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Photolithographic Patterning of C2C12 Myotubes using Vitronectin as Growth Substrate in Serum-Free Medium

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Abstract

The C2C12 cell line is frequently used as a model of skeletal muscle differentiation. In our serum-free defined culture system differentiation of C2C12 cells into myotubes required surface-bound signals such as substrate adsorbed vitronectin or laminin. Based on this substrate-requirement of myotube formation, we developed a photolithography-based method to pattern C2C12 myotubes, where myotubes formed exclusively on vitronectin surface patterns. We have determined that the optimal line width to form single myotubes is approximately 30 μm . In order to illustrate a possible application of this method, we patterned myotubes on the top of commercial substrate-embedded microelectrodes. In contrast to previous experiments where cell patterning was achieved by selective attachment of the cells to patterned surfaces in a medium that contained all the factors necessary for differentiation, this study illustrates that surface patterning of a signaling molecule, which is essential for skeletal muscle differentiation in a defined system, can result in the formation of aligned myotubes on the patterns. This technique is being developed for applications in cell biology, tissue engineering and robotics.

Keywords

C2C12; myotube formation; vitronectin; photolithography; patterning; serum-free

Introduction

Potential applications for artificial skeletal muscle includes tissue replacement, physiological and pharmacological studies, disease models and robotics (1–5). Although skeletal muscle engineering has shown significant success in the last decade, there are still several challenges hindering the development of functional artificial skeletal muscle, such as: low contractile force and lack of spatial control of myotube formation and attachment (1,2,6–8).

The goal of our investigation was to develop a method for the integration of skeletal muscle with silicon structures using the standard lithographic manufacturing steps for applications using MicroElectroMechanical Systems (MEMS) or other microdevices. As a model system, we utilized the C2C12 mouse skeletal muscle cell line because it has been frequently used for the study of myoblast differentiation, neuromuscular junction formation or muscle pharmacology (9–15). The control of the attachment of the myoblasts and their subsequent myotube formation was accomplished by adapting a photolithography-based method which

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BRIEFS: A photolithographic method was developed to pattern C2C12 skeletal muscle myotubes on vitronectin growth substrate in serum-free medium for tissue engineering and robotics applications.

has previously been applied to pattern neurons and other cell types (16,17). Besides photolithography, several other techniques (micro contact printing, micro fluidic patterning, inkjet printing, etc.) have already been established to pattern cells (18–28).

One of the common problems in patterning cells when using chemical cues on a culture surface has been the necessity of utilizing serum-free medium, because adsorbed proteins from serum may obscure the surface patterns (17,29). C2C12 cells had already been shown to form myotubes under serum-free conditions (12,30–32). Synthetic surfaces (N-(2-aminoethyl)(3-aminopropyl) trimethoxysilane/EDA/and tridecafluoro-1,1,2,2- tetrahydrooctyl-1-dimethylchlorosilane/13F/) have also been used for the study of the attachment, morphology, and proliferation of C2C12 cells (36), but myotube formation has not been reported on these surfaces. In the course of our study we have observed that in our serum-free media formulation C2C12 cells also required specific surface-bound signals to form myotubes. It was found that adsorbed Vitronectin promoted myotube formation. Vitronectin has been an important serum component and it was among the first proteins to adsorb onto hydrophilic surfaces upon exposure to serum (33). Vitronectin has been reported to play a role in myotube attachment and differentiation (34,35). The combination of a surface dependent cue for myotube formation and a serum-free medium that did not promote myotube formation by itself opened the possibility for selective control of C2C12 myotube formation on surface patterns.

Materials and Methods

Surface modification

Glass coverslips were cleaned using HCl/methanol (1:1), soaked in concentrated H₂SO₄ for 30 min then rinsed in double deionized H₂O. Coverslips were then boiled in deionized water, rinsed with acetone and oven dried. The trimethoxysilylpropyldiethylenetriamine (DETA, United Chemical Technologies) film was formed by the reaction of the cleaned surfaces with 0.1% (v/v) mixture of the organosilane in toluene. The DETA coverslips were heated to just below the boiling point of toluene, and then rinsed with toluene, reheated to just below the boiling temperature, and then oven dried. Surfaces were characterized by contact angle and X-ray photoelectron spectroscopy methods (29). In some experiments DETA coated coverslips were incubated in 1) Cell culture medium + 10% fetal bovine serum 2) 10 µg/ml (in water) vitronectin (Invitrogen) 3) 10 µg/ml (in water) laminin (Invitrogen) for 1 h at 37°C.

Surface patterning

Quartz photomasks were designed using the CleWin layout editor (WieWeb, Hengelo, The Netherlands) and fabricated through a commercial vendor (Bandwidth Foundry Pty Ltd., Australia). The surface of the protein coated coverslips, that was not covered by the photomask, were ablated using a 193 nm Ar/F LPX200i laser beam (Lambda Physik, Ft. Lauderdale, FL) combined with a beam homogenizer (Microlas, Ft. Lauderdale, FL, Energy density: 50 mJ/cm²) to create the patterns.

C2C12 culture

C2C12 cell line was obtained from ATCC. Cell stock was grown in T-75 flasks in DMEM (HyClone, SH30243.01) + 10% Fetal Bovine Serum at 5% CO₂ and 37°C. After confluence, the cells were dissociated in Cell Dissociation Solution (HyClone, HyQTase) by titration in a 5 ml pipette. After centrifugation (500g, 5 min), the C2C12 cells were replated in differentiation medium (DMEM+ 1% B27 supplement, Invitrogen) on the glass coverslips.

All cell culture experiments were repeated 3 times with 3 coverslips in each of the experiments.

Immunohistochemistry

Coverslips were fixed in -20°C methanol for 5 min and followed by permeabilization in PBS + 1% BSA + 0.05% saponin (permeabilization solution) for 5 minutes. The non-specific binding sites were blocked using permeabilization solution + 5% donkey serum (blocking solution) for 30 min at room temperature. C2C12 myotube coverslips were then incubated in anti-embryonic, myosin heavy chain, primary antibody (F1.652, Developmental Hybridoma Studies Bank), and diluted 1:4 in a blocking solution overnight at 4°C . The coverslips were then incubated in secondary antibody (Molecular Probes A-21202), diluted 1:200 in a blocking solution for 2 hrs at room temperature.

Results and Discussion

Surface-bound signals required for myotube formation

Our first observation was that, in contrast to the published results (12,30–32), C2C12 myoblasts did not differentiate into myotubes when replated in our serum-free medium formulation. However, we observed that the myotube formation occurred only when, instead of replating the cells at confluency in the serum-free medium, we just replaced the serum-containing medium with the serum-free formulation. Based upon this observation we repeated our initial experiments, but before replating, we incubated the culture surface (which was either cell-culture quality plastic or DETA coated coverslips) with the serum-containing medium for 1 h and followed that with rinsing the coverslips in serum-free medium 3 times. This short surface treatment resulted in myotube formation under serum-free conditions using our medium formulation. Our conclusion was that factors which were adsorbed from the serum-containing medium to the culture substrate were necessary for C2C12 myoblast differentiation. To support the observation, Fauxcheau et al. (2004) had reported that vitronectin (adsorbed to the culture surface from serum) was the main attachment protein mediating the initial adhesion of the cells in the presence of serum (33). Therefore, we repeated our experiments with C2C12 cells using cell-culture quality plastic and DETA-coated coverslips incubated with 10% serum, 10 $\mu\text{g}/\text{ml}$ vitronectin or 10 $\mu\text{g}/\text{ml}$ laminin (a commonly used cell-culture substrate interacting with integrin receptors) for 1 h. Although initial cell attachment was the same on all surfaces, myotube formation was not observed on the untreated surfaces, see Figure 1. Moreover, by day 5 *in vitro* C2C12 cells were detaching from these surfaces. Myotube formation was started at day 3 for the vitronectin treated surface, day 5 for serum and about day 7 for laminin. Myotube formation was strongest on the vitronectin coated surface. The effect of vitronectin on myotube formation was concentration dependent (0.01 – 10 $\mu\text{g}/\text{ml}$ range, data not shown). Both vitronectin and laminin are known substrates of integrin receptors. They have been shown to cause muscle differentiation in *Drosophila* embryo cells and follow alternate intermediate differentiation steps without affecting the final outcome (35).

Control of myotube formation through patterning of vitronectin on the surface

In our serum-free conditions the differentiation of the C2C12 cells was very sensitive to the surface coatings. Myotubes were formed only on those surface areas where vitronectin was present. Based on this observation, we adopted a photolithography-based technique (laser ablation, (37)) in order to create vitronectin patterns on the culture surface and used these patterns to subsequently control the myotube formation. Using patterns of different line-widths we determined that below a 10 μm line width myotubes did not form, at a 30 μm width single myotubes formed, and above a 30 μm width multiple myotubes formed on the lines. This observation was important because this dependence of myotube formation on the pattern dimensions makes it possible to separate different cell types; for example neurons and myotubes, just by pattern geometry (neurons grow relatively well on 5 μm lines) in future co-culture experiments.

In order to demonstrate the control of myotube formation utilizing surface patterns on a recording platform we registered these patterns with an array of substrate embedded microelectrodes (Fig. 3.). These microelectrodes could be useful for selective stimulation of single myotubes in robotics, MEMS or drug screening applications.

In summary—We have shown that myotube formation by C2C12 myoblasts is highly dependent on the culture surface under serum-free conditions. In contrast to previous experiments where surface patterns were created to guide only the initial attachment of the cells in a medium which contained all the factors necessary for differentiation, in this study patterning was achieved by the combination of a surface dependent cue for myotube formation and a serum-free medium that did not promote myotube formation by itself. This study is an example for the development of an active surface which determines the behavior of a biological system through surface-immobilized and patterned signaling molecules and could inspire a wide range of applications of this technique. In our laboratory this method is being further developed for applications in cell biology, tissue engineering and robotics.

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Figure 1. Substrate-dependence of myotube formation of the C2C12 myoblasts. Cells were cultured on the surfaces for 10 days in serum-free medium. A; Cell-culture quality plastic B: Plastic coated with serum proteins C: DETA coverslips coated with serum proteins D: DETA coverslips E: DETA coverslip coated with laminin F: DETA coverslip coated with vitronectin. Phase contrast, scale bar: 200 μm .

Figure 2.

Line-width dependence of myotube formation. A: Mask design with 50, 30, 20, 10, 5, 2 and 1 μm lines. B: Vitronectin coated coverslips were patterned using this mask and the C2C12 cells were then plated on the patterns. After 5 days single myotubes were observed on the 30 μm lines. Thicker lines resulted in the formation of multiple myotubes, whereas myotubes did not form on the thinner lines. C: Myotubes, plated on 30 μm lines, were visualized by immunostaining for the myosin heavy chain. Scale bar: 200 μm .

Figure 3.

Patterning of the C2C12 myotubes on the surface of the substrate embedded microelectrodes. The pattern consisted of 30 µm thick parallel lines connected with 2 µm thick lines at the electrodes. Myotubes formed almost exclusively on the 30 micron lines, but some myoblasts (not differentiating into myotubes) were attaching to the 2 micron lines. In rare cases myotubes formed on the 2 micron lines, which could be the result of an error in the patterning process. Phase contrast, Day 5. A: scale bar: 200 µm. B: scale bar: 100 µm.