Differentiation of multipotent skin derived precursor cells into skeletal muscle

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TABLE OF CONTENTS

Abstract

The potential use of stem cells for therapy depends on our ability to optimize differentiation conditions to best take advantage of their multipotentiality. Skin derived precursor cells (SKPs) are recently described adult stem cells that have been shown to differentiate into both neuronal and mesodermal cell types *in vitro*. We asked specifically whether they could be induced to differentiate into skeletal muscle. An accurate assay for detecting skeletal muscle was first developed, using RT-PCR, immunocytochemistry and Western blotting. We then used traditional differentiation methods, and found that SKPs could differentiate into cells expressing an immature skeletal muscle phenotype, as determined by appropriate morphology and expression of various skeletal muscle markers. By modifying this protocol, we observed a small percentage of multinucleated, fused myotubes, suggesting that SKPs have the potential to differentiate into mature skeletal muscle given appropriate conditions. Future work could enhance this population, creating an accessible, new source of cells for autologous transplants.

Résumé

Le potentiel thérapeutique des cellules souches repose sur notre capacité à optimiser les conditions de différenciation pour mieux exploiter leur multipotence. Récemment décrites, les cellules précurseurs dérivées de la peau (*Skin derived Precursor cells*, SKPs) sont des cellules souches présentes chez l'adulte dont la capacité de se différencier en types cellulaires neuronal et mésodermal a été démontré *in vitro*. Nous avons spécifiquement étudié leur potentiel de différenciation en muscle squelettique. Dans un premier temps, nous avons développé une méthode précise pour détecter la formation de muscle squelettique en combinant l'utilisation de RT-PCR, immunocytochimie, et buvardage Western. En second lieu, nous avons utilisé des méthodes traditionnelles de différenciation et trouvé que les SKPs peuvent se différencier en cellules exprimant un phénotype de muscle squelettique immature, démontré par une morphologie appropriée et par l'expression d'une variété de marqueurs de muscle squelettique. En modifiant ce protocole, nous avons observé un petit pourcentage de myotubes multinucléés et fusionnés, ce qui suggère que les SKPs possèdent le potentiel de se différencier en muscle squelettique mature en présence de conditions adéquates. La poursuite de ces études pourrait conduire à l'expansion de cette population créant une source nouvelle et accessible de muscle squelettique pour des transplantations autologues.

Chapter 1: Background Information

A. Stem cells

The recent explosion of stem cell research is largely due to the increasing awareness of its potential uses in therapeutic settings. Stem cells could be used for transplantation in degenerative diseases such as Parkinson's disease, multiple sclerosis and muscular dystrophy, or by using genetically modified stem cells as carriers for gene therapy. Stem cells have been identified in a large number of tissues in both embryonic and adult rodents and primates. The basic definition of a stem cell is an undifferentiated cell capable of self-renewal over the lifetime of its host, while maintaining the ability to differentiate into a variety of novel cell types *in vitro* (van der Kooy & Weiss, 2000). While many kinds of stem cells have been discovered, they differ with regards to their capacity to differentiate. Some can only repopulate the cells residing in the tissue from whence they came, while others can be differentiated into cell types from all three embryonic lineages (ectoderm, mesoderm and endoderm). Due to their multipotentiality, the differentiation of stem cells will generally result in a mixed population of progeny.

I. Introduction

To date, the stem cell with the broadest capacity to differentiate into functional cell types is the embryonic stem cell (ES cell). ES cells can be isolated from the inner cell mass of the preimplantation blastocyst stage of the mouse or human embryo, though cells derived from the mouse are much better classified in terms of growth and differentiation requirements than human ES cells. Mouse ES cells are maintained in an undifferentiated state in culture by the presence of leukemia inhibitory factor (LIF), while human ES cells

appear to be LIF independent (Passier & Mummery, 2003). Upon LIF withdrawal, ES cells spontaneously differentiate into a heterogeneous mixture of cells such as neurons (Bain *et al.,* 1995, Deacon *et al.,* 1998), blood (Daley, 2003), pancreatic-like cells (Lumelsky *et al.,* 2001) as well as skeletal, cardiac and smooth muscle (Myer *et al.,* 2001, Passier & Mummery, 2003). An important aspect of the *in vitro* differentiation of ES cells is that they appear to follow the same pattern of gene expression observed in the developing embryo. Undifferentiated ES cells have also been shown to form chimeras following injection into a blastocyst. These chimeras have donor cells contributing to all three primary germ layers (Andrews, 2002). While ES cells are fully totipotent and can be differentiated into any cell type of the adult organism, there are a number of issues preventing their use in therapeutic settings. One is the fear of teratomas (tumors arising from embryonic cells that do not stop dividing), which may occur once ES cells are transplanted into an adult host (Andrews, 2002). The other deterrent is concerned with the ethics surrounding the production and use of human ES cells, which has lead to an increased focus on adult derived stem cells. Even if these concerns were dealt with, the general problems with heterologous transplants and the need for immunosuppressants would still exist.

As development progresses, embryonic stem cells become more restricted with respect to their differentiation potential. First, they become committed to an ectodermal, mesodermal or endodermal lineage where they remain pluripotent and can become any cell type of that germ layer. They are then restricted further, to a multipotent, tissue-specific stem cell that can repopulate all cell types of that tissue. The most restricted are immature, precursor cells that are committed to becoming a single, specified cell type.

For example, neuronal precursors can become neurons, but not astrocytes or oligodendrocytes. In the adult, small populations of multipotent stem cells persist in a variety of tissues where they are usually quiescent and function in tissue specific cell turnover and repair (van der Kooy & Weiss, 2000). Adult stem cells have been found in the brain, bone marrow, liver, skin, and muscle. These stem cells can either divide symmetrically to increase the size of their population, or asymmetrically to maintain that population, while producing a more restricted progenitor that participates in regeneration and repair of the host tissue. When adult stem cells are isolated and cultured, they have been shown to be multipotent *in vitro*, and *in vivo* following transplants. This multipotentiality is referred to as plasticity, the ability of a cell from one lineage to make various novel cell types from another lineage (Verfaillie, 2002).

Transplantation studies have shown that hematopoietic cells isolated from the bone marrow could repopulate the hematopoietic system, as expected from an endogenous stem cell. In addition, labeled hematopoietic cells were also found in skeletal muscle, liver and the brain suggesting plasticity (Verfaillie, 2002). Plasticity has also been demonstrated *in vitro* with neural stem cells which, when co-cultured with a muscle derived cell line, were able to form mature contractile muscle in culture (Rietze *et al.*, 2001, Galli *et al.*, 2000). Recently, this apparent plasticity has been proved to be due to a phenomenon known as fusion. Fusion refers to the combining of a host and donor cell so that the host is able to reprogram the donor nucleus producing a novel tetraploid cell expressing the phenotype of the host, but genetic markers of both the host and donor simultaneously (Wang *et al.*, 2003). *In vitro* this was shown with neural stem cells, or bone marrow derived stem cells co-cultured with embryonic stem cells. The adult cells

fused with the ES cells taking on their immature characteristics and differentiation capacity (Ying *et al.,* 2002, Terada *et al.,* 2002). Upon closer examination these cells were seen to express multiple markers of both the host and donor. *In vivo*, the hematopoietic stem cells found in brain and liver following repopulation of the blood system, have been shown to be the result of fusion, as the novel cells expressed both the XY chromosomes from the male donor, and the XX chromosomes from the female host (Wang *et al.,* 2003, Weimann *et al.,* 2003). These data stress the importance of using separate markers of both host and donor cells to conclusively prove results *in vivo*, as well as using isolated culture systems that do not require co-culture with foreign cells for differentiation to occur.

Though the extent of adult stem cell plasticity is still under investigation, the benefits of adult stem cells over ES cells for therapy include a reduction in their proliferative capacity, thereby reducing the risk of tumor formation, the ability to obtain informed consent from a donor and the potential for genetically modifying the cells and using them in autologous transplants. Stem cells isolated from the dermal layer of the skin (SKPs) are novel multipotent stem cells described by Toma *et al.,* (2001). They have the additional benefit over many other adult derived stem cells of being expandable in culture, so that a large number of cells could be obtained from a small piece of skin, which is an easily accessed tissue as compared to brain, bone marrow or muscle.

II. Differentiation potential of SKPs

Skin derived precursor cells (SKPs) are recently described stem cells that appear to be multipotent, though the extent of their potential is yet to be determined (Toma *et al.,*

2001). To date, clonally derived SKPs have been shown to differentiate into neurons, glia, smooth muscle and adipocytes *in vitro*. Other cell types such as osteoblasts and chondrocytes have been observed, but have not yet been reported in clonally derived cultures (K. Fernandes, personal communication). SKPs are isolated from the dermal layer of embryonic or postnatal rodent skin and grow in culture as floating spheres that can be passaged indefinitely. These spheres express nestin, fibronectin, vimentin, and S100, and are negative for p75 neurotrophin receptor (p75NTR), and various melanocyte markers. This expression pattern indicates a novel stem cell thought to belong to the neural crest linage. Further experiments reveal the spheres to be positive for early neural crest markers such as Twist, Dermo-1, slug, SHOX2 and Pax3 (I. McKenzie, personal communication). Upon differentiation in serum, the SKPs were shown to express p75NTR, another marker of neural crest stem cells. To further support this theory of SKPs coming from the neural crest linage, specific differentiation conditions were shown to give rise to a variety of neural crest derivatives, such as osteoblasts, chondrocytes, peripheral neurons expressing TH, DβH and peripherin, smooth muscle, adipocytes and Schwann cells (F. Miller, personal communication).

Neural crest cells originate as a group of cells migrating laterally away from the developing neural tube beginning at embryonic day 9 (E9). This early migration follows a dorsal route over the somites to become the pigmented melanocytes of the skin. The second migration occurs at E16 and proceeds medially and ventrally to the somites. This occurs at the same time as dorsal skin innervation. Embryonic SKPs can only be isolated from E16 or older animals, suggesting that they may be arriving at the same time as the innervation. The number of spheres isolated from skin peaks at about E18, then declines

at birth, but a small number of spheres can still be isolated and expanded from the adult. This suggests that SKPs may be primitive neural crest cells that have remained quiescent in the skin throughout adulthood.

III. *In vivo* differentiation into skeletal muscle

An attractive property of stem cells is their ability to be transplanted into damaged tissue and to respond appropriately to a foreign microenvironment. In the case of skeletal muscle, a continuously regenerating tissue that incorporates new cells on a regular basis, stem cell therapy would be ideal. Engraftment is the result of successful incorporation of new cells into a host tissue. In skeletal muscle, this process is usually due to fusion of donor cells with the existing myofibres. To date, primary myoblasts and satellite cells have been shown to have a better rate of engraftment into damaged muscle then stem cells. Unfortunately, primary myoblasts cannot be easily expanded in culture to create a clinically useful amount for transplantation. However, immortalized myoblast cell lines can be expanded indefinitely, though they tend to form tumors after transplantation (Irintchev *et al.,* 1998). These findings prompted increased research into the use of stem cells for muscle transplants, due to their enhanced capacity for self-renewal.

Several studies have been done looking at the *in vivo* differentiation of stem cells into muscle following direct injection into regenerating muscle. Due to interference from resident satellite cells which will preferentially divide and repopulate damaged muscle, most transplants show a low rate of engraftment. By chemically inducing regeneration in the muscle prior to transplantation, engraftment by donor cells is enhanced. Galli *et al.,* (2000), has shown that neural stem cells can differentiate into muscle at an average level

of 1.5% engraftment, three weeks following transplantation into chemically damaged muscle. This was a surprising result, as neural stem cells arise from the neuroectoderm, an embryonic layer distinct from the mesoderm that gives rise to skeletal muscle. In addition, when NSCs were injected into a blastocyst, skeletal muscle was one tissue that was notably lacking in donor derived cells (Clarke *et al.,* 2000). Neural stem cells have been shown to have the ability to fuse with embryonic stem cells *in vitro* and express a myogenic phenotype as a hybrid cell. It is possible that the regenerating environment of the damaged muscle was sufficient to allow fusion of the donor nuclei at the low level observed by Galli *et al.* (2000).

Ferrari *et al.* (1998) had similar findings when they injected whole bone marrow into the muscle bed of regenerating muscle. The bone marrow was able to engraft at a low level but not as efficiently as primary myoblasts or satellite cells. Whole bone marrow contains multiple distinct subpopulations of stem cells, one of which appears to be biased towards the myogenic phenotype. It is possible that this fraction, in a myogenic environment, could contribute to muscle regeneration. Corti *et al.* (2002) demonstrated that when this myogenic fraction was isolated and transplanted into mice lacking dystrophin, the level of engraftment was superior to that obtained with transplanted whole bone marrow. Though the transplanted cells were not examined for evidence of fusion, bone marrow cells have been shown to fuse with ES cells *in vitro* as well as with hepatocytes and Purkinje cells *in vivo*, so it is likely that fusion rather then transdifferentiation accounts for the muscle phenotypes in this case.

Even with a more permissive environment created by the regenerating muscle, stem cell engraftment is still too inefficient to be a therapeutic alternative. It has been

suggested that the low engraftment following intramuscular injections may be a combination of interference from host cells, immune rejection and interactions with fibroblasts and the surrounding extracellular matrix. Irintchev *et al.* (1998) has shown that well formed mature muscle can be grown from myoblasts injected into the subcutaneous space, an area much less restrictive then the regenerating muscle mass, even though immune rejection was still an issue in this model. Obviously, conditions have yet to be defined which will optimize the engraftment of stem cells to the extent that this increase could make transplantation a viable therapeutic alternative.

Other groups have focused on intravenously injecting stem cells into lethally irradiated mice and looking at the ability of stem cells to repopulate the hematopoietic system and migrate from the blood stream into damaged or regenerating muscle. The idea of peripheral precursors being recruited is attractive, as several cell fractions isolated from bone marrow have shown multipotent properties, including myogenic ability (Corti *et al.,* 2002). This idea has been supported by studies where fetal liver cells (Fukada *et al.,* 2001), bone marrow derived stem cells (Corti *et al.,* 2002, Ferrari *et al.,* 1998), or hematopoietic stem cells (Gussoni *et al.,* 1999) demonstrated muscle engraftment following intravenous injection, but all with a low frequency, generally below 2%. LaBarge and Blau (2002) have shown the highest frequency to date of 3.5%, but more importantly showed that adult bone marrow cells passed through a satellite cell intermediate stage, prior to fusing into myotubes. Not only were these satellite cells defined anatomically *in vivo*, they were also capable of being harvested, grown clonally *in vitro* and retransplanted where they could contribute to skeletal muscle in a new host (LaBarge & Blau, 2002). This has also been demonstrated with mesenchymal stem cells

isolated from the synovial membrane, which passed through a satellite cell stage following transplantation (De Bari *et al.,* 2003). The identification of this satellite cell intermediate supports the idea that the transplanted cells could be behaving into an appropriate fashion as they participate in the regeneration of the muscle. Since no genetic analyses or karotyping was done on these cells, the possibility of fusion cannot be proven either way, though it is likely, due to recent studies looking at the genetic properties of transplanted cells. This suggests that most positive results arising from previous transplantation studies may be the result of fusion with the host cells, rather then stem cell plasticity.

IV. *In vitro* differentiation into skeletal muscle

The first conversion of a different cell type to muscle occurred in 1977 when Constantinides and colleagues added 5-azacytidine (5-azaC) to a culture of 10T1/2 cells, a murine embryonic fibroblast cell line, and saw the cells differentiate into multinucleated, contractible myotubes (As reviewed in Hauschka, 1994). 5-azaC is a cytodine analogue that causes the demethylation of cytosines in DNA. About 2-7% of all cytosines are methylated and it is believed that hypomethylation removes the inhibition of critical genes that leads to the activation of the myogenic program. 5-azaC is taken up in the cells during DNA synthesis and the newly demethylated DNA is stably propagated into daughter cells. After 10 cell doublings the myogenic phenotype begins to be expressed, as well as that of other mesodermal cells types such as adipocytes and chondrocytes (Hauschka, 1994). The use of 5-azaC to differentiate cells into muscle has been successfully demonstrated in the mouse fibroblast line 3T3 and in bone marrow

derived mesenchymal stem cells (Wakitani *et al.,* 1995, Hauschka, 1994). Mesenchymal stem cells were shown to form multinucleated myotubes one week after treatment with 5-azaC, with the maximum number of myotubes appearing after two weeks in culture. Though mature contractile muscle was formed, the conversion rate was only 5%. Another group obtained 20-30% yields of beating cardiomyocytes following 5-azaC treatments, but the overall percentages varied from culture to culture (Rangappa *et al.,* 2003).

Neural stem cells (NSCs) have been shown to have the ability to differentiate into myotubes following co-culture with the murine C2C12 myoblast cell line. NSCs obtained from transgenic mice expressing LacZ driven by a muscle specific promoter, were cultured with C2C12 cells and shown to form multinucleated myotubes. While most NSCs fused with each other into myotubes, they also fused with the C2C12 cells to form chimeric myotubes (Galli *et al.,* 2000). Rietze *et al.* (2001) also demonstrated that neural stem cells expressing the GFP transgene could undergo conversion to myotubes following co-culture with C2C12 cells. In contrast to Galli *et al.* (2000), they achieved a much higher conversion rate (57% compared to 7%) and observed the majority of the myotubes to be chimeric with C2C12 cells. This suggests that fusion was likely the cause of these chimeras rather then transdifferentiation. It is possible that the low number of NSC myotubes and the rare fusion event observed by Galli *et al.* (2000) may have been due to the muscle specific transgene not being activated once the NSCs fused with the C2C12 cells. This could account for underreporting of chimeric myotubes in this study. The GFP-NSCs would constituently express the transgene in both chimeric and stem cell only myotubes allowing for the visualization of all fusion events. Skin derived precursors (SKPs) have also shown this ability when labeled with GFP adenovirus and co-cultured

with C2C12 cells. Both chimeric and SKP only myotubes were observed, though this is still preliminary data and more a detailed analysis was not performed (M. Akhavan, personal communication).

Smooth muscle is another cell type that has been shown to differentiate into skeletal muscle both *in vitro* and *in vivo*. *In vivo* this is a naturally occurring, spontaneous transdifferentiation, which occurs in the developing mouse esophagus, beginning at E15.5 and continuing for two weeks postnatal (Kablar *et al.,* 2000). This change is dependent on the expression of myf5 and MyoD, two early muscle specific transcription factors. *In vitro*, a variety of smooth muscle cell lines have been shown to spontaneously differentiate into skeletal muscle in culture. This process is density dependent and involves the expression of MyoD, but is notably lacking myf5 expression (Graves & Yablonka-Reuveni, 2000).

Embryonic stem cells (ES cells) are able to differentiate into mature, contractile skeletal muscle *in vitro*. By culturing ES cells as aggregates known as embryoid bodies, then inducing differentiation by plating them in serum, cells will migrate outwards and form myoblasts, which then fuse into myotubes (Rohwedal *et al.,* 1994). These mature myotubes show similar contractile properties to skeletal muscle *in vivo*, expressing functional nicotinic acetylcholine receptors and appropriate electrophysiology (Rohwedal *et al.,* 1994). Transfecting ES cells with early muscle specific genes such as MyoD, and treating the cells with DMSO can enhance this differentiation process (Sumariwalla & Klein, 2001). Much can be learnt about early muscle development using this system as it mimics development *in vivo*. In addition, ES cell lines can be cultured from mice lacking

muscle specific genes to determine the effect this has on development and signaling (Myer *et al.,* 2001, Rohwedal *et al.,* 1994).

B. Skeletal muscle development

I. Introduction

In order to fully understand the differentiation of stem cells into skeletal muscle, an understanding of myogenesis, both in development and in injury repair is required. The process of muscle development in vertebrates was first described in avian models such as chick and quail, and this process has been shown to be similar in mouse (Hauschka, 1994). Muscle is often divided into three groups based on embryonic origin and final location in the adult. Epaxial and hypaxial muscle include the dorsal and ventral body muscle, as well as the limbs and both are derived exclusively from the somites. Head muscle is formed in part by the somites, along with contributions from the paraxial head mesoderm and prechordal mesoderm (Brand-Saberi & Christ, 1999). Somites are repeating segments of mesoderm lying on either side of the developing neural tube and notocord. They first appear at E8 in the mouse and are composed of two layers: a sclerotome, which will form the cartilage and bone of the skeleton, and a dermomyotome which gives rise to the skeletal muscle (Buckingham *et al.,* 2003). The dermomyotome is further divided to form an underlying structure called the myotome. The myotome is made up of post-mitotic myoblasts expressing desmin and myosin, which migrate rostral-caudally underneath the dermomyotome to become the muscles of the back (Brand-Saberi & Christ, 1999). The dermomyotome contains proliferating myoblasts, which contribute to the back and abdominal muscles, and migrate out to form the limbs.

The migration of proliferating myoblasts into the limb is controlled by a number of attractive signals. Between E11-14 the myoblasts exit the cell cycle synchronously and differentiate into primary muscle fibres (Hauschka, 1994). These are mostly slow twitch fibres, which are thought to provide a scaffold to direct the second wave of migrating muscle precursors. This secondary wave occurs between E14-16 when another round of proliferation and differentiation occurs and the myoblasts fuse asynchronously with the primary fibres. A third wave of migration and fusion is often observed in larger muscles, forming the tertiary myofibres.

II. Structure of muscle fibres

Skeletal muscle has a unique structure made up of multinucleated fibres bundled together to form a highly coordinated, contractile tissue. Following the fusion of postmitotic myoblasts, the nuclei are seen to move from a central to a more peripheral location in the cell, signifying a differentiated myofibre. Another hallmark of the mature muscle phenotype is the switching from embryonic to mature isoforms or orientations of various structural proteins such as myosin and desmin (Allen & Leinward, 2001, Li *et al.*, 1997). Some of the main structural proteins of muscle fibres include the intermediate filament desmin, the family of myosin heavy chain proteins and the clinically relevant protein dystrophin.

Desmin is one of the earliest markers to be expressed, appearing weakly in the myotome at E9 and more strongly in primary myotubes at E13. Desmin is expressed in proliferating myoblasts as well as mature myotubes, though its expression pattern differs. Initially, desmin has a longitudinal orientation, which shifts to become localized at the Z-

line resulting in the striated appearance of mature muscle (Li *et al.,* 1997). Desmin links the myofibrils at the Z-line and connects the sarcomeres to the sarcolemma membrane. This protein was once believed to be involved in muscle specification, differentiation and fusion as shown by Li *et al.* (1994) and Weitzer *et al.* (1995), who inhibited desmin expression in C2 cells and ES cells respectively, and found no muscle differentiation occurred *in vitro*. This belief was questioned following the creation of a viable desmin knockout mouse, which developed apparently normal skeletal muscle. Upon closer examination deficits in regeneration, as well as abnormal organization of the neuromuscular junction and muscle fibres were discovered (Li *et al.,* 1997, Agbulut *et al.,* 2001). This demonstrates a trend in skeletal myogenesis, in which embryonic development and *in vitro* systems are shown to have different requirements for the expression of the early muscle genes and proteins.

The myosin heavy chain (MyHC) family is made up of eight different isoforms, each of which has a specific pattern of expression. MyHC embryonic and perinatal are the first to appear during development, MyHC I (MyHC slow) is expressed in the heart and slow muscle fibres, and MyHC IIa, IIb and IId (MyHC fast) are expressed in fast muscle fibres. The eighth isoform (MyHCeo) is found in the extraocular and pharyngeal muscles (Allen & Leinward, 2001). The myosins are structural proteins that play a role in contraction and force generation, and as such are classified according to the speed of contraction of the fibre in which they are found (Allen & Leinward, 2001). Knockout mice have been created for the MyHC IIb and MyHC IId isoforms. Both show up-regulation of the remaining MyHC fast isoforms indicating some compensation, but not enough as shown by the phenotype. Knockouts of either isoform results in localized loss

of muscle fibres in the hind limbs, combined with increased muscle mass in other areas (Harrison *et al.,* 2002).

Dystrophin is the most clinically studied muscle protein, as it is the gene lacking in Duchenne's muscular dystrophy (DMD), an X-linked recessive muscle wasting disease. The dystrophin protein is located on the cytoplasmic side of the sarcolemma and plays a role in membrane stability. In muscular dystrophy, this instability in the membrane leads to a cycle of degeneration and subsequent regeneration of the muscle fibres, which continues until the limited supply of satellite cells is depleted. At this stage, damaged muscle is replaced by fatty tissue and other mesenchymal derivatives (O'Brien & Kunkel, 2001). The mdx mouse lacks dystrophin expression and is used as an animal model of DMD. These mice are viable, but demonstrate the same degeneration/regeneration cycle as seen in human DMD. Cultures of myoblasts lacking dystrophin show a reduced capacity for self renewal. This further supports the belief that there is a limited supply of satellite cells that is sufficient for muscle repair in most cases, but can become depleted in dystrophic patients (Bischoff, 1994).

III. Molecular mechanisms of muscle development

Myogenesis is controlled by the bHLH family of transcription factors known as muscle regulatory factors (MRFs). These include myf5, MyoD, MRF4 and myogenin, each of which is sufficient on its own to drive myogenesis when over-expressed in non-myogenic cells (Valdez *et al.,* 2000). The MRFs are activated by forming heterodimers with other bHLH proteins known as E-proteins. They can then bind to a specific nucleotide sequence, the E-box, which lies upstream of the promoter region of many

muscle specific genes. Though E-proteins are ubiquitously expressed, an external growth factor must first activate one of the MRFs for E-box binding to occur and for subsequent myogenesis to proceed. Another group of transcription factors is the MEF2 family of MADS box transcription factors which play an important role in myogenesis by stabilizing and enhancing the activity of the MRFs. MRF activity can be silenced by inhibitor of differentiation (Id) genes, which will preferentially form heterodimers with the MRF-HLH domain interfering with DNA binding (Brand-Saberi & Christ, 1999).

Through the generation of knockout animals, much has been learned about the role each MRF plays in development (summarized in Table 1). Interestingly, there appears to be different patterns of expression embryonically compared to those observed in primary myoblast cultures. This has led to numerous discrepancies about the order in which the MRFs are expressed. During embryonic development, the temporal order appears to be myf5, myogenin, MyoD and MRF4, though MRF4 may appear transiently following myf5. Cultured myoblasts or satellite cells first express myf5 and MyoD followed by their co-expression, while myogenin and MRF4 are activated later and have a role in muscle differentiation (Rawls *et al.,* 1998, Cornelison & Wold, 1997).

Myf5 is the earliest muscle marker to be expressed, appearing in the somites at E8.5 and persisting until E18 when is it down regulated (Hauschka, 1994). Myogenesis in the myf5 -/- mouse is delayed by about two days, but otherwise muscle develops normally. Though the reason is not fully understood, this phenotype is lethal with mice dying at birth as a result of a rib malformation, which affects the attachment of the diaphragm (Hauschka, 1994). When myf5 -/- muscle is cultured *in vitro*, the myoblasts divide and fuse into myotubes, but the process is delayed. This can be rescued with the

addition of MyoD in the culture suggesting some redundancy within the MRF family members.

MyoD appears two days after myf5 and plays a role in the initiation of differentiation in proliferating myoblasts. The MyoD -/- mouse is viable and develops normal muscle, but has a severe deficiency in regeneration following injury. The cultured myoblasts demonstrate enhanced proliferation and delayed differentiation (Yablonka-Reuveni *et al.,* 1999). *In vivo*, skeletal muscle is found to have elevated levels of myf5 and the phenotype *in vitro* can be rescued by myf5 suggesting that the two factors have overlapping functions. This idea of redundancy is further supported by the myf5-/-; MyoD-/- mouse which is lethal, with no muscle development. This appears to be concentration dependent as the myf5+/-; MyoD-/- mouse allows for the formation of some muscle with a reduced number of myoblasts, due to the presence of the single myf5 allele (Hauschka, 1994).

Myogenin -/- mice have a major deficiency in muscle development and generally die at birth. There is almost a complete lack of muscle fibres, and the fibres that do form are disorganized and show up-regulated expression of MRF4 (Rawls *et al.,* 1998). *In vitro*, myogenin -/- cultures form myoblasts, but do not terminally differentiate into myotubes, suggesting that this transcription factor is essential for fusion and terminal differentiation of myoblasts. The MRF4 -/- mouse shows no obvious deficit *in vivo* or when muscle is cultured, but a four-fold increase in myogenin expression is detected in both cases (Rawls *et al.,* 1998). Though MRF4 is expressed briefly just after myf5, it is quickly down-regulated until E10.5 where it persists as the main MRF transcript in adult muscle (Rawls *et al.,* 1998). When the MRF4 -/- and MyoD -/- are crossed, the

phenotype is similar to the myogenin -/- with respect to the disorganized and rare appearance of myotubes suggesting that MRF4 and MyoD may have an overlapping role in the differentiation of muscle (Valdez *et al.*, 2000, Rawls *et al.*, 1998).

Table 1: Phenotypes *in vivo* and *in vitro* of the MRF family knockout mice

Knockout mouse genotype	Phenotype *in vivo*	Phenotype *in vitro*
Myf5 -/-	• Normal muscle development with onset delayed for 2 days • Rib defect causes lethality	• Delayed proliferation and fusion • Rescued by MyoD
MyoD -/-	• Normal muscle development • Deficit in regeneration	• Enhanced proliferation • Delayed differentiation • Rescued by myf5
Myogenin -/-	• Lethal • Few muscle fibres • Highly disorganized	• Reduced differentiation
MRF4 -/-	• Increase in myogenin • Normal muscle development	• Increase in myogenin • Normal growth and differentiation
Myf5 -/-; MyoD -/-	• Lethal • No muscle development	• No myoblasts
Myf5 +/-; MyoD -/-	• Some muscle development	• Few myoblasts
MRF4 -/-; MyoD -/-	• Lethal • Few muscle fibres • Highly disorganized	• Reduced differentiation
MyoD -/-; MRF4 -/-; myogenin -/-	• Lethal • Myoblasts present • No muscle fibres	• No differentiation

IV. Satellite cells and regeneration of muscle

The MRFs are also involved in the regeneration of adult muscle occurring in response to damage caused by mechanical or chemical injury, exercise and overuse, or the muscular dystrophies (Cornelison & Wold, 1997). Damaged myofibres undergo a period of cell death followed by the release of growth factors such as bFGF and TGFβ, which initiate the proliferation of quiescent satellite cells and their subsequent fusion with undamaged myofibres (Bischoff, 1994). Satellite cells are a population of muscle stem cells that are located between the basal lamina and the sarcolemma of muscle fibres. They appear embryonically at E17.5 in mouse development and persist throughout the life of the animal (Seale *et al.,* 2001). Unfortunately, following frequent degeneration-regeneration cycles, as seen in muscular dystrophy, the satellite cell population can become depleted, resulting in the muscle wasting characteristic of this disease (Bischoff, 1994).

Regenerating muscle has an altered pattern of MRF expression than is seen during embryonic development. This appears to be represented in cultured satellite cells, which mimic the proliferative response to muscle injury. While quiescent, satellite cells express c-met, m-cadherin, CD34, pax7 and occasionally myf5 is seen. Once activated, satellite cells turn on the muscle regulatory factors myf5 and/or MyoD followed by their co-expression, and then MRF4 and finally myogenin as the cells become terminally differentiated (Seale *et al.,* 2001, Cornelison & Wold, 1997). The differences in MRF expression following muscle damage as compared to during development are interesting, but not entirely unexpected. Satellite cells do not appear until myogenesis is almost complete in the mouse, and questions have arisen concerning their origin. One belief is

that satellite cells may actually be hematopoietic in origin, arising from the blood circulation, rather than from mesoderm derivatives (Gussoni *et al.,* 1999, Ferrari *et al.,* 1998). If this is true, the differences in MRF expression are not surprising as a unique cell type is being investigated. To date, satellite cells are considered to be unipotent with regards to differentiation capacity, though one group has shown that satellite cell derived myoblasts have the ability to differentiate into osteocytes and adipocytes *in vitro* following treatment with bone morphogenic proteins (BMPs) or adipogenic inducers (Asakura *et al.,* 2001). Satellite cells or primary myoblasts derived from satellite cells have been an extensively studied culture system and a wide variety of extrinsic factors have been tested for their ability to promote proliferation and induce differentiation in these populations.

V. Extrinsic factors affecting muscle differentiation

The original studies performed on primary myoblasts cultures, used media containing high glucose, high serum and occasionally chick embryo extract to promote proliferation, and the withdrawal of serum and embryo extract to promote differentiation and fusion. This undefined media worked well, but the exact factors responsible for the cessation of growth and induction of fusion were unknown. Many of these factors were identified as those released from the extracellular matrix following muscle damage. The most studied are insulin-like growth factors I and II (IGF-I and II), basic fibroblast growth factor (bFGF) and transforming growth factor β (TGFβ). Platelet derived growth factor (PDGF) is also released following injury and acts to enhance the proliferation and

inhibit differentiation of cultured myoblasts. It also appears to have a role in attracting satellite cells to the location of muscle injury (Husmann *et al.,* 1996).

IGF I and II can influence both the proliferation and differentiation of primary myoblasts. *In vivo* studies have demonstrated an essential role for IGF-I in normal muscle growth and differentiation during development. Mice lacking IGF-I have a form of muscular dystrophy as well as abnormal organization of the muscle fibres of the diaphragm and intercostal muscles. The over-expression of IGF-I results in increased muscle mass and hypertrophy of fibres (Forhead *et al.,* 2002). This is further supported by *in vitro* studies where IGF-I and IGF-II have been shown to be able to substitute for serum in proliferating C2C12 cells (Yoshiko *et al.,* 2002). IGF-I appears to play more of a role in stimulating proliferation, while IGF-II has a stronger effect on enhancing differentiation. Interestingly, pure insulin has a similar effect on cultured myoblasts, because it can bind to the IGF receptor with similar specificity. The ability of one molecule to affect both phases of muscle growth is due to differential signaling. The IGFs bind to the IGF receptor, which is a tyrosine kinase activating both the MAP-kinase and PI3-kinase pathways. IGFs are thought to activate the MAP-kinase pathway during growth, while the PI3-kinase pathway is implicated in differentiation (Galvin *et al.,* 2003).

Basic fibroblast growth factor (bFGF) is a potent mitogen for many cell types. It has been shown to stimulate proliferation in neural and muscle stem cells, in primary myoblasts, and immortalized myoblast cell lines, as well as stimulating angiogenesis and wound repair. During muscle regeneration, bFGF is released from the extracellular matrix, in response to damage where it stimulates proliferation of satellite cells and

represses their differentiation (Husmann *et al.,* 1996). Mice lacking bFGF express a mild phenotype that does not appear to affect skeletal muscle, indicating compensation by other FGF family members (Ortega *et al.,* 1998). However, *in vivo* models of muscle injury in wildtype mice have shown up-regulation of bFGF expression in regenerating cells. Blocking bFGF in these cells leads to a reduction in the number of regenerating fibres (Husmann *et al.,* 1996).

Transforming growth factor (TGFβ) is also stored in the extracellular matrix and is largely responsible for inhibiting differentiation, though it has a slight negative effect on proliferation. TGFβ acts by rebuilding the basement membrane and extracellular matrix surrounding the muscle fibres following injury or muscle damage (Husmann *et al.,* 1996). It is thought to maintain the satellite cell population in a quiescent state while the extracellular matrix is being repaired. *In vitro*, myoblasts cultured in a mitogen rich media demonstrate a drastic, though reversible, suppression of differentiation in the presence of TGFβ (Stewart *et al.,* 2003). It is believed that TGFβ acts by suppressing MyoD binding which impairs the immature myoblasts from undergoing fusion. Mice lacking myostatin, a skeletal muscle specific isoform of TGFβ, have an increased muscle mass, while overexpression of myostatin results in muscle wasting, supporting the role of TGFβ in muscle growth and differentiation (Whittemore *et al.,* 2002).

With the identification of these extrinsic factors, more defined conditions have been developed for the growth of skeletal muscle *in vitro*. Though the presence of high serum is still used for growth, recent studies have shown that IGF-I and IGF-II may be sufficient to substitute for serum, at least in C2C12 cultures (Yoshiko *et al.,* 2002). Recent studies have begun to challenge the belief that mitogen withdrawal is essential for

differentiation. Yoshiko *et al.* (2002) showed that differentiation of C2C12 cells could occur in mitogen rich media by adding IGF-I and IGF-II. Though conditions for the culture of primary myoblasts are beginning to be more defined and optimized for differentiation, few of these conditions have been tested in other systems. Studies looking at stem cell differentiation into skeletal muscle in isolated culture systems use high glucose and serum levels with hydrocortisone following treatment with 5-azacytidine. The yield of myotubes from these studies is generally low but there has been no attempt to optimize conditions to specifically promote their differentiation. For my Master's project, I wanted to develop a culture system for optimal skeletal muscle differentiation from a novel source of adult stem cells isolated from the skin. My final culture conditions and results will be discussed in Chapter 3, while Chapter 2 will be focused on obtaining an accurate assay to verify skeletal muscle differentiation.

Chapter 2: Establishment of an accurate assay for skeletal muscle

I. Introduction and rationale

The existence of an accurate assay for various cell and tissue types is essential in stem cell research. Differentiating a stem cell into another cell type is the first step, while the next step is determining whether this novel cell type is actually what was expected. Previous studies showing stem cell differentiation into muscle have relied on appropriate morphology, contractile properties, and muscle specific antibody expression. While these techniques suggest differentiation has occurred, they do not always conclusively prove the presence of skeletal muscle. For instance, many common antibodies used for identifying skeletal muscle, such as desmin and some of the myosin heavy chain isoforms, are also expressed in smooth, and/or cardiac muscle cells (Agbulut *et al.,* 2001, Lyons *et al.,* 1990). Contractibility can be observed in both cardiac and skeletal muscle cultures, making it difficult to use this criterion to distinguish the two cell types, and the presence of multinucleated cells can signify skeletal, smooth or cardiac muscle, in addition to hepatocytes and fibroblasts (Wang *et al.,* 2003).

An additional technique used to show muscular differentiation is the activation of reporter genes, such as GFP and LacZ, driven by muscle specific promoters. Unfortunately, many muscle genes are transcribed without undergoing translation into a functional protein. For example, Myf5 and MyoD are both commonly used promoters to drive LacZ expression in differentiation studies of muscle *in vitro* and *in vivo*. Myf5 has been found in the mouse brain where it is transcribed, as determined by LacZ expression, but never translated into protein (Daubas *et al.,* 2000). As a muscle marker, this takes away its power as a definitive sign of differentiation down the myogenic lineage. MyoD

has also been found in numerous fetal organs in chick where it is transcribed, but not translated. Interestingly, if these MyoD positive cells are isolated and grown in culture, many will differentiate into mature skeletal muscle (Gerhart *et al.,* 2001). In addition, detection of myogenin transcripts has been observed in the developing embryo days before their translation into protein (Daubas *et al.,* 2000). This has also been shown with a few members of the myosin heavy chain family, which can be detected by *in situ* hybridization several days prior to the expression of the protein (Lyons *et al.,* 1990).

For my Master's project, I used three separate techniques to examine gene and protein expression in skeletal muscle. Gene activation was determined using RT-PCR, while protein expression was determined using Western blots, in combination with immunocytochemistry on cells with the appropriate morphology. The variety of techniques was necessary to ensure that we could accurately identify skeletal muscle from a mixed population of cells. The antibodies chosen for this study should give an indication of both early and late events in the myogenic process. I chose to compare pure skeletal muscle, brain, skin and undifferentiated SKPs growing as spheres. As SKPs readily differentiate into neurons and glia, it was essential to have muscle markers that were not expressed in the brain. Furthermore, whole skin and undifferentiated spheres were used to establish a baseline level of any muscle markers that may still be expressed from the original culture. In this chapter, I describe the methods used to determine the pattern of gene and protein expression that is unique to skeletal muscle, and those used to establish a baseline level of muscle markers in undifferentiated skin derived stem cells.

II. Materials and methods

SKP culture

SKPs were cultured from the back and abdominal skin of neonatal or adult CD1 mice. The skin was carefully cleaned under a dissecting microscope to remove fat and blood vessels, and then cut into 2x3 mm pieces. The pieces were washed twice with Hanks Buffered Salt Solution (HBSS) and put in 0.01% trypsin (Sigma) at 37°C for 45 minutes, mixing every 15 minutes to break down the tissue, allowing for easier dissociation of the cells. The trypsin was then removed and basic media (Dulbecco's Modified Eagle Medium (DMEM- Ham's F12 Nutrient Mixture (F12) 3:1 (Gibco BRL), 1% penicillin/streptomycin) containing 10% fetal bovine serum (FBS: Gibco BRL) was added to stop the trypsin reaction. The pieces were then washed 3 times with serum free media and mechanically dissociated by crushing with a plastic pipette. The resulting cell suspension was passed through a 40 μm cell strainer and spun at 1000 rpm for 4 minutes. The supernatant was removed and cells were resuspended in fresh media and placed in uncoated tissue culture flasks containing 10 mL of media supplemented with growth factors (40 ng/mL fibroblast growth factor (FGF), 20 ng/mL epidermal growth factor (EGF)), 2% B27 supplement (GIBCO BRL) and 1 μg/mL fungizone (Gibco BRL). Cells grew as floating spheres in a 37°C, 5% CO_2 tissue culture incubator and were mechanically dissociated and passaged every 5-7 days.

Primary myoblast culture

Primary myoblasts were cultured following the modified methods of Blau *et al.* (1994). Muscle was dissected from the fore and hind limbs of 2-5 day old CD1 mice and

placed in HBSS on ice. Muscle was placed in a fresh dish with a drop of HBSS and minced into a coarse slurry using razor blades. The slurry was then added to 2 mL of solution consisting of 2.4 U/mL dispase (Grade II, Roche), 1% collagenase (Class D, Roche), and 2.5 mM $CaCl_2$. This was kept at 37°C for 45 minutes and triturated with a plastic transfer pipette every 15 minutes. Cells were then passed through a 70 μm cell strainer, and spun at 1000 rpm for 4 minutes. Cells were plated on uncoated 60 mm dishes for 24 hours in Ham's F10 nutrient mixture (GIBCO BRL) with 20% FBS, 2.5 ng/mL FGF, 1% penicillin/streptomycin and 1 μg/mL fungizone. The next day the supernatant was transferred to dishes coated with 0.01% type I collagen (Sigma). This preplating technique enhanced the myoblast population as fibroblasts adhere to the uncoated dishes first. Cultures were passaged at 80-90% confluency by trypsinizing with a trypsin-versene mixture (BioWhittaker), spinning at 1000 rpm for 4 minutes, replating on plastic for 25 minutes, and then transferring the supernatant to collagen coated dishes. The preplating step continued until a relatively pure population of myoblasts had been obtained as determined by morphological criteria using phase microscopy. To differentiate myoblasts, cells were grown to confluency then media was changed to DMEM containing high glucose (GIBCO BRL) with 2% FBS, 0.5% chick embryo extract, 10 ng/mL insulin and 1% penicillin/streptomycin and 1 μg/mL fungizone. Media was changed daily.

Cortical progenitor culture

Cortical progenitors were cultured from E12-13 CD1 mice as described in Toma *et al.,* (2000). Briefly, the embryonic cortex was carefully dissected and placed in ice cold

HBSS, then the tissue was triturated with a plastic transfer pipette into small groups of cells. Cells were plated in Neurobasal media (Gibco BRL) containing 40 ng/mL bFGF, 1% N2 supplement, 2% B27 supplement, 500 μM glutamine and 1% penicillin/streptomycin on four well chamber slides coated with poly-D-lysine and laminin.

Immunocytochemistry

Immunocytochemical analysis of all cell types was performed on cells grown in four well chamber slides. Cells were washed once in HEPES buffered saline (HBS), then fixed in cold 4% paraformaldehyde (PFA) for 10 minutes, permeabilized in 0.2% Nonidet P-40 (NP-40) for 5 minutes, then blocked for 1 hour at room temperature in HBS containing 0.5% bovine serum albumin (BSA) and 0.6% normal goat serum (NGS). Cells were incubated with primary antibody overnight at 4°C, washed three times then incubated with secondary antibody for 1 hour at room temperature in the dark. Slides were washed once, treated with Hoechst 33258 (1:3000; Sigma) for 1-2 minutes at room temperature to visualize nuclei, washed twice more and then coverslipped with Geltol (Thermo Shandon). Primary antibodies were diluted in HBS containing 0.25% BSA and 0.3% NGS at the following concentrations: anti-myf5 rabbit polyclonal (1:200; Santa Cruz), anti-MRF4 rabbit polyclonal (1:200; Santa Cruz), anti-MyoD mouse monoclonal (1:50; RDI-clone 5.8A), anti-desmin rabbit polyclonal (1:400, Sigma), anti-myosin heavy chain fast mouse monoclonal (1:400; Sigma-clone MY-32), anti-myosin heavy chain slow mouse monoclonal (1:400; Sigma-clone NOQ7.5.4D) and anti-dystrophin rabbit polyclonal (1:50; kindly supplied by Dr. P. Holland). Secondary antibodies were diluted

in HBS and the following concentrations FITC-conjugated goat anti-rabbit IgG (1:50; Jackson) and CY3-conjugated goat anti-mouse IgG (1:200; Jackson).

Analysis of differentiated cells was based on morphology and positive immunostaining in comparison to cultured cortical progenitors used as negative control slides.

Western Blot Analysis

Cells and tissues were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche) and 1.5 mM sodium vanodate, scraped or broken down in a tissue homogenizer and rocked in Eppendorf tubes at 4°C for 10 minutes. Tubes were spun at 10,000 rpm for 10 minutes and the supernatant was collected and stored at -80°C. A BCA Protein Assay (Pierce Chemical Company) was performed, using bovine serum albumin (BSA) as a standard, to determine protein concentrations and ensure equal loading of samples. Equal amounts of protein were boiled in sample buffer (2% SDS, 100 mM DTT, 10% glycerol and 0.05% bromophenol blue) for 5 minutes then separated by SDS-PAGE on a 7.5-15% gradient. After electrophoresis, proteins were transferred to 0.2 μm nitrocellulose for 4 hours at 750 mA. The nitrocellulose membrane was then stained with Ponceau Red to visualize total protein and completeness of transfer. Ponceau Red was removed by washing 2-3 times with water and once with Tris buffered saline containing 1% Tween (TBS-T) followed by blocking in 5% nonfat milk (Carnation) in TBS-T for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with primary antibodies diluted in 5% nonfat milk in TBS-T. Antibodies and dilutions used are as follows: anti-myf5 rabbit polyclonal (1:500; Santa Cruz), anti-MRF4 rabbit polyclonal

(1:500; Santa Cruz), anti-MyoD mouse monoclonal (1:300; RDI-clone 5.8A), anti-desmin mouse monoclonal (1:1000, Sigma-clone DE-U-10), anti-myosin heavy chain fast mouse monoclonal (1:3000; Sigma-clone MY-32), and anti-myosin heavy chain slow mouse monoclonal (1:1000; Sigma-clone NOQ7.5.4D). After primary incubation, membranes were washed three times with TBS-T and incubated with secondary antibodies for 1-2 h at room temperature. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse (1:10,000; Boehringer Mannheim) and HRP-conjugated goat anti-rabbit (1:5000; Boehringer Mannheim). After three washes in TBS-T, detection was carried out using the ECL chemiluminescence reagent from Amersham and XAR x-ray film from Kodak.

RT-PCR

Cells and tissue were lysed with Trizol reagent and RNA was extracted using chloroform phase separation. Contaminating genomic DNA was removed from the RNA preparation with RQ1 RNase-Free DNase (Promega) for 30 minutes at 37°C, and RNA was reverse transcribed at 42°C for 2 hours using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) primed with oligo (dt) primers. PCR for GAPDH was performed following the DNAse step to rule out genomic DNA contamination, and following reverse transcription to confirm the presence of cDNA. PCR amplification was carried out for 40 or 45 cycles with an initial 5 minute denaturing step at 94°C. Each cycle consisted of 94°C for 45 seconds, 55-60°C for 1 minute, and 72°C for 90 seconds, followed by an additional 10 minute elongation period at 72°C. Optimal annealing temperatures for each primer set are listed in Table 2. Primers for myf5, MRF4,

myogenin and MyoD were as described in (Cornelison & Wold, 1997). Primers were created for desmin, dystrophin and GAPDH (Table 1). PCR products were loaded on a 1.5% agarose gel containing ethidium bromide for DNA detection and run at 80-85 V until sufficient band separation was obtained.

Table 2: Primers used for RT-PCR

Gene	Primer (5'→ 3')	Optimal Annealing Temp
Desmin	Fwd: TCCCCGCTGAGCTCTCCCGTGTTC Rev: AGCTCGCGCATCTCCTCCTCGTAG	64°C
Dystrophin	Fwd: GCTACTGCGCCAACACAAAGGAC Rev: TATAAAAACCATGCGGGAATCAGG	57.7°C
Myf5	Fwd: TGCCATCCGCTACATTGAGAG Rev: CCGGGGTAGCAGGCTGTGAGTTG	60°C
MRF4	Fwd: CTGCGCGAAAGGAGGAGACTAAAG Rev: ATGGAAGAAAGGCGCTGAAGACTG	60°C
MyoD	Fwd: GCCCGCGCTCCAACTGCTCTGAT Rev: CCTACGGTGGTGCGCCCTCTGC	66°C
Myogenin	Fwd: GGGCCCCTGGAAGAAAAG Rev: AGGAGGCGCTGTGGGAGT	66°C
GAPDH	Fwd: GTCTTCACCACCATGGAGAAG Rev: GTGATGGCATGGACTGTGGTC	55°C

III. Results

In order to develop an accurate assay for skeletal muscle, appropriate positive and negative controls were required. Using primary myoblasts and whole muscle extract as positive controls, brain and cultured cortical progenitor cells as negative controls, and skin and undifferentiated spheres to establish a baseline level of any muscle markers that may still be expressed from the original culture, a skeletal muscle specific profile was obtained. The markers selected for profiling represented both early and late events in the myogenic process. Myoblast specification was determined by expression of the bHLHs

myf5 and MyoD, while myogenin and MRF4 were used as markers for terminal differentiation. Structural proteins such as desmin, myosin heavy chain fast and slow were used for early and late markers of morphology. Dystrophin was also looked at to determine whether differentiated cells might have future therapeutic potential, in transplantations for muscular dystrophy.

Western blot analysis

Western blot analysis was performed on protein lysates from adult brain, P12 muscle, P12 skin and E18 leg to detect the presence of the various muscle specific proteins, as shown in Figure 1. Myf5 is down regulated after birth, so E18 leg was introduced as a positive control for this protein. While P12 muscle and P12 skin appear to have weak myf5 expression, this finding was not conclusive, as the antibody gave high background when the blot was overexposed. Adult brain served as an appropriate negative control for myf5. In addition to myf5, other proteins looked for included MRF4, MyoD, desmin, MyHC fast and MyHC slow. All of these markers were expressed in P12 skin, P12 muscle and E18 leg, though MRF4 was only weakly expressed in the skin. This was likely due to the small percentage of total skin protein that is specific to muscle, as well as problems with overexposing this blot. Adult brain was shown to be a good negative control for each marker. As we were not comparing the levels of protein expression between tissues, we decided to use Ponceau Red staining to show a rough relative protein level as a loading control for the Western blots. The appearance of muscle markers in the skin was not surprising as there is a thin layer of skeletal muscle,

the paniculus carnosus, which is tightly apposed to the dermal layer of the skin. This layer was not removed prior to tissue lysis, RNA extraction or primary SKP culture.

RT-PCR

We then used RT-PCR as a second method for examining muscle-specific gene expression in adult brain, P6 muscle and P7 skin. Mouse specific primers were selected for myf5, MRF4, myogenin and MyoD, as well as desmin and dystrophin (Figure 2). As expected, all muscle markers were expressed in P6 muscle, though myf5 expression was weak and required 45 amplification cycles compared to the 40 cycles used for other primers. P7 skin gave a strong band for all primers, with the exception of myf5, which was still very weakly expressed. The weak expression of myf5 in skin and muscle is likely due to its down-regulation after birth in these tissues. Unlike the Western blot data, adult brain was not as good a negative control for RT-PCR as expected, since myf5 transcripts have been detected in localized areas of the adult brain (Tajbakhsh & Buckingham, 1995) and we were not able to get a clear positive or negative result with this tissue. In addition, the primers designed for dystrophin corresponded to a region common to both the muscle and neuronal isoform, which explains the amplification of the gene in both brain, and muscle.

Immunocytochemistry

In order to determine whether we could reliably detect expression of muscle specific proteins using immunocytochemistry, I developed a culture system for primary myoblasts based on the methods of Blau *et al.* (1994). These cultures contained both

proliferating primary myoblasts and differentiated myotubes. Immunocytochemistry analysis demonstrated that the proliferating myoblasts were positive for myf5, MyoD and desmin (Figure 3 A-D), and weakly positive for MyHC fast and for MyHC slow in unfused cells (Figure 3 E, F). Differentiated myotubes showed an elongated, multinucleated morphology and were positive for MyHC fast and MyHC slow (Figure 3 E, F). Myf5 and MyoD were expressed in the unfused myoblasts that still remained in culture following 4 days of differentiation (data not shown). To determine background levels for the antibodies used, immunocytochemistry was done simultaneously on cultured cortical progenitors (Inserts Figure 3). No positive staining was observed, except for desmin, indicating that all other antibodies appear to specifically label the protein of interest. Because of the higher background obtained with the desmin antibody, and because desmin is expressed in smooth muscle, interpretation of desmin staining was based on morphological criteria or double labeling with other muscle proteins, in addition to positive desmin expression.

Analysis of SKPs spheres

Since whole skin contains muscle, and dissociated primary skin cells contain a small population of cells that can differentiate into beating myotubes *in vitro* (K. Fernandes, personal communication) it was essential to ensure that SKPs spheres formed using our routine culture procedures did not express any of the muscle markers. To examine this issue, I analyzed both early passage (3-6 weeks in culture) and late passage (15-25 weeks in culture) SKPs spheres. No differences were observed with regards to muscle marker expression in early compared to late passaged SKPs so the data presented

is representative of both populations. Western blot analyses demonstrated that spheres were negative for MyoD, desmin, MyHC fast and MyHC slow (Figure 4). Detection of myf5 and MRF4 was inconclusive due to technical difficulties combined with the high background of the antibodies. As an even more sensitive assay for the presence of muscle specific mRNAs in SKPs, I used RT-PCR. This analysis showed that SKPS spheres never expressed the mRNAs for myf5, MyoD, MRF4, myogenin, desmin and dystrophin (Figure 5). The finding that undifferentiated SKPs spheres were negative for selected early and late muscle markers, suggests that they do not contain any muscle contaminants from the original culture.

In summary, the expression of early and late muscle-specific protein and mRNA was confirmed in skeletal muscle, primary cultured myoblasts and whole skin. SKPs spheres were negative for all markers of skeletal muscle differentiation indicating that the muscle originally present in the skin, was not carried along in the cultured spheres. Most of our antibodies were observed to be specific to skeletal muscle, though interpretations of desmin must be combined with morphological criteria or double labeling to confirm its presence in the appropriate cell type. In addition, the detection of dystrophin and myf5 mRNAs must be combined with their protein expression to ensure that the presence of these markers is due to muscle, rather then neuronal differentiation.

IV. Conclusions

The development of an accurate assay of differentiation is essential in stem cell research, especially in the case of skeletal muscle detection. Previous studies looking at skeletal muscle differentiation by stem cells have encountered problems with

interpretation due to the promiscuous expression of some markers, and the delay between transcription and translation of others. The assay developed here demonstrates the strengths and weakness of the commonly used markers, and provides a baseline for future comparisons with presumptive skeletal muscle derived from differentiated SKPs.

I showed that both the early markers of myogenesis (myf5, MyoD, and desmin) and the later markers (MRF4, myogenin, MyHC fast and MyHC slow, and dystrophin) were appropriately expressed in skeletal muscle and cultured myoblasts. In addition, adult brain was shown to be an adequate negative control, though expression of myf5 mRNA by RT-PCR was inconclusive and resulted in the reliance on H_2O as a negative control for this primer set. Interestingly, whole skin had robust expression of all markers of myogenesis shown using all three techniques. Though this in itself was not surprising, the finding that both MyHC fast and slow were present in the paniculus carnosus was unusual as nothing has been reported in the literature specifying the fibre type in this muscle, so I was expecting one type or the other, but not both.

The realization that whole skin expresses significant amounts of muscle specific protein and that a subpopulation of primary skin cells can form mature muscle in culture made us unsure of whether SKPs or muscle contaminants would be revealed in our skeletal muscle differentiation conditions. However, the finding that undifferentiated spheres were negative for all markers made us more confident that our culturing process was able to select for the SKPs without carrying along any muscle contaminates. For the accurate detection of muscle differentiation from SKPs, it will be essential to rely on appropriate morphology in addition to double labeling with muscle specific proteins, and gene expression.

Chapter 3: Differentiation of SKPs into skeletal muscle

I. Introduction and rationale

Numerous studies have shown that stem cells have the ability to differentiate into skeletal muscle both *in vitro* and *in vivo*. SKPs have so far been proven to be a novel multipotent stem cell, but whether they could become skeletal muscle remained unknown. Established conditions for differentiating stem cells into skeletal muscle *in vitro* have either used a co-culture technique or treatment with 5-azacytidine (5-azaC). Preliminary studies have shown that SKPs co-cultured with C2C12 cells have the ability to fuse and form myotubes (M. Akhavan, personal communication), but recent concerns about cell fusion and labeling, have reduced the validity of co-culture studies. Instead I decided to focus on an isolated culture system. Treatment with 5-azaC has been used successfully to transform multiple cell types into skeletal or cardiac muscle *in vitro* (Rangappa *et al.,* 2003, Wakitani *et al.,* 1995). These studies were performed in basal culture conditions of DMEM supplemented with fetal bovine or horse serum. After testing SKPs in these conditions and obtaining few immature putative myoblasts, I looked at ways to optimize the conditions to increase muscle differentiation in our cells.

Treatment with different growth factors, serum or the combination of the two can bias stem cell cultures towards one fate or another, but to date homogeneous differentiated cell cultures have not been obtained. One option for increasing the yield of a preferred cell type is to identify a cell specific surface molecule and use FACS sorting to isolate a relatively pure population, or to use cell size, differential adherence, or response to treatments to separate cell types. As reliable and specific cell surface markers have not yet been identified for SKPs or for immature muscle, FACS sorting was not a

viable option. The possibility I decided on was to optimize culture conditions to such a level that a clonally derived cell could differentiate preferentially into a single desired cell type.

The final optimized conditions used for SKP differentiation were based on the literature for primary myoblast culture systems. bFGF was included for the first few days *in vitro* to enhance cell division and to increase incorporation of 5-azaC, a treatment that requires approximately 10 cell divisions to activate myogenesis (Hauschka, 1994). The addition of insulin, chick embryo extract and low serum once cells become confluent had proven an effective strategy for differentiating mouse myoblasts (Haushka, 1994). As immature myoblasts had been obtained using traditional 5-azaC treatments, I reasoned they might be responsive to similar factors that affected myogenesis in primary myoblasts. In this chapter, I show the differentiation of SKPs into immature myoblasts, and the use of optimized conditions to increase the complexity and yield of myoblasts and multinucleated myotubes.

II. Materials and methods

All materials and methods used in this study were as described in Chapter 2, with the addition of the following conditions for the differentiation of SKPs, and the maintenance of C2C12 cell lines to use as positive controls.

Differentiation of SKPs using 5-azacytidine

To differentiate SKPs, spheres were dissociated into single cells which were plated onto four well chamber slides, six well biochemistry dishes or 60 mm culture plates coated with poly-D-lysine and laminin at 3000 cells/mL. For initial muscle specific

differentiation experiments, the methods of Wakitani *et al.* (1995) were followed. Briefly, cells were plated in high glucose DMEM (GIBCO BRL) with 10% FBS, 5% horse serum (Biowhittaker), and 1% penicillin/streptomycin for 24 hours, then treated with 10 μmol/L 5-azacytidine (5-azaC) (Sigma) by changing half the media for 24 hours, then washed twice with warm HBSS and grown in fresh media containing 50 μmol/L hydrocortisone until cells were confluent. For optimized muscle differentiation conditions, cells were plated in high glucose DMEM with 10% FBS, 40 ng/mL bFGF, 50 μmol/L hydrocortisone and 1% penicillin/streptomycin for 24 hours, then treated with 10 μmol/L 5-azaC by changing half the media for 24 hours, then washed twice with warm HBSS and grown in fresh media for two days. bFGF was then removed and cells were grown until 90% confluent. At this point the media was changed to high glucose DMEM, with 2% FBS, 0.5% chick embryo extract (ICN), 10 ng/mL insulin (Sigma) and hydrocortisone to induce myoblast fusion. Media was changed every second day.

Maintenance of C2C12 cell line

The C2C12 cell line is derived from primary mouse myoblasts. These cells can be expanded in culture as a monolayer grown in high glucose DMEM and 10% FBS and passaged 1:4, every 3-4 days. C2C12 cells are passaged by trypsinizing with trypsin-versene until the cells detach, then stopping the trypsin reaction with 10% FBS and spinning down the cells at 1000 rpm for 4 minutes.

III. Results

Preliminary experiments were performed on SKPs plated in high glucose DMEM with hydrocortisone, 10% FBS and 5% horse serum for 7-10 days. This initial differentiation with 5-azacytidine (5-azaC) treatment resulted in immature myoblasts, but no multinucleated myotubes (data not shown). As traditional procedures for myogenic conversion using 5-azaC did not result in terminally differentiated myotubes as defined by the lack of myogenin and MyoD expression in addition to the lack of correct morphology, other culture conditions were tested. Since I observed myoblast-like cells in these cultures, I predicted that extrinsic factors that enhanced the proliferation and differentiation of primary myoblasts would have the same effect on putative myoblasts derived from the skin. The use of 5-azaC for skeletal muscle differentiation requires the treated cells to undergo 10 rounds of cell division. It was possible that the SKPs in culture were differentiating prematurely, so bFGF, a proven mitogen for both myoblasts and stem cells, was added to the cultures for the first four days. Once cells had become confluent, serum levels were dropped and chick embryo extract and insulin were added to induce differentiation.

Once conditions had been established, muscle marker expression was investigated using multiple techniques. Myf5 is the earliest muscle specific marker to be expressed both *in vivo* and *in vitro*. RT-PCR on 5-azaC treated SKPs showed that the gene was being transcribed (Figure 6 A). I was unable, however to detect the protein using Western blots (Figure 6 B) although immunocytochemistry revealed robust nuclear expression in a small subpopulation of cells (Figure 6 C, D). This discrepancy between the different approaches used to detect muscle-specific protein and mRNA, was likely due to the

increased sensitivity that immunocytochemistry and RT-PCR have for small populations of cells, over Western blots. Alternatively, it could be the result of the different experimental end points, or the inherent variability between the cultures analyzed by Western blot, compared to the cultures analyzed by RT- PCR and immunocytochemistry.

MyoD plays an essential role in muscle specification and differentiation and is transiently expressed in myoblasts prior to cell fusion. MyoD mRNA was not detected by RT-PCR (Figure 7 A), nor was MyoD protein detectable by Western blot (Figure 7 B). Immunocytochemistry revealed rare occurrences of MyoD expression in two cultures out of multiple cultures examined (Figure 7 C). In those rare cultures, MyoD co-labeled with desmin (Figure 7 D) and these cells demonstrated appropriate morphology (Figure 7 E). Myogenin is another transcription factor which is required for cell fusion. In 5-azaC treated SKPs, this gene was not detectable by RT-PCR (Figure 7). As we did not have an appropriate antibody for the protein, immunocytochemistry and Western blot analysis were not carried out.

The main MRF expressed in adult muscle is MRF4. In 5-azaC treated SKPs MRF4 mRNA was detectable by RT-PCR (Figure 8 A), though MRF4 protein was not detectable by Western blot analysis (Figure 8 B). However, immunocytochemistry demonstrated that MRF4 protein was detectable in a small subpopulation of differentiated SKPs (Figure 8 C), where it demonstrated an appropriate nuclear localization in cells exhibiting a mature fused morphology (Figure 8 D). The apparent discrepancy between the RT-PCRs and immunocytochemistry versus the Western blots is likely due to differences in sensitivity.

The earliest expressed structural protein found in muscle is desmin. Though it is a good early myoblast marker, desmin is also a key component of smooth muscle, emphasizing the need to rely on morphological criteria in addition to protein expression. RT-PCR showed desmin to be transcribed in SKPs treated with 5-azaC in optimized conditions for 10 days (Figure 9 A). Western blot appeared to detect a faint band in both treated and non-treated SKPs after 24 days in culture (Figure 9 B). This is likely due to the presence of smooth rather then skeletal muscle, as the level of expression appears to be the same in both treatment groups and the samples were negative for all other markers of skeletal muscle (Figures 6-10). Immunocytochemistry showed patches of positive cells with a similar expression pattern and morphology to primary myoblasts (Figure 9 C). No mature fused myotubes expressing desmin were detected.

Myosin heavy chain (MyHC) fast and slow were looked for in 5-azaC treated SKPs. Nether MyHC fast or MyHC slow were detected by Western blot (Figure 10 A) and MyHC slow was never observed by immunocytochemistry (data not shown). MyHC fast was seen reliably by immunocytochemistry in both immature myoblast-like cells, some of which were double labeled with myf5 (Figure 10 B, C) and in mature multinucleated myotubes (Figure D, E). The differentiation into a mature phenotype such as this was a rare but reproducible event, in three separate lines of SKPs.

Finally, dystrophin is the protein lacking in Duchenne's muscular dystrophy and as such has considerable potential in a clinical setting for transplants or gene therapy. 5-azaC treated SKPs have been shown to express dystrophin by RT-PCR (Figure 11 A), which is promising, but as shown previously, the primers used were also able to detect the neuronal isoform of dystrophin (Figure 2), so the data were not conclusive. By

immunocytochemistry, some positive cells were observed to be ready to fuse and double labeled with MyHC fast (Figure 11 B, C). This strongly suggests that in the rare cases of myogenic differentiation, these cells can express dystrophin.

The expression of early and late markers of skeletal muscle differentiation in SKPs is not consistent with regards to the amount, extent or timing of differentiation, but these results have been shown to be reproducible with multiple different SKP lines of both early and later passages. This suggests that myogenesis is occurring, even though we do not fully understand the regulation of this process in SKPs.

III. Discussion

Exploring the ability to differentiate SKPs into skeletal muscle *in vitro* serves two distinct purposes. First, it expands our knowledge of this novel stem cell with regards to its differentiation capacity down a mesodermal lineage such as skeletal muscle. Second, it provides a potential new source of cells for transplantation or other cell based therapies, in a variety of muscle degenerative diseases. To date there are a number of stem cells that are reported to be plastic, or able to differentiate into cell types originating from more then one embryonic lineage. Other then ES cells, which are truly totipotent, the plasticity of most adult stem cells has recently begun to be questioned. The issue of cell fusion has been brought up, suggesting that the most apparent examples of stem cell plasticity following transplantation, was due to donor cells fusing with, and being reprogrammed by the host nuclei (Wang *et al.,* 2003, Terada *et al.,* 2002, Ying *et al.,* 2002). This has been demonstrated with hematopoietic stem cells *in vivo*, and with neural and bone marrow stem cells *in vitro*. Because of concerns about cell fusion, all the *in vitro*

differentiation of SKPs in this study was performed in an isolated culture system to ensure that any muscle formed was a result of transdifferentiation of the original undifferentiated spheres.

Treatment with 5-azacytidine (5-azaC) has been proven to be an effective way of directing the differentiation of stem cells, cell lines and fibroblasts towards a myogenic phenotype in an isolated culture system. Previous work has shown successful muscle differentiation, in basal conditions of high glucose and high serum, of mesenchymal stem cells and bone marrow derived stem cells (Rangappa *et al.,* 2003,Wakitani *et al.,* 1995). Following treatment of SKPs with 5-azaC in basal conditions, immature myoblasts-like cells were obtained, but there was no evidence of the mature, contractile myotubes observed in the previous studies. By optimizing culture conditions with a variety of cytokines known to influence myoblast differentiation, we were able to obtain a small percentage of fused, multinucleated myotubes, through contractile properties were never observed. Obviously, the optimal conditions for differentiation of SKPs into fully mature skeletal muscle have not yet been determined. However, mesenchymal stem cells, and bone marrow have both been successfully differentiated into skeletal muscle in high serum following treatment with 5-azaC, without additional cytokines which raises the question of why SKPs have such different requirements (Rangappa *et al.,* 2003, Wakitani *et al.,* 1995). It is possible that the mesenchymal stem cells are closer to a myogenic lineage then SKPs are and do not require as extensive reprogramming to become a novel mesodermal cell type. SKPs are believed to originate from the neural crest (F. Miller, personal communication), which does not form skeletal muscle *in vivo*, and as such would require more specialized conditions to induce differentiation into this phenotype.

There have been no studies to date, showing neural crest stem cells with the ability to differentiate into skeletal muscle *in vivo* or *in vitro*, though both smooth and cardiac muscle are developmentally derived from the neural crest (Pietri *et al.,* 2003).

In order to determine the best conditions for SKPs to differentiate appropriately there are many issues still unresolved regarding the timing and addition of different cytokines. Time course experiments would help begin to solve this problem, by determining the best point to switch cells from proliferation to differentiation conditions, in order to increase the differentiation of SKPs.

Even though conditions were not optimal, cells with properties of skeletal muscle were observed in most cultures, but the amount was highly variable and demonstrated an unusual molecular expression profile. Members of the MRF family of transcription factors have been shown to have a great deal of overlap functionally, which can explain the formation of muscle in mice or cultured myoblasts, lacking one or more of these factors. 5-azaC treated SKPs expressed myf5 robustly at early time points as detected by RT-PCR and immunocytochemistry. When myf5 is overexpressed in other cell lines and in fibroblasts, it is sufficient to activate the other MRFs and induce myogenic differentiation (Valdez *et al.,* 2000). This has not been observed with normal myf5 expression, as demonstrated by the creation of a triple knockout mouse lacking MyoD, MRF4 and myogenin, in which myf5 was activated embryonically, but its expression was not sustained and subsequent myogenesis was not able to occur *in vivo* (Valdez *et al.,* 2000). In addition, cultured primary myoblasts from this triple knockout mouse were unable to differentiate, in contrast to myogenin -/- or MyoD-/-; MRF4-/- muscle, which can form a reduced number of apparently normal myotubes *in vitro*, likely due to the

presence of the additional MRF(s) (Rawls *et al.,* 1998). The myf5 expression in SKPs is likely not sufficient on its own to support myogenesis, but in conjunction with the expression of MRF4 and occasionally MyoD, it is conceivable that complete differentiation could occur.

The lack of myogenin expression in our cultures was a concern, as this gene is turned on at the time of terminal differentiation and is required for fusion of the myotubes (Rawls *et al.,* 1998). In the absence of myogenin, myoblasts are specified, though their differentiation into mature myotubes is rare, and thought to rely on the up-regulation of MRF4 (Rawls *et al.,* 1998). An interesting observation was noted in Hauschka (1994) that slow twitch fibres (expressing MyHC slow) had relatively more myogenin expression then fast twitch fibres (MyHC fast), which express more MyoD. This could help explain the lack of MyHC slow expression in our cultures, while MyHC fast was consistently observed in both presumptive myoblasts and fused myotubes. Though MyoD expression was rarely observed, it was likely being turned on transiently, which allowed the observed fusion of myoblasts in the absence of myogenin. It is unknown whether the presence of myf5 and MRF4 is sufficient for terminal differentiation in the absence of both MyoD and myogenin. Though differentiation may be possible in this situation, the creation and analysis of a mouse lacking MyoD and myogenin, could resolve this issue. The most plausible explanation is that myf5, MyoD and MRF4 are being expressed in a pattern similar to that seen in the myogenin -/- mouse, where myoblasts are present, and the appearance of multinucleated, fused myotubes are a rare event regulated by increases in MRF4 expression.

The percentage of skeletal muscle found in culture, was variable within and between 5-azaC treated SKPs and could be explained by the inherent differences in primary cultures or the time spent in passage. It is well documented that time in culture can effect stem cell properties. Morshead *et al.* (2002) compared neurospheres kept in passage for short or long lengths of time and found that long-term passaged neurospheres proliferated faster, had a greater tendency to adhere to the culture surface, and lost their dependency on growth factors. Importantly, they noted no changes in differentiation potential over time. Short and long-term passaged SKPs have also been examined for these property changes and it was found that long-term passaged spheres proliferated faster, but never lost their dependency on growth factors or their multipotentiality (F. Miller, personal communication). This increased growth rate was a concern, as different cultures would become confluent faster then others, which affected the time spent in culture as well as the extent of differentiation. A dependency on density for differentiation is known as the community effect and has a powerful effect on many cell types including muscle, neurons and glia. Cells tend to respond to neighboring cues and differentiate differently depending on their environment. For example, both cortical and neural crest stem cells can differentiate into neurons and glia at high densities, but into smooth muscle at low density (Tsai & McKay, 2000, Hagedorn *et al.,* 1999). Some smooth muscle cell lines also show a cell-cell contact effect as they can differentiate into skeletal muscle at high density but otherwise remain as smooth muscle (Graves & Yablonka-Reuveni, 2000). Primary cultures of skeletal muscle require a high density in order to become terminally differentiated and fuse, which lead to our decision to allow SKPs to become as dense as possible while remaining adherent. Due to differential

growth rates of the SKP lines, the time required to reach this point was highly variable resulting in inconsistent time points throughout the experiment. In our cultures, patches of muscle cells were observed, surrounded by unidentified non-muscle cells, suggesting local effects of cell-cell contact. While the density of muscle-like cells was high in these patches, the percentage of patches overall was low which likely accounted for our negative results with Western blotting and our inability to accurately quantify our data.

Another issue more specific to the skeletal muscle differentiation of SKPs, then to other adult stem cells, is one of possible muscle cell contamination in the culture. As shown in the previous chapter, skin expresses a significant amount of skeletal muscle markers by both RT-PCR and western blot, due to the presence of the paniculus carnosus. In addition, a small subpopulation of primary dissociated skin cells can differentiate into mature, contractile myotubes *in vitro*. The only way to conclusively overcome this issue of possible contamination is to clonally derive a line of SKPs that can be differentiated into neurons and glia, as well as skeletal muscle and other mesodermal derivatives. This experiment will be necessary in the future. As an alternative, we chose instead to test both early and late passaged spheres for the presence of muscle markers. This should have revealed any latent myoblasts from the original culture. As no expression was detected in any of the spheres, we felt relatively confident that our results were due to differentiation, rather then contamination in the culture.

The second reason for investigating stem cell differentiation into muscle is therapeutic in nature. Duchenne's muscular dystrophy (DMD) is a degenerative disease that affects the stability of the muscle fibres. This causes a continual cycle of degeneration and regeneration on the muscle until the satellite cell population is

eventually depleted. At this point, muscle tissue begins to be replaced by fat and other mesenchymal derivatives. The mdx mouse is an animal model of DMD, which has been used to test the ability of stem cells to engraft and restore dystrophin function *in vivo* (Gussoni *et al.,* 1999). The benefit of using SKPs for transplants is that they can be easily isolated from a living donor. Furthermore, they may even have the potential to be isolated from the dystrophic individual and be genetically modified to express functional dystrophin, after which they could be transplanted effectively without immune rejection. This study has demonstrated SKPs have the ability to become skeletal muscle *in vitro*. Though the yield is low, further optimization of culture conditions should be able to increase this number. If the skeletal muscle yield from SKPs can be increased, future studies would be required to determine whether SKPs can maintain transgene expression over time and whether they could prove effective in repopulating damaged muscle.

Summary and Conclusions

I established an accurate assay for detecting skeletal muscle differentiation *in vitro*. By utilizing three different techniques, RT-PCR, Western blotting and immunocytochemistry, I was able to determine criteria for the differentiation of SKPs into skeletal muscle. I found that whole muscle and whole skin strongly expressed all of our muscle specific markers, while whole brain proved to be negative for all markers with the exception of dystrophin. The finding that skin expressed muscle markers at a significant level, made proving that the skeletal muscle found in culture was derived from the SKPs, rather then from contamination, more difficult. I tested both early and late passage spheres for the presence of muscle specific mRNA and protein and found them to

be negative for all markers, which made me more confident of my results, though clonally derived SKPs are still required for a complete analysis of their differentiation capacity.

I treated SKPs with 5-azacytidine and found that they appear to have the potential to differentiate into immature myoblasts or satellite cells. With the addition of various cytokines, notably bFGF, insulin and chick embryo extract, the extent of differentiation was increased with the appearance of the occasional fused, multinucleated myotube. SKP derived myoblasts expressed myf5, desmin and occasionally MyoD, while the fused myotubes were positive for myosin heavy chain fast, dystrophin and MRF4. All markers were detected by immunocytochemistry and RT-PCR, while western blot was not sensitive enough to detect protein levels in such a small percentage of positive cells. While the amount of skeletal muscle derived from SKPs was highly variable both between and within cultures, a low level of skeletal muscle differentiation was occurring, suggesting a novel mesodermal cell type that can be obtained from this stem cell. The expression of dystrophin in SKP derived myotubes indicates these cells may have a therapeutic role in transplantation for individuals with muscular dystrophy in the future.

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References

Agbulut, O., Li, Z., Perie, S., Ludosky, M., Paulin, D., Cartaud, J. and Butler-Brown, G. (2001). Lack of desmin results in abortive muscle regeneration and modifications in synaptic structure. Cell Motility and the Cytoskeleton, 49, 51-66.

Andrews, P.W. (2002). From teratocarcinoma to embryonic stem cells. Philosophical Transitions of the Royal Society of London B, 357, 405-417.

Allen, D.L. and Leinwand, L.A. (2001). Postnatal myosin heavy chain isoform expression in normal mice and mice null for IIb or IId myosin heavy chains. Developmental Biology, 229, 383-395.

Asakura, A., Komaki, M. and Rudnicki, M.A. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. Differentiation, 68, 245-253.

Bain, G., Kitchens, D., Yao, M., Huettner, J.E. and Gottlieb, D.I. (1995). Embryonic stem cells express neuronal properties *in vitro*. Developmental Biology, 168, 342-357.

Bischoff, R. (1994). The Satellite Cell and Muscle Regeneration. In Myology, 2nd edition, ed. A.G. Engel & C. Franzini-Armstrong, McGraw Hill, New York, 97-112.

Brand-Saberi, B. and Christ, B. (1999). Genetic and epigenetic control of muscle development in vertebrates. Cell and Tissue Research, 296, 199-212.

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

Clarke, D.L., Johansson, C.B., Wilbertz, J., Veress, B., Nilsson, E., Karlstrom, H., Lendahl, U. and Frisen, J. (2000). Generalized potential of adult neural stem cells. Science, 288, 1660-1663.

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

Corti, S., Strazzer, S., Del Bo, R., Salani, S., Bossolasco, P., Fortunato, F., Locatelli, F., Soligo, D., Moggio, M., Ciscato, P., Prelle, A., Borsotti, C., Bresolin, N., Scarlato, G. and Comi, G.P. (2002). A subpopulation of murine bone marrow cells fully differentiate along the myogenic pathway and participates in muscle repair in the mdx dystrophic mouse. Experimental Cell Research, 277, 74-85.

Cossu, G. and Mavilio, F. (2000). Myogenic stem cells for the therapy of primary myopathies: wishful thinking or therapeutic perspectives? The Journal of Clinical Investigation, 105(12), 1669-1674.

Daley, G.Q. (2003). From embryos to embryoid bodies: Generating blood from embryonic stem cells. Annals of New York Academy of Science, 996, 122-131.

Daubas, P., Tajbakhsh, S., Hadchouel, J., Primig, M. and Buckingham, M. (2000). Myf5 is a novel early axonal marker in the mouse brain and is subjected to post-transcriptional regulation in neurons. Development, 127, 319-331.

Deacon, T, Dinsmore, J., Constantini, L.C., Ratliff, J. and Isacson, O. (1998). Blastula-stage stem cells can differentiate into dopaminergic and serotonergic neurons after transplantation. Experimental Neurology, 149, 28-41.

De Bari, C., Dell'Accio, F., Vandenabeele, F., Vermeesch, J.R., Raymackers, J. and Luyten, F.P. (2003). Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. Journal of Cell Biology, 160, 6, 909-18.

de la Haba, G., Khatami, M., Cooper, G.W., Backlund, P. and Flaks, J.G. (1999). Alanine or pyruvate is required for the development of myotubes from myoblasts and cortisol satisfies this requirement. Molecular and Cellular Biochemistry, 198, 163-170.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science, 279, 1528-1530.

Fukada, S., Miyagoe-Suzuki, Y., Tsukihara, H., Yuasa, K., Siguchi, S., Ono, S., Tsujikawa, K., Takeda, S. and Yamamoto, H. (2001). Muscle regeneration by reconstitution with bone marrow or fetal liver cells from green fluorescent protein-gene transgenic mice. Journal of Cell Science, 115, 1285-1293.

Galli, R., Borello, U., Gritti, A., Minasi, M.G., Bjornson, C., Coletta, M., Mora, M., Cusella De Angelis, M.G., Fiiocco, R., Cossu, G. and Vescovi, A.L. (2000). Skeletal myogenic potential of human and mouse neural stem cells. Nature Neuroscience, 3(10), 986-991.

Galvin, C.D., Hardiman, O. and Nolan, C.M. (2003). IGF-1 receptor mediates differentiation of primary cultures of mouse skeletal myoblasts. Molecular and Cellular Endocrinology, 200, 19-29.

Gerhart, J., Bast, B., Neely, C., Iem, S., Amegbe, P., Niewenhuis, R., Miklasz, S., Cheng, P.F. and George-Weinstein, M. (2001). MyoD-positive myoblasts are present in mature fetal organs lacking skeletal muscle. Journal of Cell Biology, 155, 3, 381-391.

Graves, D.C. and Yablonka-Reuveni, Z. (2000). Vascular smooth muscle cells spontaneously adopt a skeletal muscle phenotype: A unique Myf5-/MyoD+ myogenic program. The Journal of Histochemistry and Cytochemistry, 48(9), 1173-1193.

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

Hagedorn, L., Suter, U. and Sommer, L. (1999). P0 and PMP22 mark a multipotent neural crest-derived cell type that displays community effects in response to TGF-beta family factors. Development, 126, 17, 3781-3794.

Harrison, B.C., Bell, M.L., Allen, D.L., Byrnes, W.C. and Leinwand, L.A. (2002). Skeletal muscle adaptations in response to voluntary wheel running in myosin heavy chain null mice. Journal of Applied Physiology, 92, 313-322.

Hauschka, S.D. (1994). The Embryonic Origin of Muscle. In Myology, 2nd edition, ed. A.G. Engel & C. Franzini-Armstrong, McGraw Hill, New York, 3-73.

Husmann, I., Soulet, L., Gautron, J., Martelly, I. and Barritault, D. (1996). Growth factors in skeletal muscle regeneration. Cytokines and Growth Factor Reviews, 7, 3, 249-258.

Irintchev, A., Rosenblatt, J.D., Cullen, M.J., Zweyer, M. and Wernig, A. (1998). Ectopic skeletal muscles derived from myoblasts implanted under the skin. Journal of Cell Science, 111, 3287-3297.

Kablar, B., Tajbakhsh, S. and Rudnicki, M.A. (2000). Transdifferentiation of esophageal smooth to skeletal muscle is myogenic bHLH factor-dependent. Development, 127, 1627-1639.

LaBarge, M.A. and Blau, H.M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. Cell, 11, 589-601.

Li, Z., Mericskay, M., Agbulut, O., Butler-Brown, G., Carlsson, L., Thornell, L., Babinet, C. and Paulin, D. (1997). Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation and fusion of skeletal muscle. Journal of Cell Biology, 139, 1, 129-144.

Li, H., Choudhary, S.K., Milner, D.J., Munir, M.I., Kuisk, I.R. and Capetanaki, Y. (1994). Inhibition of desmin expression blocks myoblast fusion and interferes with the myogenic regulators MyoD and myogenin. Journal of Cell Biology, 124, 5, 827-841.

Lumelsky, N., Blondel, O., Laeng, P., Velasco, I., Ravin, R. and McKay, R. (2001). Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science, 292, 1389-1394.

Lyons, G.E., Ontell, M., Cox, R., Sassoon, D. and Buckingham, M. (1990). The expression of myosin genes in developing skeletal muscle in the mouse embryo. Journal of Cell Biology, 111, 1465-1476.

Montarras, D., Lindon, C., Pinset, C. and Domeyne, P. (2000). Culture myf5 and MyoD null muscle precursor cells display distinct growth defects. Biology of the Cell, 92, 565-572.

Morshead, C.M., Benveniste, P., Iscove, N.N. and van der Kooy, D. (2002). Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. Nature Medicine, 8, 2, 268-273.

Myer, A., Olson, E.N. and Klein, W.H. (2001). MyoD cannot compensate for the absence of myogenin during skeletal muscle differentiation in murine embryonic stem cells. Developmental Biology, 229, 340-350.

O'Brien, K.F. and Kunkel, L.M. (2001). Dystrophin and muscular dystrophy: Past, present and future. Molecular Genetics and Metabolism, 74, 75-88.

Ortega, S., Ittmann, M., Tsang, S.H., Ehrlich, M. and Basilico, C. (1998). Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. PNAS, USA, 95, 5672-5677.

Passier, R. and Mummery, C. (2003). Origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovascular Research, 58, 324-335.

Pietri, T., Eder, O., Blanche, M., Thiery, J.P., Dufour, S. (2003). The human tissue plasminogen activator-Cre mouse: a new tool for targeting specifically neural crest cells and their derivatives *in vivo*. Developmental Biology, 259, 176-187.

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

Rangappa, S., Fen, C., Lee, E.H., Bongso, A. and Wei, E.S.K. (2003). Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. The Annals of Thoracic Surgery, 75, 3, 775-779.

Rawls, A., Valdez, M.R., Zhang, W., Richardson, J. Klein, W.H. and Olson, E.N. (1998). Overlapping functions of the myogenic bHLH genes MRF4 and MyoD revealed in double mutant mice. Development, 125, 2349-2358.

Rietze, R., Valcanis, H., Brooker, G.F., Thomas, T., Voss, A.K. and Bartlett, P.F. (2001). Purification of a pluripotent neural stem cell from the adult mouse brain. Nature, 412, 736-739.

Rohwedel, J., Maltsev, V., Bober, E., Arnold, H.H., Hescheler, J. and Wobus, A.M. (1994). Muscle cell differentiation of embryonic stem cells reflects myogenesis *in vivo*: Developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Developmental Biology, 164, 87-101.

Sabourin, L.A., Girgis-Gabardo, A., Seale, P., Asakura, A. and Rudnicki, M.A. (1999). Reduced differentiation potential of primary MyoD -/- myogenic cells derived from adult skeletal muscle. Journal of Cell Biology, 144(4), 631-643.

Seale, P., Asakura, A. and Rudnicki, M.A. (2001). The potential of muscle stem cells. Developmental Cell, 1, 333-342.

Stewart, J.D., Masi, T.L., Cumming, A.E., Molnar, G.M., Wentworth, B.M., Sampath, K., McPherson, J.M. and Yaeger, P.C. (2003). Characterization of proliferating human skeletal muscle-derived cells *in vitro*: Differential modulation of myoblast markers by TGF-B2. Journal of Cellular Physiology, 196, 70-78.

Stratton C.J., Bayguinov, Y., Sanders, K.M. and Ward, S.M. (2000). Ultrastructural of the transdifferentiaition of smooth muscle to skeletal muscle in the murine esophagus. Cell and Tissue Research, 301, 283-298.

Sumariwalla, V.M. and Klein, W.H. (2001). Similar myogenic functions for myogenin and MRF4 but not MyoD in differentiated murine embryonic stem cells. Genesis, 30, 239-249.

Tajbakhsh, S and Buckingham, M.E. (1995). Lineage restriction of the myogenic conversion factor myf5 in the brain. Development, 121, 4077-4083.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E. and Scott, E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous fusion. Nature, 416, 542-545.

Toma, J.G., Akhavan, M., Fernandes, K.J.L., Barnabe-Heider, F., Sadikot, A., Kaplan, D.R. and Miller, F.D. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nature Cell Biology, 3, 778-784.

Toma, J.G., El-Bizri, H., Barnabé-Heider, F., Aloyz, R. and Miller, F.D. (2000). Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. Journal of Neuroscience, 20, 20, 7648-7656.

Tsai, R.Y.L. and McKay, R.D.G. (2000). Cell contact regulates fate choice by cortical stem cells. Journal of Neuroscience, 20, 10, 3725-3735.

Valdez, M.R, Richardson, J., Klein, W.H. and Olson, E.N. (2000). Failure of Myf5 to support myogenic differentiation without myogenin, MyoD and MRF4. Developmental Biology, 219, 287-298.

Van der Kooy, D. and Weiss, S. (2000). Why stem cells? Science, 287, 1439-1441.

Verfaillie, C.M. (2002). Adult stem cells: assessing the case for pluripotency. Trends in Cell Biology, 12, 11, 502-508.

Wakitani, S., Saito, T. and Caplan, A.I. (1995). Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle and Nerve, 18, 1417-1426.

Wang, X., Willenbring, H., Akkari, Y., Torimaru, Y., Foster, M., Al-Dhalimy, M., Lagasse, E., Finegold, M., Olson, S. and Grompe, M. (2003). Cell fusion is the principle source of bone-marrow-derived hepatocytes. Nature, 422, 897-901.

Weitzer, G., Milner, D.J., Kim, J.U., Bradley, A. and Capetanaki, Y. (1995). Cytoskeletal control of myogenesis: A desmin null mutation blocks the myogenic pathway during embryonic stem cell differentiation. Developmental Biology, 172, 422-439.

Weimann, J.M., Charlton, C.A., Brazelton, T.R., Hackman, R.C. and Blau, H.M. (2003). Contribution of transplanted bone marrow cells to Purkinje neurons in human and adult brains. PNAS, 100, 4, 2088-2093.

Whittemore, L., Song, K., Li, X., Aghajanian, J., Davies, M., Girgenrath, S., Hill, J.J., Jalenak, M., Kelley, P., Knight, A., Maylor, R., O'Hara, D., Pearson, A., Quazi, A., Ryerson, S., Tan, X., Tomkinson, K.N., Veldman, G.M., Widom, A., Wright, J.F., Wudyka, S., Zhao, L. and Wolfman, N.M. (2003). Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. Biochemical and Biophysical Research Communications, 300, 965-971.

Yablonka-Reuveni, Z., Rudnicki M.A., Rivera A.J., Primig M., Anderson J.E. and Natanson, P. (1999). The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Developmental Biology, 210, 440-455.

Ying, Q., Nichols, J., Evans, E.P. and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature, 416, 545-548.

Yoshiko, Y., Hirao, K. and Maeda, N. (2002). Differentiation in C2C12 myoblasts depends on the expression of endogenous IGFs and not serum depletion. American Journal of Physiology: Cell Physiology, 283, 4, C1278-C1286.

Figure Legends

Figure 1: Comparison of protein lysates from adult brain, P12 skin, P12 muscle and E18 leg by Western blot determined the expression of muscle specific transcription factors myf5, MRF4 and MyoD and structural proteins desmin, myosin heavy chain fast and myosin heavy chain slow. Brain lysates were negative for all markers. Myf5 was positive in embryonic leg, but only weakly expressed in skin and muscle, while all other markers were positive in all three tissues. The Ponceau Red stained membrane shows the rough relative protein levels for each tissue (bottom right).

Figure 2: Comparison of adult brain, P7 skin and P6 muscle by RT-PCR showed muscle and skin to be positive for cDNA corresponding to MyoD, myogenin, MRF4, desmin and dystrophin. Myf5 required increased amplification for detection in muscle and was unclear as to its expression in brain and skin. Otherwise, adult brain was a good negative control for all markers with the exception of dystrophin, where the primers recognized both the neuronal and the muscular isoforms of the gene. The expression of GAPDH shows the presence of DNA following reverse transcription. P7 skin was also positive for GAPDH, though this result is not shown. H_2O was used as a negative control for the RT-PCR reaction.

Figure 3: Primary myoblasts were cultured as positive controls for expression of myf5, MyoD, desmin, myosin heavy chain fast (MyHC fast) and myosin heavy chain slow (MyHC slow). Myf5 (Arrows point to a representative cell showing localization of myf5 signal (A) in the nucleus visualized by Hoechst (B)), MyoD (C) and desmin (D) are

expressed in proliferating myoblasts. MyHC fast (E) and MyHC slow (F) are expressed in differentiated myotubes. Negative control slides of cultured cortical progenitors are shown as inserts for each antibody. Most antibodies had a low background, with the exception of desmin that showed up faintly in the progenitor cultures (D insert). The presence of a high background with desmin made it essential that any interpretation of desmin expression had to be combined with the appropriate morphology or double labeling.

Figure 4: Western blot showing spheres to be negative for MyoD, desmin, MyHC fast and MyHC slow. Both early and late passaged spheres were examined. As results from early and late passaged spheres were similar, only data from the later passaged spheres is shown here. Adult brain, P6 muscle and E18 leg are included as negative and positive controls for each antibody. Ponceau Red stained membrane shows the rough relative protein levels (bottom right).

Figure 5: RT-PCR showing spheres to be negative for myf5, MyoD, MRF4, myogenin, desmin and dystrophin. Data shown is from late passaged spheres, though early passages had the same result. P6 muscle and H_2O were included as controls for the reaction. GAPDH amplification was performed on each sample to ensure the RNA had been successfully reverse transcribed.

Figure 6: Expression of myf5 in 5-azacytidine (5-azaC) treated SKPs. cDNA for myf5 was detected by RT-PCR, while cultured C2C12 cells served as a positive control (A).

GAPDH expression for each sample ensured that the RNA had been successfully reverse transcribed (A). Myf5 protein was also expressed in the nucleus by immunocytochemistry (C, D, arrow points to a positive cell double labeled with Hoechst and myf5, while arrowhead points to a Hoechst labeled cell negative for myf5). Western blot was not able to detect myf5 in SKPs cultured for either 14 or 24 days in vitro (DIV), regardless of whether they were treated or not treated (N/T) with 5-azaC (B). Adult brain, P6 muscle and E18 leg are included as controls and Ponceau Red staining of the membrane shows the rough relative protein levels in each lane.

Figure 7: Expression of MyoD in 5-azacytidine (5-azaC) treated SKPs. Through GAPDH expression shows that cDNA was present in the SKPs and in cultured C2C12 cells used as a positive control (A), RT-PCR was unable to detect MyoD or myogenin, another gene required for cell fusion (A). Western blotting was not able to detect MyoD expression in either 5-azaC treated or not treated (N/T) SKPs, at either time point (14 vs. 24 DIV) (B). Adult brain, P6 muscle and E18 leg served as controls and Ponceau Red staining of membrane shows the rough relative protein levels. Though it is an extremely rare event MyoD expression has been seen by immunocytochemistry in a small population of cells (C arrow points to nuclear expression of MyoD in a Hoechst labeled cell). Some of the MyoD positive cells double labeled with desmin (D arrow shows a double labeled cell, while the arrowhead points to a cell expressing MyoD alone. The hollow arrow shows a cell expressing desmin, but negative for MyoD). These MyoD positive cells had an appropriate, elongated morphology (E).

Figure 8: Expression of MRF4 in 5-azacytidine (5-azaC) treated SKPs. cDNA for MRF4 was detected by RT-PCR in both SKPs and C2C12 cells (A). GAPDH was performed on each sample to ensure the RNA had been successfully reverse transcribed (A). Western blotting was not sensitive enough to pick up positive cells in any of the cell lysates (B, SKPs were either treated with 5-azaC or not treated (N/T) and kept in culture for either 14 or 24 days (DIV)). Adult brain, P6 muscle and E18 leg were used as controls and Ponceau Red staining of the membrane shows the rough relative protein levels in each sample. Immunocytochemistry showed that a subpopulation of 5-azaC treated SKPs displaying the correct morphology, were positive for MRF4 (C, arrows point to positive cells in a fused muscle).

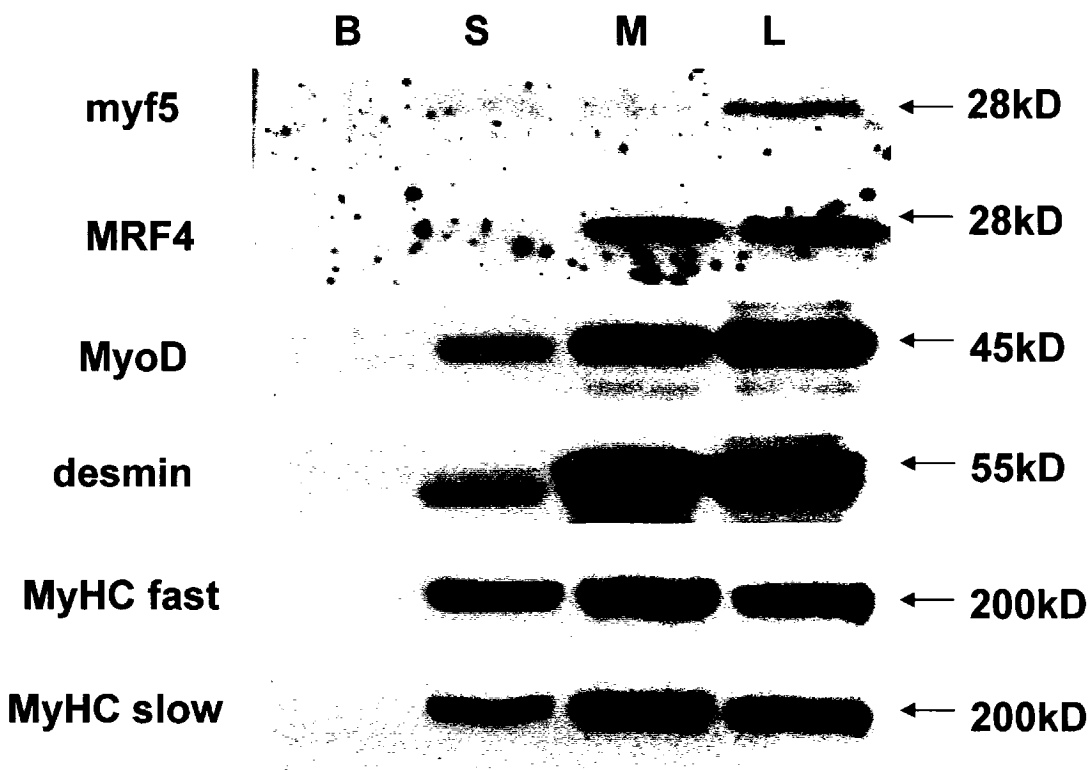
Figure 9: Expression of desmin in 5-azacytadine (5-azaC) treated SKPs. Desmin was detected by RT-PCR in SKPs and C2C12 cells (A). The expression of GAPDH shows that cDNA was present in each sample (A). As detected by Western blot, desmin may be weakly expressed in S1 and S2 SKP lanes, corresponding to cells kept in culture for 24 days and either treated (S1) or not treated (S2) with 5-azaC (B). Adult brain, P6 muscle and E18 leg were included as controls and Ponceau Red staining of the membrane shows the rough relative protein levels. Immunocytochemistry shows patches of positive cells (C, shown by arrows) and higher magnification of a separate field of view reveals desmin positive cells grouped together, appearing ready to fuse (D, arrows show positive cells).

Figure 10: Expression of myosin heavy chain fast (MyHC fast) and myosin heavy chain slow (MyHC slow) in 5-azacytidine (5-azaC) treated SKPs. Western blotting was not

able to detect MyHC fast in either 5-azaC treated or not treated (N/T) SKPs, grown in culture for 14 or 24 days (A). Adult brain, P6 muscle and E18 leg were shown as controls and Ponceau Red staining of the membrane shows the rough relative protein levels in each lane. MyHC fast is positive in both immature myoblast-like cells, which are double labeled with myf5 (B, C, arrows point to immature cells co-expressing MyHC fast and myf5, arrowheads point to MyHC fast positive cells that have down-regulated myf5 and may be ready to fuse) and more mature multinucleated fused myotubes derived from SKPs (D, E). MyHC slow was not detected by immunocytochemistry (data not shown) or by Western blot (A).

Figure 11: Expression of dystrophin in 5-azacytidine (5-azaC) treated SKPs. Dystrophin is expressed by RT-PCR in 5-azaC treated SKPs and in C2C12 cells (A) though primers are not specific for the muscular isoform and have been shown previously to be expressed in the brain (Figure 2). GAPDH expression confirms the presence of cDNA in each sample. Immunocytochemistry revealed cells to be positive for dystrophin (C), which also double labeled with MyHC fast (B). Note the 'streaming' pattern of the cells, which is characteristic of cells preparing to fuse.

Figure 1: Comparison of protein lysates by Western blot determined the expression of muscle specific porteins in brain, skin and muscle



S- P12 skin
M- P12 muscle
B- adult brain
L- E18 leg

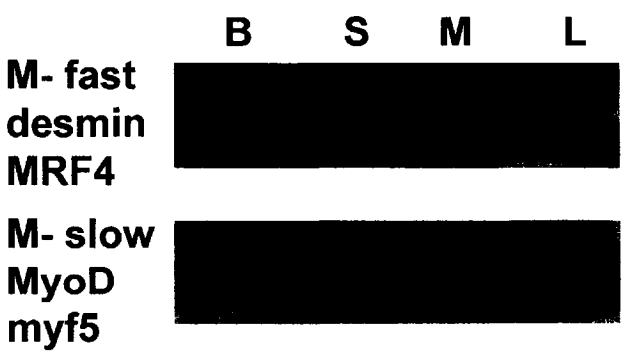


Figure 2: Comparison of cDNA by RT-PCR determined the expression of muscle specific genes in brain, skin and muscle

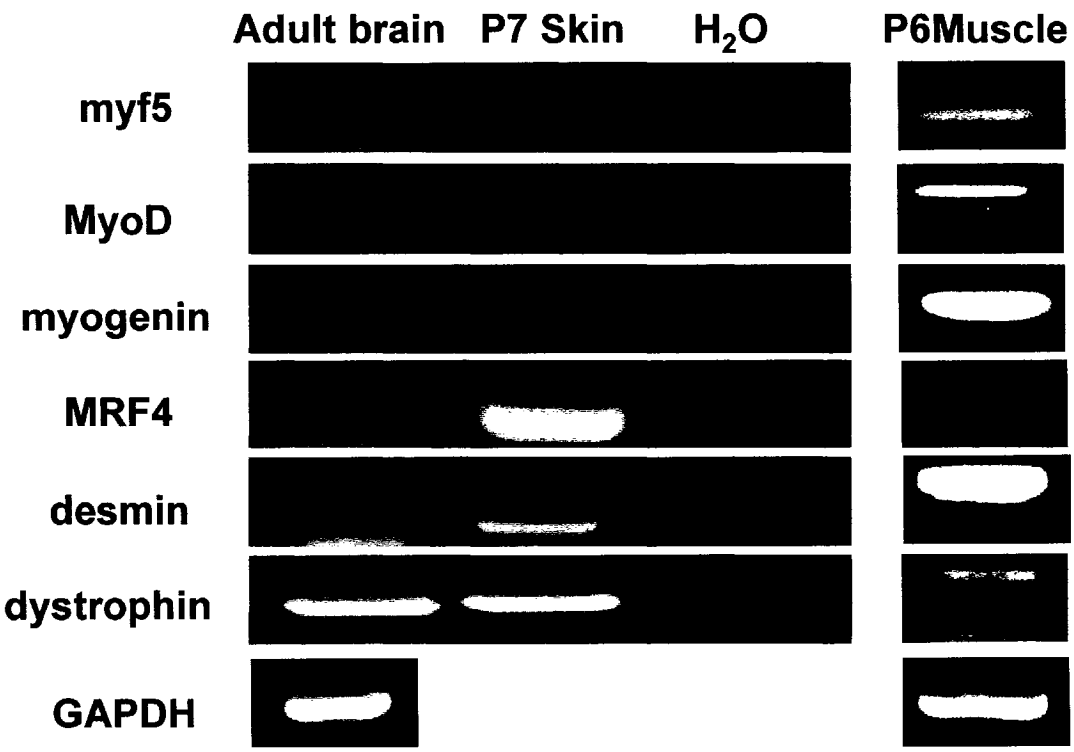


Figure 3: Expression of muscle specific proteins in primary myoblasts by immunocytochemistry

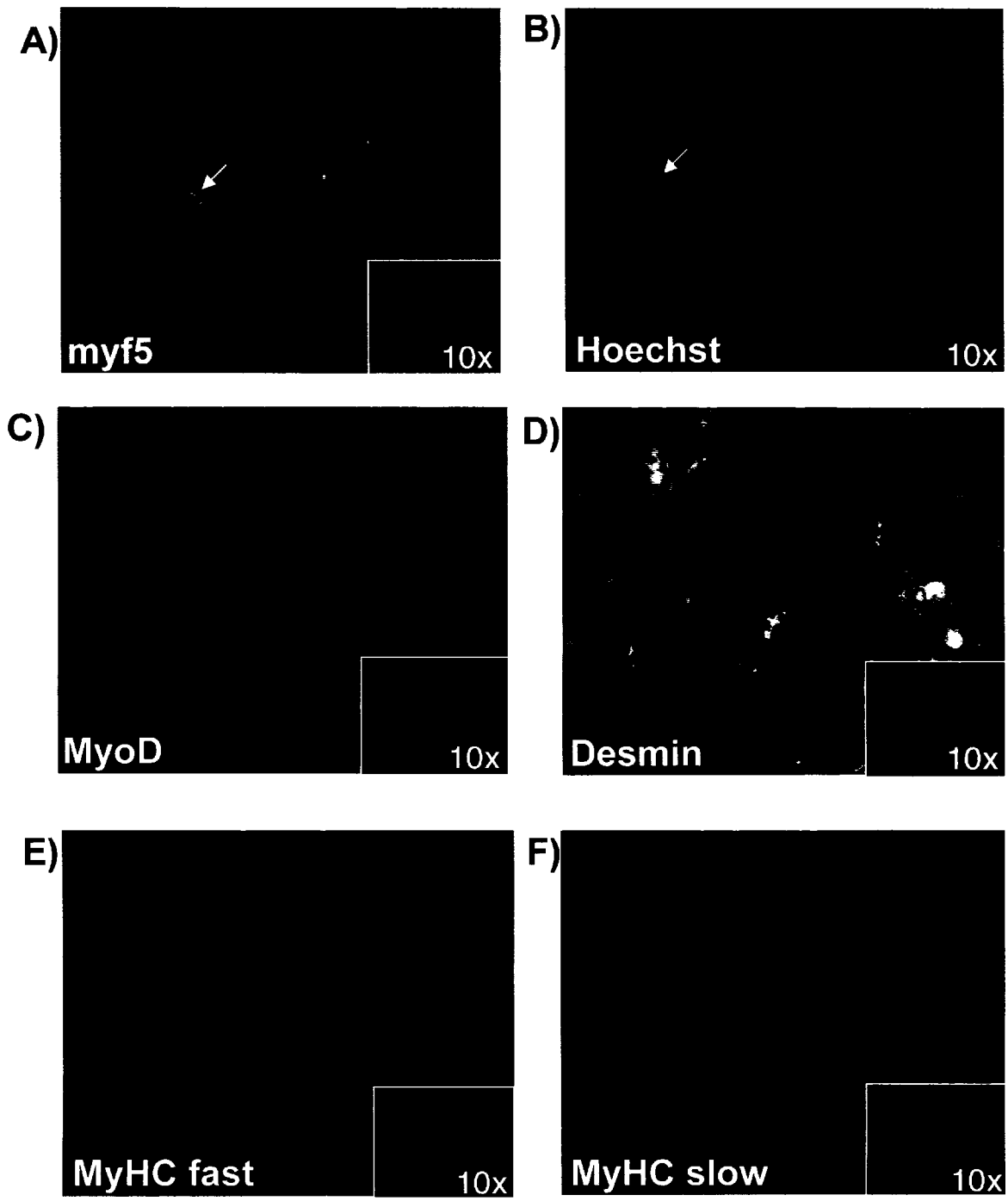
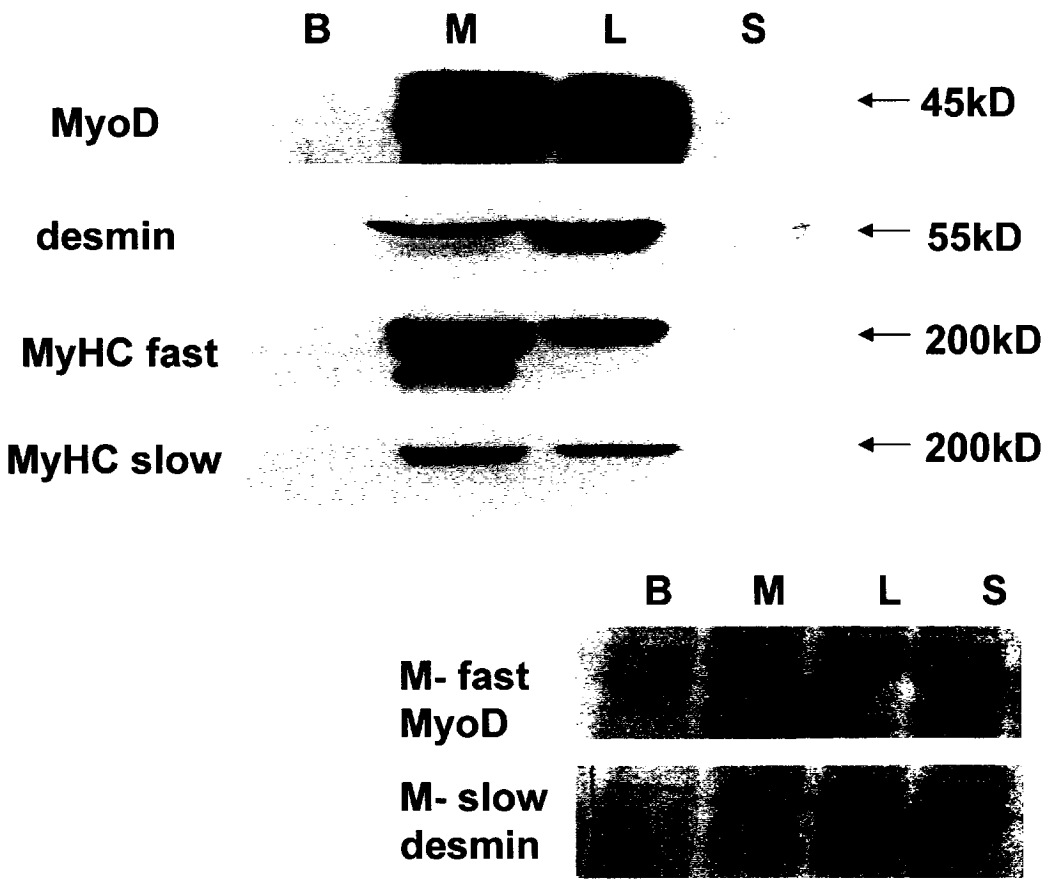


Figure 4: Expression of muscle specific proteins in undifferentiated SKPs spheres by Western blot



B- adult brain
M- P6 muscle
L- E18 leg
S- primary SKPs spheres

Figure 5: Expression of muscle specific genes by RT-PCR in undifferentiated SKPs spheres

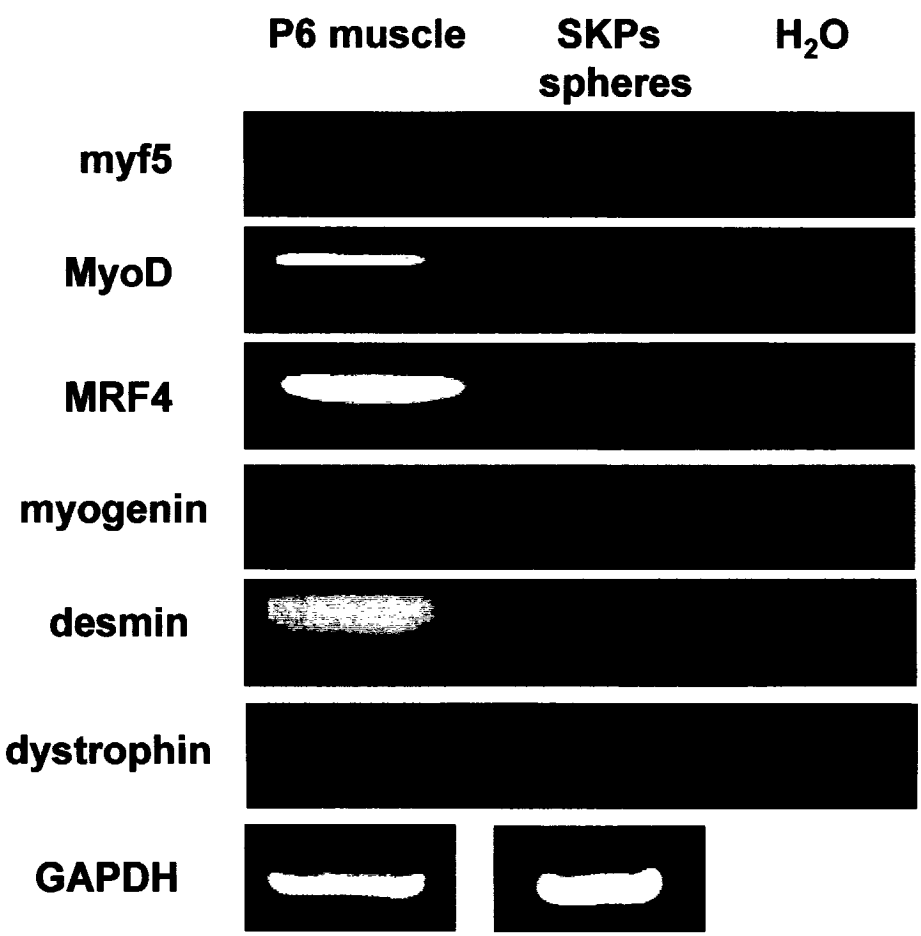


Figure 6: Expression of myf5 in 5-azacytidine treated SKPs

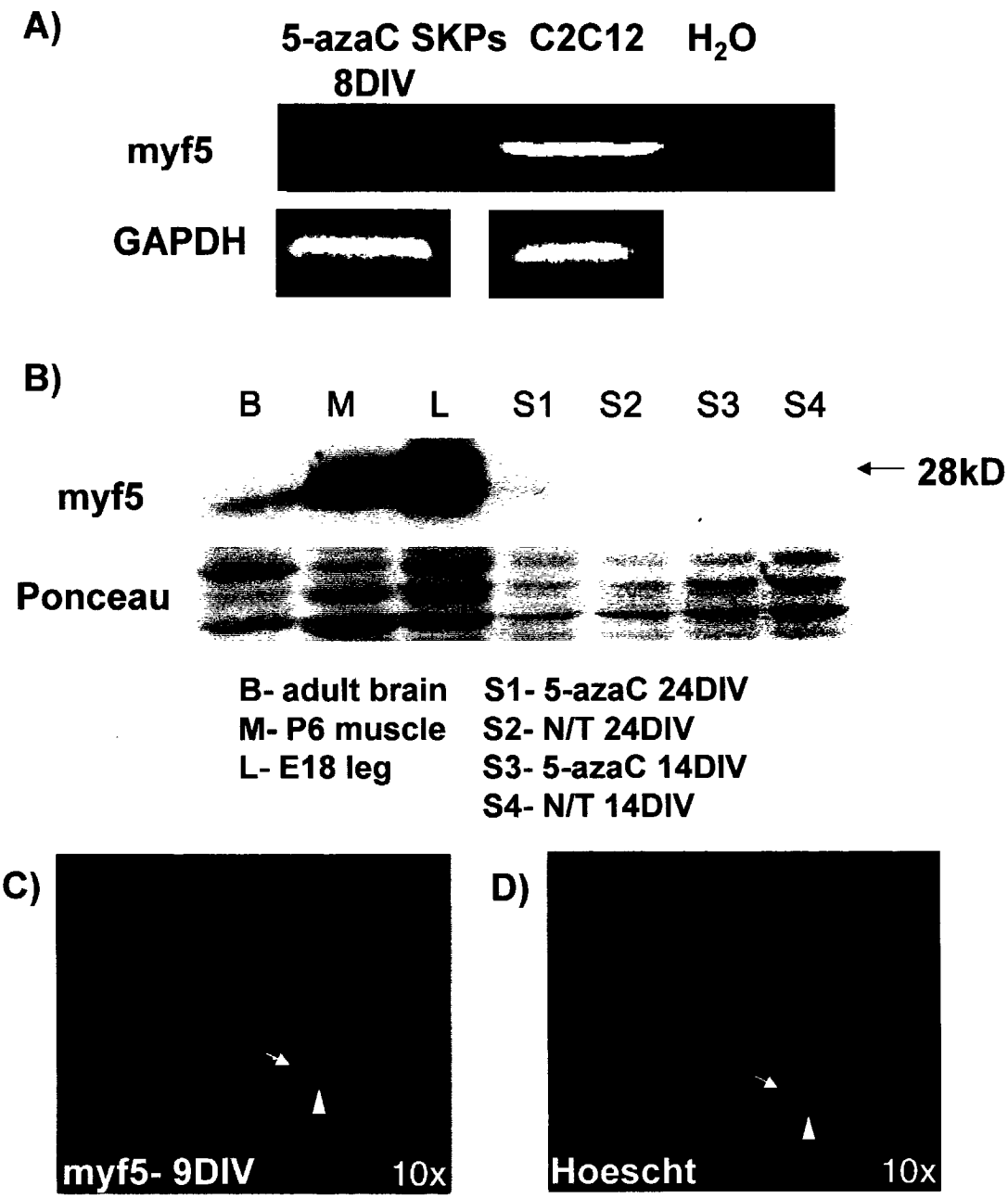


Figure 7: Expression of MyoD and myogenin in 5-azacytidine treated SKPs

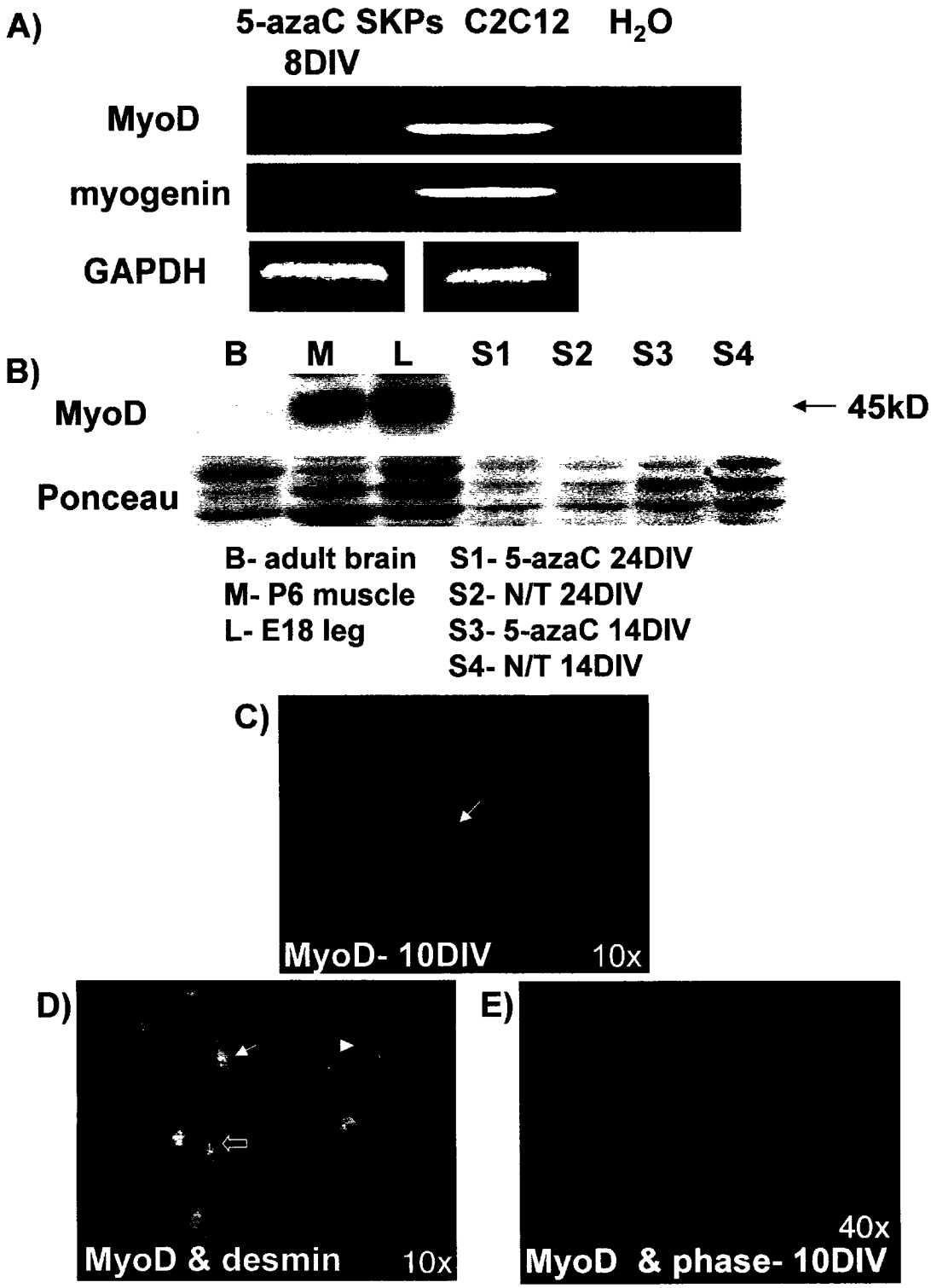


Figure 8: Expression of MRF4 in 5-azacytidine treated SKPs

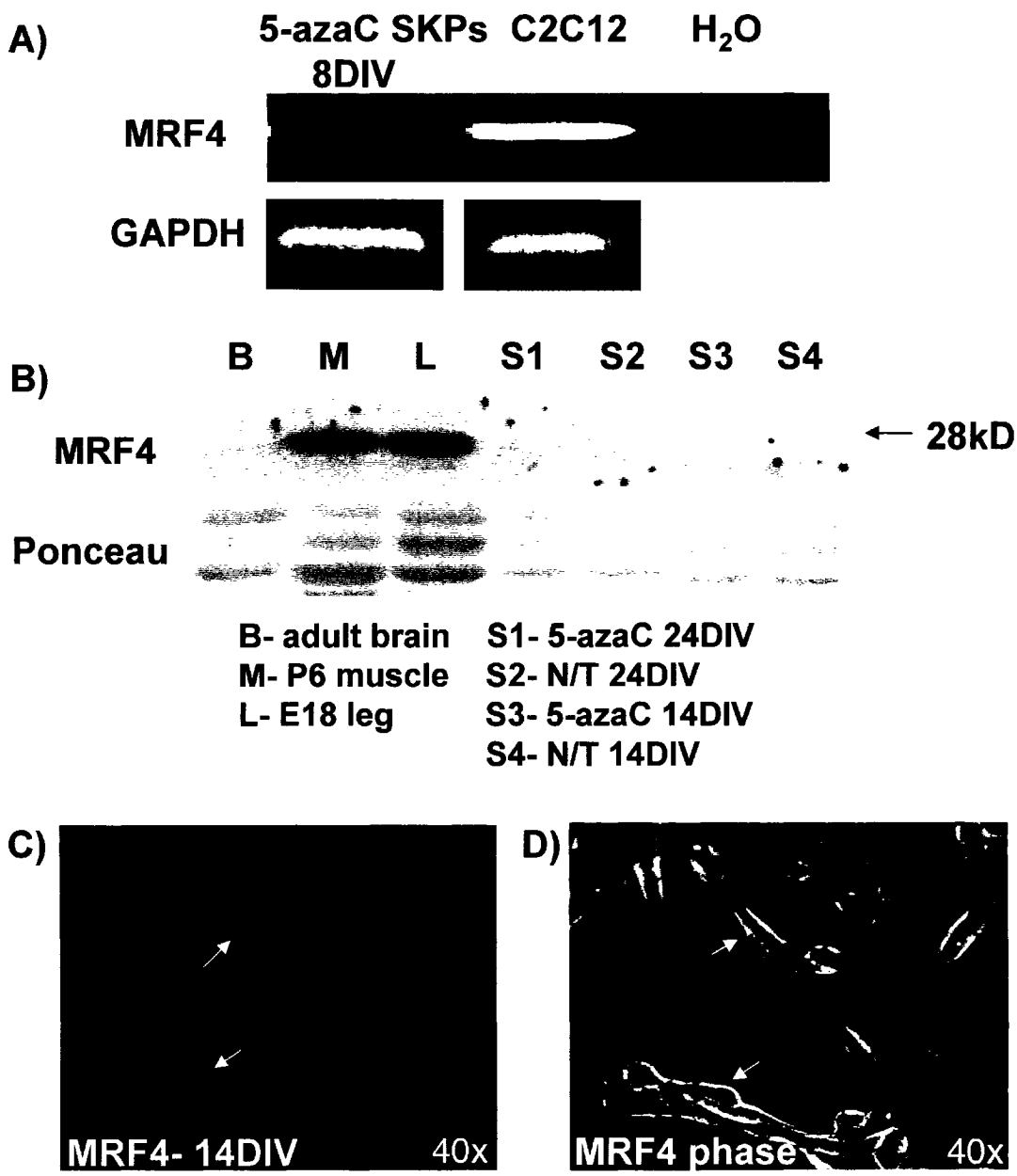


Figure 9: Expression of desmin in 5-azacytidine treated SKPs

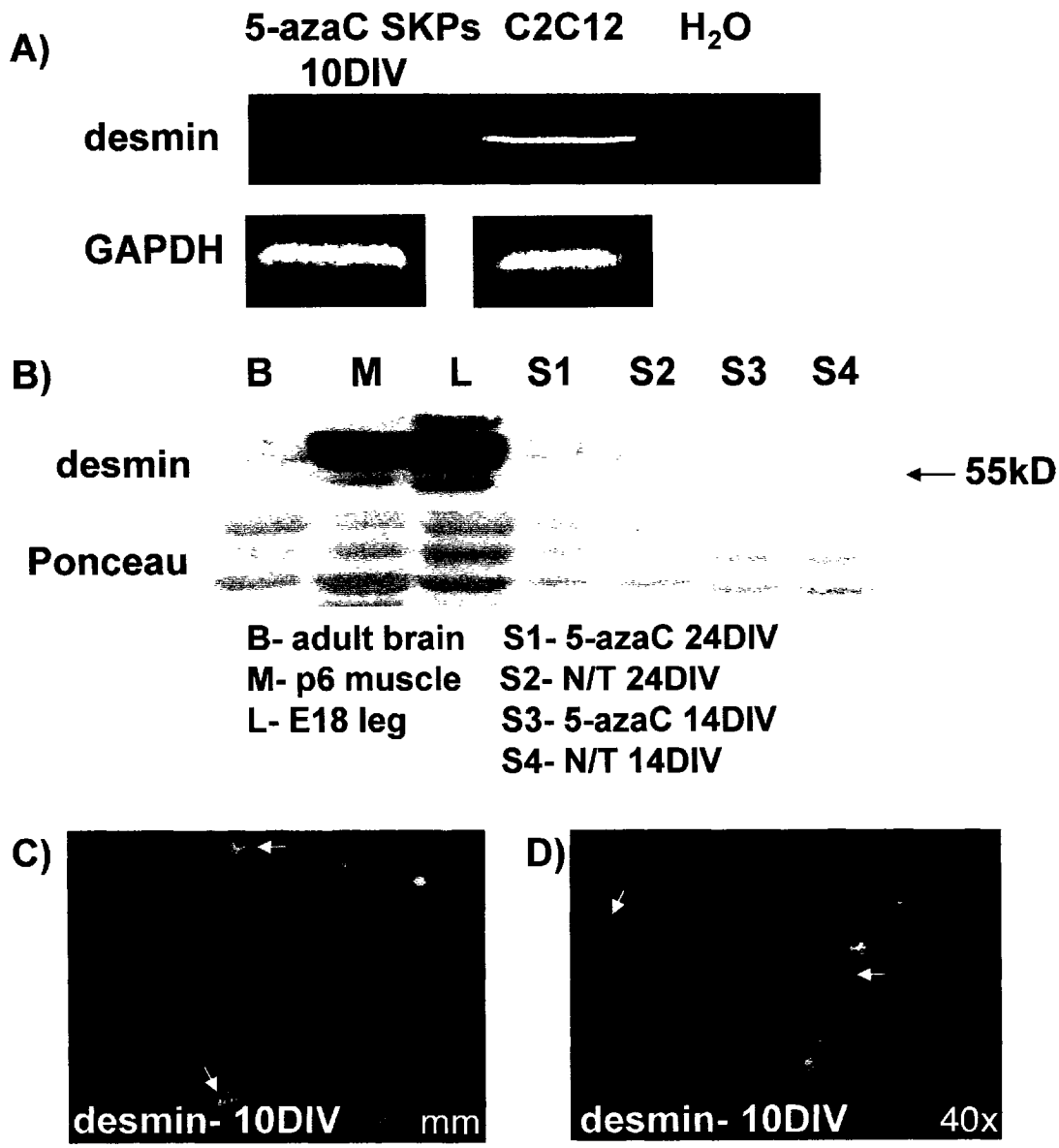


Figure 10: Expression of Myosin heavy chain fast and Myosin heavy chain slow in 5-azacytidine treated SKPs

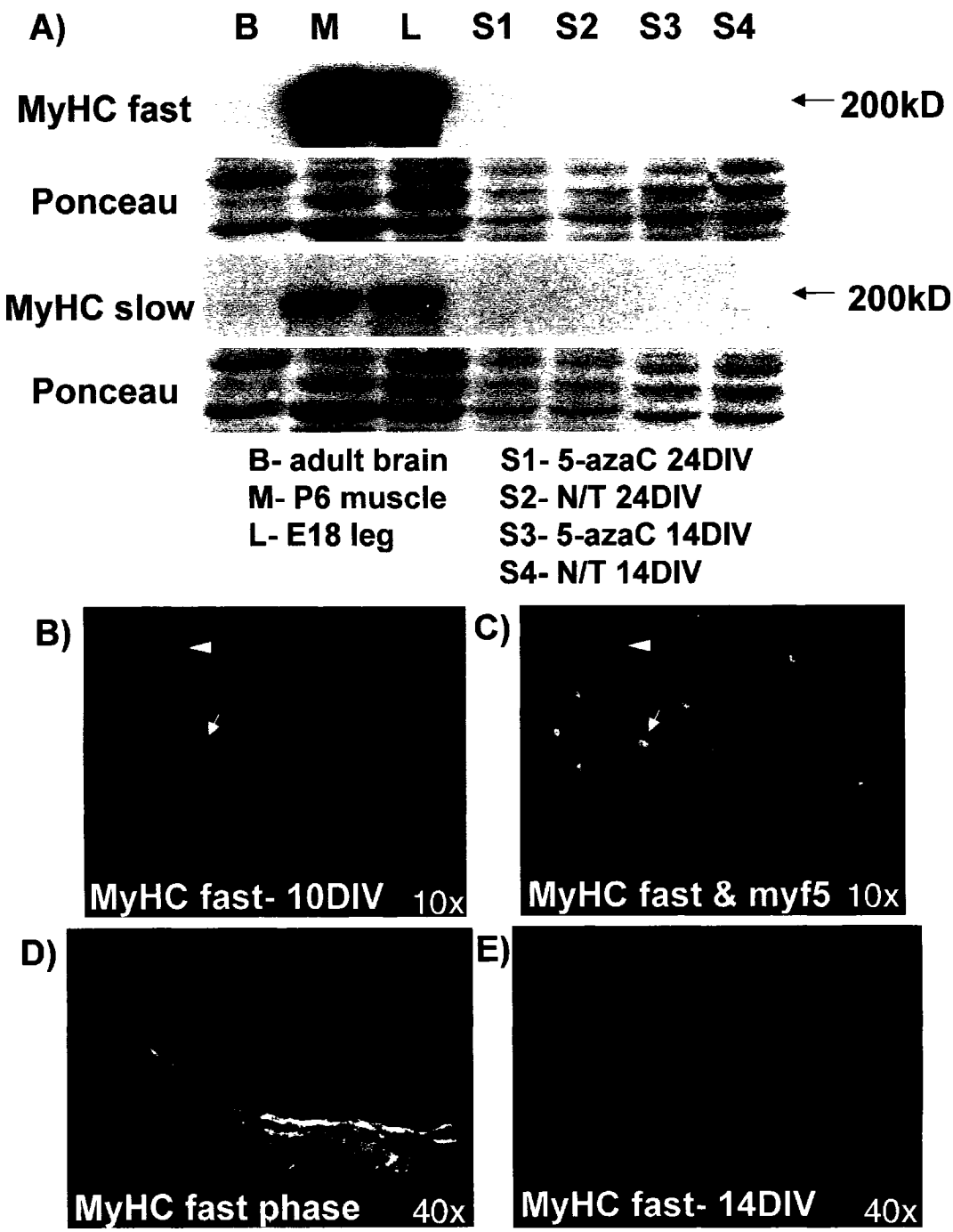


Figure 11: Expression of dystrophin in 5-azacytidine treated SKPs