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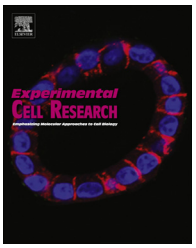

Research Article

# Potential effect of matrix stiffness on the enrichment of tumor initiating cells under three-dimensional culture conditions

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ABSTRACT

Cancer stem cell (CSC) or tumor initiating cell (TIC) plays an important role in tumor progression and metastasis. Biophysical forces in tumor microenvironment have an important effect on tumor formation and development. In this study, the potential effect of matrix stiffness on the biological characteristics of human head and neck squamous cell carcinoma (HNSCC) TICs, especially the enrichment of HNSCC TICs, was investigated under three-dimensional (3D) culture conditions by means of alginate gel (ALG) beads with different matrix stiffnesses. ALG beads with soft (21 kPa), moderate (70 kPa) and hard (105 kPa) stiffness were generated by changing alginate concentration. It was found that significant HNSCC TIC enrichment was achieved in the ALG beads with moderate matrix stiffness (70 kPa). The gene expression of stemness markers Oct3/4 and Nanog, TIC markers CD44 and ABCG2 was enhanced in cells under this moderate (70 kPa) stiffness. HNSCC TIC proportion was also highly enriched under moderate matrix stiffness, accompanying with higher tumorigenicity, metastatic ability and drug resistance. And it was also found that the possible molecular mechanism underlying the regulated TIC properties by matrix stiffness under 3D culture conditions was significantly different from 2D culture condition. Therefore, the results achieved in this study indicated that 3D biophysical microenvironment had an important effect on TIC characteristics and alginate-based biomimetic scaffolds could be utilized as a proper platform to investigate the interaction between tumor cells and 3D microenvironment.



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## Introduction

The tumor initiating cell (TIC) model, also referred to as cancer stem cell (CSC) model, proposes that tumors are hierarchically-organized tissues with TICs/CSCs at the apex [1]. TICs have the ability to self-renew and to recapitulate the hierarchy of the original tumor from which they are derived [2]. TICs have recently been reported to be involved in tumor progression, recurrence and migration [3]. A better understanding of the regulation of TICs might thus facilitate the development of improved therapeutic strategies for cancer patients.

TIC characteristics are highly dependent on their microenvironment [4,5]. Cells in living organisms are continuously exposed to physical forces such as compression, tension, hydrostatic pressure and shear stress. Cells sense physical forces in the local cellular microenvironment and modify their behaviors in response to these physical signals [6]. Solid tumors *in vivo* tend to have different matrix rigidities than normal tissues [7], and growing evidence suggests that matrix stiffness can regulate the proliferation [8], migration [9], chemotherapeutic response [10] and stem cell-like characteristics [10,11] of cancer cells. TIC characteristics may thus also be highly influenced by matrix stiffness. However, most studies examining the relationship between matrix stiffness and cell behavior have been performed in two-dimensional (2D) culture systems [12–14], while three-dimensional (3D) cultures are known to be superior to 2D cultures for recapitulating the *in vivo* cell microenvironment. 3D-cultured cells have the ability to acquire phenotypes and respond to stimuli in analogous manners to *in vivo* biological systems [15]. The results of substrate stiffness studies performed in 2D-culture systems may thus fail to reflect the *in vivo* situation. It is therefore important to develop stable 3D-culture systems with controllable matrix stiffness to investigate the potential effects of matrix stiffness on cancer cells, especially TICs.

Several 3D scaffolds have been used to study TIC properties, including collagen hydrogel [16], polyethylene glycol diacrylate (PEGDA) hydrogel [11], and soft salmon fibrin [17]. As the important constituents of the extracellular matrix (ECM), the stiffness of collagen and fibrin hydrogels can be modified by changing the concentrations of the materials [17,18]. However, it is difficult to study TIC responses to individual factors in the microenvironment, such as matrix stiffness, because of the complex interactions between biochemical signals provided by the ECM molecules and cell surface receptors. Although PEGDA hydrogels, as an inert synthetic material, can be used to elucidate the effects of some individual factors on cell fate [19–22], photochemical cross-linking strategies under non-physiological conditions may affect TIC properties.

Alginate is a naturally-occurring anionic polysaccharide with a molecular structure similar to polysaccharides in the ECM *in vivo*. As an ion-cross-linked hydrogel, the gelatinization and degradation of alginate hydrogels under physiological conditions are not associated with any toxicity, in contrast to chemically-cross-linked hydrogels. Moreover, the matrix stiffness of alginate gel (ALG) can be conveniently altered by adjusting the alginate concentration [23]. The lack of any intrinsic bioactivity of alginate molecules means that changing the matrix stiffness by adjusting the alginate concentration [23] has no effect on the biochemical signals provided by the gel. In addition, we previously showed that the metastatic ability of hepatocellular carcinoma cells was promoted in ALG beads [24]. ALG beads might thus provide a potential 3D-culture system for TIC enrichment and may also be suitable for investigating the interaction between matrix stiffness and TICs under 3D-culture conditions.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer, and is associated with a poor prognosis [25]. Recurrence and distant metastases are the main causes of death in patients with HNSCC, both of which are thought to be related to the existence of TICs [26]. Since Prince et al. initially reported the existence of TICs in HNSCC [27], they have been identified *via* surface markers [28,29], the determination of aldehyde dehydrogenase activity [30], and the ability to efflux vital dyes [31]. In addition, TICs can be maintained in an undifferentiated state in tumor spheres that are formed under serum-free conditions in the presence of growth factors, such as EGF and bFGF. These tumor spheres have been proved to be enriched for stem cell markers, CD44, Oct3/4 and Nanog [28,32]. This method has been broadly used for isolating TICs in several types of tumors [33–35]. Further understanding of TICs might contribute to improved therapeutic and survival outcomes in patients with HNSCC.

In this study, we therefore used ALG beads with different matrix stiffnesses to investigate the potential effects of 3D matrix stiffness on the biological characteristics of TICs from HNSCC, especially in terms of the enrichment of TICs. We also discussed the possible mechanisms underlying the relationship between 3D matrix stiffness and TIC characteristics.

## Materials and methods

### Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Sodium alginate (Qingdao Jingyan Bio-Tech, Qingdao, China) was purified by removing protein and endotoxin, according to the protocol used in our laboratory. The molecular weight of the sodium alginate was 500 kDa and the G:M ratio was 33:67.

Three human HNSCC cell lines were used in this study. Tca 8113 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SAS [36] and HSC-4 [37] were kindly provided by Prof. Fujii Masato (National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan).

The antibodies used in this study were ABCG2 (Santa Cruz Biotechnology, Dallas, TX, USA), CD44-FITC (eBioscience, San Diego, CA, USA), Alexa-488-phalloidin (Invitrogen, Carlsbad, CA, USA), Mouse monoclonal β-Actin (Santa Cruz Biotechnology), Rabbit polyclonal FAK (Proteintech, Wuhan, China), Rabbit polyclonal ERK1/2 (Proteintech), Rabbit polyclonal phospho-FAK (Tyr397) (Cell Signaling Technology, Danvers, Massachusetts, USA), and Rabbit monoclonal phospho-ERK1/2 (Thr202/Tyr20) (Cell Signaling Technology). The appropriate species-specific antibodies conjugated to rhodamine (Invitrogen) were used as second antibodies. Hoechst 33342 was used for nuclear staining.

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