0-O-GlcNAc on PKCζ Inhibits the FGF4-PKCζ-MEK-ERK1/2 Pathway via Inhibition of PKCζ 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. 0-linked β-N-acetylgalactosaminyl linkage (0-GlcNAcylation) is a post-translational modification characterized by the attachment of a single N-acetylgalactosamine (GlcNAc) to the serine and threonine residues of nuclear or cytoplasmic proteins. Here, we showed that the 0-GlcNAc on the phosphorylation site of PKCζ inhibits PKCζ phosphorylation (activation) and, consequently, the FGF4-PKCζ-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-PKCζ-MEK-ERK1/2 pathway by 0-GlcNAcylation on PKCζ.

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-acetylgalactosamine (0-GlcNAc) functions in the O-β-glycosidic attachment of a single N-acetylgalactosamine (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 diphosphogluco-3-phosphate (UDP-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 phosphorylation 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 (POUSF1) 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 μm.

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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...
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 cultured with or without 1 μM PD03 for 24 hr.

(D) Immunostaining using antibodies against O-GlcNAc, NANOG, and GATA6 in Ogt KD cells cultured 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 Fgf2c 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 Fgf2c 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 PKCζ 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 PKCζ, 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, PKCζ is a downstream component of FGF2 signaling (Kinehara et al., 2013). Moreover, in rat hepatocytes, PKCζ is modified by O-GlcNAc (Robles-Flores et al., 2008). Therefore, we hypothesized that PKCζ is a putative downstream component of FGF4 signaling and that PKCζ is modified by O-GlcNAc, which in turn inhibits PKCζ phosphorylation in undifferentiated ESCs. Therefore, we examined the effect of PKCζ phosphorylation on FGF4 stimulation. PKCζ phosphorylation was significantly enhanced by FGF4 stimulation (Figures 4A and 4A). The enhancement of MEK and ERK1/2 phosphorylation by FGF4 stimulation was significantly inhibited by two different PKC inhibitors, Go6983 and GF 109203X.
hydrochloride (GFX; Figures 4A and S4A). Furthermore, PKCζ coimmunoprecipitated with anti-MEK antibody, and conversely, MEK coimmunoprecipitated with anti-PKCζ antibody (Figures 4B and S5C). In Pkcζ KD cells (Pkcζ KD 1 and Pkcζ 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 PKCζ in undifferentiated ESCs (Figure 4D). PKCζ, δ, and μ also phosphorylate MEK (Wen-Sheng, 2006; Mizuguchi et al., 2011; Chen et al., 2010). However, in ESCs, PKCζ, 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 PKCζ, PKCζ phosphorylation should be promoted in Ogt KD cells. PKCζ phosphorylation was significantly higher in Ogt KD cells than in control cells (Figures 4E, 4F, and S4B), demonstrating that O-GlcNAc inhibits PKCζ phosphorylation in undifferentiated ESCs. PKCζ phosphorylation was significantly higher in PrE cells than in ESCs (Figure 4G). The upregulation of PKCζ 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 PKCζ, an upstream component of the MEK-ERK1/2 pathway, in undifferentiated ESCs. Furthermore, PKCζ 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 G66983 or GFX (Figures 4J and S4C). These results demonstrated that O-GlcNAc inhibits the FGF4-PKCζ-MEK-ERK1/2 pathway via the inhibition of PKCζ phosphorylation in undifferentiated ESCs.

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

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

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

(A) Western blot analysis using antibodies against phospho-Thr-410 of PKCζ (p-PKCζ pT410), PKCζ, 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 μM G66983, a PKC inhibitor. (B) Western blot (WB) analysis using anti-MEK and anti-PKCζ 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 Pkcζ KD cells at 2 days after the transfection of two constructs that expressed different small hairpin RNAs (shRNAs) targeting Pkcζ (Pkcζ KD 1 and Pkcζ KD 2) stimulated with 50 ng/mL FGF4 for 3 min.

(D) Schematic representation of the FGF4-PKCζ-MEK-ERK1/2 pathway.

(E) Western blot analysis using antibodies against phospho-Thr-410 of PKCζ (p-PKCζ pT410) and PKCζ 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 PKCζ (p-PKCζ pT410) in Ogt KD cells. Nuclei were stained with Hoechst (blue). Scale bars, 10 μm.

(G) Western blot analysis using antibodies against phospho-Thr-410 of PKCζ (p-PKCζ pT410) and PKCζ at 6 days after differentiation into PrE cells (PřE day 6).

(H) Western blot analysis using anti-phospho-Thr-410 of PKCζ (p-PKCζ pT410) and anti-PKCζ antibodies at 4 days after differentiation from ESCs into PrE cells in the presence or absence of 50 μM Thiamet G or 200 μM PUGNAc.

(I) Western blot analysis using antibodies against phospho-Thr-410 of PKCζ (p-PKCζ pT410) and PKCζ in Ogt KD cells stimulated with 50 ng/mL FGF4 for 3 min.

(J) Western blot analysis using antibodies against phospho-Thr-410 of PKCζ (p-PKCζ pT410), PKCζ, 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 μM G66983. Representative images of the western blot analysis and immunostaining are shown. Arrowheads indicate p-PKCζ (A, E, and G–J) and PKCζ (B). The histograms show the mean densitometric reading ± 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 G66983 (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.
coimmunoprecipitated with PKCζ were reduced (Figure 5D). Similarly, in PrE cells, the O-GlcNAc level on PKCζ and the amount of OGT that coimmunoprecipitated with PKCζ were reduced compared with ESCs (Figure 5E). These results demonstrated that (1) PKCζ is modified by O-GlcNAc in undifferentiated ESCs, (2) OGT interacts with PKCζ, and (3) a complex of OGT and PKCζ, and O-GlcNAc on PKCζ, are reduced during ESC differentiation into PrE cells.

Finally, we attempted to identify the O-GlcNAcylation site of PKCζ. Phosphorylation of Thr-410 of PKCζ is essential for the activation of PKCζ and the MEK-ERK1/2 pathway, independent of C-RAF and B-RAF (Yang et al., 2007). Therefore, we hypothesized that Thr-410 of PKCζ is modified by O-GlcNAc and that O-GlcNAc on Thr-410 of PKCζ inhibits PKCζ phosphorylation in ESCs as well. We transfected the expression vectors of PKCζ-FLAG tag and T410A-mutant PKCζ-FLAG tag (harboring a mutation of Thr-410 to Ala) to ESCs, and confirmed the expression of PKCζ-FLAG and T410A PKCζ-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 PKCζ-FLAG was significantly decreased in comparison with PKCζ-FLAG (Figure 5H). Furthermore, upon Thiamet G and PUGNac treatment, O-GlcNAc on PKCζ-FLAG was increased, and the phosphorylation of Thr-410 of PKCζ-FLAG and the amount of MEK coimmunoprecipitated with PKCζ-FLAG were decreased (Figures 5I and 56B). In addition, after FGF4 stimulation the phosphorylation levels of MEK and ERK1/2 were significantly upregulated in cells expressing PKCζ compared with control cells and cells expressing the T410A mutant of PKCζ (Figure 5J). In cells expressing PKCζ, PUGNac and Thiamet G treatment inhibited the

Figure 5. The Phosphorylation Site in PKCζ Is Modified by O-GlcNAc in Undifferentiated ESCs
(A) Western blot (WB) analysis using anti-PKCζ antibody for the fraction precipitated with succinylated wheat germ agglutinin (sWGA). ESCs were precipitated with biotinylated sWGA. PKCζ was coprecipitated with biotinylated sWGA.
(B) Western blot (WB) analysis using anti-PKCζ antibody for the fraction immunoprecipitated (IP) with anti-O-GlcNAc antibody (RL-2). Arrowhead indicates that PKCζ coimmunoprecipitated with anti-O-GlcNAc antibody.
(C) Western blot (WB) analysis using anti-PKCζ, 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-PKCζ antibody. Arrowheads indicate GlcNAc on PKCζ (LB: sWGA-HRP), O-GlcNAc on PKCζ (WB: O-GlcNAc), and MEK (WB: MEK) coimmunoprecipitated with anti-PKCζ antibody.
(D) Western blot (WB) analysis using anti-PKCζ, anti-OGT, and anti-O-GlcNAc (RL-2) antibodies for the fraction immunoprecipitated (IP) with anti-PKCζ 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 PKCζ (WB: O-GlcNAc) coimmunoprecipitated with anti-PKCζ antibody.
(E) Western blot (WB) analysis using anti-PKCζ, anti-OGT, and anti-O-GlcNAc (RL-2) antibodies for the fraction immunoprecipitated (IP) with anti-PKCζ antibody using ESCs and PrE cells at 6 days after differentiation (PrE day 6). Arrowheads indicate that OGT (WB: OGT) and O-GlcNAc on PKCζ (WB: O-GlcNAc) coimmunoprecipitated with anti-PKCζ antibody.
(F) Western blot analysis using antibodies against PKCζ in cells at 2 days after transfection with the expression vector of PKCζ-FLAG tag (as wild-type) and that of T410A mutant PKCζ-FLAG tag, having a mutation of Thr-410 to Ala.
(G) Immunostaining using anti-PKCζ antibody in cells expressing PKCζ-FLAG tag and those expressing T410A mutant of PKCζ-FLAG. Nuclei were stained with Hoechst (blue). Scale bars, 10 μm.
(H) Western blot (WB) analysis using anti-PKCζ and anti-O-GlcNAc (RL-2) antibodies for the fraction immunoprecipitated (IP) with anti-FLAG antibody. Arrowheads indicate PKCζ (WB: PKCζ) and O-GlcNAc on PKCζ (WB: O-GlcNAc) immunoprecipitated with anti-FLAG antibody. The histograms show the mean densitometric readings ± SD of (O-GlcNAc on PKCζ immunoprecipitated with anti-FLAG antibody)/(PKCζ immunoprecipitated with anti-FLAG tag) after normalization against the levels in PKCζ-FLAG-expressing cells (set to 1).
(I) Western blot (WB) analysis using anti-PKCζ, anti-O-GlcNAc (RL-2), anti-phospho-Thr-410 of PKCζ (p-PKCζ pT410), and anti-MEK antibodies for the fraction immunoprecipitated (IP) with anti-FLAG antibody using cells expressing PKCζ-FLAG tag in the presence or absence of 200 μM PUGNac for 24 hr. Arrowheads indicate OGT on PKCζ-FLAG (WB: O-GlcNAc) and the phosphorylation of Thr-410 of PKCζ-FLAG (WB: p-PKCζ 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 PKCζ-FLAG tag, cells expressing PKCζ-FLAG tag in the presence of 200 μM PUGNac for 24 hr (PKCζ + PUGNac), and cells expressing the T410A mutant of PKCζ-FLAG tag after FGF4 stimulation (50 ng/ml) for 3 min. The histograms show the mean densitometric readings ± 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 PKCζ-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-PKCζ (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 56.
phosphorylation of MEK and ERK1/2 by FGF4 stimulation (Figures S5 and S6C). These results demonstrated that Thr-410 of PKCζ was modified by O-GlcNAc, and that O-GlcNAc on Thr-410 of PKCζ competed with Thr-410 phosphorylation. Furthermore, they confirmed that O-GlcNAc on Thr-410 of PKCζ 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 PKCζ was modified by O-GlcNAc, we transfected the expression vectors for PKCζ-FLAG tag and T407A-, T408A-, or S409A-mutant PKCζ-FLAG in ESCs, then performed immunoprecipitation using an anti-FLAG tag antibody (Figures S6D and S6E). O-GlcNAc was significantly decreased only on T408A PKCζ-FLAG in comparison with PKCζ-FLAG (Figure S6E). The phosphorylation of T408A PKCζ-FLAG was not increased compared with that of PKCζ-FLAG (Figure S6F). Moreover, after FGF4 stimulation, phosphorylation of ERK1/2 in cells expressing T408A PKCζ-FLAG did not change compared with that in cells expressing PKCζ-FLAG (Figure S6G). These results indicate that Thr-408 of PKCζ was modified by O-GlcNAc in ESCs but O-GlcNAc on Thr-408 of PKCζ was not involved in the phosphorylation of PKCζ and FGF4 signaling.

Thr-560 is another phosphorylation site of PKCζ. To investigate whether O-GlcNAc on Thr-560 was modified by O-GlcNAc in ESC, we transfected expression vectors for T560A-mutant PKCζ-FLAG tag into ESCs. O-GlcNAc on T560A PKCζ-FLAG did not change compared with PKCζ-FLAG (Figure S6H), demonstrating that Thr-560 of PKCζ 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 undifferentiated state of ESCs via O-GlcNAc. In the undifferentiated state of ESCs, O-GlcNAc on Thr-410 of PKCζ inhibits its phosphorylation (activation) and consequently inhibits the MEK-ERK1/2 pathway via the inhibition of PKCζ 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 cultured 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 PKCζ.

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 Nishiharu, 2016), whereas PKCζ phosphorylation was not enhanced (data not shown). These results indicate that (1) the PKCζ-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 PKCζ phosphorylation. We showed that the OGA inhibitors Thiamet G and PUGNAc inhibited the PKCζ-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 PKCζ-MEK-ERK1/2 pathway, but not the naive-to-primed transition.

PKCζ phosphorylation is believed to be achieved via a two-step process: (1) Thr-410 of PKCζ 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 PKCζ is an essential event for PKCζ activation. Furthermore, phosphorylation of Thr-410 of PKCζ 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 PKCζ, and our results demonstrated that, in ESCs, FGF4 stimulation promotes the phosphorylation of Thr-410 of PKCζ. However, the kinase that phosphorylates Thr-410 of PKCζ was not identified in this study.

3-Phosphoinositide-dependent protein kinase 1 (PDK1) directly interacts with PKCζ 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 PKCζ via the activation of the FGF4-PI3K pathway in ESCs. Currently, we are investigating whether the PKCζ-MEK-ERK1/2 pathway is activated by the FGF4-PI3K-PDK1 pathway in ESCs.

Nuclear factor κB (NF-κB) signaling enhances the differentiation of ESCs, and inhibition of NF-κB signaling promotes the undifferentiated state of ESCs (Torres and Watt, 2008). Analysis of PKCζ-knockout mice revealed that PKCζ activation is required for the activation of NF-κB signaling (Leitges et al., 2001). In ESCs, PKCζ phosphorylates RELA, an NF-κB subunit, and phosphorylated RELA activates NF-κB signaling (Duran et al., 2003). Treatment with the PKC inhibitor and KD of PKCζ inhibited the activation of NF-κB signaling via inhibition of the phosphorylation of RELA (Duran et al., 2003). Therefore, to maintain the undifferentiated state of ESCs, O-GlcNAc on PKCζ may also inhibit NF-κB 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-PKCζ-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 PKCζ and determined the mechanism underlying the maintenance of the undifferentiated state via inhibition of the FGF4-PKCζ-Mek-Erk1/2 pathway by O-GlcNAcylatation of PKCζ. 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 μg/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 PKCζ, inducible KD analysis of Ogt, and overexpression analyses of Ogt, wild-type PKCζ-FLAG, and mutant PKCζ-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 μM Thiamet G (Sigma) or 200 μM 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 μM G66983 (Cayman Chemical), 10 μM GF 109203X hydrochloride (Sigma), or 1 μM PD0325901 (Wako). Cells were lysed with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM Na₂VO₄, 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 Na₂VO₄, 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 Na₂VO₄, 10 mM NaF, and protease inhibitors). Anti-O-GlcNAc antibody (RL-2; Abcam #2793), anti-MEK antibody (Cell Signaling Technology #9121), anti-PKCζ 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 Na₂VO₄, 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 used 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 used for immunostaining are provided in Supplemental Experimental Procedures. Primary antibodies were detected using Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (lG) 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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