

O-GlcNAc on PKC ζ Inhibits the FGF4-PKC ζ -MEK-ERK1/2 Pathway via Inhibition of PKC ζ Phosphorylation in Mouse Embryonic Stem Cells

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SUMMARY

Mouse embryonic stem cells (ESCs) differentiate into multiple cell types during organismal development. Fibroblast growth factor 4 (FGF4) signaling induces differentiation from ESCs via the phosphorylation of downstream molecules such as mitogen-activated protein kinase/extracellular signal-related kinase (MEK) and extracellular signal-related kinase 1/2 (ERK1/2). The FGF4-MEK-ERK1/2 pathway is inhibited to maintain ESCs in the undifferentiated state. However, the inhibitory mechanism of the FGF4-MEK-ERK1/2 pathway in ESCs is uncharacterized. O-linked β -N-acetylglucosaminylation (O-GlcNAcylation) is a post-translational modification characterized by the attachment of a single N-acetylglucosamine (GlcNAc) to the serine and threonine residues of nuclear or cytoplasmic proteins. Here, we showed that the O-GlcNAc on the phosphorylation site of PKC ζ inhibits PKC ζ phosphorylation (activation) and, consequently, the FGF4-PKC ζ -MEK-ERK1/2 pathway in ESCs. Our results demonstrate the mechanism for the maintenance of the undifferentiated state of ESCs via the inhibition of the FGF4-PKC ζ -MEK-ERK1/2 pathway by O-GlcNAcylation on PKC ζ .

INTRODUCTION

Mouse embryonic stem cells (ESCs) are pluripotent stem cells derived from preimplantation embryos (Evans and Kaufman, 1981). They maintain the undifferentiated state via several intracellular signaling components (signal transducer and activator of transcription 3 [STAT3], extracellular signal-regulated kinase 5 [ERK5], and β -CATENIN) (Weinberger et al., 2016; Morikawa et al., 2016). In contrast, phosphorylated ERK1/2 induces ESC differentiation (Lanner and Rossant, 2010). ERK1/2 is phosphorylated (activated) by phosphorylated mitogen-activated protein kinase/extracellular signal-related kinase (MEK). Therefore, in ESCs, the MEK-ERK1/2 pathway is inhibited to maintain the undifferentiated state. However, the inhibition mechanism of the MEK-ERK1/2 pathway in ESCs is not fully understood (Li et al., 2012; Chappell et al., 2013).

O-Linked β -N-acetylglucosamine (O-GlcNAc) functions in the O- β -glycosidic attachment of a single N-acetylglucosamine (GlcNAc) to the serine and threonine residues of nuclear or cytoplasmic proteins (Torres and Hart, 1984). O-GlcNAc modification is regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). OGT catalyzes the addition of a single O-GlcNAc residue from the donor uridine diphosphate GlcNAc to the serine and threonine residues of core proteins (Haltiwanger et al., 1992). O-GlcNAc is removed from proteins by OGA (Gao et al., 2001). O-GlcNAc modification competes with phosphory-

lation because OGT catalyzes the addition of O-GlcNAc at or in proximity to phosphorylation sites (Zeidan and Hart, 2010). Therefore, O-GlcNAc is believed to regulate signaling pathways by inhibiting the phosphorylation of their cytoplasmic components.

Several studies have reported the functions of O-GlcNAc in ESCs. OGT is required for ESC viability; *Ogt* knockout mice die during embryogenesis (Watson et al., 2014; O'Donnell et al., 2004). Embryoid body formation assays have shown that the expression levels of OGT and O-GlcNAc are reduced during ESC differentiation (Kim et al., 2009; Jang et al., 2012). Furthermore, a recent study has indicated that OCT4 (POU5F1) is modified by O-GlcNAc, and that this modification regulates transcriptional activity to maintain the undifferentiated state of ESCs (Jang et al., 2012). ESC differentiation into cardiac and neural cells is impaired by increased O-GlcNAc levels (Kim et al., 2009; Speakman et al., 2014). Our previous study demonstrated that O-GlcNAc is required for reversion from ESC-derived epiblast stem cells (ESD-EpiSCs), which correspond to the epiblast in postimplantation embryos, to ESCs (Miura and Nishihara, 2016). This suggested that O-GlcNAc maintains the undifferentiated state of ESCs and that reduced O-GlcNAc is required for differentiation. However, the relation between O-GlcNAc and signaling in ESCs remains unclear.

In this study, to clarify the mechanism underlying the maintenance of the undifferentiated state, we analyzed



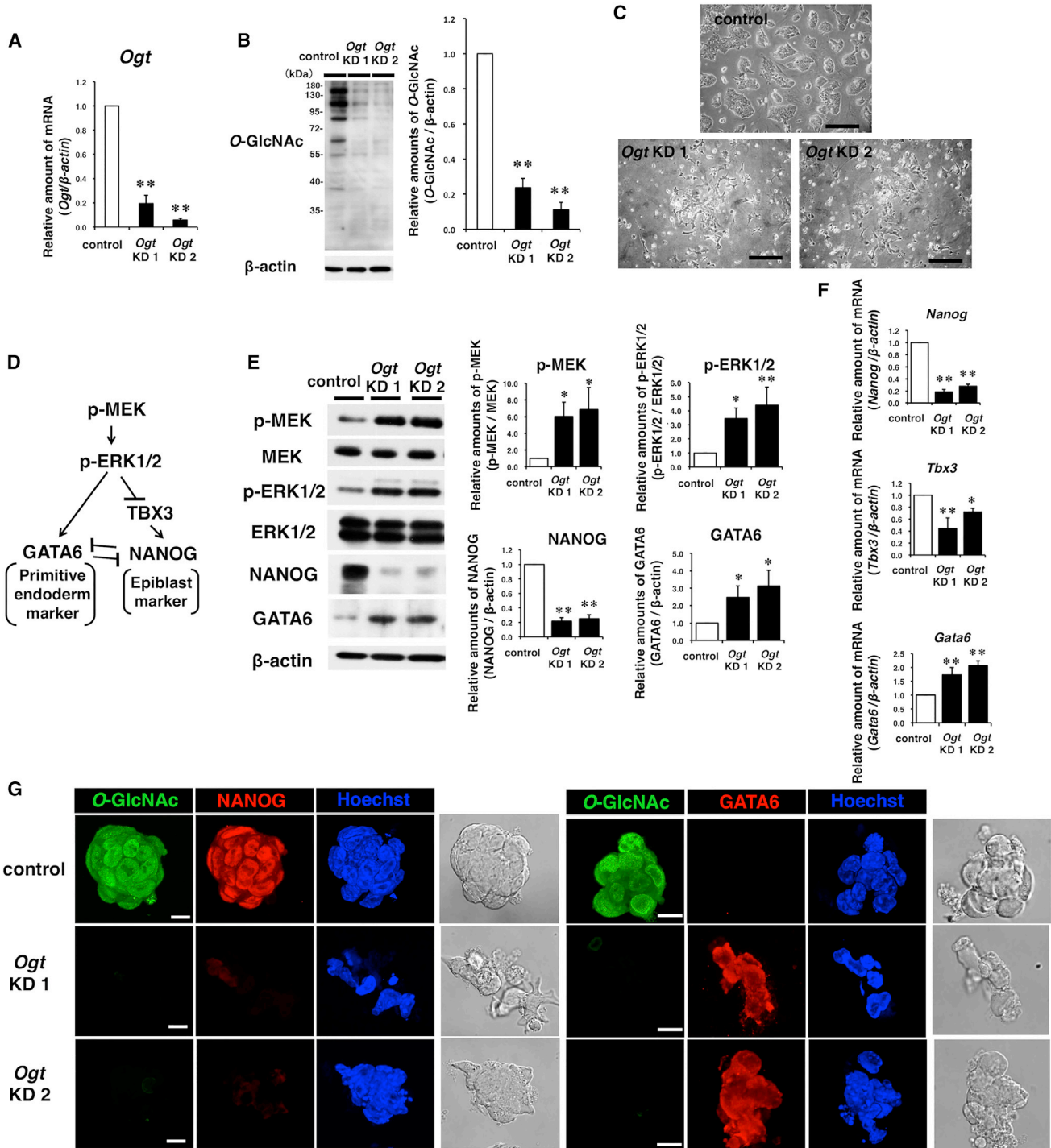


Figure 1. Reduction of O-GlcNAc Activates MEK and ERK1/2

(A) qRT-PCR analysis in *Ogt* knockdown (KD) cells at 4 days after transfection of two constructs expressing different small hairpin RNAs (shRNAs) targeting *Ogt* (*Ogt* KD 1 and *Ogt* KD 2). The amounts of *Ogt* mRNA were normalized to that of β -actin mRNA and are shown relative to control cells (set to 1).

(B) Western blot analysis using anti-O-GlcNAc antibody (RL-2) in *Ogt* KD cells.

(C) The morphology of control cells (upper panel), *Ogt* KD 1 cells (lower left panel), and *Ogt* KD 2 cells (lower right panel). Scale bars, 200 μ m.

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the regulation of the signaling pathways associated with differentiation via O-GlcNAc in ESCs. Here, we describe the mechanism for the maintenance of the undifferentiated state of ESCs.

RESULTS

Reduction of O-GlcNAc Activates MEK and ERK1/2

O-GlcNAc is reduced during embryoid body formation from ESCs (Kim et al., 2009; Jang et al., 2012), suggesting that O-GlcNAc inhibits key signaling molecules that contribute to the differentiation from ESCs. Additionally, activation of the MEK-ERK1/2 pathway promotes ESC differentiation (Lanner and Rossant, 2010). Phosphorylated MEK phosphorylates ERK1/2, which induces ESC differentiation. Therefore, we hypothesized that O-GlcNAc inhibits MEK and/or ERK1/2 phosphorylation in ESCs to maintain the undifferentiated state. We then performed a knockdown (KD) of *Ogt* mRNA using RNAi in ESCs. We designed two constructs that targeted *Ogt* (*Ogt* KD 1 and *Ogt* KD 2, which expressed different small interfering RNAs targeting *Ogt*) and one construct that targeted *Egfp* as a negative control. At 4 days after transfection, O-GlcNAc level and *Ogt* expression were lower in *Ogt* KD cells than in control cells (Figures 1A and 1B). *Ogt* KD ESCs cannot maintain the undifferentiated state (Jang et al., 2012; Shi et al., 2013). The morphology of the control cells was compact and dome-shaped, similar to undifferentiated ESCs. In contrast, *Ogt* KD cells were flat, similar to differentiated cells (Figure 1C), indicating that the *Ogt* KD cells in the current study were differentiated cells.

ERK1/2 phosphorylation induced GATA-binding factor 6 (GATA6) expression, which in turn inhibited NANOG expression (Figure 1D) (Chazaud et al., 2006). GATA6 and NANOG-positive cells function as primitive endoderm (PrE)-progenitor and epiblast-progenitor cells, respectively, in mouse embryonic development at embryonic day 3.5 (E3.5) (Chazaud et al., 2006). Phosphorylated ERK1/2 inhibits T-box transcription factor 3 (TBX3) expression, which enhances NANOG expression (Niwa et al., 2009). ERK1/2 and MEK phosphorylation was significantly higher and NANOG expression was significantly lower in *Ogt* KD

cells (Figures 1E–1G). OCT4 and SOX2, which are other markers of the undifferentiated state, were also significantly downregulated in *Ogt* KD cells (Figures S1A and S1B). These results indicated that O-GlcNAc inhibits MEK and/or ERK1/2 phosphorylation to maintain the undifferentiated state. In *Ogt* KD cells, *Tbx3* expression was significantly decreased, and *Gata6* expression was significantly increased relative to control cells (Figures 1E–1G). These results demonstrated that *Ogt* KD cells spontaneously differentiated into PrE cells, even in the presence of leukemia inhibitory factor (LIF).

In ESCs, ERK1/2 phosphorylation is inhibited by dual-specificity phosphatase 9 (DUSP9), which is induced by bone morphogenetic protein 4 (BMP4) signaling (Figure S1C) (Li et al., 2012). In *Ogt* KD cells, the levels of phosphorylated SMAD1/5/8, which are downstream components of BMP4 signaling and induce DUSP9, were not different compared with control cells (Figure S1D). Additionally, *Dusp9* expression was unchanged in *Ogt* KD cells (Figure S1E). These results indicated that the increase in phosphorylated ERK1/2 in *Ogt* KD cells was not caused by BMP4 signaling.

C-RAF and B-RAF function upstream of MEK (Galabova-Kovacs et al., 2006). Phosphorylated C-RAF and/or B-RAF phosphorylate MEK. In the current study, the levels of phosphorylated C-RAF and phosphorylated B-RAF were not increased in *Ogt* KD cells (Figure S2A). Moreover, C-RAF expression was decreased in *Ogt* KD cells (Figures S2A and S2B), suggesting that O-GlcNAc might contribute to the stability of C-RAF. These results indicated that the increased MEK and ERK1/2 phosphorylation in *Ogt* KD cells was not caused by the upregulation of C-RAF or B-RAF phosphorylation.

O-GlcNAc Inhibits ESC Differentiation into Primitive Endoderm Cells

To examine whether O-GlcNAc regulates ESC differentiation into PrE cells, we induced PrE cells from ESCs by cultivation in the absence of LIF for 6 days. The morphology of PrE cells was flat (Figure 2A). The phosphorylation levels of MEK and ERK1/2 were higher in PrE cells at 6 days than in ESCs (Figure 2B). The expression levels of *Nanog* and *Tbx3*

(D) Schematic representation of the MEK-ERK1/2 pathway during embryonic stem cell (ESC) differentiation into primitive endoderm cells. (E) Western blot analysis using antibodies against phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), ERK1/2, NANOG, and GATA6 in *Ogt* KD cells. The histograms show the mean densitometric readings \pm SD of p-MEK/MEK, p-ERK1/2/ERK1/2, NANOG/ β -actin, and GATA6/ β -actin after normalization against the levels in control cells (set to 1).

(F) qRT-PCR analysis of *Nanog*, *Tbx3*, and *Gata6* expression in *Ogt* KD cells. The amounts of each mRNA were normalized to that of β -actin mRNA and are shown relative to control cells (set to 1).

(G) Immunostaining using antibodies against O-GlcNAc, NANOG, and GATA6 in *Ogt* KD cells. Nuclei were stained with Hoechst (blue). Scale bars, 10 μ m.

Representative images of the western blot and immunostaining are shown. The values shown are the means \pm SD of three independent experiments, and significant values in comparison with control cells are indicated as * $p < 0.05$ and ** $p < 0.01$. See also Figures S1 and S2.

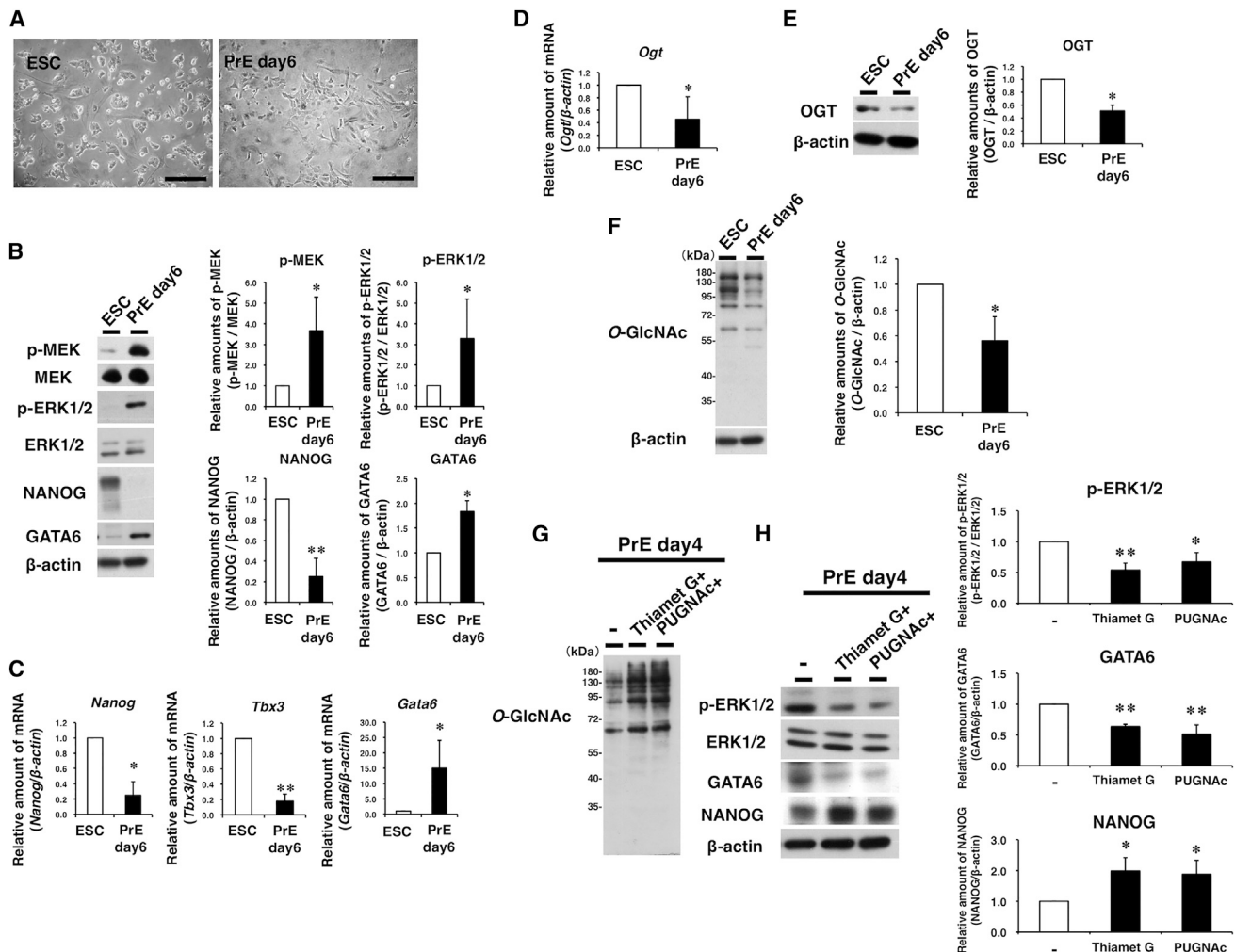


Figure 2. O-GlcNAc Inhibits ESC Differentiation into PrE Cells

(A) The morphology of ESCs (left) and PrE cells at 6 days after differentiation (PrE day 6, right). Scale bars, 200 μ m. (B) Western blot analysis using antibodies against phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), ERK1/2, NANOG, and GATA6 at 6 days after differentiation into PrE cells (PrE day 6). (C) Real-time PCR analysis of *Nanog*, *Tbx3*, and *Gata6* mRNA expression at PrE day 6. (D) Real-time PCR analysis of *Ogt* mRNA expression at PrE day 6. (E) Western blot analysis using anti-OGT antibody at PrE day 6. (F) Western blot analysis using anti-O-GlcNAc antibody (RL-2) at PrE day 6. (G) Western blot analysis using anti-O-GlcNAc antibody (RL-2) at 4 days after ESC differentiation into PrE cells in the presence or absence of 50 μ M Thiamet G or 200 μ M PUGNac, which block O-GlcNAcase. (H) Western blot analysis using anti-phospho-ERK1/2 (p-ERK1/2), anti-ERK1/2, anti-NANOG, and anti-GATA6 antibodies at 4 days after differentiation from ESCs into PrE cells in the presence or absence of 50 μ M Thiamet G or 200 μ M PUGNac. Image of a representative western blot is shown. The histograms show the mean densitometric reading \pm SD after normalization against the levels in ESCs (B–F) and in cells at 4 days after differentiation into PrE cells in the absence of Thiamet G or PUGNac (H) (set to 1). The values shown are the mean \pm SD of three independent experiments, and significant values in comparison with ESCs (B–F) or cells at 4 days after differentiation into PrE cells in the absence of Thiamet G or PUGNac (H) are indicated as * p < 0.05 and ** p < 0.01. See also Figure S2.

were decreased, while that of *Gata6* was increased in PrE cells at 6 days (Figures 2B and 2C). The expression level of *Ogt* was significantly decreased during ESC differentiation into PrE cells (Figures 2D and 2E). O-GlcNAc was also

significantly decreased in PrE cells (Figure 2F). These results suggested that O-GlcNAc inhibits the differentiation into PrE cells. Next, we examined whether O-GlcNAc inhibits ERK1/2 phosphorylation during ESC differentiation into

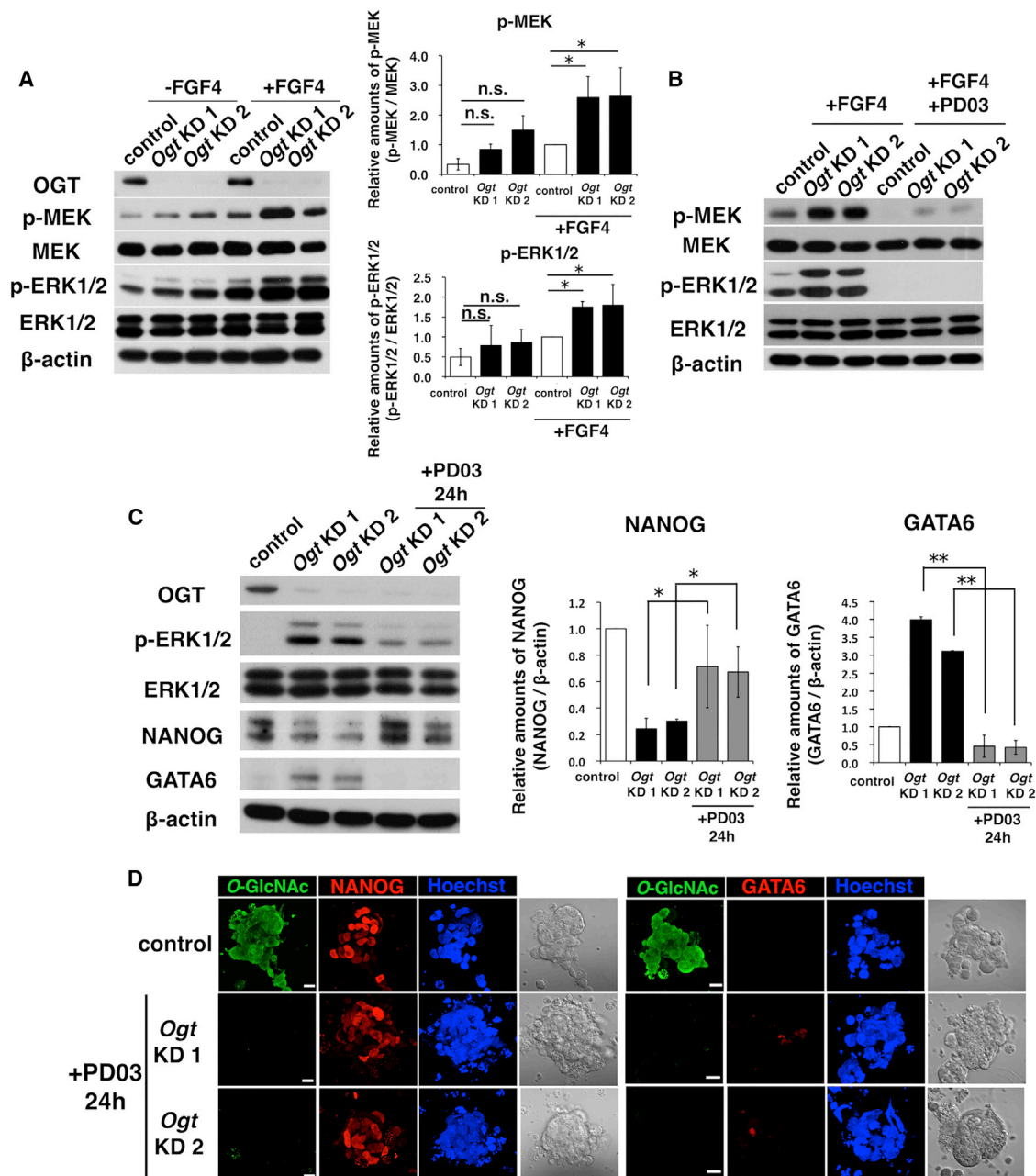


Figure 3. Knockdown of *Ogt* Enhances the FGF4-MEK-ERK1/2 Pathway

(A) Western blot analysis using antibodies against phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), ERK1/2, and OGT in *Ogt* KD cells at 4 days after transfection with two constructs expressing different shRNAs targeting *Ogt* (*Ogt* KD 1 and *Ogt* KD 2) stimulated with 50 ng/mL fibroblast growth factor 4 (FGF4) for 3 min. n.s., not significant.

(B) Western blot analysis using antibodies against phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), and ERK1/2 in *Ogt* KD cells after FGF4 stimulation (50 ng/mL) for 3 min with or without 1 μM PD0325901 (PD03), an MEK inhibitor.

(C) Western blot analysis using antibodies against OGT, phospho-ERK1/2 (p-ERK1/2), ERK1/2, NANOG, and GATA6 in *Ogt* KD cells cultivated with or without 1 μM PD03 for 24 hr.

(D) Immunostaining using antibodies against O-GlcNAc, NANOG, and GATA6 in *Ogt* KD cells cultivated with or without 1 μM PD03 for 24 hr. Nuclei were stained with Hoechst (blue). Scale bars, 10 μm.

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PrE cells. We used Thiamet G and PUGNAc, which are OGA inhibitors, during the induction of ESCs into PrE cells by cultivation in the absence of LIF for 4 days. In the presence of Thiamet G (Thiamet G⁺) and PUGNAc (PUGNAc⁺), O-GlcNAc increased (Figure 2G). The upregulation of ERK1/2 phosphorylation was significantly inhibited by Thiamet G and PUGNAc (Figure 2H). GATA6 and NANOG were upregulated and downregulated, respectively, in PrE cells at 4 days in the absence of Thiamet G and PUGNAc, and the upregulation of GATA6 and downregulation of NANOG were significantly inhibited by Thiamet G and PUGNAc. These results demonstrated that O-GlcNAc inhibits the differentiation into PrE cells by inhibiting the phosphorylation of the MEK-ERK1/2 pathway in undifferentiated ESCs.

To examine the function of OGT in the differentiation of ESCs to PrE cells, we overexpressed *Ogt* in ESCs and then induced PrE cells by cultivation in the absence of LIF for 4 days. In control cells, the MEK-ERK1/2 pathway was activated, and both OGT and markers of the undifferentiated state (OCT4, SOX2, and NANOG) were downregulated in the absence of LIF (Figure S2D). In OGT-overexpressing cells, the expression of OGT was retained despite the absence of LIF. Furthermore, activation of the MEK-ERK1/2 pathway and the downregulation of markers of the undifferentiated state by depletion of LIF were inhibited in the OGT-overexpressing cells. These results indicate that OGT inhibits activation of MEK-ERK1/2 pathway and maintains the undifferentiated state of ESCs.

Knockdown of *Ogt* Enhances the FGF4-MEK-ERK1/2 Pathway

The FGF4-MEK-ERK1/2 pathway induces ESC differentiation into PrE cells (Lanner and Rossant, 2010). Therefore, we examined the response of *Ogt* KD cells to FGF4 stimulation. After FGF4 stimulation, the phosphorylation levels of MEK and ERK1/2 were significantly higher in *Ogt* KD cells than in control cells (Figure 3A). These results demonstrated that the response of *Ogt* KD cells to FGF4 stimulation was markedly increased compared with control cells. The MEK inhibitor, PD0325901 (PD03), significantly inhibited the enhancement of ERK1/2 phosphorylation by FGF4 stimulation in *Ogt* KD cells (Figure 3B). At 24 hr after PD03 addition, markers of the undifferentiated state (OCT4, SOX2, and NANOG) and GATA6 were significantly upregulated and downregulated, respectively, in *Ogt* KD cells (Figures 3C, 3D, S1A, and S1B), indicating that the activation of MEK-ERK1/2 pathway reduced the expression

of markers of the undifferentiated state and increased GATA6 levels in *Ogt* KD cells.

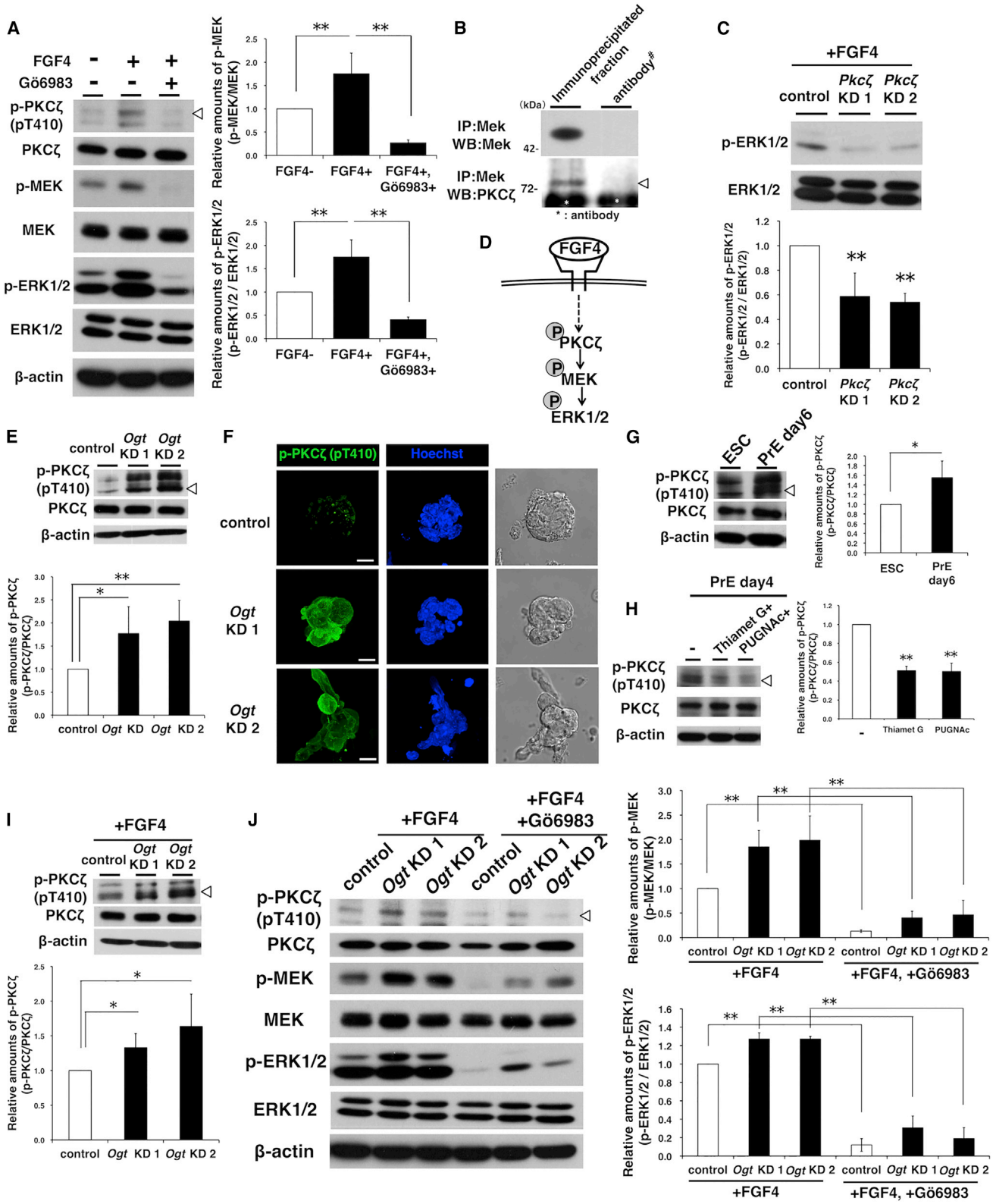
The phosphorylation levels of C-RAF and B-RAF were not increased in *Ogt* KD cells compared with control cells upon FGF4 stimulation (Figure S2B), indicating that O-GlcNAc directly/indirectly inhibits MEK phosphorylation, but not that of C-RAF and B-RAF, in ESCs. In *Ogt* KD cells, the expression levels of *Fgf4* and *Fgfr2c* were significantly decreased (Figure S2C), indicating that the activation of the FGF4-MEK-ERK1/2 pathway in *Ogt* KD cells was not caused by increased *Fgf4* or *Fgfr2c* expression. Together, these results suggest that O-GlcNAc regulates the cytoplasmic components of FGF4 signaling. Furthermore, they indicate that O-GlcNAc inhibits the FGF4-MEK-ERK1/2 pathway in ESCs and that a reduction in OGT enhances the FGF4-MEK-ERK1/2 pathway.

O-GlcNAc Inhibits the FGF4-MEK-ERK1/2 Pathway by Inhibiting PKC ζ Phosphorylation

Next, we examined whether MEK is O-GlcNAcylated and whether its phosphorylation is inhibited in ESCs. We performed precipitation assays with biotinylated succinylated wheat germ agglutinin (sWGA), a lectin that recognizes GlcNAc, and immunoprecipitation assays with an anti-O-GlcNAc antibody (RL-2), using ESC lysates (Figures S3A and S3C). MEK did not coprecipitate with biotinylated sWGA or coimmunoprecipitate with anti-O-GlcNAc antibody (Figures S3B and S3D). These results demonstrated that MEK is not modified by O-GlcNAc in undifferentiated ESCs, suggesting that O-GlcNAc inhibits the activation of upstream components of MEK, but not of C-RAF and B-RAF, in ESCs.

Phosphorylated PKC ζ , which is the active form, interacts directly with MEK and phosphorylates it, independent of C-RAF and B-RAF (Monick et al., 2000). In human pluripotent stem cells, PKC ζ is a downstream component of FGF2 signaling (Kinehara et al., 2013). Moreover, in rat hepatocytes, PKC ζ is modified by O-GlcNAc (Robles-Flores et al., 2008). Therefore, we hypothesized that PKC ζ is a putative downstream component of FGF4 signaling and that PKC ζ is modified by O-GlcNAc, which in turn inhibits PKC ζ phosphorylation in undifferentiated ESCs. Therefore, we examined the effect of PKC ζ phosphorylation on FGF4 stimulation. PKC ζ phosphorylation was significantly enhanced by FGF4 stimulation (Figures 4A and S4A). The enhancement of MEK and ERK1/2 phosphorylation by FGF4 stimulation was significantly inhibited by two different PKC inhibitors, Gö6983 and GF 109203X

Representative images of the western blots and immunostaining are shown. The histograms show the mean densitometric reading \pm SD after normalization against the levels in control cells stimulated with FGF4 (A) and in control cells (C) (set to 1). The values shown are the means \pm SD of three independent experiments, and significant values in comparison with control cells stimulated with FGF4 (A) or control cells (C) are indicated as * p < 0.05 and ** p < 0.01. See also Figures S1 and S2.



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hydrochloride (GFX; **Figures 4A and S4A**). Furthermore, PKC ζ coimmunoprecipitated with anti-MEK antibody, and conversely, MEK coimmunoprecipitated with anti-PKC ζ antibody (**Figures 4B and 5C**). In *Pkc ζ* KD cells (*Pkc ζ* KD 1 and *Pkc ζ* KD 2), at 2 days after transfection, ERK1/2 phosphorylation after FGF4 stimulation was significantly reduced compared with control cells (**Figures 4C and S5A–S5C**). These results demonstrated that FGF4 activates the MEK-ERK1/2 pathway via PKC ζ in undifferentiated ESCs (**Figure 4D**). PKC α , δ , and μ also phosphorylate MEK (Wen-Sheng, 2006; Mizuguchi et al., 2011; Chen et al., 2010). However, in ESCs, PKC α , δ , and μ were not activated by FGF4 stimulation (**Figure S6A**), indicating that they are not the downstream components of FGF4 signaling in ESCs.

If O-GlcNAc inhibits the phosphorylation of PKC ζ , PKC ζ phosphorylation should be promoted in *Ogt* KD cells. PKC ζ phosphorylation was significantly higher in *Ogt* KD cells than in control cells (**Figures 4E, 4F, and S4B**), demonstrating that O-GlcNAc inhibits PKC ζ phosphorylation in undifferentiated ESCs. PKC ζ phosphorylation was significantly higher in PrE cells than in ESCs (**Figure 4G**). The upregulation of PKC ζ phosphorylation during differentiation into PrE cells was inhibited by Thiamet G and PUGNAc (**Figure 4H**). These results suggested that O-GlcNAc inhibits the differentiation into PrE cells by inhibiting the phosphorylation of PKC ζ , an upstream

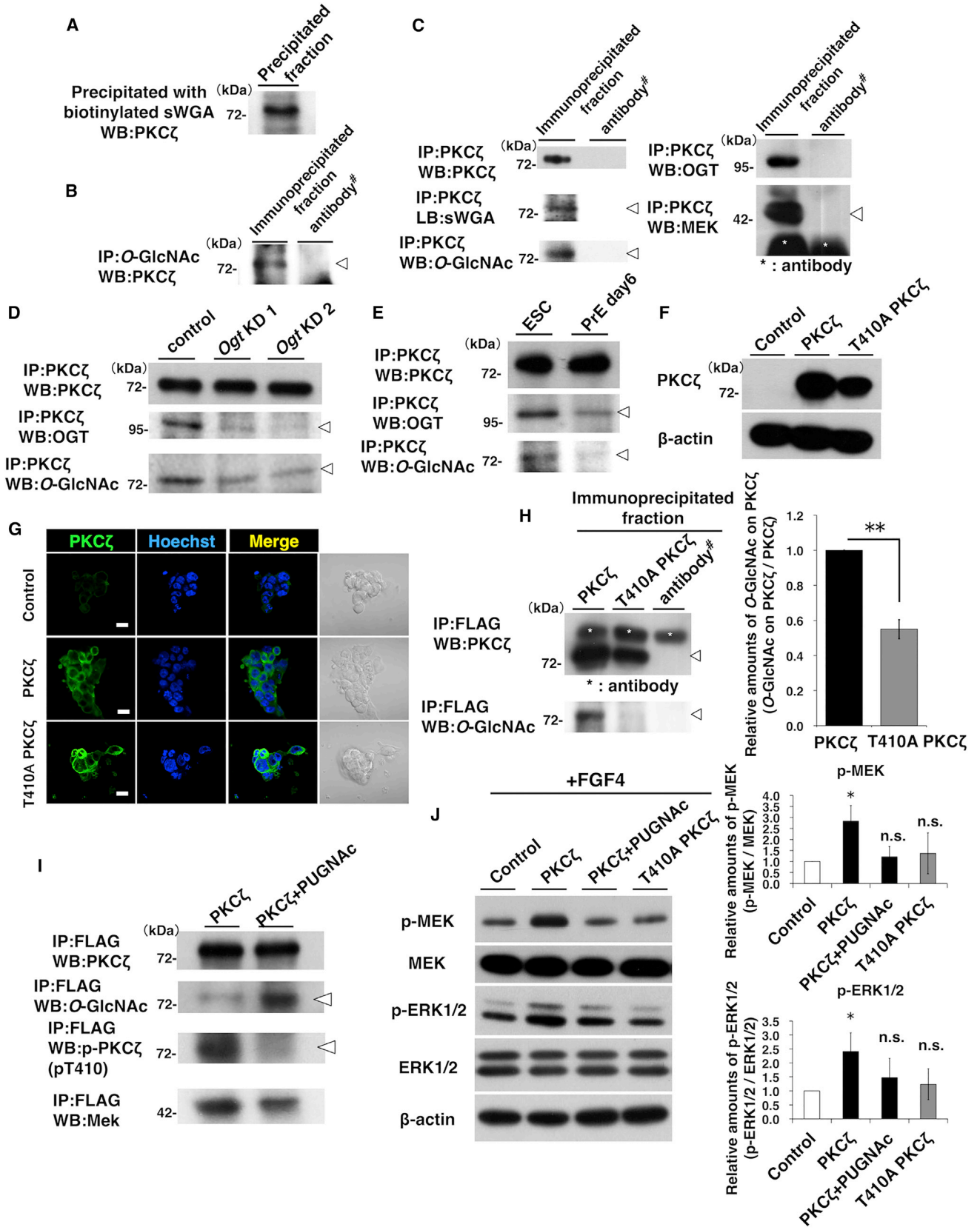
component of the MEK-ERK1/2 pathway, in undifferentiated ESCs. Furthermore, PKC ζ phosphorylation was significantly higher in *Ogt* KD cells after FGF4 stimulation than in control cells (**Figure 4I**). The increase in MEK and ERK1/2 phosphorylation after FGF4 stimulation was significantly inhibited by Gö6983 or GFX (**Figures 4J and S4C**). These results demonstrated that O-GlcNAc inhibits the FGF4-PKC ζ -MEK-ERK1/2 pathway via the inhibition of PKC ζ phosphorylation in undifferentiated ESCs.

The Phosphorylation Site in PKC ζ Is Modified by O-GlcNAc in Undifferentiated ESCs

To investigate whether PKC ζ is modified by O-GlcNAc, we precipitated ESC lysates with biotinylated sWGA. A western blot analysis of the precipitates showed that PKC ζ is modified by GlcNAc (**Figure 5A**). Next, we performed immunoprecipitation using an anti-O-GlcNAc antibody (RL-2) from the ESC lysates. PKC ζ coimmunoprecipitated with anti-O-GlcNAc antibody (**Figure 5B**). We then performed immunoprecipitation using anti-PKC ζ antibody (**Figure 5C**). Lectin blot analysis of the precipitates using sWGA-HRP showed that PKC ζ was modified by GlcNAc. Western blot analysis of the precipitates using anti-O-GlcNAc and anti-OGT antibodies showed that the GlcNAc on PKC ζ was O-GlcNAc and that OGT was coimmunoprecipitated with PKC ζ . In *Ogt* KD cells, the O-GlcNAc level on PKC ζ and the amount of OGT that

Figure 4. O-GlcNAc Inhibits the FGF4-MEK-ERK1/2 Pathway by Inhibiting PKC ζ Phosphorylation

- (A) Western blot analysis using antibodies against phospho-Thr-410 of PKC ζ (p-PKC ζ pT410), PKC ζ , phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), and ERK1/2 in ESCs after FGF4 stimulation (50 ng/mL) for 3 min with or without 10 μ M Gö6983, a PKC inhibitor.
- (B) Western blot (WB) analysis using anti-MEK and anti-PKC ζ antibodies for the fraction immunoprecipitated (IP) with anti-MEK antibody. Hash indicates that anti-MEK antibody was loaded to detect the heavy and light chains of anti-MEK antibody used for immunoprecipitation. Asterisk indicates the heavy chain of anti-MEK antibody.
- (C) Western blot analysis using antibodies against phospho-ERK1/2 (p-ERK1/2) and ERK1/2 in *Pkc ζ* KD cells at 2 days after the transfection of two constructs that expressed different small hairpin RNAs (shRNAs) targeting *Pkc ζ* (*Pkc ζ* KD 1 and *Pkc ζ* KD 2) stimulated with 50 ng/mL FGF4 for 3 min.
- (D) Schematic representation of the FGF4-PKC ζ -MEK-ERK1/2 pathway.
- (E) Western blot analysis using antibodies against phospho-Thr-410 of PKC ζ (p-PKC ζ pT410) and PKC ζ in *Ogt* KD cells at 4 days after transfection with two constructs that expressed different shRNAs targeting *Ogt* (*Ogt* KD 1 and *Ogt* KD 2).
- (F) Immunostaining using antibodies against phospho-Thr-410 of PKC ζ (p-PKC ζ pT410) in *Ogt* KD cells. Nuclei were stained with Hoechst (blue). Scale bars, 10 μ m.
- (G) Western blot analysis using antibodies against phospho-Thr-410 of PKC ζ (p-PKC ζ pT410) and PKC ζ at 6 days after differentiation into PrE cells (PrE day 6).
- (H) Western blot analysis using anti-phospho-Thr-410 of PKC ζ (p-PKC ζ pT410) and anti-PKC ζ antibodies at 4 days after differentiation from ESCs into PrE cells in the presence or absence of 50 μ M Thiamet G or 200 μ M PUGNAc.
- (I) Western blot analysis using antibodies against phospho-Thr-410 of PKC ζ (p-PKC ζ pT410) and PKC ζ in *Ogt* KD cells stimulated with 50 ng/mL FGF4 for 3 min.
- (J) Western blot analysis using antibodies against phospho-Thr-410 of PKC ζ (p-PKC ζ pT410), PKC ζ , phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), and ERK1/2 in *Ogt* KD cells after FGF4 stimulation (50 ng/mL) for 3 min with or without 10 μ M Gö6983. Representative images of the western blot analysis and immunostaining are shown. Arrowheads indicate p-PKC ζ (A, E, and G–J) and PKC ζ (B). The histograms show the mean densitometric reading \pm SD after normalization against the levels in ESCs not stimulated with FGF4 (A), in control cells stimulated with FGF4 (C and I), in control cells (E), in ESCs (G), in cells at 4 days after differentiation into PrE cells in the absence of Thiamet G or PUGNAc (H), and in control cells stimulated with FGF4 in the absence of Gö6983 (J) (set to 1). Values were obtained from three independent experiments, and significance is indicated as * p < 0.05 and ** p < 0.01. See also **Figures S3–S6**.



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coimmunoprecipitated with PKC ζ were reduced (Figure 5D). Similarly, in PrE cells, the *O*-GlcNAc level on PKC ζ and the amount of OGT that coimmunoprecipitated with PKC ζ were reduced compared with ESCs (Figure 5E). These results demonstrated that (1) PKC ζ is modified by *O*-GlcNAc in undifferentiated ESCs, (2) OGT interacts with PKC ζ , and (3) a complex of OGT and PKC ζ , and *O*-GlcNAc on PKC ζ , are reduced during ESC differentiation into PrE cells.

Finally, we attempted to identify the *O*-GlcNAcylation site of PKC ζ . Phosphorylation of Thr-410 of PKC ζ is essential for the activation of PKC ζ and the MEK-ERK1/2 pathway, independent of C-RAF and B-RAF (Yang et al., 2007). Therefore, we hypothesized that Thr-410 of PKC ζ is modified by *O*-GlcNAc and that *O*-GlcNAc on Thr-410 of PKC ζ inhibits PKC ζ phosphorylation in ESCs as well. We transfected the expression vectors of PKC ζ -FLAG tag

and T410A-mutant PKC ζ -FLAG tag (harboring a mutation of Thr-410 to Ala) to ESCs, and confirmed the expression of PKC ζ -FLAG and T410A PKC ζ -FLAG in cells at 2 days after transfection (Figures 5F and 5G). Subsequently, we performed immunoprecipitation using anti-FLAG tag antibody. The *O*-GlcNAc on T410A PKC ζ -FLAG was significantly decreased in comparison with PKC ζ -FLAG (Figure 5H). Furthermore, upon Thiamet G and PUGNAc treatment, *O*-GlcNAc on PKC ζ -FLAG was increased, and the phosphorylation of Thr-410 of PKC ζ -FLAG and the amount of MEK coimmunoprecipitated with PKC ζ -FLAG were decreased (Figures 5I and 5J). In addition, after FGF4 stimulation the phosphorylation levels of MEK and ERK1/2 were significantly upregulated in cells expressing PKC ζ compared with control cells and cells expressing the T410A mutant of PKC ζ (Figure 5J). In cells expressing PKC ζ , PUGNAc and Thiamet G treatment inhibited the

Figure 5. The Phosphorylation Site in PKC ζ Is Modified by *O*-GlcNAc in Undifferentiated ESCs

(A) Western blot (WB) analysis using anti-PKC ζ antibody for the fraction precipitated with succinylated wheat germ agglutinin (sWGA). ESCs were precipitated with biotinylated sWGA. PKC ζ was coprecipitated with biotinylated sWGA.

(B) Western blot (WB) analysis using anti-PKC ζ antibody for the fraction immunoprecipitated (IP) with anti-*O*-GlcNAc antibody (RL-2). Arrowhead indicates that PKC ζ coimmunoprecipitated with anti-*O*-GlcNAc antibody.

(C) Western blot (WB) analysis using anti-PKC ζ , anti-*O*-GlcNAc (RL-2), anti-OGT, and anti-MEK antibodies and lectin blot (LB) analysis using sWGA-HRP for the fraction immunoprecipitated (IP) with anti-PKC ζ antibody. Arrowheads indicate GlcNAc on PKC ζ (LB: sWGA-HRP), *O*-GlcNAc on PKC ζ (WB: *O*-GlcNAc), and MEK (WB: MEK) coimmunoprecipitated with anti-PKC ζ antibody.

(D) Western blot (WB) analysis using anti-PKC ζ , anti-OGT, and anti-*O*-GlcNAc (RL-2) antibodies for the fraction immunoprecipitated (IP) with anti-PKC ζ antibody using *Ogt* KD cells at 4 days after transfection with two constructs that expressed different shRNAs targeting *Ogt* (*Ogt* KD 1 and *Ogt* KD 2). Arrowheads indicate OGT (WB: OGT) and *O*-GlcNAc on PKC ζ (WB: *O*-GlcNAc) coimmunoprecipitated with anti-PKC ζ antibody.

(E) Western blot (WB) analysis using anti-PKC ζ , anti-OGT, and anti-*O*-GlcNAc (RL-2) antibodies for the fraction immunoprecipitated (IP) with anti-PKC ζ antibody using ESCs and PrE cells at 6 days after differentiation (PrE day 6). Arrowheads indicate that OGT (WB: OGT) and *O*-GlcNAc on PKC ζ (WB: *O*-GlcNAc) coimmunoprecipitated with anti-PKC ζ antibody.

(F) Western blot analysis using antibodies against PKC ζ in cells at 2 days after transfection with the expression vector of PKC ζ -FLAG tag (as wild-type) and that of T410A mutant PKC ζ -FLAG tag, having a mutation of Thr-410 to Ala.

(G) Immunostaining using anti-PKC ζ antibody in cells expressing PKC ζ -FLAG tag and those expressing T410A mutant of PKC ζ -FLAG. Nuclei were stained with Hoechst (blue). Scale bars, 10 μ m.

(H) Western blot (WB) analysis using anti-PKC ζ and anti-*O*-GlcNAc (RL-2) antibodies for the fraction immunoprecipitated (IP) with anti-FLAG antibody. Arrowheads indicate PKC ζ (WB: PKC ζ) and *O*-GlcNAc on PKC ζ (WB: *O*-GlcNAc) immunoprecipitated with anti-FLAG antibody. The histograms show the mean densitometric readings \pm SD of (*O*-GlcNAc on PKC ζ immunoprecipitated with anti-FLAG antibody)/(PKC ζ immunoprecipitated with anti-FLAG antibody) after normalization against the levels in PKC ζ -FLAG-expressing cells (set to 1).

(I) Western blot (WB) analysis using anti-PKC ζ , anti-*O*-GlcNAc (RL-2), anti-phospho-Thr-410 of PKC ζ (p-PKC ζ pT410), and anti-MEK antibodies for the fraction immunoprecipitated (IP) with anti-FLAG antibody using cells expressing PKC ζ -FLAG tag in the presence or absence of 200 μ M PUGNAc for 24 hr. Arrowheads indicate *O*-GlcNAc on PKC ζ -FLAG (WB: *O*-GlcNAc) and the phosphorylation of Thr-410 of PKC ζ -FLAG (WB: p-PKC ζ pT410).

(J) Western blot analysis using antibodies against phospho-MEK (p-MEK), MEK, phospho-ERK1/2 (p-ERK1/2), and ERK1/2 in cells expressing PKC ζ -FLAG tag, cells expressing PKC ζ -FLAG tag in the presence of 200 μ M PUGNAc for 24 hr (PKC ζ + PUGNAc), and cells expressing the T410A mutant of PKC ζ -FLAG tag after FGF4 stimulation (50 ng/mL) for 3 min. The histograms show the mean densitometric readings \pm SD of p-MEK/MEK and p-ERK1/2/ERK1/2 after normalization against the levels in control cells stimulated with FGF4 (set to 1). n.s., not significant.

Representative images of the western blots and immunostaining are shown. Values were obtained from three independent experiments, and significant values in comparison with cells expressing PKC ζ -FLAG tag (H) or control cells stimulated with FGF4 (J) are indicated as * p < 0.05 and ** p < 0.01. Hash indicates that only anti-*O*-GlcNAc (B), anti-PKC ζ (C), and anti-FLAG (H) antibodies were loaded to detect the heavy and light chains of the antibodies used for immunoprecipitation. Asterisk indicates the heavy chain or light chain of the antibodies used for immunoprecipitation. See also Figure S6.

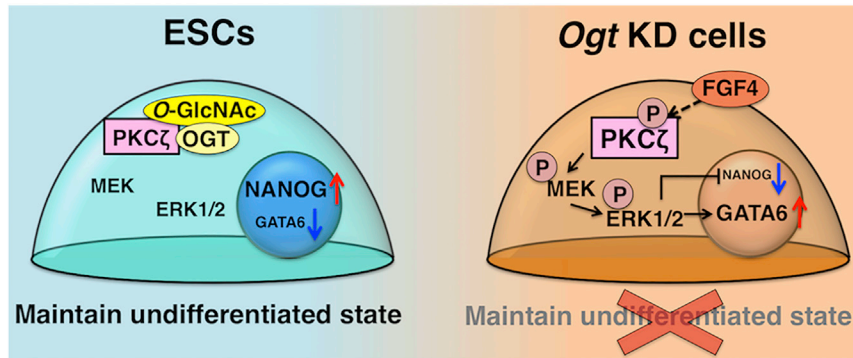


Figure 6. Schematic Representation of FGF4 Signaling Regulated by *O*-GlcNAc in ESCs

Phosphorylated PKC ζ , the active form, activates the MEK-ERK1/2 pathway by FGF4 stimulation. Thr-410, a phosphorylation site, of PKC ζ is modified by *O*-GlcNAc in undifferentiated ESCs, and *O*-GlcNAc on PKC ζ inhibits the phosphorylation of PKC ζ . In the undifferentiated state of ESCs, *O*-GlcNAc inhibits the activation of the FGF4-PKC ζ -MEK-ERK1/2 pathway by inhibiting PKC ζ phosphorylation. Reduction

of OGT induces a reduction in the *O*-GlcNAc level on PKC ζ , PKC ζ phosphorylation is enhanced by FGF4 stimulation, MEK and ERK1/2 are phosphorylated, and consequently phosphorylated ERK1/2 inhibits the maintenance of the undifferentiated state of ESCs.

phosphorylation of MEK and ERK1/2 by FGF4 stimulation (Figures 5J and S6C). These results demonstrated that Thr-410 of PKC ζ was modified by *O*-GlcNAc, and that *O*-GlcNAc on Thr-410 of PKC ζ competed with Thr-410 phosphorylation. Furthermore, they confirmed that *O*-GlcNAc on Thr-410 of PKC ζ inhibited the activation of the MEK-ERK1/2 pathway by FGF4 stimulation in undifferentiated ESCs (Figure 6).

To examine whether the serine- and threonine-rich region near Thr-410 of PKC ζ was modified by *O*-GlcNAc, we transfected the expression vectors for PKC ζ -FLAG tag and T407A-, T408A-, or S409A-mutant PKC ζ -FLAG in ESCs, then performed immunoprecipitation using an anti-FLAG tag antibody (Figures S6D and S6E). *O*-GlcNAc was significantly decreased only on T408A PKC ζ -FLAG in comparison with PKC ζ -FLAG (Figure S6E). The phosphorylation of T408A PKC ζ -FLAG was not increased compared with that of PKC ζ -FLAG (Figure S6F). Moreover, after FGF4 stimulation, phosphorylation of ERK1/2 in cells expressing T408A PKC ζ -FLAG did not change compared with that in cells expressing PKC ζ -FLAG (Figure S6G). These results indicate that Thr-408 of PKC ζ was modified by *O*-GlcNAc in ESCs but *O*-GlcNAc on Thr-408 of PKC ζ was not involved in the phosphorylation of PKC ζ and FGF4 signaling.

Thr-560 is another phosphorylation site of PKC ζ . To investigate whether *O*-GlcNAc on Thr-560 was modified by *O*-GlcNAc in ESC, we transfected expression vectors for T560A-mutant PKC ζ -FLAG tag into ESCs. *O*-GlcNAc on T560A PKC ζ -FLAG did not change compared with PKC ζ -FLAG (Figure S6H), demonstrating that Thr-560 of PKC ζ was not modified by *O*-GlcNAc in ESCs.

DISCUSSION

In the current study, we clarified one of the molecular mechanisms that promote the maintenance of the undif-

ferentiated state of ESCs via *O*-GlcNAc. In the undifferentiated state of ESCs, *O*-GlcNAc on Thr-410 of PKC ζ inhibits its phosphorylation (activation) and consequently inhibits the FGF4-PKC ζ -MEK-ERK1/2 pathway. The reduction of OGT induces the reduction of *O*-GlcNAc levels on PKC ζ , while FGF4 stimulation enhances PKC ζ phosphorylation. Phosphorylated PKC ζ (the active form) then phosphorylates MEK, which in turn phosphorylates ERK1/2. Phosphorylated ERK1/2 induces ESC differentiation into PrE cells. To maintain the undifferentiated state, *O*-GlcNAc inhibits the FGF4-PKC ζ -MEK-ERK1/2 pathway via the inhibition of PKC ζ phosphorylation, thereby inhibiting differentiation into PrE cells.

A previous study reported that the addition of the MEK inhibitor, PD03, over a short period (6 hr and 12 hr) inhibited ERK1/2 phosphorylation in ESCs cultivated in serum and LIF (Chen et al., 2015). However, after the addition of PD03 over extended periods (24 hr and 48 hr), ERK1/2 phosphorylation was recovered (Chen et al., 2015). These results indicate that treatment with the MEK inhibitor over extended intervals cannot inhibit MEK and ERK1/2 phosphorylation. Here, we showed that the addition of a PKC inhibitor for a short time (3 min) strongly inhibited the enhancement MEK and ERK1/2 phosphorylation by FGF4 stimulation in ESCs. However, another group has reported that ERK1/2 phosphorylation was not inhibited in ESCs cultivated without LIF and with the PKC inhibitor over extended periods (10 hr), similar to the results obtained with the MEK inhibitor (Dutta et al., 2011). This discrepancy between our results and those of previous studies likely resulted from the different times of PKC inhibitor treatment. Our results were confirmed by experiments involving the KD and over-expression of PKC ζ .

In this study, we focused on the role of the MEK-ERK1/2 pathway in the differentiation of ESCs, which are “naive” pluripotent stem cells, into PrE cells. Activation of the MEK-ERK1/2 pathway induces ESC differentiation into



both PrE cells and epiblast stem cells (EpiSCs), which are “primed” pluripotent stem cells. Our previous study showed that ERK1/2 phosphorylation was enhanced in primed ESC-derived EpiSCs (ESD-EpiSCs) compared with naive ESCs (Miura and Nishihara, 2016), whereas PKC ζ phosphorylation was not enhanced (data not shown). These results indicate that (1) the PKC ζ -MEK-ERK1/2 pathway was activated in the differentiation from naive ESCs to PrE cells and (2) in the differentiation from naive ESCs to primed ESD-EpiSCs, increased ERK1/2 phosphorylation was not caused by PKC ζ phosphorylation. We showed that the OGA inhibitors Thiamet G and PUGNAC inhibited the PKC ζ -MEK-ERK1/2 pathway and then inhibited the differentiation from naive ESCs to PrE cells. Speakman et al. (2014) previously showed that Thiamet G did not inhibit the naive-to-primed transition in mice, and we also found that PUGNAC did not inhibit the naive-to-primed transition in mice (data not shown). These results showed that O-GlcNAc inhibits differentiation from naive ESCs to PrE cells by inhibition of the PKC ζ -MEK-ERK1/2 pathway, but not the naive-to-primed transition.

PKC ζ phosphorylation is believed to be achieved via a two-step process: (1) Thr-410 of PKC ζ is initially phosphorylated by other kinases, and (2) this initial phosphorylation event allows the autophosphorylation of Thr-560 (Hirai and Chida, 2003). Therefore, phosphorylation of Thr-410 of PKC ζ is an essential event for PKC ζ activation. Furthermore, phosphorylation of Thr-410 of PKC ζ is essential for the activation of the MEK-ERK1/2 pathway, independent of C-RAF and B-RAF phosphorylation, in human primary monocytes, monocyte-derived macrophages, and mouse primary macrophages (Yang et al., 2007). Therefore, we focused on the phosphorylation of Thr-410 of PKC ζ , and our results demonstrated that, in ESCs, FGF4 stimulation promotes the phosphorylation of Thr-410 of PKC ζ . However, the kinase that phosphorylates Thr-410 of PKC ζ was not identified in this study.

3-Phosphoinositide-dependent protein kinase 1 (PDK1) directly interacts with PKC ζ and phosphorylates Thr-410 in ESCs (Balendran et al., 2000). PDK1 is activated by phosphatidylinositol 3-kinase (PI3K), and FGF4 stimulation activates the PI3K-PDK1 pathway (Lanner and Rossant, 2010). Therefore, PDK1 may phosphorylate Thr-410 of PKC ζ via the activation of the FGF4-PI3K pathway in ESCs. Currently, we are investigating whether the PKC ζ -MEK-ERK1/2 pathway is activated by the FGF4-PI3K-PDK1 pathway in ESCs.

Nuclear factor κ B (NF- κ B) signaling enhances the differentiation of ESCs, and inhibition of NF- κ B signaling promotes the undifferentiated state of ESCs (Torres and Watt, 2008). Analysis of PKC ζ -knockout mice revealed that PKC ζ activation is required for the activation of NF- κ B signaling (Leitges et al., 2001). In ESCs, PKC ζ

phosphorylates RELA, an NF- κ B subunit, and phosphorylated RELA activates NF- κ B signaling (Duran et al., 2003). Treatment with the PKC inhibitor and KD of PKC ζ inhibited the activation of NF- κ B signaling via inhibition of the phosphorylation of RELA (Duran et al., 2003). Therefore, to maintain the undifferentiated state of ESCs, O-GlcNAc on PKC ζ may also inhibit NF- κ B signaling, in addition to the MEK-ERK1/2 pathway.

O-GlcNAc regulates various ESC characteristics such as viability, self-renewal, differentiation, transcriptional activity, and epigenetic modifications. OCT4 is a core transcription factor involved in the maintenance of the undifferentiated state of ESCs, and a recent study has indicated that OCT4 is modified by O-GlcNAc in ESCs (Jang et al., 2012). O-GlcNAcylated OCT4 selectively transcribes several genes that are critical for the maintenance of ESCs, and O-GlcNAc on OCT4 is required for ESC maintenance. In the nucleus of ESCs, OGT interacts with the ten-eleven translocation (TET) protein TET1, which converts 5-methylcytosine to 5-hydroxymethylcytosine, an intermediate in DNA demethylation, and O-GlcNAc on TET1 stabilizes the TET1 protein (Shi et al., 2013). In addition to these functions, we found that the regulation of the FGF4-PKC ζ -MEK-ERK1/2 pathway was involved in the maintenance of the undifferentiated state of ESCs by O-GlcNAc. Our study provides additional evidence supporting the essential role of O-GlcNAc in undifferentiated ESCs.

In summary, in the current study we focused on the glycosylation of PKC ζ and determined the mechanism underlying the maintenance of the undifferentiated state via inhibition of the FGF4-PKC ζ -Mek-Erk1/2 pathway by O-GlcNAcylation of PKC ζ . Despite O-GlcNAc being essential for the maintenance of the undifferentiated state of ESCs, several factors other than O-GlcNAc, such as transcription factors and epigenetic modifications, also contribute to maintaining the undifferentiated state. These mechanisms work cooperatively to maintain the naive undifferentiated state.

EXPERIMENTAL PROCEDURES

Cell Culture

R1 ESC lines (Nagy et al., 1993) were maintained on mouse embryonic fibroblasts inactivated with 10 μ g/mL mitomycin C (Sigma-Aldrich) in ESC medium consisting of DMEM supplemented with 15% fetal bovine serum (Hyclone Laboratories), 1% penicillin/streptomycin (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 0.1 mM nonessential amino acids (Gibco), and 1,000 U/mL LIF (Chemicon International).

Transient KD analyses of *Ogt* and PKC ζ , inducible KD analysis of *Ogt*, and overexpression analyses of *Ogt*, wild-type PKC ζ -FLAG, and mutant PKC ζ -FLAG were performed. The details of these experimental procedures are provided in Supplemental Experimental Procedures.



To induce PrE, we seeded 2×10^5 ESCs on gelatin-coated 60-mm culture dishes containing ESC medium without LIF. Medium was changed daily, and cells were passaged every second day. To analyze the effects of OGA inhibition on ESC differentiation to PrEs, we seeded 2×10^5 ESCs on gelatin-coated 60-mm culture dishes in ESC medium without LIF, containing 50 μ M Thiamet G (Sigma) or 200 μ M PUGNAc (Sigma), which are OGA inhibitors.

Western Blotting

For analysis of *Ogt* KD cell response to FGF4 stimulation, the cell culture medium was replaced with serum-free ESC medium without LIF for 4 hr and cells were then stimulated for 3 min with 50 ng/mL FGF4 (R&D System) with or without 10 μ M Gö6983 (Cayman Chemical), 10 μ M GF 109203X hydrochloride (Sigma), or 1 μ M PD0325901 (Wako). Cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4 , 10 mM NaF, and protease inhibitors).

For immunoprecipitation, cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4 , 10 mM NaF, and protease inhibitors). The cell lysate, including 1 mg of proteins, was diluted 10-fold with dilution buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4 , 10 mM NaF, and protease inhibitors). Anti-O-GlcNAc antibody (RL-2; Abcam #2793), anti-MEK antibody (Cell Signaling Technology #9122), anti-PKC ζ antibody (Santa Cruz Biotechnology #sc-393218), or anti-FLAG antibody (Sigma #F3165) was added to the diluted cell lysate, after which Protein G Magnetic Beads (New England Biolabs) were added. The immunoprecipitate was washed five times with wash buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1 mM Na_3VO_4 , 10 mM NaF, and protease inhibitors).

For purification of sWGA-binding proteins, a cell lysate prepared as described above was incubated with biotinylated sWGA (EY Laboratories) and streptavidin magnetic beads (Bio-Rad), then washed with wash buffer five times.

Samples prepared as described above were separated on an SDS polyacrylamide gel with the appropriate acrylamide percentage and transferred to polyvinylidene fluoride membranes (Millipore). After blocking using BSA, the membranes were incubated with the primary antibodies. Full details of the primary antibodies using for western blotting analysis are provided in [Supplemental Experimental Procedures](#). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling), washed, and developed with ECL Plus reagents (GE Healthcare). Phos-tag biotin (Nard Institute) was used according to the manufacturer's instructions.

Real-Time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and reverse transcribed using an oligo(dT) primer (Invitrogen) and a Superscript II First Strand Synthesis Kit (Invitrogen). Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and SYBR Green Master Mix (Roche). The relative amounts of each mRNA were normalized against that of β -actin mRNA in the same sample. Primer sets for the real-time PCR are listed in [Table S1](#).

Immunostaining

Cells were fixed with 4% paraformaldehyde, washed, and blocked with PBS containing 5% BSA, 0.1% Triton X-100, and 1% normal goat serum. Full details of the primary antibodies using for immunostaining are provided in [Supplemental Experimental Procedures](#). Primary antibodies were detected using Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody (Invitrogen) and Cy5-conjugated anti-rabbit IgG secondary antibody (Invitrogen). Immunofluorescence images were taken using an LSM 700 confocal laser microscope (Carl Zeiss).

Statistical Analysis

Welch's t test and one-way ANOVA followed by Tukey's test were performed to compare two groups and three or more groups, respectively. Asterisks denote statistical significance (not significant [n.s.], $p > 0.05$; * $p < 0.05$; and ** $p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at <https://doi.org/10.1016/j.stemcr.2017.11.007>.

AUTHOR CONTRIBUTIONS

T.M.: collection/assembly of data, data analysis and interpretation, and manuscript writing. M.K., T.K., K.Y., and T.H.: collection/assembly of data, data analysis, and interpretation. S.N.: conception and design, financial support, manuscript writing, and final approval of the manuscript.

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