Life Sciences 175 (2017) 37–46

Contents lists available at ScienceDirect

Life Sciences

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

Generation of enhanced definitive endoderm from human embryonic stem cells under an albumin/insulin-free and chemically defined condition

Su Qu [a,1], Liang Yan [b,1], Bo Fang [a], Shoudong Ye [a], Ping Li [a], Shengyang Ge [b], Jian Wu [b,*], Di Qu [b,*], Houyan Song [a,**]

[a] The Key Laboratory of Molecular Medicine of Ministry of Education, School of Basic Medical Sciences, Fudan University, Shanghai, China
[b] Key Laboratory of Medical Molecular Virology of Ministry of Education and Ministry of Health, School of Basic Medical Sciences, Fudan University, Shanghai, China

ARTICLE INFO

Article history:
Received 10 December 2016
Received in revised form 12 March 2017
Accepted 21 March 2017
Available online 22 March 2017

Keywords:
Cell survival
Definitive endoderm
Human embryonic stem cell
Chemically defined condition

ABSTRACT

Aim: To enhance survival and generation of definitive endoderm cells from human embryonic stem cells in a simple and reproducible system.
Main methods: Definitive endoderm (DE) differentiation from human embryonic stem cells (hESCs) was induced under a chemical-defined condition withdrawn insulin supplement and serum albumin. We dissected influence of "alternative growth factors", WNT3A, BMP4 and bFGF in activin A-driven differentiation by detection of DE-associated genes expression and cell viability. Expression of DE-associated SOX17 and FOXA2 genes was analyzed by real time reverse transcription polymerase chain reaction (RT-PCR) and Western blot assays. Quantitative evaluation of DE efficiency was performed by flow cytometry analysis of CXCR4-expressed cell population. Cell viability during DE differentiation was analyzed by an Annexin V/PI double staining test.
Key findings: Supplementation with WNT3A, BMP4 or bFGF promoted DE generation in a dose- and time-dependent manner. Cell apoptosis elicited by activin A was significantly ameliorated by a cocktail with WNT3A, BMP4 and bFGF. This allowed for sustained cell viability without insulin-containing supplements, thereby indirectly improving the efficiency of DE generation. Therefore, the cocktail containing is optimal for efficient DE generation in the presence of activin A and an insulin/albumin-free condition.
Significance: This optimal condition facilitates the balance between the productivity and the viability maintenance, and could be valuable for mass production of DE with minimal variation.

1. Introduction

The definitive endoderm (DE) is one of the major germ layers formed during gastrulation, and gives rise to the epithelium of digestive and respiratory organs [1]. Two types of approaches, namely embryoid body (EB) and monolayer cell culture are used to generate definitive endoderm or endoderm-derived cell populations from hESCs [2,3]. Monolayer culture-based protocols have been favored in recent studies of endoderm-derived tissue production, given the latter offers a comparable condition in different culture systems, and are prone to achieve a high purity of cell lineage population [3,4]. It has been evident that two prerequisites, *i.e.* 1) activation of activin A/nodal signaling and 2) inhibition of phosphatidylinositol 3-kinase (PI3K) signaling, are critical for DE differentiation in monolayer cultures [5]. However, poor DE generation from certain hESC lines has been observed under conditions fulfilling these two requirements [6]. A better understanding of the mechanisms underlying endoderm lineage commitment would help to establish practical protocols that recapitulate the process of the definitive endoderm formation during gastrulation. Growth factors, including WNT3A, BMP4, and bFGF, are applied in several studies to mimic an *in vivo* microenvironment of soluble signals in DE generation [7,8,9]. Recently, critical role of extracellular matrix (ECM) substratum components in hESC differentiation towards specific cell lineages has been recognized with implementation of ECM array tools, and it is evident that certain ECM components are beneficial for the improvement of DE generation [10,11].

Abbreviations: DE, definitive endoderm; hESCs, human embryonic stem cells; CDM, chemical-defined medium; AA, activin A; WNT3A, wingless-type MMTV integration site family member 3A; BMP4, bone morphogenetic protein 4; bFGF, basic fibroblast growth factor.

* Corresponding authors at: Key Laboratory of Medical Molecular Virology of Ministry of Education and Ministry of Health, School of Basic Medical Sciences, Fudan University, 138 Yixue Yuan Road, Shanghai, 200032, China.
** Correspondence to: H. Song, Key Laboratory of Molecular Medicine of Ministry of Education, School of Basic Medical Sciences, Fudan University, 130 Dong'an Road, Shanghai, 200032, China.
E-mail addresses: jian.wu@fudan.edu.cn (J. Wu), dqu@shmu.edu.cn (D. Qu), hysong@shmu.edu.cn (H. Song).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.lfs.2017.03.017
0024-3205/

In the past decades, great progress has been made in efficiently deriving endoderm lineage cells from hESCs using various combinations of soluble and insoluble factors. However, excessive cell loss has also been frequently observed during endodermal or mesodermal differentiation of monolayer-cultured hESCs, particularly under a serum-free condition [12,13]. For this reason, efficient DE generation in a serum-free condition requires additional supplements to maintain cell viability, such as B27 or insulin-transferrin-selenite (ITS) [9,14,15,16]. Under this condition insulin is thought to enhance cell survival at the expense of a low transition into DE lineage cells. Obviously, there is a contradiction to induce DE differentiation by lowering PI3K/AKT signals, at the same time to maintain cell viability by adding insulin-containing supplements. Indeed, DE generation in insulin-containing systems has been achieved by the use of small molecules [5,17,18], which might import unexpected effects on the differentiation process. Serum albumin also contributes to cell survival in DE generation from hESCs [19], while unexpected lipid or growth factors carried by albumin potentially interfere with directed differentiation, and may result in substantial variations in medium components [20]. This may help to explain a wide variation under different conditions used for DE production in the literature.

The present study aimed to establish a protocol that not only efficiently induces DE differentiation, but also enhances cell viability independent of exogenous insulin/IGFs in a chemically defined system. To establish a reproducible and DE induction-friendly condition, we modified the Vallier's chemical-defined medium (CDM) formula [21] and prepared a medium that lacked insulin and serum albumin. Here we report the effectiveness and productivity of this modified culture condition.

2. Materials and methods

2.1. Cell culture condition

The human embryonic stem cell lines H7 and H9 were purchased from WiCell Research Institute (Madison, WI, USA) and maintained with mouse embryonic fibroblast-conditioned medium (MEF-CM), as described in the WiCell protocols. Cell media and supplements were purchased from Gibco (Life Technologies, Grand Island, NY, USA), except otherwise indicated. hESCs were differentiated on fibronectin and vitronectin (1:1)-coated dishes with an insulin-free, albumin-free, and chemically defined DMEM/F12 medium supplemented with Glutamax (1 mM), non-essential amino acid (NAEE) (0.1 mM), chemically defined lipid concentrate (0.1%), L-ascorbic acid (50 μg/mL) (Sigma, St. Louis, MO, USA), and polyvinyl alcohol (PVA) (0.1%, Sigma).

2.2. Differentiation of human embryonic stem cells

Growth factors for hESC differentiation included human recombinant activin A (Peprotech, Rocky Hill, NJ, USA), BMP2, BMP4, Wnt3a, FGF4, FGF10 (R&D Systems, Minneapolis, MN, USA), and bFGF (Life Technologies). In the present study, DE differentiation was induced by activin A combined with three "alternative" growth factors. When confluent levels of undifferentiated hESCs reached >50% (density ranged from 1.3×10^5 to $6 \times 10^5/cm^2$ as determined by cell counting), endoderm differentiation was initiated by incubating hESCs in induction medium with the addition of human recombination activin A at 100 ng/mL, human recombination Wnt3a (25 ng/mL, R&D Systems), human recombination BMP4 (20 ng/mL, R&D Systems), and human recombination bFGF (20 ng/mL, medium A). After 24 h, the initial medium was replaced with induction medium plus activin A at 100 ng/mL, BMP4 at 20 ng/mL and bFGF at 20 ng/mL (medium B), and cells were induced for another 48 h. If endoderm induction was extended to 4–5 days, cells were cultured in replenished medium B plus 0.1% insulin-transferrin-selenium supplementation.

After production of DE cells, we induced hepatic lineage differentiation with a protocols modified from published ones [16,18]. Briefly, cells were incubated with the following concentrations of growth factors: human recombinant BMP2 at 10 ng/mL, human recombinant BMP4 at 10 ng/mL, human recombinant bFGF at 10 ng/mL, human recombinant FGF4 at 10 ng/mL and human recombinant FGF10 at 10 ng/mL.

2.3. Gene expression evaluation by quantitative real-time polymerase chain reaction

Total RNA was extracted from samples using Trizol reagent (Life Technologies) following manufacture instructions. Single-stranded cDNA was generated using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Otsushi, Japan). Quantitative real-time PCR was performed using the ABI 7500 Fast Thermocycler with SYBR® Green Real-Time PCR Master Mixes (Life Technologies), according to the manufacturer's recommended protocol. Gene expression level was calculated using the ΔΔCT method and normalized to the housekeeping gene β-actin. Primers sequences were designed using Beacon Designer 7.0 (Palo Alto, CA, USA), which is detailed in Supplemental Table 1.

2.4. Immunofluorescence, immunocytochemistry, and flow cytometry

Immunofluorescence, immunocytochemistry, and flow cytometry were carried out as previously described [22]. For immunofluorescent staining, cells were fixed with freshly prepared 4% (weight/volume) paraformaldehyde solution for 15 min at room temperature and then permeabilized in Dulbecco's Phosphate Buffered Saline containing 0.2% Triton X-100 and 10% fetal bovine serum (FBS). Following incubation with primary antibodies at 4 °C for 12 h, samples were incubated with secondary antibodies and Hoechst (Sigma) for 30 min at room temperature. Finally, visualization was achieved using a fluorescent microscope XL31 (Olympus, Tokyo, Japan). For Western blot analyses, cells were harvested in Ripa solution (Beyotime, Nantong, China) and a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). After denaturing in a 95 °C water bath for 15 min, the cell lysates were electrophoresed and transferred onto PVDF membranes using the Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA, USA). Target protein expression was detected by chemiluminescent exposure to a C-DiGit® Blot Scanner (LI-COR Lincoln, NE, USA). For flow cytometry, the samples were subjected to a Calibur 4-color flow cytometer (BD Bioscience, San Jose, CA, USA), followed by cell dissociation and antibody staining. The details and dilutions of all antibodies in the study are listed in Supplemental Table 2.

Recorded images of immunofluorescent or immunocytochemical staining were analyzed by Image J (National Institutes of health, Bethesda, MD, USA). Flow cytometrical results were analyzed by Flowjo 7.6.2 (FlowJo, LLC, Ashland, OR, USA).

2.5. Cell apoptosis assay

Cell apoptosis was evaluated with the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Life Technologies). After induction for 24 h, the cells were washed twice with PBS and harvested with 0.05% trypsin solution. Then the cells were incubated in Annexin-binding buffer (100 μL) with anti-Annexin V-Alexa Flour® 488 (100 μL) and propidium iodide (PI, 1 μL) for 15 min on ice. The cells were then diluted in 400 μL of Annexin-binding buffer, and the samples were analyzed using a Calibur 4-color flow cytometer.

2.6. Live cell number counts

Cells were harvested and dissociated with TypLE (Life Technologies) into single-cell suspensions. Cell density and cell viability were analyzed by Vi-Cell XR (Beckman Coulter, Indianapolis, IN, USA).