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Dorsal and ventral stimuli in sandwich-like microenvironments. Effect on cell differentiation

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Running title: Cell differentiation in sandwich microenvironments

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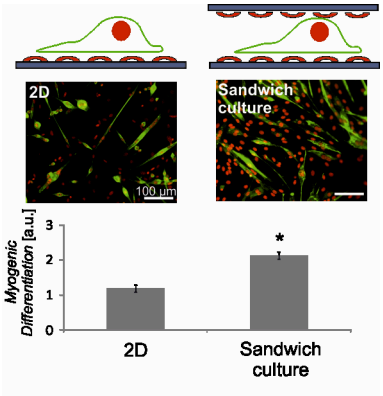
Dorsal stimulation of C2C12 myoblasts using sandwich-like microenvironments enhances cell differentiation. Dorsal integrin-mediated adhesion within sandwich culture triggers intracellular signal cascades and increase myoblast differentiation. Sandwich-like models are proposed as a versatile tool to study cell behavior in a quasi-3D environment under well-controlled conditions.

Keywords: 3D matrix adhesion, fibronectin, integrins, multilayers, myoblasts, sandwich



ABSTRACT: While most of the *in vivo* extracellular matrices are 3D, most of the *in vitro* cultures are 2D -where only ventral adhesion is permitted- thus modifying cell behavior as a way to self-adaptation to this unnatural environment. We hypothesize that the excitation of dorsal receptors in cells already attached on a 2D surface (sandwich culture) could cover the gap between 2D and 3D cell-material interactions and result in a more physiological cell behavior.

In this study we investigate the role of dorsal stimulation on myoblast differentiation within different poly(l-lactic acid) (PLLA) sandwich-like microenvironments, including plain material and aligned fibers. Enhanced cell differentiation levels were found for cells cultured with dorsal fibronectin-coated films. Seeking to understand the underlying mechanisms, experiments were carried out with (i) different types of dorsal stimuli (FN, albumin, FN after blocking the RGD integrin-binding site and activating dorsal cell integrin receptors), (ii) in the presence of an inhibitor of cell contractility and (iii) increasing the frequency of culture medium changes to assess the effect of paracrine factors. Furthermore, FAK and integrin expressions, determined by western blotting, revealed differences between cell sandwiches and 2D controls. Results show a stimuli-dependent response to dorsal excitation, proving that integrin outside-in signaling is involved in the enhanced cell differentiation. Due to their easiness and versatility, these sandwich-like systems are excellent candidates to get deeper insights into the study of 3D cell behavior and to direct cell fate within multilayer constructs.

Introduction

The nature of the cell-protein-material interaction is able to direct myogenesis by regulating cell adhesion, spreading, proliferation, migration, matrix remodeling and differentiation (Meighan and Schwarzbauer, 2008; Streuli, 2009): upon cell adhesion integrins cluster and develop focal adhesion complexes that anchor cells to the material surface and activate signaling cascades such as focal adhesion kinase (FAK). FAK can

interact with GRB2 (Schlaepfer et al., 1994), linking integrin signaling to the ras/MAP kinase pathway which plays an important role in myoblast differentiation (Bennet and Tonks, 1997). Integrin stimulation via cell adhesion controls therefore cell proliferation and differentiation. Although myogenesis is a 3D process, it has been commonly studied on flat substrates (2D) - where only ventral adhesion is permitted- thus imposing an unnatural environment that differs from the natural 3D extracellular matrix (ECM) (Lewis and Lewis, 1924; Weiss, 1959). Likewise, 3D substrates closer to the physiological environment are more and more preferred to study cellular processes *in vitro*, including matrix secretion, cell differentiation, morphogenesis, cancer research and drug development (House et al., 2012; Hutmacher, 2010; Lutolf and Hubbell, 2005; Rimann and Graf-Hausner, 2012).

We have studied myoblast differentiation in a sandwich-like culture where cells are seeded in-between two material substrates. We hypothesize that the excitation of dorsal receptors in cells already attached on a 2D surface modifies cell behavior with respect to 2D conditions, To assess this hypothesis, we study myoblast differentiation in different poly(L-lactic acid) (PLLA) sandwich-like cultures and fibronectin (FN) as extracellular matrix protein. Both flat PLLA samples (*p*) and aligned microfibers of PLLA (*a*) were used. Consequences of dorsal stimuli on FAK signaling pathway and specific integrin anchorage were furthermore determined. As sandwich culture showed to enhance C2C12 differentiation, additional experiments were designed to get further insights into this process: dorsal stimulation with non-adhesive proteins (BSA), after blocking the RGD integrin-binding domain of FN and after activating integrins with functional antibodies for α_5 and β_3 integrin subunits. Moreover, the role of RhoA pathway was studied using a contractility inhibitor, as well as the influence of paracrine factor secretion.

Materials and Methods

Materials

Thin films of PLLA (C+*p*) were prepared by spin casting a solution of 2% PLLA in chloroform (Scharlau). Moreover PLLA solution was cast in stainless steel washers and allowed to evaporate. Resulting films (Figure 1A) were thermally treated at 200ºC for 5 minutes. Solvent casted PLLA thickness was analyzed using scanning electron microscopy (JEOL JSM 6300) and Minitest coating thickness gauges (Minitest 2100 ElektroPhysics). For electrospinning, PLLA was dissolved in hexafluoroisopropanol (HFIP, Sigma) at 80 mg ml^{-1} and electrospun at a constant feed rate of 0.9 ml/h with a voltage of 30 kV (Glassman High Voltage, High Bridge, NJ, USA) and a collector distance of 12 cm. In order to obtain aligned fibers, the collector consisted of a rotating drum (rotating at 1125 cm s^{-1}) where glass coverslips were stuck (for C+*a* samples). The drum had also longitudinal holes where the aligned electrospinning was collected afterwards onto polytetrafluoroethylene (PTFE) washers for the upper sides of sandwiches (SW_p^a, Figure 1A).

Microscopy

The electrospun fibers were characterized by scanning electron microscope (SEM) JEOL JSM 6300 (JEOL Ltd., Tokyo, Japan) at 15 kV. SEM data was processed using ImageJ software (Rasband, 1997) in order to obtain quantitative data about fiber anisotropy using an external plug-in developed by O'Connell. FN distribution and surface topography of the electrospun fibers were characterized by atomic force microscopy (AFM), performed in a NanoScope III from Digital Instruments (Santa Barbara, CA) operating in the tapping mode.

Protein adsorption

FN from human plasma (Gibco) at 20 μg ml^{-1} in Dulbecco's Phosphate Saline Buffer (DPBS) or heat-denatured Bovine Serum Albumin Fraction V (BSA) (Roche) at 10 mg ml^{-1} in water were adsorbed on the different substrates during 1 h at room temperature.

Cell culture

Murine C2C12 myoblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum and 1% penicillin–streptomycin (Lonza). Prior to seeding, samples were sterilized by UV exposure for 30 min (30min each side in the case of upper substrates) and coated with FN or BSA as described before. Then C2C12 cells were seeded at 17,500 cells cm^{-2} in DMEM without serum supplemented with 1% penicillin–streptomycin and 1% insulin-transferrin-selenium-X (Invitrogen) in order to induce myogenic differentiation. Immediately after cell seeding (SW^{t0}) or 3 h later (SW), sandwich cultures were obtained by gently laying upper substrates over the bottom seeded substrate (Figure 1D). As several conditions have been studied, a specific nomenclature was used overall the study: SW_x^y with *x*-ventral and *y*-dorsal material conditions. For a detailed description of experiments including dorsal activation with antibodies, blocking of RGD specific adhesion site, contractility inhibitor, the effect of paracrine factors secretion and the timing of sandwiching please see supplementary information.

Myogenic differentiation

C2C12 cells were cultured for 4 days under differentiation conditions and immunostained for sarcomeric myosin. Briefly, cultures were fixed in 70% ethanol/37% formaldehyde/glacial acetic acid (20:2:1) and then blocked in 5% goat serum in DPBS for 1 h. Samples were sequentially incubated in MF-20 mouse antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA) and anti-mouse Cy3-conjugated secondary antibody (Jackson Immunoresearch) with 4,6-diamidino-2-phenylindole (DAPI, Sigma) for 1 hour.

Image analysis

Cell morphology was quantified by calculating cell area and roundness ($4 \times \text{area} / \pi \times [\text{major axis}]^2$), which corresponds to a value of 1 for a perfect circle, using ImageJ software of at least 20 cells for each condition. Cultures were furthermore scored by the percentage of positive cells for sarcomeric myosin using the CellC image analysis software (Selinummi et

al., 2005). In order to obtain quantitative data about cell anisotropy, the images of sarcomeric myosin staining were processed using ImageJ software following an already described procedure (Ballester-Beltrán et al., 2012 A; O´Connell 2002).

Western Blot

Cells were lysed with RIPA buffer (Tris–HCl 50 mM, 1% Nonidet P-40, 0.25% Na deoxycholate, NaCl 150 mM, EDTA 1 mM) supplemented with protease inhibitor cocktail tablets (Complete, Roche). To determine protein content (FAK, pFAK (Tyr397), α_5 and α_v integrin subunit) samples were subjected to 7% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane (GE Healthcare). Immunoreactive bands were visualized using the Supersignal West-femto Maximum Sensitivity Substrate (Thermo Scientific), analized by using ImageJ software and normalized to α-tubulin protein.

Immunofluorescence

To visualize p-FAK localization within cells, samples were fixed (4% PFA for 15 minutes), permeabilized (0.5% Triton X-100, for 5 minutes) and incubated in 1% albumin in PBS for 15 minutes and then in p-FAK (Tyr925) polyclonal antibody (Cell Signaling) diluted 1:100 in 1% albumin in PBS for 1 hour, followed by Cy3 conjugated secondary antibody (Jackson Immunoresearch) diluted 1:200. A Nikon microscope (Nikon Eclipse 80 i) was used.

Statistical analysis

Results are shown as average ± standard deviation. All experiments were performed at least three times in triplicate. Results were analyzed by one-way ANOVA and if significant differences were determined, Tukey´s post hoc test was performed. For each sandwich condition, the bottom substrate as 2D culture was used as its specific control unless otherwise noted.

Results and Discussion

Material characterization

We have engineered PLLA substrates to assess the effect of sandwich-like conditions on cell behavior. We have used plain PLLA (samples indexed with *p*) and aligned fibers of PLLA (samples indexed with *a*) seeking to obtain cell alignment and more physiological-like myotubes.

Solvent casted films with a thickness of 4.88 µm ± 1.6 µm and homogenous electrospun straight fibers of PLLA with dimension of 145.4 ±2.7 nm high and 173.8±0.7 nm wide were obtained (Figure 2A). As expected, aligned fibers display a characteristic peak after spectral analysis due to a highly anisotropic organization (Figure 2B). AFM was used to investigate FN distribution on substrates. As depicted in Figure 2C, both plain PLLA (C+*p*) and aligned PLLA fibers (C+*a*) showed globular molecules of FN.

Initial spreading and morphology; dorsal and ventral cues

To study the influence of sandwich-like culture on cell morphology, cell area and roundness were taken as morphological parameters. Unlike our previous study with NIH3T3 fibroblasts (Ballester-Beltrán et al., 2012 B), cells spread similarly on 2D and sandwich-like cultures (Figure 3A and raw data in Table S1). Figure 3A clearly shows two main groups: cells seeded on C+*a* are significantly less spread than cells seeded on C+*p* (both control and sandwich cultures). This result suggests that cell alignment on the ventral fibers limits cell spreading. This is also shown when cells seeded on C+*p* "are sandwiched" with FN-coated aligned fibers as the upper substrate (SW_p^a). As a consequence of the dorsal interaction with the aligned fibers, cells randomly spread on C+*p* start aligning in the direction of the upper fibers (Figure 3B) resulting in diminished spread area. Similarly, cell alignment influences cell roundness. Cells are more rounded on C+*p* than on C+*a*. Likewise, cells sandwiched with dorsal aligned fibers (SW_p^a) are significantly less rounded than sandwiched in-between plain

substrates (SW_p^p), with roundness close to the cells on C+*a*. By contrast, when dorsal PLLA fibers are coated with the non-adhesive bovine serum albumin (BSA) instead of FN, no cell alignment was achieved even after 2 days of culture (Figure 3B, $SW_p^{a\text{-}BSA}$). However, cells seeded on aligned fibers did not lose alignment orientation after sandwiching with plain PLLA coated with FN (SW_a^p), revealing the distinct competition between ventral and dorsal stimuli in determining cell fate. In the same way, cells seeded on C+*a* and afterwards sandwiched using aligned dorsal fibers perpendicularly oriented to the ventral ones ($SW_a^{a_\perp}$), remain aligned along the ventral fibers and do not react to the dorsal ones (Figure S1), revealing the importance of the initial ventral input.

In summary, cell morphology depends on both dorsal and ventral topological cues as sensed by the cells through the intermediate layer of proteins at the material interface. In the same way, cell alignment strongly depends on the culture condition. Whereas fast alignment is observed on 2D ventral fibers (C+*a*, 3 hours), more time is needed within SW_p^a (1d) and no alignment is observed when dorsal fibers are coated with BSA.

Dorsal stimuli enhance myogenic differentiation

We next examined the role of dorsal and ventral stimuli on cell differentiation, quantified after immunostaining for sarcomeric myosin. Similar levels of differentiation were found on both 2D substrates (C+*p* and C+*a*) and the type I collagen control (ca. 34.8% ± 7.7), which is considered to be the gold-standard substrate for myogenic differentiation (Figure S2) (García AJ et al., 1999). It is convenient to remark here that, in order to facilitate comparisons, cell differentiation levels calculated across the paper have been normalized to collagen I unless otherwise mentioned. Cell differentiation is shown in Figure 4. Even though myoblast alignment is mandatory for their fusion into myotubes (Wakelam, 1985), electrospun fibers (C+*a*) promoted C2C12 alignment (Figure 4B) without significant enhancement of cell differentiation as seen before (Ballester-Beltrán et al. 2012 A; Charest et al., 2007). In

contrast, C2C12 seeded on plain or aligned PLLA fibers and afterwards sandwiched with a plain substrate (SW_p^p and SW_a^p respectively), showed significantly enhanced differentiation levels ($p<0.001$), regardless the topology of the ventral substrate. Interestingly, there is no enhanced differentiation when fibers are used on the dorsal cell side (Figure 4C, SW_p^a), which suggests competition between cell alignment and cell differentiation, in a similar way as it happens between cell migration and cell differentiation (Ballester-Beltrán et al. 2012 A; Tse and Engler, 2011). Thus, cell differentiation might be delayed in SW_p^a as cells align along the dorsal PLLA fibers before starting the differentiation program (Figure 3). Differences in dynamics can be seen in figure 4D: while cells seeded within SW_p^p form myotubes on day 2, only isolated cells can be observed on 2D substrates at that time and not fully aligned cells are seen within SW_p^a. Sandwich culture can therefore tune differentiation dynamics -either accelerating or delaying it- regarding topological cues.

To better support this enhanced myogenic differentiation within sandwich environments, cells were cultured on C+*p* and within SW_p^p with growth medium (20% FBS), which is known to block myogenic differentiation and trigger proliferation. As Figure S3 shows, no myotubes can be seen on 2D substrates whereas long and well-developed ones are observed within SW_p^p.

Our previous results on sandwich-like cultures with fibroblasts showed the key effect of the initial ventral material interaction before dorsal stimulation in cell response (Ballester-Beltrán et al. 2012 B). Consequently, establishing the sandwich culture either immediately after cell seeding -in order to prevent any preferential role of ventral receptors- or after 3 hours of 2D culture -to permit initial cell adhesion on material surfaces using ventral receptors- may influence myoblast differentiation. Figure S4 shows that sandwiching cells immediately after cell seeding ($SW_p^{p\text{-}t0}$) results in development of longer pseudopodia without altering morphology significantly (compared to SW_p^p). We hypothesize that cells interact

symmetrically both to the ventral and the dorsal substrates when sandwiched just after cell seeding. However, when cells are sandwiched after 3 hours of ventral adhesion, strong ventral adhesions are already developed when the dorsal stimuli is received and therefore cells do not respond in the same way to the dorsal cues. This can be qualitatively explained in the context of cell tensegrity models (Ingber 2003). By contrast, cells in $SW_p^{p\text{-}t0}$ did not achieve the enhanced differentiation levels shown in sandwiches established after 3 hours of ventral adhesion (SW_p^{p}, Figure S5). As can be seen in Figure S5, small differences on cell density exist between 2D and sandwich cultures, probably due to the fact that cells are easier removed during medium changes when cultured on 2D conditions. Note that differences in cell density are not correlated with cell differentiation.

Previous studies demonstrated the effect of myoblast seeding density on the ability to fuse into myotubes in a range that difficults cell-cell contact and fusion at the lowest densities (Tanaka K et al. 2011; Smith AST et al. 2011). The same initial cell density was used in our experiments that led to modest cell density differences after 4 days of culture when cell differentiation was assessed (2D vs SW culture). Bajaj P et al. showed that this level of modest differences for cell density (while seeding at the same cell density) is poorly correlated with the differentiation level attained. We have shown typical values for cell density for the different systems used in Figure S6.

One can disregard either hypoxia or mechanical pressure within the sandwich culture as external stimuli able to account for enhanced differentiation level, since cell differentiation was not increased by sandwiching immediately after cell seeding ($SW_p^{p\text{-}t0}$). Moreover such kind of stimuli has been shown to handicap myogenic differentiation (Boonen et al., 2010; Di Carlo et al., 2004; Li et al., 2008). On the other hand, it is known that myoblast fate is closely bound to integrin involvement and expression (Sastry et al., 1999), and that changes in integrin expression and focal adhesion signaling might influence myogenic differentiation

(Belkin et al., 1996; Salmerón-Sánchez et al., 2011; Sastry et al. 1999; Liu et al., 2011). Thus we hypothesize that dorsal interactions through integrin binding to dorsal FN may trigger the enhanced cell differentiation in sandwich-like cultures. Support for this hypothesis will be afterwards provided.

Integrin expression and cell signaling

It is still unclear if the mechanisms related to cell adhesion and cytoskeleton reorganization observed in 2D, such as the formation of focal adhesions and signaling, are also observed in 3D (Cukierman et al., 2001; Yamada et al., 2003). Different nature of adhesion in 3D and 2D environments has been reported: 3D adhesions to ECM matrix as well as fibrillar adhesions do not produce phosphorylation at FAK^{Tyr397} (Cukierman et al., 2001; Ishii et al., 2001). For our sandwich-like system, an intermediate situation comes up, with cell adhesion on FN adsorbed on a rigid substrate (thus being 2D-like) but on both sides of the cell (closer to 3D-like). We thus wonder what kind of adhesion is obtained and how it can contribute to explain the enhanced cell differentiation in sandwich-like cultures.

Integrin-mediated cell adhesion triggers a cascade of intracellular signals such as the p38 mitogen-activated protein kinase (p38 MAPK) pathway which is involved in the myoblast differentiation process, by promoting the activity of several transcription factors and regulating cell cycle withdrawal (Mancini et al., 2011; Sastry et al., 1999). The intermediary step between adhesion and downstream targets, including MAPK pathways, is the phosphorylation of FAKs (Chatzizacharias et al., 2008); as a result, FAK phosphorylation at Tyr-397 plays a central role during myoblast differentiation in 2D cultures (Clemente et al., 2005; Quach and Rando, 2006). Initially, FAK phosphorylation at Tyr-397 is transiently reduced -contributing to trigger the myogenic genetic program- but it is later activated as it is central to terminal differentiation into myotubes (Quach and Rando, 2006). As cells cultured on sandwich-like cultures interact with both the ventral and dorsal substrates, FAK activation

might occur on both sides, triggering signaling pathways that differ from the standard 2D one. In order to study the activation of FAK we examined the phosphorylation of tyrosine-397 by western blot.

After 6 hours of culture (3 hours of sandwich culture), FAK phosphorylation was significantly diminished as compared to the corresponding 2D control, both for the plain (SW_p^p) and aligned PLLA ventral surfaces (SW_a^p) (Figure 5A). This result confirms the quasi-3D like adhesive behavior of cells in our sandwich model, where lack of FAK phosphorylation is also found; moreover it supports the known relationship between enhanced myogenic differentiation and pFAK decrease at the beginning of the process (Cukierman et al., 2011; Quach and Rando, 2006). Strikingly, immunodetection of FAK phosphorylation at Tyr 925 –which is known to be dependent on the autophosphorylation on Tyr397 and to trigger a Ras-dependent activation of the MAP kinase pathway (Schlaepfer et al., 1994)– shows increased brushstroke-like labeling within sandwich-like cultures (Figure 5B), in agreement with the enhanced phosphorylation of downstream MAPK showed by Yamada et al. in 3D systems (Cukierman et al., 2002). These results suggest that enhanced cell differentiation upon dorsal and ventral stimulation in our sandwich-like system may be due to a shift in response from 2D to 3D-like, and not only to the excitation of a larger number of cell adhesion receptors (due to the increased surface in contact as a result of dorsal interaction) in a mere 2D manner.

As some studies suggest that 3D matrix-adhesions of fibroblast are mainly developed through α_5 integrin subunits (Cukierman et al., 2001), integrin expression after 6 hours of culture was studied in order to determine whether dorsal interactions in sandwich-like cultures trigger any specific integrin expression. Since $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin are the major integrins anchoring to FN and are known to influence in the differentiation process, we quantified α_5 and α_v integrin subunits for each culture condition. No differences were observed, so the ratio α_v/α_5 integrin

subunits ratio was similar for 2D and sandwich-like cultures (unless SW_a^p at 6h in respect to C+*a* at 3h; Figure 5C).

Cell contractility is known to influence C2C12 differentiation under certain conditions. Concretely, FN fibrils assembled into physiological networks led to an increase in cell contractility and trigger differentiation but globular FN did not (Salmerón-Sánchez et al., 2011). In order to examine whether the enhanced myogenic differentiation within sandwiches was due to an increase in cell contractility, cells were cultured in the presence of the Rho/ROCK pathway contractility inhibitor Y-27632. As can be seen in Figure 5D, the inhibitor did not cause any significant change in cell differentiation in neither the plain (SW_p^p) nor the aligned (SW_a^p) sandwich conditions, and as expected nor on 2D substrates (since FN adopts globular conformation on PLLA). Thus enhanced myogenic differentiation in sandwich-like conditions is not a consequence of increased cell contractility due to dorsal stimuli.

Dorsal integrins and cell differentiation

To clarify whether the enhanced myogenic differentiation in sandwich-like conditions is a direct consequence of a biological cell-protein-material interaction or, by contrast, its origin must be sought as a mere physical interaction with the material surface, we have used BSA -a non-adhesive protein- (Curtis and Forrester, 1984; Tamada and Ikada, 1993; Zelzer et al., 2012) instead of FN to coat the upper substrate of the sandwich construct (using plain PLLA for both dorsal and ventral substrates). This condition (SW_{FN}^{BSA}) results in lower cell differentiation compared with the standard plain sandwich-like system (SW_p^p) coated with FN on both sides (Figure 6). Nevertheless, cell differentiation in SW_{FN}^{BSA} is still higher than on FN-coated plain 2D controls. This result suggests that the enhanced differentiation observed in (FN-coated) sandwich-like culture must be sought, at least partially, in dorsal-integrin activation and binding to the ECM. Moreover, that cell differentiation levels in

SW_{FN}^{BSA} does not match those obtained for 2D controls can be a consequence of self-secreted ECM proteins at later stages of differentiation: myogenic differentiation is a complex process lasting at least 4 days in which cells secrete their own extracellular matrix proteins such as FN or procollagen (Kislinger et al., 2005; Chan et al., 2007). Cell secreted proteins might displace previously adsorbed BSA on the dorsal material surface, and thus provide dorsal-integrin stimuli on cells. The time needed to secrete the ECM and displace the adsorbed FN might be the reason why cell differentiation levels remain below FN coated sandwiches. The final cell density was slightly higher for sandwich cultures than on 2D samples. However, note that BSA-coated sandwiches displayed similar values for cell density to FN-coated sandwiches (figure S6) while cell differentiation was statistically different (figure 6). This supports the lack of correlation between cell density and cell differentiation for the cell density range used in this work.

To get further insights into the underlying mechanism, we blocked the integrin-binding site of FN adsorbed on the dorsal substrate before cell sandwiching using the HFN7.1 antibody. This antibody binds the FN $III_{9\text{-}10}$ domain, thus blocking possible interactions with neighboring adhesion sites PSHRN and RGD (Schoen et al., 1982) and preventing $\alpha_5\beta_1$ and part of $\alpha_v\beta_3$ dorsal-integrin binding –as a new specific $\alpha_v\beta_3$ integrin binding domain was discovered (Sottile et al., 2000)-. Figure 7A shows that myoblast differentiation was significantly enhanced after blocking the integrin binding site in dorsal-FN as compared with standard sandwich conditions. It was surprising to us, as we hypothesized that blocking of dorsal interactions would result in a drop in differentiation, as observed when using albumin to coat dorsal substrates. Thus, it is likely that myoblasts have various types of interaction with FN and that one of these interactions at the dorsal side induces differentiation more than interaction at FN $III_{9\text{-}10}$ site does. Some hypothetic cases may be: (1) binding of $\alpha_v\beta_3$ to FN by the specific cell-adhesion domain (Sottile et al., 2000) and corresponding outside-in

signaling, since β_3 integrin has been linked to myogenic differentiation (Liu et al., 2011), (2) binding of other cell surface receptors (syndecans, M-cadherin, etc. (Peckham, 2002)) and corresponding outside-in signaling, or (3) interaction of vacant dorsal integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ with distinct cell-secreted factors (soluble or adsorbed to the dorsal material).

From another perspective, in order to mimic the material-driven activation of dorsal integrins within the sandwich-like culture, cells were stimulated with functional antibodies for either β_3 or α_5 shortly after seeding on 2D substrate. Antibody-activated dorsal integrins (Figure 7B) do not trigger the same signal as simply sandwiching: cell differentiation levels were significantly higher for SW_p^p than in any other condition of the experiment. Nevertheless β_3 integrin activation (without sandwiching) on the dorsal side led to a significant increase in cell differentiation compared to 2D (note that a non-significant increase in cell differentiation was obtained after stimulating the α_5 receptor). This result shows that activation of dorsal receptors is absolutely involved in enhanced cell differentiation and points towards a possible role of $\alpha_v\beta_3$ activation as previously commented. As integrin activation on 2D samples increase differentiation levels but do not level them to sandwich cultures values, we questioned the role of the dorsal substrate: was it necessary just as a physical stimulus (applying some stress on the cells for example) or as a biophysical stimuli (as cells sense the difference between mere activation by a soluble antibody and the binding to a structure resisting tension, for example the protein coating). Sandwiching cells after dorsal integrin activation did not produced any improved differentiation compared to the activation condition without sandwiching, suggesting that the increase in differentiation may be directly linked to a biophysical effect of the dorsal integrin activation state. Indeed, different states of activation may be provoked by integrin binding to FN adsorbed on a rigid surface when compared with a blocking antibody, and may not provoke the same "outside-in" signaling cascades (Wei et al., 2008). This difference in local dorsal stiffness is likely to

stimulate different mechanotransductive pathways for sandwich-like and 2D cultures, as previously reported for stem cells and C2C12 cells in 2D conditions (Engler et al., 2006; Ren et al., 2008). In summary, dorsal-antibody stimulation on cells cultured on 2D conditions partially mimics the effect of dorsal interaction with adsorbed FN in sandwich-like cultures, but the effect on cell differentiation is much lower.

Release of paracrine factors

Several growth factors are known to be able to stimulate or inhibit myogenesis in an autocrine or paracrine manner. For instance, insulin-like growth factors (IGFs) (de la Haba et al., 1966; Florini and Magri, 1989) and tumor necrosis factor (TNF) (Chen et al., 2007; Li and Schwartz, 2001) are known to stimulate myogenesis via IGF-1 receptor and p38 MAPK pathway activation respectively (Chen et al., 2007; Florini et al., 1991), whereas fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) among others inhibit myoblast differentiation (Clegg et al., 1987; Evinger-Hodges et al., 1982). As dorsal integrin activation within sandwich culture may modify growth factor expression and as medium diffusion is somehow restricted compared to standard 2D conditions, we planned a cell differentiation experiment with increased frequency of culture medium changes in order to determine if the enhanced cell differentiation was also related to the accumulation of cell-secreted factors within the sandwich structure. As seen in Figure 7C and supporting our hypothesis, extensive change of medium in control 2D conditions does not modify levels of cell differentiation but cell density is drastically reduced due to the aspiration of weakly attached cells during medium changes. On the other hand, when medium is frequently changed in sandwich cultures, cell density is maintained due to the protection of sandwich that impedes aspiration of cells, and the differentiation levels are reduced down to 2D control levels. Altogether, these results suggest that enhanced differentiation during sandwich-like experiments may be correlated with the production of soluble paracrine factors, different than

those secreted in standard 2D cultures, as a consequence of integrin-mediated dorsal stimulation since different dorsal stimuli caused different myodifferentiation levels. Besides growth factors could be retained within sandwich cultures increasing their local activity. This hypothesis will be further investigated in the future.

Conclusions

Cell morphology was easily tuned by topological cues within sandwich cultures and differentiation -upon dorsal and ventral stimulation within sandwich-like cultures- was enhanced compared to standard 2D cultures. This was shown to be a consequence of the synergistic superposition of different events that occurs upon stimulation of integrin receptors. Cell contractility was not involved in this enhanced myogenesis within the sandwich environment, as the addition of Y-27632 did not alter the differentiation level. However, the simultaneous presence of the dorsal substrate and the activation of integrins were very much related to the enhanced cell differentiation found. Coating the dorsal substrate with BSA -to prevent integrin binding- did not reach the same differentiation level than sandwiches, but it was still higher than the corresponding 2D control. Likewise, stimulating the dorsal receptors with integrin antibodies -specially with anti-β_3- increased cell differentiation above the 2D control, but below the standard sandwich conditions. This result, in context with the super-enhanced cell differentiation found after sandwiching with blocked integrin-binding domain of FN (HFN7.1 antibody), revealed the distinctive role of the β_3 integrin interaction in sandwich-like cultures, as this integrin has other domains available for interactions beyond FN $III_{9\text{-}10}$ (blocked by the HFN7.1 antibody). Finally, the role of paracrine factors distinctively released within the cell culture in sandwich conditions as a consequence of this differential integrin activation through the FN-coated dorsal material surfaces is clearly revealed. Overall, the stimulation of dorsal $\alpha_v\beta_3$ integrins seems to have a preponderant role that needs to be further explored. The fact that the myogenic process is not

fully understood yet (even on 2D substrates) and that unknown differences may exist between 2D and 3D cultures (among them differences in the interplay of the multiple cellular pathways involved) hamper the unequivocal interpretation of all the parameters involved in the differentiation. Nevertheless, this study has pointed out important relations between dorsal stimulation and cell behavior but further experiments are necessary for the better understanding of the process.

Moreover, we have shown that cell morphology and signaling within the sandwich constructs share some similarities with 3D cultures, supporting the hypothesis that sandwich-like culture can be used as an intermediate model linking 2D and 3D cultures thus providing further insights into the role of dimensionality in cell-material interactions.

The model of sandwich culture becomes therefore very interesting due to the plenty of combinations of substrates, proteins and even cells that can be studied, providing a wide spectrum of possibilities for cell and tissue engineering, as well as an interesting approach for the study of cell behavior under well-controlled conditions. Moreover, since sandwich culture is developed from two 2D substrates, more reliable controls than other 3D approaches can be obtained, making easier to elucidate the influence of each condition under each dimensionality (2D or 3D).

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