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REGULAR ARTICLE

Muscle and neuronal differentiation in primary cell culture of larval *Mytilus trossulus* (Mollusca: Bivalvia)

Nelly A. Odintsova · Vyacheslav A. Dyachuk · Leonid P. Nezlin

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Abstract Molluscan in vitro technology allows the study of the differentiation of isolated cells undergoing experimental manipulations. We have used the immunofluorescence technique and laser scanning microscopy to investigate the organization of muscle proteins (actin, myosin, paramyosin, and twitchin) and the localization of neurotransmitters (serotonin and FMRFamide) in cultured mussel larval cells. Differentiation into muscle and neuron-like cells occurs during the cultivation of mussel cells from premyogenic and prenervous larval stages. Muscle proteins are colocalized in contractile cells through all stages of cultivation. The cultivation of mussel cells on various substrates and the application of integrin receptor blockers suggest that an integrin-dependent mechanism is involved in cell adhesion and differentiation. Dissociated mussel cells aggregate and become self-organized in culture. After 20 days of cultivation, they form colonies in which serotonin- and FMRFamide-immunoreactive cells are located centrally, whereas muscle cells form a contractile network at the periphery. The pattern of thick and thin filaments in cultivated mussel cells changes according to the scenario of muscle arrangement in vivo: initially, a striated pattern of muscle filaments forms but is then replaced by a smooth muscle pattern with a diffuse distribution of muscle proteins, typical of muscles of adult molluscs. Myogenesis in molluscs thus seems to be a highly dynamic and potentially variable process. Such a "flexible" developmental program can be regarded as a prerequisite for the evolution of the wide variety of striated and smooth muscles in larval and adult molluscs.

Keywords Myogenesis · FMRFamide · Serotonin · Cell culture · *Mytilus trossulus* (Mollusca: Bivalvia)

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Introduction

During the last decade, significant progress has been achieved in the study of the regulatory mechanisms involved in the growth and differentiation of mammalian embryonic stem cells. Such cells spontaneously differentiate in vitro into many cell types but only become committed to the neuronal (Fraichard et al. 1995; Erceg et al. 2008, 2009) or muscle (Rohwedel et al. 1994; Ferreira et al. 2007; Schenke-Layland et al. 2008) lineages under specific conditions.

Invertebrate in vitro models have also significantly contributed to the study of the mechanisms of cell differentiation. Thus, flatworms have an outstanding stem cell system that seems to be an evolutionary ancient way of cell renewal in bilaterians (Pfister et al. 2008). Primary cell cultures of various invertebrates have been exploited to study mechanisms of cell differentiation (for a review, see Rinkevich 2005). The cultivation of embryonic cells isolated from living invertebrates from various taxa offer possibilities of

studying their proliferation and differentiation potentials in vitro and in vivo and should contribute to a better understanding of the evolutionary mechanisms of differentiation and regeneration in multicellular animals.

Myogenesis has become a good model for studying the way that undifferentiated cells are committed to their final fate through cell proliferation and cell differentiation following by the synthesis of specific muscle proteins and muscle fiber assembly (for a review, see Baylies and Michelson 2001). Contractile cells containing myofibrils are present in almost all animal taxa, and thus the results obtained from relatively simple invertebrate models are important for many comparative researches. To obtain greater insights into muscle development in vivo, we need to identify in vitro the protein components of contractile cells containing myofibrils and to elucidate a regulation of muscle contraction. However, studies of cell specialization mechanisms in many invertebrate taxa are limited by a lack of long-term cell lines. Today, only primary cell cultures of marine invertebrates offer opportunities to study the role of collective cell behavior in the generation of definite cell types, such as muscle cells and neurons.

So far, contracting cells in primary cultures of invertebrates have been described for larval cells of the gastropod mollusc *Haliotis rufescens* (Naganuma et al. 1994), embryonic cells from the late gastrula stage of the starfish *Asterias amurensis* (Kaneko et al. 1997), embryonic cells of insects (Lynn et al. 1998), embryonic cells of the nematode *Caenorhabditis elegans* (Strange and Morrison 2006), and larval cells of the bivalve mollusc *Mytilus trossulus* (Odintsova et al. 2000; Plotnikov et al. 2003). Furthermore, the neuronal differentiation of embryonic cells of invertebrates in vitro has been presented in studies on insects (Wu et al. 1983; Bloom 1993) and molluscs (Gruenbaum and Carew 1999; Montgomery et al. 2002). Neurotransmitters, such as acetylcholine (ACh), serotonin (5-HT), and FMRFamide-related peptides, have been suggested to regulate muscle contraction and are adequate markers of developing ganglia of the central nervous system of insects, molluscs, and flatworms (Hall and Greenspan 1979; Croll and Voronezhskaya 1995; Stewart et al. 2003; Voronezhskaya et al. 2008). The possibility of the simultaneous differentiation of neurons and myocytes in cell culture has been described for several invertebrates: the fruit fly *Drosophila* (Seecof et al. 1972), the moth *Manduca sexta* (Luedeman and Levine 1996), and the nematode *C. elegans* (Buechner et al. 1999). In addition, contacts between neurons and between neurons and muscle cells have been observed in cultivated cells of *C. elegans* (Christensen et al. 2002). By extending these data, some specific synaptic-like proteins of vesicles, synapsin, and synaptophysin that had been detected in neuromuscular junctions of mammalian cultured cells (DeCamilli et al. 1988) have been found in the neuronal cells of snails *Aplysia punctata* and *Helix pornatia* (Chin et al. 1990; Bongiovi et al. 1992; Cibelli et al. 1996). Moreover, synapse formation between the heart motor neuron and muscle fiber of the snail *Lymnaea stagnalis* has been reconstructed in vitro (Lee et al. 2002).

The biochemical and physiological properties of the muscles of *M. trossulus* have been relatively well studied in vivo. The larval muscle system contains both striated and smooth muscles (Cragg and Crisp 1991; Dyachuk and Odintsova 2009), whereas the adult musculature is simple and consists entirely of smooth muscle cells with an unusual structure of their thick filaments (Szent-Gyorgyi et al. 1971). Molluscan smooth muscles have a unique protein composition: their thick filaments are formed by paramyosin, myosin, twitchin, and myorod (Levine et al. 1976; Vibert et al. 1993; Shelud'ko et al. 1999; Odintsova et al. 2006). The last three proteins are located around the paramyosin core (Winkleman 1976; Royuela et al. 1997; Scott et al. 2008).

Here, we continue studies of the differentiation of cultivated larval cells of the mussel *M. trossulus*. The aim of this study has been to examine the onset and timing of expression of *Mytilus* muscle proteins and neurotransmitters in vitro. An immunofluorescence technique and confocal laser scanning microscopy have been applied in order to study the organization of several muscle proteins (actin, myosin, paramyosin, and twitchin) and the localization of neurotransmitters (5-HT and FMRFamide) in cultured mussel larval cells. We have also attempted to find the synapse contacts between muscle cells and neuron-like cells by using specific antibodies (Abs) against synaptophysin (a synaptic glycoprotein) and synapsin (a protein associated with synaptic vesicles), but this has proved unsuccessful. The results obtained nevertheless allow us to assert that mussel larval cells can differentiate, under specific conditions in vitro, into a definite cell type, namely, muscle cells and 5-HT- and FMRFamide-immunopositive cells, which we term neuron-like cells.

Materials and methods

Adult mussels *M. trossulus* (Mollusca: Bivalvia) were collected in the Sea of Japan (Vostok Bay). Experiments were carried out during 2005–2009 at the Marine biological station "Vostok" (A.V. Zhirmunsky Institute of Marine Biology of the Far Eastern Branch of Russian Academy of Sciences). The animals were washed three times in UV-sterilized filtered seawater. Spawning was induced by thermal shock (the temperature being first decreased to 10°C and then raised to 20°C for a 10-min period). Developing embryos were cultivated in 5-l tanks at 16–17°C and collected on a fine 30-μm nylon mesh at the trochophore stage (24 h post-fertilization, hpf).

Cell culture

Larvae were washed three times in Ca^{2+}/Mg^{2+}-free salt solution (CMFSS) and incubated in 0.125% collagenase in CMFSS at 17°C to dissociate cells, as described earlier (Odintsova et al. 2000). The cells were then seeded at a density of 2×10^6–2.5×10^6 cells/cm^2 in plastic Petri dishes (Lux Culture Dishes, ICN Biomedicals) on glass coverslips coated with carbon or adhesive proteins and cultivated in a modified (Odintsova and Khomenko 1991) L-15 medium (L-15 M), supplemented with 2% fetal bovine serum (Gibco Laboratories), insulin (50 mg/1, Sigma), and α-tocopherol-acetate (1.75 mg/1, Sigma) for 2.5 months at 17°C. Cell viability, as estimated by simultaneous staining with fluorescein diacetate with propidium iodide (Jones and Senft 1985) or by a trypan-blue exclusion test, was 90%–95% immediately after seeding and declined slightly to 75%–85% on day 10.

Adhesion assay

Adhesive substrates (Sigma) such as collagen (type I,), fibronectin, or poly-D-lysine (190 kDa) were initially applied to coverslips. The solutions (0.1–1 mg/ml) were left to settle for 12 h at room temperature (RT). After two washes in sterile seawater, the dishes with the coverslips were stored for up to 12–24 h at RT. Cell-spreading responses to the inhibitor of integrin receptors, viz., Arg-Gly-Asp-Ser (RGDS)-peptide, and control non-specific Arg-Gly-Glu-Ser (RGES)-peptide (both from Sigma) were tested. The tetrapeptides were diluted in phosphate-buffered saline (PBS, pH 7.8) to 20 mM and stored at -20°C before experiments. RGDS and RGES peptides were added to the culture medium (2 mM) before the seeding of cells on the various substrates. Mussel larval cells (2×10^6 cell/ml) were incubated in the presence or absence of these peptides on coverslips. After 6 h, 2 days, and 20 days, the cells were fixed for immunocytochemical assay.

Percoll fractionation

To avoid bacterial contamination and to enrich the culture with committed muscle and neuron-like cells, the larval cell suspension (1 ml, 8.3×10^6 cells/ml) was subjected to a Percoll density gradient (10%–36% Percoll in CMFSS). Gradients were formed by careful layering 3 ml successively less-dense Percoll solutions on top of each other in 12-ml centrifuge tubes. The tubes were centrifuged for 20 min at 1300*g* (at 4°C). Cells localized in the 10%–36% Percoll interphase were washed twice in CMFSS, twice in sterile seawater, transferred to L-15 M medium, and cultivated from 2 h to 2.5 months in a thermostat at 17°C. This approach allowed us to sort undifferentiated cells into the groups in which they later differentiated into specific cell types. The design of the experiments for obtaining the trochophore-derived primary cell culture is schematically presented in Fig. 1.

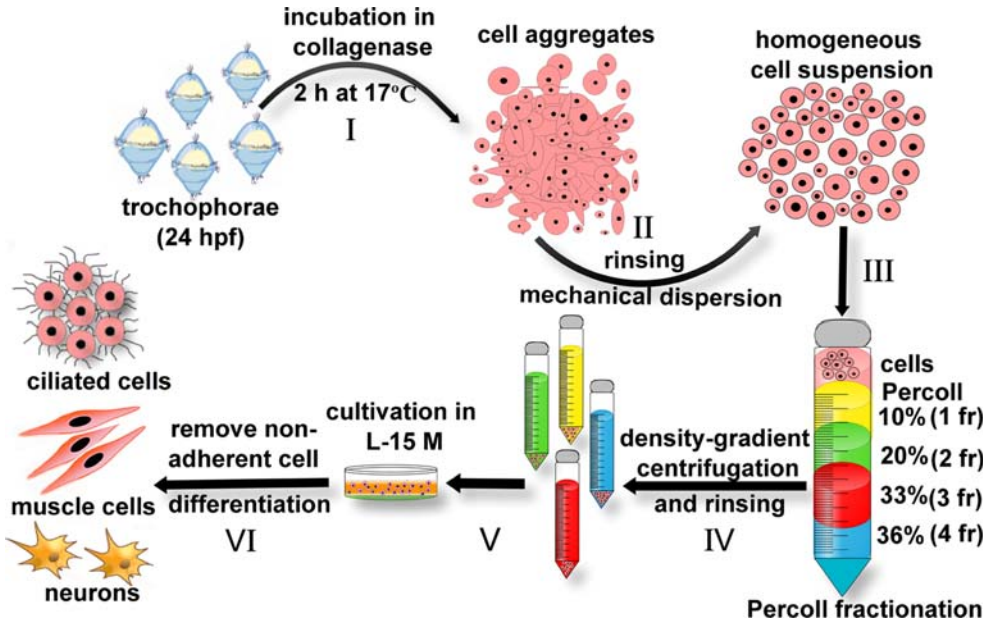

Fig. 1 Representation of the experimental procedure for establishing trochophore-derived primary cell culture of *M. trossulus* (24 h post-fertilization [hpf]). Trochophore larvae were collected and dissociated by an enzymatic treatment (*I*). Dissociated larval cells were rinsed (*II*) and fractionated on Percoll density gradient (10%–36% Percoll: 1st fraction (*1 fr*), 2nd fraction (*2 fr*), 3rd fraction (*3 fr*), 4th fraction (*4 fr*; *III*). After the Percoll medium had been washed out (*IV*), the cells were plated on glass coverslips and cultured in the L-15 M medium (Odintsova et al. 2000) overnight at 17°C (*V*). The nutrient medium was then replaced with fresh medium, at which time non-adherent cells were removed (*VI*)

Immunocytochemistry

An indirect immunofluorescent technique was utilized. After cultivation on coverslips, the cells were fixed in 4% paraformaldehyde (PFA, Sigma) in 0.1 M PBS, pH 7.8, for 7–10 min at 4°C and rinsed three times in cold PBS. The material was stored in PBS with 0.03% NaN_3 at 4°C. To reduce non-specific binding, the samples were incubated overnight in a blocking solution containing 10% normal goat serum (Sigma), 0.25% bovine serum albumin, 0.1% Triton X-100, and 0.03% NaN_3 in PBS. The preparations were then incubated for 8 h at 10°C or 2 h at RT in the blocking solution with the mixture of mouse and rabbit primary Abs. The following antibodies were used: polyclonal Abs against twitchin or paramyosin (developed in rabbit, produced in our laboratory, diluted 1:1000), monoclonal Abs against myosin (developed in mouse, Sigma, diluted 1:1000), acetylated α-tubulin Abs (developed in mouse, Sigma, diluted 1:3000), and polyclonal Abs against serotonin (5-HT) and FMRFamide (developed in rabbit, Immunostar, diluted 1:2000), synaptophysin (developed in mouse, Molecular Probes, diluted 1:500), and synapsin (developed in mouse, Sigma, diluted 1:100).

After incubation with the primary Abs, the preparations were washed in PBS (3×10 min) and incubated overnight at 10°C or 2 h at RT in the mixture of secondary Abs: goat anti-rabbit (GAR) Alexa-Fluor-488-conjugated IgG and goat anti-mouse (GAM) Alexa-Fluor-546 IgG (both Molecular Probes, diluted 1:1000) or GAR Alexa-Fluor-546 IgG and GAM Alexa-Fluor-488 IgG (Molecular Probes, diluted 1:1000) in PBS. For the simultaneous detection of paramyosin and actin, fixed cells were first incubated with the primary paramyosin Abs and then with a mixture of the secondary antibody (GAR Alexa-Fluor-546 IgG) and fluorescein-isothiocyanate-labeled phalloidin (Molecular Probes, 1:500) or the secondary antibody (GAR Alexa-Fluor-488 IgG) and tetramethylrhodamine-isothiocyanate-labeled phalloidin (Molecular Probes, 1:500). The specimens were then washed in PBS (3×10 min), stained with 0.1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI, Sigma) in PBS to reveal the nuclei, and washed again in PBS (3×10 min). The preparations were embedded in the Vectashield mounting medium (Vector), coversliped, and examined in a laser scanning microscope TCS SPE (Leica Microsystems, Germany) with 40× and 63× oil-immersion objectives and in a conventional microscope Axiovert 200 (Carl Zeiss, Germany) with 20× and 40× dry objectives. The resulting laser confocal stacks of optical sections were processed by using Leica LAS AF software. For three-dimensional reconstructions, we employed ImageJ software (NIH). The number and step size of optical sections are given in the figure legends.

The specificity of the Abs against the muscle isoforms of the myosin, twitchin, and paramyosin was tested previously (Odintsova et al. 2006). For negative controls, primary Abs were omitted from the staining protocol.

BrdU test

The 5-bromodeoxyuridine (BrdU)-labeling method is useful for studying cell cycle kinetics and DNA replication and thus is a widely used marker for cell proliferation (Zaldibar et al. 2004; Salic and Mitchison 2008). A stock solution of 10 mM BrdU (MP Biomedicals) was prepared in L-15 M medium and stored at -20°C. Cells were incubated in 1 mM BrdU in L-15 M medium for 12 h at 17°C, fixed in 4% PFA for 7–10 min at RT, and washed in cold PBS (3×10 min). An equal volume of 4 N HCl with 0.1% Triton X-100 was added, and the cells were incubated for 20 min at RT. After being washed several times in TRIS-HCI buffer (100 mM, pH 8.5) and then in PBS, the cells were incubated for 2 h at RT in the blocking solution in order to eliminate nonspecific binding. Then, the specimens were incubated in the solution of primary BrdU monoclonal Abs (MP Biomedicals, dilution 1:20) in the blocking solution overnight at +4°C, washed in PBS (3×10 min), and then incubated for 1 h at RT with GAM Alexa-Fluor-546 IgG (Molecular Probes, diluted 1:200) in PBS. The specimens were embedded, coversliped, and examined as described above.

Results

Morphology of larval mussel cells during cultivation Cells of various shapes were observed during cultivation (Fig. 2). By 2 h after seeding, the relatively small trochophore-derived cells (6–10 μm in diameter) were generally round. Nevertheless, they adhered to coverslips coated with fibronectin, poly-L-lysine, or carbon (Fig. 2a). After 12 h in culture, the cells began to spread, and bipolar cells, either single or aggregated, were detected (Fig. 2b). During the following 7 days, these cells formed multiple processes (Fig. 2c), and some of these bipolar cells showed spontaneous rhythmic contractions (see Electronic supplementary material, movie 1). The number of contractile cells increased gradually during the cultivation (but never exceeded 20% of the total cell number). After 2 weeks in culture, cell aggregates formed multilayer cell sheets that were connected to each other by long spindle-shaped cells (Fig. 2d, see Electronic supplementary material, movie 2). Contractions of the whole cell sheets were observed at late stages of cultivation (more than 2.5 months, see Electronic supplementary material, movie 3).

BrdU assay BrdU immunoreactivity was detected in cells plated on fibronectin-, poly-L-lysine-, or carbon-coated coverslips only during the first week of cultivation (Fig. 3a).

Later, no BrdU–immunoreactive cells were detected on any of the substrates tested (Fig. 3b). The BrdU-immunopositive cells were located in cell aggregates, and all of them were rounded. No BrdU immunoreaction was ever detected in spindle-shaped cells.

Distribution of muscle proteins during cell cultivation Within the first 2 h after seeding, the immunoreaction with paramyosin (as was also the case for other thick filament muscle proteins tested) was negative (Fig. 4a), whereas actin non-striated filaments were found in cultured mussel cells (Fig. 4b, c). After 6 h of cultivation, actin filaments and a diffuse distribution of paramyosin (plus that of myosin and twitchin) were observed (Fig. 4d, e, f, and insets). Following 12 h of cultivation, the reaction pattern changed: phalloidin and Abs against thick muscle proteins showed a distinct striated pattern (Figs. 4g, h, i,d 5a–f) on all substrates, except for collagen. At this time, the first contractile cells appeared in culture; they were always mononucleated (Fig. 4i).

The cells that showed positive immunoreaction to the antibodies against muscle proteins were spindle-shaped and demonstrated contractile activity after 12 h in culture (see Electronic supplementary material). Rounded cells never showed an immunoreaction against muscle proteins on any substrate, except for collagen on which all cells retained their rounded shape. The contractile cells contained thick (paramyosin) and thin (actin) filaments aligned perpendicularly to the long axis; the length of the sarcomere was about 2.5 μm (Fig. 4i, inset). However, after 20 days of cultivation, the striated pattern of thick and thin filaments in contractile cells changed to a diffuse distribution of the muscle proteins in the cytoplasm (Figs. 4j–l, insets, 5g–i).

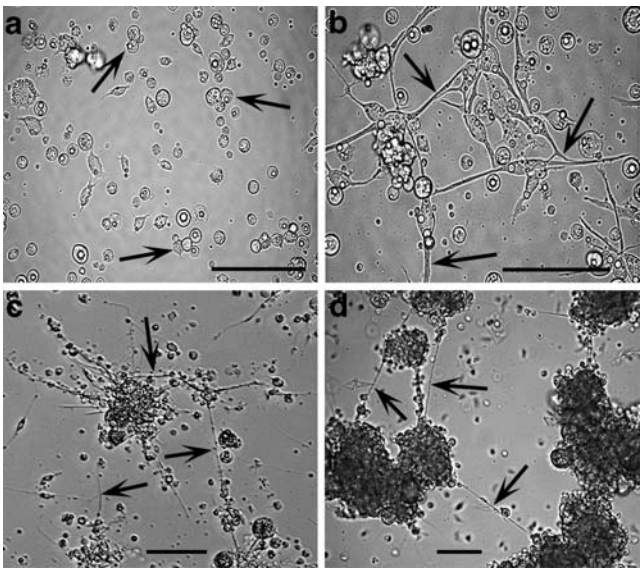

Fig. 2 Larval cells of *M. trossulus* in primary culture on carbon-coated coverslips. At 2 h (**a**), the cells were mostly rounded, rarely bipolar, and sometimes formed aggregates of 3–10 cells (*arrows*). By 12 h (**b**), spindle-shaped cells had appeared and formed multiple, rarely branching processes (*arrows*). After 7 days in culture (**c**), the cells had formed dense aggregates with fibers passing outside them and interconnecting adjacent aggregates (*arrows*). Following 20 days in culture (**d**), the cell aggregates had formed multilayer cell sheets connected with each other by long fibers of spindle-shaped cells (*arrows*). *Bars* 30 μm

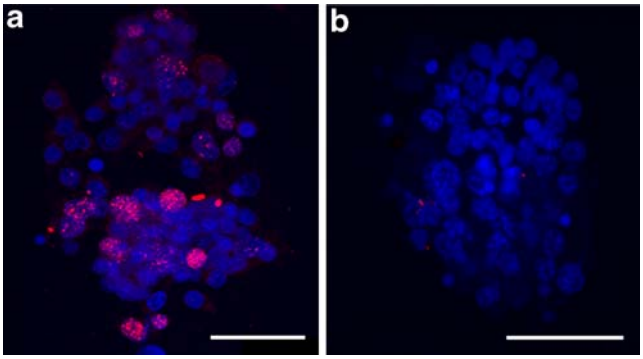

Fig. 3 BrdU-immunoreactive cells in primary culture of larval cells of *M. trossulus* after 7 days (**a**) and 14 days (**b**) of cultivation. The cells were double-labeled with BrdU antibodies (*red*) and 4′,6′-diamidino-2-phenylindole (DAPI; *blue*). Note the absence of BrdU staining in **b**. *Bars* 20 μm

Inhibition of myofibrillogenesis The cells grown on collagen type I-coated coverslips (1 mg/ml), unlike the cells plated on other substrates tested, had a rounded shape throughout the cultivation period and adhered but did not spread (Fig. 6a–c). Contractile cells were never observed. We detected the expression of actin and thick filament muscle proteins for 20 days in cells plated on collagen (Fig. 7a–c). By 2 days, single filaments of actin and paramyosin (like other thick filament muscle proteins) were found to encircle nuclei, and sometimes these filaments formed a striated-like structure (Fig. 7b, c, insets). The cells, incubated with RGDS-peptide, an inhibitor of integrin receptors, had a rounded shape (Fig. 6d–f), like those cultivated on collagen substrate; no cell contractions were registered. Only actin staining was observed in these cells (Fig. 7d–f), whereas the immunoreactions with all thick filament muscle proteins tested were negative. In contrast, the cells incubated with non-specific RGES-peptide had a morphology similar to that of control cells plated on a plastic surface (Fig. 6g–i), and sharp striated labeling of muscle proteins was observed in spreading bipolar cells at all tested stages (Fig.7g–i).

Cell fractions enriched with muscle and neuron-like cells After Percoll gradient centrifugation, the larval cell suspension was divided into four fractions: (1) 10%; (2) 20%; (3) 33%; (4) 36% (Fig. 1). The first fraction in the 10% Percoll interphase contained debris and small rounded cells with a

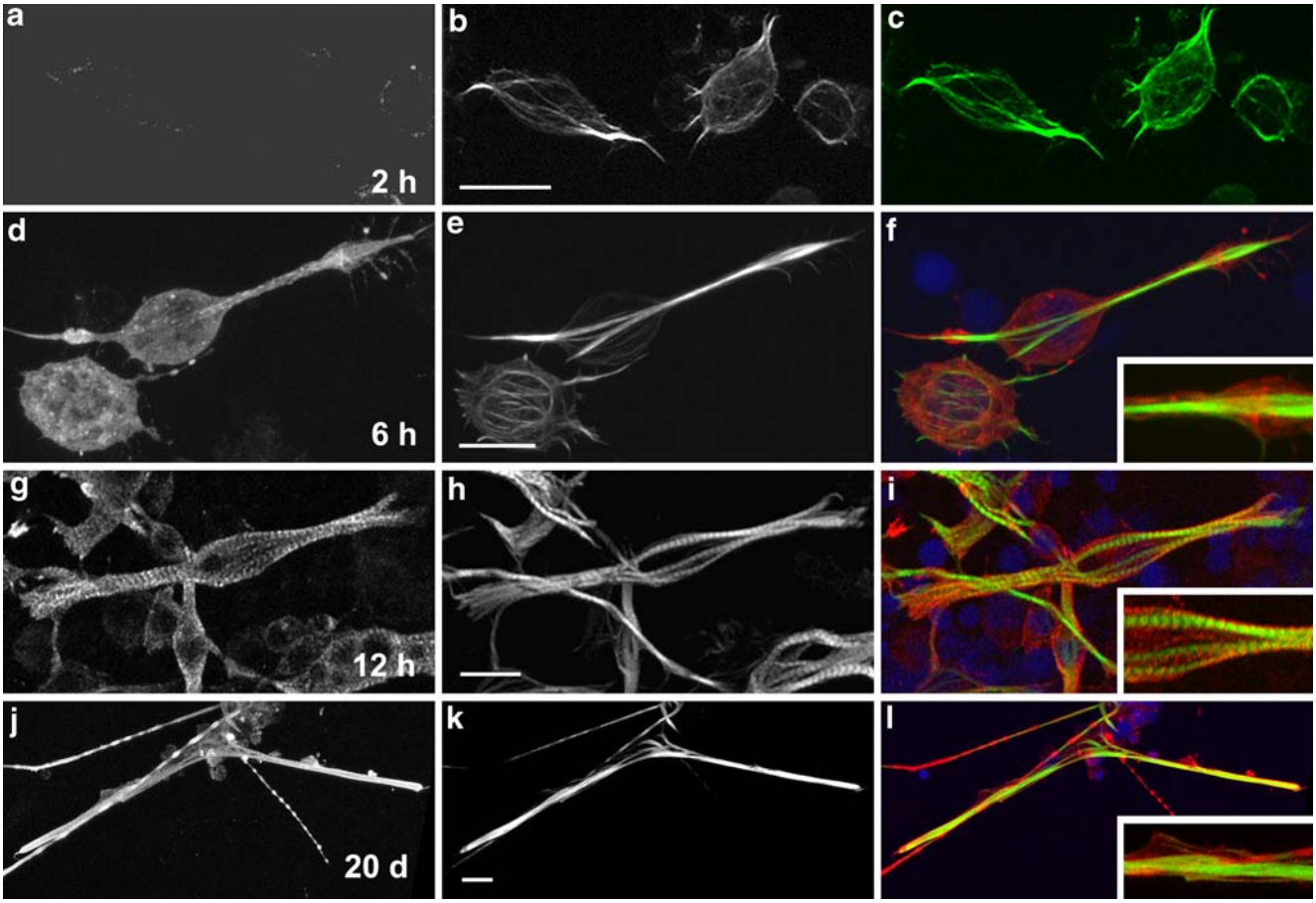

Fig. 4 Changes of muscle protein distribution during cultivation of larval cells of *M. trossulus* for 2 h (**a-c**), 6 h (**d-f**), 12 h (**g-i**), and 20 days (**j-l**). The cells were labeled with paramyosin Abs (*red* in **a**, **d**, **g**, **j**) and with phalloidin for actin detection (*green* in **b**, **e**, **h**, **k**). Merged images are presented in **c**, **f**, **i**, **l**. Nuclei were labeled with DAPI (*blue* in **f**, **i**, **l**). Note the changes of paramyosin immunoreaction during cultivation. The reaction is negative for 2 h (**a**, **c**), shows a diffuse pattern of distribution by 6 h (**d**, **f**), which interchanges to a striated pattern of distribution after 12 h (**g**, **i**), and becomes diffuse again after 20 days (**j**, **l**). *Insets* in **f**, **i**, **l**: Higher magnifications of the cells. Note the striated pattern of paramyosin and actin distribution in the cell in **i**. *Bars* 20 μm

cell viability below 60%. The cells of this fraction never attached, never spread, and degenerated within 3 days. Cell viability in other fractions was 94%–99% immediately after seeding and decreased to 75%–80% during 2.5 months of cultivation.

The majority of fractionated cells (about 70%) was concentrated in the second and the third fractions. The morphological differentiation of cells of these fractions was correlated with characteristic striations of molluscan muscle proteins, with an appearance of 5-HT- and FMRFamide-immunoreactive cells and with the expression of α-acetylated tubulin in an immunopositive reaction of ciliated cells. In the second fraction (20% of Percoll), we identified an increased number of 5-HT- and FMRFamide-expressing cells (a subset of 5%–7% cultivated cells), which began to appear approximately 6 h after seeding in aggregates consisting of ciliated cells and spindle-shaped cells. Notably, 5-HT- and FMRFamide-immunopositive cells were easily detected in cultures: the pattern of immunoreaction in 5-HT-positive cells was homogeneous (Fig. 8a), whereas FMRFamide-immunoreactive cells showed clear punctuate staining (Fig. 8b). The third fraction was enriched with the cells that differentiated in spindle-shaped cells and later were immunoreactive to Abs against muscle proteins. The fourth fraction consisted mainly of large cell aggregates.

After 48 h of cultivation, muscle cells, neuron-like (5-HT- and FMRF-amide-immunopositive) cells, and ciliated (α- acetylated tubulin-immunopositive) cells comprised 70%–75% of the total cell number. Newly formed neuron-like cells were arranged in culture randomly (Fig. 9a, b). By 20 days, cultivated cells formed compact clusters including muscle cells and 5-HT- and FMRFamide-immunoreactive cells (Fig. 9c, d). Neuron-like cells were compactly grouped inside the aggregates, whereas myocytes at the periphery of the aggregates sent fibers toward adjacent aggregates. After 2.5 months of cultivation, adjacent colonies were connected with contractile spindle-shaped fibers (Fig. 10a, b). Actin-

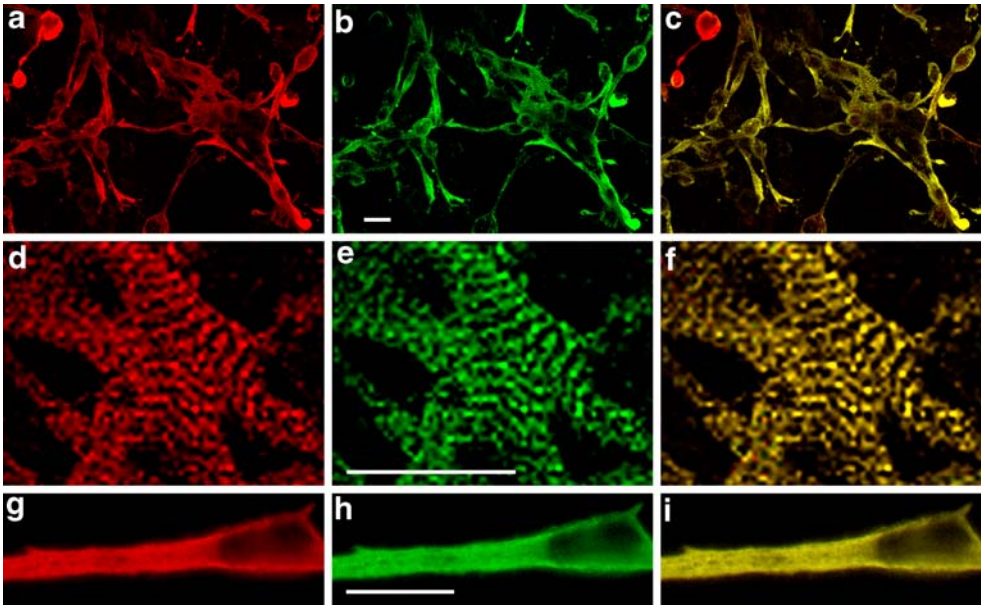

Fig. 5 Colocalization of thick filament proteins in muscle cells in primary cell cultures of *M. trossulus*. Cells were labeled with myosin Abs, produced in mouse (*red* in **a**, **d**, **g**), and twitchin Abs, produced in rabbit (*green* in **b**, **e**, **h**). Merged images are shown in **c**, **f**, **i**. Higher magnifications of the cells depicted in **a**–**c** are presented in **d**–**f** (12 h of cultivation). Myosin and twitchin are colocalized in myogenic cells. The distribution of both muscle proteins shows a sharp striated pattern (**d**–**f**), whereas after 20 days of cultivation, their distribution is diffuse (**g**–**i**). *Bars* 10 μm

and paramyosin-immunopositive cells became concentrated at the center of the colony and sent fibers to the periphery and outside of the colony (Fig. 10c). There, fibers connecting the colonies (and also single muscle cells) showed a smooth pattern of actin and paramyosin distribution (Fig. 10d, e).

Discussion

In this study, we present evidence that the differentiation of dissociated cells of a larval mussel can recapitulate the process of mussel myofibril organization in vivo. During larval metamorphosis, the initially striated pattern of mussel

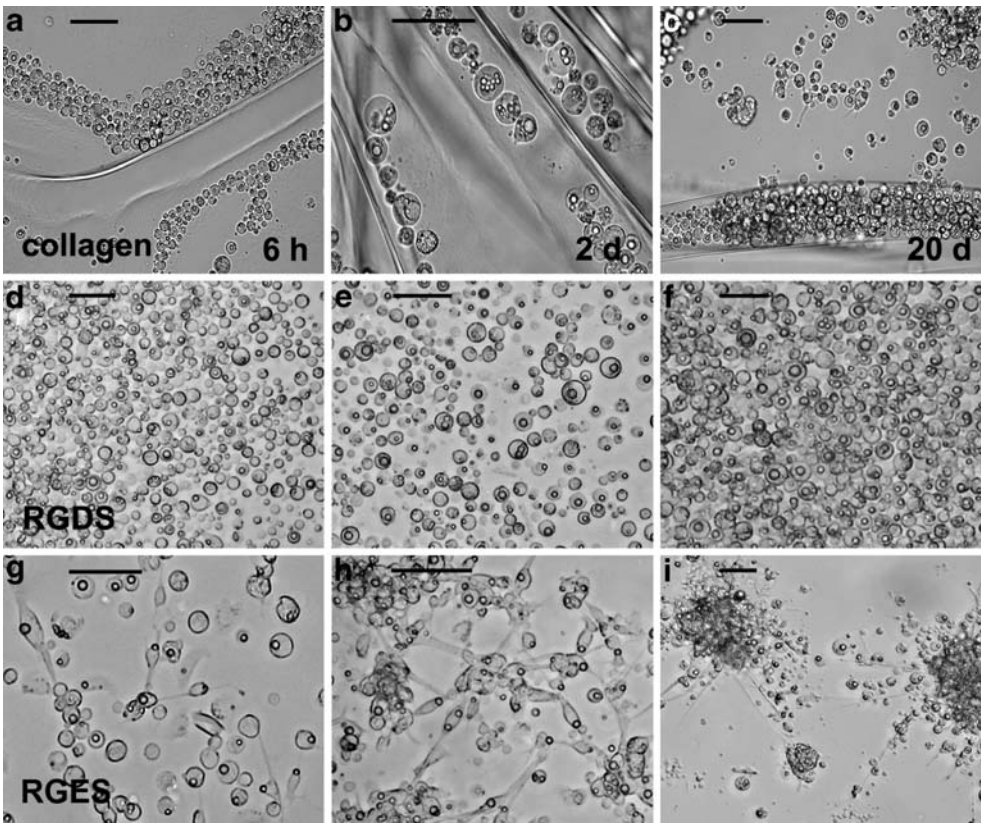

Fig. 6 Inhibition of myogenic differentiation in cultivated larval cells of *M. trossulus* by collagen and the inhibitor of integrin receptors, RGDS-peptide. The cells were cultivated on either collagen carpets (**a**–**c**) or a plastic surface with RGDS-peptide (**d**–**f**) or with non-integrin binding RGES-peptide (**g**–**i**) for 6 h (**a**, **d**, **g**), 2 days (**b**, **e**, **h**), and 20 days (**c**, **f**, **i**). Note that the cells cultivated on collagen carpets or with RGDS-peptide remained rounded, whereas the cells with RGES-peptide became spindle-shaped and formed processes. *Bars* 20 μm

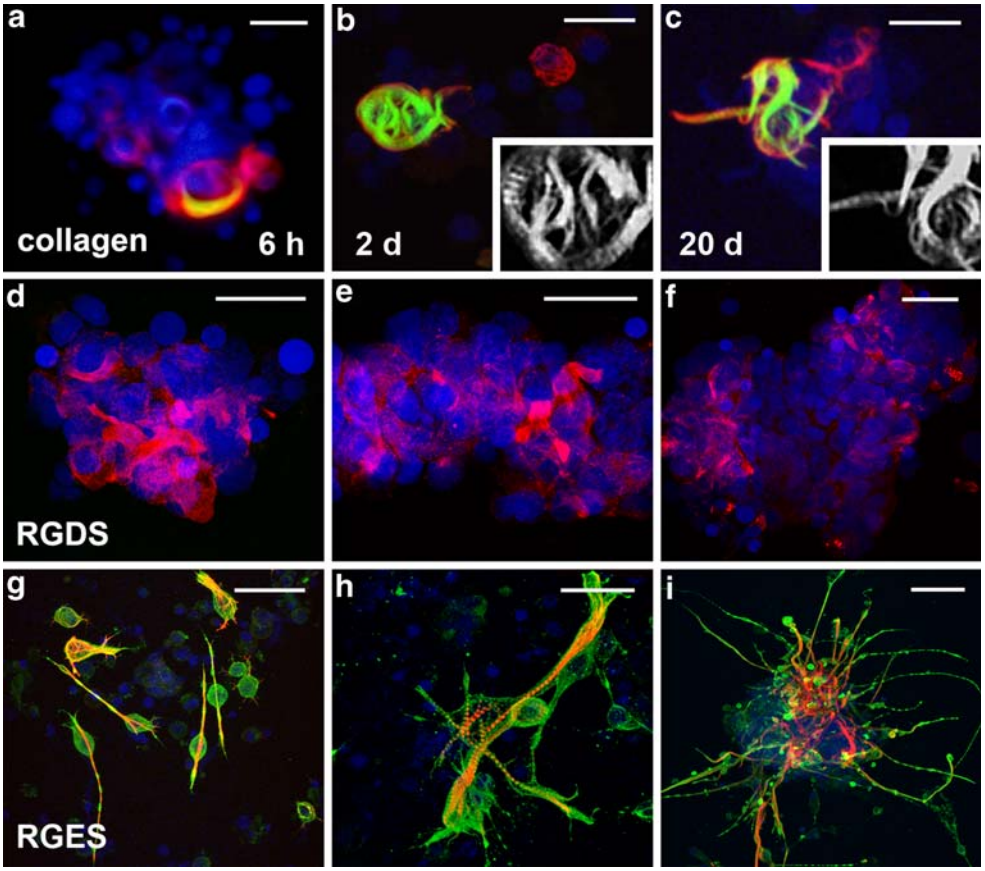

Fig. 7 Inhibition of myogenic differentiation in cultivated larval cells of *M. trossulus* by collagen and the inhibitor of integrin receptors, RGDS-peptide (continued). The cells were labeled with phalloidin for actin detection (*red*), paramyosin Abs (*green*), and DAPI (*blue*). In cells plated on collagen carpets (**a**–**c**), both actin and paramyosin fibers encircled the nuclei and had a striated pattern of distribution (**b**, **c**, *insets*). In rounded cells cultivated in the presence of RGDS-peptide, only actin staining was detected at all stages of cultivation (**d**–**f**). In the presence of non-integrin-binding RGES-peptide, both actin and paramyosin labeling was detected (**g**–**i**). Note that, at 2 days of cultivation, the organization of both actin and paramyosin was striated (**h**). *Bars* 20 μm

muscle filaments disappears after 20 days of development and changes to the smooth muscle pattern typical of adult muscles capable of the catch-contraction (Dyachuk and Odintsova 2009). Similarly, the striated pattern of thick and thin filaments in contractile cells at early stages of cultivation disappears and is replaced by the diffuse distribution of the muscle proteins in the cytoplasm of these cells after 20 days of cultivation. Cells with striated and diffuse patterns of distribution of the muscle proteins coexist for a specific period of cultivation (see Fig. 5). We suggest that cells with the striated pattern are resorbed at late stages of cultivation.

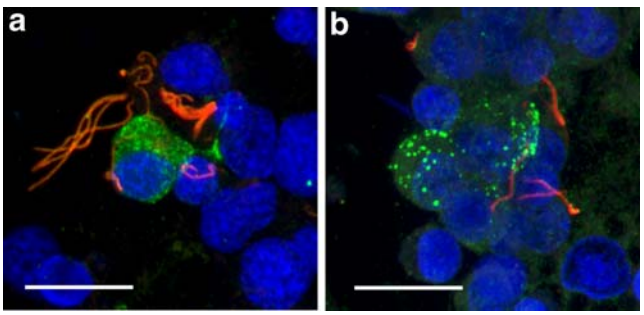

Fig. 8 Anti-5-HT (**a**) or anti-FMRFamide (**b**) immunoreactivity (*green*) together with anti-acetylated α-tubulin immunoreactivity (*red*) in cell aggregates in primary culture of larval cells of *M. trossulus*, after 6 h in culture. Nuclei were stained with DAPI (*blue*). Note that the staining of 5-HT-positive cells was homogeneous (**a**), whereas FMRFamide-immunoreactive cells showed clear punctuate staining (**b**). Both 5-HT- and FMRFamide-immunopositive cells were always associated with ciliated cells. *Bars* 10 μm

The timing of myogenesis in vitro is remarkably similar to that in vivo. Thus, in the cell culture derived from 24-h-old trochophores, the muscle proteins appear after 6 h of cultivation. Similarly, in vivo, the proteins can initially be detected at 30 hpf (Dyachuk and Odintsova 2009). The cross-striated pattern appears after 36 hpf in vivo and 12 h in culture and is replaced by the smooth arrangement after 30 days in vivo (Dyachuk and Odintsova 2009) and 20 days in culture.

An important question is whether conditions in vitro alter cell fates. Naganuma and colleagues (1994) suggested that, in the trochophore-derived cell culture of *Haliotis*, the development of myofibrils corresponded to the in vivo development of smooth muscles. However, single striated cells were detected in culture. The authors were unable to explain this, because of the lack of data concerning the cross-striated organization of larval muscles in *H. rufescens* at this time point. Only later did it become known that larval retractor muscles in this gastropod mollusc have a striated pattern (Page 1997). This finding explains the appearance of the striated cells detected in culture.

The muscle assemblage and building of a myofibril from its component proteins exhibits specific features in various

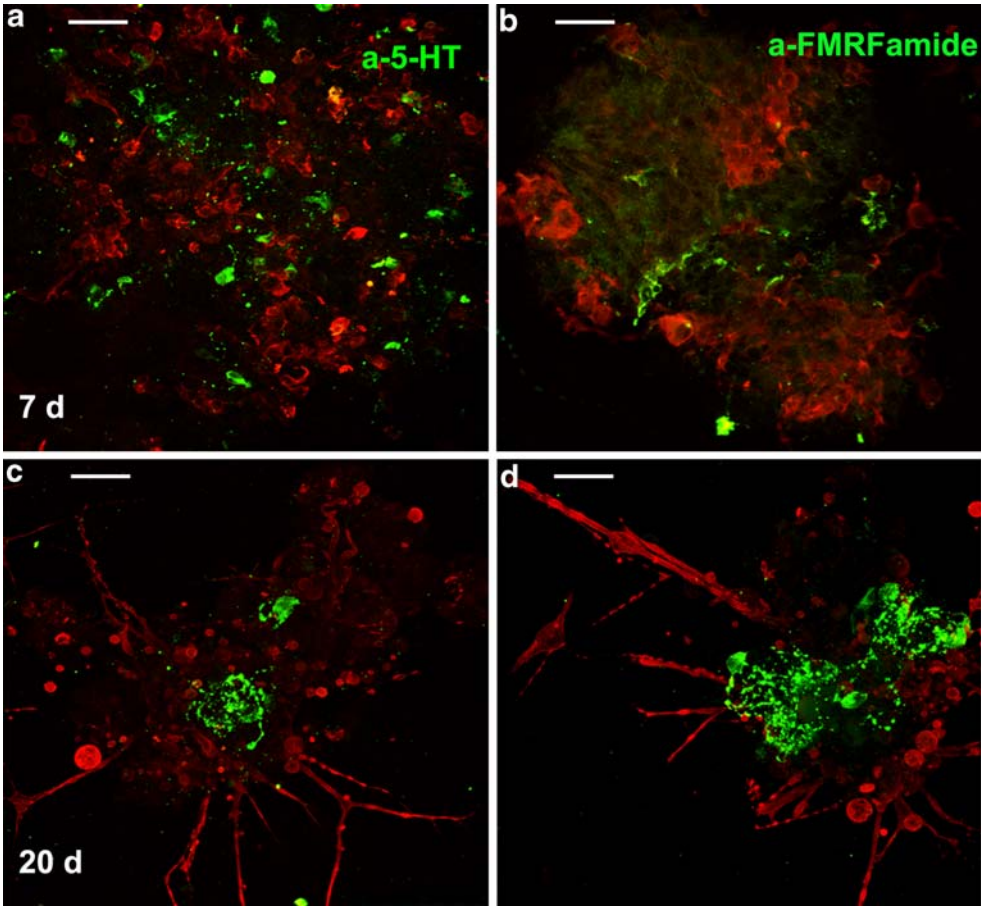

Fig. 9 Mutual alignment of muscle and neuron-like cells in primary culture of larval cells of *M. trossulus* for 7 (**a**, **b**) and 20 (**c**, **d**) days of cultivation. Double-immunostaining for 5-HT (**a**, **c**, *green*) or FMRFamide (**b**, **d**, *green*) and anti-myosin (*red*). In 7-day-old cultures, 5-HT- and FMRFamide-immunoreactive cells were randomly scattered among paramyosin-positive cells (**a**, **b**). In 20-day-old cultures, 5-HT- and FMRFamide-immunoreactive cells were compactly grouped inside the aggregates, whereas myocytes at the periphery of the aggregates sent fibers toward adjacent aggregates. *Bars* 20 μm

organisms (Sanger et al. 2006). In our experiments, the mussel larval cells differentiate into cross-striated muscle cells without forming multinuclear myotubes. A similar situation has been detected in cross-striated muscle cells of the fish *Danio rerio* (Costa et al. 2002, 2003) and the nematode *C. elegans* (Epstein et al. 1993): embryonic myocytes mature without the formation of myotubes. Our results suggest that all the tested muscle proteins act together to form the architecture of muscle cells of *M. trossulus*.

We have previously shown that, in larval *M. trossulus*, all muscle cells are mononucleated (Dyachuk and Odintsova 2009). Perhaps, myocyte maturation without the formation of multinuclear myotubes in larval mussels reflects a developmental strategy that involves the fast development of the larval body and the fast resorbtion of larval muscles during metamorphosis.

BrdU-immunopositive cells have been detected only in cell aggregates and only after 1 week in culture. Since the BrdU test is considered to be a reliable technique to visualize mussel proliferating cells (Zaldibar et al. 2004), we suggest that no cell proliferation occurs in the aggregates after 1 week. We have previously shown that the level of DNA synthesis, as revealed by H^3-thymidine labeling, drops to about zero after 10 days in cultured embryonic cells of various marine invertebrates (Odintsova 2009). Cultured cells derived from planarians reveal mitotic activity for 7 days only (Peter et al. 2004). Thus, our results are in accordance with earlier data.

The synthesis of muscle protein isoforms is known to represent the terminal stage of myogenic differentiation (Sanger et al. 2006). Thus, we suggest that the myogenic cells with contractile activity in the culture are terminal post-mitotic myocytes. However, we cannot unambiguously define the myogenic cells with contractile activity as being terminal post-mitotic myocytes because BrdU immunopositivity in cell aggregates has been detected up to 7 days, whereas muscle proteins have been detected starting from 6 h in culture. Unfortunately, technical peculiarities of the BrdU test (treatment with 2 N HCl) have not allowed us to combine the test with the antibodies against the muscle proteins. However, we have never detected the BrdU immunoreaction in the spindle-shaped myocyte-like cells. Contrariwise, the BrdU-immunopositive cells are always round, and cells of this shape never exhibit immunoreactivity to muscle proteins on any of the tested substrates, except collagen (see above).

For morphological differentiation to occur, cells must adhere to the substrate (Albelda and Buck 1990). Integrins and extracellular matrix (ECM) proteins are involved in cell adhesion (Lafrenie and Yamada 1998) and differentiation to definite cell lineages (Engler et al. 2006; Schenke-Layland et

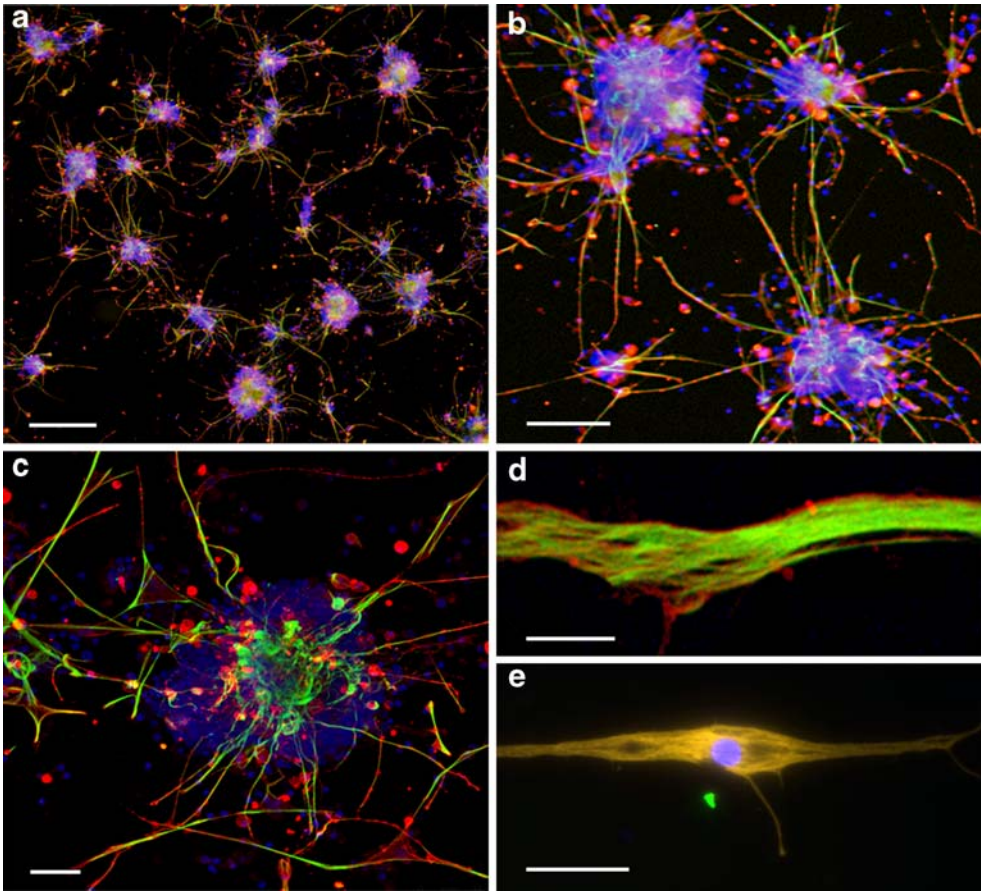

Fig. 10 Network of muscle cells in primary culture of larval cells of *M. trossulus* after 2.5 months of cultivation. The cells were visualized by paramyosin Abs (*red*) and phalloidin for actin detection (*green*). The nuclei were stained with DAPI (*blue*). Cell cultures consisted mainly of dense colonies of actin- and paramyosin-positive cells (**a**), although solitary cells were also present. Adjacent colonies were connected with contractile fibers of spindle-shaped cells (**b**). Muscle cells concentrated in the center of the colony and sent fibers to the periphery and outside of the colony (**c**; laser confocal stacks, 25×0.3 μm). The fibers connecting the colonies (**d**; laser confocal stacks, 25×0.3 μm) and solitary cells outside the colonies (**e**) were mononucleated and showed a smooth pattern of actin and paramyosin distribution. *Bars* 50 μm (**a**), 20 μm (**b**, **c**, **e**), 10 μm (**d**)

al. 2008). The results of our experiments with RGDS-peptide, an inhibitor of integrin receptors and cell adhesion, and the control non-specific RGES-peptide suggest that cultivated mussel cells use an integrin-dependent mechanism for adhesion and outgrowth on the various ECM substrates. RGDS-peptide blocks cell adhesion and inhibits myogenic differentiation, whereas incubation of the cells with RGES-peptide does not affect myodifferentiation. For many adhesion proteins, the RGD-sequence is the common site that is recognized by integrins (Ruoslahti and Pierschbacher 1987). Indeed, RGD-containing peptides are known to inhibit the force production of mouse muscles via α5β1 integrin (Sarin et al. 2005), which is involved in the adhesion of muscle cells to the ECM and defines the structure of nascent myofibrils (Lakonishok et al. 1992).

In our experiments, the ECM proteins affect the myofibril assemblage. Cell spreading and the commitment of the contractile phenotype of mussel cells cultivated on collagen carpets (1 mg/ml) are inhibited, whereas a significant increase of RNA synthesis and proliferative activity have been revealed in these very cells (Odintsova et al. 2000). Reducing the concentration of collagen over a 10-fold range, we have obtained an obvious effect on cell adhesion and differentiation: under these conditions, the mussel cells spread, and contractions have been detected for more than 2 months. These results are similar to those obtained with vertebrate cells, which differentiate on a collagen substrate at a concentration of not more than 0.1 mg/ml (Hauschka and Konigsberg 1966; Haba et al. 1975).

Our protocol, employing Percoll fractionation, efficiently supports differentiation toward not only muscle cells, but also neuron-like and ciliated cells. The cells of some fractions self-organize during culture: at first, they aggregate and then form colonies of muscle and neuron-like cells. Any contacts between muscle and neuron-like cells are difficult to detect in the interior of aggregates. We have observed rounded neuron-like cells without outgrowths situated in the colony center, whereas myocytes are located in a peripheral zone and contact other colonies by forming a contractile network.

In some cell aggregates, we have detected FMRFamide- and 5-HT-immunopositive cells with branched outgrowths that, however, do not spread over the aggregate borders. Notably, we have been unable to detect synapse formation in the mussel cell culture: immunoreaction for synaptophysin and synapsin is negative throughout the period of cultivation. Most probably, we have failed to demonstrate the presence of synaptic proteins because of the difference in the structure of the epitopes between the proteins in mammals and molluscs, since the antibodies visualize synaptic proteins in mammalian tissues but show no immunoreactivity in any of the molluscan

tissues tested. Moreover, we have found no data in the literature with regard to the successful use of these antibodies in molluscs.

Neurons and muscle cells isolated from numerous invertebrate species have previously been shown to be capable of establishing functional synapses in vitro (Seecof et al. 1972; Broadie and Bate 1993; Bi and Poo 1998; Schmidt et al. 2000). Electron microscopy and electrophysiological techniques have demonstrated that the neurons and myocytes of *Drosophila* are completely differentiated in vitro from stem cells (neuroblasts and myoblasts), and that the neuromuscular junctions thereof are morphologically complete and functional (Seecof et al. 1971, 1972; Ritzenthaler et al. 2000). Christensen and colleagues (2002) have observed contacts between neurons and between neurons and muscle cells in primary cell culture of *C. elegans* and suggest that these contacts indicate the occurrence of synapse formation in culture.

In insect and molluscan cell cultures, neurons seem to be necessary for myocyte survival. Neurons (or/and other cells) are capable of synthesizing and secreting soluble factors of cell proliferation and differentiation into the medium, as has recently been reported for arterial cells and sheath cells of the central nervous system in the snail *Aplysia californica* (Montgomery et al. 2002). A clear enhancing influence of neurons on the proliferation and differentiation of myocytes has been found in cultured embryonic cells of the moth *M. sexta* in which neurons stimulate the formation of contractile fibrils in myogenic culture (Luedeman and Levine 1996). Luedeman and Levine (1996) have emphasized that, unlike vertebrate neurons, neurons of this insect do not play a leading role at early stages of myocyte differentiation; however, cell cultures without neurons survive for 1 week only.

Our results and recent data from the literature (Haszprunar and Wanninger 2000; McDougall et al. 2006; Altnöder and Haszprunar 2008) suggest that myogenesis in molluscs is a highly dynamic and potentially variable process. Such a "flexible" developmental program of the larval cells in *M. trossulus* and other species might be regarded as a prerequisite for the evolution of the wide variety of striated and smooth muscles seen in larval and adult molluscs.

From the evolutionary perspective, the study of muscle cell differentiation in molluscs might be of great interest, since the myocyte was most probably the original mesodermal cell type in the early evolution of mesoderm (Rieger and Ladurner 2003). Muscle cells in *M. trossulus* arise from embryonic stem cells, which are committed to myogenesis and similarly transform into myocytes both in vivo and in vitro. On the contrary, in flatworms, which are likewise trochozoans and therefore relatively closely related to molluscs, muscle cells differentiate not only from embryonic totipotent stem cells, but also from neoblasts capable of giving rise to any differentiated cell type in the adults (Ladurner and Rieger 2000; Ladurner et al. 2000; Rieger and Ladurner 2003). Flatworms do not appear to segregate their germ-cell lineage during embryogenesis, unlike *Drosophila*, *C. elegans*, and molluscs. Their plasticity illustrates the enormous capacity possessed by adult planarians both to maintain and to regulate the form and function of their body and is in contrast with the rigidity displayed by adult molluscs. Further studies are necessary to determine whether totipotent embryonic stem cells exist at early stages of development in bivalves and more primitive molluscs.

We have thus shown that the differentiation of muscle and neuron-like cells occurs during the culture of mussel cells taken from premyogenic and prenervous larval stages. Dissociated mussel cells have been found to aggregate and become self-organized in culture. The pattern of thick and thin filaments in cultivated mussel cells changes according to the scenario of the muscle arrangement in vivo: initially, the striated pattern of mussel muscle filaments is formed and is then replaced by a smooth muscle pattern with a diffuse distribution of muscle proteins, typical of the muscles of adult molluscs.

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