Cell migration within confined sandwich-like nanoenvironments

Abstract

Aim: We introduced sandwich-like cultures to provide cell migration studies with representative nano-bio-environments where both ventral and dorsal cell receptors are activated. **Methods:** We have investigated different nano-environmental conditions by changing the protein coating (fibronectin, vitronectin) and/or materials (using polymers that adsorb proteins in qualitatively different conformations) of this sandwich system to show their specific role in cell migration. **Results:** Here we show that cell migration within sandwich cultures greatly differs from 2D cultures and shares some similarities with migration within 3D environments. Beyond differences in cell morphology and migration, dorsal stimulation promotes cell remodeling of the ECM over simple ventral receptor activation in traditional 2D cultures.

Keywords: sandwich culture, 3D cell migration, cell motility, lobopodia-based migration, fibronectin

Introduction

Cell migration is an essential process from embryonic development to adulthood. Hence, studying cell migration on well-defined nano-bio-environments is required to better understand cellular mechanisms, search for novel therapeutic targets and design optimal implants. Mechanisms of cell migration have been extensively studied *in vitro* on two-dimensional (2D) surfaces, where it is described as a lamellipodia-based event consisting mainly of 4 steps: extension of the leading edge, adhesion formation, traction generation and subsequent retraction of the trailing edge [1]. So, cell migration on 2D substrates is integrin-mediated and depends on the traction force cells exerce on the underlying material. Nevertheless cell migration has been shown to differ between the traditional 2D models *in vitro* and the *in vivo* situations [2-5]. When switched to three-dimensional (3D) culture systems - more representative of the *in vivo* environment - different types of single cell migration can be described: (i) *mesenchymal migration* which is proteolytic dependent and lamellipodia-based - and thus similar to 2D migration (with implication of integrins and cell contractility) -; (ii) *amoeboid migration* which is non-proteolytic dependent and where adhesions are inexistent or very weak [6-8] and (iii) *lobopodia-based migration* which is determined by large cylindrical protrusions and depends on the 3D matrix elasticity [9]. Additionally, cells of the same type can switch between different modes of cell motility in response to the physical properties of the environment, integrin impairment, degradability and soluble signalling factors [9-12].

Given the difficulty of mimicking the *in vivo* nanoenvironment of the extracellular matrix (ECM), different complex 3D culture systems have been used to study cell

46 migration [11, 13-15]. Unfortunately, differences inter- and even intra- 3D systems as
47 well as difficulties in having reliable 2D controls hinder identifying and understanding
48 the key features in 3D cell migration. Additional parameters play an important role in
49 3D migration such as (i) pore size [11], (ii) physical properties, e.g. matrix stiffness [9],
50 (iii) nutrients diffusion [16], (iv) degradability of the system [17], (v) topographical
51 cues [18], (vi) functionalities of the material (i.e. synthetic materials such as PEG
52 hydrogels should be modified with adhesion domains in order to permit cell adhesion)
53 [19] and (vii) variability of components from batch to batch [20]. Moreover, proper
54 quantitative methods for analysis of migration rates in 3D environments are still
55 lacking. New procedures to better understand cell migration within 3D cultures are
56 therefore needed.

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58 The distribution of cell receptors anchored to the ECM within 3D microenvironments
59 highly differs from that observed on traditional 2D cultures. This is thought to be one of
60 the key causes for the different cell behaviour between 2D and 3D systems since
61 integrins trigger multiple signaling pathways which determine e.g. cell growth and gene
62 expression [21-23]. Furthermore, differences in integrin localization and expression
63 between 2D and 3D cultures have been described, supporting the hypothesis that
64 different outside-in signaling may be the cause of different cell behavior [2, 24].

65

66 We have previously observed that sandwich-like microenvironments provide dorsal
67 stimulation similar to 3D systems and trigger cell signaling pathways that promote a cell
68 behavior more similar to 3D environments [25-27]. Here, we hypothesize that the
69 excitation of dorsal receptors - by sandwiching cells already attached on a 2D surface -

might switch cell migration towards 3D modes. To assess this hypothesis, we studied L929 fibroblast migration within sandwich-like cultures. Due to the high versatility of the system, different well-controlled nanoenvironments can be explored to investigate different parameters such as the protein conformation at the material interface. This technology then becomes an interesting tool to study some specific aspects of cell migration. In particular, we have varied the cell seeding procedure (culturing either isolated cells or as in wound healing assays), the chemistry of the ventral surface (using poly(ethyl acrylate), poly(methyl acrylate) and glass) and the biological input coming from the dorsal substrate (coating samples with different proteins, e.g. fibronectin (FN) and vitronectin (VN)) (see figure 1). Poly(lactic acid) (PLLA) was used as dorsal substrate in all cases. Cell morphology, adhesion and migration under different culture conditions were characterized, as well as the influence of the dorsal stimulation on the ability of cells to remodel the ECM.

Materials and methods

Materials

Polymer films of ethyl acrylate and methyl acrylate (EA and MA respectively, Sigma-Aldrich) were obtained by radical polymerization of a monomer solution using benzoin (98% pure, Scharlau) as photoinitiator at 0,35 wt% and 1 wt% respectively. The polymerization was carried out up to limiting conversion. Spin-coating was then used to produce thin films of these polymers on glass coverslips (Brewer Science, Rolla, MO). Polymer solutions were made in toluene with 2.5% PEA or 6% PMA and spin-coating at 2000 rpm for 30 s. Finally, samples were dried at 60 °C in vacuum for 1 h before its use as ventral substrates.

Thin films of PLLA were prepared by solvent casting a solution of 2% PLLA in chloroform (Scharlau) in stainless steel washers and allowed to evaporate. Resulting films were then thermally treated at 120 ºC for 5 minutes and used as dorsal substrates.

Protein adsorption

Fibronectin from human plasma (FN; Sigma) at 20 µg/ml in DPBS, vitronectin at 10 µg/ml in DPBS (VN; Sigma) or heat-denatured Bovine Serum Albumin Fraction V (BSA; Roche, Barcelona, Spain) at 10 mg/mL in MilliQ water were adsorbed on the different substrates during 1 h at room temperature. After adsorption, samples were rinsed in DPBS to eliminate the non-adsorbed protein.

Atomic Force Microscopy

Atomic force microscopy (AFM) was performed on a JPK Nanowizard 3 BioScience AFM (JPK, Germany). Images were taken operating in the AC mode and analysed by the SPM and DP 4.2 software version. Si-cantilevers with a force constant of 2.8 N/m and a resonance frequency of 75 kHz (Nanoworld AG, Switzerland) were used. The phase signal was set to zero at a frequency 5–10% lower than the resonance one. Drive amplitude was 700 mV and the amplitude set point was 700 mV.

Cell culture

L929 fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Lonza).

Prior to seeding, samples were sterilized under UV for 30 min (30 min each side in the case of the dorsal substrates) and coated as described before. Then L929 cells were seeded in DMEM without serum in order to direct specific fibronectin-cell adhesion. After 3 h of culture, sandwich cultures (SW) were obtained by gently laying the dorsal substrate over the seeded ventral substrate where cells were already adhered (figure 1). In order to better mimic SW conditions, a washer without PLLA was laid on the 2D samples. Since several conditions have been studied, a specific nomenclature was used overall the study: SW_x^y with *x*-ventral and *y*-dorsal material condition. More detailed cell culture procedures can be found in the supplementary data.

Time-lapse cell imaging

Images were acquired every 20 min for 24 h using a Leica DMI 6000 inverted microscope (Leica Microsystems) with a 10X dry objective. During the observation, the samples were maintained at 37 ºC and supplied with a 95% air and 5% CO_2 humidified gas mixture. Phase contrast images were gathered at 20 min intervals along 24 h of culture. For gap closure measurements, images were processed using the external plug-in MiToBo in ImageJ software in order to obtain quantitative data about the dynamics in cell migration [28, 29].

Immunofluorescence

Samples were fixed in 10% formalin solution (Sigma) at 4 ºC for 30 min, "de-sandwiched" and then permeabilised for 5 minutes. After that samples were incubated in 1% BSA in order to reduce the background signal. Cells were then incubated with the

primary antibody (anti-vinculin and anti-paxilin antibodies (Sigma), anti-MMP2 and anti-MMP13 (Abcam), anti-α-tubulin (Abcam), anti-β_1 integrin (BD Bioscience) or anti-α_v integrin subunit (Millipore)) for 1 hour. Samples were then rinsed in DPBS/0.5% Tween 20, followed by incubation with Cy3 or AlexaFluor 488 conjugated secondary antibody (Jackson Immunoresearch and Invitrogen respectively). If necessary, samples were incubated in BODIPY FL phallacidin (Molecular probes) diluted 1/100 in order to stain the cytoskeleton. Finally the samples were washed before being mounted in Vectashield containing 4´,6-diamidino-2-phenylindole (DAPI, Atom).

FN reorganization

The ability of cells to reorganize the FN adsorbed on the ventral material surface was evaluated after 7 h of culture. Samples were fixed, "de-sandwiched" and subjected to FN immunodetection. The monoclonal primary antibody HFN7.1 that specifically binds human FN $III_{9\text{-}10}$ domains was used (Developmental Studies Hybridoma Bank) to avoid cross-reaction with FN secreted by murine cells.

Image analysis

Cell morphology was quantified by calculating different parameters using ImageJ software. Cell area, aspect ratio (major axis/minor axis), circularity ($4\ \pi$ x area/perimeter^2) and roundness (4 x area/π x [major axis]^2) (the last 2 ones getting a value of 1 for a perfect circle) were evaluated by analyzing at least 40 cells for each condition [30]. Additionally, cell morphology and distribution of focal adhesions were analyzed by The Focal Adhesion Analysis Server [31].

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. Statistically significant differences are indicated with * $P<0.05$ and *** $P<0.001$.

Results and discussion

Phenomenology of cell migration within sandwich environments

We have studied L929 fibroblasts migration within sandwich cultures using a modified wound healing assay based on culture inserts since manual scratching stripped off the thin spin-coated polymer films (figure 1). Moreover manual scratching of cell monolayers has been correlated to changes in cell morphology, alteration of proliferation and deregulation of migration [32].

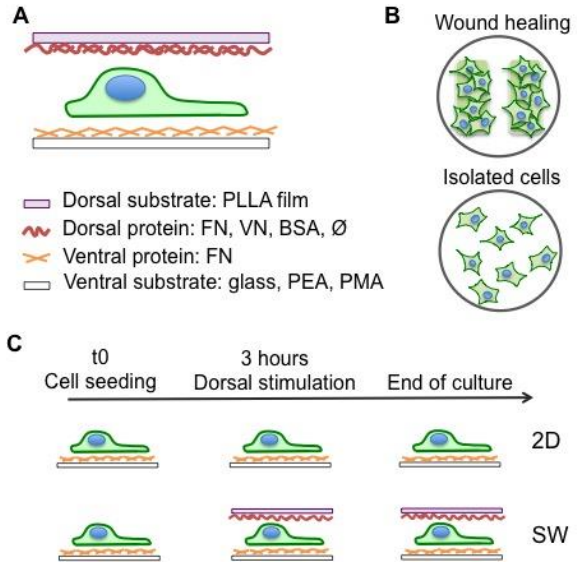

Figure 1. Summary of the sandwich-like culture conditions. (A) Sketch of the sandwich-like model and the different combinations of material and proteins. (B) Sketch of the two different cell cultures used during this work. (C) Timing diagram showing the procedure followed for sandwich culture systems.

Movie 1 (supplementary material) shows cell migration on FN coated glass coverslips (2D) and then after overlying with a FN-coated PLLA film (sandwich culture, SW). Cells on the 2D control migrate according to the classical 4 migration steps, by adopting a polygonal shape with wide lamellipodia and pseudopodia projections [33, 34]. However, cells within sandwich culture adopted an elongated morphology with fewer (usually 1 or 2) but longer pseudopodia that were more persistent in time resembling what has been observed in other 3D environments (figure 2A) [9, 35, 36]. Such elongated cell morphology of migrating cells has not been previously observed within our sandwich microenvironments [25-27]. Importantly, also note that the nuclei of cells migrating in sandwich environments hardly move from their initial position, which suggests that the last steps of the migration process (traction generation and the retraction of the trailing edge) were hindered or not coordinated [37].

One might think that the elongated morphology observed within the sandwich culture at the front edge of the 2 cell populations in a wound healing assay might be related to the biochemical crosstalk between the 2 populations (chemotaxis due to increased concentration of cell secreted signals within the SW environment), which may enhance extension directionality and persistence. However these elongated cells were observed along the whole border of the seeded fields, not only the ones facing the wound. So we first assessed whether this elongated morphology was due to the high cell density used during the wound healing-like assay rather than to the specific sandwich condition. Hence, isolated cells were cultured at low density and migration was monitored. Figure 2 depicts the morphology of selected cells after different time-points of culture so cell morphology and migration can be evaluated. Cells overlaid with the FN-coated dorsal substrate (sandwich culture, referred to as SW^{FN}) did not show such elongated morphology. Hence the cause of this elongated morphology should be sought as a consequence of combined dorsal stimuli and high cell density population, which suggests an important role of cell-cell contact and Rac1 signaling [38]. To corroborate this, high cell density areas of the wound healing were imaged and it was seen that only cells in the perifery of the population projected the long pseudopodia whilst cells inside the population conserved a polygonal shape (data not shown).

When the morphology of migrating cells within the sandwich culture was studied more in depth (figure 2) we saw that the circularity of isolated cells was similar to the circularity of cells during the wound healing assay (figure 2B) and, most importlantly, isolated cells within sandwich culture showed different roundness than isolated cells

cultured on 2D substrates. These suggest that, despite not having such long pseudopodia, cells sense the dorsal stimuli and respond to it by changing their morphology as reported for other 3D systems [39, 40]. Moreover, we have recently shown that cell morphology within sandwich culture highly depends on the substrate properties similarly as happens in 3D cultures [27]. Regarding cell motility, cells cultured on 2D substrates moved around the starting point in random open trajectories, whereas cells within SW^{FN} remain mainly static at the initial location, and only cytoplasmatic extensions were projected (figure 2C). So, sandwich culture hinders cell migration and nuclei movement both for isolated and wound healing cultures though cells do project long pseudopodia only in the latter case.

In order to investigate the role of the nature of the dorsal stimulus, cells were studied also when sandwiched with an un-coated dorsal substrate ($SW^{Ø}$). As a consequence these cells received similar biological signals than cells on 2D samples, as the only biologically driven interaction comes from the ventral substrate. As shown in figure 2C, cells within $SW^{Ø}$ can move and change their location, though less than on 2D substrates. The lack of protein coating on the dorsal substrate is therefore related to an increased cell migration compared to SW^{FN}, though cells do not behave as on the 2D control. This suggests that not only the biological but also the mechanical stimuli are important cues in sandwich environments [25-27].

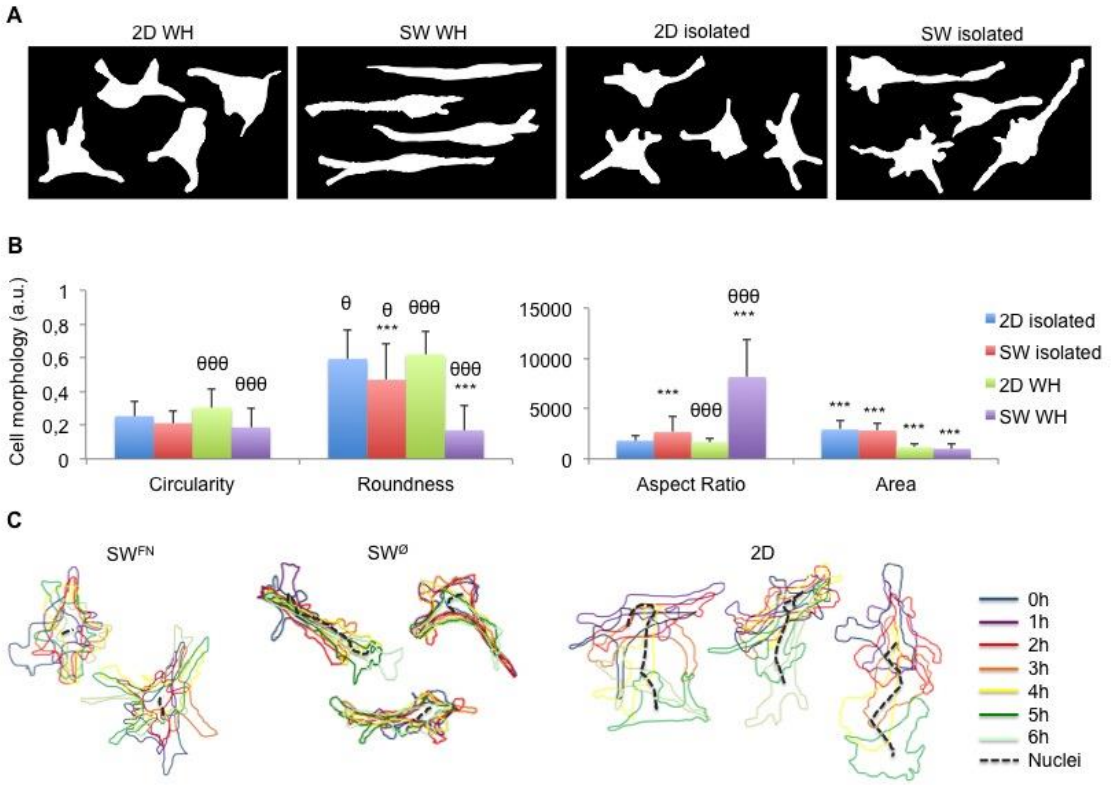

Figure 2. Cell migration and morphology. (A) Cell morphology after 12 hours of migration in a wound healing assay (WH) or as isolated cells (isolated). (B) Morphology of cells after 12 hours of culture. * shows significance comparing isolated with wound healing for the same type of dimensionality (2D or SW); and θ comparing 2D with SW for the same type of culture condition (isolated or wound healing). (C) Outlined cells after different time points of culture. Dotted lines represent the movement of cell nuclei. Coordinates are mantained for each outlined cell so that the displacement displayed in the picture is equivalent to the migration oberved during the culture.

Characterization of the migrating cells

Cells migrating in the wound healing assay were further characterized seeking to understand differences with 2D conditions. Cultures were kept for 7 h to allow cells to project these characteristic long pseudopodia within the sandwich culture. First, focal adhesion proteins and integrins were evaluated as it is well accepted that these differ

between 2D and 3D/*in vivo* environments [2, 40-42]. Focal adhesion proteins such as pFAK^{Tyr925} and vinculin were detected at the cell edge when cultured on 2D substrates (figure 3). Within sandwich microenvironments, these proteins were localized mainly at the rear part of the cell and at the tip of the long pseudopodia. Besides, colocalization between vinculin and pFAK^{Tyr925} was observed in both conditions. Further differences between vinculin and pFAK did not only occur between both 2D and SW cultures but also when comparing the leading and rear part of cells migrating within the sandwich (table 1 and 2). Cells within the sandwich culture have a higher number of focal adhesions at the rear part than at the leading edge (table 1). This might explain why cells cannot retract the trailing edge during migration and only long pseudopodia are projected. Likewise, the larger focal adhesions observed for the sandwich culture (as well as the regulation of their turnover) might explain the lower migration rate [37, 43].

Table 1. pFAK and vinculin characterization in the leading and rear part of cells migrating within sandwich culture in a wound healing assay.

	Leading	**Rear**	**P value**
pFAK			
# Focal adhesions	165.11 ± 67.33	263 ± 90.8	< 0.0001 *
Area *(μm^2)*	1.06 ± 0.23	0.81 ± 0.18	< 0.0001 *
Total Area *(μm^2)*	164.47 ± 41.92	205.80 ± 58.07	0.0065 *
Distance to edge *(μm)*	1.83 ± 0.52	4.49 ± 1.01	< 0.001 *
Major axis *(μm)*	1.46 ± 0.2	1.29 ± 0.15	0.0019 *

Minor axis *(μm)*	0.79 ± 0.09	0.68 ± 0.06	< 0.0001 *
Vinculin			
# Focal adhesions	163.27 ± 68.83	279.56 ± 106.67	0.0012 *
Area *(μm^2)*	0.84 ± 0.31	0.88 ± 0.25	0.68
Total Area *(μm^2)*	133.56 ± 65.62	234.45 ± 71.45	0.0003 *
Major axis *(μm)*	1.36 ± 0.28	1.41 ± 0.21	0.54
Minor axis *(μm)*	0.67 ± 0.11	0.71 ± 0.10	0.30

Table 2. pFAK and vinculin adhesions in 2D and sandwich cultures.

	2D	**SW**	**P value**
pFAK			
# Focal adhesions	682.22 ± 193.64	382.75 ± 98.31	0.0014 *
Area *(μm^2)*	0.57 ± 0.05	0.69 ± 0.14	0.0014 *
Total Area *(μm^2)*	387.31 ± 100.69	258.33 ± 64.95	0.0046 *
Distance to edge *(μm)*	6.39 ± 1.56	3.56 ± 0.89	0.0003 *
Major axis *(μm)*	1.03 ± 0.03	1.20 ± 0.14	< 0.0001 *
Minor axis *(μm)*	0.61 ± 0.02	0.65 ± 0.06	0.0154 *

Vinculin			
# Focal adhesions	495.44 ± 185.9	278 ± 114.29	0.0197 *
Area *(μm²)*	0.74 ± 0.10	0.66 ± 0.09	0.59
Total Area *(μm²)*	357.04 ± 102.95	240.05 ± 50.17	0.0108 *
Major axis *(μm)*	1.30 ± 0.11	1.33 ± 0.17	0.27
Minor axis *(μm)*	0.61 ± 0.03	0.64 ± 0.38	0.07

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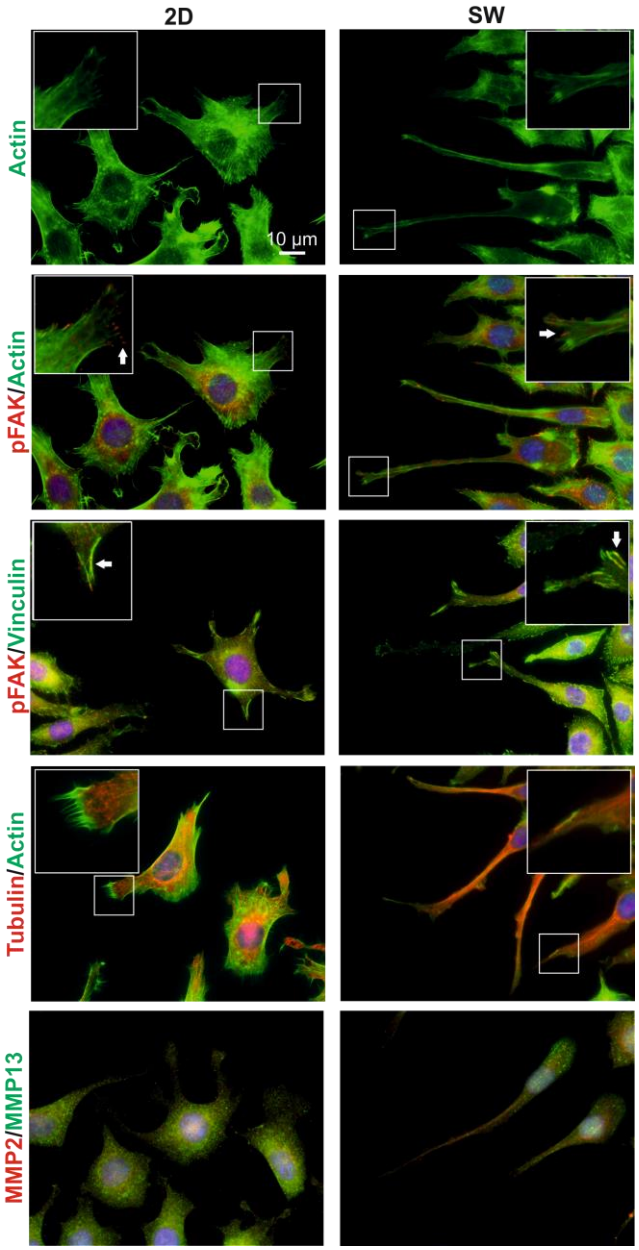

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Figure 3. Immunofluorescence images after 7 hours of culture (2D and SW). Insets show magnifications of focal adhesions and cell cytoskeleton. Arrows point out focal adhesions.

Stressed actin fibers and developed α-tubulin microtubules were assembled both on 2D and in SW cultures, but with different cell morphology that confined nuclei into a narrower space within SW cultures. Likewise, broad actin lamellipodia were seen for cells seeded on 2D substrates but not for cells within the sandwich culture (figure 3). Similar elongated fibroblast morphology has been previously described for other 3D systems. In particular, elongated cells with multiple long protrusions and small lamellipodia at their tips (that contain Rac1, cortactin, PIP3 and Cdc42) have been shown to use lamellipodia-based migration, whereas elongated cells with fewer protrusions and no lamellipodia use the lobopodia-based migration. Interestingly, cells switch between these 2 migration modes according to the elastic properties of the 3D environment, which involve RhoA-ROCK-myosin II signalling [9]. Here we observe elongated cells in SW with less lamellipodia than cells on 2D and without lateral blebs (movie 1, figure 2 and figure 4), which corresponds to migration in a lamellipodia-based mode. However, cells display only 1-2 protrusions at the leading edge, which might suggest a new mode of migration mode for cells within SW environments intermediate inbetween lamellipodia and lobopodia-based migration (figure 4). This is likely a consequence of the environment provided by the sandwich system. Likewise, changing the inputs coming from the sandwich environment could lead to different cell migration (as in 3D systems [9]) similarly as it triggers differences in cell morphology, adhesion and differentiation [25-27, 39].

ECM degradation is another key step for 3D cell migration [11, 9], as cells must literally open spaces through the fibrillar mesh of proteins that constitute their environment. We have investigated the expression of matrix metalloproteinases (MMPs) – the main enzymes secreted by cells to degrade the ECM – and focused on MMP2 and MMP13, that are known to degrade FN [44, 45]. However, no differences were found for MMP2 and MMP13 on 2D and SW cultures (figure 3).

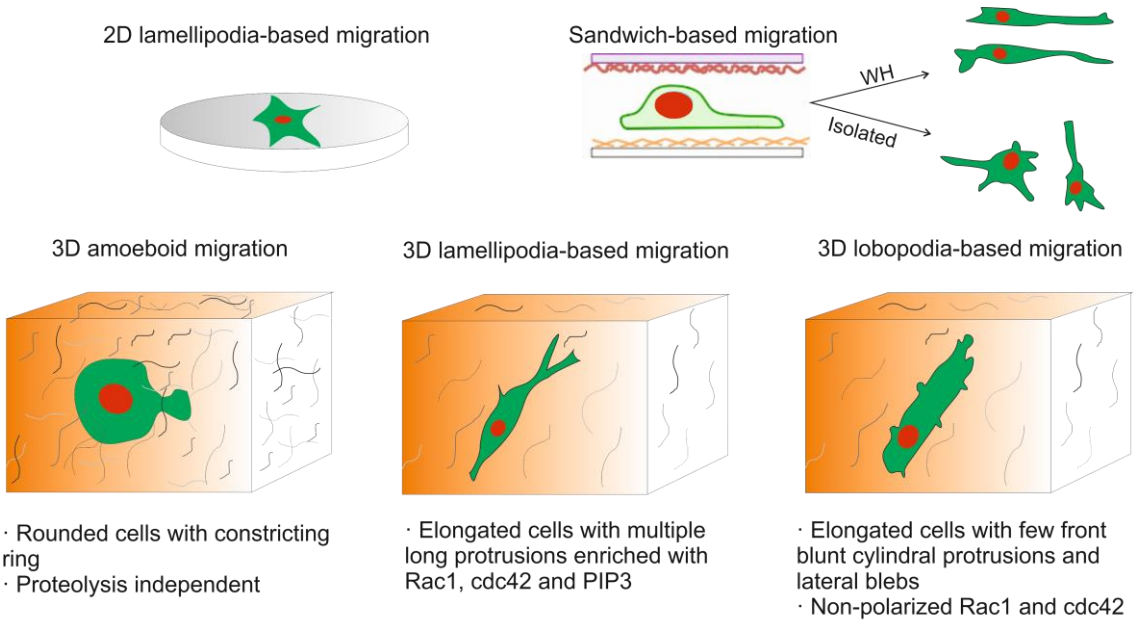

Figure 4. Schematic representation of cell migration modes in 2D, 3D and sandwich culture. Differences in the migration mode are due to dimensionality, cell lineage and ECM properties.

Ventral ECM remodeling during cell migration

It has been found that fibroblasts migrating in the lamellipodia mode remodel their matrix but those in the lobopodia mode do not [9]. Hence, we investigated the role of

SW environments in ECM remodeling. The fate of ventral FN was studied on FN-coated glass (2D) overlaid with a dorsal FN-coated PLLA film (SW). As a positive control cells were cultured with growth medium (10% FBS) as this triggers ECM reorganization [46]. It is important to remark here that for the rest of conditions cells were cultured in serum-free medium, so that the sole influence of the sandwich enviroment on cell reorganization was considered. Figure 5 shows that, regardless the migration model used (wound healing or isolated cells), FN was not remodeled on 2D substrates whilst cells formed new FN fibrils within sandwich cultures. Dorsal stimuli did therefore trigger this ECM remodeling, as reported for FN and collagen in 3D matrices [9, 47]. In order to better understand this phenomenon, we cultured cells in SW under different dorsal conditions by coating with different proteins (vitronectin, bovine serum albumin and un-coated; SW^{VN}, SW^{BSA} and $SW^{Ø}$ respectively). As controls we used 2D samples where the corresponding dorsal protein was included in the culture medium, so that dorsal receptors were biologically but not mechanically stimulated. As summarised in table 3, FN reorganization only occurred in sandwich cultures regardless of the protein coating used, even occurring when the dorsal PLLA was coated with BSA - SW^{BSA} (a non-adhesive protein) - or left uncoated ($SW^{Ø}$). Hence the mechanical dorsal input has a key role to trigger FN reorganization and determine cell fate (figure 5 and table 3).

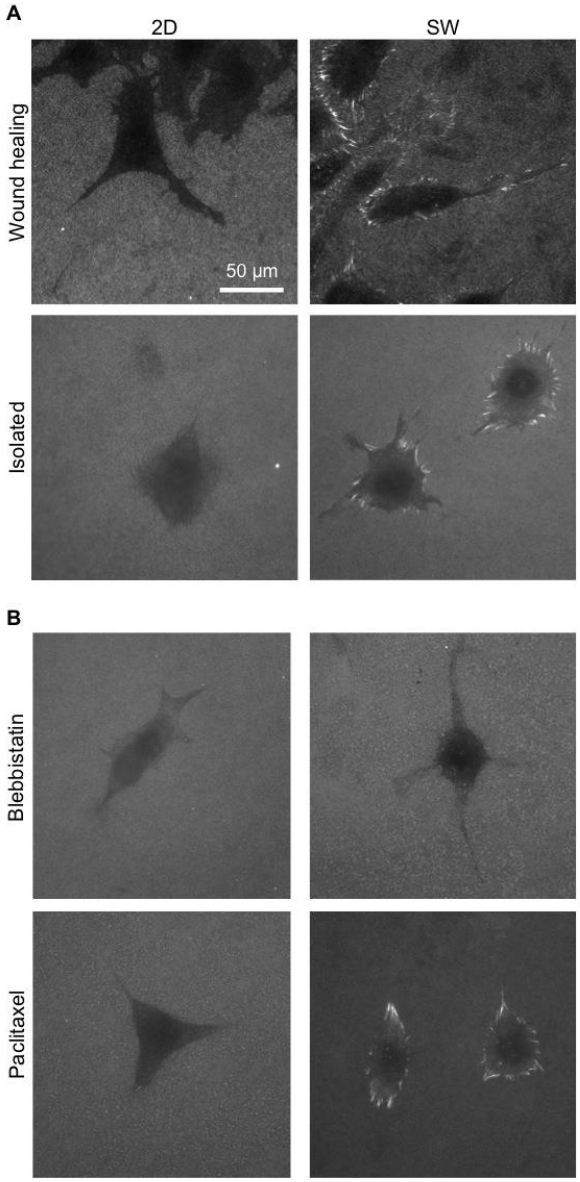

Figure 5. Ventral FN reorganization. (A) Sandwich culture triggers ventral FN reorganization by forming new fibrils both in isolated and wound healing cultures. (B) Cell contractility is needed to reorganize FN within the sandwich culture.

Cell contractility is known to influence cell migration [9, 48, 49] via phosphorylation of myosin light chain (MLC) so we examined whether this might also be related to the ECM reorganization process triggered by SW environments. To do so cells were cultured in the presence of pharmacological inhibitors that impair contractility such as

the Rho/ROCK pathway inhibitor Y-27632 and Blebbistatin, a specific inhibitor of myosin II activity [50, 51]. In addition, cells were also cultured in the presence of Paclitaxel that stabilizes microtubules thus showing an opposite effect [52]. Contractility inhibitors (Y-27632 and Blebbistatin) impaired the ventral FN reorganization within sandwich cultures whilst Paclitaxel enhanced it (figure 5B and table 3). Cell contractility is therefore needed to reorganize FN within the sandwich culture. Sandwich culture might therefore enhance cell contractility to enable the reorganization of ventral FN.

Table 3. Summary of the ventral FN reorganization. + stands for reorganization, - for no reorganization and * for the halo seen on PEA (see next section).

	2D	SW
Growth medium (+C)	+	+
Medium w/o serum	-	+
Blebbistatin/Y-27632	-	-
Paclitaxel	-	+
$SW^{FN/VN/BSA/Ø}$		+
FN/VN/BSA dissolved in medium	-	
Ventral PEA/PMA	-*	-

Role of the ventral substrate in cell migration

Next, we wanted to address if the elongated morphology and migration rates found for migrating cells within SW environments might be tuned by using different materials as ventral substrates. We used: (i) FN-coated glass, where FN adsorbs loosely in a globular conformation that can be easily reorganized by cells [53], (ii) spin-coated poly(ethyl acrylate) (PEA) on which FN assembles spontaneously into fibrillar (nano)networks [54, 55] and (iii) spin-coated poly(methyl acrylate) (PMA) on which FN adopts a globular conformation (figure 6) [56]. Figure 6A shows the cellular reorganization of adsorbed FN after 5 h of culture (in growth media) on the different 2D substrates (chemical structures shown in figure 6B). As expected, new FN fibrils were only formed on glass and PLLA (white arrows) as cells are unable to reorganize FN on PEA nor PMA due to the strength of the protein-material interactions [57]. Additionally we addressed whether the ECM reorganization triggered by the dorsal stimuli in SW cultures (using glass as ventral substrate) was also observed on PEA and PMA in the absence of serum. Cells did not reorganize ventral FN on these surfaces, which suggests that the dorsal stimulus is not strong enough to overcome the protein-material interaction (table 3). Besides, FN showed a halo around cells when cultured on 2D PEA substrates. This phenomenon has been further investigated and correlated with the strength of FN-PEA interactions [58].

After assessing that removal of the culture insert did not alter the characteristic FN adsorption and distribution on PEA and PMA (network and globular-like conformation respectively; figure 6D), cell migration on the different materials was followed using the wound healing assay. Gap closure, defined as the percentage of the initially void

surface that is colonized by cells, is shown in figure 6E. Polygonal cells were observed on every 2D substrate whilst cells adopted an elongated morphology when cultured under sandwich culture regardless of the material used. On the other hand, differences in cell migration were clearly observed between 2D and SW cultures for the same ventral substrate. Cells migrated longer distances on 2D substrates than within the SW environment, which increased gap closure (figure 6E). Finally, no differences in gap closure were oberved for 2D samples but, strikingly, significant differences were observed for the different SW cultures: gap closure occurred more efficiently using PMA than glass or PEA, which suggests that FN conformation (globular on PMA and fibrillar structures on PEA) and the strength of FN interaction with the material surface play a key role in cell migration (figure 6E). Note that these differences appear only within SW environments but not for the 2D control, which stresses the role of dimensionality in cell migration.

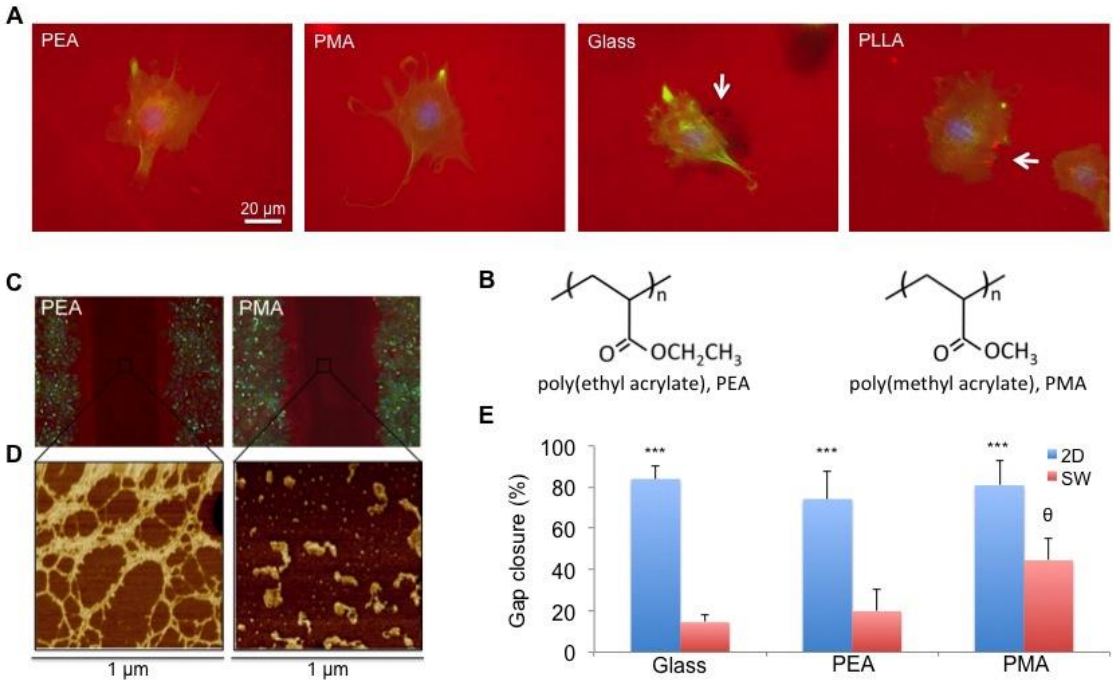

Figure 6. Cell migration using different ventral FN-coated surfaces. (A) Fibronectin (red) is reorganized on 2D glass and PLLA but not on PEA and PMA. Actin cytoskeleton is shown in green and nuclei in blue. White arrows show the formation of newly cell-reorganized FN fibrils. (B) Chemical structure of PEA and PMA. (C) Fluorescence images show the FN-coated gap (500 μm) between the two cell populations after removing the insert used for the wound healing assay. Actin cytoskeleton (green), nuclei (blue) and FN (red) are shown. (D) AFM images of FN after insert dettachment to confirm that FN distribution is not altered on the migrating area. (E) Gap closure after 24 h of wound healing assays using glass, PEA and PMA as ventral substrates. * shows significance comparing conditions with similar ventral substrate; and θ when comparing results of the same dimensionality (2D or SW).

Role of dorsal stimuli in cell migration

We next examined the role of dorsal stimuli in cell migration. To do so we used FN coated glass as ventral substrate and as dorsal substrate (i) PLLA coated with different proteins (FN or VN) to address the role of integrin-mediated interactions throughout different proteins and (ii) bare surfaces (Ø) to address the effect of pure mechanical stimuli ($SW^{Ø}$ might not induce any dorsal biological interaction but a response due to the bare surface).

As observed previously, cells adopted polygonal morphology on 2D substrates and elongated ones within sandwiched cultures. No differences in either gap closure or cell

morphology were observed regardless of the use of dorsal FN or VN coatings. Strikingly, when overlaid by a non-adhesive dorsal substrate ($SW^{Ø}$), cells maintained the characteristic elongated morphology found in SW cultures but migrated more effectively (figure 7 and movie 2). So, even when the SW provided a pure mechanical input (no protein coating), cell migration greatly differed. This observation, together with results included in figure 2 and previous works [25], suggests an important role for the dorsal mechanical input in cell behavior. Hence, we hypothesize that cell migration is increased under this condition ($SW^{Ø}$; both for isolated cells and wound healing assay) compared with SW^{FN} and SW^{VN} due to the interactions with the extra (dorsal) layer of proteins in the latter cases, and the regulation of focal adhesion formation and turnover on both dorsal and ventral substrates.

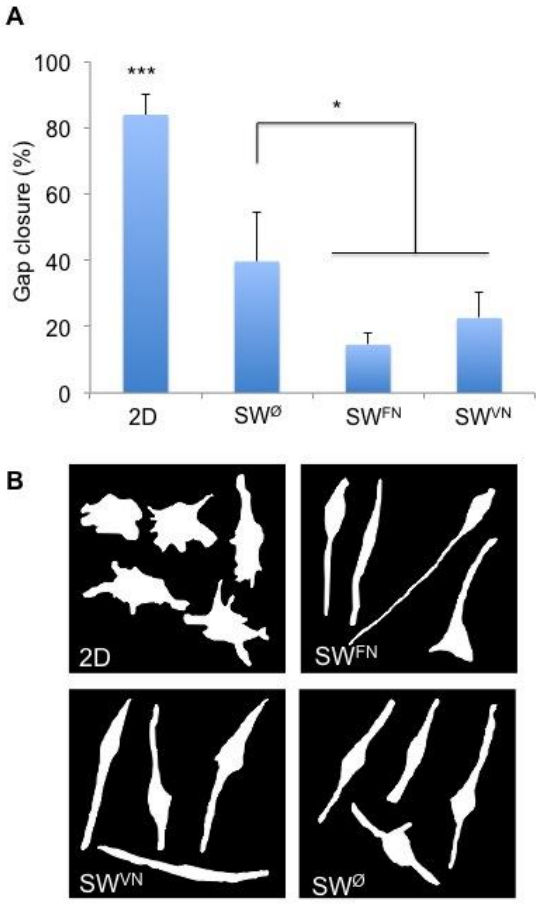

Figure 7. Wound healing assay under different dorsal stimuli (2D and SW cultures) (A) Migration rates after 24 hours of culture using FN-coated glass as ventral substrate and bare PLLA ($SW^{Ø}$) or coated with either FN or VN (SW^{FN} and SW^{VN} respetively) as dorsal substrate. (B) Representative cell morphology of migrating cells for each condition.

Executive summary

Background

- Cell migration is an essential process *in vivo*
- Robust culture systems mimicking the *in vivo* environment are needed

Results

- Sandwich culture offers a closer 3D environment and is a versatile system to mimic different environments
- Cells sense and respond to the different ventral and dorsal stimuli in terms of morphology, adhesion, ECM remodeling and migration rate
- Migration rates can be controlled by the ventral and dorsal stimuli provided by sandwich environments

Future perspective

Cell migration is an essential process in many physiological and pathological conditions. Providing novel tools to understand this process is therefore essential. Thus, future work should develop more powerful and biomimetic systems and technologies to investigate this process in *in vivo*-like conditions.

Conclusions

We have shown sandwich culture as a tool to investigate cell migration in a closer 3D environment than the traditional 2D substrates. The versatility of this system allows the study of cell fate under a wide spectrum of well-controlled conditions to better understand cell behavior within 3D cultures such as stacked layers of cells or hydrogels. Overall, cell morphology was highly influenced by the type of culture whereas cell migration was determined by the inputs coming from both the ventral and dorsal substrate. Furthermore, FN was reorganized in new fibrils when cells were dorsally stimulated within the sandwich culture, showing that the dorsal excitation triggers different signaling compared to 2D conditions. Our results suggest that both biological and mechanical stimuli play an important role in cell migration.

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