Extracellular matrix is required for muscle differentiation in primary cell cultures of larval *Mytilus trossulus* (Mollusca: Bivalvia)

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Received: 20 November 2012 / Accepted: 24 April 2013 / Published online: 9 May 2013
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Abstract Components of the extracellular matrix may modulate the growth factor effects that play important roles in the proliferation and differentiation of precursor cells. We developed an in vitro cultivation protocol for cells of the larval marine bivalve *Mytilus trossulus* to study the role that extracellular matrix components may play in myodifferentiation and replication-mediated DNA synthesis using immunofluorescence and confocal laser scanning microscopy. Here, we demonstrate that the extracellular matrix regulates the expression of muscle proteins, leading to their assembly and the terminal muscle differentiation of larval cells during cultivation. We further show that the myogenesis process progresses in cells cultivated on fibronectin, carbon or poly-L-lysine but is inhibited in cells grown on a collagen carpet. Consistent with a decrease in muscle protein expression in cells cultivated on collagen, we demonstrate an increase in the number of BrdU-positive cells in comparison with cells cultured on other substrates during the entire cultivation period. Moreover, we demonstrate that the matrix-dependent myogenic differentiation of larval mussel cells is reversible. Round-shaped cells cultivated on collagen were able to differentiate into muscle cells after reseeding on

fibronectin, carbon or poly-L-lysine. In addition, cells cultured on collagen and then transplanted to fibronectin exhibited distinct cross-striation and contractile activity. Taken together, our data suggest that the extracellular matrix participates in the regulation of the proliferation and myodifferentiation of mussel trochophore progenitor cells and validate novel approaches for successfully culturing cells from bivalves over extended periods.

Keywords Cell culture · Mussel · Myogenic differentiation · ECM

Introduction

Skeletal myogenesis is comprised of the processes of precursor cell proliferation, myoblast differentiation and migration, muscle protein synthesis, cell–cell fusion to form multinucleated myotubes, and sarcomerogenesis (Buckingham 1994; Sanger et al. 2005). These processes are transcriptionally, translationally and post-translationally regulated (Edmondson and Olson 1993; Yablonka-Reuveni and Paterson 2001). Extracellular matrix (ECM) components are central to the regulation of the differentiation-mediated morphological response of cells to their external environment and influence tissue structure, function, development and gene expression (Taipale and Keski-Oja 1997; Schwarzbauer 1999; Yurchenco et al. 2004). The ECM

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is composed of glycoproteins and proteoglycans that interact with many transmembrane cell adhesion receptors in vertebrate and invertebrate animals (Har-El and Tanzer 1993; Serpentine et al. 2000; Velleman 2002). One of the main classes of these receptors is the integrins, which are heterodimeric transmembrane receptors that transmit external mechanical forces into the intracellular space and induce dynamic reconfiguration of the cell shape and cytoarchitecture (Hynes 2002; Kim et al. 2011). Integrins interact with their ligands, which include ECM components, such as fibronectins, laminins, collagens, and vitronectin. Different tissues harbor unique extracellular matrices and display specific distributions and unique functional properties of integrins that change during development (Elangbam et al. 1997; Krieger et al. 2004). ECM components, together with integrins, have been suggested to play important functional roles in muscle differentiation (Duband et al. 1987; von der Mark and Ocalan 1989; Zagrís et al. 2000; Cachaço et al. 2005). Through integrin cell surface molecules, the ECM may participate in the myofibril assembly of skeletal muscles during the terminal differentiation stage (Katz and Yamada 1997). The ECM component/integrin complex has also been shown to participate in early muscle differentiation stages, such as proliferation, migration and myoblast fusion (McDonald et al. 1995; Gullberg and Ekblom 1995; Bray et al. 2008) because these processes depend on cytoskeleton dynamics that are regulated by the microenvironment. However, similar studies of invertebrates have been limited; in particular, little is known about ECM-dependent cell differentiation processes in invertebrates (Ishii et al. 1992; Urbano et al. 2011; Wolfstetter and Holz 2012).

Invertebrate integrin homologs have been described by Burke (1999), who concluded that invertebrate integrins exhibit a range of functions similar to those of vertebrate integrins and diverged independently within each invertebrate phylum. *Drosophila melanogaster* alpha-PS/beta-PS integrins appear to participate in cell adhesion and migration (Martin-Bermudo et al. 1998; Urbano et al. 2011). The *Caenorhabditis elegans* transmembrane integrin complexes, which form dense bodies, bridge the ECM and cytoskeletal actin filaments, performing a signaling function in muscle cells (Williams and Waterston 1994; Mercer et al. 2003; Lecroisey et al. 2007).

However, a thorough understanding of how the extracellular matrix affects mollusc muscle cell

differentiation and function remains elusive. We have previously demonstrated that muscle cell differentiation occurs during the cultivation of cells from premyogenic larval stages (Plotnikov et al. 2003; Odintsova et al. 2000). We developed a molluscan in vitro technology that allows us to study cellular differentiation (myo-, neuro- and ciliary differentiation) in isolated cells under experimental manipulations (Odintsova et al. 2010). The aims of the present study were to investigate the influence of individual ECM components on the regulation of larval mussel cell spreading and myodifferentiation and to examine whether these ECM components affect cellular DNA synthesis. To achieve these objectives, we produced and characterized specific muscle markers for mussel myogenesis research and used them, together with F-actin staining, to monitor the differentiation of larval cells cultivated on individual ECM components.

Materials and methods

Animals

Marine bivalves, specifically the mussel *Mytilus trossulus*, were collected from the Vostok Bay of the Sea of Japan (Vostok Biological Station, Institute of Marine Biology, Russian Academy of Sciences, Russia) in May 2011 and were stored in natural seawater for 10–20 days at 5–10 °C until the experiments were performed. Spawning of sexually mature specimens was induced by thermal shock. Males and females were placed in separate glass beakers containing UV-sterilized filtered seawater. Filtered seawater (FSW) containing sperm was passed through a 30-μm sterile mesh and the eggs through a 100-μm sterile mesh. The gametes were mixed immediately in FSW, and the resultant embryos were cultivated in 5-l tanks.

SDS-PAGE and WB analysis

For SDS-PAGE, we used samples of highly purified fractions of individual muscle proteins donated by Dr. N. Shelud'ko (Zhirmunsky Institute of Marine Biology FEB RAS, Vladivostok) that were isolated from the mussel *Crenomytilus grayanus*. The proteins were separated by 8 % SDS polyacrylamide slab gel electrophoresis, as described by Laemmli (1970) but with modifications described by Shelud'ko et al. (1999).

Under these conditions, the main contractile proteins (myosin, paramyosin, and twitchin) of various marine bivalve species are quite distinct and can be identified (Shelud'ko et al. 2004). For the WB analysis, we prepared total muscle (anterior adductor) extracts in a sample solution containing 2 % SDS (Merck Millipore, Darmstadt, Germany), 0.1 % 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and 0.001 % bromophenol blue (Sigma-Aldrich). The proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore), as described by Towbin et al. (1979). The membrane was blocked with 5 % (w/v) non-fat dry milk (AppliChem, Darmstadt, Germany) in Tris-buffered saline (TBS, Sigma-Aldrich) containing 0.150 M NaCl (Merck Millipore), 50 mM Tris-HCl (pH 7.5), and 0.2 % Tween-20 (MP Biomedicals, Solon, OH, USA) before the addition of the primary antibody (IgG fractions). The IgGs used for the WB analysis were purified from ascites after ammonium sulfate precipitation, and affinity chromatography was performed by passage over protein A magnetic beads (Dynabeads, Invitrogen, Carlsbad, CA, USA). The bound IgGs were eluted from the column with 0.1 M glycine (pH 2.7), immediately neutralized with 1 M Tris buffer (pH 9.0) and dialyzed against PBS (pH 7.2). The titers of the antigen-specific antibodies were measured by ELISA and absorption at 280 nm in accordance with the manufacturer's protocol (Thermo Scientific, Waltham, MA, USA). The membranes were incubated with primary antibodies diluted 1:2,000–1:3,000 and with a secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA), diluted 1:5,000–1:10,000. Color development was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (MP Biomedicals).

Primary cell culture

Primary cell cultures were prepared from early trochophore larvae (24 h post-fertilization, hpf at 18 °C) of the mussel *M. trossulus*. These primary larval cell cultures were obtained as described previously (Odintsova and Khomenko 1991) but with modifications. A combination of chemical and mechanical dissociation methods was employed for larval mussel dissociation. Larvae were collected on a fine 30- μ m nylon mesh, washed three times in filter-sterilized Ca^{2+} - and Mg^{2+} -free salt solution (CMFSS) with antibiotics (gentamycin

(40 μ g/ml, DalChemPharm, Khabarovsk, Russia)/ampicillin (100 U/ml, Biosynthesis, Penza, Russia) and were then collected and resuspended in 0.125 % collagenase (PIBOC, Vladivostok, Russia) in CMFSS at 17 °C with agitation to dissociate the cell aggregates. Next, the packed cells and small fragments of larvae were homogenized in the same solution and collected by centrifugation at 1,500 rpm for 10 min. The cell pellets were washed three times with CMFSS and two times with FSW and then resuspended in modified Leibovitz's L-15 (L-15 M, Life Technologies, Darmstadt, Germany) medium, which was modified for bivalves by Odintsova and Khomenko (1991) by adding the following supplements: 2 % fetal bovine serum (Gibco/Life Technologie, Germany), insulin (50 mg/l, Sigma-Aldrich, USA), and α -tocopherol-acetate (1.75 mg/l, Sigma-Aldrich, USA). The cell suspensions were passed through a 12- μ m sterile mesh to eliminate the larval fragments. Finally, the cells were seeded at a density of 2×10^6 cells/cm² in plastic Petri dishes (Lux Culture Dishes, ICN Biomedicals, Irvine, CA, USA) on glass coverslips coated with carbon or other adhesive substrates [collagen type I from rat tail (BD Biosciences, San Jose, CA, USA), human fibronectin (Sigma-Aldrich, USA) or poly-D-lysine (190 kDa, Sigma-Aldrich, USA)] or on uncoated coverslips as a control. The primary cell cultures were monitored daily, and the L-15M media were changed 24 h after starting the cultures and every 2–3 days thereafter. The viability of the cells in culture was assessed by the trypan blue exclusion method using a Carl Zeiss Axiovert microscope (Oberkochen, Germany) at 10 $\times$ magnification; cellular aliquots were analyzed before cell seeding and during each media change. When the larval cell viability before seeding reached 95 % or higher, we began to cultivate the cells on the substrates. The protocol for preparing the ligands [collagen type I, fibronectin or poly-D-lysine (190 kDa)] was performed as previously described (Odintsova et al. 2010). The cells were also seeded onto uncoated glass coverslips as a control. After 2 h, 12 h, 1 d, 2 d, 10 d, or 20 days, the cells were fixed for immunocytochemical assays.

Primary cell culture-reseeding

After 2 days of cultivation, the cell cultures seeded on collagen I (1 mg/ml) were treated with CMFSS in a 5 mM EDTA solution, centrifuged at 1,500 rpm for 10 min and resuspended in fresh L-15M containing

the same supplements. The cells were seeded at a density of 1×10^6 cells/cm² on the following substrates: collagen I type, fibronectin, poly-D-lysine (190 kDa), or carbon. 2 days after reseeding, the cells were fixed for immunocytochemistry.

Immunocytochemistry and microscopy

Mytilus trossulus larval cells cultivated on coverslips coated with substrates were rinsed three times with sterile FSW to eliminate the detached cells and were fixed in 3.7 % paraformaldehyde (PFA, Sigma) in 0.1 M PBS (pH 7.8) for 10 min at room temperature (RT) before being washed three times in 1× PBS. For immunostaining, we used fresh-fixed material and never stored the coverslips with cells at 4 °C. The cells were incubated for 1 h at RT in a solution containing 10 % normal goat serum (Sigma), 0.25 % BSA, and 0.1 % Triton X-100 in PBS to block non-specific binding and for permeabilization. Rabbit primary antibodies (produced in our laboratory) were added in the same buffer. The larval cells were labeled using a polyclonal antibody against myosin, paramyosin or twitchin at a dilution of 1:2,000–1:3,000 and a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 or 546 (Molecular Probes—Life Technologies) at a dilution of 1:1,000–1:2,000. In experiments aimed at revealing the early cell morphology of muscle precursor cells and non-muscle cells, we used muscle primary antibodies and Alexa Fluor 546 secondary antibodies in combination with phalloidin tagged with Alexa Fluor 488 (Molecular Probes) at a dilution of 1:500 for revealing the actin cytoskeleton. After several washes with PBS, the cells were mounted on coverslips with Vectashield containing DAPI (Vector, Novosibirsk, Russia).

Muscle (adductor) fibers from the mussel *M. trossulus* and the pecten *Chlamys nipponensis* were isolated and fixed in 4 % PFA in PBS for 3 h at 4 °C. After a washing step, the tissue was permeabilized in PBS with 1 % Triton X-100 and incubated in blocking solution overnight at 4 °C. The tissue was incubated overnight with primary polyclonal rabbit antibodies against myosin, paramyosin or twitchin at a dilution of 1:1,000 and then with Alexa Fluor 408-, 488- or 546-conjugated goat anti-rabbit IgG for 2 h at 4 °C. For imaging, the cultured cells or tissues were viewed using bright-field and fluorescence microscopy. Images were acquired for color slides using a TCS

SPE laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with the 20× and 40× dry objectives and the 63× and 100× oil-immersion objectives of a conventional Axiovert 200 microscope (Carl Zeiss, Germany). The resulting laser confocal stacks (LCS) of the optical sections were processed using Leica LAS AF software. For 3D reconstructions, ImageJ software (NIH) was used.

Larval mussel cell proliferation assay

To culture larval mussel cells, we used a standard protocol that was previously described in detail (Odintsova et al. 2010). Briefly, after 24 h, 10, or 20 days, the cell cultures were carefully rinsed with medium to remove the unattached cells. After treatment, the cells were incubated with 1 mM 5-bromo-2'-deoxyuridine (BrdU, MP Biomedicals) in L-15 M medium for 12 h at 17 °C. The incorporated BrdU was detected using an anti-BrdU monoclonal antibody (MP Biomedicals) at a 1:100 dilution. Cells undergoing DNA synthesis incorporate BrdU into their DNA. Following immunochemical processing using a BrdU-specific monoclonal antibody and a goat anti-mouse secondary Alexa Fluor 546 IgG antibody (Molecular Probes) diluted 1:1,000 in PBS, the cells were mounted on coverslips with Vectashield containing DAPI (Vector) and examined as described above.

Results

Antibody characterization

The quality of the samples obtained from the highly purified fractions of individual muscle proteins was tested by SDS-PAGE. The specificity of the polyclonal antibodies against myosin, paramyosin or twitchin was investigated by WB using total protein extracts from the anterior adductors and by indirect immunofluorescence (IF) methods using isolated muscle fibers from the mussel *M. trossulus*. The SDS-PAGE analysis revealed bands that corresponded to twitchin (TW, 530 kDa), myosin heavy chain (MHC, 200 kDa), and paramyosin (PM, 100 kDa) (Fig. 1a). The antibody against the mussel thick filament strongly recognized individual bands from the total muscle extract of the mussel *M. trossulus*

Fig. 1 Specificity of primary antibodies to mussel muscle proteins. Antibodies were raised against samples from highly purified fractions of individual muscle proteins [twitchin (530 kDa), myosin (200 kDa) and paramyosin (100 kDa)] isolated from the mussel *Crenomytilus grayanus* and were subjected to SDS-PAGE (a). The antibodies were tested by western blot analysis (b) against the total muscle (anterior adductor) protein extracts from the mussel *M. trossulus*. Immunofluorescence staining using the different muscle antibodies labeled the striated myofibrils from the pecten *Chlamys nipponensis* (c, e, g) and the smooth muscle fibers from the mussel *M. trossulus* (d, f, h) but not other tissues from adult molluscs (data not shown)

(Fig. 1b). No cross-reactivity with any other proteins was detected (data not shown). The specificity of the antibodies for the muscle proteins was also supported by IF results (Fig. 1c–h). This result demonstrated that the antibodies reacted specifically with the smooth muscle fibers from *M. trossulus* (Fig. 1d–h) and with the striated muscles from the pecten *Chlamys nipponensis* (Fig. 1c–g) but not with other tissues from adult molluscs (data not shown).

Changes in the morphology of primary larval cells during cultivation

Trochophore-dissociated, round-shaped *M. trossulus* cells were seeded onto coverslips coated with fibronectin (Fig. 2a–c), poly-L-lysine (Fig. 2d–f), carbon (Fig. 2g–i), or collagen (1 mg/ml) (Fig. 2j–l). Cells that were seeded onto uncoated coverslips (as a control) did not attach effectively to the glass, and after medium replacement, more than 90 % of the total number cells seeded onto the uncoated coverslips was lost. Within 2 h (Fig. 2a, d, g, j), the larval cells attached to all coating substrates except collagen and began to spread. The number of attached cells increased with time for all substrates. Of the cells that formed clusters, most were adherent to the fibronectin, poly-L-lysine or carbon coating. After 24 h of cultivation, the medium was changed, and the non-adherent, floating cells were collected with the withdrawn medium. After this step, two morphological types of adherent larval cells were clearly distinguishable: round-shaped, epithelial-like cells and bipolar, fibroblast-like cells (Fig. 2b, e, h). Notably, terminal differentiation, myogenic differentiation and contractile activity were detected after 24 h of culture on fibronectin, poly-L-lysine, and carbon (Fig. 2c, f, i). The first spontaneous contraction of cells seeded on fibronectin and carbon was observed after 12 h of cultivation. The number of contractile cells and the rate and frequency of contraction increased gradually during cultivation. The epithelial-like cells and fibroblast-like cells, either single or aggregated, were detected at 10 d (Fig. 2c, i) and later (data not shown). After 10 d of cell cultivation on collagen (data not shown for 20 d), only round-shaped, epithelial-like cells were observed in the culture (Fig. 2k).

Myogenesis in primary larval cell cultures

To analyze the expression of muscle proteins and myofibrillogenesis in larval muscle cells seeded on different substrates, we employed mussel polyclonal antibodies (against myosin, paramyosin, and twitchin) and fluorescent phalloidin (to detect F-actin) (Fig. 3) in all cell types. We observed that F-actin was initially expressed by the cells grown on fibronectin, poly-L-lysine or carbon at 2 h after seeding (Fig. 3a, e, i). F-actin was not detected in all of the cells in the primary mussel culture and was either homogeneously

Fig. 2 Effect of substrates on larval mussel cell morphology after 2, 24 h, or 10 days of cultivation. Cultivation of trochophore-derived cells plated on fibronectin (a–c), poly-L-lysine (d–f), carbon (g–i), or collagen I (1 mg/ml) (j–l)

localized in the cytoplasm (on fibronectin or carbon, Fig. 3a, i) or concentrated around the membrane (on poly-L-lysine, Fig. 3e). After 12 h of cultivation, we detected many bipolar cells that expressed the muscle proteins paramyosin, myosin or twitchin together with F-actin (Fig. 3b, f, j, arrows). Thick (myosin, paramyosin, and twitchin) and thin (actin) filament proteins were organized in striations that resembled cross-striated muscle cells by 24 h (Fig. 3c, g, k, arrows). By day 20, we did not observe striated muscle cells, as the pattern of thick and thin filaments in the contractile cells had changed to a diffuse distribution of muscle proteins in the cytoplasm resembling that of smooth muscle cells (Fig. 3d, h, l).

The round-shaped cells cultivated on collagen were never immunoreactive with the tested muscle protein antibodies at early stages of cultivation. The single cells in these cultures expressed muscle proteins (myosin, paramyosin or twitchin) by 20 days. However, they did not assemble functional sarcomeres and formed mononucleated myoball-like morphological structures (Fig. 3m–p). Moreover, cytoskeleton proteins (actin and tubulin) were strongly inhibited in these cells (data not shown).

Changes in larval cell morphology and myogenesis after reseeding

Cells cultivated on collagen for 2 days, which displayed a rounded morphology (Fig. 4a), were mechanically detached in CMFSS with EDTA, reseeded on fibronectin, poly-L-lysine, collagen or carbon and then cultivated for 2 days in the same L-15M medium. As expected, no changes in the morphology of the larval cells cultivated on collagen were detected. The cells that remained attached to the collagen substrate did not spread (Fig. 4b), whereas the cells reseeded on other substrates (from collagen to fibronectin, poly-L-lysine or carbon) began to spread after attaching to the substrate (Fig. 4d, f, h). Notably, more than 50 % of the resuspended cells attached and spread on the new substrates. Similarly, as previously reported, the cells cultured on fibronectin, poly-L-lysine or carbon differentiated and subsequently acquired contractile activity. However, the appearance of bipolar cells and the first contraction were shifted by at least 24 h (total cultivation time = 48 h) in comparison with the primary larval cell cultures (control; total cultivation time = 24 h).

Fig. 3 Effect of substrates on larval mussel cell myodifferentiation after 2, 12, 24 h, or 10 days of cultivation.

Immunocytochemistry of muscle proteins in larval cells cultivated on fibronectin (**a–d**), poly-L-lysine (**e–h**), carbon (**i–l**), or collagen I (1 mg/ml) (**m–p**). The cells were fixed in 4 % formaldehyde in 1× PBS and incubated with anti-myosin primary antibodies, AlexaFluor 488 (green) secondary antibodies, phalloidin tagged with AlexaFluor 546 (red) and DAPI (blue). (Color figure online)

The cells cultivated on fibronectin, poly-L-lysine or carbon expressed actin and thick filament proteins after 24 h. The cells cultivated on fibronectin displayed a cross-striation pattern of muscle proteins after 2 days (Fig. 4i). In contrast, the cells cultivated on poly-L-lysine or carbon did not form distinct striation patterns of muscle proteins (Fig. 4e, g). The cells reseeded from collagen to collagen displayed the expected inhibition of striation pattern formation (Fig. 4c). Immunostaining of primary and secondary larval cell cultures revealed the importance of ECM components in terminal myogenic differentiation and the reversible process of myogenesis control (inhibition or induction).

DNA synthesis

To investigate whether the adhered larval mussel cells could proliferate, we employed a BrdU incorporation test. BrdU incorporation was investigated at 1 days

(Fig. 5a, d, g, j), 10 days (Fig. 5b, e, h, k), and 20 days (Fig. 5c, f, i, l) after culture initiation. The BrdU signals differed among the cells and depended on the type of substrate on which the cells were cultured. Specific staining was restricted to the cell nuclei. In contrast, control dishes without BrdU exhibited no specific anti-BrdU immunostaining. Immunoreactivity to BrdU was detected in the cells plated on fibronectin, poly-L-lysine, carbon or collagen by 10 d of cultivation (Fig. 5b, e, h, k). By day 20, no BrdU-immunoreactive cells were detected on any of the substrates tested, with the exception of collagen (Fig. 5j, k, l).

Discussion

The nature of the components of the ECM dictates the activation or inhibition of the signaling pathways responsible for cellular morphogenetic patterns

Fig. 4 Restoration of the myogenic differentiation of larval mussel cells after cultivation on collagen (a). Larval cells cultivated on collagen were seeded on type I collagen (b, c), poly-L-lysine (d, e), carbon (f, g), or fibronectin (h, i). Analysis of the cellular morphology of the larval cells (b, d, f, h) and the detection of myofibrils by immunofluorescence was performed after 2 days in culture (c, e, g, i)

Fig. 5 Effect of substrates on DNA synthesis in larval mussel cells during cultivation (1, 10, or 20 days). Analysis of BrdU incorporation revealed a decreased proliferation of cells cultivated on fibronectin (a–c), poly-L-lysine (d–f), carbon (g–i), or collagen I (1 mg/ml) (j–l). By 20 days of cultivation, the larval cells plated on collagen were positive for BrdU (arrows)

in vitro. This regulates cell adhesion and spreading and results in changes to cellular morphology and the acquisition of functional cellular diversity.

In this study, we demonstrated that some components of the ECM may promote cell differentiation and proliferation processes in primary cell cultures of the

mussel *M. trossulus*. We showed that larval mussel cells attached to the substrate but did not spread when cultivated on collagen and that they differentiated into contracting muscle cells. Currently we do not know the mechanism by which collagen inhibits myogenesis in larval mussel cells. We suppose that at high concentrations, collagen, a component of the ECM, may inhibit the integrin cell receptors or that larval mussel cells do not express collagen I-associated receptors on their membranes during early differentiation. Thus, mussel cells do not spread and differentiate but retain their ability to proliferate in primary culture. Interestingly, after 20 days of cultivation on collagen, larval mussel cells developed a rounded shape, and only after passage on coverslips coated with fibronectin, poly-L-lysine or carbon, did they begin to express muscle-specific proteins and assemble into sarcomere-like structures. These observations suggest that the inhibition of larval cell myodifferentiation by collagen is a reversible process. Consistent with the decreased expression of muscle proteins that was observed in cells cultivated on collagen, we demonstrated an increase in BrdU-positive cells in comparison to cells cultured on other substrates throughout the entire cultivation period. The BrdU-positive cells were aggregated and never colocalized with diffuse, bipolar cells. If the differentiation process, particularly the mechanism controlling cell spreading, is inhibited, the potential of the cells to proliferate in culture may be elevated. However, if the cells have initiated differentiation and started to express tissue-specific markers, reverting them back to an undifferentiated stage is impossible (except for trans-differentiation). Many examples have been described that demonstrate that epithelial or epithelial-like cells from different tissue sources, such as gills, digestive tubules, intestines, feet, the body wall, gonads (Marigómez et al. 1999; Hanselmann et al. 2000) and larval material (Odintsova and Khomenko 1991; Naganuma et al. 1994; Odintsova et al. 2010), harbor a higher potential for proliferation, are present in all bivalve tissues, and can participate in regeneration processes.

It is known that a high density of substrates likely occupies the relevant receptors (such as integrins) and inhibits integrin-associated signaling pathways, leading to the reduced organization of actin into stress fibers or focal adhesions (Cukierman et al. 2001; Deroanne et al. 2001). Notably, the reactions of cells

to substrates depend on the cell type. Pelham and Wang (1997) reported increased ruffling of NRK epithelial cells and polarization without the formation of stress fibers in fibroblasts cultured on soft gels using collagen-coated polyacrylamide substrates. On more rigid substrates, both the NRK epithelial cells and 3T3 fibroblasts spread well and were indistinguishable from those cultured on glass or plastic surfaces. However, when cultured on higher concentrations of collagen, the NRK cells were more diffuse and irregularly shaped. The behavior of some cells on different substrates is characteristic of important phenotypes; for example, culturing cells on soft agar gels can be used to identify cancer cells. Thus, the reactions of cells cultured on substrates can vary greatly with respect to the expression and engagement of adhesion molecules. Integrins reportedly undergo adhesion-modulating conformational changes in response to force and induce reconfiguration of the cell shape and cytoarchitecture (Benigno et al. 2002).

The larval mussel cells that attached to fibronectin, poly-D-lysine, carbon, and plastic were round-shaped, epithelial-like and bipolar, fibroblast-like cells. The patterns of spreading and morphology of the cells cultivated on these substrates were similar. Moreover, we demonstrated that the tested ECM components promote the expression of muscle proteins, leading to the appearance of F-actin structures (stress fiber-like) after only 2 h of cultivation and following the assembly of cross-striated sarcomeres and the contraction of larval mussel cells during further cultivation. Interestingly, the morphology of these muscle structures was similar in larval cells cultivated on fibronectin, poly-D-lysine, and carbon. Trochophore cells cultivated on uncoated coverslips did not attach and were in suspension after 24 h. We concluded that glass is not an adequate substrate for mussel cell cultivation and can not be used for subsequent myogenic differentiation studies.

Several substrates have also been shown to regulate the activation of adhesion proteins, including integrins, by initiating Rho GTPase signaling, resulting in actin polymerization and the appearance of morphological structures called stress fibers, which are rarely observed in vivo (Larsen et al. 2006). These events lead to focal adhesion formation and the cell movement required for the spreading process. Thus, cells spread, and following terminal differentiation, they are impacted by the substrate type and its physical

properties (Yeung et al. 2005; Engler et al. 2006). Notably, stress fibers may be important for myofibrillogenesis. The template model of myofibrillogenesis proposes that the components of the future myofibril, the dense components of the Z-band and the thin and thick filaments, are recruited to the surfaces of stress fibers or stress fiber-like structures in developing muscle cells (Sanger et al. 2005). Stress fiber-like structures can serve as a temporary template for the elements necessary to form one myofibril (Dlugosz et al. 1984; Sanger et al. 2005). Once assembly is complete, the stress fiber template disappears and reassembles to serve as a template for a new assembling myofibril. Indeed, 2 h after seeding, the larval mussel cells were not immunoreactive for any of the tested muscle proteins, whereas F-actin filaments were observed in the cultured mussel cells.

We demonstrated that the tested substrates promoted not only the morphological differentiation but also the functional development of the cells in culture. We detected spontaneous rhythmic contractions after 24 h of culture on fibronectin, poly-L-lysine, and carbon. The number of contractile cells and the rate and frequency of contraction increased gradually during cultivation. Our data confirm previously published data that revealed changes in the contractile activities of larval mussel cells during cultivation (Plotnikov et al. 2003). Despite the contractile activity of *M. trossulus* veliger larvae muscles, they are not capable of phasic contractions; all contractions of the velum retractor muscles, the heart, and the intestine are typically tonic. In primary cell cultures derived from trochophores, the first spontaneous rhythmic contractions are phasic contractions only. After only 25 h of cultivation the cell contractile activity was shown to change, as both phasic and tonic contractions occur. After further cultivation, the cells maintain their phasic and tonic contractions, and both types are temporally regular (Plotnikov et al. 2003).

In this study, we demonstrated that components of the ECM may control cell differentiation and proliferation processes in primary cell cultures of the mussel *Mytilus trossulus*. Further analysis of the expression of ECM components, integrins, and integrin-associated proteins may elucidate the molecular mechanisms governing the specialization of marine bivalve cells.

Acknowledgments This work was supported by the Russian Foundation for Basic Research (grant no. 13-04-00946); the

study was partly performed at the “CHROMAS” center (St. Petersburg State University, Russia) and used technical resources of the “Vostok” Marine Biological Station and the core facility of IMB FEB RAS. V. Dyachuk received financial support from the Organizing Committee to attend the Marine Invertebrate Cell Culture symposium.

Conflict of interest There is absolutely no conflict of interest in this manuscript.

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