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Myogenic Skeletal Muscle Satellite Cells Communicate by Tunnelling Nanotubes

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Quiescent satellite cells sit on the surface of the muscle fibres under the basal lamina and are activated by a variety of stimuli to disengage, divide and differentiate into myoblasts that can regenerate or repair muscle fibres. Satellite cells adopt their parent's fibre type and must have some means of communication with the parent fibre. The mechanisms behind this communication are not known. We show here that satellite cells form dynamic connections with muscle fibres and other satellite cells by F-actin based tunnelling nanotubes (TNTs). Our results show that TNTs readily develop between satellite cells and muscle fibres. Once developed, TNTs permit transport of intracellular material, and even cellular organelles such as mitochondria between the muscle fibre and satellite cells. The onset of satellite cell differentiation markers Pax-7 and MyoD expression was slower in satellite cells cultured in the absence than in the presence of muscle cells. Furthermore physical contact between myofibre and satellite cell progeny is required to maintain subtype identity. Our data establish that TNTs constitute an integral part of myogenic cell communication and that physical cellular interaction control myogenic cell fate determination.

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The ability of the muscle to regenerate is based on the ability of satellite cells for self-renewal and differentiation (Collins et al., 2005; Sacco et al., 2008). Satellite cells are located on the surface of the myofibres under the basal lamina (Mauro, 1961). Upon appropriate stimuli such as muscle injury, satellite cells disengage from the myofibre surface and proliferate prior to fusing together and develop into differentiated muscle fibres. It was earlier speculated that satellite cells and the adjacent myofibres communicate directly but no electrical connection between muscle cells and satellite cells was found (Bader et al., 1988). However, early studies also suggested that physical contact between the muscle fibre and attached satellite cells affects the proliferation and responses to growth factors of the satellite cells (Bischoff, 1986, 1990a,b). Furthermore, tissue and cell culture experiments indicate that fibres resulting from fusion of satellite cells have a similar pattern of myosin isoform expression as the parent fibre (Barjot et al., 1995; Rosenblatt et al., 1996). An in vivo study suggests that a profile of the fibre type is transmitted from the muscle fibre to the satellite cells and used during muscle regeneration (Kalhovde et al., 2005). These data suggest the existence of precise communication routes between muscle fibres and satellite cells. Recently, a novel way for intercellular communication was described when it was found that specific ultrafine membrane structures named tunnelling nanotubes (TNTs) linked pheochromocytoma PC12 cells to each other (Rustom et al., 2004). Later similar structures were found to connect to a variety of different cell types (Gerdes and Carvalho, 2008) and carry a variety of cargo between cells, including soluble proteins (Koyanagi et al., 2005), mitochondria (Koyanagi et al., 2005; Onfelt et al., 2006), endosome-related organelles and lysosomes (Onfelt et al., 2006) and Ca^{2+} (Watkins and Salter, 2005).

Here we tested the hypothesis that TNTs or similar structures connect muscle fibres to satellite cells and their progeny. We demonstrate that TNTs form spontaneously between satellite cells and muscle cells and between satellite cell progeny. Transport of various cytosolic material including nucleic acids, mitochondria and Ca^{2+} was observed via these TNTs. The expression of the myoblast-specific genes, Pax-7 and MyoD, depended on TNT connecting satellite cells with their host muscle fibres. Collectively these data show myogenic communication via TNTs.

Materials and Methods

Cell cultures and immunostaining

Mice (male, CD-1, 25–40 g) were killed by rapid neck dislocation, and flexor digitorum brevis (FDB) muscles were removed. Single FDB fibres were obtained by enzymatic dissociation as described previously (Liu et al., 1997). Satellite cells were isolated from muscle cells as described earlier (Conboy et al., 2003). Briefly, satellite cells were liberated by digesting the myofibres in PBS (pH 7.4) containing 1.1 U/ml dispase (Invitrogen, Carlsbad, CA, USA) and 38 U/ml collagenase type II (Sigma-Aldrich, Helsinki, Finland) for 30 min at 37°C with agitation. After trituration with a fire-polished Pasteur pipette, the resulting digest was centrifuged at 500*g* for 1 min to pellet fibre debris, filtered through a 50-μm mesh, and finally centrifuged at 1,000*g* for 5 min to pellet satellite cells. The muscle fibres were plated in 4-well dishes and the satellite cells

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isolated from the rest of the myocytes were also plated on 4-well dishes. In some experiments, myofibre conditioned medium was collected from muscle cell cultures, filtered (Millex GP, 0.22 μm, Millipore, Espoo, Finland) and stored at −20°C for future use. Immunostaining and microinjections were performed as described earlier (Morrison et al., 2006).

Patch-clamp measurements

Satellite cells were studied 24–36 h after isolation. The whole-cell patch-clamp method was used to record whole-cell currents and membrane potential. Electrode resistances were 2–4 MΩ. The intracellular solution contained (mM): K-aspartate 120, KCl 25, $MgCl_2$ 1, Na_2 phosphocreatine 2, Na_2ATP 4, NaGTP 2, EGTA 10, HEPES 5, adjusted to pH 7.2 with KOH (Yang et al., 2005). Cells were bathed in standard Tyrode solution (Tavi et al., 2003). The membrane capacitances and resistances were measured by applying a 5 mV pulse to the holding potential. Membrane potential was measured using the current clamp mode ($I = 0$). The recordings were filtered at 2 kHz and acquired at 10 kHz. Clampex 9.2 software, Axopatch-1D amplifier and Digidata 1322A A/D-D/A (Molecular Devices, Sunnyvale, CA) were used to control the command potentials and data acquisition. All electrophysiological measurements were done at +30–33°C. Data analysis was made using Clampfit 9.2 (Axon Instruments) and Origin 7.5 (OriginLab Corporation, Northampton, MA).

Confocal imaging

An Olympus Fluoview 1000 confocal inverted microscope was used. For Ca^{2+} measurements, FDB fibres were plated onto laminin-coated glass-bottom Petri dishes (P35G-1.5-10-C, Mattek Corp., Ashland, MA) and loaded with fluo-4 (3 μM, Invitrogen) for 1 h at room temperature (Liu et al., 1997; Lanner et al., 2006). Cells were superfused with DMEM bubbled with 95% O_2/5% CO_2 at 32–34°C using a custom-made superfusion system. Fluo-4 was excited at 488 nm and the emitted light was collected with a spectral detector from 520 to 620 nm through a 20× objective lens. Fibres were stimulated with 1 msec voltage pulses, 50% over the excitation threshold, through two platinum wires located on opposite sides of the Petri dish to trigger action potential-induced Ca^{2+} release from the sarcoplasmic reticulum. Alternatively, acetylcholine (ACh; 100 μM) was rapidly applied directly to the dish. Fibres were frame-scanned at 25 Hz. Fluo-4 fluorescence intensity is expressed as an F/F_0-ratio, where F is the background subtracted fluorescence intensity and F_0 is the background subtracted minimum fluorescence value measured from each cell at rest.

UV-excited autofluorescence was assessed by exciting fibres at 351 nm with a UV-laser and the emitted light was collected through a 40× objective. Fibres were frame-scanned (1024 × 1024 pixel) at the rate of 10 μsec/pixel and emission between 400 and 600 nm was collected with 2 nm steps. The emission intensities of the regions of interest in the resultant images were plotted with Olympus fluoview 1000 software. For nucleic acid staining, cells were incubated with DAPI (14 μM, Invitrogen) for 20 min, after which the fibres were fixed with 3% paraformaldehyde for 20 min and washed three times with PBS. The fibres were left in PBS and DAPI fluorescence was excited by a 351 nm laser and emission was collected from 500 to 600 nm through a 40× objective. To visualise the mitochondria, fibres were incubated with Mitotracker Red (5 μM, Invitrogen) for 30 min and then placed into the superfusion system. Mitotracker Red was excited with a 488 argon laser and the emitted fluorescence collected with a spectral detector from 520 to 620 nm through a 20× or 60× objective lens.

RNA isolation and quantitative PCR

RNA from FDB muscles and cultured myocytes and satellite cells was isolated using the RNeasy Mini Kit (Qiagen Finland, Helsinki, Finland). cDNA was synthesised using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Finland, Helsinki, Finland), and quantitative PCR reactions were performed with the ABI 7700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA) using TaqMan chemistry. The forward and reverse primer sequences were as follows: paired box gene 7 (Pax-7) 5′-GCTCCCTCCAACCACATGAA-3′ and 5′-CTCGGGTTGC-TAAGGATGCT-3′ (GenBank accession no. NM_011039); myogenic differentiation 1 (MyoD1) 5′-CACATCCTTTTGTT-TGTCACTTTCTG-3′ and 5′-AGTGGCCTCCGCAAGCT-3′ (NM_010866); myosin heavy chain IIa (MHC-IIa) 5′-CCGAAG-CGAGGCACAAA-3′ and 5′-TTGGGCTTTTTATTTCCTTAC-AACA-3′ (NM_001039545); myosin heavy chain IIb (MHC-IIb, Myh4) 5′-GAAGAGCCGAGAGGTTCACAC-3′ and 5′-CAGG-ACAGTGACAAAGAACGTC-3′ (NM_010855); myosin heavy chain IIx (MHC-IIx, Myh1, MyhIId, MyhIIx, MyhIIx/d) 5′-GAAGA-GTGATTGATCCAAGTG-3′ and 5′-TATCTCCCAAAGTTA-TGAGTACA-3′ (NM_030679); myosin heavy chain Ib (MHC-Ib, Myo1b) 5′-AATTCACAGACCAGCAGAAACTTATTT-3′ and 5′-TGCCCAACACTAGAAGGATATAAAGC-3′ (NM_010863). The fluorogenic probes were: Pax-7, 5′-Fam-TCAGCAATGG-CCTGTCTCCTCAGGTC-Tamra-3′; MyoD1, 5′-Fam-AGCCC-TCCTGGCACCCACTTTTCC-Tamra-3′; MHC-IIa, 5′-Fam-TCA-TGCGCCTGTGTGATTCTATTCCATC-Tamra-3′; MHC-IIb, 5′-Fam-ATCCATCTTTCTGTTGAGAGGTGAC-Tamra-3′; MHC-IIx, 5′-Fam-TGACCAAAGAGATGAGCAAAATGTG-Tamra-3′; MHC-Ib, 5′-Fam-AGCTCGAGGCCAGCGAACTC-TTCAA-Tamra-3′. The results were normalised to 18S rRNA quantified from the same samples using the forward and reverse primers 5′-TGGTTGCAAAGCTGAAACTTAAAG-3′ and 5′-AGTCAAATTAAGCCGCAGGC-3′, and the fluorogenic probe 5′-Vic-CCTGGTGGTGCCCTTCCGTCA-Tamra-3′.

Results

Actin filament-containing nanotubes between myogenic cells

We isolated FDB muscle fibres together with satellite cells located at the muscle cell surface under the basal lamina (Mauro, 1961). After plating FDB muscle fibres, we observed a population of satellite cells that disengaged from the cell surface and formed distinct dividing colonies of mononucleated cells beside and between the host muscle cells. More than 90% of the fibre-derived cells expressed MyoD, indicating that they were myogenic and the progeny of satellite cells (data not shown). At 12 h, we observed tube-like structures that connected satellite cells to muscle fibres (Fig. 1A) and between satellite cell progeny (Fig. 1B). The tubes had a diameter of less than 1 μm and varied in length from a few to tens of micrometers consistent with the features of recently described TNTs (Rustom et al., 2004).

In accordance with previous descriptions intercellular connections were positive for phalloidin staining of F-actin indicating that the mononucleated satellite cells were attached to the muscle cells with F-actin and henceforth will be referred to as TNTs. Initially when satellite cells are on the muscle cells, they are connected with a single actin fibre (Fig. 1C), but as they leave the host muscle cell, they remain attached to it by single or even a bundle of actin fibres (Fig. 1C). Consistent with the model that satellite cells are connected to their parental fibres by nanotubes, fluorescent dextran injected into fibres visualised TNT-like connections to satellite cells (Fig. 1D).

The finding that satellite cells stay connected by actin containing TNTs when they move apart from their parental fibres suggests de novo synthesis of F-actin. Supporting this, when either the muscle cell/satellite cell or satellite cell cultures were exposed to different concentrations of latrunculin B, which inhibits the formation of F-actin, the cell–cell connections were destroyed and further development of the satellite cells was stopped (Fig. 2).

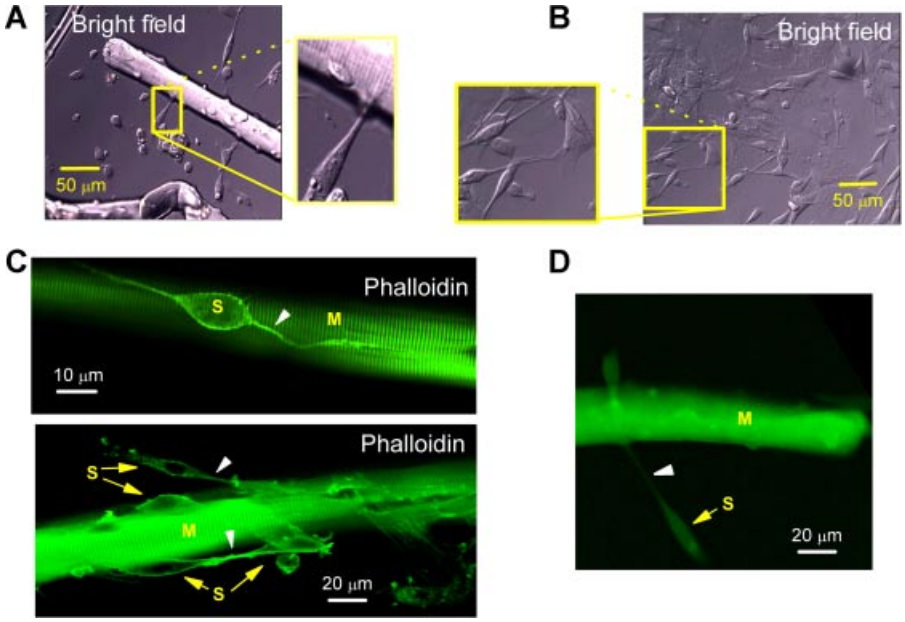

Fig. 1. TNT-like connections in muscle cell cultures. A: Light microscope picture of 24 h cultured isolated FDB muscle cells showing disengaged satellite cells (left). Some of the satellite cells remain connected to the muscle cells by nanotube-like structures (right). B: Light microscope picture of a 24 h cultured isolated FDB muscle-derived satellite cells (right), showing TNT structures between cells (left). C: Phalloidin-stained actin filaments connecting a satellite cell and underlying muscle fibre 12 h after isolation (upper) and phalloidin staining of actin filaments connecting muscle fibre and disengaging satellite cells from the surface of the muscle cell (lower). D: Confocal image from a muscle fibre injected with fluorescein conjugated dextran after 48 h in culture, showing that fluorescent dextran has diffused to attached satellite cell. In C and D yellow S marks for satellite cells and yellow M indicates myofibre. White arrowheads show examples of the connections between cells.

When we monitored muscle fibre-satellite cell progeny cultures under light microscope we noticed that formation of TNTs was a dynamic process. Satellite cells were connected from only a few to tens of minutes after which they disengaged and formed a new connection with another cell (Fig. 3).

TNTs do not couple cells electrically but transmit Ca^{2+}

Next we studied whether electrical signals were transmitted via the TNTs between muscle and satellite cells. Satellite cell progeny had a relatively depolarised resting potential of −25 mV, a high membrane resistance 2 GΩ and a membrane capacitance around 6 pF (Fig. 4A). These parameters do not differ between satellite cells connected to muscle cells and those without connections (Fig. 4A). This indicates that TNTs do not permit direct electrical coupling between cells, in line with a previous report (Bader et al., 1988). We estimated that TNTs are practically electrically uncoupled to attached cells when the TNT resistance is above 10 GΩ and the TNT resistance equals the total resistance measured from the coupled satellite cell (Fig. 4B). We saw no active electrical signals in satellite cells when action potentials were generated by current pulses in coupled muscle cells. Nevertheless, we found that satellite cells have a depolarisation-activated sodium current (Fig. 4C), which in theory makes them electrically excitable. However, the depolarised resting potential of these cells (approximately −25 mV) is likely to incapacitate the voltage-activated sodium channels and consequently prevent the generation of the action potentials.

In skeletal muscle, electrical activity of the muscle cells is triggered by ACh liberated from nerve terminals. It was previously found that activated satellite cells do not express

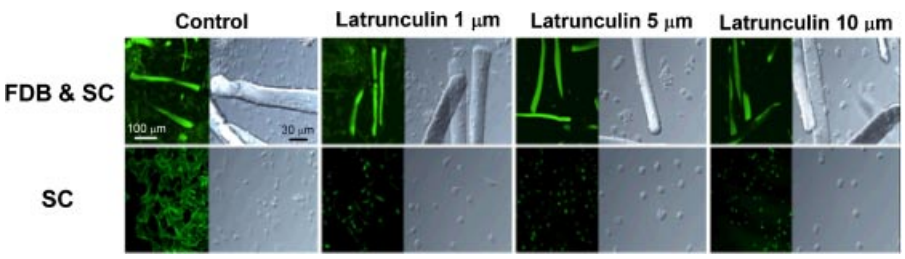

Fig. 2. Latrunculin B destroys connections in muscle fibre/satellite cell cultures and in satellite cell cultures. Cells were isolated from mouse FDB muscle, cultured 48 h and then exposed to different concentration of latrunculin (1, 5 and 10 μM) for 24 h. Upper row of images are taken form cultures containing satellite cell progeny with their parental FDB muscle fibres (FDB and SC), and lower row of images show corresponding images from satellite cell cultures (SC). In each picture the left part shows the phalloidin-stained culture and the right part shows a light microscopy image from the corresponding area.

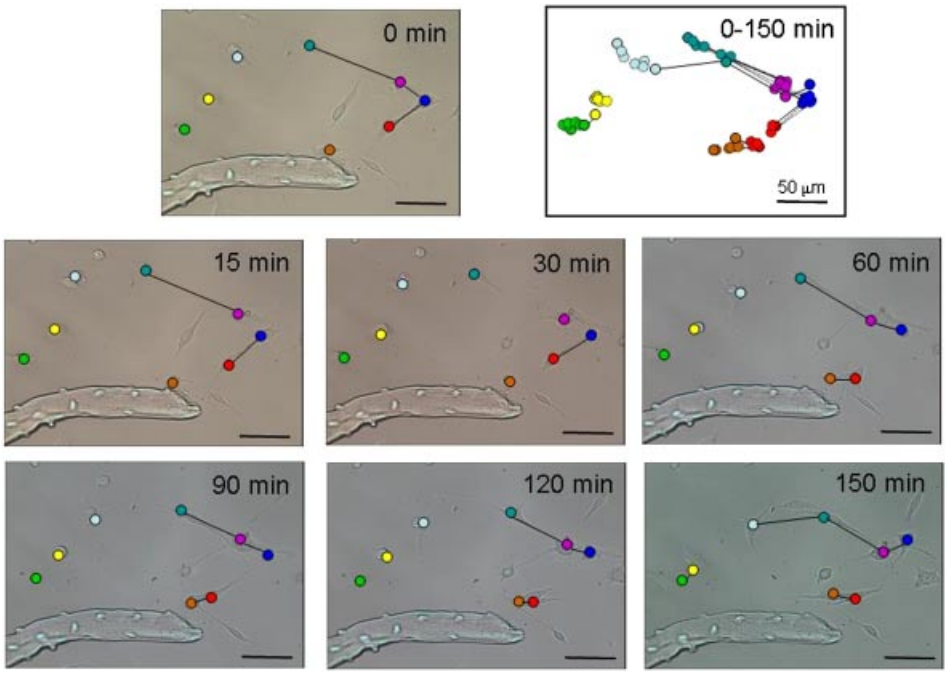

Fig. 3. Formation of the TNT connections between satellite cells in culture is a dynamic process. Time-lapse pictures from 48 h cultured muscle fibre-satellite cells co-cultures. Light microscope pictures (from 0 to 150 min) show 8 different satellite cells marked with circles of different colours. The location of each cell at all given time points is marked with the circle of corresponding colour. TNTs between cells at each time point are marked with black lines between circles. In the upper right picture, all of the locations of individual cells and connections between cells during 150 min are overlaid.

ACh receptors after isolation (Bader et al., 1988). In line with this, application of ACh did not induce any current in either solitary or attached satellite cells (Fig. 4D). Since TNTs can transmit Ca^{2+} signals between and among dendritic cells and THP-1 monocytes (Watkins and Salter, 2005), we next tested if TNTs transmit increases in muscle fibres Ca^{2+} to the attached satellite cells. Application of 100 μM ACh induced a fast and large Ca^{2+} increase in the myofibres (peak response 2.8 ± 0.25 ($\Delta F/F_0$) $n = 6$) after which Ca^{2+} increases were detected in the attached satellite cells and their progeny with a distance-related delay (Fig. 4E). These signals were transmitted through the TNTs because satellite cells not connected by TNTs to muscle fibres showed no change in Ca^{2+}. Similar results were obtained with electrical stimulation (10 Hz train), which induced large [Ca^{2+}] increase in muscle cells (peak response 2.2 ± 0.18 ($\Delta F/F_0$) $n = 5$), and a delayed prolonged increase in attached satellite cells, but no change in the non-attached cells (Fig. 4F). Some myofibre-attached satellite cells exhibited a Ca^{2+} response with an amplitude that appeared to be larger than in the adjacent muscle cell, which is suggestive of a mechanism potentiating Ca^{2+} signals in satellite cells. However, this potentially interesting observation warrants further studies with a more quantitative approach to analyse the amplitude of Ca^{2+} signals.

TNTs contain cytosolic components; nucleic acids, NADH and mitochondria

Cytosolic UV-excited emission is dominated by fluorescence from mitochondrial energy metabolites, predominantly nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) (Aubin, 1979; Andersson et al., 1998). Thus, we used UV-autofluorescence to analyse if cytosolic components are transported via TNTs. The emission spectrum of the TNT connecting two satellite cell progeny was almost identical to that measured from the satellite cell body indicating that TNTs contain material originating from the cytosol (Fig. 5A). DAPI stains several moieties (Aschar-Sobbi et al., 2008), but produces the brightest fluorescence when bound to the densely packed RNA and DNA in the nucleus (Kapuscinski, 1990). In addition to bright staining of the myonuclei and satellite cell nuclei, DAPI induced punctuate staining along the TNTs (Fig. 5B). This suggests that the TNTs contained nucleic acids. In some cell types, TNTs contain and transport mitochondria (Koyanagi et al., 2005; Onfelt et al., 2006). Labelling of satellite cell cultures with the mitochondria specific dye Mitotracker Red produced bright punctuate spots in the TNTs, indicating that they contained mitochondrial material. The Mitotracker Red staining strongly resembled the staining pattern obtained with UV-elicted autofluorescence and DAPI (Fig. 5C). When muscle-cell cultures were loaded with fluo-4, fluorescent particles were located in the TNTs and these moved unidirectionally from muscle fibre to satellite cell at average speed of 20.7 ± 2.3 μm/h (analysed in 14 TNTs) (Fig. 5D).

Impact of the nanotubes on the maturation and differentiation of the satellite cells and the myosin isoform expression of the myotubes

An interesting question is whether TNTs transmit information between muscle fibres and satellite cell progeny and how this affects cellular differentiation. To test this we compared the expressions of Pax-7 and MyoD, which are transiently co-expressed during myogenesis (Reimann et al., 2004). We found that co-culturing the satellite cell progeny with muscle cells accelerate the activation and differentiation of the satellite cells as shown by earlier expression of MyoD (Fig. 6A) as well as

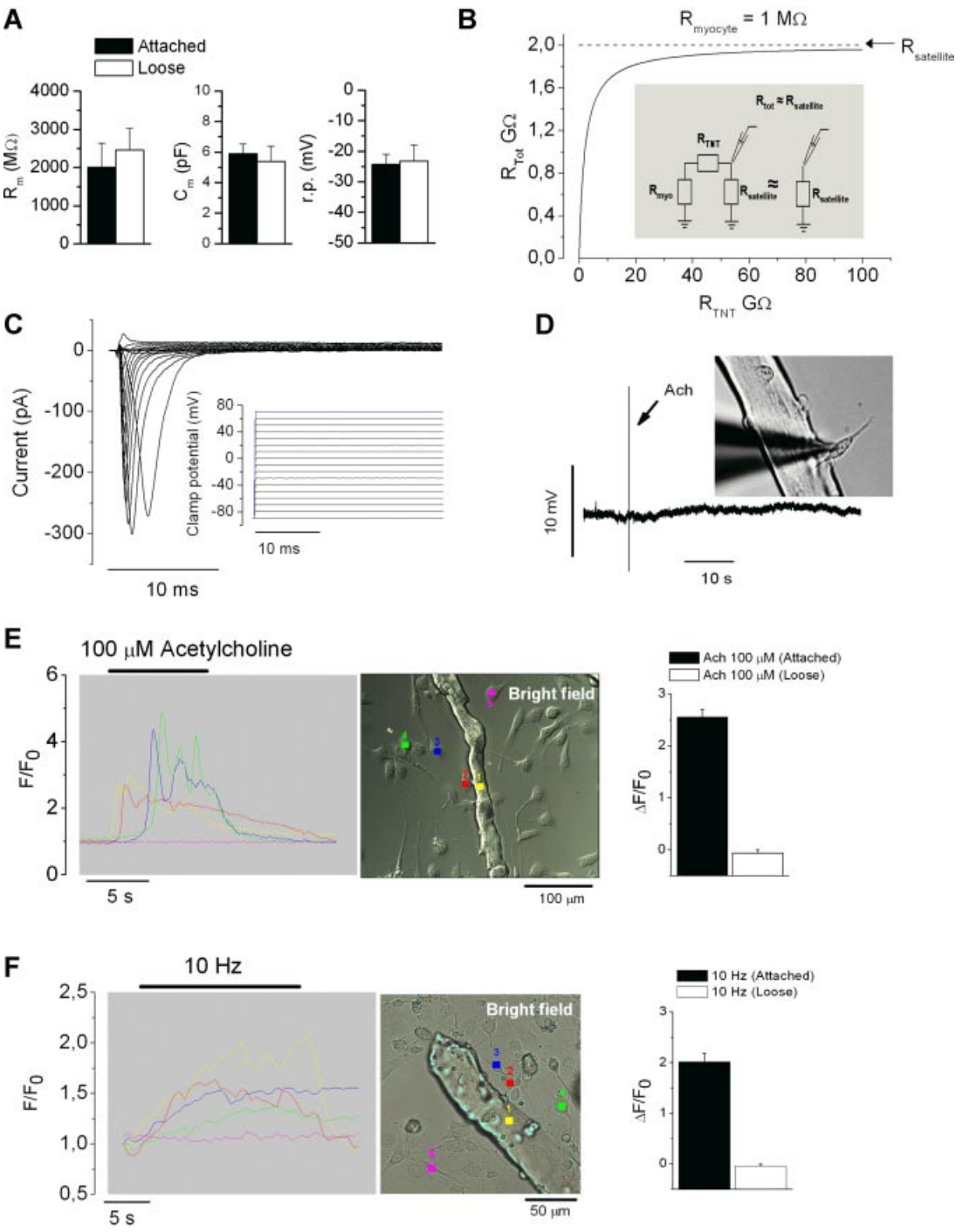

Fig. 4. TNT coupled myogenic precursors are not connected electrically but transmit Ca^{2+} signals. A: Comparison of membrane resistance (R_m, left), membrane capacitance (C_m, middle) and resting potential (r.p., right) between loose satellite cells and those attached to myocytes. B: Theoretical relationship between resistances of the TNTs (x-axis) and total resistance measured from the satellite cell connected to a muscle cell (with 1 MΩ resistance), predicting that at TNT resistances above 20 GΩ the total resistance approaches the resistance measured from the satellite cell, and TNT cannot couple cells electrically. C: Whole-cell inward currents measured from a voltage clamped (−90 to +70 mV, in 10 mV steps shown in insert) in a 24 h cultured satellite cell. D: Membrane potential of satellite cell connected to muscle cell (light microscope picture, insert before and after application of acetylcholine (100 μM)). E: Ca^{2+} signals (fluo-4 intensity) from satellite cells or muscle cell upon ACh application (left). Origin of the signals on the left part are marked with corresponding colour rectangles in the light microscope image on the middle. Note a non-connected satellite cell with no response (magenta). On the left averaged calcium responses of the satellite cells connected (attached, n = 37 cells) or non-connected (loose, n = 22) to myofibre induced by Ach application. Data were collected by frame-scanning six myofibre and surrounding satellite cells. Ach induced 2.8 ± 0.25 ($\Delta F/F_0$) peak responses in the stimulated myofibre (n = 6). F: Ca^{2+} signals (fluo-4 intensity) from satellite cells or muscle cell (left) upon 10 Hz electrical stimulation. Origin of the signals on the left part are marked with corresponding colour rectangles in the light microscope image on the middle. Note a non-connected satellite cell with no response (magenta). On the left averaged calcium responses of the satellite cells connected (attached, n = 28 cells) or non-connected (loose, n = 29) to myofibre induced by 10 Hz stimulation. Data was collected by frame-scanning five myofibre and surrounding satellite cells. Ten hertz stimulation induced 2.2 ± 0.18 ($\Delta F/F_0$) peak calcium response (n = 5) in the stimulated myofibre.

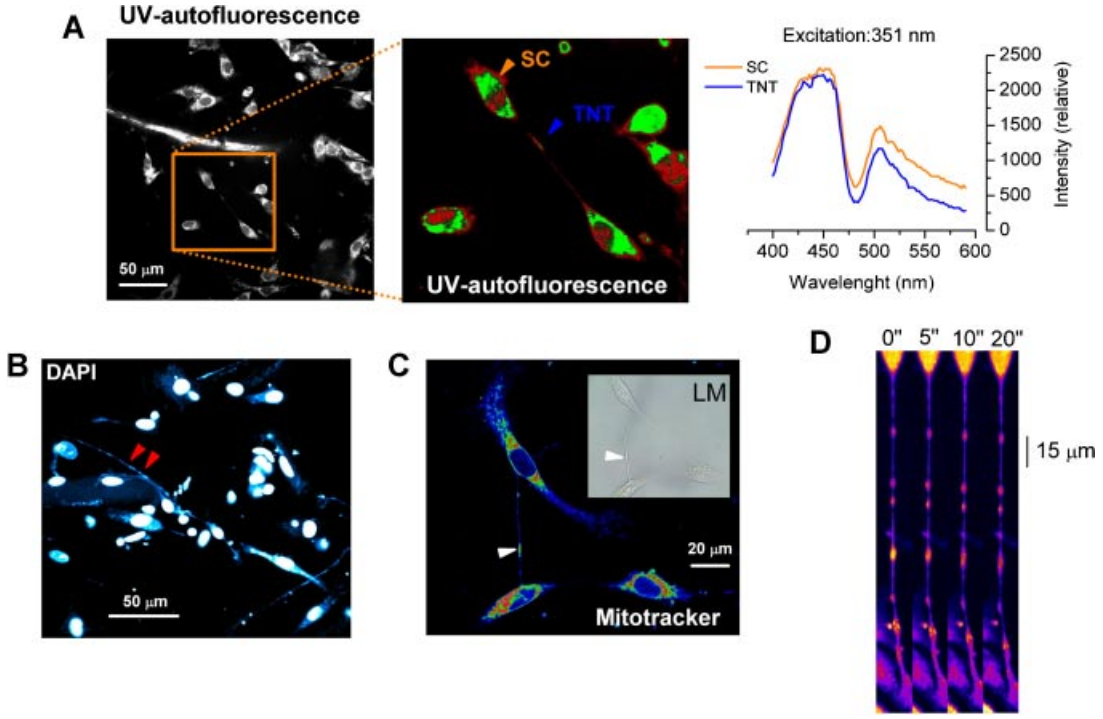

Fig. 5. TNTs actively transmit solid cargo between cells. A: UV (351 nm) excited autofluorescence (emission 400–500 nm) from muscle cell-satellite cell co-culture at 24 h (left). Picture with enlarged scale (middle) shows two satellite cells connected with a TNT. Arrowheads show satellite cell cytosol (orange) and a bulge in the TNT (blue). Emission spectrum (400–600 nm) of satellite cell cytosol and the TNT are similar (right). B: DAPI-stained (shows up as white) muscle cell-satellite cell culture, red arrowheads indicate DAPI-stained particles in TNT. C: Satellite cells in culture stained with Mitotracker Red reveals Mitotracker fluorescence in a TNT connecting two satellite cells. D: A 20 sec time-lapse series of confocal images from fluo-4-loaded satellite cells after 12 h culture showing connecting TNT with particles moving from the muscle fibre to the satellite cell.

increased expression of Pax-7 (Fig. 6B) compared to cells cultured without their parental fibres.

It is tempting to speculate that intercellular connections between myofibres and satellite cells significantly influence the properties of the developing myotubes originating from the satellite cells. To study this we cultured either muscle cells (with accompanying satellite cells) isolated from mouse FDB muscles or satellite cells and their progeny alone liberated from the surface FDB fibres. After 7 days both cultures were confluent and contained mostly multinucleated myotubes. We measured the expressions of the different myosin heavy chain isoforms (MHCs IB, IIA, IIB and IIx) in these multinucleated myotubes. The adult FDB muscle expressed predominantly type IIa myosin (Fig. 6C left). Interestingly, satellite cell progeny cultured together with FDB muscle fibres formed myotubes expressing predominantly myosins IIa, IIb and IIx, whereas cells grown without parental muscle fibres expressed mainly myosin isoforms IIx and Ib. This effect was not due to secreted factors since cultured isolated satellite cell progeny exposed to myofibre conditioned culturing medium did not shift the myosin isoform expression of the developing myotubes towards the FDB pattern of myosin isoforms. Also, the amount of MHC mRNA expression was markedly lower in satellite cells cultured without than with muscle fibres.

Discussion

In the present study we show that TNTs readily exist between the satellite cells and muscle fibres and activated myogenic cells. In culture TNTs provide a route for transport of intracellular material, including nucleic acids, ions and even cellular organelles like mitochondria between muscle cells and satellite cells. Disruption of this connection before activation of the satellite cells affects not only the speed and the extent of activation, but also the myosin expression profile of the myotubes they will subsequently form. While the precise mechanism of this phenomenon remains elusive, we propose that intercellular transport via TNTs might be involved.

TNT transport involves active transport of vesicular structures containing cytosolic material (Rustom et al., 2004; Onfelt et al., 2006; Gurke et al., 2008; Veranic et al., 2008). Here we noticed that a freely diffusible substance such as fluo-4 seems to have punctuated locations in the TNT, suggesting that fluo-4 might be trapped into vesicles rather than diffusing freely. Accepting this, it seems likely that cytosolic material such as nucleic acids and metabolites, but also solid particles like mitochondria which we saw in the TNTs, are most likely transported between cells by means of vesicular transport. However, vesicular transport is not the only form of communication via TNTs. Although TNTs do not establish a direct electrical contact between cells, they allow ions like Ca^{2+} to diffuse between coupled cells (Fig. 4E,F) (Watkins and Salter, 2005).

The ability to form TNTs seems to be an inherited property of the satellite cells. Initially when satellite cells are attached to the myofibres, at least some of them seem to have a stable TNT contact with the muscle cell. However, when satellite cells are activated TNT formation seems to be a rather dynamic process. Instead of being static structures, TNTs seem to be assembled and disassembled vigorously (Fig. 3), just like the dynamic

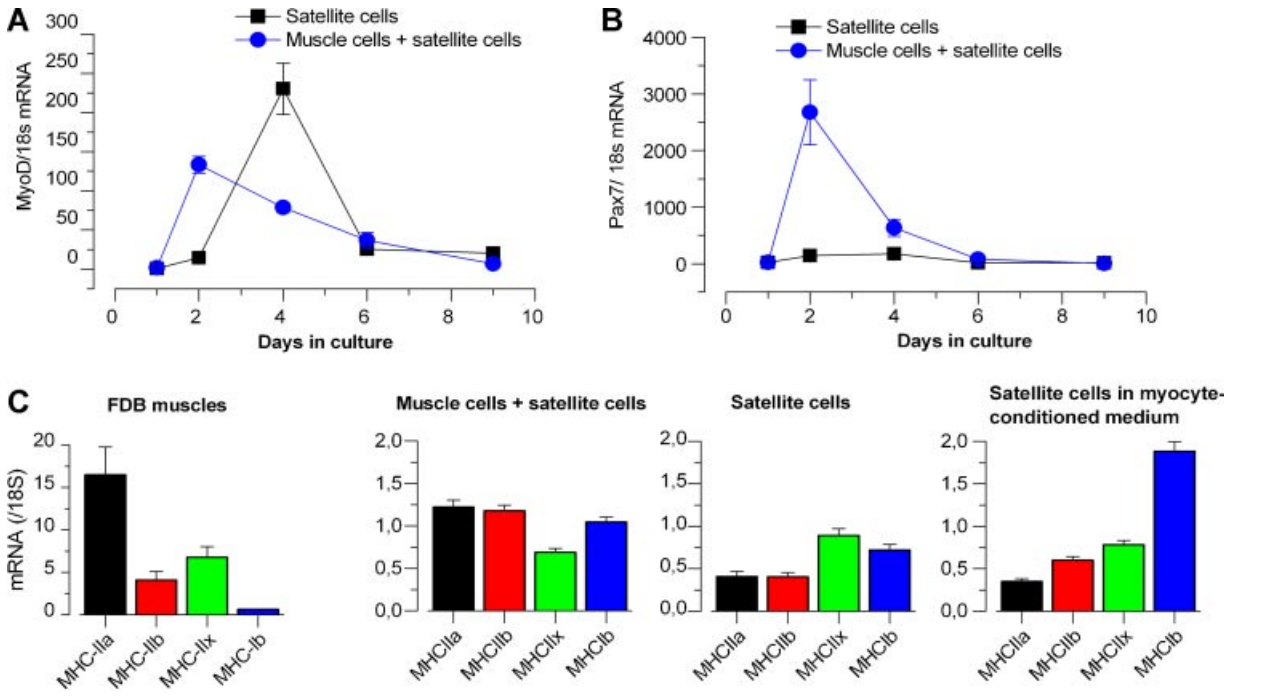

Fig. 6. Physical intercellular contacts maintain subtype identity in myogenic progenitors. Expression of (A) MyoD and (B) Pax-7 in satellite cell cultures or muscle cell-satellite cell co-cultures from 1 to 9 days in culture (n = 6–8 in each time point). C: Expressions of different myosin heavy chain isoforms (IIa, IIb, IIx and Ib) in mouse FDB muscles (left, n = 16) compared to myotubes formed after 8 days in co-cultures of FDB muscle cells and their satellite cells (n = 16), satellite cells alone (n = 16) and satellite cells exposed to muscle cell-conditioned medium (n = 11).

interaction between endothelial progenitor cells and cardiomyocytes (Koyanagi et al., 2005). As a result, a single active satellite cell may well-establish connections with several other cells in a few hours. Altogether, active transport and ion diffusion within TNTs together with dynamic assembly of new TNTs create a fast intercellular communication pathway. Since fibrous actin forms the backbone of the TNTs (Fig. 1) it is crucial for TNT to establish intercellular communication (Rustom et al., 2004; Gurke et al., 2008). We noticed that inhibition of F-actin with latrunculin inhibits the cellular connections as well as further differentiation of the satellite cells (Fig. 2). Supporting the view that F-actin is involved in the differentiation of the muscle precursors, it was found previously that latrunculin inhibits the expression of the myogenic differentiation marker MyoD in skeletal muscle myoblasts (Dhawan and Helfman, 2004). However, we noticed that F-actin inhibition has drastic effects on the morphology of the satellite cells (Fig. 2), suggesting that the latrunculin effect is not specific for MyoD expression. Instead it could be argued that latrunculin effects are due to disruption of intracellular actin structures and interference with the TNT communication of the satellite cells.

Activation of satellite cells is characterised by the expression of paired-box transcription factor Pax-7, required for the specification of satellite cells (Seale et al., 2000). Specification towards the myogenic lineage also requires the expression of myogenic regulatory factors, like MyoD, which is one of the master regulators of myogenesis (Tapscott and Weintraub, 1991; Weintraub et al., 1991). As a result, differentiating myoblasts co-express Pax-7 and MyoD (Reimann et al., 2004). We noticed that the expression of both Pax-7 and MyoD was dependent on whether satellite cells remained attached to the muscle cells at the time of their activation or if they were enzymatically isolated from the muscle cell surface before culturing (Fig. 6) This manifested as delayed expression of both MyoD and Pax-7 in isolated satellite cell cultures compared to cultures with muscle cells (Fig. 6A,B). In satellite cell–muscle cell cultures expressions of both MyoD and Pax-7 reach a maximum at day 2 in culture, whereas in satellite cell cultures the expressions peak at around day 4 (Fig. 4A,B). In addition, satellite cells express ~100-times more Pax-7 when they are cultured with muscle fibres than without (Fig. 4B), which might indicate greater activation and differentiation potential of the co-cultures. It was previously shown that the muscle fibre affects the proliferation of the attached satellite cells (Bischoff, 1986, 1990a,b). Contact with viable, mature muscle fibres tends to suppress the proliferation of the satellite cells (Bischoff, 1990b), whereas muscle fibre extract promotes proliferation and satellite cell division (Bischoff, 1990a). When muscle fibres and their satellite cells are cultured together, the muscle fibres dedifferentiate but stay intact and viable for up to ~1 week.

In regenerating adult muscle and in culture, developing myotubes tend to express the same myosin heavy chain isoforms as their parent fibres (Barjot et al., 1995; Rosenblatt et al., 1996). It is not known from where the developing satellite cells get the information about the phenotype of their parent fibre. According to the data presented in this report, it is feasible to suggest that TNTs might have a role in this. Since satellite cells are initially connected to their parent fibre by the TNTs, it is possible that transport of cytosolic material via TNTs helps them to acquire the phenotype of the parent fibre at the time of their activation. If this was true, those satellite cells that remain attached to their parental fibre at the time of their activation would eventually generate myotubes resembling the parent fibre more than counterparts cultured without parental fibres. In this study we used satellite cells and muscle cells isolated from mouse FDB muscle, which express mostly MHCIIa and MHCIIx with much lesser amounts of types Ib and IIb (Raymackers et al., 2000). After 7 days of co-culture of

satellite cells and their parent fibres, de novo myotubes express slightly more types IIa and IIb than IIx and Ib. While the MHC expression profile of the co-cultures was different than the profile of FDB muscles, it was still more similar than the MHC expression pattern of the myotubes originating from the satellite cells alone, where slow and intermediate type MHCs (Ib and IIx) were profoundly more expressed (Fig. 6C). We also showed that secreted factors from muscle cells were not responsible for this effect (Fig. 6C). Taking into account the fact that culturing as such is likely to affect the MHC expression of the developing myotubes, not least because myotubes do not reach mature muscle fibre phenotype in culture; it is not a surprise that myotubes do not have an identical MHC expression pattern to the FDB muscles. It is also possible that MHC isoform expression is affected by differences in the activation and maturation speed of the different cultures (Fig. 6A,B). However, this data shows that the presence of the parent muscle fibres at the time of satellite cell activation affects the MHC expression of the myotubes. It is feasible to assume that TNTs have a role in this process, although we cannot fully address the mechanism behind this phenomenon. TNTs are likely to be involved in the communication between muscle fibres and satellite cells, but also between satellite cells and their progeny. Communication via TNTs may thus have a crucial role in determining the fate of the differentiated muscle cells.

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