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Myogenic Progenitor Specification from Pluripotent Stem Cells

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Abstract

Pluripotent stem cells represent important tools for both basic and translational science as they enable to study mechanisms of development, model diseases in vitro and provide a potential source of tissue-specific progenitors for cell therapy. Concomitantly with the increasing knowledge of the molecular mechanisms behind activation of the skeletal myogenic program during embryonic development, novel findings in the stem cell field provided the opportunity to begin recapitulating in vitro the events occurring during specification of the myogenic lineage. In this review, we will provide a perspective of the molecular mechanisms responsible for skeletal myogenic commitment in the embryo and how this knowledge was instrumental for specifying this lineage from pluripotent stem cells. In addition, we will discuss the current limitations for properly recapitulating skeletal myogenesis in the petri dish, and we will provide insights about future applications of pluripotent stem cell-derived myogenic cells.

Graphical abstract

Since the discovery that 5-azacytidine treatment of fibroblasts was capable of inducing the activation of the myogenic program [1], it became clear that somatic cells could be forced to switch identity and, importantly, this paved the way for the future cloning of the first master regulator transcription factor: MyoD [2, 3]. As a matter of fact, myogenic conversion of fibroblasts using MyoD represented the first example of controlled cellular reprogramming, a concept that has seen its groundbreaking application with the advent of reprogramming somatic cells into induced pluripotent stem (iPS) cells by Yamanaka and colleagues [4]. The iPS cell technology represented an exceptional achievement that opened the possibility to generate patient-specific iPS cell lines which, in turn, broadened the application of

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pluripotent stem (PS) cells from primarily basic research to potentially regenerative medicine [5]. Throughout the text, we will use pluripotent stem (PS) cells for referring to studies involving both iPS and/or ES cells.

PS cells are capable of differentiating into virtually any cell type and over the years they became important tools for studying mechanisms of development or pathogenesis, and an attractive source of progenitors for the development of cell-based therapies in regenerative medicine [6]. In addition, PS cells are more permissive to genome editing through homologous recombination and this property has further enhanced their potential application in personalized medicine [7]. However, the generation of tissue-specific stem/progenitor cells with proven in vivo regenerative potential from PS cells is evidently a challenging goal, due mostly to the complex cell signaling processes occurring during embryonic development, which are difficult to recapitulate in the culture dish.

In the specific case of the skeletal muscle, signals from adjacent structures, including the neural tube and the notochord, and from migrating neural crest cells are essential for somite patterning toward the dermomyotome and ultimately the skeletal muscle commitment [8-10]. Although early attempts to induce the myogenic program from differentiating PS cells were inefficient, significant progress has been achieved in the last decade. In this review we will provide an historical perspective and an update of the recent findings in this field (Figure 1).

1. Skeletal myogenesis in the embryo

1.1 Skeletal muscle cell identity

Skeletal muscle cells are defined by the expression of Muscle Regulatory Factors (MRFs), the transcription factors regulating activation of muscle-specific genes. This family of proteins include 4 members, MyoD, Myf5, MyoG and Myf6 (also called Mrf4), which were identified based on their ability to convert fibroblasts into muscle cells, sequence similarity to MyoD, and/or subtractive hybridization [2, 11-14]. These transcription factors, which are highly conserved across mammals, result from the duplication of an ancestral gene present also in non-vertebrates (e.g. *nautilus* in the case of *Drosophila melanogaster*) [15]. Subsequent studies demonstrated that MRFs can bind the promoters of several skeletal muscle-specific genes and modulate their transcription through collaboration with multiple proteins, including other transcription factors and chromatin remodeling complexes (reviewed by [16]). The myogenic transcriptional activity of MRFs is also not equivalent, as showed by the role of Myogenin and Myf6 in the later stages of myocyte differentiation [17-19], while Myf5 and MyoD play a major role in myoblasts determination [20]. Interestingly, Myf5 and MyoD also differ on their capability to remodel chromatin and recruit RNA polymerase II [21]. The complexity of the skeletal myogenic program is also highlighted by the complex regulatory regions driving the expression of these transcription factors, with the Myf5-Myf6 and MyoD loci representing the most well characterized [22]. In the case of Myf5-Myf6, enhancer elements are dispersed in genomic regions spanning about 150Kb [23-25], only a few of which have been well characterized using transgenic animals [26, 27]. The diversity in regulatory elements responsible for the activation of a single MRF

likely reflects the need to control their expression through a mechanism involving different transcription factors restricted to specific subpopulations of myogenic progenitors.

1.2 Embryonic origin of skeletal muscles

All the skeletal muscles of the body originate from the commitment of mesodermal progenitors, which through distinct mechanisms, lead to the expression of the MRFs (reviewed by [28]). Thanks to lineage tracing studies, we now know that, except for the head muscles, all the other muscles of the body derive from the somites, aggregates of paraxial mesoderm that forms on both sides of the neural tube through segmentation of the presomitic mesoderm [28]. Mesoderm patterning represents a key step during embryogenesis, as multiple lineages arise from these uncommitted mesodermal progenitors. In mouse embryos, time and position of entry of mesodermal progenitors into the primitive streak result in the acquisition of a specific fate, and this phenomenon has been attributed to the exposure to different signals from the adjacent structures [29]. Understanding the signals responsible for the specification of paraxial mesoderm has represented a key step for the generation of myogenic cells from pluripotent stem cell cultures in vitro.

Presomitic mesoderm specification occurs in the tail bud of the mouse embryo, an important region containing neuromesodermal progenitors (NMPs) capable of generating both neural and paraxial mesoderm cells [30]. NMPs are characterized by the expression of both Sox2 and Brachyury (T) transcription factors, which define their neural and mesodermal fate, and represent a transient but extremely important population for the formation of the vertebrate trunk (reviewed by [31]). Differentiation of NMPs involves WNT, FGF and RA signals, and interference with any of these signaling pathways is associated with a truncated body axis due to depletion of NMPs [31–33]. Acquisition of the presomitic mesoderm fate involves Wnt3a and Fgf8, which exert a posteriorizing effect on NMPs by inducing expression of the Cdx transcription factors [31]. In NMPs undergoing mesoderm differentiation, Cdx proteins are important for Sox2 repression in favor of T expression [34]. As cells become presomitic mesoderm, T is downregulated while Msgn1 and Tbx6 are upregulated [35]. The expression of these two transcription factors is instrumental for the maintenance of Presomitic Mesoderm (PSM) [36, 37] since deletion of either one of these genes results in ectopic neuronal fate, as highlighted by the phenotype of Tbx6-null embryos, which consists of three neural tubes [38]. Molecular analyses of Msgn1 and Tbx6 function determined that these two transcription factors do not play redundant roles: Tbx6 deletion is associated with increased expression of Sox2 through the N1 enhancer, a regulatory element associated with ectopic expression of Sox2. Deletion of this enhancer rescues the Tbx6-dependent repression of the neuronal lineage but not the defects in the paraxial mesoderm [39]. In contrast, Msgn1 appears to represent the main inducer of PSM differentiation since it induces transcription of several paraxial mesoderm genes [40]. Since expression of T and Tbx6 is not affected in Msgn1-null embryos, it is likely that Msgn1 acts downstream of Tbx6 [37].

Following PSM specification, a sophisticated mechanism of gene expression based on signaling pathway oscillations (described by the “clock and wavefront” model) gives rise to the somites (reviewed by [41]). These aggregates of epithelial cells form on both sides of the neural tube through segmentation of the PSM. This process is regulated by NOTCH, WNT

and FGF signaling pathways which, based on local thresholds, activate the expression of segmentation genes, including the transcription factors *Mesp1/2*, thus defining the anterior and posterior borders of the future somite [41]. Newly formed somites have an epithelial cell layer surrounding a mesenchymal core, a process dependent on the Mesenchymal-to-Epithelial Transition (MET) of the anterior PSM cells [42]. After formation, these cell aggregates undergo compartmentalization into dermomyotome, which retain an epithelial organization, and sclerotome, which involves Epithelial-to-Mesenchymal Transition (EMT) of the ventral somite [42]. Importantly, the former will give rise to trunk skeletal muscles, brown adipose tissue and dermis, while the latter will form the axial skeleton. Somite compartmentalization represents another key aspect for the derivation of skeletal muscle cells from pluripotent stem cells. This process depends on signaling molecules secreted by the surrounding structures, which will direct the myogenic specification within the epithelial dermomyotome, or the acquisition of another fate, as in the case of sclerotome. WNT factors from the dorsal neural tube and the surface ectoderm, and low levels of Sonic Hedgehog (SHH) from the notochord provide positive signals for the specification of the dermomyotome [8]. In addition, recent studies from the Marcelle group highlighted also the contribution of migrating neural crest cells in the initiation of myogenesis, a process involving both WNT and NOTCH signals [9, 43, 44]. In contrast, high levels of SHH along with BMPs from the lateral plate mesoderm exert a negative effect on this process [45].

Skeletal muscle progenitors are specified in the central domain of the dermomyotome and can be identified by the expression of the Paired-domain TF Pax3 [46, 47]. Although a Myf5-dependent primitive wave of myogenesis is also observed in the newly formed somite, embryonic myogenesis requires Pax3 (reviewed by [48]). This protein regulates the expression of genes involved in cell migration (*Met*), commitment (*Myf5*), proliferation and survival of the myogenic progenitors that ultimately will contribute to the skeletal muscles of the limb, diaphragm and tongue [26, 47, 49]. Pax3-null animals display a complete lack of muscles requiring myogenic progenitor migration from the hypaxial dermomyotome, and severe impairment in hypaxial myogenesis, while epaxial myogenesis is less affected [48]. The myogenic activity of these two transcription factors depends on the ability to regulate *Myf5* expression and accordingly, Pax3:Pax7 or Pax3:Myf5 double knock-out embryos are characterized by severe impairment in skeletal muscle development, irrespective of hypaxial or epaxial origin [47, 50]. Notably, because distinct genetic hierarchies drive myogenesis in the head vs the trunk, most head muscles are spared in both Pax3:Pax7 and Pax3:Myf5 double knockout embryos [47, 50]. In addition, further studies demonstrated that Pax3:Myf5 double knockout are *de facto* triple Pax3:Myf5:Myf6 mutants due to inactivation of both *Myf5* and *Myf6* [51]. *Myf5* expression in the epaxial dermomyotome also depends on Pax3 through *Dmrt2* regulation, thus highlighting the concomitant involvement of multiple molecular mechanisms in the activation of this key gene [52]. In addition to Pax3/7, the complex Six1/4–Eya1/2 plays an important role in myogenic progenitor specification by acting upstream of Pax3 and *Myf5* expression [53]. Moreover, Six transcription factors regulate myogenesis at different levels, including fiber-type specification [54].

As mentioned above, *Myf5* expression depends on a large regulatory region, spanning about 150 kilobases, which includes the well characterized hypaxial (–57.5Kb) and trunk (–111Kb) enhancers [26, 27]. Molecular analyses demonstrated that both hypaxial and trunk

elements contain Pax3 binding sites, necessary for the activity of these enhancers, as well as other conserved motifs for the binding of Six4 (both -57.5Kb and -111Kb), Meox2 and Msx1 (-57.5Kb) and Tead (-111Kb) [27, 55–57]. Since Myf5 has a central role in the specification of the myogenic cell identity, it is not surprising that this gene is regulated in a Pax3/7-independent manner in skeletal muscles from non-somitic origin such as the facial muscles. In addition to Myf5, the MRF Myf6 located in the same genomic locus, is characterized by a distinct expression pattern during development [23, 58]. The complex regulation of this locus has been supported by the observation that attempts to knock-out only Myf5 or Myf6 resulted in impairment in the expression of both genes [25, 51, 59].

Similarly, the MyoD regulatory domain extends for several kilobases (the most distal known element is located 22Kb upstream of the transcription start site) and relies on important enhancer elements (DRR and CE) necessary for its expression [60, 61]. In addition, MyoD regulation involves also enhancer RNAs, transcribed by DRR and CE, which modulate chromatin accessibility at both MyoD and MyoG loci [62]. Although MyoG function is mainly restricted to terminal differentiation, the expression of this MRF is also controlled by proximal elements harboring multiple TF binding sites, including MyoD, Mef2 and Six4 [63, 64].

Altogether, the complex regulation of MRFs elicits how the activation of the skeletal myogenic program can be achieved through multiple avenues, culminating with the expression of the muscle specific genes (such as MyHC). This knowledge is critical for the generation of skeletal muscle from pluripotent stem cells.

1.3 From embryonic myogenesis to adult satellite cells

Formation of the myotome represents only the first of a long series of events concluding with the establishment of the definitive muscle (reviewed by [65]). In fact, the process of embryonic myogenesis gives rise to a scaffold upon which a subsequent wave of fetal progenitors participates in building the multinucleated myofibers that collectively will constitute the skeletal muscle unit. Fetal myogenesis is characterized by an independent population of progenitors expressing Pax7 arising from Pax3+ embryonic progenitors [66]. Compared to their embryonic counterpart, fetal progenitors are sensitive to external stimuli, such as TGF β and BMP signaling, and their properties rely on the expression of a different set of transcription factors (TFs), including Nfix and others [67–69]. Notably, fetal myogenesis is also important for the specification of the pool of progenitors that will later become the resident muscle stem cells (also called satellite cells). After birth, neonatal and adult myogenesis are respectively responsible for muscle growth/maturation and tissue repair. Muscle homeostasis in the adult is ensured by satellite cells, which are located beneath the muscle basal lamina and require the expression of Pax7 for their maintenance, proliferation and myogenic function [70–73]. Transplantation studies demonstrated that satellite cells hold a tremendous potential in regenerative medicine [74, 75]. However, the mechanisms behind 1) satellite cell formation during development and 2) maintenance in the adult muscle are still under investigation (reviewed by [76]). With no doubt, elucidation of the molecular determinants accounting for these aspects will definitively improve our ability to ex-vivo expand human satellite cells or generate a bona fide human satellite cells from PS

cells. When achieved, these goals will enable satellite cell-based therapy of muscle disorders.

2. Pluripotent cells as model to study muscle development

The technological advances we have experienced in the 30 years following the demonstration that MyoD can induce the skeletal myogenic conversion of 10T1/2 fibroblasts are now enabling us to manipulate cell fate decisions in the culture dish with an unprecedented accuracy. In parallel to the studies that shed light on the genetic program behind skeletal muscle differentiation, other fundamental discoveries in the field of cellular and developmental biology were instrumental for the birth of regenerative medicine. Unarguably, cloning of mouse and human ES cells followed by the development of iPS cell technology represent milestones in this process.

Before the derivation of mouse embryonic stem (ES) cells, the earliest attempt to recapitulate skeletal myogenesis in vitro took advantage of embryonic carcinoma (EC) cell lines, such as P19 cells. These cell lines, derived from mouse testicular teratomas, display the capability to differentiate into multiple adult tissues, including all three germ layers (reviewed by [77]), a feature of pluripotent stem cells. Importantly, treatment of embryonic carcinoma cells with Retinoic Acid or DMSO demonstrated that these cells were able to give rise to neuronal or muscle cells (both cardiac and skeletal), respectively [78, 79], enabling the use of this system to begin dissecting the mechanisms driving early embryonic muscle development [80].

Although EC cell lines were instrumental in the future isolation of the first mouse ES cell line, the latter attracted much more interest from the scientific community, and slowly replaced the use of EC cells. Mouse ES cells (mES) [81, 82] followed by human ES (hES) [83] and lastly iPS cells [4] became a very important tool for both basic and translational research. Importantly, derivation of mouse ES cells enabled the development of technologies to perform gene manipulation, allowing the generation of knock-out/in animals [84], which without any doubt, paved the way for the development of iPS technology, and the most recent genome editing techniques [85]. Pluripotent stem cells are now widely used to investigate new developmental biology questions and, more importantly, to identify the signals that in vitro lead to the specification of progenitors with tissue regenerative potential (reviewed by [86]). Before refining the current protocols, studies involving ES cell differentiation focused for many years on lineages that were easily recognized in the culture dish, such as the presence of red blood cells or beating cardiomyocytes [86]. Remarkably, similarly to mouse development, in vitro differentiation of mouse ES cells recapitulate both primitive and definitive hematopoiesis, allowing several investigators to use this system to study transcription factors and signaling pathways involved in early hematopoietic cell fate specification [87–89].

The first demonstration of skeletal myogenic differentiation using mES cells is dated 1994, which involved their differentiation as embryoid bodies in 15% dextran-coated charcoal treated Fetal Calf Serum (DCC-FCS) [90], which was used as the only inducing agent. Although quite crude, this work showed the possibility of generating myogenic cells under

specific culture conditions, and represented the starting point for the future development of more efficient methods for obtaining homogeneous cultures of myogenic progenitors endowed with stem cell properties.

In the absence of specific cues, serum-induced mouse ES cell differentiation produces both lateral plate and paraxial mesoderm, which can be identified by the expression of KDR (also known as FLK1) and PDGFR α respectively. FACS-sorting of embryoid body (EB)-derived PDGFR α +FLK- cells enabled the isolation of a population with paraxial mesoderm gene expression signature (enriched for Mesp2 and Tbx6 transcripts) but, unexpectedly, these displayed low myogenic potential as assessed by MyHC staining [91], thus reflecting the need of additional cues for the activation of the muscle genetic program in differentiating pluripotent stem cells. Nonetheless, this work demonstrated the possibility of defining specific lineage-restricted cells that could be isolated and used for further studies.

Serum-dependent myogenic differentiation of human ES cells was later reported by the Studer group, which involved the isolation of multipotent mesenchymal precursors from monolayer cultures using CD73-based sorting [92]. Since this initial observation, subsequent reports involving controlled-expression of transgenes or defined culture conditions improved significantly the myogenic commitment from PS cells and provided more consistent results. However, the variability resulting from different serum batches still represents an issue, as several protocols require this component for efficient cell expansion of myogenic cells [93–95].

3. Myogenic induction by controlled expression of transcription factors

The transcriptional circuit resulting in the expression of one of the MRFs, which represents the formal activation of the skeletal muscle program, has its core in the PSM. As described above, multiple transcription factors play a role in this process and it could be expected that their forced expression in differentiating PS cells will drive the differentiation toward the skeletal myogenic lineage. However, experimental observations suggest that induction of skeletal myogenesis by a single TF involves a complex interplay between transcriptional regulators and signaling pathways.

3.1 MRFs

Before the increasing interest in regenerative medicine, ES cells were mostly used for studying mechanisms of development. In the case of skeletal myogenesis, the first obvious attempt was the use of MyoD. Early attempts in both P19 EC and mouse ES cells showed that robust muscle differentiation could be achieved only upon MyoD expression in cell aggregates such as EB, thus implying the need of inductive signals produced during EB formation [96, 97]. Similar findings were later reported in human PS cells, including the observation that MYOD expression induces the formation of contractile muscle aggregates termed “myospheres” [98, 99]. Iacovino and colleagues demonstrated that MYF5 also shares the same ability to activate the myogenic program when expressed in differentiating human ES cells [100]. These studies show that MYOD and MYF5 are unable to efficiently induce myogenic conversion when expressed in ES cells, and indicate that both MRFs require transition through a mesodermal intermediate. However, although MRFs may represent the

obvious candidate genes to activate the myogenic program in differentiating pluripotent stem cells, myogenic conversion experiments using multiple PS cell lines showed that these TFs give rise to a more differentiated muscle cell, as revealed by the regulation of several terminal differentiation genes (e.g. MyoG, Desmin and Ckm) [101]. Since ES/iPS cells represent an attractive source of tissue/cells for regenerative medicine applications, it became evident that there was a need to derive a population of muscle progenitors that would be capable of participating in muscle repair and contributing to the pool of resident stem cells. In this context, due to its position in the myogenic genetic hierarchy, MyoD expression may represent a sub-optimal choice for specifying muscle progenitors from pluripotent stem cells. Nonetheless, Cossu and colleagues demonstrated that an estradiol-inducible MyoD construct positively enhances myogenic potential of iPS cell-derived mesoangioblasts [102], a cell population currently under consideration for cell therapy of muscle disorders [103, 104]. In any case, controlled MyoD expression may represent a valuable tool for deriving homogeneous cultures of terminally differentiated skeletal muscle cells for in vitro disease modeling applications using patient-specific iPS cell lines [98, 105].

3.2 Pax3 and Pax7

Myogenic progenitors during development and satellite cells in the adult are characterized by the expression of Pax3/7 (reviewed by [48]). Pax3 identifies the progenitors migrating toward the limb bud and a subset of adult satellite cells, while Pax7 is expressed in fetal myogenic progenitors and all adult satellite cells of somitic and non-somitic origin. Contrary to MRFs, expression of Pax3 or Pax7 is not restricted to myogenic cells, as these TF are also detected in migrating neural crest cells and other neural tissues [106]. Using a doxycycline-regulated system enabling the controlled expression of genes stably integrated into the genome of mouse ES cells [107], it was first demonstrated that Pax3 induction during ES cell differentiation (iPax3 ES cells) recapitulates the activation of the myogenic program that occurs during mouse embryonic development [95, 108]. Pax3 (or Pax7) expression during EB development results in increased frequency of paraxial mesoderm (PDGFR α +FLK1-) cells as well as up-regulation of several genes characteristic of developing somites and myogenic progenitors, including Meox1, Myf5 and Met [108, 109]. Importantly, following FACS-sorting, EB-derived iPax3 PDGFR α +FLK1- cells proliferate exponentially and express MyoD mRNA, thus mimicking the temporal expression of these two genes in the developing embryo [110, 111]. Molecular studies demonstrated that acquisition of the skeletal myogenic lineage identity is mostly associated with gene expression changes in the PDGFR α + fraction of the developing EBs which, in the absence of Pax3, undergo cardiac differentiation [112]. The myogenic vs. cardiac fate decision process involves Pax3-induced repression of the cardiac master regulators Nkx2-5, Gata4 and Tbx5, and this can be partially rescued solely upon Tbx5 up-regulation [112, 113]. In addition, the transition from PDGFR α +FLK1- to MyoD+ cell is accompanied by DNA demethylation at the regulatory regions of important muscle specific genes, including the Myf5 and MyoD enhancers, and the MyoG and Ckm promoters [114]. ES-derived myogenic progenitors are also characterized by Pax7-dependent chromatin remodeling at a subset of sites, which correlates with Pax7's ability to maintain the undifferentiated status of these cells [115].

The relevance of these Pax3/7-induced ES-derived myogenic progenitors is highlighted by their capability to contribute to muscle regeneration when transplanted into muscles of mouse models of Duchenne Muscular Dystrophy (*mdx* and *Dmd:Utrn* double knockout – dKO). Myogenic progenitors from Pax3- or Pax7-inducible ES cells efficiently promote muscle regeneration in vivo, as shown by restoration of components of the Dystrophin Glycoprotein Complex (DGC), and contribution to the satellite cell compartment, which is an important aspect for long-term tissue homeostasis [95, 108, 116, 117]. Importantly, the ability of Pax3 and Pax7 to drive skeletal muscle commitment from ES/iPS cell differentiation is also conserved in human cells. Similar to their murine counterparts, PAX7-induced PS cell-derived human myogenic progenitors give rise to differentiated myotubes in vitro, and contribute to muscle regeneration in vivo, as shown by myofiber and stem cell engraftment, and improvement of muscle physiological parameters [94, 118]. Further studies aimed at dissecting the molecular function of PAX7 in differentiating human ES cells identified a subset of transcripts undergoing gradual up-regulation during myogenic commitment, as well as the putative PAX7 binding sites that may be responsible for the PAX7-dependent regulation of these genes [118]. Notably, this work enabled the identification of a group of cell-adhesion molecules (CD54 or ICAM1, SYNDECAN2 and INTEGRIN $\alpha 9\beta 1$), which identify the human PAX7-derived myogenic progenitors. These findings have important therapeutic implications since surface marker-based isolation techniques are compliant with Current Good Manufacturing Practice (CGMP) procedures [118]. Since PAX7-derived myogenic progenitors can be easily expanded for multiple passages in vitro, adaptation of the purification strategy and culture conditions to CGMP standards may enable the therapeutic translation of this cell population for the treatment of muscle diseases.

3.3 Mesp1 and Msgn1

Somite formation relies on a complex signaling cascade, which ultimately results in the formation of an epithelial cell aggregate. Mesp1 and Msgn1 are expressed during this process and doxycycline-inducible expression of these two genes in differentiating mouse ES cells is able to recapitulate the gene expression changes occurring during PSM patterning. Interestingly, Mesp1 function appears to be time- and context-dependent because its induction in mesodermal cells is able to increase the frequency of hematopoietic and cardiogenic progenitors (in serum-based differentiation) or specify the skeletal myogenic lineage (in serum-free conditions) [119]. Similar to Pax3 function in PDGFR α + cells, Mesp1 regulates skeletal vs. cardiac commitment of mesodermal precursors through a process involving activation of the BMP2/4 signaling pathway [120]. The ability of mesodermal cells to acquire different lineage cell fates reflects their transcriptional plasticity and highlights the importance of maintaining the appropriate cues during in vitro ES cell differentiation.

Although less characterized, induction of Msgn1 in differentiating mouse ES cells results in the expression of several paraxial mesoderm markers, including Tbx6, Snail1 and Pdgfra [40]. Based on these data and additional in vivo results [121], it was concluded that Msgn1 may represent the master regulator of presomitic mesoderm. However, the ability of these Msgn1+ cells to further differentiate into skeletal muscle remains an open question, which,

if investigated, could help to further understand the molecular mechanisms that ultimately result in Pax3 expression within the somites.

4. Myogenic differentiation of pluripotent cells through modulation of signaling pathways

The increasing interest in the therapeutic application of pluripotent stem cell-derived progenitors has been paralleled by an extensive effort to direct their differentiation in the absence of genetic manipulations. On this line, the ability to recapitulate in the culture dish the events occurring during embryogenesis depends on the level of knowledge of the molecular events driving the development of the tissue of interest. An important aspect in this regard is the observation that mouse and human ES/iPS cells differ in their embryological status: murine ES cells are considered “naïve” because they resemble the inner cell mass, whereas human ES cells are defined as “primed” due to their similarity to cells of the epiblast stage. These differences affect both the cells’ ability to differentiate and their permissive status for genome editing (reviewed by [122]).

The first step required for the differentiation of pluripotent stem cells is the withdrawal of the signals responsible for maintenance of pluripotency, such as Leukemia Inhibitory Factor (LIF) in mouse ES cells, which induces transition toward the epiblast stage. Notably, because of their primed nature, this step is not necessary during human PS cell differentiation [123]. In the case of skeletal muscle, as mentioned in the previous section, the first lineage decision occurs at the level of neuromesodermal progenitors which, under WNT and FGF signaling, activate the expression of presomitic mesoderm markers. As we will see, all the current protocols rely on the use of a WNT activator (GSK3 inhibitor) to induce the formation of paraxial mesoderm progenitors from NMPs. However, the use of WNT activators followed by WNT inhibitors has also been previously used to induce cardiac differentiation from ES cells [124], which suggests that the mesodermal progenitors derived following treatment with GSK3 inhibitors are not uniquely committed toward the PSM.

4.1 WNT activation

The Barberi group was the first to demonstrate that treatment of ES cell colonies with CHIR99021 (a GSK3 inhibitor) and FGF2 was able to induce a cell population endowed with skeletal myogenic potential, which was defined as HNK1–AChR–MET +CXCR4+ [125]. Similar results were later reproduced by independent groups, in the absence of cell purification strategies [126–128], and applying minor modifications. For example, using small molecule screening in zebrafish, Xu and colleagues determined that a cocktail containing FGF2, GSK3 inhibitor (BIO) and PKA activator (Forskolin) was sufficient to induce skeletal myogenesis from human ES cells [128]. Although PKA signaling has been shown to regulate specification of the myogenic progenitors within the dermomyotome through CREB activation in response to non-canonical WNT pathway [129], other investigators excluded Forskolin in their myogenic differentiation protocols, thus suggesting its dispensability in this process [123, 130, 131].

4.2 WNT activation + NOTCH inhibition

Sequential treatment using CHIR99021 followed by the Notch inhibitor DAPT has also been shown to induce a myogenic population, characterized as HNK1–CD56+, capable of differentiating robustly in vitro [130]. Notch activation in migratory Pax3+ myogenic progenitor has been associated with myogenic-to-vascular cell fate transition and inhibition of this pathway increases the expression of the myogenic genes Lbx1 and Myf5 [132].

4.3 WNT activation + BMP/TGFβ inhibition

An important step toward the derivation of skeletal muscle cells from PS cells was the introduction of a BMP inhibition step during the initial PSM commitment [123]. Both Bmp2- and Bmp4-dependent signaling are responsible for paraxial-to-lateral plate mesoderm switch [133, 134] and, accordingly the skeletal vs. cardiac cell fate choice observed using iPax3 and iMesp1 PS cell lines requires repression of the Bmp2/4 pathway [112, 120]. Chal and colleagues demonstrated that epiblast-like cells derived from mouse and human ES cells acquired a posterior presomitic mesoderm fate following a combined treatment with CHIR99021 and LDN193189 (a selective BMP inhibitor). These cells, upon a short treatment with CHIR, LDN and FGF2 and no FACS-purification, were later cultured in the presence of FGF2, HGF and IGF1 until the appearance of MYOG+ and MYHC+ cells. Using a Pax7-reporter mouse ES cell line, they demonstrated that these cultures also contain Pax7+ myogenic progenitors, which may mimic the transition toward the fetal myogenic program [123].

Although previous studies have taken advantage of the knowledge obtained from development in other species (mainly mouse and chicken) to drive myogenic specification from human PS cells, transcriptional profiling of tissues isolated from human embryos provided unique insights about the signaling pathways regulating somitogenesis. Notably, this work provided the rationale for the optimization of the initial stages of myogenic differentiation protocol from PS cells. RNA-seq analysis of PSM, nascent somites and developed somites identified that both BMP and TGFβ signaling pathways are repressed in the transition from PSM to nascent somites, while WNT signaling is required for both PSM specification and the transition from nascent somites to developed somites. Following this logic, Xi and colleagues were able to derive human somite cells capable of differentiating toward myogenic, osteogenic and chondrogenic lineages [131].

In addition, Choi and colleagues showed for the first time that patient-specific DMD iPS cell lines have compromised muscle differentiation potential due to activation of BMP-TGFβ signaling, and demonstrated that this phenotype can be rescued by inhibiting these pathways in the terminal differentiation stage [130].

4.4 Lineage decisions at the single cell level

Without doubt, the remarkable technological advances in the sequencing field in the past ten years are contributing to better understanding of the mechanisms of lineage decision, as demonstrated by the transcriptomic analysis of human somitogenesis. The recent development of platforms for RNA-sequencing at the single cell level (scRNA-seq) enables the investigation of this process with an increasing accuracy. Although this technology is

still developing (including the computational analysis of the sequencing data), single cell RNA-seq studies of differentiating mouse and human ES cells are providing important clues to improve the current differentiation protocols. For example, by analyzing the response to specific extrinsic signals, the Weissman group characterized the process of lineage commitment toward multiple human mesodermal lineages, including cardiac and skeletal muscle [135], and identified a novel function for HOPX during somite development. More recently, Gouti and colleagues also applied single-cell transcriptomic data from murine NMPs isolated from the tail bud region to investigate the molecular mechanisms responsible for the cell fate choices leading to neuronal or mesoderm specification, an important aspect for vertebrate trunk development [136]. Using mES cells as model for recapitulating NMPs generation, the authors were able to validate their in vivo findings, such as the role of RA in both NMPs generation and differentiation toward neuronal progenitors, and the opposite role of *Msgn1* and *Tbx6* in controlling timing and outcome of NMP differentiation toward the paraxial mesoderm.

4.5 Muscle regeneration and characterization of myogenic progenitors from serum-free protocols

As mentioned above, the regenerative potential of PS cell-derived myogenic progenitors is a key aspect for the therapeutic application of these cell populations. With the increased availability of new mouse models recapitulating specific aspects of human diseases and capable of hosting xenotransplants (e.g. *mdx*-NSG mice [137]), the assessment of the in vivo muscle regeneration potential of specific human cell populations has become more accessible to researchers. However, although this assay has been incorporated in the routine analyses of PS cell-derived myogenic progenitors obtained using inducible transgenes [94, 95, 102, 118], engraftment data from the transgene-free differentiation protocols described in this section are still scarce. Indeed, with few exceptions [128, 130], the majority of the studies attempting to derive myogenic progenitors from human PS cells did not report whether these preparations have functional relevance for cell therapy. Although it would be expected that cells capable of differentiating in vitro are able to fuse to existing myofibers in vivo, the cell-to-cell interactions occurring in the petri dish combined with a still heterogeneous composition of the population under investigation (particularly in the case of protocols not involving cell-sorting) represent a limit for the direct translation of these PS cell-derived myogenic cell preparations. Indeed, this is supported by recent findings demonstrating the need of cell-purification strategies and robust lineage commitment for the successful engraftment of muscle progenitors derived using directed differentiation of PS cells [138].

One underestimated aspect of the protocols involving myogenic specification using defined culture conditions is the dorsalizing function of canonical WNT signaling, resulting in the generation of neural crest cells and neuroepithelium [139, 140]. The presence of contaminating neural cells has been reported by different investigators, and one reported solution has been the use of a FACS-based negative selection strategy using an anti-HNK1 antibody [93, 125, 130], which recognizes a glycoepitope expressed on migrating neural crest cells and other neural derivatives [141]. However, it should be noted that the presence of neural cells in culture may have a positive effect on the myogenic specification from

paraxial mesoderm. This is supported by the observation that migrating neural crest cells through the somites regulate both myogenic commitment, via a Notch-dependent mechanism, and maintenance of the Pax7+ pool in the central domain of the dermomyotome, which relies on Neuregulin1-ErbB3 signaling [9, 10]. These findings suggest that a controlled balance between the neuronal and myogenic differentiation in the first stages of PS cell differentiation may be beneficial for the generation of myogenic progenitors. Further elucidation of the mechanisms underlying this process during development will be instrumental to improve current methods.

5. Requirement of a mesodermal intermediate and differences between MRFs and Pax3/7

Following MyoD cloning, it was reported that not all cell lines, such as HeLa cells, were equally converted into skeletal muscle, a finding that suggested the presence of positive/inhibitory signals participating in this process [142]. Similarly, as mentioned above, mouse and human ES cells also appeared to be refractory to MyoD myogenic activity, requiring transition through a mesoderm intermediate to enable this transcription factor to be effective [96–98]. The molecular explanation for this observation became clear later when Albini and colleagues demonstrated the requirement of the SWI/SNF subunit BAF60C for MYOD-mediated activation of the myogenic program in ES cells [98]. BAF60C is not expressed in ES cells but is up-regulated during mesoderm formation, where it is required for proper cardiac development and skeletal muscle differentiation [98, 143]. The combinatorial composition of subunits within chromatin remodeling complexes of the SWI/SNF family has an important role in the modulation of the activity of these ATP-dependent enzymes (reviewed by [144]), and inclusion of BAF60C in these complexes during gastrulation may be associated with chromatin remodeling of specific sites important for mesodermal patterning. Although there are no genome wide studies validating the function of BAF60C at MYOD sites, studies from the Tapscott lab demonstrated that both Myf5 and MyoD bind similar loci and induce chromatin remodeling through an increase in acetyl-histone 4 (H4Ac) levels [21, 101], but only MyoD binding is associated with recruitment of RNA pol II and robust transcription. In addition, the Dynlacht group showed that MyoD also binds at skeletal muscle enhancers, and that this is associated with maintenance of monomethyl-lysine 4 histone 3 (H3K4me1) levels [145].

A mesodermal intermediate is also required for the Pax3- (or Pax7-) mediated activation of skeletal myogenesis [94, 95]. These TFs are instrumental for the expression of Myf5 and MyoD during embryonic development. However, unlike MRFs, Pax3 and Pax7 are not able to induce the myogenic conversion of fibroblasts into muscle cells in vitro due to their weak transactivation domains. This is supported by the observation that the oncogene PAX3-FKHR, a fusion protein between the Pax3 DNA binding module and the strong transactivation domain of FOXO1, is able to induce myogenic conversion in fibroblasts, even though not with the same efficiency as MyoD [146]. Since both MRFs and Pax3/7 activities are mainly restricted to mesodermal cells but only the former possess myogenic conversion potential in fibroblasts, it is plausible that Pax3/7 function is associated with the recruitment of different type of chromatin regulators and cooperation with other

transcription factors. In agreement with this hypothesis, it has been demonstrated that Pax7 recruits the histone methyl transferase complex Mll2 at the Myf5 regulatory regions [147], a process that requires the arginine-methyltransferase Carm1 [148]. Accordingly, recent genome wide studies reported that nucleosomes at Pax7 binding sites in mouse ES-derived myogenic progenitors are marked by H3K4me1 and H3K27Ac, which together define enhancer regions. Upon loss of Pax7 expression, H3K4me1 and H3K27Ac signals are lost at a subset of these loci thus implying Pax7 is instrumental for maintaining this chromatin feature. Interestingly, this is also paralleled by a general decrease in chromatin accessibility, with the exception of a subgroup of regions that are enriched for MyoD binding sites, which retain an active enhancer signature [115].

Taken together, these data highlight a potential sequential function of Pax7 and MyoD in the regulation of the skeletal myogenic program through regulation of a common set of enhancers. Similar findings were reported in pituitary cells, where Pax7 and Ttip regulate the corticotrope vs. melanotrope cell fate choice [149], thus suggesting the possibility that Pax3/7 function during lineage specification might be dependent on the combinatorial expression of tissue-specific TFs and activation of selected signaling pathways. Because of their ability to recapitulate embryonic development, it is likely that ES cells will play an important role in addressing this and other important open questions.

6. Current issues and future applications of pluripotent stem cell-derived myogenic progenitors

The extraordinary capability of PS cells to differentiate into all the cell types of the human body makes these cells an invaluable tool for understanding the molecular mechanisms behind tissue development and regeneration. In the case of skeletal muscle, derivation of myogenic progenitors from PS cells required the concerted effort of several investigators over the last 30 years. However, although the protocols described in this review seem quite established, there are still several aspects which, if not addressed, will limit the application of these cells in various fields. Lack of maturation and inconsistent differentiation potential among different PS cell lines represent two of the main issues affecting all the PS-cell derived lineages.

PS cell differentiation recapitulates very well the first stages of development and enables the production of large numbers of progenitors. Over the years, it became clear that these PS cell-derivatives retain an embryonic feature and do not automatically transit into the fetal stage [150, 151]. This process is associated with changes in gene expression and physiological properties, as exemplified by the transition from primitive to definitive erythroid colony-forming-units during hematopoiesis [86]. However, from a therapeutic point of view, derivation of progenitors with adult hematopoietic stem cell features still represents a challenge that only recently witnessed substantial improvement [152]. Because of the similarities between cardiac and skeletal muscles, studies on cardiomyocytes provide important insights for maturation of PS cell-derived skeletal muscle. During heart development, cardiomyocytes undergo a complex maturation process including changes in cell morphology (mature cells have a rod shape), binucleation, and switch in the expression

of specific sarcomeric proteins (such as ssTnI to cTnI transition). These features of cardiomyocytes have been difficult to recapitulate in PS cell-derived cultures and resulted in the incapability to reproduce in vitro the pathophysiological conditions of the adult human heart, which ultimately is preventing their application in drug discovery (reviewed by [153]).

Embryonic skeletal muscle is characterized by a low fusion rate and expression of specific gene isoforms such as the embryonic Myosin Heavy chain (Myh3). Transition toward the adult stage (through fetal then neonatal) is associated with increased myoblast fusion, switch in myosin isoforms, and full maturation of the excitation-contraction coupling machinery. As seen in cardiomyocytes, iPAX7 PS cell-derived skeletal myocytes have an embryonic signature. Only upon extended culture (30 days), these cells display sarcomeric organization and functional excitation-contraction coupling, but still an incomplete electrophysiological maturation [154]. Sarcomeric maturation has been also observed in differentiated muscle cells obtained through modulation of signaling pathways [123]. Interestingly, these authors were able to detect expression of the secondary myogenesis marker Nfix [69], transition from the embryonic slow myosin to the perinatal isoform, and an overall high myofibril organization. Multiple groups have interpreted the incomplete electrophysiological maturation as a consequence of the lack of innervation and establishment of co-culture protocols could represent an alternative for overcoming this issue [155]. Interestingly, lack of maturation seems to be a less relevant issue for cell therapy applications. Following transplantation of PS cell-derived cardiac progenitors into the adult heart, cells were able to couple to the resident adult cardiomyocytes, and display signs of maturation [156, 157], although development of arrhythmia was also reported. Similarly, upon transplantation, PS cell-derived skeletal myogenic progenitors demonstrated the capability to engraft in the skeletal muscles of both dystrophic and non-dystrophic mice, express adult myosin isoforms, and improve physiological muscle functions of dystrophic animals [94, 95, 116, 158]. Thus, the improved maturation of PS-cell derived progenitors observed in vivo implies that cues provided by the environment may overcome the roadblocks observed in vitro.

Regarding the issues associated with variability among PS cell lines, one aspect to be noted is that characterization of PS cell lines is often limited to expression of pluripotency markers and ability to produce teratomas when injected in immunocompromised mice. Nonetheless, epigenetic differences among lines due to somatic memory and/or aberrations during the reprogramming process may affect their multiple-lineage differentiation ability [159–162]. This represents a major obstacle in order to avoid biases in the interpretation of results obtained using multiple clones, such as mechanisms of pathogenesis deduced through comparison of normal- and disease-specific PS-cell derived progenitors. An obvious example of the variation among lines is the requirement of variable concentration of the GSK3i inhibitor for mesoderm induction [124]. In the case of skeletal myogenesis, it has been shown that human PS cell lines display a variable capability to differentiate using a chemically defined protocol, thus implying the need of further improvements before the generalization of current protocols [138]. As in the case of maturation, detailed analysis of the epigenetic mechanisms behind cell differentiation will definitively help to understand why some PS cell lines are more permissive than others. Indeed, following this approach, a recent study published by the Yoshida group identified that IGF2 levels produced by human PS cells correlate with their hematopoietic potential [163].

The recent advancements in genome editing technologies have greatly contributed to an increasing availability of human reporter ES/iPS cell lines, which will ultimately contribute to the optimization of culture conditions driving myogenic specification from PS cells. In the case of skeletal muscle, the Darabi group has developed both PAX7-GFP and MYF5-GFP human ES cell lines, in which a 2A-GFP sequence is integrated before the stop codon of these genes [164, 165]. They demonstrated that GFP+(MYF5+) cells are endowed with myogenic potential in vitro. These ES reporter lines will be powerful tools for testing new differentiation protocols as well as disease modeling and drug screening. On the same topic, tissue engineering will likely play an important role on the application of PS cell-derived myogenic progenitors to develop three-dimensional skeletal muscle tissue [166]. In vitro engineered skeletal muscle constructs represent attractive models to test therapies and drugs, or potentially they could be used as mini-organs for replacement therapy (reviewed by [167]).

One of the main drivers of stem cell research in the last decades has been the possibility of using PS cell-derived progenitors for cell therapy. However, before this becomes a reality, several important aspects need further investigation: scalability, purity of cell preparations (for safety), methods of delivery, immunological response, and efficacy. As more protocols for deriving myogenic progenitors become available, it will be fundamental to unbiasedly assess the translation potential of each cell preparation by taking in consideration the above mentioned points. An important advance in this direction was the recent identification of the surface receptors that will enable the purification using current good manufacturing practices (cGMP)-compatible methods of iPAX7 PS-cell derived myogenic progenitor endowed with in vivo regenerative potential, a mandatory required step for preventing the injection of potentially harmful cell preparations [118]. Nonetheless, although promising, further studies will definitively assess their validity in cell therapy.

7. Conclusions

One of the most famous songs from the Rolling Stones says “You can’t always get what you want, but if you try sometimes, well, you might find, you get what you need”. Nicely, this lyric quite well resumes the efforts that took us to understand and successfully recapitulate myogenesis in vitro using pluripotent stem cells. Nonetheless, the same approach will be instrumental for understanding the mechanisms driving maturation of skeletal muscle cells, and further studies will be necessary to evaluate the translational potential of the various PS-cell derived myogenic progenitor preparations for cell therapy of muscle diseases.

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Highlights

- Pluripotent stem (PS) cells recapitulate skeletal myogenic lineage specification
- Controlled expression of transcription factors in PS cells induces myogenesis
- Myogenic commitment of PS cells can be achieved by manipulating signaling pathways
- PS-derived myogenic cells as potential tools for cell therapy and disease modeling

Figure 1. timeline of selected findings that contributed to the actual status of the field. References are indicated by the numbers in parentheses.