shown that satellite cells from aged donors regenerate host muscle with similar efficiency as young donors in our model of transplantation (see section 1.7). These data stress the importance of a conceptual dissociation between satellite cells for muscle growth and routine muscle maintenance and satellite cells for muscle regeneration after injury.

Here, satellite cell populations have been studied across the life span of an animal that is largely sedentary. This has enabled conclusions regarding muscle maintenance and homeostasis and is arguably a good model for 21st century humans. Yet it remains possible that muscle fibre hypertrophy in response to exercise works via different mechanisms. Mice lacking functional Pax7 expressing cells show a significant decrease in muscle mass and dramatic increase in fibrosis after strenuous exercise, compared to exercised wild type and un-exercised controls (Lepper et al., 2011). Although strenuous exercise can also cause muscle damage and therefore regeneration, these studies indicate that hypertrophy in response to exercise, as with normal
muscle growth, is usually associated with satellite cell activity.

Blockade of Myostatin has been proposed as a therapeutic strategy for muscle wasting disorders (see section 1.6) (Wagner et al., 2002, Bogdanovich et al., 2002). Here we show that normal healthy muscle, when it increases in size also increases in myonuclei number. However myostatin blockade is known to increase muscle size without satellite cell activity (Amthor et al., 2009). It is possible that an increase in muscle size without associated satellite cell activity reflects a change in the association between myofibre, myonuclei and satellite cell and thus represents a pathological condition. It would be of interest to observe if hypertrophy without myonuclei addition could be sustained over long time periods or whether the fibre tends towards its previous homeostasis. In conclusion, agents that increase cytoplasmic volume without increasing myonuclei number should be approached with caution as a therapeutic strategy.

Based on the data reported here, I hypothesise that there are at least two distinct satellite cell populations. The first population is responsible for myonuclei addition during growth and general muscle maintenance throughout life. These satellite cells are present in greater numbers in growing muscle, are diminished with age, and are more numerous in adult males compared to females. The second population is formed by those satellite cells that are activated by severe muscle injury and survive transplantation. These cells are present in similar numbers from birth to old age and do not differ between males and females (Neal et al., 2012).

Chapter 4

Radio Resistant Satellite Cells

4.1 Introduction

That certain elements emit radiation was described by Pierre and Marie Curie, following their isolation of the radioactive elements Polonium and Radium. The Curie’s elements and the theory of radioactivity that they developed received two Nobel Prizes: the 1903 prize for physics, and the 1911 prize for chemistry. From smoke detectors to nuclear power plants, from sterilising food to nuclear weapons, the use of radioactive elements shapes our modern world. Radiation is widely applied across the biological sciences and is fundamental to the field of stem cell biology.

Nuclei are stable or unstable depending on their ratio of protons to neutrons. In unstable nuclei, binding energy is not strong enough to hold the nucleus together. Thus, sub-nucleic particles are emitted until a stable state is reached: radioactive decay. This decay, can take one of three forms: the emission of neutrons (beta radiation), emission of 2 proton and 2 neutrons (He$_4$) (alpha radiation) or the highest energy form of electro magnetic radiation, the emission of photons (gamma radiation). As sub-atomic par-
particles emitted from an unstable nucleus travel, they interact with the atoms they encounter. Energy transfer from travelling radiation excites valence electrons, resulting either in their associated nucleus releasing its own radiation, or, given a high enough energy transfer, the complete dissociation of that electron from its nucleus and the formation of ions (Wikipedia contributors, 2012). In tissue, this process can be damaging in two ways: the energy of passing radioactive particles can directly ionize atoms of the DNA chain, or, of higher probability, due its compromising a larger volume of the cell, radiation can ionize cytoplasmic water (reviewed Hall et al. (1988)). Ionization of water causes the formation of free H and OH radicals which can recombine to form the damaging H$_2$O$_2$ (reviewed (Begg, 2010)). These free radicals cause DNA damage (see section 1.7). Exposure of cells to a radioactive source therefore results in DNA double strand breaks, apoptosis and cell death (reviewed (Begg, 2010)).

Not all cells are equally sensitive to radiation. Although both dividing and non dividing cells are likely to accrue a similar amount of radiation induced damage, radiation generally results in the specific loss of dividing cells. Radiation causes damage to cellular components vital for cell division, and, due to improperly repaired chromosomal damage (Dewey et al., 1970), this leads to cell death in the first cell division after irradiation (Thompson & Suit, 1969). As an alternative to cell death, dividing cells may undergo terminal differentiation rather than proliferation. Radiation therefore results either in the loss of stem cells or a reduction in their capacity to form colonies in a dose dependent manner (reviewed (Prise & Saran, 2011, Reya et al., 2001)).

Ionising radiation has been developed as a cancer therapy to kill rapidly dividing tumour cells (Thompson & Suit, 1969). Tumorogenesis is thought to be driven by a rare population of cells that are capable of extensive self renewal: cancer stem cells (reviewed (Reya et al., 2001, Vlashi et al., 2009)).
Aside from the detrimental side effects caused by the non specificity of radiation therapy (reviewed (Stone et al., 2003)) its major limitation is that cancer stem cells are relatively resistant to ionising radiation compared to the majority of rapidly proliferating tumour cells. When radiation therapy stops, radio resistant cancer stem cells are able to re enter the cell cycle and tumours can reoccur (reviewed (Vlashi et al., 2009)). Research in glioblastoma cells suggests that cancer stem cells preferentially activate DNA damage checkpoint responses, arrest mitosis and undergo damage repair in response to radiation (Bao et al., 2006). Similarly in the haematopoietic system, research has shown that the most primitive stem cell subpopulation is the population that shows the highest degree of radio resistance (Meijne et al., 1991). It is conceivable that, due to their critical importance in tissue maintenance, long lived stem cells have evolved to undergo damage repair, whilst replaceable cells either terminally differentiate or die. It is possible that the higher up the stem cell hierarchy a cell is, the more resistance it will have to ionising radiation.

In muscle, exposure to gamma irradiation does not have a major detrimental effect on the post mitotic myofibre, but causes a satellite cell specific impairment (Lewis, 1954, Quinlan et al., 1997). Using the damaging effects of radiation to worsen the phenotype of the *mdx* mouse (see section 1.9.2) so as to better resemble that of DMD, it has been shown that exposure of the hind limb to 16Gy radiation causes the progressive loss of fibres and a loss of regenerative capacity (Wakeford et al., 1991). Furthermore, in wild type mice, exposure to irradiation results in stunted growth (Quinlan et al., 1997).

The detriment in muscle growth and regeneration observed following exposure to ionising radiation, suggests that radiation incapacitates the satellite cell. However, there is evidence to suggest that not all satellite cells are equally radio sensitive. Surprisingly, 18Gy irradiated wild type mouse hind
limbs, when injected with the muscle damaging agent notexin, were seen to undergo muscle regeneration (Heslop et al., 2000). When single fibres are isolated from irradiated mice, as expected, they have very few associated satellite cells and those that are present do not proliferate. However, if fibres are isolated from irradiated and notexin treated muscles, fibres have significantly more fibre associated satellite cells and these are able to proliferate rapidly when cultured (Heslop et al., 2000).

Together these experiments suggest that, although the majority of satellite cells are sensitive to high dose ionising radiation, a sub population of satellite cells are radio resistant, but require exposure to an extreme muscle damage in order to become activated. The apparent disparity between regeneration observed in the notexin treated wild type (Heslop et al., 2000), but not observed after degeneration in the mdx mouse (Wakeford et al., 1991, Quinlan et al., 1997) may be due to the depletion of a radio resistant satellite cell subpopulation due to the continuous cycles of regeneration induced by the mdx pathology (see sections 1.9.2).

There is good evidence to suggest that satellite cells that proliferate extensively in vivo, do not proliferate in vitro (Beauchamp et al., 1999) (see section 1.8). That radio resistant satellite cells do not proliferate in vitro but can be activated in vivo may therefore suggest that radiation may be selecting for a functionally relevant satellite cell population.

As a subset of satellite cells are known to survive high dose ionising radiation I asked whether, as observed in cancer stem cells and the haematopoietic system, ionising radiation could be used to separate stem cells with differing properties. Furthermore, I wanted to investigate if the resultant radio resistant satellite cell populations related to the hypothesised regeneration and muscle maintenance specific populations hypothesised in chapter 3. In this chapter I investigate radio resistant satellite cells in vitro, in normal muscle growth and in muscle regeneration after transplantation. I then attempt
to characterise radio resistant satellite cells in comparison to radio sensitive satellite cells at the RNA level.

4.1.1 Aims

- To separate satellite cells that are able to survive transplantation from satellite cells that are unable to survive transplantation by using high dose ionising radiation.

- To investigate radio-resistant satellite cells and their contribution to muscle growth in unchallenged muscle.

- To investigate the contribution of radio resistant satellite cells to muscle regeneration after transplantation.

- To characterise radio resistant satellite cells at the RNA level.

4.2 Radio Resistant Satellite Cells *in vitro*

To investigate the *in vitro* proliferative capacity of satellite cells exposed to ionising radiation, male and female mouse hind limbs were exposed to 18Gy radiation (see section 2.5) and satellite cells isolated by the single fibre method 3 days later. Satellite cells were plated at clonal density and kept for 7 days, then fixed and stained with trypan blue for visualisation (see section 2.4.8). Cells were observed in just 4% of wells and those wells that did (see section 2.4.1) contain cells did not contain more than one. Therefore, a rare few satellite cells were able to survive 18Gy irradiation, but these did not undergo any proliferation at clonal density (see figure 4.1.A).

It is possible that association with the myofibre may aid radiation resistant satellite cell proliferation. To investigate satellite cell dose response to radiation, single fibres were isolated from mice irradiated with 4.5Gy,
Figure 4.1: Irradiated satellite cells *in vitro* (Caption on page 107)
Figure 4.1: Irradiated satellite cells in vitro. Satellite Cells isolated from single fibres of mice irradiated with 18Gy radiation 3 days previously, kept at clonal density for 7 days before staining with trypan blue. Single cells were observed in 4% of wells. B. Single fibres isolated from mouse EDL muscle irradiated with 4.5Gy 3 days previously Pax7+/MyoD− satellite cells are observed at T0 and these proliferate to form colonies of Pax7+/MyoD+ cells at T72, although nuclei appear swollen and nuclear specific antibodies are spread throughout the cytoplasm. C. Single fibres isolated from mouse EDL muscle irradiated with 18Gy 3 days previously shows the presence of Pax7+/MyoD+ cells at T0 and T24 although nuclei appear unhealthy. Scale bars 20µm 9Gy, 18Gy and 25Gy radiation and analysed for Pax7 and MyoD expression immediately following isolation or after 24, 48 and 72 hours of culture. Single fibres isolated from muscles irradiated with 4.5Gy and 9Gy irradiation had associated Pax7 positive nuclei in numbers comparable with control fibres. These satellite cells were seen to express MyoD at 24 hours in culture and had proliferated to form myofibre associated colonies after 72 hours (see figure 4.1.B). However, the morphology of these colonies was not as expected, with many nuclei elongated in appearance (see figure 4.1.B). Strangely, MyoD and Pax7 appeared not to be restricted to their normal nuclear location but were found to be more diffuse throughout the cell. The reason for this change in morphology is unclear, it was initially thought to be due to experimental error, but upon experiment replication similar staining patterns were observed. Furthermore, T0 fibres were immuno-stained in parallel and these show expected patterns of expression (see 4.1.B, top panel). Possibly this is an indication of cell or nuclear membrane damage.

Single fibres isolated from 18Gy irradiated muscles were found to have associated Pax7 expressing cells immediately following isolation (see figure...
4.1. C). These cells were seen to activate MyoD after 24 hours in culture. However, by T72 there were very few myofibre associated satellite cells, and those that were present had not divided and looked swollen and unhealthy (see figure 4.1.C). After 25Gy radiation there were no Pax7 expressing cells observed at any time point. Thus, a subpopulation of satellite cells are able to survive 18Gy irradiation. These cells activate MyoD but are then lost (see figure 4.1.C). Satellite cells that are resistant to 18Gy irradiation do not survive 25Gy irradiation.

It is possible that satellite cells on 18Gy irradiated fibres activate MyoD and then apoptose due to radiation induced DNA damage. To investigate this, 18Gy irradiated single fibres were analysed for apoptosing nuclei at T48 with TUNEL (see section 2.4.5). However, less than 0.5% of nuclei were identified with TUNEL, suggesting either that satellite cells are not lost by apoptosis or they apoptose before 48 hours but after 24 hours in culture and thus were already gone from the fibres analysed.

4.3 Radio Resistant Satellite Cells in Growth

To investigate the in vivo persistence and functional capacity of radiation resistant satellite cells in normal muscle growth and maintenance, actively growing 4 week old wild type mice were irradiated with 4.5, 9, 18 or 25Gy radiation. TA and EDL muscles were harvested and weighed at 4 weeks, 6 months or 1 year post radiation. Single fibres were isolated from EDL muscles and were assessed for Pax7 expression. TA muscles were frozen and cryosectioned for analysis of gross morphology.

4.3.1 The Satellite Cell Pool

A two-way ANOVA revealed a significant effect of time and radiation dose on the number of satellite cells per fibre (p<0.001) and a significant interaction
between the two variables. Bonferroni post hoc tests show that, as observed previously (see figure 3.2), in control EDL muscles satellite cell number decreases with increasing age (0Gy, 4 weeks compared to 1 year p<0.001) (see figure 4.2). Mice exposed to ionising radiation showed a significant decrease in the number of satellite cells per fibre at all time points and at all radiation doses compared to control fibres (all p<0.001).

After 4.5Gy radiation, the number of satellite cells per fibre was significantly reduced compared to control muscle at all time points (p<0.001) (see figure 4.2.A). However, there was no significant difference between the number of satellite cells per fibre at 4 weeks, 6 month or 1 year post 4.5Gy radiation. Thus, after the initial loss of satellite cells, 4.5Gy irradiated muscle maintains its satellite cell numbers. Similarly, single fibres from muscles irradiated with 9Gy radiation have a reduced number of satellite cells per fibre compared to control, but show no significant difference in the number of satellite cells per fibre at 4 weeks or 1 year post radiation.

Single fibres from 18Gy irradiated muscles show a significant reduction in satellite cells per fibre compared to controls at 4 weeks post radiation. Unlike 4.5Gy or 9Gy radiation, the number of satellite cells per fibre continues to fall between 4 weeks and 6 months (p<0.001) and still further between 4 weeks and 1 year post 18Gy radiation (p<0.001). These results appear to support the in vitro data (see section 4.2) that suggests that radio sensitive satellite cells are lost only once they enter the cell cycle and activate MyoD.

Following exposure to 25Gy radiation, the number of satellite cells per fibre was severely reduced, even in comparison to 18Gy radiation (p < 0.05). Pax7 positive cells were difficult to find at 4 weeks, and this remained the case for 6 months and 1 year post irradiation.

Results suggest that, after any dose of ionising radiation some satellite cells die and the satellite cell pool is subsequently unable to recover its previous numbers. After the initial loss of satellite cells following 4.5 and
9Gy radiation, numbers remain fairly constant and there is no further loss of satellite cells. However, after 18Gy radiation, satellite cells continue to be lost between 4 weeks and 6 months, leaving only a very rare population of cells that are able to survive up to 1 year. In contrast, 25Gy radiation ablates almost all the satellite cell pool by 4 weeks, and these numbers can not subsequently be recovered. Results therefore suggest that circulating cells do not contribute to the satellite cell pool.

4.3.2 Maintenance of Myonuclear Numbers

The number of myonuclei per fibre reduces with increasing age in non irradiated EDL muscle (see figure 3.2). A two-way ANOVA on myonuclei per fibre after 4.5, 9, 18 and 25Gy radiation at 4 weeks, 6 months and 1 year post radiation reveals a significant main effect of time, radiation dose and interaction between these variables ($p < 0.001$). Post hoc Bonferroni tests show that there was a significant reduction in the number of myonuclei per fibre at 4 weeks post radiation at all radiation doses. However, in the 4.5Gy and 9Gy irradiation conditions myonuclei per fibre were comparable to control fibres at 1 year post irradiation. This is in contrast to 18Gy irradiated fibres, which show a further significant loss of myonuclei between 4 weeks and 6 months and between 6 months and 1 year post radiation ($p < 0.001$). 25Gy irradiated fibres lose significantly more myonuclei than any other radiation dose by 4 weeks (18Gy compared to 25Gy at 4 weeks $p < 0.001$). These decreased numbers remain constant at 6 months and 1 year post radiation. This suggests that radiation results in the loss of some myonuclei, but that satellite cells subjected to 4.5Gy and 9Gy radiation are able to replace these nuclei, while 18Gy and 25Gy irradiated satellite cells are unable to do so.
Figure 4.2: Satellite cell and myonuclei numbers in irradiated muscles 4 weeks, 6 months and 1 year post radiation. (Caption on page 112)
Figure 4.2: Satellite cell and myonuclei numbers in irradiated muscles 4 weeks, 6 months and 1 year post radiation. A. Satellite cells per fibre on single fibres from muscle irradiated with 4.5Gy, 9Gy, 18Gy and 25Gy radiation, isolated 4 weeks, 6 months and 1 year post radiation. All irradiated fibres had less satellite cells and less myonuclei per fibre than controls. After 18Gy irradiation there was a significant decrease in satellite cell and myonuclei number between 4 weeks and 1 year of age. B. As in A but shows myonuclei per fibre. All irradiated fibres had less myonuclei per fibre than controls at 4 weeks. At 1 year post radiation only 18 and 25Gy irradiated fibres are significantly different from control. 18Gy irradiated fibres show a significant decline in satellite cells numbers between 4 weeks and 1 year post radiation.

4.3.3 TA and EDL weights and Gross Morphology

Cryosections of TA muscles from irradiated mice were stained with H&E (see section 2.3.3) and assessed for gross morphological changes. Fibres appeared to have a uniform size, did not show an apparent necrosis, increase in central nucleation, monocyte infiltration or an increase in fat content (see figure 4.3). In accordance with previous reports (Quinlan et al., 1997, Pagel & Partridge, 1999), ionising radiation did not result in any obvious changes to muscle morphology.

TA and EDL weights were taken at the time of muscle harvest and are reported in figure 4.4. For TA weights, a two-way ANOVA reveals a significant main effect of radiation dose and time and an interaction between the two variables (\(p < 0.001\)). Post hoc bonferroni tests shows that in control muscle, TA weight increases significantly between 4 weeks and 6 months of age (all \(p < .001\)) and this weight is maintained between 6 months and 1 year of age.
Figure 4.3: H&E on muscle cross section 4 weeks 6 months and 1 year post radiation TA cross sections from mice irradiated with 4.5, 18 or 25 Gy irradiation isolated 4 weeks 6 months and 1 year post radiation and stained with H&E. Sections show no clear changes in muscle morphology or signs of de or regeneration. 20x magnification.
Figure 4.4: TA and EDL weights from irradiated mice. A. TA weights after 0 4.5, 9 18 and 25Gy irradiation 4 weeks 6 months and 1 year post irradiation. Control TA muscles significantly increase between 4 weeks and 1 year. 4.5 and 9Gy irradiated TAs are significantly smaller than control at 6 months but not at 1 year. 18 and 25Gy irradiated TA muscles are significantly smaller than controls at 6 months and 1 year post radiation. B. As in A, but shows EDL weights. Non irradiated TA weights significantly increase between 4 wks and 1 year. 25Gy irradiated muscles were significantly smaller than controls at 6 months and 1 year post radiation and 18Gy irradiated muscles show a trend in the same direction.
After all doses of radiation, TA weights were significantly smaller than controls 6 months post radiation (18Gy and control p <0.001, 4.5Gy and control p= 0.03). However, TA muscles exposed to 4.5Gy or 9Gy radiation were comparable to control muscle by 1 year post radiation. This is in contrast to 18Gy and 25Gy irradiated TA muscles, which remained significantly smaller than control muscles at 1 year (18Gy and control p=0.01, 25Gy and control p=0.004). The recovery of TA weight compared to control observed in 4.5Gy and 9Gy irradiated TA muscles 1 year post radiation is consistent with the observed changes in myonuclear numbers observed in the EDL muscles.

For EDL weights, a two-way ANOVA reveals a significant main effect of time (p <0.001), radiation dose (p = 0.01) and an interaction between the two (p <0.01). Control EDL weights increase significantly between 4 weeks and 1 year of age. There was no difference in EDL weights between control and any dose of radiation 4 weeks post radiation. At 1 year post radiation 4.5Gy and 9Gy EDL weights remained comparable with control EDLs. However, 25Gy irradiated EDLs showed a significant reduction in weight compared to controls at 6 months and 1 year post irradiation (p <0.005), and 18Gy irradiated muscle shows a trend in the same direction (p=0.08). Together with myonuclei per fibre counts and TA weights, these results suggest that following 18Gy and 25Gy irradiation muscles undergo a degree of atrophy indicative of a loss in satellite cell function. This atrophy is not apparent at lower radiation doses. Thus, it seems that, although there are some satellite cells present on 18Gy irradiated myofibres, they do not contribute to muscle growth (4 weeks - 6 months) or to muscle maintenance (6 months - 1 year).
4.4 Radio Resistant Satellite Cells in Regeneration

Thus far, experiments demonstrate that a rare subset of satellite cells survive 18Gy radiation and are present on fibres 1 year post radiation. However, these satellite cells do not proliferate in culture, do not contribute to muscle growth and cannot replace lost myonuclei. Interestingly changes observed in myonuclei number and muscle weight after 18Gy radiation are all comparable to those observed after 25Gy radiation. Yet after 25Gy irradiation, there are no satellite cells present within the muscle (see figure 4.2). This seems to demonstrate convincingly that although a subpopulation of satellite cells are present on fibres following 18Gy radiation, they are not functional.

However, in the previous chapter I hypothesised that there exists two distinct populations of satellite cells, one for muscle growth and routine muscle maintenance and one for muscle regeneration after severe injury. The above experiments investigate the effects of radiation on satellite cells in situ in unchallenged muscles. If my previous conjecture were to be true, it remains possible that, although they do not contribute to muscle maintenance, radio resistant satellite cells can contribute to muscle regeneration. To test this, I compared the capacity of satellite cells isolated from mice hind limbs irradiated with 4.5Gy, 9Gy, 18Gy or 25Gy to contribute to host muscle regeneration in the mdx nude mouse after transplantation.

Myosin 3F-nlacZ-2E positive donor mice (see section 2.1.1) were irradiated 3 days prior to satellite cell isolation and transplanted into host mdx nude TAs (see section 2.6). Results show that, 4 weeks post injection, there were no donor-derived fibres produced from 25Gy irradiated donors. However, donors irradiated with 4.5, 9 or 18Gy irradiation were able to give rise to small clusters (25 ± 11, 26 ± 5, 20 ± 5 respectively) of dystrophin positive fibres that, due to colocalisation with βgal, were clearly donor derived.
Figure 4.5: Engraftment efficiencies of irradiated satellite cells
(Caption on page 118)
Figure 4.5: Engraftment efficiencies of irradiated satellite cells A.

(i) TA cross sections from host mice engrafted with irradiated donors show small clusters of donor derived $\beta$gal$^+$/dystrophin$^+$ fibres A(ii). A higher magnification of dystrophin positive fibres in host TAs from satellite cells isolated from 18Gy irradiated donors. Scale bar = 50$\mu$m. B. Quantification of donor derived fibres from in host TAs engrafted with donor cells isolated from 4.5, 9, 18 and 25Gy irradiated donors shows all donors were able to produce small clusters of donor derived fibres except for TAs engrafted with 25Gy irradiated donors in which no donor derived fibres were found. Scale bar 50$\mu$m (see figure 4.5.A).

These results demonstrate that, despite their incapacity to replace myonuclei in non challenged muscle, 18Gy irradiated satellite cells are regeneration competent. In accordance with results from irradiation followed by notexin challenge in wild type mice (Heslop et al., 2000) (see section 4.1), the experiments reported here suggest that 18Gy gamma radiation selects for a sub population of satellite cells that are activated only upon exposure to a regenerating muscle environment.

4.5 Characterising Radio Resistant Satellite Cells

It is possible that survival of ionising radiation could be conferred by one of two mechanisms: either a subset of satellite cells are better protected against radiation damage, or a subset of satellite cells are better able to repair damage caused by radiation. Although research in other systems is indicative of the latter (Mohrin et al., 2010, Bao et al., 2006) (see section 4.1), it must be considered that these experiments were carried out on stem cell populations outside their niche, and may not be relevant to the quiescent
adult satellite cell.

Radiation can damage DNA by two pathways, either by directly ionising DNA atoms or by ionising atoms within the cell resulting in DNA damaging free radicals (see section 4.1. It is possible that a low oxygen niche and a small cytoplasm may protect satellite cells from radiation damage. Therefore, satellite cells with a small cytoplasm may suffer less damage from ionising radiation giving them a survival advantage.

To investigate this, 3 days after exposure of mouse hind limbs to 0, 18 or 25Gy irradiation, single fibres were isolated and immunostained with Pax7 and a polyclonal antibody against γH2AX (see table 2.1). H2AX is one of a number of genes that codes for histone H2A. After double strand breaks (DSBs), a reaction involving kinases of the PI-3 family causes phosphorylation of H2AX at serine 139, then termed γH2AX. Focal γH2AX staining can be used to quantify DSBs in DNA.

On single fibres isolated from irradiated mice, although there was clearly more binding in irradiated compared to control nuclei (see figure 4.6), γH2AX could not be used to give a reliable quantification of DSBs. This was principally due to the high level of background observed from the fibre. Numerous strategies were tried to reduce this background, including longer permeabilisation, antibodies from different companies (see table 2.1), longer washes, the use of detergent at various concentrations (triton X-100, 0.5% to 2%) and confocal imaging (see figure 4.6.B). The most specific staining is shown in figure 4.6, yet this was still deemed not specific enough for quantification due to occasional cross reaction with the monoclonal antibody (figure 4.6.A) and to the presence of large ‘blobs’ in positive nuclei rather than the expected focal points.

Previous successful γH2AX immunostaining has used isolated cells, and it is likely that in these efforts it is the presence of the fibre that renders the technique unreliable. I conclude that γH2AX would require satellite cells
Figure 4.6: $\gamma$H2AX immunofluorescence on single fibres from irradiated and control mice

A. fluorescent microscope images show high background and non specific staining of $\gamma$H2AX on control and 18Gy irradiated fibres. B. Confocal images show a myonucleus with high levels background, non specific staining and the presence of $\gamma$H2AX$^+$ ‘blobs’ that were not quantifiable. Scale bar 20µm
stripped from the fibre in order to be successful (see section 2.4.6). However, this would not be appropriate in this instance due the fact that the majority of irradiated satellite cells, even those that are highly proliferative *in vivo*, do not survive in cell culture dissociated from the fibre (see section 4.1).

### 4.5.1 Whole Transcript Analysis with Microarray

Results described thus far suggest that high dose ionising radiation selects for a population of satellite cells that are specifically involved in regeneration and are able to survive transplantation. However, radiation has widespread and largely unknown effects not only on the musculature but also on supporting cells (see section 5.1). Furthermore, radiation resistant satellite cells are contaminated with radio sensitive satellite cells until sensitive cells are activated (see figure 4.1) and are therefore very difficult to isolate. Additionally, these cells grow well in specific *in vivo* environments (see figure 4.5) (Heslop et al., 2000) but do not grow in culture (see figure 4.1). Indeed, it is difficult to imagine a cell type less disposed to experimental investigation. It is clear that in order to better understand these cells it is necessary to select for them by an alternative method. To this end, whole transcript analysis was carried out on irradiated and non irradiated satellite cells.

Wild type mouse hind limbs were exposed to 18Gy radiation at 4 weeks of age (see section 2.5) and satellite cells were isolated 4 weeks later by the single fibre method (see section 2.4.1). It was hoped that because mice were irradiated during a period of active growth (White et al., 2010), many of the radio sensitive population would be activated by growth requirements and would die, leaving the majority of satellite cells within irradiated muscle as radio resistant satellite cells. Previous results suggest that 4 weeks post radiation there are still some radio sensitive satellite cells present on fibres (see figure 4.2). However, microarray comparisons of cells of different purities has shown that gene expression profiles from mixed cell types yield
the profile of the dominating cell type. Cell mixtures of 75% purity are indistinguishable from pure cell populations in their overall gene expression profiles after microarray (Szaniszlo et al., 2004). Therefore RNA was isolated from satellite cells from 18Gy irradiated muscles (see section 2.5) and compared to 8 week old non irradiated litter mate controls.

Satellite cells are a rare cell population (see section 4.2) and radio resistant satellite cells even rarer still, this combined with the single fibre method of satellite cell isolation, required due to its purity, but limited by its small cell yield, means that only small concentrations of RNA could be isolated. To overcome this, RNA was pooled such that one sample contained the RNA of 3 sex-matched litter mates. The final RNA concentration for pooled samples ranged from 16 to 30 ng/µl. Such concentrations are still not sufficient for microarray analysis, so RNA was pre-amplified prior to library preparation (see section 2.7.2).

Positive control probes are a set of probes against housekeeping genes whilst negative controls probe putative intronic regions. Binding to positive and negative control probes therefore gives a value to what is highly expressed compared to what is very weakly expressed. On a scale from 0 to the maximum positive control binding = 1 the relative mean intensity value across all positive control probes was >0.8 of maximum binding, whilst mean negative probe value was <0.2 for all samples, indicating excellent hybridization across the chip. Intensity distributions were similar across samples indicating no outlying samples. All data were carried forward for analysis.

Figure 4.7 shows a grayscale image of micro array chip raw intensity values for one sample. In this representation one pixel is equal to one oligonucleotide probe and the colour represents the intensity of fluorescence emitted from that probe (i.e. the amount of hybridization, a proxy for the amount of RNA present). Raw intensity data (figure 4.7A(i)) were converted to a lo-
Algorithmic scale (figure 4.7A(ii)) with a range of 1.07 to 13.88. Figure 4.7.B shows the distribution of intensity measurements for all samples. These intensity measurements were used for comparative metrics.

Using log intensity measurements (figure 4.7.Ai, 4.7.B), table 4.1 lists the known genes most highly expressed, across irradiated and non-irradiated samples. The number of mitochondrial (Cox8b, Cox6a2, Atp5e) and muscle specific genes (e.g. troponins and Acta1) expressed at a relatively high level is perhaps surprising given the undifferentiated cell population being profiled. These results are perhaps indicative of the presence of myonuclei in the satellite cell preparation. However, these results are consistent with previous microarray data from satellite cell preparations isolated by FACs sorting on the basis of GFP expression from the Pax3\(^{GFP}\) mouse, which also show the expression of these muscle specific genes (Pallafacchina et al., 2010). Although it is clear that these genes are expressed in both studies, levels of expression cannot be compared between studies as Pallafacchina et al. do not report mean expression, but only expression in relation to other samples of that study. Consistent with results in table 4.1, genes thought to be characteristic of differentiated muscle cells (e.g. Acta1, Mylpf, Sepw1, Tnnc2) are seen to be up regulated in quiescent compared to mdx or cultured (i.e. activated) satellite cells (Pallafacchina et al., 2010).

Log transformed normalised intensity measurements (see figure 4.7.B) were used in an ANOVA to test for differences between 0Gy and 18Gy irradiated satellite cells. Using Benjamini and Hochberg adjustment for multiple comparisons ANOVA revealed no significant changes (p<0.05) in the RNA levels of genes between groups. Mean F ratio within group = 2.14, experimental error mean F ratio = 1, suggesting that the inherent variation between samples was too great to reveal a between group difference. The same was true when data were analysed for alternative splicing.

False discovery rate correction tests such as the Benjamini and Hochberg
Figure 4.7: Affymetrix Microarray Chip, Raw and Logarithm Intensity Data (Caption on page 125)
**Figure 4.7: Affymetrix Microarray Chip, Raw and Logarithmic Intensity Data**

A. Raw chip data from a single sample. 
   i. Grid of 2560 x 2560 pixels where each pixel represents one probe and the brightness of the pixel corresponds to the intensity measurement for that probe. 
   II. As in I but data is logged. 
   III. Zoom of area within the red box of II.

B. Histogram shows log intensity measurements for each probeset, line colour denotes sample.

The test used here are designed to account for the number of positive results that would be expected to be observed by chance when performing the same test multiple times. Such adjustments reduce the number of false positives but can eliminate potential genes of interest. If ANOVA is applied to the data set without adjustment, 496 significant changes in intensity levels are observed between the transcriptome of 18Gy and 0Gy irradiated satellite cells. The 10 most up regulated and 10 most down regulated genes are presented in table 4.2 and table 4.3.

Perhaps the most interesting of the up and down regulated genes are the highly upregulated Arl15 and the significantly down regulated Ahsg, both mediators of insulin signalling pathways. Arl15 encodes ADP-ribosylation factor-like 15, which is more abundantly expressed in skeletal muscle than any other tissue (Richards et al., 2009). The function of Arl15 is unclear; however SNPs in this gene have been associated with type 2 diabetes, coronary heart disease and reduced adiponectin levels in humans (Richards et al., 2009). As Arl15 is expressed primarily in skeletal muscle which does not synthesise adiponectin, it is thought to play a role in insulin resistance via an intra cellular trafficking function. This is supported by its similarity in structure to other proteins also important in intracellular trafficking e.g. the ADP-ribosylation factors and Ras-related GTP-binding proteins (Richards et al., 2009). Arl15 has been shown to be marginally upregulated in quies-
cent compared to activated satellite cells (Pallafacchina et al., 2010).

Ahsg encodes the Alpha-2-HS-glycoprotein also known as fetuin-A (Oswawa et al., 1997). Fetuin is known to be secreted by the liver, is found in blood plasma and is an inhibitor of the insulin stimulated insulin receptor tyrosine kinase. In the human, fetuin is produced exclusively by the liver, tongue and placenta, however in the mouse, fetuin-A RNA is detected in skeletal muscle (Denecke et al., 2003). Fetuin mutant mice display improved insulin sensitivity and are resistant to weight gain in response to a high fat diet (Mathews et al., 2002).

Insulin signalling pathways are known to be important in ageing: caloric restriction, reduced insulin levels and reduced insulin like growth factor signalling have been directly related to an increased lifespan (reviewed (Gems & Partridge, 2001)). Ageing is inextricably linked with stem cell function (see section 1.7). The down-regulation of a gene known to increase insulin resistance and the upregulation of a gene that may protect against insulin resistance in radio resistant satellite cells presents a good case for the further investigation of these genes and insulin signalling in the satellite cell population. It is possible that mediators of insulin play an as yet unexplored role in the maintenance of stem cell function and the state of quiescence.

Results show that the most significantly up regulated gene in the microarray is Tnrc6a (see table 4.3) which encodes trinucleotide repeat-containing 6A protein and is known to play a role in post transcriptional gene silencing via RNA interference and microRNA pathways. This may suggest a role for Tnrc6a in radio resistance. Confusingly however, Tnrc6a has previously been reported to be down regulated in quiescent compared to activated satellite cells (Pallafacchina et al., 2010). The down regulated Atp1b1b (see table 4.2), encodes the beta1 subunit of an integral membrane protein important for establishing and maintaining gradients of Na⁺/K⁺ across the membrane. Atp1b1b is essential for electroexcitability of skeletal muscle.
However Atp1b1b has no known function in the satellite cells.

Olfr183 encodes the olfactory receptor 183 (see table 4.3) a transmembrane protein involved in the G-protein coupled receptor signaling pathway. Olfactory receptors respond to molecules in the nose to initiate neuronal responses. The expression of these receptors in satellite cells is entirely unexpected and is likely to be a false positive. Similarly, the highly down regulated Rims4 (see table 4.2), regulating synaptic membrane exocytosis protein 4, is involved in neurotransmitter transport and exocytosis, and there is no evidence that it is expressed in skeletal muscle.

Although some of the genes identified by uncorrected ANOVA on microarray data suggest avenues of investigation, the small fold changes and the appearance of two genes, Olfr183 and Rims4, in the 6 most highly up or down regulated gene list that are thought not to be expressed in skeletal muscle, caution against over-interpretation of these findings. As none of these genes stand up against multiple testing corrections it is entirely possible that they are false positives. Western blot and qPCR analysis is necessary to verify the satellite cell specific expression of any of these genes and to observe differences in expression between radio resistant and radio sensitive satellite cells. However, these analyses are not viable for the study of satellite cell protein expression as it is not possible to obtain radio-resistant satellite cells in large enough numbers to isolate the necessary amount of protein or RNA.

To gain more information about the radio resistant and radio sensitive cell populations, a number of genes were selected based on their known roles in the maintenance of satellite cell quiescence, the cell cycle, myogenic differentiation, oxidative stress response or DNA damage repair. Results are presented in table 4.4. As expected, samples express genes known to be involved in the maintenance of quiescence Pax7, Dach1, and Sox genes (see table 4.4). However, results show high expression of genes indicative of satellite cell activation and entrance into the cell cycle. Immunofluorescence
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>mean log intensity</th>
<th>p(18Gy v 0Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acta1</td>
<td>actin, alpha 1, skeletal muscle</td>
<td>13.541</td>
<td>0.909</td>
</tr>
<tr>
<td>Aldoa</td>
<td>aldolase A, fructose-bisphosphate</td>
<td>13.432</td>
<td>0.183</td>
</tr>
<tr>
<td>Cox8b</td>
<td>cytochrome c oxidase, subunit VIIIb</td>
<td>13.287</td>
<td>0.641</td>
</tr>
<tr>
<td>Cox6a2</td>
<td>cytochrome c oxidase, subunit VI a, polypeptide 2</td>
<td>13.173</td>
<td>0.291</td>
</tr>
<tr>
<td>Atp2a1</td>
<td>ATPase, Ca++ transporting, cardiac muscle, fast</td>
<td>13.167</td>
<td>0.731</td>
</tr>
<tr>
<td>Tnnc2</td>
<td>troponin C2, fast</td>
<td>13.143</td>
<td>0.565</td>
</tr>
<tr>
<td>Tnt3</td>
<td>troponin T3, skeletal, fast</td>
<td>13.112</td>
<td>0.154</td>
</tr>
<tr>
<td>Tnnt2</td>
<td>troponin I, skeletal, fast 2</td>
<td>13.057</td>
<td>0.024</td>
</tr>
<tr>
<td>Mylpf</td>
<td>myosin light chain, phosphorylatable, fast skeletal muscle</td>
<td>13.027</td>
<td>0.604</td>
</tr>
<tr>
<td>Clm</td>
<td>creatine kinase, muscle</td>
<td>13.062</td>
<td>0.482</td>
</tr>
<tr>
<td>Hspb6</td>
<td>heat shock protein, alpha-crystallin-related, B6</td>
<td>12.855</td>
<td>0.544</td>
</tr>
<tr>
<td>Atp5e</td>
<td>ATP synthase, H+ transporting, mitochondrial F1 complex</td>
<td>12.815</td>
<td>0.148</td>
</tr>
<tr>
<td>Sepw1</td>
<td>selenoprotein W, muscle 1</td>
<td>12.775</td>
<td>0.0672</td>
</tr>
<tr>
<td>Pvalb</td>
<td>parvalbumin</td>
<td>12.783</td>
<td>0.0609</td>
</tr>
<tr>
<td>Enol3</td>
<td>enolase 3, beta muscle</td>
<td>12.652</td>
<td>0.538</td>
</tr>
<tr>
<td>Uqcr10</td>
<td>ubiquinol-cytochrome c reductase, complex III subunit X</td>
<td>12.626</td>
<td>0.091</td>
</tr>
<tr>
<td>Pygm</td>
<td>muscle glycogen phosphorylase</td>
<td>12.603</td>
<td>0.658</td>
</tr>
<tr>
<td>Myh4</td>
<td>myosin, heavy polypeptide 4, skeletal muscle</td>
<td>12.617</td>
<td>0.683</td>
</tr>
<tr>
<td>Myl1</td>
<td>myosin, light polypeptide 1</td>
<td>12.593</td>
<td>0.412</td>
</tr>
<tr>
<td>Pgam2</td>
<td>phosphoglycerate mutase 2</td>
<td>12.562</td>
<td>0.684</td>
</tr>
<tr>
<td>Ccdec50</td>
<td>coiled-coil domain containing 50</td>
<td>12.513</td>
<td>0.536</td>
</tr>
<tr>
<td>Mdh2</td>
<td>malate dehydrogenase 2, NAD (mitochondrial)</td>
<td>12.481</td>
<td>0.012</td>
</tr>
<tr>
<td>Tpm2</td>
<td>tropomyosin 2, beta</td>
<td>12.465</td>
<td>0.432</td>
</tr>
<tr>
<td>Mt2</td>
<td>metallothionein 2</td>
<td>12.445</td>
<td>0.867</td>
</tr>
<tr>
<td>Atf3</td>
<td>activating transcription factor 3</td>
<td>12.247</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Table 4.1: 25 most abundant RNA transcripts across samples
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>p(18Gy v 0Gy)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rims4</td>
<td>regulating synaptic membrane exocytosis</td>
<td>8.38E-05</td>
<td>-1.340</td>
</tr>
<tr>
<td>Ahsq</td>
<td>alpha-2-HS-glycoprotein</td>
<td>0.0001</td>
<td>-1.436</td>
</tr>
<tr>
<td>Atp1b1</td>
<td>ATPase, beta 1 polypeptide</td>
<td>0.0002</td>
<td>-1.710</td>
</tr>
<tr>
<td>Krtap5-3</td>
<td>keratin associated protein 3-3</td>
<td>0.0002</td>
<td>-1.909</td>
</tr>
<tr>
<td>Fgf4</td>
<td>fibroblast growth factor 4</td>
<td>0.0002</td>
<td>-1.357</td>
</tr>
<tr>
<td>Olfr1302</td>
<td>olfactory receptor 1302</td>
<td>0.0003</td>
<td>-1.503</td>
</tr>
<tr>
<td>1700034E13Rik</td>
<td>RIKEN cDNA 1700034E13</td>
<td>0.0003</td>
<td>-1.608</td>
</tr>
<tr>
<td>Atp5g3</td>
<td>ATP synthase, H+ transporting, mitochondrial</td>
<td>0.0007</td>
<td>-1.259</td>
</tr>
<tr>
<td>Pabpn1l</td>
<td>poly(A)binding protein nuclear 1-like</td>
<td>0.0009</td>
<td>-1.259</td>
</tr>
<tr>
<td>Car4</td>
<td>carbonic anhydrase 4</td>
<td>0.0010</td>
<td>-3.308</td>
</tr>
</tbody>
</table>

**Table 4.2:** 10 most significant genes down-regulated in irradiated compared to control satellite cells in uncorrected ANOVA comparisons

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>p(18Gy v 0Gy)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnrc6a</td>
<td>trinucleotide repeat containing 6a</td>
<td>0.0001</td>
<td>1.385</td>
</tr>
<tr>
<td>Arl15</td>
<td>ADP-ribosylation factor-like 15</td>
<td>0.0002</td>
<td>1.572</td>
</tr>
<tr>
<td>Olfr183</td>
<td>olfactory receptor 183</td>
<td>0.0003</td>
<td>1.567</td>
</tr>
<tr>
<td>Slc35b3</td>
<td>solute carrier family 35</td>
<td>0.0003</td>
<td>1.269</td>
</tr>
<tr>
<td>Rif1</td>
<td>Rap1 interacting factor 1</td>
<td>0.0003</td>
<td>1.406</td>
</tr>
<tr>
<td>Atf7ip</td>
<td>activating transcription factor 7 interacting protein</td>
<td>0.0007</td>
<td>1.286</td>
</tr>
<tr>
<td>Esyt1</td>
<td>extended synaptotagmin-like protein 1</td>
<td>0.0009</td>
<td>1.661</td>
</tr>
<tr>
<td>Eef2</td>
<td>eukaryotic translation elongation factor 2</td>
<td>0.0009</td>
<td>1.154</td>
</tr>
<tr>
<td>Vmn1r29</td>
<td>vomeronasal 1 receptor 29</td>
<td>0.0010</td>
<td>1.319</td>
</tr>
<tr>
<td>Med14</td>
<td>mediator complex subunit 14</td>
<td>0.0010</td>
<td>1.191</td>
</tr>
</tbody>
</table>

**Table 4.3:** 10 most significant genes up-regulated in irradiated compared to control satellite cells in uncorrected ANOVA comparisons

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analysis on single fibres suggests that, if satellite cells are isolated with a trituration time of less than 20 minutes, the majority of satellite cells does not express MyoD (see figures 2.2 and 3.4). Immunofluorescence analyses expression at the protein level, whilst microarray analyses at the mRNA level and is therefore more sensitive. Upon activation, mRNA will be upregulated prior to strong expression of the protein and this may explain the apparent disparity in results. MyoD is seen to have a higher intensity measurement than Pax7, strongly suggesting that the the majority of the satellite cell population were activated at the time of RNA isolation (see table 4.4).

The ATP-binding cassette, sub-family B (Abcb1a) and flavin containing monoxygenase 2 (Fmo2) have previously been shown to be up regulated in quiescent compared to activated satellite cells (Pallafacchina et al., 2010) and are known stress resistance genes. Abcb1a is a protein coding gene for an ATP-binding cassette transporter involved in transporting molecules across extra- and intra-cellular membranes. Fmo2 is an NADH dependant enzyme that catalyses the oxidation of many drugs. Results show that both genes are expressed in satellite cells and there is no difference between irradiated and control groups.

Genes associated with the oxidative stress response (Srxn1, Gpx3, Txnrld1 and HIF1) were expressed at varying levels, and showed no consistent direction of up or down regulation between groups. Genes involved in double strand break repair (Kanaar et al., 1998) (Rad51, Kn70, DNA ligase IV) are expressed at fairly low levels and are not different in control compared to irradiated satellite cells. In accordance with results from single fibres (see section 4.2) irradiated satellite cells expressed very little Caspase 3 and showed no difference compared to non irradiated satellite cells indicating little apoptosis in either group.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene Name</th>
<th>mean log intensity</th>
<th>p(18Gy v 0Gy)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax7</td>
<td>paired box gene 7</td>
<td>5.338</td>
<td>0.334</td>
<td>-1.608</td>
</tr>
<tr>
<td>Dach1</td>
<td>dachshund 1</td>
<td>5.292</td>
<td>0.824</td>
<td>-1.0471</td>
</tr>
<tr>
<td>Sox7</td>
<td>SRY-box containing gene 7</td>
<td>6.537</td>
<td>0.325</td>
<td>1.724</td>
</tr>
<tr>
<td>Sox17</td>
<td>SRY-box containing gene 17</td>
<td>6.378</td>
<td>0.782</td>
<td>1.086</td>
</tr>
<tr>
<td>Sox18</td>
<td>SRY-box containing gene 18</td>
<td>6.395</td>
<td>0.781</td>
<td>1.079</td>
</tr>
<tr>
<td>Myf5</td>
<td>myogenic factor 5</td>
<td>7.23</td>
<td>0.139</td>
<td>-1.656</td>
</tr>
<tr>
<td>MyoD</td>
<td>myogenic differentiation 1</td>
<td>8.725</td>
<td>0.547</td>
<td>1.473</td>
</tr>
<tr>
<td>Mki67</td>
<td>Identified by monoclonal antibody Ki 67</td>
<td>3.089</td>
<td>0.627</td>
<td>-1.145</td>
</tr>
<tr>
<td>Smarcc1</td>
<td>SNF related, matrix associated, actin dependent regulator</td>
<td>6.897</td>
<td>0.658</td>
<td>1.066</td>
</tr>
<tr>
<td>Smarca4</td>
<td>SNF related, matrix associated, actin dependent regulator</td>
<td>7.725</td>
<td>0.327</td>
<td>1.074</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>cyclin D1</td>
<td>6.843</td>
<td>0.210</td>
<td>1.842</td>
</tr>
<tr>
<td>Ccne1</td>
<td>cyclin E1</td>
<td>4.393</td>
<td>0.031</td>
<td>1.201</td>
</tr>
<tr>
<td>Abcb1a</td>
<td>ATP-binding cassette, subfamily B(MDR/TAP),1A</td>
<td>5.637</td>
<td>0.803</td>
<td>-1.089</td>
</tr>
<tr>
<td>Fmo2</td>
<td>flavin containing monooxygenase 2</td>
<td>5.132</td>
<td>0.437</td>
<td>-1.297</td>
</tr>
<tr>
<td>Srxn1</td>
<td>sulfiredoxin 1 homolog</td>
<td>8.717</td>
<td>0.276</td>
<td>2.047</td>
</tr>
<tr>
<td>Gpx3</td>
<td>glutathione peroxidase 3</td>
<td>0.396</td>
<td>-1.125</td>
<td></td>
</tr>
<tr>
<td>Txnrd1</td>
<td>thioredoxin reductase 1</td>
<td>8.172</td>
<td>0.249</td>
<td>1.675</td>
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<tr>
<td>Hif1a</td>
<td>hypoxia inducible factor 1, alpha subunit</td>
<td>7.424</td>
<td>0.741</td>
<td>1.063</td>
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<tr>
<td>Rad51</td>
<td>RAD51 homolog</td>
<td>3.5</td>
<td>0.584</td>
<td>1.0763</td>
</tr>
<tr>
<td>Xrcc6</td>
<td>Ku70</td>
<td>5.11</td>
<td>0.887</td>
<td>-1.061</td>
</tr>
<tr>
<td>Lig4</td>
<td>DNA ligase IV</td>
<td>4.87</td>
<td>0.636</td>
<td>1.307</td>
</tr>
<tr>
<td>Casp3</td>
<td>caspase 3</td>
<td>2.232</td>
<td>0.134</td>
<td>1.382</td>
</tr>
</tbody>
</table>

Table 4.4: Expression levels and fold change in irradiated compared to non irradiated satellite cells of potential genes of interest. The mean fluorescence intensity is presented as an average across samples to give a measure of the relative abundance of that transcript together with p values from a Benjamani & Hochberg corrected ANOVA.
4.6 Discussion

Experiments in this chapter have shown that radiation does not directly cause the loss of myonuclei, as there was no significant reduction in the number of myonuclei per fibre 4 weeks post radiation (see figure 4.2). Ionizing radiation causes the loss of satellite cells in a dose dependant manner. At 4.5 and 9Gy radiation, enough satellite cells survive to partially replenish the satellite cell pool and these are able to maintain myonuclear numbers. However, after 18 and 25Gy radiation, all but a rare few satellite cells are lost, and those that remain are unable to contribute to the replacement of myonuclei during normal nuclear turnover (see figure 4.2). Thus, after 18 and 25Gy radiation, myonuclei are gradually lost between 6 months and 1 year post radiation. The gradual decline in satellite cell number per fibre with increasing time post radiation (see figure 4.2) is consistent with in vitro data which shows that 18Gy irradiated satellite cells are lost only once they have activated MyoD (see figure 4.1).

Results show that satellite cell numbers are severely reduced and almost undetectable 4 weeks post 25Gy irradiation and these numbers are not increased even a year post radiation. Thus, once the satellite cell pool is depleted, it cannot be renewed from another cell source. This is in accordance with previous research that shows satellite cells are specified before birth (Relaix et al., 2005, Gros et al., 2005)(section 1.5) and that they are a self sustaining population that cannot be replaced by cells from a different lineage (Lepper et al., 2011) (section 1.3).

Control TA and EDL weights increase between 4 weeks and 1 year of age (see figure 4.4). This is in accordance with previous results showing an increase in myonuclei number and myofibre volume during this time period (see figures 3.2 and 3.7). In the EDL, 4.5Gy and 9Gy radiation appears to delay but not ablate muscle growth, as at 6 months muscles are significantly smaller than controls, but at 1 year weights are comparable with
control muscle (see figure 4.4). Satellite cells per fibre seem to follow a similar pattern, increasing between 6 months and 1 year in 4.5 and 9Gy irradiated mice (see figure 4.2). Together, these data suggest that 4.5Gy and 9Gy radiation results in the loss of some satellite cells when they subsequently activate MyoD. However, the majority of satellite cells are able to survive this damage and can replace those satellite cells that are lost and subsequently contribute to the addition of myonuclei (see figure 4.2) and an increase in muscle weight (see figure 4.4).

Results reported here converge to show that, after exposure to 18 or 25Gy radiation, satellite cells cannot contribute to myonuclei addition during muscle growth (4 weeks to 6 months) or muscle maintenance (6 months to 1 year) in an unchallenged system. However, when satellite cells were prepared from 18Gy irradiated muscle, they were able to contribute to host muscle regeneration (figure 4.5). Clusters of donor derived fibres from irradiated donors were considerably smaller than those previously observed from non irradiated donors (compare figure 4.5 with figures 3.5 and 3.6) (Collins et al., 2005, 2007, Boldrin et al., 2009, 2012). However this can be accounted for by the very small number of cells injected. Satellite cells were isolated from 100 fibres for each muscle injection: from 18Gy irradiated donors this would give less than 100 viable satellite cells (see figure 4.2). These cells are also likely to have undergone considerable DNA damage and it is possible that even these small numbers of cells, if they could be selected for by another method, could give rise to much larger clusters of donor derived fibres.

Results show that within muscle there exist a population of satellite cells that are resistant to 18Gy ionising radiation; these cells do not contribute to muscle growth but are able to contribute to muscle regeneration. This supports previous findings that show that following 18Gy radiation, muscle growth is stunted in the mouse (Quinlan et al., 1997, Pagel & Partridge,
1999) and isolated single fibres show no viable satellite cells in vitro (Heslop et al., 2000). However, if 18Gy irradiated muscle is challenged with notexin, a regenerative response is observed and subsequently isolated fibres show viable satellite cells (Heslop et al., 2000). Data presented here show that the frequency of radiation-resistant, regeneration-competent satellite cells is very low (figure 4.2). This may provide an explanation of findings which show that the transplantation of just one single myofibre isolated from an 18Gy irradiated donor results in no donor contribution to muscle regeneration (Collins et al., 2005). As radio resistant satellite cells are so rare, when performing only a small number of transplantations the probability of the transplanted fibre having even a single associated radio resistant satellite cell is low.

Microarray analysis of satellite cells isolated from 18Gy irradiated muscle compared to satellite cells from non irradiated muscle showed no significant difference in single gene expression levels. Extracting and analysing genes of potential interest, shows that the majority of the satellite cell population were activated at the point of sequencing. As 6 muscles, and the maximum numbers of fibres possible were required for RNA extraction (see section 2.7.1), isolation time was longer than that in the single fibre analysis. Increased trituration time increases the percentage of the satellite cell population that is activated (see figure 2.2) and this may have contributed to satellite cell activation. However, trituration time did not exceed 2 hours and it was therefore expected that no more than 50% of the population would be activated (see figure 2.2). It is possible that MyoD levels at this time point are below the detection levels of immunostaining but are detected by microarray, but this is not consistent with the results observed for Pax7 which is detectable by immunostaining and is expressed at a lower level according to microarray data. Results therefore suggest that the process of stripping the satellite cells and/or the process of RNA extraction results in
their activation. Activation of satellite cells may explain the lack of between group differences. It is possible that the radio resistant and radio sensitive satellite cell populations differ only in their quiescent state.

ANOVA analysis of microarray data shows a high within group variance, resulting in a lack of group clustering rendering between group differences unobservable. Within sample noise is increased by RNA amplification, sample pooling and by mixed cell populations. Although each source of noise is in itself relatively minor, results show that the combination of this noise has in this instance resulted in a level of within group noise that is too great to detect significant between group changes with ANOVA.

From a review of microarray papers it has been concluded that total RNA required for a successful microarray must be in the region of 50-200µg. 1 cell yields between 10 to 30pg RNA (Duggan et al., 1999) meaning that the required number of cells for microarray analysis is in the range of $1.6 \times 10^6$ and $2 \times 10^7$ cells. Satellite cells cannot be isolated purely in these numbers, therefore sample pooling and RNA amplification were necessary to undertake microarray analysis. Although RNA was pooled from litter mates, each mouse will have slightly different expression levels of different genes and different genetic variations.

In addition to sample pooling, samples were pre amplified prior to microarray analysis. With RNA amplification comes many problems: SMART PCR technology used here employs a PCR based amplification method which introduces PCR priming sites at each end of a reverse transcribed cDNA molecule and amplifies using DNA polymerase. Amplification is thus a random process, and although fairly good correlations are reported between pre and post amplification RNA transcripts DNA polymerase is known to show amplification bias - mis incorporation of bases, a bias towards short transcripts and differential amplification depending on GC composition (reviewed (Nygaard & Hovig, 2006)).
These experiments show that a subpopulation of satellite cells survive high dose ionising radiation. Radio-resistant satellite cells do not contribute to muscle growth or muscle maintenance, but they can be activated to contribute to muscle regeneration after transplantation. I hypothesise that radio resistant satellite cells are the subpopulation of satellite cells that in non irradiated donors are able to contribute to muscle regeneration post transplantation. Thus, high dose ionising radiation selects for the rare regeneration competent satellite cell that is necessary for successful satellite cell transplantation. The mechanism by which this satellite cell sub population is able to survive radiation is unclear.

Figure 4.5 is published in: Boldrin, L., Neal, A., Zammit, P.S., Muntoni, F., Morgan, J.E. (2012) Donor Satellite Cell Engraftment is Significantly Augmented When the Host Niche is Preserved and Endogenous Satellite Cells are Incapacitated. Stem cells. 1549-4918
Chapter 5

Radiation as A Muscle
Pre-treatment for Improved
Satellite Cell Transplantation

5.1 Introduction

In both mdx and wild type mouse muscle, myoblast transplantation produces more donor derived fibres over a larger area in pre irradiated compared to non irradiated mdx and wild type hosts (Morgan et al., 2002). Satellite cell transplantation into muscles that have received no pre treatment results in very poor engraftment (Boldrin et al., 2012).

Commonly, mouse muscle is treated with cardiotoxin, notexin, barium chloride (reviewed (Harris, 2003)) or cryoinjury (reviewed (Grounds & Yablonka-Reuveni, 1993)) prior to transplantation of donor cells into skeletal muscle (Pye et al., 2004, Wernig et al., 2005, Brimah et al., 2004, Cooper et al., 2001, Hall et al., 2010). These treatments cause muscle necrosis and therefore initiate a regenerative response allowing donor cells to contribute to myofibre formation. Irradiation differs from other muscle injury regimes in
that it does not cause myofibre damage (see section 4.3 and figure 4.3). Presumably in the mdx mouse the naturally occurring degeneration is sufficient to allow donor cell contribution to host myofibre repair (see section 1.9.2). A comprehensive investigation of injuries in our model system shows that in the mdx nude mouse, host muscle pre treatment with high dose ionising radiation results in considerably greater numbers of donor derived fibres than any other treatment (Boldrin et al., 2012). Pre irradiation is effective if it is performed 1, 2 or 3 days prior to donor cell transplantation (Boldrin et al., 2012) and its subsequent improvement in the number of donor derived fibres observed is related to the rate at which the irradiation is delivered (Gross et al., 1999).

Irradiation causes DNA double strand breaks (see section 4.1) and prevents the majority of mitotically active cells from subsequent division. In the haematopoietic system, successful transplantations require homing of cells to their niche within the bone marrow. For bone marrow transplantation, pre irradiation is necessary as it empties the host niche and thereby allows niche reconstitution by donor cells (reviewed (Whetton & Graham, 1999)). The mechanism by which irradiation improves donor stem cell engraftment in skeletal muscle is unknown.

Muscle irradiation kills the majority of satellite cells upon their subsequent cell division (see section 4.2 and 4.3) and thereby prevent muscle growth in wild type (see figure 4.4) and mdx mice and prevents regeneration in the mdx mouse (Wakeford et al., 1991). It is likely that, as seen in the haematopoietic system, irradiation improves engraftment efficiency because it incapacitates the host stem cell pool and thereby eliminates endogenous competition. In the absence of host satellite cells transplanted cells become the sole source of new myonuclei and are able to make a greater contribution to host muscle regeneration.

There appears to be a complex relationship between the irradiated host
environment and the donor satellite cell that is currently not well understood. It seems that the irradiated muscle environment affects donor satellite cell fate decisions independent of its damaging effects on the host satellite cell pool. The myogenic cell line C2C12 (Yaffe & Saxel, 1977, Blau et al., 1983a) produce tumours more rapidly in irradiated compared to non irradiated hosts (Morgan et al., 2002). Interestingly, this phenotype is reversible, as these same tumour forming cells when re-isolated and subsequently injected into non irradiated hosts do not proliferate to form tumours but differentiate to produce donor derived muscle (Morgan et al., 2002). This suggests that the irradiated muscle environment has pro proliferative effects on non committed cells. The improved donor satellite cell engraftment observed in irradiated host muscle is therefore likely due to a combination of the effects of irradiation on the host stem cell pool and radiation induced changes to the host muscle environment that improves donor cell proliferation.

Skeletal muscle injury is accompanied by an inflammatory response. Neutrophils invade myofibres within an hour of damage and their numbers remain elevated for up to 5 days post injury (reviewed (Tidball, 2005)). Rapid neutrophil infiltration is followed by an invasion of macrophages which act as scavengers of tissue debris (Tidball & Wehling-Henricks, 2007). Macrophages are known for their heterogeneity (reviewed (Gordon & Taylor, 2005), and their functional relevance is difficult to unravel due to evidence to demonstrate their apparently contradictory effects i.e. pro inflammatory and anti inflammatory (Stout & Suttles, 2004). Nevertheless, it is possible that radiation improves donor satellite cell engraftment via changes to the monocyte cell infiltrate.

Macrophages have been shown to enhance satellite cell proliferation in macrophage-satellite cell co cultures (Merly et al., 1999). Furthermore, Inhibition of macrophage function has been shown to impair muscle mem-
brane repair after muscle loading in mice (Tidball, 2005) and to moderately increase muscle fat accumulation post cryoinjury (Summan et al., 2006). There is some evidence to show that cellular free radicals formed by irradiation can interact with the phospholipids of the myofibre membrane and increase its permeability (Canaday et al., 1994). However, these findings were not supported in an investigation of myofibre membranes in vivo in mdx mice (Pagel & Partridge, 1999).

Irradiation-induced improvement in donor cell engraftment may be due to the secretion of pro-proliferation factors or the inhibition of anti proliferative factors. Cytokines are known mediators of satellite cell function and irradiation has previously been shown to alter cytokine expression within skeletal muscle (Han et al., 2006, Liu et al., 2011, Shin et al., 2010). However, a cytokine array on this model system showed no evidence of a cytokine differentially expressed in the same direction in both males and females at different time points post radiation. This strongly suggests that changes in cytokine expression are not critical to radiation induced improvement in donor cell engraftment (Boldrin et al., 2012).

In tumours, it is known that radiation therapy not only directly kills tumour cells but also destroys the tumour feeding vasculature via endothelial cell apoptosis (Garcia-Barros et al., 2003). In the rabbit, radiation has been shown to cause capillary damage after a dose of just 2Gy (Krishnan et al., 1987). Measurements of capillary length in the inner ear of the rabbit reveal a dose dependent decrease in capillary density upon exposure to radiation (Dimitrievich et al., 1984). Small changes to the vascular network can have large ramifications for the tissues they supply: destruction of just a few capillaries is sufficient to cause the death of small tumours (Garcia-Barros et al., 2003). Endothelial cells are considerably more radio sensitive than muscle cells, therefore it is likely that high dose radiation delivered to the mouse hind limb causes damage to the vasculature and a reduction in muscle
Stem cells reside in specialised micro environments that are known to maintain a low oxygen tension (reviewed (Moore & Lemischka, 2006)). This oxygen tension is critical for the maintenance of the undifferentiated state (reviewed (Mohyeldin et al., 2010)). *In vitro*, low oxygen has been shown to prevent haematopoietic stem cell exhaustion (reviewed (Eliasson & Jansson, 2010)), inhibit cell death pathways (Clarke & van der Kooy, 2009) and increase proliferation (Krinner et al., 2009). Low oxygen tension is associated with tumour stem cell proliferation and self renewal (Das et al., 2008), migration (Sahlgren et al., 2008) and, intriguingly, with radio resistance (reviewed (Chapman et al., 1998)).

In satellite cells, low oxygen culture conditions have been shown to promote satellite cell proliferation and differentiation (Majmundar et al., 2012). Furthermore, myoblasts exposed to hypoxic conditions prior to transplantation produce more donor derived fibres and show a greater amount of self renewal (Liu et al., 2012b). Due to the established importance of oxygen tension in stem cell survival, and the radio sensitivity of endothelial cells it is possible that pre irradiation of host muscles improves engraftment efficiency via changes in muscle oxygen tension.

### 5.1.1 Aims

- To establish the optimal dose of exposure of host muscle to radiation prior to engraftment for the most efficient donor cell contribution to host muscle regeneration.

- To elucidate the mechanism by which exposure of host muscle to high dose ionising radiation prior to engraftment improves donor cell contribution to host muscle regeneration.

- To investigate hypoxia as a potential mediator of radiation induced
improvement in engraftment efficiency.

- To investigate devascularisation as a host muscle injury model for donor cell engraftment.

### 5.2 The Host Satellite Cell Pool

In the hematopoietic system, pre irradiation allows donor cell engraftment by emptying the host bone marrow niche resulting in the homing of donor cells (reviewed (Whetton & Graham, 1999)). Previous results show that in the wild type mouse, exposure to 18Gy irradiation damages satellite cells such that they are lost after their first attempt at cell division post radiation (see figure 4.1). 18Gy irradiation therefore results in the continual loss of satellite cells for 6 months post radiation (see figure 4.2).

The \textit{mdx} mouse model is a different muscle environment than that of the wild type, and in this context radiation is known to have different consequences for the satellite cell pool (Wakeford et al., 1991, Heslop et al., 2000) (see section 4.1). In transplantation procedures, \textit{mdx} host mice are irradiated at 3-4 weeks of age (see section 2.6), during the peak of their muscle degeneration and regeneration (McGeachie et al., 1993, Grounds & Torrisi, 2004).

To investigate satellite cell number at the point of engraftment, wild type and \textit{mdx} nude mice were exposed to 18 or 25Gy irradiation and single fibres were isolated either immediately (T0) or at 3 days (T72) post radiation.

A two way ANOVA shows a significant main effect of radiation dose and time, and interaction between the two (p <0.001). In the wild type mouse there was no difference in the number of satellite cells per fibre after exposure to 25Gy or 18Gy radiation at T0 or T72 (p >0.05).

However, in contrast to the wild type, fibres from \textit{mdx} mice showed a significant reduction in the number of satellite cells per fibre at T72 compared
to T0 in both 18Gy (p <0.01) and 25Gy fibres (p <0.001) (see figure 5.1). Additionally, at T72, fibres from muscle exposed to 25Gy irradiation had significantly fewer satellite cells per fibre than fibres from 18Gy irradiated muscle (p <0.001) (see figure 5.1).

At 3 weeks of age mdx mouse muscles are undergoing regeneration (see section 1.9.2). As irradiated satellite cells are lost after the activation of MyoD (see figure 4.2), the difference in post radiation satellite cell numbers between wild type and mdx mice is likely due to their activation status.
Figure 5.1: Satellite cells per fibre mdx and WT mice immediately following and 3 days post radiation (Caption on page 145)
Figure 5.1: Satellite cells per fibre *mdx* and WT mice immediately following and 3 days post radiation. A. Satellite cells per fibre from *mdx* nude and wild type mice on fibres isolated immediately following or 72 hours post radiation doses of 18 or 25Gy. Results show a significant decline in satellite cells per fibre from irradiated *mdx* mice at T72 compared to and that 25Gy irradiated fibres have significantly less satellite cells per fibre than 18Gy irradiated fibres at T72. There were no significant changes in satellite cell numbers observed on fibres from irradiated wild type mice at these time points (* = p<0.05). B. Example images showing Pax7+/DAPI+ cells, satellite cells. Scale bar = 50µm

5.3 Dose Response of Host Muscle Irradiation on Donor Satellite Cell Engraftment

At 3 days post radiation, 25Gy irradiated muscles have significantly fewer satellite cells per fibre than 18Gy irradiated muscle (see figure 5.1). It follows therefore, that if pre irradiation improves satellite cell engraftment via its effects on the host satellite cell pool, pre irradiating host muscle with 25Gy should produce superior engraftment.

To test this, host *mdx* nude mice were exposed to 4.5, 9, 18 or 25Gy radiation and injected with donor satellite cells 3 days later. One way ANOVA shows a significant main effect of radiation dose on the number of donor derived fibres observed (p=0.001) (see figure 5.2). Results reveal a dose response curve such that 18Gy irradiated hosts were seen to have significantly more donor derived fibres than 4.5Gy (p=0.01). Crucially however, at 25Gy irradiation there were significantly less donor derived fibres observed than at 18Gy pre irradiation (p<0.001).

These results show that, when injecting 3 days post radiation delivery, a dose of 18Gy is optimal for donor satellite cell engraftment. Although the
Figure 5.2: Host muscle regeneration with irradiated donor cells
(Caption on page 147)
majority of host satellite cells will be non functional at this time point, host mice still have a pool of satellite cells present in the myofibre niche (figure 5.1). In contrast, after 25Gy, when engraftment was not successful, most host satellite cells have already been lost. This provides strong support to the conclusion that pre irradiation of host muscle does not augment satellite cell engraftment solely by eliminating host satellite cell competition. Indeed, these results present the intriguing possibility that the presence of incapacitated host satellite cells within host muscle actually improves donor cell engraftment.

5.4 Potential Irradiation Induced Changes in Skeletal Muscle Oxygen Tension

Previous data have shown that irradiation does not cause damage to the muscle fibre. Irradiation does not result in loss of myonuclei or cause a change in skeletal muscle gross morphology (see section 4.3.2 and 4.3.3). Irradiation is not a focal damage, and, of course, does not only affect the skeletal muscle cells of the exposed hind limb. For example, it has been shown that exposure to radiation prevents subsequent bone growth in the mdx mouse (Pagel & Partridge, 1999). It is likely that the mediating effect of pre irradiation on host engraftment occurs via muscle extrinsic factors, such
as damage to other cell types or by the induction of signaling cascades or
growth factors that aid satellite cell survival or proliferation. As a cytokine
array on this system revealed no radiation induced cytokine changes that
could obviously affect these pathways (Boldrin et al., 2012) and given the
established importance of oxygen availability in stem cell fate decisions (see
section 5.1), I looked for evidence of potential radiation related changes in
skeletal muscle oxygen tension as a potential mechanism by which 18Gy
significantly improves engraftment efficiency (see figure 5.2).

5.4.1 HIF1

During development, responses to hypoxia are mediated by the activation
of Hypoxia Inducible Factor 1 (HIF1) (Iyer et al., 1998). HIF1 is a basic
helix-loop-helix-PAS transcription factor, the expression of which directly
increases in response to decreasing cellular oxygen (reviewed (Semenza,
1999)). HIF1 transcription initiates the expression of many target genes
including glucose transporters, glycolytic enzymes and endothelial growth
factors. These factors either decrease cytoplasmic oxygen or produce an
adaptive cellular response (reviewed (Liu et al., 2012a)). Loss of function
experiments demonstrate the critical importance for HIF1 as a master regu-
lator of the hypoxic response. Adaptive responses to hypoxic conditions are
impaired in mice with a mutated HIF1α subunit, and the HIF1 null mouse
is embryonic lethal by E11 (Iyer et al., 1998).

Skeletal muscle expresses high levels of HIF1 even in normoxic conditions
(Kubis et al., 2005, Stroka et al., 2001). However, in normoxic conditions the
HIF1α subunit is localised exclusively in the cytoplasm. Under hypoxic con-
ditions, the cytoplasmic HIF1α subunit translocates to the nucleus, forms a
DNA binding complex with the HIF1β subunit and mediates transcription
(reviewed (Semenza, 2000, Liu et al., 2012a)). Thus, in normoxic conditions
HIF1α is located in the cytoplasm, whilst in hypoxic conditions HIF1α is
found in the nucleus.

In order to observe changes in oxygen tension in response to irradiation, single fibres were isolated from irradiated and control wild type mice and analysed for location of the HIF1α subunit. It was reasoned that if irradiation affects skeletal muscle oxygen tension, then a translocation of HIF1α to the nucleus would be observed.

As expected, control single fibres were seen to express high levels of HIF1α localised to the cytoplasm (figure 5.3.A). As the anti-HIF1α antibody was monoclonal (see table 2.1) these experiments utilised the satellite cell specificity of Caveolin 1 (Gnocchi et al., 2009) to distinguish satellite cells from myonuclei. Interestingly it was observed that HIF1α was more strongly expressed in the satellite cells compared to the myofibre cytoplasm and the majority of satellite cells appeared to show both nuclear and cytoplasmic localisation (see figure 5.3).

Single fibres were isolated from 18Gy irradiated mice three days post radiation. Immunofluorescence shows that as in control fibres, HIF1α was localised to the cytoplasm, and there was no evidence of its trans location to the nucleus, (see figure 5.3.A). Analysis of HIF1 expression therefore suggests that irradiation does not affect oxygen tension in skeletal muscle. As in control fibres, satellite cells could be distinguished from myonuclei based on a visibly stronger expression of HIF1α, which appeared to have both a cytoplasmic and nuclear localisation (see figure 5.3.B).

### 5.4.2 Devascularisation as an Injury Model

Low oxygen tensions have been shown to mediate satellite cell self renewal and proliferation and pre conditioning of cultured myoblasts in low oxygen improves their subsequent engraftment efficiency (Majmundar et al., 2012, Liu et al., 2012b). It is possible that a hypoxic host environment could improve donor cell survival and/or proliferation post engraftment and thus
**Figure 5.3:** HIF1α and Caveolin-1 staining on single fibres from control and 18Gy irradiated mice. A. Fluorescent microscope images of single fibres show Caveolin1+ nuclei (DAPI+) and a cytoplasmic localisation of HIF1α in control and 3 days post 18Gy irradiation. B. Confocal image shows a nuclear and cytoplasmic location of HIF1α exclusively in Caveolin1+ nuclei. Scale bar = 50µm.
provide an effective alternative to 18Gy irradiation.

To investigate the effect of oxygen tension on donor satellite cell engraftment, host mdx nude TA muscles were devascularised prior to donor cell transplantation (for methods see section 2.6.2). Engraftment efficiency was compared to that of 18Gy irradiated host mice. 4 weeks post transplantation, devascularised muscle showed very few donor-derived fibres (see figure 5.4). A one way student T-test showed significantly more donor derived fibres in 18Gy pre-irradiated muscles compared to devascularised host muscles ($p<0.01$).

Results show that devascularisation is not an effective injury model for subsequent donor cell engraftment and converge with HIF1α immuno fluorescence to suggest that 18Gy irradiation does not improve donor cell engraftment via mediation of oxygen tension.

5.4.3 The Effects of Irradiation on Cell Infiltrate and Vessel Density

Ionizing radiation has been shown to alter myofibre permeability (Canaday et al., 1994) and therefore may cause an increase in cell infiltrate. In order to quantify cell infiltrate within irradiated skeletal muscle, TA sections were analysed for expression of Cluster of Differentiation 45 (CD45). Also known as Protein tyrosine phosphatase receptor type C (PTPRC), CD45 is a transmembrane protein expressed exclusively on haematopoietic cells and is an essential regulator of T and B cell antigen signaling (reviewed (Holmes, 2006)).

Exposure to radiation has been shown to alter capillary density in other tissues (Dimitrievich et al., 1984). To observe any potential changes in the number of vessels and their morphology, irradiated mdx and wild type TA cross sections were stained with Cluster of differentiation 31 (CD31). CD31 also known as platelet endothelial cell adhesion molecule 1 (PECAM1), is
Figure 5.4: Donor derived fibres in devascularised and irradiated host *mdx* nude TA muscles. A. Dystrophin+ fibres on TA cross sections from host mice pre treated with 18Gy irradiation or devascularisation shows significantly more dystrophin+ fibres in irradiated compared to devascularised muscle (p<0.01). B. Example cross sections show clusters of donor derived fibres by colocalisation of dystrophin and βgal. Scale bar = 50µm.
a protein of the immunoglobin superfamily expressed on the surface of cells within the vasculature: platelets, monocytes, neutrophils, and some T-cells (Newman, 1997). Critically CD31 is also expressed on endothelial cell junctions, and can therefore be used to visualise blood vessel density (Lehmann et al., 2009).

*Mdx* nude mouse hind limbs were irradiated with 18 or 25Gy irradiation and harvested either on the day of irradiation or 3 days afterwards. TA sections were co stained with CD45 and CD31 antibodies (see table 2.1). The area of positive staining is expressed as a percentage of the area of the whole section, ascertained from 6 images taken randomly at 20x across a single section. A one-way ANOVA showed no differences in the area of CD31 positive staining in control 18Gy or 25Gy irradiated sections either immediately following or 3 days post radiation (p=0.108) (see figure 5.5). This suggests that irradiation does not affect vascular morphology or vessel density within skeletal muscle. Similarly one-way ANOVA showed no difference in CD45+ area between control 18 or 25Gy irradiated sections, at T0 or T72 (p=0.169) (see figures 5.5,5.6 and 5.7).

Results show that irradiation does not significantly increase immune cell infiltrate in *mdx* nude mice and that at the point of engraftment neither vessel density nor immune infiltrate is altered compared to control. There were no observed differences in CD31 or CD45 expression where engraftment does not work (0Gy and 25Gy) and when engraftment is successful (18Gy). Therefore, altered immune infiltrate, or changes within the vasculature, cannot account for irradiation induced improvement in donor cell engraftment within the *mdx* nude mouse model.
Figure 5.5: % of section area positive for CD45 or CD31 in irradiated and control mice. A. The % of TA cross section area positive for CD45 in mdx nude muscle irradiated with 18 or 25Gy irradiation and harvested immediately following or 3 days post irradiation. There were no significant differences in CD45+ areas between irradiation doses or at different time points. B. As in A. but TA sections were analysed for CD31 expression. Shows no difference in CD31+ area between conditions. Scale bar 20µm
Figure 5.6: 18Gy Irradiated mdx nude TA cross sections analysed for CD31 and CD45 expression A. Example images of control TA muscles from non irradiated mdx nude mice co stained with CD31 and CD45 shows the presence of some mononucleated cells and micro vessels. B. As in A. but TA cross sections are taken from mdx nude irradiated with 18Gy and harvested immediately following or 72 hours post radiation, shows little difference in the amount of CD31 or CD45 expression between conditions. Scale bar 50µm
Figure 5.7: 25Gy Irradiated mdx nude TA cross sections analysed for CD31 and CD45 expression

A. Control TAs from non irradiated mdx nude mice co stained with CD31 and CD45. TAs from non irradiated mdx nude mice B. As in A but TA cross sections are taken from mdx nude irradiated with 18Gy and harvested immediately following or 72 hours post radiation, shows a similar pattern of CD31 and CD45 expression between conditions. Scale bar 50µm
5.5 Discussion

In this chapter I have shown that best donor cell engraftment efficiency is achieved with a dose of 18Gy irradiation (see figure 5.2). At a dose of 25Gy, the irradiation mediated advantage for donor satellite cells is lost and engraftment efficiency falls to that observed at 4.5Gy (see figure 5.2). The observed dose response curve demonstrates that the loss of host satellite cells is not sufficient to explain the effects of radiation on donor satellite cell engraftment. Experiments show that donor cell engraftment cannot be improved by a reduction in skeletal muscle oxygen supply (see figure 5.4), and that HIF1 expression (see figure 5.3) or vessel density (see figure 5.5) do not change in response to irradiation. Furthermore results suggest that immune cell infiltrate is not increased in irradiated compared to control or 25Gy compared to 18Gy irradiated mdx nude muscles. In combination, these experiments convincingly demonstrate that oxygen availability is not the mechanism by which irradiation improves engraftment efficiency.

Satellite cell transplants into mdx nude TA muscles pre irradiated at different doses demonstrate that donor cells make the most contribution to host muscle regeneration at a dose of 18Gy. Conversely, donor cells produced the least amount of donor derived muscle at the lowest dose, 4.5Gy and the highest dose 25Gy. 3 days post radiation, 25Gy irradiated muscles have fewer satellite cells per fibre than 18Gy irradiated muscles (see figure 5.1). If the loss of host satellite cells were the mechanism by which irradiation improves engraftment, then it would therefore be expected that 25Gy would produce greater engraftment efficiencies than 18Gy. Clearly this is not the case (see figure 5.4). However it must be considered that although I have shown that 25Gy irradiation does not result in major changes to myofibre morphology (see figure 4.3) it is possible that it causes irreparable damage to cells outside of the myofibre that are critical for donor cell survival e.g. endothelial cells, blood cells and/or immune cells.
Irradiation has previously been shown to increase cell membrane permeability (Tidball & Wehling-Henricks, 2007). Some research has suggested that an increase in immune cell infiltrate within the myofibre improves resident satellite cell proliferation (Merly et al., 1999). An investigation of CD31 positive monocytes within irradiated mdx nude TAs demonstrated that cell infiltration did not increase in response to irradiation. It is therefore unlikely that irradiation improved engraftment is a secondary consequence of its effects either on the cell membrane or on immune cells.

Oxygen concentrations are known to be critical for the maintenance of the undifferentiated state within the stem cell niche (reviewed (Mohyeldin et al., 2010)) and for the continuance of cell proliferation within tumours (Das et al., 2008). Here I have investigated the role of oxygen concentration as a potential mediator of donor satellite cell engraftment efficiency. However, using three different strategies, there was no evidence to support the hypothesis that irradiation alters skeletal muscle oxygen tension.

In accordance with previous research (Stroka et al., 2001, Kubis et al., 2005), my results show that the HIF1α subunit is highly expressed in skeletal muscle fibre cytoplasm (see figure 5.3). This was also seen to be the case after 18Gy radiation (see figure 5.3). Under hypoxic conditions the HIF1α subunit translocates to the nucleus where it forms a DNA binding complex with HIF1β to affect transcription (reviewed (Semenza, 2000)) Interestingly it was observed that unlike myofibres, satellite cells show a nuclear location of HIF1 (see figure 5.3). This suggests that the satellite cell niche, as is the case for other stem cell niches (reviewed (Moore & Lemischka, 2006)), maintains an hypoxic environment. Given that cultured satellite cells, under conditions of low oxygen availability, maintain their undifferentiated state and their proliferative capacity (Majmundar et al., 2012, Liu et al., 2012b), the hypoxic environment of the satellite cell niche likely plays a critical role in the maintenance of satellite cell ‘stemness’.
Results show that 3 days post 18Gy irradiation, host satellite cells are still present within the satellite cell niche (see figure 5.1). If donor satellite cells are injected at this point, excellent engraftment is observed (see figure 5.2). At 25Gy irradiation, there is a significant reduction in the number of host satellite cells within the host satellite cell niche (see figure 5.1). If donor cells are injected at this point, engraftment is poor (see figure 5.2). In combination, these results present the intriguing possibility that perhaps via secreted factors, signaling pathways or the maintenance of the physical structure of the niche itself, the presence of host satellite cells at the point of engraftment is advantageous for donor cell survival and/or proliferation.

In other systems, it is known that for efficient donor cell engraftment competition must be eliminated by incapacitating host stem cell (see section 5.1). Results show that in this system, after 18Gy irradiation host satellite cells will still be present within the host myofibre at the point of engraftment (see figure 4.2). However, the majority of these cells, although present, are incapacitated, and will be lost after the activation of MyoD (see figure 4.1). Thus, after 18Gy, satellite cells are present but do not constitute competition for donor cells. After 4.5Gy, satellite cells are present but engraftment is poor. This may be because host satellite cells are able to out compete donor cells (see figure 4.1). After 25Gy, it is possible that engraftment is poor because there are no advantage-giving host cells.

This hypothesis is given strong support by results that show in this system, if 25Gy irradiated mdx nude mice are injected at T0 rather than T72 post radiation, i.e. when host satellite cells are present but incapacitated, engraftment is equally as efficient as satellite cells injected into 18Gy irradiated muscles (Boldrin et al., 2012). In summary, results support the conclusion that the presence of host satellite cells within injected myofibres improves satellite cell engraftment, but only if these cells are incapacitated such that they do not constitute competition for donor cells but are able to
preserve the niche (Boldrin et al., 2012).

Although high dose ionising irradiation is the most effective muscle pre-treatment for successful donor cell engraftment, due to the non specific nature of irradiation induced damage and the debilitating side effects to more radio sensitive tissues, irradiation cannot be considered as a viable therapeutic option. It is therefore necessary to understand the mechanism by which irradiation improves satellite cell engraftment in order to identify relevant targets for therapeutic intervention and improvements in engraftment efficiency. Results reported here suggest that altering oxygen tension, introducing ROS scavengers or intervening at the cellular response to hypoxia, would not benefit satellite cell engraftment. Instead, research must focus on unraveling the mechanisms by which an incapacitated host satellite cell is able to mediate donor cell engraftment.

*Figures 5.1 and 5.2 are published in: Boldrin, L., Neal, A., Zammit, P.S., Muntoni, F., Morgan, J.E. (2012) Donor Satellite Cell Engraftment is Significantly Augmented When the Host Niche is Preserved and Endogenous Satellite Cells are Incapacitated. Stem Cells. 1549-4918. (Figure 5)*
Chapter 6

Discussion

6.1 Thesis Summary

This thesis has investigated the mouse satellite cell in muscle growth, maintenance, ageing and regeneration. I hypothesised that there exist two distinct satellite cell subpopulations: one for muscle growth and one for muscle regeneration. I show that a sub population of satellite cells is able to survive high dose ionising radiation; these cells do not contribute to growth or maintain myonuclear numbers in non-injured muscle but can successfully engraft and produce donor derived fibres in a permissive environment in host muscle. Therefore, I suggest that radio resistant satellite cells are the rare satellite cell sub population that contributes to host muscle regeneration post transplantation. These regeneration competent satellite cells make the greatest contribution to host muscle regeneration in 18Gy irradiated \textit{mdx} nu/nu host muscle environments and this effect is not mediated by changes to cell infiltrate or oxygen concentrations within host muscle. I suggest that the regenerative donor satellite cell subpopulation requires signaling from resident host satellite cells in order to engraft efficiently.
6.2 The Satellite Cell for Muscle Growth and for Muscle Regeneration

In chapter 3, analysis of single fibres from male and female mice across the lifespan shows that satellite cell number is increased in male compared to female adult mice and in young compared to aged mice (figure 3.2). In contrast to myoblast transplantation (Montarras et al., 2005), satellite cell transplant experiments show that the number of satellite cells injected is not linearly related to the amount of donor derived fibres subsequently found (figures 3.9 and 3.10). I therefore suggest that there is a distinct satellite cell subpopulation that is present in comparable numbers across these populations of mice and that is responsible for donor derived host muscle regeneration post engraftment. This is supported in chapter 4 where I show that a subpopulation of satellite cells are resistant to 18Gy irradiation (figure 4.2). These radio resistant cells do not contribute to muscle growth or maintenance in situ (figure 4.4), but can regenerate host muscle after engraftment (figure 4.5). These findings highlight an important conceptual dissociation between muscle growth and muscle regeneration and strongly indicate that different satellite cell sub types are activated by these different processes. Furthermore, these results suggest that regenerated donor derived fibres derive from a rare satellite cell sub population.

In all satellite cell transplant experiments reported within this thesis, there are some injections that did not result in any donor derived muscle fibres. The engraftment efficiency reported here is similar to other papers that inject freshly isolated satellite cells derived from single fibres. For example, Boldrin et al. show between 25 and 30% of injections result in no donor derived fibres (Boldrin et al., 2009), whilst Ono et al. report 14% non engraftment (Ono et al., 2010). It is possible that due to the microenvironment in which they find themselves upon grafting satellite cells have
gone directly into quiescence without proliferating and thus no donor derived muscle is observed. However, it has previously been shown in this system that the number of donor derived fibres is related to the amount of self renewal, such that where one is observed so is the other (Boldrin et al., 2012). Therefore, this possibility is not considered probable. A more likely explanation is that non engraftment is due to the very small cell numbers injected per engraftment (e.g. 400 cells per muscle). If, as hypothesised, the number of cells that can contribute to regeneration is only a fraction of these, then it is possible that hosts with no donor derived muscle did not receive any effective cells.

Within this thesis, results converge to suggest that only a small percentage of injected satellite cells gives rise to all donor derived fibres. This is consistent with previous data investigating satellite cell engraftment. Individual EDL derived myofibres engrafted with their associated 7 myofibres show donor derived fibres in just 1 out of every 8 engraftments (Collins et al., 2005). Furthermore, transplantation of individual satellite cells show donor derived muscle in 4% of engraftments (Sacco et al., 2008).

By using radio labeled donor myoblasts, Beauchamp et al. 1999 demonstrate that the amount of radiolabel sharply declines immediately following injection, thus the majority of donor cells die upon engraftment. However, 4 weeks post engraftment, clusters of donor derived fibres were observed in host TA muscle. Due to the initial rapid loss of radio label, this suggests that donor derived fibres are produced by the proliferation of a rare few dividing cells (Beauchamp et al., 1999).

This is supported by evidence showing that, after inverse PCR analysis of retrovirally marked satellite cell derived myoblasts, the diversity of retroviral integration sites within a myoblast population is considerably reduced in donor derived muscle after transplantation (Cousins et al., 2004). This indicates that only a subset of the initial starting population were able to
engraft. Confusingly however, transplantation of individual clones shows that all clones were able to give rise to donor derived muscle (Cousins et al., 2004). This suggests that all cultured myoblasts are able to contribute to some donor derived muscle formation, although some contribute more than others (Cousins et al., 2004). This is supported by research that shows a linear relationship between the number of injected myoblasts and the subsequent amount of donor derived muscle obtained (Montarras et al., 2005).

However, it must be considered that these experiments can only draw conclusions about cultured myoblasts, not satellite cells. Satellite cells must be plated and cultured for at least one cell division in order to be labeled. As those cells that proliferate rapidly in vivo are known to divide slowly in vitro (Beauchamp et al., 1999), only a minority of plated cells are able to give rise to clones in vitro (Cousins et al., 2004) and as radio resistant cells proliferate in vivo (figure 4.5) but do not survive in vitro (figure 4.1), it is highly likely that a period in culture changes both population and individual satellite cell parameters. Thus, the oligoclonal nature of donor derived fibres after satellite cell transplantation remains to be expressly demonstrated by experimental investigation.

It might be postulated that the ideal way to directly demonstrate the origin of donor derived myonuclei would be to give each donor satellite cell a unique identifying mark. If all donor derived myonuclei found within the host also had the same identifying mark, then this would demonstrate that they were derived from a single cell. Importantly, to avoid in vitro modulation, it would be necessary to mark donor satellite cells prior to their isolation.

Such an experiment could perhaps be achieved by using the DNA damaging effects of radiation. In chapter 4 of this thesis I show that, after exposure to low doses of ionising radiation, both of my hypothesised satellite cell subpopulations - those that contribute to either maintenance or regen-
eration - remain viable (see sections 4.2 and 4.3). However, exposure to low dose ionising radiation causes chromosome aberrations (Bedford, 1991). As the location of DNA damage is random and the possible locations are enormously large, the probability of the same aberration occurring in two different satellite cells is extremely small. It follows then that, if donor mice were exposed to low dose ionising radiation prior to satellite cell isolation and engraftment, and donor derived myonuclei all contained the same chromosome aberration, then one could conclude that they were of clonal origin. Such an experimental method has been used to demonstrate the clonal nature of spleen nodules formed by bone marrow transplants: a seminal experiment demonstrating the stem cell nature of bone marrow derived cells (Becker et al., 1963).

Actually, such an experiment is impossible for the assessment of donor derived myonuclei, as cells must be in mitotic meta phase for aberrations to be identified. However, it may be used to demonstrate the clonal nature of donor derived satellite cells. The Myf5\textsuperscript{nLacZ} mouse expresses nuclear $\beta$gal under the Myf5 promotor and therefore if used as a donor provides a method of identifying donor derived satellite cells. Yet, there remain major practical hurdles: firstly, in order to identify chromosomal aberrations nuclei must be manually scored by microscopic examination. This would be an enormous task to undertake for donor derived satellite cells. Secondly, Becker et al. report that they frequently observe colonies with no identifiable aberrations (Becker et al., 1963). They conclude that this is due to experimental error (Becker et al., 1963). Microscopes have improved considerably since this original report, but still, there is certainly no guarantee that all donor satellite cells will incur an identifiable chromosomal aberration after exposure to low dose radiation. Although a mixture of chromosome aberration and non aberration containing donor derived satellite cells would be informative, a possible outcome is that no chromosomal aberrations are observed in donor
derived nuclei, in which eventuality no conclusions could be drawn.

Perhaps a more realistic approach to demonstrating the clonal nature of donor derived satellite cells would be to make use of the recently described Brainbow mouse (Livet et al., 2007). The Brainbow mouse uses Cre/lox-based transgenes to create mosaic patterns of cells expressing different fluorescent proteins within the same tissue. The Brainbow cassette randomly recombines fluorescent protein genes such that cells express one or more of the fluorescent proteins green, red, yellow or blue. The possibility of randomly expressing one or more of these fluorescent proteins generates cell populations with multiple distinct hues. These distinct hues have been used to observe dense networks of cells and elucidate their interactions within the nervous system (Livet et al., 2007). The original Brainbow mouse uses the neuronal specific Thy1 promoter, but a variation, the R26-confetti mouse, adds a neomycin roadblock which inhibits protein expression until cre is activated (Snippert et al., 2010). If cre is placed under a tissue specific promoter, then Brainbow can be expressed in any tissue. Importantly, once tamoxifen induced cre-mediated recombination has occurred and fluorescent proteins are expressed, the trans gene is stabilised. The same hue will therefore be passed to a cell’s daughter cell.

The R26-Confetti mouse crossed with the Pax7-Cre mouse (Lepper et al., 2009, 2011, McCarthy et al., 2011) may provide a system to identify the survival of satellite cell subpopulations post transplantation. If all donor derived myofibres were the same colour, they are likely all to be from the same cell. Indeed, a similar strategy has been used to show a clonal drift during the culture of intestinal crypt cells demonstrating that clonal patches are easily identified (Snippert et al., 2010).
6.3 Satellite Cells in Muscle Growth, Maintenance and Ageing

I have hypothesised that there exists a distinct satellite cell sub population for skeletal muscle regeneration and for muscle growth and maintenance (chapter 3). Regenerative satellite cells are defined as cells with the ability to contribute to muscle regeneration after severe injury, survive high dose ionising radiation and give rise to muscle after transplantation. However, a maintenance satellite cell is more difficult to specify. It is unclear precisely what is required of a satellite cell in order to maintain muscle mass and myonuclear number (i.e. how often must a myonucleus be replaced?). In addition, activity of the maintenance satellite cell pool is likely to change according to the demands placed on the muscle. In this thesis I have shown that, in the wild type mouse, a reduction in satellite cell number caused by high doses of ionising irradiation is associated with a loss of myonuclei and a reduction in myofibre volume (figure 3.7). In chapter 4, I demonstrate that wild type mouse TA and EDL muscles decrease in weight (figure 4.4) and myonuclei number (figure 4.2) 1 year after the majority of satellite cells are incapacitated (figures 4.1 and 4.2). Together, these data suggest that satellite cell activity is required throughout life for myofibre growth and maintenance even in non-exercised muscle.

25Gy ablates the satellite cell pool and these numbers do not recover even a year post radiation (figure 4.3). Contrary to some reports (Ferrari et al., 1998, Gussoni et al., 1999, Torrente et al., 2004, Sampaolesi et al., 2003, Dellavalle et al., 2007), these data suggest that satellite cells cannot be renewed by another stem cell source (see section 1.3). Of course, it must be considered that this is an unchallenged muscle and it is possible that other cell sources are able to contribute to the satellite cell pool, but only do so in a regenerative context. However, 25Gy irradiated muscles treated with
notexin showed little regeneration and no myogenic cells associated with the fibres (Heslop et al., 2000), suggesting that even during regeneration, satellite cells cannot be renewed from a circulating cell source. Furthermore, conditional ablation of Pax7 expressing cells convincingly demonstrates that skeletal muscle regeneration does not occur in the absence of Pax7 and that exercising satellite cell ablated muscle results in a severe muscle damage (Lepper et al., 2011). This supports the conclusions of this thesis that satellite cells cannot be renewed by another cell source and that satellite cell activity is required for routine muscle maintenance throughout life.

Although a degree of muscle atrophy is observed in irradiated mouse hind limbs, it is perhaps not as dramatic as one might expect given that 25Gy completely abolishes the satellite cell population (figure 4.2). 25Gy irradiated mouse hindlimbs were still mobile even a year post radiation, with no obvious gait deficit and no morphological changes in muscle cross sections (figure 4.3). This is in contrast to findings in the mdx mouse which show that exposure to 16 or 18Gy irradiation results in the progressive loss of muscle fibres and their replacement with connective tissue and the worsening of the mdx muscle phenotype (Wakeford et al., 1991, Pagel & Partridge, 1999). The absence of centrally nucleated fibres in irradiated mdx mice suggests that irradiation completely ablates muscle regeneration (Wakeford et al., 1991). If single fibres are isolated from 18Gy irradiated and notexin treated mdx and wild type muscles, functional colony forming satellite cells are observed from wild type, but not mdx, fibres (Heslop et al., 2000). Therefore the effects of irradiation observed in the mdx mouse are likely due to a failure of regeneration in response to the pathological degeneration, due to the exhaustion of a radio resistant satellite cell subpopulation. It must be considered that the mice studied here are sedentary animals with unchallenged muscle.

Throughout this thesis no significant differences in donor derived fibres
were observed across a range of injected satellite cell numbers (figures 3.9 and 3.10), these results suggest that the number of donor cells able to contribute to regeneration is a constant. If this is assumed, then the changes in satellite cell number observed between male and female, young and aged muscle (figure 3.2) demonstrate that the number of cells that do not contribute to donor derived muscle formation fluctuates.

Studies of MRF expression on single fibres isolated from aged and young mouse muscle suggest that the reduction in satellite cell number observed on aged fibres is due to a reduced capacity for self renewal (see figure 3.4). This is supported by other single fibre (Collins et al., 2007) and clonal culture studies (Day et al., 2010). The hypothesis of distinct satellite cell populations for muscle maintenance and muscle regeneration explains these findings in the context of other data showing no impairment in satellite cells from aged donors when transplanted to young mdx nude hosts (Collins et al., 2007). I suggest that satellite cells for muscle maintenance are lost with increasing age, yet regeneration competent satellite cells remain in comparable numbers in young adult and aged muscle.

The reduction in overall satellite cell numbers with increasing age (figure 3.2) suggests that muscle maintenance satellite cells have a finite number of cell divisions. It is likely that regeneration satellite cells, as with their more numerous counterparts, maintenance satellite cells, also have a limiting mitotic clock. This is supported by the observation that mdx mice have less radio resistant (regeneration competent) satellite cells compared to wild type muscle (Heslop et al., 2000). As the mdx mouse undergoes many cycles of degeneration, the regeneration competent (radio resistant) satellite cell may well be exhausted in mdx muscle. The existence of distinct satellite cell populations may explain why patient derived myoblasts from 80 year old donors are able to undergo a similar number of divisions as myoblasts from 20 year old donors (Renault et al., 2000) despite the fact that muscle
atrophy is a defining feature of the ageing phenotype. Muscle atrophy in vivo may be due to a reduced number of muscle maintenance satellite cells, but regeneration competent cells will be activated by isolation and are still mitotically young.

If as I have suggested, satellite cells have a finite number of cell divisions, they are required to maintain myonuclei number and myonuclei are required to maintain cytoplasmic volume, it follows that, if exercise required satellite cell activation, then exercised muscle would undergo a greater degree of muscle atrophy than non exercised muscle in old age. So, in terms of muscle atrophy in old age, is exercise that increases muscle mass throughout life bad for you? Of course, this is not necessarily the case. It is highly probable that increases in myofibre volume in response to exercise work via different mechanisms to those of growth and may occur without myonuclei addition. Myofibre volume increase is known to occur in the absence of satellite cell activity in myostatin inhibitors (Amthor et al., 2009), the GF1/AKT signalling pathway (Schiaffino & Mammucari, 2011, Blaauw et al., 2009) and after synergist ablation in satellite cell ablated muscle (McCarthy et al., 2011).

It must be borne in mind that results in this thesis represent a sedentary mouse model and the relationships between satellite cell activity, exercise and muscle ageing are likely to be complex.

6.4 Satellite Cell Heterogeneity. Towards a Unifying Hypotheses

It is well established that satellite cells are a heterogeneous population. Much research is aimed at uncovering the mechanism(s) that give(s) rise to satellite cell heterogeneity and bio markers that could be indicative of a particular satellite cells proliferative/self renewal or engraftment capacity
How the functional subpopulations delineated in this thesis relate to the heterogeneity detailed elsewhere is an important and exciting question.

A popular current hypothesis within the skeletal muscle stem cell field maintains that the satellite cell population is a hierarchy of cell subtypes at the top of which sits a distinct satellite cell that gives rise to all the other satellite cell subtypes: a stem satellite cell (reviewed (Partridge, 2003)) (see section 1.8). Many studies have proposed that this stem satellite cell can be identified by the expression or exclusion of various markers e.g. Myf5, CD34, M-Cadherin, CXCR4 (Beauchamp et al., 2000, Kuang et al., 2007, Cerletti et al., 2008), however results have been inconsistent and frequently contradictory (Kuang et al., 2007, Kanisicak et al., 2009) (see section 1.8). The most convincing evidence for a stem satellite cell population comes from the observation that only a small percentage of cells survive to engraft successfully post transplantation (Beauchamp et al., 1999, Collins et al., 2005, Sacco et al., 2008). However these experiments do not answer whether this phenomenon is due to true heterogeneity or due to a transient cell state, an artifact of cell preparation or to factors within the host environment.

In this thesis I have hypothesised that there exists a distinct satellite cell sub population for growth and for muscle regeneration. I have separated regeneration competent satellite cells from growth satellite cells using ionising radiation. However, a critical question remains how do the ‘regenerative’ satellite cells relate to the ‘maintenance’ satellite cells? Does one give rise to the other? and is the regeneration competent cell the same stem satellite cell that has been hypothesised elsewhere? By exposing growing wild type muscle to high dose ionising radiation, I show that the majority of satellite cells are lost by 6 months post radiation (figure 4.2) and those that remain are regeneration competent cells (figure 4.5). Importantly however, in situ, satellite cell numbers were not recovered even 1 year post radiation (see fig-
ure 4.2) and the muscle was left to atrophy (see figure 4.4). This strongly suggests that in the absence of signals from the regenerating muscle, regeneration competent satellite cells do not give rise to the more numerous maintenance satellite cells. Furthermore, the observation that a sub population of satellite cells are quiescent as early as 2 weeks of age (figure 3.5) together with previous studies showing that regeneration competent cells survive into old age whilst maintenance satellite cells are lost (Collins et al., 2007) suggests that these sub populations do not relate to one another at all, but rather are separate populations from birth to death.

However, previous studies have shown that post engraftment satellite cells undergo extensive self renewal to re-populate the host stem cell niche. These satellite cells are able to contribute to host muscle upon subsequent rounds of regeneration (Collins et al., 2005). The extensive self renewal of donor cells upon engraftment (Collins et al., 2007, Boldrin et al., 2009, 2012) supports a model in which the satellite cell subpopulation that survives, i.e. the constant regeneration competent sub population, is able to give rise to a numerous functional satellite cell population. It is likely that the relationship between the regeneration and the maintenance satellite cell populations is changed upon exposure to a regenerating muscle environment.

It is highly probable that the regeneration competent satellite cells described here overlap with other satellite cell sub populations described elsewhere. For example, a rare satellite cell sub population has been described as undergoing asymmetric strand segregation such that the cell that remains as a stem cell retains the template DNA strand, whilst newly synthesised strands are passed to the daughter cell that adopts a more differentiated state (Shinin et al., 2006, Conboy et al., 2007). This may relate to subpopulations delineated here in a number of ways. Perhaps regeneration competent satellite cells undergo asymmetric strand segregation and thereby retain their extensive proliferative capacity. Perhaps muscle maintenance satellite
cells undergo template strand segregation as they more regularly enter the cell cycle and therefore need to be protected from mutagenesis. Or perhaps, if these cell sub population are entirely separate from birth, a sub population of each sub population undergoes template strand segregation. Clearly experiments reported in this thesis cannot answer such questions, however they represent interesting avenues of investigation for future research.

6.5 Isolating Satellite Cell Subpopulations

In this thesis I propose that satellite cell sub populations for regeneration and maintenance can be separated by ionising radiation (chapter 4). Satellite cells can be identified according to their small size and granularity (Montarras et al., 2005), and some evidence suggests that the smallest most non granular satellite cells make the most muscle upon engraftment compared to larger satellite cells (Collins et al., 2007). A small cytoplasmic volume and tightly packed chromatin is likely to give considerable protection against damaging radiation (see section 4.5). It is likely that the radio-resistant satellite cells reported in this thesis are smaller than the average satellite cell size. This could be tested experimentally by measuring the volume of satellite cells isolated from muscle 6 months post radiation (figure 4.2). However, as radio resistant satellite cells are rare, a large number of fibres would be required in order to obtain significance. Importantly, obtaining a control would also be difficult, as of course, non irradiated fibres contain both radio resistant and radio sensitive satellite cell populations. As evidenced in the microarray data in chapter 5, the cell mixture within the control population adds noise to statistical methods making significance difficult to obtain.

To overcome similar problems, epidemiology research has developed statistical methods whereby the mean of ‘part’ is subtracted from the mean of the ‘whole’ prior to statistical testing (Hayes & Berry, 2006). This may be useful in future analysis of irradiated and non irradiated satellite cells.
I suggest that radio sensitive and radio resistant satellite cells have unique transcripts at the RNA level (section 4.5.1). Disappointingly, experiments failed to identify any RNA transcripts differentially expressed between irradiated and non irradiated satellite cells. I propose that rather than there being no differences, this is due to a failure to identify these differences due to the difficulty of identifying and separating these populations. The rarity of the radio resistant satellite cell, combined with the single fibre method of isolation, means that sufficient concentrations of RNA could not be isolated. Enzymatic disaggregation has been used as an alternative to the single fibre method of satellite cell isolation (Bischoff, 1975, Blau & Webster, 1981, Yablonka-Reuveni et al., 1987), however, cell yield is still not sufficient for microarray analysis and as muscle tissue also contains a variety of other cell types, vessels, fibroblasts, nerves, fat etc. the purity of the cell preparation is significantly reduced compared to single fibre methods.

The difference between radio resistant and radio sensitive satellite cells needs to be elucidated in transcriptional as well as functional terms in order for understanding to progress. Perhaps microarray experiments would be fruitful if the starting populations were of greater purity. In the microarray experiment in chapter 4, satellite cells were isolated 4 weeks post radiation. At this time point there would still be some radio sensitive satellite cells present within the satellite cell pool (figure 4.2) as they had not undergone activation since radiation (figure 4.1). Results show that 6 months post radiation only radio resistant satellite cells remain (figure 4.2). Perhaps if satellite cells were harvested from muscle irradiated 6 months previously, the satellite cell population would be purer and differences in RNA therefore more easily identified with ANOVA.

However, this does not address the biggest obstacle to microarray techniques for radio resistant satellite cell analysis: isolating a sufficient concentration of RNA. As the cell population is so rare, samples require pooling
and RNA requires amplification (see section 2.7.2). The slight variations between cells from different mice, the random nature of the amplification process and the bias of DNA polymerase (reviewed (Nygaard & Hovig, 2006)) contribute considerable noise into the data. Perhaps transcript analysis of radio resistant satellite cells will not be possible until single cell RNA analysis becomes more accessible and/or microarray techniques become more sensitive and allow for the analysis of lower concentrations of RNA.

Identifying RNA transcripts expressed specifically in radio resistant or radio sensitive satellite cells would open the door to further charaterisation of this cell sub type. However, still the question would remain: does this pertain to heterogeneity or plasticity? Because, at a given time, a population can be separated based on its expression of a given gene, it does not necessarily follow that that cell will always express that gene. The experiments reported in this thesis represent a series of snapshots throughout the life of the mouse. Much can be extracted from the data that they provide, but there is nothing herein to demonstrate that the same quiescent cell observed at two weeks is the same population still present in old age. Functionally it is the same, but it may be a different cell expressing that phenotype. Therefore, what I have delineated as regenerative cells and maintenance cells, may actually represent different cell states that every satellite cell may or may not enter and exit throughout its lifespan.

Microarray analysis of isolated satellite cells suggests that after the single fibre method of satellite cell isolation the majority of satellite cells are activated (table 4.4). It is possible that engraftment survival is conferred by a cell’s position in the cell cycle. In the haematopoietic system, self renewal has been related to a stem cells quiescence at the time of isolation: stem cells with low amounts of cytoplasmic ROS were shown to be a more quiescent stem cell population than a ROS-high stem cell fraction and this quiescent population showed a higher amount of self renewal after transplantation and
Radio resistance too, is likely related to quiescence. Cells visibly expand when they express MyoD (compare figure 3.2 to figure 3.3) and exit their niche. A tightly packed chromatin, small cytoplasm and location in a low oxygenic niche can reasonably be assumed to confer considerable radio resistance and transplantation survival.

However, if transplant survival is conferred by cell cycle position, this would not in itself be indicative of plasticity as opposed to heterogeneity. I would suggest that the point in the cell cycle a particular satellite cell finds itself in upon engraftment is not a stochastic process, but is the consequence of intrinsic heterogeneity. Data reported here show that a subset of satellite cells are quiescent at 2 weeks of age (figure 3.5) and therefore are not contributing to the growth programme. Previous research has demonstrated that those myoblasts that divide slowly in culture are the cells that proliferate rapidly upon transplantation (Beauchamp et al., 1999) and that slowly dividing cells retain self renewal capacity (Ono et al., 2012). This suggests that those cells that are able to survive transplantation have internally specified cell cycle kinetics that differ from cells that do not survive.

The amount of cell division a cell undergoes can be measured using an analogue of thymidine: 5-Ethynyl-2-deoxyuridine (EdU). EdU is able to substitute for thymidine in DNA and therefore becomes incorporated into a cells DNA upon cell division. Incorporated EdU is detected with a fluorescent azide which is preferable to the more common 5-bromo-2’-deoxyuridine (BrdU) as it does not require heat or acid treatment and is therefore more easily used in conjunction with immunohistochemistry (reviewed (Salic & Mitchison, 2008)). Upon subsequent cell divisions, EdU will be divided equally between each daughter cell and thus with each cell division the label becomes diluted until it is undetectable (reviewed (Yan et al., 2007)). If, as I suggest, regeneration competent satellite cells are present in quiescence from
birth until old age, then these cells should not lose EdU label. Therefore a conceivable experimental strategy would be to administer EdU in embryogenesis and analyse skeletal muscles for the presence of label retaining cells at time points throughout life. In such an experiment the timing of EdU administration would be of critical importance, if it were administered too early, before quiescent satellite cells were specified, then extensive proliferation would dilute the label and, in adulthood, no label retaining cells would be found. On the other hand, if the EdU were administered too late, then cells would already be specified i.e. in quiescence, and would therefore not incorporate the label.

Satellite cells are first specified in the somitic myotome when proliferating cells migrate from the somitic dermomyotome and become trapped by the formation of the primitive basal lamina (Relaix et al., 2005, Gros et al., 2005) (see section 1.5). This process occurs at different times in different somites and it is unknown if or when a subset of these cells stop proliferating. Therefore, perhaps injecting EdU every day between E14 and E20 would ensure that all satellite cells were labelled at the right time. However, the cost of the required amount of EdU soon renders such an experiment highly impractical. Nevertheless, I would hypothesise that label retaining satellite cells would be identifiable throughout life, but lost if they were exposed to a regenerating muscle environment (therefore absent in the mdx mouse).

6.6 Permissive Environment for Grafting

In chapter 5 of this thesis, I investigate the role of the host muscle environment in mediating donor satellite cell engraftment. Previous research has shown that irradiated host muscle produces more donor derived myofibres from grafted myoblasts (Morgan et al., 2002) and satellite cells (Boldrin et al., 2012) than non irradiated host muscle and that irradiation is signif-
icantly more effective than any other muscle pre treatment for subsequent
donor derived fibres from satellite cell transplants (Boldrin et al., 2012).
In this thesis I show that donor satellite cell engraftment improves as pre-
irradiation dose increases up to the optimal does of 18Gy (see figure 5.2).
However, 3 days after 25Gy irradiation, the radiation mediated improve-
ment in donor satellite cell engraftment efficiency is lost and no engraftment
is observed (see figure 5.2). To investigate potential mediators of irradiation
induced improvement in donor cell engraftment I compare the host oxy-
gen tension and cell infiltrate between 18Gy and 25Gy host pre-irradiation
doses. Results show that radiation does not mediate donor cell engraftment
via these intermediaries. I suggest that the effect of host muscle pre irradi-
atation is mediated by the presence of non functional irradiated host satellite
cells (figure 5.1 and section 5.5). However, the mechanism by which pre
irradiation improves engraftment efficiency remains unclear.

Exposure of a cell to ionising radiation does not only have consequences
for that cell itself, but also for the cells that it subsequently has direct
or indirect interactions with (bystander effects). Exposure of non irradi-
ated cells to irradiated cell populations result in expression of stress related
genes, malignant transformation, formation of micronuclei and increased cell
mortality (reviewed (Azzam et al., 2004)) in the non irradiated population.
People who have been accidentally exposed to ionising radiation in the 1986
Chrenobyl disaster or A-bomb survivors have been shown to carry factors
within their blood plasma capable of causing chromosomal damage (elas-
togenic factors) to other cells more than 10 years after radiation exposure
(Emerit et al., 1994, Pant & Kamada, 1977, Emerit et al., 1997). Amazingly,
co-cultures of irradiated and non irradiated cells show that the irradiated
cells can induce double strand breaks in non irradiated cells (Sokolov et al.,
2005). Even a single cell traversed with 1 helium ion has been shown to in-
duce an increase in micronuclei formation in surrounding cells (Shao et al.,
Not all bystander effects are necessarily cytotoxic or detrimental. Previous research suggests that pre-irradiation of host graft sites has a pro-proliferative effect on donor cells. Tumour formation from donor mammary epithelial cells is increased in pre-irradiated compared to non-irradiated host mammary glands (Barcellos-Hoff & Ravani, 2000). Survival, migration and proliferation are improved in rat spinal cord grafts by pre-irradiation (Franklin et al., 1996). In the rat liver, it has been shown that these pro-proliferative effects can occur at doses as low as 0.5Gy radiation (Gerashchenko & Howell, 2003b). Interestingly, it is not just the irradiation of the graft site that confers this pro-proliferative response; exposure of non-irradiated cells to irradiated cells in a co-culture has been shown to induce the proliferation of the non-irradiated cells in human lung fibroblasts upon their subsequent engraftment (Iyer et al., 2000). Furthermore, transplantation of mouse tumor cells show increased tumor size in hosts engrafted with donor cells that had been co cultured with irradiated cells compared to non-irradiated donor cells (Revesz, 1956, 1958).

It is possible that the irradiation bystander effect is due to an excreted factor, specifically transforming growth factor beta 1 has been shown to increase in irradiated cell supernatant (Iyer et al., 2000). However, there is also research to suggest that direct cell-cell contact is necessary for the bystander effect on proliferation. Co-culturing irradiated and non-irradiated cells separated by a porous low-protein binding membrane prevents the pro-proliferative bystander effects in rat spleen cells (Gerashchenko & Howell, 2003a) and human keratinocytes (Mothersill & Seymour, 1998). It is possible that the mechanism by which irradiated cells mediate proliferation in non-irradiated cells is cell type specific.

In this thesis I have shown that the 18Gy irradiated host muscle is the optimal environment for donor cell engraftment (figure 5.2). It is possible
that the irradiated host environment effects host cells via secreted factors either by the muscle itself, or by neighboring cells. However, a cytokine array on this system showed no cytokine expression consistently differentially regulated that could obviously affect satellite cell proliferation (Boldrin et al., 2012). The growth factors FGF and Hepatocyte growth factor (HGF) have been shown to increase proliferation and depress differentiation of both myoblasts and satellite cells (Bischoff, 1997, El Fahime et al., 2000, Kstner et al., 2000, Allen et al., 1984, Sheehan & Allen, 1999). However, immunoblotting analysis showed no change in the expression of FGF, FGF-4, FGF-6 or HGF between irradiated and non irradiated muscle (Morgan et al., 2002). Matrix Metalloproteinase (MMP) are a family of structurally related proteinases associated with the extra cellular matrix involved in matrix degradation, receptor cleavage, initiation of apoptosis and chemokine/cytokine mediation (reviewed (Page-McCaw et al., 2007)). An increase in expression of MMPs in skeletal muscle is associated with an increase in satellite cell proliferation and migration (El Fahime et al., 2000). Investigation has shown that expression of MMP-2 or MMP-9 is not altered between irradiated and non irradiated muscle (Morgan et al., 2002), suggesting that, as with growth factors, these are not crucial mediators of radiation induced improvement in donor satellite cell engraftment efficiency.

Experiments reported here suggests that incapacitated, but present, host satellite cells are necessary for successful donor satellite cell engraftment (section 5.5). It is possible that physical cell-cell interaction of donor cells with irradiated host satellite cells effects host satellite cell proliferation. Such a mechanism would require the establishment of gap junctions between satellite cells (Gerashchenko & Howell, 2003a). However, satellite cells in host 18Gy irradiated mdx nude muscle are at a frequency of just 2 satellite cells per fibre (figure 5.1), therefore it is improbable that an injected donor cell would be able to establish direct contact with a host satellite cell. It seems
unlikely that the dramatic pro proliferative effect of pre irradiation is induced by the small probability of direct donor-host (irradiated-non irradiated) cell interaction.

It is possible that, in this system, the observed pro proliferative effect of irradiated host muscle on non irradiated donor cells is micro-RNA mediated. Micro-RNAs are small (typically just 21-25 nucleotides) non coding RNAs that can cleave complementary sequences of gene coding RNA and induce translational repression or post transcriptional gene silencing (reviewed (He & Hannon, 2004)). Micro-RNAs are critical in development and in embryonic stem cell survival (Murchison et al., 2005, Wang et al., 2007), have been observed in various cancers (Volinia et al., 2006) (reviewed Wiemer (2007)) and are associated with muscular dystrophies (Eisenberg et al., 2007). Changes in Micro-RNA expression have been observed after exposure to radiation in cultured rat fibroblasts (Maes et al., 2008), in mouse embryonic stem cells (Ishii & Saito, 2006) and in various cancer cells (Josson et al., 2008, Chaudhry et al., 2010) (reviewed (Esquela-Kerscher & Slack, 2006)). Furthermore, Micro-RNAs have also been associated with irradiation induced bystander effects (reviewed (Kovalchuk & Baulch, 2008).

As a cytokine array (Boldrin et al., 2012), investigation of immune infiltrate (figure 5.5) and oxygen tension (figures 5.3 and 5.4) suggest that these are not mediators of irradiation induced improvement in donor satellite cell proliferation, changes in Micro-RNA levels may be a good next target for investigation. Micro-RNAs are known to be important in skeletal muscle development (reviewed (van Rooij et al., 2008)). MiR-133 promotes proliferation in cultured myoblast and xenopus laevis embryos (Chen et al., 2006). MiR-1 and MiR-206 have been shown to negatively regulate Pax7 expression and therefore satellite cell proliferation. In vivo, knockdown of MiR-1 and MiR-206 in new born mice enhances satellite cell proliferation and increases Pax7 expression (Chen et al., 2010). It is possible that irradiation induced
changes in Micro-RNA secretion from host satellite cells may have a pro
proliferative effect on donor cells.

Such an hypothesis could be easily addressed by experimental investi-
gation in vitro. Micro-RNA expression can be assessed in vitro from cell
supernatant of irradiated satellite cells or in vivo from blood serum of ir-
radiated mice and assessed with qPCR. Given results in chapter 4 of this
thesis, MicroRNAs present after 18Gy but not present 3 days after 25Gy
irradiation would be good candidates for mediators of donor satellite cell
proliferation.

6.7 Conclusions and Future Work

For efficient satellite cell engraftment it is necessary both to find the best
cell and give this cell the optimal environment. This thesis has aimed to elu-
cidate these factors through a detailed study of the satellite cell population
and its radio resistance, heterogeneity with age, heterogeneity between sexes
and its performance in irradiated and devascularised muscle environments.
Results suggest that the majority of satellite cells do not engraft successfully
and that donor derived muscle post transplantation derives from a rare satel-
lite cell subpopulation. This is in accordance with much previous research
(Beauchamp et al., 1999, Collins et al., 2005, Sacco et al., 2008).

In order for satellite cell transplantation to become a reality, it is nec-
essary to understand this rare sub population of cells such that we might
specifically select for them and ideally determine methods that allow for
their in vitro expansion. It is unlikely that even in the optimal environ-
ment the small fraction of the satellite cell population that is able to survive
transplantation would be able to give rise to enough donor derived muscle
for real therapeutic gain, without prior expansion. Research must therefore
aim towards methods of separating regeneration competent from mainte-
nance satellite cells such that in vitro expansion does not select for mainte-
nance cells (which divide more quickly in culture (Beauchamp et al., 1999, Ono et al., 2012)) and define culture conditions that do not impede their subsequent in vivo proliferation.

Exposure of muscle to high dose ionising radiation is necessary for satellite cell engraftment in the mouse (Boldrin et al., 2012) and radiation is most effective at the dose of 18Gy (figure 5.2). In the human, exposure of even a single muscle to such a high dose of radiation is not therapeutically viable. As well as directly causing widespread cell death of dividing cells, irradiation affects cells that are not directly targeted, many years after exposure e.g. blood vessels, nerves, bones etc. These effects can cause chromosomal damage, loss of tissue function and malignant transformations. Clearly an alternative muscle pre-treatment must be sought.

These experiments have investigated immune infiltrate and oxygen tension, as it is conceivable that they may be therapeutically regulated (e.g. by ROS scavengers or steroids). However, results suggest that such interventions would not improve satellite cell engraftment. This thesis presents a straightforward experimental method for the investigation of potential mediators of donor cell proliferation. As 3 days after irradiation, 18Gy is the optimal environment and 25Gy the most obstructive, experiments need only look for factors that are different between these two muscle environments. Future research must now use our detailed knowledge of the effects of radiation on donor satellite cell engraftment efficiencies to elucidate therapeutically relevant factors that can improve satellite cell proliferation post engraftment.
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