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Murine pluripotent stem cells derived scaffold-free cartilage grafts from a micro-cavitary hydrogel platform

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ABSTRACT

By means of appropriate cell type and scaffold, tissue-engineering approaches aim to construct grafts for cartilage repair. Pluripotent stem cells especially induced pluripotent stem cells (iPSCs) are of promising cell candidates due to the pluripotent plasticity and abundant cell source. We explored three dimensional (3D) culture and chondrogenesis of murine iPSCs (miPSCs) on an alginate-based micro-cavity hydrogel (MCG) platform in pursuit of fabricating synthetic-scaffold-free cartilage grafts. Murine embryonic stem cells (mESCs) were employed in parallel as the control. Chondrogenesis was fulfilled using a consecutive protocol via mesoderm differentiation followed by chondrogenic differentiation; subsequently, miPSC and mESC-seeded constructs were further respectively cultured in chondrocyte culture (CC) medium. Alginate phase in the constructs was then removed to generate a graft only comprised of induced chondrocytic cells and cartilaginous extracellular matrix (ECMs). We found that from the mESC-seeded constructs, formation of intact grafts could be achieved in greater sizes with relatively fewer chondrocytic cells and abundant ECMs; from miPSC-seeded constructs, relatively smaller sized cartilaginous grafts could be formed by cells with chondrocytic phenotype wrapped by abundant and better assembled collagen type II. This study demonstrated successful creation of pluripotent stem cells-derived cartilage/-chondroid graft from a 3D MCG interim platform. By the support of materials and methodologies established from this study, particularly given the autologous availability of iPSCs, engineered autologous cartilage engraftment may be potentially fulfilled without relying on the limited and invasive autologous chondrocytes acquisition.

Statement of Significance

In this study, we explored chondrogenic differentiation of pluripotent stem cells on a 3D micro-cavitary hydrogel interim platform and creation of pluripotent stem cells-derived cartilage/chondroid graft via a consecutive procedure. Our results demonstrated chondrogenic differentiation could be realized on the platform via mesoderm differentiation. The mESCs/miPSCs derived chondrocytic cells were further cultured to finally generate a pluripotent stem cells-derived scaffold-free construct based on the micro-cavitary hydrogel platform, in which alginate hydrogel could be removed finally. Our results showed that miPSC-derived graft could be formed by cells with chondrocytic phenotype wrapped by abundant and assembled collagen type II. To our knowledge, this study is the first study that initials from pluripotent stem cell seeding on 3D scaffold environment and ends with a scaffold-free chondrogenic micro-tissue. By the support of materials and methodologies established from this study, engineered autologous iPSC-derived cartilage engraftment may be potentially developed instead of autologous chondrocytes grafts that have limited source.

1. Introduction

Articular cartilage plays important roles in smooth transfer of loading between the joints. Cartilage belongs to avascular and

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aneural tissue type, characterized by significant constituents of collagen type II and a water-absorbing proteoglycan component, aggrecan, in abundant extracellular matrix (ECM). Due to the avascular nature of cartilage, cartilage lesions or injuries are hard to self-heal. With the development of tissue engineering, researchers are trying to construct cartilage graft by means of appropriate cell sources and scaffolds for cartilage repair. Autologous chondrocytes are of the first choice, but it is constrained by limited cell sourcing availability and invasive procedure of cell acquisition. Therefore, autologous stem cells become the most promising candidates. Somatic stem cells derived from bone marrow, synovium, adipose have been explored as multi-potent autologous candidates [1]. Bone marrow-derived stromal cells (BMSCs) are widely adopted for chondrogenes is by stimuli of growth factors such as transforming growth factor (TGF)-β1 or 3 as well as bone morphogenic proteins (BMPs) [2–4]. However, such induced chondrogenesis often results in commitments with hypertrophic cartilage phenotype [5]. In recent decades, pluripotent stem cells have become more and more attractive candidates for regenerative medicine. In comparison with embryonic pluripotent stem cells, such as embryonic stem cells (ESCs), embryonic germ cells (EGCs) or embryonal carcinoma cells (ECCs), more recently developed induced pluripotent stem cells (iPSCs) possess further superior and promising strengths that they are available from autologous sources and much free of ethical quandary [6–9].

The possible approaches for pluripotent stem cells' *in vitro* chondrogenesis have been explored in several studies, respectively, through the pathway via commitment of mesenchymal stem/precursor cells (MSCs) or more directly via primitive streak mesoderm, or via direct differentiation under stimuli of growth factor [10–13]. MSCs have been obtained by co-culturing ESCs with murine OP-9 cells or medium supplemented with growth factors such as β-FGF [14,15]. MSCs can also be obtained by flow cytometry sorting from spontaneously formed embryoid bodies (EBs) [16]. To apply the pathway via primitive streak mesoderm, iPSCs/ESCs are differentiated under stimuli of defined medium with a series of growth factors [17,18]. During embryogenesis, the formation of PS marks the beginning of downstream from gastrulation [19] typically represented by Brachyury (T) gene expression [20], which was found being controlled by Wnt, Nodal and bone morphogenetic protein (BMP)-signaling pathways [21,22]. The differentiation from primitive streak to mesoderm is marked by the up-regulation of platelet-derived growth factor receptors (PDGFR) and Flk-1 (KDR, Kinase Insert Domain Receptor). BMP signaling is required to induce Flk-1$^+$ hematopoietic mesoderm [23]. Previous study showed that the combination of Wnt signaling with BMP inhibition could successfully catalyze the generation of mesoderm cell population with chondrogenic potential, marked by the expression of PDGFRα while lack of expression of Flk-1 [24].

Currently, most of studies on chondrogenic differentiation of pluripotent stem cells ESCs/iPSCs are established on basis of two dimensional (2D) cultures. 2D culture system is appropriate and ideal for investigating and monitoring the chondrogenic differentiation pathway of pluripotent stem cells. However, 2D culture system is not feasible for tissue repair purpose and application as a tissue-engineering construct. Compared with commonly adopted chondrocytes or MSCs derived 3D tissue-engineered construct, ESCs/iPSCs-derived (especially iPSCs) 3D tissue-engineered graft is more challenging but more promising. In this study, in order to fabricate a miPSCs-derived tissue engineered graft for cartilage repair, we first established an applicable three dimensional (3D) iPSCs/ESCs' chondrogenesis cultural system via mesoderm pathway, further from which a product of synthetic-scaffold-free cartilage or chondroid graft (SfCG) is fabricated via this newly developed continuous processes. The whole differentiation process will be established on a micro-cavitary hydrogel (MCG), which has been demonstrated to be a superior platform for 3D culture of non-anchorage dependent cells (ADCs), by previous studies on chondrocytes-derived cartilage graft [25]. In this study, since the both starting and targeted ending phenotypes of murine iPSCs/ESCs (miPSCs/mESCs) always remain as non-ADCs, similar MCG platform is adopted by encapsulation of miPSCs/mESCs to explore the chondrogenic differentiation. The differentiation was conducted in turn via two main stages: mesoderm differentiation (*mdf*) and chondrogenic differentiation (*cdf*), followed by a chondrogenic development (*cdp*) stage to establish final PSC-d-SfCGs. The designed differentiation route is indicated in Fig. 1 and described in materials and methods. Based on the unique platform, 3D pluripotent stem cells derived synthetic scaffold-free products (PSC-d-SfCGs inclusive of miPSC-d-SfCGs and mESC-d-SfCGs) would be generated using a consecutive process.

2. Materials and methods

Unless stated, all cell-culture reagents were purchased from Invitrogen, Life technologies, and all chemicals were purchased from Sigma Aldrich.

2.1. Pluripotent stem cell culture

miPSCs (APS0003) were purchased from RIKEN Bio Resource Centre (Ibaraki, Japan) and mESCs (E14tg2A) were purchased from American Type Culture Collection (ATCC, USA). Both miPSCs and mESCs were respectively cultured on a 0.1% gelatin-coated petri-dish under feeder cell-free conditions. miPSCs and mESCs were cultured in "stem cell medium" – Dulbecco's modification of Eagle's medium (DMEM) supplemented with 15% Fetal Bovine Serum (FBS, PAA laboratories), 0.1 mM 2-Mercaptoethanol and 1000 U/ml leukemia inhibitory factor (LIF, Millipore).

2.2. Establishment of 3D cell-laden MCG constructs

miPSCs and mESCs that were cultured on gelatin-coated petri-dish, respectively, were re-suspended and co-encapsulated with gelatin beads (micro-spheroidal porogens) in alginate-based MCG constructs. Gelatin (type B gelatin from bovine skin) and alginic acid sodium (from brown algae) was purchased from Sigma. Non-crosslinked gelatin beads (about 200 μm in diameter) were fabricated via double emulsion method as described before [26]. Briefly, at 4 °C, the fabricated micro-spheroidal beads were soaked in 10× concentration of penicillin/streptomycin solution overnight for sterilization and then transferred into DMEM for usage. miPSCs and mESCs that were cultured on gelatin-coated petri-dish, respectively, were trypsinized and centrifuged. Cells were then suspended in cold alginate solution (1.5% in 0.15 M NaCl made from alginic acid sodium) at a density of 10^7 cells/ml. The cell-alginate suspension was then mixed gently with pre-measured gelatin beads (0.3 g gelatin per 1 ml alginate solution). 80 μl of the mixture was injected into a circular silicon mold (with a diameter of 6 mm and thickness of 4 mm). Gelation of alginate hydrogel was induced by adding 10 μl of 102 mM $CaCl_2$ solution onto the surface of the mixture and leaving the construct at 4 °C for 4 mins. The constructs were then unloaded from the molds and cultured in 12-well plate (Corning, the bottom was pre-coated with agarose gel) at 37 °C in 5% CO_2 incubator. When incubated at 37 °C, the gelatin beads inside the construct were dissolved and diffused from the alginate hydrogel, leaving cavities in the construct. These