Review Article

Gene Networks during Skeletal Myogenesis

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Mammalian skeletal muscles are derived from mesoderm segments flanking the embryonic midline. Upon receiving inductive cues from the adjacent neural tube, lateral plate mesoderm, and surface ectoderm, muscle precursors start to delaminate, migrate to their final destinations and proliferate. Muscle precursor cells become committed to the myogenic fate, become differentiated muscle cells, and fuse to form myofibers. Myofibers then fuse together to form the muscle groups. Muscle precursor cells have the ability to proliferate, and differentiate during development, while a subset remains capable of regeneration and repair of local injuries in adulthood. When the process of muscle development is perturbed such as in muscular dystrophies and injuries, ways to intervene and allow for proper muscle development or repair are the focus of regenerative medicine. Thus, understanding the developmental program of muscle at the genetic, cellular, and molecular levels has become a major focus of skeletal muscle regeneration research in the last few years.

1. Introduction

Movement and locomotion are common characteristics of all living animals. The vertebrate musculature system that includes cardiac, smooth, and skeletal muscles makes both activities possible. Skeletal muscle is the dominant type of musculature making up about 40% of the adult human body weight, and it is a key player in energy metabolism. Skeletal muscles are attached to bones in the body and their contractions are under voluntary control, unlike cardiac and smooth muscles. Skeletal muscles are composed of many bundles of long, thin, multinucleated myofibers that are made up from cells that systemically proliferate and differentiate throughout development. Adult mature muscles maintain a population of self-renewing cells, satellite cells, which are capable of regeneration and repair.

2. The Genesis of the Muscle

Skeletal muscles of the trunk derive from the paraxial somitic mesoderm, whereas skeletal muscles of the head derive mostly from the prechordal and nonsomitic paraxial head mesoderm. Somites develop in a rostral (head) to caudal (tail) order as a result of the segmentation of the presomitic (paraxial) mesoderm that lies on each side of the neural tube and notochord [1]. Somites give rise to the progenitor cells of muscle, cartilage, bone, connective tissue, and dermis, forming between embryonic day (E) E8.0 and E13.0 in mice. As individual somites mature, they receive signals from the adjacent notochord, neural tube, ectoderm, and lateral plate mesoderm (LPM) [2].

In the trunk, the medial and lateral somites give rise to the hypaxial and epaxial muscles, respectively. Hypaxial muscles develop in the context of LPM and include the ventral muscles of the body and the appendicular and abdominal body wall muscles. Epaxial muscles develop outside of the LPM and include the deep back muscles. Hypaxial muscles are derived from molecularly specified muscle progenitor cells in the ventrolateral dermomyotome. Appendicular muscle precursors delaminate from limb level somites, migrate into the LPM-derived limb mesenchyme as it is being patterned, and reaggregate in specific locations to form the muscle anlagen that presage the distinct muscles observed in adults.

The ventral-medial part of the somite, the sclerotome, receives signals from the paracrine factor sonic hedgehog (Shh), which is secreted by the notochord and the floor plate of the neural tube [3, 4]. The sclerotome breaks up into the mesenchyme, begins to express the paired box 1 (Pax1), and
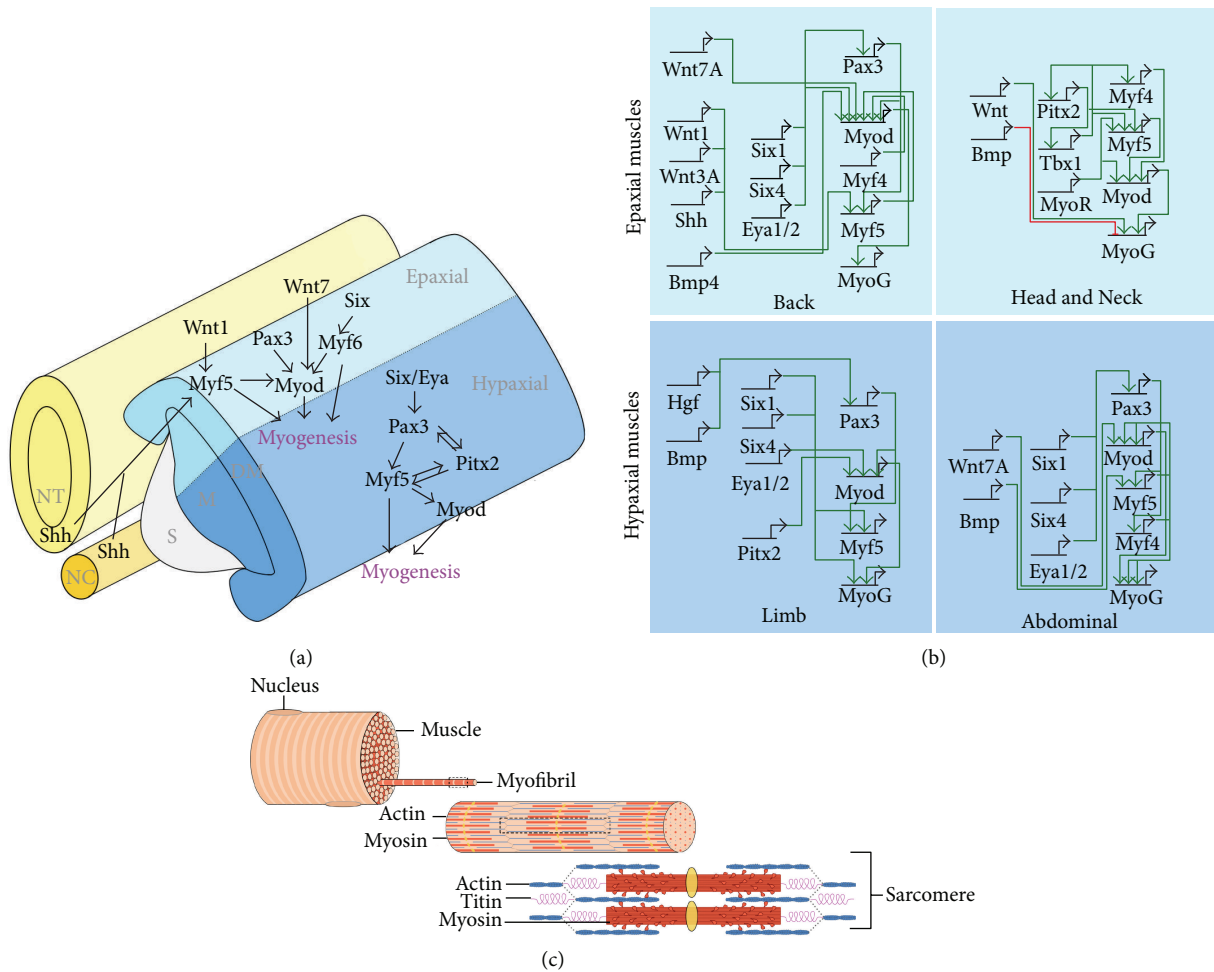
FIGURE 1: Skeletal myogenesis. (a) Induction of Myogenesis. Somites form in a rostral to caudal manner as a result of segmentation of the paraxial mesoderm. They lie on each side of the neural tube and notochord and are the source of body musculature. As somites mature, they receive signals from the adjacent neural tube (NT) and notochord (NC). These signals result in the division of the somite into the sclerotome (S), dermomyotome (DM), and myotome (M). Cells in the epaxial region will give rise to the muscles of the deep back, while cells in the hypaxial region will give rise to the muscles of the abdomen and limbs. Shh from the floor plate of the neural tube and from the notochord and Wnt1 from the dorsal neural tube activate Myf5, which in turn activates Myod. Wnt7 and Pax3 from the dorsal neural tube also activate Myod to initiate myogenesis of the epaxial somites. Activation of Pax3, by Pitx2 and Six/Eya, activates Myf5 and Myod for initiation of hypaxial myogenesis. (b) Gene Regulatory Networks. Interactive networks of SSTFs in anatomically distinct muscle groups as modeled in biotapestry [78]. Developmental networks regulating myogenesis depend on the anatomical location of the myogenic population. The MRFs are universal nodes in muscle development, but the upstream effectors are what define each population. Myogenesis of the head and neck depends on MyoR and Pitx2 and is distinct from the myogenesis of the trunk which all depends on the genes Six1/4 or Eya1/2. Within the trunk, the abdomen, limb, and back can be considered distinct regulatory regions, each with their own set of upstream regulators. Green arrows indicate a positive downstream effect, while red arrows indicate a negative downstream effect. (c) Contractile Apparatus of the Muscle. Myoblasts fuse to form a multinucleated muscle fiber, the smallest complete contractile system. Muscle fibers or muscle cells consist of many contractile unites, the myofibrils. Myofibrils consist of an ordered arrangement of long molecules, the thick (myosin) and thin (actin) myofilaments, which one called sarcomeres. Myosin is responsible for force generation. Actin is responsible for motion generation. Titin maintains the order of the striation patterning by anchoring the myosin network to the actin network.

forms the cartilage and the axial skeleton [5]. The dorsal part of the somite becomes the dermomyotome that retains its epithelial structure, gives rise to the dermis, and is the source for body musculature [6, 7]. The myotome located underneath the dermomyotome is formed by dermomyotomal delaminated cells. The epaxial myotome is specified by a combination of Wnt1 and Wnt3a from the dorsal neural tube, and Shh from the floor plate of the neural tube and notochord,

to give rise to the muscle groups of the back [2, 8, 9]. The hypaxial myotome is specified by bone morphogenetic protein 4 (BMP4) signals from the LPM and Wnt7a signals from the surface ectoderm, to give rise to the limb and ventral trunk musculature (Figure 1(a)) [10]. Limb muscles originate from progenitors that delaminate from the ventrolateral lip of the dermomyotome and migrate into the prepatterned limb [11, 12]. The most rostral somites contribute to the skull

and some musculature of the head and neck, but for the most part, craniofacial musculature is derived from the cranial mesoderm [13]. Abdominal muscle precursors proliferate without delaminating and thereby expand ventrally into the LPM-derived somatopleure. The somatopleure and splanchnopleure are derivatives of the LPM, which provide inductive cues for hypaxial muscle specification [14–17].

3. The Gene Networks of the Muscle

Sequence specific transcription factors (SSTFs) are required for molecular specification, movement, and myogenic progression during muscle development, and they represent the nodes of the gene network. Combinatorial codes of SSTFs mark specific regions of the somite and dermomyotome before the onset of myogenic progression [10]. The cell types of myoblasts are specified by a different combination of SSTFs; yet, the timing, location, and nature of this specification process are not yet known. The paired domain homeobox transcription factors Pax3 and Pax7 are expressed in somitic cells, and the Pax3$^+$Pax7$^+$ cells form the muscle progenitor pool of the limb [18, 19]. Pax3 is required for somite segmentation and formation of the dermomyotomal lips [20–22]. When Pax3 is mutated or absent, dermomyotomal cells fail to delaminate and hypaxial muscles fail to form [23]. Pax7 is required for proper fetal myogenesis and the maintenance of "juvenile" adult satellite cells, which are a self-renewing population of myogenic precursors [18]. The sine oculis-related homeobox family members Six1 and Six4 are expressed in overlapping domains in the dermomyotome, myotome, and limb buds (Figure 1(a)). Double mutant Six1/4 mice exhibit a complete lack of musculature in the limbs and abdomen, with gross deep back muscle defects [24]. Similarly, double mutant Eya1/2 mice exhibit muscleless limbs [25]. This phenotype is a result of the necessary interaction of the Six and Eya proteins for the expression of Pax3 in the dermomyotome. Six1/4 and Eya1/2 are involved in the developmental network of fast versus slow adult muscle types [26]. The four Myogenic Regulatory Factors (MRFs) are highly conserved basic helix-loop-helix transcription factors, and they include the Myogenic factor 5 (Myf5), Myogenic differentiation 1 (Myod), Myogenic factor 6 (Mrf4), and Myogenin (MyoG). They are most notably associated with myogenesis because of their ability to transform nonmuscle cell lines into a muscle cell fate, albeit being downstream of the Pax and Six genes [27].

A complex set of genetic interactions has been well documented between different MRFs and between Pax3 and MRFs in anatomically distinct muscle groups (Figure 1(b)) [28–32]. In general, the myogenic progression in limbs proceeds from Pax3 and Lbx1 expressions [33], to Myod or Myf5 expression, and then to MyoG expression. MyoG expression is required for classic muscle differentiation [34] and precedes the expression of the proteins of the contractile apparatus. Pax3 and Pax7 [21] and Myod and Myf5 [35] can contribute to different muscle lineages. These genes mark the progression of different muscle lineages that get incorporated into most, if not all, muscle anlagen, rather than different muscle lineages that each creates different anlagen. Myf5 and Myod are first expressed in the myogenic network to specify the myogenic fate during embryonic development, while MyoG is expressed later to control differentiation. The Myod/ Myf5 double knockout mice that completely lack skeletal muscle despite having Pax3 progenitors, verifying the redundancy of Myod and Myf5 and their role in myoblast specification [29]. MyoG is considered to be a differentiation factor, as KO mice have myoblasts, but these fail to differentiate and fuse [34]. When MyoG is placed in a context of commitment as driven by Myf5 in Myod/Myf5 knockout mice, muscle development was rescued but there was an overall reduction in myofiber size [36]. Most SSTFs regulate gene expression by interacting with transcriptional coregulators and remodel the chromatin structure. During myogenesis, Myod and Myf5 are cooperating with histone acetyltransferase (HAT) p300 and CBP to mediate activation of MyoG and Mrf4 [37]. Myod directly binds the HAT p300, and p300 recruits another HAT, the p300/CBP associated factor (PCAF), to form the Myod complex with two distinct HAT activities [38, 39]. Myod negatively regulates the transcription of some genes in the myoblast stage, and muscle differentiation is initiated when Myod switches from its association with repressive factors to activating factors.

Fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), BMP inhibitors, sonic hedgehog (Shh), hepatocyte growth factor (SF/HGF), and wingless-related MMTV integration site proteins (Wnts) have all been implicated in balancing differentiation and proliferation in limb muscle precursors. A general consensus suggests that BMPs, FGFs, Shh, and SF/HGF inhibit differentiation and promote proliferation [11, 40]. The same set of inductive molecules has also been invoked in patterning the limb bud itself. The precise spatial distributions of all the specific members of these inducer families together produce a set of molecularly defined networks into which limb muscle precursors migrate and grow into muscles. Cells of the muscle lineage appear to retain a common ability to balance growth and differentiation within the individual molecular networks. The complex set of positive and negative cues from both the growing muscle anlagen itself and its local environment influence this balance.

4. The Growth of the Muscle

All muscle precursor cells undergo specification to become myoblasts that will differentiate into myocytes, and later will fuse to become myofibers and myotubes. In mice, there are four stages of skeletal myogenesis, embryonic (E10.5–E12.5), fetal (E14.5-P0), postnatal (P)/neonatal (P0–P21), and adult (>P21) [41]. Fetal myoblasts have arrived in the premuscle clusters by E12.5. However, the secondary myofibers that derive from them only become anatomically detectable after a two-day delay, at E14.5. Neuromuscular contacts develop on primary embryonic myotubes during this delay. Secondary myofibers then gradually expand over the site of the initial neuromuscular interaction to form syncytia of up to 50 nuclei. These nuclei are placed peripherally around the centrally developing molecular contractile apparatus. Each myofiber expresses myofilaments (actin, myosin) and assembles them into myofibrils along its longitudinal core (Figure 1(c)).

It uses other specialized cytoskeletal components to bundle myofibrils and create an interwoven sarcoplasmic reticulum that can uniformly deposit Ca^{2+} ions onto the myofibrils once a neural stimulus arrives. Myofibers are bundled and surrounded by a perimysium and these bundles are further bundled and surrounded by the epimysium. During this process, collaterals sprout from the primary innervating axons and establish the motor units by making neuromuscular junctions on affiliated secondary myofibers. In addition, spindle cells must be incorporated into the muscle to provide somatosensory feedback and the muscle must be integrated with the tendon on either end. In response to physiological stimuli, the muscle alters its myofiber composition. Skeletal muscles are characterized by the tandem arrangements of individual sarcomeric contractile units (Figure 1(c)). The sarcomere is a highly ordered cytoskeletal structure composed of skeletal muscle myosin, actin, and a set of regulatory proteins, which include the troponins and tropomyosin. Contraction of the sarcomere occurs when the thick filament myosins bind the actin thin filaments and hydrolyze ATP to generate a stroke. This activity is regulated by the calcium-dependent troponin-tropomyosin complex. Skeletal muscles consist of heterologous populations of slow and fast myofibers. Slow type I myofibers express slow isoforms of myosin-heavy chain 7 (Myh7) and exhibit oxidative metabolism. Fast type Ia, IIx/d, and IIb myofibers express fast myosin-heavy chain isoforms Myh2, Myh1, and Myg4, respectively, and exhibit glycolytic metabolism. Muscle activities are determined by genetic and epigenetic mechanisms that control their development and postnatal remodeling.

Abdominal muscles begin to form at E8.5 when a thin sheet of LPM-derived cells cover the abdomen. LPM cells express the paired-like homeodomain transcription factor 2 (Pitx2) [42–46]. By E9.5, the Pitx2$^+$ ventrally located somatopleure cells will give rise to the abdominal wall musculature [46]. Shortly after, at E10, the Pax3$^+$ cells of the ventrolateral lip of the interlimb somites begin to proliferate and migrate toward the embryo's midline and start to express Pitx2 [47]. The Pax3$^+$Pitx2$^+$ cells begin to express MRFs and expand ventrally around the abdomen. Pitx2 represses T-box genes by recruiting corepressors and HDACs and activates Hox genes during abdominal wall development [48, 49]. Pitx2 enables the growth factor dependent control of myoblast proliferation by altering the chromatin state of these genes from a repressed to an activatable state [50]. Pitx2 mutant mice develop limb muscles but fail to form higher order muscle assembly [51].

Limb muscles derive from a similar population of migratory Pax3$^+$ cells in the limb level somites that do not express MRFs until they reach the limb bud. They delaminate from the ventrolateral lip of the dermomyotome upon activation of the met proto-oncogene (c-Met) and the associated ligand hepatocyte growth factor (HGF/SF). Without either of these signaling factors, cells are unable to delaminate [14, 52]. These migratory cells require the proper expression of Lbx1 to properly locate and move into the limb buds [33] and start to express Pitx2 [46]. The delamination process begins at E9.5 in the forelimb where the Pax3$^+$Lbx1$^+$ migratory muscle precursors reach the limb buds, begin to express Myod and Myf5,

proliferate, and position themselves along the developing bone anlagen. Soon thereafter, a subpopulation of muscle precursors begin to express Mrf4 and MyoG, differentiate, and fuse into myofibers that form premuscle clusters in the shape and position of adult musculature. Pitx2 assists the expression of Pax7 in a subset of the postmigratory Pax3$^+$ cell population [53].

At E14.5, all premuscle clusters have formed from embryonic myoblasts. The differentiation and fusion of fetal myoblasts with each other or with the primary fibers contribute to the growth and maturation of skeletal muscles. Instead of synchronously withdrawing from the cell cycle, fetal myoblasts proliferate as they deposit postmitotic cells onto secondary myofibers that form on the surface of primary myotubes from E14.5 to E17.5 [54]. Secondary myofibers are composed of 10–50 nuclei and expand around the innervation zones. Their nuclei are located at the cellular periphery, around a central core of myofibrils. In this process, Pax3 expression declines while Pax7 takes over as an important node in this stage of development. Genetic ablation of Pax7 expressing cells does not affect the formation of primary myotubes, but it does lead to a nearly complete loss of secondary myofibers, showing that Pax7$^+$ cells are absolutely required for fetal myogenesis [55]. Pax7 activates the transcription factor nuclear factor 1/X (Nfix) that in turn, activates fetal specific genes, including muscle creatine kinase (MCK) and β-enolase, while repressing embryonic genes, such as slow myosin [56]. In Pax7 null mice, satellite cells progressively die and Pax3 is not able to compensate for the loss. However, despite Pax3 not being absolutely required after establishment and execution of the embryonic myogenic network, both Pax3$^+$ and Pax7$^+$ cells contribute to fetal myofibers.

5. The Repair of the Muscle

After birth, muscles follow the body's growth and become the adult muscles. The repair of an adult muscle can occur by muscular hypertrophy or through de novo fiber formation and regeneration, with the latter dependent on satellite cells [57]. Satellite cells reside around the fibers in a quiescent state, and stay poised for muscle homeostasis and regeneration. The fetal Pax3$^+$Pax7$^+$ myoblasts can further give rise to Myf5$^+$Myod$^+$ cells or to Pax3$^+$Pax7$^+$MRF$^-$ cells. The Pax3$^+$Pax7$^+$MRF$^-$ cells align with the nascent myotubes and these satellite cells develop in the sublaminar position [18] and become Pax3$^-$Pax7$^+$Myf5$^+$ [19]. Pax7 recruits the histone methyltransferase complex to promoter elements of myogenic genes for their activation [58]. Similar to embryonic myoblasts, these adult satellite cells also express c-Met and associated ligand HGF/SF, of which HGF can induce the exit from quiescence in vitro [59]. Their expression in adult satellite cells likely allows for the delamination and migration of activated cells from the basal lamina of adult muscle fibers, to the site of injury. When activated, these satellite cells become Pax7$^-$, proliferate, relocate, begin to express Myod, and proceed down the path of terminal differentiation and fusion with existing muscle. As a self-renewing population, a subset of these activated cells returns back to an undifferentiated Pax7$^+$Myod$^-$ state [19]. When Pax7 is ablated

in juvenile mice, Pax3 does not compensate for Pax7 in maintaining a satellite cell state, as seen during fetal and early postnatal myogenesis [60]. Dual inactivation of Pax3 and Pax7 in adult mice did not affect their muscle regenerative capacity, suggesting that the network regulating satellite cell state changes depending on the level of maturation [60]. Later findings identified that after ablation of satellite cells in adult muscles, muscle fibers were able to compensate for damage through hypertrophy [57].

Mobility disabilities can be caused by genetic (dystrophies) and environmental insults (injuries). The repair mechanism in adults which is based on fiber regeneration uses many of the same nodes involved during embryonic and fetal myogenesis. In response to injury, skeletal muscle undergoes an orderly process of regeneration that results in the reformation of an orderly and functional contractile tissue. The trigger for regeneration is inflammation due to death of existing muscle cells that compromise the muscle fiber integrity and release muscle specific proteins into the extracellular space [61]. As a result, satellite cells are activated, proliferate, differentiate, and fuse with existing muscle. De novo regeneration and repair of adult muscles rely on the activation of satellite cell precursors for integration into existing muscle structure, which is similar to the transition between embryonic and fetal myogeneses. Thus, clarifying the molecular and cellular mechanisms that govern skeletal muscle development, with an emphasis on this transition stage, can be very useful in developing feasible ways to repair debilitated muscles of patients with myopathies or injuries for which one's own system cannot compensate.

6. MicroRNA and Muscle

Several miRNAs have been shown to be involved in myogenesis and are often located in close proximity of their targets; on noncoding regions within or around muscle-specific genes [62]. When transcribed from DNA, premiRNAs are processed with the Drosha/Dgcr8 complex, exported out of the nucleus, processed by Dicer, and then associated with the RNA-induced silencing complex (RISC) to bind the $3'$UTR of their targets. Primarily, miRNAs act as general negative regulators of gene function though mRNA destabilization and the degradation of its targets [63]. When this pathway is disrupted in Dicer knockout mice, resulting in no functional miRNA, embryos had early mortality and no evidence of the development of a primitive streak [64]. When Dicer is conditionally knocked out under control of Myod[Cre], the mice die at birth due to major muscular defects [65]. The mutant limbs are smaller with a significant muscular hypoplasia and an increased muscle cell apoptosis.

There are several miRNAs enriched in skeletal muscles and are linked to the network nodes of myogenesis [62, 66]. Several miRNAs (miR-206, miR-486, and miR-378) contain both of the Myod binding sites and can induce myoblast differentiation [67, 68], down regulate Pax7 [69] with miR-206, as a skeletal muscle specific target of Myod [70]. miR-486, which is enriched in skeletal muscles, was also shown to target Pax7 in a similar developmental context, exemplifying overlapping roles in regulatory nodes during complex

developmental processes to ensure proper development [67]. Other targets of miR-1/miR-206 are involved in cellular fusion that allows myocytes to form myofibers and myotubes including Cx43 which must be repressed prior to cell fusion, and HDAC4 as part of the mTor-MyoD-mir1-HDAC4-follistatin pathway for myocyte fusion [71, 72]. Other miRNAs involved in myogenesis are not necessarily muscle specific, but are still involved in the myogenic network. miR-26a and miR-214 are ubiquitously expressed and target Ezh2, a known myogenic inhibitor that works in association with the transcription factor YY1 to silence muscle gene expression, thus promoting differentiation of myoblasts [73, 74]. YY1 itself is also bound by miR-29, and it is suspected to have a role in cardiac fibrosis [75, 76]. miR-27b is ubiquitously expressed and targets Pax3, which needs to be repressed to allow the onset of fetal myoblasts [77]. One of the overlying themes in miRNA regulation is that they often have many targets and act cooperatively with a high level of redundancy.

7. Reconstruction of the Muscle

Diseases such as muscular dystrophy or traumatic injuries of skeletal muscles are often a debilitating problem for affected individuals as large portions of one or more anatomical muscles are lost or nonfunctional. Designing treatments to help rebuild muscles to replace damaged or diseased tissue would improve patient mobility and increase the well-being of the afflicted individuals. However, before this can be feasible, the molecules and their interactions in time and space during myogenesis must be identified. The goal of regenerative medicine which is to "build" the defective body parts of the patient is getting closer.

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