Journal of Industrial and Engineering Chemistry 37 (2016) 147–155

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

Osteogenic commitment of human induced pluripotent stem cell-derived mesenchymal progenitor-like cells on biomimetic scaffolds

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ARTICLE INFO

Article history:
Received 1 January 2016
Received in revised form 6 March 2016
Accepted 8 March 2016
Available online 16 March 2016

Keywords:
Bone tissue engineering
Hydroxyapatite
Retinoic acid
Human induced pluripotent stem cells
Mesenchymal progenitor-like cells

ABSTRACT

In this study, mesenchymal progenitor-like cells (MPLCs) were derived from human induced pluripotent stem cells (hiPSCs) by retinoic acid (RA) treatment. In particular, RA induced hiPSC–derived MPLCs were able to efficiently committed into osteogenic lineage cells. In addition, hiPSC–MPLCs were seeded onto three-dimensional biomimetic scaffolds composed of hydroxyapatite and poly(L-lactic acid)/poly(lactic-*co*-glycolide) for assessing osteoinductivity, and enhanced osteogenic activities were perceived in RA-treated cells *in vitro* and *in vivo*. In conclusion, addition of RA increased the osteogenic commitment potential of hiPSCs–MPLCs and further applications with biomimetic composite scaffolds showed a promising combination for bone tissue engineering applications.

Introduction

Bone defects can occur after an unexpected accident or a pathological process deteriorating essential parts of the bone. Lack of suitable bone tissues on defect sites provokes risks of infection, hemorrhage, loss of function, nerve damage and cosmetic disability [1]. Conventional methods to treat these bone defects include autologous bone grafts and the use of metals/ceramics as alternatives. Autologous bone grafting has presented an effective bone replacement [2], but body site limitation for harvesting bones without functional loss and donor site morbidity are still major hurdles for its clinical applications [3]. Other than autologous bone grafting, use of artificial materials such as metals can provide strong mechanical supports but display poor cell migration at the implantation site and raise risks of infection. Another type of artificial material, like biocompatible ceramics, is not optimal under strong tension due to its poor fracture toughness [4]. Therefore, bone tissue engineering techniques can potentially provide better solutions for severe bone injury.

In terms of cell sources for bone tissue engineering, mesenchymal stem cells (MSCs) and mesenchymal progenitor cells (MPCs) can be isolated from several tissue types such as bone marrow [5], umbilical cord blood [6], or adipose tissues [7] either from patients or donors. While bone marrow is the most accessible source for these cell types [8], aging-dependent decrease in the total number of MSCs [9,10] and the invasive implantation procedures are yet to be overcome [11]. Recent emergence of pluripotent stem cells such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) provided alternative ways for derivation of MSCs/MPCs [11–13]. Specifically, hiPSCs can provide additional advantages over ES cells by enabling derivation of autologous MSCs/MPCs on top of minimizing ethical issues and possible immune rejections.

Differentiation of pluripotent stem cells can be induced by the formation of three-dimensional cell aggregates called embryoid bodies (EBs) in a suspension culture [14]. MSCs could be derived by outgrowing hEBs in human bone marrow-derived mesenchymal stem cells media [15]. It has been reported that theses derived MSCs were able to differentiate into mesenchymal cell types such as osteoblasts and adipocytes, and exhibited similar immunophenotype ($CD73^+$,

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http://dx.doi.org/10.1016/j.jiec.2016.03.017
1226-086X/

STRO-1$^+$ and CD45$^-$) to bone marrow-derived MSCs [15]. In case of deriving mouse MSCs, Takashima et al. previously reported that an earliest wave of differentiation was not directly derived from the mesoderm layer but from the Sox1$^+$/PDGFRα expressing neuroepithelial cells [16] by exposing mouse ES cell culture to retinoic acid (RA) during the EB formation. RA belongs to the group of retinoids which are responsible for cell proliferation, differentiation and apoptosis [17] and serves as important metabolites of retinol, functioning in reproduction, embryogenesis, bone modeling, the immune system, and the epithelial differentiation [18]. RA is also frequently used to induce neural differentiation *via* treating onto mouse EBs [19]. Albeit the treatment of RA is one of the common methods for differentiating mESCs and miPSCs into different lineages, its application onto hiPSCs for hMSC derivation is yet to be reported.

Upon achieving cell sources, it is essential to substitute a bone-mimicking scaffold for structural support, cell interactions and formation of native bone-extracellular matrix to a newly formed tissue at repair sites. New bone materials become gradually deposited at scaffolds from adjacent bones while scaffolds serve as osteoconductive moieties for optimized integration [20]. Biomimetic materials used in scaffold fabrication include a wide variety of ceramics and polymeric materials from either natural or synthetic origins. Hydroxyapatite (HAP) ($Ca_{10}(PO_4)_6(OH)_2$) is a prominent candidate satisfying key properties as a scaffold due to its chemical similarity to the inorganic component of the bone matrix with great affinity to host hard tissues, leading to a strong chemical bonding which could be more advantageous in clinical applications than other types of bone substitutes [21]. The basic resorption of HAP can buffer the acidic resorption by-products of aliphatic polyester to avoid the formation of an unfavorable environment for cells from a decreased pH [22]. The application of HAP possesses advantages on biocompatibility, degradation rate *in situ* and osteoinductivity [23], yet the low mechanical strength from brittleness can inhibit the high load-bearing. To overcome this problem, HAP can be hybridized with synthetic polymers which are biocompatible and degradable without significant cytotoxicity. These synthetic polymers mainly consist of polyesters: poly(lactic acid) [24], poly(L-lactic acid) [25], and poly (lactic-*co*-glycolide) [26]. These HAP/polymer composite scaffolds can support cell growth and tissue formation by mimicking the natural environment of the bone to some extent.

Here, we derived proliferative MPLCs from hiPSCs by treating RA during embryoid body formation and used HAP/PLLA/PLGA scaffolds to observe the effect of retinoic acid-treated cells in osteogenesis *in vitro* and *in vivo*. Our results indicated that the derived MPLCs with the treatment of RA exhibited higher osteogenic lineage commitment than hBM-MSCs and non-RA treated control cells *in vitro*, and *in vivo* experiment utilizing HAP/PLLA/PLGA scaffolds demonstrated an efficient bone regeneration in a mouse cranial critical-sized defect model.

Materials and methods

Cell culture

Human induced pluripotent stem cells (hiPSCs) WT-3 derived from human dermal fibroblasts were kindly gifted from iPS bank at Yonsei Stem Cell Research Center, Seoul, South Korea. hiPSCs were cultured on mouse STO feeder layer and maintained in DMEM/F-12 (11320-033, Gibco), 20% (w/v) Knockout serum replacement (10828-028, Gibco), 0.1 mM β-mercaptoethanol (M7522, Sigma), 10 ng/ml bFGF (13256-029, Gibco), 1% (w/v) Non-essential amino acids (11140-050, Gibco) and 0.1% (w/v) penicillin–streptomycin (15140, Gibco). The medium was changed every day. When the size of hiPSC colonies reached at confluency, the cells were detached by collagenase (17104-019, Gibco) and suspended into the hiPSCs culture medium without bFGF (hEB medium) on the Petri dish. For the experimental group, 10^{-7} M of retinoic acid (RA) (R2625, Sigma) was supplemented into the medium from day 2 to day 6, while the control group was maintained in the hEB medium. On day 7, control and RA treated hEBs were transferred to gelatin-coated tissue culture plate for outgrowth. Outgrowth cells and human mesenchymal stem cells were maintained in High-glucose DMEM (10566-016, Gibco), 10% (w/v) fetal bovine serum (US1520, Biowest) and 1% penicillin–streptomycin (15140-122, Gibco). Characterization of cells was taken at passage 5.

Reverse-transcriptase PCR

Cells were lysed with Trizol® reagent and collected in 1.5 ml-tube for total RNA extraction. Extracted total RNA samples were reversely transcribed into cDNA by M-MLV cDNA synthesis kit (EZ006S, Enzynomics, Korea). Primers targeting Sox1, PDGFRα, BMPR-IA, BMPR-IB, BMPR-II, cbfa1, smad8, Oct4 and 18s were utilized to observe gene expression levels of these markers using AccuPower® PCR PreMix (K-2016, Bioneer, Korea) *via* gel electrophoresis. Primer sequences are listed in Table 1.

CD surface marker analysis via *flow cytometry*

To compare expressed CD surface markers of human EB derived cells to human mesenchymal stem cells, all of the cells were stained with CD90, CD44, CD105 and CD73 by BD Stemflow™ hMSC Analysis Kit (562245, BD Sciences) according to manufacturer's instruction. After filtering with 35 μm cell-strainer (352235, BD Falcon, Canaan, CT, USA), the cells were analyzed using FACScalibur (BD Biosciences) and FlowJo software (FlowJo, LLC).

Differentiation assay

As a way of evaluating differentiation ability into osteogenic lineage, cells at day 7 and 14 were fixed in 10% formalin for 15 min at room temperature. To observe osteogenic differentiation by Ca^{2+} deposition, fixed cells were immersed by Alizarin Red S solution (A5533, Sigma) at pH 4.3 for 20 min at RT after fixation and washed three times with PBS.

Table 1
Primer sequences for reverse-transcriptase PCR.

Primer name	Sequence (5′–3′)
Sox1 F	TAC AGC CCC ATC TCC AAC TC
Sox1 R	GCT CCG ACT TCA CCA GAG AG
PDGFRα F	GAG CAT CTT TGA CAA CCT CTA CAC
PDGFRα R	CCG GTA CCC ACT CTT GAT CTT ATT G
cbfa1 F	AGG CAG TTC CCA AGC ATT TC
cbfa1 R	GGT CGG CCA AAC AGA TTC ATC
Smad1 F	GAG ACA GCT TTA TTT CAC CAT ATC C
Smad1 R	CAT AGT AGA CAA TAG AGC ACC AGT GTT TT
Smad5 F	CGG TAG CCA CTG ACT TTG AGT TAC
Smad5 R	AGC TGA AAT GGA CTT CCT GGT C
Smad8 F	TGC TGT GGC CTC TTA TGC AC
Smad8 R	GTC TCC ACC CGG CGG
BMPR-IA F	TAA AGC TGA CAG TAC ACA GGA ACA
BMPR-IA R	TCT ATG ATG GCA AAG CAA TGT CC
BMPR-IB F	TAC AAG CCT GCC ATA AGT GAA GAA CC
BMPR-IB R	ATC ATC GTG AAA CAA TAT CCG TCT G
BMPR-II F	TCC TCT CAT CAG CCA TTG TCC TTC
BMPR-II R	AGT TAC TAC ACA TTC TTC ATA G
OCT4 F	ACC CCT GGT GCC GTG AA
OCT4 R	GGC TGA ATA CCT TCC CAA ATA
18s F	CCC TGT AAT TGG AAT GAG TCC ACT T
18s R	ACG CTA TTG GAG CTG GAA TTA C