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Original Article

Self-assembling peptide RADA16-I hydrogel for three-dimensional culture of hepatocarcinoma $HepG_2$ cells

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Abstract: The aim of this study is to evaluate the biological behaviors of human hepatocarcinoma cell $HepG_2$ in self-assembling peptide RADA16-I hydrogel. The proliferation, viabilities, densities, configuration and microstructures, the expression of extracellular matrix proteins, migration rate of hepatocarcinoma cells cultured in the hydrogels were investigated. Our results showed that $HepG_2$ cells embedded in RADR16-I, Matrigel and Collagen-I 3-D matrices are evenly distributed with a great viability and compatibility. The cells cultured in RADR16-I hydrogels showed a spindle-shaped phenotype with irregular and radial nuclei, and cells cultured in Matrigel formed multicellular spheroids with disorganized nuclei. However, no cell clusters were observed when the cells were cultured in Collagen-I hydrogel. Immunohistochemical results showed that the expression of fibronectin in $HepG_2$ cells is positive cultured in RADR16-I, Matrigel and Collagen-I hydrogels, and the expression levels of laminin are weakly positive. DNA contents cultured in RADA16-I hydrogel gradually increased up to Day 6, and consistently raised with the rising culture time in Collagen-I and Matrigel hydrogels. The levels of VEGF, EGF and FGF2 cultured in three hydrogels showed no statistically significant differences ($P>0.05$), and the levels of IGF-1 in RADA16-I and Collagen-I were significantly lower than those of in the Matrigel hydrogel ($P\leq0.05$). Moreover, the migration rate of $HepG_2$ cells cultured in RADA16-I hydrogel were significantly lower than those of cultured in the Collagen-I and Matrigel ($P\leq0.05$). The present results suggested that 3-D culture system for $HepG_2$ cells *in vitro* was successfully established used self-assembling nano-oligopeptide RADA16-I, and $HepG_2$ cells may maintain the better biological characteristics when they were cultured in RADA16-I hydrogel compared to those of cultured in the Collagen-I and Matrigel hydrogels. Moreover, the proliferation and migration rate of $HepG_2$ cells significantly reduced compared to those of in the Collagen-I and Matrigel hydrogels.

Keywords: Three-dimensional culture, self-assembling peptide, hydrogel, $HepG_2$ cells, biological behaviors

Introduction

Adherent monolayer culture has been used as a conventional method for traditional cells culture *in vitro*. However, many studies showed that cells cultured in two-dimensional system often lead to ectopic alternations in biological characterizations, even missing some significant phenotypes and functions [1]. Recently, a variety of extracellular microenvironment signals are actively involved in the regulation of cell growth, differentiation, proliferation and apoptosis, such as cytokines, neurotransmitters and extracellular matrix (ECM) [2-4]. Thus, it has become a hot spot to study how cultured cells response to extracellular microenvironment. Bissell et al. report showed that teratoma cells became normal when they were transplanted into normal embryos, suggesting that cell microenvironment may affect tumor characterization [3]. Although animal model show morphological characteristics of the tumor and mimic microenvironment of tumor cells *in vivo*, it is difficult for large-scale screening of molecular mechanisms. Three-dimensional (3D) cultured cells have showed great potential to tumorigenesis studies, with exquisite cell-to-cell signal transductions and verisimilar cell differentiation and ultrastructures [5]. 3D cell culture system may provide scaffold or matrix for

the proliferation and migration of tumor cells. Moreover, ingredients and alternations of ECM in the 3D culture system may have a significant impact on biological behaviors of tumor cells, such as polarization, migration and invasion [6, 7]. Thus, there have been a variety of *in vitro* systems to investigate interactions between tumor characterizations and ECM microenvironment [6-8].

Nowadays, self-assembling nano-oligopeptides provide an opportunity to address how ECM microenvironment interacts with cultured cells. These polypeptides which have hydrophilic and hydrophobic surface and self-assemble into different structures are called "molecular building blocks". There have been a series of designed ionic self-assembling nano-oligopeptides to be used in biomaterials, tissue engineering and biomedical fields [8-10]. Self-assembling nano-oligopeptides are also widely used in regeneration and repair of neural stem cells [11], bone repair and remodeling [12], and scalded skin repair [13, 14]. Recent studies have shown that self-assembling nano-oligopeptide RADA16-I hydrogels have excellent structures and properties, and therefore could provide an applausive environment for maintaining the phenotype or redifferentiate of many cancer cells, such as breast [15] and lung cancer cells [16].

There is a high incidence of liver cancer in China, which is about 55% of world's total liver cancer [17]. Despite great progress have been made, there are still cancer metastasis and recurrence of 60~70% hepatocellular carcinoma patients. Reports have showed that host microenvironment is involved during the process of tumor metastasis [1-3, 18]. Thus, mimetic 3D microenvironment *in vitro* will help to understand the molecular mechanism of tumor metastasis in hepatocellular carcinoma. In the present study, self-assembling peptide RADA16-I was designed as biomaterial for developing a 3D culture system to explore the cell morphology, proliferation, migration, ECM proteins and cytokines in human hepatoma cell line. To accomplish this, we adopted Matrigel and Collagen-I hydrogels as positive control. The present results will provide a novel experimental method to study the biological characterizations of liver cancer cells, and afford experimental and theoretical basis for standardization and engineering of hepatoma cell 3D culture systems.

Materials and methods

Materials and reagents

Human hepatoma cell line was obtained from Department of Microbiology, Zunyi Medical University (Introduced from ATCC). RADA16-I was purchased from Shanghai Bootech Bioscience & Technology. Collagen-I and Matrigel were from Becton, Dickinson and Company. Trypsin-EDTA, calcein-AM, phalloidin, BrdU, BrdU antibody and DNA fluorescence quantitative detection Kit were from Sigma-Aldric (St. Louis, MO, USA). Goat anti-mouse IgG-FITC was from Solarbio Science Technology. Other chemicals were of the highest purity available.

Cells culture

$HepG_2$ cells were planted in culture flasks with culture medium RPMI 1640 containing 10% FBS, then cultured in 5% CO_2 incubator. The cells were digested and centrifuged with 0.02% EDTA and 0.25% trypsin after reached 80% confluence, and cells were washed and resuspended with 10% sucrose. The peptide solution was mixed thoroughly with the cell suspension by 9:1, with the final concentration of RADA16-I reached to 0.5% (w/v) and final cell concentration 1×10^6/ml. Similarly, Collagen-I solution and cell suspension were thoroughly mixed by 0.79:1, and the final concentrations of Collagen-I and cells were 1.5 mg/ml and 1×10^6/ml, respectively. Matrigel solution and cell suspension were thoroughly mixed by 1:1 under ice bath, and the final concentration of Matrigel and cells were 0.5% (W/V) and 1×10^6/ml, respectively. These mixtures were dripped quickly into the homemade molds, and incubated in 37°C incubator with RPMI 1640 media for 20 min. After gel formation, the molds were removed and RPMI 1640 medium was added. Finally, cells were cultured in 37°C incubator, and the media were changed every other day.

Assay of Calcein-AM staining

Cells were cultured in three hydrogels for 72 hours, and rinsed with PBS for three times. 2 μM calcein (50 μl) was added in the cell, and incubated in the dark for 45 min, rinsed for several times with PBS. Finally, these cells were

photographed under a fluorescence microscope at room temperature.

Assay of phalloidin/DAPI staining

Cells were cultured in three hydrogels for 3 days, and rinsed with pre-warmed PBS buffer for several times. Cells were fixed with 4% paraformaldehyde for 15 min, and washed with PBS. Then, phalloidin (8.3 μg/ml, 10 μl) were added to incubate with cell clumps for 20 min, and washed with PBS. Finally, these cells were treated with DAPI (2.5 μg/ml, 10 μl) for nuclei staining, and photographed by fluorescence microscope.

Assays of DNA contents

At Day 3, 6 and 9, Cells were collected and washed with PBS buffer for several, and then placed in citrate buffer with 5 mM sodium citrate and 100 mM sodium chloride. Cells were repeatedly frozen, thawed and shook until clumps completely dissolved. DNA content was analyzed using the DNA fluorescence assay kit.

Assay of BrdU labeling

At Day 3, 6 and 9, cells were collected and an equal amount of 10 mM of BrdU reagent was added. Next, these cells were incubated for 12 h. Cells were washed and treated with 4% paraformaldehyde for 30 min, and washed thrice with PBS. Then, PBS buffer with 0.3% TritonX-100 was added, and washed thrice with PBS. 60% HCl was added and incubated for 30 min at 37°C, and then washed thrice with PBS. PBS with 3% BSA, 0.2% TritonX-100 and 2% donkey serum were added and incubated for 60 min, and then washed thrice with PBS. BrdU antibody and goat anti-mouse FITC successively added, and then DAPI (2.5 μg/ml, 10 μl) was added at room temperature. Photographs were recorded under a fluorescence microscope, and FITC-labelled cells were counted.

Immunohistochemistry of ECM

After incubated with antibodies, and stained by hematoxylin, cells were washed with water and differentiated with 0.1% hydrochloric acid and ethanol. Finally, cells were washed by tap water, dehydrated with graded alcohol, sealed with gum, dyed and observed.

Assay of ELISA

After culturing at day 6, the cells were centrifuged at 2000 g for 20 min, and the supernatants were collected for the expressions levels analysis of VEGF, EGF, FGF-2 and IGF-1 using ELISA assays. The results were recorded at 450 nm using microplate reader.

Assay of cell scratch

To examine the cell migration rate of $HepG_2$ cells in there hydrogels, cell scratch assays were carried out. Cells density was adjusted to 5×10^5/ml, and cells were seeded on the well plates coated with RADA16-I, Collagen-I and Matrigel. The cells were cultured up to the formation of monolayer cells, and scratched into a linear shape along the bottom of culture plate, and then washed several times with serum-free medium. Photographs were recorded under the microscope, and the relative distance of scratch was measured. After cultured for 24 h, the relative distance of cell migration towards the injured area was measured, and the actual cell migration distance may be calculated from original injured area. The migration rate was defined as the ratio of the distance between actual migration distance and original injured area.

Statistical analysis

SPSS 17.0 software was used for statistical analysis of experimental data. All the results were presented as mean ± standard deviation ($x \pm SD$), and One-Way ANOVA test was conducted between groups. Values 0.05 (*) and 0.01 (**) were assumed as levels of significance for the statistic tests carried out.

Results

Morphology and aggregation states of $HepG_2$ cells in different culture systems

As shown in **Figure 1**, cells in 2D culture conditions began to adhere after 4 h, and up to completely adhere after 24 h. These cells shape like polygon or stars with uniform cell size. The cell confluence may run up to 80% when they were cultured on second day. Compared with 2D culture system, $HepG_2$ cells cultured in 3D system showed different characterizations, and grow suspendedly with slow proliferation rate. The cell clumps aggregation

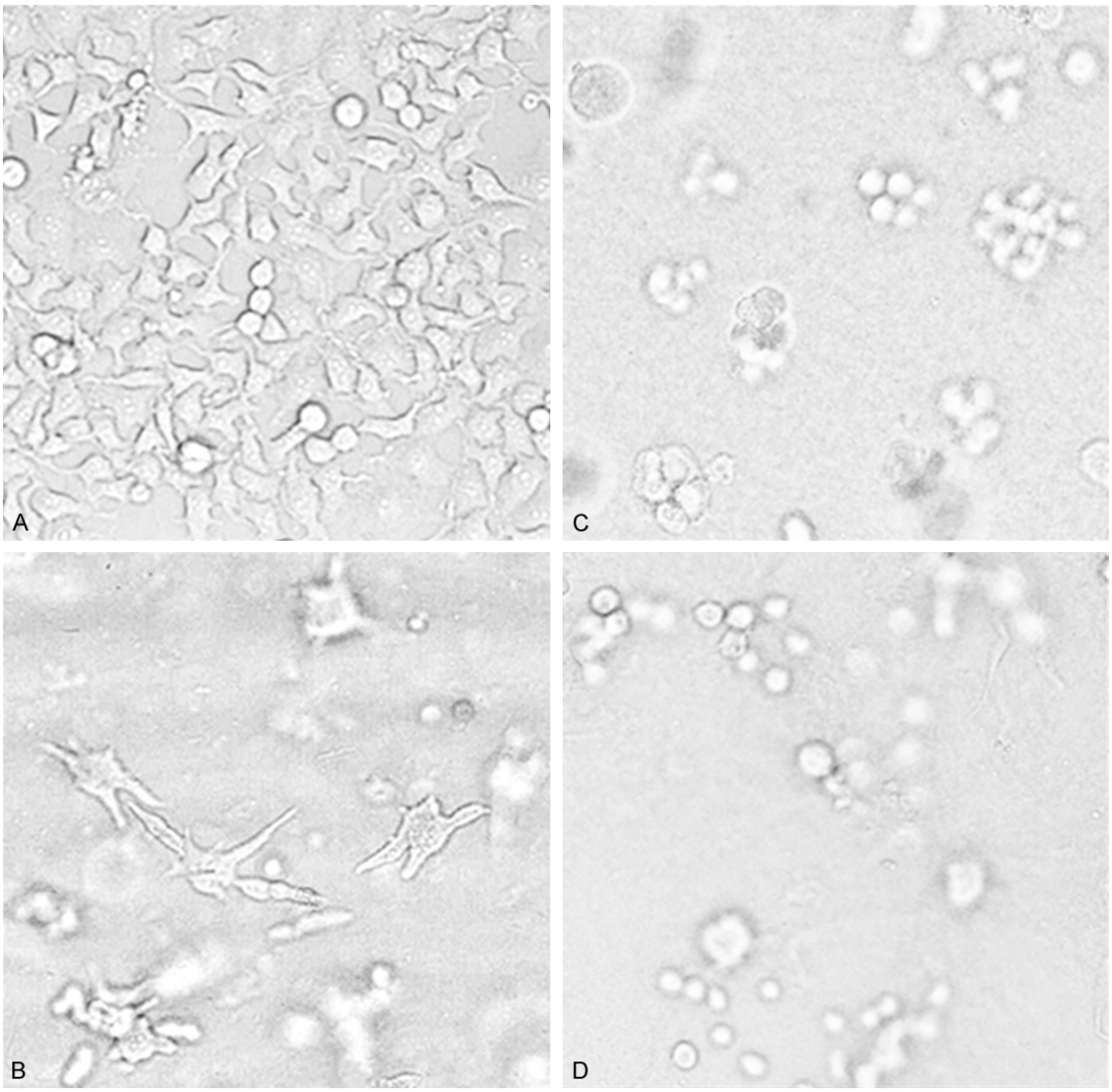

Figure 1. Light microscopy images of $HepG_2$ cells encapsulated in 2D (A), Matrigel (B), RADA16-I (C) and Collagen-I (D) systems.

can even be observed on the third day. Notably, cells in RADA16-I and Matrigel hydrogels proliferated to large aggregates. In RADA16-I hydrogels, cell grew to be radial or multiangular and star like clusters. However, cells in Matrigel hydrogels were like grapes clusters or spheres. $HepG_2$ cells in Collagen-I gels showed no significant large aggregates, with only cell density increasing. Moreover, the cell shape was extended like irregular spindles in RADA16-I hydrogels, and maintained round or spherical in Matrigel and Collagen-I gels (**Figure 1**).

Staining morphology of $HepG_2$ cells in different culture systems

As shown in **Figure 2A**, $HepG_2$ cells grew well in RADA16-I, Matrigel and Collagen-I hydrogels, suggesting that these scaffolds have excellent biocompatibility to $HepG_2$ cells. Moreover, different cell shapes and aggregations indicate that microenvironment may directly affect cell morphology. To further detect cell morphology of $HepG_2$ cells in different culture systems, we performed phalloidin/DAPI staining. Images showed the morphology of $HepG_2$ cells, blue for the nuclei and red for cytoskeletons. Cells in

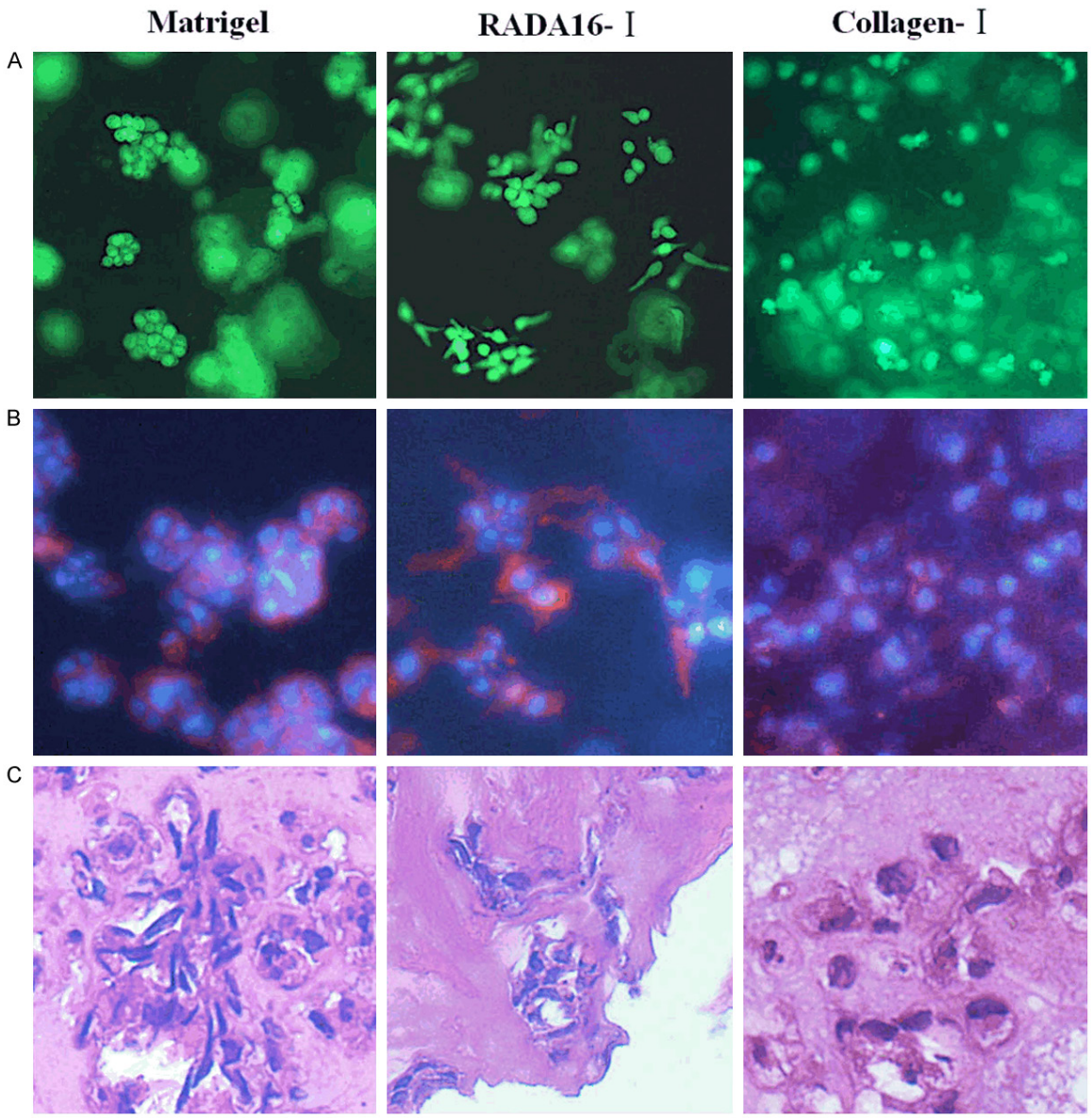

Figure 2. Morphology of $HepG_2$ cells cultured in Matrigel, RADA16-I and Collagen-I hydrogels. A. Cultured for 3 d, calcein-AM staining, ×100; B. Cultured for 3 d, phalloidin/DAPI staining, ×200, F-actin (red) and nuclear (blue); C. Cultured for 7 d, H-E staining, ×400.

RADA16-I showed elongated cell bodies and polarized cell mass, while those in Matrigel and Collagen-I had disordered cell arrangement, with various cell size and scattered sphere or grapes (**Figure 2B**). By H-E staining, we further examined the cell status in different cultured systems. As shown in **Figure 2C**, after paraffin sections of $HepG_2$ cells in different hydrogels on the seventh day, irregular polarized cell aggregation was observed in RADA16-I hydrogels, and their nuclear were heavily stained. Those cells in Matrigel showed clear nuclear staining and sphere or grapes like cell mass, and those cells in Collagen-I kept scattered spherical or round shapes, and the nuclear staining were also heavily stained (**Figure 2C**).

Proliferation rate of $HepG_2$ cells in different culture systems

To detect the cell proliferation rate of $HepG_2$ cells in different systems, we carried out DNA fluorescent quantification assays. As shown in **Figure 3**, it can be found that cell proliferated

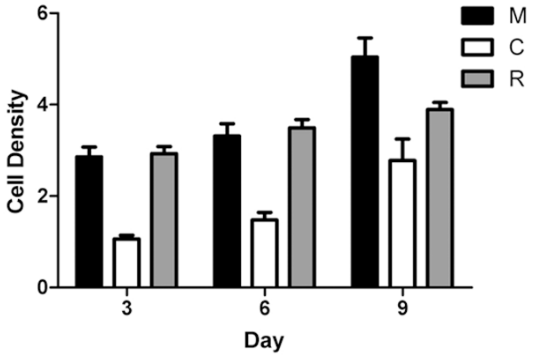

Figure 3. $HepG_2$ cells proliferation in different 3D systems by determination of DNA contents in cultured 3, 6 and 9 days. M, Matrigel; C, Collagen-I; R, RADA16-I.

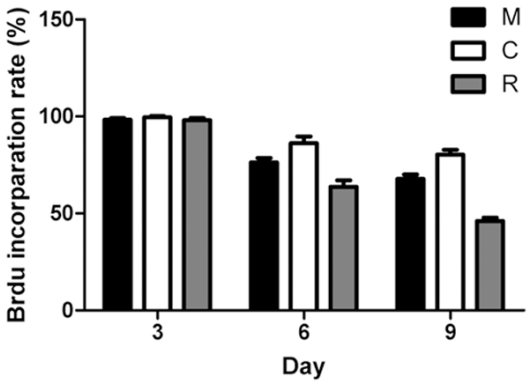

Figure 4. Percentage of BrdU labelling in $HepG_2$ cells grown in different 3D systems in cultured 3, 6 and 9 days. M, Matrigel; C, Collagen-I; R, RADA16-I.

well in these three systems. Notably, $HepG_2$ cells in Matrigel and Collagen-I gels kept a rapid growth during these culture processes, but those cells in RADA16-I maintained active proliferation rate from the beginning to sixth day, and declined from then on (**Figure 3**). To confirm the cell proliferation status, we further applied BrdU labeling assays to these 3D cultured cells. As shown in **Figure 4**, penetration by BrdU in $HepG_2$ cells were all gradually reduced, and the reduction was more significant in RADA16-I gels than those of in the Matrigel and Collagen-I systems (**Figure 4**).

Different ECM protein expression of $HepG_2$ cells in different culture systems

The expression levels of extracellular matrix (ECM) protein were analyzed for the understand the characterize $HepG_2$ cell status in 3D cultured systems. In the present study, our results suggested that the expression levels of fibronectin (FN) were observed in these three culture systems. Especially, the expression levels of FN were extremely highest in Collagen-I hydrogels, and lowest in Matrigel hydrogels (**Figure 5A**). As shown in **Figure 5B**, the expression levels of laminin (LN) showed no significant differences in the three cultured systems.

Cell factors expressions of $HepG_2$ cells in different culture systems

The expression levels of VEGFA, EGF, FGF2, and IGF1 were also analyzed to understand the tumorigenesis state of $HepG_2$ cells when they were cultured in different 3D culture systems. As shown in **Table 1**, our results showed that there were no significant differences in VEGFA, EGF, FGF2 levels of $HepG_2$ cells in the three cultured systems ($P>0.05$). However, IGF1 levels of $HepG_2$ cells cultured in RADA16-I and Collagen-I systems were lower than that those of in Matrigel system, with significant differences (*$P\leq0.05$, **$P\leq0.01$).

Migration rate of $HepG_2$ cells in different culture systems

To investigate the ability of $HepG_2$ cell migration in 3D cultured systems, we examined the cell migration rate by cell scratch assays. As shown in **Figure 6A**, we found that cell migration rate was lower cultured in RADA16-I system than those of cultured in Matrigel and Collagen-I systems (*$P\leq0.05$), suggesting that RADA16-I hydrogels may affect the cell migration of HepG2 cells. As shown in **Figure 6B**, results of cell scratch suggested that the cells cultured in RADA16-I system have similar trend with those cultured in Matrigel and Collagen-I systems.

Discussion

Recently, many studies have showed that 3D culture may reflect biological behaviors of cultured cells better than those of in 2D culture systems [19]. To establish ideal external 3D cell culture models, a variety of biomaterials have been used to mimic internal microenvironment, especially for synthetic polymer scaffold or animal-derived matrix [20-23]. However, the diameter of traditional synthetic scaffold is approxi-

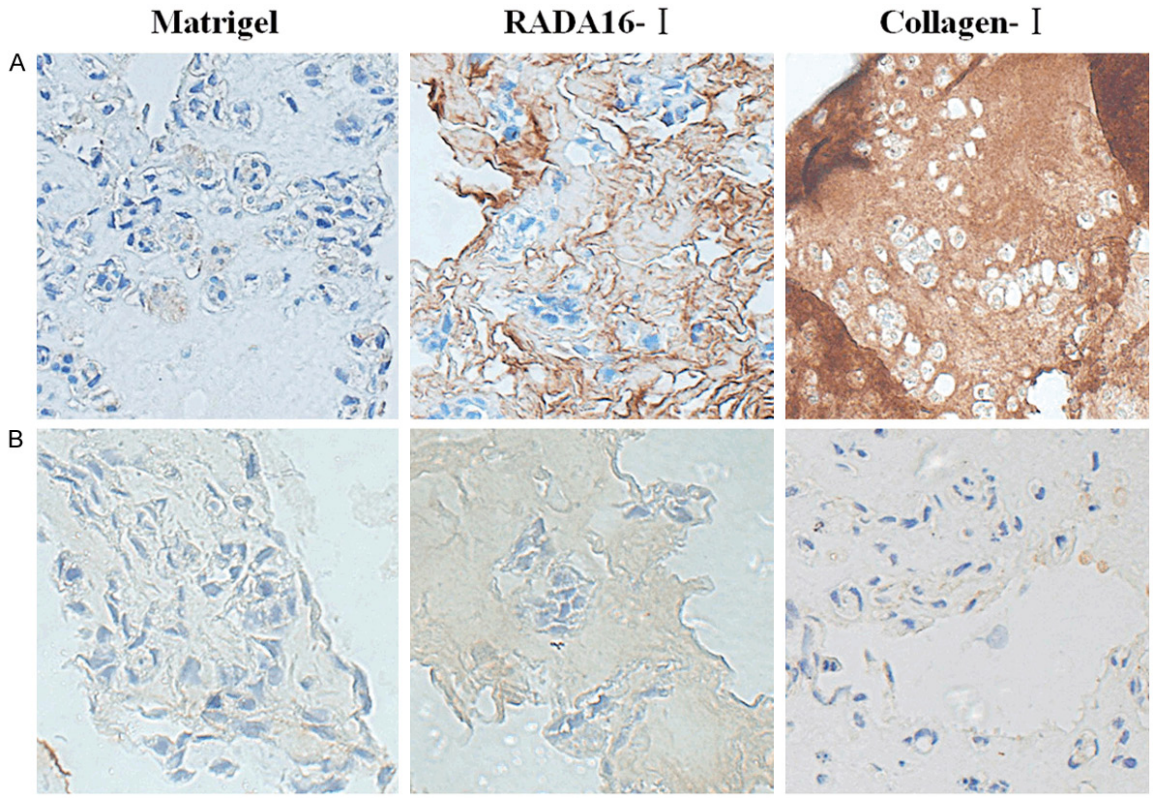

Figure 5. Distribution and expression pattern of Fibronectin (FN) (A) and Laminin (LN) (B) of HepG$_2$ cells in cultured Matrigel, RADA16-I and Collagen-I systems (7 d, ×200).

Table 1. Expression levels of VEGFA, EGF, FGF2 and IGF1 in HepG$_2$ cells in Matrigel, RADA16-I and Collagen-I systems (mean ± SD, ng/L)

Group	RADA16-I	Matrigel	Collagen
VEGFA	31.95 ± 0.01	39.46 ± 3.44	32.46 ± 1.51
EGF	6.87 ± 0.05	6.78 ± 0.03	6.90 ± 0.04
FGF2	2.11 ± 0.06	2.48 ± 0.35	1.89 ± 0.18
IGF1	2723.00 ± 20.40	5024.00 ± 41.50**	3376.00 ± 18.50*

Data represent mean ± SEM. *P<0.01, **P<0.05.

mately 10-100 μm, similar to most cell size. Thus, these cultured cells were actually still in the 2D environment, although they would adhere to the fiber surface [24, 25]. Some novel artificial polymer fibers have solved the above problems, but these materials may produce free radicals and other toxic chemicals in the process of polymer degradation. Thus, animal-derived matrix materials, such as Matrigel and Collagen-I, are regarded as ideal 3D culture materials by their excellent biocompatibility, abundant source and suitable mechanical properties [26]. However, these animal-derived biological materials contain various components. For example, Matrigel is the basement membrane matrix that isolated from EHS mouse tumors, including laminin, collagen-IV, nidogen, heparin, sulfate glycoprotein and other components. Different batches of Matrigel may have different amounts of growth factors and matrix metalloproteinases, reducing the reproducibility in cell cultures [23]. On the other hand, these materials are expensive and temperature-sensitive. These limitations bring great difficulties in 3D cell cultures.

Self-assembling peptides RADA16-I is synthesized by natural amino acids, and the fiber pore size is 5-200 nm. RADA16-I may assemble into a stable β-folding structure, forming nanofiber mesh stent structure similar to extracellular matrix with the water content as high as 99.5% [27]. Based on its suitable porosity rate and high water contents, RADA16-I may provide an adequate 3D microenvironment for cell cul-

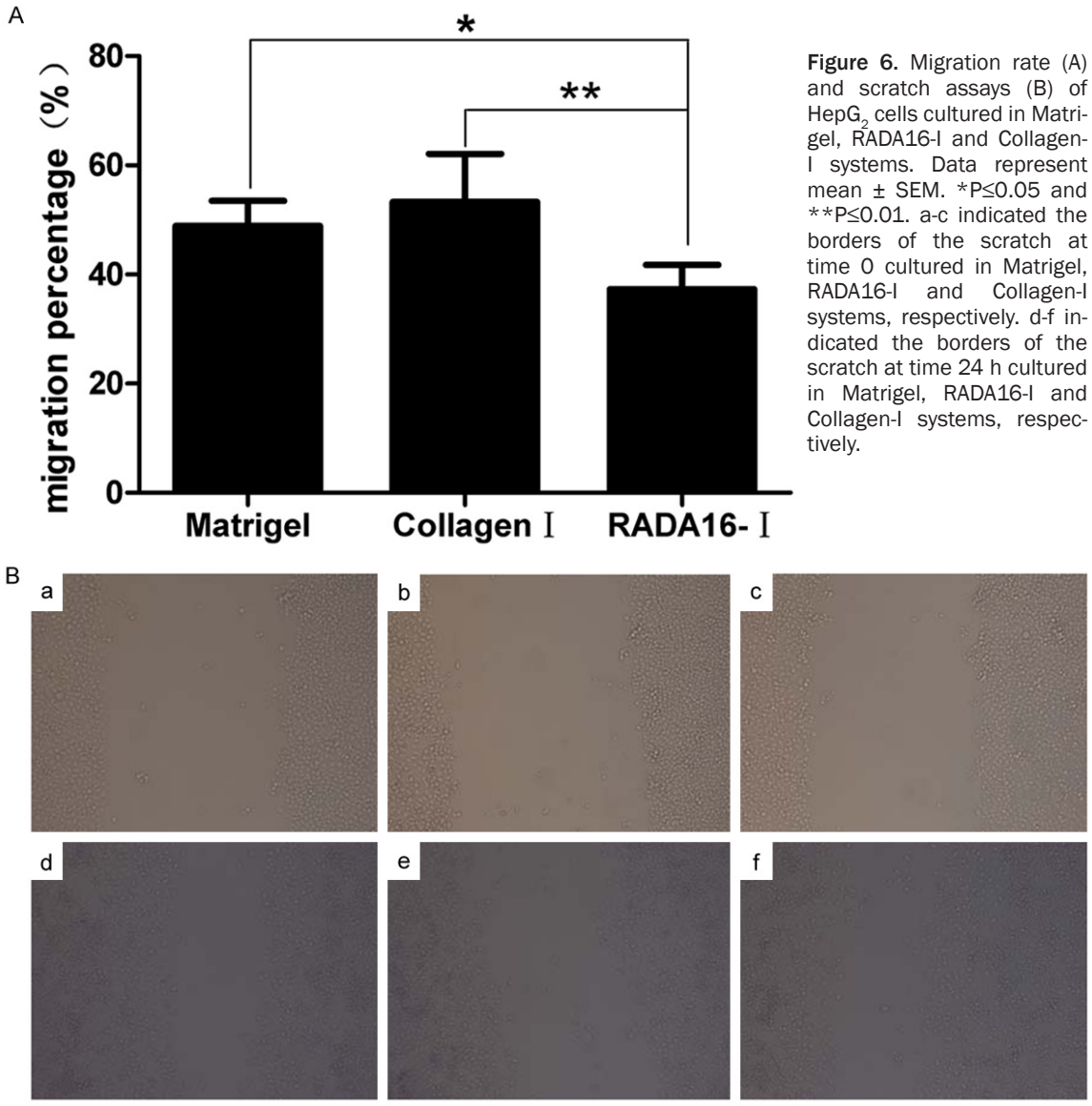

Figure 6. Migration rate (A) and scratch assays (B) of $HepG_2$ cells cultured in Matrigel, RADA16-I and Collagen-I systems. Data represent mean ± SEM. *P≤0.05 and **P≤0.01. a-c indicated the borders of the scratch at time 0 cultured in Matrigel, RADA16-I and Collagen-I systems, respectively. d-f indicated the borders of the scratch at time 24 h cultured in Matrigel, RADA16-I and Collagen-I systems, respectively.

tures. RADA16-I hydrogel is conducive to cell adhesion and growth, as well as microenvironment nutrients diffusion and absorption [28, 29].

Self-assembling peptide hydrogel RADA16-I has unique advantages Compared to Matrigel and Collagen-I. It may design to scaffolds for cells according to cell characteristics. Moreover, RADA16-I component is single without interference of impurities. It can be synthesized with large scales and lower cost. The degradation products are natural amino acids, with no significant immunogenicity [30]. There is no animal-derived pollution in RADA16-I gels, excluding the impact of animal-derived toxicity [31]. Compared with the temperature-sensitive materials, RADA16-I may not be affected by temperature changes during gelation, reducing operation difficulties.

In the present study, our results that $HepG_2$ cells grew well in these three scaffolds, suggesting that hepatoma cells have excellent biocompatibility with these three biomaterials. Our findings that $HepG_2$ cells exhibited different morphology and aggregation state in different 3D microenvironments during the culture processes, indicating that microenvironment might have a direct impact on the cell morphology and characterizations. During the cell cultures, we also found that $HepG_2$ cells exhibited different growth phenomena. For example, they showed a sustained and rapid growth In

Matrigel and Collagen-I gels, but a decline of proliferation rate in RADA16-I gels when grew rapidly up to the sixth day. The reason might be that Matrigel and Collagen-I were derived from animal materials, containing biological components such as growth factors, which may promote cell growth and proliferation. However, RADA16-I is a hydrogel self-assembled by natural amino acids, which provide a single microenvironment for cells. Thus, it was observed that rapid proliferation rates began to decrease with prolonging culture times. Moreover, the BrdU test further confirmed that cell proliferation in RADA16-I gels was inhibited, but there were still more than 65% of cells still proliferating in Matrigel and Collagen-I gels.

When $HepG_2$ cells were cultured in RADA16-I hydrogels, they may maintain the expression and secretion of various related factors such as fibronectin, laminin, VEGF, EGF, FGF-2 and IGF-1, which are similar to the other two hydrogels. These results indicated that RADA16-I was suitable for 3D culture of $HepG_2$ cells *in vitro*, which will help to maintain the stability of biological functions in $HepG_2$ cells. Results of cell scratch test suggested that cell migration rate cultured in RADA16-I gels were lower than those of cultured in Matrigel and Collagen-I gels. This may be due to the unique interaction between $HepG_2$ cells and RADA16-I gels. Zhang et al. reports had showed that one amino acid was a charged residue in these nanomaterials, and the charged residues may interact with cell surface components, regulating cell adhesion, proliferation and migration by certain signal pathways, and then RADA16 may limit certain cell behaviors [9]. Although many progresses have been made, the detailed mechanism between nano fiber matrixs and cells in microenvironment remain unclear. In the present study, 3D culture system of $HepG_2$ cells using RADA16-I hydrogel has been successfully established, which may provide not only a new tool for studies on signal transductions and drug resistance, but also afford us experimental and theoretical basis for the treatment of tumor by altering their microenvironment.

Conclusion

The present results showed that the 3-D culture system of $HepG_2$ cells using self-assembling nano-peptide RADA16-I has successfully established. Cells cultured in RADA16-I system may exhibit excellent stabilities of biological characterizations comparing with those of cultured in Matrigel and Collagen-I systems. However, cell proliferation, migration and other malignant phenotypes cultured in RADA16-I system were significantly restricted. Thus, the RADA16-I peptide and present findings may serve as a promising scaffold for the biological studies of cells in 3-D culture systems. Future studies will focus on the activating and/or inhibiting of signal pathways on the cell proliferation, migration and malignant phenotypes when $HepG_2$ cells were cultured in RADA16-I system.

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Disclosure of conflict of interest

None.

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