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Knockdown of Archvillin by siRNA Inhibits Myofibril Assembly in Cultured Skeletal Myoblast

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A myofiber of skeletal muscle is composed of myofibrils, sarcolemma (plasma membrane), and constameres, which anchor the myofibrils to the sarcolemma. Achvillin is a recently identified F-actin binding muscle protein, co-isolates with dystrophin and caveolin-3 in low-density sarcolemma of striated muscle, and colocalizes with dystrophin at costameres, the specialized adhesion sites in muscle. Archvillin also binds to nebulin and localizes at myofibrillar Z-discs, the lateral boundaries of the sarcomere in muscle. However other roles of archvillin on the dynamics of myofibrillogenesis remain to be defined. The goal of this study is, by using siRNA-mediated gene silencing technique, to investigate the effect of archvillin on the dynamics of myofibrillogenesis in cell culture of a mouse skeletal myogenic cell line (C2C12), where presumptive myoblasts withdraw from the cell cycle, fuse, undergo de novo myofibrillogenesis, and differentiate into mature myotubes. The roles of archvillin in the assembly and maintenance of myofibril and during the progression of myofibrillogenesis induced in skeletal myoblast following gene silencing in the cell culture were investigated. Fluorescence microscopy demonstrated that the distribution of archvillin was changed along the course of myofibril assembly with nebulin, vinculin and F-actin and then located at Z-lines with nebulin. Fluorescence microscopy demonstrated that knockdown of mouse archvillin expression led to an impaired assembly of new myofibrillar clusters and delayed fusion and myofibrillogenesis although the mouse archvillin siRNA did not affect those expressions of archvillin binding proteins, such as nebulin and F-actin. This result is corresponded with that of RT-PCR and western blots. When the perturbed archvillin was rescued by co-transfection with GFP or Red tagged human archvillin construct, the inhibited cell fusion and myotube formation was recovered. By using siRNA technique, archvillin was found to be involved in early stage of myofibrillogenesis. Therefore, the current data suggest the idea that archvillin plays critical roles on cell fusion and dynamic myofibril assembly.

Key Words: Archvillin, Myofibril assembly, Sarcolemma, Costameres, Sarcomere, C2C12, siRNA

INTRODUCTION

Skeletal muscle development is a multistep pathway that myoblasts fuse to form long multinucleated myotubes then the muscle-specific proteins are produced (Gregorio et al., 2000). During the cell differentiation, thousands of structural and regulatory molecules are assembled into the sarcomeric contractile units (Sanger et al., 2002; Lu et al. 2001). Studies on cytoskeletal protein interactions and functions in muscle cells have mainly been carried out with myoblasts (Gregorio et al., 2000). Experiments on skeletal myoblasts have a three-step model (Myofibrillogenesis) for the formation of mature skeletal myofibrils: premyofibrils to nascent myofibrils to mature myofibril (Sanger et al., 2002). Premyo-

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fibrils are deposited at the ends of elongating and widening myotubes. Premyofibrils are composed of mini-sarcomeres, the ends of which are α-actinin-rich Z-bodies to which actin filaments are attached. Mini-A-bands composed of nonmuscle myosin II filaments are localized between the Z-bodies. As groups of premyofibrils begin to align with one another, titin and muscle-myosin II filaments are recruited to form nascent myofibrils. The mature myofibrils form from the fusion of the Z-bodies into Z-bands, the elimination of the nonmuscle myosin II filaments and the alignment of the muscle-myosin filaments into A-bands (Sanger et al., 2002).

The subcellular architecture of skeletal muscle is very different from that of mononucleated cell (Kaisto, 2003; Lodish et al., 2001). A skeletal muscle comprises a bundle of muscle cells, or myofibers. A myofiber has a long cylindrical structure surrounded by a plasma membrane, sarcolemma and contains up to several thousand nuclei derived from the fusion of myoblasts in fetal and postnatal life (Kaisto, 2003; Gregorio et al., 2003). A myofiber is composed of myofibrils, sarcolemma, and constameres, which anchor the myofibrils to the sarcolemma (Gregorio et al., 2000). A myofiber is packed with myofibrils, bundles of filaments that run from one end of the cell to the other end lines (Lodish et al., 2001). Myofibrils are further subdivided into A- and I-bands and M- and Z-lines. The filaments consist of actin-containing thin filament, myosin-containing thick filament, titin, and nebulin (Clark et al., 2002). The sarcomere, the basic contractile unit of myofibrils, attaches to sarcolemma at costameres that co-distribute with the Z-line (Pardo et al., 1983b) and the M-line (Porter et al., 1992). The Z-lines represent the lateral boundaries of the sarcomere where the thin, titin, and nebulin filaments are anchored but how the filaments are attached is not certain. Because of this anchoring property, Z-lines are the primary conduits of the force generated by contraction (Clark et al., 2002). Alpha-actinin is an actin-binding protein that is localized in all Z-lines of the sarcomeres. Costameres coordinately transduce contractile force from the Z-line to the sarcolemma, where the force is transmitted laterally to the muscle termini (Danowski et al., 1992). In addition, costameres are organizational points for the membrane cytoskeleton, which maintains the structural integrity of the membrane during contraction. These features are a result of at least three different cytoskeletal networks (integrins/focal adhesion complexes, the dystroglycan complex, and the spectrin-based cytoskeleton (Clark et al., 2002).

The representative one among skeletal muscle proteins between costamere and nebulin is archvillin discovered in 2003. Archvillin is a 250 kDa F-actin binding protein and a muscle specific isoform of supervillin found in myogenic cell lines and in cardiac and skeletal muscles. The archvillin includes an initial muscle-specific exon containing an extended 5' leader sequence with potential post-transcriptional control elements and four exons encoding 47 kDa of additional muscle-specific protein sequence in the form of two inserts within the NH2-terminus and three potential actin binding sequences in the COOH-terminus (Fig. 1). The first of these muscle-specific inserts contains two conserved nuclear targeting signals. The gene encoding this protein, 'archvillin' (Latin, archi; Greek, árchos; 'principal' or 'chief'), contains an evolutionarily conserved, muscle-specific 5' leader sequence (Oh et al., 2003). Archvillin contains sequences that activate the transcriptional activity of the androgen receptor (Ting et al., 2002), and nuclear localization has been observed (Oh et al., 2003; Pestonjamasp et al., 1997; Ting et al., 2002). Archvillin binds tightly to membranes, F-actin and smooth-muscle and nonmuscle myosin II (Chen et al., 2003; Oh et al., 2003; Pestonjamasp et al., 1995; Pestonjamasp et al., 1997; Fig. 1). Archvillin localizes primarily within nuclei but is also concentrated at the plasma membrane with F-actin, nonmuscle myosin II and vinculin in myoblast and at costamere with dystrophin in myotube (Oh et al., 2003). In addition, the nebulin Ser-rich region interacts with the COOH terminus of archvillin at Z-line in skeletal muscle (Fig. 1). In a dissected skeletal muscle, the COOH terminal region of nebulin colocalized with archvillin. The interaction between nebulin and archvillin may provide a direct link between the sarcolemma and myofibrillar Z-discs (Lee et al., 2003).

Two transcript variants encoding different isoforms of archvillin are supervillin and smooth muscle archvillin. Smooth muscle achvillin (SmAV), a new splice variant of archvillin, is a recently discovered protein found in fellet

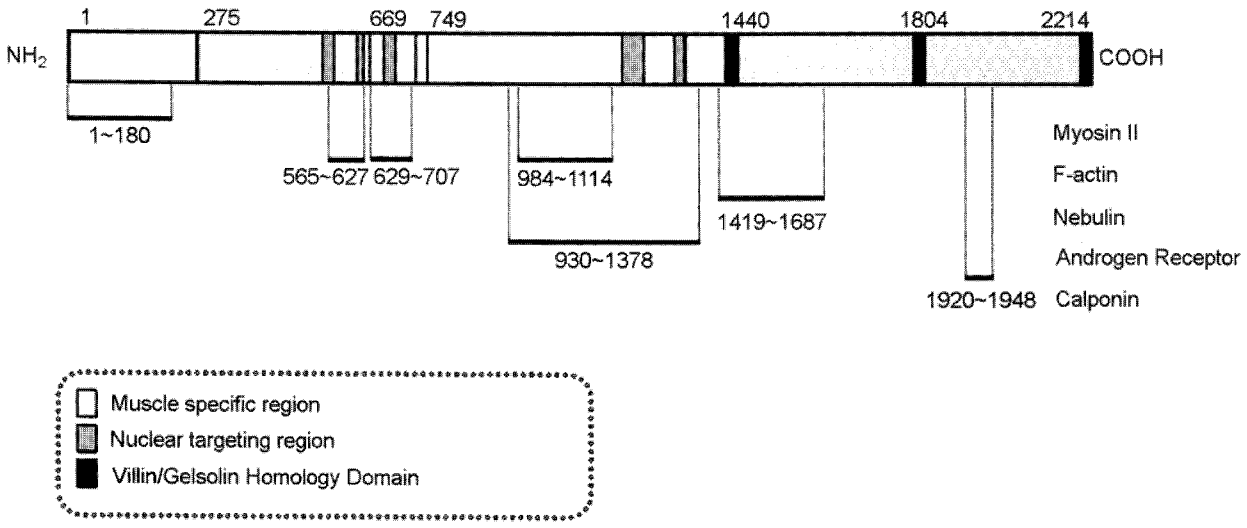

Fig. 1. The schematic domain structure of archvillin. Schematic representation of the domain structures of human archvillin showing the N-terminal region with putative nuclear targeting regions and muscle specific region. The C-terminal domain shows extensive similarity to villin and gelsolin, with three regions of F-actin binding sites (Pestonjamasp et al., 1997 Oh et al., 2003; Gangopadhyay et al., 2004). Domains are marked with residue numbers. Sequences corresponding to the predicted binding sites for myosin II, F-actin, nebulin, androgen receptor, and calponin (Pestonjamasp et al., 1997 Oh et al., 2003; Gangopadhyay et al., 2004 Lee et al., 2004) are shown.

aorta and was identified as an interacting protein with calponin (Fig. 1). SmAV appears to be the predominant archvillin homolog in smooth muscle. SmAV sequence contains several predicted nuclear targeting sequences and several potentially significant regulatory sites (Gangopadhyay et al., 2004). One of the two nuclear targeting sequences within the first insert in archvillin of skeletal muscle is conserved in SmAV (Oh et al., 2003; Gangopadhyay et al., 2004). C-terminal sequences homologous to six domains in gelsolin and villin are also present in SmAV (Pestonjamasp et al., 1997; Oh et al., 2003; Gangopadhyay et al., 2004). Additionally, several kinase-binding sites and phosphorylation sites are predicted. Because calponin has been linked with PKC and ERK-dependent signaling pathways, it is noteworthy that the SmAV sequence predicts the presence of two ERK-binding sites and a motif consistent with phosphorylation by PKC and a proline-directed kinase. SmAV contributes to the regulation of contractility through a calponin-mediated signaling pathway, involving PKC activation and phosphorylation of ERK1/2 (Gangopadhyay et al., 2004).

Supervillin initially reported a major 205 kDa F-actin binding protein in HeLa cervical carcinoma cells (Luna et al., 1997) and in bovine neutrophil plasma membranes complex containing non-erythrocyte spectrin (fodrin), actin, non-muscle myosin-IIA (Nebl et al., 2002; Pestonjamasp et al., 1995; Pestonjamasp et al., 1999). Supervillin mRNAs are expressed in all human tissues tested, but are most abundant in bone marrow, thyroid gland, and salivary gland; comparatively little message is found in brain. The human supervillin gene from normal human kidney and from the cervical carcinoma HeLa S3 is localized to a single chromosomal locus at 10p11.2, a region that is deleted in some prostate tumors (Pope et al., 1998). Supervillin encodes a bipartite protein with three potential nuclear localization signals in the NH2-terminus and three potential actin binding sequences in the COOH-terminus except for muscle specific regions. Supervillin is a T-down-regulated gene in dermal papilloma cells, which may contribute to male baldness syndrome (Pan et al., 1999). Supervillin homologue plays a role in sex determination (Fraser et al., 2000). Also supervillin interacts with androgen receptor (AR) protein and functions as an AR coregulator by enhancing AR transactivation (Wulfkuhle et al., 1999; Fig. 1).

The goal of this study is, by using siRNA-mediated gene silencing technique, to investigate the effect of archvillin on the dynamics of myofibrillogenesis in cell culture of a mouse skeletal myogenic cell line (C2C12), where presum-

ptive myoblasts withdraw from the cell cycle, fuse, undergo de novo myofibrillogenesis, and differentiate into mature myotubes.

MATERIALS AND METHODS

1. Synthetic oligonucleotides

The pSuper-neo-GFP plasmid was used to drive the cellular expression of specific siRNA. Two siRNA duplexes were prepared, mouse archvillin siRNA and control siRNA against human archvillin. So the 19-mer target sequences for the siRNA against the human and mouse archvillin were determined by the siRNA Target Designer program (http://bioinfo.clontech.com/siRNAdesigner/sirnaSequence/DesignInit.do). Sixty-mer oligonucleotides (mArch1, 5-gatccccggatcgcaagatacaaagcttcaagagaggatcgcaagatacaaagctttta-3; mArch2, 5-agcttaaaaagctttgtatcttgcgatcctctcttgaagctttgtatcttgcgatccggg-3; hArch1, 5-gatccccgccgaaagaattgcaaggtttcaagagagccgaaagaattgcaaggtttttta-3; hArch2, 5-agcttaaaaaaccttgcaattctttcggctctcttgaaaccttgcaattctttcggcggg-3) were annealed and cloned as described into the pSuper-neo-GFP plasmid (Brummelkamp et al., 2002). Two pairs of oligos were assembled by mixing 1 μl of each oligo (forward + reverse) with 48 μl annealing buffer, incubated the mixture at 90℃ for 4 min, and then at 70℃ for 10 minutes. The annealed oligo inserts were used immediately in a ligation reaction with 1 μl of the pSuper-neo-GFP vector linearized with BglII and HindII and transformed into competent cells of DH5α according to the transformation protocol routinely used in our laboratory. In addition, the presence of the correct insert within pSuper-neo-GFP vector was confirmed by sequencing prior to transfection in mammalian cells.

2. EGFPC1 or DsRedC1-tagged human archvillin

Five sets of gene specific primers were designed from human archvillin cDNA sequences (AF109135) to generate human archvillin (lym12, 5-cgagctcgaatgaaaagaaaagaaagaa-3; min15, 5-gctccgggaggctgcctgctgcagggag-3; min16, 5-cgcccacagccctccctgcagcaggca-3; lym23, 5-accttgtgtccagcatgct-3; lym24, 5-agcatgctggacacaaaggt-3; lym 25, 5-gcagcctgtccctcaagtcc-3; lym22, 5-ggggatgaaccgaaggaatt-3; lym27, 5-tgcattctccaggatccagg-3 lym26, 5-cctggatcctggagaatgca-3; lym13, 5-acgcgtcgactcagaacaggccttttgc-3). The complete human archvillin coding sequence was also amplified from a human skeletal muscle cDNA library (HL4010AB; CLONTECH Laboratories, Inc.) as a template in PCR reactions with Advantage KlenTaq polymerase (Clontech, Palo Alto, CA). LYM12 was modified at the 5'-end by the replacement of the Sac I site at +745 nt of archvillin cDNA sequences and LYM13 also was modified at the 3'-end by the replacement of the Sal I site at +7409 nt of human archvillin cDNA sequences. The PCR products were obtained by PCR amplification with five sets of gene specific primers encompassing unique restriction sites along the archvillin coding sequence. All Clones were completely sequenced in both directions by M13F and SP6 primer. These individual fragments were recovered by digestion with SacI, NcoI, BamHI, and SalI for assembling consensus matching regions of them and subcloned into pDsRedC1 or pEGFPC1-tagged vector (Clontech, Palo Alto, CA) to generate a human archvillin cDNA fragment. All clones were also sequenced to ensure the maintenance of the proper reading frame before being amplified and purified with the Qiagen Midi Prep Kit (Qiagen, Santa Clarita, CA).

3. Preparation of C2C12 cells cultures and siRNA transfection of myoblast

C2C12 cells were grown at 37℃ in DMEM/high glucose (HG) supplemented with 10% (v/v) fetal bovine serum, 1× antibiotics (0.1 units/ml of penicillin and 0.1 g/ml of streptomycin; HyClone) in a 5% CO_2 humidified chamber. Cultures were maintained in growth medium for 24 h and subsequently were switched to differentiation medium (DM) consisting of DMEM/HG supplemented with 2% horse serum (HS; HyClone) and 1× antibiotics and 5% CO_2 for 1~9 days, which was replaced after the first day and every other day thereafter. Cells were transfected with 1 μg of siRNA plasmid by using FuGENE6 transfection kit (Roche Diagnostic Manheim, Germany) according to the manufacturer's protocol. Cells were transfected with siRNA in 12 well or 35-mm plates 24 hours after plating. For rescue experiments 200 ng of the rescue plasmid was mixed with 1 g of siRNA plasmid and then transfected.

4. RNA isolation and RT-PCR

C2C12 cells were harvested for total RNA (50 μl) immediately after treatment with oligonucleotides. C2C12 cultured on 35-mm dishes were rinsed thoroughly with cold DPBS, then overlaid with 0.5 ml of 1× trypsin in DPBS. After cell detachment was mostly complete (2 min), the supernatants were collected and each dish was rinsed with 1.5 ml of culture medium. This rinse was pooled with the previously collected supernatant. The supernatants were centrifuged for 5 min at 2500 g, and the pelleted cells were washed in cold 1× DPBS and again pelleted by centrifugation. Total RNA was carried out using TRI reagent (Sigma-Aldrich). The amount of total RNA isolated from the cells was quantified using spectrophotometric OD260 measurements on a GeneQuant pro (Amersharm Biosciences). First strand cDNA synthesis was prepared from 2 μg total RNA using the MMLV Reverse Transcriptase (Promega) using random hexamer and at a reaction volume of 25 μl as per the manufacturer's protocol. A 2 μl volume of this reaction product was PCR amplified in 20 μl reactions using e-Taq PCR kits (Takara) with these sense and antisense primers of mouse archvillin. Amplifications were performed using a thermocycler (Eppendorff) and a program as follow: 94℃ for 2 minutes (1 cycle); 94℃ for 20 seconds, 57℃ for 30 seconds, 72℃ for 3 mins (35 cycles); and 72℃ 2 minutes (1 cycle). The reaction was then held at 4℃ overnight. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also was amplified as a loading control. Products were analysed on a 2% agarose gel and photographed under UV light.

5. Protein isolation and western blots

Homogenates of mouse skeletal muscle and lysates from C2C12 cells cultured for 7 days in DM were prepared. After treatment as indicated, cells were washed twice with phosphate-buffered saline and extracted with PRO-PREPTM Protein Extraction Solution (iNtRON Biotechnology) according to the manufacturer's protocol. Equal amounts of protein were subjected to electrophoresis SDS-PAGE and transferred to polyvinylidenedifluoride (PVDF) membranes (Amersharm) in a transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 0.1% SDS, and 10 mM β-mercaptoethanol using a Trans Blot Cell (Bio-Rad, Hercules, CA). After blocking in 3% BSA, the membranes were incubated with each primary antibody, followed by incubation with a AP-conjugated secondary antibody. The protein bands were visualized using the NBT/BCIP system (Bio-Rad). Protein preparations were sufficient to produce several blots which were probed with the various primary antibodies. All blots show results from a single blot which was sequentially probed with each of the primary antibodies.

6. Immunostaining and fluorescence microscopy

The following immunostaining procedure was modified from a previous report (Carroll et al., 2000). The C2C12 cells induced to differentiate for various times (1~8 days) were removed from the incubator and rinsed with 1× DPBS. Subsequently, the cells were fixed with methanol (Merk) at -20℃ for 10 min, rinsed with two times (15 min each wash), and blocked in 1× DPBS and 3% BSA (Sigma) for 30 min at room temperature before being immunolabeled with primary antibodies. Fixed cells were incubated with primary antibodies to the Z disk protein, nebulin overnight at 4℃ or at room temperature for 1 h in humidified chamber. After twice washes in 1× DPBS for 15 min to minimize nonspecific antibody, samples were counterstained with the secondary antibodies: Alexa 488 anti-rat IgG, Alexa 488 anti-goat IgG (1:100; Molecular Probes, Eugene), TRITC conjugated anti-mouse IgG (1:50; Sigma) and TRITC conjugated phalloidin (1:200; Sigma) for 1 h at room temperature. Cells were washed extensively with 1× DPBS and mounted with Vectashield™ (Vector Laboratories, Burlingame, CA). Vectashield is common used to stained cell to prevent photobleaching. Images were digitally collected using an Olympus BX50 fluorescence microscope (Olympus, Melville, NY) and confocal microscope equipped with a 100×, 40×, or 10× magnifications. Appropriate filters were used for visualizing Alexa Fluor 488, FITC, GFP, TRITC or DAPI. Recording of the samples was done electronically using a digital camera DP70 (Olympus) connected to DP70-BSW Version 01.02 software.

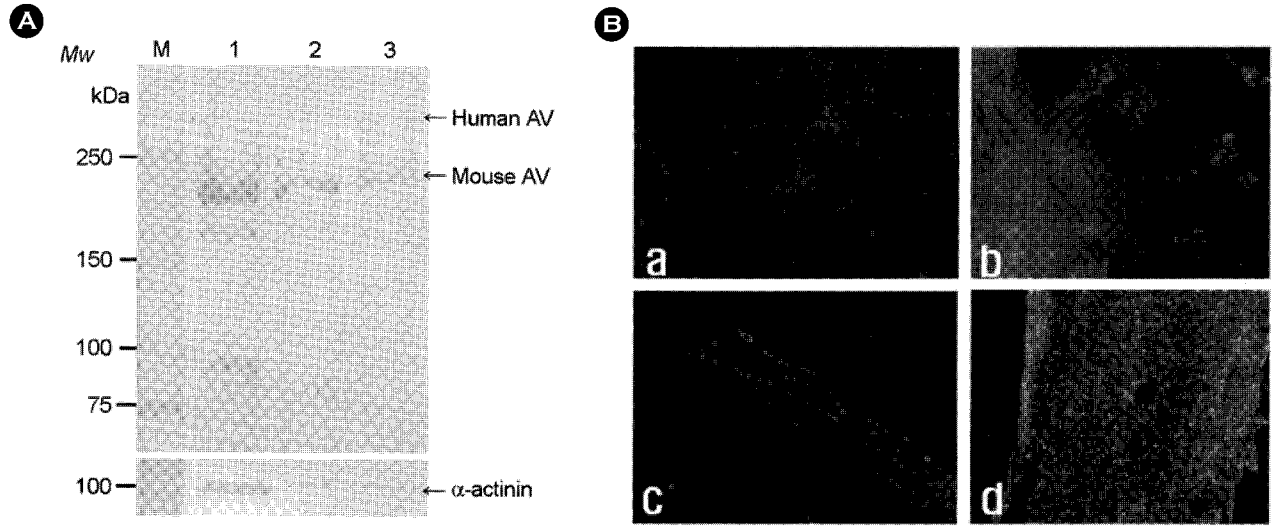

Fig. 2. Archvillin expression in C2C12 cells. **(A)** Western blots of archvillin in mouse skeletal muscle and C2C12 cells. The proteins were separated by SDS-PAGE in a 6% gel and 10% gel. Molecular weight (Mw) markers are indicated to the left of the figure. Homogenate of adult mouse skeletal muscle (lane 1), lysate from C2C12 cells cultured for 7 days (lane 2), and C2C12 cells transfected EGFPC1-human full archvillin fusion protein cultured for 7 days (lane 3) were loaded with 2 mg total protein and separated by SDS-PAGE in a 6% gel and a 10% gel. The samples were probed with the archvillin antibody (lanes 1-3). **(B)** Immunostaining of archvillin in differentiated C2C12 cells. The archvillin appeared as strong punctae at cytoplasm (a) and tip (b) in myoblast. The archvillin was also detected weakly along associated stress fiber (c) and strongly in the central region (d) of mature myofibrils after 2, 4, and 8 days from induction of differentiation. Differentiating myoblasts were stained with the archvillin antibody. Magnifications, ×1000.

RESULTS

1. Mouse archvillin in C2C12 cells

In the test to identify presence of archvillin protein in mouse skeletal muscle cell, only the 243 kDa band was detected in lysates from C2C12 cells (Fig. 2A). The size of the 243 kDa band in lane 1 and lane 2 is consistent with the size of mouse archvillin (AF317423) predicted from the deduced amino acid sequence. Alpha-actinin as a muscle specific protein marker was used as a loading control. The result was indicated the C2C12 cells express endogenous archvillin as mouse skeletal myoblast. The archvillin antibody also recognized supervillin expressed in non-muscle tissues because of conserved sequence at the COOH terminus of supervillin and archvillin (not shown). DsRedC1 or EGFPC1-human full archvillin (EGFPC1-AV) were constructed with PCR technique using appropriate designed primers, for better defining the distribution of archvillin in muscle. Expression of human archvillin protein in C2C12 cells following transfection of EGFPC1-AV was confirmed by the archvillin antibody (Fig. 2A, lane 3). Fig. 2B shows the distribution of endogenous mouse archvillin using the archvillin antibody. Archvillin shown in green revealed as punctate pattern near cell nuclei, in cytoplasm, and especially at tip and also as linear arrays throughout the cell (Fig. 2B, a-b). After identification of expression of archvillin in myoblasts, study of the dynamics of myofibril formation and myogenesis was important. This experiment was performed to investigate the apparent amounts and localizations of archvillin during differentiation along the myogenic pathway. Archvillin was dramatically induced during the course of differentiation of C2C12 cells from myoblasts to myotubes on western blots and Real Time-PCR (not shown). As differentiation progresses, the protein were observed in patterns that had the appearance of aligning filaments at peripheries of cell (Fig. 2B, c). In the stage of mature, archvillin finally appeared the narrow striated bundles throughout cells (Fig. 2B, d). The data represented the distribution of archvillin was changed along the course of myofibril assembly.

2. Knockdown of mouse archvillin and rescue by human archvillin in C2C12 cells

The siRNA technique was employed to specifically knockdown archvillin mRNA. The pSuper-neo-GFP plasmid

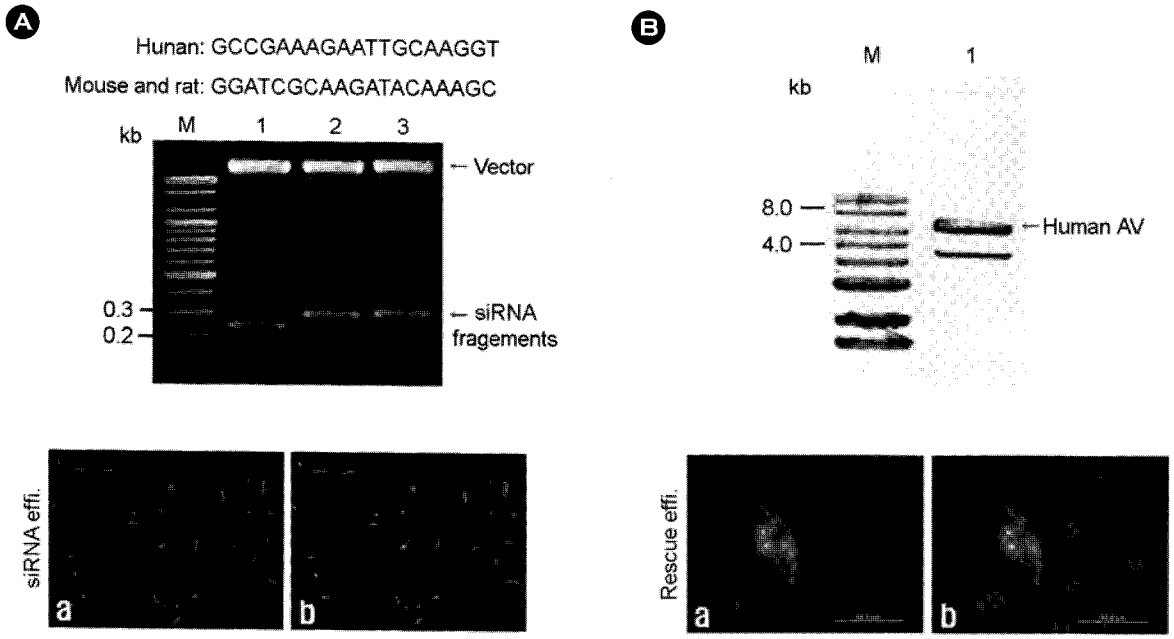

Fig. 3. The siRNA synthesis and cloning for knockdown and rescue of archvillin. **(A)** 19-mer of siRNA regions encoded mouse and human archvillin. Each pSuper-neo-GFP plasmid inserted with the specific target sequences of mouse or human archvillin was analyzed on 2% agarose gel. The samples were treated with EcoRI and XhoI. 1 kb DNA ladder marker(M); pSuper-neo-GFP vector, 248 bp (lane 1); pSuper-neo-GFP-msiRNA, 298 bp (lane 2); and pSuper-neo-GFP-hsiRNA, 298 bp (lane 3). The pSuper-neo-GFP plasmid (green) and DAPI-stained nuclei (blue) were visualized by fluorescence microscopy after transfection. The plasmid exhibited through the cells with a transfection efficiency of ~90%. Magnifications, ×200. **(B)** Human full archvillin constructed for rescue. DsRedC1 plasmid and fusion human full archvillin treated by SacI/SalI was fractionated on a 0.8% agarose gel stained with EtBr. The bands of 6.6 kb and 4.7 kb are the full-length human archvillin and DsRedC1 vector (lane 1). Markers (kb) are indicated to the left of the figure. M, 1 kb DNA ladder marker. Cells cotransfected with the pSuper-neo-GFP-msi plasmid and DsRedC1-human full archvillin in the ratio of 10:1 were stained with DAPI. Magnifications, ×1000.

was used to drive the cellular expression of specific siRNA. The 19-mer target sequences for the siRNA from homologous regions of the human and mouse archvillin were determined (Fig. 3A). The human archvillin specific siRNA was used as an experimental control to monitor nonspecific effects of expressing the mouse archvillin specific siRNA in cells in all the following experiments. The plasmids directing expression of siRNA targeting human and mouse archvillin were designated both pSuper-neo-GFP-hsiRNA (negative control and mock) and pSuper-neo-GFP-msiRNA respectively (Fig. 3A). The constructs were first tested in transient transfection assays with C2C12 cells. The cells were transfected with pSuper-neo-GFP-msiRNA, mock, and vector alone 24 h after plating and observed for 1 days after transfection (Fig. 3A, a). The transient transfection efficiency of the construct into cells was quantified by measuring the number of cells containing pSuper-neo-GFP and by comparing these with the total number of cells in the same fields visualized either by fluorescence observation of DAPI stained nuclei or by bright field illumination. pSuper-neo-GFP was visible in ≤90% of the cells after transfection (Fig. 3A, b). The plasmid of pSuper-neo-GFP was diffusely distributed in transected cells. The effect of mouse archvillin siRNA on cell viability was tested using a tryphan blue dye exclusion assay. But the cell death was undetected in the results of cell viability (not shown). The EGFPC1-AV or DsRedC1-AV construct was used to be resistant to knockdown by the mouse archvillin specific siRNA. The transient transfection efficiency of the construct into cells was quantified by measuring the number of cells containing pSuper-neo-GFP and by comparing these with the total number of cells in the same fields visualized either by fluorescence observation of DAPI stained nuclei. DsRedC1-AV was visible in ≤10% of the cells after transfection (Fig. 3B, a-b).

To evaluate the levels of archvillin protein and mRNA expression, protein and RNA were isolated from C2C12 treated with each of siRNA against mouse archvillin, mock

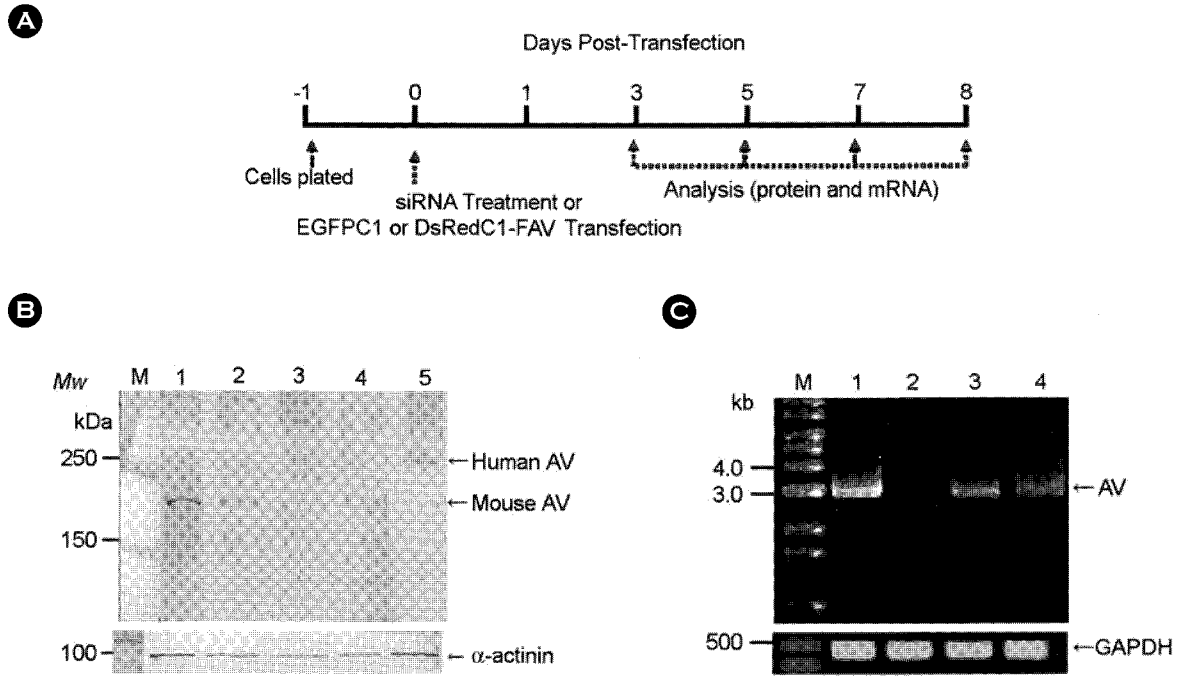

Fig. 4. Knockdown of mouse archvillin by siRNA in C2C12 cells. **(A)** Diagram illustrating the protocol for archvillin expression following siRNA treatment. **(B)** Representative western blots showing archvillin and α-actinin protein levels on day 5 after the induction of myogenic differentiation. M, protein marker; lane 1, lysates from untransfected C2C12 cells; lane 2, lysates from C2C12 cells transfected with pSuper-neo-GFP-hsiRNA plasmid. Lane 3, lysates from C2C12 cells transfected with pSuper-neo-GFP-msiRNA plasmid lane 4, lysates from C2C12 cells with transfected pSuper-neo-GFP-hsiRNA plasmid. lane 5, lysates from C2C12 cells with transfected pSuper-neo-GFP-msiRNA plasmid and EGFPC1-human full archvillin. **(C)** RT-4; lane 1, cDNA from untransfected C2C12 cells; lane 2, cDNA from C2C12 cells transfected with pSuper-neo-GFP plasmid; lane 3, cDNA from C2C12 cells transfected with pSuper-neo-GFP-msiRNA; lane 4, cDNA from C2C12 cells transfected with pSuper-neo-GFP-hsiRNA plasmid.

and vector alone on 5 days and 3 days (Fig. 4A). The mRNA expression was measured by RT-PCR using a pair of primer and the proteins were analyzed by western blots using the archvillin antibody. The expression of mouse archvillin protein was remarkably reduced by siRNA against mouse archvillin. In contrast, the transfection with mock and vector alone did not result in changed archvillin protein levels (Fig. 4B). The results of the protein levels are equal to that of the mRNA levels. Fig. 4C shows the mRNA expression of mouse archvillin is significantly inhibited in the cells treated siRNA against mouse archvillin, in comparison to the cells treated pSuper-neo-GFP-hsiRNA and pSuper-no-GFP each. The decrease persisted for 5 days after transfection and returned to normal levels of mRNA (not shown).

With the antibodies against archvillin and nebulin, its expression in cells treated archvillin, mock and vector alone was identified (Fig. 5). Dual immunolabeling for nebulin and archvillin in the cells treated pSuper-neo-GFP-hsiRNA showed that the archvillin revealed normally in the striated myofibrils with nebulin in myotube (Fig. 5a-c) But the cells treated pSuper-neo-GFP-msiRNA observed the shape shown in the early stage of myofibril assembly and myotubes did not mostly undetectable (Fig. 5d-f). The data presented the knockdown of archvillin by siRNA can inhibit the formation of the myofibril assembly as effectively. After the test of transfection efficiency, Cells were cotransfected with a mixture of the pSuper-neo-GFP-msiRNA plasmid and the EGFPC1-AV plasmid and the effect of rescue was studied with the antibodies of archvillin and nebulin. Some myotubes was partly detected in the cells and archvillin appeared as clusters along the myofibrils with nebulin. After 8 days in DM, the percentages of total fluorescent cells present as multinucleated myotubes were scored as a measure of the relative efficiency of differentiation (Fig. 5g-i). In the cells expressing either pSuper-neo-GFP alone or pSuper-neo-GFP-hsiRNA, the percentages generated the myotube are 40% or 35%. By contrast, cells expressing

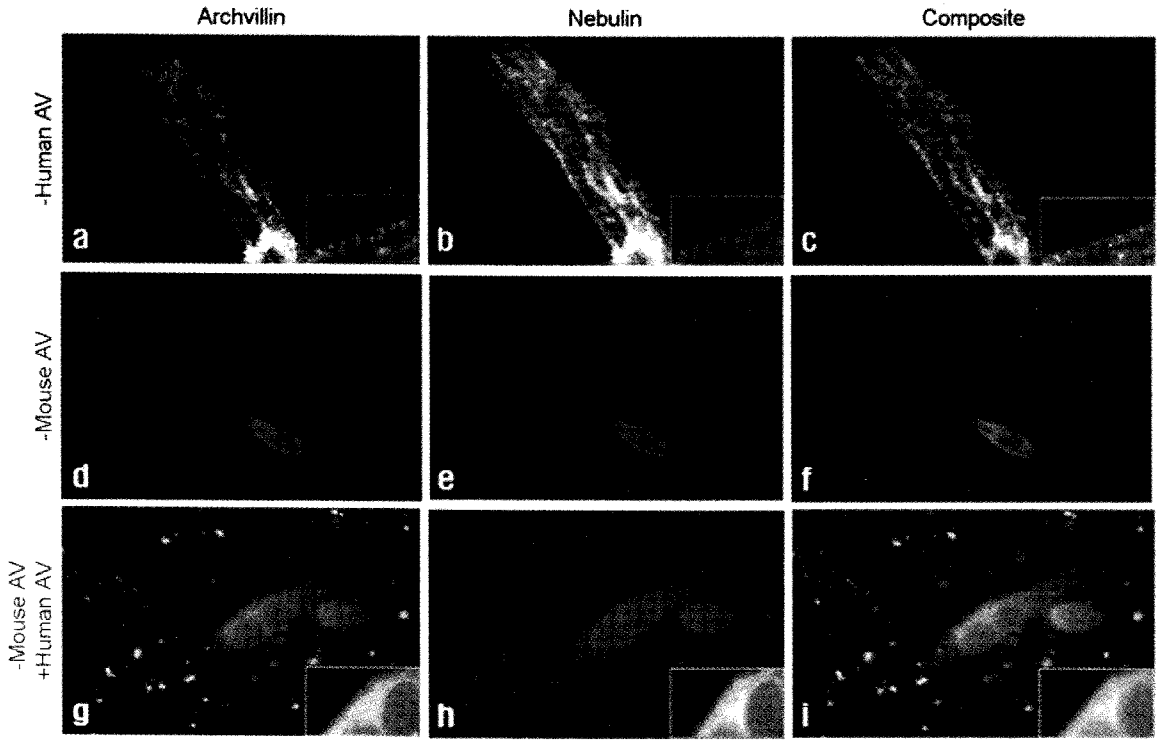

Fig. 5. Inhibition of myofibrils by siRNA against mouse archvillin. Immunostaining of archvillin and nebulin in differentiated cells. C2C12 cells separately transfected with the pSuper-neo-GFP-hsiRNA (negative control, -Human AV) and pSuper-neo-GFP-msiRNA (-Mouse AV) and induced myogenic differentiation by 8 days. In the C2C12 cells transfected with the pSuper-neo-GFP-hsiRNA, multinucleated myotubes were clearly visible (a-c). Archvillin appeared as clusters along the stress fibers and partly striated bands at peripheries of cells. The distributions of archvillin did overlap with that of sarcomeric nebulin (yellow). In the cells treated with the pSuper-neo-GFP-msiRNA, multinucleated myotube was almost undetectable (d-f). Signals of archvillin were diffused in the cell. But in the cell cotransfected with pSuper-neo-GFP-msiRNA and EGFPC1-human full archvillin in the ratio of 10:1 (-Mouse AV +Human AV), myotube formation was recovery by human archvillin (g-i). Magnifications, ×400.

pSuper-neo-GFP-msiRNA were far less efficient at forming myotubes under identical conditions. The cells contransfected with pSuper-neo-GFP-msiRNA and EGFPC1-AV fused into multinucleated cells are under 25% (not shown). The data clearly support the idea that archvillin is required for myogenesis and myofibrillogenesis, and a costameric protein involved in myofibril assembly.

DISCUSSION

In agreement with previous biochemical and immunological observations (Oh et al., 2003; Lee et al., 2003), this has been shown that archvillin associates with nebullin at the myofilbrils and expression patterns of archvillin in myogenic differentiation. In addition, this paper has been identified that knockdown of archvillin directly inhibited the myofibril assembly and myotube formation by treatment of the siRNA of archvillin in early myoblast differentiation and recovered the cell fusion and myofibril assembly by induction of human archvillin. Prior to this, the common C-terminus region of archvillin, supervillin, and smooth muscle archvillin has the villin headpiece domain (aa 2179~2214 in AF109135.1; www.scansite.mit.edu) so the analysis of archvillin isoforms suggests that archvillin can be the dimmer formation at Z-disc and costamere.

Up to now, three isoforms of archvillin were discovered and the difference of these is existence of muscle specific regions of N-terminus. Archvillin (AF109135.1) has 4 exons in muscle specific insert 1 (18~20 exons) and muscle specific insert 2 (24 exon) in comparison with supervillin (AF051850.1). Previous report described that smooth muscle archvillin (AY380816) found fellet aorta revealed 82% similarity (78% identity) with human archvillin and 1416 residues of the C-terminus are 88% identical to the corre-

sponding sequence of archvillin (Gangopadhyay et al., 2004). But considering the protein from fellet as human, it would be expected that the protein has the lack of 2 exons of muscle specific insert 1 (18 exons) and insert 2 (24 exons) of N-terminus and a perfectly correspond of C-terminus of archvillin in comparison with 10p11.2 based on human genome searching program (http://www.ncbi.nlm.nih.gov/). The data also suggest that C-terminus of archvillin also can interact with the calponin, PKC substrate and mediator of signaling through ERK1/2 like smooth muscle archvillin (Gangopadhyay et al., 2004).

Overt differentiation is indicated by the assembly of multi-nucleated syncytia, which commences 24 h after the cells are switched to low mitogen media (Galbiati et al., 1999). The results in Fig. 2 have indicated that archvillin is not expressed in nuclei in myoblast and myotube however a previous study was shown that archvillin is primarily within nuclei. By using the different antibody existence of archvillin in nuclei can be or not be. This data in Fig. 4 and 5 have presented that archvillin is an important muscle protein in myofibrillogenesis. Archvillin can strongly be therefore an important one of costameric proteins, essential and expressed at an early myofibrillogenesis stage. Also these data provide the first experimental evidence of role of archvillin in muscle differentiation with the cell culture model and siRNA depletion of archvillin. This system can be also used to study the function of other genes that are essential and expressed at an early myofibrillogenesis stage.

Finally, According to an earlier report, archvillin was expected as a costameric protein and can interact with DCG complex because of co-isolation with dystrophin and caveolin-3 and co-location of dystrophin, a representative costameric protein (Oh et al., 2003). But data of archvillin binding with dystrophin or caveolin-3 directly are not yet. According to the result of binding with C-terminus dystrophin and caveolin-3 in yeast two hybrid assays in our lab archvillin did not associate with them. So this data suggest the possibility of other proteins at costamere. The costamere-associated sarcolemmal membrane has been proposed to consist of a mosaic of domains (Rahkila et al., 2001; Williams et al., 1999a) containing dystrophin, integrins, spectrin and associated proteins, and cholesterolrich, low-density membrane domains called caveolae (Oh et al., 2003). Future study is especially required to search the archvillin binding proteins related to the costamere-associated sarcolemma because of the reason why archvillin locates at costameres. And then it is can explain the archvillin is a linker between Z-line and sarcolemma.

REFERENCES

Chen Y, Takizawa N, Crowley JL, Oh SW, Gatto CL, Kambara T, Sato O, Li XD, Ikebe M, Luna EJ. F-actin and myosin II binding domains in Supervillin. J Biol Chem. 2003. 278: 46094-46106.

Clark KA, McElhinny AS, Beckerle MC, Gregorio CC. Striated muscle cytoarchitecture: an intricate web of form and function. Annu Rev Cell Dev Biol. 2002. 18: 637-706.

Danowski BA, Imanaka-Yoshida K, Sanger JM, Sanger JW. Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. J Cell Biol. 1992. 118: 1411 -1420.

Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature. 2000. 408: 325-330.

Galbiati F, Volonte D, Brown AM, Weinstein DE, Ben-Ze'ev A, Pestell RG, Lisanti MP. Caveolin-1 expression inhibits Wnt/beta-catenin/Lef-1 signaling by recruiting beta-catenin to caveolae membrane domains. J Biol Chem. 2000. 275: 23368 -23377.

Gangopadhyay SS, Takizawa N, Gallant C, Barber AL, Je HD, Smith TC, Luna EJ, Morgan KG. Smooth muscle Archvillin: a novel regulator of signaling and contractility in vascular smooth muscle. J Cell Sci. 2004. 117: 5043-5057.

Gregorio CC, Antin PB. To the heart of myofibril assembly. Trends Cell Biol. 2000. 10: 355-362.

Gregorio GV, Choudhuri K, Ma Y, Pensati P, Iorio R, Grant P, Garson J, Bogdanos DP, Vegnente A, Mieli-Vergani G, Vergani D. Mimicry between the hepatitis C virus polyprotein and antigenic targets of nuclear and smooth muscle antibodies in chronic hepatitis C virus infection. Clin Exp Immunol. 2003. 133: 404-413.

Kaisto T. Special features of vesicle trafficking in skeletal muscle cells. Oulu. University of Oulu. 2003. 33-39.

Lee MA, Park SW, Moon HT, Ko HS, Lee YM, Kim SY, Joo YM,

Kim CR. Nebulin in Z-discs and Costameres. J Biomed Lab Sci. 2003. 9: 231-240.

Lodish H, Berk A, Kaiser CA, Krieger M. Molecular Cell Biology (5 edition). 2003. p.1152. W. H. Freeman. NY, USA.

Lu Z, Joseph D, Bugnard E, Zaal KJ, Ralston E. Golgi complex reorganization during muscle differentiation: visualization in living cells and mechanism. Mol Biol Cell. 2001. 12: 795 -808.

Oh SW, Pope R, Smith KP, Crowley JL, Nebl T, Lawrence JB, Luna EJ. Archvillin, a muscle-specific isoform of Supervillin, is a nearly expressed component of the costameric membraneskeleton. J Cell Sci. 2003. 116: 2261-2275.

Pan HJ, Uno H, Inui S, Fulmer NO, Chang C. Roles of testosterone in the growth of keratinocytes through bald frontal dermal papilla cells. Endocrine. 1999. 3: 321-327.

Pardo JV, Siliciano JD, Craig SW. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc Natl Acad Sci. 1983. 80: 1008-1012.

Pestonjamasp KN, Amieva MR, Strassel CP, Nauseef WM, Furthmayr H, Luna EJ. Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes. in the villin/gelsolin superfamily. Mol Biol Cell. 1995. 6: 247 -259.

Pestonjamasp KN, Pope RK, Wulfkuhle JD, Luna EJ. Supervillin (p205): A novel membrane-associated, F-actin-binding protein in the villin/gelsolin superfamily. J Cell Biol. 1997. 139: 1255 -1269.

Porter GA, Dmytrenko GM, Winkelmann JC, Bloch RJ. Dystrophin colocalizes with beta-spectrin in distinct subsarcolemmal domains in mammalian skeletal muscle. J Cell Biol. 1992. 117: 997-1005.

Rahkila P, Takala TE, Parton RG, Metsikko K. Protein targeting to the plasma membrane of adult skeletal muscle fiber: an organized mosaic of functional domains. Exp Cell Res. 2001. 267: 61-72.

Sanger JW, Chowrashi P, Shaner NC, Spalthoff S, Wang J, Freeman NL, Sanger JM. Myofibrillogenesis in skeletal muscle cells. Clin Orthop Relat Res. 2002. (403 Suppl): S153 -162.

Ting HJ, Yeh S, Nishimura K, Chang C. Supervillin associates with androgen receptor and modulates its transcriptional activity. Proc Natl Acad Sci. 2002. 99: 661-666.

Williams MW, Bloch RJ. Differential distribution of dystrophin and beta-spectrin at the sarcolemma of fast twitch skeletal muscle fibers. J Muscle Res Cell Motil. 1999a. 20: 383-393.

Wulfkuhle JD, Donina IE, Stark NH, Pope RK, Pestonjamasp KN, Niswonger ML, Luna EJ. Domain analysis of Supervillin, an F-actin bundling plasma membrane protein with functional nuclear localization signals. J Cell Sci. 1999. 112: 2125-2136.