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Focal adhesion kinase is essential for costamerogenesis in cultured skeletal muscle cells

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Abstract

A central question in muscle biology is how costameres are formed and become aligned with underlying myofibrils in mature tissues. Costameres are composed of focal adhesion proteins, including vinculin and paxillin, and anchor myofibril Z-bands to the sarcolemma. In the present study, we investigated the process of costamere formation ("costamerogenesis") in differentiating primary mouse myoblasts. Using vinculin and paxillin as costameric markers, we found that two additional focal adhesion components, $\alpha5\beta1$ integrin and focal adhesion kinase (FAK), are associated with costameres. We have characterized costamerogenesis as occurring in three distinct stages based on the organizational pattern of these costameric proteins. We show that both costamerogenesis and myofibrillogenesis are initiated at sites of membrane contacts with the extracellular matrix and that their maturation is tightly coupled. To test the importance of FAK signaling in these processes, we analyzed cells expressing a dominant negative form of FAK (dnFAK). When cells expressing dnFAK were induced to differentiate, both costamerogenesis and myofibrillogenesis were disrupted although the expression of constituent proteins was not inhibited. Likewise, inhibiting FAK activity by reducing FAK levels using an siRNA approach also resulted in an inhibition of costamerogenesis and myofibrillogenesis. The relationship between costamere and myofibril formation was tested further by treating myotube cultures with potassium or tetrodotoxin to block contraction and disrupt myofibril organization. This also resulted in inhibition of costamere maturation. We present a model of costamerogenesis whereby signaling through FAK is essential for both normal costamerogenesis and normal myofibrillogenesis which are tightly coupled during skeletal myogenesis.
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Keywords: FAK; Focal adhesion; Myofibrillogenesis; Costamerogenesis; "precostamere"; "nascent costamere"; "mature costamere"; Skeletal muscle

Introduction

In contractile cells, the interactions between the cytoskeleton and the plasma membrane require complex protein structures. In skeletal and cardiac muscles, costameres are important components of those structures. Costameres were originally described as electron-dense plaques rich in the focal adhesion (FA) protein vinculin and located between the plasma membrane and myofibrils (Koteliansky and Gneushev, 1983; Pardo et al., 1983; Shear and Bloch, 1985; Bloch et al., 2002). Besides vinculin, FA proteins that localize at costameres include integrins (McDonald et al., 1995; Belkin et al., 1996; Spence et al., 2002), talin (Belkin et al., 1986; Tidball et al., 1986), paxillin and Cas (Kovacic-Milivojevic et al., 2001). Integrins are the major transmembrane component of both costameres and FAs where they serve as a link between cytoplasmic proteins and proteins in the extracellular matrix (ECM) (Spence et al., 2002; Carragher and Frame, 2004). Proteins in the ECM that are known to bind to specific integrins and that align with costameres include laminin-2 (Imanaka-Yoshida et al., 1999; Bezakova and Lomo, 2001), collagens (Borg et al., 1983) and fibronectin (Kami et al., 1993). Based on their location and associations, it has been proposed that the functions of

Abbreviations: DM, differentiation medium; dnFAK, dominant negative for FAK; ECM, extracellular matrix; EDL, extensor digitorum longus; FA, focal adhesion; FAK, focal adhesion kinase; FAT, focal adhesion targeting; GM, growth medium; MAPK, mitogen activated protein kinase; siRNA, small interfering RNA; TTX, tetrodotoxin.

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costameres are to transmit contractile force laterally from the myofibrils across the sarcolemma to the ECM, to maintain the spatial organization of the myofibrils, and to insure the integrity of the muscle fibers during cycles of contraction and relaxation (Pardo et al., 1983; Shear and Bloch, 1985; Danowski et al., 1992). However, the molecular mechanisms that regulate the organization and alignment of costameres are poorly understood.

Mechanical tension is critical in regulating costameric protein expression, stability, and organization (Ervasti, 2003). The costameric constituents talin and vinculin are upregulated in response to muscle contraction through a mechanism involving nitric oxide synthase and a protein kinase G dependent pathway (Tidball et al., 1999). In cardiomyocytes in which contraction has been inhibited with a calcium channel antagonist, costameric staining for vinculin and β1 integrin is abrogated but can be recovered by washout of the inhibitor or application of a static stretch (Sharp et al., 1997). Similarly, the assembly of costameres parallels the reassembly of myofibrils in isoproterenol-stimulated quiescent cardiomyocytes (Simpson et al., 1993). Moreover, the normally transverse banded pattern of several costameric proteins is disrupted in skeletal muscle after 3 days of denervation, but is restored when the muscles are electrically stimulated (Bezakova and Lomo, 2001). In an α-actinin null *Drosophila* mutant in which sarcomeres fail to form correctly, myofibrillar attachments to the plasma membrane are disrupted (Fyrberg et al., 1990). Clearly, the processes directing costamere formation, maturation, and stability are linked to myofibril organization and muscle contraction.

The formation of aligned myofibrils, a prerequisite for productive contraction in striated muscle, represents an exquisite example of supramolecular assembly in eukaryotic cells (Clark et al., 2002). Several lines of evidence indicate that the plasma membrane and its associated cytoskeletal elements are directly involved in myofibril assembly (Dlugosz et al., 1984; Epstein and Fischman, 1991; Rhee et al., 1994; Sanger et al., 2002) and FAs may provide nucleation sites for new sarcomere addition during myofibril elongation (Lin et al., 1989; Epstein and Fischman, 1991). Thus, there appears to be an interdependence between myofibril formation and costamere formation, with FA protein signaling directing myofibril maturation and thus regulating costamere maturation and alignment, in part through contractile function.

Focal adhesion kinase (FAK) is the major cytoplasmic non-receptor tyrosine kinase that transmits bi-directional signals at FAs between the ECM and the intracellular milieu (Schlaepfer et al., 2004). One key mechanism of FAK activation is the binding of integrins, clustered at FAs, to their ECM ligands (Hanks et al., 1992; Schlaepfer et al., 2004). The localization of FAK at FAs is dependent on its C-terminal focal adhesion targeting (FAT) domain (Hildebrand et al., 1993; Parsons, 2003). The FAT domain of FAK interacts with integrin-associated proteins such as paxillin (Hildebrand et al., 1995; Schaller, 2001) and talin (Chen et al., 1995; Schaller, 2001). After integrin binding to ECM proteins, FAK undergoes autophosphorylation at Tyr397, creating a docking site for Src-family kinases and other proteins (Schaller, 2001). Src further phosphorylates FAK at several tyrosines including Tyr576, which enhances FAK catalytic activity (Calalb et al., 1995; Parsons, 2003). Activation of FAK initiates intracellular signal transduction cascades, including those involved in the mitogen-activated protein kinase (MAPK) effector cascades (Schlaepfer et al., 1994; Mitra et al., 2005) and cytoskeleton remodeling (Mitra et al., 2005). These effects, in turn, regulate cellular processes such as migration, growth, and differentiation (Schaller, 2001; Schlaepfer et al., 2004).

In differentiated muscle, FAK is present at the sarcolemma and myotendinous junction of skeletal myofibers in vivo (Baker et al., 1994; Fluck et al., 2002) and displays a striated pattern of distribution in cultured cardiomyocytes (Kovacic-Milivojevic et al., 2001). FAK is phosphorylated during myoblast adhesion to fibronectin via an α5β1 integrin dependent pathway and regulates the cell cycle (Disatnik and Rando, 1999; Sastry et al., 1999). FAK phosphorylation is biphasic during C2C12 myoblast differentiation, and disruption of this temporal pattern interferes with normal myogenic differentiation (Clemente et al., 2005). The level and phosphorylation of FAK are increased during skeletal and cardiac muscle induced hypertrophy, which is associated with increased myofibrillar content in vivo and in vitro (Fluck et al., 1999; Carson and Wei, 2000; Eble et al., 2000; Laser et al., 2000; Taylor et al., 2000; Kovacic-Milivojevic et al., 2001).

In this report, we characterize costamerogenesis in skeletal muscle using an in vitro system of primary mouse myoblast differentiation and maturation and we explore the role of FAK signaling in costamerogenesis. Our findings demonstrate that FAK signaling is essential for both costamerogenesis and myofibrillogenesis in differentiated skeletal muscle cells in vitro, and we present a model of costamerogenesis as a multistep process tightly coupled to myofibrillogenesis.

Methods

Animal

The mouse SV129 strain was obtained from Charles River Laboratories (Hollister, CA). All animals were handled in accordance with guidelines of the Administrative Panel on Laboratory Animal Care of Stanford University.

Reagents

For immunoblot and immunostaining analysis, the following antibodies were used: rabbit polyclonal anti-α-actin (Sigma Biosciences, St. Louis, MO), mouse monoclonal anti-sarcomeric α-actinin (Sigma Biosciences), rabbit polyclonal anti-FAK (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-FAK (BD Transduction Laboratories, San Jose, CA), rabbit polyclonal anti-FAK phosphotyrosine 397 (Biosource, Camarillo, CA), rabbit polyclonal anti-FAK phosphotyrosine 576 (Biosource), mouse monoclonal anti-GFP (Santa Cruz), rabbit polyclonal anti-α5 integrin (Chemicon International, Temecula, CA), mouse monoclonal anti-myosin heavy chain (Sigma Biosciences), mouse monoclonal anti-paxillin (BD Transduction Laboratories), mouse monoclonal anti-paxillin phosphotyrosine 118 (Cell Signaling, Beverley, MA), mouse monoclonal anti-vinculin (Sigma Biosciences), horseradish peroxidase-linked sheep-anti-mouse or donkey-anti-rabbit (Amersham Corp., Arlington Heights, IL), Cy3-conjugated goat anti-mouse (Amersham Biosciences, Piscataway, NJ), and FITC-conjugated goat anti-rabbit (ICN, Aurora, OH) antibodies. Tetrodotoxin was purchased from Calbiochem (San Diego, CA).

Single fibers isolation

Single fibers were isolated as described (Rosenblatt et al., 1995). Briefly, extensor digitorum longus (EDL) muscles from 1- to 2-month-old mice were removed and incubated for 1 h at 37°C in DMEM containing 0.2% collagenase I (Sigma Biosciences). Following enzymatic digestion, muscles were placed in DMEM containing 10% horse serum plus 0.5% chick embryo extract and triturated to liberate single fibers. Intact single fibers were placed in 4-chamber glass slides coated with 10% ECM gel (Sigma Biosciences) and incubated at 37°C for 6 h before fixation with 4% paraformaldehyde.

Primary cultures of myoblasts

Limb muscles from either neonatal mice (4–7 days old) or young mice (14–30 days old) that had been previously injured with a 30-gauge needle were dissociated to isolate pure populations of myoblasts as described previously (Rando et al., 1998). Primary cultures were plated on 5 μg/ml laminin-1 (Invitrogen, Carlsbad, CA)/collagen-coated dishes and amplified in growth medium (GM) consisting of Ham's F-10 (Mediatech, Inc., Herndon, VA) supplemented with 20% fetal bovine serum (Mediatech, Inc.), 2.5 ng/ml basic fibroblast growth factor (Promega Corp., Madison, WI) and penicillin (200 U/ml)/streptomycin (200 μg/ml) (GIBCO BRL). To induce differentiation, myoblast cultures were maintained in DMEM supplemented with 2% horse serum and penicillin/streptomycin. Myotubes were defined as cells with 3 or more nuclei.

Adenoviral infection

Adenoviruses expressing GFP (University of Iowa) or a FAT-GFP fusion protein (a generous gift from D. Ilic, UCSF) were amplified by infecting HEK 293 cells. HEK cells and medium were collected, frozen and thawed three times, centrifuged, and the supernatant containing adenoviruses was then aliquoted and stored. The viral titer in the supernatants was determined to be approximately $\sim 10^9$ infectious units per ml (Adeno-X™ Rapid Titer Kit; Clontech). Myoblasts were plated in GM at a density of 4×10^5 cells per 60 mm dish, on dishes coated with 5 μg/ml fibronectin (Calbiochem). The cells were infected with adenoviral constructs 6 h after plating. The medium was replaced after 24 h and cells were left for 24 h in GM before switching to differentiation medium (DM).

Silencing of FAK protein expression

Two pairs of siRNA oligonucleotides targeting different region of FAK transcript (sense (GCCCUUGGGUCAAGUUGGAUCAUUU) and antisense (AAAUGAUCCAACUUGACCCAAGGGC); sense (GGCUGUCAUCGA-GAUGUCCAGCAAA) and antisense (UUUGCUGGACAUCUCGAUGA-CAGCC)), as well as a negative control siRNA oligonucleotide (Stealth select RNAi from Invitrogen) were used in these studies. Myoblasts were plated at a density of $1.2–1.5 \times 10^5$ cells per 35 mm dish the day before the transfection. siRNA oligonucleotides (100–500 pmol/well) were transfected into myoblasts using Lipofectamine 2000 (Invitrogen). Cells were analyzed at different times after transfection, either in GM or after the induction of differentiation in DM.

Western blot analysis

After trypsinization, cells were plated on fibronectin or laminin-collagen. Cells were lysed in RIPA buffer (50 mM Tris–HCL, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, and 1% Nonidet P-40) containing aprotinin (20 μg/ml), leupeptin (20 μg/ml), phenylmethylsulfonyl fluoride (10 μg/ml), sodium orthovanadate (1 mM), sodium pyrophosphate (10 mM), and sodium fluoride (10 mM). Protein (60 μg) from total cell extract was electrophoresed on 10% SDS-PAGE gels under reducing conditions and transferred to nitrocellulose membranes (Osmonics Inc.). The membranes were blocked for 1 h at room temperature with PBS containing 0.05% Tween 20 and 1% BSA (blocking buffer) or 5% BSA for antibodies against phosphorylated proteins (anti-phosphorylated paxillin and anti-phosphorylated FAK antibodies) and then probed overnight at 4°C with primary antibody diluted in blocking buffer. All primary antibody incubations were followed by incubation with an appropriate horseradish peroxidase-coupled secondary antibody for 1 h at room temperature. An enhanced chemiluminescence (Amersham) system was used to visualize the specific secondary antibody binding.

Immunofluorescence

For immunofluorescence analysis, cultures were fixed for 10 min in 4% paraformaldehyde. Permeabilization and blocking of non-specific binding were done for 1 h with 1% normal goat serum in PBS containing 0.1% Triton X-100 (blocking buffer). Samples were incubated overnight at 4°C with primary antibody diluted in blocking buffer. Specimens were washed with PBS containing 0.1% Triton X-100, then incubated with secondary antibody for 2 h at room temperature. Hoechst dye was added to the secondary antibody incubation to label nuclei. After washes, coverslips were mounted on specimens with Vectashield (Vector, Burlingame, CA). Fluorescence was viewed with a

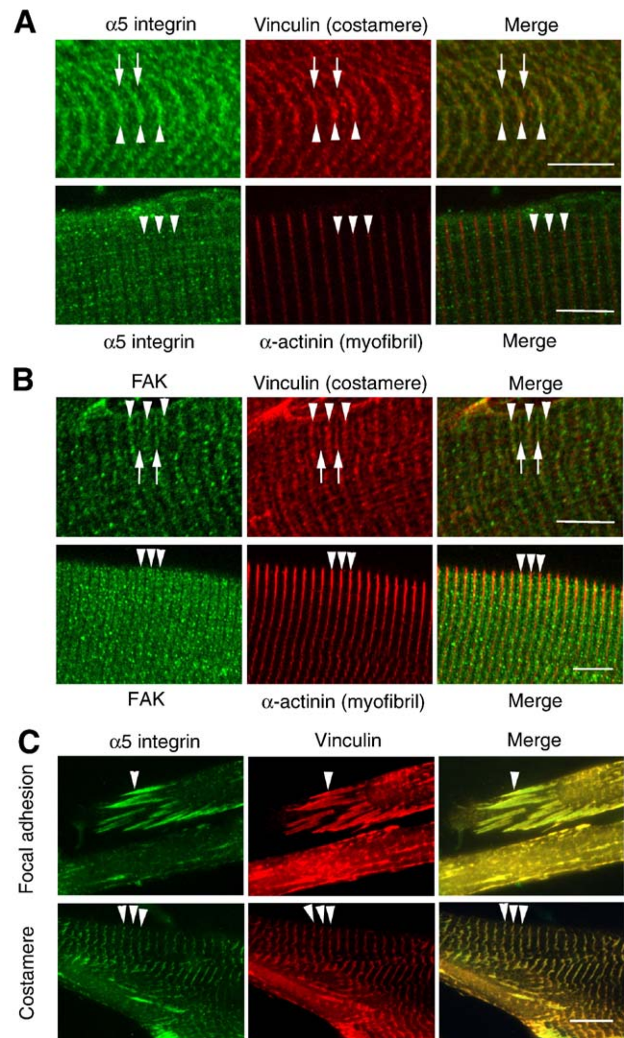

Fig. 1. Localization of α5 integrin and FAK at costameres in skeletal muscle. (A, B) Single fibers isolated from EDL muscle of 4-month-old mice were immunostained to determine α5 integrin (green) and FAK (green) localization with regard to costameric and sarcomeric striations. Co-immunostaining with vinculin (defining costameres, red) or sarcomeric α-actinin (defining Z-bands of myofibril, red) showed α5 integrin and FAK were concentrated at costameres and aligned with Z-bands (arrowheads). To a lesser extent, vinculin, α5 integrin and FAK were also present at M-bands (arrows). Imaging was performed by confocal microscopy. Scale bar, 8 μm. (C) Muscle cells undergoing differentiation in vitro were immunostained with α5 integrin (green) and vinculin (red) to determine the pattern of integrin expression with respect to FAs and costameres (arrowheads). α5 integrin co-localized with vinculin at both structures. Scale bar, 20 μm.

Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) using a 100× oil immersion objective.

Quantitative analysis of costamere and myofibril organization

Cells were fixed at the indicated times after the induction of differentiation and immunostained either with an antibody directed against vinculin to evaluate costamere organization or with an antibody against sarcomeric α-actinin to evaluate myofibril organization (Sanger et al., 2002). Quantitative analysis of costamere organization was performed by determining the percentage of differentiated cells that displayed a precostamere, nascent costamere, or mature costamere pattern (see Fig. 2). Likewise, quantitative analysis of myofibril organization was performed by determining the percentage of differentiated cells that displayed a premyofibril, nascent myofibril, or mature myofibril pattern (Sanger et al., 2002). In each case, analyses were done in randomly selected microscopic fields, in which all cells present were evaluated using a 100× objective. Between 100 and 200 cells were analyzed in three or more independent experiments. When more than one pattern of distribution was observed in a single differentiated cell, the cell was classified by the most advanced stage of costamerogenesis or myofibrillogenesis. In each experiment, half of the culture dishes were analyzed for costamere formation and half of the dishes were analyzed for myofibril formation to study these processes in parallel.

Confocal imaging and 3D reconstruction

Fluorescently labeled cells or isolated fibers were viewed using a Zeiss model LSM 510 inverted laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss, Oberkochen, Germany), equipped with both argon ion and helium-neon lasers. For double labeling, images from two different channels, one green (excited at 488 nm) and the other red (excited at 543 nm) were collected sequentially. For each image, 3D reconstruction was performed first by optically sectioning the cells from the upper to the basal surface at intervals of 0.2 μm. By using the Volocity software (Improvision), surface rendering of each section stack yielded the reconstructed images.

Statistical analysis

All quantitative data are presented as means ± SD. Statistical analysis to determine significance was performed using paired Student's t tests. Differences were considered to be statistically significant at the $P < 0.05$ level.

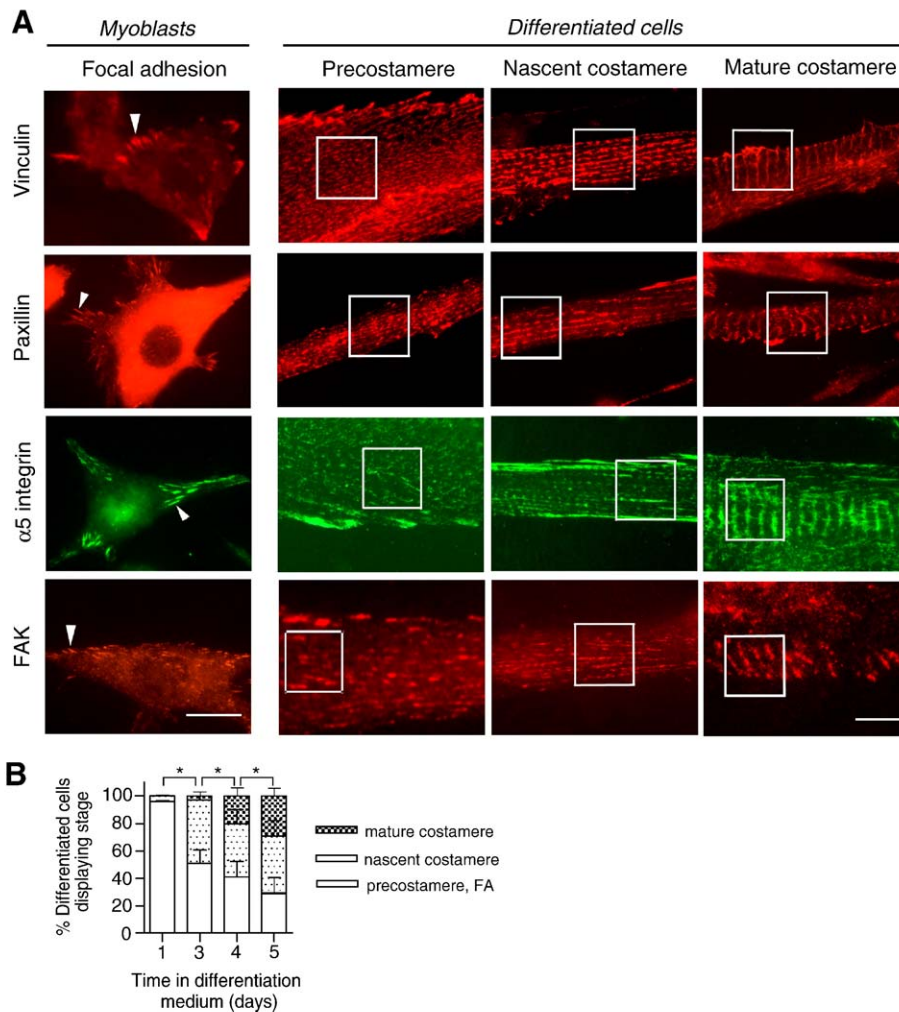

Fig. 2. Ontogeny of costameres in vitro. (A) Myoblasts and myotubes were immunostained for vinculin, paxillin, α5 integrin or FAK in order to identify the stages leading to the formation of banded costameres during myogenic differentiation. In myoblasts, only FAs were detected (arrowhead). In myotubes, costamerogenesis could be observed to proceed in distinct stages (as highlighted in boxes), starting from a finely punctate distribution ("precostameres"), progressing to longitudinal aggregates that formed along fibrillar structures ("nascent costameres"), and finally maturing to a transverse and periodic banded pattern ("mature costameres"). Scale bar, 20 μm. (B) The ontogeny of costameres was quantified by counting the number of myotubes exhibiting costameric organization, as shown in panel A, as a function of time of differentiation. Briefly, cultures were maintained for 1, 3, 4 or 5 days in DM and then were immunostained with an antibody to vinculin to identify different phases of costameric maturation. These data demonstrate quantitatively the maturation of costameres over time in culture (*$P < 0.05$, comparing levels of mature costameres).

Results

α5 integrin and FAK in mature and developing costameres

We previously demonstrated the importance of α5β1 integrin expression for skeletal muscle growth and survival in vivo and also α5β1 integrin signaling through FAK for myoblast spreading on fibronectin in vitro (Taverna et al., 1998; Disatnik and Rando, 1999). In order to explore the potential role of this integrin signaling pathway in costamerogenesis, we first examined the distribution of α5 integrin and FAK in myofibers in vivo. In isolated single fibers from adult EDL muscles, immunofluorescence analysis revealed a banded pattern of distribution of α5 integrin and FAK that co-localized with vinculin at I-bands and flanked α-actinin Z-bands of underlying myofibrils (Figs. 1A, B). The co-localization with vinculin suggested that α5β1 integrin and FAK are components of costameres in mature skeletal muscle. To a lesser extent, vinculin, α5β1 integrin and FAK could also be detected overlying M-bands, as previously observed for vinculin (Porter et al., 1992) and αν integrin (McDonald et al., 1995). α5 integrin also co-localized at FAs and costameres in mature myotubes in vitro (Fig. 1C).

To study the dynamics of costamere formation and maturation, we studied these processes during the myogenic differentiation in vitro. In the presence of low serum, mononucleated myoblasts fuse to form multinucleated myotubes and reorganize their cytoskeleton. This provides a convenient experimental system in a controlled environment to analyze the stages of formation and maturation of costameres and myofibrils. To follow the formation of costameres, we studied the differential distribution of costamere markers vinculin and paxillin, as well as α5 integrin and FAK. In myoblasts, these proteins were present at FAs (Fig. 2A). During myogenic differentiation and myotube maturation, vinculin, paxillin, α5 integrin and FAK were distributed in an abundant and finely punctate lattice ("precostamere") (Fig. 2A). In more mature myotubes, these proteins were observed in patterns that had the appearance of aligning filaments ("nascent costameres"). In the most mature myotubes, these same proteins were detected in a regular, striated pattern ("mature costameres"), typical of costameres in myofibers in vivo. A sequence of events leading to mature costameres could thus be observed in our in vitro system. Quantitative analysis was performed to determine the proportion of differentiated cells in each stage of costamere formation at different times and demonstrated that, although costamere maturation did not occur synchronously in all cells, it could be followed quantitatively in the population to study the regulation of costamerogenesis (Fig. 2B).

Temporal relationship of myofibrillogenesis and costamerogenesis

We used the same culture system to define the spatiotemporal relationship between myofibrillogenesis and costamerogenesis. In previous studies, three phases of myofibril maturation have been identified in striated muscles based on their subcellular localization and protein composition: (1) "premyofibrils" that are located at the periphery in immature cells and contain muscle specific actin and α-actinin, but not muscle specific myosin II or titin; (2) "nascent myofibrils" that are located between premyofibrils and mature myofibrils and contain titin, muscle specific myosin II and non-muscle myosin II; and (3) "mature myofibrils" that are centrally positioned in mature cells and contain muscle myosin II in A-bands but do not contain non-muscle myosin II (Rhee et al., 1994; Sanger et al., 2002). Myofibrillogenesis was assessed using an antibody against sarcomeric α-actinin, a component of the sarcomeric Z-bands (Sanger et al., 2002), in differentiating cells. In early cultures, α-actinin immunostaining revealed myotubes in which the myofibrillar organization represented different stages of myofibrillogenesis, ranging from punctate filaments (premyofibrils), to filaments that had begun to align and become periodic (nascent myofibrils), and finally to cross-striated myofibrils (mature myofibrils) (Fig. 3A). Quantitative analysis of myofibril maturation highlighted an increased myofibril organization over time (Fig. 3B), with more than 50% of cells containing aligned myofibrils by day 4 of differentiation. Thus, myofibrillogenesis and costamerogenesis proceed contemporaneously (see Fig. 2B), but with the most mature stages of myofibril formation consistently preceding that of costamere formation. At days 3, 4, and 5, the percentage of differentiated cells displaying mature myofibrils was significantly ($P < 0.05$) greater than the percentage displaying mature costameres.

Comparative distribution of FAs, costameres, and myofibrils

To understand the relationship between myofibrils, the membrane associated FAs, and costameres, we examined the

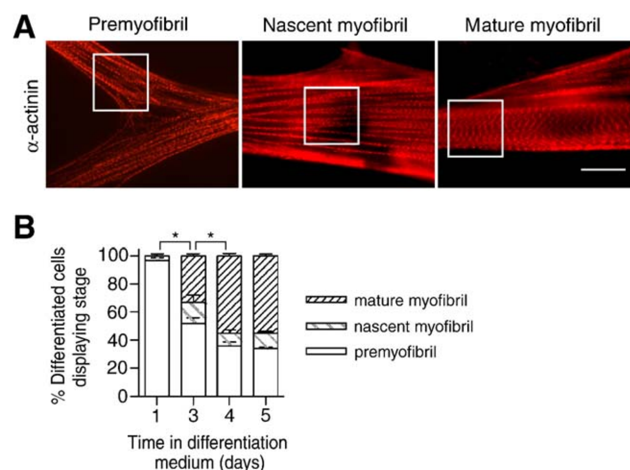

Fig. 3. Temporal pattern of myofibrillogenesis during skeletal muscle cell differentiation. (A) Cultures of differentiated cells were immunostained for sarcomeric α-actinin to examine myofibril formation in vitro. The different stages of myofibril maturation (premyofibrils, nascent myofibrils, and mature myofibrils), characterized by their morphology (Sanger et al., 2002) and highlighted in the boxed regions, were observed during myotube maturation. Scale bar, 20 μm. (B) Quantitative analysis of myofibrillogenesis during myogenic maturation in vitro. Cultures were maintained for 1, 3, 4, or 5 days in DM were then immunostained for sarcomeric α-actinin to quantify the number of differentiated cells displaying each stage of myofibrillogenesis as a function of time (*$P < 0.05$, comparing levels of mature myofibrils).

cellular distribution of these structures. FAs were particularly abundant at the ends of the myotubes (Fig. 4A). Co-immunostaining for sarcomeric α-actinin demonstrated that the extremities of premyofibrils and mature myofibrils ended in FAs (Fig. 4A), suggesting that FAs may serve as nucleation sites for sarcomere assembly and elongation as previously suggested based on studies of cultured skeletal and cardiac myocytes (Epstein and Fischman, 1991).

In order to determine precisely the cellular localization of myofibrils and costameres in myotubes in culture, confocal imaging and 3D reconstruction were performed. We analyzed separately both the "basal" surface in contact with the solid matrix and the "apical" surface that lacks any contact with the solid surface. Immunofluorescent analysis revealed that developing costameres were concentrated at the basal cell membrane but not at the apical membrane (Fig. 4B), strongly implicating the need for solid matrix contact for costamere assembly.

In early myotubes, nascent myofibrils began to align thereby creating a cross-striated pattern at the basal side of the sarcolemma in contact with the ECM, whereas non-aligned premyofibrils could be observed throughout the cytoplasm, away from the basal membrane (Fig. 4C). This observation supports the idea of the sarcolemma as an active site for myofibrillogenesis, providing the structural elements or the signaling necessary for this process.

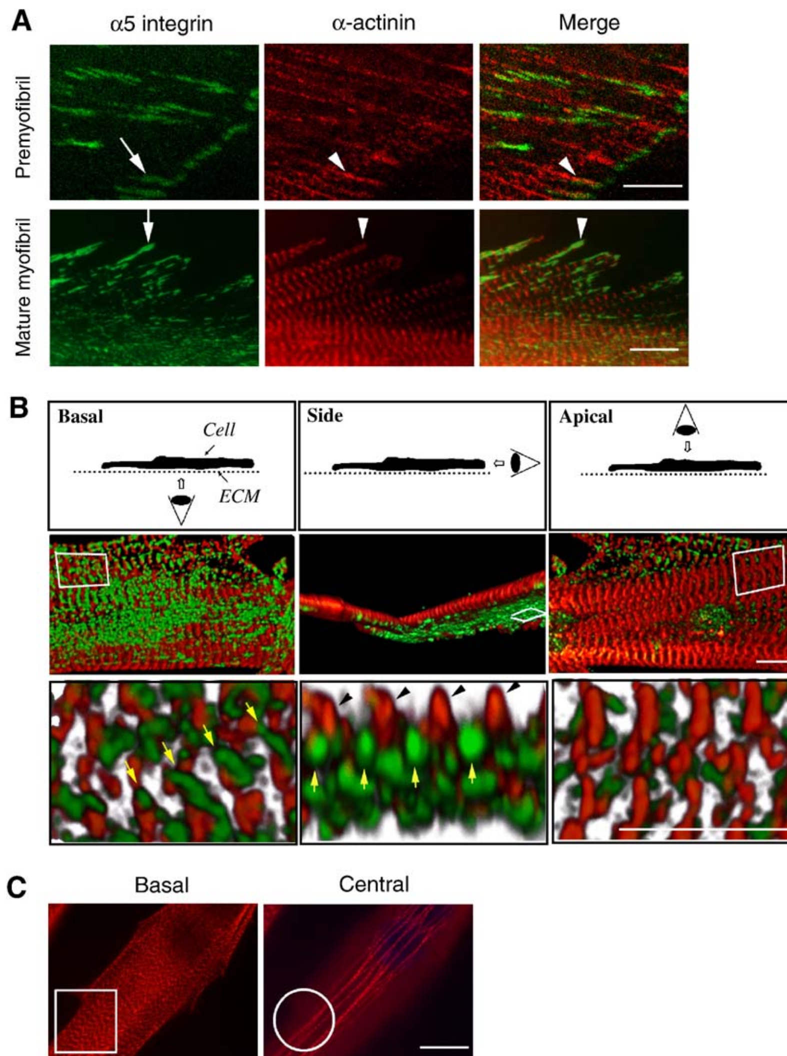

Fig. 4. Subcellular compartmentalization of myofibrillogenesis and costamerogenesis. (A) To examine the spatial relationship between maturing myofibrils and membrane-associated FAs, cultures of differentiated cells were immunostained with sarcomeric α-actinin (red) and α5 integrin (green). The ends of premyofibrils and even mature myofibrils (arrowhead) were found to associate with FAs (arrow), consistent with the idea that FAs may serve as nucleation sites for myofibrils (Dlugosz et al., 1984; Epstein and Fischman, 1991). Scale bar, 20 μm. (B) Cultures of differentiated cells were co-immunostained for α5 integrin (green) and sarcomeric α-actinin (red), followed by confocal imaging and 3D reconstruction. Costameres were concentrated at the basal surfaces of the myotubes in contact with the ECM. The lower panels are higher magnification views of the rectangular sections outlined in white in the panels in the middle row. Developing costameres (arrows) formed over underlying mature myofibrils (arrowheads). Scale bar, 8 μm. (C) Cultures of differentiated cells were stained as in panel A and examined for myofibril organization along the *z* axis of the cells. Aligned myofibrils could be observed on the basal surface of the sarcolemma in contact with the ECM (left panel, box), whereas premyofibrils were found in the central region, away from the basal surface (right panel, circle). These data further illustrate the essential role of contact with the ECM for myofibril organization during myotube maturation. Scale bar, 20 μm.

Myofibrillogenesis and costamerogenesis are independent of myoblast fusion

During skeletal muscle development or regeneration, the assembly of myofibrils and costameres follows the fusion of myoblasts into multinucleated myofibers. In order to determine whether fusion is required for costamerogenesis, we analyzed muscle cells maintained in DM. Although most myoblasts had fused to become multinucleated myotubes after 3 days, unfused cells remained. Interestingly, cross-striated myofibrils and banded costameres could be detected in many of these mononucleated cells (Fig. 5). These observations demonstrate that fusion is not required for costamere formation, consistent with previous studies showing that myoblast fusion is not necessary for myofibril maturation (Sanger et al., 1971).

Effects of focal adhesion targeting (FAT) domain expression on FA structure and muscle differentiation

These data demonstrate that proteins present at FAs redistribute to costameres upon muscle differentiation. It is well documented that FAs are important sites of mechanotransduction and that FAK is a major signaling protein present at FAs (Parsons, 2003). We thus investigated whether FAK was essential for costamerogenesis by expressing a dominant negative form of FAK, termed FAT, which is portion of the C-terminal domain of FAK. The FAT region of FAK interacts with integrin-associated proteins such as paxillin and talin (Bellis et al., 1995; Chen et al., 1995) and acts as a dominant negative inhibitor of FAK by displacing FAK from focal adhesion sites, thereby blocking its signaling functions

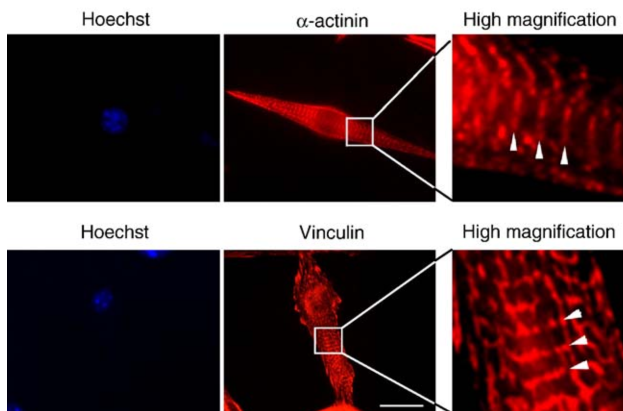

Fig. 5. Myoblast fusion is not required for myofibril and costamere formation. Myoblast cultures were induced to differentiate for 3 days, and the resulting cultures were composed of multinucleated myotubes and unfused, mononucleated myocytes. Cultures were stained (blue) with Hoechst dye (left panels) to label all nuclei and distinguish between mononucleated and multinucleated cells. Cultures were also stained (red) for either sarcomeric α-actinin or vinculin (middle panels, as labeled) to assess myofibril and costamere formation, respectively. These studies revealed that mononucleated cells contained mature myofibrils and mature costameres, most clearly seen in the boxed regions of the middle panels and shown at higher magnification in the panels on the far right. Arrowheads show mature myofibrils (top) and costameres (bottom). These data demonstrate that costamerogenesis is not dependent on myoblast fusion. Scale bar, 20 μm.

(Gilmore and Romer, 1996; Ilic et al., 1998; Almeida et al., 2000; Schaller, 2001). Myoblasts were infected with an adenovirus expressing FAT as a GFP fusion protein (Ad-FAT-GFP) or an adenovirus expressing GFP alone (Ad-GFP). Viral infections were established so that similar levels of GFP and FAT-GFP proteins were expressed 48 h after infection (Fig. 6A). Phosphorylation of FAK at Tyr576, which correlates with FAK kinase activity (Calalb et al., 1995; Parsons, 2003), and phosphorylation of paxillin at Tyr118, which represents the principal site of phosphorylation by FAK in vitro (Bellis et al., 1995), were reduced in cells expressing FAT compared to controls, confirming the inhibition of endogenous FAK activity (Fig. 6A).

FAT-GFP was detected diffusely but was concentrated at FAs (Fig. 6B). Staining of FAT-GFP expressing cells with an anti-FAK antibody that recognizes the N-terminal region of FAK (and therefore not FAT) showed that endogenous FAK was displaced from FAs (Fig. 6B), consistent with previous studies reporting a dominant negative effect of FAT by displacing FAK from FA sites. Interestingly, FAs, identified by paxillin and vinculin staining, were indistinguishable in FAT-expressing and control cells (Fig. 6C), showing that FAT inhibited FAK localization and activity without disrupting basic FA structures (Hildebrand et al., 1993).

Although FAT displaced FAK and inhibited its signaling functions, it did not disrupt the biochemical changes associated with myogenic differentiation. The early induction of myogenin was not significantly different in FAT-expressing cells compared to control cells (Fig. 6D). Even at increasing levels of FAT expression that were associated with diminishing levels of phosphorylation of FAK at Tyr397, FAT did not alter the levels of expression of differentiation-specific proteins such as myosin heavy chain and sarcomeric α-actinin after 3 days of differentiation (Fig. 6E). Therefore, inhibition of FAK signaling did not block biochemical differentiation of skeletal muscle cells in vitro.

Effect of FAK inhibition on costamere formation and myofibrillogenesis

FAK localizes primarily to FAs in myoblasts and later to costameres in mature myotubes. To test whether inhibition and displacement of FAK from FAs would affect costamere formation, myoblasts were infected with Ad-FAT-GFP or Ad-GFP and then induced to differentiate. After 6 days in differentiation, cultures were immunostained for vinculin (Fig. 7A). Costameres appeared less organized in FAT-expressing myotubes compared to control cells. Quantitative analysis revealed that the proportion of differentiated cells containing nascent and mature costameres was dramatically decreased by FAT expression. Thus, FAK localization and signaling at FAs are essential for costamere formation.

Previous studies suggested that FAs provide nucleation sites for sarcomere assembly (Epstein and Fischman, 1991) and are increased during induced cardiac hypertrophy (Eble et al., 2000). We therefore tested whether expression of FAT would also alter myofibrillogenesis. We examined whether FAK

signaling is essential for de novo myofibril assembly during skeletal muscle differentiation. Since sarcomeric proteins are induced and begin to assemble rapidly during differentiation, we expressed FAT at the myoblast stage. Following differentiation, myotubes were fixed and immunostained for α-actinin to evaluate the level of myofibril organization. Most myotubes expressing FAT contained immature myofibrils (Fig. 7B). Quantitative analysis showed more than 40% of control cells contained mature myofibrils compared to only 13% of differentiated cells that expressed FAT. These results suggested that FAK activity is critical for normal myofibril assembly during skeletal muscle differentiation and that disruption of

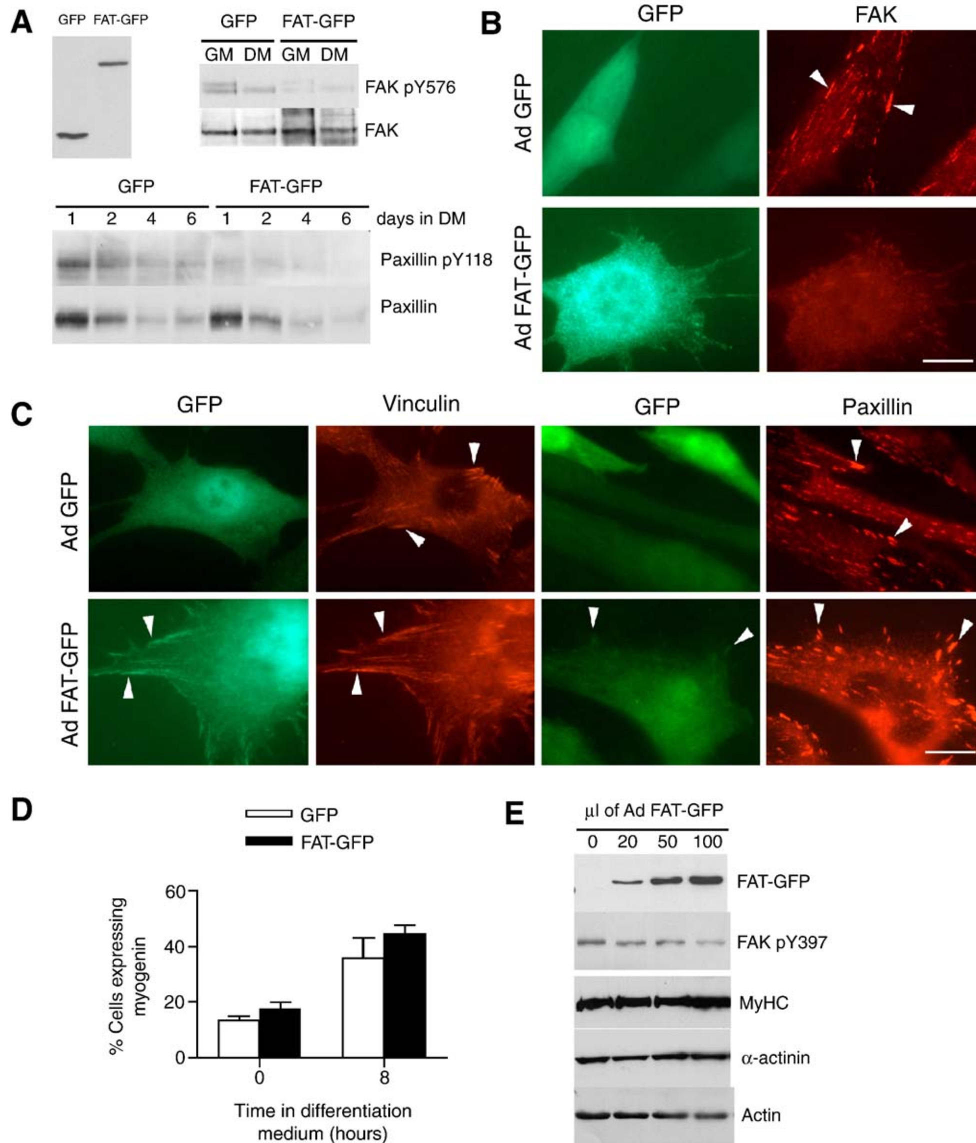

Fig. 6. FAT disrupts endogenous FAK signaling and localization. (A) Myoblasts were infected with Ad-GFP or Ad-FAT-GFP to examine the effect of FAK inhibition on costamerogenesis. Two days after infection, similar levels of GFP and FAT-GFP proteins were expressed, as assayed by Western blot using an anti-GFP antibody. FAT expression inhibited FAK phosphorylation at Tyr576 (which is correlated with FAK catalytic activity) in myoblasts in GM and after 2 days in DM. Likewise, the phosphorylation of paxillin, a FAK substrate, was inhibited by FAT expression during differentiation (up to 6 days) compared to control cells. These data confirm the inhibition of FAK activity and signaling by expression of FAT. (B) FAT- or GFP-expressing myoblasts (green) were immunostained with an antibody directed against the FAK N-terminal domain to visualize endogenous FAK (red) in the presence of FAT. Endogenous FAK was present at FAs (arrowheads) in control cells but rarely in FAT-expressing cells in which FAT was detected at FAs instead, demonstrating the displacement of endogenous FAK from FAs in the presence of FAT. Scale bar, 20 μm. (C) FAs, identified by paxillin and vinculin immunostaining (red), were examined in FAT- and GFP-expressing muscle cell cultures (green) to determine whether expression of FAT disrupted FAs structures. FAs (arrowheads) were comparable in FAT- and GFP-expressing cells, indicating that displacement of endogenous FAK from FAs did not significantly alter FA structures. Scale bar, 20 μm. (D) Skeletal myoblasts maintained in GM or in DM for 8 h were analyzed for effects of FAT expression on early biochemical changes associated with the induction of differentiation. There was no significant difference in the level of myogenin induction in FAT-expressing cells compared to control cells. (E) Myoblasts were infected with increasing amounts of Ad-FAT-GFP and then maintained in DM for 3 days to assess biochemical differentiation. Western blot analysis demonstrated that FAK phosphorylation was reduced as FAT expression increased. However, the levels of myosin heavy chain (MyHC) or sarcomeric α-actinin were comparable in FAT-expressing and control cells, further demonstrating that expression of FAT did not inhibit biochemical differentiation. Actin was used as a loading control.

FAK activity results in a marked inhibition, at least temporally, in both myofibrillogenesis and costamerogenesis.

Inhibition of FAK expression by siRNA results in impaired costamere and myofibril maturation

As a complementary approach to test the importance of normal FAK signaling in costamerogenesis and myofibrillogenesis, we used an siRNA approach to reduce FAK levels and activity. Myoblasts were transfected with different doses (100–500 pmol/well) of siRNA oligonucleotides targeting FAK or control siRNA. Two days after transfection, the level of FAK was dramatically downregulated in myoblasts treated with FAK siRNA compared to controls. FAK level remained almost undetectable after the induction of differentiation (Fig. 8A). After 5 days in differentiation medium, cells were co-immunostained for FAK and vinculin to assess costamere formation, or FAK and sarcomeric α-actinin to assess myofibril formation. Inhibition of FAK by siRNA resulted in defective maturation of both costameres (Fig. 8B) and myofibrils (Fig. 8C). These results confirm the requirement of FAK signaling for normal costamerogenesis and myofibrillogenesis.

Effect of myofibril disassembly on costamere organization in skeletal muscle

To further analyze the importance of the relationship between myofibril formation and costamere maturation, we treated cultures of differentiated cells with 20 mM KCl or 3 μM tetrodotoxin (TTX) for 3 days, both of which block contraction and lead to myofibril disruption (Bandman and Strohman, 1982; De Deyne, 2000). Whereas KCl blocks contraction by generalized membrane depolarization, TTX blocks contraction by the highly specific inhibition of voltage-gated sodium channels. Induced arrest of contraction by the addition of either KCl or TTX was observed immediately. Contraction arrest was accompanied by disruption of myofibril organization (Figs. 9A, B), as expected, and also by the disruption of mature costameres (Figs. 9A, C). These data suggest that myofibril integrity is necessary for the maintenance of mature costamere structure and alignment, and further support evidence of the tight coupling between costamere and myofibril maturation. A model of the interdependence of costamerogenesis and myofibrillogenesis, both of which are dependent on FAK signaling as shown here, is presented in the Discussion (see Fig. 10).

Discussion

How the proteins in skeletal muscle, including myofibrillar, costameric and ECM proteins, that are involved in contractile function achieve such a highly ordered structure is a fundamental question in muscle biology. In the present report, we investigated costamere formation and propose a model that describes the progressive alignment of costameric complexes with myofibrils. We have characterized the ontogeny of costameres, progressing from protein complexes at FAs to the

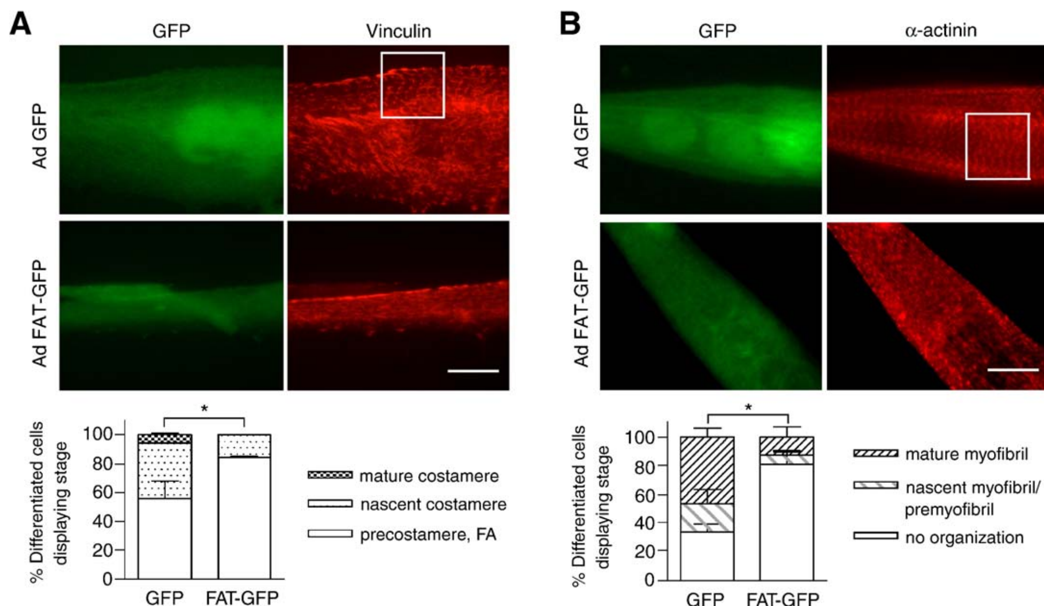

Fig. 7. FAK signaling is essential for costamerogenesis and myofibrillogenesis. (A) Cultures of differentiated cells expressing GFP or FAT (green) were immunostained with vinculin (red) to determine whether FAK signaling is necessary for normal costamere formation. Mature costameres were observed in controls (box) but only rarely detected in FAT-expressing myotubes. Scale bar, 20 μm. Quantitative analysis confirmed that costamere maturation was markedly inhibited in myotubes in which FAK signaling was inhibited. We determined the percentage of differentiated cells displaying costameres at different stages of maturation (see Fig. 2). (*$P < 0.05$, comparing levels of mature costameres). (B) Since myofibril organization begins at FAs, myofibril structures were analyzed in FAT-expressing cells (green) to determine whether the inhibition of FAK signaling would also affect myofibrillogenesis. Mature myofibrils were observed in controls (box), but myofibril organization (determined by sarcomeric α-actinin immunostaining, red) was impaired in FAT-expressing myotubes. Scale bar, 20 μm. Quantitative analysis confirmed the inhibition of myofibrillogenesis by FAT expression. We determined the percentage of differentiated cells displaying myofibrils at different stages of maturation (see Fig. 3) (*$P < 0.05$, comparing levels of mature myofibrils).

mature, striated structures seen in mature muscle fibers in vivo. This process developed in a clear sequential pattern in differentiating muscle cells in culture. Based on our studies, we found that myofibrillogenesis and costamerogenesis are tightly coupled. We observed that the basal surface of the sarcolemma associated with the ECM in culture was the primary site for both myofibribrillogenesis and costamerogenesis. Most significantly, we have found that FAK signaling at FA is essential for both myofibril organization and costamere formation.

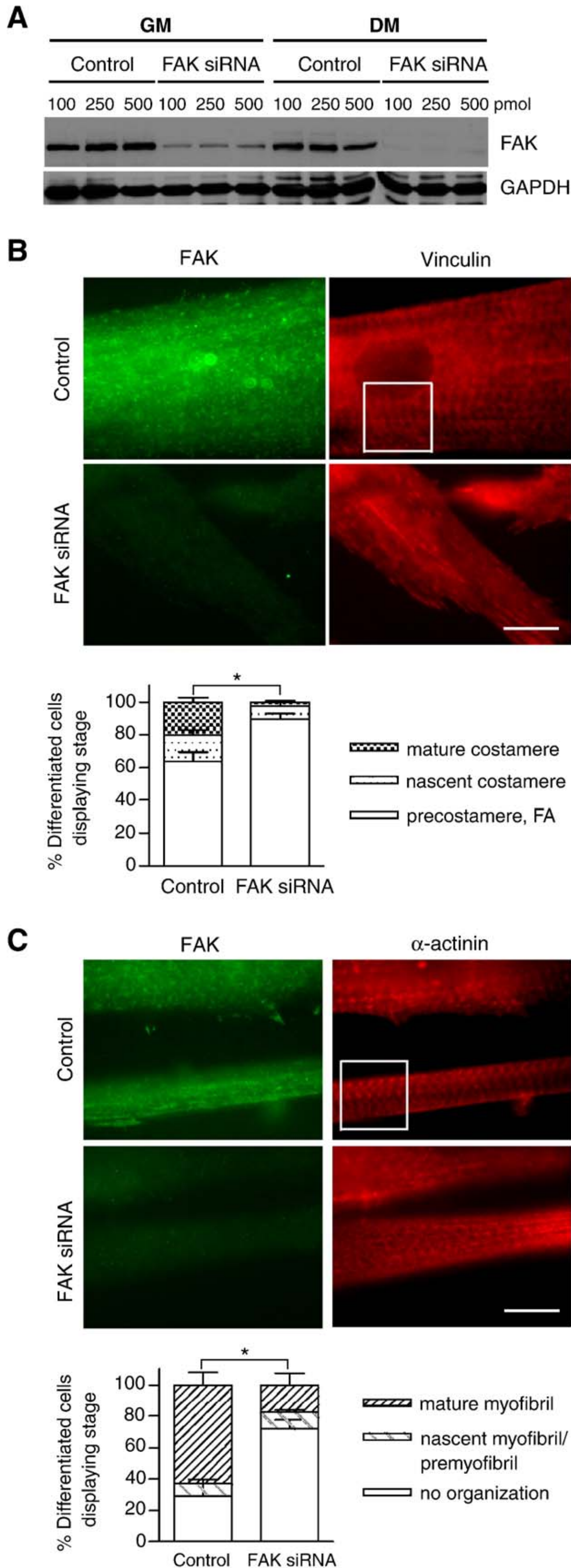

Fig. 8. Downregulation of FAK by siRNA inhibits costamere formation. (A) siRNA was used to downregulate the expression of FAK and study the effect on costamere formation. Myoblasts were transfected with different amounts of siRNA oligonucleotides (100, 250 or 500 pmol/well). Two days after transfection, cultures maintained in GM were assessed for levels of FAK or were cultured in DM for 2 days and then analyzed for levels of FAK. GAPDH was used as loading control. (B) Costamere formation was analyzed in siRNA-treated cells. Myoblasts were transfected with 100 pmol siRNA/well. After 5 days in DM, mature costameres (box) were observed in control cells but were only rarely observed in cells treated with siRNA oligonucleotides targeting FAK. Scale bar, 20 μm. Quantitative analysis (graph below) confirmed the inhibition of costamerogenesis in cells in which FAK was downregulated by siRNA (*$P < 0.05$; comparing levels of mature costameres). (C) Myofibrillogenesis in siRNA-treated cells. As with costameres, the number of cells displaying mature myofibrils was much higher in control cultures compared to cultures in which FAK was downregulated. Scale bar, 20 μm. This was confirmed by quantitative analysis presented graphically below (*$P < 0.05$; comparing levels of mature costameres).

At FAs and costameres, integrins serve as the primary transmembrane proteins that interact with ECM proteins extracellularly and both cytoskeletal and signaling proteins intracellularly (Geiger et al., 2001; Spence et al., 2002; Hynes, 2002). Deletion studies in *Drosophila* and *C. elegans* have shown that integrins are essential for myofibrillogenesis (Volk et al., 1990; Brown, 2000). In mice, muscle specific deletion of β1 integrin results in defects of myofibrils and costameres (Schwander et al., 2003). Chimeric mice with a deficiency in α5 integrin in skeletal muscle develop a muscular dystrophy (Taverna et al., 1998), and mice deficient in α7 integrin develop a muscular dystrophy with prominent myotendinous junction defects (Mayer et al., 1997). Human mutations in the α7 integrin are also associated with a myopathy (Hayashi et al., 1998). However, the downstream effectors of integrins that regulate cytoskeletal organization in muscle are unclear. Among the most well-studied intracellular signaling proteins that mediate the signal transduction initiated by integrins binding to their ECM receptors is FAK (Mitra et al., 2005). Our data showing that FAK is essential for myofibrillogenesis and costamerogenesis are consistent with the hypothesis that integrin signaling through FAK is important for both processes.

Does FAK play a critical role in myofibril and costamere assembly in vivo?

There is limited evidence as to the importance of FAK for myofibrillogenesis and costamerogenesis in vivo because mice that are homozygous null for FAK die early in embryogenesis (Furuta et al., 1995). Interestingly, FAK knockout mice die from defects in mesoderm development that are similar to those caused by the knockouts of α5 integrin (Yang et al., 1993) and its ligand fibronectin (George et al., 1993), supporting the idea of a common signaling pathway. The integrity of sarcomeres and costameres was not examined in these mice. From studies of *Drosophila*, flies completely lacking FAK56 were viable and no

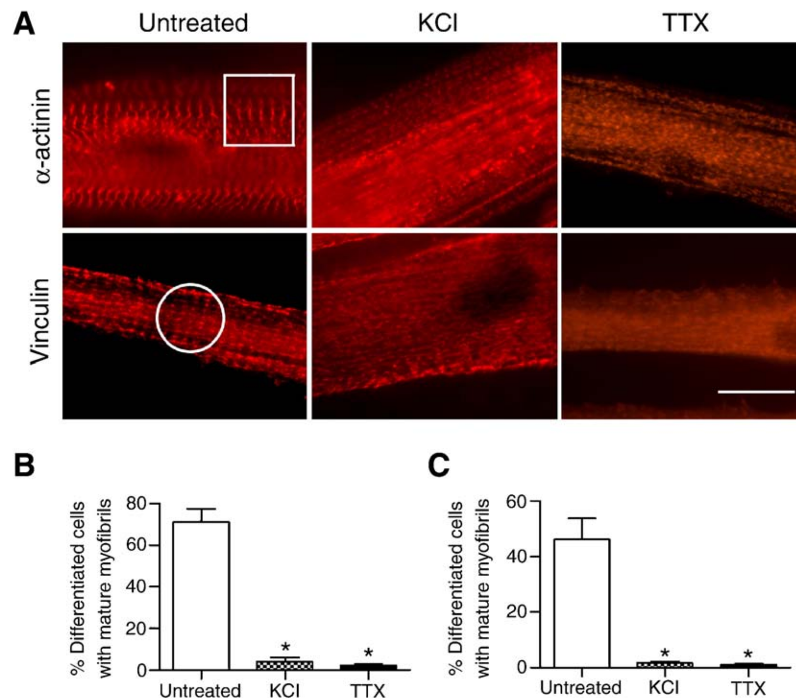

Fig. 9. Myofibril integrity is essential for costamere formation. (A) Cultures of differentiated cells were treated with 20 mM KCl or 3 μM TTX for 3 days to inhibit contractile activity. The cells were immunostained for sarcomeric α-actinin or vinculin to determine myofibril and costamere organization in the absence of contraction. Mature myofibrils (box) and mature costameres (circle) were observed in untreated cells. Myofibril organization was disrupted in cells in which contraction was blocked. In those cells with disrupted myofibrillar structure, costamere organization was similarly disrupted, consistent with the hypothesis that myofibrillogenesis and costamerogenesis are tightly coupled. Scale bar, 20 μm. (B) Quantitative analysis of the percentage of differentiated cells displaying aligned myofibrils (determined by sarcomeric α-actinin immunostaining) in untreated and in KCl- or TTX-treated cultures (*$P < 0.05$, compared to untreated). (C) Quantitative analysis of the percentage of differentiated cells displaying aligned costameres (determined by vinculin immunostaining) in untreated and in KCl- or TTX-treated cultures (*$P < 0.05$, compared to untreated).

developmental defects could be detected (Grabbe et al., 2004), suggesting that the functions of the FAK family of protein tyrosine kinases are not totally conserved between *Drosophila* and vertebrates. The results presented here predict that disruption of FAK signaling will alter myofibrillogenesis and costamerogenesis in vivo in vertebrates, but that remains to be determined.

Study of de novo myofibril and costamere organization in skeletal muscle

Most studies on myofibril and costamere formation have been performed in terminally differentiated cardiomyocytes or adult skeletal muscle, focusing on the re-assembly of myofibrils and costameres that have been physiologically or pharmacologically disrupted (Simpson et al., 1993; Sharp et al., 1997; Bezakova and Lomo, 2001). In the present study, we used myoblasts undergoing differentiation. Unlike previous studies, we were thus able to investigate de novo myofibrillogenesis (upon primary induction of myofibrillar genes expression) and costamerogenesis using an in vitro model of myogenic differentiation. Our data suggest that FAK is essential for both myogenic differentiation and myotube maturation in vitro. Previous data reported a role for FAK in myofibrillar organization in cardiomyocytes (Taylor et al., 2000). The downstream effectors of FAK signaling that regulate myofibrillogenesis are unclear. Several intracellular signaling molecules have been proposed to mediate sarcomeric organization. These include the small G proteins Ras (Thorburn et al., 1993), Rac1 (Pracyk et al., 1998) and RhoA (Hoshijima et al., 1998), MAPK (Clerk et al., 1998), and myosin light chain kinase (Aoki et al., 2000) in cardiomyocytes. Studies in other systems have documented functional interactions of a number of these signaling molecules with FAK (Schaller, 2001). Interaction of FAK with myosin heavy chain has been proposed to activate Src signaling in response to mechanical stress in cardiomyocytes (Fonseca et al., 2005). In cardiomyocytes, inhibition of the interaction between FAK and Cas, which is a substrate of FAK, impaired sarcomere stability and endothelin-induced expression of atrial natriuretic peptide (Kovacic-Milivojevic et al., 2001). In skeletal muscle cells, the increase of myofibrillar content that occurs during hypertrophy involves FAK-mediated induction of α-actin expression via a signaling cascade involving RhoA (Wei et al., 2000). As most previous studies of costamere formation in vitro have been performed using cardiomyocytes, it should be noted that significant differences in protein components, myofibrillar development, and physiologic activity exist between skeletal and cardiac muscle. The very basic process of myogenic differentiation is different in that skeletal muscle progenitors fuse to form multinucleated syncitia of hundreds or thousands of nuclei, whereas cardiomyocytes do not fuse during differentiation. It is thus likely that there are significant differences between these two tissues in terms of costamere and myofibril formation.

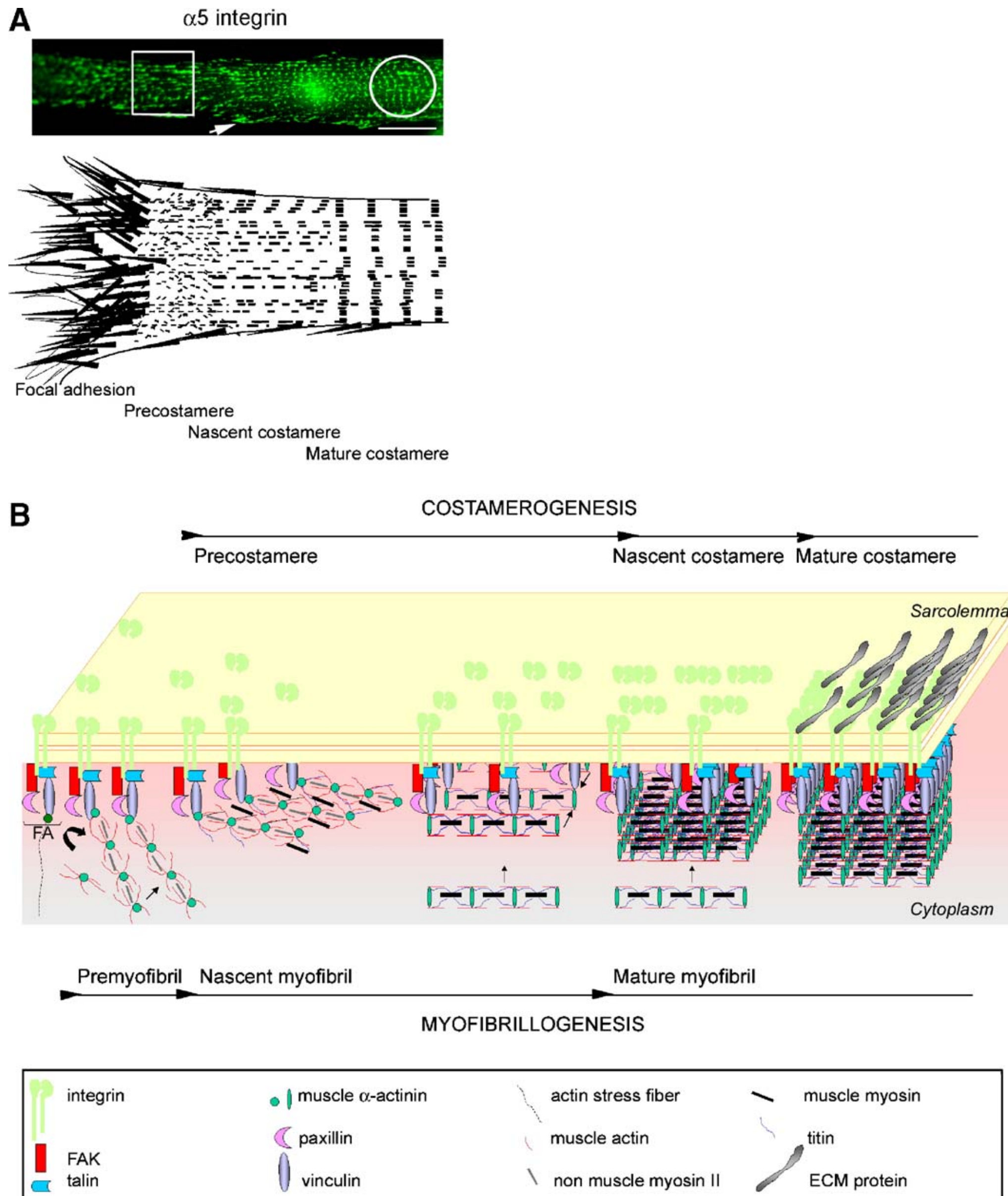

Fig. 10. Model of costamerogenesis. (A) Cultures of differentiated cells were immunostained for α5 integrin. Different phases of costamere maturation could be identified in single myotubes, including FAs (arrow), nascent costameres (box), and mature costameres (circle). Scale bar, 20 μm. This transition is illustrated diagrammatically below (precostamere stage added). (B) A model of the temporal and spatial coordination of myofibrillogenesis and costamerogenesis is presented. In this model, FAs serve as nucleation sites for sarcomere assembly and premyofibril elongation. As myofibril organization increases, premyofibrils align to form nascent myofibrils in regions adjacent to the membrane where FA proteins are recruited to form precostameres. As maturation proceeds, nascent myofibrils progressively align to form striated, mature myofibrils while nascent costameres appear using myofibrils as a scaffolding structure. Finally, costameric proteins redistributes over myofibril striations to form banded, mature costameres. The presence of integrins at costameres subsequently guides the assembly of their ECM ligands in a costameric pattern. In this model, FAK is involved in bi-directional signaling between the ECM and the cytoskeleton. In a first step, integrin binding to the ECM activates FAK and associated molecules that in turn remodel the actin cytoskeleton and regulate myofibril assembly. In a second step, maturation of myofibrils associated with contractile activity feeds back to establish a banded distribution of integrins at costameres, which subsequently directs matrix assembly into a similar costameric pattern outside the myofiber.

A dynamic model of costamerogenesis

We observed distinct phases of costamere formation and maturation based upon the localization of the major proteins constituents of FAs and costameres. First, a punctate lattice distribution was observed on the basal surface of the myotube. Then, aggregates along filamentous structures appeared progressively in a “pearl on the string” manner. As maturation proceeded, the longitudinal punctate distribution became periodic and progressively reoriented in a transverse banded pattern (Fig. 10A).

Based on our data demonstrating a tight coupling between myofibrillogenesis and costamere formation and the critical role FAK signaling in these processes, we present a model of

costamerogenesis tightly coupled to myofibrillogenesis (Fig. 10B). In cultured myoblasts, the distribution of α5 integrin, vinculin, FAK and paxillin are located predominantly at FAs. During early differentiation, sarcomeres form and assemble into premyofibrils at FAs, which are nucleation sites where FAK may transduce signals. At the sarcolemma in contact with the ECM, abundant FAs are present and premyofibrils align laterally to form nascent myofibrils. In addition to FAs that are present throughout differentiation, mesh-like precostameres begin to form and condense into nascent costameres. The progressive alignment of adjacent nascent myofibrils gives rise to cross-striated mature myofibrils. Interestingly, the later transition from nascent to mature costameres is marked by the transition from a longitudinal to a transverse pattern of distribution.

Although focal adhesion proteins regulate cytoskeletal dynamics and transduce signals from the ECM, they have equally important roles in the organization of the ECM itself (Geiger et al., 2001). The involvement of FAK signaling for ECM assembly was demonstrated by defects of fibronectin matrix organization in FAK-deficient cultures. The absence of FAK results in failure to translocate supramolecular complexes of integrin-bound fibronectin and FA proteins along actin filaments to form mature fibrillar adhesions (Ilic et al., 2004). In muscle, integrins localized at costameres mediate assembly of their ECM ligands in a costameric pattern (Borg et al., 1983; Imanaka-Yoshida et al., 1999; Bezakova and Lomo, 2001). Thus, FAK is involved in a bi-directional signaling between the ECM and the cytoskeleton. Integrin binding to the ECM activates FAK and associated molecules that regulate cytoskeletal and myofibril assembly. Then, contraction feeds back to establish a striated pattern of integrins at costameres, which in turn organize ECM proteins into a corresponding pattern outside of the myofiber. FAK is thus involved in an essential process establishing the connectivity between the contractile myofibrils, the sarcolemma, and the ECM.

In summary, we have examined the molecular mechanisms that direct costamere formation during myogenesis in vitro, and we propose a model in which FAK signaling directs both myofibril maturation and costamere alignment. Clearly, the signaling events initiated by FAK activation that regulate these processes will be of great interest in terms of understanding how these highly ordered protein complexes in skeletal muscle are formed. Such a characterization will also be important for understanding the pathophysiologic basis of myopathies and muscular dystrophies that arise when these protein complexes are disrupted.

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