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Fibronectin-matrix sandwich-like microenvironments to manipulate cell fate

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Conventional 2D substrates fail to represent the natural environment of cells surrounded by the 3D extracellular matrix (ECM). We have proposed sandwich-like microenvironments as a versatile tool to study cell behaviour under quasi-3D conditions. This is a system that provides a broad range of dorsal and ventral independent spatio-temporal stimuli. Here, we use this sandwich technology to address the role of dorsal stimuli in cell adhesion, cell proliferation and ECM reorganisation. Under certain conditions, dorsal stimuli within sandwich microenvironments prevent the formation of focal plaques as well as the development of the actin cytoskeleton, whereas α_5 *versus* α_v integrin expression is increased compared to the corresponding 2D controls. Cell signaling is similarly enhanced after dorsal stimuli (measured by the pFAK/FAK level) for cells sandwiched after 3 h of 2D ventral adhesion, but not when sandwiched immediately after cell seeding (similar levels to the 2D control). Cell proliferation, studied by the 5-bromo-2-deoxyuridine (BrdU) incorporation assay, was significantly reduced within sandwich conditions as compared to 2D substrates. In addition, these results were found to depend on the ability of cells to reorganise the dorsal layer of proteins at the material interface, which could be tuned by adsorbing FN on material surfaces that results in a qualitatively different conformation and distribution of FN. Overall, sandwich-like microenvironments switch cell behaviour (cell adhesion, morphology and proliferation) towards 3D-like patterns, demonstrating the importance of this versatile, simple and robust approach to mimic cell microenvironments *in vivo*.

1 Introduction

Cells within tissues are surrounded by the extracellular matrix (ECM), a three-dimensional (3D) complex fibrous matrix that provides mechanical support as well as key biochemical and biophysical signals to direct cell fate in multicellular organisms.[1–3] Standard two-dimensional (2D) culture involves mechanical, physical and biochemical signals that differ dramatically from the physiological ECM. Cell behaviour is then altered under these non-physiological conditions.[4,5] Hence, approaches have been developed to study a variety of cellular physiological and pathological processes (*e.g.*, matrix secretion, cell differentiation, cell migration, morphogenesis, cancer research and drug development) in more physiological-like 3D environments.[6–10] These strategies include complex systems such as cell aggregates, 3D scaffolds, gels and cell multilayers that involve a broad range of variables and do not allow one to deconstruct the origin of the dimensionality conflict (2D *vs.* 3D).

Cell–matrix interactions differ from 2D and 3D environments. On flat substrates, cells adhere using only the ventral plane, while *in vivo* cells interact with the surrounding environment in a three-dimensional configuration.[11,12] The initial cell–matrix interaction includes cell adhesion, and absolutely determines cell fate in the long term,[13,14] which is important in the design of tissue engineering strategies, as well as for other biomedical fields (*e.g.* cancer research).

We have recently hypothesised that excitation of ventral and dorsal receptors using a sandwich-based culture system is a versatile tool to provide a broad range of dorsal and ventral independent spatio-temporal cues under quasi-3D conditions (Fig. 1).[15,16] This study investigates cell interactions in sandwich-like microenvironments. Within 3D environments, cell adhesion is a $\alpha_5\beta_1$ integrin-dependent process stimulated by the presence of FN fibrils within the ECM.[11,17,18] To mimic this, we have used poly(ethyl acrylate) (PEA) as one of the material components of the sandwich system, as this is able to direct the organization of FN into physiological-like fibrils upon simple adsorption in a process called material-driven FN fibrillogenesis.[14,19,20] In addition, we have included three other dorsal material–protein combinations seeking to alter the ability of cells to reorganise fibronectin, to correlate with cell morphology/adhesion. We have studied focal adhesion assembly, the development of the actin cytoskeleton, integrin

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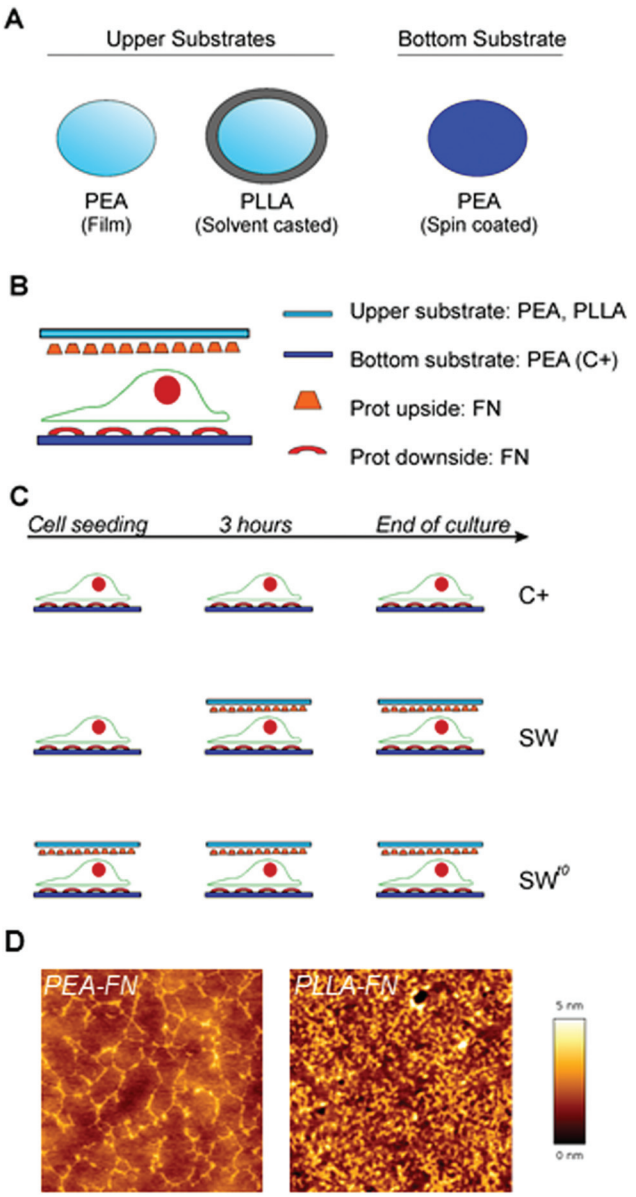

Fig. 1 Sandwich-like culture. (A) Representation of the upper (film of PEA and solvent-casted PLLA) and the bottom substrates (spin-coated PEA) used in the sandwich-like culture. (B) Sketch of the sandwich-like model and the different combination of materials and proteins. (C) Timing diagram showing the cell culture procedure and the nomenclature for every condition. (D) Fibronectin distribution on the material surfaces as observed by AFM after adsorption from a solution of concentration 20 μg ml^{-1}.

expression, the phosphorylation of focal adhesion kinase (pFAK) and cell proliferation *via* the 5-bromo-2-deoxyuridine (BrdU) incorporation assay for different sandwich conditions.

2 Materials and methods

2.1 Materials

Polymer films of ethyl acrylate (EA) (Sigma-Aldrich, Steinheim, Germany) were obtained by radical polymerization of a solution of EA using 0.2 wt% benzoin (98% pure, Scharlau, Barcelona, Spain) as a photoinitiator. The polymerization was carried out up to limiting conversion. Rounded samples were cut from the polymerized film in order to be used as the top substrates of the sandwich (Fig. 1A). The day before cell culture, rounded poly(ethyl acrylate) films were washed in an ultrasonic bath for 5 min, sterilized by UV exposure for 30 min and hydrated overnight in Dulbecco's phosphate buffered saline (DPBS, Invitrogen).

PEA was spin-coated (Brewer Science, Rolla, USA) to be used as ventral substrates (Fig. 1B). PEA was dissolved in toluene at a concentration of 2.5 wt% and deposited on glass coverslips at 2000 rpm for 30 s. Samples were dried under vacuum before use.

Poly(lactic acid) films (PLLA) were prepared by casting a solution of 2% PLLA in chloroform (Scharlau) in stainless steel washers. Films were then thermally treated at 200 °C for 5 min to obtain a transparent and fully amorphous film.

2.2 Protein adsorption

Fibronectin (FN) from human plasma (Sigma) at 20 μg mL^{-1} in DPBS was adsorbed on the different substrates by immersing the sample in the protein solution for 1 h. After adsorption, samples were rinsed in DPBS to eliminate the non-adsorbed protein.

2.3 Atomic force microscopy

Atomic force microscopy (AFM) was performed on a JPK Nanowizard 3 BioScience AFM (JPK, Germany) operating in the AC mode; the SPM and DP 4.2 software version was used for image processing and analysis. Si-cantilevers from Nanoworld AG (Switzerland) were used with a force constant of 2.8 N m^{-1} and a resonance frequency of 75 kHz. 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.

2.4 Cell culture

NIH3T3 fibroblasts (European Collection of Cell Cultures) were maintained in DMEM medium. Before cell culture, both top and bottom substrates were UV-sterilized for 30 min and then coated with FN as explained before. Sandwich cultures were established as described elsewhere.[15] Briefly, 7000 cells per cm^2 were seeded under serum free conditions on bottom substrates. Afterwards the upper substrate was gently laid over the bottom substrate either immediately (SW^{t0}) or after 3 h of culture (SW) (see Fig. 1C). A highly concentrated cellular suspension was used to avoid cell loss after laying the upper substrate (for SW^{t0}). Likewise, for the SW condition, excess of medium on the bottom surface was removed before laying the upper substrate. After assembling the sandwich, a gentle pressure of approx. 10^3 Pa was applied for 3 min on the top surface to facilitate the initial stability of the system. Finally, pressure was released and the medium replenished. Sandwich-like cultures were then maintained at 37 °C under a humidified atmosphere under 5% CO_2 for 6 h unless otherwise noted.

For longer cultures the medium was replaced by a serum-containing medium after 3 h of culture.

For experiments including dorsal activation of cells seeded on 2D substrates, blocking antibodies or fibronectin were added after 5 min (t_0) or 3 h of culture. Anti-α_5 integrin antibody (CD 49e, clone SAM-01, Immunotech) was used at 1.2 μg ml^{-1} and FN at 50 μg ml^{-1}.

2.5 Image processing

All of the image processing and analysis was done using Adobe Photoshop CS5 and ImageJ as commented elsewhere.[15] Cell area and circularity ($4\pi \times$ area/perimeter^2), which corresponds to a value of 1 for a perfect circle, were quantified using ImageJ software. $n \geq 25$ cells for each condition.

2.6 Immunofluorescence

Immunofluorescence was carried out in sandwich cultures without "de-sandwiching" the constructs to preserve cell morphology. For cell adhesion studies, cells were washed in DPBS and fixed in 10% formalin solution (Sigma) at 4 °C for 30 min. Samples were then rinsed, permeabilised (saccharose 0.3 M, NaCl (Sigma) 50 mM, $MgCl_2$ hexahydrate (Scharlab) 3 mM, Hepes (Sigma) 20 mM and Triton X-100 (Sigma) 0.5%) for 5 minutes and washed with DPBS. Then samples were incubated in DPBS/1% BSA to reduce the background signal. Cells were then incubated with anti-vinculin antibody (Sigma) diluted 1/400 for 1 hour. Samples were then rinsed in DPBS/0.5% Tween 20, followed by incubation with Cy3 conjugated secondary antibody (Jackson Immunoresearch) diluted 1/200, BODIPY FL phallacidin (Molecular probes) diluted 1/100 and DAPI.

FN reorganisation was studied after 5 hours of culture on PEA and PLLA spin coated samples. Samples were coated with FN and NIH3T3 fibroblasts seeded in DMEM containing 10% FBS. Once the culture was finished, cells were fixed with 10% formalin. Then samples were washed with DPBS and cells were permeabilised for 5 min at room temperature. Afterwards, samples were blocked in DPBS/1% BSA and incubated with HFN7.1 antibody (Developmental Studies Hybridoma Bank), which only recognises human FN. After 1 hour of incubation, samples were rinsed in DPBS/0.5% Tween 20, and then incubated in Cy3-conjugated secondary antibody (Jackson Immunoresearch, 1/400), BODIPY FL phallacidin and DAPI. Heat maps were performed using an external plug-in of ImageJ (HeatMap Histogram).

2.7 Western blotting

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). Subsequently proteins were concentrated using Microcon YM-30 Centrifugal Filter devices (Millipore) as the manufacturer described.

To determine FAKs and its phosphorylated forms at Tyr397 (pFAK), as well as α_v and α_5 integrin subunits, samples were subjected to 7% SDS-PAGE gel electrophoresis. Proteins were then transferred to a positively charged polyvinylidene difluoride nylon membrane (PVDF, GE Healthcare) using a semidry transfer cell system (Biorad) and blocked by immersion in 5% skimmed milk in PBS for 30 minutes at room temperature. The blot was then incubated with primary antibodies in PBS containing 0.1% Tween 20 and 2% skimmed milk (anti-FAK antibody (Upstate) and anti-pFAK antibody (Millipore) diluted 1 : 2500; anti-α_v integrin (Millipore) diluted 1/1000 and anti-α_5 integrin (Santa Cruz Biotechnology) diluted 1/100). After several washes with PBS/0.1% Tween 20, the blot was incubated in horseradish peroxidase-conjugated antibody (GE Healthcare) in PBS containing 0.1% Tween 20 and 2% milk for 1 hour at room temperature. After several washes with PBS containing 0.1% Tween 20 and 2% milk, immunoreactive bands were visualized using Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

2.8 Proliferation assay

Cell proliferation was evaluated based on the measurement of 5-bromodeoxyuridine (BrdU, Sigma-Aldrich) uptake during DNA synthesis in proliferating cells. 10 μg mL^{-1} of BrdU was added to the medium after 2 h of cell seeding. After 6 or 24 h of culture, cells were fixed with 10% formalin solution (Sigma-Aldrich) for 30 min at 4 °C and then washed three times in PBS. DNA was then denatured by incubating in 1 N HCl at 65 °C for 15 min with agitation. After washing three times in neutralizing buffer (50 mM NaCl, 100 mM Tris-HCl, pH 7.4) for 20 min each and twice in PBS, cells were blocked with 1% BSA and 5% FBS in DPBS at room temperature for 10 min. After that, cells were incubated with mouse monoclonal antibody against BrdU (Sigma) diluted 1 : 1000 at 37 °C for 1 h and then washed twice with PBS. Cells were then incubated with Cy3 conjugated secondary antibody (Jackson Immunoresearch) diluted 1/200 and DAPI (Sigma-Aldrich) at room temperature for 1 h. After further washing in PBS, cells were mounted and observed under fluorescent microscopy (Nikon Eclipse 80i).

2.9 Statistical analysis

Results are shown as average ± standard deviation. All experiments were performed in triplicate. Results were analyzed by one-way ANOVA. If treatment level differences were determined to be significant, a Tukey's *post hoc* test was performed.

3 Results and discussion

Cells are surrounded by a 3D extracellular matrix (ECM) that consists of interconnected FN fibrils assembled through an integrin mediated FN-fibrillogenesis process.[11,17] To mimic this environment we have used a sandwich culture system that provides dorsal and ventral FN (nano)networks organised at the material interface. This material-driven FN fibrillogenesis is a process biomimetic with the naturally assembled ECM that occurs in certain chemistries, *e.g.* poly(ethyl acrylate) PEA.[14,19,20] To do so, we have sandwiched cells in between two biointerfaces that consist of FN fibrils assembled on PEA at

the material interface (we will use FN-matrix to unequivocally identify this condition). Only when we wanted to compare the effect of the FN nano(network) with more conventional states of FN on biomaterials (globular-like) we have changed the dorsal substrate to PLLA (Fig. 1A and D).

3.1 Focal adhesion formation and cytoskeleton organization in FN-matrix sandwich microenvironments

Notwithstanding controversial results and discussions,[21,22] it is well accepted that cells both *in vivo* and within 3D *in vitro* cultures do not develop focal adhesions as they do on 2D substrates. Rather, cells organise randomly distributed 3D matrix adhesions with increased intra-cytoplasmatic labeling,[23] different molecular composition and broader distribution of sizes,[11,24] revealing the key role of dimensionality on cell adhesion and signaling.[25]

We have previously observed good cell viability, significant increase in myogenic differentiation[16] as well as altered cell morphology within sandwich cultures.[15] In this work we have used poly(ethyl acrylate) (PEA) as a ventral component of the sandwich system as it is a material that drives the organization of FN into physiological-like fibrils[14,19,20] and provide a ECM-mimetic environment, while a variety of dorsal stimuli (material/protein) have been considered to assess the influence on cell behavior (Fig. 1). Sandwich cultures were performed immediately after cell seeding (SW^{t0}) or after 3 hours of 2D culture (SW) (Fig. 1C).

Fig. 2 shows the formation of focal adhesion plaques and the development of the actin cytoskeleton under different sandwiching conditions. As expected, mature actin fibers inserted into well-defined focal adhesions (vinculin) were found on the 2D controls, the ventral (spin casted PEA; C+) and the dorsal (film of PEA) one. In contrast, vinculin is not organized into focal plaques within sandwich cultures but remains homogeneously distributed throughout the cytoplasm. Accordingly, the actin cytoskeleton is not completely developed and only a peripheral staining is shown, which is characteristic of early stages of actin polymerization[26] and reported for other 3D systems.[23,27] Poor cell adhesion is then correlated to the altered cell morphology observed under these sandwich conditions[15] –significantly less spread cells–, especially when sandwiching immediately after seeding, SW^{t0} (Fig. 2), as reported for 3D matrices both *in vitro* and *in vivo*.[15,24,25]

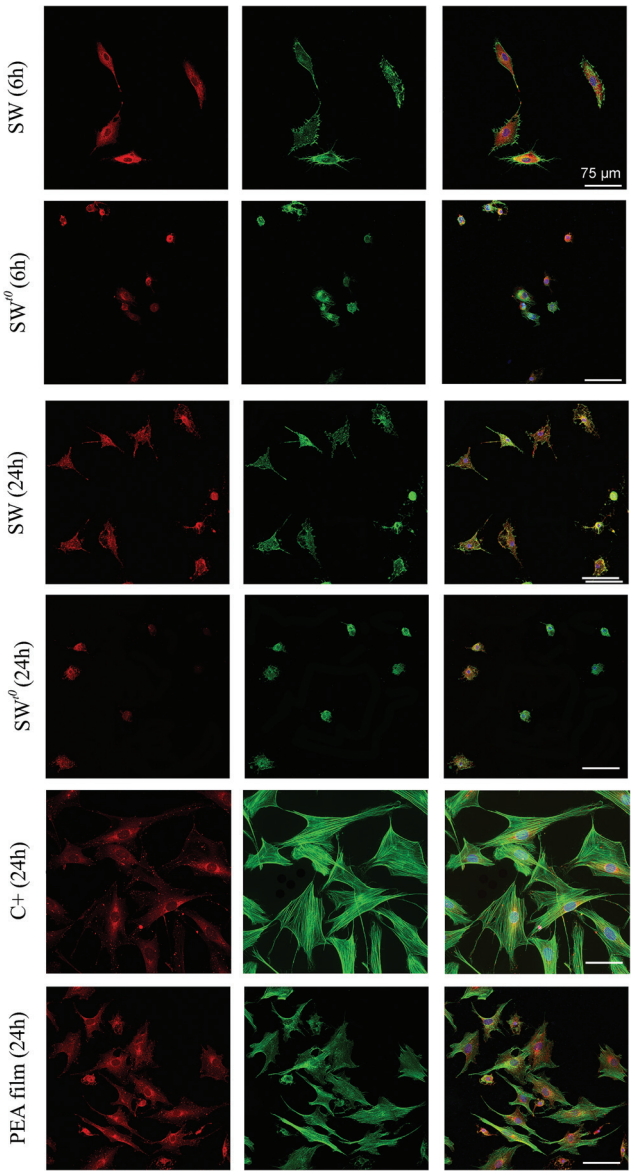

Fig. 2 Cytoskeleton organisation and focal adhesion formation on 2D controls and sandwich cultures (with both dorsal and ventral interfaces consisting of FN fibrils assembled on PEA). Differences in cell morphology can be clearly perceived. Cells were cultured within sandwiches for 6 and 24 hours. Fluorescence images show vinculin (red), F-actin cytoskeleton (green) and nuclei (blue).

3.2 FN-matrix sandwich microenvironments trigger $\alpha_5\beta_1$ integrin-mediated adhesion and cell signaling

Cell adhesion to biomaterials takes place primarily by anchorage of integrins – a family of transmembrane cell adhesion receptors – to adsorbed proteins at the material interface. $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins are the main FN receptors,[28] so we quantified α_5 and α_v integrin subunits for different sandwich conditions. As shown in Fig. 3, higher α_5 levels were found for cells within sandwich conditions, which suggest altered cell adhesion compared to the equivalent 2D culture. Although the highest α_5 level was for cells dorsally and ventrally stimulated from the beginning of the culture (SW^{t0}), it is remarkable that further increase in integrin expression occurs if cells are dorsally stimulated after 3 h of ventral adhesion (SW) (Fig. 3A and B). Differences in integrin expression between SW and SW^{t0} might be sought in the duration of the dorsal stimuli and/or the existence of a "ventral memory" for SW conditions (3h-2D + 3h-sandwich). However, higher α_5 integrin levels within sandwich cultures are not merely a consequence of the higher surface available for cell contact (dorsal and ventral), as no differences in α_v integrin were detected (Fig. 3). Hence this result supports the claim that cells behave in sandwich-like

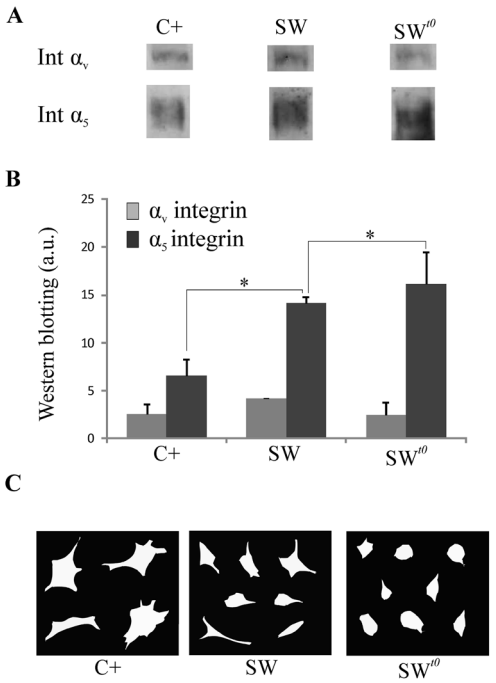

Fig. 3 Integrin quantification for α_5 and α_v subunits, major fibronectin receptors, under different conditions after 6 h of culture. (A) Representative western blot bands for α_5 and α_v integrin subunits. (B) Quantification of α_v and α_5 integrin subunits. (C) Representative outlined cells from contrast phase microscopy for each one of the conditions. * Stands for $p < 0.05$.

systems closer to 3D environments, where cell adhesion has been shown to be mostly $\alpha_5\beta_1$ dependent.[11,29]

After ligand binding, integrins cluster and develop focal adhesion complexes that anchor cells to the material surface. These complexes link the extracellular matrix with the actin cytoskeleton and direct the subsequent cellular response by triggering the phosphorylation of focal adhesion kinases (FAKs). FAK is a key signaling protein contributing to integrin control of cell motility, invasion, survival, and proliferation (see Fig. 4D).[30] As a consequence FAK has been explored as a target molecule in regenerative therapies and pathological processes (*e.g.* cancer research).[31] Here we address whether FAK phosphorylation is altered by the dorsal stimuli within the sandwich microenvironments. Fig. 4 shows that phosphorylation of FAK is enhanced when dorsal stimuli are applied after 3 h of ventral adhesion (SW) compared to both the simultaneous sandwiching (SW^{t0}) and 2D controls. These results reveal the difference between the simultaneous (SW^{t0}) and sequential (after 3 h of 2D culture, SW) stimulation of dorsal and ventral receptors. It is interesting to note that when the level of FAK phosphorylation is normalized relative to α_5 integrin expression, the value obtained is lower within the sandwich conditions compared to the equivalent 2D system, which suggests that even if α_5 level is higher within sandwich systems (Fig. 3), these integrins are less effective in phosphorylating FAK (Fig. 4C) than in a 2D culture based on the same material–protein interface. It cannot be disregarded that this

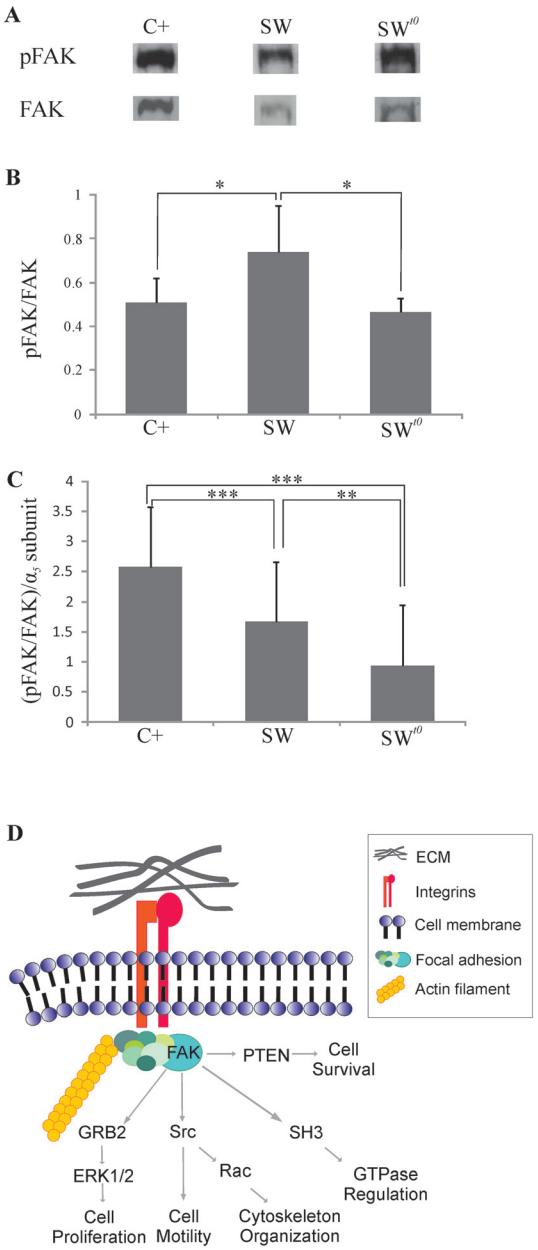

Fig. 4 FAKs phosphorylation under different culture conditions. (A) Representative western blot for phosphorylated tyrosine-397 residue on FAK and total FAK (pFAK and FAK respectively). (B) Quantification of the fraction of phosphorylated FAKs by image analysis of the western blot bands. (C) Normalised FAK activation relative to α_5 integrin subunit expression. (D) Schematic FAK pathways involving cell adhesion and cell fate. Error bars represent the standard deviation of three independent experiments. * Stands for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

higher phosphorylation of FAK for SW (Fig. 4B) is related to other activation processes such as growth factor receptor signalling or other β_1 integrins able to bind FN. However, our experiments were performed under serum-free conditions (so without added growth factors) and it is well documented that $\alpha_5\beta_1$ is the main FN receptor. This result is also supported by the less effective integrin clustering into focal adhesions observed in these sandwiched systems (Fig. 2).

3.3 Cell proliferation in FN-matrix sandwich microenvironments

FAK signalling is a key pathway linking cell adhesion to cell proliferation, cell motility and cytoskeleton reorganization (Fig. 4D). The assembly of the actin cytoskeleton is altered within FN-matrix sandwich microenvironments (Fig. 2). We addressed cell proliferation after 6 and 24 h of culture. As can be seen in Fig. 5A, cell proliferation was similar for both 2D and sandwich microenvironments after 6 h of culture, but significantly lower within sandwich conditions after 24 h, as reported to occur *in vivo*.[32] Smaller but significant differences can be also observed after 24 hours depending on whether the sandwich was assembled immediately (SW^{t0}) or after 3 h of culture (SW).

To clarify the role of dorsal adhesion in cell proliferation, the integrin binding site of fibronectin was blocked (with HFN7.1 antibody) on the dorsal substrate before sandwiching. HFN7.1 binds to the flexible linker between the 9th and 10th type III repeats of FN where the RGD and synergy sequences are located.[33] Fig. 5B shows a slight increase in cell proliferation after blocking (SW^{t0} +HFN7.1) that is not statistically significant in SW environments (3 h dorsal + 3 h ventral). Blocking RGD domains of dorsal FN – and therefore limiting dorsal integrin binding[34] – does not restore cell proliferation to the levels observed in 2D cultures. In addition, had this happened, cell morphology should be the same for cells within the sandwiches and the 2D controls, which is not the case (Fig. 5C). The same behaviour had been observed using serum albumin – a non-adhesive protein – as a dorsal protein coating.[15] This suggests that altered proliferation within sandwich cultures is not directly a consequence of $\alpha_5\beta_1$ integrin-mediated adhesion with the dorsal substrate.

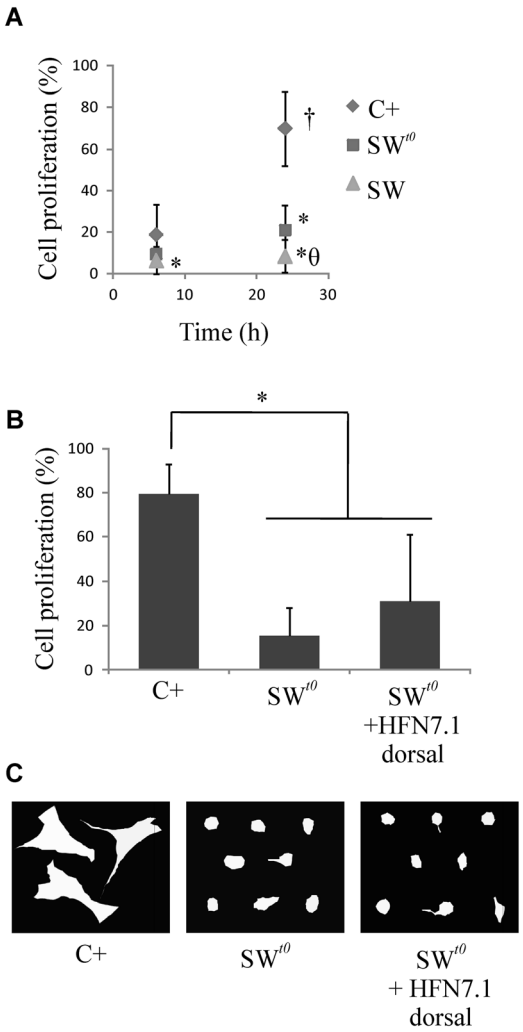

Fig. 5 Cell proliferation within sandwich cultures. (A) Cell proliferation on 2D substrates and sandwich cultures assembled immediately after cell seeding (SW^{t0}) or 3 hour later (SW). † Stands for significant difference with time, * differences with the control (same time) and θ differences with the sandwich assembled immediately after cell seeding. (B) Cell proliferation for culture conditions where HFN7.1 antibody was used to block dorsal FN cell adhesion domain so that cells cannot use $\alpha_5\beta_1$ integrin to interact with the dorsal substrate. Total culture time 24 h. * Stands for $p < 0.05$. (C) Representative outlined cells for each one of the conditions.

3.4 Effect of dorsal fibronectin reorganisation in sandwich-like microenvironments

In the first part of this work, we have sandwiched cells using FN coated PEA, so that the interface consisted of a physiological-like FN network driven by FN–material interactions.[19] In previous studies, we observed that cells cannot mechanically remodel (reorganise) this material-driven FN network (on 2D substrates) due to the strength of the protein–material interaction.[35] As a consequence, cells cultured on 2D PEA substrates, unable to reorganise FN, showed increased proteolytic activity and remodelled the provisional matrix by increasing the expression of MMPs.[36] Consequences in sandwich culture might be more severe as cells are not able to spread properly and remain "frozen" in the attained morphology (rounded and smaller for SW^{t0}; smaller for SW) resembling non-degradable 3D hydrogels.[15] As this was in fact observed using PEA as a dorsal stimulus, we wanted to inquire about the influence of dorsal protein remodelling on cell adhesion, morphology and proliferation in sandwich cultures.

To this end, we have performed sandwich experiments using PLLA as the dorsal substrate, on which FN adopts a globular conformation (Fig. 1D). This globular FN at the PLLA interface can be mechanically reorganised upon cell adhesion due to the lower intensity of the protein–material interaction. Fig. 6 shows cellular reorganisation of adsorbed FN after 5 h of culture on the different 2D surfaces. It is clearly observed that cells are able to reorganise FN on PLLA as revealed by the dark areas observed nearby the cell that lead to the formation of fibronectin fibrils (brighter areas pointed out by arrows). As expected, this does not happen on FN-matrix assembled on PEA.[35] Fig. 7 and 8 show that cells sandwiched with FN coated PLLA adopted a morphology similar to that observed for the 2D controls (data for PEA have been included in Fig. 8 to facilitate reading and are extracted from ref. 15). To further

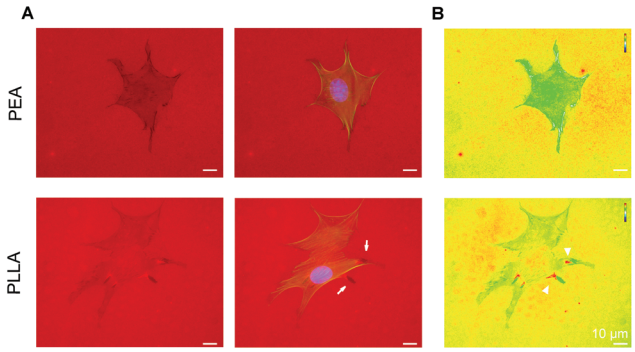

Fig. 6 Fibronectin reorganisation on 2D PEA and PLLA spin coated samples after 5 hours of culture. (A) Fluorescence staining for fibronectin (red), actin cytoskeleton (green) and nuclei (blue). (B) Heat maps for fibronectin reorganisation and fibril formation. Arrows point out reorganised fibronectin fibrils.

correlate the role of the mobility of the dorsal FN layer, we investigated cytoskeleton assembly and focal adhesion formation. Cells seeded within the PLLA sandwiches showed a matured cytoskeleton inserted into well-defined focal adhesions, similarly as it is shown for 2D controls (Fig. 7) and unlike cells sandwiched within the FN-matrix sandwich (Fig. 2).

As cell morphology is known to direct cell fate,[37,38] cell proliferation was also studied for sandwich cultures with PLLA as the dorsal substrate. The ability to reorganise dorsal FN was correlated with increased cell proliferation after 24 hours in PLLA sandwiches (note that this did not occur in FN-matrix PEA sandwiches; results are included in Fig. 8D to facilitate reading). Interestingly, cell proliferation with PLLA as the dorsal substrate was still lower than on 2D controls where cells only need to reorganise proteins at the ventral plane. Hence, dorsal stimuli within sandwich cultures highly influence cell morphology, adhesion and proliferation and correlates with the ability of cells to reorganise dorsal FN.

To support this hypothesis, cells seeded on standard 2D surfaces were coated with either FN or anti-α_5 antibody dissolved in the culture medium to promote the activation of dorsal integrins without any material (physical) support. Hence this culture mimics the dorsal receptors excitation and can be considered a condition where dorsal remodelling is not constrained by any protein–material interaction. No changes in either cell morphology or cell adhesion were observed as compared to the 2D controls (Fig. 9). Actually, spread cells with well-defined focal plaques containing vinculin were observed, revealing the distinctive mechanism of the sandwich microenvironment to trigger cell adhesion depending on the dorsal stimuli. Besides, a similar level of proliferation was achieved for every condition (Fig. 10), supporting the importance of the biophysical cues. Dorsal stimulation is therefore a key parameter for cell behaviour and understanding its role will allow us to tune cell fate.

4 Conclusions

We have used sandwich-like microenvironments to perform a systematic investigation of cell–material interactions beyond standard 2D cultures, seeking to stimulate both dorsal and ventral receptors and establish a link between 2D and 3D cell

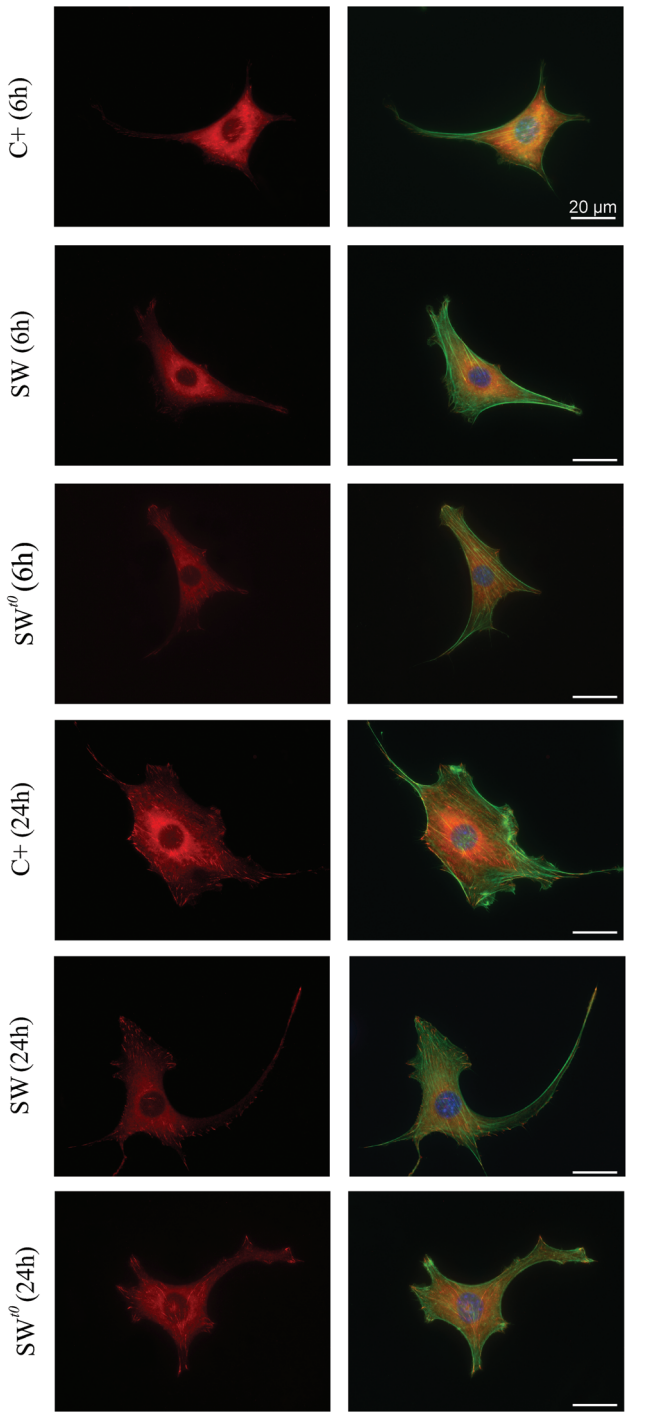

Fig. 7 Cytoskeleton organization and focal adhesion assembly for cells seeded on 2D controls and sandwich cultures (with dorsal PLLA). Fluorescence images show vinculin (red), F-actin cytoskeleton (green) and nuclei (blue).

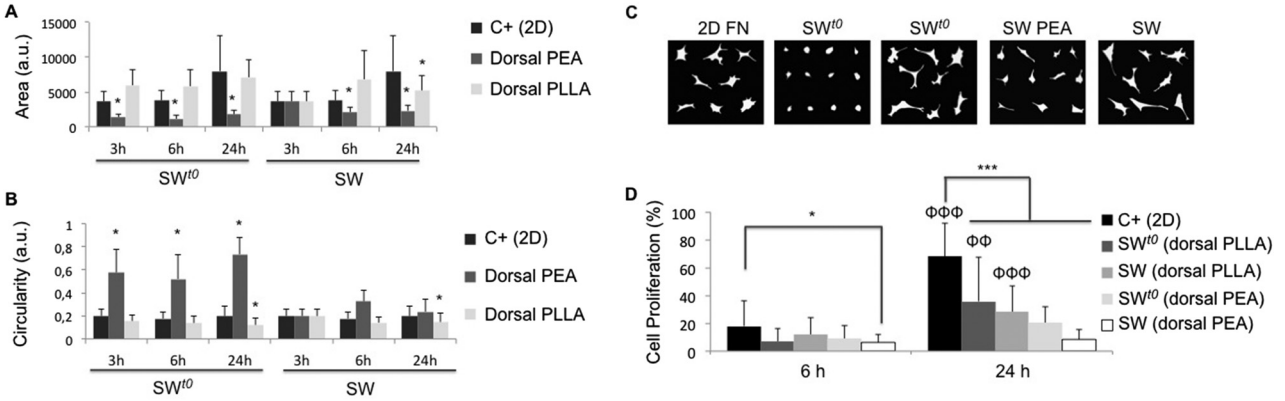

Fig. 8 Cell morphology within sandwich cultures with different dorsal stimuli including sandwich with PEA film or PLLA film as the upper substrate, both coated with FN. (A) Cell area depends on the dorsal stimuli. Since sandwich cultures differed only on the dorsal stimuli, spin coated PEA was the control for every condition. (B) Cell circularity depends on the dorsal stimuli. Statistical differences with respect to the control for the same culture time are marked with * p-value < 0.05 for (A) and (B). (C) Representative outlined cells from contrast phase microscopy for each one of the conditions at 6 hours of culture. (D) Cell proliferation. Φ Stands for significant differences with time and * differences with the control (for the same culture time).

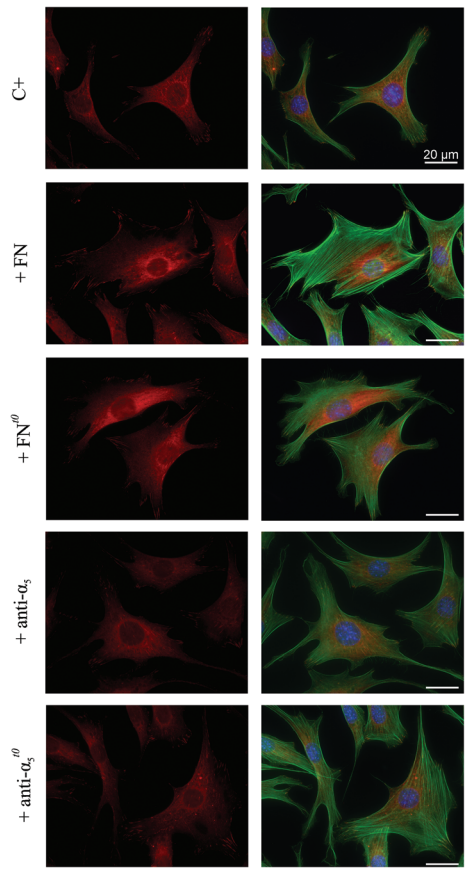

Fig. 9 Cytoskeleton organization and focal adhesion formation on 2D (C+) and after addition of FN (50 μg ml^{-1}) or anti-α_5 antibody (1.2 μg ml^{-1}) to activate dorsal receptors immediately after cell seeding (t_0) or 3 hours later; the total culture time was 6 h. Fluorescence images show vinculin (red), F-actin cytoskeleton (green) and nuclei (blue). No differences were observed after 24 hours of culture.

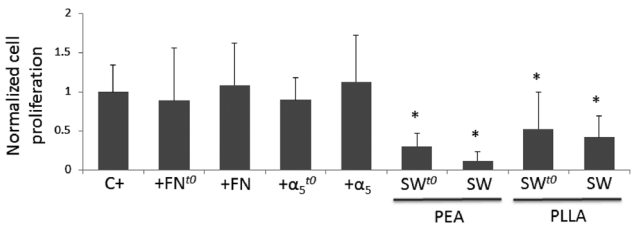

Fig. 10 Cell proliferation after 24 h of culture for samples where FN or anti-α_5 antibody was added to the medium in order to mimic the sandwich environment. Results for PEA and PLLA sandwiches are included for the sake of clarity. Data were normalized to the 2D control. Statistical differences with respect to the control are marked with * p-value < 0.05.

environments. This is then a versatile tool that allows the combination of different protein coatings, surface topology, and materials for both the dorsal and ventral substrates. Besides, representative 2D controls can be easily performed.

Overall, cell adhesion within FN-matrix (PEA) sandwiches showed enhanced $\alpha_5\beta_1$ integrin-mediated adhesion and lower activation of FAK signaling pathways, as a consequence of the dorsal material stimuli. This is correlated with the lack of well-developed focal adhesion plaques within sandwich-like cultures. Cell proliferation decreased within sandwich cultures compared to 2D control. Moreover, blocking the integrin-FN dorsal interaction with HFN7.1 antibody within the sandwich culture did not result in the same proliferation rate as in 2D, which suggests the importance of the dorsal material stimulation. In this way, cells sandwiched with PLLA – where FN adopts a globular morphology and can be mechanically remodelled by cells – showed differences in cell morphology, adhesion and proliferation (with respect to sandwiches with dorsal FN-matrix, PEA), suggesting the key role of protein reorganisation to tune cell fate into a closer 3D environmental manner. Dorsal activation of receptors without any physical/material support does not reproduce results found in

sandwich cultures, which makes this system suitable to mimic complex cell microenvironments.

The ability to reorganize the ECM is therefore fundamental to understand cell adhesion, cytoskeleton development and cell morphology within sandwich culture, as well as in both 2D and 3D systems.[39,40] Our results suggest that cells behave within sandwich culture similarly as within 3D hydrogels. Cells sandwiched with FN-matrix (that cannot be reorganised) adopted a rounded morphology with diffused adhesions (resembling cells in non-degradable hydrogels) while cells adopted a spread morphology with well-defined adhesion when sandwiched with a reorganisable FN layer (resembling cells in MMP-sensitive hydrogels),[27,29,41] These results support the hypothesis that sandwich cultures can mimic 3D environments.

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Notes and references

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