Mucin-type O-glycosylation controls pluripotency in mouse embryonic stem cells via Wnt receptor endocytosis

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ABSTRACT

Mouse embryonic stem cells (ESCs) can differentiate into a range of cell types during development, and this pluripotency is regulated by various extrinsic and intrinsic factors. Mucin-type O-glycosylation has been suggested to be a potential factor in the control of ESC pluripotency, and is characterized by the addition of N-acetylgalactosamine (GalNAc) to serine or threonine residues of membrane-anchored proteins and secreted proteins. To date, the relationship between mucin-type O-glycosylation and signaling in ESCs remains undefined. Here, we identify the elongation pathway via C1GalT1 that synthesizes T antigen (Galβ1-3GalNAc) as the most prominent among mucin-type O-glycosylation modifications in ESCs. Moreover, we show that mucin-type O-glycosylation on the Wnt signaling receptor frizzled-5 (Fzd5) regulates its endocytosis via galectin-3 binding to T antigen, and that reduction of T antigen results in the exit of the ESCs from pluripotency via canonical Wnt signaling activation. Our findings reveal a novel regulatory mechanism that modulates Wnt signaling and, consequently, ESC pluripotency.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: C1GalT1, Embryonic stem cells, Glycosylation, Pluripotency, T antigen, Wnt signaling pathway

INTRODUCTION

Mouse embryonic stem cells (ESCs) are pluripotent stem cells derived from preimplantation embryos and have the capability of differentiating into cells of the three germ layers (Evans and Kaufman, 1981). A large number of studies have explored the potential applications of ESCs for research into developmental biology and regenerative medicine therapies (Murry and Keller, 2008; Shahbazi and Zernicka-Goetz, 2018). Particularly, pluripotent stem cells can aggregate in three-dimensional culture and potentially reconstruct any organ when cultured under specific conditions. These organotypic structures, known as organoids, retain their organ identity and are genetically stable over long periods of time, thus representing a powerful tool to dissect the mechanisms underpinning development and for disease modeling (Lancaster and Knoblich, 2014). To date, several signaling pathways that regulate ESC pluripotency have been identified. Leukemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) signaling are strongly involved in the maintenance of ESC pluripotency via the LIF receptor and BMP receptor families, respectively (Niwa et al., 2009; Ying et al., 2003). In contrast, fibroblast growth factor (FGF) signaling triggers ESC differentiation via the FGF receptor family (Lanner and Rossant, 2010). Wingless-type (Wnt) signaling has been identified as an essential pathway for maintaining pluripotency in ESCs via interaction of Wnt ligands with frizzled (Fzd) proteins, which are seven-transmembrane domain G-protein-coupled receptors (ten Berge et al., 2011). Enforced activation by Wnt through CHIR99021 (CHIR) in combination with the FGF signaling inhibitor PD0325901 (PD) is commonly used to culture ESCs in an undifferentiated state (Ying et al., 2008; Ying and Smith, 2017). However, Wnt activation requires complex signaling translation via a large family of protein ligands, receptors, co-receptors, agonists and antagonists; as a consequence, Wnt signaling regulation is still poorly understood. Indeed, in contrast to the generally accepted view of Wnt signaling, some studies have reported that Wnt activation results in ESC differentiation (He et al., 2008; Price et al., 2013; Zhang et al., 2013). In addition, Wnt signaling plays a crucial role in embry development, tumor initiation, and organoid growth, maturation and morphogenesis (McCauley and Wells, 2017; Steinhart and Angers, 2018; Zhan et al., 2017). These observations emphasize the importance of clarifying the mechanisms that modulate Wnt signaling.

Mucin-type O-glycosylation (hereafter just referred to as O-glycosylation) is an evolutionarily conserved protein modification and, together with N-linked glycosylation, is one of the most abundant forms of glycosylation present on membrane proteins and secreted proteins (Tran and Ten Hagen, 2013). Indeed, 83% of human proteins are O-glycosylated (Steentoft et al., 2013). O-glycosylation is a stepwise process characterized by the initial attachment of N-acetylgalactosamine (GalNAc) to the hydroxyl group of serine or threonine residues of the target protein by a large family of up to 19 polypeptide α-N-acetylgalactosaminyltransferases (ppGalNAc-Ts) in mouse; the attachment of GalNAc forms the so-called Tn antigen (Fig. 1A). Galactose (Gal), sialic acid (NeuAc) or N-acetylgalactosamine (GlcNAc) can be successively added to Tn antigen by core 1 β1,3-galactosyltransferase (C1GalT1), GalNAc α2,6-sialyltransferase-I (ST6GalNAc-I), and β1,3-N-
acetylglucosaminyltransferase-6 (β3GnT6), to form three main structures – T antigen (Galβ1-3GalNAc), sialyl Tn antigen (NeuAcα2-6GalNAc) and the core 3 structure (GlcNAcβ1-3GalNAc) (Bennet et al., 2012). Several studies have demonstrated that O-glycosylation plays a vital role in development and tumorigenesis. The importance of this modification during embryonic development is highlighted by the fact that disruption of the C1GalT1 gene causes embryonic lethality in mice (Xia and McEver, 2006). Galnt1 is the most abundantly expressed of the 19 ppGalNAc-Ts during murine embryonic submandibular gland development; mice deficient for Galnt1 show a reduction in FGF signaling (Tian et al., 2012). We previously reported that O-glycosylation is required for normal development in Drosophila (Fuwa et al., 2015; Itoh et al., 2016, 2018). Furthermore, aberrant glycoprofiles are observed in essentially all types of cancers (Pinho and Reis, 2015). Recently, a novel culture medium that includes the primitive growth factor NME7AB, which binds to the extracellular domain of the cleaved form of the O-glycosylated protein MUC1, was shown to maintain human ESCs in an undifferentiated state (Carter et al., 2016), suggesting that O-glycosylation is involved in the pluripotency network. However, the function of O-glycosylation in ESC pluripotency network and its relationship to signaling in ESCs remain unknown.

The present study was initiated to clarify the role of mucin-type O-glycosylation in the ESC pluripotency network. We identified the most prominent mucin-type O-glycosylation elongation pathway in ESCs and manipulated the expression of the enzyme involved in its formation. Our results provide the first demonstration that O-glycosylation controls ESC pluripotency by directly regulating Wnt signaling receptor endocytosis.

**RESULTS**

**The mucin-type O-glycosylation elongation pathway via C1GalT1 is the most prominent in ESCs**

Tn antigen formation, which can be catalyzed by 19 different ppGalNAc-Ts in mouse, is the initial step of mucin-type O-glycosylation. Tn antigen is further elongated by ST6GalNAc-I, C1GalT1 or β3GnT6 to synthesize sTn antigen, T antigen or the core 3 structure, respectively (Fig. 1A). The partial functional redundancy of ppGalNAc-Ts makes analysis of their function highly complex (Bennet et al., 2012); we therefore focused on the catalyzation step following Tn antigen formation that is selectively performed by ST6GalNAc-I, C1GalT1 or β3GnT6 to synthesize sTn antigen, T antigen or the core 3 structure, respectively (Fig. 1A). The partial functional redundancy of ppGalNAc-Ts makes analysis of their function highly complex (Bennet et al., 2012); we therefore focused on the catalyzation step following Tn antigen formation that is selectively performed by ST6GalNAc-I, C1GalT1 or β3GnT6. In addition to sTn antigen, T antigen and the core 3 structure, the Tn antigen can also be extended to form core 5 to 8 structures. However, core 5 to 8 structures have an extremely restricted occurrence and the glycosyltransferases involved in their formation remain to be fully characterized (Brockhausen et al., 2009); thus, they are not considered in this study.

We analyzed mRNA expression in ESCs and found that C1GalT1 was the most highly expressed of the three enzymes (Fig. 1B). Quantitative analysis of O-glycans in ESCs was performed by mass spectrometry using optimized microwave-assisted β-elimination in the presence of a pyrazolone (BEP) method for O-glycomic analysis that was validated with a mixture of equal quantities of four glycans.
We here identified T antigen, and its further modifications, as the only detectable structure among sTn antigen, T antigen and the core 3 structure (Fig. 1C). Interestingly, the absolute amount of T antigen, and C1GalT1-mediated elongation pathway modifications, were consistent among different ESC lines, suggesting that the C1GalT1 elongation pathway might play a crucial role in ESCs (Fig. S1A, B). Next, we investigated the O-glycosyltransferase dynamics during early and late differentiation by using an embryoid body (EB) assay. C1GalT1 showed the highest level of expression among the three O-glycosyltransferases between day 0 and day 4, suggesting that the C1GalT1-mediated elongation pathway might have a role during early differentiation of ESCs. However, at later differentiation stages, expression of all three enzymes increased dramatically, indicating that the ESC surface might be decorated by several types of O-glycan structure during late differentiation (Fig. 1B; Fig. S1C–E).

**C1GalT1 knockdown causes ESCs to exit from pluripotency**

To elucidate the role of the C1GalT1 elongation pathway in determining pluripotency, we performed a RNAi knockdown (KD) of C1GalT1. Two constructs were designed that expressed different siRNAs targeting C1GalT1 (C1GalT1 KD 1 and C1GalT1 KD 2). At 4 days after transfection, C1GalT1 KD cells showed decreased C1GalT1 expression (Fig. 2A) followed by a reduction of T antigen at the cell membrane and internally, as shown by peanut agglutinin lectin (PNA) staining, which binds specifically to T antigen (Fig. 2B–F). C1GalT1 KD resulted in a significant increase in Tn antigen and a mild decrease in sTn antigen at the cell surface, as shown by Helix pomatia agglutinin lectin (HPA) and Sambucus nigra lectin (SNA) staining, respectively (Fig. S2A–D). C1GalT1 KD cells exhibited a flat shape, which is typical of differentiated cells, compared to the dome-shape morphology of undifferentiated cells (Fig. 3A); this observation demonstrated that C1GalT1 KD cells were differentiated cells. Next, we analyzed the expression of the core pluripotency markers Oct3/4 (also known as POU5F1) and Sox2, and of SSEA-1, a pluripotent cell surface marker (Nakai-Futatsugi and Niwa, 2013). The levels of Oct3/4 and Sox2 mRNAs were significantly decreased in C1GalT1 KD cells compared to the control; a similar decrease at the protein level was also observed (Fig. 3B–D). The SSEA-1+ cell population was reduced in C1GalT1 KD cells compared to the control (Fig. 3E). To assess the ability of C1GalT1 KD cells to self-renew, we performed a clonogenicity assay at 4 days post transfection. The number of alkaline phosphatase (ALP)-positive colonies was markedly decreased in C1GalT1 KD cells, demonstrating that the self-renewing ability of C1GalT1 KD cells was compromised (Fig. 3F). ESC pluripotency and self-renewal loss following C1GalT1 KD was further confirmed by using stable C1GalT1 KD ESCs (Fig. S3A–D). An analysis of the expression of differentiation markers showed that C1GalT1 KD resulted in an upregulation of the trophoblast markers Cdx2 and Gata3, suggesting that C1GalT1 KD induces ESC differentiation.

**Fig. 2. Knockdown of C1GalT1 results in T antigen reduction.** (A) Real-time PCR analysis of C1GalT1 KD cells. The amount of C1GalT1 was normalized against that of β-actin. (B) FACS analysis of C1GalT1 KD cells stained by PNA–FITC. (C) Histogram representing fluorescence mean intensity of data in B. The fold change is presented relative to that of control cells. (D) Representative image of cell surfaces of C1GalT1 KD cells after immunostaining using PNA–biotin. Nuclei were stained with Hoechst. Scale bars: 10 μm. (E) Representative image of a maximum intensity projection of internal molecules in C1GalT1 KD cells after immunostaining using PNA–biotin. Nuclei were stained with Hoechst. Scale bars: 10 μm. (F) Internal PNA mean intensity of images in E. The mean intensity is shown as fold change relative to that of the control. Quantitative values are shown as means±s.e.m. of three independent experiments. *P<0.05; **P<0.01; ***P<0.001 (unpaired two-tailed Student’s t-test).
transdifferentiation toward the trophectoderm (Fig. 3G). Consistent with this, an EB assay showed an enhanced differentiation potential in C1GalT1 KD ESCs (Fig. S3E,F). These data demonstrate that C1GalT1 KD cells spontaneously exit from pluripotency, even in the presence of LIF.

C1GalT1 knockdown promotes canonical Wnt signaling in ESCs

Previous studies have reported that mucins, which are heavily O-glycosylated proteins, interact with β-catenin, the key mediator of the canonical Wnt signaling, during many malignancies (Pai et al., 2016). Therefore, we hypothesized that C1GalT1 KD might alter canonical Wnt signaling in ESCs. Activation of Wnt signaling results in inhibition of β-catenin phosphorylation, thereby preventing its degradation. Stabilized β-catenin accumulates in the cytoplasm and translocates to the nucleus where, together with Tcf/Lef, it triggers Wnt-specific target gene transcription (Nusse and Clevers, 2017). Here, we found that C1GalT1 KD cells exhibited a reduction in phospho-β-catenin (p-β-catenin) and an increase in total β-catenin (Fig. 4A,B). A reduction in the level of β-catenin mRNA was observed, suggesting that C1GalT1 KD resulted in increased β-catenin protein stability (Fig. 4C). A luciferase assay...
using a β-catenin/Tcf responsive element (TOP) showed marked upregulation of Wnt signaling (Fig. 4D), followed by an increased expression of the Wnt-specific target gene Axin2 (Fig. 4E). Moreover, immunostaining using an anti-β-catenin antibody (Ab) in permeabilized C1GalT1 KD cells showed an increased localization of β-catenin in the nucleus compared to what was seen in the control (Fig. 4F,G). These results demonstrate that C1GalT1 KD promotes canonical Wnt signaling activation in ESCs.
Mucins have also been reported to activate phosphoinositide 3-kinase (PI3K)/Akt and Fgf signaling in breast cancer cells (Woo et al., 2012; Hiraki et al., 2016). However, C1GalT1 KD did not result in activation of PI3K/Akt and Fgf signaling pathways in ESCs (Fig. S4A–D). Previously published ChIP-seq analysis of wild-type, untreated ESCs [available at NCBI Sequence Read Archive; accession numbers: SRX1204276 (Dahl et al., 2016), SRX191012 (Mouse ENCODE et al., 2012) and SRX1080398 (Wang et al., 2017)] revealed the following characteristics in the C1GalT1 promoter region: an active promoter as shown by the presence of the marker H3K4me3 (Tserel et al., 2010), an open conformation as shown by DNase-seq, and a Wnt effector as shown by Tcf3 binding (Fig. S4E). These findings suggest that Wnt signaling may regulate C1GalT1 expression (Oki et al., 2018).

**Fzd5 carries T antigen**

Canonical Wnt signaling is initiated by the binding of the Wnt ligand to its Fzd receptors, which then dimerize with the co-receptor LRP5–LRP6 and results in downstream signaling (Nusse and Clevers, 2017). Among the Fzd receptor family involved in the canonical Wnt signaling in ESCs, Fzd5 is the most highly expressed and plays an essential role during development (Hao et al., 2006). Support for this conclusion comes from the fact that Fzd5 depletion causes embryonic lethality in mice (Ishikawa et al., 2001). Here, to investigate whether Wnt signaling receptors are O-glycosylated, we precipitated Fzd5 as a putative candidate. A pulldown assay using biotinylated PNA followed by western blotting using an anti-Fzd5 Ab showed that Fzd5 was successfully precipitated, and demonstrated that Fzd5 carries T antigen (Fig. 5A). Next, we performed an immunoprecipitation analysis on an ESC lysate using an anti-Fzd5 Ab. A lectin blot analysis of the immunoprecipitates using PNA confirmed that Fzd5 was modified by T antigen (Fig. 5B). Moreover, the amount of Fzd5 protein precipitated in C1GalT1 KD cells decreased compared to the control, further confirming that Fzd5 is O-glycosylated (Fig. 5C, D). However, T antigen was not detected by lectin blot analysis for LRP5 and LRP6 immunoprecipitates, demonstrating that the Fzd co-receptors LRP5 and LRP6 do not carry T antigen (Fig. S5). Together, these results demonstrate, for the first time, that Wnt receptor Fzd5 carries T antigen. Thus, other Fzd receptors, including secreted Fzd molecules, may also be O-glycosylated.

**T antigen on Fzd5 regulates its galectin-3-mediated endocytosis**

O-glycosylation has multiple functions, such as in protein–protein interaction, and in trafficking and turnover of cell surface proteins (Razawi et al., 2013; Karabasheva et al., 2014). We therefore analyzed the role of T antigen on Fzd5. Immunostaining using an anti-Fzd5 Ab in permeabilized C1GalT1 KD cells revealed that there was a striking reduction of internalized Fzd5, as shown by puncta staining, compared with what was seen in the control (Fig. 6A–C; Movies 1–3). In contrast, Fzd5 staining at the cell surface increased in C1GalT1 KD cells (Fig. 6D). A surface biotinylation assay indicated that Fzd5 was markedly increased on the surface of C1GalT1 KD cells (Fig. 6D). Moreover, ultrastructural localization of Fzd5 by immunoelectron microscopy showed that colloidal gold labeling was scarcely present on the plasma membrane of control cells compared to in C1GalT1 KD cells (Fig. 6F), further demonstrating that Fzd5 is retained at the cell membrane after C1GalT1 KD. To confirm these observations, we treated ESCs with the O-glycosylation inhibitor benzyl 2-acetoamido-2-deoxy-α-D-galactopyranoside (GalNAC-Bn) and then stained the cells using an anti-Fzd5 Ab. A similar effect was found to that seen in C1GalT1 KD cells; namely, we observed a dramatic reduction of internalized Fzd5 and an increase at the cell surface after O-glycosylation inhibition (Fig. S6A–D; Movies 4 and 5). These observations confirmed that T antigen on Fzd5 is involved in Fzd5 internalization. The ultrastructural localization of Fzd5 was investigated using two different Abs against Fzd5. Colloidal gold labeling was observed inside and outside the endosomes of control cells, as shown by using anti-Fzd5 Abs against the N-terminal and C-terminal regions, respectively (Fig. 6G). In addition, immunostaining showed that the early endosome marker Rab5 colocalized with Fzd5 in ESCs (Fig. 6H; Movie 6), demonstrating that Fzd5 is cleared from the plasma membrane by endocytosis.

**Fig. 5. Fzd5 carries T antigen.** (A) Representative image of a western blot (IB) using an anti-Fzd5 Ab on the lectin-precipitated fraction (LP) precipitated with biotinylated PNA. The input represents the total ESC lysate. Similar results were obtained in four independent experiments. (B) Representative image of a western blot (IB) using an anti-Fzd5 Ab and lectin blot (LB) by PNA–HRP on the immunoprecipitated fraction (IP) precipitated with an antibody against Fzd5. The input represents the total ESC lysate. The arrowhead indicates the Fzd5 protein. Similar results were obtained in three independent experiments. (C) Representative image of a western blot (IB) using an anti-Fzd5 Ab on the lectin precipitated fraction precipitated with biotinylated PNA in C1GalT1 KD cells. Similar results were obtained in three independent experiments. Band intensity fold change relative to control is shown below the western blot image. (D) Representative image of western blot using an anti-Fzd5 Ab in C1GalT1 KD cells. Similar results were obtained in three independent experiments.
Galectins (Lgals) belong to a family of carbohydrate-binding proteins that bind to β-galactose-containing glycoproteins (Johannes et al., 2018). Previous studies have reported that Lgals, in particular Lgals3, have a stimulatory effect on endocytosis (Merlin et al., 2011; Gao et al., 2012; Lepur et al., 2012; Lakshminarayan et al., 2014). Moreover, frontal affinity

Fig. 6. T antigen on Fzd5 regulates its endocytosis. (A) Representative image (n=3) of a maximum intensity projection of intracellular molecules using PNA–biotin and an anti-Fzd5 Ab in C1GalT1 KD cells. Nuclei were stained with Hoechst. Arrowheads indicate Fzd5 puncta staining. Scale bars: 10 μm. (B) 3D reconstruction of images in A using Imaris version 9.3.1. Scale bars: 10 μm. (C) Quantification of Fzd5 puncta staining normalized against the number of nuclei and shown as a fold change relative to control. (D) Magnification of areas highlighted in A. Scale bar: 2.5 μm. (E) Assessment of Fzd5 surface protein in C1GalT1 KD cells using a biotinylation assay. Band intensity fold change relative to control is shown below the western blot image. Representative of three independent experiments. (F) Representative transmission electron micrographs from C1GalT1 KD cells. Arrowheads indicate Fzd5 staining at the plasma membrane. Scale bars: 500 nm. The micrographs were obtained from a single biological replicate. (G) Representative transmission electron micrographs from control cells showing the ultrastructural localization of Fzd5 in the endosome. Colloidal gold labeling is inside the endosome using an anti-Fzd5 Ab against the N-terminal region (upper panel), and outside the endosome using an anti-Fzd5 Ab against the C-terminal region (lower panel). Scale bars: 10 nm. The micrographs were obtained from a single biological replicate. (H) Representative image (n=3) of a maximum intensity projection of intracellular molecules using an anti-Fzd5 Ab and an anti-Rab5 Ab in ESCs. Arrowheads indicate colocalization of Fzd5 puncta and Rab5. Scale bars: 10 μm. Quantitative values are shown as means±s.e.m. of three independent experiments. **P<0.01; ***P<0.001 (unpaired two-tailed Student’s t-test).
chromatography analysis has demonstrated that T antigen is a ligand for Lgals9, followed by Lgals2, Lgals3 and Lgals4 (Iwaki and Hirabayashi, 2018). Therefore, we hypothesized that Lgals are involved in Fzd5 endocytosis. Immunostaining using an anti-Fzd5 Ab in permeabilized ESCs showed a marked reduction of internalized Fzd5 in ESCs treated with 50 mM lactose monohydrate for 30 min. Arrowheads indicate colocalization of Fzd5 puncta and Lgals3. Scale bars: 10 μm. (B) Quantification of Fzd5 puncta staining of image in A normalized against the number of nuclei and shown as a fold change relative to control. (C) Galectin expression in ESCs analyzed by RNA-seq shown as normalized read count. RNA-seq data were obtained from a single technical and biological replicate. (D) Representative image (n=3) of a maximum intensity projection of intracellular molecules using an anti-Fzd5 Ab and an anti-Lgals3 Ab in ESCs treated with 15 μg/ml of recombinant Lgals3 for 30 min. Arrowheads indicate colocalization of Fzd5 puncta and Lgals3. Scale bars: 10 μm. (E) Quantification of Fzd5 puncta staining in image D normalized against the number of nuclei and shown as a fold change relative to control. Quantitative values are shown as means±s.e.m. of three independent experiments. **P<0.01 and ***P<0.001 (unpaired two-tailed Student’s t-test).

(F) Schematic representation of ESC pluripotency regulation by mucin-type O-glycosylation. Mucin-type O-glycosylated Wnt receptor Fzd5 on wild-type (WT) ESCs is cleared from the plasma membrane by endocytosis via the binding of galectin-3 to T antigen, resulting in constitutive β-catenin signaling, which promotes the maintenance of ESC pluripotency. By contrast, C1GalT1 KD ESCs show disrupted galectin-3-mediated endocytosis of Fzd5 leading to its retention at the plasma membrane. As a result, Wnt signaling, mediated by β-catenin, is enhanced and culminates in the loss of ESC pluripotency.
human embryonic kidney cells and mouse intestinal stem cells (Hao et al., 2012; Koo et al., 2012). Thus, reduction of T antigen on Fzd5 may alter its interaction with Znrf3 and or Rnf43, prolonging Wnt receptor availability at the cell surface. RNA-seq analysis showed that Znf3 is highly expressed in ESCs compared to Rnf43 (Fig. S7A). However, Znf3 knockdown in ESCs did not affect Fzd5 endocytosis (Fig. S7B–D). In conclusion, our findings demonstrate that reduction of T antigen results in disruption of Lgals3-mediated endocytosis of the Wnt receptor Fzd5. As a result, Fzd5 is retained at the plasma membrane, thereby prolonging the activation of Wnt signaling (Fig. 7F).

**DISCUSSION**

It is becoming clear that glycosylation acts as a pivotal regulatory switch of pluripotency in a range of cell types in different organisms (Nishihara, 2018). In the present study, we characterized the function of mucin-type O-glycosylation in the pluripotency network and clarified the relationship between O-glycosylation and signaling in ESCs. The C1GaT1-mediated elongation pathway is the most prominent in ESCs. Knockdown of C1GaT1 expression results in a decrease of T antigen on the Wnt receptor Fzd5, reducing the level of Lgals3-mediated Fzd5 endocytosis. The retention of Fzd5 on the surface, in turn, promotes excessive canonical Wnt signaling activation via β-catenin stabilization, resulting in the exit from pluripotency (Fig. 7F).

The expression of O-glycosyltransferases increases after ESC differentiation into embryoid bodies (EBs) or extraembryonic endoderm cells, suggesting that O-glycosylation may play a key role during early commitment (Nairn et al., 2012). A recent study has shown that O-glycosylation plays a critical role in maintaining the epithelial state of trophoblast stem cells, which are derived from the first embryo lineage commitment (Raghu et al., 2019). Here, we demonstrated that O-glycosylation is crucial for the maintenance of ESC pluripotency; our findings provide further insights into the roles of O-glycosylation in early embryonic development. Furthermore, we showed that C1GaT1 was most highly expressed during early commitment in ESCs (Fig. 1B; Fig. S1C–E) and that C1GaT1 KD induced expression of the trophectoderm markers Cdx2 and Gata3 (Fig. 3G). Thus, it will be of particular interest to investigate the role of mucin-type O-glycosylation in the early commitment of ESCs and in early embryo development in vivo.

A previous comprehensive study of the glycomes of various cell lines reported that the T antigen elongation pathway is the most prominent in conventional human ESCs, similar to what is found in mouse ESCs (Fujitani et al., 2013). However, mouse and conventional human ESCs reflect two different pluripotent states, namely the naïve and the primed state, respectively, which rely on different signaling pathways to maintain the pluripotent state and induce differentiation (Weinberger et al., 2016). Thus, the influence of T antigen on the pluripotency network and differentiation potential is likely to differ between mouse and human ESCs. Currently, we are exploring the role of C1GaT1-mediated elongation pathway in the conventional human ESC pluripotency network.

In the past decade, Wnt signaling has been shown to be a key factor in the maintenance of the undifferentiated state. The addition of Wnt3a and LIF is sufficient to support self-renewal and allows derivation of ESCs from non-permissive strains (ten Berge et al., 2011). The induction of Wnt signaling through treatment with CHIR together with the FGF signaling inhibitor PD is commonly used to maintain the undifferentiated state in cultured ESCs (Ying et al., 2008; Ying and Smith, 2017). However, the Wnt signaling outcome depends on its interactions with other signaling pathways. CHIR alone induces ESC differentiation (Ying et al., 2008). In addition, other groups have reported that β-catenin promotes the expression of genes associated with both pluripotency and differentiation (Kelly et al., 2011; Price et al., 2013; Zhang et al., 2013). Therefore, Wnt signaling maintains cells in the undifferentiated state while at the same time priming cells for differentiation. Here, Wnt signaling dysregulation led to a loss of ESC pluripotency. Our previous study characterized the role of heparan sulfate proteoglycans in Wnt signaling regulation in ESCs (Sasaki et al., 2008). The present study identifies another mechanism through which glycosylation regulates Wnt signaling.

Mucin-type O-glycosylation plays multiple roles, including protection from shedding, protein–protein interactions, and protein turnover and trafficking (Tian and Ten Hagen, 2009; Razawi et al., 2013; Karabasheva et al., 2014). In this study, we demonstrated that T antigen on the Wnt receptor Fzd5 modulates endocytosis of Fzd5 via Lgals3 (Fig. 7F). Fzd endocytosis modulates Wnt signaling positively or negatively depending on the cellular context (Brunt and Scholpp, 2018). In the present study, a reduction in Fzd5 endocytosis resulted in Wnt signaling activation. Abnormalities in O-glycosylation and Wnt signaling are hallmarks of tumorigenesis (Pinho and Reis, 2015; Zhan et al., 2017). Here, we demonstrated for the first time a direct connection between O-glycosylation and Wnt signaling.

Pluripotent stem cell-derived organoids are a valuable tool for investigating the mechanisms of development and for disease modeling (Lancaster and Knoblich, 2014). However, improving organoid maturity remains one of the greatest challenges in this field. Recent studies identified Wnt as one of the central signaling molecules in organoid growth, patterning and morphogenesis (McCaulay and Wells, 2017). In the present study, we identified a novel Wnt signaling regulatory mechanism regulated by mucin-type O-glycosylation, providing an additional approach of manipulation of organoid culture conditions. It will be of particular interest to understand the mechanistic role of mucin-type O-glycosylation with regard to Wnt signaling during organoid maturation. In conclusion, our observations provide a significant contribution to research in developmental biology and to the development of future therapeutic applications.

**MATERIALS AND METHODS**

**Cell culture**

R1 (Nagy et al., 1993) and E14TG2a (Smith and Hooper, 1987) ESC lines were maintained on mouse embryonic fibroblasts that were prepared from embryos at embryonic day (E)14.5 and inactivated with 10 μg mitomycin C (Sigma-Aldrich). The cell lines were maintained in ESC medium, consisting of DMEM (Gibco) supplemented with 15% fetal bovine serum (FBS) (Nihirei Biosciences), 1% penicillin/streptomycin (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 0.1 mM nonessential amino acids (Gibco) and 1000 U/ml LIF (Chemicon International). The R1 ESC line was used for the experiments unless stated otherwise. ESC lines were recently tested for mycoplasma contamination and authenticated by ATCC cell line authentication service - mouse STR profiling.

To induce embryoid body (EB) formation, ESCs were transferred to 35-mm Low Cell Binding dishes (Nunc) and cultured in ESC medium in absence of LIF for 2.5, 4, 8 or 12 days. The EB assay from transient C1GaT1 KD ESCs was performed 2 days post transfection and cells were collected at day 0 (ESCs), 2, 3, and 4.

For knockdown (KD) of the C1GaT1 and Znf3 genes in ESCs, we generated small hairpin RNA (shRNA) expression vectors targeting two different regions of each gene [and also enhanced green fluorescent protein (Egfp) as the control] by inserting the appropriate double-stranded DNA between the BamH1 and HindIII sites of pSilencer 3.1-H1 (Ambion) or
medium (without FBS). For galectin-3 (Lgals3) addition, ESCs were cultured in ESC medium containing 50 mM lactose monohydrate (Kanto Chemical) for 30 min.

- For transient KD, 10^6 ESCs were plated prior to transfection in gelatin-coated feeder-free 60-mm culture dishes (TrueLine) in ESC medium with LIF. The next day, the cells were transfected with 4 µg of expression vector using Lipofectamine 2000 (Invitrogen). From 1 day after transfection, the cells were selected with 2 µg/ml puromycin (Sigma-Aldrich). C1GalT1 KD 1, C1GalT1 KD 2, Znr3 KD 1, and Znr3 KD 2 transiently transfected cells were cultured for 4 days post transfection. For stable C1GalT1 KD, the appropriate constructs were transfected into ecotropic virus-packaging (PLAT-E) cells. Supernatants containing retrovirus were collected 48 h following PLAT-E transfection, mixed with 8 µg/ml polybrene (Sigma-Aldrich) and incubated with ESCs for 24 h. The following day, ESCs were replated with ESC medium containing LIF and 2 µg/ml puromycin, and selected for 7 days prior to use.

- For the clonogenicity assay, 3×10^4 KD cells were plated on gelatin-coated 60-mm dishes in ESC medium supplemented with LIF. The cells were fixed and stained for ALP with 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (Nacalai Tesque) 4 days after replating. The colonies were counted by microscopy and scored manually.

- To analyze the effect of O-glycosylation inhibition, ESCs were cultured in ESC medium containing 2 mM GalNac-Bn (Sigma-Aldrich), in the presence of LIF for 48 h.

- For galecitin dissociation, ESCs were cultured in ESC medium (without FBS) containing 50 mM lactose monohydrate (Kanto Chemical) for 30 min. For galecitin-3 (Lgals3) addition, ESCs were cultured in ESC medium (without FBS) for 2 h before addition of 15 µg/ml Lgals3 for 30 min in ESC medium (without FBS).

**Real-time PCR and RNA-seq analysis**

- Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and reverse transcribed using a Superscript II First Strand Synthesis Kit (Invitrogen) and oligo-dT primers. Real-time PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and SYBR Green Master Mix (Roche). The absolute amount of each mRNA was normalized against the β-actin or Gapdh mRNA level in the same sample.

**Cellular O-glycome analysis**

ESCs (~1×10^6) were homogenized using an Ultrasonic Homogenizer (Taitec Corp) as previously described (Furukawa et al., 2015a). The concentrated cellular proteins were then subjected to microwave-assisted β-elimination in the presence of a pyrazolone (BEP) reaction using a microwave reactor (Monowave 300, Anton Paar). After the reaction, 25 pmol bis-PMP-labeled N, N', N'-tetraacetyl chitotetraose (GN4) was added as an external standard to the reaction mixture for glycan quantification. PMP-labeled O-glycans were purified and subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis.

**Tandem MALDI-TOF MS analysis**

- MALDI-TOF MS analysis was carried out as previously described (Furukawa et al., 2015b). Briefly, all measurements were performed using an Ultraflex II TOF/TOF mass spectrometer equipped with a reflector and controlled by the FlexControl 3.0 software package (Bruker Daltonics) in accordance with the standard protocols. All spectra were obtained in reflector mode with an acceleration voltage of 25 kV, a reflector voltage of 26.3 kV, and a pulsed ion extraction of 160 ns in positive ion mode. Absolute quantification was performed by comparative analyses between the areas of the MS signals derived from O-glycans and a known amount of the internal standard bis-PMP-labeled N, N', N'-tetraacetyl chitotetraose (GN4).

**FACS analysis**

- A single-cell suspension was obtained using 0.02% EDTA in PBS. Subsequently, 2×10^5–3×10^6 cells were collected and washed in fluorescence-activated cell sorting (FACS) buffer [0.5% BSA (Iwai), 0.1% sodium azide (Sigma-Aldrich) in PBS]. After washing, the cell suspension was incubated with PE-conjugated anti-SSEA-1 Ab (Immunoetch; 1954; 1:3), FITC-conjugated PNA (Cosmo Bio; J514; 1:100), FITC-conjugated HPA (EY Laboratories; F-3601-1; 1:100) or FIT-conjugated SNA (EY Laboratories; F-6802-1; 1:100) in an FACS buffer. The cells were then analyzed using a BD FACSAria III Cell Sorter (Becton Dickinson). Cells were gated to exclude debris, dead cells (identified by propidium iodide staining; Sigma-Aldrich) and doublets.

**Immunostaining**

- Cells were fixed with 4% paraformaldehyde in PBS (PFA/PBS) and washed in PBS. The fixed cells were blocked with 10% Block Ace (Dainihon Pharmaceutical) in PBS (Block Ace/PBS), 10% Block Ace/PBS or 1% BSA/PBS with 0.3% Triton X-100 (Sigma-Aldrich) for the analysis of cell surface or intracellular molecules, respectively. For primary labeling, the cells were incubated with PNA conjugated to biotin (Biotin–avidin–biotin; Sigma-Aldrich; J514; 1:100), anti-Fzd5 Ab (Sigma-Aldrich; SAB4503132; 1:100), anti-β-catenin Ab (Cell Signaling; #9562; 1:100), anti-Rab5 Ab (Cell Signaling; #46449; 1:100), anti-Lgals3 Ab (Santa Cruz Biotechnology; sc-32790; 1:100) or anti-Znrf3 Ab (Bioss; bs-9141R; 1:100). Subsequently, the cells were stained with Alexa Fluor 555-conjugated streptavidin (Life Technologies; H33750; 5 µg/ml). Images were obtained using an LSM 700 confocal laser microscope (Carl Zeiss).

**Western and lectin blotting**

- Cells were lysed with lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 50 mM EDTA, 1 mM Na₂VO₃, 10 mM NaF, 1 mM PMSF (Sigma-Aldrich), 2 µg/ml leupeptin (Sigma-Aldrich), 0.7 µg/ml pepstatin A (Sigma-Aldrich), 2 µg/ml aprotinin (Sigma-Aldrich), and 1 µg/ml antipain (Sigma-Aldrich)]. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore). For western blotting, membranes were blocked using 1% BSA and incubated with the following primary antibodies: anti-Sox2 Ab (R&D Systems; MAB2018; 1:1000), anti-Oct3/4 Ab (Santa Cruz Biotechnology; sc-5279; 1:1000), anti-β-actin Ab (Sigma-Aldrich; A5441; 1:10,000), anti-p-β-catenin Ab (Cell Signaling; #9561; 1:1000), anti-β-catenin Ab (Cell Signaling; #9562; 1:1000), anti-Fzd5 Ab (Sigma-Aldrich; SAB4503134; 1:1000), anti-Erk1/2 Ab (Cell Signaling; #9102L; 1:1000), anti-p-Erk1/2 Ab (Cell Signaling; #9101L; 1:1000), anti-Akt Ab (BD Biosciences; #9272; 1:1000), and anti-Znrf3 Ab (Bioss; bs-9141R; 1:100). Membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature and then washed. After washing, the membranes were incubated with ECL reagent. Membranes were then imaged using a digital imaging system.
610836; 1:1000), anti-p-Akt Ab (Cell Signaling; #9275; 1:1000), anti-LRP5 Ab (Cell Signaling; #5731; 1:500), or anti-LRP6 Ab (Cell Signaling; #3395; 1:1000). The membranes were then incubated with HRP-conjugated anti-rabbit IgG (Cell Signaling; #7074; 1:10,000), anti-rabbit IgG light chain specific (Jackson ImmunoResearch; 211-032-171; 1:10,000) or anti-mouse IgG (Cell Signaling; #7076; 1:10,000) secondary Abs. For lectin analysis, membranes were probed with PNA conjugated to horseradish peroxidase (HRP) (Cosmo Bio; J414; 1:10,000). The membranes were then washed, and developed with ECL Plus reagents (GE Healthcare).

Luciferase assay

A shRNA expression vector targeting C1GalT1 (2 μg) was co-transfected using Lipofectamine 2000 with the reporter plasmid TOPFLASH (Upstate Biotechnology; 2 μg) or FOPFLASH (Upstate Biotechnology; 2 μg); pHCh110 (GE Healthcare; 0.2 μg) was used as a control for transfection efficiency. Cells were lysed 4 days after transfection. Luciferase activity was measured by Dual-Light System (Applied Biosystems). Luminescence was measured with a Lumat LB9501 luminometer (Berthold).

Immunoprecipitation and pull-down assay

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 Na3VO4, 10 mM NaF, and protease inhibitors). A sample (100–500 μg) of proteins was diluted 10-fold with wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 1 mM PMSF (Sigma-Aldrich), 2 μg/ml leupeptin (Sigma-Aldrich), 0.7 μg/ml pepstatin A (Sigma-Aldrich), 2 μg/ml aprotinin (Sigma-Aldrich), and 1 μg/ml antipain (Sigma-Aldrich)]. For immunoprecipitation, anti-Fzd5 Ab (Sigma-Aldrich; SAB4503134; 1 μg), anti-LRP5 Ab (Cell Signaling; #5731; 1 μg), anti-LRP6 Ab (Cell Signaling; #3395; 1 μg), or normal rabbit IgG (R&D Systems; AB-105-C; 1 μg) was added to the diluted cell lysate. Protein G Magnetic Beads (New England Biolabs) were then added. For the pulldown assay, the diluted cell lysate was incubated with PNA–biotin (Cosmo Bio; J214; 20 μg); streptavidin magnetic beads (Bio-Rad) were then added. The precipitated fractions were then washed five times with wash buffer. The surface biotinylation assay was performed using the Pierce cell surface protein isolation kit (Thermo Scientific) following the manufacturer’s instructions.

Immunoelectron microscopy

ESCs (5×105) were plated on gelatin-coated 35-mm plastic dishes (TruPet) in ESC medium with LIF. At 4 days after transfection, cells were fixed with 4% PFA and 0.01% glutaraldehyde in PBS for 1 h at room temperature and washed in PBS. The fixed cells were permeabilized with 0.5% saponin in PBS for 5 min and untreated aldehyde functional groups were quenched using 10 mM glycine in PBS for 5 min. After PBS washing, the cells were blocked with 5% donkey serum (Jackson Immunoresearch) in 0.5% saponin in PBS for 5 min and unreacted aldehyde functional groups were fixed with 4% PFA and 0.01% glutaraldehyde in PBS for 1 h at room temperature and washed with PBS. The cells were then incubated with PNA–biotin (Cosmo Bio; J214; 20 μg); streptavidin magnetic beads (Bio-Rad) were then added. The precipitated fractions were then washed five times with wash buffer. The surface biotinylation assay was performed using the Pierce cell surface protein isolation kit (Thermo Scientific) following the manufacturer’s instructions.

Statistical analysis

An unpaired two-tailed Student’s t-test was used to compare data with the control groups. The experiments were performed in triplicate unless stated otherwise. Statistical significance is denoted by asterisks: *P<0.05, **P<0.01, ***P<0.001.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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