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Mini Review

Podosomes in muscle cells and their role in the remodeling of neuromuscular postsynaptic machinery

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ABSTRACT

Podosomes are adhesive, matrix remodeling organelles that have been described in numerous cell types, including all three vertebrate muscle cell lineages. Podosomes have been intensively studied in smooth muscle cells, but they have also been described in cardiac myocytes and skeletal muscle cells where they are proposed to play a role in developmental remodeling of neuromuscular junction postsynaptic machinery. In this review, we summarize the current state of knowledge of podosomes in muscle cells, with a focus on their potential function at the maturing synapse.

Introduction

Podosomes are dynamic organelles formed by many motile and invasive cells. These specialized structures have a characteristic distribution of components that are segregated into two distinct domains. A centrally located core region is rich in actin and actin-organizing proteins, including the Arp2/3 complex, cortactin, and cofilin, and surrounded by a thin cortex that contains focal adhesion marker proteins, including talin, paxilin, and vinculin (Linder et al., 2011; Murphy and Courtneidge, 2011). Individual podosomes are usually approximately 0.5–1 μm in diameter, but they can form larger assemblies, in which subunits coalesce together to form structures of various shapes. In some cells, these shapes may span up to 50 μm. Podosomes are usually formed at the ventral surface of cultured cells where the plasma membrane is in contact with the substratum, and one of their functions is to promote cellular adhesion (Linder et al., 2011; Murphy and Courtneidge, 2011; Spinardi and Marchisio, 2006). At the same time, the local exocytosis of proteases allows podosomes to remodel or degrade the extracellular matrix (ECM) to facilitate cellular migration and invasion through tissue and influence intercellular interactions (Linder et al., 2011; Murphy and Courtneidge, 2011). Another intriguing feature of podosomes is their dynamics. These organelles may appear and disappear within minutes. They undergo lateral mobility and may fuse together into larger structures or split into smaller subunits. Podosomes often appear in clusters, in which individual podosomes are connected by actin cables and exhibit synchronized dynamics. Podosomes were originally discovered in Rous sarcoma virus-transformed fibroblasts and subsequently described in many other cells types, including osteoclasts, macrophages, dendritic cells, smooth muscle cells (SMCs), and endothelial cells (Calle et al., 2006; Evans and Matsudaira, 2006; Jurdic et al., 2006; Lener et al., 2006; Linder et al., 2011; Marchisio, 2012; Murphy and Courtneidge, 2011; Ory et al., 2008; Tarone et al., 1985). Many cancer cells form structures called invadopodia, which are closely related to podosomes that are present in non-transformed cells (Linder et al., 2011; Murphy and Courtneidge, 2011). Various cells in different tissues apparently utilize podosomes as "remodeling devices" to facilitate various biological processes, including cell migration, trans-cellular diapedesis at sites of inflammation, cancer metastasis, antigen recognition, bone resorption, and cell fusion

Abbreviations: NMJ, neuromuscular junction; AChR, acetylcholine receptor; SMC, smooth muscle cell; LDL, low-density lipoprotein; PKC, protein kinase C; ECM, extracellular matrix; ACh, acetylcholine.

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(Carman et al., 2007; Le Roux-Goglin et al., 2012; Linder et al., 2011; Murphy and Courtneidge, 2011; Sage et al., 2012; Sens et al., 2010; Sibony-Benyamini and Gil-Henn, 2012). In this review, we describe the current state of knowledge of podosomes in muscle cells. We summarize past reports that described them in smooth and cardiac myocytes and focus on studies of podosomes in skeletal muscles and at the neuromuscular junction (NMJ). Please note, that in this review, we use term "in vitro" for experiments performed on cultured cells and "in vivo" for observations performed on a living animal or on tissues dissected and fixed without culturing; we reserve the term "ex-vivo" for cultured undissociated tissues.

Podosomes in muscle cells

Smooth muscle cells

The best studied muscle podosomes are the ones formed by smooth muscle cells. They have been proposed to play a role in the pathogenesis of atherosclerosis, one of the major sources of mortality in developed countries (Lener et al., 2006; Libby et al., 2011). This disease is caused by the accumulation of cholesterol-containing low-density lipoprotein (LDL) deposits, known as "plaques" (atheroma), in the inner-most layer of arteries, the *tunica intima* (Doran et al., 2008; Libby et al., 2011). These deposits attract inflammatory cells that secrete cytokines and growth factors that stimulate the invasion of SMCs from their normal location in the adjacent *tunica media* into the *tunica intima* (Doran et al., 2008; Libby et al., 2011). These invasive SMCs that produce ECM components, constitute an important part of the fibrous cap that is a major component of plaques, impeding blood flow and leading to ischemia. Rupture of the fibrous cap may produce thrombi, potentially resulting in myocardial infarction or stroke (Doran et al., 2008; Libby et al., 2011). Therefore, the switch in behavior of SMCs and their migration to the inner layer are fundamental steps in the pathology of atherosclerosis. The process of SMC invasion is thought to be facilitated by podosomes in the same way that the invasion of metastatic cancer cells depends on invadopodia (Lener et al., 2006; Quintavalle et al., 2010). The role of podosomes in SMC invasion is thought to consist of "clearing the path" through the degradation of ECM components by podosome-associated proteases (Lener et al., 2006).

After a decade of studies, we now start to understand major mechanisms that regulate podosomes in smooth muscle cells. At the molecular level, two opposing pathways – Src-STAT3 and p53-PTEN – regulate the switch from contractile to invasive behavior in a protein kinase C (PKC)-dependent manner. The Src-STAT3 pathway promotes the assembly of podosomes and invasion by activating a podosome-specific scaffold protein, Tks5, which in turn recruits the actin-organizing proteins AFAP-110, 190RhoGAP, and cortactin (Crimaldi et al., 2009; Lener et al., 2006; Mak, 2011; Mukhopadhyay et al., 2010). Additionally, AFAP-110 is activated through phosphorylation by PKC, and this modification influences the lifespan of podosomes in various cells, including SMCs (Dorfleutner et al., 2008).

The second pathway, p53-PTEN, is known for its tumor-suppressive activity. It blocks podosome formation, inhibits migration, and promotes the contractile phenotype of SMCs (Lener et al., 2006; Mak, 2011; Mukhopadhyay et al., 2010; Poon et al., 2010). The p53 protein additionally regulates the actin-binding protein caldesmon, which inhibits podosome assembly by competing with the Arp2/3 complex for actin binding (Morita et al., 2007; Mukhopadhyay et al., 2009). Podosomes and the proliferation of SMCs are also negatively regulated by two microRNAs, miR-143 and miR-145, that control the expression of PKCε, the podosome-associated protein fascin, and platelet-derived growth factor receptor (PDGFR) (Quintavalle et al., 2010). Interestingly, both miRs are produced from the same gene, whose expression is activated by p53, further illustrating the role of p53 in suppression of the invasive SMC phenotype (Cordes et al., 2009; Quintavalle et al., 2010; Xin et al., 2009).

Notably, an electron microscopic study of SMCs in the miR-143/miR-145 knockout mouse aorta provided one of the strongest cases for the existence of podosomes *in vivo* (Quintavalle et al., 2010). These structures were subsequently characterized by the presence of cortactin and Tks5, a protein that is believed to be specific for podosomes and invadopodia.

Cardiac myocytes

Studies by Hilenski et al. (1991) reported the presence of podosomes in cardiac myocytes. When neonatal rat cardiac myocytes were cultured on collagens I and III, these cells formed up to 20 structures with a 1.5–2 μm diameter, which had actin-rich cores and cortex domains that contained vinculin, α-actinin, and β_1 integrins (Hilenski et al., 1991). Also VanWinkle et al. (1995) reported the formation of podosome-like organelles in cultured neonatal rat cardiomyocytes in response to stress. They suggested that podosome formation could release the tension imposed by contractile forces. Interestingly, cellular stress was induced by hypoxia, which also occurs *in situ* during myocardial ischemia (VanWinkle et al., 1995). It would be beneficial to better characterize these organelles in cardiac myocytes. For instance, do they form when cells are cultured on other surfaces and how does it relate to mechanotransduction? Do podosomes also play an important role in the physiology and pathology of human cardiomyocytes? Interestingly, a mutation in the gene that encodes the podosome adaptor protein Tks4 causes Frank-Ter Haar Syndrome, the symptoms of which include cardiac abnormalities (Iqbal et al., 2010). Similarly, the deletion of p130Cas, which encodes a protein required for the Src-induced formation of invadopodia in fibroblasts, causes severe defects in heart development (Honda et al., 1998; Nakamoto et al., 1997). However, in both cases the direct link to podosomes as part of the etiology of the disease is missing.

Presence of podosome-like organelles was also reported in *Caenorhabditis elegans* muscle cells. Worms contain body wall muscles that are involved in locomotion and a pharyngeal muscle that is similar to the vertebrate cardiac muscle. The pharyngeal muscle contains actin-rich structures that are surrounded by areas enriched in PXL-1 and DEB-1, nematode orthologs of the vertebrate proteins paxillin and vinculin, respectively (Warner et al., 2011). Whether these structures also contain other podosome markers and whether they are involved in ECM remodeling are currently unknown.

Cell–cell fusion in Drosophila melanogaster myoblasts

Another interesting example of podosomes in muscle cells is myoblast fusion in *Drosophila melanogaster*. In flies, as in vertebrates, myoblasts fuse to form long, polynucleated muscle fibers that allow for force transduction over a long distance. The fusion of two myoblasts involves the organization of a complex, actin-containing machinery that facilitates the adhesion of cells and the close apposition and subsequent fusion of the lipid bilayer (Chen and Olson, 2005). Myoblasts have been proposed to utilize podosome-like organelles for the formation of the fusion pore. In their elegant work, Elizabeth H. Chen and her group showed that a fusion-competent myoblast forms an actin-rich, invasive protrusion toward its fusion partner. This process is regulated by WASP, WIP, and PAK, and is required for the cells to coalesce (Sens et al., 2010). The role of podosomes in this process is unconventional:

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instead of degrading the ECM, protrusions formed by *Drosophila* myoblasts are utilized to invade the other cell in a process that resembles transcellular diapedesis by leukocytes (Carman et al., 2007).

Skeletal muscle cells

Primary myoblasts and immortalized C2C12 cells also form adhesive podosome-like structures that degrade the ECM. These structures, like conventional podosomes, are rich in actin and contain other typical markers, including the podosome-specific protein Tks5, vinculin, talin, and FAK (Thompson et al., 2008). They are also enriched in phospho-tyrosine, a podosome and focal adhesion marker. Interestingly, podosomes in myoblasts contain dystroglycan and utrophin, two components of the dystrophin-associated glycoprotein complex (DGC), a major laminin receptor in the muscle (Thompson et al., 2008). The formation of actin-rich puncta in cultured myoblasts depends on Tks5, dystroglycan, and Src. Upon phosphorylation of dystroglycan by Src, these three proteins form a ternary complex, the assembly of which is essential for podosome formation (Thompson et al., 2008).

Development of the postsynaptic machinery in skeletal muscle

Apart from myoblasts, podosome-like organelles were described in differentiated myotubes where they were proposed to play a role in the remodeling of neuromuscular junction (NMJ) postsynaptic machinery (Proszynski et al., 2009; Proszynski and Sanes, 2013). NMJs are specialized synapses in the peripheral nervous system that are formed between motor neurons and skeletal muscle fibers. To ensure the efficient release and detection of the secreted neurotransmitter acetylcholine (ACh), the presynaptic nerve terminal precisely opposes and mirrors the size and shape of the postsynaptic machinery on the surface of muscle fibers (Sanes and Lichtman, 2001). In mammals, the adult NMJ has a complex topology that resembles an approximately 50 μm diameter pretzel.

The elaborate organization of the NMJ is achieved through a series of transformations during postnatal development (Fig. 1A, top). At birth, NMJs are simple, plaque-like structures. Over the course of the next few days, they grow in size and start to acquire approximately 5 μm diameter perforations. With time, these perforations become more numerous and fuse together, creating cracks and indentations between AChR-rich areas and transforming the "plaque" into a "pretzel" (Sanes and Lichtman, 2001; Shi et al., 2012; Slater, 1982).

Abnormalities in the development and maintenance of the NMJ are often observed in many neuromuscular disorders, including neuromuscular dystrophies, but the molecular mechanisms that orchestrate NMJ development are poorly understood (Shi et al., 2012). Previous research revealed that the presynaptic nerve terminal plays a crucial role in synaptic remodeling, influencing the organization of postsynaptic AChRs by secreting the glycoprotein agrin and neurotransmitter acetylcholine, which promote clustering/stability and the dispersion/internalization of AChRs, respectively (Kummer et al., 2006; Misgeld et al., 2005; Nitkin et al., 1987; Wu et al., 2010). At the same time, the nerve terminal mirrors the precise localization of the postsynaptic machinery and retracts from areas that do not contain elements of the postsynaptic apparatus (Sanes and Lichtman, 2001). Interestingly, after nerve injury, the morphology of the re-growing nerve terminal is dictated by the shape of the postsynaptic apparatus and ECM components (Rich and Lichtman, 1989; Sanes et al., 1978).

Recent observations suggest that muscle cells have an intrinsic, nerve-independent mechanism for remodeling the postsynaptic machinery. When differentiated myotubes (formed either from C2C12 cells or from primary myoblasts) are cultured on unpatterned laminin-coated surfaces, they form complex clusters of postsynaptic machinery that resemble the postsynaptic specialization of the NMJ with regard to its molecular composition and general organization (Kummer et al., 2004; Proszynski et al., 2009; Proszynski and Sanes, 2013). More importantly, clusters of AChRs in aneurally cultured myotubes undergo very similar developmental remodeling as the postsynaptic machinery at the NMJ. Initially, AChRs are assembled into simple oval clusters that subsequently become perforated with scattered openings, which transform the "plaque" into a pretzel-like structure (Fig. 1A, bottom) (Kummer et al., 2004; Proszynski et al., 2009; Proszynski and Sanes, 2013). These observations imply that the muscle has an intrinsic mechanism for postsynaptic remodeling. Importantly, the proposed muscle-intrinsic program for the development of the postsynaptic machinery does not exclude the critical role of the nerve and nerve-derived factors in synaptic remodeling. It is likely that proper development of neuromuscular synapses is controlled by various mechanisms that partially compensate for defects in one another and mutually coordinate each other to ensure correct synaptic function. In fact, neither cultured muscle cells nor denervated muscle fibers develop complex shapes of synaptic specializations without the addition of stimuli that initiate the remodeling. *In vivo* such stimuli are supplied by the nerve terminal. In cultured myotubes they are generated by laminin-dependent signaling and possibly mechanical factors such as cell adhesion tensile forces. These factors together mimic the presence of the nerve and trigger the remodeling in the absence of neurons (Kummer et al., 2004). Such a muscle-intrinsic program for synaptic development would be difficult to study *in vivo* in the presence of the nerve. Therefore, the discovery that myotubes cultured on laminin develop complex AChR clusters provides a simple system where muscle-based remodeling of the postsynaptic machinery can be investigated.

Involvement of podosomes in remodeling the postsynaptic specialization

Further studies on laminin-cultured myotubes led to the unexpected discovery that perforations that appear in developing AChR clusters contain podosome-like organelles, referred to as "synaptic podosomes" (Proszynski et al., 2009; Proszynski and Sanes, 2013). These newly identified structures closely resemble conventional podosomes. They are rich in actin and often have a visible actin cloud in their close vicinity. Synaptic podosomes are usually approximately 5 μm in diameter, but they may also form smaller or larger structures of various shapes (Fig. 1B–D). At high resolution, actin-rich puncta appear to be composed of smaller subunits. The synaptic podosomes have a typical podosome-like molecular composition and core-cortex organization (Fig. 1C and D) (Proszynski et al., 2009; Proszynski and Sanes, 2013). Finally, like their counterparts in other cells, synaptic podosomes are very dynamic (Proszynski et al., 2009). They appear and disappear within minutes, undergo fusion and fission, and exhibit lateral mobility. Importantly, time-lapse analysis revealed that synaptic podosomes form below juvenile, continuous clusters of AChRs, which precedes the formation of perforations at these locations (Proszynski et al., 2009). This observation suggests that podosomes are actively involved in remodeling the postsynaptic machinery.

Several functional properties of podosomes, also shared by synaptic podosomes, make them particularly suitable for locally remodeling the postsynaptic specialization. First, podosomes are sites of enhanced adhesion to the substratum (Linder et al., 2011;

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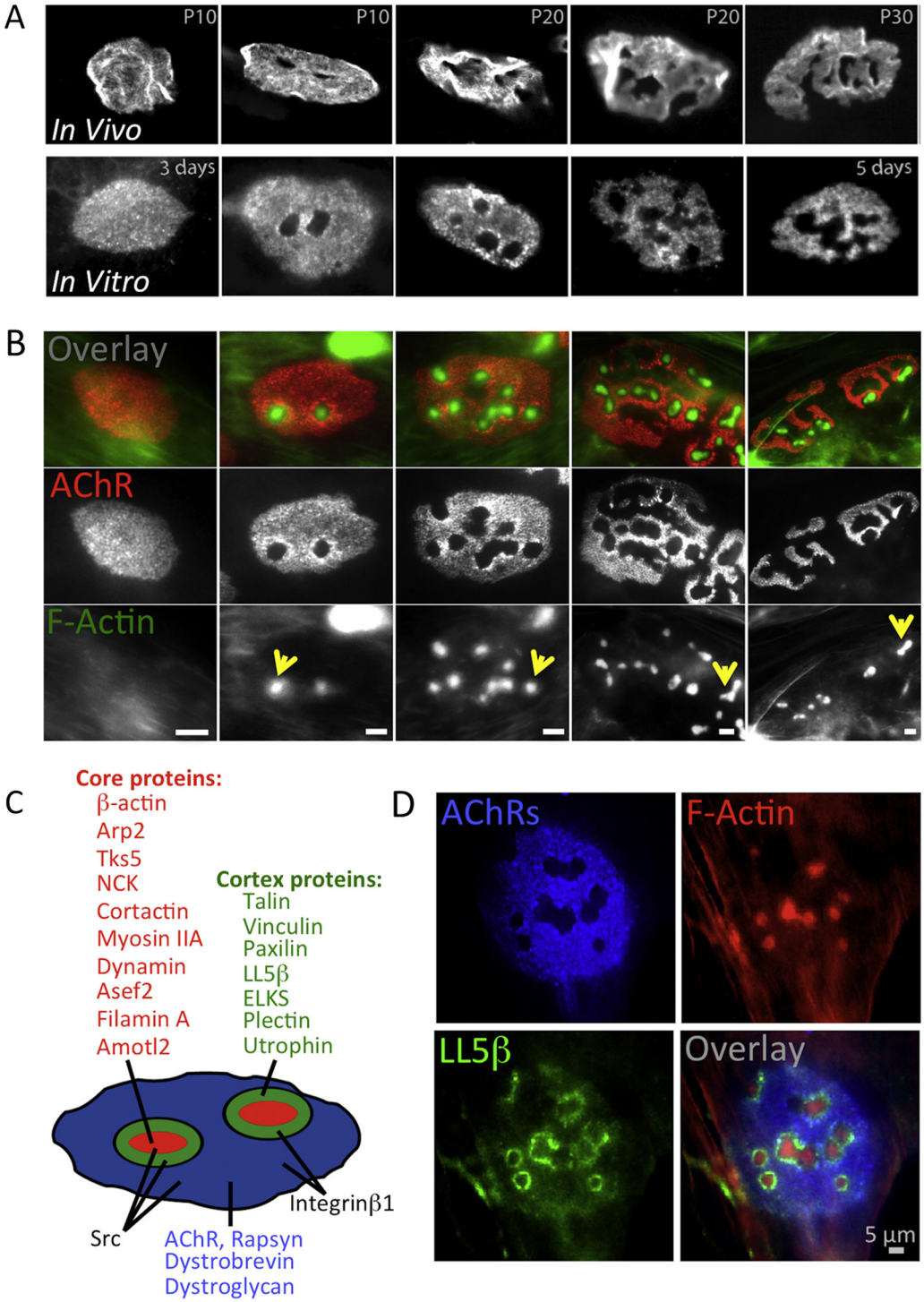

Fig. 1. Developmental remodeling of the postsynaptic machinery in muscle cells. (A) Maturation of the postsynaptic specialization at the neuromuscular junction (*in vivo* – top) and in laminin-cultured myotubes (*in vitro* – bottom). Postsynaptic AChRs were labeled with bungarotoxin-Alexa-488. Postnatal days in development are indicated for *in vivo* panels. Days after differentiation are indicated for *in vitro* panels. (B) Podosome association with AChR (red) clusters at various stages of development. Arrows indicate individual podosomes (F-actin, green). Scale bar = 5 μm. (C) Schematic illustration of components of podosomes and the postsynaptic machinery. (D) Representative illustration of the distribution of podosome core (F-actin, red) and cortex (LL5β, green) marker proteins at the AChR cluster (AChRs, blue) in cultured C2C12 myotubes. Scale bar = 5 μm.

Murphy and Courtneidge, 2011; Proszynski et al., 2009). As they form, these rigid structures push AChR molecules sideways, contributing to the appearance of "holes" in AChR plaque (Proszynski et al., 2009).

Second, synaptic podosomes remodel the ECM components that are needed to stabilize the postsynaptic machinery (Proszynski et al., 2009). Importantly, the NMJ differs from synapses in the central nervous system by the significant amount of ECM deposited in the synaptic cleft (Singhal and Martin, 2011). The presence of ECM components is crucial for the assembly, development, and maintenance of proper postsynaptic machinery *in vivo* and *in vitro* (Nishimune et al., 2008). In aneurally cultured myotubes, the clustering of AChR into complex assemblies relies on the presence of laminin in the substratum (Nishimune et al., 2008).

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Interestingly, maturation of the postsynaptic machinery from a "plaque" to "pretzel" is compromised in double knockouts of laminin α4 and α5 or in the absence of dystroglycan, a component of the dystrophin-associated glycoprotein complex, which is the major laminin receptor in muscle cells (Nishimune et al., 2008). Thus, synaptic podosomes attach to the ECM, providing signals that are crucial for synaptic remodeling. At the same time, podosomes actively remodel the extracellular scaffolds to which the postsynaptic machinery is anchored.

Third, enhanced endocytosis occurs in the close vicinity of synaptic podosomes, which may facilitate the resorption of degraded material or components of the postsynaptic membrane (Proszynski et al., 2009).

Like in the case of traditional podosomes, the formation of podosomes in cultured myotubes depends on dynamin and Src tyrosine kinase activity (Proszynski et al., 2009). Recent studies identified a number of additional components of synaptic podosomes, which were subsequently shown to be present at podosomes in other cells (Proszynski and Sanes, 2013). Specifically, the protein LL5β, which organizes the cytoskeleton and regulates exocytosis, was first proposed as a candidate protein for synaptic remodeling (Grigoriev et al., 2007; Hotta et al., 2010; Kishi et al., 2005; Lansbergen et al., 2006). It was then found to be a component of synaptic podosomes and also enriched in the close vicinity of podosomes in RAW.297 macrophages and invadopodia in Src-transformed 3T3 cells (Proszynski et al., 2009; Proszynski and Sanes, 2013). An LL5β-interacting protein, Amotl2, was also shown to play a role in synaptic podosome formation and remodeling of the postsynaptic machinery (Proszynski and Sanes, 2013). Amotl2 is a scaffold for signaling and polarity organizing proteins (Moleirinho et al., 2014). Myotubes depleted of Amotl2 formed exuberant podosomes that were on average 10-times larger than in control cells (Proszynski and Sanes, 2013). Several, but not all, LL5β-interacting proteins, including a scaffold protein Amotl2, a Rab-6-binding protein ELKS, and Asef, a guanine nucleotide exchange factor (GEF) for Rac and Cdc42 GTPases, were then found to be associated with podosomes in myotubes, macrophages, and Src-transformed fibroblasts, suggesting their involvement in the organization of these organelles in various cells and revealing additional parallels between these structures (Proszynski and Sanes, 2013).

Most of the information on the organization and function of synaptic podosomes has come from studies on cultured rodent muscle cells. The formation of synaptic podosomes, however, is not limited to the mammalian muscle postsynaptic specialization. Very similar actin and cofilin-rich puncta were observed in perforations in developing AChR clusters in cultured frog myotubes (Lee et al., 2009). Moreover, chick primary myotubes, when stimulated by phorbol esters, form numerous adhesion structures that have core domains that are rich in actin and α-actinin, surrounded by cortical areas that contain vinculin and talin (Lin et al., 1989). These structures appear on electron micrographs as 3-μm-long cellular protrusions formed by invagination of the plasma membrane. Therefore, skeletal muscle cells in various vertebrates appear to form podosomes. However, there may be some molecular differences between these structures in various species or muscle types. For example, unlike chick primary myotubes, mouse C2C12 myotubes do not form more podosomes after phorbol ester stimulation (unpublished data).

Studies on podosomes *in vivo*

Podosome structure and function have been well defined in adherent cell cultures. However, evidence that confirms their existence in a three-dimensional environment, particularly their *in vivo* relevance, is still limited (Linder et al., 2011; Murphy and Courtneidge, 2011). The characterization of podosomes *in vivo* is complicated because of difficulties in precisely visualizing the distribution of marker proteins in three-dimensional tissues. Studies of the functional relevance of podosomes are difficult because much of the same machinery that is important for podosome formation also regulates other cellular processes, such as focal adhesion formation and actin dynamics. To address the difference between two-dimensional cell culture systems and the *in vivo* environment, several groups observed podosome-like structures in cells that were grown in three-dimensional matrices (Hotary et al., 2000; Saltel et al., 2011; Tolde et al., 2010; Van Goethem et al., 2011; Wolf and Friedl, 2009). The first piece of evidence for the existence of podosomes in the tissue came from studies on cultured explants from arterial vessels (Rottiers et al., 2009). Some data also support the existence of podosome-like structures within tissue collected from mice and fixed without culturing (Quintavalle et al., 2010). To dissect the function of podosomes/invadopodia *in vivo*, several groups disrupted their formation by genetically manipulating the genes that are crucial for their assembly, such as Tks5 (Blouw et al., 2008; Murphy et al., 2011), N-WASP (Gligorijevic et al., 2012), and Arg kinase (Gil-Henn et al., 2013). Nevertheless, evidence of podosome function *in vivo* is still scarce. This makes the NMJ a potentially attractive model for studying podosomes in living organisms.

Several lines of evidence suggest the existence of podosomes at the developing NMJ *in vivo*. Proteins associated with synaptic podosomes in cultured C2C12 myotubes (*e.g.*, actin, a scaffold protein Amotl2, and a cytoskeletal cross-linking protein plectin (Proszynski et al., 2009; Proszynski and Sanes, 2013; and unpublished data) are also localized to postsynaptic machinery at the NMJ. Plectin Amotl2 and actin are concentrated at perforations in the developing postsynaptic apparatus, sites of intense NMJ remodeling, which is consistent with the potential involvement of podosomes in this process. Importantly, Amotl2 and plectin are virtually absent at the juvenile, unperforated postsynaptic machinery, and their abundance at the synapse increases during developmental remodeling (Proszynski and Sanes, 2013; and unpublished data).

The developing postsynaptic machinery in the muscle provides a small and very well defined area where podosomes are expected to form within a narrow time period. A particular advantage of this system is that peripherally located skeletal muscles are relatively accessible for manipulations and microscopy in living animals. Therefore, with the support of more basic biochemical experiments and genetic screening in cultured myotubes, the NMJ promises to be a valuable model for the study of podosomes.

Although localization of podosome components at sites of postsynaptic membrane remodeling suggests that synaptic podosomes are present at the developing NMJ *in vivo*, we must consider the possibility that these structures may turn out to be different from those observed in cultured myotubes. For instance, conventional podosomes are usually small and dynamic. In contrast, the actin and Amotl2-rich structures at the NMJ *in vivo* are much larger and relatively stable, surviving until adulthood (Proszynski et al., 2009; Proszynski and Sanes, 2013; and unpublished data). To counter this argument, although individual podosomes in osteoclasts and invadopodia in Src transformed fibroblasts are small, they coalesce together to form much larger and more stable structures. Likewise, the structures observed in the adult NMJ may originate from podosomes present at the perforations in the juvenile NMJ, but their organization and dynamics may change with time. Nevertheless, evidence for the relevance of podosomes *in vivo* in various systems is scarce and specifically a definitive proof of the existence of synaptic podosomes at the NMJ is still missing.

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Conclusions and future perspectives

Not fully understood is why NMJs develop their conserved, complex morphology. It is assumed to be required for high-fidelity function of the neuromuscular system and the quick and precise control of our movements. Although mammals can move their limbs already in the prenatal stage, when neuromuscular contacts are in their juvenile, plaque-like stage, these movements are very slow. The complex topology of the NMJ can be beneficial for the efficient propagation of the action potential along the muscle fiber. Alternatively, the actin- and Amotl2-rich regions that lie between AChR-rich areas may have a function that is not directly related to signal transmission, for example in cell adhesion or structural stabilization. Many specialized cell–cell contacts have domains of distinct function such as the supra-molecular activation complexes (SMACs) at immunological synapses (Huppa and Davis, 2003; Monks et al., 1998) puncta adherentia and synaptic active zones at synapses in the CNS, which also sometimes acquire a complex, pretzel-like morphology (Spacek and Harris, 1998).

We are now beginning to appreciate that NMJ remodeling is much more complex than previously thought. First, this important process is likely controlled not only by nerves, but also by a muscle-intrinsic program, with the two synaptic partners cooperating in the restructuring of the junction. Second, muscle cells may use specialized podosome-like organelles to determine the shape of the postsynaptic specialization (Proszynski et al., 2009; Proszynski and Sanes, 2013). Podosomes have been described in a plethora of different cell types, and their discovery in differentiated skeletal muscle cells is yet another example of their diverse functions, depending on the cellular context.

Can studies on synaptic podosomes contribute to our understanding of conventional podosomes and invadopodia? This seems likely, and there are some examples of the molecular machinery that was first identified on synaptic podosomes and subsequently discovered in other cells (Proszynski and Sanes, 2013). Nevertheless, these organelles have a certain degree of variability in different cells. Some proteins that are found on synaptic podosomes were not observed on their counterparts in other cells. For example, the depletion of the synaptic podosome component Amotl2 has opposite effects in myoblasts and Src-3T3 cells (Proszynski and Sanes, 2013).

Several key questions about the relationship between podosomes and NMJ remodeling remain unanswered. First, more evidence is still needed to confirm that the structures at the NMJ are indeed podosomes. Second, it would be interesting to know how they preferentially form at AChRs clusters and at a specific time during synapse development. The machinery for podosome formation is likely connected to the one responsible for assembly of the postsynaptic specialization, but the molecular link is still unknown. A potential candidate is the molecular pathway that is responsible for the formation of focal adhesions, structures known to give rise to podosomes under some circumstances (Kaverina et al., 2003) and at the same time abundantly present at the NMJ (Turner et al., 1991). An interesting observation would be that NMJ focal adhesions become transformed into synaptic podosomes and contribute to the plaque-to-pretzel transition.

Another important issue is the coordination between pre- and postsynaptic machinery in NMJ remodeling. Are podosomes sites of enhanced contact between the muscle and nerve? Their protrusive shape and capacity to degrade the ECM suggest that this indeed might be the case. Alternatively, podosome-like perforations might be sites where the postsynaptic membrane contacts Schwann cells to coordinate remodeling (Smith et al., 2013). To answer these questions, we may need to resort to electron microscopy because light microscopy, which we have used to study synaptic podosomes, lacks the required resolution.

Currently, we assume that the major function of synaptic podosomes is remodeling the postsynaptic machinery. However, they might also play a role in the stabilization of synaptic structure. Importantly, the actin cytoskeleton at the NMJ is crucial for its maintenance. Therefore, another interesting line of investigation would be to determine how long synaptic podosomes persist at the NMJ and whether they are also present at fully mature junctions. Could they potentially play a role in the aging or diseased NMJ, when postsynaptic machinery becomes fragmented and disassembles? Defects in the maturation and maintenance of the NMJ are often observed in various neuromuscular disorders, potentially implicating synaptic podosomes in the pathogenesis of these diseases.

At the molecular level, an important gap in our knowledge of synaptic podosomes is the identity of proteases that are responsible for ECM remodeling at their locations. Proteases of the ADAM family are potential candidates because their subcellular localization can be regulated by Tks5, a podosome and invadopodia-specific scaffold protein that was also found to be associated with synaptic podosomes (Abram et al., 2003). Novel, genetically engineered sensors of protease activity may help us understand how different proteases cooperate in time to remodel the postsynaptic machinery (Stawarski et al., 2014).

Another interesting issue is whether the LL5β/ELKS complex that was recently shown to be associated with podosomes in different cell types, including cultured myotubes, could be involved in the regulation of exocytosis at synaptic podosomes, similar to its involvement in HeLa cells, and, if so, what cargo is transported in the LL5β exocytic pathway (Grigoriev et al., 2007; Hotta et al., 2010; Lansbergen et al., 2006; Proszynski and Sanes, 2013). Podosome-associated proteases, adhesion molecules, and components of the ECM are all potential candidates.

Most of our knowledge of podosomes comes from studies on cultured cells, therefore, learning the degree to which synaptic podosomes *in vivo* are different from their counterparts *in vitro* will be interesting. For example, podosomes in cultured cells can assemble and disassemble within minutes. Do podosomes exhibit the same behavior *in vivo*? The environment is very different, and these organelles, which are responsible for interactions between the cell and surrounding environment, may have different properties. If this is the case, then can we still call them *bona fide* podosomes? Ultimately, however, the important issue will be to determine their functional properties and biological relevance, using the concept of "podosomes" as a useful paradigm to facilitate the discovery of new and exciting biology.

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