Directed derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells

Bianca Borchin

BSc. (Hons)

Australian Regenerative Medicine Institute (ARMI)

Faculty of Medicine

Monash University, Australia

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To my parents

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ABSTRACT

Human pluripotent stem cells (hPSCs) constitute a promising resource for use in cell-based therapies and a valuable in vitro model for studying early human development and disease. Despite significant advancements in the derivation of specific fates from hPSCs, the generation of skeletal muscle remains challenging and is mostly dependent on transgene expression. Here, we describe a method based on the use of a small-molecule GSK3β inhibitor to derive skeletal muscle from several hPSC lines. We show that early GSK3β inhibition is sufficient to create the conditions necessary for highly effective derivation of muscle cells. Moreover, we developed a strategy for stringent fluorescence-activated cell sorting-based purification of emerging PAX3+/PAX7+ muscle precursors that are able to differentiate in postsort cultures into mature myocytes. This transgene-free, efficient protocol provides an essential tool for producing myogenic cells for in vivo preclinical studies, in vitro screenings, and disease modeling.

ABBREVIATIONS

AChR, acetylcholine receptor
APC, allophycocyanin
BMP, bone morphogenetic protein
C-MET, Hepatocyte Growth Factor receptor
CXCR4, chemokine (C-X-C motif) receptor 4
DAPI, 4'- 6'diamidino - 2- phenylindole
DLL, delta like ligand
DMD, Duchenne Muscular Dystrophy
DMEM dulbecco's modified eagle medium
DML, dorsal medial lip
EYA, eyes absent
FACS, fluorescence activated cell sorting
FGF, fibroblast growth factor
FITC, fluorescein isothiocyanate
FSC, forward light scatter
GFP, green fluorescent protein
GSK3β, glycogen synthase kinase 3 beta
hESC, human embryonic stem cell
HGF, hepatocyte growth factor
hiPSC, human induced pluripotent stem cell
hPSC, pluripotent stem cell
ITS, insulin transferrin selenium
LMX1A, LIM homeobox transcription factor
MEF, mouse embryonic fibroblast
mM, millimole
MRF, myogenic regulatory factor
MYF5, myogenic regulatory factor 5
MYF6, myogenic regulatory factor 6
MYOG, myogenin

NCAM, neural cell adhesion molecule

PAX, paired box gene

PCR, polymerase chain reaction

PE, R - Phycoerythrin

PS, primitive streak

qPCR, quantitative - PCR

RT-PCR, reverse transcriptase – PCR

SDF-1, stromal cell-derived factor 1

SIX, sine oculis homeobox

SOX, SRY- box containing gene

SSC, side light scatter

VLL, Ventral Lateral Lip

μl, microlitre

μg, microgram

Wnt, wingless - related MMTV integration site

2D, 2 dimensional

1. INTRODUCTION

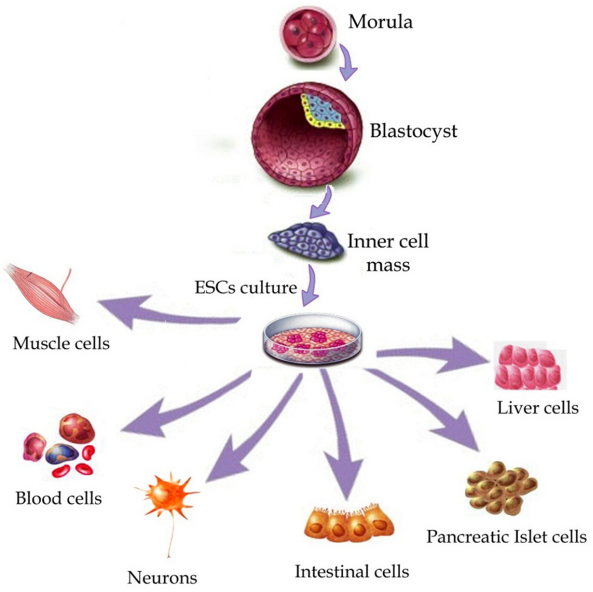
1.1 Stem cells: definition

Stem cells are primitive cells that can either divide to reproduce themselves (self-renewal) or give rise to more specialized (differentiated) cells, such as muscle cells, nerve cells or red blood cells. Stem cells can theoretically divide without limit to replenish other cells (stemcells.nih.gov). Therefore, the most important characteristics of stem cells that distinguish them from other cell types are:

- Self-renewal: cellular division maintains stem cell potential
- Indefinite expansion: undifferentiated cells can be maintained in culture for long periods of time.
- Pluripotency : potential to differentiate into all cell types of the embryo
- Multipotency: potential to differentiate into multiple but limited number of cell types of the embryo.

The two kinds of stem cells from animals and humans currently being used in research are: embryonic stem cells (ESC) and adult (somatic) stem cells (Fig. 1). Fundamental questions in stem cell research focus on understanding how stem cells remain unspecialized and able to self-renew for long periods of time and on identifying the signals the cause stem cells to differentiate into specialized cells.

Human embryonic stem cells



Adult stem cells from the bone marrow

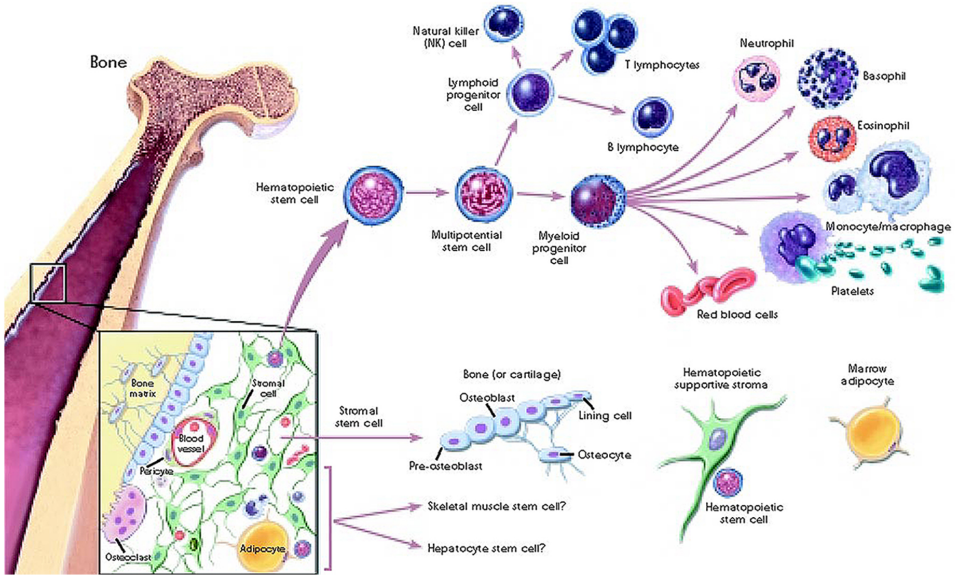


Figure 1. Origin and differentiation potential of human embryonic stem cells (top) and bone marrow stem cells as an example of adult/somatic stem cells (bottom).

(modified from stemcells.nih.gov)

1.2 Adult (somatic) stem cells

An adult stem cell is a resident undifferentiated progenitor cell found within a tissue or organ. Residing in specific areas, referred to as the “stem cell niche”, they remain quiescent until activated by disease or injury, whereby then can differentiate into specialized cell types belonging to their resident tissue or organ. Therefore, the primary role of adult stem cells is to maintain tissue homeostasis and repair the tissue in which they are found. These cells have been found in numerous tissues such as the brain, bone marrow, liver and skeletal muscle (Young and Black, 2004). As such, adult stem cells have become important tools for therapeutic applications. For instance, adult blood-forming stem cells from bone marrow have been used in transplants for more than 40 years (Young and Black, 2004). In the 1960’s, researchers discovered that the bone marrow contains at least two kinds of stem cells: hematopoietic stem cells (Moore and Metcalf, 1970; Till, 1967), which form all types of circulating blood cells, and bone marrow stromal cells discovered a few years later, which are a mixed population of cells capable of generating bone, cartilage and fat (Caplan, 1970; Owen, 1988). Since this discovery, research has advanced towards the successful use of adult hematopoietic stem cells for the treatment of hematological malignancies (Verfaillie, 2002), as well as the evaluation of bone marrow stromal cells for the treatment of a variety of conditions such as cartilage and bone repair (Wei et al., 2013). A significant advantage of using stem cells from an adult is that the patients own cells could be expanded in culture and then reintroduced into the patient. The use of patient’s own autologous cells would circumvent the issue of immune rejection. However, unlike resident bone marrow stem cells, adult stem cells are rare in other tissues with limited cell turnover. In addition, depending on their location, isolation is difficult and their extraction requires invasive procedures. Moreover, methods for the in vitro expansion of these cells for clinical purposes have revealed to be challenging.

1.3 Human embryonic stem cells

The derivation of stem cells from early mouse embryos was reported for the first time more than 25 years ago (Evans and Kaufman, 1981; Martin, 1981). This discovery led to the 2007 Nobel prize in Medicine and Physiology for the applications of ESC in the

generation of genetic mutant animals. The tenacious exploration of mouse ESCs, led to the discovery of methods to isolate and grow the equivalent stem cells from human embryos (hESCs) (Thomson et al., 1998). Accordingly, investigators utilized 4-5 days old fertilized eggs from in vitro fertilization (IVF) clinics. At that stage the embryo is typically composed of a hollow ball of cells termed the blastocyst. The human blastocyst is comprised of two different cell types: the trophoectoderm outer layer and the inner cell mass (ICM), which is a group of approximately 30-50 cells within the blastocoel cavity. Human ESC lines were established from isolated ICM cultured onto a feeder layer of irradiated mouse embryonic fibroblasts (MEFs). The role of such a feeder layer is to provide an adherent surface to which the ICM can attach as well as to release soluble proteins and growth factors required for their survival and maintenance. The cells of the ICM are subcultured repeatedly, resulting in the original 30-50 cells of the ICM yielding millions of ESC colonies. Colonies of cells that have proliferated in culture for >6 months without differentiating, thus retaining pluripotency, and appear genetically normal are referred to as an established hESC line. The original cell lines established by Thomson's group are still in use today, however since then, many labs worldwide have derived additional cell lines by employing a similar protocol. To confirm the true nature of hESC lines, cells within the colonies have to: a) remain undifferentiated upon expansion and subculturing for many months in vitro, to ensure that they are capable of long term self-renewal, b) express surface markers characteristic of undifferentiated cells along with the pluripotent marker gene Oct3/4. In addition, pluripotency of hESCs is evaluated by the ability of the cells to spontaneously differentiate in culture into derivatives of all three germ layers; as well as form a benign tumor called teratoma after injection of the cells into an immune-compromised mouse. When ESCs are cultured under defined conditions, they remain undifferentiated, retaining pluripotency. However, under altered cell culture conditions, these cells can theoretically differentiate into all specialized cell types of the embryo. These unique features award these cells remarkable potential for use in cell-based therapies as well as a tool to model early human development in vitro. Nevertheless, the use of fertilized eggs to derive hESC lines raise major ethical concerns that may hinder their use.

1.4 Induced pluripotent stem cells (iPSC)

A way to circumvent the ethical problems surrounding hESCs came from the establishment of methods to induce pluripotency in somatic cells. The ground-breaking work of Shinya Yamanaka demonstrated that ESC-like cells can be generated from adult somatic cells by forcing the expression of four key factors involved in the control of pluripotency and cell proliferation: Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007) (Fig. 2).

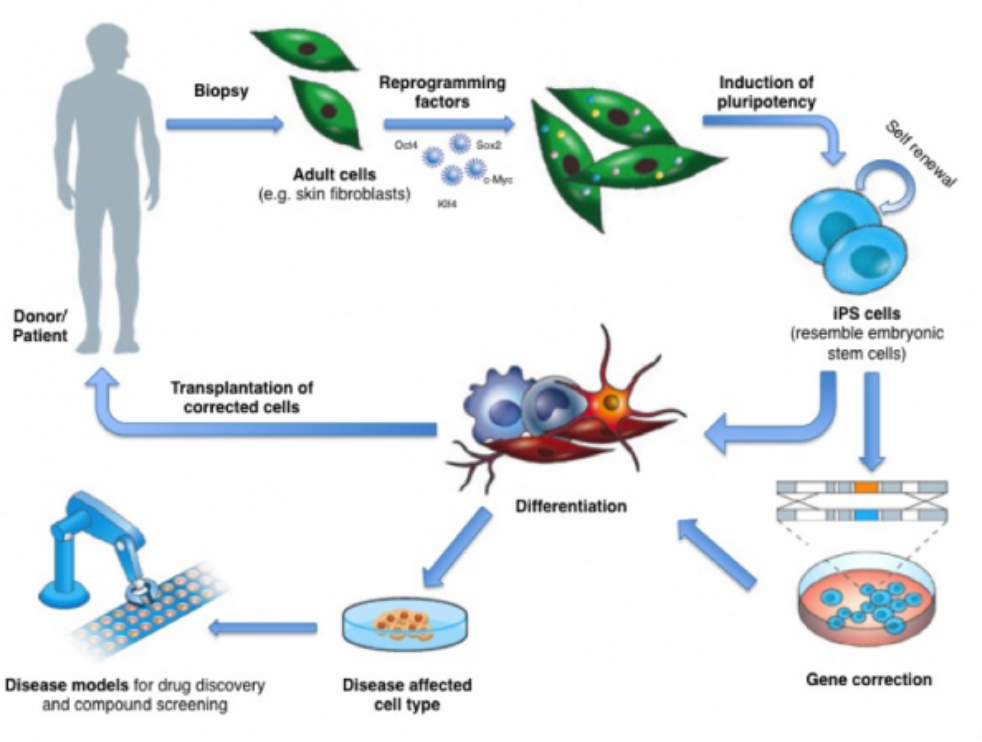


Figure 2. Schematic representation of iPSC derivation with potential applications in regenerative medicine and in vitro disease modeling.

(modified from eurostemcell.org)

Induced pluripotent stem cells (iPSC) have been shown to be comparable if not identical to hESC in regard to morphology, proliferation ability, gene expression and, more importantly, to differentiation potential. As such, there is great expectation that iPSCs will offer the same therapeutic potential as hESCs. Since the technology to make iPSC was developed in 2007, many changes were made to generate these cells. Initial safety concerns have been addressed, for instance c-myc has been

removed from the original “recipe” because of its potential tumorigenic role, and non-viral delivery of the factors has been used to develop new lines (Malik and Rao, 2013). Those and other changes contributed to making iPSC the present elective source of PSCs for in vitro and in vivo studies.

One other significant advantage of iPSCs is that they can be generated from patients with several complex genetic disorders. This will allow studying human disease directly in the petri dish. In vitro disease modeling will accelerate drug and active molecule screenings and in some cases will lead to individualized cell replacement therapy after in vitro genetic correction of the diseased cells. Several examples of the differentiation of patient-specific iPSCs into the cell types that are affected by the disease have been already reported, and therefore this technology is particularly attractive for diseases for which animal models are either not available or do not accurately represent the human disease etiology (Grskovic et al., 2011).

Even though our understanding of the full characteristics of iPSCs such as the detailed mechanism of reprogramming is yet to be complete, and fundamental questions such as genomic integrity and latent epigenetic memory remain to be elucidated, these cells are likely to replace hESC as primary source of PSCs in the near future.

1.5 Pluripotent stem cell maintenance and culture

Maintenance of the pluripotent state is a prerequisite for the effective use of PSCs for any in vitro or in vivo applications. Only PSCs that preserve pluripotency retain the ability to differentiate into all derivatives of the three germ layers. This status is highly dependent on culture techniques employed. A well-established way to maintain hPSCs undifferentiated is when the cells are cultured on a feeder layer of MEFs. In this condition, hPSCs may be continually propagated without losing their potential for up to one year (Reubinoff et al., 2000). Although this is the easiest way to propagate PSCs, there is a risk that viruses or other macromolecules in the MEFs may be transmitted to human cells. In addition, the specific membrane-bound or soluble factors released by these cells are unknown. Alternative methods have been sought to replace the feeders with acellular substrates composed of extracellular matrix (ECM) proteins. Although

their use does not eliminate the presence of xenogeneic proteins, these ECM substrates are theoretically chemically defined, allowing a more controlled and simplified culture technique for PSC propagation. A widely used substrate to grow hPSCs is Matrigel, a soluble basement membrane extract from the Engelbreth-Holm-Swarm (EHS) mouse tumor containing several ECM components and low dose growth factors. Licensed by Becton and Dickinson (BD), Matrigel became the gold standard substrate for the expansion of PSCs and special media were formulated to work in conjunction with it, such as mTeSR (Borowski et al., 2008). Other ECM and different kind of proteins have been recently proposed as substrates for culturing hPSCs. In particular, Vitronectin and E-Cadherin have been tested as possible candidates (Valamehr et al., 2011). I tested Vitronectin as a replacement substrate for Matrigel in my culture system. Even though hPSC colony morphology was preserved, suggestive of proper maintenance of pluripotency, dissociation of colonies for serial passaging revealed to be difficult. Prolonged enzymatic treatment was required to detach the colonies, which resulted in cell damage and consequent inefficient passaging.

In addition to the substrate, culture medium plays an essential role in the maintenance of PSCs. Although there are many media on the market there are still mixed results on their use for long-term expansion of hPSCs. Interestingly, media alone do not work at their best with all substrates and the search for the best medium-substrate combination can be time consuming and laborious. In a recent report, a medium formulation based on 5 small molecule inhibitors has been proposed for the long-term maintenance of hPSCs (Tsutsui et al., 2011). Although this is the first formulation entirely based on small molecules acting in concert as repressors of differentiation pathways and regulators of self-renewal, the results obtained in different hPSC lines were not uniform.

Another important factor for the correct maintenance of hPSC lines is the method used for cell passaging. This procedure can be done essentially in two ways, mechanically or enzymatically (Hoffman and Carpenter, 2005). Passaging by mechanical means involves manually excising and isolating cell colonies and transferring them to a new culture plate with fresh substrate. A benefit of this method is that it better preserves cell integrity and therefore cells may be passaged almost indefinitely. However, a

disadvantage is that cell density is not maintained constant after every passage and manual passaging is very time consuming and laborious. Cells cultured at high densities (colonies in close proximity or contact) exhibit an increased rate of spontaneous differentiation. Also, hPSCs not maintained as clusters or “colonies” but passaged as single cells do not receive correct survival signals from neighbouring cells and soon undergo apoptosis. On the other hand, enzymatic passaging involves hPSC colony dissociation with an enzyme (trypsin, collagenase, dispase, accutase, etc.). This method when correctly executed allows for a more uniformed dissociation of colonies with similar number of cells within each plate. However, exposing cells to enzymes for long-term passaging has been shown to possibly result in an increased number of spontaneous mutations such as aneuploidies (Brimble et al., 2004; Maitra et al., 2005)

Ultimately, an important point to consider is that the type of culture system used must be compatible with the desired experimental endpoint of the cells. While in vitro studies can be performed using a variety of substrates and media that contain animal products, it is a requirement for clinical applications to remove potential contaminants. To this end, a variety of reagents that do not contain xenogeneic components are now available.

1.6 Differentiation of pluripotent stem cells in vitro

The successful use of hPSCs is based on the assumption that it will be possible to obtain pure populations of a defined cell type from these cells via their differentiation in vitro. The simplest approach is to rely on spontaneous differentiation to develop the cell type of interest. Spontaneous differentiation occurs routinely in PSC cultures and produces a wide variety of cell types, most of which are uncharacterized. This process is triggered by exposing PSCs to conditions that are sub-optimal for self-renewal but support cell survival. Among the protocols for the induction of differentiation, a widely used method is embryoid body (EB) formation. When cultured in suspension PSC form cell aggregates consisting of two initial layers: an outer layer of cells with properties of extra-embryonic endoderm and an inner cell layer that represents pluripotent tissue.

Then differentiation proceeds to form derivatives of all three germ layers. Although very popular to differentiate mouse ESCs, this method has not been used extensively with hPSCs. Mainly because hPSCs don't form EBs easily unless small aggregates of 50-100 cells are used. Also, my personal view is that because of the size and tightness of the aggregates, treatment with growth factors/small molecules to direct differentiation could be ineffective in EBs, as cells at the center would not be exposed to the appropriate dose.

Another method of differentiation is based on the co-culture of hPSCs on a layer of tissue-derived primary or immortalized cells that can provide the needed soluble and cell-mediated factors to promote the specification of a distinct cell type. Although the co-culture method has been successfully used to obtain neural (Barberi et al., 2003) and other cell types (Ishii et al., 2013; Talavera-Adame et al., 2011; Yue et al., 2010) as the field becomes more mechanistic and translational, the uncontrolled use of primary cells influence the results in an inconsistent manner, which is unacceptable for the generation of cells for pre-clinical studies. The best-controlled method of differentiation is where PSCs are allowed to overgrow in a monolayer culture resulting in spontaneous differentiation accompanied by complex cell stratification. To drive differentiation preferentially along a given lineage selective culture conditions may be employed to promote the growth of a particular cell type at the expense of another. This system is the most suitable for the use of growth factor and/or small molecules to direct differentiation. The absence of feeder or inducing cells and the 2D distribution of cells also facilitate monitoring the progress towards the lineage of interest. I was able to partially control the fate of cells by modifying cell density of hPSC cultures prior to induction of differentiation. Cultures induced at medium to low cell density were observed to yield a mixed population consisting of both neural and non-neural cell types. Likewise, starting induction at high or very low cell density yields almost exclusively, neural or non-neural cell types respectively. Therefore the monolayer method provided the base for development of my project.

1.7 Embryonic skeletal myogenesis in vivo

1.7.1 Development of the embryonic myotome

All skeletal muscle with the exception of craniofacial muscle derives from the somites (Christ and Ordahl, 1995). The somites are epithelial structures that develop as metameric divisions of paraxial mesoderm, flanking both sides of the neural tube (Fig. 3A). As development proceeds, the somites become more specialized and are sub-divided into a dorsal and ventral compartment (Fig. 3B). The ventral portion of the somite de-epithelializes and becomes mesenchyme forming what is referred to as the sclerotome. The sclerotome will give rise to parts of the axial skeleton such as the vertebrae and ribs. Conversely, the dorsal portion of the somite remains epithelial and is known as the dermomyotome. This region will form the most primitive skeletal muscle the myotome (Bentzinger et al., 2012). Formation of the myotome involves two phases (Gros et al., 2004) (Fig. 3C). Firstly, muscle progenitors located at the dorsal medial lip (DML) of the dermomyotome translocate underneath the dermomyotome and elongate bi-directionally along the rostral and caudal borders of the somite. While the dermomyotome is composed of epithelial-like skeletal muscle progenitors, the early myotome comprises post-mitotic bipolar myocytes aligned parallel to the embryonic axis. As this process continues, older myocytes are displaced laterally by new cells translocating from the DML, and consequently the myotome expands incrementally. Myocytes derived from the DML, contribute only to the expansion of the epaxial region of the myotome (Fig. 3D). This region will give rise to the inter-costal and deep muscles of the back. During the second phase, myotome growth continues through the translocation of skeletal muscle progenitors from the other three borders of the dermomyotome, the rostral/caudal and ventral-lateral (VL) borders (Fig. 3C). Skeletal muscle progenitors emanating from the VLL elongate bi-directionally and contribute to the growth of the hypaxial domain of the myotome (Fig. 3D). The hypaxial domain will form the musculature of the ventral body wall, limbs, tongue and diaphragm. However, myocytes originating from the rostral/caudal border preferentially colonize the central region of the myotome, contributing only little to both epaxial and hypaxial domains (Fig. 3D). It is now understood that not all cells of the

dermomyotome terminally differentiate during early myotome formation. A reserve population of muscle progenitors remain in the centre of the dermomyotome contributing to muscle growth throughout prenatal development (Gros et al., 2005; Relaix et al., 2005). After formation of the primary myotome, muscle progenitors migrate from the central dermomyotome to the underlying myotome and integrate with resident mono-nucleated myocytes, forming secondary muscle fibers. Furthermore, this reserve population of muscle progenitors originating from the dermomytome, maintain their myogenic potential well into postnatal life, adopting a peripheral position between the muscle fiber and basal lamina. These satellite cells represent a replenishing source of muscle progenitors required for the regeneration of muscle fibers throughout adult life.

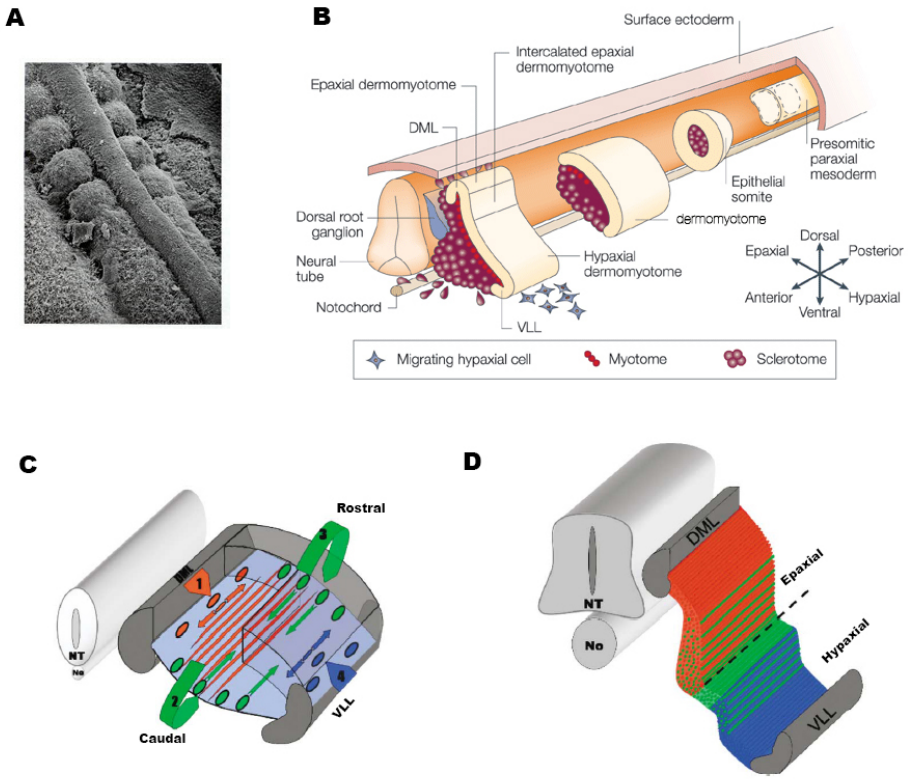


Figure 3.(A,B) Morphological organization of the somites. **(A)** Electronmicroscopic image showing a dorsal view of the developing chick somites. **(B)** 3D representation of the different stages of somite development and specialization. **(C,D)** A model for the formation of the myotome. **(C)** Progenitors from the DML translocate first under the dermomyotome to form bipolar myocytes (red). Later, bipolar myocytes arise from the rostral and caudal borders (green). Lastly, myocytes from the VLL complete the structure of the primary myotome (blue). **(D)** Preferential contribution of different borders of the dermomyotome to the epaxial and hypaxial domains of the myotome.

modified from UNSW embryology, Parker et al., 2003; Gros et al., 2004.

1.7.2 Intrinsic control of myogenesis

The process of generating skeletal muscle during embryogenesis can be divided in several distinct phases. An elaborate interplay of extrinsic and intrinsic regulatory mechanisms controls myogenesis at all stages of development (Figure 4.)
Muscle determination relies on a complex transcriptional regulatory network, based on the expression of the basic helix-loop-helix muscle regulatory factors (MRFs) (Fig. 4A) These myogenic transcription factors are organized in hierarchical gene expression networks that are spatiotemporally induced or repressed during lineage progression. This MRF network is composed of myogenic factor 5 (*Myf5*), myogenic differentiation (*MyoD*), myogenic factor 6 (*Myf6*) and myogenin (*Myog*). Numerous studies have been undertaken to elucidate the hierarchical network governing expression of MRFs. *Myf5* is the first MRF to be expressed within the dermomyotome, just prior to *MyoD* activation (Buckingham and Rigby, 2014). Mice devoid of either Myf5 or MyoD develop muscle, although somewhat delayed, whereas mice carrying a double knockout of *Myf5* and *MyoD* exhibit a complete loss of skeletal myogenesis, highlighting the compensatory action between *Myf5* and *MyoD* in skeletal muscle determination (Rudnicki et al., 1993). In contrast to *Myf5* and *MyoD*, Myog and *Myf6* are involved in the terminal differentiation of skeletal myoblasts, acting downstream to regulate the expression of myotube contractile protein genes. Analysis of *Myog-/-* and *Myf6-/-* knockout mice reveal a loss of myocytes and terminally differentiated myofibers with unaltered formation of MYF5+ and MYOD + skeletal myoblasts (Venuti et al., 1995).

The genetic hierarchy controlling myogenesis is dominated by the paired-box transcription factors *Pax3* and *Pax7* (Buckingham and Rigby, 2014) (Fig. 4A). These transcription factors are critical in the determination of myogenic cell fate by acting upstream of the MRF, *Myf5* (Relaix et al., 2005). *Pax3* is first expressed in the pre-somitic mesoderm and its expression is maintained in the muscle progenitors throughout the myotome stage of development. *Pax7* on the other hand, is first expressed in the central dermomyotome and then co-localizes with *Pax3* positive cells in the myotome (Horst et al., 2006). Mice lacking *Pax3* expression fail to develop the

hypaxial domain of the myotome resulting in the absence of limb, tongue and diaphragm muscles (Tajbakhsh et al., 1997). However, muscle derived from the epaxial domain of the myotome, remain unaffected. During epaxial myogenesis, as muscle precursors delaminate from the dermomyotome to form the dorsal medial domain of the myotome, *Myf5* can act independently from *Pax3* to activate *MyoD* expression. This can also be seen in mutants bearing a hypomorphic form of the *Pax3* allele, which show a lack of hypaxial but not epaxial *Myf5* expression (Bajard et al., 2006). In contrast, *Pax7* is dispensable for early muscle development. Knock-out of *Pax7* results in defects occurring at a later stage of development during fetal myogenesis, with a reduction in the number of functional satellite cells (Seale et al., 2000). However, *Pax3:Pax7* double mutants show more defective muscle formation than the *Pax3* mutant alone. In addition to the lack of hypaxial muscle, epaxial musculature of the double mutant does not progress further than the myotome stage. Interestingly, *Pax7* knock-in at the *Pax3* locus can rescue the *Pax3*-null phenotype seen during somitogenesis and early mytome formation, suggesting a partial compensatory role of *Pax7* for *Pax3.* However, it does not fully rescue the failure in delamination and long-range migration of muscle progenitors to the limb bud of *Pax3* mutants (Relaix et al., 2004). Following their contribution to embryonic myogenesis, PAX3+/PAX7+ cells acquire a satellite cell position within the developed fetal muscle generating a stem cell pool of muscle progenitors throughout fetal and postnatal life (Gros et al., 2005).

Other transcription factors implicated in the regulation of MRFs are the sine oculus-related homeoproteins 1 (*Six1*) and *Six4* (Buckingham and Rigby, 2014) (Fig. 4A). Both factors govern at the apex of the genetic regulatory cascade that directs dermomyotomal progenitors toward the myogenic lineage. Along with cofactors eyes absent homologue 1 and 2 (EYA1, EYA2), SIX proteins bind to and activate hypaxially active enhancer element of *Pax3* and *Myf5*. Consequently, *Six1:Six4* mouse mutants are devoid of Pax3 and Myf5 in the hypaxial dermomyotome, leading to a loss of limb, tongue and diaphragm musculature. As *Myf5* expression in the epaxial domain is

independent of both SIX and PAX3 factors, dorsal muscles arising from this structure remain unaffected (Giordani et al., 2007).

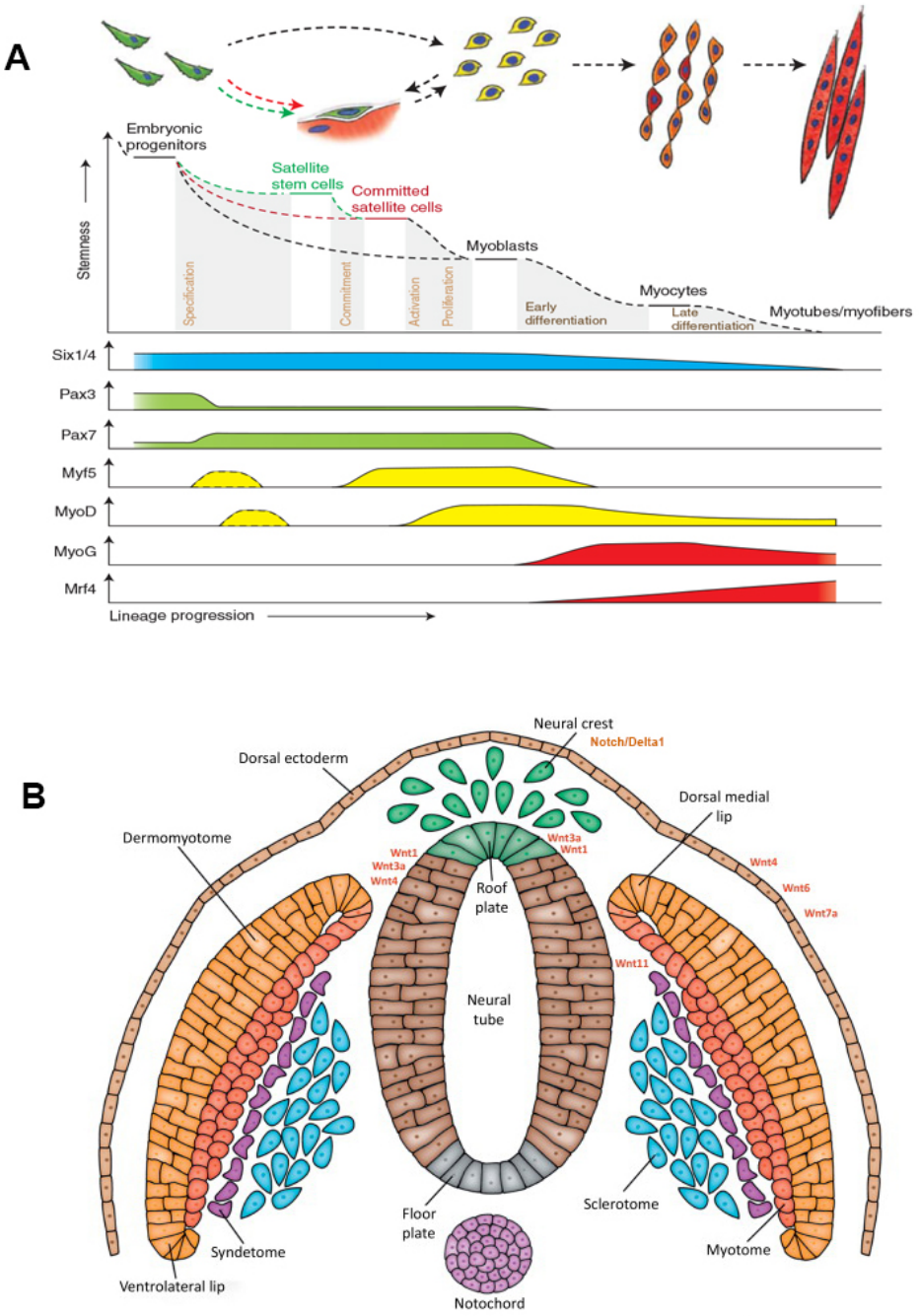


Figure 4. Extrinsic and intrinsic regulatory mechanisms regulating the embryonic myogenic program. (A) Hierarchical gene expression network governing myogenesis.**(B)** Dorsal tissue origin of known extrinsic signals required for myogenic specification of the dermomyotome.

(modified from Bentzinger et al., 2012; Maltzahn et al., 2012)

1.7.3 Extrinsic control of myogenesis : role of wnt and fgf signaling

During embryonic myogenesis, early cell fate decisions are guided by a broad spectrum of signaling molecules that control the intrinsic genetic networks patterning tissue morphogenesis (Fig. 4B). Members of the wingless-type MMTC integration site (WNT) and Fibroblast growth factor (FGF) family of proteins play crucial roles during this process from the pre-patterned embryo to myotome formation (Aulehla and Pourquie, 2010). The embryonic mesoderm of the mammalian embryo is first formed by a series of interactions at the primitive streak (PS) (Chuai and Weijer, 2008). In wild-type embryos, pluripotent epithelial epiblast cells of the primitive streak ingress through the streak and become mesenchymal upon exposure to mesoderm- promoting factors such as WNT3A (Yamaguchi et al., 1999). The specification and maturation of paraxial mesoderm is controlled by the synergism between WNT3A, acting through the effectors LEF1 and TCF7, and Brachyury (T) to regulate expression of target genes involved in mesoderm cell type identity, such as *Tbx6* (Chapman et al., 1996; Yamaguchi et al., 1999). In the absence of either *Wnt3a* or *T*, epithelial epiblast cells continue to ingress through the primitive streak however these cells retain their epithelial identity and assume a neural fate to eventually form ectopic neural tube (Chapman and Papaioannou, 1998; Yoshikawa et al., 1997). Extrapolating from genetic studies, reports have demonstrated human ESC/iPSC differentiation towards PS and paraxial mesoderm under the effect of WNT signaling (Nakanishi et al., 2009). Short-term activation of the WNT/bcatenin pathway through a small molecule inhibition of the GSK-3β signaling has been shown to be sufficient to induce PS-like cells from human ESC/iPSC (Bone et al., 2011).

Following ingression through the PS, the paraxial mesoderm is arranged as mesenchymal, still unsegmented tissue called pre-somitic mesoderm. Subsequent maturation and metameric division to form the definitive somite is controlled through a combinatorial gradient system of FGF and WNT signaling present in the pre-somitic mesoderm. Local oscillations of WNT signaling in synergy with a posterior to anterior FGF morphogen gradient, control expression of Delta-like ligand 1 (Dll1), the ligand of NOTCH1 essential for somite boundary formation (Aulehla and Pourquie, 2010).

At the onset of somitogenesis, cell fate of the epithelial somite is not yet determined. Epithelial cells in the newly formed somite initially have the potential of adopting a sclerotomal or a dermomyotomal fate. Classic in vivo transplantation or ablation experiments have shown the requirement of axial structures (neural tube, notochord, surface ectoderm) to specify dorso-ventral polarity of the epithelial somite (Aoyama and Asamoto, 1988; Dockter and Ordahl, 2000). Extrinsic signaling molecules emanating from these neighboring tissues coordinate the spatio-temporal expression of intrinsic transcription pathways required for dorsal dermomyotome or ventral sclerotome cell identities (Bentzinger et al., 2012; Yusuf and Brand-Saberi, 2006). A sclerotomal cell fate is first specified within the ventral somite by soluble morphogens such as sonic hedgehog (SHH) produced by the notochord, and floor plate of the neural tube. SHH in conjunction with BMP4 secreted from the lateral mesoderm, activate *Pax1* in the sclerotome, thus initiating chondrogenesis. Conversely myogenic patterning of the dermomytome is reliant on dorsal WNT signaling. WNT members involved in somite patterning are WNT1 and WNT3A (canonical WNT signaling), which are secreted from the dorsal neural tube and WNT4, WNT6, and WNT7A (non-canonical WNT signaling), which are produced by the overlying surface ectoderm (Bentzinger et al., 2012; Yusuf and Brand-Saberi, 2006). The positive specification of myogenesis by WNT signaling within the dermoyotome is achieved through the induction of expression of transcription factors *Pax3* and *Myf5* (Borello et al., 2006; Cossu and Borello, 1999). Ablation of *Wnt1* and *Wnt3a* in the mouse results in a reduction in Myf5 expression and the absence of the epaxial myotome domain (Ikeya and Takada, 1998). Similarly, in explant cultures of mouse pre-somitic mesoderm, WNT7A and WNT6 induce expression of MyoD (Tajbakhsh et al., 1998).

Along with WNT and FGF signaling, another extrinsic signaling pathway critical for myogenic specification is NOTCH (Bentzinger et al., 2012). NOTCH mediates juxtacrine cell-to-cell cross talk through ligand receptor signaling with Dll1 receptors present on neighboring cells (Baron, 2003). During myogenic development, Dll1 expressing neural crest cells transiently bind to myogenic progenitor cells within the dermomyotome (Rios et al., 2011). Only those PAX3, PAX7 positive progenitor cells

that are presented with Dll1 and thus activate NOTCH signaling are induced to undergo myogenic differentiation. Activated NOTCH signaling directly suppresses *MyoD* expression thereby preventing differentiation of myogenic precursors (Jarriault et al., 1995). During embryonic myogenesis, the balance between maintenance and differentiation is critically important to retain the progenitor cell pool. Along with NOTCH signaling, members of the FGF family have been shown to control the balance between muscle progenitor proliferation and differentiation (Lagha et al., 2008). In vitro, members of the FGF family of ligands possess mitogenic activity, stimulating proliferation of cultured muscle progenitors (Bischoff, 1986; Sheehan and Allen, 1999). Moreover, loss of function experiments in mouse embryos revealed that perturbation of *Sprouty* gene, a negative feedback modulator within the FGF signaling pathway, results in an inhibition of myogenic progenitor differentiation (Michailovici et al., 2014).

In summary, the process of myogenesis involves an elaborate interplay of extrinsic and intrinsic regulatory mechanisms governing cell fate specification. In vitro recapitulation of these events requires a comprehensive understanding of these mechanisms and the use of the appropriate combination of morphogenic factors and/or small molecules to mimic this process.

1.8 Potential sources of cells for muscle regeneration

Replacement of defective muscle fibers to restore muscle function could have a significant impact in regenerative medicine. Such an approach would be indispensable to treat a variety of congenital and acquired conditions affecting the muscular system such as muscular dystrophies (MDs) or volumetric muscle loss after trauma or cancer (Turner and Badylak, 2012). Although a subset of genetic conditions, including some forms of MD, could profit from a molecular therapy approach, such as exon skipping to remove the mutated part of the affected gene (Touznik et al., 2014) or from gene therapy to correct the affected gene (Braun et al., 2014), the majority of acquired and inherited disorders affecting the muscles in our body remain incurable. A cell-based therapy aimed at replacing defective resident stem cells is an option to be explored.

Over the past two decades, numerous studies have exploited the potential of cell-mediated therapy to promote muscle regeneration. Initial work involved transplantation of adult murine myoblasts in a mouse model of MD, the *mdx* mouse, with encouraging results (Huard et al., 1991; Partridge et al., 1989). The potential of these cells was then tested in early clinical trials, where human myoblasts obtained from healthy donors were transplanted into specific muscles of patients with Duchenne MD (DMD) (Gussoni et al., 1992; Mendell et al., 1995). The expected outcome was the formation of hybrid myofibers containing nuclei of both donor and recipient origin. Some trials reported that transplanting normal human myoblasts into DMD patients resulted in a limited and transient restoration of dystrophin expression; normally absent in those patients. However, those transplants proved to be inefficient due to reduced survival, limited migratory potential, and above all, the inability of myoblasts to sustain long-term muscle turnover (Mouly et al., 2005).

The characterization and isolation of stem cells residing in the adult skeletal muscle, the satellite cells (SCs) has given support to potential cell therapies (Mauro and Adams, 1961; Peault et al., 2007). SCs are present in adult mammalian muscle as quiescent pool of stem cells constituting 2-6% of cells within a muscle fiber. In healthy muscle these cells are able to self-renew while also generating new myofibers in response to injury.

Several studies in injured and disease mouse models such as the *mdx* mouse have shown that transplanted SCs are able to contribute extensively to the regeneration of new muscle fibers, and to the restoration of dystrophin expression (Cerletti et al., 2008; Collins et al., 2005; Sacco et al., 2008). SCs have been successfully characterized and isolated using a combination of surface markers; CXCR4, CD34, integrin β1 and syndecan 3/4, allowing investigation of their myogenic potential both in vitro and in vivo (Cerletti et al., 2008; Sherwood et al., 2004; Tanaka et al., 2009). However, despite these results in the mouse, these surface markers have not been proven applicable to human SCs, with the marker neural cell adhesion molecule (NCAM) being the only likely candidate to date (Kadi et al., 2004; Maier and Bornemann, 1999).

Moreover, only freshly isolated cells are capable of contributing to the formation of new muscle fibers. In vitro expansion of SCs results in decreased proliferative capacity and their differentiation towards a mature muscle phenotype, thus reducing their ability to repair damaged fibers (Montarras et al., 2005; Shadrach and Wagers, 2011). In addition, SCs are unable to cross the endothelial wall, making systemic delivery difficult (Tedesco and Cossu, 2012). These characteristics may limit the use of SCs in a clinical setting.

Concurrent with SCs, the regenerative potential of alternative cell types has been investigated. Mesangioblast cells were first identified as a population of stem cells within the embryonic mouse aorta (De Angelis et al., 1999). These embryonic cells were shown to express endothelial surface markers (CD34, Sca-1, Flk1, VCAM-1) and possess the ability to differentiate into many mesodermal cell derivatives, including skeletal muscle (Minasi et al., 2002). In contrast, in adult tissues, these cells have been found to surround endothelial cells in small vessels and capillaries and exhibit expression of pericyte markers, such as NG2 proteoglycan and alkaline phosphatase (ALP) (Dellavalle et al., 2007). Successfully isolated from mouse, dog and human adult tissues, these pericyte-like cells have demonstrated robust ability to engraft and generate new muscle fibers following transplantation in models of MD (Tonlorenzi et al., 2007). In one study, pericytes isolated from the muscle microvasculature of healthy dogs were transplanted into GRDMD dogs, a DMD animal model considered to be the closest in resembling the human pathology. These transplanted pericytes demonstrated robust engraftment, restoration of dystrophin expression and an improvement in muscle function (Sampaolesi et al., 2006). Furthermore, transplantation of human adult pericytes into *mdx* mice have been shown to assume a satellite cell position within the muscle fiber, contributing to formation of new muscle fibers expressing human dystrophin (Dellavalle et al., 2011; Dellavalle et al., 2007). Although similar to SCs, these cells have the distinct ability to proliferate in culture without losing in vivo regenerative ability. Extrapolating from these findings, a phase I/II clinical trial is being undertaken using HLA-identical allogeneic pericytes to treat DMD (Noviello et al., 2014). Nonetheless, a hindrance to their use in the clinical setting is that pericytes have a limited lifespan in culture, which is a problem when large

numbers of cells are required for patient treatment. Overall, these sources of stem cells are difficult to isolate from donor tissue, and due to the characteristics described above, their use in the clinical setting could be challenging.

An additional potential source of myogenic cells could come from human PSCs. The self-renewal ability of PSCs together with their broad differentiation potential, make these cells a promising candidate for the derivation of myogenic progenitors to be used in muscle repair. However, few methods for the derivation of skeletal muscle from hPSCs have been proposed. The successful use of hPSC-derived progeny for regenerative medicine requires tight control of the cell differentiation process and isolation of pure, specialized cell types. At present, the controlled derivation and efficient isolation of hPSC-derived myogenic precursors equivalent to an in vivo PAX3+/PAX7+ satellite cells has not been accomplished. Challenges that arise in the derivation of these cells include the lack of knowledge of the essential factors required to recapitulate the in vivo patterning of myogenic somitic mesoderm and the timing of their distribution.

The first protocol demonstrating derivation of skeletal muscle from hPSCs has been reported by Barberi and colleagues. Their method was based on the initial derivation of multipotent mesenchymal precursors (MMPs) that were able to differentiate into a variety of cell types, including myogenic cells. Mesenchymal differentiation was induced by co-culturing undifferentiated hESCs with murine stromal cells (OP9), without the addition of exogenous growth factors, suggesting that mesenchymal progenitors derive from a spontaneous process driven by contact with OP9 cells. The obtained mesenchymal cells were isolated by FACS sorting using the adult mesechymal stem cell marker, CD73. These purified MMPs could be differentiated, using standard protocols, into bone, cartilage and fat cells (Pittenger et al., 1999). Importantly, a small fraction of these MMPs started to spontaneously express muscle markers during prolonged culture in serum-containing medium. Although this is the first report of muscle derivation, there was no direct control in the process leading to its differentiation (Barberi et al., 2005).

Successful modification of this protocol was achieved using a stroma-free induction

system involving a two-step purification process (Barberi et al., 2007). By sequentially changing media conditions, Barberi and colleagues increased efficiency of induction towards a mesenchymal fate resulting in enrichment of CD73+ MMPs. However, the efficiency of myogenic differentiation was low with only 2%-10% of cells within the CD73+ cell population, expressing skeletal muscle markers such as Pax7 and MYOD. To further restrict lineage specification towards a myogenic fate, CD73+ cells were sorted for the neural cell adhesion molecule (NCAM), expressed on embryonic skeletal muscle (Charlton et al., 2000). Exposure of NCAM+ cells to serum free medium resulted in the differentiation of these cells into PAX7+ and MYOD+ muscle cells followed by their maturation into MYOG+ bipolar myocytes. Upon transplantation, NCAM+ cells successfully engrafted and participated in muscle regeneration in a mouse model of artificial injury. These experiments demonstrate the feasibility of generating engraftable muscle cells from hPSCs without using a co-culture system. However, a fundamental issue of MMP based protocols to derive skeletal muscle cells is that MMPs are a poorly characterized cell population with limited skeletal muscle potential.

The derivation of skeletal myogenic progenitors from ESCs has proven to be less efficient in vitro because of the absence of somitogenesis or paraxial mesoderm patterning, two essential mechanisms in normal muscle differentiation. This failure can be compensated by the overexpression of genes, which promote myogenesis in vitro. Darabi and colleagues have reported the successful derivation of skeletal myocytes from mouse embryonic stem cells (mESCs) utilizing a *Pax-3* inducible ESC line (iPax3) (Darabi et al., 2008) The same approach was later used to induce muscle differentiation from hPSCs (Darabi et al., 2012). A dox-inducible *PAX7* construct was expressed in EB-derived monolayer cultures for 4 days. The resulting PAX7+ GFP+ population, comprising only 14% of total cell population was then FACS sorted and expanded in vitro. Upon withdrawal of dox and exposure to serum-containing medium, these cells differentiated into mature myosin heavy chain+ (MHC) muscle cells. The ability of these cells to repair damaged fibers was tested by local transplantation into *mdx* mice. Expression of human dystrophin indicated successful engraftment and

integration in the host muscle. In addition, presence of human cells expressing PAX7 was confirmed by co-expression of human Lamin AC in the chimeric fibers. Similarly, successful induction of myogenic cells from hPSCs was demonstrated by Goudenege and colleagues following transfection of hPSC-derived MMPS with an adenovirus ubiquitously expressing MYOD. After 3 days of transfection, 31% of total cells were positive for MYOD with 60% of these cells capable of further differentiation into MHC+ mature myocytes. When transplanted in the muscle of *mdx* mice, MYOD-hPSCs participated in muscle regeneration, fusing with existing mouse muscle fibers as confirmed by labeling with human-specific anti-dystrophin (Goudenege et al., 2012).

The use of a transgene based differentiation protocol demonstrates proof of principle that skeletal myocytes can be efficiently and robustly induced from hPSCs. However the use of transgene overexpression to induce selective differentiation of hPSCs, bypasses key early developmental stages during myogenesis, thereby limiting the use of these cells as a platform for developmental/disease modeling. Moreover, genetically modified PSCs cannot be used as a valid therapeutic treatment approach. Alternative protocols aimed at transgene-free derivation of skeletal muscle cells from hPSCs utilize the formation of embryoid bodies (EBs), cellular aggregates that are formed by preventing surface adhesion. EB differentiation proceeds in a manner that mimics embryonic development, occasionally achieving a high level of organization cells.

Ryan and colleagues generated skeletal muscle cells by exposing hESC- derived EBs to increasing concentrations of retinoic acid (RA) from day 0 to 10 of differentiation. Following growth on adherent plates, expression of MRFs MYOD and MYOG were observed by day 25. By day 39 of differentiation, total cell culture comprised of an average of only 4% MYHC+ skeletal myocytes (Ryan et al., 2012). In addition to RA, exposure to high FGF2 levels have been found to promote myogenesis within EB cultures (Hosoyama et al., 2014). After 6 weeks of culture, EBs comprised of an average 30% of MYOD+ skeletal myocytes.

Despite the advantages held by a transgene-free system, a significant caveat to these EB culture methods is the lack of a purification method to isolate skeletal muscle from the mix of cell types. This is essential if we are to apply these cells to drug discovery and therapeutic efforts.

1.9 Rationale of study

Due to their exceptional developmental potential and extensive proliferative capabilities, hPSCs represent a promising unlimited source of cells for the treatment of degenerative diseases and cancer. Despite the progress made in obtaining a vast variety of specialized cells from hPSC (Fox et al., 2014), the controlled derivation and efficient isolation of hPSC-derived myogenic precursors equivalent to an in vivo PAX3+/PAX7+ satellite cell has not been accomplished. The difficulty lies in the limited knowledge of the specific inductive signals and their timing of expression required for myogenic induction of paraxial mesoderm. Also the appropriate combination of markers for the purification of skeletal muscle progenitors has not been reported. As such, there are only a few studies reporting the derivation of skeletal muscle cells from hPSCs and they mostly utilize an approach reliant on forced transgene expression to induce myogenesis. The goal of my PhD project is to develop a tightly-controlled, small-molecule based approach to direct hPSCs through defined developmental events leading to the derivation of committed skeletal muscle precursors. A developmental based approach relies on the knowledge of embryogenesis to recapitulate in vitro, the necessary conditions for the induction and myogenic patterning of paraxial mesoderm. This will not only improve efficiency of skeletal muscle derivation but also enable us to track in vitro, the myogenic progression from undifferentiated ESC to a committed muscle precursor, allowing examination of each developmental milestone. This chemically defined protocol would be highly reproducible, readily adaptable to a broad number of cell lines ultimately facilitating the use of hPSC-derived muscle cells for a plethora of regenerative and basic applications.

2. EXPERIMENTAL PROCEDURES

2.1 Culture of hPSCs

Human PSC lines [WA-09 (H9), Mel1, HES3 (Monash University StemCore) and PDL-iPS, LMX1A-GFP] passages p40-65 were passaged and maintained under feeder-free conditions (Ludwig and Thomson, 2007). Human PSCs were cultured on hESC-qualified Matrix (BDMatrigel; BD Biosciences, San Diego, CA) in the presence of mTESR1 medium (Stem Cell Technologies, Vancouver BC, Canada). Cells were passaged when the majority of colonies were large (~1mm size) and colonies were dissociated enzymatically following a 20 minute incubation in 1 U/mL Dispase solution (Stem Cell Technologies, Vancouver BC, Canada). When the edges of the colonies began to lift from the dish, cells were dissociated by gentle pipetting and transferred to a 15ml tube containing 10 ml of DMEM/F12 medium (Gibco, Grand Island, NY). After 5 minutes centrifugation at 200g and medium aspiration, cell pellets were resuspended in mTESR1 medium and replated in BDMatrigel coated dishes as small clumps at a split ratio of 1:4 at a final cell density of $3X10^5$ cells dish. Cells were grown in 5% CO2 with 95% humidity incubators.

For cryopreservation, hPSCs were dissociated and collected as described above. Cells were frozen (one culture dish/vial) in 1ml freezing medium (mFreSR, Stem Cell Technologies, Vancouver BC, Canada). Vials of hPSCs were temporarily frozen at -80°C and then transferred into liquid nitrogen tank for long-term storage. Cryopreserved stocks of hPSCs were recovered by partially submerging the vial in water at 37°C until thawed, then transferred to a 15ml tube containing DMEM/F12 medium. Cells were spun down for 5 minutes at 200g and the supernatant discarded. Cells were resuspended in 3ml mTESR1 medium containing 10μM ROCK inhibitor Y-27632 (Sigma, St. Louis, MO). The cell suspension was then transferred to a BDMatrigel-coated 6cm dish for culturing. The experiments in this study were approved by the Monash University Human Research Ethics Committee (CF09/2725).

2.2 Directed differentiation of hPSCs into skeletal muscle cells

Experiments were performed using all four hPSC lines. When colony size reached >600 μm in diameter and colony density on the plate was approximately 30-40%, we induced differentiation of hPSC by switching the culture medium from mTESR1 to a chemically defined, serum-free medium DMEM-F12 supplemented with insulin-transferrin-selenium (ITS) (all from Sigma-Aldrich, St. Louis, MO). Starting at day 0 of differentiation, cells were cultured in the presence of 3 μM CHIR 99021 (Miltenyi Biotech, Auburn, CA) for 4 days. Culture medium was then replaced by ITS containing 20 ng/ml of FGF2 (Miltenyi Biotech) for a further 14 days. For each experimental control condition, hPSCs differentiation was induced as follows: 1) CHIR only: ITS medium containing 3 μM CHIR from days 0-4, followed by ITS medium only until day of analysis; and 2) FGF2 only: ITS medium only between days 0-4, followed by ITS medium containing 20 ng/ml FGF2 for 14 days. Medium was replaced daily until the day of the analysis.

2.3 Fluorescence activated cell sorting (FACS)

Cells were dissociated with 0.05% trypsin or TrypLE Select (Invitrogen, Carlsbad, CA) to a single cell suspension and incubated with the appropriate fluorochrome-labelled antibodies (Table S2) at a concentration of 10^7 cells/ml for 30 minutes on ice. Indirect labeling of HNK and AChR antibodies was done using goat anti-mouse Alexa Fluor 488 and goat anti-mouse PE (both from Molecular Probes, Invitrogen) as secondary antibodies. Labelled cells were sorted through a BD Influx (five lasers) flow sorter (BD Biosciences) according to the excitation requirements of the fluorochromes. Sorted populations were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.4 Immunocytochemistry

For cytospin preparations of FACS sorted populations, cells were spun onto glass slides using Cytospin 4 (Shandon, Thermofisher, Waltham, MA). Cells were then fixed with 100% cold methanol for 5 minutes and subsequently rehydrated in phosphate buffered solution (PBS) for 15-20 minutes. Cultured cells were fixed with 4%

paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 30 minutes. A complete list of primary and fluorochrome-labelled secondary antibodies used in this study is provided in the Supporting Information (Table 2). Incubations with primary and subsequently secondary antibodies were performed in Incubation Buffer (0.1% BSA, 2% FBS, 0.1% Triton X-100 in PBS) for 40 minutes at 37°C. Image acquisition was performed on an inverted Nikon Eclipse Ti epifluorescence microscope with the appropriate filter sets using single channel acquisition on a Nikon Digital sight DS-U2 camera. Images were analyzed with Nikon NIS-Elements 3.2 software. All immunofluorescence images are representative of one individual experiment. Experiments were performed n=3 per cell line. Similar results were obtained in all cell lines.

2.5 Gene expression analysis

Total RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany), and DNAse I treatment (Qiagen) was performed to avoid genomic DNA contamination. The Ambion RETROscript First Strand Synthesis Kit (Invitrogen) was used to reverse-transcribe total RNA (500 ng each sample). Polymerase chain reaction (PCR) was performed using the Mastercyler proS (Eppendorf AG, Hamburg, Germany). I optimized the PCR conditions and determined the linear amplification range for each primer by varying annealing temperature and cycle number. Primer sequences, cycle numbers and annealing temperatures are provided in Supporting information Table 1. All RT-PCR data shown are representative of one individual experiment. Experiments were performed n= 3 per cell line. Similar results were obtained in all cell lines.

For quantitative PCR, GAPDH was used as a reference gene and reactions were run using LightCycler480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN) on a LightCycler 480 system (Roche Applied Science). Target gene expression was normalized to the reference gene (GAPDH), and subsequent quantification of gene expression was compared relative to day 0 undifferentiated hPSCs (Pfaffl, 2001).

2.6 Culture of FACS-isolated cell populations

FACS purified ACHR+ myocytes, CXCR4-/c-MET+ and CXCR4+/c-MET+ precursors were plated onto tissue culture wells coated with 2 μg/ml fibronectin and 2 μg/ml laminin (both from Invitrogen) in ITS medium supplemented with 10 μM Rock Inhibitor Y-27632 (Sigma Aldrich). Myocytes were maintained in ITS medium in the presence of 50 ng/ml IGF1 (Peprotech, Rocky Hill, NJ) until analyzed. Progenitor cell populations were cultured in ITS medium until terminal muscle differentiation.

2.7 Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's post-test to calculate p values. Analyses were performed using statistical software (GraphPad Prism 5.04; GraphPad Software, San Diego, CA). Probability values <0.05 were considered statistically significant. Error bars in each figure represent the standard error of the mean of three or more individual experiments. For qPCR data, p values were calculated for changes in expression of markers over time compared to day 0. For quantitative analysis of FACS sorting data, the percentage of myogenic cells relative to the total number of cells were obtained for each experimental culture treatment, and p values were calculated for differences between the means of each experimental condition.

3. RESULTS

3.1 Derivation of skeletal muscle cells from hPSCs

I initiated differentiation on hPSCs cultured on matrigel at medium to high colony size and low colony density, using a serum-free medium consisting of DMEM-F12 supplemented with insulin, transferrin and selenium (ITS). As stated in the introduction of this thesis, the medium to high colony size would allow hPSCs to differentiate towards both neural and non-neural (mesendodermal) fates in the absence of a specific treatment (ITS only). Paraxial mesoderm specification of hPSCs was achieved through the activation of WNT/ beta-catenin signaling by exposure to the small molecule GSK-3β inhibitor CHIR 99021 (Cohen and Goedert, 2004; Lindsley et al., 2006; Tan et al., 2013). GSK-3β is known to target a number of substrates for phosphorylation, one of which is beta-catenin, an integral transducer within the canonical WNT signaling pathway. Therefore, inhibition of GSK-3β activity prevents the targeted phosphorylation of beta-catenin, rendering it resistant to degradation and thus leading to activation of T cell factor (TCF)-mediated transcription of downstream target genes (Wu and Pan, 2010).

During hPSC differentiation, inhibition of GSK-3β under feeder-free conditions have been shown to up-regulate primitive streak (PS) – like genes (Tan et al., 2013). Since the formation of PS marks the initial specification step that generates all mesodermal lineages during early embryogenesis (Chuai and Weijer, 2008), efficient differentiation toward the PS during in vitro PSC differentiation is an important first step toward increasing the yield of paraxial mesoderm and as such, myogenic cells derivation.

Even though paraxial mesoderm can be easily recapitulated during in vitro differentiation of hPSC, the differentiation of this tissue towards the myogenic lineage remained elusive. The challenge is due to a lack of inductive signals during hPSC differentiation that are necessary for proper myogenic patterning of paraxial mesoderm. The addition of appropriate growth factors required to recapitulate in vitro the specification of paraxial mesoderm into muscle, proved also to be challenging due to the lack of knowledge of the appropriate combination of factors and timing of their

delivery. Since it is necessary to simultaneously have both paraxial mesoderm and key embryonic-like structures such as neural tube and dorsal ectoderm to provide endogenous signaling necessary for the proper patterning of paraxial mesoderm and activation of the myogenic program (Bentzinger et al., 2012; Tajbakhsh and Buckingham, 2000), I decided to start the differentiation with medium to large colonies.

In addition to paraxial mesoderm, WNT signaling is a potent inducer of dorsal cell fates such as roof plate, neural crest and non-neural ectoderm (Menendez et al., 2011). Therefore, exposure to GSK-3β inhibition led to expression of dorsal markers LMX1A, SOX10 and AP2a (Gammill and Bronner-Fraser, 2003; Millonig et al., 2000). in differentiating hPSCs. This is in contrast to cultures differentiated in ITS alone which showed no expression of dorsal neural tube and neural crest markers LMX1A and SOX10, respectively (Fig. 5).

To optimize the differentiation of hPSCs towards a myogenic phenotype I tested different CHIR concentrations and found high toxicity at >3 μM and inefficient induction at doses of <3 μM (data not shown). Moreover, prolonged exposure to CHIR for up to 10 days demonstrated to have a negative effect on muscle derivation. Prolonged exposure resulted in reduced cell viability and an absence if muscle cell differentiation (data not shown). Therefore a balance must be sought between achieving robust differentiation and cell viability.

Following hPSC induction in the presence of 3 μM CHIR for 4 days, the small molecule was replaced with 20 ng/ml of FGF2 for an additional 2 weeks (Fig. 6A). The FGF signaling pathway has been identified to regulate several developmental processes of muscle formation. During somitogenesis, segmentation determination is mediated by an FGF signaling gradient within the presomitic mesoderm (Aulehla and Pourquie, 2010). Significantly, FGF molecules such as FGF2 have been described as potent inducers of mitogenic activity in both embryonic skeletal muscle precursors and adult satellite cells (Chakkalakal et al., 2012; Lagha et al., 2008; Sheehan and Allen, 1999). As such, the primary reason for the addition of FGF2 was to drive expansion of the muscle progenitor compartment within my culture system. Following withdrawal of FGF2 cells were cultured in ITS medium alone for a further 17 days.

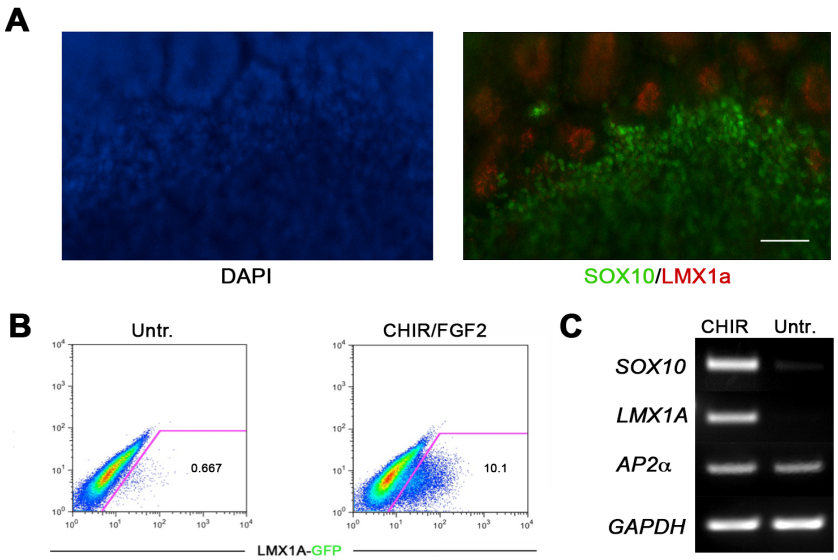


Figure 5. CHIR treatment promotes induction of dorsal tissues in differentiating hPSCs. (A) Immunocytochemical analysis of hESC (H9) at day 12 of differentiation under treatment conditions, showing neural crest marker SOX10 (green), dorsal neural tube/roof plate marker LMX1A (red) Scale bar: 50um. **(B)** FACS analysis on the hESC line LMX1A:GFP under ITS or CHIR/FGF2 conditions at day 10 of differentiation; LMX1A-driven GFP expression is present almost exclusively in CHIR/FGF2 treated cells. **(C)** RT-PCR analysis for *LMX1A*, *SOX10* and *AP2α* at day 10 of hESC (HES3) differentiation. *SOX10* and *LMX1A* are expressed only in CHIR treated cells while *AP2α* expression is detected also in untreated cells.

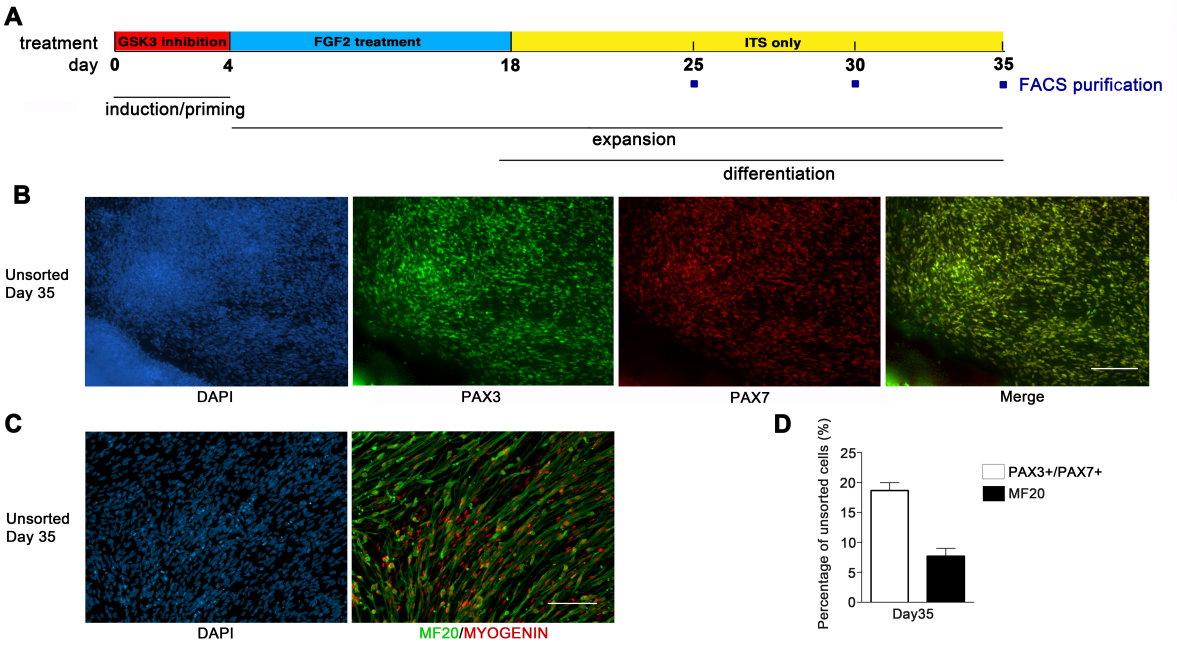


Figure 6. Derivation of skeletal muscle from hPSCs. (A) Schematic diagram summarizing the treatment protocol for the induction of myogenic differentiation from hPSCs. **(B)** Immunocytochemical detection of representative fields of PAX3+ and PAX7+ skeletal muscle precursors and **(C)** MF20+, Myogenin+ mature skeletal myocytes in unsorted cultures at day 35 of hESC (H9) differentiation, under treatment conditions Scale bar = 50 um. **(D)** Quantitative analysis of PAX3+/7+ nuclei and MF20+ cells at day 35 of hPSC differentiation (H9, HES3, MEL1,DPL-iPS n=4) in unsorted cultures.

Prior to FACS analysis, areas with skeletal muscle cells were scored in treated culture dishes, and identified by immunocytochemistry as PAX3+ and PAX7+ precursors (Fig. 6B) (Relaix et al., 2005) and bipolar skeletal myocytes, positive for myogenin and sarcomeric myosin (MF20) (Fig. 6C). Quantitative analysis revealed the percentage of total PAX3+/PAX7+ and MF20+ muscle cells within the cell culture to be >18% and >8%, respectively, demonstrating the robustness of the treatment strategy (Fig. 6D). To further profile the efficacy of the treatment, I analyzed the expression of key regulatory genes associated with the acquisition of a myogenic cell fate by quantitative PCR (qPCR). Data were acquired during a fixed 3-day interval starting at day 0 and ending at day 30 of in vitro differentiation in CHIR+FGF2 compared with untreated hPSCs (Fig. 7). Expression profiling of differentiating hPSCs over the course of treatment showed the guided progression of hPSCs through key myogenic milestones. Inhibition of GSK-3β resulted in a marked increase in the expression of paraxial/presomitic mesoderm genes such as *TBX6*, *Mesogenin* (*MSGN1*) (Wittler et al., 2007), and *MESP1* (Chan et al., 2013), with an early peak at day 3 of differentiation. Subsequent *PARAXIS* (Burgess et al., 1996) activation starting at day 9 of differentiation indicated progression toward somitic mesoderm. Significantly, expression of the muscle specification genes *SIX1* and *SIX4* (Grifone et al., 2005), *PAX3*, *PAX7*, and the migratory muscle progenitor marker *LBX1* (Gross et al., 2000; Schafer and Braun, 1999) exhibited marked activation at day 21 of differentiation under the treatment conditions. Expression of the myogenic regulatory factors *MYF5* and *MYOD* indicated muscle commitment and progression of the myogenic differentiation program (Rudnicki et al., 1993). In contrast, an insignificant activation of myogenic-specifier genes occurred during differentiation of untreated (ITS only) hPSCs. Interestingly, *PARAXIS* exhibited a second peak of expression beginning at day 21, correlating with the activation of *SIX1*, *SIX4*, *PAX3*, and *PAX7*. Although it is known to regulate somite epithelization, *Paraxis* has also been shown to be expressed in migratory hypaxial muscle progenitors (Delfini and Duprez, 2000). Therefore, secondary activation of *PARAXIS* expression, in conjunction with expression of *LBX1*, suggests a bias toward hypaxial myogenesis within my system.

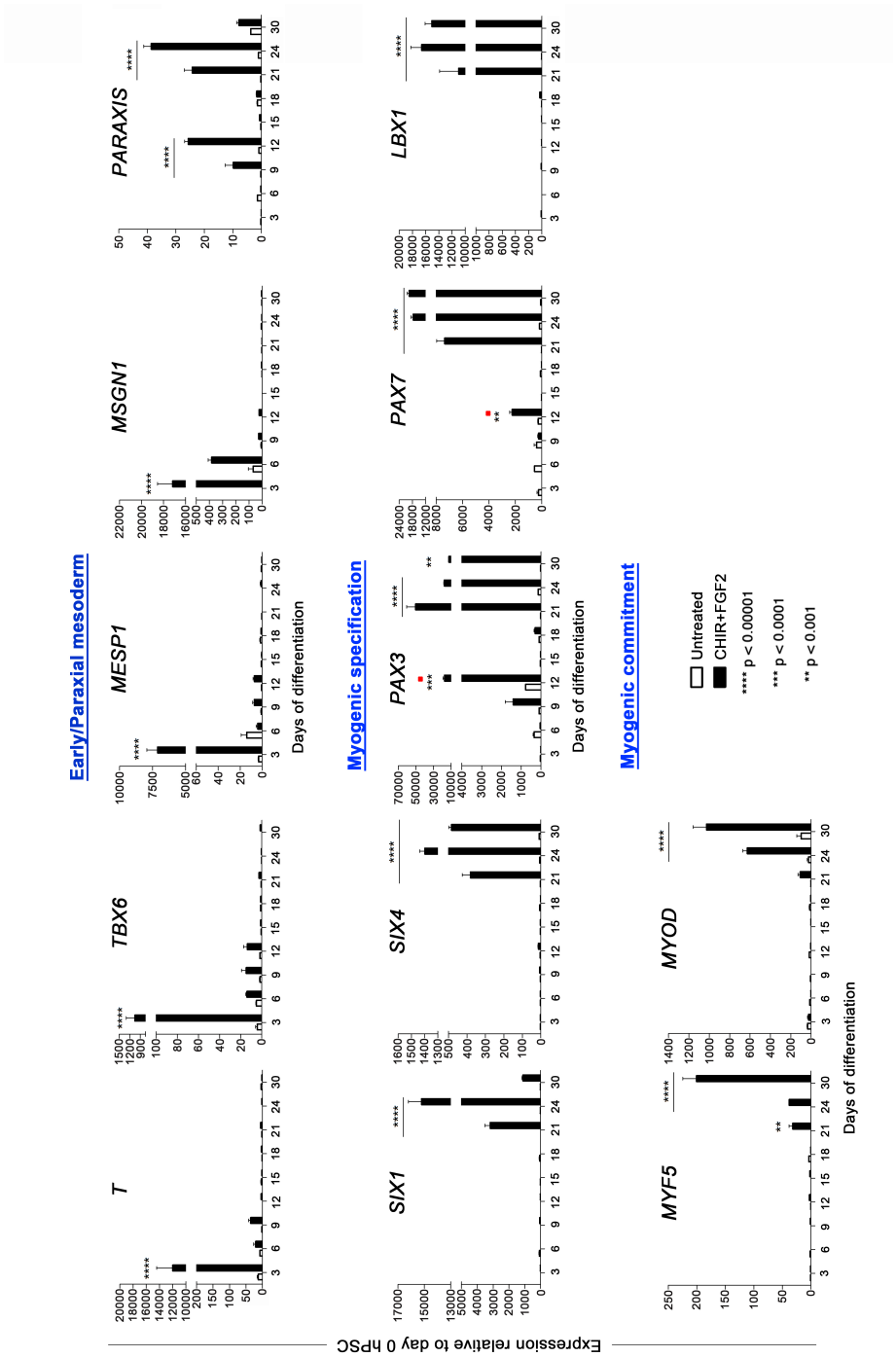


Figure 7. Detection of gene transcripts relevant to the acquisition of a myogenic cell fate. Quantitative polymerase chain reaction (qPCR) analysis showing transcript levels of key muscle development genes from hPSCs (DPL-iPS, H9, MEL1, HES3 n=4) differentiating under treatmentconditions vs medium alone. cells were collected and analyzed at three day intervals between days 0 and 30 of hPSC differentiation. The relative expression level of each gene is calibrated to its expression at day 0 (represented on the Y axis). Ct. values for each gene are normalized to Ct values of the reference gene, GAPDH. Values represent mean +/- SEM of four independent experiments. Red dots mark early peaks of PAX3 and PAX7 expression corresponding to the timing of development of early dorsal tissues (roof plate/neural crest).

3.2 FACS isolation of hypaxial skeletal muscle precursors

The expression of the migratory skeletal muscle progenitor marker *LBX1* I observed in CHIR-treated differentiating hPSCs, led me to consider purification of this putative migratory muscle cells by using two cell surface markers, which together are reported to define migratory muscle precursors: the CXC chemokine receptor-4 (CXCR4) and c-MET tyrosine kinase.

Within the hypaxial domain of the embryonic dermomyotome, c-MET expression is first observed in specified, PAX3+ muscle progenitors. Along with its ligand Scatter Factor/ Hepatocyte Growth Factor (SF/HGF), c-MET is critical for the delamination of PAX3+ LBX1+ migratory muscle precursors from the dermomyotome (Bladt et al., 1995; Dietrich, 1999). In the absence of c-MET, PAX3+ LBX1+ muscle progenitors are specified but fail to de-epithelialize and migrate, thus remaining aggregated within the dermomytome. This causes a complete absence of all muscle groups derived from these migratory precursors, such as limbs, tongue and diaphragm (Bladt et al., 1995; Dietrich et al., 1999).

During mouse and chick development, CXCR4 expression is first observed on muscle progenitors following their delamination from the dermomytome. At the time muscle progenitors migrate away from the dermomyotome, CXCR4+ cells co-express Pax3 and Lbx1. Along with its ligand SDF1, CXCR4 provides potent chemotactic signaling for the proper distribution and survival of muscle progenitors along migratory paths to target sites. This is demonstrated in CXCR4 null mice, which show pronounced reduction in the number of Lbx1+ muscle progenitors within the dorsal limb anlage as well as in additional migration routes such as those of the tongue and diaphragm (Buckingham, 2006; Vasyutina et al., 2005)

Once Pax3+/Lbx1+ muscle precursors reach their target sites, both c-MET and CXCR4 expression is extinguished prior to their differentiation and up regulation of Myogenic regulatory factor MyoD(Dietrich et al., 1999; Vasyutina et al., 2005).

In conjunction with the positive selection of muscle precursors by CXCR4 and c-MET, I used of a negative selection marker to eliminate unwanted cells which is fundamental for the development of a stringent purification stategy. CXCR4 and c-MET may also be

expressed in cells of different origins such as neural and neural crest cells, respectively (Kos et al., 1999; Sun et al., 2000; Zhu and Murakami, 2012). Therefore, to exclude these cell types, which represent a fraction of the total hPSC-derived cell populations in the dish, I used HNK-1 (human natural killer-1) carbohydrate, as a negative selection marker (Lee et al., 2007; Morita et al., 2008). This carbohydrate moiety is expressed on a number of cell adhesion and extracellular matrix proteins such as NCAM and tenascin-R. During development HNK-1 is widely expressed on neural crest cells as well as on cells of the central and peripheral nervous system. Although CXCR4 can be used to define and isolate definitive endoderm from hPSCs (Drukker et al., 2012; Teo et al., 2012) the CHIR treatment used in my protocol was not permissive for the generation of endoderm cells, as confirmed by the lack of specific endodermal markers in the cultures (data not shown).

Lastly, due to the presence in the dishes of muscle cells at different developmental stages, I used the acetylcholine receptor (AChR) as additional negative selection marker to eliminate mature muscle cells present in the culture. Exclusion of AChR+ cells allowed me to select for the earliest muscle progenitors expressing solely CXCR4 and c-MET.

In light of the observed activation of myogenic specification genes beginning at day 21 of hPSC differentiation (Fig. 7), I decided to isolate skeletal muscle precursors by FACS at three different time-points, day 25, 30 and 35. The following cell populations were isolated: HNK-/ACHR-/CXCR4-/c-MET- (all negative), HNK-/ACHR-/CXCR4+/ c-MET- (CXCR4+/c-MET-); HNK-/ACHR-/CXCR4+/c-MET+ (CXCR4+/c-MET+) and HNK-/ACHR-/CXCR4-/c-MET+ (CXCR4-/c-MET+). A detailed gating strategy used for the FACS protocol is shown in (Fig. 8).

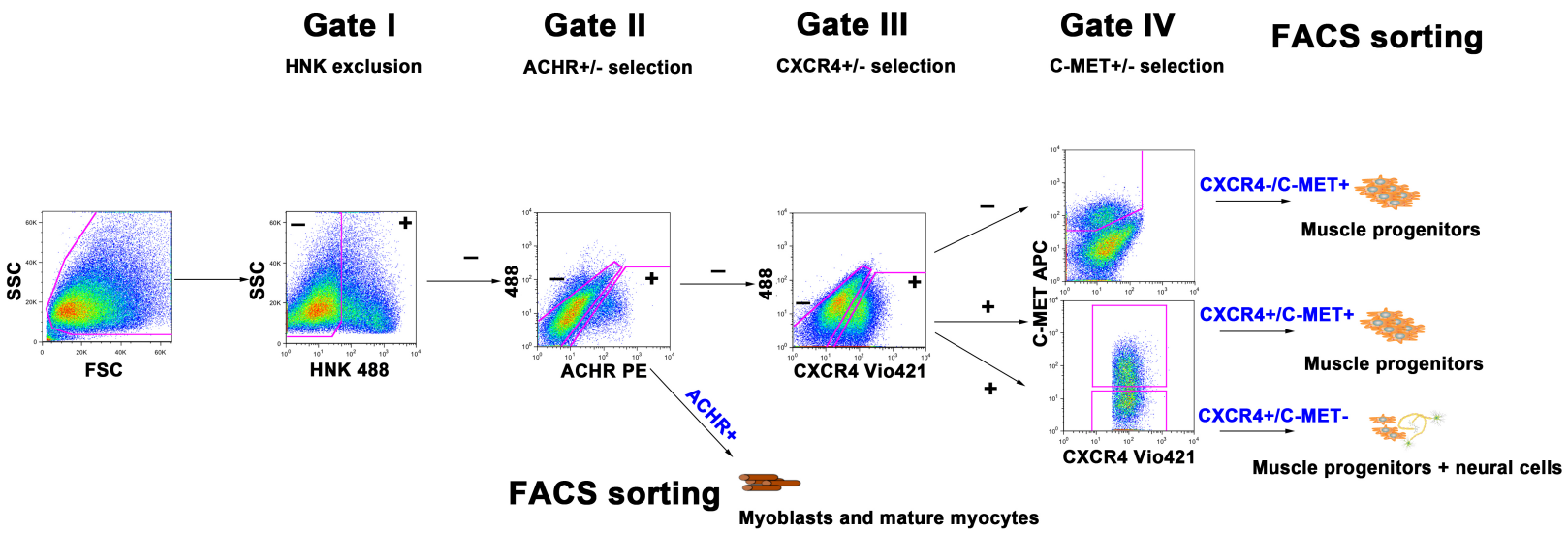


Figure 8. FACS strategy for the isolation of myogenic cell populations. Shown is a representative experiment in which hESCs (MEL1) differentiating for 35 days under treatment conditions were sorted based on their HNK, AChR,CXCR4 and c-Met surface marker expression. The gates in each dot plot designate the cell fraction analyzed for the prospective steps. +/- is indicative of either positive or negative expression of each surface antigen. The myogenic cell populations collected from sorting were asfollows: (HNK-/AChR+), (HNK-/AChR-/CXCR4-/c-Met+), (HNK-/AChR-/CXCR4+/c-Met+), (HNK-/AChR-/CXCR4+/c-Met-). Gate I: HNK- cells were selected to exclude HNK+ neural/neural crest component. Gate II: selection of HNK-/AChR- cells for myogenic progenitor isolation at subsequent steps or direct isolation of HNK-/AChR+ mature myocytes. Gate III: selection of CXCR4+/- cells. Gate IV: Isolation of myogenic progenitor cell populations: HNK-/AChR-/CXCR4-/c-Met+ from gated CXCR4- cells and HNK-/AChR-/CXCR4+/c-Met+, HNK-/AChR-/CXCR4+/c-Met- from gated CXCR4+ cells.

Post-sorting analysis revealed the presence of myogenic cells only in the populations where CXCR4 and/or c-MET were present (CXCR4+/ c-MET-, CXCR4+/c-MET+ and CXCR4-/c-MET+) (Fig. 9). To quantify the level of purity in these populations, I performed an immediate post-sort immunocytochemical analysis on cytospin preparations. The cytocentrifugation technique spins a cell suspension onto a defined area of a glass slide, creating a monolayer of flattened cells and thus allowing prominent nuclear presentation. Based on nuclear staining, only CXCR4-/c-MET+ and CXCR4+/c-MET+ cell populations allowed the isolation of highly pure skeletal muscle precursors. At day 35, the percentage of total cells immunoreactive for the muscle stem cell marker PAX3 was 97 ± 0.5% in CXCR4-/c-MET+ and 98 ± 0.2% in CXCR4+/c-MET+. The percentage of PAX7 was 84 ± 1.7% in CXCR4-/c-MET+ and 96 ± 2.8 in CXCR4+/c-MET+ (Fig. 9A). Immunocytochemical analysis of precursor populations sorted at earlier time-points revealed developmental progression of the myogenic program. CXCR4-/c-MET+ and CXCR4+/c-MET+ cells sorted at day 23 were characterized by expression of early myogenic specifier genes SIX4 and PAX3 prior to PAX7 expression. Subsequent acquisition of PAX7 expression, starting at day 25, marked lineage progression (Fig. 10). By day 35, close to all CXCR4-/c-MET+ CXCR4+/c-MET+ cells co-expressed PAX3 and PAX7 (Fig. 9A). However, an overall lower expression of PAX7 was observed in CXCR4-/c-MET+ cells compared to CXCR4+/c-MET+ cells. Given the earlier activation of Pax3 (Horst et al. 2006) and the expression of CXCR4 in late-stage migratory precursors (Vasyutina et al., 2005)) during muscle development, I speculate that CXCR4-/c-MET+ cells could represent a more primitive progenitor population.

Post-sorting cultures of CXCR4-/c-MET+ and CXCR4+/c-MET+ cells isolated at day 35 of hPSC differentiation confirmed the validity of the sorting strategy, with all plated cells from both populations undergoing progressive terminal muscle differentiation as shown by expression of MYF5, Myogenin and MF20 (Fig. 9B). After 3 days of culture few cells retained expression of PAX7 while all cells expressed MYF5 indicating muscle commitment and progression. By day 9 the majority of cells were in an advanced stage of muscle differentiation. Gene expression analysis by RT-PCR confirmed the immunocytochemical data, demonstrating the presence of *PAX3* and

PAX7 mRNA transcripts together with *LBX1* in both CXCR4-/c-MET+ and CXCR4+/c-MET+ sorted populations and, importantly, their absence in all negative cell population (Fig. 9C). Although enriched in muscle precursors, the CXCR4+/c-MET- cell population showed heterogeneity, with gene expression analysis revealing the presence of muscle-specific transcripts together with SOX1+ (pan-neural marker) expressing cells (Fig. 9C) and thus would not be useful for my present and future in vitro and in vivo studies.

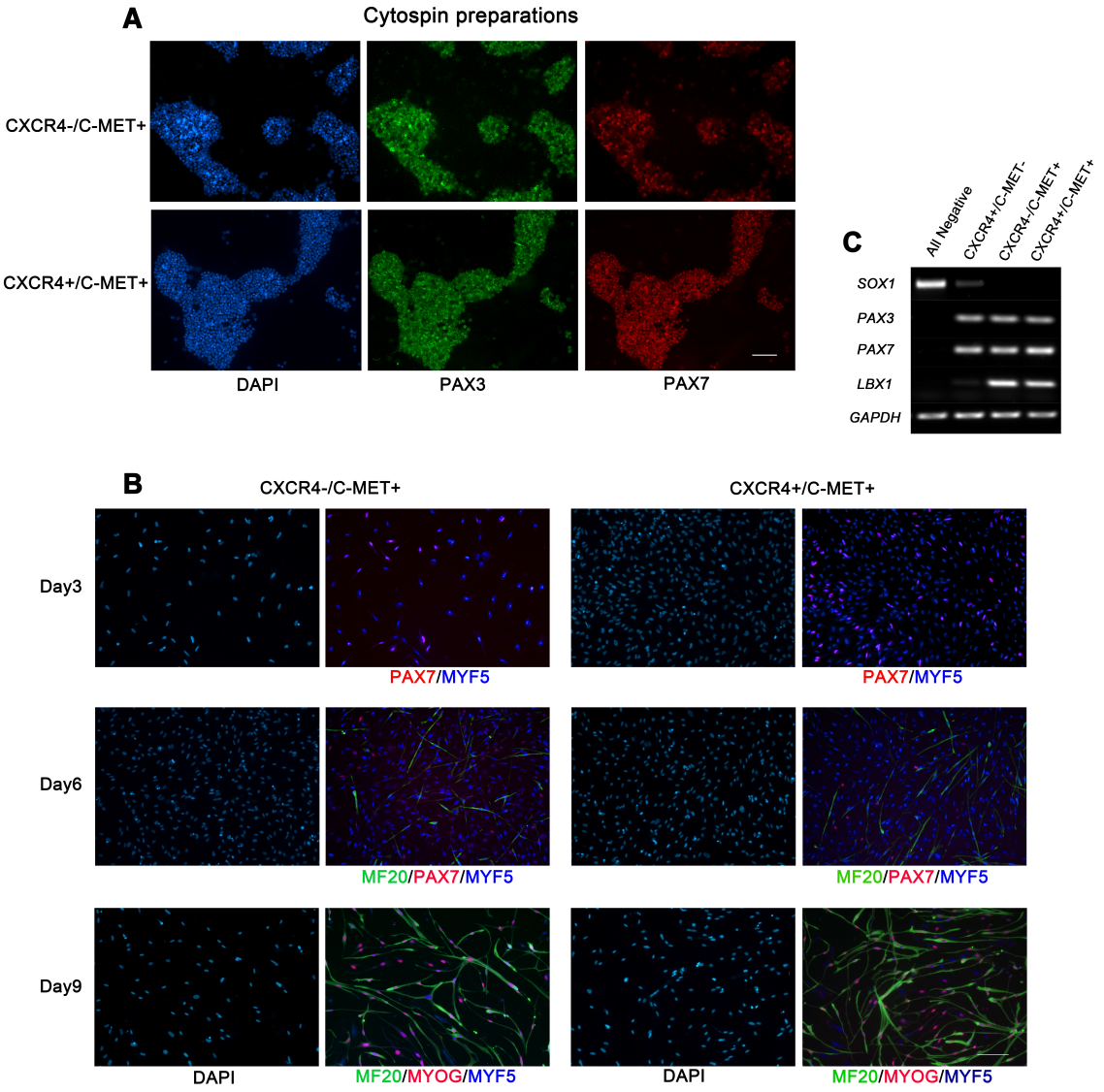


Figure 9. Characterization of CXCR4-/c-Met+ and CXCR4+/c-Met+ sorted populations. (A) Cytospin preparations of muscle progenitpr cell populations CXCR4-/c-Met+ (top) and CXCR4+/c-Met+ (bottom) sorted at day 35 of hESC (HES3) differentiation. Cells were cytospun onto glass slides and analyzed by immunocytochemistry for myogenic stem cell markers PAX3 (green) and PAX7 (red) immediately following sorting. Each dot represents one nucleus as confirmed by DAPI counterstaining **(B).** Immunostaining of replated muscle progenitors CXCR4-/c-Met+ (left) and CXCR4+/c-Met+ (right) (from hESCs-MEL1) at days 3,6 and 9 of post-sorting cultures showing progression towards a muscle terminal differentiation phenotype. **(C)** RT-PCR analysis of skeletal muscle progenitor genes (*PAX3, PAX7,LBX*1) and neural gene (*SOX1*) in all sorted populations (from DPL-iPS) derived under treatment conditions. Abbreviations: myogenin (Myog); sarcomeric myosin (MF20). Scale bars = 50 μm.

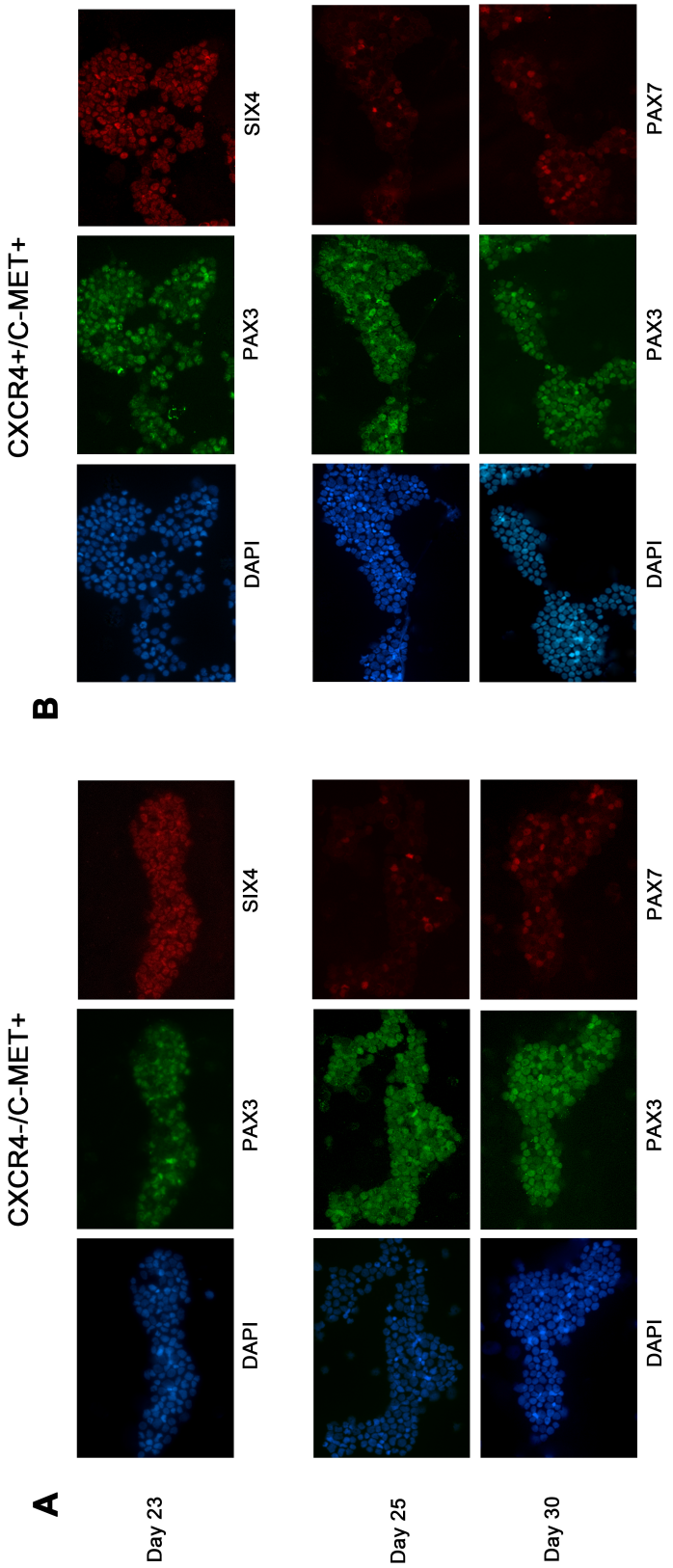


Figure 10. Developmental progression of hPSC-derived myogenic cell populations. Representative immunocytochemical analysis on cytospin preparations of progenitor populations' CXCR4-/c-Met+ and CXCR4+/c-Met+ at days 23, 25, and 30 of hESC (MEL1) differentiation. Expression of early myogenic regulatory genes SIX4 and PAX3 is detectable as early as day 23, prior to PAX7 expression as expected during myogenic lineage progression. As development proceeds (days 25-30), an increase in PAX7 expression is observed. Scale bars = 50 μm.

3.3 Single-step FACS isolation of mature skeletal myocytes

In addition to the central role in regenerative medicine, hPSCs and their derivatives also provide a platform for large-scale compound screening for drug development and toxicity testing. The cell types that offer the greatest value for drug screening are those that would be difficult to isolate from patients and are commonly post-mitotic. For instance, human primary skeletal muscle is not easily obtained and myoblasts cannot be expanded in vitro for a prolonged amount of time. As such due to their theoretical unlimited self-renewal and great differentiation capabilities, hPSC are replacing primary adult tissues as a platform to obtain mature cells for large-scale compound screenings.

Extrapolating from work performed with hESC, the generation of hiPSC has provided an indispensable source of patient-derived pluripotent cells for the generation of an in vitro disease model assay. As hiPSC carry the same genotype as the donor patient, disease relevant phenotypes can be recapitulated in vitro. The aim of this approach is to elucidate the molecular mechanisms of disease pathogenesis and a disease-based approach screening of novel therapeutic compounds. A major advantage is that hiPSC may be derived from patients carrying low penetrant mutations allowing generation of pluripotent cells from genetically complex disorders. Furthermore, hiPSC-derived cells allow assessment of the human-impact profile of a drug's toxicological effect, which otherwise would not be predicted using an animal model. In support of this, Yasuno and colleagues showed that skeletal myocytes derived from patients with carnitine palmitoyltransferase 2 (CPT2) deficiency, a disorder which leads to rhabdomyolysis, develop an accumulation of palmitoyl carnitine (C16) a biomarker that could be used to diagnose CPT2 deficiency (Yasuno et al., 2014). This is a proof of concept that hiPSC-derived myocytes recapitulated the metabolic manifestations of the disease.

Particularly for high throughput applications, it is essential to be able to generate hPSC derived skeletal myocytes at high purity. However, a challenge in generating a large-scale drug screening platform is that the appropriate combination of markers for the efficient purification of skeletal muscle cells remains unaccomplished. Moreover, the population of skeletal muscle cells isolated represents a heterogeneous mix of cells

belonging to all developmental stages, from progenitor to mature cells. This maybe hold a disadvantage if a compound's effect is required to be analyzed on a specific subset of cells, as data may be diluted if the readout is taken from the entire cell population. Therefore it is important to discriminately isolate different sub-populations of skeletal muscle cells.

To this end, I set up a simple single-antigen strategy for the direct isolation and purification of mature skeletal myocytes. In my two-step culture system, bipolar skeletal myocytes appeared at approximately 4 weeks of hPSC differentiation. Immunocytochemical analysis revealed strong expression of the muscle-specific nicotinic acetylcholine receptor (nAChR) on these cells (Fig. 11A). Nicotinic acetylcholine receptors form part of a larger family of ligand gated membrane ion-channels, which respond to the neurotransmitter acetylcholine, released by cholinergic motor neurons (Kalamida et al., 2007). This sub-family of receptors may be distinguished from other members of the superfamily by their additional responsiveness to the alkaloid, nicotine. AChR is composed of four different but highly related subunits that form a pentameric structure in which the subunits are selected from a pool of 17 homologous polypeptides (α1–10, β1–4, γ, δ, and ε) (Kalamida et al., 2007; Karlin, 2002) There are many AChR subtypes, each composed of a specific combination of sub-units, which mediate diverse physiological functions. Depending on subunit conformation, AChR's facilitate fast, excitatory synaptic transmission within the central nervous system or at the neuromuscular junction of developing muscle fibers and ganglia. During embryonic skeletal muscle development, receptors are distributed throughout the plasma membrane of myoblasts at a relatively low density and are composed of two α1 subunits and one each of β1, γ, and δ subunits. As maturation proceeds, receptor expression becomes highly concentrated within the future synaptic site (Franklin et al., 1980; Missias et al., 1996). Concurrent with these findings, immunocytochemical analysis against the α subunit of AChR revealed punctated expression throughout the plasma membrane of hPSC-derived skeletal myocytes.
As such, for the direct purification of hPSC-derived skeletal myocytes, I utilized an antibody specifically recognizing the muscle specific $\alpha 1$ subunits of AChR. At days 30

and 35 of hPSC differentiation, an easily distinguishable AChR+ population (up to 8% of total cells) was identified and isolated by FACS (Fig. 11B). Gene expression analysis on both AChR+ and AChR- fractions showed that expression of the mature muscle marker myosin heavy chain 2a (MYHC2) was restricted only to AChR+ cells (Fig. 11C). Following isolation, the AChR+ cells were plated onto fibronectin/laminin coated plates in the presence of ITS medium. At 24 hours after plating, as expected, all AChR+ cells were immunoreactive for the mature muscle markers myogenin and MF20 (Fig. 11D). Prolonged cell culture (>20 days) of AChR+ cells led to the progressive fusion of myocytes into multinucleated myotubes (Fig. 11E).

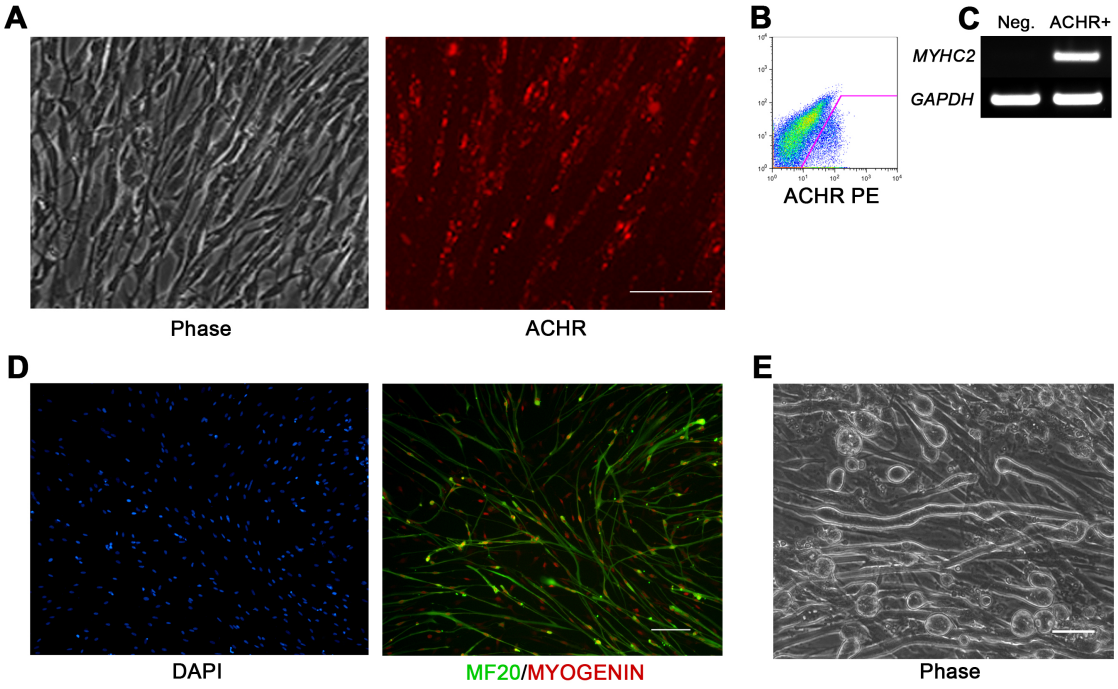


Figure 11. Isolation of AChR+ skeletal myocytes. (A) Phase contrast image (left) and immuno-cytochemical analysis for AChR expression (right) on hESC-derived (MEL1) skeletal myocytes prior to FACS isolation. **(B)** FACS profile of AChR+ cell population (from hESC-H9). **(C)** RT-PCR analysis of mature skeletal muscle marker *MYH2* in AChR negative cells (Neg) and positive cells (AChR+) (from HES3). **(D)** Immunocytochemical analysis of hESC-derived AChR+ myocytes (H9) 24 hours post-sort, expressing mature skeletal muscle proteins, MF20 and MYOG. **(E)** Phase contrast image showing morphology of AChR+myocyte-derived myotubes (from H9) after prolonged cell culture (>20 days). Scale bars= 50 μm. Abbreviation: myosin heavy chain 2 (MYH2).

3.4 GSK-3β inhibition is required for efficient muscle derivation

I next determined the efficacy of the two-step protocol by comparing the following sorted populations: AChR+; CXCR4+/c-MET+; CXCR4-/c-MET+ and CXCR4+/c-MET- derived under different culture conditions at different days of hPSC differentiation. The conditions were: CHIR+FGF2, CHIR only, FGF2 only, and untreated. Muscle precursors were already present at day 25 of hPSC differentiation as indicated by the presence of both CXCR4+/c-MET+ and CXCR4-/c-MET+ cell populations (12A II, III).

As expected, the overall percentage of each myogenic population increased overtime and thus at day 35, under CHIR+FGF2 treatment I obtained collectively from AChR+, CXCR4+/c-MET+ and CXCR4-/c-MET+ cell populations, a total of up to 20% of muscle cells (Fig. 12A I, II, III). Significantly, a large component of these cells were PAX3+ and PAX7+ precursors (CXCR4+/c-MET+; CXCR4-/c-MET+) comprising of more than 12% of total cells (Fig. 12A II, III). A similar robust percentage of muscle cells was observed across all the four cell lines I tested (3 hESC and 1 hiPS), demonstrating the efficiency of the protocol (Fig. 13). To note, the sorting conditions I used were very stringent, and to avoid any contamination with negative or low positive cells, gating were set in a very conservative way. In addition, the trypsin treatment I used to dissociate differentiating hPSCs had some effect on the AChR. A partial disruption of the epitope recognized by the anti AChR antibody resulted in a lower percentage of cells isolated by FACS.

Treatment with CHIR+FGF2 resulted in the efficient derivation of muscle precursors; however, exposure of hPSCs to CHIR only was sufficient for myogenic induction. Reduction in the percentage of CXCR4+/c-MET+ and AChR+ populations compared to CHIR+FGF2 cultures indicated an active role for FGF2 in the expansion of the myogenic compartment (Fig. 12A I, II 12B top and middle). In stark contrast, the absence of CHIR treatment resulted in almost complete loss of both of these cell fractions (Fig. 12A I, II, 12B top, middle). Interestingly, the overall percentage of CXCR4+/c-MET- and CXCR4-/c-MET+ cells did not change significantly among the four different treatment conditions (Fig. 12A III, IV, 12B middle, bottom).

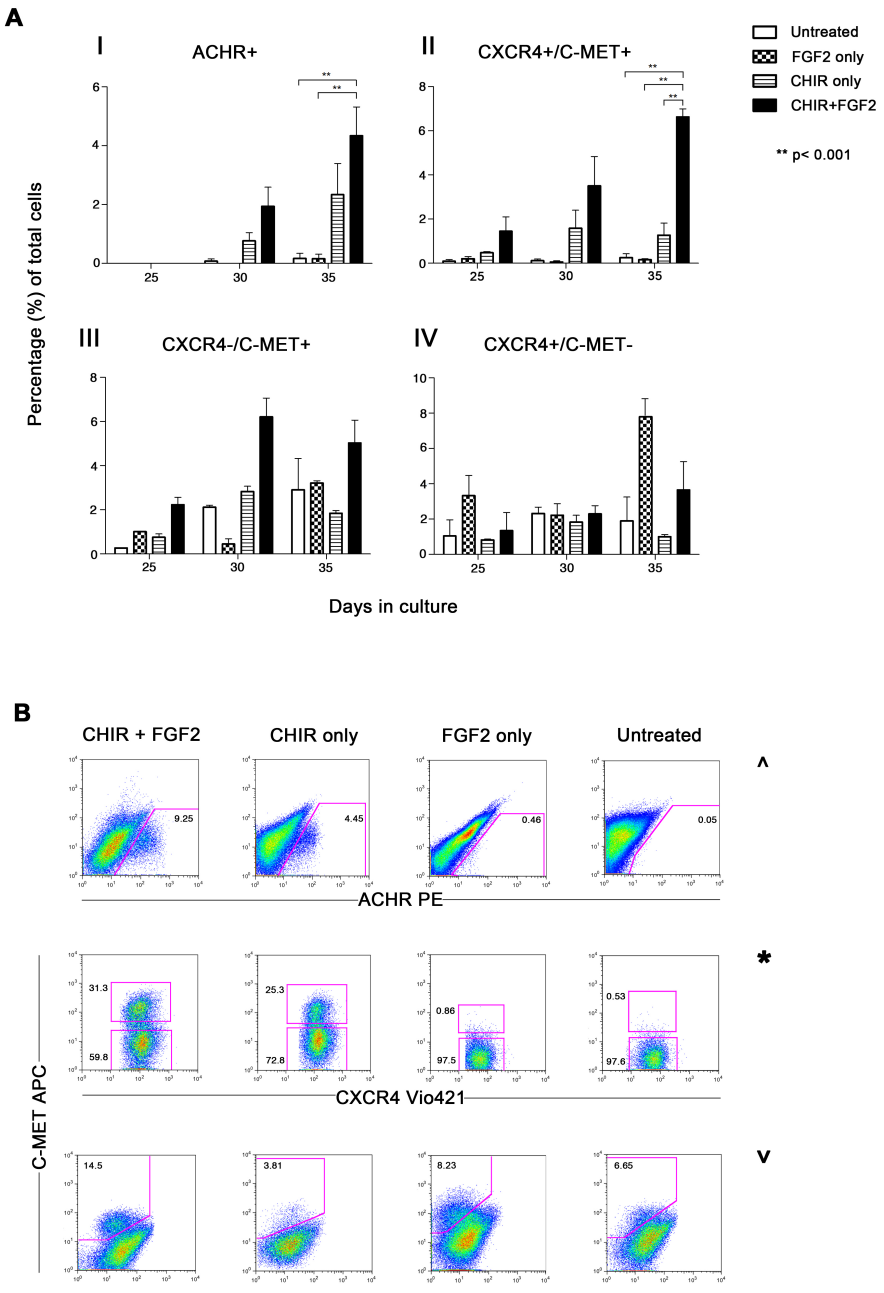


Figure 12. Quantification of AChR+, CXCR4+/c-Met+, CXCR4+/c-Met- and CXCR4+/c-Met- cell populations derived from hPSCs differentiated under 4 treatment conditions: CHIR+FGF2; CHIR only; FGF2 only; Untreated. **(A)** Percentage of AChR+ myocytes (I) CXCR4+/c-Met+ (II) CXCR4-/c-Met+ myogenic precursors (III) and CXCR4+/c-Met- mixed cell population (IV) from multiple FACS purification experiments at three different time-points. Results shown for each treatment condition represent (n=3) experiments averaged from each of the 4 hPSC lines. (I, II) Fold change difference is observed between CHIR+FGF2 treatment and all other conditions at each time-point. CHIR+FGF2 treatment significantly ($p<0.001$) improves induction of both cell populations at day 35 of differentiation compared to FGF2 only or untreated cultures. (III, IV) Percentage of cells is independent of treatment, however cell composition is altered (refer to Figure 14) **(B)** Shown is a representative FACS profile of hPSCs (H9) at day 35 of differentiation. The sorted populations are represented as percentage fractions of respective parent population. AChR+ (top) and CXCR4+/c-Met+; CXCR4+/c-Met- (middle) and CXCR4-/c-Met+ (bottom) cell populations are present under all conditions. **^** % of AChR+ gated populations are based on HNK- gated fractions. ***** % of CXCR4+/c-Met+ and CXCR4+/c-Met- gated populations are based on HNK-/AChR-/CXCR4+ gated fractions. **v** % of CXCR4-/c-Met+ gated populations are based on HNK-/AChR-/CXCR4- gated fractions.

However, comparative analysis of cell composition between these cell fractions isolated from CHIR+FGF2 or FGF2 only treated cultures revealed a fundamental shift from a myogenic to a non-myogenic cell fate in the absence of CHIR treatment (Fig. 14). These data illustrate a key requirement of CHIR-mediated GSK-3β inhibition for the robust induction of muscle cells from hPSCs. The addition of FGF2 is then necessary to achieve optimal expansion of skeletal muscle precursors.

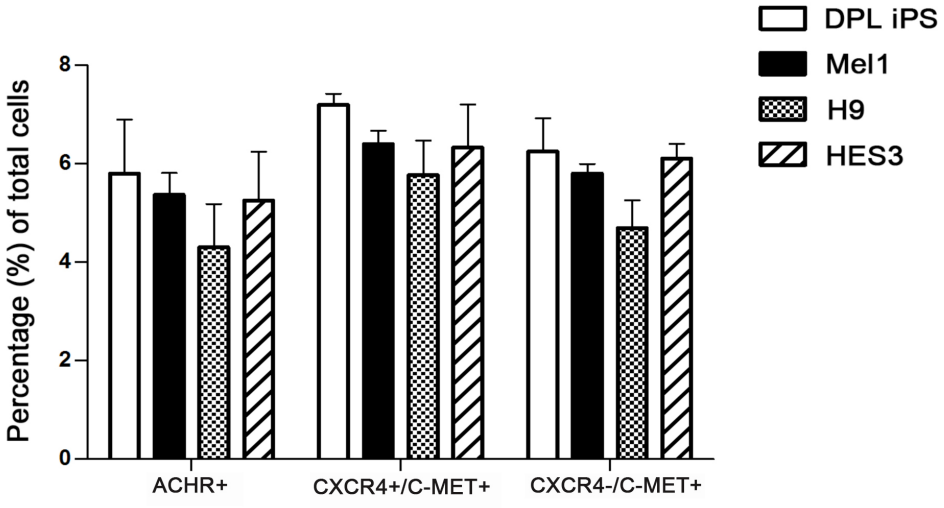


Figure 13. Quantification of ACHR+, CXCR4+/C-MET+ and CXCR4-/C-MET+ cell populations derived across hPSC independent cell lines. Percentage of myogenic cell populations isolated at day 35 from four hPSC lines differentiated under treatment conditions (CHIR+FGF2). Efficiency of myogenic differentiation was similar across all cell lines. Results shown for each cell popualtion represents n = 3 experiments averaged for each of the 4 hPSC lines.

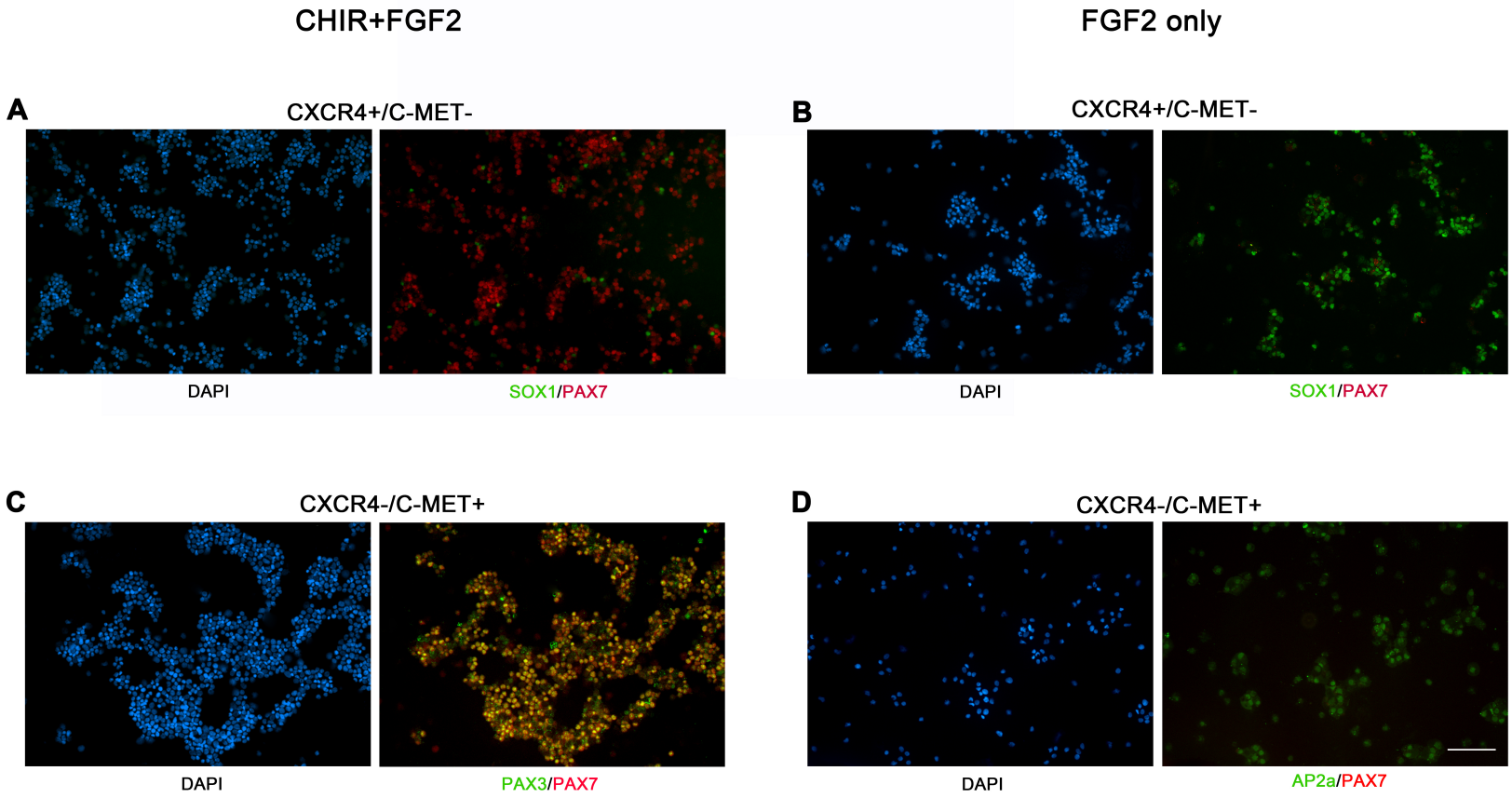


Figure 14. Lack of CHIR treatment during hPSC differentiation results in the absence of a muscle phenotype. Immunocytochemical analysis from cytospin preparations of CXCR4+/c-Met- **(A,B)** and CXCR4-/c-Met+ **(C,D)** sorted cells. **(A)** Under CHIR+FGF2 treatment, majority of CXCR4+/c-Met- cells are PAX7+ indicating a predominant muscle phenotype. **(B)** A complete switch towards SOX1 expression is observed in FGF2 only conditions. **(C)** CXCR4-/c-Met+ cell population derived from CHIR+FGF2 treated hPSCs is composed of highly enriched PAX3+/PAX7+ muscle precursors **(D)** PAX3+ and PAX7+ cells are not present under FGF2 only conditions, with a large number of cells instead expressing the non-neural ectoderm marker AP2α. Scale bars = 50μm. All images from hESC-HES3.

4. DISCUSSION

The successful use of hPSC-derived progeny for in vitro screening (e.g., for disease modeling, drug development and toxicity studies) or regenerative medicine, requires tight control of the cell differentiation process and isolation of pure, specialized cell types. At present, the controlled derivation and efficient isolation of hPSC-derived myogenic precursors equivalent to in vivo PAX3+/PAX7+ satellite cells has not been accomplished. Challenges that arose in the derivation of these cells include the lack of knowledge about the essential factors required in vitro to recapitulate the in vivo patterning of myogenic somitic mesoderm and the timing of their distribution. This may explain the previous limited success and the large number of published protocols relying on an artificial system of derivation utilizing forced transgene expression (Darabi et al., 2012; Goudenege et al., 2012). In spite of demonstrating proof of principle results that skeletal muscle progenitors may be derived from hPSCs, these protocols are not representing a significant progress in the field. The use of transgenes to induce selective differentiation limits the potential applications of the derived cells for both basic and clinical applications. In contrast, a developmental based approach to muscle derivation from hPSCs relies on the knowledge of embryogenesis to recapitulate in vitro, using signaling molecules, the necessary conditions for the differentiation into the muscle lineage. This includes examination of all developmental milestones, some of which are bypassed by transgene overexpression. A controlled, chemically defined protocol would be highly reproducible, readily adaptable to a broad number of cell lines and applicable for drug discovery studies and cell-based therapies. As such, a chemical-based approach has been increasingly used in recent years as a method of controlling lineage specification during PSC differentiation. The use of small molecules may address past challenges associated with genetic or growth factor based approaches. Firstly, their biological effects are specific, dose-dependent, rapid and reversible, allowing for precise control of cell fate specification. Secondly, they can be easily manufactured allowing for structural diversity and cost effectiveness (Zhang et al., 2012). To this end, I developed a simple small-molecule based, two-step differentiation method that recapitulates the early events of

embryogenesis to efficiently derive PAX3+/PAX7+ skeletal muscle precursors from hPSCs. I also demonstrate the feasibility of deriving robust numbers of skeletal muscle cells without the aid of transgene driven differentiation. Central to this method was the activation of canonical WNT signaling by the GSK-3β inhibitor CHIR. Expression profiling of hPSCs over the course of guided differentiation showed progression through defined developmental milestones leading to myogenesis. This transition was initiated by a strong induction of *TBX6, MESP1 and MSGN1* in CHIR-treated hPSCs, followed by high levels of *PARAXIS* expression, indicating progression into somitic mesoderm. In addition to the induction of paraxial mesoderm, activation of WNT signaling by CHIR was responsible for the generation of dorsal tissues, such as dorsal neural tube cells marked by LMX1A expression, along with SOX10+ neural crest cells and AP2α+ non-neural ectoderm (Fig. 5). It has been established that myogenic patterning of the dermomyotome requires WNT signaling from the dorsal neural tube and overlying ectoderm (Tajbakhsh and Buckingham, 2000), together with transient, neural crest-mediated notch activation of myogenic precursors (Rios et al., 2011). Early GSK-3β inhibition during hPSC differentiation allowed reproduction of the conditions necessary for the specification of skeletal muscle cells, closely replicating the events that occur during normal development in vivo. I speculate that the generation of dorsal tissues played an essential role in delivering the appropriate signals required for the patterning of the pre-somitic mesoderm within the culture system. Conversely, prolonged exposure to CHIR for up to 10 days demonstrated to have a negative effect on muscle derivation and no muscle cells were identified in the treated dishes (data not shown). Although I show that CHIR alone is sufficient for myogenic induction, prolonged FGF2 exposure proved to play a proliferative role by significantly increasing the number of myogenic precursors. The robustness of my protocol was validated by obtaining similar results with 4 hPSC lines, confirming that small molecule-mediated GSK-3β inhibition is a simple but highly efficient approach to direct differentiation of hPSCs into skeletal muscle precursors. Concomitant with the publishing of my findings, two additional studies were published utilizing small molecule-mediated GSK-3β inhibition to promote skeletal myogenesis in hPSCs (Shelton et al., 2014; Xu et al., 2013). By performing a high-throughput screening of

more than 2,000 compounds, Xu and colleagues found GSK-3β inhibition together with FGF2 treatment to be the only combination to efficiently activate Myf5 expression in explant cultures of somitic mesoderm from a *myf5*:GFP transgenic zebrafish. Their findings were then successfully translated to hIPSCs, where differentiation was induced in the presence of both the WNT/b-catenin signaling agonist and FGF2. Similarly, Shelton and colleagues generated a robust number of skeletal myogenic cells from hESCs utilizing both factors. Ultimately, these two studies significantly confirm the validity of my approach and the essential requirement of activated WNT/β-catenin signaling to promote myogenesis in hPSCs. This treatment finally allows for a tightly controlled and reproducible derivation of myogenic precursors from hPSCs thereby accelerating evaluation of the therapeutic potential of hPSC-derived muscle cells in preclinical models. In addition to the challenges faced in developing a standardized differentiation protocol the consideration of hPSC-derived muscle as a valid source of cells for basic and translational research applications has been hindered by the lack of an efficient method to isolate muscle precursors. Regardless of the efficiency of a differentiation protocol, a heterogeneous population of cells will always be present. Therefore, a stringent purification strategy must be developed in order to eliminate unwanted cells. Moreover, it is a requirement to be able to discriminate between all developmental stages of the target cell type (i.e. muscle progenitors, myoblast, myocytes), by isolating different sub-populations from progenitors to mature cells. To address this problem, I also developed a FACS strategy to purify the muscle precursors I generate with my differentiation system. Since I detected *LBX1* transcripts (a marker of hypaxial muscle), during directed myogenic commitment of hPSCs, I considered the use of two markers, c-MET and CXCR4, that are also known to be highly expressed in hypaxial migratory muscle precursors during development (Dietrich et al., 1999; Gross et al., 2000; Vasyutina et al., 2005). FACS selection of two populations, CXCR4-/c-MET+ and CXCR4+/c-MET+, allowed the isolation of PAX3+/PAX7+ precursors at high purity. Notably, the negative cell population (HNK-/AChR-/CXCR4-/c-MET-) was devoid of any muscle markers indicating not only that my sorting strategy is sufficient to isolate all skeletal muscle cells generated in my culture system but that all PAX3+/PAX7+ precursors are of

hypaxial origin. The specificity of this strategy is also confirmed by the complete absence of CXCR4+/c-MET+ cells and by a non-muscle identity of CXCR4-/c-MET+ cells in the absence of early GSK-3β inhibition during hPSC differentiation.

In addition to the isolation of skeletal muscle precursors, I set up a simple strategy for the direct isolation of mature skeletal myocytes through the positive selection of AChR+ cells. This highly efficient derivation and direct isolation of mature embryonic stage skeletal myocytes provides a platform for developmental modeling and candidate drug screening. I consider the work I have done during my PhD, where I successfully developed a method for the derivation of skeletal mucle precursors from hPSCs, the perfect groundwork to establish myself in the stem cell arena. I have therefore started planning the continuation of my studies. Expanding from these findings as first step I will further characterize these sorted myogenic cell populations. Firstly, co-expression of *LBX1* with PAX3+/PAX7+ muscle precursors, strongly suggests that these cells correspond to hypaxial/migratory muscle precursors (Dietrich et al., 1999; Gross et al., 2000; Vasyutina et al., 2005). It is necessary to perform an in vitro migratory assay to investigate the ability of sorted cells (CXCR4-/c-MET+; CXCR4+/c-MET+) to respond to HGF and SDF-1, the ligands of c-MET and CXCR4, respectively. During skeletal myogenesis in vivo, HGF and SDF-1 are expressed by the mesenchyme surrounding the developing myotome, where they direct, migration of hypaxial muscle progenitors to target destinations (limb, diaphragm) (Dietrich et al., 1999; Vasyutina and Birchmeier, 2006; Vasyutina et al., 2005). Furthermore as c-MET is expressed on myogenic progenitors prior to their delamination from the myotome and before expression of CXCR4, I can infer that the CXCR4-/c-MET+ cell population represents a more primitive myogenic progenitor population than the double positive CXCR4+/c-MET+ cells. To address this question, I will compare the gene expression profile between the CXCR4-/c-MET+ and CXCR4+/c-MET+ cell populations using deep-sequencing of the transcriptome (RNA-Seq).

Lastly, I will perform a co-culture assay composed of hPSC-derived skeletal myocytes and hPSC-derived motor neurons to monitor structural changes and localization of the AChR, and eventual in vitro formation of neuromuscular junctions (NMJ). A defining feature in the maturation of muscle and the formation of the NMJ is the accumulation

of AChR channels at the site of nerve-muscle contact as well as the conformational change of AChR subunits from the embryonic (ψ-AChR) to the adult (ε-AChR) types (Madhavan and Peng, 2005; Missias et al., 1996; Yumoto et al., 2005). This switch occurs gradually from late fetal to postnatal life. The presence of the ε-AChR is critical for correct development of synaptic structure and maintenance of neuro-muscular transmission (Croxen et al., 2001; Schwarz et al., 2000). An in vitro model of synaptogenesis will allow determination of the functional relevancy of hPSC-derived skeletal muscle by investigating its capability to a) switch expression from fetal to adult AChR subunits b) form functional neuromuscular synapses. Moreover, the nerve and muscle derived molecules that mediate development of the NMJ in humans could also be determined.

Concomitant with the in vitro characterization of hPSC-derived muscle cells, it is my priority to use this method of derivation to generate muscle progenitors for in vivo functional studies. The isolation of a large number of pure PAX3+/PAX7+/LBX1+ muscle progenitors from normal hPSCs will allow us to test their ability to replace/repair dystrophic fibers in a canine model of Duchenne MD (DMD) (Kornegay et al., 1988). These dogs represent the most appropriate animal model prior to clinical testing, as they present the same fatal disease progression observed in young boys affected by DMD (Verma et al., 2010). The expression of muscle stem cell markers PAX3 and PAX7 together with migratory muscle markers *LBX1*, c-MET and CXCR4 on hPSC-derived muscle progenitors (Dietrich et al., 1999; Relaix et al., 2005; Vasyutina et al., 2005), strongly support the use of these cells for therapeutic inventions due to their likelihood to a) migrate to target muscle sites following intra-arterial injection b) home into the endogenous satellite cell niche. In addition, the regional identity of the hPSC-derived muscle precursors make them the perfect match for transplantation studies in models of DMD where limb and diaphragm muscles are the most severely affected. While many inherited muscle disorders can profit from a cell-centric based approach (replacement of defective resident stem cells), some conditions where large volumes of muscle are lost or damaged such as volumetric muscle loss (VML), cannot be compensated via an inherent regenerative mechanisms (Grogan et al., 2011). Common causes of VML include trauma, tumor ablation or degenerative disease. One

therapeutic option for VML would be to engineer 3D muscle tissue in vitro with the hope of replacing the injured tissue to recover functional and structural integrity (Cittadella Vigodarzere and Mantero, 2014). Strategies to engineer muscle tissue involve collective use of different cell sources, small molecules and scaffold materials. Collaborative work with biomaterial engineering labs would be required to face the challenges associated with the incorporation of myogenic cells within an appropriate three-dimensional biomaterial scaffold. Ideal biomaterials for skeletal muscle engineering should be able to support the propagation and expansion of myogenic progenitor cells in vitro and also, for in vivo implantation purposes, be able to integrate into host tissue (Wolf et al., 2014). Use of hiPSC-derived skeletal muscle progenitors may overcome challenges observed when developing cellular constructs such as cell survival, proliferation and differentiation (Atala et al., 2012; Cittadella Vigodarzere and Mantero, 2014) In addition to the potential clinical application of hPSC-derived skeletal muscle progenitors in muscle replacement or repair, these cells represent a tool for studying muscle development.

My findings demonstrated that undifferentiated hPSCs can be directed to differentiate into hypaxial-like myogenic cells through small-molecule activation of WNT signaling. However, some interesting data I acquired during the writing of my thesis indicate that through the addition of a specific morphogen in conjunction with WNT activation, I was able to manipulate the system and obtain muscle cells with a different positional identity. Gene expression analysis on myogenic cells differentiated in the presence of the morphogen revealed an absence of posterior HOX gene expression (HOX10-13) compared to myogenic cells derived under CHIR/FGF2 treatment alone (data not shown) (Alexander et al., 2009). This anterior shift in axial identity corresponds with the trunk in a developing embryo. Together with the HOX gene expression shift, the lack of LBX1 expression strongly suggests a non-migratory/epaxial identity for the derived muscle. Modulation of this protocol would allow me to derive myogenic cells with alternative regional identities. This is very significant and may provide new insights to the development of different muscle groups in vitro. Furthermore, it may be applied to disease modeling using hiPSCs from muscle syndromes that are tissue regionalized such as Facioscapulohumeral muscular dystrophy (Shieh, 2013).

5. CONCLUDING REMARKS

Muscle loss and atrophy can result from a wide range of genetic (Muscular Dystrophies) or acquired (trauma, cancer) conditions but share the same dramatic outcome. Despite an extraordinary worldwide effort to find a treatment and a possible cure for these conditions, the results are very modest with no effective solutions available. Promising results from molecular, gene and cell therapeutic approaches are now being tested in the clinic but more research is needed to continue this progress towards a cure. To this end, I have established a highly reproducible method for muscle derivation from human pluripotent stem cells (hPSC). This method is the first non-transgenic based system to allow an efficient derivation and isolation of pure skeletal muscle progenitors. By treating hPSCs with a WNT signaling agonist, I created the conditions to efficiently derive skeletal muscle at two levels: 1) by promoting the formation of paraxial mesoderm from which the somites and skeletal muscle originates; and 2) by inducing the formation of dorsal neural/neural crest cell phenotypes that provide the soluble and contact-mediated signaling to the paraxial/somitic mesoderm to initiate the myogenic program. This tightly controlled system enabled us to use Fluorescence Activated Cell Sorting (FACS) to isolate the emerging muscle progenitors at high purity. A combination of surface antigens that includes the Hepatocyte Growth Factor receptor c-MET and the chemokine receptor CXCR4 have been used to purify PAX3+/PAX7+/LBX1+ hypaxial muscle progenitors able to terminally differentiate in post-sorting cultures. This efficient protocol provides a platform for developing a standardized production of myogenic cells from hPSCs, to be used for in vivo preclinical studies, in vitro screenings and disease modeling.

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7. ADDENDUM

7.1 List of primers

GENE	SENSE	SEQUENCE 5' to 3'	ANNEALING TEMPERATURE °C	AMPLICON LENGTH bp
*LBX1**	Forward	AAAGTCGCGCACGGCCTTCA	59	249
	Reverse	GCCAGCGCCACGATGTCCAT		
*PAX7**	Forward	ACCCCTGCCTAACCACATC	60	121
	Reverse	GCGGCAAAGAATCTTGGAGAC		
*PAX3**	Forward	TACAGGTCTGGTTTAGCAAC	57	183
	Reverse	GATCTGACACAGCTTGTGGA		
MYF5	Forward	TTCTCCCCATCCCTCTCGCT	59	235
	Reverse	AGCCTGGTTGACCTTCTTCAG		
MYH2	Forward	CCGCCCTTGACAAAAAGCAA	55	220
	Reverse	GCGCAGGATCTTTCCCTCTT		
ACHR	Forward	GCTAACCCTCACCAACCTCAT	59	346
	Reverse	GGTTGCTGCACTTTGGTCC		
*MYOD**	Forward	GCGCGCTCCTGAAACCCGAA	60	166
	Reverse	TCGGCGTTGGTGGTCTTGCG		
*TBX6**	Forward	TTCCCGGCTCTCACCTCCGT	60	143
	Reverse	TGGCCTGCACCAGTGTGTGT		
*MESP1**	Forward	CACACCTCGGGCTCGGCATAAA	60	119
	Reverse	CAGGCCGCAGAGAGCATCCAG		
*GAPDH**	Forward	CCCCTTCATTGACCTCAACTACA	60	342
	reverse	TTGCTGATGATCTTGAGGCTGT		
*SIX4**	Forward	CCATGCTGCTGGCTGTGGGAT	60	164
	Reverse	AGCAGTACAACACAGGTGCTCTTGC		

*PARAXIS**	Forward	AGGGCCACGGAGATGAGCCT	61	120
	reverse	GGTCCCCCGGTCCCTACACA		
SLUG	forward	GGCACTTGGAAGGGGTATTGT	52	377
	reverse	TACATGTCGGTTGTCTGGTTGC		
SOX10	forward	CCCACACTACACCGACCAG	59	143
	reverse	GGCCATAATAGGGTCCTGAGG		
SOX9	forward	CATGAACGCCTTCATGGTGTG	60	200
	reverse	GGTACTTGTAATCCGGGTGGT		
PAX6	forward	CCATCTTTGCTTGGGAAATC	60	213
	reverse	GAAGTCCCCGGATACCAAC		
SOX1	forward	GAGCTGCAACTTGGCCACGAC	60	271
	reverse	GAGACGGAGAGGAATTCAGAC		
AP2a	forward	AGGCAGAGCCAGGAGTCTGGGCT		
	reverse	CGGAGCACTCCGCCCAGCAGCGA	60	464
LMX1A	forward	TCCTAGCCTTGGAGAAGCAACT		
	reverse	CAGTGACTGGAGCAGAGAGAA	59	271

* Primers used for Quantitative PCR

7.2 List of antibodies

* (Developmental Hybridoma Studies Bank)

PRIMARY ANTIBODIES				
ANTIGEN	**FLUOROCHROME**	**HOST/CLONALITY**	**DILUTION**	**COMPANY**
Human HGF/C-MET	APC	mouse IgG1	1:50	R&D Systems
CXCR4 (CD184)	Brilliant Violet 421	Mouse IgG2a	1:200	BioLegend
Mab 35 (ACHR)		Mouse IgG1	1:100	DHSB*
Human CD57 (HNK-1)		mouse IgM	1:200	Sigma Aldrich
PAX3		mouse IgG2a	1:100	R&D Systems
PAX7		mouse IgG1	1:100	DHSB
MYF5		rabbit polyclonal	1:100	Santa Cruz Biotechnology
MYOG		mouse IgG1	1:100	Santa Cruz Biotechnology
MYOD		rabbit polyclonal	1:100	Santa Cruz Biotechnology
MF20		mouse IgG2b	1:100	DSHB
MYHC2		mouse IgG2a	1:100	DHSB
SOX1		goat IgG	1:100	R&D Systems
PARAXIS		goat IgG	1:100	Santa Cruz Biotechnology
SOX10		goat polyclonal	1:100	R&D Systems
AP2α		mouse IgG2b	1:100	DHSB
LMX1A		rabbit polyclonal	1:200	Abnova
SIX4		Mouse IgG1	1:100	Abnova
SECONDARY ANTIBODIES				
Anti-rabbit IgG (H+L)	Alexa Fluor 647	donkey	1:400	Molecular Probes (Invitrogen)
Anti-goat IgG (H+L)	Alexa Fluor 647	donkey	1:400	Molecular Probes (Invitrogen)
Anti-goat IgG (H+L)	Alexa Fluor 488	donkey	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgG2a	Alexa Fluor 555	donkey	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgG(H+L)	Alexa Fluor 488	Goat	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgG2b	Alexa Fluor 555	donkey	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgG1	Alexa Fluor 647	Goat	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgM	Alexa Fluor 555	Goat	1:400	Molecular Probes (Invitrogen)
Anti-rabbit IgG (H+L)	Alexa Fluor 488	Goat	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgG1 (H+L)	Alexa Fluor 555	Goat	1:400	Molecular Probes (Invitrogen)
Anti-mouse IgG1	PE	Goat	1:400	Molecular Probes (Invitrogen)

7.3 Isolation and preliminary characterization of a sclerotome-like cell population from hPSCs.

In parallel to the work described in the main body of my thesis, I have identified a cell population with myogenic potential forming after prolonged hPSC differentiation. This cell population spontaneously forms during ITS-induced differentiation started at a medium cell density (3000 cells/cm^2) to achieve induction of both neural and non-neural cell types.

After 30-35 days of hPSC differentiation I observed the emergence of a distinct cell population, easily identified as a cluster of rounded, tightly packed cells (Fig. 1A). Taking advantage of their compact and unique morphology, this cell population could be easily mechanically isolated from the rest of the culture, allowing characterization.

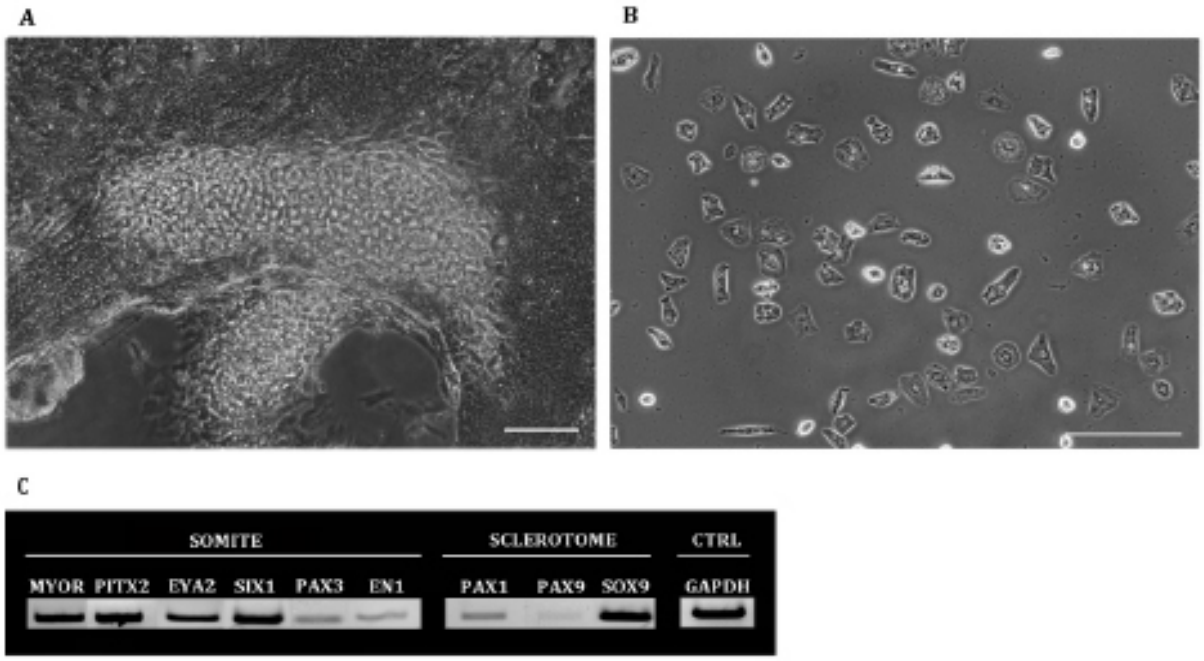

Figure 1. Characterization of hESC-derived somitic-like cells. **(A)** Phase contrast image showing a compact cell cluster with bone/myogenic potential arising after day 30 of hESC-differentiation. **(B)** Phase contrast image of replated single cells after mechanical isolation. **(C)** RT-PCR analysis on mechanically isolated clusters showing expression of somite genes. Scale bar: 50 um.

When replated after enzymatic dissociation, single cells from these clusters displayed distinctive granules and a polygonal shape (Fig. 1B).

A preliminary gene expression analysis performed on mechanically isolated cells, revealed expression of markers corresponding to a somitic–like cell identity, such as Six1, Pitx2, and Pax3. However, most significant, was the expression of distinct sclerotomal markers Sox9, Pax1 and to a lesser extent Pax9 (Fig. 1C). Forming part of the ventral compartment of the developing somite, the sclerotome comprises vertebral and rib precursor cells (Christ and Scaal, 2008; Shih et al., 2007).

Once replated, dissociated cluster cells develop a mesenchymal phenotype in the first 24-48 hours. When cell confluency is reached, condensation of these mesenchymal cells is coupled with the acquisition of bone specific markers (Fig. 2A,B). This

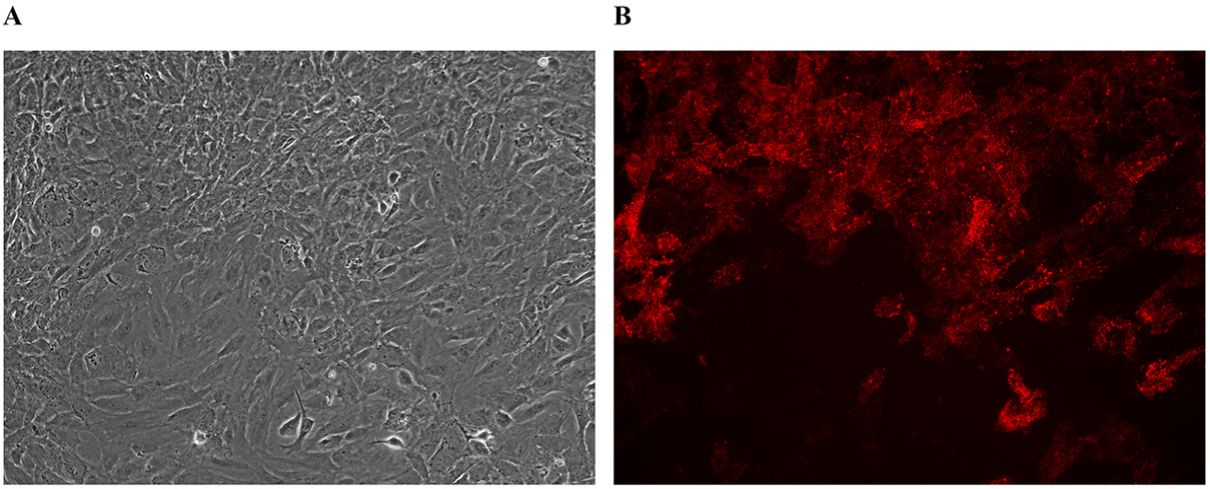

Figure 2. Rapid acquisition of a mesenchymal cell morphology in replated cells originating from mechanically isolated cell clusters. **(A)** Phase image showing condensation of mesenchymal cells. **(B)** Immunostaining for bone-specific alkaline phosphatase revealing progression towards the osteocytic lineage

spontaneous differentiation is in agreement with a sclerotome-like cell identity as suggested by RT-PCR data. Interestingly, when left in the original culture for a prolonged time (>40 days), bipolar cells resembling embryonic skeletal myocytes are sometimes observed emanating from these cell clusters. Immunocytochemical analysis confirmed the identity of these cells as mature MYOG+ skeletal myocytes (Venuti et al., 1995). (Fig. 3)

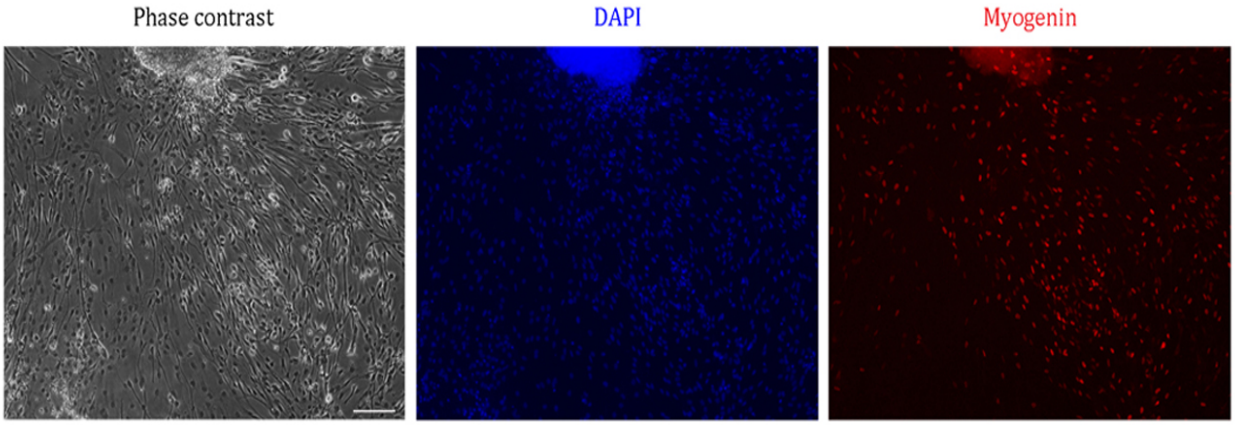

Figure 3. Characterization of skeletal myocytes arising at day 42 of hESC differentiation (**left)** Phase contrast image of hESC-derived skeletal myocytes differentiating from a cell cluster of somitic like cells **(centre)** DAPI staining marking cell nuclei. **(right)** Immunocytochemistry for mature skeletal muscle marker myogenin. Scale bar: 50um.

Although being preliminary findings, the incongruous expression of SOX9 and PAX1 with cells possessing myogenic potential, makes this cell population worthy to be investigated further. It has been established in murine and chick models that *Pax3* and *Sox9* define either dermomytome or sclerotome lineages respectively (Cairns et al., 2008) Whether this cell population is an equivalent to the in vivo sclerotome but retains cellular plasticity, remains to be determined.

Preliminary screening for surface antigens have allowed FACS mediated isolation of this putative sclerotome-like cell population by revealing expression of the fibronectin receptor, integrin beta 1 (CD29) (Brakebusch et al., 1997) However as differentiation proceeds, when cells within the cluster adopt a mesenchymal-like phenotype, CD29+ cells start to co-express the low affinity nerve growth factor receptor (p75) (Locksley et al., 2001) (data not shown). Given these observations, I isolated the sclerotome-like cells based on the preliminary, CD29+ p75- profile. As CD29 also marks neural cells, I isolated the target population by first removing HNK-1+ cells (Morita et al., 2008) that could correspond to a possible neural sub-population (Fig. 4A).

Comparative analyses between mechanically isolated and CD29+p75-HNK-1- sorted cells to determine whether the sorting strategy successfully isolated the target cell type from the general culture. FACS isolated cells were replated on fibronectin-coated plates in the presence of ITS medium. Sorted cells exhibited a cell morphology and gene expression profile, comparable to the mechanically isolated cells suggesting that this cell sorting strategy selects the sclerotome-like compartment within the culture system (Fig. 4B-C).

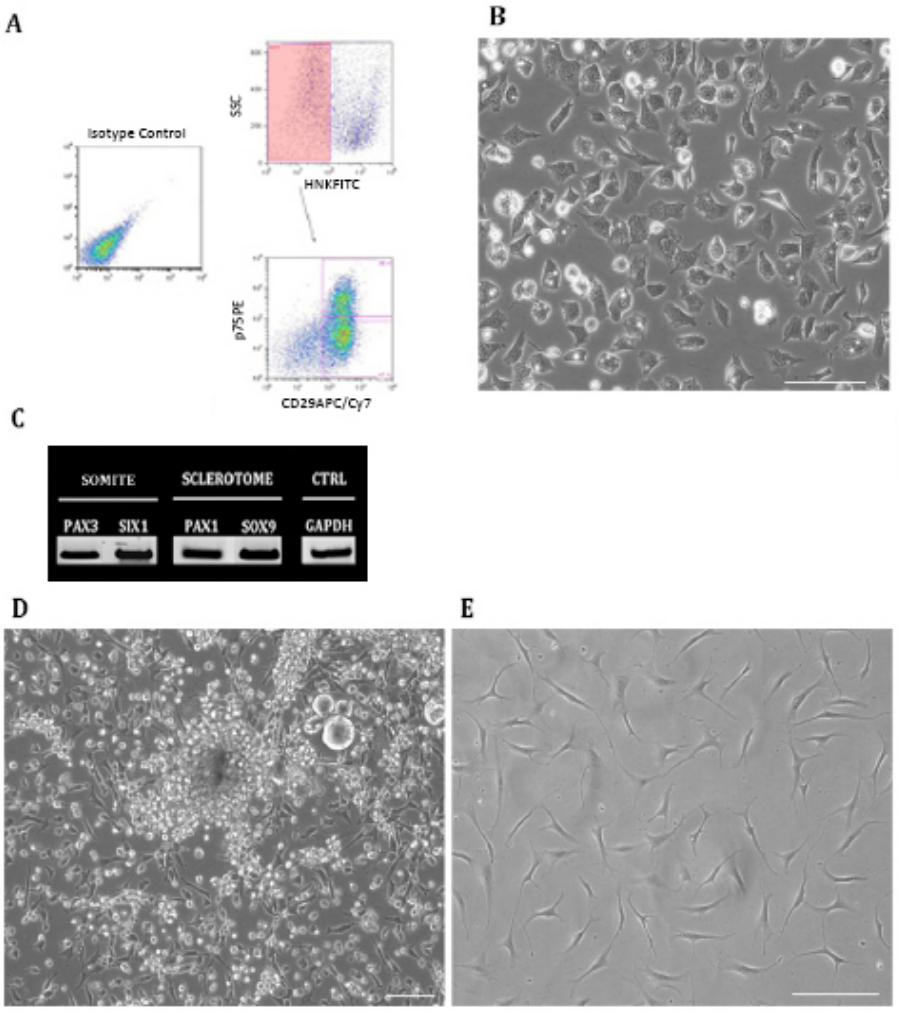

Figure 4. Isolation and characterization of hESC-derived somitic-like cells generated under stroma-free conditions. **(A)** Representative FACS sorting for the isolation of somitic-like cell populations: CD29+ p75- ; CD29+ p75+. Both populations were sorted against an HNK-1 negative background. **(B)** Phase contrast image of CD29+p75-HNK-1- cells after sorting. Note: Sorted cells have similar morphology to the somitic-like cells, mechanically isolated from differentiating hESC cultures (Fig. 1B) **(C)** RT-PCR analysis on CD29+p75-HNK-1- cells reveal somitic gene expression profile, similar to mechanically isolated cells (Fig. 1C). **(D)** Phase contrast image depicting CD29+p75-HNK-1- cells, cultured in the presence of 10 ng/ml BMP2, 10ng/ml BMP4 for 3 days. **(E)** Phase contrast image depicting the same cells cultured in N2 medium only. Scale bar: 50um

Immunocytochemistry analysis and in particular, genome wide microarray on mechanically isolated cells, will identify additional markers, allowing me to build a comprehensive surface antigen profile to better define this target cell population. When replated in the presence of ITS medium supplemented with 10ng/ml of BMP2 and BMP4, these cells retain their original morphology and proliferate, forming loosely adhered cells, which float up into the media (Fig. 4D). In contrast, when replated, in ITS medium alone, CD29+ p75-HNK-1- cells begin to differentiate, losing their distinct form and adopting the mesenchymal morphology (Fig. 4E). During development lateral sclerotome cells are known to respond to BMP signaling, initiating the chondrogenic program (Christ and Scaal, 2008).

Based on the previous immunostaining analysis, I also collected an additional cell population: CD29+p75+HNK-1-. This cell population, as expected, was composed of mesenchymal cells (Fig. 4A).

Interestingly, I have observed the appearance of cell clusters with identical morphology to the hPSC-derived sclerotome-like cells in differentiating mouse embryonic stem cells (mESCs) (Fig. 5A-B). These cells started to appear after 14 days of mESC differentiation in N2 medium supplemented with B27. Given the striking similarity, I mechanically isolated these clusters and performed an RT-PCR analysis for genes I previously found expressed on the hPSC-derived clusters. Based on the small subset of genes analyzed, the cells isolated from mESC had an identical gene expression profile to the hPSC-derived ones (Fig. 5C) strongly suggesting that this could be the murine counterpart. Due to the many advantages of mESC such as a shorter developmental time and greater availability of research tools, this system could serve to further characterize these cells.

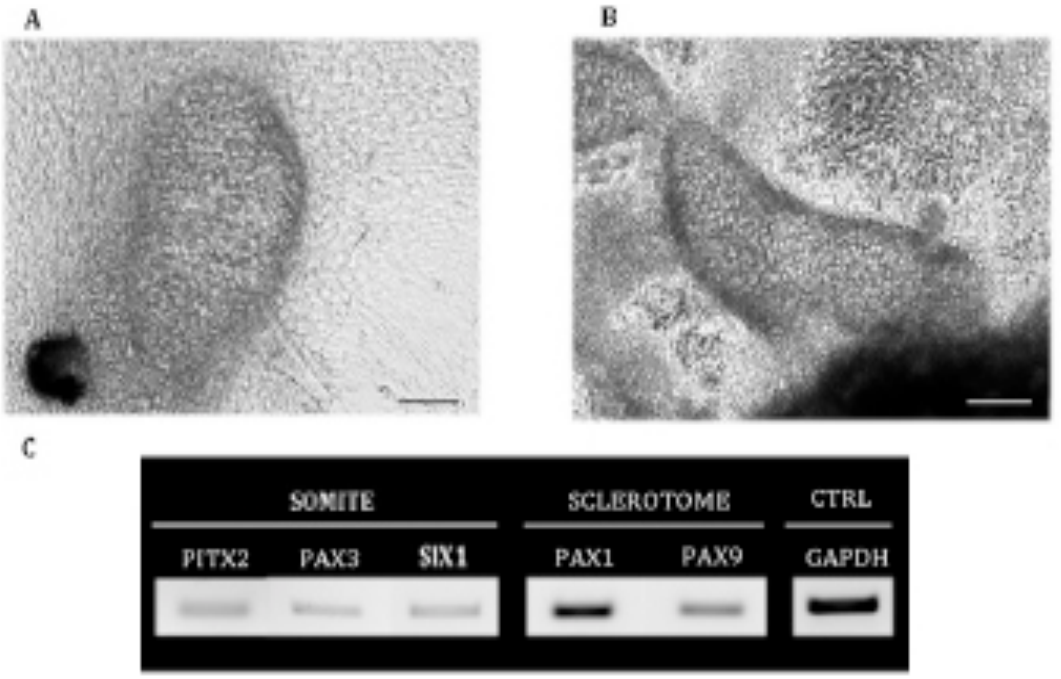

Figure 5. Characterization of mESC-derived somitic-like cells. (A) Morphological comparison between hESC-derived cell clusters and (B) mESC-derived cell clusters. (C) RT-PCR analysis showing expression of somite genes.

In conclusion, I have identified and isolated a cell population from differentiating human and mouse PSCs, which express markers suggestive of a sclerotome-like cell identity. Although the sclerotome is described as responsible of forming the axial skeleton in vivo, this cell population retains a degree of myogenic potential, in vitro. This could be explained by an incomplete commitment of these cells towards a sclerotome fate together with a dependence on specific environmental signaling from dorsal tissues occasionally present within the culture. These putative sclerotome-like cells were not observed in CHIR-treated hPSCs. It is likely that the early effect of WNT signaling biases the system towards dorsal fates of the paraxial mesoderm. A larger set of genes needs to be analyzed to confirm whether or not these are indeed an in vitro equivalent of a ventral somite.

7.3.1 References

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