

## Acknowledgments

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## Author Contributions

Conceived and designed the experiments: HW K. Takayama MI MT K. Katayama K. Kawabata HM. Performed the experiments: HW K. Takayama MI NM. Analyzed the data: HW K. Takayama MI MT K. Katayama K. Tashiro YN FS K. Kawabata MKF HM. Wrote the paper: HW K. Takayama HM. Contributed equally to this work: HW K. Takayama.

# Protein Kinase C-Induced Early Growth Response Protein-1 Binding to *SNAIL* Promoter in Epithelial–Mesenchymal Transition of Human Embryonic Stem Cells

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Epithelial–mesenchymal transition (EMT) has been thought to occur during early embryogenesis, and also the differentiation process of human embryonic stem (hES) cells. Spontaneous differentiation is sometimes observed at the peripheral of the hES cell colonies in conventional culture conditions, indicating that EMT occurs in hES cell culture. However, the triggering mechanism of EMT is not yet fully understood. The balance between self-renewal and differentiation of human pluripotent stem (hPS) cells is controlled by various signal pathways, including the fibroblast growth factor (FGF)-2. However, FGF-2 has a complex role for self-renewal of hES cells. FGF-2 activates phosphatidylinositol-3 kinase/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase-1/2 kinase, and also protein kinase C (PKC). Here, we showed that a PKC rapidly induced an early growth response protein-1 (EGR-1) in hES cells, which was followed by upregulation of EMT-related genes. Before the induction of EMT-related genes, EGR-1 was translocated into the nucleus, and then bound directly to the promoter region of *SNAIL*, which is a master regulator of EMT. *SNAIL* expression was attenuated by knockdown of *EGR-1*, but upregulated by ectopic expression of *EGR-1*. EGR-1 as the downstream signal of PKC might play a key role in EMT initiation during early differentiation of hES cells. This study would lead to a more robust understanding of the mechanisms underlying the balance between self-renewal and initiation of differentiation in hPS cells.

## Introduction

THE BALANCE BETWEEN self-renewal and differentiation of human pluripotent stem (hPS) cells, including embryonic stem (hES) and induced pluripotent stem cells, is controlled by various signal pathways [1–8], including transforming growth factor- $\beta$ /activin A/Nodal [9–11], sphingosine-1-phosphate/platelet-derived growth factor [12], insulin growth factor (IGF)/insulin [13], and fibroblast growth factor-2 (FGF-2) [14–17]. In particular, FGF-2 signaling appears indispensable to maintain the undifferentiated state of hPS cells [18–20]. However, FGF-2 has a complex role for self-renewal of hES cells [21]. FGF-2 activates phosphatidylinositol-3 kinase (PI3K)/AKT and mitogen-activated protein kinase/extracellular signal-regulated kinase-1/2 (ERK-1/2) kinase. The PI3K/AKT pathway plays important roles in the proliferation and survival of hPS cells [22–24]. On the other hand, the

ERK-1/2 pathway has reported to have diverse roles in hPS cells, such as self-renewal, cell attachment, and cell differentiation [2,25–27]. FGF-2 also induces inactive phosphorylation of glycogen synthase kinase-3 (GSK-3) [1,3,4,28] of which function has been reported to be controversial for self-renewal of hPS cells [29,30]. Moreover, we have recently reported that FGF-2 induces protein kinase C (PKC), which leads to phosphorylation of GSK-3 $\beta$  [4]. An activator of PKC, phorbol 12-myristate 13-acetate (PMA) changes the sharp-edged, flat, and tightly packed colonies of hPS cells to scattered cells, indicating that PKC induces the epithelial–mesenchymal transition (EMT) in hPS cells [4]. Consistent with our findings, Thomson's group reported that activation of PKC is associated with EMT, leading the hPS cell differentiation into extraembryonic endodermal (ExEn) cells [5]. However, the downstream of the PKC pathway in regulating the EMT initiation is not yet understood.

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In early embryogenesis, the EMT plays crucial roles in the differentiation of multiple tissues and organs and the formation of a body plan [31]. During the EMT process, E-cadherin is downregulated. Several transcription factors have been implicated in this repression, including SNAIL, SLUG, and TWIST1 [32–37]. Actually, in hPS cell culture, spontaneous differentiation is sometimes observed with the expression of SNAIL and VIMENTIN at the peripheral of the hPS cell colonies in conventional culture conditions, indicating that EMT occurs in hPS cell culture [38–41].

In this study, we investigated molecules involved in the cascade of PKC-induced EMT in hES cells using the minimal growth factor-defined culture medium hESF9, which includes FGF-2 as the sole growth factor to remove the effects of other growth factors on EMT [17]. The results showed that the early growth response protein-1 (EGR-1; also known as NGFI-A, KROX-24, ZIF268, or TIS8) was induced by a PKC activator, PMA, and bound to the regulatory region of *SNAIL*, resulting in the upregulation of *SNAIL* and EMT-related genes. PMA-induced *SNAIL* expression was attenuated by knockdown of *EGR-1*, whereas ectopic expression of *EGR-1* induced EMT-related genes expression. These results indicated that a downstream effector of PKC signaling, EGR-1, contributed to the induction of EMT in hES cell differentiation. This study would lead to a more robust understanding of the mechanisms underlying the balance between self-renewal and initiation of differentiation in hPS cells.

## Materials and Methods

### Cell culture

The hES cell line, H9 [19,42] (WA09, WISC Bank; WiCell Research Institute), was routinely maintained as previously described [19]. For the experiment, the cells were seeded on a six-well plate (BD Falcon) coated with bovine fibronectin (FN; Sigma; 2  $\mu\text{g}/\text{cm}^2$ ) in the hESF9 medium [17] consisting of the ESF basal medium (CSTI) [43] without 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid supplemented with L-ascorbic acid-2-phosphate (Wako), 2-mercaptoethanol, 2-ethanolamine, sodium selenite, insulin, transferrin, oleic acid conjugated with bovine fatty acid-free albumin, heparan sulfate sodium salt (all from Sigma), and FGF-2 (Katayama Kagaku Kogyo Ltd.). PMA dissolved in dimethyl sulfoxide (DMSO) was added into the medium at a final concentration of 10 nM (containing a final concentration of 0.1% DMSO). The experiments using hES cells were performed following the Guidelines for utilization of hES cells of the Ministry of Education, Culture, Sports, Science and Technology of Japan with the approval by the institutional research ethics committee.

### Immunocytochemistry

Immunocytochemistry was performed as previously described [4]. The image analysis was performed by IN Cell Analyzer 2000 and IN Cell Developer Toolbox software (GE Healthcare). The primary and secondary antibodies used are listed in Supplementary Table S1 (Supplementary Data are available online at [www.liebertpub.com/scd](http://www.liebertpub.com/scd)).

### Real-time quantitative reverse transcription–polymerase chain reaction

Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and real-time quantitative PCR (qPCR) were performed based on the SYBR Green gene expression technology in a 7300 Real Time PCR System (Applied Biosystems), according to the manufacturer's instructions. Specific primers used are listed in Supplementary Table S2.

### DNA microarray

DNA microarray analysis was performed using the whole human genome DNA microarray 4x44K kit (ver.2.0) and a microarray scanner G2565BA (Agilent) according to the manufacturer's instructions (Agilent). The signal intensity data produced for each of the spots were analyzed using feature extraction (Agilent) and GeneSpring GX software (Agilent).

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express kit (Active Motif) according to the manufacturer's instructions. Chromatin was precipitated with EGR-1 antibodies (Cell Signaling Technology) or H3K9ac antibodies (MAB Institute). The immunoprecipitated DNA samples were analyzed by qPCR. The *SNAIL* promoter was amplified with the primer pairs listed in Supplementary Table S2.

### Construction of *EGR-1* expression vector

The *EGR-1* expression vector was constructed as follows. The EGR1-2A-eGFP fragment coding *EGR-1* (NM\_001964.2), a self-cleaving 2A peptide [44], and the enhanced green fluorescent protein (eGFP) were synthesized by the GeneArt gene synthesis service (Life Technologies). The synthesized fragment was inserted into the *Xho*I and *Not*I sites of the episomal pEBmulti-Hyg plasmid (Wako Pure Chemical Industries) to create the pEGR1-2A-eGFP plasmid. To generate the pEGFP control vector, the pEGR1-2A-eGFP plasmid was digested with *Xho*I and *Sal*II restriction enzymes, and the EGR1 fragment was removed from the pEGR1-2A-eGFP plasmid. The plasmids were verified by sequencing.

### Transfection

Before transfection, the hES cells were incubated with ROCK inhibitor Y-27632 (10  $\mu\text{M}$ ) for 1 h and dissociated into single cells by a cell dissociation solution, TrypLE™ Select (Life Technologies). Pellets of  $1 \times 10^6$  cells were mixed with 2  $\mu\text{g}$  of the EGR-1 expression plasmid (pEGR1-2A-eGFP) or control plasmid (pEGFP) in 100  $\mu\text{L}$  of Neon R buffer solution (Life Technologies). The cell suspension was transferred to a cuvette and electroporated using a Neon Transfection system (Life Technologies) with program (voltage 1,050, width 30, and pulses 2) according to the manufacturer's protocol. The electroporated cells were plated onto a six-well plate coated with FN in the hESF9 medium supplemented with ROCK inhibitor (10  $\mu\text{M}$ ). The eGFP-positive cells were selected on hygromycin B (200  $\mu\text{g}/\text{mL}$ ) for 6 days in the hESF9 medium.

### 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 dodecyl 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<sub>2</sub>. 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 *MSII*, *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- $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\zeta$  negated PMA-induced differentiation of hPS cells, whereas Gö6976, which is a selective inhibitor of PKC- $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms could not counteract the effect of PMA [4]. Therefore, among genes influenced by PMA (fold change  $\geq 5.0$ ), genes of which expression were different between the cells treated PMA or together with GFX (fold change  $\geq 5.0$ ) 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].

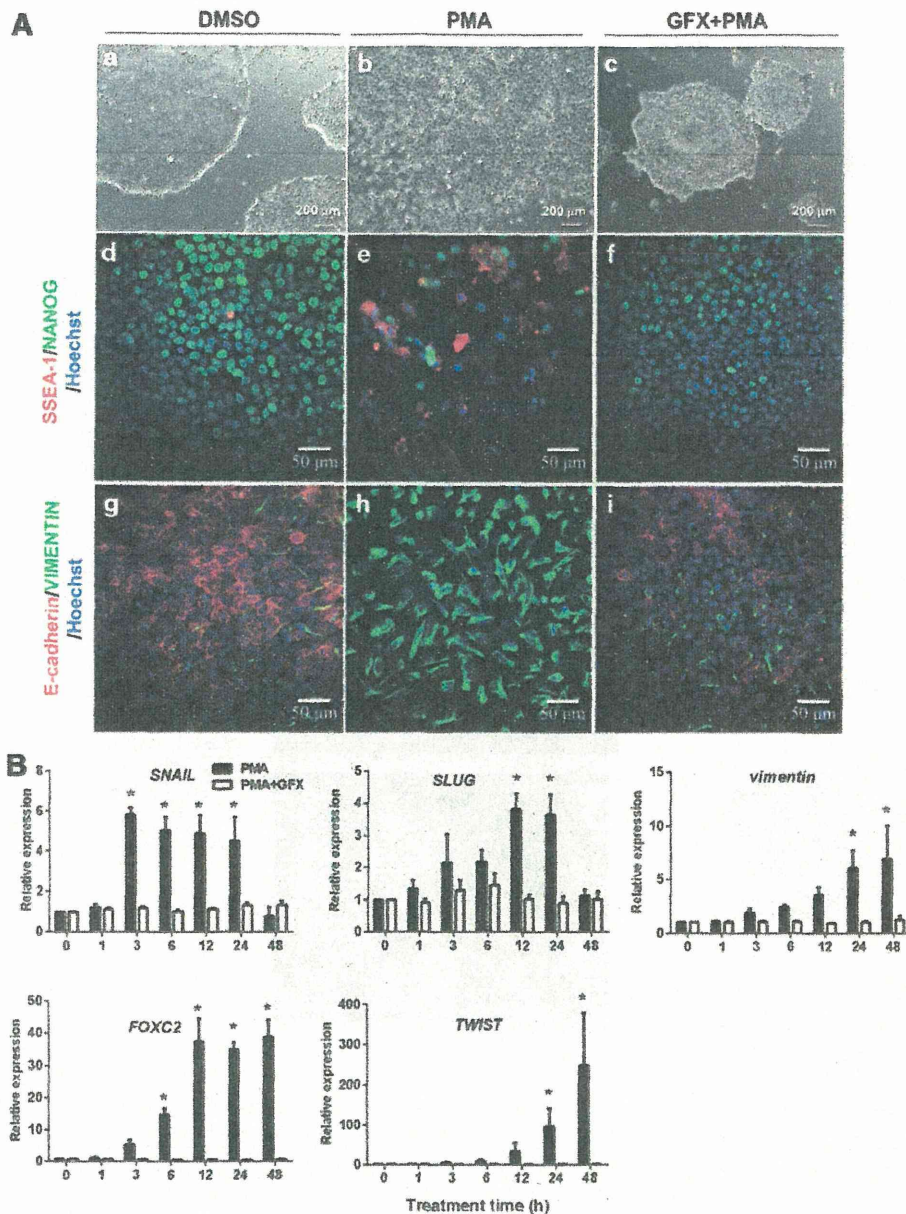
To confirm the expression of *EGR-1* in the cells treated with PMA, immunocytochemistry analysis was performed using the anti-*EGR-1* antibody (Fig. 2E). The result indicated *EGR-1* protein expression in the cells 1 h after PMA treatment and accumulation of *EGR-1* in the nucleus. *EGR-1* protein expression 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



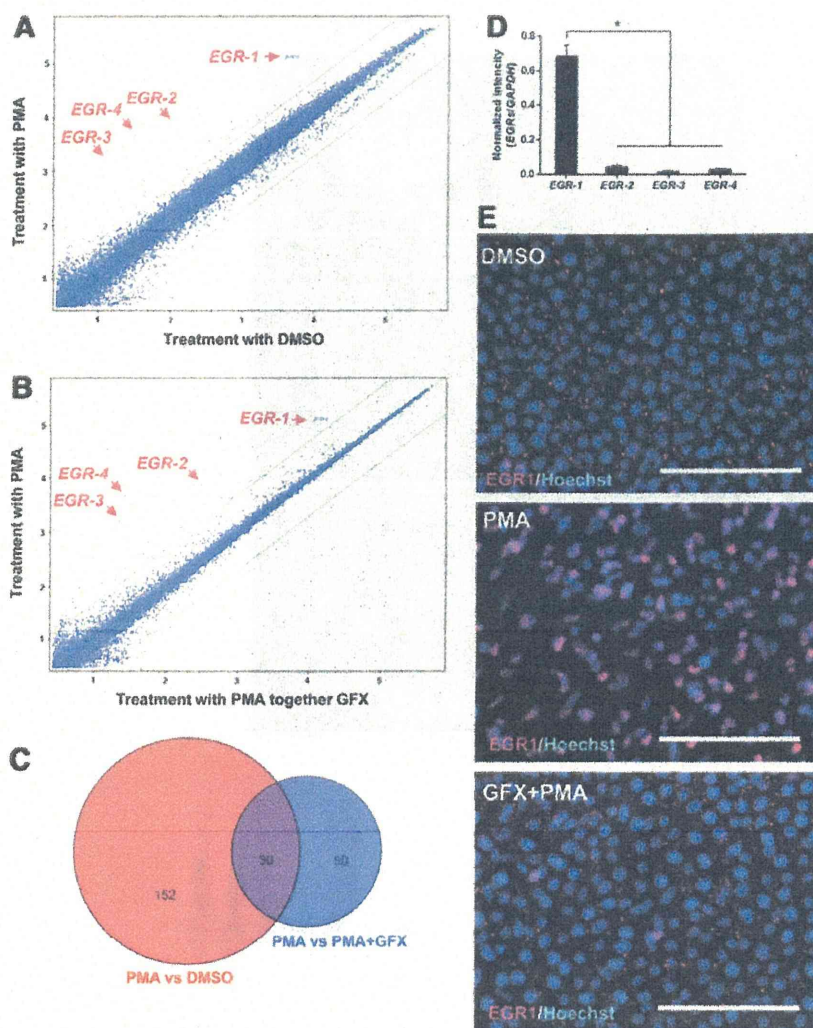


**FIG. 1.** Phorbol 12-myristate 13-acetate (PMA)-induced epithelial–mesenchymal transition (EMT) process in human embryonic stem (hES) cells. **(A)** Phase-contrast images and immunostaining of H9 hES cells 24 h after addition of PMA (10 nM) with or without GFX (5  $\mu$ M) in the defined medium hESF9. **(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  $\mu$ m. **(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  $\mu$ m. **(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  $\pm$  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  $\geq 5.0$ ) 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  $\pm$  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  $\mu$ M). Nuclei were stained with Hoechst 33342 (blue). Scale bars = 50  $\mu$ m.

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

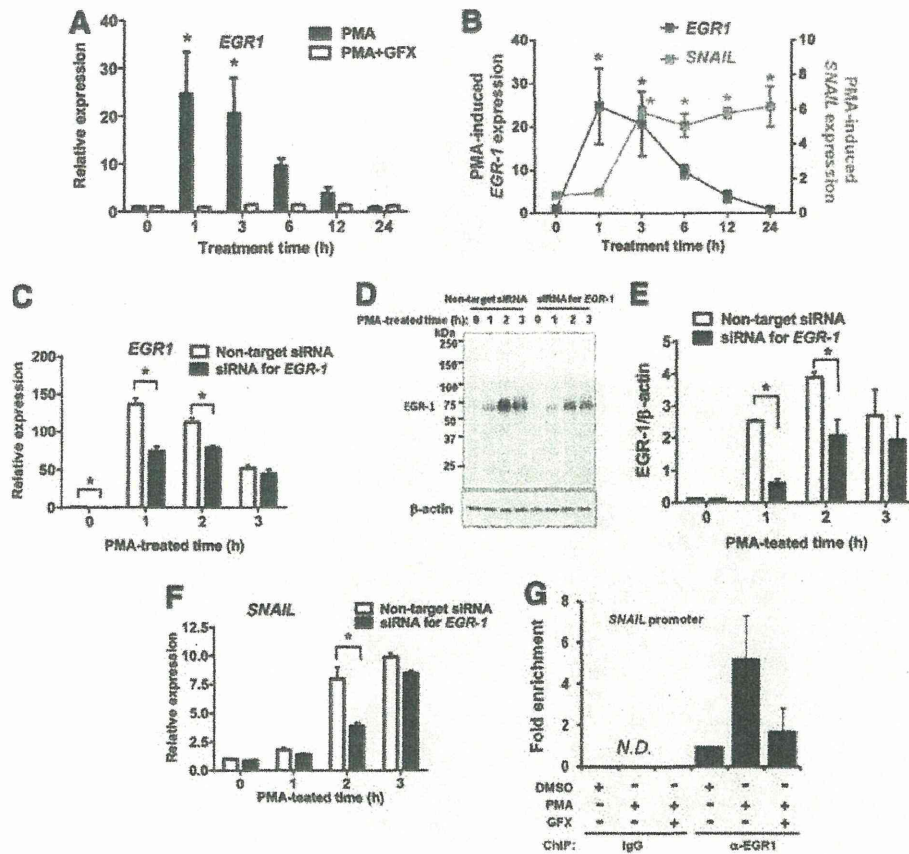
To confirm the function of EGR-1 as an EMT inducer in hES cells, an episomal vector carrying a CAG promoter-driven EGR1-2A-eGFP was transiently transfected into H9 cells. The expression of ectopic EGR-1 in H9 hES cells 24 h after the transfection was confirmed by western blotting

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



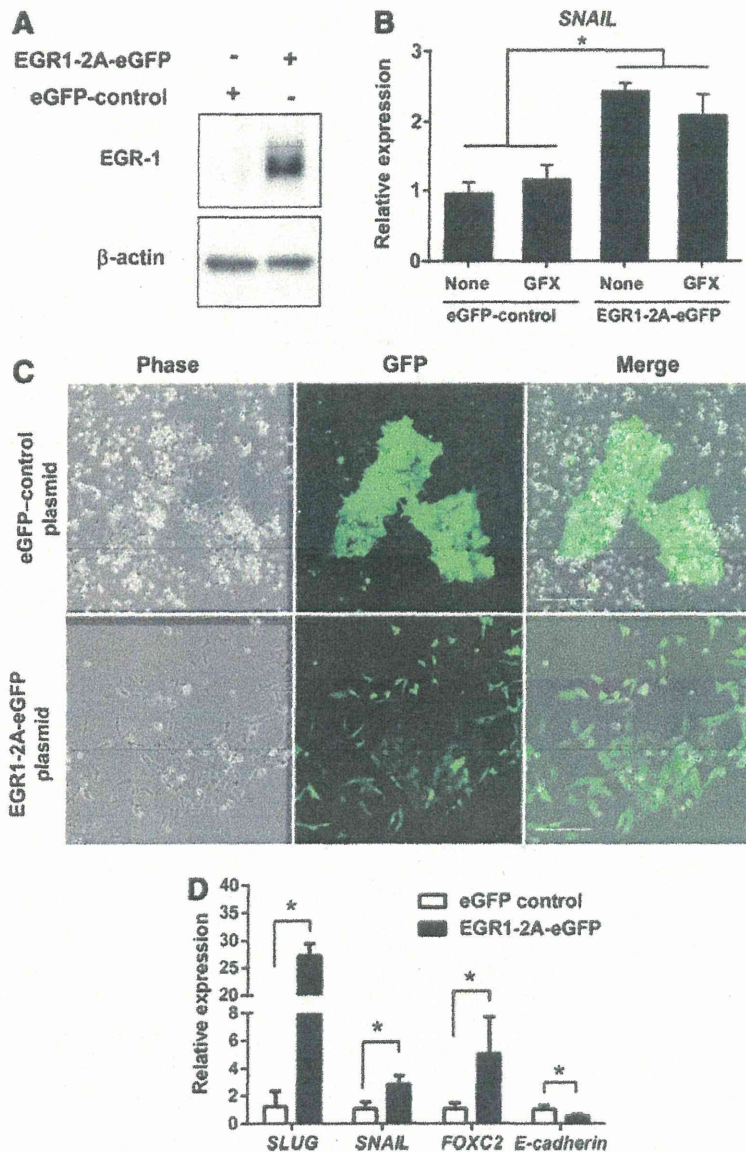


**FIG. 3.** PMA-induced *EGR-1* expression in hES cells. (A) qRT-PCR analysis of *EGR-1* expression in the cells treated with PMA (black bars) or PMA together with GFX (white bars) for 24 h. (B) qRT-PCR analysis of *EGR-1* (black square) or *SNAIL* (gray square) expression in the cells treated with PMA 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  $\pm$  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  $\pm$  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  $\pm$  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  $\pm$  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  $\mu$ M) 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-3 $\beta$ , 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-3 $\beta$  in the nucleus [48]. Although GSK-3 $\beta$  is generally considered to be a cytoplasmic protein, previous reports suggest that GSK-3 $\beta$  also functions in the nucleus [49–51]. It means that Wnt signaling promotes EMT in hPS cells. We have previously reported that phosphorylation of



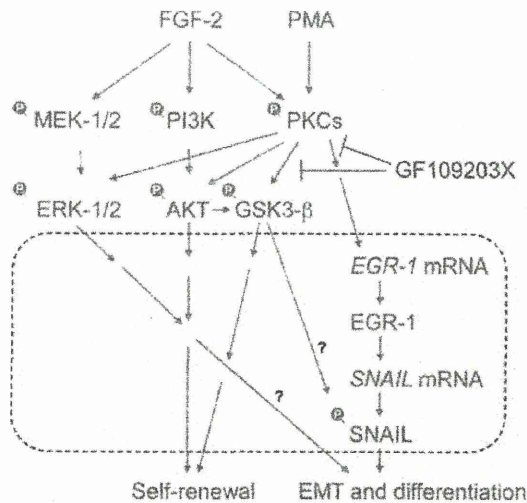
**FIG. 4.** EMT-related gene expression by ectopic EGR-1. **(A)** Western blot analysis of ectopic EGR-1 expression in the cells transfected with EGR1-2A-eGFP or with enhanced green fluorescent protein (eGFP) control plasmid. Transiently ectopic EGR-1 expression was confirmed by the specific EGR-1 antibody 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  $\pm$  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  $\mu$ g/mL) in the hESF9 medium for 6 days. Scale bars = 200  $\mu$ m. **(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  $\pm$  SE ( $n=3$ ).  $*P < 0.05$ .

GSK-3 $\beta$  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-3 $\beta$  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-3 $\beta$  phosphorylation is inhibited in the pres-

ence of the PKC inhibitor, resulting in the inhibition of the PKC/GSK-3 $\beta$  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-3 $\beta$  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-3 $\beta$  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-3 $\beta$  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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