

Oncostatin M in William's E medium is suitable for initiation of hepatocyte differentiation in human induced pluripotent stem cells

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Abstract. William's E (WE) is a suitable medium for the differentiation of human induced pluripotent stem (iPS) cells to the hepatocyte lineage. The aim of the present study was to investigate various growth factors in their ability to promote hepatocyte differentiation of iPS cells in WE medium. Human iPS 201B7 cells were cultured in WE medium supplemented with growth factors, and mRNA expression levels and promoter activities of α -fetoprotein (AFP) and albumin were examined by reverse transcription-quantitative polymerase chain reaction and luciferase assay, respectively. In addition, time course analysis of AFP mRNA expression was performed in 201B7 cells cultured in WE medium supplemented with oncostatin M. The results demonstrated that mRNA expression levels of AFP were significantly elevated by most growth factors tested as supplements in WE medium, except all-trans retinoic acid, compared with cells cultured in ReproFF (a medium that maintains pluripotency). The highest increase in AFP mRNA expression levels was observed by oncostatin M stimulation. Albumin mRNA expression levels were increased by all-trans retinoic acid and insulin-transferrin-selenium supplementation in WE medium compared with cells cultured in ReproFF. Oncostatin M supplementation significantly stimulated the promoter activity of the AFP gene, but no growth factor tested significantly stimulated the promoter activity of the albumin gene. By time course analysis, significant increase of AFP mRNA expression was observed on the sixth day post-stimulation, compared with cells cultured in WE medium alone. In conclusion, the present study demonstrated that oncostatin M supplementation in WE medium was sufficient to initiate hepatocyte differentiation in iPS cells.

Introduction

Human induced pluripotent stem (iPS) cells are established with the introduction of reprogramming factors (1). iPS cells have the potential to differentiate into hepatocyte-like cells by stimulation with growth factors or by introduction of transcription factors (2,3). It is hypothesized that hepatocytes generated from iPS cells could be used in the future to treat liver failure, a fatal condition due to major loss of hepatocytes, by transplantation into patients (4). Current protocols, however, have many limitations, including that the hepatocytes generated from iPS cells remain at an immature state (5). In addition, current protocols require several weeks to obtain hepatocyte-like cells.

Growth factors and transcription factors are important for differentiation of iPS cells to hepatocyte lineage. Transcription factor protocols efficacy depends on the efficiency of the method used for the introduction of the gene of interest to target cells. This problem has been, partly, overcome by the use of adenovirus vectors (6). Transcription factors have also been introduced into iPS cells with conventional reagents (7,8). Alternatively, a combination of growth factors and media has been demonstrated to affect the differentiation of iPS cells to hepatocyte lineage. A recent study reported that William's E medium (WE) is suitable for hepatocyte differentiation of iPS cells when followed by culturing in hepatocyte differentiation inducer medium (9). WE medium was originally formulated to culture primary hepatocytes, and it has been demonstrated to maintain hepatocyte-specific drug metabolism (10,11). However, whether growth factor supplementation in WE medium is sufficient to initiate hepatocyte differentiation of iPS cells remains unknown.

Oncostatin M, a member of interleukin 6 family, is expressed in fetal liver, and its expression decreases after birth (12). Oncostatin M promotes maturation of hepatocyte differentiation from fetal hepatocytes *in vitro* (13). The resulting hepatocytes express hepatocyte-specific genes, accumulate glycogen, and remove ammonia (13). Based on these properties, it is hypothesized that oncostatin M may promote hepatocyte differentiation of iPS cells, but its effect as a supplement in WE medium remains unknown.

The present study, therefore, investigated whether supplementation of WE medium with various growth factors, including oncostatin M, was a suitable method for hepatocyte

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differentiation of iPS cells. The results may provide a novel method for the successful differentiation of iPS cells to hepatocytes, which could be potentially useful in the future for therapeutic transplantation into patients.

Materials and methods

Cell culture. Human iPS 201B7 cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in ReproFF (Reprocell, Inc., Yokohama, Japan) medium in 6-well plates coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) under feeder-free conditions. The cells were then harvested using Accutase (Innovative Cell Technologies, Inc., San Diego, CA, USA) and centrifuged at $100 \times g$ for 3 min at 4°C . The cells were spread onto new 6-well plates or 96-well plates coated with Matrigel. For experiments, cells were cultured in ReproFF or in WE (Thermo Fisher Scientific, Inc., Waltham, MA, USA) media supplemented with nicotinamide (1.2 mg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan), proline (30 ng/ml; Wako Pure Chemical Industries, Ltd.) and 10% Knockout serum replacement (Thermo Fisher Scientific, Inc.).

Growth factors. The growth factors used in the present study were: Basic fibroblast growth factor (5 ng/ml; Wako Pure Chemical Industries, Ltd.), bone morphogenetic protein 4 (20 ng/ml; Wako Pure Chemical Industries, Ltd.), oncostatin M (20 ng/ml; Wako Pure Chemical Industries, Ltd.), epidermal growth factor (20 ng/ml; Wako Pure Chemical Industries, Ltd.), β -nerve growth factor (100 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA), all-trans retinoic acid (1 μM ; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), transforming growth factor- β 1 (2 ng/ml; R&D Systems, Inc.), hepatocyte growth factor (20 ng/ml; Wako Pure Chemical Industries, Ltd.), dexamethasone (10^{-7} M; Wako Pure Chemical Industries, Ltd.), and insulin-transferrin-sodium selenite media supplement (100x; Sigma-Aldrich; Merck KGaA).

Plasmid construction. The EcoRI-SalI fragment of the human α -fetoprotein (AFP) promoter (Switchgear Genomics, Carlsbad, CA, USA) was subcloned into the pMetLuc2-reporter plasmid (Clontech Laboratories, Inc., Mountainview, CA, USA) to produce the pMetLuc2/AFP promoter reporter plasmid. Similarly, the SacI-Hind3 fragment of the human albumin promoter (Switchgear Genomics) was subcloned into the pMetLuc2-reporter plasmid to produce the pMetLuc2/albumin promoter reporter plasmid.

Transfection and luciferase assay. 201B7 cells were cultured in 96-well plates coated with Matrigel. Cells in each well (5×10^5 cells/well) were transfected with 100 ng pMetLuc2/AFP or pMetLuc2/albumin plasmid using FuGENE HD transfection reagent (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. As a control, 10 ng pSEAP2-control plasmid (Clontech Laboratories, Inc.), which expresses secreted alkaline phosphatase (SEAP), was transfected into the cells in parallel to monitor the transfection efficiency. The transfected cells were then cultured in ReproFF (FF) or WE media, with or without growth factors. Following two days of culture, media samples were evaluated by a Ready-To-Glow secreted luciferase assay (Clontech

Laboratories, Inc.) and a SEAP assay (Clontech Laboratories, Inc.). The luciferase measurement was divided by the SEAP measurement to calculate the relative gene promoter activity.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA ($\sim 5 \mu\text{g}$) was isolated from cultured cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and was utilized for the synthesis of first-strand cDNA using SuperScript III reverse transcriptase and oligo (dT) primers (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Total RNA from human fetal liver was purchased from Clontech Laboratories, Inc. RT-qPCR was performed using Fast SYBR-Green Master Mix (Thermo Fisher Scientific, Inc.) and the results were analyzed using the MiniOpticon Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was performed in a volume of 20 μl for 40 cycles, with thermocycling conditions according to the Fast SYBR-Green Master Mix suggested protocol. Primer sequences are listed in Table I. RPL19 was used as an endogenous reference control because it is a constitutively expressed housekeeping gene (14). The gene expression levels were analyzed automatically using the MiniOpticon System, based on the $2^{-\Delta\Delta C_q}$ method (15). The relative expression was calculated as the expression level of a specific gene divided by that of RPL19.

Statistical analysis. Data are presented as means \pm standard deviation of three independent repeats. Results were analyzed for statistical significance by one-way analysis of variance followed by Turkey's post hoc test, using JMP version 10.0.2 software (SAS Institute, Inc., Cary, NC, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Human iPS 201B7 cells were cultured in WE medium supplemented with various growth factors for 7 days and then mRNA expression levels of AFP and albumin were analyzed by RT-qPCR. As a control, cells were also cultured in WE medium alone and in ReproFF, a medium that maintains pluripotency. The mRNA expression levels of AFP were significantly increased in cells cultured in WE with most of the growth factors tested, except all-trans retinoic acid, compared with cells cultured in ReproFF (Fig. 1A). Out of all the factors tested, Oncostatin M supplementation resulted in the highest increase in AFP mRNA (Fig. 1A). The mRNA expression levels of albumin was increased in cells cultured in WE medium supplemented with all-trans retinoic acid and insulin-transferrin-selenium compared with cells cultured in ReproFF (Fig. 1B).

A luciferase reporter assay was performed to investigate the effect of the growth factors on the promoter activity of the AFP and albumin genes. 201B7 cells were transfected with either the pMetLuc2/AFP (Fig. 2A) or pMetLuc2/albumin (Fig. 2B) promoter reporter plasmid, in parallel with pSEAP2-control plasmid, which expresses alkaline phosphatase, as a control for transfection efficiency. Following 2 days of culturing the transfected cells in ReproFF or WE with or without growth factors, samples from the culture media were subjected to luciferase and SEAP assays. Oncostatin M supplementation in WE media was observed to stimulate AFP promoter activity most strongly and significantly (Fig. 1A), while no growth

Table I. Primer sequences used for quantitative polymerase chain reaction.

Gene	Primer	Sequence (5'-3')	Product size (bp)	GenBank accession number
AFP	F	ACACAAAAAGCCCACTCCAG	147	NM_001134
	R	GGTGCATACAGGAAGGGATG		
RPL19	F	CGAATGCCAGAGAAGGTCAC	157	BC095445
	R	CCATGAGAATCCGCTTGTTT		
Albumin	F	GCTCGTGAAACACAAGCCCAAG	114	NM_000477
	R	GCAAAGCAGGTCTCCTTATCGTC		

Primers were designed to have an annealing temperature of 60°C and underwent 40 cycles of quantitative polymerase chain reaction. AFP, α -fetoprotein; RPL19, ribosomal protein L19.

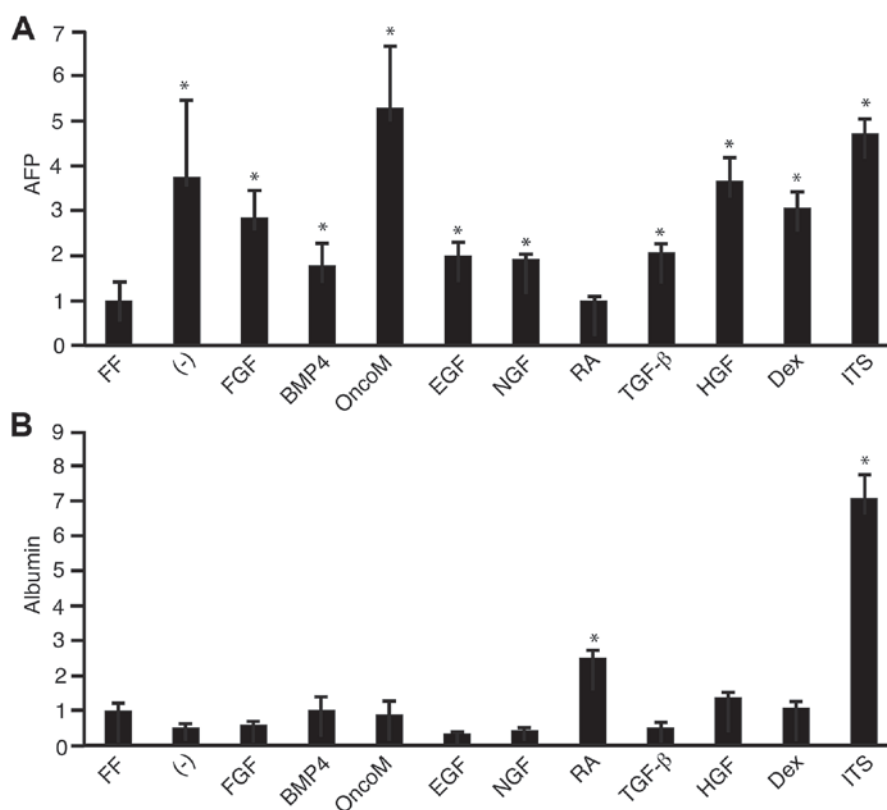


Figure 1. Effect of growth factors on AFP and albumin mRNA expression. 201B7 cells were cultured in FF medium or in WE medium with or without (-) growth factors for 7 days. Reverse transcription-quantitative polymerase chain reaction was then performed to analyze the mRNA expression levels of (A) AFP and (B) albumin. * $P < 0.05$ vs. FF. AFP, α -fetoprotein; FF, ReproFF; WE, William's E; FGF, fibroblast growth factor; BMP4, bone morphogenetic protein 4; OncoM, oncostatin M; EGF, epidermal growth factor; NGF, nerve growth factor; RA, retinoic acid; TGF- β , transforming growth factor β ; HGF, hepatocyte growth factor; Dex, dexamethasone; ITS, insulin-transferrin-selenium.

factor tested significantly affected the promoter activity of the albumin gene (Fig. 1B).

The present results suggested that oncostatin M may be suitable for the initiation of hepatocyte differentiation in iPS cells. Time course analysis of the AFP mRNA expression pattern was performed to identify the timing of the stimulation of 201B7 cells towards the initiation of hepatocyte lineage differentiation (Fig. 3). The mRNA expression levels of AFP

increased on the sixth day following the initiation of culture in the oncostatin M-supplemented WE medium, compared with cells cultured in WE medium alone (Fig. 3).

Discussion

Oncostatin M belongs to the interleukin-6 subfamily (16) and it is secreted by hematopoietic cells during endoderm

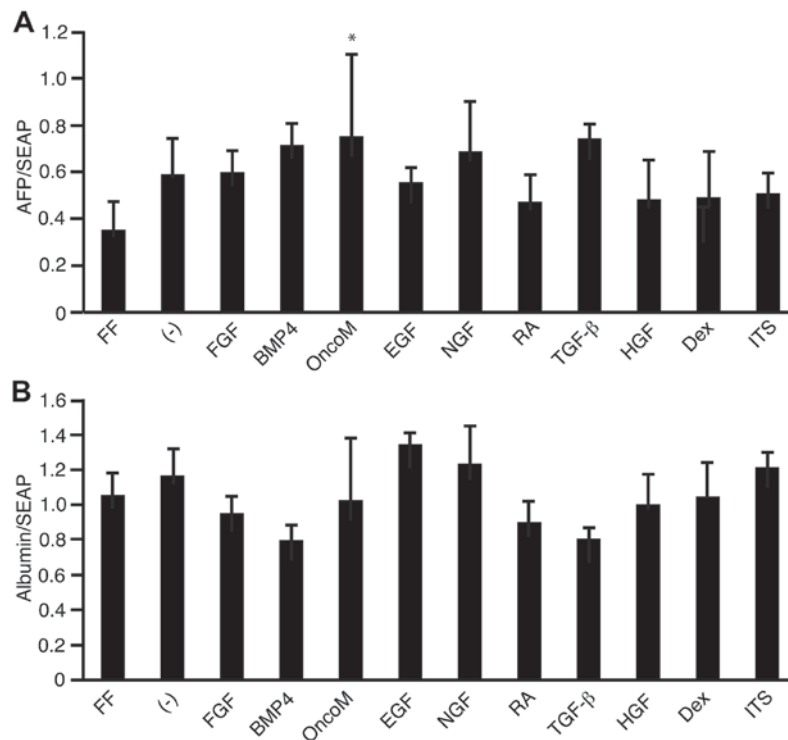


Figure 2. Effect of growth factors on AFP and albumin gene promoter activities. 201B7 cells were transfected with luciferase reporter plasmids driven by either the promoter of (A) AFP or (B) albumin. A pSEAP2-control vector was transfected in parallel to monitor transfection efficiency. Following transfection, cells were cultured in FF medium or in WE medium with or without (-) growth factors for 2 days, and analyzed by luciferase and alkaline phosphatase assays. Gene promoter activity is reported as the relative ratio of luciferase vs. SEAP measurement for each gene. * $P < 0.05$ vs. FF. AFP, α -fetoprotein; SEAP, secreted alkaline phosphatase; FF, ReproFF; WE, William's E; FGF, fibroblast growth factor; BMP4, bone morphogenetic protein 4; OncoM, oncostatin M; EGF, epidermal growth factor; NGF, nerve growth factor; RA, retinoic acid; TGF- β , transforming growth factor β ; HGF, hepatocyte growth factor; Dex, dexamethasone; ITS, insulin-transferrin-selenium.

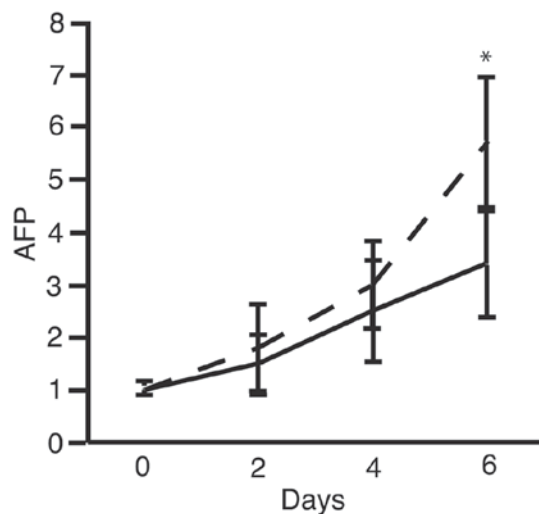


Figure 3. Time course analysis of the AFP mRNA expression. 201B7 cells were cultured in William's E medium without (solid line) or with oncostatin M (broken line) for up to 6 days, and AFP mRNA expression was analyzed at 0, 2, 4 and 6 days post stimulation. * $P < 0.05$ vs. day 0. AFP, α -fetoprotein.

formation (17). Oncostatin M is important in the maturation of immature hepatocytes (18) and in the long-term culture of human primary hepatocytes (19). In the present study, it was demonstrated that oncostatin M initiated the differentiation of iPS cells to hepatocyte lineage. The mechanism of the initiation of hepatocyte differentiation of iPS cells remains

unclear. The present data is consistent with previous studies from our own group (5,20) and a study from another group that has demonstrated that oncostatin M promotes differentiation of iPS cells to hepatocytes (21). Therefore, oncostatin M is suggested to be suitable for the initiation of hepatocyte differentiation from iPS cells.

There is the potential that some of the immature iPS cells cultured in WE medium supplemented with oncostatin M remain after differentiation initiation. To counter this, hepatocyte selection medium (HSM) could be used to eliminate undifferentiated iPS cells from the culture. Cells do not survive in media without glucose or arginine, because these two ingredients are essential for their survival. However, the gluconeogenesis pathway and the urea cycle occur in hepatocytes, thereby allowing them to produce glucose from galactose and arginine from ornithine, respectively (18,19). Hence, HSM is routinely prepared without glucose or arginine, but supplemented with galactose and ornithine (22), and culturing in HSM results in elimination of undifferentiated iPS cells, however survival of hepatocytes (23). Since iPS cells cannot survive in media without glucose, it is hypothesized that the potentially remaining iPS cells in the differentiation conditions tested in the present study may be eliminated by subsequent culture in HSM.

A limitation of the present study is that the expression levels of AFP and albumin were analyzed only at the mRNA level, and not at the protein level. In addition, the expression levels of liver-specific genes, including cytochrome P450 3A4,

aldehyde dehydrogenase 2, and glucose-6-phosphatase were not analyzed. Finally, Fig. 3 demonstrated that AFP expression increased on the sixth day of culture post-stimulation with oncostatin M in WE medium compared with WE medium alone, however, the time course analysis was only performed up to 6 days and therefore, it remains unclear whether longer culture would increase AFP expression further or whether this was the maximum. Further studies are required to culture iPS cells in WE supplemented with oncostatin M for longer than 6 days, and to analyze the protein expression of AFP and albumin, in addition to the expression of liver-specific gene markers.

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