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endoderm (DE) differentiation from human embryonic stem cells (hESCs) was induced under a chemical-defined condition withdrawn insulin supplement and serum albumin.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).

2.7. Enzyme linked immunosorbent assay

ALB in culture media at day 24 of hepatocyte differentiation from hESCs was analyzed using an ALB ELISA assay kit (Bethyl Laboratories Inc) according to the manufacturer's instructions. ALB in culture media at day 24 of hepatocyte differentiation from hESCs was analyzed using an ALB ELISA assay kit (Bethyl Laboratories Inc) according to the manufacturer's instructions.

Human serum albumin secreted from differentiated hESCs in culture medium at day 15 of differentiation was analyzed by Human Serum Albumin DuoSet ELISA kit (R&D Systems), according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analysis was carried out with SSPS16.0 (IBM, Armonk, NY, USA). Unpaired *t*-test was used to analysis data from two groups. When the experimental design was >2 groups, one-way ANOVA was initially used for evaluation of general difference between groups. Least-significant difference test was used for multiple comparisons between any two given groups. A *P* value less 0.01 was considered statistically significant.

3. Results

3.1. Impaired endoderm differentiation under insulin-free, serum albumin-free and a chemically defined condition

To establish a culture condition optimal for DE induction, we prepared an albumin/insulin-free CDM by modifying Vallier's CDM [21]. Details of different culture media used in the experiment were summarized in Table 1. After initiating differentiation of hES cell line H9 by supplementation with activin A at 100 ng/mL, we observed that hESCs gradually exhibited a loose colony structure and morphologically changed to a spiky shape. Expression of a mesendoderm marker *T* (*BRACHURY*) increased at 24 h, but declined to basal levels by 96 h, as determined by qRT-PCR. *GSC*, another indicator of mesendoderm, was highly expressed between 24 and 48 h, but expression was declined between 72 and 96 h. Expression of endoderm-associated genes, such as *SOX17* and *FOXA2*, reached a peak at 48 h and was maintained for additional 48 h. In contrast, iCDM containing insulin at 10 μg/mL and activin A induced a minimal level of dynamic expression of DE-specific genes (Fig. 1A). The dynamic expression of DE-associated genes suggested that a 3-day duration in the above-described condition was sufficient for the transition from primitive streak into DE.

It has been proved that CXCR4 expression is in parallel with expression of DE markers, such as FOXA2 and SOX17 in hESCs exposed to a high concentration of activin A [3]. We further evaluated the extent of DE generation by detecting CXCR4-expressing cells using flow cytometry. The proportion of $CXCR4^+$ cells was augmented from 1.49 ± 0.23% in undifferentiated hESCs to 19.43 ± 0.67% in a population after exposure to activin A for three days (Fig. 1B). Extended activin A driven-differentiation (up to 4 days) did not result in a further increase in the output of CXCR4-positive cells (Fig. S1A). Additionally, a dramatic cell loss occurred in the process of activin A-driven differentiation, particularly during the first 48 h. Cell viability significantly declined after replacing the hESC culture medium with the induction medium and gradually was recovered in the differentiation (Fig. S1B), as determined by PI-Annexin V double staining. The survived population only was accounted for 34.23 ± 2.23% of seeded cells after activin A induction for 24 h; whereas it reached 64.13 ± 4.25% of hESCs cultured in MEF-conditioned medium (Fig. 1C).

3.2. Optimizing endodermal differentiation by supplementation of BMP4, bFGF or WNT3A with activin A under an albumin/insulin-free condition

To efficiently obtain DE cells under an albumin/insulin-free condition, we performed a comparative analysis of differentiation efficacy by a combination of growth factors (listed in the schema in Fig. 2) from previously published protocols for DE differentiation from H9 [8,9,23,24].

Treatment with BMP4 at 20 ng/mL plus activin A at 100 ng/mL significantly elevated *T* expression at 24 h, compared with treatment with activin A alone (as a control) or BMP4 at 10 ng/mL plus activin A. However, supplementation with BMP4 at a higher concentration (50 ng/mL) did not further increase *T* expression. Exogenous BMP4 exerted a similar dose-dependent effect on *GSC* expression at 48 h and on *FOXA2* expression at 72 h; whereas, the greatest *SOX17* expression was observed in the condition with BMP4 at 50 ng/mL (Fig. 2A). At 24 h, the combination of bFGF at 10 ng/mL or 20 ng/mL with activin A induced much more *T* expression than in cells treated only with activin A. The strongest expression of *GSC*, *SOX17* or *FOXA2* was detected in cells cultured with bFGF at 20 ng/mL plus activin A. However, treatment with bFGF at 100 ng/mL plus activin A failed to improve expression of DE-associated marker genes as compared to the controls (Fig. 2B). Treatment with WNT3A at 10 or 25 ng/mL plus activin A resulted in significantly higher *T* or *GSC* expression than in the controls. However, there was no significant improvement in *SOX17* or *FOXA2* mRNA expression following exposure to WNT3A. Therefore, we shortened the duration of WNT3A treatment as previously described [4]. HESCs were induced with WNT3A and activin A during the first 24 h, followed an induction by activin A alone for 48 h. A shortened exposure to WNT3A at 10 or 25 ng/mL resulted in greater *SOX17* and *FOXA2* expression than continuous treatment with WNT3A in the presence of activin A (Fig. 2C). These results suggested that a moderate concentration of BMP4, bFGF or WNT3A is sufficient to promote activin A-driven DE induction under a chemically defined condition.

To corroborate the qRT-PCR results, SOX17 and FOXA2 protein expression in hESCs induced by various combinations for 72 h was determined by Western blot analysis (Fig. 2D). We observed that WNT3A, BMP4 or bFGF exerted dose-dependent or time-dependent effects on SOX17 and FOXA2 protein expression in the presence of activin A, similar to what was detected at the mRNA levels. *SOX17* and *FOXA2* expression in ESCs with a short exposure (one day) to WNT3A at 10 or

Table 1
Medium components of different culture conditions.

Culture medium	Basal medium	Supplements	Treatment	Usage
Conditioned medium (CM)	Knockout-DMEM/F12	20% Knockout serum replacement	Conditioned by mouse embryonic fibroblast	Human embryonic stem cell maintenance
Chemical defined medium (CDM)	DMEM/F12	1 mM Glutamax, 0.1 mM non-essential amino acid, 0.1% chemical defined lipid concentrate, 50 μg/mL L-ascorbic acid, 0.1% polyvinyl alcohol		Definitive endoderm differentiation
Insulin-contained chemical defined medium (iCDM)	DMEM/F12	1 mM Glutamax, 0.1 mM non-essential amino acid, 0.1% chemical defined lipid concentrate, 50 μg/mL L-ascorbic acid, 0.1% polyvinyl alcohol, 1% insulin-transferrin-selenium		

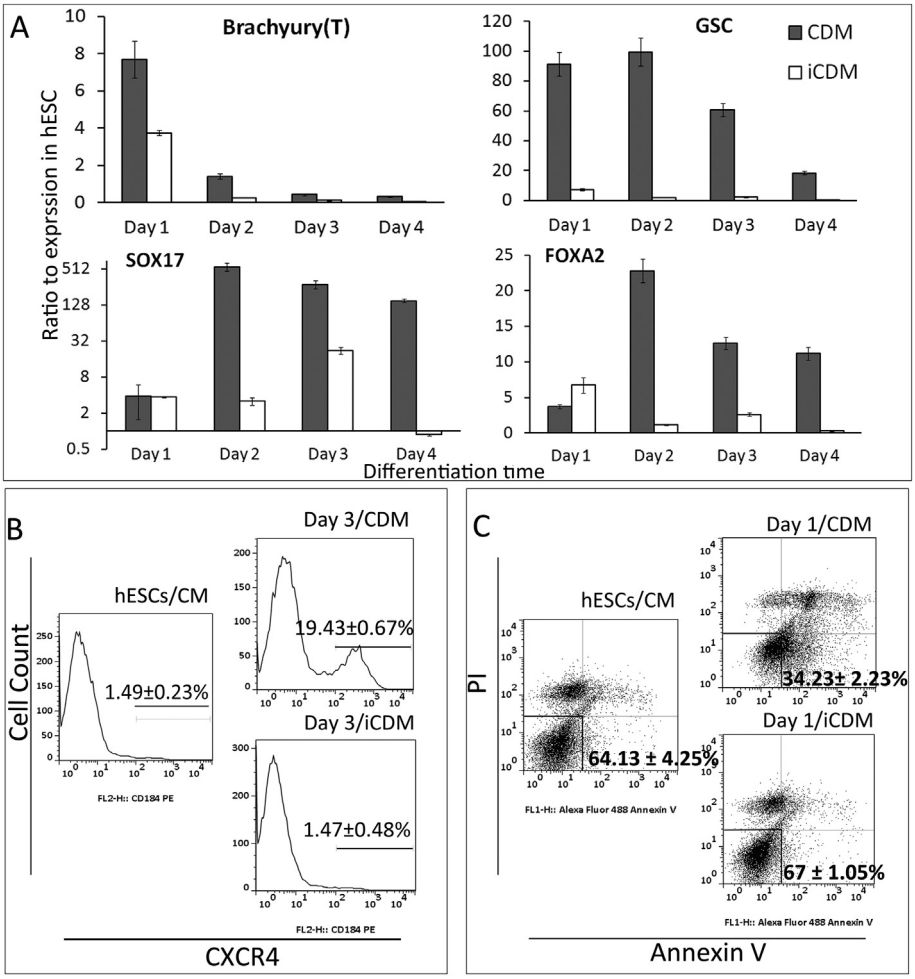

Fig. 1. Inefficient definitive endoderm differentiation and high cell loss in the presence of activin A under an albumin/insulin-free and chemically defined condition. (A) Transcriptional levels of primitive streak or DE marker genes (*BRACHURY*, *GSC*, *SOX17*, and *FOXA2*) after a 4-day differentiation from hESCs (H9) were determined by qRT-PCR. Bar illustrates SD from biological replicates ($n = 3$). $^{*}P < 0.01$, $^{**}P < 0.005$ according to Student's *t*-test. (B) Proportion of cells expressing the DE-specific surface antigen CXCR4, as measured by flow cytometry at day 3. Numbers in boxes represent percentages of cell populations positive for CXCR4. (C) Annexin V/PI double staining assay was used to evaluate cell viability at day 1 of activin A-driven differentiation. Cells in bottom left region (Annexin −/PI − population) are classified as surviving cells. Numbers in boxes represent percentages of survival cell populations. AA: activin A (100 ng/mL); CDM: chemically defined medium without insulin supplement; iCDM: chemically defined medium with 10 μg/mL insulin.

25 ng/mL was higher than 3-day treatment with WNT3A. The highest *SOX17* and *FOXA2* expression was seen at the combination of BMP4 (20 ng/mL), bFGF (20 ng/mL) or Wnt3a (10 or 25 ng/mL) with activin A.

3.3. Dual improvement on DE induction and cell survival using a cocktail containing BMP4, bFGF and WNT3A

Basing on the results shown above, we created several combinations of growth factor, and utilized *SOX17* and *FOXA2* expression as readouts for evaluating their effects on DE differentiation. Of all the combinations tested, the cocktail containing activin A, WNT3A, BMP4 and bFGF (AWBF combination) showed the highest efficacy on *SOX17* expression at 72 h, but it did not have the same effect on *FOXA2* expression. AWBF-induced *FOXA2* expression was comparable to AFW (activin A, WNT3A and bFGF) or AF (activin A and bFGF), but greater than the remaining combinations (Fig. 3A). In addition, it was determined that expression of *KDR* (the mesoderm marker) and *SOX7* (the extraembryonic endoderm marker) was barely induced while *HHEX* (the anterior definitive endoderm marker) expression was gradually increased in the present of AWBF combination (Fig. S2), by qRT-PCR assays. It suggested that AWBF combination did not promote the differentiation towards mesoderm or extraembryonic endoderm lineages under the chemical defined condition. Western blot analysis confirmed a similar expression pattern of SOX17 and FOXA2 protein in cells induced for 3 days (Fig. 3B).

The influence of the combination of WNT3A, BMP4 and bFGF on activin A-driven DE generation was further evaluated by CXCR4 expression with flow cytometry analysis. This combination increased $CXCR4^{+}$ cell production from 27.6 ± 1.5% (induction by activin A alone) to 88.2 ± 0.9% 3 days after differentiation. An induction duration up to 5 days resulted in increased homogeneous $CXCR4^{+}$ cell generation (97.67 ± 1.4%) in comparison to a diminished $CXCR4^{+}$ cell output by activin A alone (14.3 ± 2.16%) (Fig. 3C). We also evaluated the expression of another DE marker, FOXA2 with immunocytochemical staing. It was observed that the condition with activin A, WNT3A, BMP4 and bFGF resulted in over 90% CXCR4-positive DE cell outputs (91.9 ± 2.2% and 95.2 ± 0.9% at day 3 and 5), compared to a relatively lower CXCR4-positive cell induction (32.6 ± 9.4% and 21.2 ± 8.5% at day 3 and 5) by activin A alone (Fig. 3D). Furthermore, the Annexin V/PI double-binding flow cytometric assay revealed that after exposure to a WNT3A BMP4 and bFGF cocktail, hESC showed a significantly increased double-negative population (47.93 ± 4.4%), indicating improved cell viability with the combination in activin A-driven differentiation under an albumin/insulin-free condition (Fig. 3E). As mentioned above, major cell loss happened in the first 48 h of activin A induction, especially in the first 24 h (Fig. 3F). The supplementation of WNT3A BMP4 and bFGF

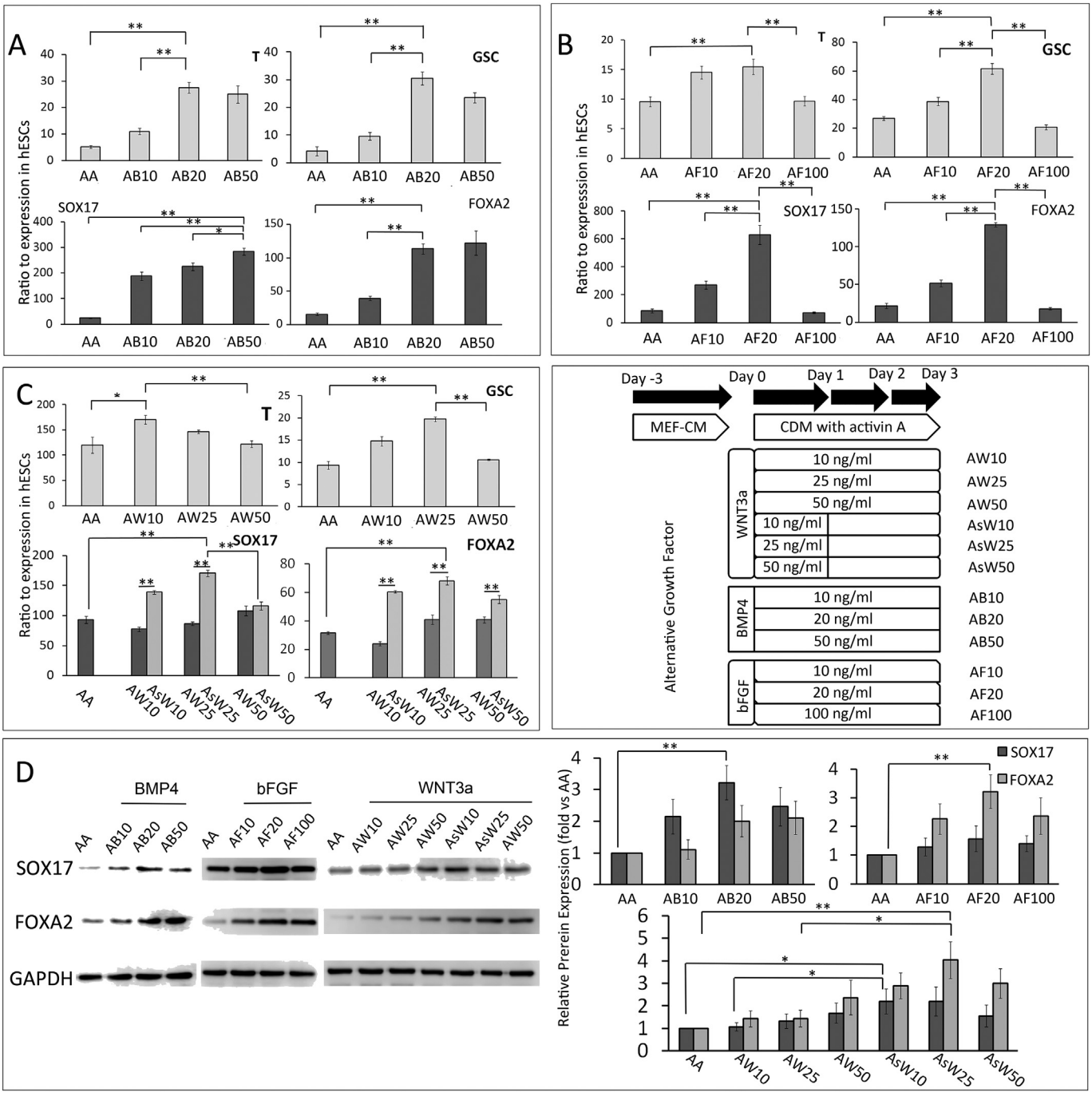

Fig. 2. BMP4, bFGF, or WNT3A dose dependence of DE-associated gene induction in activin A-driven differentiation. DE Differentiation from hESCs (H9) was induced by 100 ng/mL activin A and BMP4, bFGF, or WNT3A). Expressions of the primitive streak markers (T and GSC) at 24 h or 48 h, and definitive endoderm markers (SOX17 and FOXA2) at 72 h, respectively, in the presence of activin A combined with BMP4 (A), bFGF (B), or WNT3A (C). Bar illustrates SD from biological replicates ($n = 3$). $^{*}P < 0.01$, $^{**}P < 0.005$. Western blot analyses were performed to determined SOX17 and FOXA2 protein expression in cells induced by BMP4, bFGF, or WNT3A in the presence of activin A for 72 h (D). Error bars indicate SD from biological replicates ($n = 3$). $^{*}P < 0.01$, $^{**}P < 0.005$. The schema illustrates multiple stages of DE differentiation from hESCs induced by different growth factors. The illustration also shows details of medium and growth factors used at different stages of DE differentiation.

mitigated cell loss and increased cell viability in comparison with activin A-alone-induced differentiation.

To rule out the possibility of strain-specific effects of alternative growth factors on DE differentiation, we tested the growth factors on another hESC line, H7. The presence of the cocktail containing WNT3A, BMP4 and bFGF under a chemically defined condition resulted in improved DE production and viability maintenance in H7 DE differentiation (Fig. S3). These results verified that this differentiation protocol is optimal to drive DE differentiation from different hESCs at a high efficiency.

3.4. Synergism of WNT3A, BMP4 and bFGF on cell maintenance in activin A-driven differentiation

The observation that the presence of WNT3A, BMP4 and bFGF significantly improved cell viability raised a question which factor in the

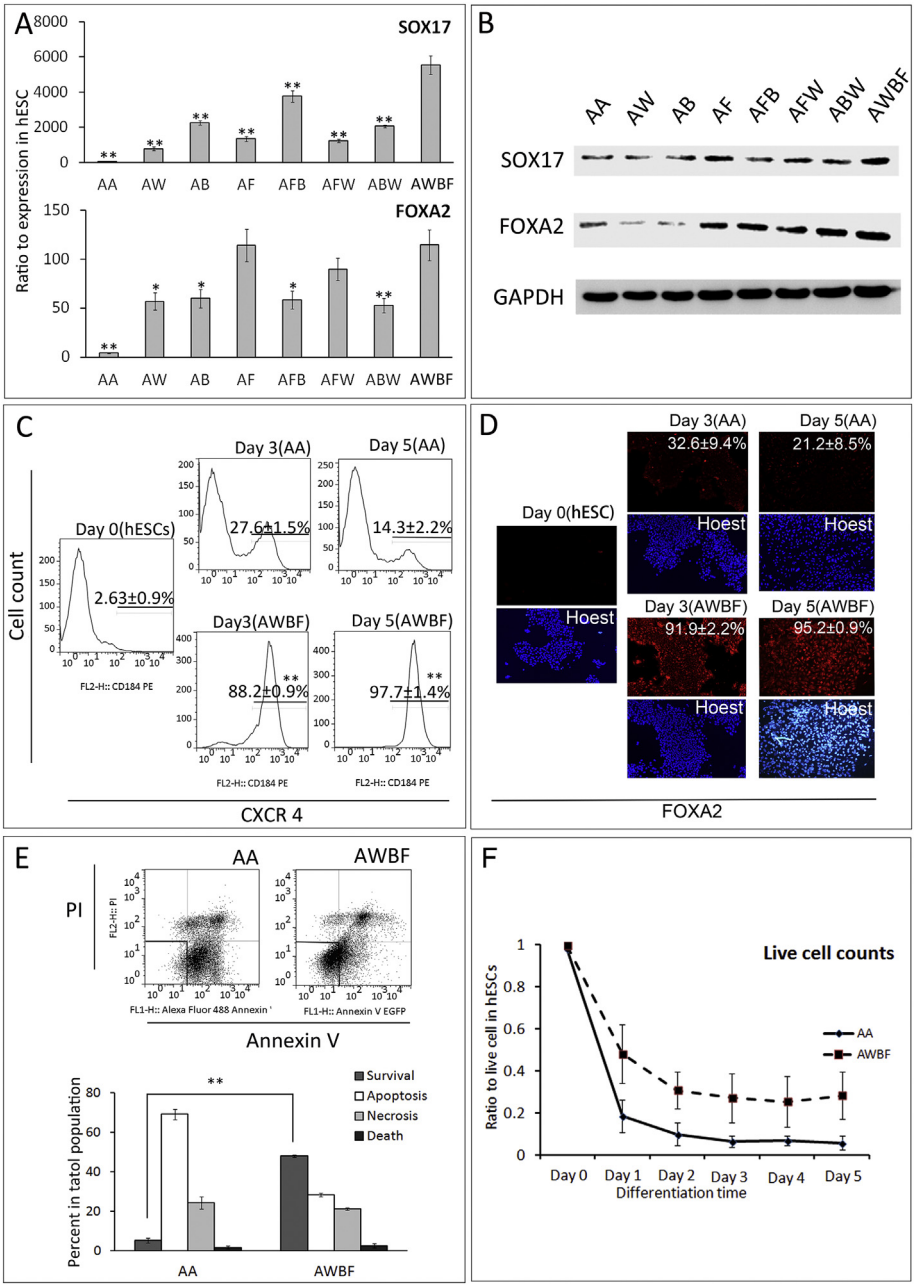

Fig. 3. Enhanced DE differentiation using a BMP4, bFGF, and WNT3A cocktail in the presence of activin A. (A) Transcriptional expression of *SOX17* and *FOXA2* during 3-day differentiation of hESCs (H9) induced by activin A alone or growth factor combinations, as detected by real-time qRT-PCR. Data were normalized to β-actin and are expressed as folds of expression in undifferentiated hESCs ($n = 3$ biological replicates; bar illustrates SD). $^{*}P < 0.01$, $^{**}P < 0.005$, *vs* AWBF. (B) Western blot analyses were performed to detect SOX17 and FOXA2 protein expression in cells exposed to different growth factor combinations for 3 days. (C) Flow cytometric analysis of CXCR4 expression in H9 induced by AWBF combinations or activin A for 3 or 5 days ($n = 3$ biological replicates), $^{**}P < 0.005$, AA *vs* AWBF, using Student's *t*-test. (D) Immunofluoresent staining was performed to determine FOXA2 expression in H9 cells treated by an AWBF combination or activin A alone for 3 or 5 days. Results are presented as a mean of positive cells in six fields (mean ± SD). (E) Annexin V/PI double staining assay was used to evaluate H9 cell viability after 24 h activin A-driven differentiation. Cells in bottom left region (Annexin^{-}/PI− population) are classified as viable; cells in bottom right region (Annexin+/PI− population) are classified as apoptotic; cells in upper left region (Annexin−/PI+ population) are classified as dead; cells in upper right region (Annexin+/PI+ population) are classified as necrotic. Three biological replicates are shown in the column chart and the bar illustrates SD from biological replicates. $^{**}P < 0.005$, AA *vs* AWBF, using Student's *t*-test. Cytokine combinations are represented as follows: control (activin A, AA), activin A + WNT3A (AW), activin A + BMP4 (AB), activin A + bFGF (AF), and activin A + 24 h-treatment with WNT3A + BMP4 + bFGF (AWBF) combination. (F) Percentage of total live cell was calculated every 24 h during DE induction. Three biological replicates were normalized to percentage of total live cells in undifferentiated cells.

cocktail contributed most to cell survival in DE differentiation. Hence, an Annexin V/PI double-binding assay was performed to evaluate cell viability under various conditions for DE induction. When hESCs underwent spontaneous differentiation in the control CDM, a moderate amount of cell death (apoptosis and necrosis) was observed, although a large portion of cells remained viable (Annexin V^{-}/PI^{-}) at 24 h. Exposure to a high concentration of activin A dramatically increased cell apoptosis and reduced cell viability, although insulin treatment restored cell viability upon spontaneous or activin A-driven differentiation (Fig. 4A).

Subsequently, we examined the effect of WNT3A, BMP4 or bFGF on cell viability in activin A-driven differentiation. Supplementation

with WNT3A (25 ng/mL), BMP4 (20 ng/mL) or bFGF (20 ng/mL) slightly elevated the rate of viable cells in the presence of activin A, whereas cell apoptosis remained high. Following exposure to a WNT3A, BMP4 and bFGF cocktail, hESCs significantly seemed to be resistant to apoptosis, and the percentage of viable cells significantly increased up to 50% (Fig. 4B). To rule out the possibility that an increase in viable cells could be a consequence of enhanced cell proliferation, we performed Ki67 staining to evaluate cell proliferation rate under the above-mentioned conditions. After 24 h of activin A-induced differentiation, there was only a slight decrease in the percentage of Ki67^{high}-cells in comparison to undifferentiated hESCs; and this effect was impeded by insulin supplementation.

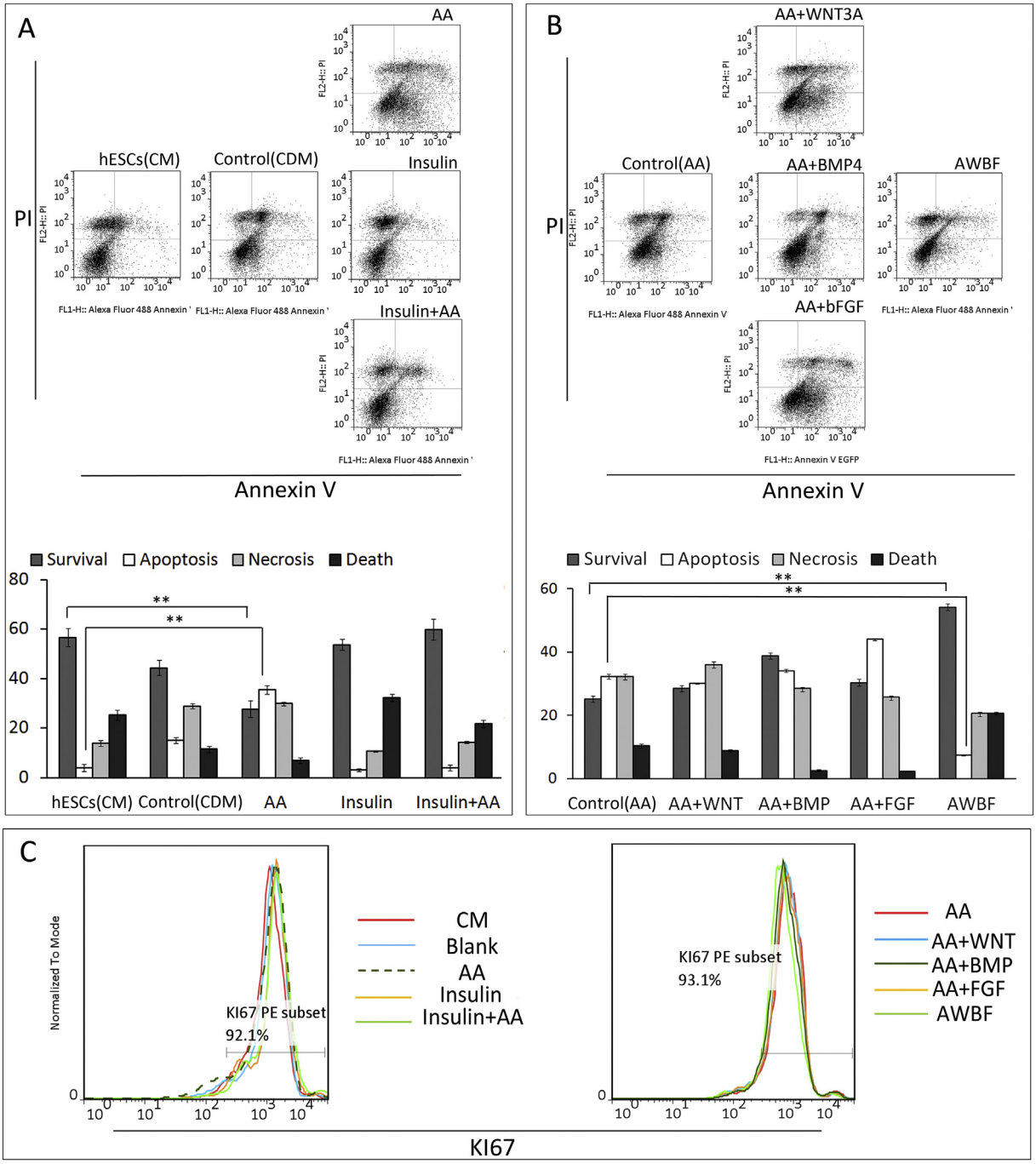

Fig. 4. WNT3, BMP4, and bFGF improved cell survival during activin A-driven differentiation under an insulin-free condition. (A) Annexin V/PI double staining assay was used to evaluate viability of H9 cells after 24 h of activin A-driven differentiation. (B) The Annexin V/PI double staining assay was used to evaluate the effect of alternative growth factors on cell viability after 24 h of activin A-driven differentiation. Three biological replicates are shown in the column chart, and the bar illustrates SD from biological replicates. $^{**}P < 0.005$, surviving cells; $^{**}P < 0.005$, apoptotic cells; CM *vs.* AA, or AA *vs.* AWBF, using Student's *t*-test. Cells in bottom left region (Annexin −/PI − population) are classified as surviving; cells in right region (Annexin +/PI − population) are classified as apoptotic; cells in upper left region (Annexin −/PI + population) are classified as dead; cells in upper right region (Annexin +/PI + population) are classified as necrotic. (C) Effect of WNT3a, BMP4, and bFGF on cell proliferation after 24 h of activin A-induced DE differentiation, as evaluated by Ki67 staining. Cells were maintained in or induced by MEF-conditioned medium (CM), chemically defined medium (blank), 100 ng/mL activin A (AA), and 50 μg/mL insulin and activin A (AA + Insulin). Cytokine combinations are represented as follows: control (activin A, AA), activin A + WNT3A (AW), activin A + BMP4 (AB), activin A + bFGF (AF), and activin A + WNT3A + BMP4 + bFGF (AWBF) combination.

Additionally, there was no significant difference in the percentage of Ki67^{high}-cells between conditions with or without WNT3A, BMP4 and bFGF supplementation (Fig. 4C), suggesting that these growth factors did not significantly affect cell proliferation during the initial stages of DE differentiation. These results also indicated that there is a synergistic effect of WNT3A, BMP4 and bFGF on activin A-induced

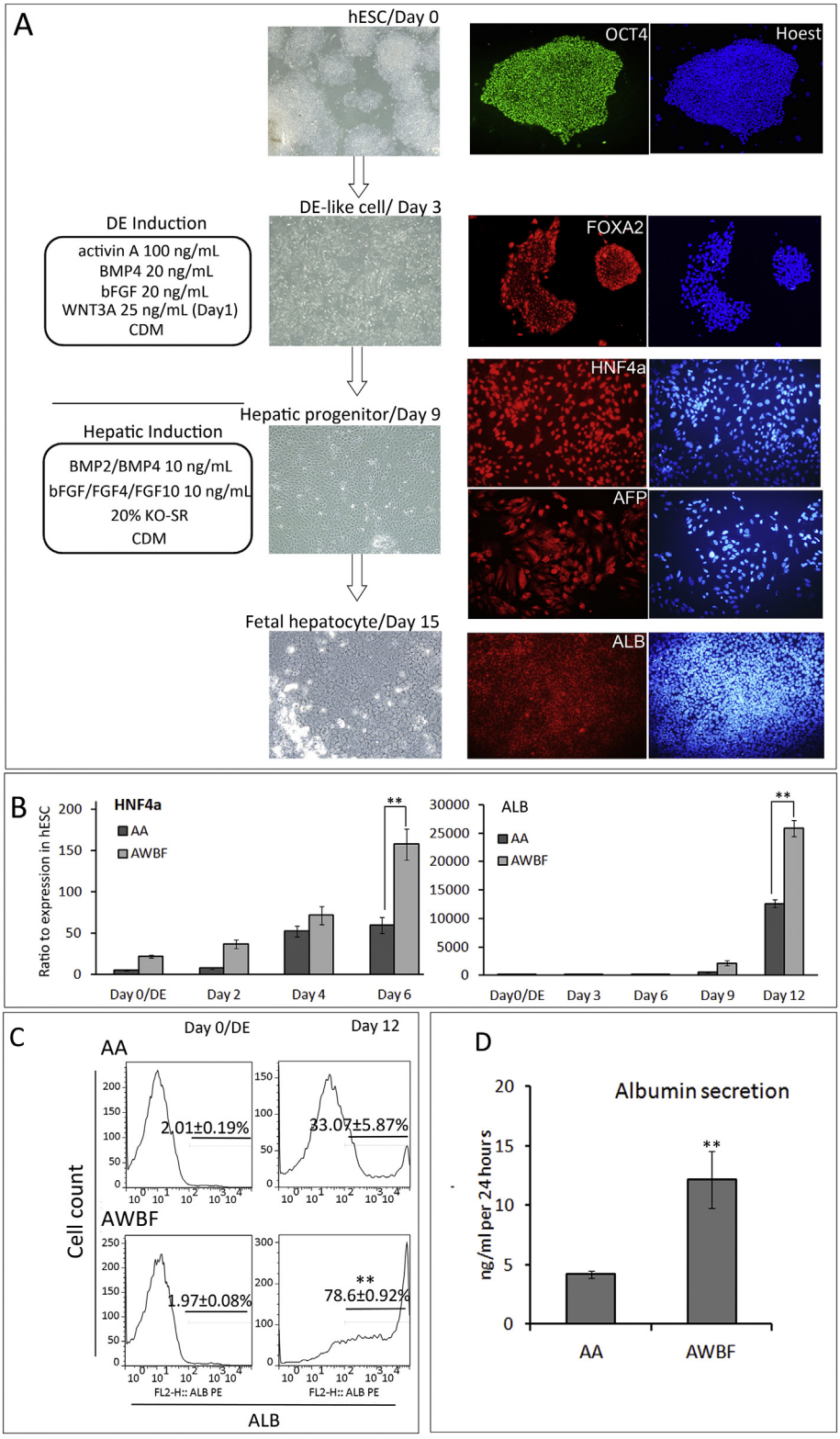

Fig. 5. Differentiation competency of BMP4, bFGF, and WNT3A-induced DE cells into a hepatic lineage. (A) Schematic illustrates DE differentiation from hESCs and further differentiation towards hepatic lineage cells. Images show temporal and sequential changes in cell morphology, and immunofluorescence analysis presents expression of key markers at different stages of differentiation. (B) QRT-PCR shows expression of hepatocyte-specific genes (*HNF4a* and *ALB*) in DE cells cultured under hepatic induction conditions, and the bar illustrates SD from biological replicates ($n = 3$), $^{**}P < 0.005$, AA *vs.* AWBF, using Student's *t*-test. (C) Flow cytometry detection of albumin expression in AWBF combination or activin A-induced DE cells after 12-day hepatic induction, $n = 3$, $^{**}P < 0.005$, AA *vs.* AWBF, using Student's *t*-test. (D) Albumin protein secretion was measured by an ELISA kit on day 15 of differentiation and is expressed as ng/ml/24 h, $n = 3$, $^{**}P < 0.005$, AA *vs.* AWBF, using Student's *t*-test.

apoptosis, which may contribute to improved cell survival under an insulin-free condition.

3.5. Hepatic differentiation of activin A, WNT3A, BMP4 and bFGF combination-induced DE cells

A further differentiation assay of hESCs towards a hepatic lineage was performed to determine the potential of DE cells induced by the AWBF combination cocktail containing activin A, WNT3A, BMP4 and bFGF. The schematic representation illustrates the procedure of DE generation and further differentiation of DE cells along the hepatic lineage (Fig. 5A). Characteristic morphological changes and expression of key hepatic markers in hESCs-derived cells at different stages were also shown in this panel. Under the hepatic induction by BMPs/FGFs/KO-SR, higher expression of *HNF4* or *ALB* mRNA levels was observed than those in activin A-induced DE cells at Day 6 or Day 12 with exposure to the same hepatic induction regimen (Fig. 5B). Detection of ALB^+ cells by FACS showed that the percentage of fetal hepatocyte increased up to ~80% 12 days under the hepatic induction in the AWBF-induced DE cells, in contrast to ~30% in activin A-induced DE cells (Fig. 5C). Hepatic progenitor or hepatoblasts induced by AWBF protocol also released a greater amount of albumin than that induced by Activin A-alone protocol (Fig. 5D). These results suggested that AWBF-induced DE cells are sufficiently competent to derive specialized lineages from the endoderm *in vitro*.

4. Discussion

It is common that chemically defined conditions for DE differentiation from hESCs contain serum albumin, insulin and other supplements [21,23,25]. Those conditions are insufficient to achieve high endoderm productivity, and potentially result in unexpected variations in endoderm induction, which may be attributed to a variety of lipids or growth factors associated with serum albumin preparation [20]. Insulin/IGFs serve as a blockade of endoderm fate specification by activating PI3K/AKT signaling [5]. Therefore, we initially modified the Vallier's hESC culture medium by withdrawing insulin to establish a fully defined condition for DE production. Our efforts faced the challenge of a low DE yield in the presence of activin A under an albumin/insulin-free condition, even though DE differentiation was evidenced by temporal and sequential up-regulation of T/GSC and SOX17/FOXA2 expression. Thus, it appears to us that an efficient DE generation requires inducing factors other than activin A under an albumin/insulin-free condition.

From literature it is clear that several growth factors, including BMP4, bFGF, and WNT3A, promote DE cell fate determination from hESCs in the presence of activin A [8,9,23,25]. In these studies, supplementation with moderate concentrations of BMP4, bFGF, and WNT3A was sufficient to up-regulate expression of DE key markers during activin A-driven differentiation. Additionally, the effect of WNT3A on DE differentiation was dependent on exposure duration under the chemically defined condition. A shortened duration of WNT3A treatment during the first 24 h seemed to result in a better synergistic effect of WNT3A and activin A on DE differentiation in comparison to continuous WNT3A treatment. This was consistent with the fact that *in vitro* hESC differentiation into DE initially requires activation of the canonical Wnt-signaling pathway [26]. However, current approaches to direct DE differentiation show a quite difference in the use of inducing factors that cooperate with activin A. One explanation may be the batch-to-batch variation in serum supplements whose components gave rise to a substantial difference in medium components [20]. These variations might alter the optimal inducing capability, including growth factor concentrations, the combined effects and sustainability, and ultimately compromise reproducible DE generation. Therefore, a large-scale of reproducible DE generation will require an optimal serum or serum albumin-free condition to minimize these variations.

As shown in other studies, insulin-free conditions caused severe cell death and impaired the yield of desired cells [12,13]. In addition, we found that inhibition of the insulin/PI3K pathway using the chemical LY294002 potentiated apoptotic cell death and eliminated most cells within 12 h under insulin-free conditions (data not shown). In contrast, supplementation with insulin ameliorated hESC apoptosis and significantly improved cell viability during activin A-driven differentiation. Therefore, it is conceiving that the viability of hESCs undergoing DE differentiation is dependent on activation of insulin/PI3K-dependent signaling, which is consistent with previous studies on signaling networks in embryonic stem cells [27,28]. Insulin-containing supplements have been prevalently used to maintain cell viability under serum-free conditions, because the high cell density is believed to be important for efficient DE generation. Unfortunately, maintenance of cell survival with insulin addition is inconsistent with the DE cell commitment that requires reduced insulin-PI3K/AKT signaling. In the present study, dramatic cell loss was observed during DE induction, particularly during the first 24 h, which was consistent with observations of hESC differentiation towards endodermal or mesodermal pathways in serum-independent conditions [12,13]. The presence of WNT3A, BMP4, or bFGF resulted in improved cell viability at first 24 h of DE induction under an albumin/insulin-free condition. Moreover, there was a strong synergism with WNT3A, BMP4, and bFGF on cell viability during activin A-induced DE differentiation, which significantly minimized cell apoptosis by circumventing the insulin/IGF signaling pathway and indirectly contributed to DE induction in the absence of insulin. This might explain why some growth factors foster activin A-driven differentiation into endodermal lineages *in vitro*, even though there is no evidence of their implication in mesendoderm/endoderm formation *in vivo* [8,29]. However, insulin supplementation was indispensable for viability maintenance and DE cell production in the case where induction duration was extended to 5 days under the insulin/albumin-free condition. A recent study revealed that PI3K/AKT signaling promotes the transition from naïve endoderm precursors into anterior endoderm [30], which suggests that the growth factor cocktail is a short-term and partial substitute for exogenous PI3K signaling during DE differentiation. Further optimization of cell maintenance requires a deep understanding of the balance between cell death and cell survival during DE differentiation *in vitro*. Our ongoing investigation focuses on the mechanisms underlying the choice between cell viability and survival. Indeed, highly efficient DE generation from hESCs has been achieved without alternative growth factors [3], which may be due to the application of serum or serum albumin that carries some of the growth factors required for DE differentiation. Mclean et al. observed that DE differentiation results in dramatic up-regulation of FGF/BMP signal-associated gene expression in hESCs [5], it is speculating that these signaling pathways could be stimulated in an autocrine or paracrine manner. This could explain why the maintenance of an appropriate cell density is required for efficient DE differentiation. Manipulating signaling pathways is not the only way to drive hESCs towards a DE cell fate. It has been recently shown that output of DE population generation was enhanced by creating a low oxygen culture condition in the process of directed differentiation. However, prolonged severe hypoxia (treatment with 1.5% O_2) compromised cell proliferation and survival [31]. The dual impacts of hypoxia suggest that the maintenance of cell viability should be seriously considered in the development of a refined protocol for DE differentiation.

5. Conclusions

Our results indicate that a cocktail containing WNT3A, BMP4 and bFGF promotes DE differentiation under a chemically defined condition in two manners. First, exogenous WNT3A, BMP4 and bFGF directly promote DE differentiation in a dose- or time-dependent manner. Second, as a consequence of increased cell viability following exposure to the cocktail, DE generation is indirectly improved by minimal

supplementation with insulin/IGFs. This optimal protocol confers efficient production of DE cells in an albumin/insulin-free and chemically defined system, and could facilitate the establishment of a DE generation system feasible for future clinic and pharmaceutical applications.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2017.03.017.

Conflict of interest

None to declare.

Funding

This work was supported by the National Science and Technology Major Project of China (2012ZX10002002; 2012ZX10003008-010; http://www.nmp.gov) to D.Q, and the National Natural Science Foundation of China (NSFC 81572356) and the Ministry of Science & Technology (#2016YFE0107400) to J.W.

Acknowledgement

We thank Professor QiLong Ying for help in providing technical support and advice in the beginning of the study.

References

[1] P.P. Tam, R.S. Beddington, Establishment and organization of germ layers in the gastrulating mouse embryo, CIBA Found. Symp. 165 (1992) 27–41 (discussion 42–29).
[2] H. Shirahashi, J. Wu, N. Yamamoto, A. Catana, H. Wege, B. Wager, K. Okita, M.A. Zern, Differentiation of human and mouse embryonic stem cells along a hepatocyte lineage, Cell Transplant. 13 (2004) 197–211.
[3] K.A. D'Amour, A.D. Agulnick, S. Eliazer, O.G. Kelly, E. Kroon, E.E. Baetge, Efficient differentiation of human embryonic stem cells to definitive endoderm, Nat. Biotechnol. 23 (2005) 1534–1541.
[4] K.A. D'Amour, A.G. Bang, S. Eliazer, O.G. Kelly, A.D. Agulnick, N.G. Smart, M.A. Moorman, E. Kroon, M.K. Carpenter, E.E. Baetge, Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells, Nat. Biotechnol. 24 (2006) 1392–1401.
[5] A.B. McLean, K.A. D'Amour, K.L. Jones, M. Krishnamoorthy, M.J. Kulik, D.M. Reynolds, A.M. Sheppard, H. Liu, Y. Xu, E.E. Baetge, S. Dalton, Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed, Stem Cells 25 (2007) 29–38.
[6] J.K. Mfopou, B. Chen, I. Mateizel, K. Sermon, L. Bouwens, Noggin, retinoids, and fibroblast growth factor regulate hepatic or pancreatic fate of human embryonic stem cells, Gastroenterology 138 (2010) (2233–2245, 2245 e2231–2214).
[7] D.C. Hay, J. Fletcher, C. Payne, J.D. Terrace, R.C. Gallagher, J. Snoeys, J.R. Black, D. Wojtacha, K. Samuel, Z. Hannoun, A. Pryde, C. Filippi, I.S. Currie, S.J. Forbes, J.A. Ross, P.N. Newsome, J.P. Iredale, Highly efficient differentiation of hESCs to functional hepatic endoderm requires Activin A and Wnt3a signaling, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 12301–12306.
[8] G.M. Morrison, I. Oikonomopoulou, R.P. Migueles, S. Soneji, A. Livigni, T. Enver, J.M. Brickman, Anterior definitive endoderm from ESCs reveals a role for FGF signaling, Cell Stem Cell 3 (2008) 402–415.
[9] A.K. Teo, Y. Ali, K.Y. Wong, H. Chipperfield, A. Sadasivam, Y. Poobalan, E.K. Tan, S.T. Wang, S. Abraham, N. Tsuneyoshi, L.W. Stanton, N.R. Dunn, Activin and BMP4 synergistically promote formation of definitive endoderm in human embryonic stem cells, Stem Cells 30 (2012) 631–642.
[10] D.A. Brafman, C. Phung, N. Kumar, K. Willert, Regulation of endodermal differentiation of human embryonic stem cells through integrin-ECM interactions, Cell Death Differ. 20 (2013) 369–381.
[11] D.F. Malta, N.E. Reticker-Flynn, C.L. da Silva, J.M. Cabral, H.E. Fleming, K.S. Zaret, S.N. Bhatia, G.H. Underhill, Extracellular matrix microarrays to study inductive signaling for endoderm specification, Acta Biomater. 34 (2016) 30–40.
[12] B.R. Lawton, J.A. Sosa, S. Roman, D.S. Krause, Effect of a matrigel sandwich on endodermal differentiation of human embryonic stem cells, Stem Cell Rev. 9 (2013) 578–585.
[13] J. Zhang, M. Klos, G.F. Wilson, A.M. Herman, X. Lian, K.K. Raval, M.R. Barron, L. Hou, A.G. Soerens, J. Yu, S.P. Palecek, G.E. Lyons, J.A. Thomson, T.J. Herron, J. Jalife, T.J. Kamp, Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method, Circ. Res. 111 (2012) 1125–1136.
[14] D.C. Hay, D. Zhao, J. Fletcher, Z.A. Hewitt, D. McLean, A. Urruticoechea-Uriguen, J.R. Black, C. Elcombe, J.A. Ross, R. Wolf, W. Cui, Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo, Stem Cells 26 (2008) 894–902.
[15] N. Shiraki, T. Yamazoe, Z. Qin, K. Ohgomori, K. Mochitate, K. Kume, S. Kume, Efficient differentiation of embryonic stem cells into hepatic cells in vitro using a feeder-free basement membrane substratum, PLoS One 6 (2011), e24228. .
[16] J. Cai, Y. Zhao, Y. Liu, F. Ye, Z. Song, H. Qin, S. Meng, Y. Chen, R. Zhou, X. Song, Y. Guo, M. Ding, H. Deng, Directed differentiation of human embryonic stem cells into functional hepatic cells, Hepatology 45 (2007) 1229–1239.
[17] M. Borowiak, R. Maehr, S. Chen, A.E. Chen, W. Tang, J.L. Fox, S.L. Schreiber, D.A. Melton, Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells, Cell Stem Cell 4 (2009) 348–358.
[18] T. Touboul, N.R. Hannan, S. Corbineau, A. Martinez, C. Martinet, S. Branchereau, S. Mainot, H. Strick-Marchand, R. Pedersen, J. Di Santo, A. Weber, L. Vallier, Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development, Hepatology 51 (2010) 1754–1765.
[19] H. Wang, X. Luo, L. Yao, D.M. Lehman, P. Wang, Improvement of cell survival during human pluripotent stem cell definitive endoderm differentiation, Stem Cells Dev. 24 (2015) 2536–2546.
[20] G. Chen, D.R. Gulbranson, Z. Hou, J.M. Bolin, V. Ruotti, M.D. Probasco, K. Smuga-Otto, S.E. Howden, N.R. Diol, N.E. Propson, R. Wagner, G.O. Lee, J. Antosiewicz-Bourget, J.M. Teng, J.A. Thomson, Chemically defined conditions for human iPSC derivation and culture, Nat. Methods 8 (2011) 424–429.
[21] L. Vallier, T. Touboul, S. Brown, C. Cho, B. Bilican, M. Alexander, J. Cedervall, S. Chandran, L. Ahrlund-Richter, A. Weber, R.A. Pedersen, Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells, Stem Cells 27 (2009) 2655–2666.
[22] S. Ye, L. Tan, R. Yang, B. Fang, S. Qu, E.N. Schulze, H. Song, Q. Ying, P. Li, Pleiotropy of glycogen synthase kinase-3 inhibition by CHIR99021 promotes self-renewal of embryonic stem cells from refractory mouse strains, PLoS One 7 (2012), e35892. .
[23] A.K. Teo, I.A. Valdez, E. Dirice, R.N. Kulkarni, Comparable generation of activin-induced definitive endoderm via additive Wnt or BMP signaling in absence of serum, Stem Cell Rep. 3 (2014) 5–14.
[24] P. Gadue, T.L. Huber, P.J. Paddison, G.M. Keller, Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16806–16811.
[25] L. Sui, J.K. Mfopou, M. Geens, K. Sermon, L. Bouwens, FGF signaling via MAPK is required early and improves Activin A-induced definitive endoderm formation from human embryonic stem cells, Biochem. Biophys. Res. Commun. 426 (2012) 380–385.
[26] O. Naujok, U. Diekmann, S. Lenzen, The generation of definitive endoderm from human embryonic stem cells is initially independent from activin A but requires canonical Wnt-signaling, Stem Cell Rev. 10 (2014) 480–493.
[27] L. Armstrong, O. Hughes, S. Yung, L. Hyslop, R. Stewart, I. Wappler, H. Peters, T. Walter, P. Stojkovic, J. Evans, M. Stojkovic, M. Lako, The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis, Hum. Mol. Genet. 15 (2006) 1894–1913.
[28] K. Takahashi, M. Murakami, S. Yamanaka, Role of the phosphoinositide 3-kinase pathway in mouse embryonic stem (ES) cells, Biochem. Soc. Trans. 33 (2005) 1522–1525.
[29] Y.F. Chen, C.Y. Tseng, H.W. Wang, H.C. Kuo, V.W. Yang, O.K. Lee, Rapid generation of mature hepatocyte-like cells from human induced pluripotent stem cells by an efficient three-step protocol, Hepatology 55 (2012) 1193–1203.
[30] S.N. Villegas, M. Rothova, M.E. Barrios-Llerena, M. Pulina, A.K. Hadjantonakis, T. Le Bihan, S. Astrof, J.M. Brickman, PI3K/Akt1 signalling specifies foregut precursors by generating regionalized extra-cellular matrix, elife 2 (2013), e00806. .
[31] L.F. Chu, N. Leng, J. Zhang, Z. Hou, D. Mamott, D.T. Vereide, J. Choi, C. Kendziorski, R. Stewart, J.A. Thomson, Single-cell RNA-seq reveals novel regulators of human embryonic stem cell differentiation to definitive endoderm, Genome Biol. 17 (2016) 173.