Spatial and functional restriction of regulatory molecules during mammalian myoblast fusion

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Abstract

Myoblast fusion is a highly regulated process that is key for forming skeletal muscle during development and regeneration in mammals. Much remains to be understood about the molecular regulation of myoblast fusion. Some molecules that influence mammalian muscle fusion display specific cellular localization during myogenesis. Such molecules can be localized to the contact region between two fusing cells either in both cells or only in one of the cells. How distinct localization of molecules contributes to fusion is not clear. Further complexity exists as other molecules are functionally restricted to myoblasts at later stages of myogenesis to regulate their fusion with multinucleated myotubes. This review examines these three categories of molecules and discusses how spatial and functional restriction may contribute to the formation of a multinucleated cell. Understanding how and why molecules become restricted in location or function is likely to provide further insights into the mechanisms regulating mammalian muscle fusion.

Keywords

myoblast fusion; myogenesis; myotube; muscle regeneration; satellite cells; vertebrate

Myoblast fusion is important not only for skeletal muscle formation during development, but also for post-natal muscle growth and regeneration of skeletal muscle. Satellite cells, the myogenic stem cells responsible for post-natal muscle growth and repair, proliferate in the presence of growth factors and give rise to myoblasts. Myoblast fusion follows an ordered set of cellular events, including elongation, cell migration, recognition/adhesion, and membrane fusion [1]. Upon mitogen withdrawal, myoblasts in culture differentiate and they become elongated, spindle shaped cells that migrate towards other differentiated myoblasts to form groups of aligned cells (Figure 1A). Differentiated myoblasts fuse to form nascent myotubes and later, further fusion of differentiated myoblasts with nascent myotubes gives rise to mature myotubes with many nuclei. A variety of extracellular, cell surface and intracellular molecules act to finely coordinate the cellular and molecular events that influence the ability of mammalian myoblasts to fuse [2].

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Spatial or functional restriction of regulatory molecules during myoblast fusion

During *Drosophila* developmental myogenesis, small binucleate myotubes form by the fusion of two distinct types of myoblasts, founder myoblasts and fusion-competent myoblasts. In some cases, molecules critical for cell fusion are asymmetrically expressed in founder and fusion-competent myoblasts and are likely to subserve specific roles in each cell type for eventual membrane fusion [3]. No evidence exists for these specialized myoblast types in mammalian myofiber formation and regeneration. However, a number of molecules that influence mammalian myoblast fusion are localized to presumptive sites of fusion in both cells of a pair of fusing cells, whereas other such localized molecules are present only in one of the cells of the pair (Table 1). Furthermore, at later stages of myogenesis other molecules are only functionally required for fusion in differentiated myoblasts not in nascent myotubes (Table 2). In this review, these three categories of molecules are discussed in further detail.

Molecules concentrated at the border of contacting myogenic cells: symmetrical localization

Immunofluorescence assays of cultured myoblasts reveal that several types of molecules are localized to the contact region of closely apposed myogenic cells in *both* cells (Figure 1B, left; Table 1). Cell adhesion molecules as M-cadherin, integrins and *a d*isintegrin *a*nd *m*etalloprotease 12 (ADAM12) are commonly found localized in this manner [4–7]. M-cadherin function is required for myotube formation *in vitro* [8, 9] but not *in vivo* [10] suggesting other cadherins or cell adhesion molecules may functionally compensate for the lack of M-cadherin. Eliminating the function of β1 integrin, α3 integrin and α9 integrin through genetic ablation or function blocking antibodies decreases myoblast fusion *in vitro* [4–6] and *in vivo* [5]. ADAM12 is a transmembrane protein that includes an integrin-binding site and can bind α9β1 integrin in muscle cells [6]. Antisense oligonucleotides to ADAM12 preferentially inhibit myoblast fusion with myotubes [6]. Molecules that associate with the intracellular domains of specific cell adhesion molecules such as β-catenin [11] and kindlin-2 [12] are also localized at cell contact sites. Given the importance of integrin signaling for myogenesis [13], the necessity of kindlin-2, an integrin-associated cytoplasmic adaptor, for myoblast fusion is not unexpected [12]. Kindlin-2 is highly enriched along the plasma membrane between contacting myogenic cells and in the absence of kindlin-2, myoblasts fail to elongate and form myotubes.

Localized transmembrane molecules and their intracellular adaptors most likely activate signal transduction pathways in aligned myogenic cells that ultimately lead to membrane fusion [11,14]. Extensive cytoskeletal reorganization occurs before and after fusion [15]. A number of studies indicate remodeling of both the actin [16] and microtubule [17] cytoskeletal network are critical for myoblast fusion. The structure of the actin cytoskeleton at the contact site of fusing myoblasts is highly regulated by a complex signal cascade initiated by cell adhesion. The actions of these signaling molecules and actin regulators lead to the recruitment of vinculin and cytoskeletal proteins to contact sites of fusing myoblasts [11], which is hypothesized to be essential to localize Golgi-derived prefusion vesicles [18] and/or fusion pore formation/ expansion in *Drosphila* [19]. Both actin and EB3, a microtubule regulatory protein, are localized near the plasma membrane of both aligned myoblasts [20,21]; in some studies, actin is localized asymmetrically in pairs of myogenic cells [22,23] as discussed below although the reasons for this discrepancy are unknown.

Myoferlin, a member of the ferlin family that contain C2 domains with a role in calcium-mediated membrane fusion events, is an intracellular phospholipid binding protein that

associates with the plasma membrane [24]. Myoferlin is concentrated at the sites of juxtaposed myoblast-myotube as well as myotube-myotube membranes in both cells [25]. Although myoferlin is expressed throughout myogenesis, myoferlin null myoblasts fuse normally to form nascent myotubes but further nuclear accretion in nascent myotubes is blocked. The muscles of myoferlin null mice are significantly smaller than wild type and fail to fully regenerate after injury [25]. More recently, myoferlin was found to interact with the endocytic recycling protein EHD2 and is hypothesized to help regulate membrane fusion at cell-cell contact sites [26].

The presence of localized molecules at cell-cell contacts is not just limited to proteins. Phosphatidylserine is transiently exposed at the sites of cell-cell contact [27] and is functionally required for myotube formation as inhibition of phosphatidylserine by annexin V inhibits fusion. Multiple roles for this transient exposure of phosphatidylserine are proposed including cell recognition and cell signaling. Additionally, lipid rafts containing cholesterol accumulate at cell contact sites and are required for the accumulation of adhesion molecules at these sites [28]. Soon after cell contact, dynamic redistribution of the lipid rafts occurs and membrane fusion ensues. Cholesterol is proposed to help maintain the proper rigidity of the lipid bilayers necessary for adhesion between the two myogenic cells.

Molecules concentrated at the border of contacting myogenic cells: asymmetrical localization

Molecules in this category localize to the contacting region between two opposing muscle cells, but strikingly in *only one* of the cells (Figure 1B, right; Table 1). The exact role these asymmetrically localized molecules play during the fusion process is unknown. A greater variety exists among the molecules that are asymmetrically localized than those that are symmetrically localized. In one *in vitro* study, electron microscopy revealed that actin forms a dense wall structure in one cell, paralleling the long axis of aligned myoblasts [22]. As fusion proceeds, gaps appear in the actin wall at sites of vesicle accumulation, vesicles pair in both cells along the membrane and fusion pores form. Non-muscle myosin 2A associates with the subplasmalemmal actin wall and is required for its formation as well as appearance of the vesicles at the membrane and myoblast fusion. This actin wall may provide membrane rigidity needed for cell fusion or serves as a barrier to temporally impede vesicle movement to the membrane. Although such vesicles are associated with sites of membrane fusion in myoblasts their role is unclear [18].

Time-lapse microscopy reveals that myoblast elongation during differentiation is followed by extension of pseudopodia and filopodia, dynamic cell extensions composed of actin filaments, which make contact with neighboring muscle cells [21,28–30]. The role of these dynamic cell structures in the fusion process is unknown but they may receive and/or send intercellular signals. Localization of diverse molecules occurs at the distal tip of a pseudopodium in one cell when in close proximity to another myoblast [23]. These molecules include diacylglycerol kinase ζ, an enzyme that generates phosphatidic acid, syntrophin, a scaffold protein that binds diacylglycerol kinase ζ, and the GTPase Rac1. These three molecules may be involved in actin rearrangements required for pseudopodia and filopodia extension. Unknown is whether protein accumulation occurs before a filopodial connection is established or if contact-mediated signaling induces it. Curiously, the adhesion molecule N-cadherin is co-localized with diacylglycerol kinase ζ at nascent intercellular contacts but not at established cell contacts indicating temporal regulation also occurs in these localized molecular components [23].

As myogenic differentiation proceeds the enzyme creatine kinase B becomes prominently localized to the ends of nascent and mature myotubes [31]. This localization is most frequently observed in one myotube of a pair of neighboring myotubes. Cytosolic isoforms of creatine kinase such as creatine kinase B replenish local ATP levels at sites of high ATPase activity by

catalyzing the transfer of phosphate from the high-energy intermediate phosphocreatine to ADP thereby preventing ATP depletion [32–34]. These data suggest that ATP-dependent reactions are highly localized at the ends of myotubes. Myonuclear addition to myotubes may be a higher energy process compared to myoblast-myoblast fusion due to the more organized cytoskeletal structure of myotubes. Alternatively, the ends of myotubes may consume ATP too rapidly for replenishment by diffusion due to the larger size and sarcomeric structures of myotubes and therefore, these regions of the myotube may need to rely on localized production of ATP.

Molecules with functional localization during myoblast fusion

Some molecules regulate fusion only at later stages of myogenesis where they are functionally required in differentiated myoblasts and regulate their fusion with nascent myotubes (Figure 1C, Table 2). However, the expression of these molecules is not restricted just to myoblasts. Some molecules are expressed by nascent myotubes only [35], whereas others are expressed by both types of cells [36, 37].

NFATc2 is a calcium-activated transcription factor that plays a key role in orchestrating myoblast-myotube fusion [37,38]. Loss of NFATc2 results in normal formation of nascent myotubes both *in vitro* and *in vivo* but further myonuclear accretion is inhibited due to defects in the recruitment and/or fusion of myoblasts with nascent myotubes. Further studies demonstrated NFATc2 controls myoblast fusion by regulating the expression of IL4 by nascent myotubes [35]. IL4 promotes fusion in part by regulating expression of the mannose receptor (MR), a cell surface endocytic C-type lectin. MR null myoblasts display a general reduction in general motility as well as an impairment of directed migration to unidentified factors released by fusing muscle cells [36]. Collagen uptake is also decreased in MR null muscle cells supporting a role for this endocytic receptor in helping to clear extracellular matrix from the leading edge of migrating cells. Additionally, IL4 may have MR-independent effects on migration [39]. Mutations in either IL4 or mannose MR leads to similar defects in growth of nascent myotubes *in vitro* and *in vivo* as observed for NFATc2 mutants [35,36].

Nephrin is a transmembrane protein of the Ig superfamily whose expression is induced in muscle cells both *in vitro* during myotube formation and *in vivo* during development or myofiber regeneration, at times associated with myoblast fusion [40]. In the absence of nephrin, nascent myotubes form normally but further myoblasts are unable to fuse with these myotubes. Nephrin function is required in differentiated myoblasts not in myotubes, although the relative expression pattern between these two cell types is unknown. Nephrin shares structural similarities with the *Drosophila* transmembrane protein, Sticks and Stones (Sns). During the recognition and adhesion of founder myoblasts and fusion-competent myoblasts, Sns, which is expressed only by fusion-competent myoblasts, interacts with the transmembrane protein kirre/duf on founder myoblasts to initiate intracellular signaling necessary for cell fusion [3]. The mechanism by which nephrin regulates fusion is unknown but it is likely to be part of a larger protein complex that initiates intracellular signaling in myoblasts because of its structural similarity to Sns.

The question arises of why the myoblast-myotube fusion step in myogenesis requires additional unique molecules compared to myoblast-myoblast fusion. Potentially special challenges may be encountered during cell fusion to a multinucleated cell containing sarcomeres. Additionally, these molecules could represent a fine-tuning mechanism for controlling the ultimate number of nuclei within a myotube/myofiber. Furthermore, these molecules may direct myoblast fusion to specific sites along the myotube or with specific myotubes. Finally, since muscle regeneration *in vivo* is asynchronous, these molecules could specifically control growth of

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regenerating myofibers rather than allowing new myofibers to form and thus be a means of controlling the number of regenerated myofibers after muscle injury.

Summary

In recent years the number of molecules that regulate mammalian myoblast fusion has greatly expanded and the molecular steps that govern this finely orchestrated process are being revealed in greater detail. An emphasis should be placed on determining where specific molecules are expressed, localized and function in terms of both myoblast-myoblast as well as myoblast-myotube fusion. Understanding how and why such molecules become restricted in location or function is likely to provide further insights into the mechanisms regulating mammalian muscle fusion.

Acknowledgments

Abbreviations

ADAM 12	a Disintegrin and Metalloprotease 12
IL4	Interleukin 4
IL13	Interleukin 13
MR	mannose receptor
Mb	myoblast
Mt	myotube
NFATc2	Nuclear Factor of Activated T cells c2
Sns	Sticks and Stones

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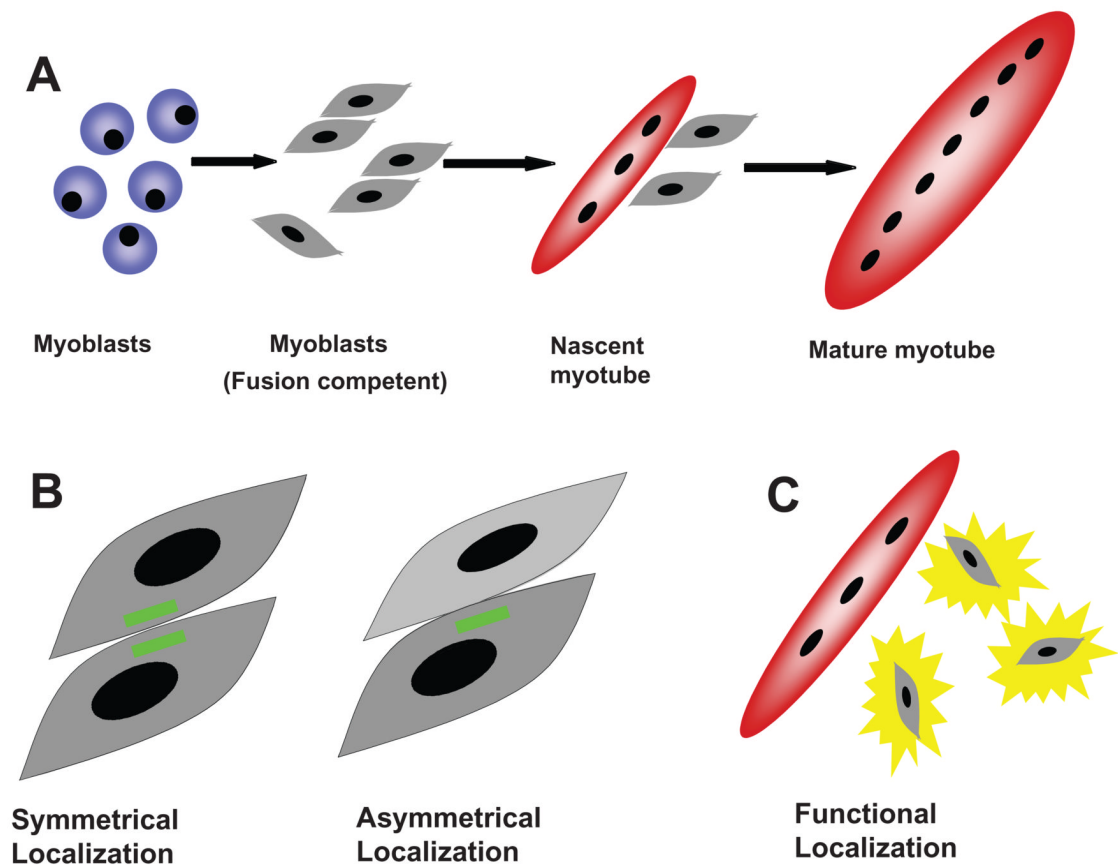
Figure 1. Myoblast fusion is associated with spatial and functional localization of molecules
A) Myoblasts proliferate and then, in response to environmental cues, differentiate to become fusion-competent. Myoblast fusion occurs in two stages: in the first phase, a subset of differentiated myoblasts fuse together to form a nascent myotube with a limited number of nuclei. Subsequently, additional differentiated myoblasts fuse with a nascent myotube to generate a large, mature myotube with many nuclei. B) During fusion, myogenic cells are closely juxtaposed. Two differentiated myoblasts are shown here. Some molecules that influence fusion are localized to the contact region between the two cells either in both cells (symmetrical localization) or only in one of the cells (asymmetrical localization). C) Some molecules that influence fusion are only functionally required in differentiated myoblasts as they fuse with nascent myotubes. This is an example of functional localization.

Table 1

Molecules that are localized to cell-cell contact regions during myoblast fusion

	Molecule	Location	Proposed Function/Activity	References
Symmetrical Localization	Actin	Intracellular	Cytoskeleton, fusion pore	[21]
	ADAM12	Cell surface, Intracellular	Cell-cell adhesion	[6]
	Alpha3 integrin	Cell surface	Cell-cell adhesion	[4]
	Alpha9 integrin	Cell surface	Cell-cell adhesion	[6]
	β–catenin	Intracellular	Intracellular signaling	[8]
	β1 integrin	Cell surface	Recruitment of CD9	[5]
	Cholesterol	Membrane	Membrane rigidity, accumulation of adhesion molecules	[28]
	EB3	Intracellular	Microtubule regulation at cell cortex	[20]
	Kindlin-2	Intracellular	Integrin-associated cytoplasmic adaptor	[9]
	M-cadherin	Cell surface	Cell-cell adhesion	[7]
	Myoferlin	Intracellular	Phospholipid binding; membrane repair	[25]
	Phosphatidylserine	Membrane	Cell recognition,signaling	[27]
Asymmetrical Localization	Actin	Intracellular	Cytoskeleton, fusion pore	[22]
	Creatine kinase B	Intracellular	ATP production	[31]
	Diacylglycerol kinase ζ	Intracellular	Diacylglycerol metabolism	[23]
	N-cadherin	Cell surface	Cell-cell adhesion	[21,23]
	Non-muscle myosin 2A	Intracellular	Associates with actin	[22]
	Rac1	Intracellular	GTPase: WAVE complex formation	[23]
	Syntrophin	Intracellular	Scaffold protein; binds diacylglycerol kinase ζ	[23]

ADAM 12, a Disintegrin and Metalloprotease 12

WAVE, WASP family Verprolin-homologous protein

Table 2

Molecules functionally required in myoblasts during myoblast-myotube fusion

Molecule	Location	Cell type expressed by	Proposed Function/Activity	References
Interleukin 4	Secreted	Mt	Stimulates expression of mannose receptor; migration	[35][[39]
Mannose receptor	Cell surface	Mb, Mt	Directed migration; collagen clearance	[36]
Nephrin	Cell surface	n.d.	Cell-cell adhesion; signaling	[40]
NFATc2	Intracellular	Mb, Mt[1]	Regulates gene expression in nascent myotube	[37]

Mb, myoblast

Mt, myotube

n.d., not determined

NFATc2, Nuclear Factor of Activated T cells, c2

[1] Only becomes activated and undergoes nuclear translocation in nascent myotubes