Essential environmental cues from the satellite cell niche: optimizing proliferation and differentiation


1Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven; and 2Department of Physiology, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands

Submitted 12 January 2009; accepted in final form 24 March 2009

{Am J Physiol Cell Physiol 296: C1338–C1345, 2009. First published March 25, 2009; doi:10.1152/ajpcell.00015.2009.—The use of muscle progenitor cells (MPCs) for regenerative medicine has been severely compromised by their decreased proliferative and differentiative capacity after being cultured in vitro. We hypothesized the loss of pivotal niche factors to be the cause. Therefore, we investigated the proliferative and differentiative response of passage 0 murine MPCs to varying substrate elasticities and protein coatings and found that proliferation was influenced only by elasticity, whereas differentiation was influenced by both elasticity and protein coating. A stiffness of 21 kPa optimally increased the proliferation of MPCs. Regarding differentiation, we demonstrated that fusion of MPCs into myotubes takes place regardless of elasticity. However, ongoing maturation with cross-striations and contractions occurred only on elasticities higher than 3 kPa. Furthermore, maturation was fastest on poly-d-lysine and laminin coatings.

SATELLITE CELLS (SCs) can be considered adult muscle stem cells and are the most promising candidate for regenerative medicine in skeletal muscle disorders (1, 5, 10, 24, 28). In the body, when muscles are damaged or otherwise compromised, these ordinarily quiescent cells are activated and become proliferating myoblasts. Myoblasts then migrate to the designated site to form new myofibers by fusing with each other or with damaged fibers. In addition, they can give rise to new SCs by self-renewal (35). Thus, in theory, these cells are an inexhaustible source of cells for muscle regeneration throughout life. It is therefore not surprising that they have been used for regenerative medicine purposes, such as myoblast transfer therapy for muscular dystrophies (12, 14–16, 27, 32) and tissue engineering for replacement therapy (2, 8, 9, 17, 22, 23, 29). For both types of therapies, cells will need to be expanded to reach required cell numbers.

In the case of the SC and its proliferating progeny, the myoblast or muscle progenitor cell (MPC), it has been shown that in vitro culturing is deleterious to its performance as a stem cell (5), leading to a loss of proliferative capacity and failure to differentiate into functional muscle tissue. It has been hypothesized that this phenotypical change is caused by loss of the “niche” environment that surrounds these cells in an in vivo situation (3). Clearly, cultured cells encounter a two-dimensional environment that is very different from the in vivo environment. First, in vivo, the SC is located in between the sarcolemma of a muscle fiber and a basement membrane (BM). The importance of the close contact of the SC with its parent fiber is underscored by the fact that implantation of SCs together with their parent fiber results in higher regenerative capacity than implantation of enzymatically isolated “single” SCs, suggesting that either the parent fiber or the ability of the SC to bind to the parent fiber is pivotal to its stem cell nature and that this is compromised by in vitro culturing or enzymatic isolation (5). The BM is made up of networks of laminin and collagen type IV, linked by entactin, which provide attachment sites but also serve as a reservoir and regulator of growth factor supply to SCs. In vitro experiments have shown that several matrix proteins, including those present in the BM, affect myoblast proliferation and differentiation (13, 18, 20). Second, the elasticity of an in vitro environment (tissue culture plastic) is very different from the in vivo environment, which could also affect the stem cell phenotype. Several observations have suggested that muscle differentiation is strongly influenced by substrate stiffness: C2C12 myoblasts only showed striations characteristic of maturation on a small range of soft gels or on top of a layer of myotubes of approximately equal stiffness (11). In addition, the proliferation of myoblasts has been shown to be influenced by substrate stiffness, with higher stiffness leading to higher proliferation rates (4).

In this study, we investigated the influence of substrate elasticity and protein coating alone or in combination on primary myoblast proliferation and differentiation. For this reason, we isolated murine MPCs and cultured them without prior expansion on coverslips or polyacrylamide (PA) gels with different elastic properties and BM protein coatings, after which the proliferation and differentiation capacities of these cells were analyzed.

MATERIALS AND METHODS

MPC isolation and culture. MPCs were prepared from single fibers from intact hindlimb muscles (extensor digitorum longus, tibialis posterior, tibialis anterior, and soleus muscles) as previously described (5, 30). Briefly, whole muscles were dissected from 10- to 12-wk-old male C57BL/6 mice and digested for 1 h with 2 mg/ml (567 U/ml) collagenase type I (Sigma-Aldrich, St. Louis, MO). Afterward, muscles were passed through pipettes of decreasing diameter in culture medium consisting of DMEM Advanced (Invitrogen, Carlsbad, CA) containing 20% FBS) Greiner Bio-One, Frickenhausen, Germany), 10% horse serum (Invitrogen), 100,000 IU/l penicillin and 100 mg/l streptomycin (Lonza), and 4 mM l-glutamine (Lonza), resulting in a single fiber suspension. Single fibers were then triturated with a 19-gauge needle for 5 min and passed through a 40-μm cell sieve. The resulting single cell suspension was frozen in culture medium containing 10% DMSO (Merck, Schiphol-Rijk, The Netherlands) in liquid nitrogen until use. This animal study with
number 2007-152 was authorized by the Dutch Institutional Animal Care and Usage Committee.

Coated PA gels and coverslips. PA gels were created using a method adapted from Ref. 25. N,N',N''-methylene-bis-acrylamide (0.03%, 0.13%, 0.26%, or 0.3%, Sigma-Aldrich) was mixed with acrylamide (5% or 10%, Sigma-Aldrich) and cross-linked with 10% ammonium persulfate [1/200 (vol/vol); Fisher, Pittsburgh, PA] and N,N,N',N''-tetramethylethylenediamine [1/200 (vol/vol), Merck]. Droplets of the solution (100 μl) were placed on a Teflon surface and covered by 13-mm 6-aminoacridine [with (3-aminopropyl)trimethoxysilane, Sigma-Aldrich] glass coverslips (Menzel, Braunschweig, Germany) for polymerization. Protein coatings of growth factor-reduced Matrigel (15 µg/cm², BD Biosciences), entactin-collagen-laminin (ECL) gel (15 µg/cm², Millipore), collagen type IV (15 µg/cm², BD Biosciences), or poly-D-lysine (180 µg/cm², Sigma-Aldrich) were cross-linked to the inert surface of the gels using heterobifunctional sulfosuccinimidyld-6-(4'-azido-2'-nitrophenylamino)hexanoate (Pierce Bio-technology). Laminin (15 µg/cm², BD Biosciences) was adsorbed to poly-D-lysine (Sigma-Aldrich) because it was not suitable for cross-linking. Adhesive was also used for coating coverslips without gels. All proteins were added in excess to ensure equal coating.

Indentation tests of PA gels. The elastic modulus of the PA gels was determined by indentation tests (7). Indentation was applied to the center of the gels with a spherical indenter while force and depth were measured. Afterward, a numerical model was iteratively fitted to these experimental results using a parameter estimation algorithm.

Bromodeoxyuridine assay. Cells were labeled for 16 h with bromodeoxyuridine (BrdU) and treated according to the manufacturer’s protocol (Roche Diagnostics). In brief, cells were fixed with ethanol 5 min, and mounted with mowiol (Calbiochem, San Diego, CA) on coverslips. Substrates were coated with Matrigel, ECL gel, collagen type IV, poly-D-lysine, and laminin. Cells were analyzed after 1, 4, 6, 8, 11, or 15 days. Gels were created at least 1 wk in advance to allow for swelling. After stabilization of hydrogel swelling, the stiffness of the gels was measured and calculated using indentation tests (Table 1).

Matrigel coating and myoblast proliferation. Proliferation was assessed by a BrdU assay in cells cultured on Matrigel-coated coverslips or gels of 3, 14, 21, 48, or 80 kPa after 1, 4, 8, 11, or 15 days. Sixteen hours before each time point, BrdU was added to the medium. Proliferation did not start immediately after cells had been seeded. However, on day 4, MPCs had proliferated on all substrates, with BrdU ratios varying between 0.5 and 0.9, meaning that 50–90% of the cells proliferated. Over time, proliferation gradually decreased, with a BrdU ratio between 0.1 and 0.2 at the end of the culture period. Proliferation was found to be significantly higher and lasted longer (P < 0.01) on glass and 21-kPa gels (close to physiological elasticity of skeletal muscle) compared with 3-, 14-, 48-, and 80-kPa gels (Fig. 1).

Matrigel coating and differentiation. Spontaneous differentiation of MPCs into muscle was analyzed on Matrigel-coated 3-, 21-, or 80-kPa gels or coverslips by examining the expression of differentiation markers (MyoD and MHC). As cells started to differentiate from day 4 onward, we evaluated differentiation 4, 6, 8, 11, or 15 days after cells had been plated. MyoD is a transcription factor involved in early differentiation, but early MyoD-expressing myoblasts are still capable of proliferation. After expression of MHC and fusion into myotubes, MyoD expression continues for a short period of time. However, fused cells can no longer proliferate.

In the present study, differentiation had already started by day 4, with expression of the differentiation markers clearly visible, although not in all cells. At this time point, only a small number of cells were MyoD positive/MHC positive, but in time, the proportion of double-positive cells increased as differentiation and maturation progressed (Fig. 2). In addition, a

<table>
<thead>
<tr>
<th>Table 1. Stiffness of gels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acrylamide, %</strong></td>
</tr>
<tr>
<td>3 kPa</td>
</tr>
<tr>
<td>14 kPa</td>
</tr>
<tr>
<td>21 kPa</td>
</tr>
<tr>
<td>48 kPa</td>
</tr>
<tr>
<td>80 kPa</td>
</tr>
</tbody>
</table>

The stiffness of gels was measured and calculated by indentation tests.
large proportion of single cells was MyoD positive, representing active myoblasts. Because of low seeding density, cells grew in colonies. Only few colonies were completely MyoD negative, whereas the majority of the colonies contained both MyoD-positive and MyoD-negative cells or only MyoD-positive cells. No differences were found in the percentage of MyoD-positive colonies across the different elasticities.

Maturation was analyzed by assessing the presence of cross-striations and spontaneous contractions. Myotubes cultured on gels never demonstrated spontaneous contractions, and myotubes cultured on coverslips only sporadically contracted. Occasionally, contractions caused myotubes to detach, but only from coverslips. Cross-striations were almost always present in myotubes grown on coverslips but never in myotubes grown on gels (Table 2).

**BM components and myoblast proliferation.** MPCs were cultured on gels with a stiffness of 3, 21, or 80 kPa or on coverslips. Gels and coverslips were coated with ECL gel (similar to Matrigel but purified for specific proteins), collagen type IV, poly-D-lysine, or laminin, and cultures were analyzed after 4, 6, 8, 11 or 15 days. Proliferation of MPCs significantly differed between all elasticities, both in level and duration. Proliferation was highest on coverslips followed by 21-gels and then 80- and 3-kPa gels. No differences were found between the different coatings (Fig. 3). However, proliferation ratios on these purified BM components were lower than on Matrigel (0.5 vs. 0.8 for Matrigel; Figs. 1 and 3).

**BM components and myoblast differentiation.** Differentiation of MPCs into myotubes was analyzed on 3-, 21-, or 80-kPa gels or coverslips and after 4, 6, 8, 11, or 15 days by MyoD and MHC expression. In agreement with cells growing on Matrigel, differentiation of MPCs on the different coatings also started on day 4, with detectable expression of MyoD in the nucleus and MHC in the cytoplasm. Cells grew in colonies as a consequence of low density seeding. The proportion of MyoD-positive and MyoD-negative cells was similar for different coatings and elasticities and comparable with the results with Matrigel. In addition, no differences in the timing of differentiation were seen between different coatings and elasticities. However, at later time points, the number of myotubes formed was higher on laminin- and poly-D-lysine-coated substrates compared with cells grown on ECL gel- and collagen type IV-coated substrates (Fig. 4). Formation of more myotubes resulted in smaller myotubes (Fig. 4).

Maturation, as analyzed by MHC cross-striations and spontaneous contractions, depended on substrate elasticity. Irrespective of coating, spontaneous contractions were observed in myotubes cultured on coverslips (Supplemental Material), sometimes in myotubes on gels of 80 and 21 kPa, and were never observed in myotubes on 3-kPa gels (Table 2). The incidence of cross-striations and contractions was noticeably affected by coating. Myotubes grown on laminin- and poly-D-lysine-coated substrates had the highest presence of spontaneous contractions, whereas myotubes on ECL-gel-, collagen

---

**Fig. 1.** A: proliferation of muscle progenitor cells (MPCs) on Matrigel-coated gels (3, 14, 21, 48, and 80 kPa) or on coverslips. The bromodeoxyuridine (BrdU) ratio was calculated in cells cultured on different elasticities at several time points (1, 4, 8, 11, and 15 days). Curves were fitted through the data to visualize the progress of proliferation over time. B: statistical analyses of differences in both the level and duration of proliferation of MPCs cultured on different elasticities. Data were analyzed with two-way ANOVA; n = 5 for all groups.
type IV-, and Matrigel-coated substrates only incidentally demonstrated spontaneous contractions (Table 2). In addition, none of the myotubes on gels coated with collagen type IV or Matrigel spontaneously contracted (Table 2). Spontaneous contractions also incidentally caused cell sheets to detach from coated coverslips, which did not occur on gels. Cross-striations were observed from day 11 onward in cells cultured at elasticities of $>$3 kPa and never at 3 kPa (Fig. 5). On coverslips, cross-striations were invariably present, regardless of protein coating. On 21- and 80-kPa gels, their presence depended on the coating. On these elasticities, Matrigel or collagen type IV coating never resulted in cross-striations (Table 2).

**DISCUSSION**

MPCs are a promising cell source for regenerative medicine. However, in vitro, they have shown decreased proliferation and differentiation potential after being passaged (19). In addition, repair of damaged muscles is much less successful when implanted cells have been cultured in vitro compared with

**Table 2. Cross-striations and spontaneous contractions of myotubes**

<table>
<thead>
<tr>
<th></th>
<th>Matrigel</th>
<th>Entactin-Collagen-Laminin Gel</th>
<th>Collagen Type IV</th>
<th>Poly-α-Lysine</th>
<th>Laminin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 kPa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21 kPa</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>17</td>
<td>66</td>
</tr>
<tr>
<td>80 kPa</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td>Coverslip</td>
<td>66</td>
<td>17</td>
<td>100</td>
<td>17</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are percentage of samples showing cross-striations ($n = 3$) and spontaneous contractions ($n = 6$) of myotubes on gels and coverslips with different coatings on day 15.
Implantation directly after isolation (5). It was hypothesized that changes in these cells occur because of the loss of pivotal stem cell niche components (3). For applicability of MPCs in regenerative medicine both proliferation and differentiation need to be improved. To assess the role of biochemical and biophysical cues from the SC niche in MPC proliferation and differentiation, the effects of substrate elasticity and matrix proteins were investigated separately and combined.

Proliferation was primarily influenced by substrate elasticity and not by protein coating. Proliferation of MPCs was significantly higher and for a longer duration on coverslips and 21-kPa gels compared with 3-, 14-, 48-, and 80-kPa gels. The optimum elasticity of ~21 kPa corresponds nicely to the physiological elasticity of skeletal muscle (6). Substrate elasticity has previously been shown to influence the proliferation of MPCs (4, 21). Boontheekul and coworkers (4) compared the proliferation of MPCs on 1-, 13-, and 45-kPa gels and showed increasing proliferation with increasing stiffness, whereas we found the optimum to be 21 kPa. Our data suggest that the temporal profile of proliferation might vary between surfaces of different elasticity. In the experiment by Boontheekul et al., the cell number on 13-kPa gels was still increasing at day 8, whereas it had already stabilized on 45-kPa gels. We observed similar results at day 8, after which the curves for 14 and 48 kPa crossed each other, eventually ending up with higher proliferation in the 14-kPa gels. Although an optimal elasticity can be observed, cells on coverslips grow equally well or even better. For cell expansion purposes, culturing on classic surfaces therefore seems optimal.

---

**Fig. 3.** Proliferation after 4 days (A), 6 days (B), 8 days (C), 11 days (D), or 15 days (E) of MPCs on 3-, 21-, or 80-kPa gels and on coverslips with coatings of entactin-collagen-laminin (ECL) gel, collagen type IV, poly-β-lysine, and laminin. F: statistical analysis of differences in both the level and duration of MPC proliferation on different elasticities. Data were analyzed with two-way ANOVA; n = 3 for all groups. No significant differences were found between coatings.
In our hands, MPC proliferation was independent of coating. Reported effects of coating on MPC proliferation are controversial. Similar to our findings, mouse myoblasts at passage 0 had a proliferation rate that was independent of protein coating on tissue culture plastic (collagen type IV, laminin, and Matrigel) (20). In contrast, it has also been reported that proliferation was stimulated by different laminin isoforms (13, 18, 31, 34) and collagen type IV (18) compared with BSA (31) or tissue culture plastic (18). The reason for this discrepancy is currently not clear.

Once expanded, MPCs need to differentiate into myotubes, develop typical cross-striations, and show contraction patterns. Early effectors in this differentiation track are MyoD and MHC, which are expressed when myoblasts start to differentiate, although MyoD-positive cells can stay in this phase and proliferate for some time. We observed no differences in

![Fig. 4. Cross-striations in myotubes cultured on different elasticities. Satellite cells cultured on different elasticities were stained after 11 or 15 days for MHC (green), MyoD (red), and nuclei (blue). Arrows show to cross-striations. Magnification: ×400. Bar = 50 μm.](image)

![Fig. 5. Staining of myotubes on coverslips with different coatings cultured for 11 days. Myosin (green), MyoD (red), and nuclei (blue) are visualized. Magnification: ×200. Bar = 100 μm.](image)
temporal profiles of MyoD and MHC expression and fusion of myoblasts into myotubes by MPCs cultured on varying elasticities and coatings. As a consequence of growth in colonies, we could distinguish that MyoD/MHC-positive cells form populations that were MyoD negative/MHC negative, suggesting differentiation of MPCs along different lineages or some level of heterogeneity in these primary cells. Obviously, a certain degree of heterogeneity already existed after the isolation of the cells. Although the type of isolation efficiently gives rise to a relatively pure MPC population, one cannot rule out other types of cells (e.g., fibroblasts) being coisolated. In a clinical study (33), for instance, a “pure” myoblast population still consisted of 20% cells that were negative for CD56 or desmin. These cells are probably responsible for part of the MyoD-negative/MHC-negative colonies. In addition, myoblasts from different muscle groups may consist of populations with distinct phenotypes (26). However, because of the existence of mixed colonies, differentiation of MPCs into MyoD-negative/MHC-negative cells cannot be excluded.

Spontaneous contractions and typical myosin cross-striations (necessary for contractions and indicative of maturation of myotubes) occurred in myotubes on gels independent of elasticity but not in myotubes on 3-kPa gels. On coverslips, cross-striations and contractions were most prominent, although contractions sometimes caused cell sheets to detach from coated coverslips. Since this never happened on gels, this is an advantage of culturing cells on gels. As myoblasts differentiate by confluence, the higher proliferation rates on coverslips might have accounted for the difference in maturation. However, local confluency in colonies is just as high, suggesting other mechanisms to be more important. Maturation of C2C12 myoblasts (11) has previously been shown to be influenced by substrate elasticity. Comparable to our results, Engler and coworkers found that, although early fusing of myoblasts into myotubes took place regardless of the stiffness, differentiation and especially maturation of C2C12 myoblasts depended on the stiffness of the substrate. However, they demonstrated that cross-striations were only formed in myotubes cultured on 8- and 11-kPa gels and not in myotubes on 1- and 17-kPa gels, suggesting a very narrow elasticity window. One potentially important difference with our setup is the method of measuring the elastance modulus of gels. With similar ratios of acrylamide-bisacrylamide, our tests showed different elastance moduli. For example, 5% acrylamide mixed with 0.3% bisacrylamide resulted in 8.1 kPa in their experimental setup, whereas in our hands, an elastance modulus of 21 kPa was measured after the stabilization of hydrogel swelling. At these apparent higher stiffness values, we observed cross-striations. From both studies, it has become clear that a minimum stiffness is required to mature myotubes into cross-striated muscle.

For different coatings, we demonstrated that MPCs grown on laminin- and poly-D-lysine-coated substrates resulted in more and thinner myotubes compared with myotubes on ECL gel, collagen type IV, and Matrigel coating. Previously, the opposite has been shown in mouse primary cultures, where Matrigel gave rise to more myotubes than laminin and collagen type IV after 4 days of culture, whereas a myogenic cell line formed equal amounts of myotubes on all coatings (20). Direct comparison with our experiments is confounded by the difference in culture time and the use of nongrowth factor-reduced Matrigel. More recently, and in line with our results, it was shown that mechanical stimulation through the laminin receptor increased differentiation compared with stimulation through the fibronectin receptor, inducing more and smaller myotubes in C2C12 myoblasts (13). The positive influence of mechanical stimulation indicates a role for mechanotransduction in this part of the differentiation process.

However, the most important difference between these studies is passage number. We used cells without prior expansion, hence passage 0, at the time of seeding. Apparent from implantation experiments, even short periods of culturing on tissue culture plastic changes the MPC phenotype and causes a loss of regenerative capacity (5). In addition, when C2C12 cells were compared with primary (precultured) cells during culture on substrates of different elasticities, the effect of substrate elasticity was found to be much larger on primary cells compared with C2C12 myoblasts (4). Moreover, in another experiment, it has been shown that a discrepancy exists between primary cells and cell lines in effects of coating on proliferation and differentiation (20). These results indicate that a significant difference possibly exists between primary cultures and cell lines that could also explain the difference between the results of Engler and coworkers and ours.

The effects of various coating and elasticity conditions were mainly temporal with the exception of the lack of maturation at very low elasticity. For instance, on day 11, it became clear that the laminin and poly-D-lysine coating gave rise to more and smaller myotubes and that ECL gel, Matrigel, and collagen type IV resulted in larger but fewer myotubes. In addition, on gels coated with Matrigel and collagen type IV, myotubes never showed any cross-striations, whereas myotubes on coated coverslips did. However, contractions were seen on Matrigel-coated 21-kPa gels that were cultured longer (unpublished observations), so we believe that progression and speed of maturation are the most important distinctions between the different conditions. Overall, we provided evidence that laminin- or poly-D-lysine-coated coverslips and 21- and 80-kPa gels are the best stimuli for the differentiation of myotubes.

In conclusion, substrate elasticity influences both myoblast proliferation and differentiation, whereas BM protein coating of these substrates only has an additive effect on the differentiation/maturation of these cells. The data presented here can be used to optimize culture conditions for future regenerative medicine applications.

ACKNOWLEDGMENTS

The authors are grateful to Martijn A. J. Cox for performing indentation tests on the gels. The authors also thank Marina Doeselaar for technical assistance and Marloes L. P. Langelaan for critical reading of the manuscript. In addition, the authors thank Leonie B. P. Niesen and Jo W. Habets for taking care of the animals. The MF20 antibody developed by Donald A. Fishman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by Department of Biological Sciences of The University of Iowa (Iowa City, IA).

GRANTS

This work was financially supported by SenterNovem Grant ISO 42022.

REFERENCES


