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# Effects of spreading areas and aspect ratios of single cells on dedifferentiation of chondrocytes

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ABSTRACT

Dedifferentiation of chondrocytes is common in culturing, and seriously affects the restorative efficacy of cartilage repair. The present study examines the effect of initial cell shapes on dedifferentiation of chondrocytes *in vitro*. The cell shape was controlled with a unique material micropatterning technique. With this technique, a series of microarrays of cell-adhesive peptide arginine-glycine-aspartate (RGD) were generated on a persistent non-fouling poly(ethylene glycol) (PEG) hydrogel. After culturing chondrocytes derived from rats on the micropatterned surfaces, the cell shapes were adapted by the geometries of adhesive microislands with pre-defined diameters (10, 15, 20 and 30 μm) for round ones and aspect ratios (1, 1.2, 1.5, 2, 4 and 6) for elliptical ones. After 10 days, collagen II staining was demonstrated to identify normal chondrocytes and dedifferentiated cells for those single cells on microislands. Furthermore, the gene expression of collagen II, collagen I, aggrecan and SOX9 were detected by qRT-PCR. The statistical results illustrated that dedifferentiation of chondrocytes happened more probably in the cases of larger sizes and higher aspect ratios. The conclusions stand under circumstances of both normoxia (21% $O_2$) and hypoxia (5% $O_2$) atmospheres.



## 1. Introduction

Articular cartilage has, once damaged, poor ability to recover. Meanwhile, as increasing with age, more and more people suffer joint degradation. Cartilage repair has been a challenging topic for a long time [1–6]. In the treatment of osteoarthritis, a common therapy is autologous chondrocyte implantation (ACI). In order to obtain sufficient chondrocytes, autologous chondrocytes need to go through expansion several times *in vitro*. However, chondrocytes are easy to dedifferentiate during culturing [7,8], and lose their ability to form normal cartilage after implanting [9,10]. So it is very meaningful to elucidate the cues to regulate the dedifferentiation of chondrocytes during *in vitro* culture.

It has been well known that cytokines such as transforming growth factor-β, bone morphogenetic protein, and insulin-like growth factor are beneficial for maintaining the normal chondrocyte phenotype [11–14]. Besides soluble factors, physical factors have recently been found to tune the dedifferentiation of chondrocytes. For instance, mechanical stimulations including compression, shear, flow perfusion and stiffness influence the phenotype of chondrocytes [15–19]; three-dimensional cultures partially prevent chondrocytes from dedifferentiation [20–23]. These studies imply that cell shapes might change after chondrocyte dedifferentiation. The initial shapes of chondrocytes prior to dedifferentiation are diverse themselves, as schematically indicated in Fig. 1A. We herein address a question whether or not a relatively fixed initial cell shape is a regulator of maintaining the chondrocyte phenotype.

The present article is aimed to answer this question on the level of single cells in two-dimensional *in vitro* culture. Chondrocytes isolated from articular cartilage of Sprague Dawley (SD) rats will be examined. The key is to generate and maintain the pre-defined cell shapes for a long time. To this end, a micropatterning technique was employed. Various micropatterning techniques have recently been put forward for cell research [24–27]. In this study, we design an array of microislands of cell-adhesive peptide arginine-glycine-aspartate (RGD) on a persistent non-fouling poly(ethylene glycol) (PEG) hydrogel, as schematically represented in Fig. 1B. This patterning is enabled by combining several techniques including photolithography, lift-off technology, macromonomer polymerization plus transfer lithography, and self-assembly monolayer (SAM) of active ligands. The material technique itself has been reported and applied to investigate cell adhesion and differentiation of mesenchymal stem cells [28,29]. On such micropatterned

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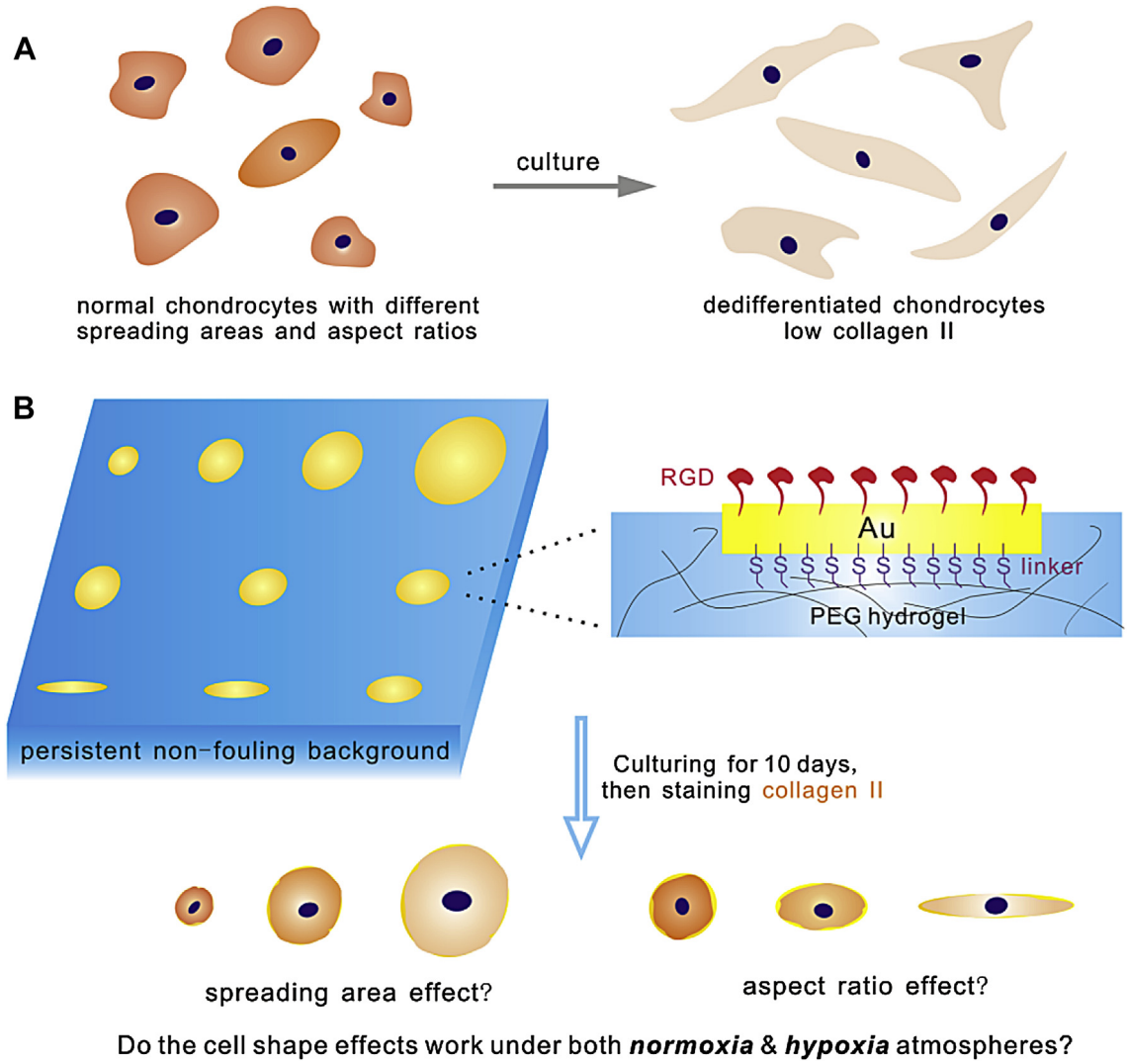


**Fig. 1.** Schematic presentation of the idea to explore whether spreading areas and aspect ratios of initial chondrocytes affect the maintenance of the normal chondrocyte phenotype. A) Articular chondrocytes are easy to dedifferentiate when culturing *in vitro*, from expressing high levels of collagen II to low levels. Normal chondrocytes exhibit globally less spread and less elongated than dedifferentiated cells. B) A unique micropattern with cell-adhesive microislands of varied sizes and shapes on a persistent non-fouling background of the poly(ethylene glycol) (PEG) hydrogel. Chondrocytes were seeded on these microislands for 10-day culture in normoxic (21% $O_2$) or hypoxic (5% $O_2$) conditions. The levels of collagen II were used to identify the cell phenotypes on different microislands. The effects of initial cell areas, aspect ratios and oxygen conditions on chondrocyte dedifferentiation will be examined on the single cell level.

surfaces, high probable single cell adhesion can be achieved under appropriate microisland areas; the cells can be localized on microislands for more than one week; the shapes of single cells can be pre-designed by those of microislands [30]. The present study investigates the dedifferentiation of chondrocytes during 10-day culture in normal growth medium. Two series of microislands with varied spreading areas of diameters (10, 15, 20 and 30 μm) and varied aspect ratios (1, 1.2, 1.5, 2, 4 and 6) will be prepared; the shape effects on maintaining chondrocyte phenotype will thus be investigated in a well-defined system.

In addition, since low oxygen tension has or has not a notable influence on preserving the chondrocyte phenotype as reported in the literature [31–33], the potential cell shape effects on cell dedifferentiation will be examined in both normal (21% $O_2$) and hypoxia (5% $O_2$) oxygen conditions. We would like to see whether or not there is a significant difference of chondrocyte dedifferentiation under different oxygen tensions at the single cell level.

## 2. Materials and methods

### 2.1. Preparation of micropatterns with a persistent non-fouling background

The RGD microarrays on PEG hydrogels were prepared by a photolithography and transferring technology [34]. Briefly, the preparation contained three steps: first, using lithography to fabricate a gold microarray on a glass slide; then transferring the gold microarray from the glass slide onto a PEG hydrogel; at last, grafting RGD peptides to the gold microarray on the PEG hydrogel.

In the first step, glass surface was spin-coated by photoresist (RZJ-304, Ruihong, Suzhou). Then a photomask with a pre-designed micropattern was applied on the glass surface. The photoresist on glass was exposed to UV light and developed in a developer solution (RZX-3038, Ruihong, Suzhou), and further sprayed with gold. After the extra photoresist was lift-off, a gold microarray was obtained on the glass slide.

The second step is the key to successfully fabricate micropatterns. In this transfer step, the virgin microarray on an inorganic substance was transferred to a polymeric hydrogel. While physical hydrogels are quite unique in medical applications [35–37], here chemical hydrogels formed by crosslinking macromonomers via free-radical polymerization [38,39] were employed to obtain stable hydrogels under cell culture environment. To enable a successful transfer, a bifunctional linker N, N′-bis(acryloyl) cystamine (Sigma) was introduced. It was dissolved in absolute ethanol