Simplified microenvironments and reduced cell culture size influence the cell differentiation outcome in cellular microarrays

Santiago A. Rodríguez-Seguí, María José Ortuño, Francesc Ventura, Elena Martínez & Josep Samitier

Journal of Materials Science: Materials in Medicine
Official Journal of the European Society for Biomaterials

ISSN 0957-4530
Volume 24
Number 1

J Mater Sci: Mater Med (2013)
24:189-198
DOI 10.1007/s10856-012-4785-1

Springer

J Mater Sci: Mater Med (2013) 24:189–198
DOI 10.1007/s10856-012-4785-1

Simplified microenvironments and reduced cell culture size influence the cell differentiation outcome in cellular microarrays

Santiago A. Rodríguez-Seguí · María José Ortuño · Francesc Ventura · Elena Martínez · Josep Samitier

Received: 27 March 2012 / Accepted: 27 September 2012 / Published online: 19 October 2012

Abstract Cellular microarrays present a promising tool for multiplex evaluation of the signalling effect of substrate-immobilized factors on cellular differentiation. In this paper, we compare the early myoblast-to-osteoblast cell commitment steps in response to a growth factor stimulus using standard well plate differentiation assays or cellular microarrays. Our results show that restraints on the cell culture size, inherent to cellular microarrays, impair the differentiation outcome. Also, while cells growing on spots with immobilised BMP-2 are early biased towards the osteoblast fate, longer periods of cell culturing in the microarrays result in cell proliferation and blockage of osteoblast differentiation. The results presented here raise concerns about the efficiency of cell differentiation when the cell culture dimensions are reduced to a simplified microspot environment. Also, these results suggest that further efforts should be devoted to increasing the complexity of the microspots composition, aiming to replace signalling cues missing in this system.

Electronic supplementary material The online version of this article (doi:10.1007/s10856-012-4785-1) contains supplementary material, which is available to authorized users.

S. A. Rodríguez-Seguí (✉) · E. Martínez · J. Samitier
Nanobioengineering Group, Institute for Bioengineering of Catalonia, Baldiri i Reixac 10-12, 08028 Barcelona, Spain
e-mail: sarodris@clinic.ub.es; srodriguez@fbmc.fcen.uba.ar

J. Samitier
e-mail: jsamitier@ub.edu

S. A. Rodríguez-Seguí · J. Samitier
Department of Electronics, University of Barcelona, Martí i Franquès 1, 08028 Barcelona, Spain

S. A. Rodríguez-Seguí · E. Martínez · J. Samitier
Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, 08028, Barcelona, Spain

Present Address:
S. A. Rodríguez-Seguí
Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE)-Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Intendente Guiraldes 2160, Pab. II, 2do piso, C1428EGA Buenos Aires, Argentina

M. J. Ortuño · F. Ventura
Departament de Ciències Fisiològiques II, IDIBELL, Campus de Bellvitge, Universitat de Barcelona, L'Hospitalet de Llobregat, Barcelona, Spain

1 Introduction

A cellular microarray consists of an array of cell adhesive features immobilised on a substrate in which cells are seeded and cultured, with the final aim of introducing cell signalling through the immobilised factors [1–3]. When aimed at the study of stem cell differentiation, this technique would allow discovering signalling pathways ultimately leading to improved cell differentiation protocols [4–9]. In particular, for the assessment of cell response to growth factor stimuli, it has been suggested that factor covalent immobilisation is highly desired in order to improve signalling [10, 11], and some approaches have been reported previously for the study of stem cell differentiation [4–6]. These reports have also shown that critical parameters for cellular microarray fabrication, such as the surface passivation and ligand immobilisation, vary depending on the cell type and the differentiation pathway analysed [12]. Thus, it is expected that growth factor signalling requirements are different (both in intensity of signalling and in time of exposure) for different cell types. The effectiveness of signalling can also be affected by cell–cell contacts, as suggested by recent

studies which highlight the important influence of cell–cell signalling in cell differentiation [13–15]. Therefore, further characterization of cellular microarrays is needed to gain insights into the previously exposed facts.

In the present work, we used cellular microarrays to evaluate the early cell commitment events of an extensively characterised cell differentiation model, consisting in the myoblast-to-osteoblast differentiation in response to bone morphogenetic protein 2 (BMP-2) [16]. The C2C12 cells used are by default differentiated to myocytes when reducing the serum content of the culture medium, but its differentiation in switched to osteoblasts in the presence of BMP-2 [17, 18]. Thus, a single factor (BMP-2) serves as the key to decide which pathway is followed [17, 19–22]. C2C12 cell differentiation in response to printed BMP-2 has been previously described by the group of P. Campbell using a completely different approach to the one presented here [23]. In this report, a single layer of cells was cultured on uniformly-coated fibrin glass slides containing BMP-2 patterns. The advantage of using a cellular microarray consisting of mutually isolated cell microspots is the multiplex evaluation of cell response to several BMP-2-ECM protein combinations in a single cell culture experiment.

In this work, two checkpoints of the BMP-2 signalling pathway were chosen to evaluate short- and medium-term cell differentiation in response to soluble or immobilised BMP-2. To provide a comprehensible interpretation of the results obtained from cellular microarrays with immobilised BMP-2, control cellular microarrays with protein spots composed only of fibronectin (Fn) or laminin (Ln) were exposed to soluble BMP-2. This approach allowed evaluating the individual effect of culturing cells in isolated spots. Our results show that restrains on the cell culture size, inherent to cellular microarrays, impair the differentiation outcome. Also, while cells growing on spots with immobilised BMP-2 are early biased towards the osteoblast fate, longer periods of cell culturing in the microarrays result in cell proliferation and blockage of osteoblast differentiation. The results presented here raise concerns about the efficiency of cell differentiation when the cell culture dimensions are reduced to a simplified microspot environment. Also, these results suggest that further efforts should be devoted to increasing the complexity of the microspots composition, aiming to replace signalling cues missing in this system.

2 Materials and methods

2.1 Proteins and chemicals

Human cellular fibronectin and laminin from Engelbreth-Holm-Swarm murine sarcoma were obtained from Sigma (Spain). Recombinant human BMP-2 (expressed in chinese hamster ovary, CHO, cells) was obtained from R&D. This growth factor was provided by the manufacturer as a lyophilized powder containing 50 μg of BSA per 1 μg of BMP-2 and was reconstituted using a sterilised 4 mM HCl solution prepared in Milli-Q water, following the provider suggestions. When subsequently referring to the BMP-2 concentrations used here, it is understood that these solutions also contain BSA in a 50:1 relation (i.e. 50 μg of BSA per 1 μg of BMP-2). Unless otherwise specified, all other chemicals were purchased from Sigma (Spain).

2.2 Protein microarray fabrication and characterisation

Aldehyde-derivatized glass (AD-Glass) slides (SuperAldehyde 2, Telechem, USA) were used to fabricate the microarrays as previously described [11, 24]. In brief, protein solutions of Fn or Ln at different concentrations (200 or 360 μg/mL, subsequently called Fn200, Fn360, Ln200 and Ln360) were prepared in PBS. In order to test the effect of delaying spot dry out after printing, Fn360 and Ln360 protein solutions were also prepared in PBS with glycerol (2 % v/v, final concentration). All the protein solutions assayed were prepared with and without BMP-2 (100 μg/mL of BMP-2, subsequently called B100, which also contained BSA at a concentration of 5 mg/mL). The BMP-2 concentration spotted was of the same order of magnitude of the values previously reported in the literature (70 μg/mL) for the immobilisation of a similar growth factor (BMP-4) in AD-Glass [4]. A robotic non-contact piezoelectric plotter (Nano-Plotter, GeSiM GmbH, Germany) was used to dispense the protein solutions on the substrates (protein layout is shown in Suppl. Fig. 1a). Two spot sizes were produced by overprinting 5 and 10 consecutive drops, 0.4 nL in volume each. Control microarrays, in which no BMP-2 was spotted, were also produced by printing 5 and 10 drops of Fn360 and Ln360 protein solutions prepared in PBS (eight replicate spots per composition were printed). The temperature of the workplate was set to 4 °C to delay protein dry out when printed. Once spotted, the slides were incubated overnight at 4 °C to prevent fast evaporation of the buffer solution and to allow proteins to react with the AD-Glass surface chemistry.

The printed protein microarrays were characterised by immunostaining of Ln and Fn. For this purpose, the slides were blocked with BSA (1 % in PBS-Gly) for 20 min and then incubated for 1 h with primary antibodies: either rabbit anti-Ln or rabbit anti-Fn (Sigma, Spain), followed by 1 h incubation the secondary antibody: goat anti-rabbit Alexa Fluor 647. Next, the samples were dried by centrifugation and scanned using a GenePix 4000B microarray scanner device (Molecular Devices Corp., USA). The Alexa Fluor 647 signal (red) was successfully detected using the scanner excitation laser at 635 nm with the ~650–690 nm emission filter.

2.3 Cell culture

C2C12 mouse cells were expanded in Dulbecco's modified Eagle's medium (DMEM, provided by GIBCO, Spain) containing 10 % foetal bovine serum (FBS), 1 % penicillin/streptomycin, 1 % L-Glutamine and 1 % sodium pyruvate, subsequently called "growth medium".

To produce the semi-confluent cell cultures, used as controls in this work, cells were seeded in standard well plates and cultured for 1–2 days in growth medium. For short-term differentiation experiments, consisting in the analysis of Osx expression at 24 h, semi-confluent cells were subsequently incubated in "serum-free medium" (composed of DMEM, 1 % penicillin/streptomycin, 1 % L-Glutamine and 1 % sodium pyruvate), supplemented or not with soluble BMP-2 (50 ng/mL). Alternatively, for medium-term differentiation experiments in which ALP activity was evaluated after 4–6 days of cell culture, semi-confluent cells were cultured with "low serum medium" (composed of DMEM containing 2 % horse serum (HS), 1 % penicillin/streptomycin, 1 % L-Glutamine and 1 % sodium pyruvate), supplemented or not with soluble BMP-2 (50 ng/mL). Medium was changed every 2 or 3 days. The use of low serum medium for the later experiments has been previously reported in the literature [25], and in our case was also necessary to allow viable cell experiments in the microarray format for up to 6 days.

For production of the cellular microarrays, previous to cell seeding, the printed protein slides were incubated in a 2 % BSA solution for 1 h to passivate non-printed areas. Afterwards, the slides were washed twice with PBS and C2C12 cells were seeded at a 20,000 cells/cm^2 density for 15 min. Cells in the microarrays were then cultured either in serum-free medium (for Osx expression assessment at 24 h) or low serum medium (for ALP activity assessment at day 4 or day 6). For control microarrays (those in which no BMP-2 was printed on the spots), the culture media was supplemented or not with soluble BMP-2 (50 ng/mL).

2.4 Characterisation of cell cultures

Cell viability was assessed using a cell Viability/Cytotoxicity Assay Kit For Animal Live & Dead Cells (Biotium, Inc) following the manufacturer instructions. Using this kit, the cytoplasm of viable cells is fluorescently stained in green and non-viable cells have nuclei stained in red.

To quantify the number of cells attached in the cellular microarray spots, cells were fixed (4 % paraformaldehyde or 10 % formalin) and nuclei were stained with Hoechst. The fixed cells were imaged using a standard fluorescence microscope. Cell counting was performed with the aid of ImageJ software [26], and adjusted manually for regions with poor ImageJ counting resolution due to crowded nuclei.

To assess the early cell commitment steps to osteoblasts, the expression of Osx was evaluated by fluorescence immunostaining after 24 h of cell culture. For this purpose, cells were fixed in 4 % paraformaldehyde, permeabilized for 4 min in Triton 100X (0,2 % (v/v) solution in PBS-Gly) and blocked with goat serum (15 % in PBS-Gly) for 45 min. Afterwards, the slides were incubated overnight at 4 °C with the primary antibody (rabbit anti-Osterix, Abcam, USA), followed by incubation with the secondary antibody (goat anti-rabbit Alexa Fluor 488, Molecular Probes, USA) for 1 h at room temperature. Finally, samples were mounted using Fluoromount (Sigma, Spain) and imaged using standard or confocal fluorescence microscopes.

For ALP activity determination, at days 4 and 6 cells were fixed in 10 % formalin for 1 h. Then a solution of naphtol AS-MX phosphate and fast blue RR in Milli-Q water was used to stain the cells according to the manufacturer instructions (Sigma Kit #85). Cells were incubated in the dark for 30 min and rinsed with water. Fixed cells were imaged using a bright field microscope or a magnifying glass.

2.5 Statistics

All experiments were repeated at least twice. For microarrays, the results were based on quantification from at least 8 spots from two biological replicates. Graphical data is reported as mean ± standard deviation. Non-parametric Wilcoxon rank-sum tests were performed on the statistical analysis of variables plotted using R [27]. Significance levels were set at $P < 0.05$.

3 Results

3.1 Microarray characterisation

Analysis of Fn and Ln immunostaining images showed that the protein spots in the microarray remained clearly identifiable after repeated washing steps and no protein cross-contamination was detected (Suppl. Fig. 1). Next, cell attachment to these features was evaluated. Despite cells attached to all the spot compositions assayed, the Fn spots yielded a better reproducibility in the number of cells captured at day 0 (Suppl. Fig. 2a), with ~20 cells in 5 drop spots and ~25 cells in 10 drop spots (Suppl. Fig. 2b). Noteworthy, despite previous reports have shown that BSA might modulate the cell adhesion activity of ECM proteins [28–30], we did not see any important differences in the number of cells attached on the features when BMP-2 (which also contained BSA, see Sect. 2.1) was included in the spot composition. Thus, the following analyses were mainly focused on Fn and BMP-2 protein combinations.

Cellular microarrays were next characterised for cell proliferation in the spots. For this purpose, cells were cultured for 24 h in serum-free medium or, alternatively, in low serum medium for 4 days. The rationale for this choice was to reproduce the culture conditions chosen for the induction of short- or medium-term osteoblast differentiation (see Sect. 2.3). Noteworthy, cell viability was not affected after 24 h of cell culture in either serum-free or low serum medium (Fig. 1a). The proliferation results showed that, while the number of cells attached on the spots at days 0 and 1 was of the same order of magnitude, an important increment was observed in the microarrays that were cultured for 4 days in low serum medium (Fig. 1b, c; Suppl. Fig. 2c). In the later case, the number of cells per spot increased from ~25 cells at day 0 to more than 300 cells at day 4, with cells eventually exceeding the spot premises. This behaviour was independent of the inclusion of BMP-2 on the features. Thus, cells continued to proliferate in the microarrays in the medium-term differentiation experiments and this presented a limitation for the objective evaluation of cell differentiation after 4 days of culture, as exposed further in this work.

3.2 Cell differentiation at 24 h: Osterix gene expression analysis

Osx gene expression was chosen as an early checkpoint in the induction of osteoblast commitment, and therefore as an indicator of active BMP-2 signalling. In order to provide relevant control and test experiments, the BMP-2 was either added in solution to the culture medium or immobilised on the microarray spots as follows.

3.2.1 Control cell cultures: BMP-2 added in the culture medium

As expected, standard well plate cell differentiation assays showed the initial C2C12 cell commitment to osteoblasts in response to soluble BMP-2. While more than 99 % of the cells had green stained nuclei (indicative of Osx expression) in BMP-2 treated cultures, cells in control cultures without BMP-2 did not express this marker (Fig. 2a). Thus, the C2C12 cells used in these experiments showed an early and measurable response to soluble BMP-2 added to the culture medium.

3.2.2 Control cellular microarrays: BMP-2 added in the culture medium

To analyse the effect of BMP-2 on Osx expression when the cell culture is restricted to microspots, control cellular microarrays (see Sect. 2.2) were exposed to soluble BMP-2. The results showed that ~37 % (in average 7 out of 19 cells, Ln360 spots) and ~53 % (average 11 out of 21 cells, Fn360 spots) of the cells attached to the control microarray spots expressed Osx (Fig. 2b and d). Interestingly, Ln spots yielded slightly lower numbers of Osx-expressing cells, in agreement with previous reports that associate Ln with myoblast differentiation to myocytes [31–33]. Similar results were obtained for the different spot sizes assayed (Suppl. Fig. 3a). Osx expression was negligible (average 0.2 out of 15 cells, Fn360 spots) in cells cultured without BMP-2. These data points out that, independently of the spot composition, the expression of Osx is induced by BMP-2 when added to the culture medium. However, these results also show that the culture of cells on isolated microspots produces a decrease in Osx expression when compared to the well plate experiments.

3.2.3 Cellular microarrays with BMP-2 immobilised on the spots

The Osx expression was next analysed in cellular microarrays with immobilised BMP-2. It was found that, while ~20 % (in average 3 out of 15 cells, Ln360B100 spots) to 24 % (average 5 out of 21 cells, Fn360B100 spots) of the cells attached on the BMP-2 containing spots expressed Osx, the expression of this marker was negligible (average 0.1 out of 20 cells) in features without BMP-2 (Fig 2c and e). Similar results were obtained for the different spot sizes assayed (Suppl. Fig. 3b). These findings indicate that Osx expression is induced by the BMP-2 factor immobilised on the substrate surface. Noteworthy, the soluble and printed BMP-2 also contained BSA and thus the cell differentiation results account for the response to the BMP-2/BSA combination. No significant differences could be established in the relative number of Osx-expressing cells for the different spot compositions which included BMP-2. Further studies are envisaged to assess with more detail whether BMP-2 signalling is importantly affected by the ECM protein co-spotted in the microarrays. On the other hand, cells growing in spots printed with glycerol showed an additional impairment on the Osx expression (<14 %, Fig. 2f and Suppl. Fig. 3c) when compared to spots printed with PBS only.

3.3 Cell differentiation after 4 days: ALP activity analysis

3.3.1 Control cell cultures: BMP-2 added in the culture medium

As expected, the ALP activity in response to soluble BMP-2 was detectable at day 4 and strongly increased at day 6 of cell culture in standard well plate assays (Fig. 3a and b, see "24 well plate" panels). No ALP activity was detected in the control well plates, in agreement with previous reports showing that C2C12 cells differentiate to

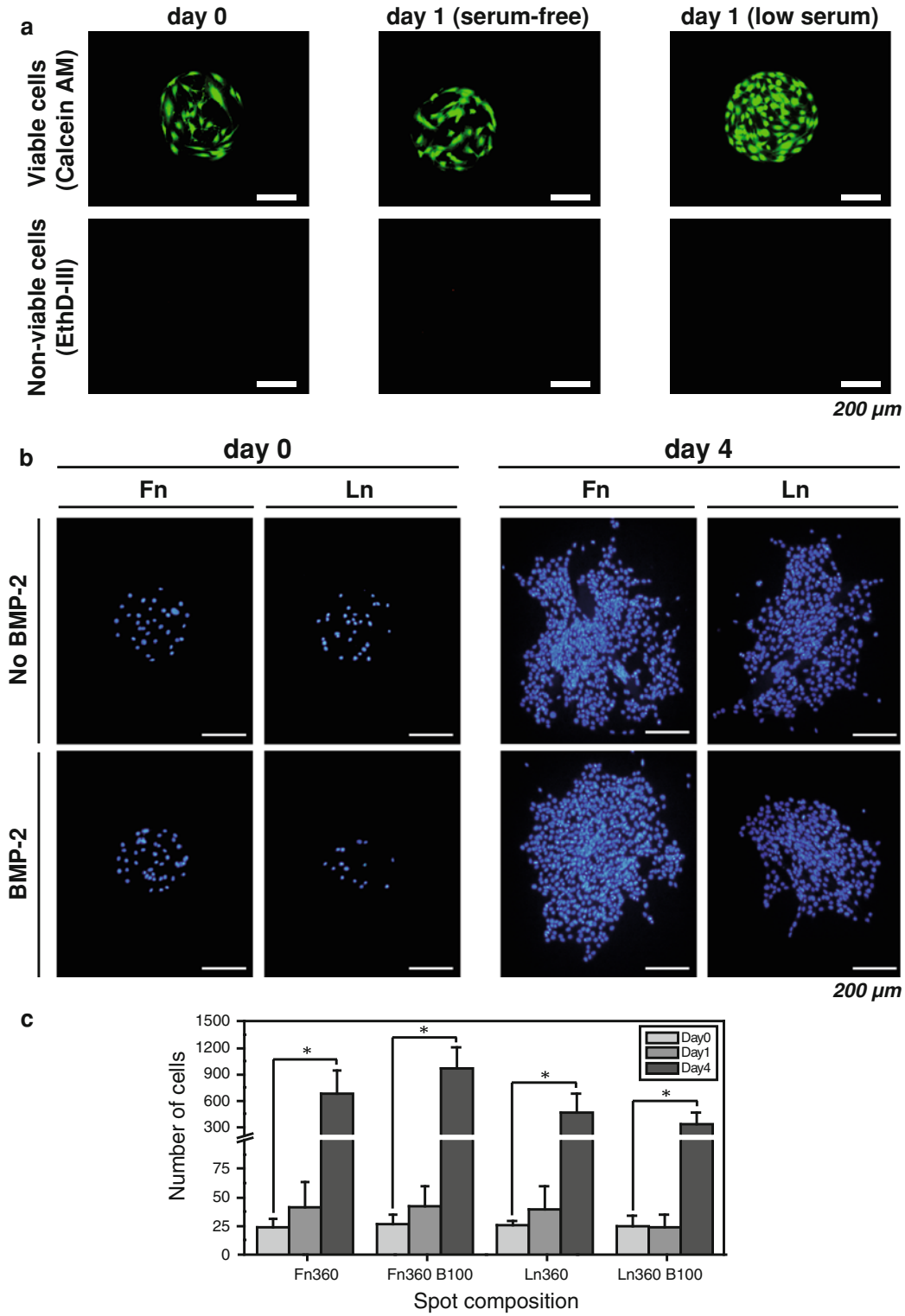

Fig. 1 Cell viability and proliferation analysis. **a** Representative fluorescence images of cell viability for cells attached on Fn360 features (10 drops spot size). Cell viability was assessed at day 0 and after 24 h of cell culture in either serum-free or low serum medium. Calcein signal, in *green*, stains viable cells. EthD-III signal, in *red*, indicates non-viable cells. Most cells attached on the spots remained viable after 24 h of cell culture in serum-free or low serum medium. **b** Proliferation analysis. Cell nuclei were fluorescently stained in blue and imaged by fluorescence microscopy. Representative images of cells at day 0 and day 4 are presented. **c.** The average number of cells on the microarray spots was evaluated at days 0, 1 and 4 of cell culture. The number of cells attached on the spots increased more than 20-fold from day 0 to day 4. *indicates $P < 0.05$ (Wilcoxon rank-sum test) (Color figure online)

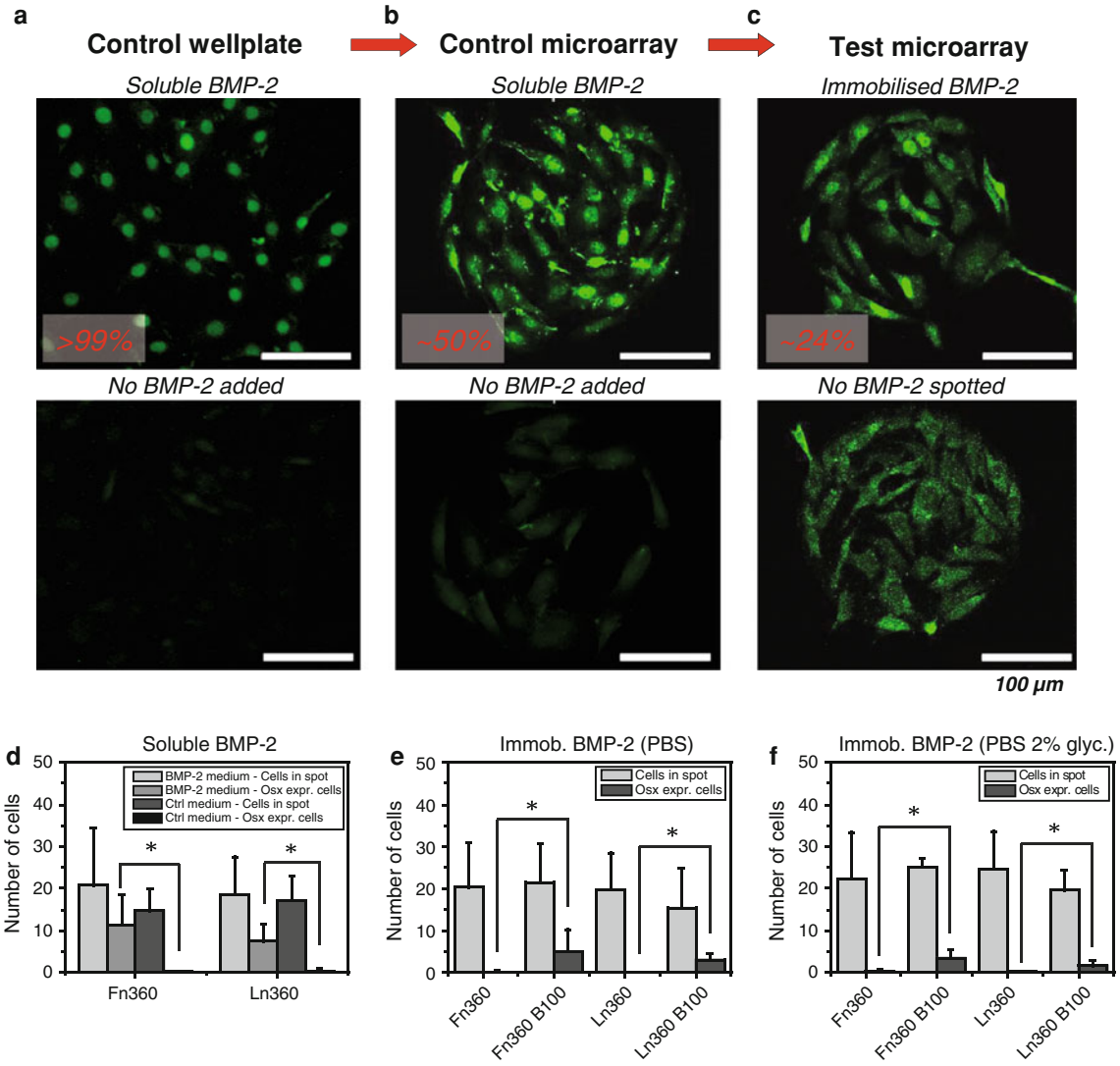

Fig. 2 Short-term cell differentiation results. **a** In the well plate experiments, it was observed that most cells showed Osx expression after 24 h of cell culture with soluble BMP-2, as indicated by cell nuclei immuno stained *green* in this image. Osx expression was not detected in control well plates, which were not exposed to BMP2. **b** A second control, consisting of cells attached on the control microarray Fn spots, yielded Osx expression in ~50 % of the cells, indicating that osteoblast cell commitment was decreased by culturing cells in Fn microspot. Osx expression was not detected in control microarrays which were not exposed to BMP-2. Spot size for images shown is from 5 drops. **c** When cells were cultured on microarray spots with immobilised BMP-2, it was observed that Osx expression was ~24 %, and it was exclusively detected on BMP-2 containing spots. Spot size for images shown is 5 from drops. **d**–**f** Quantification of the total number of cells attached on the spots at day 1, and the number of these cells which showed Osx expression. In **d** the control microarrays were cultured in serum-free medium with (BMP-2 medium) or without (Ctrl medium) soluble BMP-2. In **e**, **f** cells were cultured in microarray spots composed of Fn or Ln co-spotted with and without BMP-2, and which were printed with **e** PBS only or **f** PBS 2 % glycerol buffer. Data plotted for 5 drop spots. *Indicates $P < 0.05$ (Wilcoxon rank-sum test) (Color figure online)

myocytes when cultured in low serum medium [34, 35]. Thus, C2C12 responded to BMP-2 by increasing the ALP activity, which can be detected from day 4.

3.3.2 *Cellular microarrays with BMP-2 immobilised on the spots*

We next sought to evaluate the ALP activity in cells cultured for 4–6 days in the microarrays. As expected, cells on control spots did not show any positive staining for ALP. However, in stark contrast to the well plate controls, ALP activity in spots printed with BMP-2 was very low (Fig. 3a and b, see "Microarray" panels). As previously noted, an important rate of cell proliferation was detected in cells attached on the spots after 4 days of cell culture, with cells eventually exceeding the spot premises. Therefore, not all the cells stained at day 4 or 6 were actually exposed to the signalling from the spot. It was not possible to evaluate

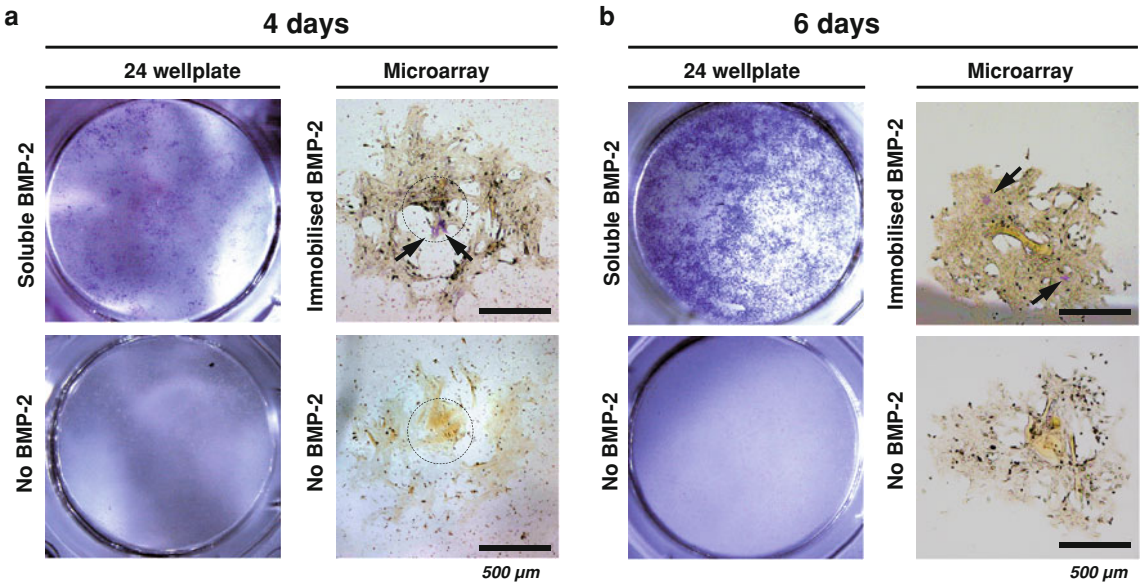

Fig. 3 Medium-term cell differentiation results. **a** A mild ALP activity (seen in these images as a blue cell staining) was detected after 4 days of cell culture in standard well plates in response to BMP-2 added in the medium ("24 well plate" panel). No ALP activity was detected in control cultures. The ALP activity was very low in cells cultured for 4 days in the microarrays, as shown by representative images presented in the "Microarray" panel. **b** The ALP activity presented an important increase at day 6 of cell culture in the well plate assays, but this marker continued to be very low in the cellular microarrays. *Arrows* indicate cells showing ALP activity in the microarrays. As expected, this was only detected in cells attached on BMP-2 containing spots

with precision the original limits of the spotted feature from the histological ALP staining in the images presented. Thus, the few ALP-expressing cells could account for differentiated cells from a smaller, undifferentiated, cell population of ~25 cells which were initially attached on the spots at day 0.

4 Discussion

The C2C12 myoblast cell line has been widely used as a paradigm of cell differentiation. Simply lowering the serum content of the culture medium is enough for a single growth factor (BMP-2) to switch the default differentiation pathway from myocytes to osteoblasts [34, 36, 37]. More recently, this model has been exploited for validation of novel techniques used in cell differentiation studies [23, 38]. In the work presented here, we utilised the C2C12 cell differentiation model to evaluate the cellular microarray performance.

The relatively high proliferation rate which led to the increase in the number of cells on the microarrays at day 4 was partially attributed to the presence of the small percentage of serum added to the culture medium (i.e. 2 % HS), which could include growth factors and hormones that enhance cell proliferation. It has been previously shown the cell doubling time is dependent on the amount of serum content in the culture medium. More specifically, when C2C12 cells were cultured in 2 % serum, their doubling time was ~26 h [39]. With this rate, 25 cells would yield ~200 cells in 4 days. This value is closer to the number of cells found in our experiments, especially for the Ln-containing spots at day 4. The Fn-containing spots, on the other hand, showed a larger number of attached cells at day 4, suggesting an additional role of the Fn coating in cell proliferation. This is in agreement with previous reports showing that, by clustering to different integrin subunits, Fn promotes myoblast proliferation and Ln decreases cell proliferation, enhancing myoblast differentiation [31–33, 40]. Since our goal was to assess cell differentiation to osteoblasts in response to BMP-2, we did not further evaluate this effect. Nevertheless, these preliminary findings show that cellular microarrays provide an ideal tool to evaluate the molecular mechanisms of cell proliferation versus differentiation in a better defined cell microenvironment. Future work should be devoted to finding suitable replacements for the low serum content in the culture media, thus allowing cell culturing on microarrays under better controlled conditions.

4.1 Cell differentiation at 24 h: Osterix gene expression analysis

An important decrease in the C2C12 osteoblast commitment was observed when comparing cells exposed to soluble BMP-2 in well plates (>99 % Osx-expressing cells) to control microarrays exposed to soluble BMP-2 (~53 %

Osx-expressing cells). This finding can be accounted in part to the effect of culturing small numbers of cells in the control microarrays. In this case, the cell culture size (limited by the spot dimensions) and the dispersion of cell islands in the microarrays is far from providing a semi-confluent environment, as usually happens in the well plate differentiation experiments. The enhancement of cell–cell signalling and the limitation of cell spreading in the well plate experiments are very important factors that could significantly affect cell differentiation [15, 41]. Another fact to be taken into account is that, previous to BMP-2 exposure, the cells cultured in well plates were expanded to form a semi-confluent layer, as previously reported for cell differentiation assays. On the other hand, cells cultured in the microarrays were seeded and exposed to BMP-2 medium from the beginning of the experiment. It is known that the amount of ECM proteins produced by mesenchymal cells is increasing proportionally to the time spent in culture [42]. Thus, while cell attachment on the microarray spots was mediated by a simple ECM composed of Fn or Ln, the attachment of cells on a more complex ECM, like the one arising in the well plate assays, could optimise the signalling effect of the soluble BMP-2. Optimisation of growth factor signalling by increasing the complexity of the underlying ECM has been shown to be mediated by the recruitment of additional integrin types, as well as by providing additional binding sites for BMP-2 due to the presence of other ECM proteins such as collagens [43–45]. Altogether, the results presented here suggest that, when few cells are cultured in isolated spots, the osteoblast differentiation process is impaired (with Osx expression changes larger than 50 % in some cases) due to the lack of additional cell–cell signalling or complex microenvironments [45].

For the cellular microarrays with BMP-2 immobilised in the spots, it was found that 20–24 % of the cells expressed Osx after 24 h of cell culture, indicating osteoblast commitment in response to the immobilised growth factor. The differentiation outcome turned out to be decreased when compared with the control microarrays exposed to soluble BMP-2, in which the Osx expression was up to 53 %. These findings can be partially attributed to the effect of immobilisation of the growth factor. BMP-2 immobilisation can lead to decreased activity due to incorrect orientation or partial denaturation. However, other adverse effects of protein immobilisation, such as the restriction of ligand-receptor diffusion along the cell membrane, cannot be ruled out. In this sense, it has been shown that integrins might mediate growth factor signalling by enhancing or inhibiting their effects through the coupling of intracellular signals triggered by the ECM proteins and some growth factors [43, 46]. Thus, diffusion restriction of BMP2 bound to its receptor in the cell membrane could result in loss of the intracellular signal coupling mediated by integrins.

4.2 Cell differentiation after 4 days: ALP activity analysis

The ALP activity has been previously established as a suitable marker for osteoblast differentiation assessment [23, 34]. In this work, we used control well plate experiments to show that ALP activity can be detected at day 4 of cell culture in BMP-2 enriched medium, and that the presence of this marker importantly increases at day 6 of cell culture. On the other hand, the ALP positive staining obtained for the BMP-2 containing spots after 4–6 days of cell culture in the microarrays was very low. These results contrasted with findings of Phillippi et al. [23] that made their assessment as soon as 3 days after cell seeding in monolayer on top of BMP-2 patterns. This suggests that cell differentiation in response to printed BMP-2 is decreased when culturing cells on individual and mutually isolated cell spots, as presented here. This rationale was supported by the fact that in this work an important rate of cell proliferation was detected in cells attached on the spots after 4 days of cell culture. It is well-known that cell proliferation and cell differentiation are mutually excluded pathways, exit from the cell cycle being a prerequisite for cell differentiation [43]. This is the reason why cell differentiation assays are usually performed in semi-confluent cell cultures. Therefore, it could be argued that the effect of culturing cells in a semi-confluent monolayer (as reported by Phillippi et al.) could influence the rate of ALP activity by adequately providing additional signals which would allow exit from the cell cycle for the whole cell culture and subsequent osteoblast differentiation. Finally, other reasons that could further account for the low ALP activity detected in the microarrays presented here cannot be discarded. These could include the need for co-immobilisation of BMP-2 with other ECM proteins which were not studied in this work (such as collagens) [44] or the need for a more complex ECM microenvironment.

5 Conclusion

In this work, we used an extensively validated cell differentiation model to evaluate the cellular microarray performance in reproducing the early myoblast-to-osteoblast cell commitment steps in response to a growth factor stimulus. Our results show that the cell culture size restraints inherent of cellular microarrays impair the differentiation outcome. Importantly, cells growing on spots with immobilised BMP-2 were early biased towards the osteoblast fate. Cell culture for longer periods of time

resulted in cell proliferation and blockage of osteoblast differentiation. Overall, the results presented here suggest that even for one of the simplest and best characterised cell differentiation models, additional signalling cues are required to reproduce the differentiation outcomes on a well defined differentiation set-up. The results presented here raise concerns about the efficiency of cell differentiation when the cell culture dimensions are reduced to a simplified microspot environment. Also, these results suggest that further efforts must be devoted to increasing the complexity of the microspots composition, aiming to replace signalling cues missing in this system. Such improvements should include the immobilisation of mixtures of several ECM proteins on the microspots, as well as the use of an enhanced serum-free medium composition allowing cell culturing on the microarrays for several days.

References

1. Chen DS, Davis MM. Molecular and functional analysis using live cell microarrays. Curr Opin Chem Biol. 2006;10(1):28–34.
2. Mei Y, Goldberg M, Anderson D. The development of high-throughput screening approaches for stem cell engineering. Curr Opin Chem Biol. 2007;11(4):388–93.
3. Underhill GH, Bhatia SN. High-throughput analysis of signals regulating stem cell fate and function. Curr Opin Chem Biol. 2007;11(4):357–66.
4. Soen Y, Mori A, Palmer TD, Brown PO. Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments. Mol Syst Biol. 2006;2:37.
5. Nakajima M, Ishimuro T, Kato K, Ko IK, Hirata I, Arima Y, et al. Combinatorial protein display for the cell-based screening of biomaterials that direct neural stem cell differentiation. Biomaterials. 2007;28(6):1048–60.
6. LaBarge MA, Nelson CM, Villadsen R, Fridriksdottir A, Ruth JR, Stampfer MR, et al. Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. Integr Biol. 2009;1(1):70–9.
7. Brafman DA, Shah KD, Fellner T, Chien S, Willert K. Defining long-term maintenance conditions of human embryonic stem cells with arrayed cellular microenvironment technology. Stem Cells Dev. 2009;18(8):1141–54.
8. Brafman DA, de Minicis S, Seki E, Shah KD, Teng DY, Brenner D, et al. Investigating the role of the extracellular environment in modulating hepatic stellate cell biology with arrayed combinatorial microenvironments. Integr Biol. 2009;1(8–9):513–24.
9. Jones CN, Tuleuova N, Lee JY, Ramanculov E, Reddi AH, Zern MA, et al. Cultivating liver cells on printed arrays of hepatocyte growth factor. Biomaterials. 2009;30(22):3733–41.
10. Ito Y. Covalently immobilized biosignal molecule materials for tissue engineering. Soft Matter. 2008;4(1):46–56.
11. Rodriguez-Segui SA, Pons Ximenez JI, Sevilla L, Ruiz A, Colpo P, Rossi F, et al. Quantification of protein immobilization on substrates for cellular microarray applications. J Biomed Mater Res A. 2011;98(2):245–56.
12. Toh YC, Blagovic K, Voldman J. Advancing stem cell research with microtechnologies: opportunities and challenges. Integr Biol. 2010;2(7–8):305–25.
13. Tang J, Peng R, Ding JD. The regulation of stem cell differentiation by cell–cell contact on micropatterned material surfaces. Biomaterials. 2010;31(9):2470–6.
14. Nelson CM, Chen CS. Cell-cell signaling by direct contact increases cell proliferation via a PI3 K-dependent signal. FEBS Lett. 2002;514(2–3):238–42.
15. Gobaa S, Hoehnel S, Roccio M, Negro A, Kobel S, Lutolf MP. Artificial niche microarrays for probing single stem cell fate in high throughput. Nat Methods. 2011;8(11):949–55.
16. Ryoo HM, Lee MH, Kim YJ. Critical molecular switches involved in BMP-2-induced osteogenic differentiation of mesenchymal cells. Gene. 2006;366(1):51–7.
17. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol. 1994;127(6 Pt 1):1755–66.
18. Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem. 2002;277(5):3176–85.
19. Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R. Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes Cells. 2002;7(9):949–60.
20. Vinals F, Ventura F. Myogenin protein stability is decreased by BMP-2 through a mechanism implicating Id1. J Biol Chem. 2004;279(44):45766–72.
21. Celil AB, Campbell PG. BMP-2 and insulin-like growth factor-I mediate Osterix (Osx) expression in human mesenchymal stem cells via the MAPK and protein kinase D signaling pathways. J Biol Chem. 2005;280(36):31353–9.
22. Celil AB, Hollinger JO, Campbell PG. Osx transcriptional regulation is mediated by additional pathways to BMP2/Smad signaling. J Cell Biochem. 2005;95(3):518–28.
23. Phillippi JA, Miller E, Weiss L, Huard J, Waggoner A, Campbell P. Microenvironments engineered by inkjet bioprinting spatially direct adult stem cells toward muscle- and bone-like subpopulations. Stem Cells. 2008;26(1):127–34.
24. Rodriguez-Segui SA, Pla-Roca M, Engel E, Planell JA, Martinez E, Samitier J. Influence of fabrication parameters in cellular microarrays for stem cell studies. J Mater Sci Mater Med. 2009;20(7):1525–33.
25. Vinals F, Ventura F. Myogenin protein stability is decreased by BMP-2 through a mechanism implicating Id1. J Biol Chem. 2004;279(44):45766–72.
26. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9:671–5.
27. R Development Core Team. R: A language and environment for statistical computing. R foundation for statistical computing. Vienna, Austria 2008.
28. Buchanan LA, El-Ghannam A. Effect of bioactive glass crystallization on the conformation and bioactivity of adsorbed proteins. J Biomed Mater Res A. 2010;93(2):537–46.
29. Soman P, Siedlecki CA. Effects of protein solution composition on the time-dependent functional activity of fibrinogen on surfaces. Langmuir. 2011;27(17):10814–9.
30. Tagaya M, Ikoma T, Takemura T, Hanagata N, Yoshioka T, Tanaka J. Effect of interfacial proteins on osteoblast-like cell adhesion to hydroxyapatite nanocrystals. Langmuir. 2011;27(12):7645–53.
31. von der Mark K, Ocalan M. Antagonistic effects of laminin and fibronectin on the expression of the myogenic phenotype. Differentiation. 1989;40(2):150–7.
32. Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. Development. 1993;117(4):1183–98.
33. Sastry SK, Lakonishok M, Wu S, Truong TQ, Huttenlocher A, Turner CE, et al. Quantitative changes in integrin and focal

adhesion signaling regulate myoblast cell cycle withdrawal. J Cell Biol. 1999;144(6):1295–309.
34. Katagiri T, Yamaguchi A, Komaki M, Abe E, Takahashi N, Ikeda T, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol. 1994;127(6 Pt 1):1755–66.
35. Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F. Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem. 2002;277(5):3176–85.
36. Katagiri T, Akiyama S, Namiki M, Komaki M, Yamaguchi A, Rosen V, et al. Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin. Exp Cell Res. 1997;230(2):342–51.
37. Matsubara T, Kida K, Yamaguchi A, Hata K, Ichida F, Meguro H, et al. BMP2 regulates Osterix through Msx2 and Runx2 during osteoblast differentiation. J Biol Chem. 2008;283(43):29119–25.
38. Tourovskaia A, Figueroa-Masot X, Folch A. Differentiation-on-a-chip: a microfluidic platform for long-term cell culture studies. Lab Chip. 2005;5(1):14–9.
39. Liu JM, Burkin DJ, Kaufman SJ. Increasing alpha(7)beta(1)-integrin promotes muscle cell proliferation, adhesion, and resistance to apoptosis without changing gene expression. Am J Physiol Cell Physiol. 2008;294(2):C627–40.
40. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673–87.
41. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell. 2004;6(4):483–95.
42. Beach RL, Burton WV, Hendricks WJ, Festoff BW. Extracellular-matrix synthesis by skeletal-muscle in culture. Proteins and effect of enzyme degradation. J Biol Chem. 1982;257(19):1437–42.
43. Giancotti FG, Ruoslahti E. Integrin signaling. Science. 1999;285(5430):1028–32.
44. Xiao GZ, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. J Bone Miner Res. 2002;17(1):101–10.
45. Stern MM, Myers RL, Hammam N, Stern KA, Eberli D, Kritchevsky SB, et al. The influence of extracellular matrix derived from skeletal muscle tissue on the proliferation and differentiation of myogenic progenitor cells ex vivo. Biomaterials. 2009;30(12):2393–9.
46. Boudreau J, Jones PL. Extracellular matrix and integrin signalling: the shape of things to come. Biochem J. 1999;339:481–8.