Three-dimensional tumor-stroma co-culture system development using self-assembling peptide scaffolds

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Desarrollo de un sistema tridimensional de co-cultivo tumor-estroma en scaffolds peptídicos auto-ensamblantes

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RECEIVED: 11 MARCH 2019; ACCEPTED: 27 APRIL 2019

SUMMARY

Cancer research has traditionally relied on 2D cell culture, focusing mainly on cancer cells and its disrupted genetics. However, tumors have been accepted as complex tissues, and as such, they need signals from a 3D environment to form tissue structures in vitro. Moreover, cancer cells behavior can only be deciphered considering the contribution of the cells existing in the tumor stroma as well as its complex microenvironment. Since the tumor microenvironment plays an important role in cancer progression, it is widely accepted that culturing cells in 3D scaffolds, which mimic the native extracellular matrix, represents a more realistic scenario. In the present work we aim to develop an in vitro 3D co-culture system that would comprise both cancer and stromal cells. For that, HeLa cells were injected into a RAD16-I peptide scaffold containing fibroblasts, resulting in a 3D system were cancer cells were embedded within a stromal cells matrix. With this system, we were able to study cancer cells behavior in a 3D context in terms of survival, migration and proliferation. Moreover, we have demonstrated that the anti-cancer effect of different pharmaceutical drugs (Gemcitabine, 5-Fluorouracil and the ROCK inhibitor Y-27632) can be qualitatively and quantitatively evaluated on the 3D co-culture system developed.

Keywords: Cancer; self-assembling peptides; three-dimensional culture; tumor microenvironment.

RESUMEN

La investigación en cáncer se ha basado tradicionalmente en los cultivos celulares en 2D, centrándose mayoritariamente en las células cancerígenas y su genética anómala. No obstante, los tumores son actualmente aceptados como tejidos complejos, y como tales, necesitan señales de un ambiente 3D para formar estructuras tisulares in viro. Además, el comportamiento de las células tumorales solo puede ser descifrado considerando también la contribución de las células existentes en el estroma del tumor, así como de su complejo microambiente. Debido a que el microambiente del tumor tiene un papel importante en la progresión del cáncer, es ampliamente aceptado que cultivar las células en scaffolds 3D, que imitan la matriz extracelular nativa, representa un escenario más realista. En este trabajo tenemos como objetivo desarrollar un sistema in vitro de co-cultivo 3D, que comprensa tanto células cancerígenas como células estromales. Para esto, las células HeLa fueron inyectadas en el scaffold peptídico RAD16-I, que contenía fibroblastos, resultando en un sistema 3D donde las células cancerígenas quedaban rodeadas por una matriz de células estromales. Con este sistema hemos sido capaces de estudiar el comportamiento de las células tumorales en un contexto 3D, en términos de supervivencia, migración y proliferación. Además, hemos demostrado que el efecto anticancerígeno de

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diferentes fármacos (Gemcitabina, 5-Fluorouracilo y el inhibidor de ROCK Y-27632) pueden ser cualitativamente i cuantitativamente evaluado en el sistema de co-cultivo 3D desarrollado.

Palabras clave: Cáncer; peptidos auto-ensamblantes; cultivo tridimensional; microambiente tumoral.

RESUM

La recerca en càncer s'ha basat tradicionalment en els cultius cel·lulars en 2D, centrant-se sobretot en les cèl·lules cancerígenes i la seva genètica anòmala. No obstant, els tumors són actualment acceptats com a teixits complexos, i com a tals, necessiten senyals d'un ambient 3D per a formar estructures tissulars in vitro. A més, el comportament de les cèl·lules tumorals només pot ser desxifrat considerant també la contribució de les cèl·lules existents en l'estroma del tumor, així com del seu complex microambient. Ja que el microambient del tumor juga un paper important en la progressió del càncer, és àmpliament acceptat que cultivar les cèl·lules en scaffolds 3D, que imiten la matriu extracel·lular nativa, representa un escenari més realista. En aquest treball tenim com a objectiu desenvolupar un sistema in vitro de co-cultiu 3D, que comprengui tant cèl·lules cancerígenes com cèl·lules estromals. Per a això, les cèl·lules HeLa varen ser injectades en l'scaffold peptídic RAD16-I que contenia fibroblasts, resultant en un sistema 3D on les cèl·lules cancerígenes quedaven rodejades per una matriu de cèl·lules estromals. Amb aquests sistema hem sigut capaços d'estudiar el comportament de les cèl·lules tumorals en un context 3D, en termes de supervivència, migració i proliferació. A més a més, hem demostrat que l'efecte anti-cancerigen de diferents fàrmacs (Gemcitabina, 5-Fluorouracil i l'inhibidor de ROCK Y-27632) por ser qualitativament i quantitativament avaluat en el sistema de co-cultiu 3D desenvolupat.

Paraules clau: Càncer; pèptids auto-ensamblants; cultiu tridimensional; microambient tumoral.

INTRODUCTION

Significantly, 90% of cancers have an epithelial origin mainly including the epidermis and the gastrointestinal tract. Epithelial tissues comprise two parts: a layer of polarized epithelial cells supported on a basal membrane, and the stroma, rich in extracellular matrix (ECM), that contains fibroblasts, immune cells and vessels. The stroma serves as a structural support for epithelial cells and also interacts biochemically with them through soluble signaling molecules and the ECM. The ECM provides both a mechanical and a chemical microenvironment, through its own signaling moieties and its ability to bind molecules such as cytokines, growth factors and enzymes[1]. Traditionally, cancer research has relied on 2D cell culture, but given the complex interactions existing within a tissue, important biological features may be missed if they are only studied in unnatural and constraining 2D cell cultures. In these systems, cells are forced to polarize, which leads to an excessive nutrition and oxygenation, while molecular gradients found in vivo are not reproduced. The ECM composition and configuration are strongly modified, and consequently, cells do not receive the proper signals that provides a normal ECM configuration2. Moreover, the 2D expansion of cancer cells over time results in the selection of those cells with the highest proliferative capacity. These cells not only do not represent the whole tumor but also are very susceptible to therapies targeting rapidly proliferating cells[3].

Three-dimensional cell cultures can satisfy the need for more representative cancer models[4]. These systems can capture the phenotypic heterogeneity found in a tumor, which is in part due to environmental differences[5] (stress, nutrient gradients, hypoxia, etc.). Different cell behavior means different drug targets, fact that makes the destruction of the entire tumor more challenging[6]. Since 3D cultures recreate molecular gradients, these models can be used to test drug efficacy above the heterogenous cell population. Finally, *in vitro* 3D cancer models allow to mimic the tumor microenvironment and manipulate each component in order to study its implications in tumor progression[7–9].

Considering that the microenvironment has a critical role in tumorigenesis10, ECM analogs, also called "scaffolds", have been introduced in 3D cell culture systems. In these systems, cells are embedded in a natural or synthetic scaffold that mimics the ECM, providing thus, the chemical, mechanical and physical cues that cells need to form physiological tissue structures *in vitro*.

Self-assembling peptide scaffolds (SAPS) are promising synthetic biomaterials which have minimal variation from batch to batch production and present low biodegradability *in vitro*. Therefore, SAPS provide a reproducible cellular microenvironment with constant structural and mechanical properties over time [14–18] proliferate and migrate interacting among each other and with the surrounding matrix in a three-dimensional (3D. In particular, RAD16-I is a short peptide constituted by the sequence AcN-$(RADA)_4$-$CONH_2$, which alternates hydrophilic and hydrophobic amino acids. The peptide undergoes self-assembly into a nanofiber network with antiparallel β-sheet configuration by increasing ionic strength or adjusting pH to neutrality[19]. The nanoscale fiber architecture (around 10 nm diameter and 5-200 nm pore size) allow the cells to experience a truly 3D environment. Besides, biomolecules in such nanoscale environment diffuse slowly and are likely to establish a local molecular gradient. Since stiffness can be controlled by changing peptide concentration these hydrogels can be tuned up to embed cells but not to entrap them [19]. Moreover, they do not contain chemical cues *per se*, providing thus a non-instructive environment, from the point of view of receptor recognition/activation. Non-covalent interactions allow cell growth, migration, con-

tact with other cells, shape changes and a properly exposition of membrane receptors[19–22]

In the present work, we have developed a 3D co-culture system based on the self-assembling peptide scaffold RAD16-I, comprising HeLa as cancer cells and dermal fibroblasts as stromal cells. With this system, is our intention to recreate a more realistic scenario, in which cancer cells would receive from the 3D environment the mechanical and physical signals needed to undergo their biological functions while stromal cells would contribute with the chemical signals that support tumorigenesis.

MATERIALS AND METHODS

Mammalian cell culture

Cervical adenocarcinoma-derived cells (HeLa) and human Normal Dermal Fibroblast (hNDF) were cultured and expanded in Dulbecco's modified Eagle's medium (DMEM, 12491-023; Gibco,) supplemented with 10% fetal bovine serum (FBS, S1810; Biowest), 1% penicillin/streptomycin (L0022; Biowest) and 1% L-Glutamine (X0550; Biowest) in a humidified atmosphere at 37°C and 5% CO_2.

3D culture technique in SAPS

The commercial peptide RAD16-I (1% in water) (PuraMatrix™, 354250; Corning) was prepared at a final concentration of 0.3% (w/v) in 10% sucrose (S0389; Sigma) and sonicated for 20 min at room temperature. Meanwhile, cells expanded in 2D monolayer were harvested by trypsinization and resuspended to 4·106 cells/mL in 10% sucrose. The 0.3% (w/v) peptide solution was mixed with and equal volume of cell suspension to obtain a final concentration of 2·106 cells/mL and 0,15% (w/v) RAD16-I in 10% (w/v) sucrose. Next, 40 µL of the mixture (80,000 cells) were loaded into wells containing prewarmed media, which induced the spontaneous self-assembling of the peptide. The constructs were left in the flow cabinet for 20 min to let them gel. Then, the media was changed, to favor the leaching of sucrose and the equilibration of cells, and the plate containing the 3D constructs was placed in the incubator (37°C, 5% CO2, humidified atmosphere). Cells were maintained in the incubator and medium was changed every other day.

3D co-culture technique in 3D SAPS

hNDF growing in 2D monolayer were harvested and resuspended to $2.5{\cdot}10^5$ cells/mL in 10% sucrose. The RAD16-I peptide solution (0.3% (w/v)) was mixed with an equal volume of cell suspension to obtain a final concentration of $1.25{\cdot}10^5$ cells/mL and 0.15 % (w/v) RAD16-I in 10% sucrose. Next, 40 µL of the mixture (5,000 cells) were loaded into wells of a 12-well plate. Medium was added in each well to induce the self-assembling of the peptide and the constructs were left in the flow cabinet for 20 min to let them gel. In this case, the resulting gel was attached on the well surface. By doing this, the mechanical contraction produced by fibroblasts growing in 3D configuration was avoided. Fibroblasts were cultured for 3 days to allow them to adapt to the 3D environment. To incorporate HeLa cells to the 3D system, medium was removed and 1 µL of a mixture of HeLa (final concentration $2{\cdot}10^6$ cells/mL; 2,000 cells) and 0.3% RAD16-I (final concentration 0.15%), was injected into the 3D constructs containing hNDF (Figure 1A) or into an empty hydrogel (Figure 1B).

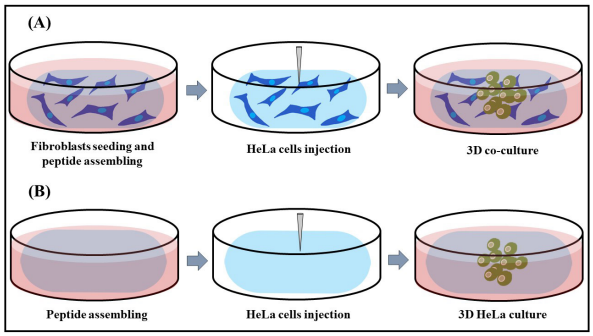


Figure 1. Schematic representation of the 3D system construction.

(A) Co-culture construction. hNDF were encapsulated into the SAPS and HeLa cells were injected into the fibroblasts-containing hydrogel matrix. The result was a 3D environment with cancer cells embedded into a fibroblasts network. **(B) Cancer cells injection into an empty peptide hydrogel.** HeLa cells were injected into a preformed hydrogel matrix.

MTS assay

MTS Cell Proliferation Assay Kit (ab197010; Abcam) was used to assess cell viability and proliferation in 3D cultures. 3D constructs (80,000 cells) were assembled by triplicate in wells of a 48 well-plate not treated for cell culture. Briefly, medium was removed, and cells were incubated with 1 mL of a mixture containing MTS reagent and medium (20:100) for 3h in the dark, at 37°C and 5% CO_2 in a humidified atmosphere. For the blank, MTS and medium were incubated without cells. Absorbance was read at 490 nm in a microplate reader.

Cell viability

Cell viability was determined using the Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen; L3224). 3D constructs were rinsed three times with 1x PBS and incubated for 40 min in the dark with a fresh solution of 2 µM calcein and 2 µM ethidium homodimer-1 in PBS. Finally, the samples were rinsed with 1x PBS and visualized under an epifluorescent microscope (Nikon Eclipse TE2000-1) to detect live cells (in green) and dead cells (in red).

Toluidine blue staining

Toluidine blue (TB) is an indicator of proteoglycans production. However, in the present work, TB staining has been used to evaluate cell density in 3D cul-

tures. For that, 3D constructs were washed with 1x PBS and fixed with 3.7% formaldehyde in PBS for 30 min. Then, constructs were washed with 1x PBS and incubated with 0.05% (w/w) toluidine blue in water during 20 min. Samples were washed several times with distilled water during 24 h and visualized under contrast phase microscopy (Nikon Eclipse TE2000-1 epifluorescent microscope).

Drug incubation

The chemotherapeutic effect of different drugs was tested in 3D co-cultures. The drugs and its concentrations used were: 10 µM Gemcitabine23 (G6423; Sigma), 10 µM 5-Fluorouracil24 (F6627; Sigma) and 10 µM ROCK Inhibitor Y-2763225 (SCM075; Sigma). Drugs were added 24 h after cell injection and incubated during 72 h. 3D co-cultures were then assessed for viability with Live/Dead staining and MTS assay. Cell density was evaluated under phase contrast microscopy (Zeiss Axiocam 503 microscope) with toluidine blue staining.

Statistics

Data are presented as mean ± Standard Deviation. Statistical differences between groups were analyzed using One-Way ANOVA at a significance level of 5%. The statistical software used was GraphPad Prism 6.0.

RESULTS AND DISCUSSION

Cellular viability and proliferation in 3D SAPS

As a previous step before the development of the 3D co-culture system, HeLa and hNDF cells were assessed for viability and proliferation within the 3D nanofiber peptide scaffold RAD16-I. Live/Dead staining showed some dead cells after the encapsulation into the peptide scaffold, probably due to the fact that cells are in a hostile environment during the seeding process, since the peptide pH is approximately 4-5. Nevertheless, most of the cells were found to be alive at day 10 of culture (Figure 2A). Proliferation in 3D constructs was characterized through the MTS assay (Figure 2B). Growth curves for HeLa cells in 3D constructs presented approximately a doubling time of 7 days. Interestingly, no proliferation was observed in the case of hNDF, suggesting that these cells cultured in 3D configuration enter in G0 phase of the cell cycle. Consequently, cellular density increased slowly for HeLa cells and it was maintained constant for fibroblasts. These last ones adopted an elongated shape extending cellular processes within the space of the scaffold, and as a consequence, contracted the entire peptide matrix.

One could have expected that due to the neoplastic origin of HeLa cells, they would have a higher proliferation rate in 3D. However, it is necessary to keep in mind different facts. First, the doubling time is, by definition, the period required to double the entire cell population. While in 2D monolayer all the cells are under the same nutrient and oxygen levels, in 3D constructs exist molecular gradients that produce environmental differences within the 3D system. Thus, the cells located in the inner part of the 3D construct are under lower nutrient and oxygen levels, fact that may lead to a decrease in the proliferation rate of these cells. Second, cells were cultured in a peptide scaffold that did not contain any signaling motif, so the environment was non-instructive from the point of view of cell receptor recognition/activation. Probably, HeLa cells would have a higher proliferation rate cultured in a peptide scaffold functionalized with integrin binding sites (RGD sequences), since proliferation depends on the activation of integrins 26–29. It is important to remind that, when culturing cells in a 3D environment, cellular behavior should be interpreted in the context of each experimental design.

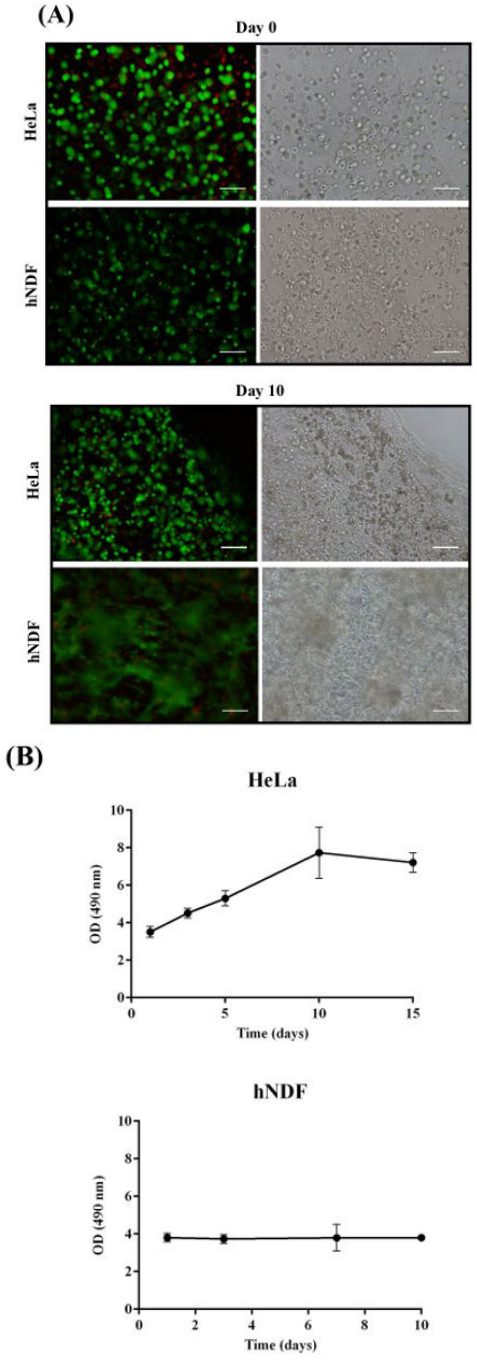


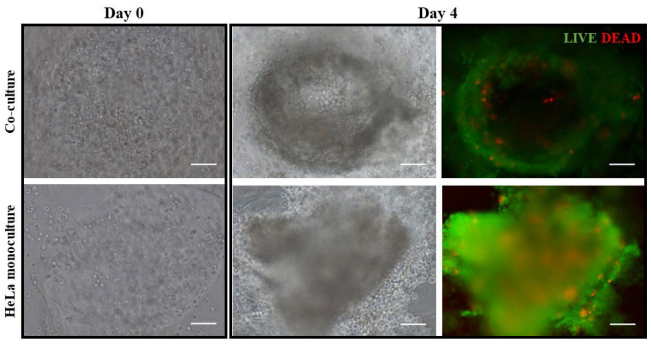
Figure 2. *Cellular viability and proliferation in 3D SAPS.*

(A) Viability assessment of HeLa and hNDF 3D cultures. Viability was assessed at day 0 and 10 of encapsulation into the self-assembling peptide RAD16-I. Live cells are stained in green, while dead cells are stained in red. Scale bars 100 µm. **B) HeLa and hNDF growth curves in 3D culture.** The duplication time obtained for HeLa was 7 days while hNDF did not show proliferation.

Three-Dimensional co-culture development and characterization

The field of cancer research has largely been guided by a reductionist view focused on cancer cells and its disrupted genetics[30]. However, over the past decade, tumors have been accepted as complex tissues, being thus the reductionist view too simplistic30. It is increasingly apparent that tumor growth can only be explained considering the contribution of the cells existing in the tumor stroma as well as the tumor microenvironment that they construct during the course of multistep tumorigenesis. In the present work, a 3D co-culture system using human cervical adenocarcinoma cells (HeLa) and human normal dermal fibroblasts (hNDF) was developed with the aim of recreating a more realistic environment in which cancer cells were embedded. In other words, to obtain the simplest representation of a complex system such as a tumor tissue. With this system, we aimed to study cancer cells survival, proliferation and migration, as well as testing well known drugs to characterize our system, in a 3D context which not only included the peptide network and cancer cells itself, but also stromal cells.

Progression of the HeLa/hNDF co-culture and the HeLa monoculture along seven days could be easily monitored under phase contrast microscopy (Figure 3). HeLa cell density was significantly increased when co-cultured with hNDF as well as in monoculture. Interestingly, the central area of the tumor in the co-culture system presented areas with fewer cellular density which were not found in the monoculture. The differential color intensity in the tumor core of 3D co-cultures stained with toluidine blue (TB, which stains for proteoglycans) at day 7 from the injection exposed the same phenomenon (Figure 3). These results suggest that HeLa cells migrated from the center when co-cultured with fibroblasts, but not when monocultured.



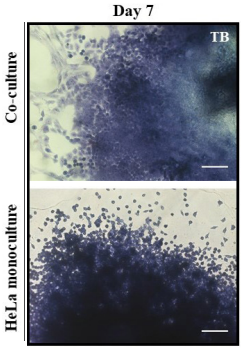


Figure 3. *HeLa and hNDF 3D co-culture progression over time.*

HeLa cells were injected into a gel containing fibroblasts (top) or an empty gel (bottom) and cultured for 7 days. Cells were tested for viability (Live/Dead assay) at day 4 from the injection. Toluidine blue staining at day 7 revealed HeLa cells migration when co-cultured with fibroblasts but not when monocultured. Images represent the tumor core. Scale bars 100 µm.

Moreover, Live/Dead staining at day 4 revealed the presence of some dead cells in the tumor core (Figure 3). This fact was probably due to hypoxia and nutrient depletion conditions in the inner part of the tumor, as previously described for 3D spheroids[9]. This phenomenon was brought to an extreme when cells were cultured for 7 days (Figure 4). In this case, a clear necrotic core, surrounded by a presumably quiescent viable zone could be observed. In contrast, HeLa cells located in the outer part were actively proliferating. These results were considered when testing drug candidates with this system, as cell death occurred naturally over certain time in culture.

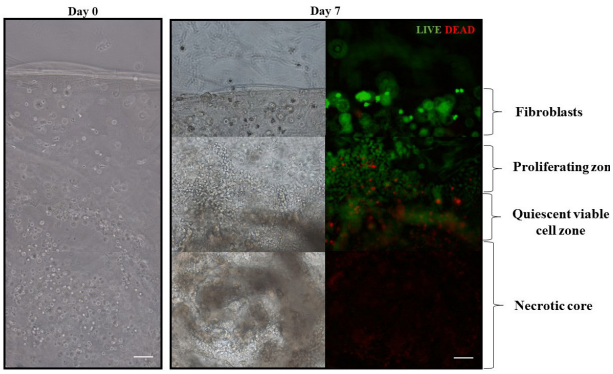


Figure 4. *Differential proliferation zones in the 3D co-culture system at day 7.*

The necrotic tumor core was surrounded by a quiescent viable zone. Cells in the outer part of the tumor were actively proliferating. Pictures of three consecutive areas were taken and merged. Scale bars 100 µm.

Drug testing

The effect of Gemcitabine, 5-fluorouracil (5-FU) and Y-27632 was analyzed in the 3D co-culture system developed. HeLa cells resuspended in a RAD16-I pep-

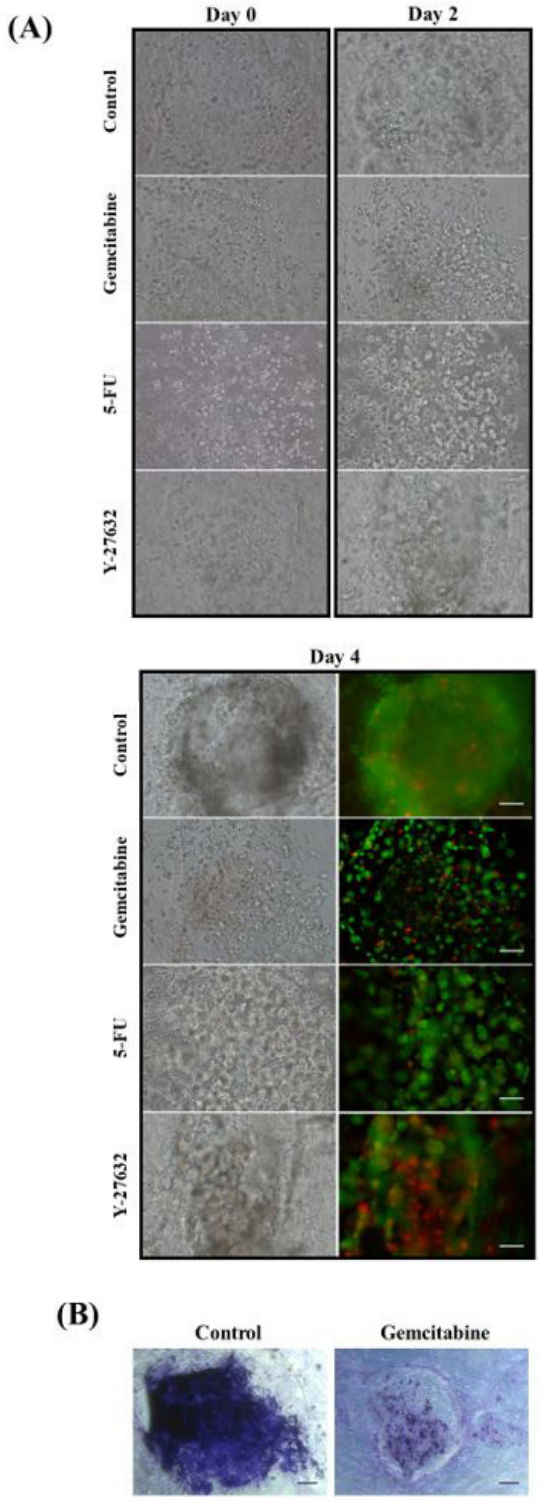
tide solution were injected into a preformed RAD16-I peptide matrix containing fibroblasts (see **Materials and Methods**). Test compounds were added 24 h after the injection followed by 72 h of culture. Cell density in the 3D co-culture system was qualitatively assessed by visual inspection at different time points using phase contrast microscopy (**Figure 5A**) and by toluidine blue staining at the end of culture time (**Figure 5B**). Cells were also tested for viability using the Live/Dead staining (**Figure 5A**) and the MTS assay (**Figure 5C**).

As expected, both Gemcitabine[23] and 5-FU[24], which are considered anti-proliferative drugs, prevented HeLa cell proliferation, compared to the control group (**Figure 5A** and **Figure 5B**). Visual inspection of the 3D co-cultures treated with these drugs revealed that Gemcitabine had a stronger effect over proliferation and viability than 5-FU (**Figure 5A**). These results were quantitatively confirmed by the MTS assay (**Figure 5C**).

On the other hand, cells treated with the ROCK inhibitor Y-27632[25] were likely to proliferate but not to migrate, since cells were found to be accumulated forming compacted clusters (**Figure 5A**). This result agrees with the fact that ROCK inhibitor abrogate cytoskeleton remodeling during migration but does not influence spindle formation during cell cycle progression. Moreover, the presence of dead cells within the tumor core could be explained by the great accumulation of cells at one site which could increase unfavorable mass transfer conditions including formation of a hypoxic environment (**Figure 5A**). However, MTS assay indicated no differences between the control group and the Y-2632 treated group (**Figure 5C**). The reason of this unexpected result could be that, even though cell death in the tumor core in presence of Y-27632 seemed to be quite high, the MTS assay performed over the co-culture may not be sensitive enough to detect these differences.

We have demonstrated that the effect of different drugs can be quantified in our 3D co-culture system with MTS assay. Optical density values obtained for 3D co-cultures agreed with HeLa cell density in the 3D constructs incubated with each drug. However, it is important to consider that the results obtained with MTS assay include both tumor and stromal cell types. Further experiments should be performed to test the cytotoxic effect of the compounds on non-cancer cells. Still, this co-culture system is a valuable tool to test drug efficacy in preliminary experiments, since drug response is strongly affected by the tumor microenvironment, which includes other cell types that exist within it, situation that is not recreated in conventional 2D monolayer cultures.

HeLa cells resuspended in a RAD16-I peptide solution were injected into a 3D culture of hNDF (day 0). Next, Gemcitabine, 5-FU or Y-27632 were added 24 h after the injection. Cells were incubated with the drugs for 72 h and assessed for cellular density and viability. (A) Co-culture tumor core progression over time. Cellular density was evaluated at different time points with phase contrast microscopy, while cellular viability was tested with Live/Dead staining at day 4 from the injection. Scale bars 100 μm. (B) Toluidine blue staining of 3D co-cultures. Cellular density was evaluated at the end of culture (day 4) time using toluidine blue staining. Scale bars 200 μm. (C) MTS Assay. MTS assay was performed over 3D co-cultures at day 4. Data are presented as mean ± Standard Deviation. Statistical differences between groups were analyzed using One-Way ANOVA at a significance level of 5%. Significative differences are indicated as **** for $p<0.0001$ and ns for $p>0.05$.



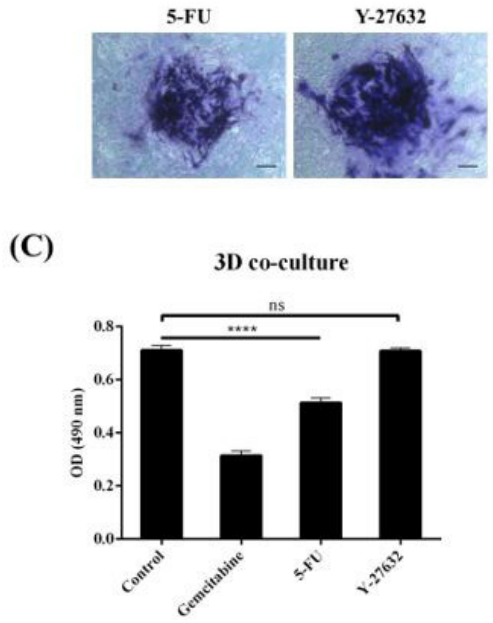


Figure 5. *Effect of Gemcitabine, 5-FU and Y-27632 on the 3D co-culture system.*

CONCLUSION

We report the development of a 3D tumor-stroma co-culture system using a self-assembling peptide scaffold. With this system, we were able to study cancer cell behavior in terms of proliferation and survival, in a 3D context including not only cancer cells itself but also stromal cells. This co-culture system is a useful tool to test drug efficacy, since drug response is strongly affected by the other cell types existing in the tumor microenvironment.

ACKNOWLEDGMENTS

The authors thank to the research funding from the Department of Bioengineering, IQS-School of Engineering, URL and Hebe Biolab S.L. to CES.

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