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A Review

Advances in myogenic differentiation: Role of stem cells, RNA-binding proteins, Molecular pathways, and Detection techniques

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The demand for effective muscle regeneration therapies has grown with the prevalence of muscle-degenerative conditions like muscular dystrophies which lack curative treatments. Although stem cells, including induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs), have great promise for muscle regeneration, their therapeutic application is constrained in the absence of a thorough comprehension of myogenic differentiation mechanisms. A complicated network of molecular pathways, including Wnt, Notch, and Hedgehog, controls the myogenic differentiation process of the muscle progenitors. Various transcription regulators, including MyoD1, Pax 3 and 7, Myf5, and Mrf4, and the Cis-regulatory sites near the promoters, orchestrate the intricate mechanisms of gene regulation for differentiation. Activation of the signaling cascades conveys the message of muscle tissue-specific gene expression *via* regulating the genes at the transcriptional, epigenetic, post-transcriptional, and translational stages. Muscle-specific DNA methylation, histone acetylation and deacetylation, histone methylation, *etc*., and their regulators also play crucial roles in the chromatin accessibility of myogenic gene expression. In addition, The RNA-binding protein families, especially the MBNL-family, hnRNP-family, and the CELF-family, which modulate RNA-splicing, mRNA stability, and translation, are also major players in this process. Misregulation of these regulatory factors may cause muscle pathogenesis in patients. Metabolic alterations in glycolysis and OXPHOS due to changes in energy demand are crucial regulatory steps of myogenic differentiation of stem cells. Changes in mitochondrial biogenesis and metabolic pathways meet the shifting energy needs while controlling the levels of reactive oxygen species affecting the fate of the stem cells. In this review article, we discussed the advances made in the recent past of all the above aspects of myogenic differentiation of stem cells along with cutting-edge technologies, including real-time imaging, metabolic imaging, gene editing tools, and organoid cultures to study the dynamic changes at the cellular and subcellular levels during muscle differentiation.

Keywords: 3D-Culture, CRISPR-Cas, hnRNPs, Imaging, MBNL, Notch, Shh, Wnt

Introduction

Myogenic differentiation is the process by which stem cells differentiate into muscle cells or myocytes. A subset of small adult stem cells known as satellite cells are essential for muscle regeneration. Satellite cells are quiescent at rest, but during wound or upon receiving growth signals, they can rapidly enter the cell cycle and multiply widely[1]. The activated satellite cells next develop into multinucleated myotubes through mitosis. The impaired signaling pathways in satellite cells contribute to reduced muscle regeneration in clinical conditions like muscular dystrophies (MDs) and muscle atrophy. Addressing these deficiencies through targeted therapies or biomaterials could enhance satellite cell function, offering potential applications in regenerative medicine and the treatment of muscle-related disorders[2]. A complex gene regulatory network tightly regulates the multi-step muscle development process.

The scientific community has explored myogenesis across various stem cell types, owing to the rapid advancements in stem cell research, each with distinct potentials and certain limitations. Mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), and different progenitor

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Abbreviations: AMPK, AMP-activated protein kinase; ARE, AU-rich elements; AUF, AU-rich element binding factor; CREB, Cyclic AMP response element binding protein; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DM, Myotonic dystrophy type; DMD, Duchenne muscular dystrophy; FAD, Flavin adenine dinucleotide; hiPSCs, Human Induced pluripotent stem cells; hnRNP, Heterogeneous nuclear ribonucleoproteins; MBNL, Muscleblind Like Splicing Regulator; MD, Muscular dystrophy; MEF, Myocyte enhancer factor; MRF, Myogenic regulatory factor; MSCs, Mesenchymal stem cells; MyoD, Myoblast determination protein; MyoG- myogenin, Myogenic factor; MyHC, Myosin heavy chain; iMPCs, induced Myogenic Progenitor Cells; RBPs, RNA Binding proteins; siRNA, Small interfering RNA; UTR, Untranslated region

cells are some of the critical participants. While each cell type has unique benefits in myogenic differentiation, much remains to explore about the fidelity and efficiency of their differentiation potential due to ongoing challenges in improving reproducibility and protocol optimisation[3].

A complicated network of molecular pathways controls the myogenic differentiation process, with majorly contributing signaling cascades including Wnt, Notch, and Hedgehog propelling the growth and differentiation of muscle progenitors. Myogenic determination gene number 1 (MyoD1) and other myogenic regulatory factors (MRFs), such as Myf5, Mrf4, Pax7, are also essential for generating the myogenic lineage, which commits stem cells to develop into functional muscle cells[4]. Anetwork of transcriptional activators like Pax3, Six1 and transcriptional repressors like Sim2, and DELTEX2 bind different regulatory sequences near the promoter to influence the expression of MyoD1. In addition to transcription factors and epigenetic modulators, RNA-binding proteins (RBPs), the modifiers of post-transcriptional processing, especially alternative splicing, are also involved in maintaining muscle homeostasis. Significant contributors include the muscle blind-like protein family (MBNL), heteronuclear nuclear ribonucleoproteins (hnRNPs), and CELF-family (CUG-BP and ETR-3-like factor proteins) of proteins as they manage various post-transcriptional processes like alternative splicing, mRNA stability, and translation. The CELF family of RNA-binding proteins not only regulates gene expression during normal muscle development but also contributes to muscle pathologies, such as those seen in DM1, by disrupting splicing and other post-transcriptional processes. Understanding their role could open new avenues for developing therapeutic strategies targeting aberrant gene regulation in muscle disorders[5].

Cellular metabolism, which changes significantly as stem cells advance along the myogenic lineage, is another crucial component of myogenic differentiation. Knowing the changes in metabolism provides significant insights into how they control myogenic differentiation. myogenic differentiation research has been transformed by the developments in imaging techniques, which enable researchers to view cellular and molecular processes in real-time with high accuracy. Advanced methods such as fluorescence, confocal microscopy, and metabolic imaging, when paired with live-cell imaging, monitoring the metabolic changes non-invasively became possible in stem cells[6].

Gene-editing methods, including CRISPR/Cas9, may manipulate myogenesis-related genes, opening up new possibilities for researching gene function and fixing defects that cause muscular diseases. Researchers may now investigate muscle growth and disease in a controlled, tissue-like environment due to the development of 3D culture systems and organoid models, which offer a chance to produce more physiologically accurate models of myogenesis. With the potential to restore muscle function in disorders like muscular dystrophy, stem cell-based treatments for degenerative diseases of the muscles are also significantly developing[7].

In this review article, we discussed the advances made in the recent past of all the above aspects of myogenic differentiation of stem cells, which have broadened the understanding of the scientific community in this field. Here, we are exploring the involvement of stem cells undergoing differentiation, gene regulators at the transcriptional, epigenetic, and post-transcriptional levels, signaling cascades, metabolic status, and their roles in muscle differentiation and disease pathogenesis. Additionally, we tried to understand cutting-edge technologies that help study dynamic changes at the cellular and subcellular levels during muscle differentiation. These findings and resources greatly expand our knowledge of myogenic differentiation and treatment approaches in regenerative medicine.

The impact of stem cell types on myogenic differentiation

Various types of stem cells commit to and develop into muscle cells by differentiation, each with different origins, plasticity, and regenerative potential. Below are key stem cell types involved in myogenesis:

Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into various cells such as osteoblasts (bone cells), chondrocytes (cartilage cells), and adipocytes (fat cells) derived from the mesodermal lineage. Unlike pluripotent stem cells, MSCs have limited differentiation potential and are essential in tissue repair, making them a prime focus for regenerative medicine and cell-based therapies. MSCs derived from bone marrow can support haematopoiesis and also differentiate into mesodermal layer cells such as osteoblasts, chondrocytes, adipocytes, and myoblasts[8].

Three donor tissues of MSCs, Adipose Tissue (AT), Synovial Membrane (SM), and Bone Marrow (BM), were compared for their ability to differentiate and proliferation capabilities. The division rate of AT-derived MSCs is much higher than the other two. As shown by De La Garza-Rodea *et al.* (2011), when cardiotoxin-damaged tibialis anterior muscles (TAMs) of immunodeficient mice were transplanted with human AT-, BM-, or SM-MSCs, the frequency of hybrid myofibers was highest in the TAMs treated with AT-MSCs. AT-MSCs appear to be the best choice for the further development of MSC-based treatments of myopathies due to their efficient contribution to myo-regeneration and high *ex vivo* expansion potential[9].

Human umbilical cord blood (UCB) is an alternative cell therapy and transplantation source because of its nonhematopoietic (mesenchymal) and hematopoietic potential. MSC-like cells can be derived from human UCB and can be differentiated into various cells of mesodermal origin. MSCs isolated from human UCB can also differentiate into skeletal muscle cells. Both flow cytometric and gene expression analyses have shown that two early myogenic markers, myogenin and MyoD, are expressed after 3 days of incubation and a late myogenic marker, Myosin heavy chain(MyHC), after 6 weeks. These findings highlight the potential of UCB-derived MSCs for skeletal myogenic differentiation, suggesting their suitability for regenerative applications[10].

In their 2010 study, Beier *et al.* (2010) explored the potential of MSCs to undergo myogenic differentiation when co-cultured with primary myoblasts. Co-culturing also stimulates the formation of the multinucleated myotubes that improve myogenic differentiation compared to MSCs cultured alone. These findings highlighted the importance of cell-cell interactions in directing MSC differentiation. MSCs could serve as a viable therapeutic approach for muscle regeneration by contributing to muscle tissue repair by offering potential applications in treating muscle injuries and degenerative diseases. This work supports the use of MSCs in muscle regeneration therapies, where their differentiation into myogenic lineages can be enhanced through co-culture with myoblasts[11].

Induced pluripotent stem cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are pluripotent stem cells that can be generated directly from somatic cells. These cells possess the remarkable ability to differentiate into virtually any cell type in the body, similar to embryonic stem cells, making them invaluable for regenerative medicine and research. iPSCs offer several advantages along with the potential to create patient-specific cells for personalized medicine and thereby reducing the risk of immune rejection. They have enormous potential applications, particularly in muscle repair and regeneration[12].

Shoji *et al*. (2015) have established a myogenic differentiation protocol mediated by inducible transcription factor expression that can reproducibly and efficiently drive human iPSCs into myocytes. This differentiation protocol yielded a homogenous skeletal muscle cell population with efficiencies as high as 70-90%. MyoD1-induced myocytes demonstrated characteristics of mature myocytes, such as cell twitching and cell fusion within 14 days of differentiation[13].

Tanaka *et al*. utilized a PiggyBac transposon system to integrate MyoD cDNA into the genome of hiPSCs for overexpression. Within 5 days, a large number of myogenic cells were obtained due to the integration. MyoD-induced iPSCs showed suppression of pluripotency markers while a transient increase in the Pax7 and Pax3 genes, known as early markers for myogenesis, which belong to the paraxial mesodermal/myogenic progenitor cells, were obtained[14].

Mapping myogenic differentiation: Key signaling pathways and their functional roles

When mononucleated myoblasts fuse during development, mature multinucleated skeletal myofibers are formed. The conversion of myoblasts to myofibers is a two-step process; first is a proliferation phase, when myoblast cells divide, and second is a differentiation phase, when post-mitotic myoblasts fuse to form fully contractile and multinucleated myofibers. An intricate network of signaling pathways regulates this complex two-step process, including Wnt, Notch, and Sonic Hedgehog (Shh).

Figure 1 is a flowchart which gives an overview of stem cell types involved in myogenic differentiation, their origins, and their applications in muscle regeneration.

Wnt signaling pathway

The Wnt signaling pathway is a critical regulator of various cellular processes, including development, cell proliferation, and differentiation. It is involved in switching from cell proliferation to myogenic

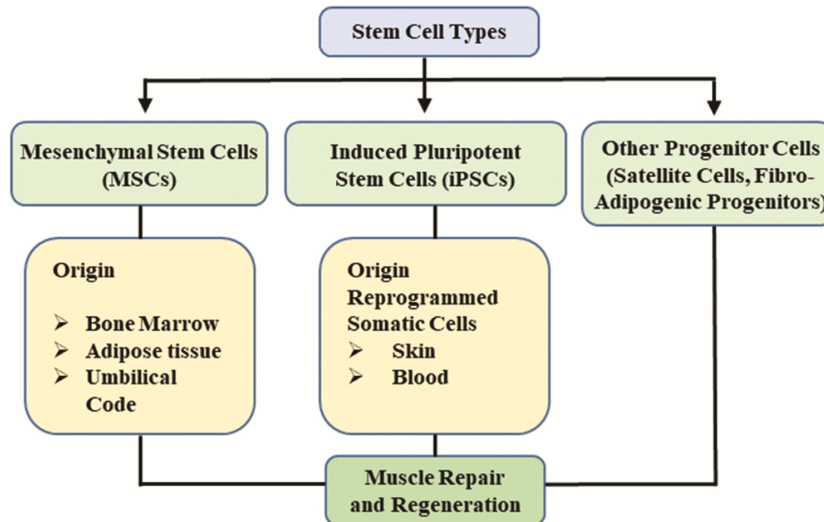

Fig. 1 — A flowchart presenting an overview of the various stem cell types involved in myogenic differentiation, their origin, and their roles in muscle repair and regeneration

differentiation in myoblasts and also plays a critical role in maintaining stem cells and adult tissue homeostasis. In addition, Wnt signaling controls cell movements and the establishment of tissue polarity. Due to such a diverse array of involvement, dysregulation of the Wnt pathway can induce degenerative and cancerous disorders[15].

Based on the downstream intracellular signaling, the Wnt family proteins consist of two subfamilies involving canonical and non-canonical pathways. The non-canonical Wnt pathways are not dependent on the β-catenin-and the T-cell factor/lymphoid enhancer-binding factors (TCF/LEF) and can enable regulation of cell polarity and migration. On the contrary, the canonical Wnt pathway involves the nuclear translocation of β-catenin and activation of target genes *via* TCF/LEF transcription factors, which predominantly controls cell proliferation. Both these pathways share a network of mutual regulation.

Wnt signaling plays a crucial role in embryonic muscle development and adult skeletal muscle homeostasis maintenance. Wnt ligands regulate the MRFs, including Myf5 and MyoD, which are essential for progressing the myogenic lineage during embryogenesis, involving both the canonical and non-canonical pathways. In adults, non-canonical Wnt signaling is involved in satellite cell self-renewal and muscle fiber growth, while canonical Wnt signaling controls muscle stem cell differentiation[16].

The balance between canonical and non-canonical Wnt signaling is essential for proper muscle regeneration and prevention of fibrosis. The increase in muscle mass due to the enlargement of myofiber size is called muscle hypertrophy. This process is mediated by the canonical Wnt signaling. Wnt4 has also been found to induce hypertrophy through canonical signaling in myogenesis models[17]. This hypertrophic effect is related to the downregulation of myostatin, leading to enhanced differentiation marked by increased expression of myogenic markers (Myf5, myogenin, MRF4) and proliferation. Interestingly, in C2C12 cells, Wnt4 seems to counteract canonical Wnt signaling, suggesting that Wnt4 acts through both canonical and non-canonical pathways. In contrast, Wnt7a has been shown to induce hypertrophy through a non-canonical pathway in adult skeletal muscle, which is distinct from the typical canonical signaling[18].

Wnt5a and Wnt11 influence the development of slow and fast myosin heavy chain positive fibers, respectively. β-catenin is essential for fiber type specification and myofiber number during vertebrate development. Increased β-catenin activity leads to more slow myosin-positive fibers[19]. While both canonical and non-canonical Wnt signaling pathways contribute to muscle hypertrophy and fiber type specification, their roles vary based on the specific Wnt ligand, driven by increased differentiation or direct growth signaling.

Vertebrate skeletal muscle forms somites along the embryo's anterior-posterior axis and its development originates from the paraxial mesoderm. Other than certain head muscles, most skeletal muscles develop from somites, eventually differentiating into the

mesenchymal sclerotome at the ventral end. These mesenchymal sclerotomes form bones and cartilage. At the dorsal end, the epithelial dermomyotome develops into the dermis and skeletal muscles of the trunk and limbs. myogenic precursor cells arise from the dermomyotome and are specified by the transcription factors Pax7 and Pax3. Wnt signaling induces somitic myogenesis upon interaction with the Shh signaling (discussed below). Wnt1, Wnt3a, and Wnt4 are expressed in the dorsal neural tube, essential for muscle formation[20].

Notch signaling pathway

Notch signaling plays an essential role in the development and regeneration of skeletal muscle and in maintaining muscle homeostasis.

Notch functions as a receptor that is activated by ligands from neighboring cells. In mammals, there are four Notch receptors (Notch1–4) and five ligands (Jagged 1 and 2, Delta-like (Dll)- 1, 3, and 4.Notch signaling plays a major role in both embryonic and post-natal skeletal myogenesis. When Numb, a Notch inhibitor, is asymmetrically distributed in dividing myogenic progenitors with the help of cytoskeleton components affecting cell polarity, Numb-positive cells exhibit markers associated with myogenic progression, such as Myf-5 and Desmin. On the contrary, the Numb-negative cells show the opposite pattern, expressing early markers like Pax3. However, a definitive genetic role for the Numb in muscle regeneration is still unclear, as forced expression of the Numb could not alter Notch activity or induce premature differentiation in somites[21].

The activation of the Notch pathway in cultured myoblasts suppresses their differentiation. Notch signaling also plays a role in distinguishing muscle stem cells (Pax7+/Myf5−) from committed progenitors (Pax7+/Myf5+). The progenitors express Dll1, whereas the muscle stem cells are located near the muscle fiber, expressing elevated levels of Notch3. Notch pathway allows the maintenance and expansion of progenitors. Mutations of the notch pathway players lead to severe muscle defects due to premature differentiation and loss of the Pax3+/Pax7+ progenitor pool. This is called hypertrophy, where premature differentiation leads to an initial excess of myogenic cells, but this myogenesis ceases early, preventing the development of normal musculature[22].

Sonic hedgehog signaling pathway

The Sonic Hedgehog (Shh) pathway is a crucial regulator of myogenesis, muscle formation, and differentiation. Shh signaling influences the differentiation, proliferation and fate of myogenic progenitor cells during embryonic development. Shh is secreted by the floor plate and notochord, signaling through its receptor Patched (PTCH1), which normally inhibits Smoothened (SMO), a key protein in the pathway. Upon Shh binding to PTCH1, the inhibition of SMO is lifted, allowing a cascade that activates the Gli family of transcription factors (Gli1, Gli2, and Gli3) in the target cells. Activated Gli transcription factors move to the nucleus and initiate transcription of genes involved in cell cycle regulation and myogenic differentiation. This signaling is essential for the expansion of myogenic precursor cells in somites, which later differentiate into muscle fibers[23].

Shh signaling promotes the expression of MRFs such as Myf5 and MyoD, which are important for initiating the muscle differentiation program during muscle injury and regeneration. Muscle injury typically triggers the activation of these MRFs, which serve as markers of muscle regeneration. Inhibition of the Shh signaling in cyclopamine-treated mice not only reduces the expression of these MRFs but also reduces the count of activated satellite cells at the injury site.

Shh signaling regulates MyoD expression during muscle repair and development. Gli2, a key Shh effector, controls MyoD by interacting with its gene elements. Experiments in P19 cells showed Gli2 is necessary and sufficient for MyoD expression in skeletal muscle development. Inhibiting Shh signaling reduces MyoD expression during satellite cell activation and impacts MyoD's transcriptional activity. MyoD, Gli2, and MEF2C form a complex that enhances MyoD activity on muscle-specific promoters, creating a feedback loop where MyoD further activates Shh signalling[24].

Figure 2 depicts the key signaling pathways influencing myogenic differentiation by modulating gene expression.

Role of transcription factors in myogenic differentiation

In this section, the crucial roles of the transcription factors influencing the MRF gene expression are discussed. The transcription activators, repressors, and epigenetic modulators bind to various cis-acting elements near the promoter region of MyoD, influencing its chromatin accessibility and, thus, its expression. Apart from them, their role of various

RNA binding proteins, which primarily modulate post-transcriptional events such as alternative splicing, mRNA stability, translation, *etc*., for myogenic differentiation is also discussed in detail.

MyoD1 is a key regulator of skeletal muscle differentiation, which belongs to the family of MRFs that control the differentiation and determination of skeletal muscle cells. Other than MyoD, the main MRFs include myogenic Factor 5 (Myf5) and myogenic Regulatory Factor 4 (Mrf4 or Myf6). During muscle regeneration, MyoD is reactivated in satellite cells. MyoD and Myf5 can compensate for each other during myogenesis, as Mrf4 alone is unable to initiate fetal skeletal muscle differentiation without the presence of either. The fourth member of the MRF family, myogenin (MyoG), plays an essential role in terminal differentiation and is activated by MyoD, Myf5, and Mrf4[25].

The 24 kb region upstream of the MyoD gene controls its expression patterns throughout different developmental stages. This regulation is primarily attributed to two key enhancers, the Core Enhancer (CE) and the Distal Regulatory Region (DRR), as well as a Proximal Regulatory Region (PRR) located near the transcription start site (TSS). The CE is responsible for initiating MyoD expression in muscle precursor cells and early myocytes across diverse anatomical regions. Deleting the CE in a 24 kb reporter construct containing the regulatory elements of the MyoD gene reduces but does not completely eliminate MyoD expression, suggesting that other regulatory regions can partially compensate for its loss. Although the CE effectively drives early MyoD expression, it is insufficient for maintaining expression in differentiated skeletal muscle[26].

Conversely, the DRR regulates MyoD expression during later developmental stages, *i.e*., in differentiated muscle cells, and continues this expression pattern into adulthood. The DRR also actively participates during the regeneration of muscle satellite cells and is essential for maintaining MyoD levels in adult skeletal muscle. The DRR does not affect MyoD expression during embryonic and fetal development. The PRR, present upstream of the TSS, acts as a minimal promoter when combined with the DRR or CE. Various activators and repressors interact with the CE, DRR, and other regulatory regions in the proximal upstream region of MyoD, allowing precise regulation of its expression based on cell origin and stages of myogenesis. This regulation is crucial for targeted applications such as enhancing muscle regeneration in degenerative diseases or improving cell-based therapies for muscle repair[27].

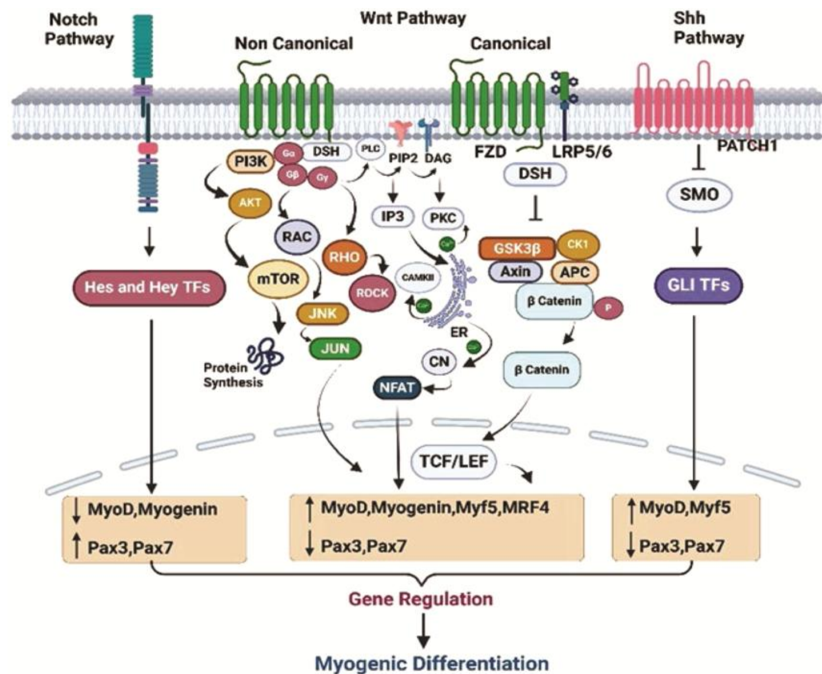

Fig. 2 — An overview of molecular signaling pathways influencing myogenic differentiation through gene expression modulation. The figure illustrates the Wnt signaling pathways (both canonical and non-canonical), as well as the Notch and Shh pathways, highlighting their roles in regulating gene expression to drive myogenic differentiation

Activators binding to MyoD cis-regulatory elements

The activator proteins Pax3 and Myf5 work together during early myogenesis, with Pax3 being expressed before MyoD in the dermomyotome and limb cells. Pax7, though expressed a little later in the central dermomyotome, contributes redundantly with Pax3. Mutations in both result in a significant absence of MyoD expression during later stages of development[28].

Both Myf5 and Mrf4 are expressed before MyoD; they play compensatory roles in regulating MyoD; thus, the loss of both leads to delayed MyoD expression. Activator protein Pitx2 regulates MyoD expression differently in various muscle groups; it cooperates with both in the limb while binding to the CE in limb buds, whereas, in extraocular muscles, it acts upstream of Myf5 and Mrf4.Wnt signaling influences the activity of the transcription factors Lef1 and Pitx2, which influence the Pax3/Pax7-positive cell number[29].

Other transcriptional activators also aid MyoD expression. For example, Six1/Six4 and Eya1/Eya bind to both the CE and DRR sequences of the MyoD gene and induce its expression in muscle precursor cells. On the other hand, MUSCULIN and TCF21 bind to the PRR and DRR and are involved in branchial arch-derived muscle formation. GLI2 binds upstream of the MyoD TSS and is necessary for MyoD expression in the myotome and during muscle regeneration. Transcriptional activators, like SRF, collaborate with factors like MASTR and MRTF, which bind the DRR, contributing to adult muscle homeostasis. It initiates MyoD expression, while MEF2 controls the rest of the muscle differentiation[30].

Repressors binding to MyoD cis-regulatory elements

Other than the activators, repressor proteins are also crucial for the regulated MyoD expression in different developmental stages. Sim2 repressor binds to the CE sequence and inhibits MyoD transcription upon overexpression in the limb bud myoblasts. It represses MyoD to prevent premature entry into the myogenic program.DELTEX2 binds to the DRR and PRR and inhibits a demethylase, leading to increased demethylation of histone H3 at the MyoD transcription start site, causing its suppression. CHOP binds at 3 kb upstream of the MyoD TSS and regulates the histone deacetylase HDAC1. Knockdown of CHOP causes premature expression of myogenic genes in mouse myoblast C2C12 cells, whereas its overexpression inhibits myogenic development and reduces nuclei numbers in myotubes[31].

Epigenetic influence in myogenesis: Essential modulators of muscle differentiation and regeneration

MyoD gene regulation is greatly influenced by the DNA methylation near the promoter sites and the modified histones. Methylation at the CpG dinucleotides near the promoters generally represses gene expression by attracting repressor proteins and not giving access to RNA polymerase. Low levels of methylation at the CE site have been observed in myoblasts in the MyoD gene, allowing the activation of transcription, contrasting with non-muscle tissues, which exhibit higher levels of methylation. DNA looping has been observed in Rhabdomyosarcoma cells, with the help of RAD21, to bring enhancers like the CE into proximity with the MyoD promoter, enhancing the gene expression. Histone modifications, for example, H3K4me3 and H3K27ac, are associated with transcriptional activation and are seen in myoblasts and myotube, while repressive modifications such as H3K27me3 and H3K9me2/3, which are linked to silencing of genes, are seen in non-muscle cells[32].

The histone variant H3.3 is linked to active gene expression and is incorporated at the MyoD regulatory regions during differentiation. In C2C12 cells at the MyoD locus, recruitment of H3.3 increases at the CE, DRR, and PRR sites when the myoblasts are differentiating. This recruitment at the specific sites is mediated by the H3/H4 histone chaperone, Histone regulator A (HIR A). It has been observed that inhibition of HIR A reduces the levels of H3.3 at these regulatory regions, causing a reduction in MyoD transcription[33].

Transcribed from the CE, non-coding enhancer RNAs (CEeRNAs), known to transcribe from the active enhancers, are seen to promote MyoD expression by increasing chromatin accessibility near the MyoD promoter. Upon depletion *via* siRNA, the chromatin accessibility, as measured by DNAse1 sensitivity, is reduced, leading to a reduction in MyoD transcription. A super-enhancer region 40-50 kb upstream of MyoD has been identified, and PAX3 has been observed to enhance chromatin accessibility in this region during muscle differentiation[34].

Figure 3 shows the timeline outlining the stages of differentiation from stem cells to mature myofibers, highlighting the key transcription factors and

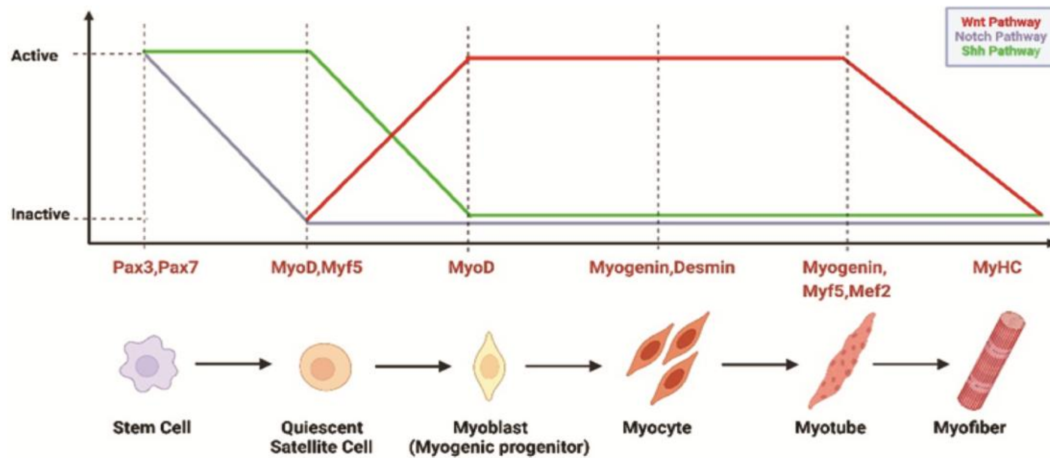

Fig. 3 — Timeline depicting the stages of differentiation from stem cell to mature myofiber, with key transcription factors and signaling pathways activated at each phase. The progression includes stages from stem cell activation, commitment to the myogenic lineage, myoblast formation, and myotube fusion, to mature myofiber formation. Key signaling pathways and transcription factors are highlighted to emphasize their roles in promoting and regulating myogenic differentiation at each stage

signaling pathways activated at each stage. These pathways and factors are emphasized to showcase their roles in promoting and regulating myogenic differentiation throughout the process.

RNA-Binding Proteins: Master controllers of transcriptional and translational regulation

In this section, the role of three major groups of RNA binding proteins, Muscle blind-like (MBNL), Heterogeneous nuclear ribonucleoproteins (hnRNPs), and CUG-BP and ETR-3-like factors (CELF), in muscle homeostasis and pathogenesis mainly by influencing post-transcriptional events of gene expression, are discussed.

Muscle blind-like proteins

Muscle blind-like (MBNL) protein family members, consisting of MBNL1, MBNL2, and MBNL3, are involved in alternative splicing of gene expression. Loss of MBNL splicing factors is a central pathological event associated with the DM1 disease[35]. DM1 is an autosomal dominant neuromuscular disease caused by abnormal expansion of CUG repeats in the 3′ untranslated region of the Dystrophia myotonia protein kinase (*DMPK*) gene. Following transcription, these abnormal transcripts from double-stranded hairpin structures that trap MBNL proteins within nuclear foci. This sequestration disrupts both the cytoplasmic and nuclear availability of the MBNL proteins, hindering their essential role as alternative splicing regulators during postnatal development. This functional loss of MBNL proteins leads to misregulated alternative splicing events and causes abnormal exclusion or inclusion of specific exons and defective mRNA translation and mRNA localization. This may give rise to symptoms such as insulin resistance, muscle weakness, and myotonia[36].

Antoine Mérien *et al.* used a CRISPR/Cas9 strategy to generate knockouts of MBNL proteins in hiPSC cells to evaluate and study the impact of this group of protein loss at the molecular and cellular level during the different developmental stages of myogenesis. The results showed no major loss of the early myogenic commitment in the absence of MBNL proteins. Thus, MBNL1 and MBNL2 were dispensable for the early myogenic commitment. But at the same time, they played an essential role during the late stages of myogenesis. MBNL1 and MBNL2 depletion made a severe impact on late myogenesis and reproduced the features associated with DM1 disease[37].

In DM1, reduced activity of MBNL proteins disrupts the splicing pattern of the muscle-specific chloride voltage-gated channel 1 gene (*CLC-1*), which is essential for regulating muscle contraction and relaxation. Additionally, the splicing regulator CUG-binding protein (CUG-BP), whose levels are elevated in DM1 striated muscle, binds to *CLC-1* pre-mRNA and contributes to its abnormal splicing. Overexpression of CUG-BP in normal cells mimics the aberrant *CLC-1* splicing pattern seen in DM1 skeletal muscle. This alteration in *CLC-1* alternative splicing results in prolonged muscle contractions, a hallmark pathological feature of DM1[38]. The *Bridging integrator-1* (*BIN1*) gene, another target of disrupted

MBNL1 activity, encodes a protein critical for forming tubular invaginations in muscle membranes and for the development of muscle T-tubules. These T-tubules are vital for excitation-contraction coupling in muscle cells. In DM1 and DM2, abnormal alternative splicing of *BIN1* pre-mRNA caused by impaired MBNL1 function leads to the production of an inactive form of the BIN1 protein. This defective BIN1 protein lacks the phosphatidylinositol 5-phosphate binding site and membrane-tubulating functions, resulting in muscle weakness in both DM1 and DM2[39]. Lee *et al*. (2007) showed that MBNL3 is involved in downregulating myogenesis. The constitutive expression of MBNL3 antagonizes myosin heavy chain expression and myogenin, and it also inhibits myotube formation. MBNL3 inhibits muscle differentiation by suppressing or downregulating MyoD and MEF2D (myocyte enhancer factor 2D) expression levels to prevent unwanted myogenic gene transcription. Also, Mbnl3 knockout myoblasts show defective myogenesis[40]. These findings demonstrate that MBNL proteins play regulatory roles at the non-pathogenic level in muscle differentiation and myogenesis.

HnRNP proteins

Heterogeneous nuclear ribonucleoproteins (hnRNPs) form a diverse group of RNA-binding proteins (RBPs) with shared general features but distinct structural domains, target sequence preferences, and functions. Their roles in post-transcriptional regulation of gene expression are highly dependent on their cellular location. hnRNPs can move from the nucleus to the cytoplasm in response to post-translational modifications or due to interactions with other hnRNPs. In differentiated cells, most hnRNPs are primarily found in the nucleus, but under cellular stress, they shuttle to the cytoplasm to influence mRNA stability and translation. Mutations or malfunctions in many hnRNPs are linked to neuromuscular diseases such as ALS, SMA, and FXS, highlighting their critical roles in muscle development and maintenance. Understanding these connections could pave the way for developing targeted therapies to address muscle-related dysfunctions in both embryonic and adult stages[41].

In the following sections, we discuss different hnRNPs and their roles in maintaining muscle homeostasis:

The hnRNP Dprotein, also known as AU-binding factor 1 (AUF1), binds to AU-rich sequences on the RNA and has four isoforms that are produced through alternative splicing of its pre-mRNA. These isoforms contain two RNA recognition motifs (RRMs). They can be complexed with AU-rich elements (ARE) in the 3′-UTR of target mRNAs by promoting their degradation through the deadenylation-dependent mRNA decay pathway. In this process, the removal of the mRNA poly(A) tail from the 3′ end by specific cellular deadenylases serves as the initial catalytic and rate-limiting step. Once the poly(A) tail is removed, the remaining mRNA is quickly degraded either in the 3′ → 5′ direction by nucleases in the exosome complex or in the 5′ → 3′ direction following decapping by the Xrn1 exoribonuclease[42]. AUF1 is expressed at low levels in adult skeletal muscle, but its transcription is upregulated by CCCTC-binding factor (CTCF) in activated satellite cells during muscle repair. AUF1 plays a role in destabilizing key checkpoint mRNAs that encode proteins involved in regulating satellite cell growth and differentiation. It also targets and destabilizes *Twist*, *Cyclin D1*, and *RGS5*, the latter being an inhibitor of the Sonic Hedgehog pathway[43]. In C2C12 myoblasts, AUF1 binds to the 3′-UTR of Mef2C (Myocyte enhancer factor 2C) mRNA, which encodes the key myogenic transcription factor MEF2C and enhances its translation. MEF2C is activated in response to signaling pathways, such as MAPK and calcium/calmodulin-dependent protein kinases, triggered during muscle cell formation and regeneration. Consequently, decreased AUF1 expression delays myogenesis. Therefore, AUF1 supports muscle growth and repair by facilitating stage-specific mRNA degradation of checkpoint genes and boosting MEF2C protein synthesis, a crucial factor in skeletal muscle differentiation[44].

Transactive response DNA-binding protein 43 (*TDP43* or *TARDBP*), a member of the hnRNP family, binds to both single-stranded DNA and RNA. While it is predominantly found in the nucleus, it also shuttles to the cytoplasm, enabling it to regulate multiple aspects of muscle development. It was found that *Tardbp* gene knockout leads to muscle degeneration in mice, which suggests its role in muscle maintenance. Mutations of this gene also impact muscle functions; hence, its role is linked to familial ALS and frontotemporal dementia. Loss of *TARDBP* in C2C12 cells results in impaired myogenesis. Also, mice with one *Tardbp* allele missing develop smaller myofibers during muscle repair. All this evidence highlights *TARDBP*'s importance in myogenic differentiation and skeletal muscle repair[45].

The hnRNP A1 protein has two RRMs in its N-terminal region, which bind to UAGGGA/U sequences in target RNAs. The expression of hnRNP A1 decreases following birth but rises in regenerating and DM1-affected skeletal muscles. The overexpression of hnRNP A1 in differentiated myoblasts has been shown to impair muscle function by counteracting MBNL1 activity and promoting fetal-specific splicing patterns associated with DM1 (discussed later). This suggests the importance of a delicate balance of MBNL1 and hnRNP A1 in maintaining muscle development in healthy individuals[46].

The hnRNP K is a highly versatile hnRNP containing three KH domains and binding to poly(C) sequences. The expression of this hnRNP decreases during muscle formation. Expression of a mutated version of hnRNP K in C2C12 myoblast resulted in a reduced proliferation rate, indicating a compromised proliferation phenotype. These mutated cell lines also showed an increase in the G2/M phase, while a reduction in the S phase and a rise in the sub-G1 phase of the cell cycle. Expression analysis of key cell cycle regulators revealed a significant increase in Cyclin A2 mRNA in the mutant myoblasts compared to controls, whereas Cyclin B1, Cdc25b, and Cdc25c levels were reduced. These mutated cells also displayed defects in myotube formation. Myosin heavy chain expression, a myotube formation marker, was significantly lower in hnRNPK-mutated cells than in control myoblasts during differentiation[47].

Additionally, hnRNPK deficiency led to the downregulation of MyoG, an essential regulator of myogenic differentiation. Therefore, the loss of hnRNP K function has been associated with a delay in both cell cycle progression and muscle differentiation due to inhibition of the expression of cell cycle regulators and myogenic factors, indicating the importance of hnRNP K in myoblast proliferation and differentiation.

The hnRNP L shows an increase in expression during myogenic differentiation in human myoblasts. This hnRNP contains four RRMs that bind to the CA repeats at the target RNAs. Knockdown of this hnRNP in animal cell lines showed impaired muscle structure and myoblast fusion, respectively. hnRNP L interacts with MBNL1 and forms nuclear protein aggregates in DM1 myoblasts, which partially overlap with the toxic CUG repeats. This suggests that hnRNP L plays an essential role in the pathology of DM1. Bioinformatic data suggest hnRNP L regulates numerous genes that are abnormally spliced due to MBNL1 loss in DM1 muscle[48].

Polypyrimidine tract binding protein 1 (PTBP1), also known as hnRNP I, is a ubiquitously expressed protein that is found both in the nucleus and cytoplasm. PTBP1 competes with other RNA-binding proteins for binding at the regulatory sites and inhibits splicing events. Studies have shown that PTBP1 counteracts the actions of RBM4 and CELF1, promoting myotube-specific inclusion of exons of the α-tropomyosin (α-TM) and β-tropomyosin (β-TM) mRNAs, respectively.

α-TM mRNA exhibits a skeletal muscle cell-specific splicing pattern, including the alternatively spliced exons of this mRNA, aided by the splicing factor RBM4 (RNA-binding motif protein 4). RBM4 executes this job, most likely by binding to pyrimidine-rich elements within the regulatory introns[49].

β-TM pre-mRNA undergoes alternative splicing during myogenic differentiation. Exon 6B of this gene, is alternatively spliced and excluded in myoblasts but included in the mRNA in myotubes. The latter is aided by the CELF proteins (CUG-BP and ETR-3-like factor proteins, discussed in detail in the next section), critical for switching splicing from exon 6A to exon 6B during differentiation. Overexpressing CELF proteins in myoblasts promotes the splicing of exon 6B, while on the contrary, PTBP1 is essential for repressing exon 6B splicing in myoblasts. Together, CELF and PTBP1 exhibit antagonistic properties on exon 6B inclusion throughout myogenic differentiation[50].

PTBP1 also represses the inclusion of exon 9 of the Capzb gene by acting in opposition to Quaking (QK) protein. QK is known to regulate mRNA function through binding to the ACUAA motifs found in the exon9 of Capzb. Capzb codes for the muscle Z-line actin capping protein beta subunit that eventually helps in regulating the growth of actin filaments and the assembly of myofibrils. These findings suggest that PTBP1 inhibits muscle differentiation by competing with various RBPs with overlapping regulatory activities, which in turn control splicing of muscle regulatory gene expression during myogenesis[51].

The hnRNP E1, also known as Poly(C)-binding protein 1 (PCBP1), binds to the poly(C) sequences *via* the KH RNA-binding domains. It is also known to interact with the components of the miRNA-processing pathway, one such component being argonaute 2. hnRNP E1 modulates the maturation of muscle-specific miRNAs such as miR-1, miR-133, and miR-206 and interferes with the myogenic

differentiation of the C2C12 mouse myoblasts. hnRNP E1 is highly abundant in skeletal muscles at the embryonic stage, and null mutants for this gene cause embryonic lethality in mice. Conditional knockout of this gene induces defects in proliferation and in the rate of differentiation of myoblasts and muscle satellite cells into multinucleated myofibers[52].

Figure 4 illustrates the diverse roles of RBPs in regulating alternative splicing, mRNA decay, translation, gene expression, and mRNA stability, as well as their interaction with signaling pathways that influence myogenic differentiation.

CELF family of proteins

CELF proteins are a family of RNA-binding proteins with three RNA recognition motifs that primarily bind to the UG-rich sequences in RNA. These proteins are important for gene regulation during development. They are involved in alternative splicing, mRNA decay, and translation. CELF1 is highly expressed in muscles at the embryonic stage, and its expression decreases postnatally.

CELF1 is upregulated in DM1 myoblasts due to increased stability from hyper-phosphorylation by protein kinase C. It was found that the majority of CELF1 is free under normal conditions, whereas in DM patients, they are primarily associated with the RNAs containing the CUG repeats[74]. In undifferentiated C2C12 myoblasts, CELF1 is found predominantly in the nucleus but translocates to the cytoplasm upon differentiation. This changing expression and localization suggest the potential roles of CELF1 in managing splicing patterns of the genes crucial for skeletal muscle development. Overexpression or nuclear localization of CELF1 in adult muscle alters alternative splicing of the target genes and leads to conditions like DM and muscle wasting[53].

CELF1 and MBNL1 share overlapping and distinct RNA targets but regulate pre-mRNA splicing and mRNA stability in opposing ways. It has been established in DM1 that CELF1 over-activity and loss of MBNL1 simultaneously revert adult muscle-specific splicing to fetal muscle-specific splicing phenomenon. Fetal-specific splicing patterns refer to the alternative splicing profile typical of fetal or embryonic muscle cells, as opposed to adult muscle cells. During normal development, muscles switch from fetal to adult-specific splicing patterns, which optimizes the expression of proteins necessary for mature muscle function. This switch is regulated by proteins like MBNL1. In DM1 and under certain conditions, such as overexpression of CELF1, opposes the action of MBNL1, and the splicing switch does not fully occur. As a result, the adult muscle cells revert to a splicing pattern that is more characteristic of fetal development, which is associated with a lack of functional differentiation in muscle cells. This contributes to muscle weakness and dysfunction characteristics of DM[54].

Figure 5 shows a Flow chart which describes the key molecular mechanisms involved in myogenic differentiation, highlighting signaling pathways, transcription factors, and epigenetic regulation.

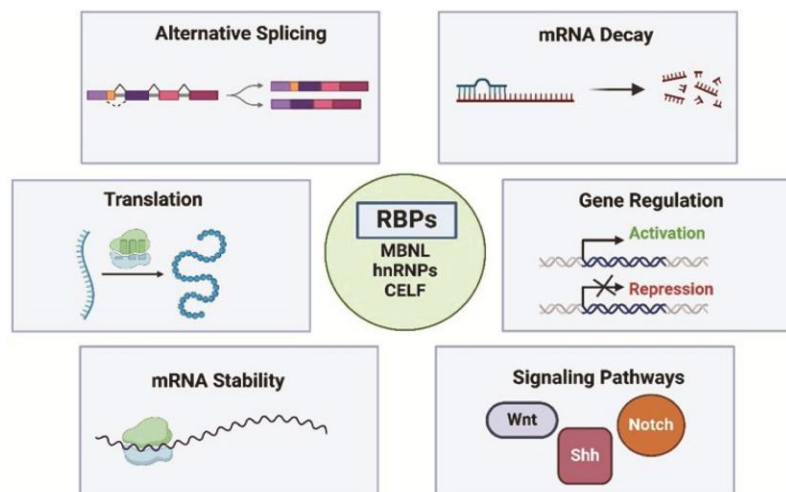

Fig. 4 — Role of RNA-binding proteins (RBPs) in regulating alternative splicing, mRNA decay, translation, gene expression, mRNA stability, and the cross-talk with signaling pathways in influencing myogenic differentiation. This figure highlights the multidimensional functions of RBPs in controlling post-transcriptional processes essential for myogenic differentiation, including the modulation of key transcripts involved in myogenic signaling pathways and muscle-specific gene expression

Metabolic pathways influencing myogenic differentiation

In this section, we discuss the complex network of cellular pathways that intricately regulate the metabolism and energy production in the cell and the impact on myogenesis. Researchers are concentrating on identifying which subcellular compartments are engaged in stem cell differentiation because of the theory that metabolic decisions may precede or accompany decisions on cell fate, including myogenic differentiation.

Energy metabolism in muscle stem cells

Energy metabolism plays an important role in muscle cell (MuSC) function and differentiation. Glycolysis and oxidative phosphorylation (OXPHOS) are two critical metabolic pathways that determine the metabolic state of these cells. Changes in intracellular redox balance can shift metabolism toward glycolysis, a hallmark of cancer and other proliferating cells. Increased glycolysis results in free cytosolic NADH, and the pentose phosphate pathway increases cytosolic NADPH. At the same time, mitochondrial pathways, such as the TCA cycle and pyruvate dehydrogenase, produce more NADH. These changes decrease the ratio of bound NAD(P)H to free NAD(P)H[55].

The dynamic role of NAD(P)H and FAD, two coenzymes important in cellular metabolism, provides important insights into stem cell differentiation. Studies observe metabolic changes without invasion can be used to understand the dynamics of stem cell differentiation by autofluorescence measurements of NAD(P)H and FAD[56].

Changes in the differentiation of rabbit bone marrow-derived stem cells were studied by Lamia *et al* ; focusing on cell size, mitochondrial distribution, and nucleotide content. The study found that in cells enlarged during differentiation, mitochondria changed from perinuclear to cytoplasmic distribution, and NAD(P)H fluorescence increased, reflecting metabolic changes. NADH triggered early fluorescence, while NADPH levels increased later in differentiation. These findings suggest that NAD(P)H fluorescence may serve as a non-invasive marker to monitor the initiation and progression of myoblast differentiation. According to another study by Purohit *et al*., the interaction between SIRT1 (Sirtuin 1 - a class III Histone deacetylase) and AMPK (AMP-activated protein kinase) is important for the regulation of metabolic processes, especially during muscle contraction and stress response[57].

The role of mitochondrial biogenesis in muscle stem cell function and renewal

Mitochondria play a central role in myogenic differentiation, cellular metabolism, and muscle regeneration. During differentiation, mitochondrial biogenesis increases, increasing the number and activity of mitochondria so that myoblasts can adapt to stress and maintain cell viability. This mitochondrial remodelling is essential for muscle differentiation and repair, although it is less critical during the proliferative phase. Mitochondrial stress responses (MSRs) promote bioenergetic remodelling, promoting myoblast survival and differentiation. The ATP produced by OXPHOS in the inner mitochondrial membrane by transferring electrons from NADH/FADH2 to oxygen provides energy for muscle regeneration and repair.

In addition to energy production, mitochondria regulate reactive oxygen species (ROS) levels, fatty acid metabolism, and epigenetic remodelling, all of which influence stem cell fate and plasticity. Environmental signals modulate mitochondrial function, affecting OXPHOS activity, ROS generation, and TCA cycle intermediates driving muscle stem cell differentiation.

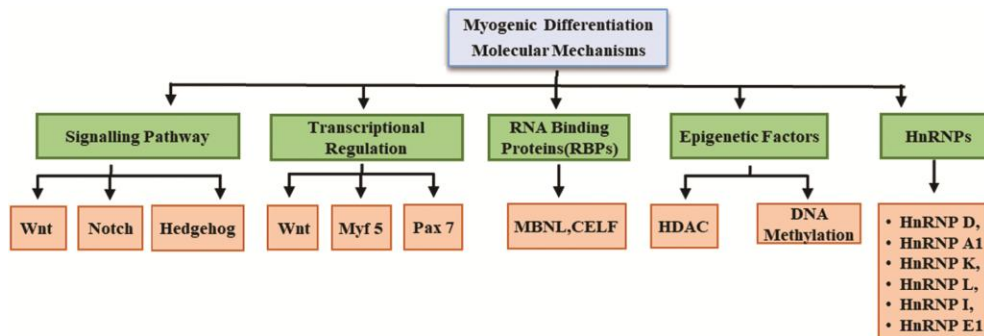

Fig. 5 — Illustrates a flowchart that outlines the essential molecular processes driving myogenic differentiation, emphasizing key signaling pathways, regulatory transcription factors, and epigenetic modifications

Stem cells and differentiated cells show distinct metabolic profiles. While stem cells primarily rely on glycolysis to rapidly generate energy, avoid excessive ROS, and adapt to hypoxia, differentiated cells shift to OXPHOS and establish a more robust mitochondrial network. A study of muscle satellite cells, a subset of muscle stem cells, revealed that their metabolic requirements differ depending on their state – growing, resting, or regenerating. Notably, quiescent and senescent satellite cells show lower levels of ATP and rely more on glycolysis than on mitochondrial OXPHOS. Despite the abundance of mitochondria during growth, proliferating cells are surprisingly not strongly dependent on OXPHOS, suggesting unique metabolic strategies. In addition, peroxisomal fatty acid oxidation serves as a metabolic switch promoting differentiation efforts, and blocking this can impair regeneration. In quiescent MuSCs, energy conservation is achieved by suppressing anabolic processes and activating catabolic pathways such as autophagy and fatty acid oxidation[58].

High levels of the histone deacetylase, SIRT1, in quiescent MuSCs maintain histone H4 in a deacetylated state, thereby reducing the overall transcription of the myogenic genes. When MuSCs are active, SIRT1 deactivates, permitting H4K16 acetylation, chromatin relaxation, and the start of myogenic gene transcription. This ensures that myogenic differentiation genes are repressed until MuSCs become activated. Additionally, H4K20me3-mediated heterochromatinization compacts the genome and limits transcriptional activity. Upon activation, MuSCs rapidly shift from fatty acid oxidation to glycolysis to meet anabolic demands, inactivate SIRT1, and increase H4K16 acetylation at myogenic loci, thereby promoting gene activation and differentiation[69].Activated MuSCs also show increased mTORC1 signaling and autophagy to promote growth. Metabolic reprogramming of MuSCs is closely linked to mitochondrial function, with AMPK playing a role by activating the transcriptional coactivator PGC1A (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha) to control mitochondrial biogenesis[60]. On the contrary, aging impairs autophagosome clearance, accumulating damaged mitochondria and metabolic waste, compromising stem cell function.

Key signaling pathways such as PTEN (Phosphatase and Tensin Homolog), TSC1 (tuberous sclerosis complex subunit 1), and SIRT7 (Sirtuin 7) regulate mitochondrial function to maintain stem cell properties of MuSC. PTEN negatively regulates the PI3K-Akt signaling pathway, which modulates mTOR, a key regulator of cell growth and mitochondrial function. Restricting mTOR signaling helps maintain quiescence and prevention of excessive mitochondrial activity. TSC1 slows down mitochondrial biogenesis by suppressing mTORC1. When muscle stem cells are activated, TSC1 inhibition causes mTORC1 to be released, which enhances mitochondrial activity and produces the energy needed for myogenic differentiation[61].

Caloric restriction (CR) can improve mitochondrial function, increase stem cell abundance, and activate regulators such as AMPK, SIRT1, and mTOR, all promoting healthy aging. During myogenic differentiation, AMPK works together with SIRT1to control mitochondrial and metabolic balance. By deacetylating PGC1A and other targets, AMPK can raise NAD+ levels and improve SIRT1 activity, which in turn stimulates mitochondrial biogenesis and myogenic gene expression[62].

These findings highlight the pivotal role of mitochondrial biogenesis, metabolic shifts, and redox regulation in optimizing muscle stem cell function, differentiation, and regeneration. Understanding these processes can pave the way for developing targeted therapies to enhance muscle repair and combat degenerative muscle diseases[63].

Innovative approaches to myogenic differentiation: live-cell imaging, metabolic imaging, and gene editing

Live-cell imaging

Live-cell imaging is one of the effective methods that allows researchers to track cellular processes in real-time. In combination with fluorescent microscopy, this technique offers great sensitivity and specificity when tracking changes at the cellular level. Fluorescence lifetime imaging (FLIM) enables precise distinction between NADH and NADPH levels in the cell. The primary factors influencing the redox state of the cell are the nicotinamide adenine dinucleotide (NAD+/NADH) and nicotinamide adenine dinucleotide phosphate (NADP+/NADPH) redox couples. Depending on their energy requirements, cells alternate between different pathways, such as glycolytic or oxidative pathways. Studies targeting these pathways indicate that these metabolic shifts do not significantly impact NAD(P)H fluorescence decay rates. Mathematical models are useful to quantify

NADH and NADPH levels across various cell types within complex tissues to monitor their metabolic state[64].

Time-lapse images are usually captured at fixed intervals (*e.g.*, every 20 min) with an incubator microscope kept at 37°C and 5% CO_2. The growth medium of the cultured cells is usually replaced with the differentiation medium, which is replenished every five days until differentiation is complete, depending on the specific cell line. Autofluorescence of the cells can be utilized, or a fluorescent protein tag may be employed to enhance visualization[65].

Live-cell imaging can be used for studying muscle differentiation because it enables real-time observation of individual myoblast behaviors, allowing researchers to analyse variability in cell cycle duration, viability, and lineage contributions during the differentiation process. This technique was used to define myoblast dynamics. When EGFP-positive myoblasts under the regulation of a constitutively active EF-1α promoter were visualized at specified time intervals using an automated cell counting method, thorough comprehension of lineage variability and information on how the behavior of EGFP+ cells differ from that of the general population was achieved[66].

While highlighting the inherent variety of cultured myoblasts and offering crucial insights into the regulation of muscle differentiation, this dynamic analysis also demonstrates the importance of live-cell imaging in defining these processes.

Metabolic imaging techniques - fluorescence and confocal microscopy

As described in the previous section, glycolysis is essential for maintaining stem cells, characterized by low NADH, which rises with differentiation and the prominence of OXPHOS. This phenomenon is useful for studying the fate and function of stem cells since it allows label-free imaging and helps in the detection of metabolic abnormalities that may hinder appropriate differentiation. Variations in NADH/FAD fluorescence provide details regarding early and late phases of differentiation by reflecting the cellular redox state[67].

Using the inherent fluorescence of biological constituents such as NAD(P)H, flavoproteins, and lipofuscin, two-photon excited fluorescence (TPEF) and second harmonic generation (SHG) provide non-invasive methods for monitoring the development of human MSCs. Researchers can track dynamic changes in metabolic activity within the same cell population over time and uncover unique metabolic patterns during differentiation by extracting these signals from TPEF images and computing the redox ratio (NAD(P)H/flavoproteins).

Microscopy determines the optical redox ratio ((NAD+ (NADH+NAD+) or (FAD / (NADH + FAD)) due to metabolic changes that occur while undergoing differentiation. Better depth resolution, less photodamage, deeper tissue penetration (up to 0.5-1 mm), and increased signal collection make it ideal for non-invasive imaging of living biological samples in three dimensions[68].

Additional information could be obtained by sophisticated methods like SHG (Second Harmonic Generation), THG(Third Harmonic Generation), SRS (Stimulated Raman Scattering), and MRI (Magnetic Resonance Imaging), which aid in the isolation of internal lipids, extracellular matrix proteins, and redox alterations in cancer and stem cells. PSC-CMs (PSC-derived cardio-myocytes) can be distinguished from undifferentiated pluripotent stem cells using SHG, a possible label-free, non-genetic method[69].

MRI imaging provides useful information for non-invasive *in vivo* monitoring of myogenic differentiation from muscle precursor cells to mature skeletal muscle tissue, especially through relaxation and diffusion studies. Studies on cancer, angiogenesis, and the brain have demonstrated that imaging modalities such as OCT (optical coherence tomography) when paired with NAD(P)H and FAD spectroscopy, provide improved metabolic and structural insights. Such studies could also be used to monitor myogenic differentiation of stem cells. Klyen *et al.* described OCT as an effective method for studying the morphology of mouse skeletal muscle in the Duchenne muscular dystrophy (DMD) model. It indicates that OCT can efficiently visualize the boundaries between grafted and host muscles in a whole-muscle autograft paradigm, providing insight into tissue integration following injury and inflammation[70]. Additionally, by linking redox measurements to particular metabolic pathways and validating fluorescent biomarkers, the integration of optical imaging with genomics, proteomics, and metabolomics will be advantageous for disease detection and treatment monitoring.

Figure 6 highlights metabolic changes during myogenic differentiation, focusing on fluctuations in NADH, NAD^+, FAD, and $FADH_2$ levels linked to shifts in glycolysis, oxidative phosphorylation (OXPHOS), and the TCA cycle.

CRISPR/Cas9 and gene editing in myogenic differentiation

CRISPR/Cas9, a type II clustered regularly interspaced short palindromic repeats (CRISPR) system, is a genome-editing technology that can regulate myogenic differentiation by modulating myogenic factors. This technique is a promising approach for treating diseases connected to the muscles in the future as it provides a precise way to target genes involved in muscle development and repair. For example, CRISPR/Cas9 holds the potential as a therapeutic approach for DMD, which is suggested to be a stem cell-related disease, by regulating dystrophin expression. Dystrophin plays a crucial part in stem cell activity, which implies that dystrophin restoration on stem cell function using CRISPR Technology may improve cellular energetics and stress tolerance, which in turn may improve the function of muscle progenitors[71].

CRISPR/Cas9 has been used to generate MyoG-deficient hiPSCs to study its effect on myogenic differentiation, achieving an impressive 80% knockout efficiency of the MyoG gene[72]. Additionally, the Synergistic Activation Mediator (SAM) CRISPR-Cas9 system has been applied to overexpress IGF-I mRNA variants in human and mouse precursor cells, leading to an increase in muscle mass. As this technology is also capable of upregulating gene expression, it has been used to activate the transcription factor Pax7, facilitating the differentiation of both human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) into skeletal muscle progenitors. In order to evaluate the molecular impact of MBNL protein loss across different phases of myogenic development, researchers used the CRISPR/Cas9 technique to produce knockouts of these proteins in hiPSCs. The knockout is generated using sgRNA to target the coding exons and a PAM restriction enzymatic site. The sgRNA is complexed with SpCas9 protein to perform ribonucleoprotein transfection of dissociated hiPSCs. Sanger DNA sequencing and restriction fragment length polymorphism were performed in order to detect CRISPR gene editing according to total or partial loss of restriction sites.

In another study by Domenig *et al.*, embryonic fibroblasts from DMD mouse models were reprogrammed into induced myogenic progenitor cells by overexpressing MyoD using small molecule treatment. To address this, CRISPR/Cas9-mediated exon skipping was employed successfully, restoring dystrophin expression *in vitro*[73].

Li *et al*. have shown that *dystrophin* gene correction by knocking-in in hiPSCs, derived from a DMD patient, is possible by the TALEN and CRISPR-Cas9 gene-editing systems. The patient-

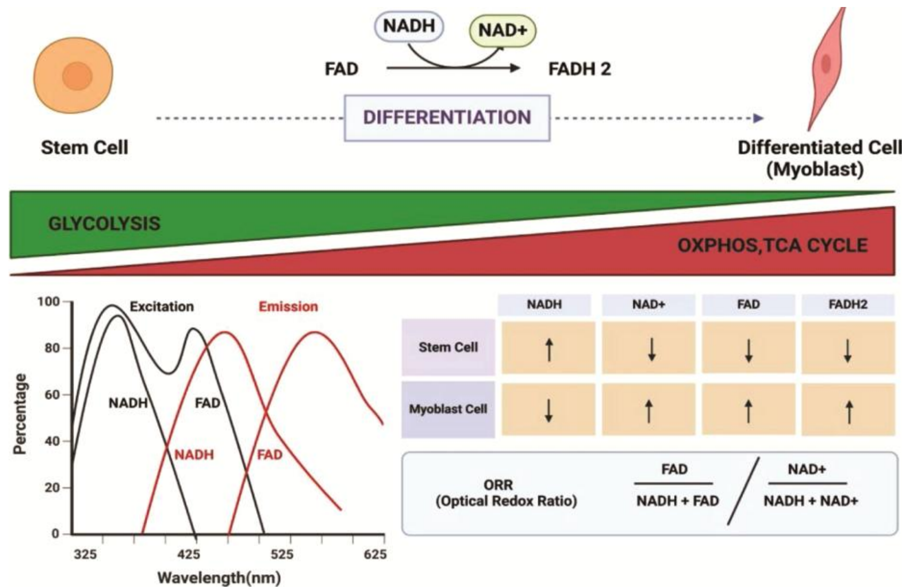

Fig. 6 — Metabolic changes associated with myogenic differentiation, highlighting fluctuations in NADH, NAD^+, FAD, and $FADH_2$ levels due to shifts in glycolysis, oxidative phosphorylation (OXPHOS), and the Tricarboxylic Acid (TCA) cycle. By measuring specific wavelengths associated with these metabolic cofactors, the progression through different stages of myogenic differentiation can be tracked using advanced microscopic techniques.Optical redox ratio can be calculated using the formula - ((NAD+ (NADH+NAD+)or(FAD / (NADH + FAD)) which will aid in detecting the metabolic state of the cell

derived hiPSCs originally lacked exon 44 of the *dystrophin* gene, which caused severe symptoms in the patient. Using gene editing tools, the authors successfully knocked in the correct version of the *dystrophin* gene in these cells. Subsequently, they overexpressed MyoD in these corrected cells for myogenic differentiation, and eventually, full-length dystrophin protein expression was achieved[74].

Organoids and 3D Cultures in myogenesis Research

Culturing cells in three dimensions can recreate a stem cell niche condition for various tissues, enabling the expansion of adult stem cells while retaining their capacity to differentiate and mature[75]. Scaffolds and hydrogels are instrumental in this process, improving cell alignment and spatial organization. Commonly used hydrogels are made from synthetic polymers like PEG(Polyethylene glycol) or PLGA(Poly Lactic-co-Glycolic Acid) and natural ones like collagen and fibrin.

To support cell attachment, proliferation, and differentiation, this technique employs three-dimensional scaffolds—typically hydrogels—to cultivate stem cells, such as MSCs or hPSCs. Incorporating biological elements like growth factors like IGF and HGF within these 3D cultures further drives cells toward the myogenic lineage[76].

Another promising approach within 3D myogenesis is the development of skeletal muscle organoids (SkMOs) from myoblasts. These organoids contain cells resembling satellite cells, known as *in vitro*-derived satellite cells (idSCs), which, when transplanted into damaged or diseased muscle, can regenerate muscle similarly to natural satellite cells[130]. In a notable study by Pinton *et al.*, bioengineered multilineage skeletal muscle cells were obtained from hiPSCs. Following differentiation into progenitor cells, the 3D hydrogels were compressed to produce aligned myofiber scaffolds containing motor neurons and vascular networks. The resulting bioengineered muscles replicated the anatomical and functional characteristics of human skeletal muscle, including the formation of a cell pool that expresses markers associated with muscle stem cells[77].

A technique was developed using four distinct human pluripotent stem cell sources to generate myogenic progenitors, termed induced myogenic Progenitor Cells (iMPCs). In 2D cultures, iMPCs effectively differentiate into multinucleated myotubes. When cultured in a 3D hydrogel environment, they further develop into aligned, functional skeletal muscle tissues, known as iSKM bundles. These bundles can generate calcium transients and exhibit twitch and tetanic contractions in response to electrical and neurotransmitter stimulation, thereby offering a reliable platform for the *in vitro* production of functional muscle tissues[78].

By producing *in vitro* conditions that closely resemble native muscle tissue, organoids, and 3D cultures have proved to be applicable for promoting realistic cell interactions and effective muscle differentiation. It improves the understanding of illnesses affecting the muscles and possible treatments by facilitating disease modelling, differentiation, and muscle regeneration.

Figure 7 outlines key factors and advanced techniques used to study and promote myogenic differentiation in stem cells. It includes differentiation media with growth factors, chromatin modifiers, siRNA, shRNA, and CRISPR/Cas9 for gene expression manipulation. Culture methods such as 3D, co-culture, and organoid systems support differentiation, while advanced imaging techniques like confocal microscopy, two-photon microscopy,

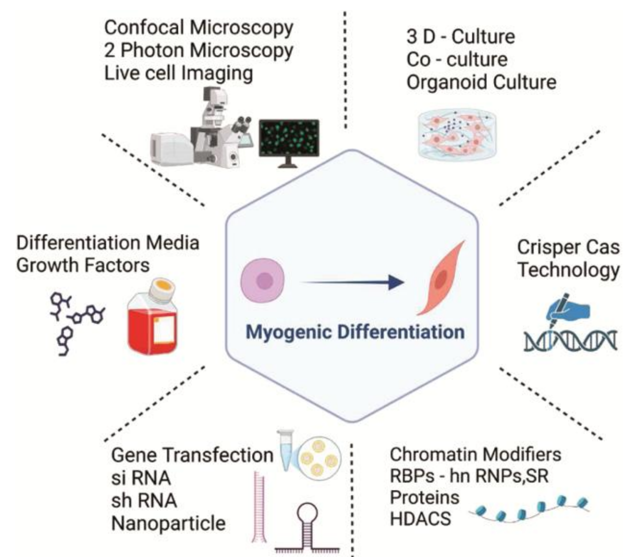

Fig. 7 — This schematic illustrates the factors and advanced techniques influencing and detecting myogenic differentiation in stem cells. Key components include differentiation media with growth factors, chromatin modifiers, siRNA, shRNA, *etc* for manipulation of gene expression and CRISPR/Cas9 for gene editing, and innovative culture methods, including 3D, co-culture, and organoid systems for promoting differentiation. In addition, advanced imaging techniques like confocal, two-photon microscopy, and live-cell imaging, are highlighted for their roles in real-time monitoring and detection of myogenic differentiation

and live-cell imaging enable real-time monitoring and detection of the process.

Conclusion

Muscle regeneration depends on the complex process of myogenic differentiation, which involves a closely controlled series of cellular, molecular, and metabolic processes that direct stem cells toward a myocyte lineage. Potential therapies in regenerative medicine, especially for diseases and injuries affecting the muscles, depend on this intricate interaction of pathways. The effect of different stem cell types in myogenic differentiation, including MSCs, iPSCs, and MESCs, has been discussed. We have also looked at the improvements in myogenic differentiation protocols, which increase the potential of these cells for muscle regeneration and repair.

The crucial regulatory functions of transcription factors, such as MyoD, Pax7, and Myf5, as well as epigenetic modulators that either activate or suppress particular genes to promote precise muscle development, are highlighted in this article. It has been found that post-transcriptional, especially the alternative splicing modifier RBPs such as the MBNLs, hnRNPs, and CELF families, play crucial roles in myogenic gene expression and contribute to pathogenesis due to their misregulation and thus, can potentially be therapeutic targets.

As stem cells undergo a transition from a proliferative to a differentiated state, there is an energy demand alongside metabolic alterations like changes in glycolysis and OXPHOS, which become essential causes driving myogenesis. During muscle development, mitochondrial biogenesis and ROS control are essential for preserving cellular health and energy balance. Knowing the changes in the energy state in metabolism has made it possible to identify the effective differentiation process, which is crucial for creating focused treatments and has opened up new research directions for stem cell fate.

Advancements in technologies have made real-time imaging and analysis of myogenic differentiation possible. Energy demands, lineage expansion, and differentiation dynamics can all be better understood by non-invasive monitoring of cellular changes by techniques like metabolic imaging by fluorescence and confocal microscopy. While 3D culture systems and organoid models offer a more physiologically appropriate platform for studying muscle growth and pathology *in vitro*, gene-editing techniques like CRISPR/Cas9 offer exciting potential for correcting mutations that may lead to muscle-related disorders.

In summary, advancements in myogenic differentiation research have expanded the understanding of the involvement of cellular signaling pathways, regulatory proteins, metabolic changes, *etc*. Cutting-edge detection techniques made it possible to monitor these changes in real time. Together, these developments set the stage for bringing fundamental research into clinical settings, with stem cell-based therapies combined with gene editing and organoid culture, providing hope for treating degenerative muscle disorders and injuries. Advancements in these fields will enable the medical and scientific community to address disease-specific issues and improve therapeutic approaches in regenerative medicine for muscle repair.

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Conflict of interest

All authors declare no conflicts of interest.

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