

Advances in DNA Methylation and Demethylation Regulating Muscle Development (Postprint)

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Abstract

Muscle development is a complex biological process, and its regulatory mechanisms remain incompletely elucidated. However, in recent years, the regulatory role of epigenetic modifications in muscle development has emerged as a research hotspot, with studies demonstrating that DNA methylation and demethylation modifications play crucial regulatory roles in myogenesis and muscle development. DNA methylation modifications at specific loci in muscle stem cells affect the expression of key genes during muscle development, thereby regulating the myogenic process in early development. This article primarily discusses the dynamic changes in DNA methylation and demethylation modifications during muscle development, important methyltransferases and demethylases, and the role of nutrients in influencing myogenesis through DNA methylation modifications.

Full Text

Advances in the Regulation of DNA Methylation and Demethylation on Muscle Development

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Abstract: Muscle development is a complex biological process, and its regulatory mechanisms remain incompletely understood. In recent years, the regulatory role of epigenetic modifications in muscle development has emerged as a hot research area. Studies have revealed that DNA methylation and demethylation modifications play crucial regulatory roles in myogenesis and muscle development. DNA methylation at specific sites in muscle stem cells affects the expression of key genes during muscle development, thereby regulating the myogenic process in early development. This review focuses on the dynamic changes of

DNA methylation and demethylation modifications during muscle development, important methyltransferases and demethylases, and the effects of nutrients on myogenesis through DNA methylation modifications.

Keywords: DNA methylation; demethylation; myogenesis; enzymes; nutrient

With the development of epigenetics, the corresponding epigenome has emerged. The epigenome's hereditary modification mechanisms primarily include three major components: DNA methylation [?], histone modifications [?], and non-coding RNAs [?]. Discovered in 1975, DNA methylation as an epigenetic modification [?] is currently the most extensively studied and deeply understood epigenetic mechanism. DNA methylation modification involves the covalent attachment of a methyl group from a methyl donor to the 5-carbon of cytosine nucleotides, forming 5-methylcytosine (5mC). This modification can exist relatively stably in plant, animal, and fungal models [?]. In animal models, this modification typically occurs in CpG-rich and symmetric regions of the DNA strand, known as CpG islands, which are predominantly found in the promoter regions of housekeeping and developmental genes [?]. DNA methylation in these regions inhibits transcription by preventing transcription factors and RNA polymerase from binding to the template strand through methyl-CpG-binding domain proteins, thereby affecting gene expression levels in corresponding regions and ultimately altering biological functions in the organism.

As a vital component of the organism, muscle tissue development is also regulated by DNA methylation levels. Muscle tissue originates from mesodermal progenitor cells that proliferate, differentiate, fuse, and mature to ultimately form skeletal muscle fibers—a process known as myogenesis. Brunk et al. [?] demonstrated that the expression activity of the myogenic differentiation factor MyoD gene depends on the demethylation status of DNA, establishing the first direct link between DNA methylation and muscle cell differentiation. Subsequent studies on the transcriptional regulation of myogenesis by DNA methylation have attracted considerable research interest, gradually clarifying the modification status of key genes and critical regulatory enzymes in this process. Moreover, with increasing emphasis on nutrition, studies have found that nutrients can also influence muscle-related gene expression by affecting DNA methylation, potentially altering physiological states. Therefore, exploring the relationship between DNA methylation and muscle development enhances our understanding of muscle developmental processes and the regulatory effects of nutrition on muscle development.

1.1 DNA Methylation Mechanism

Numerous studies have confirmed that DNA methylation is a crucial epigenetic modification in mammals, participating in the regulation of X chromosome inactivation [?], genomic imprinting [?], silencing of transposons and other genomic elements to ensure genome stability [?], and altering the transcriptional levels of numerous genes [?]. Among these, the mechanism by which DNA

methylation regulates gene expression has received the most attention. Early studies demonstrated that DNA methylation inhibits gene expression by preventing transcription factors from binding to transcription start sites, a finding widely accepted by scholars. More recently, Schüebeler [?] proposed five potential mechanisms by which DNA methylation may regulate gene expression (Fig. 1 [Figure 1: see original paper]): (a) methylation-insensitive transcription factors binding to transcription start sites can inhibit DNA methylation in that region; (b) transcription factors bind to methylated start sites through a specific binding mode to enable transcription; (c) methylation-sensitive transcription factors cannot bind to regions where cytosines in the start site are methylated, thereby inhibiting transcription; (d) in CpG-rich start site regions (shaded in the figure), methylation facilitates binding to methyl-CpG-binding domain proteins (MBD), indirectly preventing transcription factor binding; and (e) methylation-insensitive transcription factors first bind to start sites, creating a hypomethylated binding site, after which methylation-sensitive transcription factors bind to ensure proper transcription initiation. Among these five models, models c and d are relatively well-established, while the remaining three require further validation.

DNA methylation can occur in multiple regions of the DNA strand, including both intragenic and intergenic regions, with different consequences depending on the location. Research indicates that intragenic methylation has diverse effects on transcription and can also regulate alternative splicing [?], whereas intergenic DNA methylation suppresses gene activity by inhibiting enhancers [?]. However, the function of non-CpG site methylation remains unclear. Thus, DNA methylation modification plays a vital role in regulating gene expression and consequently influences tissue development in organisms.

1.2 Methyltransferases and Demethylases

DNA methylation and demethylation represent a dynamic and plastic process during organismal development. In this process, DNA methyltransferases (DNMTs) and demethylases—the ten-eleven translocation (TET) enzyme family—play essential roles in establishing, maintaining, and removing methyl groups.

1.2.1 Methyltransferase Family

The primary function of DNMTs is to catalyze the transfer of methyl groups from S-adenosylmethionine (SAM) to the 5-carbon of cytosine, forming 5mC [?]. The DNMT family mainly includes DNMT1, DNMT3a, DNMT3b, and DNMT3L. DNMT1 primarily functions during mitosis to maintain the DNA methylation pattern on the newly synthesized strand identical to that of the template strand during replication. DNMT3a and DNMT3b share high homology and are mainly responsible for establishing new DNA methylation, though they act at specific developmental stages. DNMT3b is the key enzyme for establishing new DNA methylation during early embryonic development, particularly during implantation, whereas DNMT3a mainly functions during later

embryonic development and cell differentiation [?]. Additionally, some studies suggest that DNMT3a and DNMT3b also serve to hinder DNMT1 activity during the maintenance of genomic methylation patterns in offspring [?]. DNMT3L assists DNMT3a and DNMT3b in establishing new methylation and promotes their action on chromatin [?]. Thus, the DNMT family plays a crucial regulatory role in establishing and maintaining DNA methylation states.

1.2.2 Demethylation Regulatory Pathway

DNA methylation modifications can be eliminated in organisms through demethylation pathways. In this process, the TET family (including three isoforms: TET1, TET2, and TET3) plays a critical regulatory role. TET enzymes first hydroxylate 5mC to 5-hydroxymethylcytosine (5hmC), then further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [?], thereby eliminating 5mC. However, whether 5caC can be converted back to cytosine through decarboxylation remains unclear. Additionally, the intermediate products of TET-catalyzed reactions, 5fC and 5caC, can be excised by thymine DNA glycosylase (TDG) through the base excision repair mechanism [?]. Seisenberger et al. [?] further proposed a potential mechanism for methyl group clearance in organisms (Fig. 2 [Figure 2: see original paper]), which includes two primary pathways: passive demethylation and active demethylation. Some processes in this pathway require further validation to complete the demethylation pathway. In organisms, both DNA methylation and demethylation processes are regulated by key enzymes, and their specific regulatory mechanisms may vary across different developmental stages to achieve precise epigenetic modifications that maintain normal development. During myogenesis, DNA methylation and demethylation are also regulated, and key myogenic regulatory genes are subject to epigenetic modifications.

2.1 Gene Network Regulating Muscle Development

Skeletal muscle originates from mesodermal progenitor cells during embryonic development. These cell populations undergo proliferation, differentiation, fusion, and maturation to form skeletal muscle fibers. During muscle formation, cells progress through stages including embryonic progenitor cells, satellite stem cells, committed satellite cells, myoblasts, myocytes, and myotubes/muscle fibers. Two main classes of regulatory factors participate in muscle formation: myogenic regulatory factors (primarily Myf5, MyoD, Myf6, and MyoG) and upstream regulators Pax3 and Pax7.

Pax3 and Pax7 belong to the evolutionarily conserved Pax family and play critical roles in regulating tissue differentiation and organ development, with expression that is not tissue-specific. During embryonic development, Pax3 participates in the formation and delamination of hypaxial trunk muscles, and is also required for the migration of myogenic progenitor cells to other myogenic regions such as the limbs [?]. Migrating cells expressing Pax3 give rise to cells

expressing Myf5 and MyoD, further promoting muscle generation and establishing the satellite cell pool. During fetal muscle formation, Pax3 expression is downregulated, and Pax7 becomes the primary factor regulating all myogenic stem cells. In the limbs, both Pax7 and Pax3 are initially expressed, and lineage tracing experiments have shown that all cells expressing Pax7 at later stages originate from cells that previously expressed Pax3 [?]. Pax7 is also essential for maintaining adult stem cell populations. Before birth, Pax7 is not indispensable for fetal muscle development, likely because Pax3 can partially compensate for Pax7 function; however, after birth, Pax7 becomes essential for muscle development, possibly due to functional divergence between Pax7 and Pax3, with each assuming different regulatory roles in myogenesis. Thus, Pax3 and Pax7 are interdependent yet functionally independent during myogenesis, jointly regulating muscle development.

Myf5, Myf6, and MyoD are myogenic determination factors and necessary for muscle generation, while MyoG acts as a differentiation factor that, together with Myf6 and MyoD, regulates the differentiation of myotubes into mature muscle fibers [?]. Myf5 is the first myogenic regulatory factor expressed during muscle development and is crucial for skeletal muscle cell determination and differentiation during embryogenesis. Myf6 functions not only in skeletal muscle cell determination but is also activated during myotube differentiation to participate in this process. MyoD expression follows Myf5 expression in both epaxial and hypaxial dermomyotomes, with its initial expression activity depending on Myf5 and Pax3, after which it can activate its own expression through a feedback mechanism [?]. MyoG expression is regulated by MyoD and Myf5, and during differentiation, MyoG and MyoD jointly activate terminal differentiation genes. As myogenic differentiation progresses, MyoG activates the expression of genes encoding structural and enzymatic muscle proteins such as α -actin, troponin, tropomyosin, and creatine kinase. Therefore, myogenic determination factors and differentiation factors work together to promote myogenesis, with their distinct expression patterns and interactions finely regulating the muscle formation process.

2.2 DNA Methylation and Demethylation During Myogenesis

During myogenesis, the methylation status of corresponding genes undergoes a dynamic and complex process. As cellular differentiation progresses, the overall methylation level increases; however, gene expression is cell-type specific, and methylation levels at certain specific sites decrease in different cells. This may be because the increase in global DNA methylation serves to reduce cellular pluripotency, while the expression of different cell type-specific genes during organism development requires decreased methylation at specific sites to ensure normal expression of those genes.

2.2.1 DNA Methylation

DNA methylation patterns exhibit specificity across different tissues and cell types, and comparing these patterns with muscle tissue helps researchers understand how DNA methylation regulates muscle development. Comparison of methylation patterns between skeletal muscle and blood cells, sperm, brain tissue, and spleen cells revealed 178 specifically hypermethylated sites in skeletal muscle [?]. Subsequently, Calvanese et al. [?] identified 47 significantly hypomethylated genes in skeletal muscle, some of which encode contractile proteins such as obscurin, muscle contraction proteins, and slow-contracting proteins. These findings demonstrate that different tissues display distinct methylation patterns to adapt to their unique structures and functions. Tsumagari et al. [?] compared methylation patterns between muscle cells and 30 non-muscle tissues, finding that 94% of differentially methylated sites showed hypomethylation in muscle, with 47% of these hypomethylated sites also present in myoblasts or myotubes, while only 3% of differentially methylated sites were hypermethylated in myogenic progenitor cells. This indicates that muscle methylation patterns are specific and dynamically change across different myogenic stages to regulate stage-specific gene expression and ensure proper muscle development. The study also revealed that de novo methylation in muscle tissue occurs primarily before the myoblast stage, while demethylation occurs around the myotube formation stage, thereby regulating transcription. During demethylation, TET1 and TET2 play important roles in activating the demethylation process and forming stable 5hmC products. Tsumagari et al. [?] also reported that the Pax3 gene remains hypermethylated in both myogenic cells and mature skeletal muscle, which may affect cell migration during myogenesis. However, Miyata et al. [?] found that during myogenesis, the genome-wide methylation level showed only minor changes but significant increases from the myoblast to myotube stage. Further analysis revealed that these significantly hypermethylated sites were in promoter regions of genes (transcription factors ID4 and ZNF238) related to muscle contraction and other myogenic processes. These results differ from previous reports, possibly because the study used Illumina's 450K DNA methylation bead chip, which does not distinguish between 5mC and 5hmC in its measurements. These studies collectively demonstrate that DNA methylation is specific to myogenic cells at different differentiation stages and represents a dynamic process, with methylation patterns varying even within the same stage, requiring further validation. Additionally, as myogenesis progresses, DNA demethylation at specific sites ensures normal expression of corresponding genes, attracting increasing research interest. Regarding the timing of DNA methylation modifications, most scholars agree that methylation changes occur primarily before cell fate determination, with only minor modifications after cell maturation. However, the effects of environmental stimuli after maturation remain rarely reported.

2.2.2 DNA Demethylation

During myogenesis, different myogenic regulatory factors participate at various differentiation stages, and their expression is closely related to DNA methylation status. Studies have shown that the 110 kb enhancer region of the myogenic regulatory factors Myf5/Myf6 contains numerous enhancer elements. Carrió et al. [?] compared this region across embryonic stem cells, skeletal muscle stem cells, myoblasts, and myotubes, finding that embryonic stem cells exhibited hypermethylation, while skeletal muscle stem cells, myoblasts, and myotubes all showed hypomethylation. This hypomethylation increased Myf5 gene expression and confirmed that DNA methylation levels specifically regulate the Myf5 enhancer during cell differentiation stages, primarily controlling the myogenic process. MyoD is a key gene in myogenesis, and its function is also associated with methylation status. Brunk et al. [?] found that the distal enhancer region of MyoD was unmethylated in all myogenic cells but showed an average methylation level of 50% in non-muscle cells and tissues (liver, heart, brain, etc.). The study also indicated that while methylation of the MyoD distal enhancer does not directly block embryonic MyoD activity, maintaining a demethylated state may be necessary to generate specific developmental signals, enabling the enhancer to respond to further signaling cues and activate the MyoD gene. MyoG activity is also closely linked to methylation status. Lucarelli et al. [?] demonstrated that MyoG expression in differentiated muscle cells depends on a hypomethylated state, whereas it remains methylated in non-muscle tissues (such as spleen and brain) and in proliferating myotubes. Treatment of proliferating myotubes with methylation inhibitors increased MyoG expression due to reduced methylation levels. The MyoG promoter region requires a demethylated state to bind transcription factors, while in the methylated state, MBD proteins bind to the promoter region and prevent normal transcription [?]. Recent studies have also identified methylation and demethylation in non-CpG regions of myogenic cells, but the regulatory role of methylation status in these regions during myogenesis remains unclear and requires further investigation.

2.2.3 Role of DNMT and TET in Myogenesis

During myogenesis, the activities of DNMTs and TETs are crucial for understanding methylation and demethylation processes and are essential for normal organismal development. Due to their functional specificity, DNMTs and TETs exhibit distinct expression patterns during development. DNMT1 expression is downregulated during myogenic cell differentiation, consistent with its maintenance function. Gene knockout experiments have confirmed that the DNMT family is essential for normal development: embryos with DNMT1 knockout show approximately 70% reduced methylation levels and cannot develop normally [?]; mice with DNMT3a knockout survive only four weeks during embryonic stages, while DNMT3b knockout mice are not viable [?], demonstrating that DNA methylation is indispensable for development. Dawlaty et al. [?] knocked out TET family genes and observed increased overall 5mC levels, indicating

enhanced methylation. Further analysis of hypermethylated promoters and correspondingly downregulated genes revealed that the downregulated genes are primarily involved in embryonic development and differentiation and are significantly enriched in skeletal muscle developmental pathways. Thus, DNMTs and TETs can regulate gene expression by influencing methylation and demethylation states, thereby affecting normal developmental processes, and myogenesis requires the coordinated participation of both.

3 Effects of Nutrients on Muscle DNA Methylation

Tissue and cell development depends on nutrient supply, making nutrients important regulators of developmental processes. In recent years, research on nutrients regulating organismal development through epigenetic modifications has gradually increased, particularly during embryonic development and the pre-weaning period. Factors such as maternal protein levels, high-fat diets, imbalanced nutrition during pregnancy, and overfeeding can affect offspring development through DNA methylation changes, predisposing them to diseases such as diabetes, obesity, and hypertension [?]. However, studies on the effects of nutrients on DNA methylation modifications during myogenesis remain relatively limited. During DNA methylation, methyl donors are provided by nutrients such as folate, methionine, choline, and betaine, which participate in regulating DNA methylation levels. This process also requires ATP, which is generated during nutrient metabolism and may consequently influence methylation. During demethylation, TET enzymes require the tricarboxylic acid cycle intermediate α -ketoglutarate for participation in the demethylation process. Thus, nutrients may affect DNA methylation by altering cellular metabolic pathways.

Studies have shown that protein restriction during pregnancy may affect oxidative phosphorylation in offspring muscle through DNA methylation modifications, causing muscle dysfunction [?]. Human health research indicates that short-term high-fat intake significantly increases DNMT3a and DNMT1 expression in muscle and substantially alters DNA methylation patterns, with poor plasticity of these changes [?]. This suggests that the methylation status of mature muscle cells is significantly influenced by nutritional regulation, and such effects are long-lasting. Oster et al. [?] demonstrated that supplementing methyl donors such as methionine, folate, choline, and vitamin B6 to pregnant pigs can regulate the insulin-like growth factor pathway in offspring muscle, increase offspring birth weight, and confirmed that these changes in muscle have poor plasticity to dietary stimuli and can persistently affect offspring development. As an important component of organismal development, muscle development is substantially influenced by nutrients. However, many unknowns remain regarding how nutrients affect muscle development through DNA methylation pathways. Research in this area will help deepen our understanding of nutritional effects on muscle development and enable better regulation of muscle growth.

During muscle development, DNA methylation plays an important regulatory role. Through the catalysis of methyltransferases and demethylases, appropriate

levels of DNA methylation at specific sites in cells at different myogenic stages ensure the temporal and spatial specificity of key gene expression, maintaining normal myogenesis. However, because DNA methylation levels during muscle development constitute a complex dynamic process, current research remains incomplete. Several aspects require deeper investigation, including the factors or signals that stimulate methylation and demethylation during myogenic cell differentiation, the role of non-CpG site methylation in regulating muscle development, and the mechanisms by which nutrients regulate muscle development through DNA methylation. Exploring epigenetic modifications during muscle development will help us precisely understand the regulatory mechanisms of muscle development and promote healthy animal growth and increased meat production.

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Figure Legends

Fig. 1 Interplay functions between DNA methylation and transcription-factors [16]

Fig. 2 DNA demethylation pathways [27]

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