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Retinoic Acid Receptor Signaling in the Differentiation of Pluripotent Stem Cells into Skeletal Muscle Lineage

Jihong Chen and Qiao Li

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Abstract

Pluripotent stem cells have the capacity to differentiate into many types of cell lineages including skeletal myocytes. Nevertheless, the frequency of pluripotent stem cells generating skeletal myocytes in the absence of developmental cues is very low, and signaling molecules are required to commit them to muscle lineage. Thereby, *in vitro* stem cell differentiation has been used for decades to study molecular mechanisms of myogenic specification. Similar to human embryonic stem (ES) cells, various mouse pluripotent stem cells respond well to development cues *in vitro* to differentiate into cell types of all three primary germ layers. In tissue cultures, they can be induced into myogenic differentiation with an aggregation protocol which involves the formation of embryoid bodies (EBs). Their commitment into the skeletal muscle lineage recapitulates closely the cellular and molecular processes occurring in the early embryogenesis. Treatment of these stem cells with regulatory signals important for embryonic development, such as ligands of nuclear receptors, during EB formation markedly enhances the efficiency of myogenic differentiation. However, many challenges remain. Understanding on a molecular level, how different signaling pathways and chromatin dynamics converge during stem cell differentiation to specify the muscle lineage is imperative for identifying effective signaling molecules to generate sufficient amount of muscle progenitor cells for potential therapeutics. To this end, mouse stem cells will continue to serve as valuable model systems due to their close resemblance to skeletal myogenesis *in vivo*, and the ease of manipulation in experimental procedures. In this chapter, we will focus on recent research findings on nuclear receptor signaling in the specification of skeletal muscle lineage.

Keywords: Histone acetylation, Ligand, Myogenic specification, Nuclear receptor, Stem cells

1. Introduction

Pluripotent stem cells are valuable systems for delineating mechanisms of cellular differentiation due to their abilities to differentiate into virtually all cell types *in vitro*. Based on their derivative origins, there are ES cells, adult stem (AS) cells, and induced pluripotent stem (iPS) cells. ES cells are derived from the inner cell mass of blastocysts, have the capacity of unlimited self-renewal, and can give rise to derivatives of all three primary germ layers. AS cells, known as somatic stem cells, have limited ability to proliferate and can give rise to multiple cell lineages of the organ from which they are originated, but not all lineages of the three germ layers. iPS cells are generated from somatic cells by inducing conditions which reprogram the cells from a nonpluripotent state into a pluripotent, or ES cell-like state.

The first evidence for the pluripotent nature of embryonic cells was obtained from studies of mouse embryonal carcinoma (EC) cells, decades before the isolation of mouse ES cells [1]. Subcloned from teratocarcinomas, the EC cells grow as adherent cells in tissue-culture dishes and proliferate indefinitely [2]. When cultured in Petri dishes, they are unable to adhere and thereby form cell aggregates that contain a core of stem cells surrounded by epithelial cells. These cell aggregates are known as embryoid bodies (EBs), because they resemble the inner cell mass of embryo and develop extensive cavities and different cell types when subsequently grown as adhesive culture [3]. Since then, many EC cell lines have been generated and provided valuable experimental systems for studies of early development and cellular differentiation. More importantly, they paved the way for the isolation of mouse ES cells. Although pluripotent EC cells are much less used today, they remain an invaluable system for the studies of myogenic differentiation [4].

Mouse ES cells were first isolated in the early 1980s, from blastocysts grown on feeder-layer of division-incompetent mouse fibroblasts cells [5, 6]. These ES cells express all markers of EC cells and can differentiate extensively *in vivo and in vitro*. The requirement for ES cell to differentiate *in vitro* is, in essence, the same as for the EC cells, going through the stage of cell aggregation or EB formation [7]. However, ES cells need to be cultured in an inhibitory condition, such as in the presence of leukemia inhibitory factor (LIF), to retain the undifferentiated state, since they are prone to spontaneously differentiation [8, 9]. If grown in suspension without the LIF, the ES cells readily form EBs and differentiate as a result.

2. Ligand-enhanced myogenic differentiation

P19 pluripotent stem cell line, isolated from an experimental teratocarcinoma induced in the C3H/HC mice, exhibits typical EC morphology and normal karyotype [10]. Like other EC cell lines, the P19 cells can be grown as undifferentiated monolayer in tissue-culture dishes indefinitely and induced into differentiation to form cell lineages of all three germ layers [11]. In addition, they are amenable for genetic manipulation to incorporate and express ectopic genes and for selection of subclones or stable clones that retain their ability to differentiate [12].

All these characteristics have made them an excellent model system for mechanistic studies of early development.

One particular trait is the capacity of the P19 stem cells to generate skeletal myocytes in response to developmental cues. If grown in Petri dishes, the P19 cells readily form aggregates and develop into EBs [13]. Mesoderm specification takes place at the early stage of EB formation, coinciding with expression of Brachyury T, a member of the T-box family of transcription factors [14, 15]. However, EB formation *per se* does not result in myogenic differentiation, which requires additional regulatory signals. When cultured in the presence of signaling molecules, such as dimethyl sulfoxide (DMSO) or all-*trans* retinoic acid (RA), during EB formation, the P19 cells differentiate into skeletal myocytes in a relative low frequency [16, 17]. Treatment with combination of inducers, such as with both DMSO and RA, markedly enhances the myogenic conversion of P19 stem cells [18].

The differentiation of P19 stem cells is affected by the concentration of RA treatment. Cells exposed to high concentrations of RA ($>10^{-7}$ M) develop into neurons and astrocytes, but fail to differentiate into skeletal myocytes [19–21]. On the other hand, EBs formed at low concentrations of RA ($<10^{-7}$ M) develop into striated muscle, wherein the working concentration of RA is typically below the Kd, about 5–30 nM [4, 21–23]. The efficiency of P19 myogenic commitment is also affected by the timing and duration of RA treatments. As shown in **Figure 1**, cells treated with RA and DMSO during the full 4-day period of EB formation generated about 10% of myocytes by day 9 of differentiation, as determined by quantitative immnuofluorescence microscopy. However, if the EBs were allowed to form for 1 day in the absence of any treatment and then treated for the remaining 3-day period, the efficiency of myogenic differentiation increased by about twofold (**Figure 1**). When EBs were treated just for the last 2-day of EB formation, only about 5% of skeletal myocytes were generated (**Figure 1**). Finally, the ability of P19 cells to undergo myogenesis is also influenced by other factors in the serum, and EB formation is a prerequisite for myogenic differentiation of the pluripotent P19 EC cells [24].

Most interestingly, recent studies have identified bexarotene, a selective ligand of retinoid X receptor (RXR), to be an effective enhancer for the generation of skeletal myocytes by pluri-
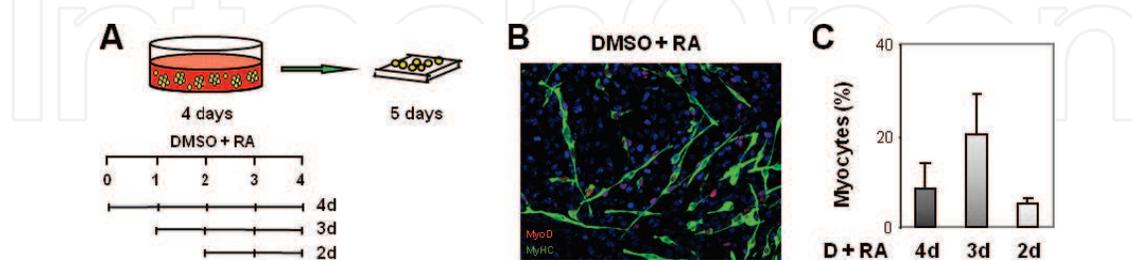

Figure 1. Effects of time course treatments on myogenic differentiation. (A) P19 stem cells were treated with DMSO and RA for the indicated times in Petri dishes during EB formation, maintained on coverslips for additional 5 days without any treatments and then stained for microscopic analysis. (B) Representative image of myosin heavy chain (green), MyoD (red), and DNA (blue) co-staining. (C) Quantification of myocytes is presented as the fractions of cells stained positively for myosin heavy chain in relation to the total cell populations. Error bars are the standard deviations of three independent experiments.

potent stem cells [4, 23]. Particularly, bexarotene enhances myogenic differentiation in a concentration dependent manner. The range of working concentration is wide (10–1000 nM) and fits the kinetics of its affinity to RXR as a ligand [4, 23]. More importantly, high concentrations of bexarotene do not inhibit the differentiation of P19 cells into skeletal myocytes [4, 23], which is in marked contrast with the dose effects of RA on myogenic conversion [21]. Nevertheless, the below Kd narrow concentration range also applies to the enhancement effect of arotinoid acid, a selective ligand for retinoic acid receptor (RAR), on myogenic specification [25]. In addition, the efficacy of bexarotene in P19 myogenic differentiation is comparable to RA and arotinoid acid [4, 23].

Early events of embryonic myogenesis are also closely recapitulated by the differentiation of ES cells into skeletal muscle lineage [26, 27]. RA is able to enhance myogenic differentiation of ES cells. More specifically, RA also affects the differentiation of ES cells into skeletal myocytes in a time- and concentration-dependant manner, similar manner as in pluripotent P19 EC cells. High concentrations of RA ($>10^{-7}$ M) induce neuronal differentiation in the ES cells, but inhibit myogenic commitment. Treatment of EBs with low concentrations of RA ($<10^{-7}$ M) at day 2–5 of differentiation leads to the induction of skeletal myogenesis, but the inhibition of cardiomyogenesis [28]. However, when low concentrations of RA are administered at day 5–7 of differentiation, skeletal myogenesis is inhibited, whereas cardiomyogenesis is induced [28].

Since ES cells respond poorly to RA regarding myogenic differentiation, the effect of bexarotene on the differentiation of ES cells into skeletal muscle lineage thus becomes critical [24]. A hanging-drop procedure was used to form the EBs which leads to ES cell differentiation. DMSO was omitted from the medium due to the toxicity to ES cells, and RA was administered in parallel as a comparison. Consistent with literature, RA had a low efficacy, about 3%, at converting the ES cells into skeletal muscle lineage [4]. However, bexarotene is about fivefold more efficient than RA and significantly increased the specification of skeletal muscle lineage [4]. Taken together, these data demonstrate that the RXR ligand is a more effective signaling molecule than RA to enhance the differentiation of ES cells into skeletal muscle lineage [4].

3. Ligand-inducible transcription factors

Vitamin A plays important roles in patterning and development during vertebrate embryogenesis [29]. Proper distribution and metabolism of vitamin A is fundamental for normal embryonic development and growth. Deficiency in vitamin A during early stage of embryogenesis results in congenital malformations affecting the patterning and development of many organ systems [30]. On the other hand, high concentrations of vitamin A or pharmacological concentration of RA, a potent derivative of vitamin A, have severe teratogenic consequences [31]. The diversified effects of RA are mediated by multiple levels of effectors, including enzymes that control the synthesis and degradation of RA, the cytoplasmic RA-binding proteins, and the nuclear receptors that are activated by RA [32].

The RARs are ligand-inducible transcription factors mediating the effects of RA on cellular activities [33]. There are three subtypes, namely RARα, RARβ, and RARγ, which can all be activated by both all-*trans*- and 9-*cis* RA [33]. Single subtype of RAR knockout mice is viable and appears normal, exhibiting few developmental defects [34, 35]. Nevertheless, double RAR knockout mice present a wide range of developmental abnormalities which resemble the vitamin A deficiency syndrome [36–39]. In fact, there appears to be a large degree of functional redundancy between RARs which play important roles in many distinct stages of embryonic patterning and organogenesis [33].

As a transcription factor, the RAR binds to RA-responsive element constitutively as a heterodimer with RXR (**Figure 2**). In the absence of ligand, the DNA-bound RAR/RXR heterodimer functions as a transcription repressor by associating with the NCoR co-repressor complex, but, upon RA induction, it acts as an activator by recruiting the p300 coactivator complexes to activate transcription [40, 41]. Often, RA-responsive promoters are classified as pre-set or poised promoters, as the TBP and Pol II complex associate with the TATA box constitutively [42, [43]. In this bimodal mode, ligand induction is through the RAR, wherein RXR is generally considered as a silent partner [44]. However, RXR is also amenable to RXR ligand induction and to form RXR homodimers or permissive heterodimers via dimerization with other nuclear receptors [45–47].
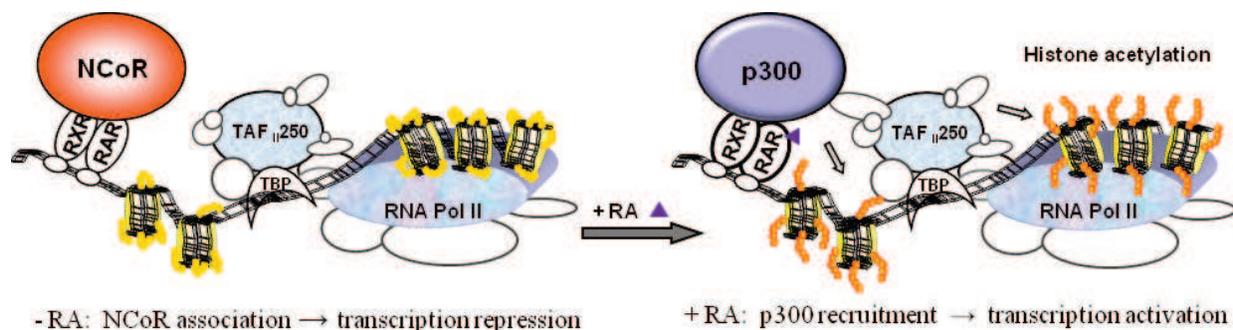

Figure 2. Schematic presentation of the bimodal function of RAR/RAR. In the absence of RA, the DNA bound RAR/RXR heterodimer associates with the NCoR corepressor complex to repress gene transcription. Upon RA induction, the corepressor complex is released, and the p300 coactivator complex is recruited by RAR/RXR to activate gene transcription.

The RXRs also consist of three subtypes, namely RXRα, RXRβ, and RXRγ, which bind to 9-*cis* RA [33]. Mice with subtypes of RXRs knocked out are also well characterized. RXRβ and RXRγ null mice are viable and mostly normal [48, [49]. In contrast, RXRα null mutants die in utero and display myocardial and ocular malformations [48]. Most interestingly, the RXRα null mutants exhibit developmental defects similar to the fetal vitamin A deficiency syndrome [50, [51]. Furthermore, the compound RXR and RAR knockout mice recapitulate most of the defects observed in RAR double mutants [48, [52]. Therefore, RXRα is the main subtype involved in embryonic development, and RXRα/RAR is the main functional unit to mediate RA signals during embryonic development [53, 54]. In addition, RXR is involved in an array of signal-

ing cascades and has the capacity to converge multiple pathways as an liganded receptor [55, 56].

4. Transcription networks of myogenesis

Skeletal myogenesis is a complex process coordinated temporally by multiple myogenic regulatory factors including Myf5, MyoD, myogenin, and Mrf4 [57, 58]. While Myf5 and MyoD initiate the expression of muscle-specific genes and commit the progenitor cells into the muscle lineage [59–61], myogenin and Mrf4 mainly regulate the late stage of differentiation, such as the fusion of myoblasts into myotubes [62–65]. At the upstream, Wnt signaling and Shh from the dorsal neural tube and notochord act as the positive regulators of Myf5 gene expression, whereas the expression of MyoD depends on the function of progenitor factor Pax3 and Myf5 [66]. Although both mesoderm factor Meox1 and Pax3 are important for myogenesis, overexpression of Mexo1 *per se* is not sufficient to induce P19 myogenic differentiation [67, 68].

During P19 myogenic specification, Meox1 and Pax3 expression are upregulated by RA by day 4 of differentiation [4, 22, 23]. Similarly, Myf5 transcripts can also be detected by day 4 of differentiation following RA treatment [23, 69]. Interestingly, bexarotene increases the transcript level of Meox1 with a greater efficiency than RA (about twofold), whereas RA has a larger impact than bexarotene on gene expression of Pax3 and Myf5 [4, 23]. In addition, the temporal gene expression pattern induced by bexarotene during P19 myogenic differentiation is similar to during myogenesis *in vivo,* and RXR ligand acts as an effective enhancer for the specification of muscle lineage [4]. It worth noting that bexarotene and RA have comparable efficacies at enhancing P19 myogenic differentiation [4]. While RA enhances skeletal myogenesis by expanding the progenitor population [22], bexarotene may affect germ layer fate determinations, particularly promoting mesoderm differentiation [4].

Intriguingly, bexarotene is a more efficient enhancer than RA for myogenesis in the ES cell system [4, 23]. Similar as in the P19 stem cells, bexarotene augments Meox1 transcripts more potently than RA in ES cell system, whereas RA is more efficient at increasing Pax3 transcripts [4, 23]. Nonetheless, bexarotene alone is able to induce the expression of early differentiation marker meox1, whereas RA requires additional signaling molecules to induce Meox1 expression. Hence, bexarotene may enhance the commitment of skeletal muscle lineage by fine-tuning premyogenic transcriptional networks which then preferentially affect the downstream myogenic program. Comprehensive and condition-specific gene expression profiling will uncover additional early regulators activated by RXR selective signaling during mesoderm differentiation, identify novel regulators of myogenic differentiation, and determine why RXR agonist is an effective inducer for ES myogenic specification.

Genetic manipulation can also be used as an approach to induce myogenic differentiation in ES cells. The premyogenic factor Pax3 plays a critical role in embryonic muscle formation by acting upstream of the myogenic-specific program [70–72], whereas Pax7 is important for the maintenance of the muscle satellite cells [73–75]. Consequently, ectopic expression of Pax3 during EB differentiation enhances mesoderm formation and increases the myogenic poten-

tial of Pax3-induced ES cells [76]. Similarly, forced Pax7 expression promotes the expansion of myogenic progenitors which possess muscle regeneration potentials [77]. In any events, activating the myogenic signaling pathway with small molecular inducers, which can be easily supplemented into, or removed from differentiation media, to direct myogenic commitment remains a more practical and attractive approach in view of potential cell-based therapies. Most intriguingly, the premyogenic factor Pax3 is also an inhibitor of cardiac differentiation in lineage specification [23]. The activation of Pax3 by RA or bexarotene during myogenic differentiation coincides with inhibiting the expression of early cardiac factors including GATA4, Tbx5, and Nkx2.5, and the inhibitory effect of bexarotene or RA on cardiac differentiation depends on the function of Pax3 [23]. Thus, the premyogenic factor Pax3 plays dual roles in stem cell fate determination by regulating and integrating different signaling pathways.

5. Enhancer elements of myogenesis

In eukaryotic cells, the regulatory DNA elements, such as the enhancers and promoters, are organized with histones to form nucleosomes which are further packaged into a higher-order chromatin structure [78–81]. The organization of chromatin structure not only establishes hierarchical platforms, but also provides epigenetic information including histone acetylation, for the intricate interactions amongst regulatory-proteins in cell fate determination, and ultimately for the control of specific transcription networks [82]. Thus, chromatin signatures are valuable signatures to identify novel regulatory elements, in addition to the sequences of DNA binding elements [83–85].

Recent genome-wide analyses have revealed an apparent functional relationship between chromatin dynamics and transcriptional activation in lineage specification. For instance, active promoters are often associated with multiple histone modifications, whereas enhancers are generally associated with the transcriptional coactivator p300 and histone acetylation [86, 87]. Moreover, genetic evidence in mouse and ES cell model systems has demonstrated that the expression of Myf5 and MyoD genes specifically depends on the histone acetyltransferase (HAT) activity of p300 [88]. The question is, on a molecular level, how different signaling pathways and chromatin dynamics converge to direct cell fate determination. Understanding the molecular mechanisms of myogenic specification is imperative for manipulating stem cell fate determinations in view of cell-based therapies.

A long-range RAR-binding site has been identified within the *Pax3* locus [22]. Both RAR and RXR are found at this region at the early stage of myogenic specification regardless of RA treatment, as determined by a real-time PCR-based chromatin immunoprecipitation (ChIP-qPCR) analysis [4]. In addition, the association of transcriptional coactivator p300 to this RAR-binding region increased markedly following treatment with RA (**Figure 3**) or arotinoid acid, a RAR selective ligand [25]. More interestingly, the *Myf5* epaxial enhancer is also a direct target of p300, as determined by the ChIP-qPCR analysis (**Figure 3B**).
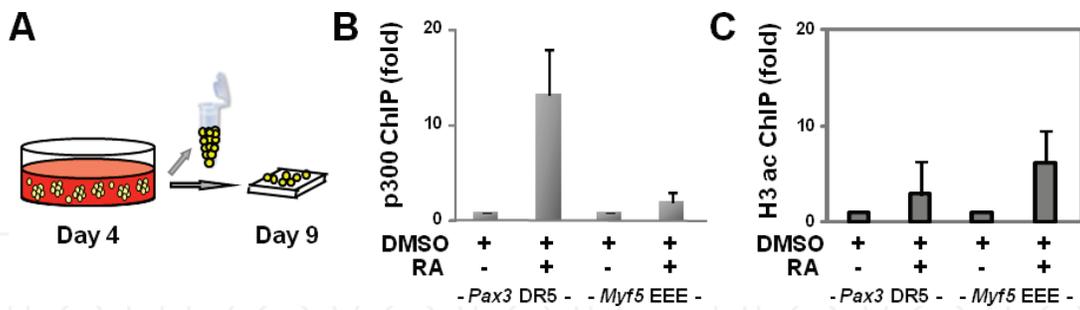
Figure 3. Occupancy of transcriptional coactivator p300 at myogenic loci. (A) Time line of the ChIP-qPCR procedure. Cells were cultivated in Petri dishes and treated with RA in the presence or absence of DMSO during EB formation. On day 4, aliquot of EBs was seeded on coverslips to assess the efficiency of myogenic differentiation on day 9. The remaining EBs was used for ChIP. (B) Association of p300 to the *Pax3* locus (*Pax3* DR5) and the *Myf5* early epaxial enhancer (*Myf5* EEE) was determined by ChIP-qPCR analysis, which is presented as the fold change in relation to DMSO controls. Input DNA was used as internal controls. Error bars are the standard deviations of three independent experiments. (C) The levels of H3K27 acetylation were also analyzed in parallel using the ChIP-qPCR assay.
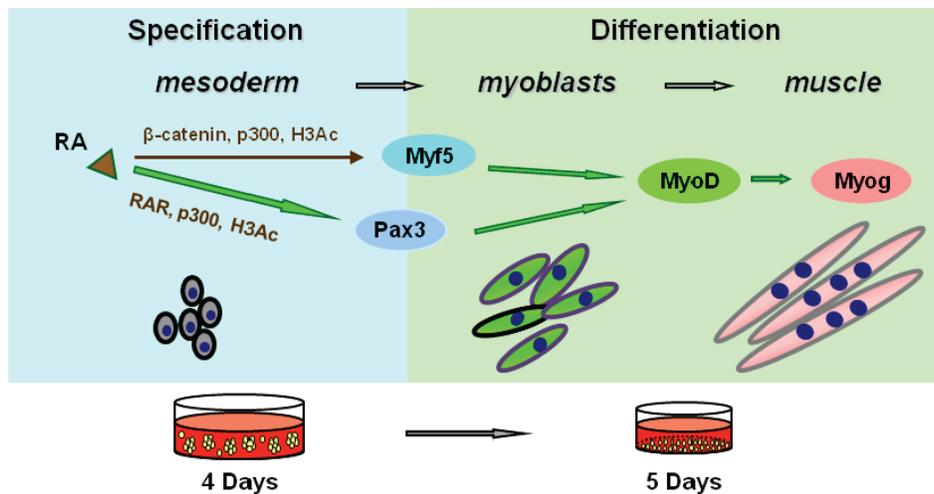

Figure 4. RA signaling in myogenic differentiation. RA enhances myogenic differentiation by augmenting Pax3 and Myf5 gene expression partly through promoting p300 occupancy and histone acetylation at the RA-responsive loci (solid brown and open green arrows).

The association of p300 to the *Pax3* locus is dependent on liganded RAR in an on-and-off mode [4] and increased about 15-fold following RA addition (**Figure 3B**). Intriguingly, the occupancy of p300 at the *Myf5* early enhancer, which does not harbor RAR binding sites, increased only about twofold following RA treatment (**Figure 3B**). Nevertheless, histones acetylation increased at both the *Pax3* locus and the *Myf5* early enhancer following RA treatment (**Figure 3C**). Therefore, RA regulates myogenic differentiation through p300-instigated histone acetylation in either RAR-binding dependent or independent manner (**Figure 4**). The relevance of histone acetylation during myogenic differentiation is additionally reinforced by the fact that valproic acid, a histone deacetylase inhibitor, is able to act in concert with RA to enhance the commitment of stem cells into the skeletal muscle lineage [89]. Nonetheless, chromatin signature for bexarotene to activate myogenic networks, particular-

ly how RXR selective signaling is transmitted during myogenic differentiation, remains to be defined. Systemic ChIP-seq analysis will identify additional p300-dependent myogenic enhancers and uncover novel regulatory elements to confer p300 function and histone acetylation in RXR-mediated stem cell differentiation.

6. Cell-based therapies for muscle-related diseases

Many diseases and conditions, including cancer, AIDS, muscular dystrophies, and chronological aging develop severe muscle wasting and would benefit enormously from muscle regeneration therapies. The unique architecture of skeletal muscle tissue makes it difficult to obtain differentiated skeletal muscle for tissue transplantation. Hence, muscle repair or regeneration may be best achieved through transplantation of the progenitor cells which are already committed into muscle lineage but not yet differentiated into skeletal myocytes. These progenitor cells will differentiate into functional skeletal muscle *in vivo* following transplantation. However, there are many challenges with respect to the effectiveness of myogenic differentiation, and the safety and long-term engraftment of transplanted progenitor cells. Some specific issues include what type of stem cells is best suited for production of progenitor cells and how to enrich the desired progenitor cells for clinical application.

Successful long-term therapeutics for skeletal muscle regeneration requires the contribution of transplanted progenitors to both the muscle fibers and the muscle stem cell pool. Muscle satellite cells may be an idea cell source of muscle regeneration, because they are not only able to generate muscle efficiently, but also able to establish the satellite cell pool following transplantation [90, 91]. However, their therapeutic potentials are greatly limited by their low abundance in the muscle. Another limitation is that the expansion of these cells *in vitro* reduces subsequently their regeneration capacity *in vivo* [92]. Similar limitation has also been found in murine and human hematopoietic stem cells [93, 94]. Finally, in the severe cases of muscular dystrophies, the regenerative source of satellite cells is unfortunately exhausted [95].

The ES cells can be expanded unlimitedly in tissue cultures, while maintaining their pluripotent differentiation potential. In addition, ES-derived myogenic progenitors have the ability to seed in the skeletal muscle stem cell compartment [76, 77]. Thus, ES cell-based muscle regeneration has unique advantage and presents immense promise. However, the use of ES cells in muscle-related disease is curtailed by the low frequency of myogenic differentiation in cultures and the difficulty in identifying and isolating the progenitor cells. The low frequency of ES cells to commit into skeletal muscle lineage is mostly attributed to the paucity of mesoderm formation during EB-differentiation in the absence of inducing signals.

To harness the full potential of ES cells in muscle regeneration, it is imperative that we identify small signaling molecules capable of efficiently directing ES cells into skeletal muscle lineage. Attempts at using RA in ES cell cultures have yielded moderate results, while RXR ligand appears to be a better inducer for myogenic differentiation. However, the molecular pathways involved have not yet been fully defined. A comprehensive deciphering of the differentiation cues in ES cultures and a better understanding of the regulation of the myogenic

pathway *in vivo* will help us to identify additional small molecule inducers and devise optimal protocols to generate desirable myogenic progenitors for muscle regeneration or repair.

Small molecule inducers have been used to reprogram somatic cells, to maintain induced pluripotent states and to directly control lineage specification. They have the potential to enable the development of viable cell-based therapies and to control endogenous cell populations for supporting regeneration events. The impact of chemical biology on emerging regenerative medicine will only increase with time, with a greater comprehension of the signaling pathways regulating cell fate determination, and the molecular mechanisms promoting nascent cell survival and engraftment.

7. Perspectives

Pluripotent stem cells, regardless of their origin, possess a tremendous potential for use in the treatment of muscle-related disease, because of their capacities to differentiate into skeletal muscle lineage. However, small molecule inducers are required to specify myogenic differentiation *in vitro* with an efficacy suitable for cell-based therapies. Recent studies have uncovered the ability of RXR ligand to efficiently induce the commitment of ES cells into skeletal muscle lineage, but the molecular pathways involved remain to be determined. Concerted RNA-seq and ChIP-seq analysis with stem cell differentiation as a model system will uncover novel early regulators and epigenetic signatures important for myogenic differentiation. A systematic understanding on a molecular level, how different signaling pathways and chromatin dynamics converge to direct cell fate determination, is imperative for identifying additional small signaling molecules and developing nontoxic protocols with optimal combination of signaling molecules and treatment conditions to direct the specification of skeletal muscle lineage. The strategies and protocols devised in mouse stem cell systems can then be transferred to human ES cells and to other types of pluripotent stem cells in view of generating muscle progenitors for clinical applications.

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