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Generating *in vitro* solid tumor models on gelatin-alginate scaffolds

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Abstract:

In recent years, three-dimensional (3D) in vitro tumor models have become more common in cancer research across the world. Selecting scaffolds, seeding cells into the scaffolds, and culturing them are pivotal for the morphologies and proliferation of cancer cell lines. Therefore, this experience has developed a 3D tumor model based on co-culturing MCF-7 cells and fibroblasts (3:1) into gelatin-alginate scaffolds (3x3x5 mm^3). Quantity, proliferation, and morphologies of tumors were evaluated through cell yield on a scaffold, enzymatic assay, cell number, hematoxylin and eosin stains (H&E), and scanning electron microscope (SEM). The results indicate that the complex of MCF-7 cell lines and fibroblasts in scaffolds proliferated and increased in terms of cell mass number and cell mass size. Their morphologies align with those of the in vivo figures demonstrated previously. This research facilitates new opportunities for the study of cancer biology and drug screening in Vietnam.

Keywords: **co-culture, fibroblast, Gelatin-alginate scaffold, MCF-7 cell line, three-dimensional (3D) *in vitro.***

Classification number: **3.2**

Introduction

Breast cancer is a leading cause of cancer deaths among women globally, following lung cancer [1]. Understanding cancer biology, definitively confirming diagnoses, and prescribing effective treatments are currently major challenges. Although it is common in cancer cell research, a monolayer culture system does not resemble characteristics of a typical tumor *in vivo,* such as adhesions and interactions among cells and extracellular matrices (ECM). Additionally, cell proliferation and increases in gene expression, which are caused by a deposition of ECM, have also been demonstrated to decline dramatically in 2D cell culture [2]. In addition, although the organ culture remains in terms of properties such as interactions among cells, matrices, and interstitial fluids, which results in acquiring extraordinary abilities in cellular differentiation, it encounters challenges in maintaining specimens and poor viability of the tissues [3]. The three-dimensional (3D) cell culture can overcome the obstacles of the 2D cell culture system *in vitro*, in the animal tumor, and cancer model. Firstly, ECM, stromal elements, and initial avascular regions support building the models and mimicking the tumors *in vivo*. Secondly, tumor-stromal interactions can create a more realistic environment, thereby eliciting an *in vivo* condition such as responses to cancer therapies. Thirdly, 3D cultures more closely resemble cell morphologies and their surroundings, which define the characteristics of these cells in the tumor mass [3, 4].

Gelatin is a natural polymer produced by the partial hydrolysis of collagen, which is the most common component in the connective tissues of mammals [5]. Because of its thermal sensitivity, gelatin can deposit multiple layers due to a process of gel transformation into sol [6]. Gelatin has been more commonly used than collagen across the globe; collagen presents challenges in terms of creating a concentrated collagen solution from its parent substance and is also more expensive than gelatin [5]. However, gelatin has poor mechanical properties, which limits its broader potential applications; using covalent crosslinking with alginate enhances the stiffness of the model [6]. Alginate is a naturally occurring nontoxic polysaccharide found in brown algae. Alginate is hydrophilic, biocompatible, and relatively economical [7]. While original alginate does not promote adherence and attachment among cells, gelatin does promote this [6]. Therefore, the mixing between gelatin and alginate help avoid their drawbacks.

MCF-7 (Michigan cancer foundation - 7) is the most

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commonly used breast cancer cell line in the world, with T-47D and MDA-MB-231 cell lines. MCF-7 is an ideal breast cancer cell line because it expresses Estrogen receptors (ER), which are sensitive to hormone response. Therefore, models that use MCF-7 have become common in recent years [8]. MCF-7 cultured in chitosan scaffold, collagen-alginate hydrogel, agarose [9-11] achieves effective results, but these disadvantages are the limits of the culture time (about eight days) due to the death of these cells.

To improve the probability of survival, researchers often combine MCF-7 with stromal cells, such as fibroblasts, adipose cells, etc. Therefore, this study aims to create 3D *in vitro* cancer models based on tissue engineering principles by combining MCF-7 cell lines with fibroblasts on gelatin-alginate scaffolds. Fibroblasts are used because they are the most abundant element in connective tissues. In addition, they secrete extracellular components to form a tissue scaffold [1]. Furthermore, fibroblasts have been demonstrated to be activated in cancer and create cancer-associated fibroblasts (CAF) [12], which facilitate the development, progression, invasion, and metastasis of breast cancer [1].

The connections between cell masses are the major advantage of this research; these are similar to the *in vivo* human tumors, while most three-dimensional models from previous research concerning gel often produce spherical spheres, which limit the development of tumor mass.

Materials and methods

Materials

MCF-7 cell lines, fibroblasts, and gelatin-alginate scaffolds were supplied by TEBM lab, University of Science, Vietnam National University, Ho Chi Minh city.

Trypsin/EDTA, DMEM/F12, FBS, penicillin-streptomycin, gelatin, and alginate were purchased from Sigma-Aldrich (St. Louis, MO).

Cell maintenance

Human mammary epithelial cells MCF-7 and fibroblast were cultured in Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 containing 10% FBS and 1% penicillin or streptomycin in plastic flasks to help maintain and expand them. Cells were incubated at 37°C and 5% CO_2. A sub-confluent culture (80-90% confluence) was utilized in the following experiments.

Preparation of gelatin or alginate sponges (GA sponges) as scaffolds for cells

Gelatin or alginate sponges were prepared based on the previously established protocol. Gelatin and alginate (Sigma) were completely dissolved in distilled water at 50°C to create 1% gelatin and 1% alginate solutions. These solutions were then mixed with a ratio of 8 gelatin per 2 alginates in terms of volume and incubated at -80°C for 24 hours. These frozen blocks were then incubated in 0.3% EDC for 24 hours at 4°C in dark conditions and freeze-dried. Finally, GA sponges were sterilized by irradiation in 25 kGy and cut into small pieces of 5x5x3 mm^3.

The cell culture on GA scaffold

MCF-7 breast cancer cells and fibroblasts were maintained in a T-flask in an incubator at 37°C and 5% CO_2. We used an incubation method to inject cells into scaffolds. The inoculum concentration consisted of $2x10^4$ cells and $2x10^5$ cells for all experiments involving cell culture with the ratio of 1 fibroblast per 3 MCF-7. Fibroblasts ($0.5x10^4$ cells) and scaffold complexes were cultured in a 96-well dish at 37°C and 5% CO_2 for 20 minutes. Then, 200 µl were added to the dish. After three days, MCF-7 ($1.5x10^4$ cells) was injected into these scaffolds and maintained at 37°C and 5% CO_2. The procedure was similar to the sample of $2x10^5$ cells.

Cell seeding efficiency was determined by counting the cell density in the supernatant.

After seeding cells into scaffolds, shaking culture was maintained for four weeks. The culture medium was changed every two days.

Control samples were used without scaffolds. First, fibroblasts ($0.5x10^4$ cells) were seeded into a 96-well dish before being supplemented with a 200 µl culture medium at 37°C and 5% CO_2 for 20 minutes. Second, MCF-7 cells ($1.5x10^4$ cells) were seeded in a 96 well plate three days later. The procedure is similar to the sample of $2x10^5$ cells.

Cell Proliferation Assay

After two days, the supernatant media were collected for glucose estimation. Glucose concentration in the culture supernatants was determined by enzymatic assays; the changes in optical density were measured and compared with standard solutions.

Cell morphology analysis

Scanning electron microscopy (SEM, S-3000N) and hematoxylin and eosin (H&E) staining were used to evaluate the cell morphology in the GA scaffold. First, tumor masses were washed three times with cold D-PBS and fixed with 3% Glutaraldehyde for 24 hours. The cells were then dehydrated in a range of ethanol concentrations (50, 70, 80, 90, and 100%). For H&E staining, the samples were fixed in 10% formalin and embedded in paraffin. The specimens were cut into slices and strained by hematoxylin and eosin (H&E) to analyse the morphologies.

Immunocytochemistry staining

The specimen was stained with vimentin to determine

changes in the characteristics of the tumor mass.

Statistical analysis and image processing

Statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). ($P<0.05$), which determined statistical significance.

Images were developed by ImageJ version 1.52e (National Institutes of Health, Bethesda, Maryland, U.S.).

Results

The method of seeding MCF-7 and fibroblasts into GA scaffolds

Incubation is regarded as a common method because cell adhesion is more stable on the scaffold relative to centrifugation (Fig. 1). Although simple, this method is overly tedious and is difficult to execute in low concentrations.

We have selected a $2x10^4$ cells scaffold based on the research of Pan, et al. (2016), Zheng, et al. (2012), and Dhiman, et al. (2005) [6, 9, 10]. The purpose of the increase in the number of cells to a $2x10^5$ cell scaffold is to assess the cell adhesion of these tumors and form the extraordinary tumor structures. The result is that a low concentration is fairly similar to a high concentration ($p^*>0.005$). Therefore, we implemented both concentrations for the following experiments.

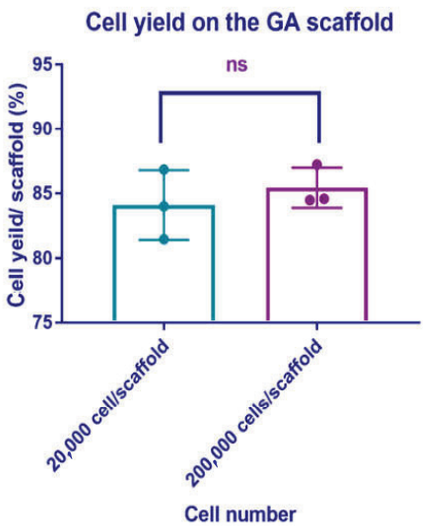

Fig. 1. The yield of cells on the GA scaffolds using a low concentration ($2x10^4$ cell scaffold) and a high concentration ($2x10^5$ cell scaffold).

The growth of MCF-7 cells on the GA scaffolds

Warburg et al. assume that tumor cells rapidly use the glucose and convert it into lactate. This is the most fundamental and enduring observation related to tumor metabolism [6]. Therefore, the determination of the glucose concentration remaining in the solution after the culture is based on OD assay. Glucose concentration and proliferation are inversely correlated.

In the $2x10^4$ cell scaffold samples (low-concentration samples), cell mass continuously proliferated to day 4 and was then stable from day 6 to day 18; it then decreased by day 22. Particularly after day 22, cell proliferation generally resurfaced but declined after day 26 (Fig. 2). This result aligns slightly with Dhiman's research, in which glucose concentration decreased dramatically by day 6 (rapid cell proliferation by day 6) [10]. In addition, Zheng, et al. (2012) have demonstrated that the decrease in glucose concentration by day 4 aligns with this experiment [9]. However, the decrease in proliferation from day 18 to 22 results from breaking through cancer cell masses and cell death, which results from long-term hypoxia in the tumor. The repeated proliferation clearly reveals that unequal nutrition distribution in the scaffold is responsible for a heterogeneous tumor in these scaffolds. Although loss occurs in some cell masses, the others begin to proliferate drastically. However, these masses decline rapidly without a stable stage. This is because of acid accumulation, which causes an unstable scaffold (weak mechanical property) and reduced cell adhesion with ECM. It has been that a GA scaffold supported adhesion and proliferation of the MCF-7 cell line in about 18 days.

The $2x10^5$ cell scaffold samples (high-concentration samples) facilitated cell proliferation effectively on the GA scaffold as well; however, they did not do so more effectively than the low concentration samples. This is because the initial number of cells seeding into the scaffold was considerably excessive relative to the scaffold space and the culture medium. It is insufficient for cells to proliferate and grow; cells compete with each other, and these cells may be dead. Cell adherence to the scaffold then continuously proliferates and grows but is limited relative to the other.

The cancer cells slightly proliferated up to day 12. However, the proliferation of the tumor increased dramatically from day 12 to day 18 and had then declined by day 22. After day 22, its figure resembled a low concentration sample. The extremely high cell concentration caused a vast number of cells to not create masses, and the shortage of nutrition caused the cell death. After creating stable cell masses, it began to proliferate rapidly.

Relative to control samples, tumor proliferation in the scaffold was more significant after day 8; the scaffold supported the samples to allow them to be maintained and developed effectively.

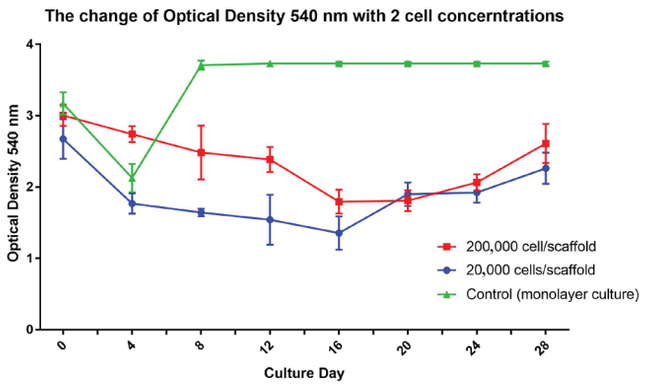

Fig. 2. Cell growth in 28 days. A blue line of the $2x10^4$ cell sample, a red line of the $2x10^5$ cell sample, and a green line of monolayer samples.

The cells adhered to the scaffold and proliferated after seeding into the scaffold. In the microscope slides, cells created clusters like spheroids, which were disseminated uniformly in the scaffold. It was observed that round-like cells gathered in the 'tumorid' (Figs. 3A, 3B). This aligns with Remedios Castello-Cros' research, which describes round and less-spread cancer cells on the scaffold [13].

While cells in the monolayer culture spread on the surface of TPCS dishes and exhibited sheet-like trigonal or polygonal morphologies, those on the scaffold displayed a spheroid shape (Fig. 3C). Cells and their interactions filled and formed a multi-layer tumor cell mass. The findings of Lei Chen, et al. (2012) [2] and Hongzhou Huang, et al. (2013) [14] have demonstrated similar outcomes.

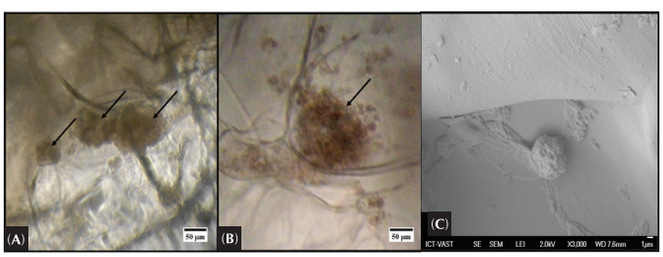

Fig. 3. Images of cell mass on the GA scaffold from the microscope. (**A**) $2x10^4$ cell scaffold sample (20x), (**B**) $2x10^5$ cell scaffold sample (20x). The black arrow indicates cell mass, (**C**) SEM image of the cancer cell mass on the GA scaffold (3000x).

With routine fibroblast H&E staining, only the highly thin, elongated nuclei of the cells are clearly visible [15]. In the tumor, the cancer cell was stained dark violet and had a round nuclei structure [2].

With H&E staining, cells in the $2x10^4$ cell scaffold sample created round cell masses or were spheroid at day 0 (Figs. 4A, 4D). In addition, oblong cell masses appeared (Figs. 4B, 4C). These masses often had marge round dark-violet-stained cell nucleus and fibroblast whose nuclei are spindle-shaped. The nucleus is bigger than ordinary ones because DNA nuclear is double in dozens of time. Particularly, Fig. 4D displays many cells whose nuclei were elongated and stained darker than other fibroblasts (black arrows).

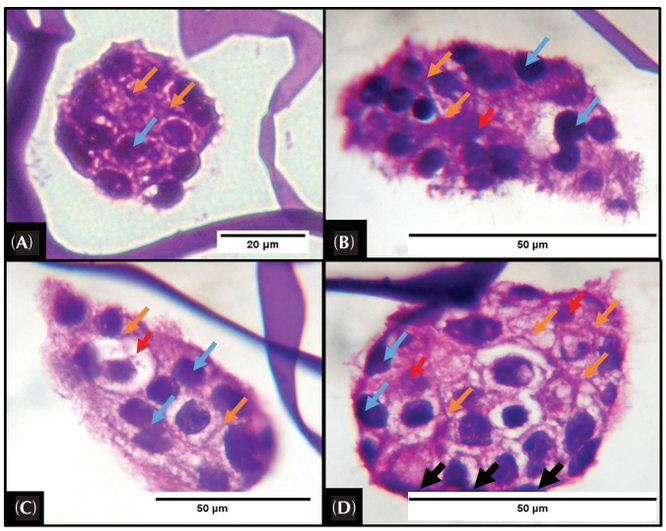

Fig. 4. Images of cancer cell masses with a $2x10^4$ cell scaffold at day 0. Orange arrows indicate cell membranes, blue arrows indicate nuclei of cancer cells, red arrows indicate necrosis area, and black arrows indicate elongated nuclei.

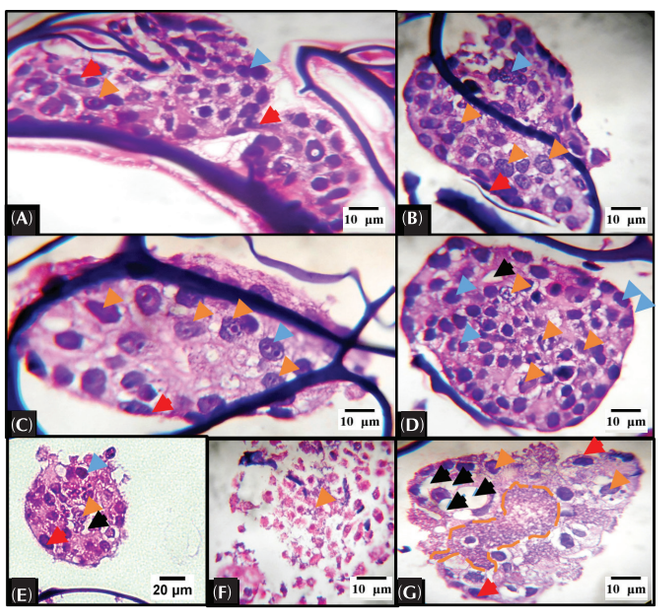

Fig. 5. Images of cancer cell mass of a $2x10^4$ cell scaffold at different culture times.
(**A**+**B**+**C**+**D**) Day 7; (**E**+**F**) Day 14; (**G**) Day 21. Blue arrows indicate normal nuclei of cancer cells (particularly cell division at image B and image C), orange arrows indicate necrotic cells, red arrows indicate the elongated nuclei (image A displays the bridge between two masses), and black arrows indicate lumen in tumors.

Cell morphology had changed dramatically by day 7; the nuclei were paler than on sample day 0 and were multi-lobed (some granules) (Fig. 5). Fig. 5A illustrates the connection of two cell masses; this is a reason why there were larger tumors. These masses' nuclei were the same as a sample at day 0; therefore, combining masses helped the cells to develop better. A particular structure in Fig. 5B had nuclei which were divided into two or four lobes.

Tumors tended to break up into smaller cell masses after two weeks (Fig. 5E); the cancer cell masses were not as dense and solid as at day 0. Xiong, et al. cultured an MDA-MB-231 cell line on the bacterial cellulose. Xiong, et al. described the lumina of the cell mass, which is the space inside of a tubular structure, as being covered by one or two cell layers [16]. At day 21, tumor masses have a fibroblast area which strains denser than other areas (an orange circle at Fig. 5G). This may be an area which contains CAF that has interacted with a normal cancer cell.

For the $1.5x10^5$ cell scaffold sample, the structure of the cell mass is similar to the $1.5x10^4$ cell scaffold sample. However, the structure of cell mass at the former is less solid than the latter at day 7 and day 14.

According to the $2x10^5$ cell scaffold sample, cells were dead at day 0 because of extremely high density (Fig. 6A). Dead cells were rejected after eliminating old mediums and supplying new ones every two days. As with the low concentration sample, after the cell masses were broken apart, cells at day 7 tended to create a larger mass. In addition, cell masses had many extraordinary structures, such as pyknosis, karyorrhexis, karyolysis, etc. (Fig. 6B).

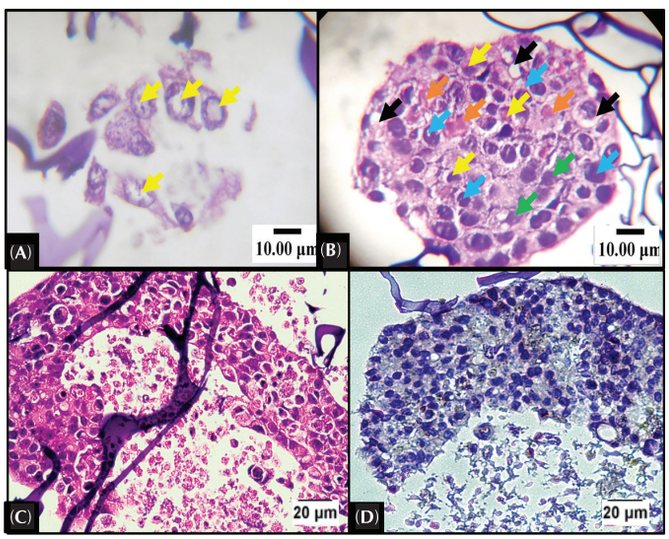

Fig. 6. Images of cancer cell masses with a $2x10^5$ cell scaffold at different culture times. (**A**) Day 0; (**B**) Day 7; (**C**) Day 28; (**D**) Day 28 (vimetin strain). Necrosis cell: green arrows (pyknosis), yellow arrows (karyorrhexis), and orange arrows (karyolysis). Blue arrows indicate the normal nuclei of cancer cells, and black arrows indicate lumen in tumors.

At days 14 and 21, no cell masses were recorded in the scaffold, but at day 28, we observed the special structure, which had non-necrosis areas and necrosis areas (Figs. 6C, 6D). When straining with vimentin, the substance confirmed the mesenchymal origin of some tumors; this was expressed in the tumor masses (Fig. 6D).

Discussion

Although numerous cells proliferated drastically in the shake culture condition, other cells which were located deeper inside the tumor tended to lack nutrients and oxygen (hypoxia). Evidence suggests that tissue hypoxia is a crucial element in initiating tumor necrosis during tumor progression because it contributes to tumors' rapid growth and the inadequate supply of blood [17]. Tumor hypoxia is an important feature of aggressive cancers [18]. Cancer cells in necrotic areas can express different characteristics, such as dedifferentiation and malignancy. This causes specific features of the tumor, such as high-grade structure and resistance to radiation and chemotherapy [19].

Nuclear changes assume one of three patterns, which all result from the breakdown of DNA and chromatin. The first pattern is pyknosis, which is characterised by nuclear shrinkage and increased basophilia, in which the DNA condenses into a dark shrunken mass. In karyorrhexis, the pyknotic nucleus can undergo fragmentation (Figs. 5D, 6B). Lastly, the basophilia fades due to fragment DNA by DNase activity (karyolysis). In one to two days, the nucleus in a dead cell completely disappears (Fig. 6B) [17].

The changes in glucose consumption were suitable for the tumor proliferation indicated by histological images. For example, breakdowns in tumor mass are correlated with decreasing glucose consumption after day 18 and the creation of new clusters after day 21, which caused an increase in glucose consumption at day 22 in the low concentration sample. After breaking down the cancer cell mass on day 14 at a low concentration sample and on day 0 at a high concentration sample, new tumors were formed with extraordinary structures. This demonstrates that the tumor changed its properties, which may be the primary reason for drug resistance and tumor aggression (Figs. 5G, 6C).

Notably, at higher concentrations, there were masses at day 28, although they were observed at days 14 and 21. Cells in broken masses could perhaps gather to create new masses. In addition, the result of the vimentin strain (Fig. 6D) demonstrated that these cells produced a mesenchymal characteristic, which rendered the tumor masses more malignant.

The optimal duration of the development of cells on the GA scaffold was about seven days. To increase the productivity of the tumors, cells were grown in the rotating cell culture system (RCCS). It was rotated in a gentle fluid orbit so that they could make contact with the others, form

complex 3D structures, and attain a tissue-like phenotype. Therefore, cells in the RCCS functionally resembled tissues in the human body [3].

Tumor tissues have three zones, including the tumor, interface, and normal ones. Normal tissues contained intermingled fibro-fatty tissue, which is an interface area composed of altered fibroblast and a tumor sector characterised by an invasive ductal type of carcinoma [20]. A sample day 21, we recorded the altered fibroblast area (orange area), which contained CAF with darker straining relative to the surrounding areas (Fig. 5G). The appearance of lobular ducts indicated that these fibroblasts are altered into breast cancer-specific ones.

Through SEM images, scaffold structures with multiple layers indicated that the formation of masses was caused by the connection of cell clusters and adhesive elements which these cells secrete. The development and acid secretion of tumor masses imposed pressure on the GA scaffold, which spurred the collapse into the layers of the scaffold network. The gelatin and alginate provided the micro-environment in which cells could stably settle.

Because of the lower number of cells, the morphology of the MCF-7 line was spheroid, while there were many morphologies for a higher number of cells (luminal, spheroid). Subsequently, 3D models in previous research often formed separate spheroids. The advantages of this model include the cell-cell interactions, as well as tumorid-tumorid interactions, which mimic breast tumors *in vivo*.

The tumor microenvironment is regarded as a temporal and spatial 3D structure with distinctly physiochemical and histopathological properties, which are pivotal in cell molecular signaling, morphology, motility, differentiation and proliferation [4]. However, the technique applied in this research is not simple, and the morphology of cells is heterogeneous; this causes problems research in terms of the effect of drugs on cells.

Conclusions

Seeding MCF-7 cells and fibroblasts into the scaffold through incubation at 37°C and 5% CO_2 in 20 minutes with a $2x10^4$ cell scaffold and a $2x10^5$ cell scaffold enables cells to grow and proliferate on the scaffold.

The development of the tumor in this research is similar to the tumor mass *in vivo*. The appearance of hypoxia necrosis is an indispensable tendency and helps cancer invade and metastasis.

The cell-scaffold complex created through incubation has a form which is similar to human breast tumor masses.

The authors declare that there is no conflict of interest regarding the publication of this article.

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