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Chemically defined serum-free conditions for cartilage regeneration from human embryonic stem cells

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ABSTRACT

Aims: The aim of this study was to improve a method that induce cartilage differentiation of human embryoid stem cells (hESCs) *in vitro*, and test the effect of *in vivo* environments on the further maturation of hESCs derived cells.

Main methods: Embryoid bodies (EBs) formed from hESCs, with serum-free KSR-based medium and mesodermal specification related factors, CHIR, and Noggin for first 8 days. Then cells were digested and cultured as micropellets in serum-free KSR-based chondrogenic medium that was supplemented with PDGF-BB, TGF β3, BMP4 in sequence for 24 days. The morphology, FACS, histological staining as well as the expression of chondrogenic specific genes were detected in each stage, and further *in vivo* experiments, cell injections and tissue transplantations, further verified the formation of chondrocytes.

Key findings: We were able to obtain chondrocyte/cartilage from hESCs using serum-free KSR-based conditioned medium. qPCR analysis showed that expression of the chondroprogenitor genes and the chondrocyte/cartilage matrix genes. Morphology analysis demonstrated we got PG + COL2 + COL1-particles. It indicated we obtained hyaline cartilage-like particles. 32-Day differential cells were injected subcutaneous. Staining results showed grafts developed further mature *in vivo*. But when transplanted in subrenal capsule, their effect was not good as in subcutaneous. Microenvironment might affect the cartilage formation.

Significance: The results of this study provide an absolute serum-free and efficient approach for generation of hESC-derived chondrocytes, and cells will become further maturation *in vivo*. It provides evidence and technology for the hypothesis that hESCs may be a promising therapy for the treatment of cartilage disease.

1. Introduction

Articular cartilage is vitally important in the joints, providing smooth articulation and sustaining skeletal mobility [1]. It is highly susceptible to be damaged in injury or degenerative conditions. Currently, cartilage degeneration that caused by primary osteoarthritis (OA) or trauma becomes a major healthcare problem affecting people of all ages [2–4]. Due to its avascular and hypocellular nature, the cartilage has been widely confirmed to be lack of the automatic regeneration potential [1,5,6]. The injury tissue cannot be effectively repaired by itself to form normal hyaline cartilage, which leads to the disorder of the joint function or even disability. Cell-based therapies have been shown to reverse the symptoms and pathophysiology of OA [7]. The dedifferentiated chondrocytes and mesenchymal stem/stromal cells (MSCs), were once regarded as candidate cell sources [8]. However, these cell sources are non-ideal since they require *ex vivo* expansion to be used in current cellular therapies, showing the increased risk of oncogenic transformation [9], and the production of fibrocartilage with poor biomechanical properties [10]. Embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells (designated collectively, pluripotent stem cells or PS cells), due to their relative abundance and multipotent capabilities, specifically their ability to undergo chondrogenesis, have become an attractive therapeutic alternative treatment for OA.

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Pioneering studies in ESCs involved differentiation *via* serum-containing medium. Many growth factors and supplements, such as TGF-β family, PDGF-bb, IGF-1, SHH, were applied at different stages during the chondrogenic differentiation [11–14]. Serum can provide rich nutrients during the *in vitro* culture of chondrocytes and cartilage. However, many recent studies have shown that exposing cartilage to blood or serum can induce extracellular matrix damage. The adverse effect was partially attributed to cytotoxic oxygen metabolites [15], Cytokines in serum, such as COMP, IL-1, TNF-α, may also induce the inflammatory processes [16,17], which could further produce the degradation of cartilage matrix [18]. Recently, chemically defined serum-free medium has been used for cartilage differentiation *in vitro* culture. One method was embryoid body (EB) formation [19], which mimicked the early embryonic development with the formation of three germs, offering a supply of cells for cartilage tissue engineering. But few studies reported pluripotent stem cells (PSCs) were subjected to cartilage differentiation with EB-based method and absolute serum-free medium. More recent study showed direct differentiation to generate chondroprogenitors from PSCs without EB, and they can regenerate hyaline cartilage in an osteochondral defect model in nude rats [20,21]. This chondrogenic protocol has the advantages of rapid and efficient differentiation, but its repair effect needs to be further verified *in vivo*.

As known, sclerotome (from somite/rostral paraxial mesoderm) was responsible for hyaline cartilage during embryogenesis. Several reports have revealed that mesodermal genes are expressed in parallel during the generation of chondrocytes or their precursors [20,22]. It was also clearly demonstrated the promoters of Wnt signaling and inhibitors of BMP signaling were able to promote the chondrogenic differentiation from ESCs *in vitro* [23–25]. During specification, mESCs and hESCs derived omitic/rostral presomitic mesoderm-like progeny (also known as rostral paraxial mesoderm) was widely confirmed to express platelet-derived growth factor receptor-α (PDGFRα) but not vascular endothelial growth factor receptor-2 (VEGFR2, also known as FLK1 or KDR) [23–29]. However, the differentiation media used in these studies still contained a small amount of serum, restricting to the future application in clinic. Meanwhile, the role of the specification cells *in vivo* during chondrogenesis was still unclear in these studies.

In our study, we revised a differential protocol for cartilage regeneration from human ESCs. Cells were initially employed mesoderm specification with EB formation, and were subsequently induced with 3D micropellet for chondrogenesis. We used serum-free KSR-based medium with addition of several important factors during differentiation for 32 days *in vitro* (Fig. 1A). Furthermore, the 32-day differentiated derivatives were respectively injected subcutaneously, or directly transplanted into subcutaneous, or the renal subcapsular layer of adult nude mice for 8 weeks to identify the developmental potential *in vivo*.

2. Materials and methods

2.1. Cell culture

Cell line hESCs H1(Wicell, Madison, WI, USA) was used in this study. hESCs were cultured as previously described [30,31]. They were maintained in mTeSR1 medium (Stemcell Technologies, Vancouver, BC, Canada) on the plates coated with Matrigel (BD Biosciences, SanJose, CA, USA) using. The hESCs were routinely passaged by 0.5 M EDTA every 3–5 days.

2.2. Chondrogenic differentiation

The process of hESCs differentiation was a two-stage procedure, mesodermal specification and chondrocyte development. The culture was initiated with the embryoid body (EB)-forming. H1 hESCs colonies were detached with 2 mg/ml Dispase (Gibco) and grown in suspension in EB medium [DMEM/F12, 20% knockout serum replacement (KSR) (Gibco), 1%L-glutamine, 1% non-essential amino acid (NEAA), penicillin/streptomycin and 0.1 mM beta-mercaptoethanol] for 24 h. hESC-derived EBs were then treated with KSR-based Serum-free Medium (KSR-SFM) for 8 days as previous described with slight modifications [23,24]. To induce mesodermal differentiation, EBs were replated on a flask coated with 1% Agarose, in an induction medium (DMEM/F12, 2% KSR, 5 mg/ml BSA (Invitrogen), 1% NEAA, 2 mM GlutaMax, 1% Insulin-Transferrin-Selenium (ITS), 0.17 mM ascorbic acid 2-phosphate (AA2P), 0.45 mM MTG, 0.5 μM Thi) with inducing factors 10 ng/ml Noggin, and CHIR99021 (2 μM at first 3 days, 5 μM for the other 5 days). At day 8, the EBs were dissociated with 0.5 mM EDTA and reaggregated to form the pellets (5.0×10^5cells/pellet) by centrifugation at 800*g*. These aggregated specimens were incubated in KSR-SFM chondrogenic medium (DMEM/High, 1% KSR, 1% NEAA, 2 mM GlutaMax, 1% ITS, 0.17 mM AA2P, 0.05 mM MTG, 0.5 μM Thi, 0.1 μM Dexamethasone, 0.35 mM Proline, 1 mM Sodium pyruvate) supplemented with 40 ng/ml PDGF-BB, 10 ng/ml TGF β3, and 50 ng/ml BMP4 subsequently as described till day 32 (Fig. 1A) [24]. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.3. Cell injections and tissue transplantations

32-Day differentiated derivatives were dissociated by 2 mg/ml Collagenase II. 5.0×10^5 dissociated cells mixed with 50 μl matrigel were injected subcutaneously into the dorsal region of adult nude mice as described previously [29]. Alternatively, 32-day differentiated derivatives were directly transplanted into subcutaneous and the renal subcapsular layer of adult nude mice respectively. The host mice were sacrificed to harvest the transplanted specimens 8 weeks post-implantation as indicated.

2.4. Flow cytometry analysis

hESC-derived EBs were dissociated by EDTA, fixed in PFA, and stained with the following antibodies: mouse anti-human KDR and rabbit anti-human PDGFRα monoclonal antibodies. All analyses were performed on the Accri C6 flow cytometer (BD Biosciences). Details of the antibodies we used are given in Supplementary Table S1.

2.5. Real-time quantitative PCR

Total RNA was extracted with Trizol (Invitrogen). 2 μg of RNA was reverse transcribed using RT-PCR kit (Takara) and qPCR was performed using a Thermal Cycler DiceTM Real Time System and SYBR Green Premix EX TaqTM (Takara).

GAPDH was used for normalization, and the data were measured in triplicate. The gene expression level of D0 group was set as "1", and the fold changes of other groups relative to the reference sample was further analyzed. Details of the qPCR primers are given in Supplementary Table S2.

2.6. Histology and immunohistochemistry

In vitro 32-day differentiation cultures, and decalcified *in vivo* 8-week engraftments were fixed in 4% PFA and sectioned in a 7 μm thickness. Sections were stained with Alcian Blue, Hematoxylin and Eosin (H&E), Safranin O/Methyl green, and Toluidine Blue as indicated [20,29,32]. For immunohistochemistry, the primary antibody information could be found in Supplementary Table S1.

3. Results

3.1. Chondrogenic differentiation of hESCs with KSR-SFM

We improved a method to induce chondrogenesis from hESCs with KSR-SFM (Fig. 1A). The process consisted of two stages, mesoderm differentiation (Day 0–8) and Chondrogenesis from mesoderm cells (Day