Confined sandwich-like microenvironments tune myogenic differentiation

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Abstract

Sandwich-like cultures are engineered as a multilayer technology to simultaneously stimulate dorsal and ventral cell receptors, seeking to mimic cell adhesion in 3D environments in a reductionist manner. The effect of this environment on cell differentiation was investigated for several cell types cultured in standard growth media (DMEM with FBS), which promotes proliferation on 2D surfaces and avoids any preferential differentiation. First, murine C2C12 myoblasts showed specific myogenic differentiation. Then human mesenchymal stem cells (hMSCs) of adipose and bone marrow origin, which can differentiate towards a wider variety of lineages, showed again myodifferentiation. Overall, this study shows myogenic differentiation in normal growth media for several cell types under sandwich conditions, avoiding the use of growth factors and cytokines, i.e. just by culturing cells within the sandwich environment. Mechanistically, it provides further insights into the balance between integrin adhesion to the dorsal substrate and the confinement imposed by the sandwich system.

Keywords: 3D culture, confinement, myodifferentiation, C2C12, ~~c2c12,~~ hMSC

1. Introduction

Multipotent cells remain in their niche as slow proliferating and metabolically quiescent cells.[1-2] Strategies to maintain multipotency or induce differentiation *in vitro* commonly rely on complex cocktails of soluble factors that either promote quiescence to maintain multipotency or induce cell differentiation. More recent strategies aim to control cell fate by mimicking the physiological niche in order to recapitulate *in vivo* signalling.[3-7] Because most of the *in vivo* cell niches are 3D environments, these new strategies culture cells *in vitro* within 3D systems using different technologies such as spheroids, hydrogels, cell multi-layers

and 3D printing.[8] These culture systems provide new features that dictate cell fate such as 3D adhesion to extracellular matrix (ECM), 3D interaction with other cells, mechanical stimulation and altered diffusion (i.e. Oxygen, nutrients, cytokines and waste).[9-10] As a consequence, these strategies contribute towards the development of more relevant culture systems for tissue engineering and stem cell technologies.[10-11]

Collagen sandwich is a good example of functional 3D cell culture for hepatocytes, which quickly lose cell polarity and viability when cultured on 2D substrates. However, the culture between 2 collagen sheets (sandwich culture) improves morphology, viability and function maintenance.[12-13] We propose the sandwich (SW)-like culture, which built from 2D substrates, provides independent ventral and dorsal stimulation (figure 1A). Previous studies showed that cells interact with the dorsal substrate upon sandwiching and respond accordingly.[14-16] Hence the sandwich culture has the potential to recapitulate the dynamic interactions within relevant 3D environments such as the extracellular matrix (ECM). This 3D-like cell interaction triggers specific adhesion signaling that differs from the 2D, resulting in cell behavior closer to what is described for 3D cultures.[14, 16] Cell behavior is further determined by the nature of substrates chosen as this modulate key parameters for 2D cultures such as protein adsorption, including protein conformation and strength of interactions.[14-16]

Our previous studies showed the ability of murine C2C12 myoblasts to differentiate on poly-L-lactic (PLLA) substrates towards the myogenic lineage when differentiation media was used, for both 2D conditions and sandwich cultures.[14, 17] We wondered how the confinement provided by the sandwich-like culture modulates cell differentiation, a process highly dependent on cell/ECM/material interactions.[3, 5-6, 18] Cell differentiation was therefore investigated using standard growth media in order to prevent any preferential/targeted differentiation process. For instance, C2C12 cells proliferate when cultured on 2D substrates under growth media but can differentiate towards osteogenic and myogenic lineages if the

appropriate cytokines are provided. Therefore, we investigated whether having C2C12 cells confined between ventral and dorsal PLLA substrates, the sandwich-like environment, directs preferential differentiation under growth media. Then, we used human mesenchymal stem cells (MSCs) from bone marrow and adipose origin because of their potential to differentiate into several lineages (i.e chondrogenic, adipogenic, osteogenic, myogenic and reticular).[19-22] MSCs cultured *in vitro* on standard 2D tissue culture plastics (very different to the niche environment) tend to spontaneously differentiate resulting in a heterogeneous population with diminished multipotency.[23] Topography, stiffness, contractility, mechanical stimulation and culture media among others have the potential to direct cell differentiation.[6, 24] Previous studies showed MSCs of different origins behave differently under the same external conditions (physical and chemical environments).[25] Hence, we investigated whether sandwich environments promote MSCs differentiation towards preferential lineages in four different cell types.

2. Materials and methods

2.1 Materials

Spin coated and solvent casted poly(lactic acid) (PLLA, 4042D NatureWorks) samples were used as ventral and dorsal substrates respectively (figure 1A). Briefly, spin coated samples were obtained by spin casting a solution of 2% PLLA in chloroform (Scharlau, Barcelona, Spain) on glass coverslips for 5 seconds at 2000 rpm (SPS-Europe). On the other hand, solvent casted samples were obtained by casting 200 μL of the PLLA solution in stainless steel washers as explained elsewhere (figure 1B and 1C).[26] After solvent evaporation, resulting films were thermally treated at 120°C for 5 min in order to evaporate solvent traces. Note that due to the glass coverslip, PLLA spin coated samples are not permeable to media and then not useful to be used as dorsal substrates. Additionally, as dorsal PLLA is casted into

a washer prevents PLLA from floating. [26] Spin coated and solvent casted PLLA samples were UV sterilised for 30 min.

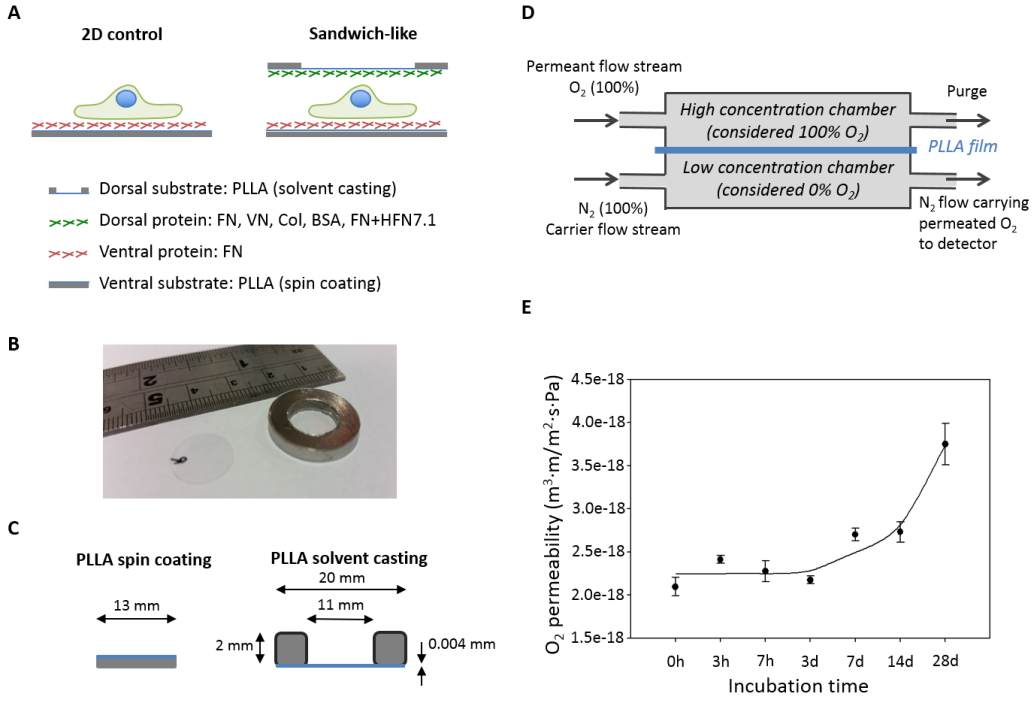

Figure 1. Presentation of the sandwich cell culture system. (A) Sketch of the 2D control culture (left) and the sandwich-like culture (right) including conditions used for the sandwich-like culture. PLLA is shown in blue, glass coverslip and stainless steel washer are depicted in gray. (B) Images of the PLLA spin coated on a glass coverslip and PLLA solvent casted samples on a washer. (C) Geometry and dimensions of samples, blue represents PLLA. (D) Sketch of the device used to measure oxygen permeability. (D) Permeability of the dorsal PLLA film to oxygen at different time points ($n \geq 3$).

2.2 Protein adsorption

Ventral and dorsal substrates were coated with proteins in order to direct specific cell/protein adhesion in the culture environment. Fibronectin (FN, Gibco) from human plasma was used at 20 µg/mL in Dulbecco's Phosphate Saline Buffer (DPBS) to coat the ventral substrate. Dorsal substrates were coated with either FN, vitronectin (VN, Sigma) at 10 µg/mL, heat-denatured Bovine Serum Albumin Fraction V (BSA, Roche) at 10 µg/mL in water or type I Collagen 1

mg/mL (Col I, STEMCELL Technologies). Adsorption was carried out for 1 h at room temperature and then samples were rinsed twice in DPBS to eliminate the non-adsorbed protein. For those experiments involving blocking of the RGD adhesion domain in FN, dorsal substrates were further incubated (after FN adsorption) with the monoclonal antibody HFN7.1 (Developmental Studies Hybridoma Bank) at 7.3 µg/mL during 1 hour and then washed twice in DPBS before cell culture.

2.3 Oxygen permeability measurements

Solvent casted PLLA films were prepared by casting a solution of 2% PLLA in chloroform on a petri dish. Resulting films were thermally treated (120°C for 5 min) to evaporate solvent traces and UV sterilised. Films were then incubated ~~in~~ at 37 °C in MilliQ water, which was changed every 2-3 days to mimic different time points of the culture. Oxygen permeability through PLLA films was measured in controlled conditions of temperature and relative humidity by following the procedures based on the ASTM D1434-82(2009) standard method.[27] In this method the transport of oxygen through the films was analyzed with an OX-TRAN model 2/21 ML permeation system (Paul Lippke Handels–GmbH, Neuwied, Germany), programmed to measure oxygen transmission rates at 23 ºC and 90% relative humidity (RH), and to subsequently convert them into permeability data. For this, an isostactic permeation apparatus with a stainless-steel cell containing two chambers separated by the sample to be tested was used (figure 1D). A constant gas stream was passed through each chamber at the required RH. The permeant gas, oxygen, flowed through the upper chamber while the carrier gas, nitrogen, flowed through the lower chamber and drove the permeated molecules to the detector system.[28] All measurements were made at 23 ºC and oxygen permeability was calculated from the average of three transmissibility values. We note that little effect of temperature is expected at 37 ºC as both temperatures are well below

the glass transition temperature of PLLA (~ 65 ºC). The average thickness of each sample was calculated from ten measurements using a micrometer, one in each of different zones of the central area of the sample, which is equal to the area of the cathode. The values found were about 32 ± 2 μm.

2.4 Cell culture

Murine C2C12 myoblasts (ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S, Lonza). C2C12 cells were seeded at 17,500 cells/cm^2 in serum-free DMEM in order to direct specific adhesion to the ventral FN coating. After 3 hours of culture, culture media was removed and sandwich cultures were obtained by directly overlaying the protein-coated PLLA film on top of the cells unless otherwise noted (details in [26]). Then growth media was added and cells were cultured for 4 days. Media was changed every 2 days carefully to avoid disturbing the assembled sandwich. Differentiation towards osteogenic and myogenic lineages was assessed by alkaline phosphatase (ALP) staining and sarcomeric myosin immunodetection respectively.

Human mesenchymal stem cells (hMSCs) were obtained either from PromoCell (C-12975 and C-12978; Germany) or isolated from normal patients undergoing surgery after providing informed written consent (approved by the NHS Glasgow and Greater Clyde Biorepository board) (table 1). hMSCs were extracted from bone marrow samples (hMSC-BM) obtained from haematologically normal patients undergoing routine hip-replacement surgery and from lipoaspirates (hMSC-AT). Harvested cells (hMSC-BM and hMSC-AT) were selected by plastic adherence alone whereas commercial cells (hMSC-BM selected and hMSC-AT selected) were already pre-selected using adherence and CD31-, CD44+, CD45-, CD105+ markers, providing a more enriched multipotent population. hMSCs were maintained in

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% (P/S). hMSCs between passage 1 and 4 were seeded at 5,000 cells/cm^2 in serum-free DMEM in order to direct specific adhesion to the ventral FN coating. Three hours after cell seeding, cells were adhered to FN-coated PLLA and then media was removed. Sandwich cultures were obtained by directly overlaying the FN-coated PLLA film on top of the cells, followed by the addition of the specific culture media. Positive differentiation controls were performed using induction media (table 2). In the case of the adipogenic differentiation, 2 different media were used alternatively and continuously during the culture: induction media (for 3 days) and then maintenance media (for 4 days). Media was changed every 2-3 days carefully to avoid disturbing the assembled sandwich.

Table 1. Mesenchymal stem cells used during this work.

Cells	Source	Markers
hMSC-AT (selected)	Adipose tissue	Adherence and selection with CD31-, CD44+, CD45-, CD105+
hMSC-BM (selected)	Bone marrow	Adherence and selection with CD31-, CD44+, CD45-, CD105+
hMSC-AT	Adipose tissue	Adherence
hMSC-BM	Bone marrow	Adherence

2.5 Alkaline phosphatase (ALP) staining

C2C12 osteogenic differentiation was determined by ALP detection. ALP staining (Sigma-aldrich) was performed following manufacturer´s suggestions. Briefly, cells were fixed in citrate-acetone-formaldehyde solution and then incubated in Sodium Nitrite/Naphtol alkaline solution for 15 min and protected from direct light. Then samples were washed in deionized water and counterstained with Neutral Red. Consequently, cells undergoing osteogenic differentiation showed a blue stain while the rest showed a red stain. Finally, cultures were scored quantifying the area (% of the total image) covered by the positive staining (blue) using ImageJ (National Institutes of Health, US).

2.6 Myogenic differentiation

Myodifferentiation was determined by the immunodetection of sarcomeric myosin, a myogenic differentiation marker. Briefly, C2C12 cells were fixed in 70% ethanol/37% formaldehyde/glacial acetic acid (20:2:1 V/V) and then blocked in 5% goat serum for 1 h. Afterwards 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 each. Samples were imaged at 5 randomly chosen positions maintaining the acquisition settings. Myodifferentiation was then scored by the percentage of positive sarcomeric myosin cells using the CellC image analysis software ($n \geq 15$ images per condition).[29] Myotube thickness and fusion index (nuclei per myotube) were quantified manually using ImageJ ($n \geq 100$ myotubes).

2.7 Immunofluorescence

MSCs were fixed in 4% Formaldehyde (Sigma) with 2% Sucrose (VWR) and permeabilised for 5 min at room temperature using 0.5% Triton X-100 (Sigma) in 20 mM HEPES buffer (Sigma) supplemented with 0.3 M saccharose, 50 mM NaCl (Sigma) and 3 mM $MgCl_2$ hexahydrate (Scharlab). Then samples were incubated in blocking buffer (1% BSA) for 15 min at 37 ºC, followed by incubation with MF-20 mouse antibody (Developmental Studies Hybridoma Bank, University of Iowa, USA). After washing in 0.5% Tween 20, samples were sequentially incubated in biotinilated secondary antibodies and Streptavidin-FITC conjugate. Finally, samples were washed and mounted in Vectashield containing DAPI (Vector Laboratories, UK).

Table 2. Growth and induction media used during the culture of the different cell types.

hMSCs	All conditions include 1% P/S
Growth	DMEM with 10% FBS
Myogenic	DMEM with 2% FBS, and 1% ITS-X
Osteogenic	DMEM with 10% FBS, 0.1 µM dexamethasone and 350 µM L-ascorbic acid 2-phosphate
Adipogenic (induction)	DMEM with 10% FBS, 1 µM dexamethasone, 1.7 µM insulin, 200 µM indomethacin and 500 µM isobutylmethylxanthine
Adipogenic (maintenance)	DMEM with 10% FBS and 1.7 µM insulin
C2C12	All conditions include 1% P/S
Growth	DMEM with 20% FBS
Myogenic	DMEM with 1 % ITS-X
Osteogenic	DMEM with 150 ng/mL BMP2

2.8 Gene expression analysis

Gene expression of several differentiation markers (supplementary table 1) was analysed by qPCR. First, RNA was extracted using RNeasy micro kit (Qiagen). Then 200 ng of RNA were reverse transcribed using the QuantiTect Rev Transcription Kit (Qiagen) and finally gene expression was quantified by real-time qPCR (7500 Real Time PCR system from Applied Biosystems). Results were normalized to GAPDH expression, used as the house-keeping gene, and then to the 2D value so that the final results show the fold increase over the 2D condition.

2.9 Statistical analysis

Results are shown as average ± standard deviation with *n* representing the number of biological replicates. Normal distributed data were analysed by one-way ANOVA followed by a Tukey's post hoc test whereas heteroscedastic data were analysed by Kruskal-Wallis followed by Dunn's post hoc test. p-values were corrected for the multiple comparisons. Statistically significant differences are indicated with * $P<0.05$, ** $P < 0.01$ and *** $P<0.001$.

3. Results

3.1 Sandwich-like culture characterization

The sandwich-like system has been developed as a technology that, grounded on 2D cultures, provides additional dorsal stimulation and thus has the potential to recapitulate the 3D environment in a sequential and controlled manner (figure 1A).[14-15] PLLA was used as ventral and dorsal substrates. PLLA samples remained stable and with no microscopic signs of degradation for up to 3 d. SEM images after 7 d show a higher number of small holes on the surface of the sample (supplementary figure S1). Samples were coated with fibronectin at 20 μg/ml (unless otherwise noted), which results in surface density of 1600-1800 $\mu g/m^2$.[30] Hence, both sides of the SW culture provided the same biological input (i.e. fibronectin coated PLLA surfaces). Oxygen permeability of PLLA was measured as a function of time using the device sketched in figure 1D. Figure 1E shows that PLLA permeability to oxygen increases during the culture time.

3.2 Sandwich-like culture triggers C2C12 myodifferentiation

C2C12 were cultured in growth media (i.e. without any supplement but with 20% FBS) to assess the only effect of the confinement provided by the sandwich environment. As expected when using growth media, cells on 2D substrates (2D) proliferated and hardly differentiated towards either osteogenic or myogenic lineages (figure 2A, 2B and 2C). On the other hand, sandwich-like microenvironments (SW) triggered the formation of long myotubes and promoted myogenesis (figure 2). Note that the sandwich-like system cultured with growth media resulted in higher myogenic differentiation levels than the 2D myogenic control (C+ myo) cultured in differentiation media (figure 2B and 2C). Additionally sandwich cultures did not promote osteogenic differentiation and cell proliferation was lower in sandwich cultures

compared to the 2D condition (figure 2C). Thus the SW environment specifically and efficiently triggered myogenic differentiation of C2C12 myoblasts.

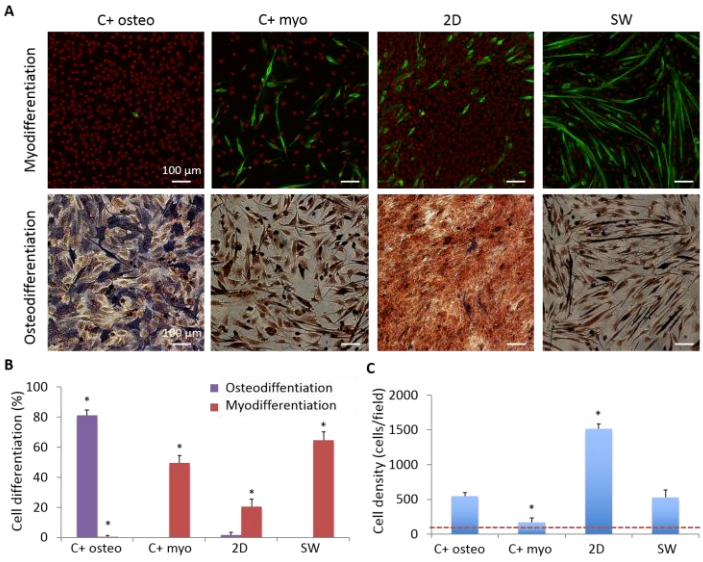

Figure 2. C2C12 differentiation after 4 days of culture. The bidimensional control (2D) and the sandwich-like (SW) samples were cultured in growth media (DMEM with 20% FBS) whereas the 2D control for osteogenesis and myogenesis were cultured in differentiation media (table 2). (A) Myodifferentiation was assessed by fluorescence staining of sarcomeric myosin positive cells (green) and cell nuclei (red). Alkaline phosphatase staining shows osteodifferentiation in blue. (B) Osteogenic and myogenic differentiation quantification for every condition. (C) Cell density after 4 days of culture. Dashed line represents cell density after 3 h of culture (prior to sandwich assembling). Statistically significant differences with the rest of the conditions are indicated with *$P < 0.05$ ($n \geq 3$).

3.3 Role of cell confinement in sandwich-like cultures

To gain more insights into the role of dorsal stimuli, C2C12 cells were cultured in SW using growth media using different dorsal protein coatings. We used fibronectin, vitronectin, collagen type I, fibronectin with the integrin binding region (FNIII9) blocked using the antibody HFN7.1 and bovine serum albumin (FN, VN, Col I, FN-HFN7.1 and BSA respectively). As shown in supplementary figure S2, FN, VN and Col I coatings triggered cell adhesion and spreading in control 2D substrates. However, FN-HFN7.1 (where the RGD

domain of FN is blocked and cells can only interact with the synergy domain) and BSA (that is a non-adhesive protein) coatings did not support cell spreading. These protein coatings mediate cell adhesion through different integrins. Figure 3 shows C2C12 differentiation under these different conditions. As seen before, the 2D condition resulted in low cell differentiation and high cell proliferation, contrary to the SW cultures (figure 3A and 3B). Particularly, SW cultures promoted C2C12 myogenesis regardless of the protein coating used. However, different dorsal protein stimuli resulted in different differentiation and maturation levels, as assessed by myotube thickness and fusion index (the number of nuclei per myotube) (figure 3C and 3D~~C~~).

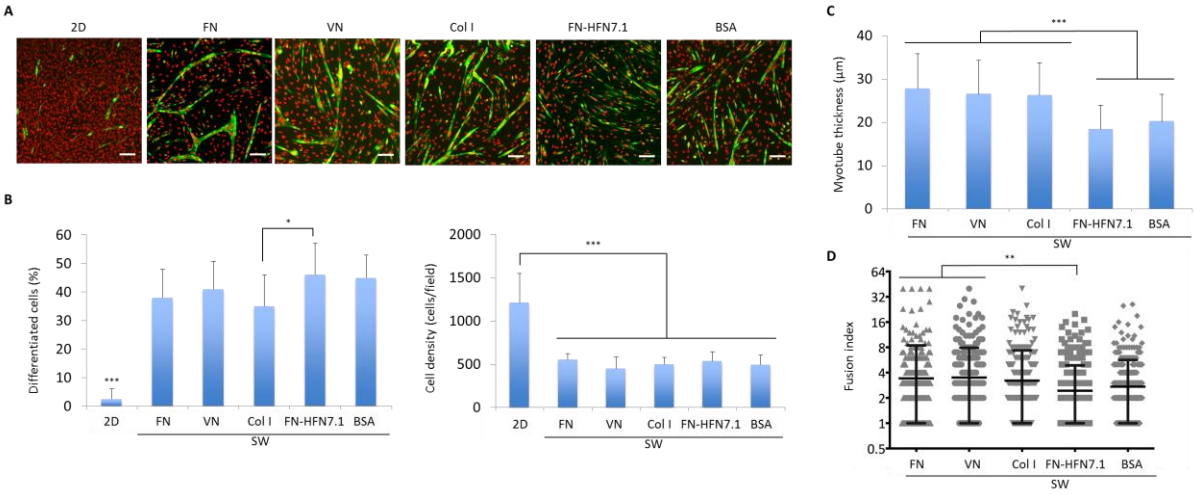

Figure 3. C2C12 differentiation under different dorsal stimulation. (A) Myodifferentiation was assessed by fluorescence staining of sarcomeric myosin positive cells (green) and cell nuclei (red). Scale bar 100 µm. (B) C2C12 myodifferentiation and cell density after 4 days of culture under the different environments. (C) Myotube thickness and (D) fusion index analysis for the SW culture with different dorsal protein coating. Graph D represents the fusion index for differentiated C2C12 cells as gray points and the average ± standard deviation in black ($n \geq 3$).

FBS contains a large and batch-dependent amount of proteins and cytokines that may influence cell differentiation and can displace BSA from the coating. Therefore C2C12 differentiation using BSA as dorsal substrate was also studied in myogenic media, which

lacks FBS (1% ITS, table 2). Again, SW with dorsal fibronectin resulted in higher levels of differentiation than the 2D control. Myodifferentiation for SW with dorsal BSA, though lower than the observed for the SW with dorsal FN, was still higher than the 2D control (figure 4).

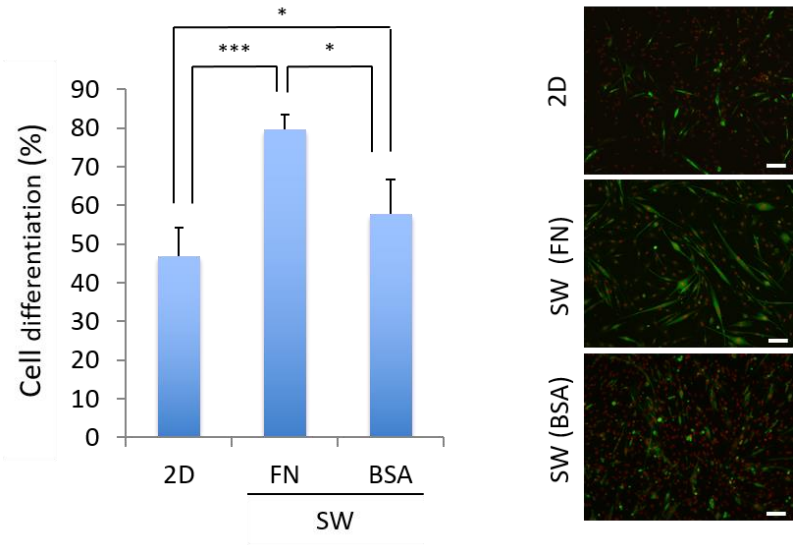

Figure 4. C2C12 differentiation under myogenic media (no FBS) for sandwich-like cultures with FN or BSA dorsal coating. Myodifferentiation was assessed by fluorescence staining of sarcomeric myosin positive cells (green) and cell nuclei (red). Scale bar 100 μm ($n \geq 3$).

We have previously observed that the dorsal stimulation provided by the SW culture modulates Focal Adhesion Kinase (FAK) signalling pathway, a key step to trigger the myogenic genetic program.[14, 31] Thus, we hypothesised that modulating FAK activation during the culture would result in different myodifferentiation levels. Figure 5 shows myodifferentiation increases monotonically with the time of sandwiching, reaching the highest value for those cultures sandwiched after 4 hours (SW^{t4}) and then it decreases monotonically keeping higher values than the 2D condition. We noted that cells spread on 2D substrates fast enough to avoid differences in cell area after 1h of adhesion (supplementary figure S3).

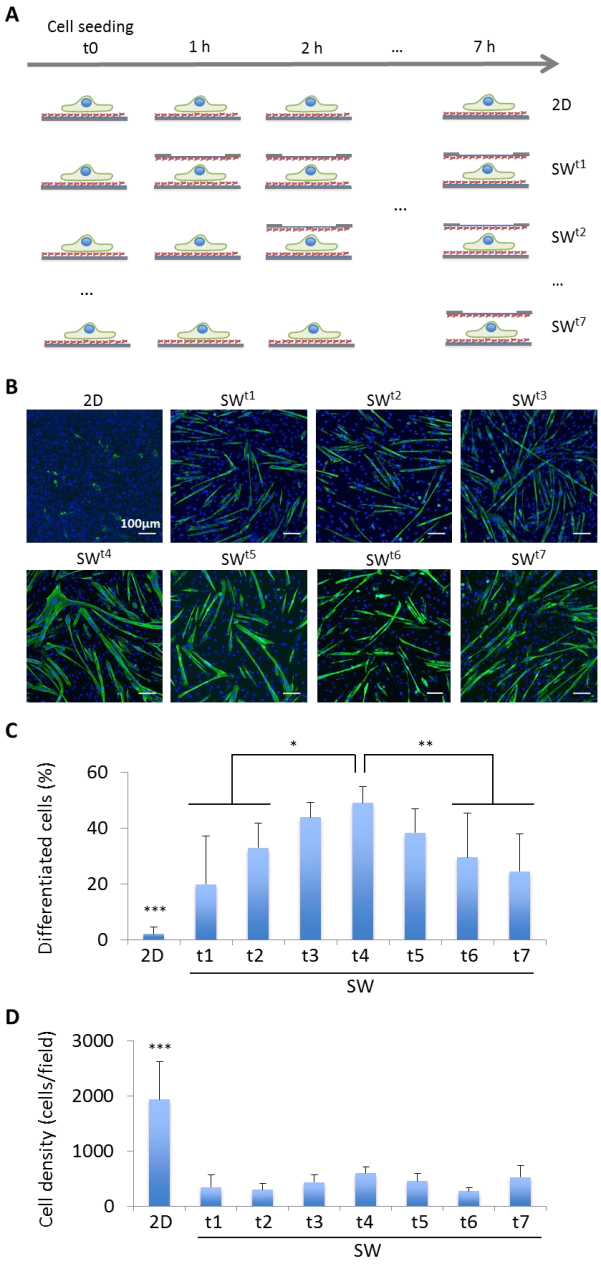

Figure 5. C2C12 myodifferentiation after assembling sandwich-like cultures at different time points after cell seeding. (A) Sketch depicting the cell culture procedure and the nomenclature (SW^{tx}, "x" being the time when the sandwich was assembled). (B) Myogenic differentiation after 4 days of culture in growth media assessed by staining for sarcomeric myosin (green). Cell nuclei labelled in blue. (C) C2C12 myodifferentiation as determined by the fraction of sarcomeric myosin positive cells. (D) Cell density after 4 days of culture ($n \geq 3$).

3.4 hMSC differentiation within SW cultures

Cell differentiation within the SW environment was further studied with human MSCs due to their multipotent potential and their relevance in clinical applications. There is a wide spectrum of MSCs types, which furthermore consist of sub-populations of multipotent cells with distinct differentiation potential.[32] Here 4 different types of hMSCs were studied: two of them isolated from the bone marrow and two from adipose tissue. At the same time, two of them were pre-selected commercially and the other two were primary cultures established by us (table 1). Similarly as with the C2C12 cultures, substrates were coated with fibronectin.

Dorsal stimuli provided by the sandwich-like culture modulated hMSCs differentiation towards different lineages in a cell-source dependent manner (figure 6A). For example, hMSC-BM showed overexpression of self-renewal, adipogenic, myogenic and chondrogenic markers for the sandwich-like culture whereas hMSC-AT (selected) only showed preference for osteogenesis. Three of the four hMSCs overexpressed MyoD in the SW culture. This was confirmed by immunofluorescence of sarcomeric myosin, with higher expression in SW cultures compared to 2D cultures (figure 6B).

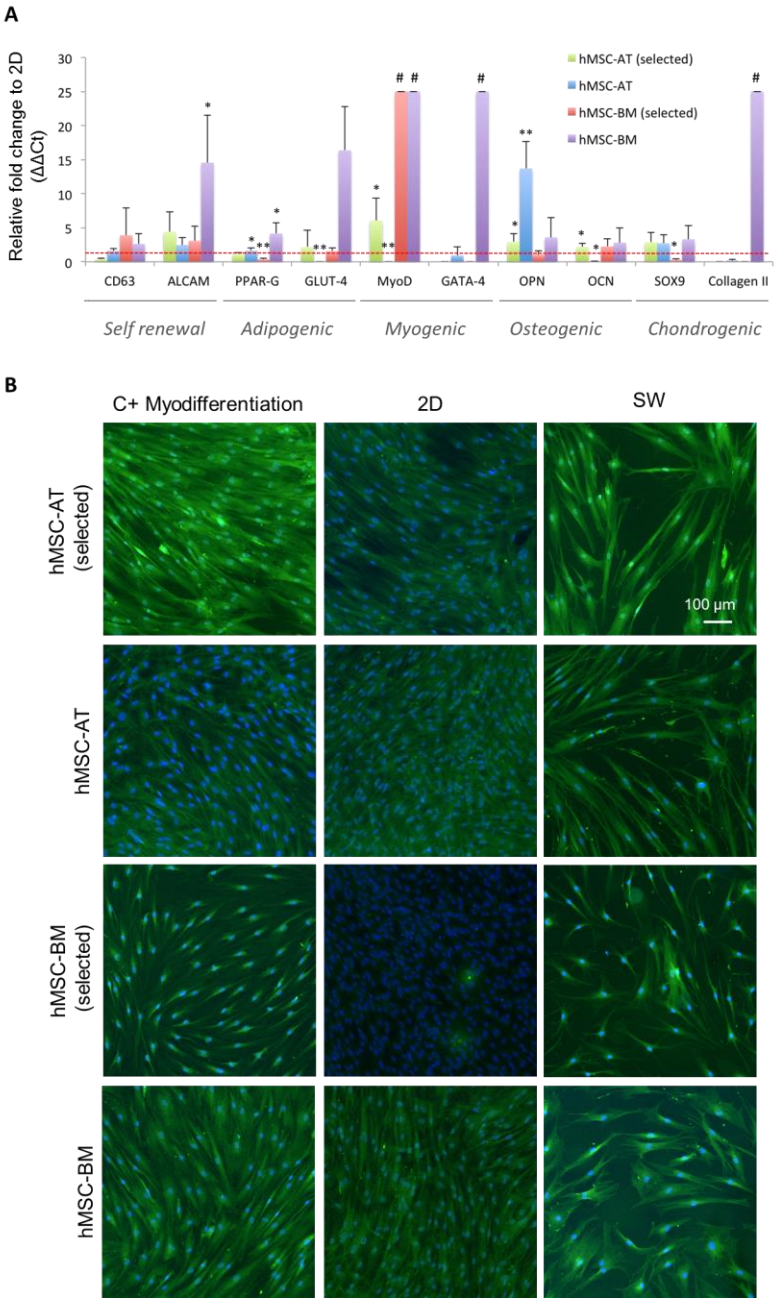

Figure 6. hMSCs differentiation after 14 days of sandwich culture in growth media (DMEM with 10% FBS). (A) Differentiation was assessed by qPCR. Data was first normalized to GAPDH gene expression and then to the 2D condition level that was assigned a value of 1 (red dashed line). Not detected markers were assigned value of 0. # designates markers detected for SW but not for 2D (and thus unable to normalize), which we assigned an arbitrary value of 25 to stress overexpression. (B) Myogenic differentiation was assessed by the immunodetection of sarcomeric myosin in green (a myogenic marker) and cell nuclei in blue ($n \geq 3$).

4. Discussion

This study describes a robust and versatile technology to investigate the relationship between cells and their environment in more relevant physiological conditions. Since 2D cultures are far from the *in vivo* environment, we have engineered sandwich-like cultures that provide both ventral and dorsal interaction to recapitulate 3D environments. Several systems that stimulate dorsal interactions have been used before to study cell behaviour in a more relevant 3D condition, such as overlaying cells with polyacrylamide sheets, protein gels or cell sheets (review in [10]).[33-35] We have overlaid a flat poly-lactic acid (PLLA) substrate since this would allow in addition to finely tune several relevant properties in the future such as topography, thickness and degradation rate of the dorsal substrate, all parameters that influence cell fate. [36] To our knowledge, this is the first time that differentiation of 5 different cell types (C2C12 myoblasts and 4 hMSCs) has being investigated in the same 3D-like environment under growth conditions.

We have previously shown that C2C12 cultured in myogenic media (1% ITS, no FBS) differentiate within the sandwich-like culture, with percentages of differentiation significantly above the 2D control.[14] In this study cells were cultured in growth media (DMEM with FBS) to avoid any preferential differentiation role of the biochemical environment. Figure 2 shows that sandwich-like culture specifically directs C2C12 differentiation towards the myogenic lineage, hindering osteogenic differentiation and slowing cell proliferation compared with the corresponding 2D system in the same culture conditions (figure 2). Additionally, differentiation levels for SW were higher than those for C+ myo. We note that C2C12 myogenic commitment in 20% FBS is highly unusual and quite unique to SW environments. This evidence supports the strong influence of

the sandwich environment on cell fate and highlights the use of 3D environments as an alternative to complex culture media to direct cell fate.

C2C12 differentiation is a complex process determined by adhesion signaling and paracrine factors among others. Several features of the SW culture environment could trigger (in combination) this specific differentiation towards myogenic lineages: i) altered media diffusion leading to hypoxia/starvation and/or paracrine factors retention, ii) mechanical pressure provided by the dorsal substrate and iii) the biological input of the dorsal cell/protein interaction.

i) As observed in 3D environments, including *in vivo*, the spatial confinement provided by the SW culture system may induce hypoxia and cell starvation along with increased local concentration of paracrine factors when compared to 2D cultures. In the latter, nutrients and waste products diffuse freely and oxygen pressure is high and constant.[37-39] Our previous results show that cells receive enough nutrients to proliferate, migrate and differentiate within the SW system, ruling out drastic hypoxic or starvation effects in SW cultures.[14, 16, 26] Additionally, hypoxia has been related to diminished C2C12 myogenesis whereas our results clearly show SW environments enhanced myogenesis, suggesting drastic hypoxia is not related to this SW environment.[40-41] On the other hand, fFigure 1EC shows that PLLA permeability to oxygen is low, but it increases with time in culture, likely due to the PLLA hydrolytic degradation.[42-43] Finally, we have already shown that the confined environment provided by the SW culture retains paracrine factors during the culture (i.e. after 12 hours), which also plays a positive role in C2C12 myodifferentiation.[14]

ii) Pure mechanical confinement could also play a role in C2C12 differentiation regardless of cell adhesion. It is now well known that not only biological but also mechanical cues have a significant effect on cell fate, including differentiation.[3, 44] The effect of mechanical

compression in osteogenic, chondrogenic and cartilage differentiation is well characterized but there is a lack of studies regarding myogenic differentiation.[24] However, multiple studies have shown mechanical strain has a significant impact on myogenic differentiation, suggesting myodifferentiation might be positively altered by mechanical cues.[24, 45] In this case, dorsal substrates weight 3.08 g and thus provide a compression pressure of 227 Pa to the cells. To assess the effect of the compression load in the sandwich-like culture dorsal substrates were coated with bovine serum albumin (BSA), a non-adhesive protein that does not support cell adhesion.[46] As a consequence, cells sandwiched with dorsal BSA do not biologically adhere to the dorsal substrate but are immediately subjected to a mechanical compression that has the potential to alter cell fate.[47] We note that once adsorbed on PLLA, and in the presence of medium containing serum, BSA can be displaced by serum proteins (e.g. fibronectin or vitronecin), – the so-called Vroman effect.[48] To address this issue, C2C12 differentiation was investigated in medium that contained 1% ITS but no FBS, so that there were not adhesive proteins in the culture media that could displace dorsal BSA. As shown in figure 4, the SW culture with dorsal BSA resulted in lower cell differentiation than those with dorsal FN. Note that using BSA on the dorsal side did not completely revert differentiation to 2D values. This suggests that not only dorsal adhesion but also confinement imposed by the SW system may play an important role in cell differentiation.

iii) The biological input provided by the SW culture could influence cell differentiation by exciting ventral and dorsal receptors simultaneously, which results in a different cell adhesion signaling than that occurred in 2D. Figure 3 shows different dorsal proteins resulted in different maturity and differentiation levels. These results are in line with our previous studies that show the effect of the dorsal protein coating in cell morphology, cell migration and ECM reorganization.[14-16] The SW culture is therefore a simple system to study cell fate in 3D-like environments with heterogeneous signaling. Blocking the integrin binding region (FNIII9) of

dorsal FN or using dorsal BSA (and thus hindering initial dorsal adhesion) resulted in similar number of differentiated cells but with less mature myotubes than those with dorsal FN, VN or Col I as shown by the lower fusion index and myotube thickness (figure 3~~A and 3C~~). This result suggests that the initial dorsal adhesion plays an important role on the development of mature (thick) myotubes.

Myogenic differentiation has been related to a transient reduction of phosphorylated FAK (pFAK) during the first hours of the process followed by a later activation to achieve terminal differentiation and the formation of myotubes.[31] We have previously shown that the dorsal stimulation provided by the SW culture modulates this signaling pathway decreasing pFAK after 3 h.[14, 31] Thus, we hypothesised that sandwiching C2C12 cells at different times would induce FAK signaling differentially and this would result in altered myodifferentiation levels. Results shown in figure 5 support this hypothesis and show that the confinement provided by the sandwich-like culture modulates myogenic differentiation.[14] This result supports furthermore our previous observations regarding the rapid interaction with the dorsal substrate since sandwiching with only 1 hour delay has dramatic effects on cell differentiation 96 hours afterwards.[14-16] Altogether these results suggest that engineering the architecture of the cell microenvironment *in vitro* might replace the use of cytokines and growth factors, a major limitation to scale up culture systems due to e.g. increased costings.

To investigate whether these results could be replicated in cells with more translational potential and whether the myogenic differentiation input provided by the SW environment could trigger differentiation in more potent cells, human MSCs were tested. Notwithstanding the clear effect that SW culture had on C2C12, specific differentiation towards myogenic lineages was not universally observed for hMSCs. Actually, the same sandwich environment triggered different cell responses on MSCs

of different origins and isolated under different protocols. However, myogenic differentiation, a less-often achieved phenotype from MSCs, was noted within the SW cultures in three out of four hMSC types, which did not occur for any of the other markers (self-renewal, adipogenic, osteogenic and chondrogenic). The different effect of SW confinement on MSC and C2C12 is likely to be related to the broad number of lineages into which MSCs can differentiate, whereas only osteogenic and myogenic lineages are linked to C2C12 cells. This result stresses the differential effect of the cell microenvironment on MSCs from different origins and highlights the need to personalize *in vitro* systems for every specific cell type in order to understand and manipulate cell behavior. This links well with current efforts in precision medicine where personalized biomaterials and environments are engineered in response to demands imposed by the type of cell and even the conditions of the patient from which these cells have been isolated.[49]

4. Conclusions

This study shows the novelty of triggering myogenic differentiation in normal growth media for several cell types when cultured under sandwich conditions. In addition, more mature myotubes obtained when this physical confinement also promotes excitation of dorsal receptors. Mechanistically, it provides further insights into the balance between integrin adhesion on the dorsal substrate and the confinement imposed by the SW system. We note that although MSCs culture within the SW system expressed preferentially myogenic markers, they also showed phenotypes compatible with other lineages~~,~~ in dependence of cell source and isolation protocol. These results highlight the need of studying cell biology in culture conditions closer to the *in vivo*.

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Supporting Information Available

The following files are available free of charge:

- Supplementary table 1. Primer sequences used for the quantitative real time PCR.
- Supplementary figure S1: Microscopic evolution of PLLA membranes after different times in hydrated conditions using SEM.
- Supplementary figure S2. C2C12 cell adhesion and spreading on different protein coatings.
- Supplementary figure S3. C2C12 adhesion and spreading on FN coated PLLA after different time points.

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Confined sandwich-like microenvironments tune myogenic differentiation

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