Transfections with siRNA

Transfections with siRNA targeting human *EGR-1* (SMARTpool ON-TARGETplus, L-006526-00) or non-targeting control siRNA (ON-TARGETplus Non-targeting Pool, D-001810-10) were performed using Dharmafect1 (Dharmacon) as previously described [4]. Total RNAs or proteins were extracted for analysis 72 h after the fast transfection.

Western blot analyses

Western blot analyses were performed as described previously [4]. The protein was separated by 12.5% sodium dodeyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were reacted with primary antibodies, peroxidase-conjugated secondary antibodies, and ECL Plus reagent (GE Healthcare). Protein bands were visualized using the LAS-4000 imager (Fujifilm). The primary antibodies used are listed in the Supporting Information Table S2.

Cell imaging analysis

The images of eGFP-positive cells in culture were captured in a cell imaging system, BioStation CT (Nikon Instruments, Inc.) at 37°C 10% CO_2. The images were analyzed by a software CL-Quant (Nikon Instruments, Inc.).

Results

EMT induction of hES cells by PMA

To confirm whether a PKC activator, PMA, induces EMT in hES cells, PMA was added into the culture of H9 hES cells grown in the defined culture conditions. The colony morphology of H9 cells was changed within 24 h after PMA addition. Compared with the control (Fig. 1A[a]), the packed colonies of undifferentiated H9 hES cells came loose and flatter cells scattered out (Fig. 1A[b]). PMA addition together with the PKC inhibitor GFX did not change the colony morphology of H9 hES cells (Fig. 1A[c]). The scattered cells were positive for SSEA-1 and VIMENTIN, and negative for NANOG or E-cadherin, and vice versa for the cells treated with PMA together with GFX (Fig. 1A[d–i]). These results indicated that the activation of PKC induced the EMT process.

To determine whether PMA promotes the expression of EMT-related genes or lineage-specific cell differentiation-associated genes, H9 hES cells were cultured in the presence of PMA with or without GFX for 48 h, and then for 4 more days after changing to a fresh medium without PMA or GFX (Supplementary Fig. S1A). qRT-PCR analysis showed that the expression of *SNAIL* rapidly increased 3 h after PMA treatment (Fig. 1B). After the *SNAIL* expression, the expression of *SLUG*, *VIMENTIN*, *FOXC2*, and *TWIST* significantly increased in a time-dependent manner in the cells treated with PMA. After the gene expression, the expression of an ExEn marker gene *SOX7* and endoderm marker genes *SOX17*, *EOMES*, *GATA4*, and *GATA6* was significantly increased in the cells (Supplementary Fig. S1B, C). On the contrary, the expression of a primitive streak marker gene *GSC* (Supplementary Fig. S1C) or ectoderm genes *MSI1*, *SOX9*, *MAP2*, *Nestin*, and *PAX3* was not induced by PMA (Supplementary Fig. S1D). PMA addition together with GFX did not change the expression profile of EMT-related genes or cell differentiation marker genes. These results confirmed that the activation of PKC induced EMT-related genes and led to ExEn differentiation.

The expression of EGR-1

To determine PKC downstream molecules involved in the EMT initiation, differences in gene expression profiles among the cells treated with DMSO (control), PMA, or PMA together with GFX were analyzed by using DNA microarrays (Fig. 2A, B). The cells were treated with PMA for 1 h, in which time period, the *SNAIL* expression was before increase as shown in Fig. 1B. We previously reported that GFX, which is a selective inhibitor of PKC-α, β, γ, δ, and ζ negated PMA-induced differentiation of hPS cells, whereas Gö6976, which is a selective inhibitor of PKC-α, β, and γ isoforms could not counteract the effect of PMA [4]. Therefore, among genes influenced by PMA (fold change ≥5.0), genes of which expression were different between the cells treated PMA or together with GFX (fold change ≥5.0) were considered to be involved in EMT (Fig. 2C). Accordingly, the analysis nominated 26 activated genes and 2 repressed genes as the candidates for PKC downstream genes (Supplementary Table S3). Among them, we found that a zinc finger transcription factor *EGR* gene family members, *EGR-1*, *EGR-2*, *EGR-3*, and *EGR-4* were increased in the PMA-treated cells. The expression levels of *EGR-1* were significantly higher in the *EGR* gene family members (Fig. 2D). Therefore, we focused on *EGR-1*, which is reported to be involved in the EMT process in epithelial cells [45,46].

To confirm the expression of *EGR-1* in the cells treated with PMA, immunocytochemistry analysis was performed using the anti-EGR-1 antibody (Fig. 2E). The result indicated EGR-1 protein expression in the cells 1 h after PMA treatment and accumulation of EGR-1 in the nucleus. EGR-1 protein expression was at low levels in the cells treated with PMA together with GFX. From these results, it was confirmed that EGR-1 expression was induced by PMA in hES cells.

Relation between EGR-1 and SNAIL

To investigate the induced expression level of EGR-1 in the cells treated with PMA, qRT-PCR analysis was performed. The results showed that the *EGR-1* expression peaked at 1 h after PMA treatment (Fig. 3A) and the induction of *SNAIL* expression peaked at 3 h after PMA treatment (Fig. 3B).

From these data above, it was predicted that the PMA-induced *EGR-1* might directly regulate the induction of *SNAIL*. To determine the relationship between *EGR-1* and *SNAIL*, the cells were transfected with siRNA targeting *EGR-1*. When the cells were treated with PMA for 3 h, the expression of PMA-induced *EGR-1* mRNA (Fig. 3C) and its protein levels (Fig. 3D, E) were significantly reduced at 1–2 h PMA treatment time, and then *SNAIL* expression (Fig. 3F) was significantly reduced at 2 h PMA treatment time, in the cells transfected with siRNA of *EGR-1* compared with those in the cells transfected with nontarget siRNA. Then, we searched the ChIP-on-chip data for EGR-1, provided by

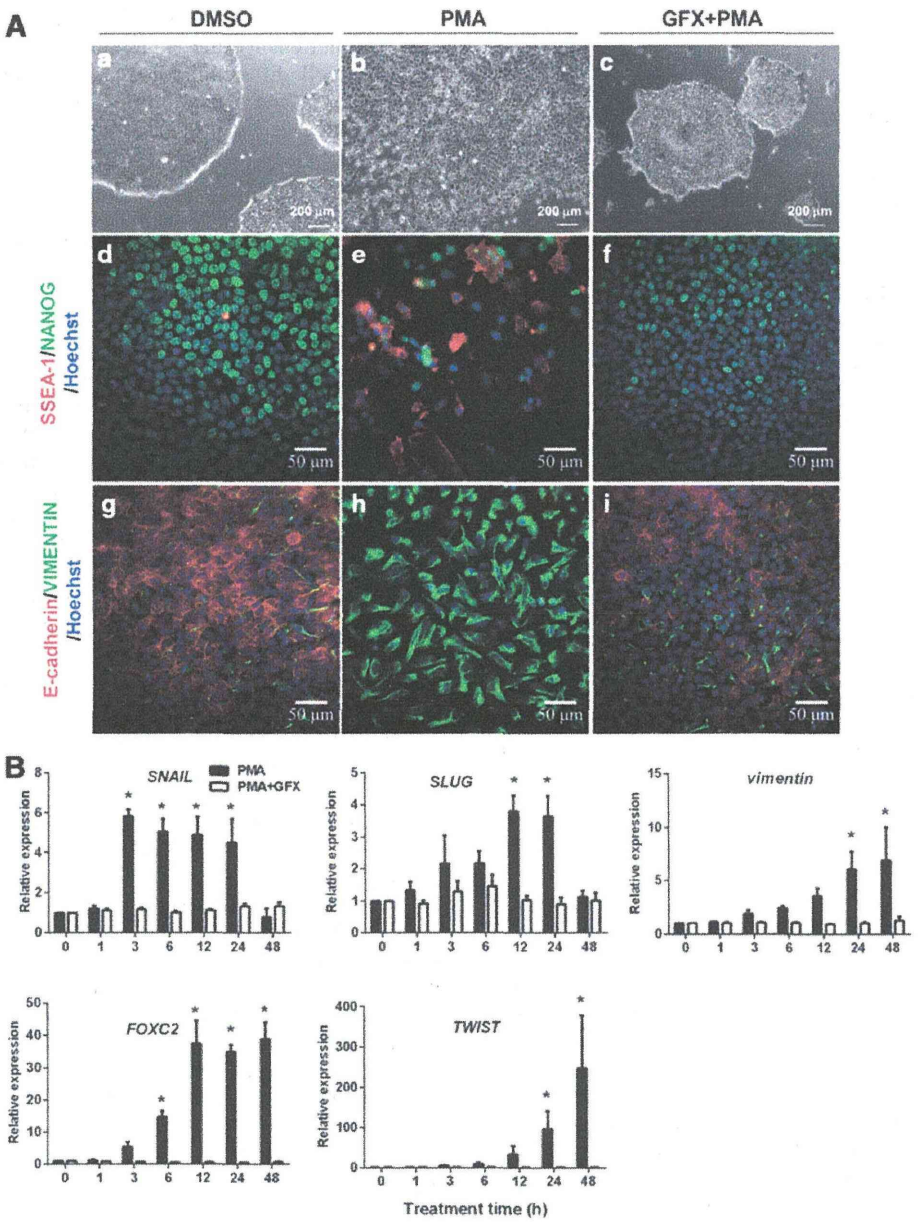

FIG. 1. Phorbol 12-myristate 13-acetate (PMA)-induced epithelial–mesenchymal transition (EMT) process in human embryonic stem (hES) cells. **(A)** Phase-contrast images and immunostaining of H9 hES cells 24 h after addition of PMA (10 nM) with or without GFX (5 μM) in the defined medium hESF9. **(a–c)** Phase-contrast images of H9 cells. **(a)** The cells in the presence of dimethyl sulfoxide (DMSO) (control). **(b)** The cells treated with PMA. **(c)** The cells treated with PMA and GFX. Scale bars = 200 μm. **(d–f)** Immunostaining for NANOG (*green*) and SSEA-1 (*red*) of the cells with DMSO **(d)**, PMA **(e)**, and PMA and GFX **(f)**. **(g–i)** Immunostaining for E-cadherin (*red*) and VIMENTIN (*green*) of the cells with DMSO **(g)**, PMA **(h)**, and PMA and GFX **(i)**. Nuclei were stained with Hoechst 33342 (*blue*). Scale bars = 50 μm. **(B)** Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis of the EMT-related gene expression. The cells were treated with PMA (*black bars*) or PMA in the presence of GFX (*white bars*) in the hESF9 medium for 48 h. Expression levels were all normalized against *GAPDH*. The relative expression levels of each gene were calculated from the undifferentiated H9 hES cells untreated with PMA or PMA together with GFX. The data are represented as mean ± standard error (SE, $n = 3$). $^*P < 0.05$.

the FANTOM 4 database (http://fantom.gsc.riken.jp/4/) [47]. Data showed that EGR-1 binds to the region of the *SNAIL* promoter in human THP-1 cells (Supplementary Fig. S2A). Thus, ChIP analysis was performed to determine whether EGR-1 could actually bind to the promoter region of *SNAIL*, which contains the putative EGR-1 binding site in hES H9 cells (Supplementary Fig. S2B). The result showed that EGR-1 bound to the promoter region of *SNAIL*, and that the amounts of immunoprecipitated DNA with the EGR-1 antibody was increased by PMA treatment, but decreased by

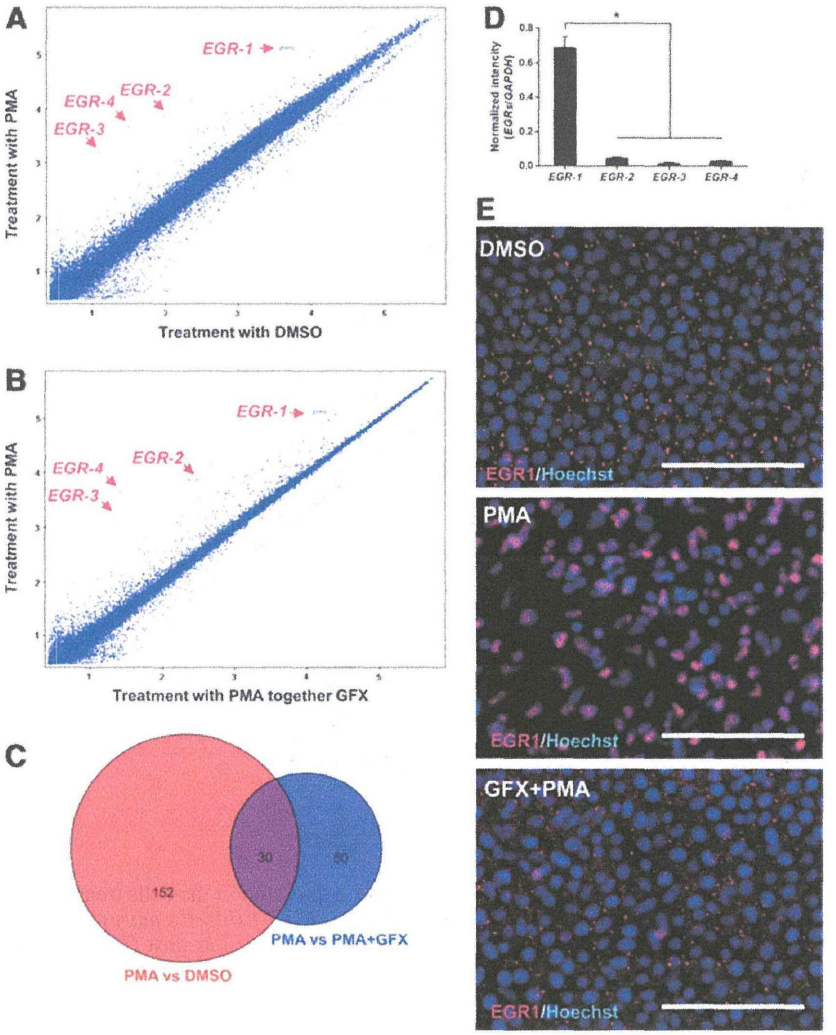

FIG. 2. Early growth response protein-1 (*EGR-1*) increases in the hES cells treated with PMA. **(A)** DNA microarray analysis of H9 hES cells treated with or without PMA for 1 h. **(B)** DNA microarray analysis of H9 hES cells treated with PMA or PMA together with GFX for 1 h. **(C)** Venn diagram of probes (*purple*) shared by the PMA-influenced probes (*red*) and GFX-influenced probes (*blue*) in DNA microarray analysis. Values in Venn diagram indicated the number of probes changed (fold change ≥5.0) by DNA microarray analysis at **(A)** and **(B)**. **(D)** The fluorescence intensity of each spot of *EGR* genes in the microarray was normalized against the fluorescence intensity of *GAPDH* gene. The data are represented as mean ± standard error (SE, n = 3). $*P < 0.05$. **(E)** Immunostaining for EGR-1 (Alexa fluor 647; *red*) in H9 hES cells treated with PMA (10 nM) or PMA together with GFX (5 μM). Nuclei were stained with Hoechst 33342 (*blue*). Scale bars = 50 μm.

PMA treatment together with GFX (Fig. 3G). It is reported that EGR-1 is colocalized with histone H3 lysine 9 acetylation (H3K9ac) sites, which is tightly associated with the transcription start sites of genes [47]. Thus, H3K9ac is important for EGR-1 target site selection for gene activation. To confirm H3K9ac modification around the EGR-1 binding site in the *SNAIL* promoter, ChIP analysis was performed using the anti-H3K9ac antibody (Supplementary Fig. S2C). The result indicated that the EGR-1 binding region in the *SNAIL* promoter was colocalized with the H3K9ac site. These above results demonstrated that the PMA-induced EGR-1 was directly bound to the *SNAIL* promoter region and it resulted in the expression of *SNAIL*, suggesting that EGR-1 may play a role in EMT initiation during early differentiation of hES cells.

EMT induction by ectopic EGR-1

To confirm the function of EGR-1 as an EMT inducer in hES cells, an episomal vector carrying a CAG promoter-driven EGR1-2A-eGFP was transiently transfected into H9 cells. The expression of ectopic EGR-1 in H9 hES cells 24 h after the transfection was confirmed by western blotting analysis using the specific anti-EGR-1 antibody (Fig. 4A). The expression of *SNAIL* was significantly increased in the cells expressing ectopic EGR-1 with or without GF109203X (GFX) (Fig. 4B). Furthermore, the cells expressing ectopic EGR-1-2A-eGFP were selected on hygromycin B for 6 days, colonies became loose and scattered flatter cells. Control cell colonies maintained undifferentiated morphology (Fig. 4C). The result by qRT-PCR showed that the expression of EMT-related genes *SNAIL*, *SLUG*, and *FOXC2* were significantly upregulated in the cells expressing ectopic EGR-1, but the expression of *E-cadherin* was significantly downregulated (Fig. 4D). These results indicated that the expression of EGR-1 induced EMT in hES cells.

Discussion

In this study, we showed that PMA rapidly induced the expression of EGR-1 and accumulation of EGR-1 into the nucleus, which resulted in the expression of *SNAIL* in hES cells. PMA-induced *SNAIL* expression was attenuated by knockdown of *EGR-1*, whereas ectopic *EGR-1* expression induced EMT-related genes expression, resulting in the

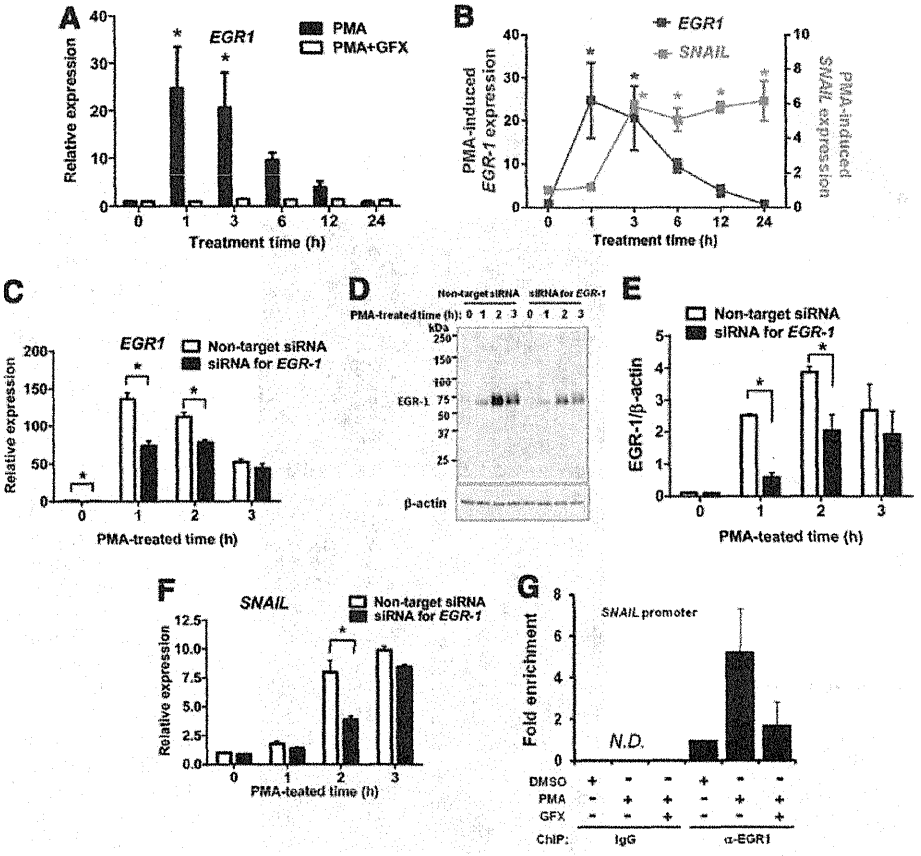

FIG. 3. PMA-induced *EGR-1* expression in hES cells. **(A)** qRT-PCR analysis of *EGR-1* expression in the cells treated with PMA (*black bars*) or PMA together with GFX (*white bars*) for 24 h. **(B)** qRT-PCR analysis of *EGR-1* (*black square*) or *SNAIL* (*gray square*) expression in the cells treated with PMA for 24 h. Expression levels were all normalized against *GAPDH*. The relative expression levels of each gene were calculated from the undifferentiated H9 hES cells untreated with PMA or PMA together with GFX. The data are represented as mean ± SE ($n = 3$). $^*P < 0.05$. **(C)** qRT-PCR analysis of PMA-induced *EGR-1* expression in the cells transfected with siRNA targeting *EGR-1* (*black bars*) or nontarget control (*white bars*). Cells were treated with PMA for 3 h. Expression levels were all normalized against *GAPDH*. The relative expression levels of *EGR-1* were calculated from the nontarget siRNA-transfected H9 hES cells. The data are represented as mean ± SE ($n = 3$). $^*P < 0.05$. **(D)** Western blot analysis of PMA-induced EGR-1 expression in the cells transfected with siRNA targeting *EGR-1* or nontarget control. Cells were treated with PMA for 3 h. **(E)** Quantification of the western blot result at **(D)**. Cells were transfected with siRNA targeting *EGR-1* (*black bars*) or nontarget control (*white bars*). The data are represented as mean ± SE ($n = 3$). $^*P < 0.05$. **(F)** qRT-PCR analysis of PMA-induced *SNAIL* expression in the cells transfected with siRNA targeting *EGR-1* (*black bars*) or nontarget control (*white bars*). Cells were treated with PMA for 3 h. Expression levels were all normalized against *GAPDH*. The relative expression levels of *SNAIL* were calculated from the nontarget siRNA-transfected H9 hES cells. The data are represented as mean ± SE ($n = 3$). $^*P < 0.05$. **(G)** Chromatin immunoprecipitation (ChIP) assay. The cells were treated with PMA (10 nM) or PMA together with GFX (5 μM) for 1 h. N.D., not detected.

induction of the EMT process in hES cells. Furthermore, EGR-1 may function in hES-specific lineage cell differentiation. The ChIP-on-ChIP database of EGR-1, provided by the FANTOM 4 database (http://fantom.gsc.riken.jp/4/) [47], showed that EGR-1 binds not only to the region of *SNAIL* promoter, but also to the promoter and intragenic regions of a number of genes. For example, EGR-1 binding sites are present in the promoter region of EMT-related genes; *E-cadherin*, *TWIST*, *VIMENTIN*, and *MMP2*, and extraembryonic and/or definitive endodermal cell lineage-associated genes; *EOMES*, *GATA6*, *SOX7*, *FOXA2*, and *CXCR4* in human THP-1 cells (Supplementary Fig. S3). These findings are consistent with our results that PMA induced EMT and also cell differentiation into the extra-embryonic endoderm in hPS cells.

The previous study reported that the SNAIL protein is phosphorylated by GSK-3β, and then the phosphorylated SNAIL protein binds to the promoter region of E-cadherin and downregulates the transcript of *E-cadherin*, leading to the EMT process [48]. These findings implied that the function of SNAIL protein induced by EGR-1 might be regulated by GSK-3β in the nucleus [48]. Although GSK-3β is generally considered to be a cytoplasmic protein, previous reports suggest that GSK-3β also functions in the nucleus [49–51]. It means that Wnt signaling promotes EMT in hPS cells. We have previously reported that phosphorylation of

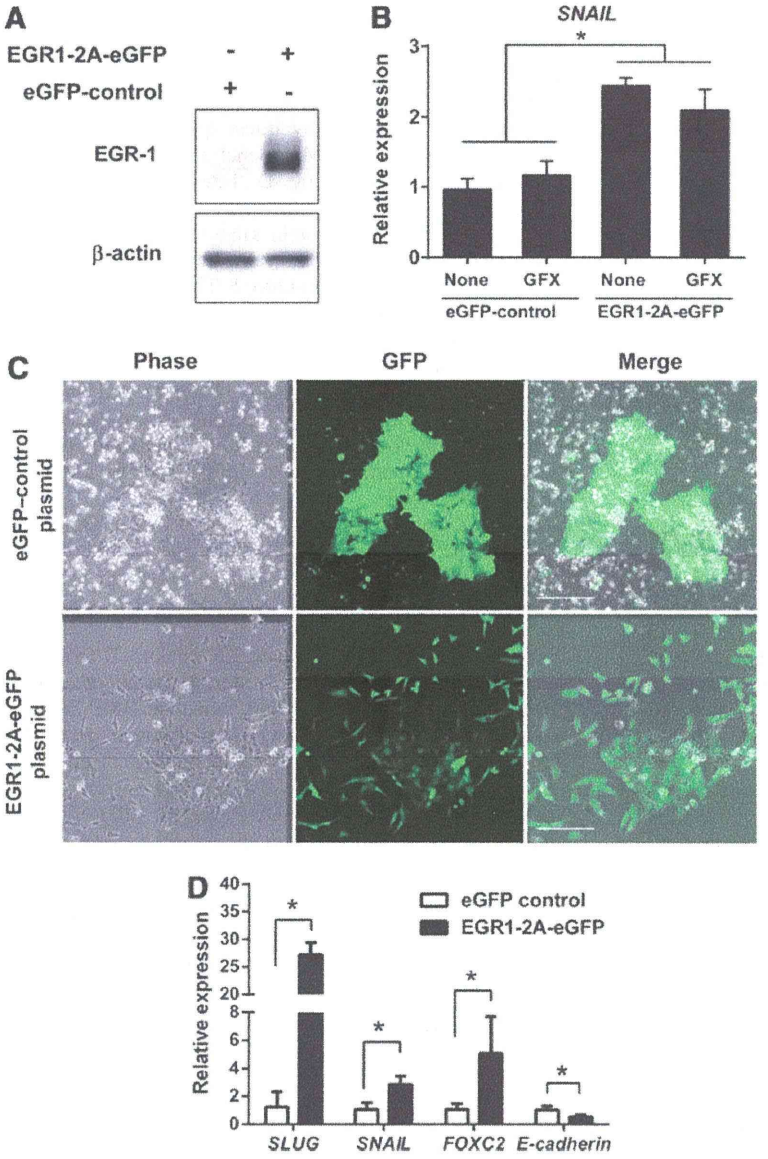

FIG. 4. EMT-related gene expression by ectopic EGR-1. **(A)** Western blot analysis of ectopic EGR-1 expression in the cells transfected with EGR1-2A-eGFP or with enhanced green fluorescent protein (eGFP) control plasmid. Transiently ectopic EGR-1 expression was confirmed by the specific EGR-1 antibody at 24 h after transfection. **(B)** qRT-PCR analysis of *SNAIL* in the cells expressing EGR1-2A-eGFP or eGFP alone in the presence or absence of GFX. Expression levels of the *SNAIL* gene were all normalized against *GAPDH*. The data are represented as mean ± SE ($n=3$). $^*P<0.05$. **(C)** Phase-contrast and fluorescence images of H9 hES cells transfected with pEGR1-2A-eGFP or peGFP control plasmid. The EGR1-2A-eGFP or eGFP alone expressing cells were selected on hygromycin B (200 μg/mL) in the hESF9 medium for 6 days. Scale bars = 200 μm. **(D)** qRT-PCR analysis of the EMT-related genes. Expression levels of genes in the cells expressing EGR1-2A-eGFP (*black bars*) or eGFP alone (*white bars*) were all normalized against *GAPDH*. The data are represented as mean ± SE ($n=3$). $^*P<0.05$.

GSK-3β is induced by FGF-2-activated PKC and that this process might be related to hPS cell differentiation [4]. This is consistent with previous studies that canonical Wnt signaling does not appear to promote stem cell maintenance [52,53]. However, this conclusion contradicts the findings of previous studies, which demonstrated that canonical Wnt signaling supports self-renewal of stem cells [6,38]. A recent study has shown the dual function of Wnt signaling in hES cells, suggesting that the pathways of self-renewal or differentiation are dependent on the presence of hES cell supporting factors [7,30,54,55]. From these studies, GSK-3 has emerged as an important regulator of undifferentiated state in hPS cells.

GSK-3β has been reported to be controlled by various growth factors, including insulin, IGF, FGF-2, and WNT3A [4,22,29,56,57]. We reported that in hPS cells, FGF-2-induced GSK-3β phosphorylation is inhibited in the presence of the PKC inhibitor, resulting in the inhibition of the PKC/GSK-3β signaling pathway to support the maintenance of an undifferentiated state [4]. From the findings above, it is implied that FGF-2 induces the expression of EGR-1 through the PKC signaling pathway, EGR-1 induced the SNAIL protein, and then PKC/GSK-3β activates SNAIL, leading to the EMT process [48] (Fig. 5). However, if FGF-2 continues to amplify the EMT process, hPS cells should rapidly differentiate. Inhibitory signaling related to the dual function of GSK-3β might exist in hPS cell culture and it can balance the FGF-2-induced EMT process to maintain the undifferentiated state although the PKC inhibitor can artificially stabilize the balance between undifferentiated state and cell differentiation. Further investigation of mechanisms involved in the regulation of PKC/EGR1/SNAIL and PKC/GSK-3β activity should be performed in future.

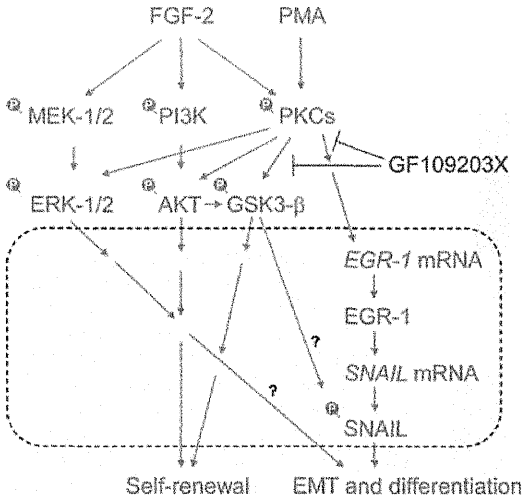

FIG. 5. A model of EMT-triggering pathways using protein kinase C (PKC) in fibroblast growth factor (FGF-2) signaling. PMA induces PKCs, and then induces EGR-1. EGR-1 directly induces the expression of *SNAIL* mRNA, leading to the EMT process [48]. GF109203X, a selective inhibitor of PKCs, negated the PKC-induced *EGR-1/SNAIL* expression, EMT process, and differentiation of human pluripotent stem cells. PKC-EGR-1-SNAIL pathways promote EMT and cell differentiation. *Dot line box* indicates the process in the nucleus.

This study clarified that PKC activation induced the EMT process through the EGR-1/SNAIL pathway suggesting that EGR-1 plays a role in the differentiation of hES cells. This finding would lead to a better understanding of the precise mechanism regulating the balance between undifferentiated state and cell differentiation in hPS cells.

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Author Disclosure Statement

All the authors have read and approved the article, and hereby declare that none of them has any competing interest.

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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

CCAAT/enhancer binding protein-mediated regulation of TGFβ receptor 2 expression determines the hepatoblast fate decision

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ABSTRACT

Human embryonic stem cells (hESCs) and their derivatives are expected to be used in drug discovery, regenerative medicine and the study of human embryogenesis. Because hepatocyte differentiation from hESCs has the potential to recapitulate human liver development *in vivo*, we employed this differentiation method to investigate the molecular mechanisms underlying human hepatocyte differentiation. A previous study has shown that a gradient of transforming growth factor beta (TGFβ) signaling is required to segregate hepatocyte and cholangiocyte lineages from hepatoblasts. Although CCAAT/enhancer binding proteins (c/EBPs) are known to be important transcription factors in liver development, the relationship between TGFβ signaling and c/EBP-mediated transcriptional regulation in the hepatoblast fate decision is not well known. To clarify this relationship, we examined whether c/EBPs could determine the hepatoblast fate decision via regulation of TGFβ receptor 2 (TGFBR2) expression in the hepatoblast-like cells differentiated from hESCs. We found that *TGFBR2* promoter activity was negatively regulated by c/EBPα and positively regulated by c/EBPβ. Moreover, c/EBPα overexpression could promote hepatocyte differentiation by suppressing TGFBR2 expression, whereas c/EBPβ overexpression could promote cholangiocyte differentiation by enhancing TGFBR2 expression. Our findings demonstrated that c/EBPα and c/EBPβ determine the lineage commitment of hepatoblasts by negatively and positively regulating the expression of a common target gene, *TGFBR2*, respectively.

KEY WORDS: Hepatoblasts, c/EBP, CEBP, Human ESCs

INTRODUCTION

Many animal models, such as chick, *Xenopus*, zebrafish and mouse, have been used to investigate the molecular mechanisms of liver development. Because many functions of the key molecules in liver development are conserved in these species, studies on liver development in these animals can be highly informative with respect that in humans. However, some functions of important molecules in liver development might differ between human and other species. Although analysis using genetically modified mice has been successfully performed, it is not of course possible to perform genetic experiments to elucidate molecular mechanisms of liver development in human. Pluripotent stem cells, such as human embryonic stem cells (hESCs), are expected to overcome some of these problems in the study of human embryogenesis, including liver development, because the gene expression profiles of this model are similar to those in normal liver development (Agarwal et al., 2008; DeLaForest et al., 2011).

During liver development, hepatoblasts differentiate into hepatocytes and cholangiocytes. A previous study has shown that a high concentration of transforming growth factor beta (TGFβ) could give rise to cholangiocyte differentiation from hepatoblasts (Clotman et al., 2005). To transmit the TGFβ signaling, TGFβ receptor 2 (TGFBR2) has to be stimulated by TGFβ1, TGFβ2 or TGFβ3 (Kitisin et al., 2007). TGFβ binding to the extracellular domain of TGFBR2 induces a conformational change, resulting in the phosphorylation and activation of TGFBR1. TGFBR1 phosphorylates SMAD2 or SMAD3, which binds to SMAD4, and then the SMAD complexes move into the nucleus and function as transcription factors to express various kinds of differentiation-related genes (Kitisin et al., 2007). Although the function of TGFBR2 in regeneration of the adult liver has been thoroughly examined (Oe et al., 2004), the function of TGFBR2 in the hepatoblast fate decision has not been elucidated.

CCAAT/enhancer binding protein (c/EBP) transcription factors play decisive roles in the differentiation of various cell types, including hepatocytes (Tomizawa et al., 1998; Yamasaki et al., 2006). The analysis of *c/EBPα* (*Cebpa*) knockout mice has shown that many abnormal pseudoglandular structures, which co-express antigens specific for both hepatocytes and cholangiocytes, are present in the liver parenchyma (Tomizawa et al., 1998). These data demonstrated that c/EBPα plays an important role in hepatocyte differentiation. It is also known that the suppression of c/EBPα expression in periportal hepatoblasts stimulates cholangiocyte differentiation (Yamasaki et al., 2006). Although the function of c/EBPα in liver development is well known, the relationship between TGFβ signaling and c/EBPα-mediated transcriptional regulation in the hepatoblast fate decision is poorly understood. c/EBPβ is also known to be an important factor for liver function (Chen et al., 2000), although the function of c/EBPβ in the cell fate decision of hepatoblasts is not well known. c/EBPα and c/EBPβ bind to the same DNA binding site. However, the promoter activity of hepatocyte-specific genes, such as those encoding hepatocyte nuclear factor 6 (HNF6, also known as ONECUT1) and UGT2B1,

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is positively regulated by c/EBPα but not c/EBPβ (Hansen et al., 1998; Plumb-Rudewiez et al., 2004), suggesting that the functions of c/EBPα and c/EBPβ in the hepatoblast fate decision might be different.

In the present study, we first examined the function of TGFBR2 in the hepatoblast fate decision using hESC-derived hepatoblast-like cells, which have the ability to self-replicate, differentiate into both hepatocyte and cholangiocyte lineages, and repopulate the liver of carbon tetrachloride (CCl_4)-treated immunodeficient mice. *In vitro* gain- and loss-of-function analyses and *in vivo* transplantation analysis were performed. Next, we investigated how TGFBR2 expression is regulated in the hepatoblast fate decision. Finally, we examined whether our findings could be reproduced in delta-like 1 homolog (Dlk1)-positive hepatoblasts obtained from the liver of E13.5 mice. To the best of our knowledge, this study provides the first evidence of c/EBP-mediated regulation of TGFBR2 expression in the human hepatoblast fate decision.

RESULTS

Hepatoblast-like cells are generated from hESCs

First, we investigated whether the hepatoblast-like cells (HBCs), which were differentiated from hESCs as described in supplementary material Fig. S1A, have similar characteristics to human hepatoblasts. We recently found that hESC-derived HBCs could be purified and maintained on human laminin 111 (LN111)-coated dishes (Takayama et al., 2013). The long-term cultured HBC population (HBCs passaged more than three times were used in this study) were nearly homogeneous and expressed human hepatoblast markers such as alpha-fetoprotein (AFP), albumin (ALB), cytokeratin 19 (CK19, also known as KRT19) and EPCAM (Schmelzer et al., 2007) (supplementary material Fig. S1B). In addition, most of the colonies observed on human LN111-coated plates were ALB and CK19 double positive, although a few colonies were ALB single positive, CK19 single positive, or ALB and CK19 double negative (supplementary material Fig. S1C). To examine the hepatocyte differentiation capacity of the HBCs *in vivo*, these cells were transplanted into CCl_4-treated immunodeficient mice. The hepatocyte functionality of the transplanted cells was assessed by measuring secreted human ALB levels in the recipient mice (supplementary material Fig. S1D). Human ALB serum was detected in the mice that were transplanted with the HBCs, but not in the control mice. These results demonstrated that the HBCs generated from hESCs have similar characteristics to human hepatoblasts and would therefore provide a valuable tool to investigate the mechanisms of human liver development. In the present study, HBCs generated from hESCs were used to elucidate the mechanisms of the hepatoblast fate decision.

TGFBR2 expression is decreased in hepatocyte differentiation but increased in cholangiocyte differentiation

The HBCs used in this study have the ability to differentiate into both hepatocyte-like cells [cytochrome P450 3A4 (CYP3A4) positive; Fig. 1B] and cholangiocyte-like cells (CK19 positive; Fig. 1C) (the protocols are described in Fig. 1A). Because the expression pattern of TGFBR2 during differentiation from hepatoblasts is not well known, we examined it in hepatocyte and cholangiocyte differentiation from HBCs. *TGFBR2* was downregulated during hepatocyte differentiation from HBCs (Fig. 1D), but upregulated in cholangiocyte differentiation from HBCs (Fig. 1E). After the HBCs were cultured on Matrigel, the cells were fractionated into three populations according to the level of TGFBR2 expression (TGFBR2-negative, -lo or -hi; Fig. 1F). The HBC-derived TGFBR2-lo cells strongly expressed *αAT* and *CYP3A4* (hepatocyte markers), whereas the HBC-derived TGFBR2-hi cells strongly expressed *SOX9* and integrin β4 (*ITGB4*) (cholangiocyte markers). These data suggest that the TGFBR2 expression level is decreased in hepatic differentiation, but increased in biliary differentiation of the HBCs.

The cell fate decision of HBCs is regulated by TGFβ signals

To examine the function of TGFβ1, β2 and β3 (all of which are ligands of TGFBR2) in the hepatoblast fate decision, HBCs were cultured in medium containing TGFβ1, β2 or β3 (Fig. 2A,B). The expression levels of cholangiocyte marker genes were upregulated by addition of TGFβ1 or TGFβ2, but not TGFβ3 (Fig. 2A), whereas those of hepatocyte markers were downregulated by addition of TGFβ1 or TGFβ2 (Fig. 2B). To ascertain that TGFBR2 is also important in the hepatoblast fate decision, HBCs were cultured in medium containing SB-431542, which inhibits TGFβ signaling (Fig. 2C,D). Hepatocyte marker genes were upregulated by inhibition of TGFβ signaling (Fig. 2C), whereas cholangiocyte markers were downregulated (Fig. 2D). To confirm the function of TGFβ1, β2 and β3 in the hepatoblast fate decision, colony assays of the HBCs were performed in the presence or absence of TGFβ1, β2 or β3 (Fig. 2E). The number of CK19 single-positive colonies was significantly increased in TGFβ1- or β2-treated HBCs. By contrast, the number of ALB and CK19 double-positive colonies was reduced in TGFβ1-, β2- or β3-treated HBCs. These data indicated that TGFβ1 and β2 positively regulate the biliary differentiation of HBCs. Taken together, the findings suggested that TGFBR2 might be a key molecule in the regulation of hepato-bilary lineage segregation.

TGFBR2 plays an important role in the cell fate decision of HBCs

To examine whether TGFBR2 plays an important role in the hepatoblast fate decision, *in vitro* gain- and loss-of-function analysis of TGFBR2 was performed in the HBCs. We used siRNA in knockdown experiments (supplementary material Fig. S2) during HBC differentiation on Matrigel. Whereas TGFBR2-suppressing siRNA (si-TGFBR2) transfection upregulated the expression of hepatocyte markers, it downregulated cholangiocyte markers (Fig. 3A). si-TGFBR2 transfection increased the percentage of asialoglycoprotein receptor 1 (ASGR1)-positive hepatocyte-like cells (Fig. 3B). By contrast, it decreased the percentage of aquaporin 1 (AQP1)-positive cholangiocyte-like cells. These results suggest that TGFBR2 knockdown promotes hepatocyte differentiation, whereas it inhibits cholangiocyte differentiation. Next, we used Ad vector to perform efficient transduction into the HBCs (supplementary material Fig. S3) and ascertained *TGFBR2* gene expression in TGFBR2-expressing Ad vector (Ad-TGFBR2)-transduced cells (supplementary material Fig. S4). Ad-TGFBR2 transduction downregulated the expression of hepatocyte markers, whereas it upregulated cholangiocyte markers (Fig. 3C). Ad-TGFBR2 transduction decreased the percentage of ASGR1-positive hepatocyte-like cells but increased the percentage of AQP1-positive cholangiocyte-like cells (Fig. 3D). These results suggest that TGFBR2 overexpression inhibits hepatocyte differentiation, whereas it promotes cholangiocyte differentiation. Taken together, these results suggest that TGFBR2 plays an important role in deciding the differentiation lineage of HBCs.

To investigate whether hepatoblasts would undergo differentiation in a TGFBR2-associated manner *in vivo*, HBCs transfected/transduced with si-control, si-TGFBR2, Ad-LacZ or Ad-