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An alginate-based platform for cancer stem cell research

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ABSTRACT

As the primary determinants of the clinical behaviors of human cancers, the discovery of cancer stem cells (CSCs) represents an ideal target for novel anti-cancer therapies (Kievit et al., 2014). Notably, CSCs are difficult to propagate *in vitro*, which severely restricts the study of CSC biology and the development of therapeutic agents. Emerging evidence indicates that CSCs rely on a niche that controls their differentiation and proliferation, as is the case with normal stem cells (NSCs). Replicating the *in vivo* CSC microenvironment *in vitro* using three-dimensional (3D) porous scaffolds can provide means to effectively generate CSCs, thus enabling the discovery of CSC biology. This paper presents our study on a novel alginate-based platform for mimicking the CSC niche to promote CSC proliferation and enrichment. In this study, we used a versatile mouse 4T1 breast cancer model to independently evaluate the matrix parameters of a CSC niche – including the material's mechanical properties, cytokine immobilization, and the composition of the extracellular matrix's (ECM's) molecular impact – on CSC proliferation and enrichment. On this basis, the optimal stiffness and concentration of hyaluronic acid (HA), as well as epidermal growth factor and basic fibroblast growth factor immobilization, were identified to establish the platform for mimicking the 4T1 breast CSCs (4T1 CSCs) niche. The 4T1 CSCs obtained from the platform show increased expression of the genes involved in breast CSC and NSC, as compared to general 2D or 3D culture, and 4T1 CSCs were also demonstrated to have the ability to quickly form a subcutaneous tumor in homologous Balb/c mice *in vivo*. In addition, the platform can be adjusted according to different parameters for CSC screening. Our results indicate that our platform offers a simple and efficient means to isolate and enrich CSCs *in vitro*, which can help researchers better understand CSC biology and thus develop more effective therapeutic agents to treat cancer.

Statement of Significance

As the primary determinants of the clinical behaviors of human cancers, the discovery of cancer stem cells (CSCs) represents an ideal target for novel anti-cancer therapies. However, CSCs are difficult to propagate *in vitro*, which severely restricts the study of CSC biology and the development of therapeutic agents. Emerging evidence indicates that CSCs rely on a niche that controls their differentiation and proliferation, as is the case with normal stem cells (NSCs). Replicating the *in vivo* CSC microenvironment *in vitro* using three-dimensional (3D) porous scaffolds can provide means to effectively generate CSCs, thus enabling the discovery of CSC biology. In our study, a novel alginate-based platform were developed for mimicking the CSC niche to promote CSC proliferation and enrichment.

1. Introduction

Cancer is a major disease with detrimental health effects and is also associated with high incidence and mortality rates worldwide [2]. There have been significant challenges in developing effective

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treatments for cancer due to the complexity of its pathogenesis and molecular mechanisms, which have not been fully understood [3,4]. Growing evidence has suggested that cancer is a stem cell-based disease, and it is analogous to the growth of normal proliferative tissues such as bone marrow, skin and intestinal epithelium, and that the growth of cancer tumors is fated by the cancer stem cells (CSCs), which possess the capacity for self-renewal, unlimited proliferation, and multidrug and radiotherapy resistance [5]. As such, discovering the CSCs will greatly facilitate the development of novel research strategies to investigate the occurrence, development, and recurrence of tumors [6–8].

Although much progress has been made in the study of CSCs, many issues remain to be addressed by research. One issue is the difficulties to isolate and propagate CSCs owing to the small percentage of CSCs found in tumors. For example, only about 2% of breast tumor cells are comprised of CSCs, and 0.1–1% of acute myeloid leukemia (AML) cells are CSCs [9,10]. The most common methods used to isolate CSCs are serum-free culture and fluorescence-activated cell sorting (FACS); however, both methods have certain shortcomings. Serum-free culture requires large volumes of expensive and specialized media and is frequently unsuccessful due to the small percentage of CSCs found in the tumor of origin. The FACS approach requires costly antibodies and dedicated equipment, yielding low numbers of viable cells. Therefore, there is an essential need to establish new and extensive screening methods to isolate and propagate CSCs *in vitro* [11,12].

Normal stem cells (NSCs) have been found to reside within a "stem cell niche", which plays critical roles in the maintenance of stem cell characteristics, such as pluripotency and self-renewal [13]. Recent data imply that CSCs also share a similar niche as NSCs, referred to as the "CSC niche", which regulates their stemness and proliferation [14,15]. The CSC niche is complex and includes diverse stromal cells, the extracellular matrix (ECM), and soluble factors secreted from the niche's cells [15]. Mounting evidence suggests that the ECM is an essential noncellular component of the adult stem cell niche and that its rigidity and organization play important roles in stem cell differentiation and wound healing, as well as in cancer pathologies [16–20]. Porous hydrogel is widely used to mimic NSCs or the tumor microenvironment's ECM, and substantial evidence from recent studies has suggested that it can regulate stem cell differentiation, while promoting CSC selection and proliferation by adjusting the stiffness of the hydrogel [21–23]. Furthermore, emerging evidence has supported the idea that three-dimensional (3D) culture can promote cell reprogramming and tumor malignancy; and that in the tumor microenvironment where CSCs and non-CSCs maintain balanced, 3D culture can promote the reprogramming of non-CSCs to CSCs [20,24]. Evidence from recent studies has also shown that it is feasible to proliferate and enrich CSCs by culturing the tumor cells on the porous hydrogel [1,25].

As a major glycosaminoglycan of the ECM, hyaluronic acid (HA) has been described as one of the components of the stem cell niche. HA has been found enriched in many types of tumors and associated with tumor growth and invasion [26–29]. Growing evidence has demonstrated that CSCs share many similar signaling pathways with NSCs; however, there is an imbalance in these pathways [30]. Researchers investigated the use of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) in maintaining the stemness and proliferation of NSCs [31]. Subsequently, many researchers have isolated CSCs from various tumors via serum-free culture combined with EGF and bFGF [32,33]. As cytokines of the CSC niche, EGF and bFGF can stimulate CSC self-renewal and stemness [14].

In this paper, we present the synthesis and material characterization, and perform *in vitro* studies on alginate-based 3D porous hydrogels for mimicking the CSC microenvironment ECM. In our study, we also demonstrate that the stiffness of the hydrogels can be modulated by changing the concentration of alginate used for cross-linking. HA is added to alginate-based hydrogels at different molecular weights and concentrations, with limited influence on the stiffness of the hydrogel, and that EGF and bFGF, cytokines of the CSC niche, can be covalently linked to the oxidized alginate hydrogel and the cytokines which chosen to link to the oxidized alginate hydrogel could be changed for different needs. On this basis, a platform that features with the optimized stiffness and HA concentration as well as immobilized EGF and bFGF was developed and used for the rapid and efficient isolation of 4T1 breast CSCs with high expression of CD44 and Sca-1 as well as low expression of CD24, which were proven as breast CSCs markers and often used for breast CSCs isolation from 4T1 breast cancer cells [33]. Taken together, we illustrate the novel *in vitro* platform comprised of the ECM with appropriate stiffness, cytokines, and HA is promising to mimic the *in vivo* CSC niche for isolating thus discovering and treating the CSCs.

2. Materials and methods

2.1. Cell lines and materials

Mouse 4T1 breast cancer cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, People's Republic of China). 2B11 hybridoma cells for secreting the crypito-1 antibody, were purchased from the Biosynthesis Biotechnology Company (Beijing, People's Republic of China). Dulbecco's Modified Eagle'sMedium:Nutrient Mixture F-12 (DMEM/F-12), Roswell Park Memorial Institute (RPMI) 1640 medium, B-27 supplement, GlutaMAX™ supplement, and fetal bovine serum(FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Propidium iodide(PI), calcein, phalloidin, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). bFGF and EGF were purchased from Peprotech(RockyHill, NJ, USA). Mouse monoclonal CD44 primary antibodies conjugated with FITC, mouse monoclonal CD24 primary antibodies conjugated with phycoerythrin (PE), mouse monoclonal MDR1 primary antibodies, mouse monoclonal Dclk1 primary antibodies, FITC-conjugated goat polyclonal secondary antibodies to rabbit immunoglobulin (Ig)G, and rhodamine-conjugated rat polyclonal secondary antibodies to rabbit IgG were purchased from Abcamplc (Cambridge, MA, USA).

2.2. Experimental animals

Balb/c female mice (SPF), 6–8 weeks of age, were provided by the Laboratory Animal Center of Harbin Medical University. All animals were fed ad libitumand kept under the normal 12-h light/12-hourdark cycle. All procedures were approved by the University Ethics Committee of the Harbin Institute of Technology.

2.3. Synthesis of hydrogel and its physicochemical characterization

Alginate (low viscosity) and HA were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Alginate hydrogels were prepared as previously reported [34,35]. Briefly, alginate was dissolved in 100 mL of distilled water to a concentration of 20 mg/mL. The alginate was purified by the addition of ethanol. Then, the precipitated alginate was dialyzed, lyophilized, and dissolved in distilled water to obtain different concentrations. The alginate hydrogel was prepared through cross-linking with calcium ions, and the alginate–HA hydrogel was formed through the addition of HA. The gelation process and the mechanical properties of the alginate hydrogels were evaluated by examining the time of gelation onset and the evolution of elasticity at 37 °C in constant strain mode by means