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Muscle cell communication in development and repair

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Abstract

Under basal conditions, postnatal skeletal muscle displays little cell turnover. With injury, muscle initiates a rapid repair response to reseal damaged membrane, reactivating many developmental pathways to facilitate muscle regeneration and prevent tissue loss. Muscle precursor cells become activated accompanied by differentiation and fusion during both muscle growth and regeneration; inter-cellular communication is required for successful completion of these processes. Cellular communication is mediated by lipids, fusogenic membrane proteins, and exosomes. Muscle-derived exosomes carry proteins and micro RNAs as cargo. Secreted factors such as IGF-1, TGF β , and myostatin are also released by muscle cells providing local signaling cues to modulate muscle fusion and regeneration. Proteins that regulate myoblast fusion also participate in membrane repair and regeneration. Here we will review methods of muscle cell communication focusing on proteins that mediate membrane fusion, exosomes, and autocrine factors.

Keywords

fusion; repair; membrane; myoblast; muscle; development; cell communication; exosome

Introduction

Skeletal muscle is the largest tissue in the human body comprising approximately 30-40% of total body weight [1]. Due to the elongated nature of individual myofibers and their role in muscle contraction, skeletal muscle is prone to injury. Muscle continuously adapts to environmental and physical challenges through regeneration and membrane repair to ensure tissue survival [2,3]. Muscle growth and regeneration are multi-step processes requiring cellular activation and cell-cell fusion. Myoblast and myofiber differentiation occur both before and after cellular fusion (Figure 1), including the expression of cell surface markers and secreted factors that coordinate these activities. Membrane lipids, exosome-borne proteins/microRNAs (miRNAs), and autocrine cytokines help orchestrate differentiation and

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fusion. This review will evaluate the current knowledge of muscle cell communication during growth, repair and regeneration.

Membrane Phospholipids

The muscle plasma membrane, known as the sarcolemma, is a lipid bilayer containing phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidic acid (PA). The composition of lipids varies along the sarcolemma, clustering in lipid rafts and forming sites with a specific enrichment of PS and a reduction in PC at the specialized membrane of transverse tubules (T-tubules) [4,5]. As in other cells, phospholipid clustering is temporally dynamic and depends on the cell state. PS is normally found within the inner lipid bilayer and flips to the outer bilayer with plasma membrane disruption during injury, and this configuration serves as a signal to invading macrophages to remove the damaged myofiber [6]. However, external PS need not always signaling injury, as PS also localizes to the site of cell-cell contact during myoblast fusion and at the site of membrane repair [7-11]. Blocking PS exposure with an anti-PS antibody inhibits muscle cell fusion [9,12] indicating the necessity of external PS in cell-cell communication during membrane fusion.

Phosphoinositides (PI), specifically phosphatidylinositol (4,5)-bisphosphate (PIP₂) and phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) comprise approximately 1% of the lipid content within the membrane, yet are critical components that direct membrane and cytoskeleton reorganization [13]. PIP₂ is enriched at the site of muscle cell contact providing a local cue for fusogenic proteins and actin regulators [14]. Disruption of PIP₂ expression in C2C12 myoblasts, a myogenic cell line, with calcimycin, LiCl, neomycin, or genetic reduction of PIP5K1 γ , reduced myoblast fusion suggesting PIP₂ contributes to the fusogenic signaling pathway [14]. Additionally, manipulation of PIP₃ levels improved the severe muscular dystrophy phenotype in myotubularin (*Mtm1*) deficient mice and zebrafish [15]. Therefore, expression and localization of phosphoinositides serve as a lipid code to direct cellular activity, and a unique balance of lipids is required by muscle for proper function.

Regulators of membrane fusion in development and repair

Ferlins—The Ferlin family of proteins is a family of six related proteins that regulate membrane fusion, vesicle trafficking and membrane repair [16-21]. Dysferlin (also known as Fer1L1) was the first mammalian ferlin family member identified and is broadly expressed, including in mature skeletal muscle where it localizes to the plasma membrane and T-tubules [22-24]. Loss-of-function mutations in the dysferlin gene result in muscle disease, specifically muscular dystrophy [25,26]. Dysferlin contains seven C2 domains and a carboxyl-terminal transmembrane domain [27]. These C2 domains are highly related to the C2 domains found in synaptotagmin, a Ca²⁺-sensing regulator of membrane fusion. C2A, the most amino-terminal C2 domain, binds phospholipids in the presence of Ca²⁺ likely contributing to the localization and fusogenic potential of dysferlin [16,28,29].

After membrane damage, it is thought that membrane patches, derived from intracellular vesicles, form to reseal the disrupted membrane [30]. This model does not preclude that

resealing also uses additional sources of membrane-bilayer. For example, labeling studies have suggested that plasma membrane components adjacent to the disruption contribute to resealing in what has been referred as “lateral contribution” of membranes to repair [10,23]. Dysferlin's role in muscle membrane repair was first observed utilizing laser-ablation to induce membrane injury [16]. Dysferlin-null myofibers reseal membrane disruptions much more slowly than normal myofibers, illustrated by an increase in fluorescent FM 1-43 dye uptake after sarcolemmal disruption [16,31]. Immunofluorescence microscopy was used to demonstrate dysferlin's recruitment to the site of membrane insult [16]. With high-resolution live-cell imaging of multiple components mediating the repair process, dysferlin was observed to localize in the membrane immediately adjacent to sites of disruption [10]. This position may indicate that dysferlin regulates membrane incorporation at the site of damage through its interaction with phospholipids and other repair proteins [11].

In addition to a role in mediating plasma membrane resealing, dysferlin regulates vesicle trafficking. Dysferlin strongly localizes to discrete cytoplasmic puncta ranging from 200nm to >1 μ m in size. Dysferlin specifically colocalizes with the early endosomal marker Rab5 and the late endosomal marker Rab7. Furthermore, dysferlin colocalizes with endocytosed transferrin [32,33]. Pulse chase studies of Alexa-488 labeled transferrin in dysferlin-null myoblasts demonstrate a delay in recycling [17], consistent with a broader membrane and vesicle trafficking role within cells.

Myoferlin is highly homologous to dysferlin, but myoferlin is most highly expressed during early muscle development and expression decreases as muscle matures [19,34]. Myoferlin is upregulated after muscle injury, and myoferlin is enriched at the site of cell-cell contact during myoblast fusion [19]. Similar to dysferlin, myoferlin regulates endocytic recycling of multiple receptors including transferrin and the insulin like growth factor receptor (IGF1R) [18]. Indicative of decreased IGF-1 signaling and ineffective myoblast fusion, myoferlin-null mice exhibit reduced myofiber size and delayed muscle regeneration [18,19]. Myoferlin-null myofibers reseal more slowly than normal fibers after laser-induced membrane injury [10], consistent with a role in mature myofibers. Myofibers lacking both myoferlin and dysferlin have enhanced repair defects compared to either single mutation. Curiously, transgenic overexpression of myoferlin in dysferlin-null mice was sufficient to improve resealing, however, dystrophic pathology remained [35].

Myomaker—Myomaker (also known as TMEM8C) is enriched in developing muscle; the myomaker protein contains seven transmembrane domains and is localized to the plasma membrane [36]. Consistent with the reactivation of developmental gene programs in response to muscle injury, myomaker is re-expressed after muscle insult while it is minimally expressed in mature uninjured muscle. Similar to myoferlin, myomaker localizes to the site of cell-cell contact during myoblast fusion. Through co-culture experiments, it was found that myomaker-null myoblasts fuse more efficiently with wildtype myoblasts than myomaker-null myoblasts, consistent with a model where at least one cellular partner must expose myomaker for membrane coalescence [36,37]. Correspondingly, myomaker-null mice die during gestation with a severe defect in skeletal muscle formation [37]. Overexpression of myomaker in the C2C12 myoblast cell line or even in fibroblasts results in enhanced fusion potential. Deletion of myomaker's carboxy-terminal 8 amino acids

(aa214-221) interferes with its fusogenic potential. Expression of the related proteins TMEM8A and TMEM8B had no effect on fusion, suggesting distinct cellular roles compared to TMEM8C [36].

Exosomal proteins and miRNAs in muscle communication

Exosomes are small membranous vesicles, 50–150-nm in diameter, derived from the late endosomal system, specifically the multivesicular bodies (MVBs). Exosomes are released from cells through exocytic fusion with the plasma membrane. Multiple cell types, including skeletal muscle, shed exosomes into the extracellular space or circulation under both normal and pathological conditions [38,39]. Exosomes, as high as $\sim 10^{10}$ /ml, can be found in serum of healthy subjects suggesting a biological function beyond disease [40]. Released exosomes contain cargo proteins, miRNAs and lipids that are thought to be involved in trans-cellular and probably even trans-tissue communication (Figure 2). Efforts have been directed at characterizing the cargo contained within exosomes to better understand their biological function.

Proteomic analysis of exosome-like vesicles released from C2C12 myoblasts and myotubes revealed distinct specific subsets of proteins released throughout muscle differentiation [41]. Vesicles from both cultures showed enrichment in annexins, EHDs, LAMPs, Rabs, VAMPs, and VPSs (ESCRT complex members) consistent with the proposed multi-vesicular body / endosomal origin. Exosome-like vesicles derived from myotube cultures were also enriched in proteins involved in muscle contraction including DAG1, FLNC, TLN1, TTN, VCL, and VIM. Incubation of myoblasts with exosomes from myotubes inhibited myoblast proliferation and promoted differentiation. To determine if this increase in fusion was due to uptake of exosomal contents by myoblasts, GFP-positive myotube exosomes were incubated with non-fluorescent myoblasts [41]. After 24hrs, GFP fluorescence was detected within the myoblasts confirming myotube-to-myoblast transfer of exosomal cargo. These data support a role for exosomes actively participating in muscle cell communication through dispersion of released contents. In addition, differentiation in the presence of exosome-depleted serum, reduces the efficiency of myoblast differentiation implicating exosomes in cross-talk between mature muscle and myoblasts [42].

The mechanism by which exosomes interact with and are taken up by target cells is not well understood. This is further complicated by the idea that tissue type specificity may exist between the target cell and released exosome. Multiple mechanisms between exosomes and recipient cells have been documented including interaction with cell surface receptors, plasma membrane fusion, and phagocytosis [43-45]. Both myoferlin and dysferlin interact with Eps15 homology domain containing proteins, EHD1 and EHD2, in muscle and loss of EHD1 results in deficient myoblast fusion [20,46,47]. Proteins identified in exosomes derived from myoblasts and myotubes include myoferlin, annexins and the EHD proteins [41,48]. EHDs bind phospholipids and have been implicated in endocytic recycling in multiple cell types, acting as membrane “pinchases” [49,50]. Annexins have also been implicated in muscle cell fusion, membrane repair, and ferlin binding [10,51-53]. The annexin family includes twelve different members, and the annexins are implicated in Ca^{2+} -dependent phospholipid binding with the actin cytoskeleton [54]. Annexin expression

increases during muscle differentiation and inhibition of annexin A1 or A5 decreases myoblast fusion [12,55]. Additionally, annexins participate in membrane repair and can be observed to aggregate at the membrane lesion [10,53]. Enrichment of EHD proteins and annexins within exosomes may reflect a role for these proteins in exosome release or docking on target membranes. Alternatively, these proteins may simply passively enrich in the exosomal cargo due to their natural abundance on the sarcolemma. Koumangoye et. al. found that shRNA-mediated reduction of annexin A2 and A6 in a breast cancer cell line was sufficient to reduce the internalization of exosomes, suggesting a functional role for the annexin proteins in exosomal uptake [56].

microRNAs (miRNAs) are small non-coding RNAs approximately 19-24 nucleotides in length that function as negative regulators of gene expression through binding mRNA [57-59]. miRNAs are evolutionarily conserved and global loss of miRNA production is inconsistent with life [60]. miRNAs are temporally regulated with unique tissue and/or disease signatures facilitating their role as biomarkers. Dysregulation of miRNA expression has been noted in many disease states including muscular dystrophy [61]. Forterre et al. tested whether miRNA incorporation into exosomes could act as a viable means of cellular communication regulating gene transcription, as miRNAs modulate muscle development [62,63]. Using C2C12 myoblasts and myotubes, miRNAs contained within exosomes were analyzed [63]. Over 170 miRNAs were found within muscle exosomes including miR-1, miR-133a, miR-133b miR-206, which regulate muscle differentiation [64-66]. To demonstrate physical transfer of miRNAs as means of muscle cell communication, the *C.elegans* miRNA, cel-miRNA-238, which is not normally expressed in C2C12 myoblasts, was expressed in myotubes. After incubation with exosomes from transfected myotubes, cel-miRNA-238 miRNA was detected within myoblasts, illustrating miRNA transfer by muscle cells [63]. Additionally, Fry et. al. demonstrated that co-culture of exosomes isolated from muscle precursor cells (MPCs) with fibrogenic muscle cells was sufficient to downregulate *Col1a2*, *Col3a1*, *Col6a2* and *Fibronectin* [67]. MPC depletion of the myo-miR, miR-206, resulted in an increase in *Rrbp1*, a master regulator of collagen synthesis, and increased fibrogenic collagen expression. *In vivo*, miR-206 is highly expressed in activated satellite cells. Using the Pax7/DTA mouse model, satellite cells were depleted and upregulation of miR-206 after mechanical overload was lost. This correlated with an increase in *Rrbp1* and upregulation of *Col1a2*, *Col3a1*, and *Col12a1*. These findings are consistent with the idea that exosomes release miRNAs which regulate gene expression in target cells. Engineered exosomal miRNAs are now being considered as a circulating tool to promote myogenesis and regeneration.

Autocrine signaling during skeletal muscle development and regeneration

Skeletal muscle secretes cytokines to regulate its own growth and regeneration. These factors are referred to as myokines and include IGF-1 and transforming growth factor beta (TGF β) superfamily members TGF β and myostatin (also known as GDF8) (Figure 3). IGF-1 modulates muscle growth promoting muscle cell activation, differentiation and hypertrophy [68-70]. IGF-1 binds the IGF1 receptor (IGF1-R) initiating AKT activation promoting protein synthesis and inhibiting protein degradation [71]. In mature muscle, TGF β negatively regulates muscle differentiation inhibiting fusion through binding its receptor,

TGF β R, and activating SMAD signaling, which inhibits AKT-induced muscle growth. TGF β inhibition through neutralizing antibodies or nonspecific drugs like losartan improve muscle performance and disease pathology in mouse models of muscular dystrophy [72,73]. Myostatin is a negative regulator of muscle growth suppressing muscle maturation. Overexpression of myostatin, in mice, results in decreased myofiber area and muscle mass [74,75]. Inhibition of myostatin or genetic loss of myostatin results in muscle hyperplasia and hypertrophy, with a profound increase in muscle mass [76,77]. Myostatin binds the activin receptor II β (ActRIIB) inhibiting myogenesis through SMAD activation and inhibition of AKT induced hypertrophy [78]. miRNAs have been identified that modulate these signaling pathways or are modulated by these autocrine factors providing an additional layer of regulation [79-81]. Manipulating the IGF-1, TGF β , and myostatin signaling pathways to promote muscle growth and improve regenerative capacity are currently being investigated for treating muscle disease.

Conclusion

Modulation of membrane lipids, localization and expression of fusogenic proteins, and secretion of exosomal proteins / miRNAs are key modes of communication utilized by muscle cells to regulate skeletal muscle growth, regeneration, and repair. Understanding how muscle cells communicate will allow the development of better therapeutics to promote muscle growth and regeneration.

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Abbreviations

EHD	Eps15 Homology Domain
PIP2	Phosphatidylinositol 4,5-bisphosphate
PS	phosphatidylserine
T-tubule	transverse tubule
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PA	phosphatidic acid
IGF1	insulin like growth factor 1

Highlights

- Cell-cell communication facilitates fusion and regulates muscle growth, repair, and regeneration
- Exosomes contain protein and micro RNAs that facilitate communication among muscle cells
- Autocrine factors including IGF-1, TGF β , and myostatin modulate muscle growth and regeneration
- Phospholipid composition within the membrane influences cell fusion
- Myoferlin and myomaker localize to the site of muscle cell contact promoting fusion

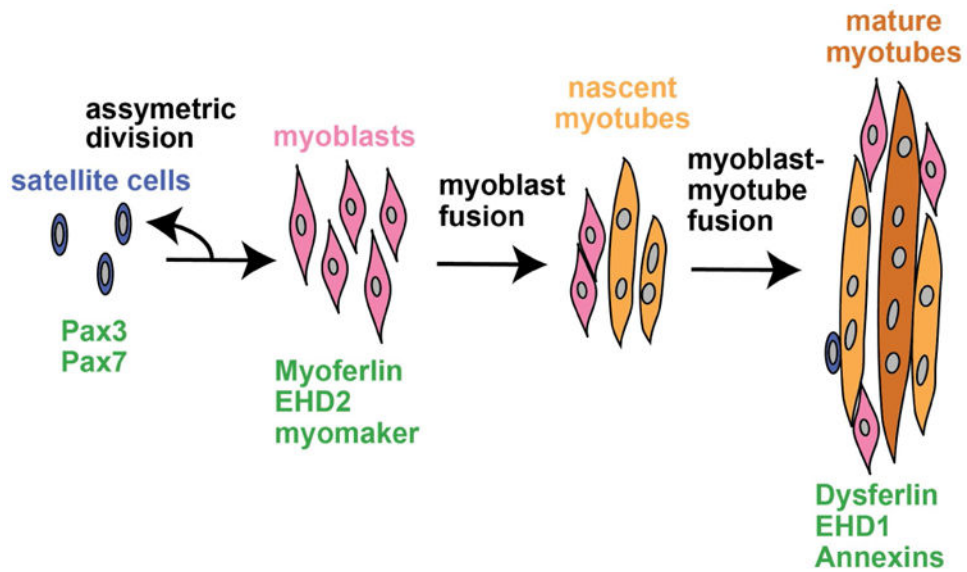


Figure 1. Muscle fusion

During muscle growth and regeneration satellite cells (blue) asymmetrically divide giving rise to mono-nucleated muscle precursor cells, myoblasts (pink). Myoblasts are activated and fuse with one-another to generate nascent myotubes (orange). Myoblasts fuse with existing myotubes to promote muscle growth and regeneration (dark orange). Pax3 and Pax7 are highly expressed in satellite cells. Fusogenic proteins myoferlin, EHD2, and myomaker are highly expressed in myoblasts with expression decreasing as differentiation proceeds. Dysferlin, EHD1, and annexins have low-level expression in myoblasts with increased expression in mature muscle.

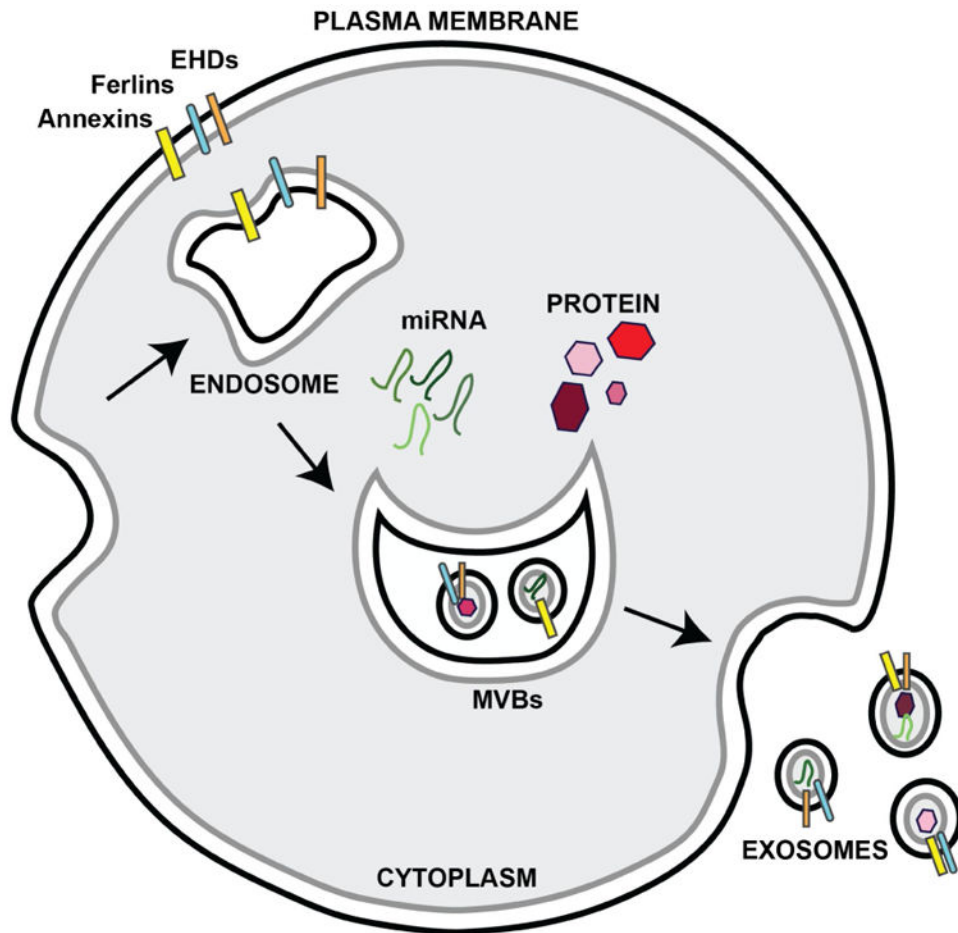


Figure 2. Exosome biogenesis and release

Exosomes are formed from multi-vesicular bodies (MVBs), which are formed from endosomes. Exosomes are released as MVBs fuse with the plasma membrane. Exosome membranes are enriched with fusogenic proteins annexins (yellow), ferlins (teal), and EHDs (orange). Exosomes contain both miRNA (green) and cytosolic proteins (red) thought to participate in cell-cell communication.

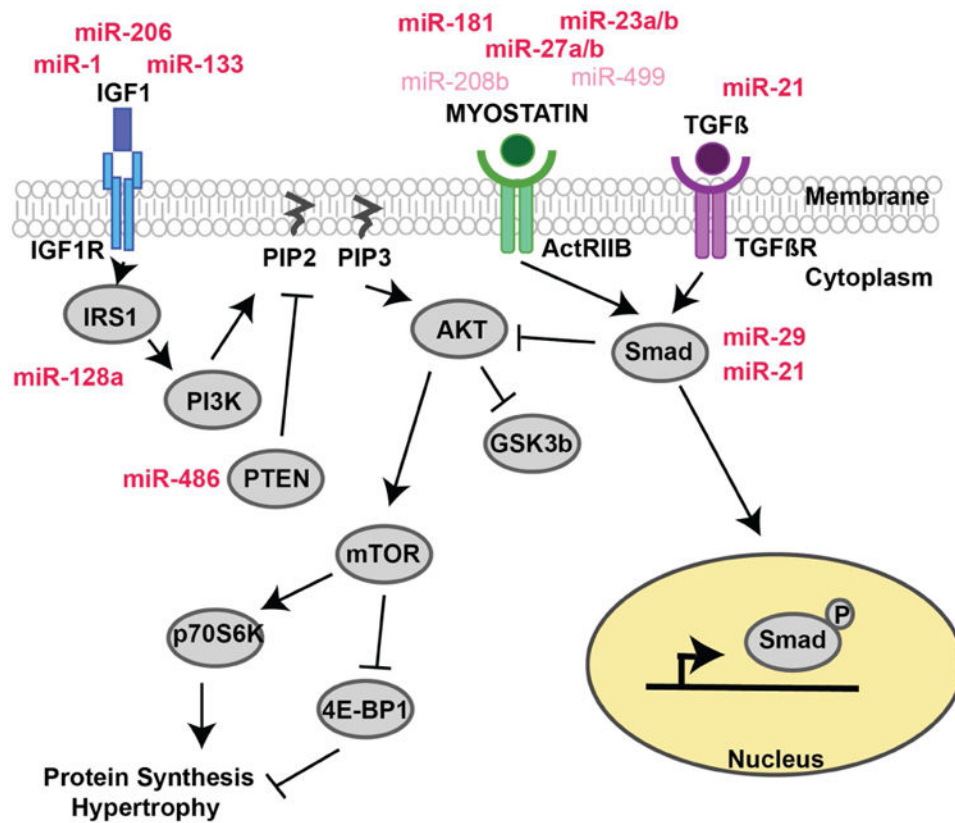


Figure 3. Autocrine factors regulating muscle growth and regeneration
 IGF1 is a potent muscle growth factor that binds IGF1-R activating PI3K/AKT signaling to promote protein synthesis and muscle hypertrophy. Myostatin and TGFβ are negative regulators of muscle growth inhibiting AKT induced hypertrophy through SMAD activation. miRNAs (mi-R) both regulate and are regulated by these signaling pathways adding additional layers of control to the system. mi-Rs (dark red) have been isolated from skeletal muscle derived exosomes, suggesting a mechanism by which cells can communicate and control muscle growth in a synchronous fashion. mi-Rs (pink) have been implicated in autocrine regulation but have yet to be isolated from skeletal muscle derived exosomes.