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LacdiNAc (GalNAcβ1-4GlcNAc) Contributes to Self-Renewal of Mouse Embryonic Stem Cells by Regulating Leukemia Inhibitory Factor/STAT3 Signaling

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Key Words. LacdiNAc • Leukemia inhibitory factor/STAT3 signaling • Embryonic stem cell • Self-renewal

ABSTRACT

Self-renewal of mouse embryonic stem cells (mESCs) is maintained by leukemia inhibitory factor (LIF)/signal transducer and activator of transcription (STAT3) signaling. However, this signaling control does not function in neither mouse epiblast stem cells (mEpiSCs) nor human ESCs (hESCs) or human induced pluripotent stem cells (hiPSCs). To date, the underlying molecular mechanisms that determine this differential LIF-responsiveness have not been clarified. Here, we show that the cell surface glycan LacdiNAc (GalNAcβ1-4GlcNAc) is required for LIF/STAT3 signaling. Undifferentiated state mESCs expressed LacdiNAc at a higher level than differentiated state cells. Knockdown of *β4GalNAc-T3* reduced LacdiNAc expression and caused a decrease in LIF/STAT3 signaling that lessened the rate of self-renewal of mESCs. A biochemical analysis showed that LacdiNAc expression on LIF receptor (LIFR) and gp130 was required for the stable localization of the receptors with lipid raft/caveolar components, such as caveolin-1. This localization is required for transduction of a sufficiently strong LIF/STAT3 signal. In primed state pluripotent stem cells, such as hiPSCs and mEpiSC-like cells produced from mESCs, LacdiNAc expression on LIFR and gp130 was extremely weak and the level of localization of these receptors on rafts/caveolae was also low. Furthermore, knockdown of *β4GalNAc-T3* decreased LacdiNAc expression and reduced the efficiency of reversion of primed state mEpiSC-like cells into naïve state mESCs. These findings show that the different LIF-responsiveness of naïve state (mESCs) and primed state (mEpiSCs, hESCs, and hiPSCs) cells is dependent on the expression of LacdiNAc on LIFR and gp130 and that this expression is required for the induction and maintenance of the naïve state. STEM CELLS *2011;29:641–650*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem cells (ESCs) [1, 2] have considerable promise for exploitation in biotechnology applications; in particular, the pluripotency of human ESCs (hESCs) will be valuable in cell replacement therapies [3, 4]. One possible route for use of ESCs in the development of regenerative medicine therapies might be through exploitation of human induced pluripotent stem cells (hiPSCs) [5]. In order to be able to exploit the rapid culture methods and safety of hESCs and hiPSCs for therapeutic purposes, it will be important to elucidate the molecular mechanisms that control the self-renewal and differentiation of ESCs. Many factors that control ESC pluripotency have now been identified and it has been demonstrated that an appropriate balance among several extrinsic signaling pathways is required for the maintenance of pluripotency [6–8]. However, the underlying molecular mechanisms that determine the regulation of these extrinsic signaling pathways in ESCs remain unknown.

In recent years, several signaling pathways required for self-renewal have been identified in mouse ESCs (mESCs) and hESCs. One confounding factor, however, is that mouse and hESCs show differences in their responses to the extrinsic signal factors required for self-renewal. For example, leukemia inhibitory factor (LIF)/signal transducer and activator of transcription (STAT3) signaling contributes to the maintenance of self-renewal in mESCs but not hESCs [4, 9–13]. Bone morphogenic protein 4 (BMP4)/Smad signaling also contributes to the maintenance of mESC self-renewal in synergy with LIF [14, 15]. By contrast, in hESCs, BMP signaling induces differentiation [16, 17]. Wnt/β-catenin signaling contributes to the regulation of self-renewal in both mESCs and hESCs [18–21], whereas fibroblast growth factor 2 (FGF2) and activin/nodal signaling contribute only to the maintenance of hESC self-renewal [22–24]. Thus, mESCs and hESCs maintain their pluripotency using different signaling factors. Recently, it was reported that primed state hESCs could be induced to revert to a naïve state similar to that of mESCs

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and that these different states reflected differences in signaling required for maintenance of self-renewal [25]. However, the key factors that underlie the differences in signaling between mESCs and hESCs have yet to be clarified. One possibility is that cell surface factors may be important for regulation of extrinsic signaling. On this basis, we speculated that cell surface glycans might be a key factor in the different signaling pathways for self-renewal in mESCs and hESCs.

Several types of glycan are expressed on the cell surface and are present as components of glycoproteins, glycolipids, and proteoglycans; these glycans thereby contribute significantly to biologically important functions such as the regulation of signaling pathways [26]. It has recently been demonstrated that the cell surface glycan heparan sulfate (HS) contributes to self-renewal and differentiation of mESCs by regulating BMP, Wnt, and FGF signaling [21, 27–29]. The expression patterns of several other cell surface glycans have now been described in undifferentiated and differentiated mESCs and hESCs [30–34]. However, although these glycans may be involved in the regulation of the signaling required for ESC self-renewal, with the exception of HS, their functional roles have not been demonstrated.

To test our speculation that cell surface glycans might contribute to ESC self-renewal, we performed an RNA interference (RNAi) screen using short hairpin RNAs (shRNAs) that targeted specific glycan-related genes in mESCs and sought to identify cell surface glycans that were essential for ESC self-renewal. By evaluation of alkaline phosphatase activity in RNAi-mediated knockdown mESCs, we identified glycans essential for self-renewal. One of the glycan sequences of interest is the LacdiNAc (GalNAcβ1-4GlcNAc) motif, which is synthesized by β4GalNAc-T3 [35]. LacdiNAc is frequently present on glycoproteins and glycolipids in invertebrates but is only present on a limited number of glycoproteins and glycolipids, such as glycoprotein hormones, in vertebrates [36, 37]. At present, the roles of LacdiNAc in mammalian cells are not fully understood. Here, we investigated the role of LacdiNAc and show that it contributes to the maintenance of the naïve state in pluripotent cells by regulating LIF/STAT3 signaling.

Materials and Methods

Cell Culture

R1 mESC lines [38] were maintained on mouse embryonic fibroblasts (MEFs) inactivated with 10 μg/ml mitomycin C (Sigma, Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com) in mESC medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 15% fetal bovine serum [Hyclone Laboratories, South Logan, UT, www.hyclone.com], 1% penicillin/streptomycin [Invitrogen, Invitrogen, Carlsbad, CA, www.invitrogen.com], 0.1 mM 2-mercaptoethanol [Invitrogen], 0.1 mM nonessential amino acids [Invitrogen]) and 1,000 U/ml LIF [Chemicon International, Temecula, CA, www.chemicon.com]. hiPSC clones (MRC-hiPS_-Tic [JCRB1331]) [39] were maintained on inactivated MEFs in hiPSC medium (DMEM/F12 supplemented with 20% Knockout Serum Replacement [Invitrogen], 2 mM L-glutamine [Invitrogen], 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids) with 10 ng/ml FGF2 (Wako, Osaka, Japan, www.wako-chem.co.jp).

Knockdown of *β4GalNAc-T3* was performed as described previously [21]. We generated shRNA expression vectors targeting *β4GalNAc-T3* or *enhanced green fluorescent protein* (*EGFP*) (negative control) by inserting the appropriate double-stranded DNAs between the BamHI and HindIII sites of pSilencer 3.1-H1 (Ambion, Austin, TX, www.ambion.com). The sequences used for RNAi were designed, as described previously [40], using "siDirect (http://sidirect.jp/esd/modules/modsiperfect/)" and are listed in supporting information Table 1. Transient transfection was performed as described previously [21]. After selection of cells carrying shRNA, they were harvested and analyzed as described below. Stable transfection was performed as described previously [29].

Mouse epiblast stem cell (mEpiSC)–like cells were induced from mESCs as described previously [41]. In brief, mESCs were harvested and 2×10^5 cells per dish were replated on 35 mm tissue culture dishes precoated with 15 μg/ml Fibronectin (Sigma-Aldrich). After 24 hours of culture, the medium was replaced with serum-free medium consisting of DMEM/F12 supplemented with 48% Neurobasal (Invitrogen), 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 1 mM L-glutamine, 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 5 mg/ml bovine serum albumin (BSA), with 20 ng/ml activin A (Wako) and 12 ng/ml FGF2. Thereafter, cells were passaged every 2–3 days using collagenase IV (Invitrogen). Cells that had been passaged three times were used for almost all experiments.

mEpiSC-like cells were induced to revert to mESCs as described previously [25, 42]. In brief, the EpiSC-like cells were seeded on inactivated MEFs as small clumps and cultured with serum-free medium containing 1,000 U/ml LIF, 1 μM PD0325901 (Wako), 3 μM CHIR99021 (Wako), 10 μM forskolin (Stemgent, San Diego, CA, www.stemgent.com), and 2 μM SB431542 (Wako).

A proliferation assay was performed as described previously [21]. Briefly, transfected cells were replated in 96-well gelatin-coated plates in mESC medium with LIF, and a solution of WST-8 from a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan, www.dojindo.co.jp) was added after 0, 24, or 48 hours culture. WST-8 is reduced by cellular dehydrogenases to produce a soluble formazan product, which can be detected colorimetrically at 450 nm. The intensity of staining is proportional to cell numbers. Manual cell counts were also performed to confirm the results of the proliferation assay.

Alkaline phosphatase staining was performed as described previously [21]. Briefly, transfected cells were replated at clonal density in gelatin-coated 60 mm tissue culture dish in ESC medium with LIF. After 5 days of culture, the cells were stained with 5-bromo-4-chloro-3-indoxyl phosphate-nitro blue tetrazolium chloride (Nacalai Tesque, Kyoto, Japan, www.nacalai.co.jp), and alkaline phosphatase positive colonies were counted under a microscope.

Fluorescence-Activated Cell Sorting (FACS) Analysis

In general, trypsinization reduces the expression of some cell surface antigens. Therefore, to avoid this effect, cells were harvested with accutase (Millipore, Billerica, MA, www.millipore.com) and these dissociated single cells were incubated with fluorescein isothiocyanate–conjugated *Wistaria floribunda* (WFA) (EY Laboratories, San Mateo, CA, www.eylabs.com) in FACS buffer (0.5% BSA and 0.1% sodium azide in phosphate-buffered saline (PBS)) for 30 minutes on ice. After washing, cell sorting and analysis were performed using a FACSAria Cell Sorter (Becton Dickinson, Franklin Lakes, NJ, www.bd.com).

Analysis of Proteins by Immunoblotting

For observation of cell signaling, the cell culture medium was replaced with serum-free ESC medium without LIF for 4 hours and the cells were stimulated for 20 minutes with 1,000 U/ml LIF or 10 ng/ml BMP4 (R&D Systems, Minneapolis, MN, www.rndsystems.com) or for 5 minutes with 10 ng/ml FGF4 (R&D Systems). 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, 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, protease inhibitors), and immunoprecipitation was performed with appropriate antibody and Protein G Magnetic Beads (New England Biolabs, Ipswich, MA, www.neb.com).

Fractionation of lipid rafts/caveolae and nonrafts was performed as described previously [43]. Harvested cells were incubated with 0.5 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Brij58 (Sigma-Aldrich), 1 mM Na_3VO_4, 10 mM

NaF, protease inhibitors) for 30 minutes on ice and homogenized 50 times with a tight Dounce homogenizer. The extract (1 ml) was mixed with 85% sucrose solution (50 mM Tris-HCl pH 7.4 and 150 mM NaCl) to produce a 42.5% sucrose solution, transferred to a centrifuge tube (Beckman Coulter, Fullerton, CA, www.beckmancoulter.com) and overlaid with 5 ml of 30% sucrose solution and 3 ml of 5% sucrose solution. The discontinuous sucrose gradients were centrifuged at 4°C for 16 hours in an SW41 Ti rotor at 30,000 rpm. The gradient was divided into nine fractions from the bottom to the top.

For extraction of WFA-binding proteins, a cell lysate prepared as described above was incubated with WFA-agarose (Vector Laboratories, Burlingame, CA, www.vectorlabs.com) at 4°C, washed with 0.1% Triton X-100 in tris-buffered saline, and WFA-binding proteins were extracted with 0.2 M lactose (Sigma-Aldrich).

SDS–polyacrylamide gel electrophoresis (PAGE) samples prepared as described above were separated by the appropriate percentage SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore). After blocking, the membranes were incubated with the following primary antibodies: anti-STAT3 (Becton Dickinson), anti-phospho-STAT3 (Tyr705; Becton Dickinson), anti–extracellular signal-regulated kinase 1/2 (anti-ERK1/2) (Cell Signaling Technology, Danvers, MA, www.cellsignal.com), anti-phosphorylated ERK1/2 (Thr183/Thr185; Cell Signaling Technology), anti-phospho-Smad1 (Ser463/Ser465; Cell Signaling Technology), anti-β-actin (Sigma-Aldrich), anti-Flotillin-1 (Becton Dickinson), anti-Transferrin receptor (Zymed, San Francisco, CA, www.zymed.com), anti-LIF receptor (LIFR) (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, www.scbt.com), anti-gp130 (M-20; Santa Cruz Biotechnology), anti-caveolin-1 (7C8; Santa Cruz Biotechnology), anti-Oct3/4 (C-10; Santa Cruz Biotechnology), anti-Nanog (ReproCELL, Yokohama, Japan, www.reprocell.com), or anti-Sox2 (R&D Systems). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology), washed, and developed with ECL Plus reagents (GE Healthcare, Waukesha, WI, www.gehealthcare.com). For lectin blot analysis, the membranes were incubated with peroxidase-conjugated WFA (EY Laboratories), washed, and developed with ECL Plus reagents.

Cell surface expression of LIFR and gp130 was examined by biotinylation of cell surface proteins followed by immunoprecipitation. Biotinylation of cell surface proteins was performed using EZ-Link sulfosuccinimidyl-6-(biotin-amido) hexanoate (PIERCE, Rockford, IL, www.piercenet.com) in accordance with the manufacturer's protocol. Then, biotinylated cell surface proteins were immunoprecipitated with anti-LIFR antibody or anti-gp130 antibody as described above. Immunoprecipitated proteins were subjected to Western blot analysis using peroxidase-conjugated streptavidin (Wako). Similar to the FACS analysis above, we did not use trypsinization to harvest the cells used for analyzing cell surface proteins. Instead, the cells were harvested using 0.05% EDTA and a cell scraper.

Immunostaining of Nonpermeabilized Cells

mESCs, mEpiSC-like cells, or hiPSCs were fixed with 2% paraformaldehyde, washed, and subsequently blocked with PBS containing 3% BSA and 1% normal goat serum. After washing, nonpermeabilized cells were double-stained with an anti-caveolin-1 antibody and an anti-LIFR or anti-gp130 antibody. After washing, cells were stained with Alexa488-conjugated secondary antibody (Invitrogen) and Alexa594-conjugated secondary antibody (Invitrogen). Immunofluorescence images were taken using an LSM5Pascal confocal laser scanning microscope (Carl Zeiss, Thornwood, NY, www.zeiss.com) with 63x/1.3 objective at room temperature.

Real-Time PCR

Real-time PCR was performed as described previously [21]. For some genes, FastStart Universal SYBR Green Master (Roche, Basel, Switzerland, www.roche-applied-science.com) was used. Primer sets and probes for real-time PCR are listed in supporting information Tables 2 and 3, respectively.

Results

LacdiNAc Contributes to Self-Renewal of mESCs

To identify cell surface glycans involving in self-renewal of mESCs, we performed an RNAi screen using shRNAs that targeted various glycan-related genes in mESCs. By evaluating alkaline phosphatase activity in glycan-related gene knockdown mESCs, LacdiNAc, which is synthesized by β4GalNAc-T3 [35], was identified as a candidate cell surface glycan required for self-renewal of mESCs. We designed two constructs that expressed different shRNAs targeting *β4GalNAc-T3* (*β4GalNAc-T3-1* and *β4GalNAc-T3-2*), as described previously [40], and additionally constructs that expressed shRNAs targeting *EGFP* as negative controls. We describe mESCs that have been transfected with *EGFP* shRNA expression plasmids as "control cells" throughout this article. *β4GalNAc-T3*-knockdown (KD) cells showed an approximately 70% reduction in *β4GalNAc-T3* mRNA when compared with control cells (Fig. 1A). FACS analysis using WFA lectin, which recognizes LacdiNAc [44], showed that cell surface expression of LacdiNAc was also reduced in *β4GalNAc-T3*-KD cells (Fig. 1B).

The proportion of alkaline phosphatase positive colonies in cultures of *β4GalNAc-T3*-KD cells after replating was markedly decreased when compared with those of control cells even in the presence of LIF and serum, demonstrating that the *β4GalNAc-T3*-KD cells had lost this stem cell trait (Fig. 1C). Furthermore, after 4 days of culture, even at normal density, the expression of Oct3/4, Nanog, and Sox2 markers of the undifferentiated state was significantly decreased in *β4GalNAc-T3*-KD cells when compared with control cells at both the mRNA and protein levels (Fig. 1D, 1E); thus, the ability for self-renewal in *β4GalNAc-T3*-KD cells had been abolished. These results indicate that LacdiNAc contributes to self-renewal of mESCs. As shown in Figure 1F, cell surface expression of LacdiNAc was decreased following LIF withdrawal, suggesting that LacdiNAc plays functionally important roles in undifferentiated mESCs. In addition, the rate of proliferation of *β4GalNAc-T3*-KD cells decreased significantly when compared with that of control cells (Fig. 1G; supporting information Fig. S1). Taken together, these results demonstrate that LacdiNAc functions in undifferentiated mESCs and is required for their self-renewal and proliferation.

LacdiNAc Regulates LIF/STAT3 Signaling

Next, we examined whether LacdiNAc is involved in the extrinsic signaling pathways required for maintenance of self-renewal. A Western blot analysis showed that LIF-induced phosphorylation of STAT3 occurred at a substantially reduced rate in *β4GalNAc-T3*-KD cells when compared with control cells, demonstrating that LacdiNAc has a role in LIF/STAT3 signaling (Fig. 2A). In contrast, we observed similar increases in the level of phosphorylation of Smad1 or ERK in control and *β4GalNAc-T3*-KD cells following exposure to BMP4 or FGF4 (supporting information Fig. S2), indicating that LacdiNAc does not participate in BMP4/Smad or FGF4/ERK signaling.

We then addressed the question of how LacdiNAc might regulate LIF/STAT3 signaling. Signal transduction via the LIF/STAT3 pathway is mediated by the binding of LIF to LIFR, and heterodimerization of LIFR and gp130 is subsequently induced [45]. This heterodimerization triggers the activation of associated Janus kinase tyrosine kinases followed by phosphorylation of gp130 and results in the activation of STAT3. Here, we first examined the levels of LIFR and gp130 on the cell surface by a Western blot analysis. LIFR and gp130 located on the cell surface were biotinylated and then immunoprecipitated; the amounts of biotinylated LIFR or biotinylated

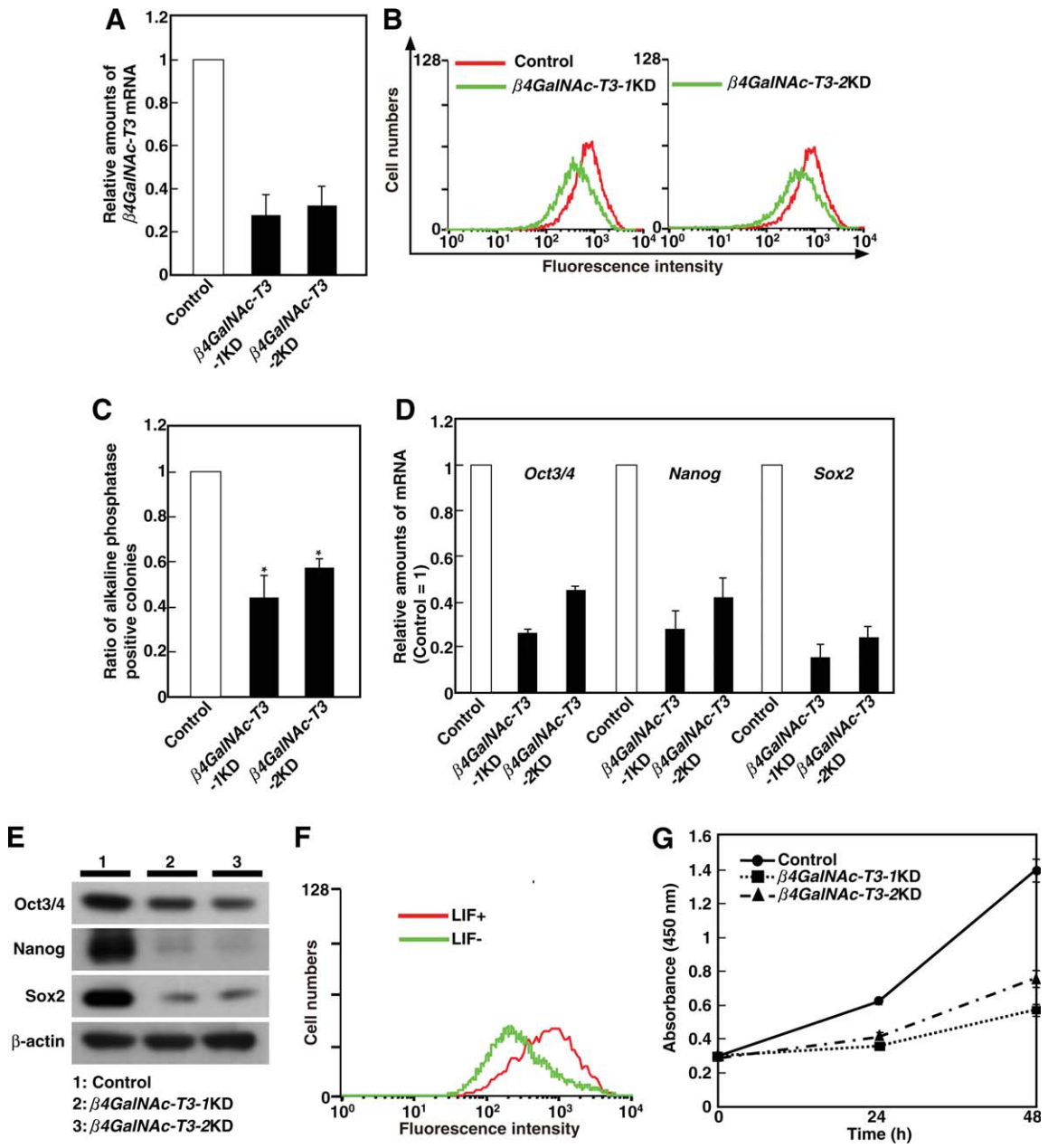

Figure 1. Self-renewal and proliferation is reduced in *β4GalNAc-T3*-KD cells. (**A**): Real-time PCR analysis of cells 2 days after transfection. Relative amounts of *β4GalNAc-T3* mRNA were calculated after normalization against *β-actin* mRNA. The results are shown after normalization against the value obtained in control cells (value = 1). The values shown are the means ± SD of three independent experiments. (**B**): FACS analysis of cells 2 days after transfection using FITC-*Wistaria floribunda*. Three independent experiments were performed and representative results are shown. The mean intensities of FITC staining were 23,947, 17,400, and 18,204 in the control, *β4GalNAc-T3-1*-KD, and *β4GalNAc-T3-2*-KD cells, respectively. (**C**): Alkaline phosphatase staining. The ratio of alkaline phosphatase positive colonies is shown after normalization to the ratio obtained in control cells (value = 1). The values shown are the means ± SD from three independent experiments and significant values are indicated, *, $p < .01$, in comparison to the control. (**D**): Real-time PCR analysis of *Oct3/4*, *Nanog*, and *Sox2* markers of the undifferentiated state at 4 days after transfection. The results are shown after normalization against the values obtained for control cells (value = 1). The values shown are the means ± SD from two independent experiments. (**E**): Western blot analysis of Oct3/4, Nanog, and Sox2 at 4 days after transfection. (**F**): FACS analysis of cells 4 days after culture with or without leukemia inhibitory factor (LIF). Three independent experiments were performed and representative results are shown. The mean intensities of FITC staining were 22,954 and 13,315 in LIF+ and LIF− cells, respectively. (**G**): Proliferation assay using cell counting kit-8. The values shown are the means ± SD from three independent experiments. Abbreviations: KD, knockdown; LIF, leukemia inhibitory factor.

gp130 were determined by a Western blot analysis. As shown in Figure 2B, there were no significant differences in cell surface expression of LIFR and gp130 between control and *β4GalNAc-T3*-KD cells. This result indicates that the reduction in LIF/STAT3 signaling in *β4GalNAc-T3*-KD cells was not caused by variation in the relative expression levels of LIFR and gp130 on the cell surface and that LacdiNAc did not affect the levels of these receptors on the cell surface.

We then investigated whether LacdiNAc is required for the heterodimerization of LIFR and gp130. A Western blot analysis showed that gp130 on control cells could be detected in immunoprecipitates using an anti-LIFR antibody and,

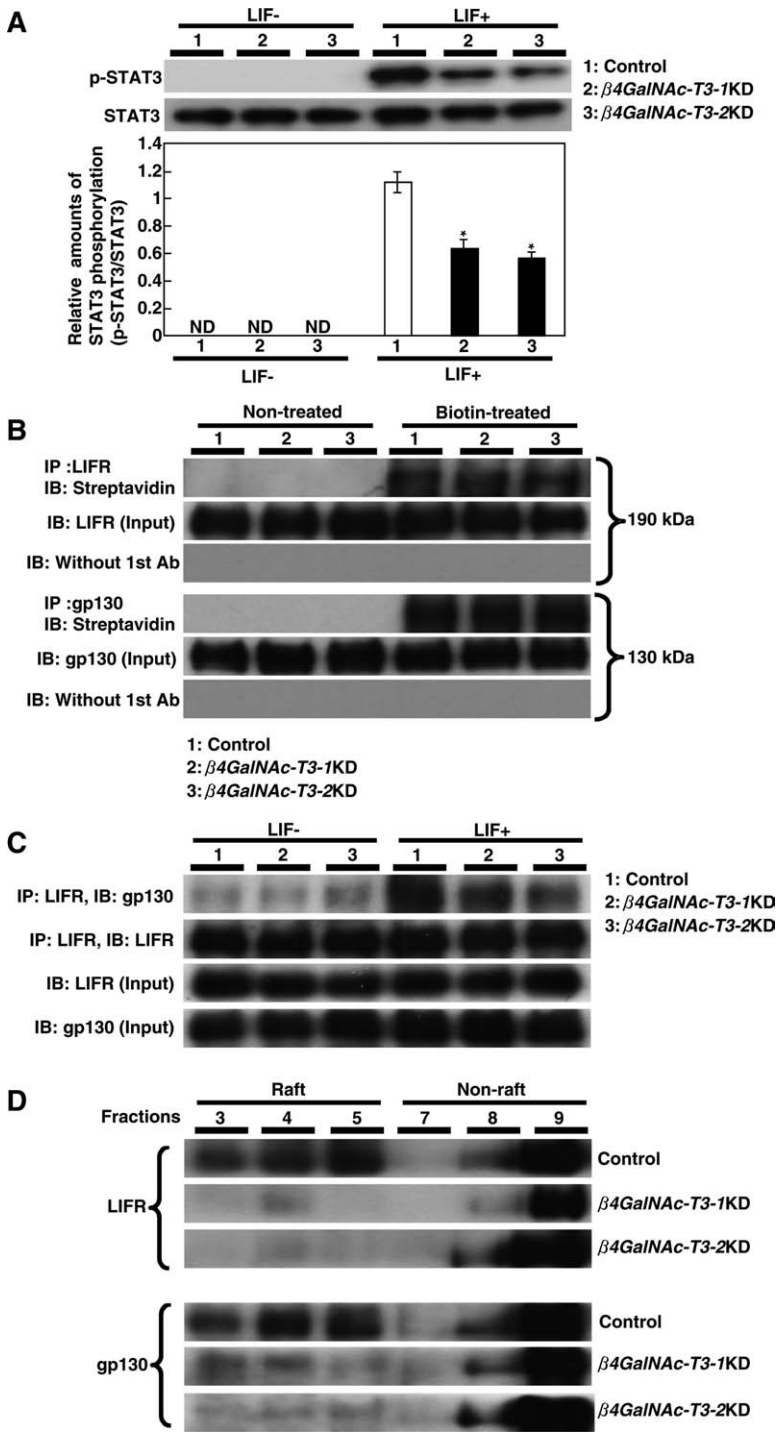

Figure 2. Leukemia inhibitory factor (LIF)/STAT3 signaling is reduced in *β4GalNAc-T3*-KD cells. (**A**): Western blot analysis of mouse embryonic stem cells stimulated with LIF 2 days after transfection. The histograms show mean densitometric readings ± SD of the phosphorylated protein/loading controls. Values were obtained from duplicate measurements of three independent experiments and significant values are indicated, *, $p < .01$, in comparison to the stimulated control. (**B**): Western blot analysis of cell surface LIF receptor (LIFR) and gp130. Two days after transfection, cells were biotinylated for detection of cell surface LIFR and gp130. After lysis, biotinylated cell surface proteins were immunoprecipitated with anti-LIFR or anti-gp130 antibody. Immunoprecipitates were detected by horseradish peroxidase–streptavidin (first and fourth panels). Second and fifth panels represent total expression levels of LIFR and gp130. Negative control staining without the primary antibody did not show nonspecific bands of the size expected of either LIFR or gp130 (third and sixth panels). (**C**): Western blot analysis of heterodimerization of LIFR and gp130 in response to LIF 2 days after transfection. Top panel shows Western blot analysis of gp130 coimmunoprecipitated with LIFR. Middle top panel shows amounts of immunoprecipitated LIFR, and middle lower and lower panels show total expression levels of LIFR and gp130. (**D**): Western blot analysis of LIFR and gp130 in raft (Fr.3–5) and nonraft (Fr.7–9) fractions 2 days after transfection. Abbreviations: Ab, antibody; IB, immunoblot; IP, immunoprecipitate; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; ND, not detected; p-STAT3, phospho-STAT3; STAT3, signal transducer and activator of transcription 3.

moreover, that the amount of gp130 varied with the concentration of LIF used to stimulate the cells. This observation confirms that gp130 forms a heterodimer with LIFR in response to LIF (Fig. 2C; top panel; first and fourth lanes). In contrast, in *β4GalNAc-T3*-KD cells, the levels of gp130 present in immunoprecipitates with the anti-LIFR antibody were significantly reduced when compared with control cells (Fig. 2C; top panel; fifth and sixth lanes), although the total amounts of LIFR and gp130 did not change (Fig. 2C; middle lower and lower panels). This result clearly indicates that the ability of LIFR and gp130 to heterodimerize in response to LIF was reduced in *β4GalNAc-T3*-KD cells.

A recent study demonstrated that LIFR and gp130 are localized in lipid rafts/caveolae and that this localization is required for LIF/STAT3 signaling in mESCs [46]. Therefore, we examined the localization of LIFR and gp130 by isolating lipid rafts. The separation of lipid rafts and nonrafts was confirmed by Western blot analysis using an anti-Flotillin-1 antibody for the raft fractions (fractions 3–5) and an antitransferrin receptor antibody for the nonraft fractions (fractions 7–9) (supporting information Fig. S3). As shown in Figure 2D, the relative levels of LIFR and gp130 in raft fractions (fractions 3–5) were considerably reduced in *β4GalNAc-T3*-KD cells when compared with control cells. This result indicates that LIFR and gp130 were dispersed from lipid rafts/caveolae in *β4GalNAc-T3*-KD cells.

Overall, the findings in these various experiments demonstrate that LacdiNAc is involved in LIF/STAT3 signaling in mESCs through regulating the localization of LIFR and gp130 to rafts/caveolae and through controlling their heterodimerization.

LacdiNAc on LIFR and Gp130 Is Required for Raft/Caveolar Localization

The results described above indicate that LacdiNAc sequences on LIFR and gp130 influence their localization to rafts/caveolae. However, to date, the occurrence of this motif on LIFR and gp130 has not been demonstrated. We investigated whether LacdiNAc sequences are present on LIFR and gp130 using WFA-binding proteins (LacdiNAc-expressing proteins) purified from mESCs using WFA-agarose. As shown in Figure 3A, both LIFR and gp130 were observed in the fractions eluted using the WFA-binding proteins, indicating that LacdiNAc is expressed on LIFR and gp130. We also found that the amounts of LIFR and gp130 that could be purified from *β4GalNAc-T3*-KD cells were reduced when compared with control cells (Fig. 3B) indicating that expression of LacdiNAc on LIFR and gp130 was reduced in *β4GalNAc-T3*-KD cells.

\The localization of signal receptors to lipid rafts is believed to be regulated by the association of signal receptors with components of the rafts, such as caveolin-1 and glycolipids. We found that both LIFR and gp130 colocalized with caveolin-1 on the surfaces of mESCs (supporting information Fig. S5). Thus, we carried out biochemical analysis to determine whether LIFR and gp130 interact specifically with particular components of the rafts/caveolae, such as caveolin-1, and also whether this interaction is dependent on LacdiNAc expression. A Western blot analysis of immunoprecipitates obtained using the anti-caveolin-1 antibody identified comparatively low amounts of LIFR and gp130 from *β4GalNAc-T3*-KD cells when compared with control cells, demonstrating that interaction of caveolin-1 with LIFR and gp130 was reduced in *β4GalNAc-T3*-KD cells (Fig. 3C). This result indicates that caveolin-1 binds to LIFR and gp130 by recognizing LacdiNAc sequences on these receptors.

On the basis of these results, we propose that LacdiNAc sequences on LIFR and gp130 are required for the localization of these receptors to the rafts/caveolae by interaction with components such as caveolin-1.

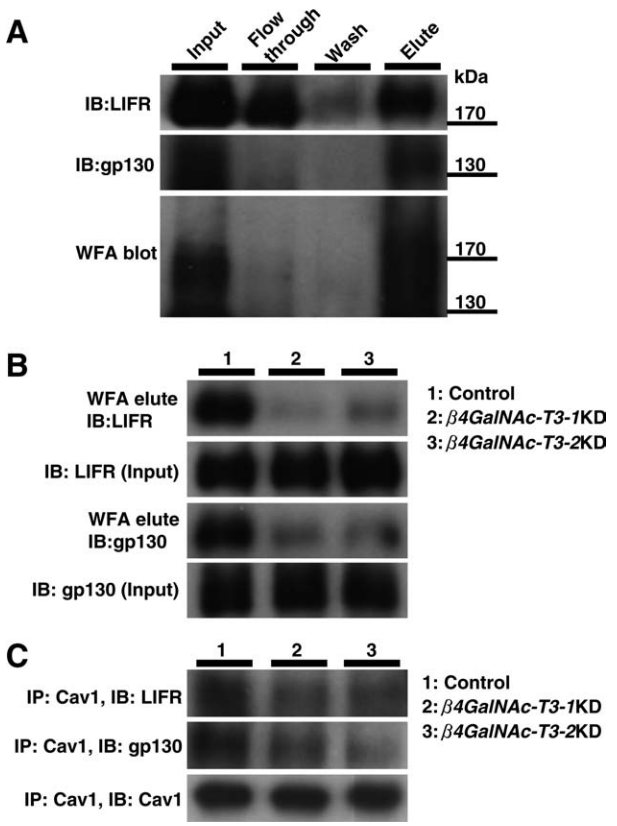

Figure 3. LacdiNAc on leukemia inhibitory factor receptor (LIFR) and gp130 contributes to interaction of these receptors with caveolin-1. (**A**): Western blot analysis of *Wistaria floribunda* (WFA)-binding proteins. Immunoblots with anti-LIFR or anti-gp130 antibody or lectin blot with horseradish peroxidase–WFA are shown. Input, flow-through, wash, and eluted fractions were prepared from mouse embryonic stem cells as described in the Materials and Methods section. (**B**): Western blot analysis of WFA-binding LIFR or gp130 2 days after transfection. Immunoblot with anti-LIFR or anti-gp130 antibody are shown. Eluted fractions from WFA-binding proteins were prepared as described in the Materials and Methods section. (**C**): Western blot analysis of interaction between caveolin-1 and LIFR or gp130 2 days after transfection. Immunoblot of LIFR or gp130 coimmunoprecipitated with caveolin-1 are shown. Abbreviations: Cav1, caveolin-1; IB, immunoblot; IP, immunoprecipitate; LIFR, leukemia inhibitory factor receptor; WFA, *Wistaria floribunda*.

LacdiNAc on LIFR and gp130 Is a Key Factor for LIF/STAT3 Signaling in Naïve State Pluripotent Stem Cells

It is known that although LIF/STAT3 signaling maintains self-renewal of mESCs, it does not function in either mEpiSCs or hESCs [4, 13, 47, 48]. The underlying molecular mechanisms of this differential response to LIF have yet to be clarified. We examined this question by first comparing the levels of LacdiNAc on mESCs and mEpiSC-like cells (produced from mESCs in EpiSC culture conditions as previously reported [41]). mEpiSC-like cells show characteristic features of mEpiSCs such as a monolayer morphology and marker gene expression (supporting information Fig. S4). We found that cell surface expression of LacdiNAc was lower in mEpiSC-like cells than in mESCs (Fig. 4A). Moreover, expression of *β4GalNAc-T3* was also lower in mEpiSC-like cells (Fig. 4B). Similar to mEpiSCs and hESCs, the mEpiSC-like cells showed a very weak response to LIF (Fig. 4C). The presence of LacdiNAc sequences on LIFR and gp130 is required for LIF/STAT3 signaling in

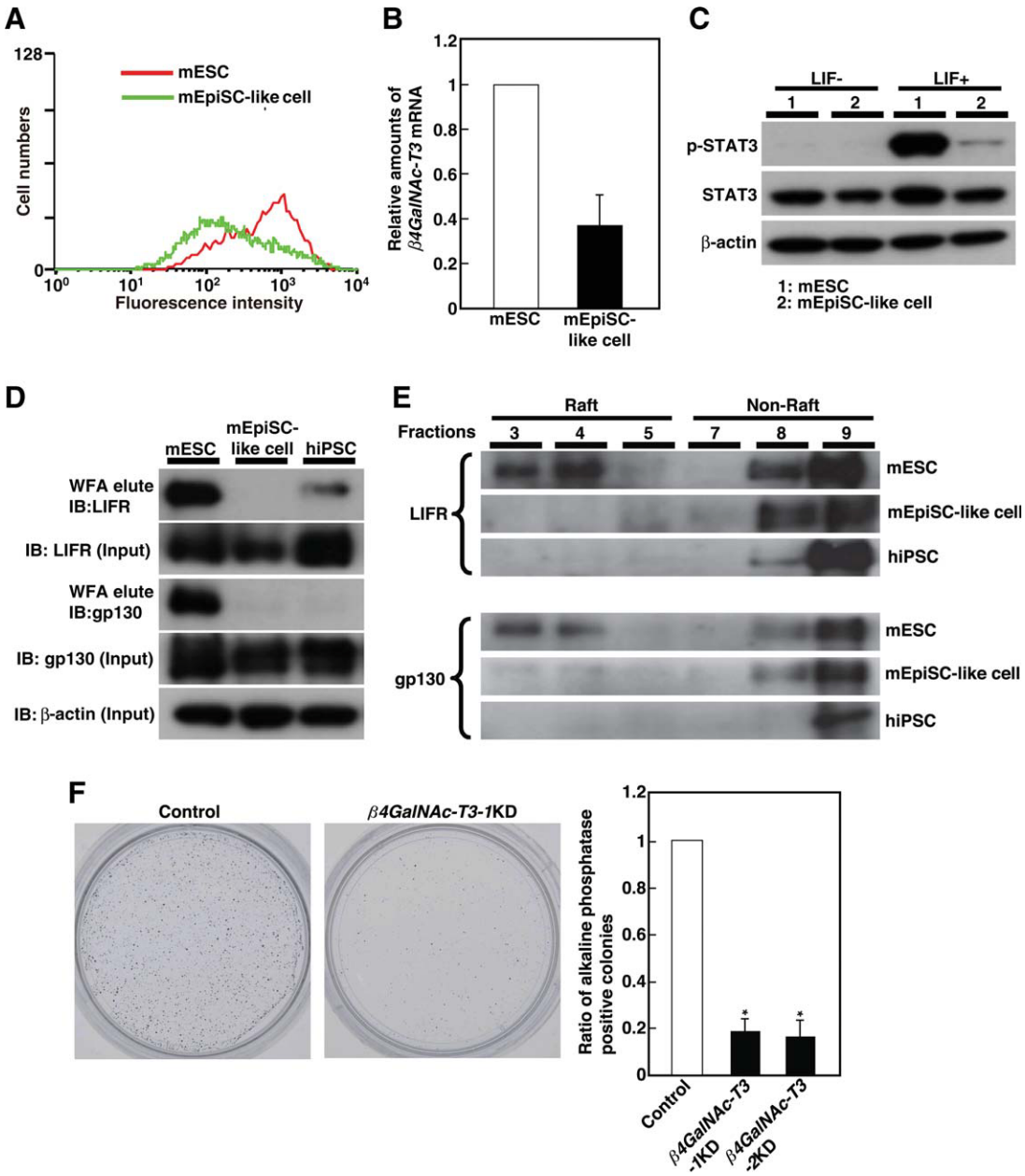

Figure 4. The presence of LacdiNAc on leukemia inhibitory factor receptor (LIFR) and gp130 is required for LIF/STAT3 signaling in naïve state. (**A**): FACS analysis of LacdiNAc on mouse embryonic stem cell (mESCs) and mouse epiblast stem cell (mEpiSC)-like cells using FITC-*Wistaria floribunda* (WFA). Mean FITC staining intensities of 20,959 and 13,476 were obtained for mESCs and mEpiSC-like cells, respectively. (**B**): Real-time PCR analysis of *β4GalNAc-T3* in mESCs and mEpiSC-like cells. Relative amounts of *β4GalNAc-T3* were calculated after normalization to *β-actin* mRNA and shown after normalization against the value obtained in mESCs (value = 1). The values shown are the means ± SD of duplicate measurements. (**C**): Western blot analysis of mESCs and mEpiSC-like cells stimulated with LIF. (**D**): Western blot analysis of WFA-binding LIFR or gp130 in mESCs, mEpiSC-like cells, and human induced pluripotent stem cells (hiPSCs). Immunoblot with anti-LIFR, anti-gp130, or anti-β-actin antibody is shown. Eluted fractions from WFA-binding proteins were prepared as described in the Materials and Methods section. (**E**): Western blot analysis of LIFR and gp130 in raft (Fr.3–5) and nonraft (Fr.7–9) fractions in mESCs, mEpiSC-like cells, and hiPSCs. (**F**): Reversion of *β4GalNAc-T3*-KD EpiSC-like cells. Left panel, macroscopic view of alkaline phosphatase-stained dishes 4 days after reversion is shown. Right panel, relative number of alkaline phosphatase-positive colonies after normalization against the value obtained in control cells (value = 1) is shown. The values shown are the means ± SD from three independent experiments and significant values are indicated, *, $p < .01$, in comparison to the control. Abbreviations: hiPSC, human induced pluripotent stem cell; IB, immunoblot; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; mESC, mouse embryonic stem cell; mEpiSC, mouse epiblast stem cell; p-STAT3, phospho-STAT3; STAT3, signal transducer and activator of transcription 3; WFA, *Wistaria floribunda*.

mESCs (Figs. 2 and 3 and above description). We postulated that expression of LacdiNAc on these receptors was reduced in EpiSC-like cells. Although the total levels of LIFR and gp130 expression were lower in mEpiSC-like cells than mESCs, the rate of LacdiNAc expression on LIFR and gp130 was absolutely lower in mEpiSC-like cells than mESCs (Fig. 4D). This result indicates that expression of LacdiNAc sequences on LIFR and gp130 was reduced in EpiSC-like cells. Furthermore, the levels of LacdiNAc on LIFR and gp130 were also extremely low in hiPSCs (Fig.

4D). Next, we compared the localization of LIFR and gp130 on rafts/caveolae in mEpiSC-like cells, hiPSCs, and mESCs. Expression of LIFR and gp130 in the raft fractions was low in both mEpiSC-like cells and hiPSCs when compared to mESCs, indicating that raft/caveolar localization of LIFR and gp130 was weak in both mEpiSC-like cells and hiPSCs (Fig. 4E). In addition, immunocytostaining showed that both LIFR and gp130 colocalized poorly with caveolin-1 in mEpiSC-like cells and hiPSCs (supporting information Fig. S5). From these results, we propose the following molecular mechanism for the weak response to LIF in primed state pluripotent stem cells, such as EpiSCs and hESCs including hiPSCs: LIFR and gp130 do not localize in rafts/caveolae because of the low level of LacdiNAc, which results in the weak transduction of LIF/STAT3 signaling.

A recent study reported that LIF/STAT3 signaling contributes to reversion from the primed state to the naïve state [49–51]. Therefore, we investigated whether the regulation of LIF/STAT3 signaling by LacdiNAc expression contributed to reversion. We used mEpiSC-like cells produced from stable *β4GalNAc-T3*-KD cells. *β4GalNAc-T3*-KD cells produced mEpiSC-like cells similar to control cells in morphology and marker gene expression (supporting information Fig. S6). We found that the rate of reversion in mEpiSC-like cells derived from *β4GalNAc-T3*-KD cells was markedly lower than in control cells (Fig. 4F). This result indicates that upregulation of LacdiNAc is required for reversion from the primed state to the naïve state. We conclude that one factor in the differential response of naïve and primed state pluripotent cells to LIF is the levels of LacdiNAc sequences on LIFR and gp130 and that expression of LacdiNAc is required for the induction and maintenance of the naïve state in pluripotent stem cells.

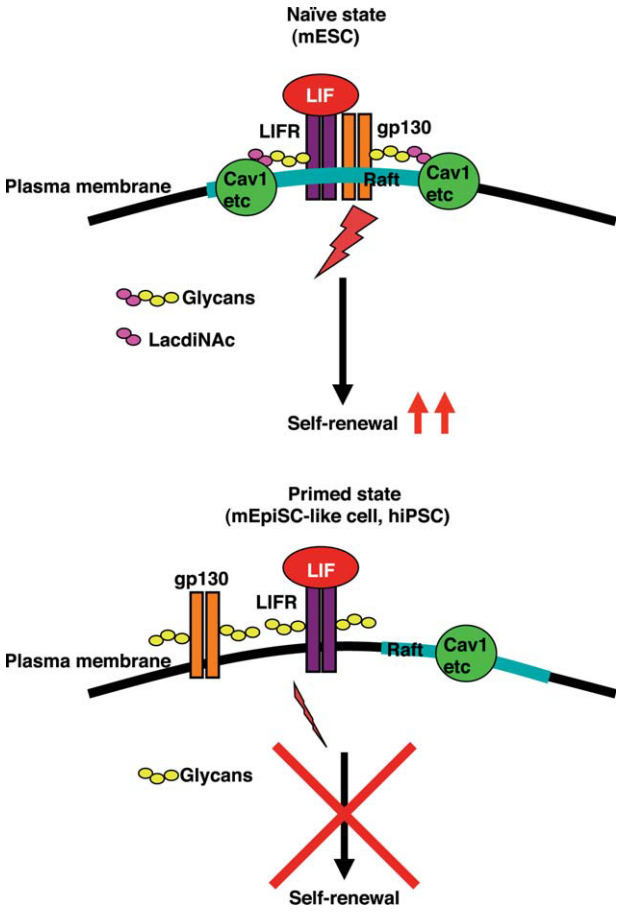

Figure 5. Schematic representation of leukemia inhibitory factor (LIF)/STAT3 signaling regulated by LacdiNAc in naïve and primed state cells. In the naïve state, LacdiNAc expressed on LIF receptor (LIFR) and gp130 contributes to the interaction with raft/caveolar components, such as caveolin-1, and this interaction stabilizes the raft/caveolar localization of LIFR and gp130 and results in strong transduction of the LIF/STAT3 signal. Thus, naïve state mouse embryonic stem cells and presumably converted cells can be maintained in an undifferentiated state by LIF/STAT3 signaling. In the primed state, LIFR and gp130 are weakly localized to raft/caveolae due to the lack of LacdiNAc on the receptors, which results in weak transduction of the LIF/STAT3 signal. Therefore, self-renewal of mouse epiblast stem cells, human induced pluripotent stem cells, and presumably human ESCs cannot be maintained by LIF/STAT3 signaling. Abbreviations: Cav1, caveolin-1; hiPSC, human induced pluripotent stem cell; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; mESC, mouse embryonic stem cell; mEpiSC, mouse epiblast stem cell.

Discussion

The extrinsic signals required for self-renewal differ between mESCs and hESCs. However, the molecular mechanism that underlies this differential behavior has not yet been clarified. In recent years, pluripotent stem cells at different developmental stages have been identified, such as EpiSCs and FGF/activin/Bio-stem cells (FAB-SCs) in the mouse [46, 47, 52]. EpiSCs are derived from the postimplantation epiblast and have similar features to hESCs. In particular, both cell types are maintained by the same extrinsic signals, namely, activin/nodal and FGF2 signaling. Recent studies have demonstrated that EpiSCs, which are more advanced developmentally than mESCs, can be induced to revert to mESC-like cells [41, 42, 49–51]. FAB-SCs are generated from the preimplantation epiblast under defined culture conditions, including the presence of FGF2 and activin, and they can be induced to revert to the mESC-like state. The relative developmental stage of FAB-SCs is uncertain but they are thought to be more developmentally advanced than mESCs. Thus, the information obtained from several studies, using various mouse stem cells derived from developmentally different stages, indicates that hESCs are at a developmentally later stage that corresponds to the postimplantation epiblast (primed state). By contrast, mESCs are derived from ICM and are maintained in the naïve state. Recent reports indicate that hESCs (primed state) can be induced to revert to mESC-like cells (naïve state) under the specific culture conditions that are required for maintenance of mESCs; after reversion, these naïve hESCs can be maintained under culture conditions containing LIF [25, 53]. Thus, these reports have reinforced the view that hESCs and mESCs are at developmentally different stages. This difference in developmental stage between hESCs and mESCs is also reflected in their different responses to extrinsic signals. In particular, while LIF/STAT3 signaling contributes to the maintenance of self-renewal in mESCs, it does not do so for hESCs [4, 13]. However, the factors responsible for this variation in LIF-dependency have yet to be identified. In this study, we have shown that expression of LacdiNAc on LIFR and gp130 is required for their localization to rafts/caveolae; this expression is required for strong transduction of LIF/STAT3 signaling in naïve state cells, and the low expression of primed states cells is associated with weak signal transduction. To the best of our knowledge, this is the first demonstration that differential expression of LacdiNAc on LIFR and gp130 in naïve and primed state cells underlies the different responses of mESCs (naïve state) and hESCs, including mouse EpiSCs (primed state), to LIF/STAT3 signaling.

The molecular mechanisms of signal transduction mediated by interleukin-6 and related cytokines, such as LIF, are well-defined [45]. More recently, the contribution of glycans to LIF/STAT3 signaling has been reported in neural stem cells, in which *N*-glycans contribute to the heterodimerization of LIFR and gp130 [54]. However, the molecular mechanisms by which *N*-glycans mediate heterodimerization have not been clarified. In this study, we demonstrated that reduction of LacdiNAc, which can be present on *N*-glycans or *O*-glycans, resulted in the inhibition of heterodimerization and a decrease in LIF/STAT3 signaling. Furthermore, we found that a reduction in LacdiNAc sequences induced defective localization of LIFR and gp130 to rafts/caveolae. The rafts/caveolae provide an effective platform for signal transduction by enrichment of several signal receptors, thereby forming an efficient complex of receptors and other signal-related factors. Therefore, we propose that deficient heterodimerization of LIFR and gp130 as a consequence of a reduction in LacdiNAc is dependent on a reduction in the raft/caveolar localization of these receptors. Nevertheless, it is also possible that LacdiNAc sequences directly contribute to the interaction between LIFR and gp130; further study will be required to examine this possibility.

It was recently reported that LIFR and gp130 localization in rafts/caveolae in mESCs is required for LIF/STAT3 signaling [46]. This study found that knockdown of *caveolin-1* did not affect raft/caveolar localization of LIFR and gp130. Here, we demonstrated that the interaction of LIFR and gp130 with caveolin-1 was mediated by LacdiNAc. Therefore, we propose that LacdiNAc mediates the interaction of LIFR and gp130 not only with caveolin-1 but also with other raft/caveolar components, such as polymerase I transcript release factor/cavin-1, caveolin-2, and glycosylphosphatidylinositol-anchored proteins, to stabilize the localization of these receptors. We shall examine this proposal in future studies. To date, it has been demonstrated that the binding of some raft/caveolar associated proteins to caveolin-1 is mediated by the caveolin binding motif [55]. We found that LIFR and gp130 have the caveolin binding motifs YGTVVFAGY (amino acids 318–326) and FTFTTPKF (amino acids 604–611), respectively. Both motifs contain threonine residues that might possibly be modified by *O*-linked glycosylation. It is possible that the *O*-glycans modified on the threonine residues of LIFR or gp130 have LacdiNAc sequences. Thus, we suggest that LacdiNAc modification on the caveolin binding motif might affect the interaction between caveolin-1 and LIFR or gp130; further investigation will be needed to confirm this possibility.

It has been reported that LIF/STAT3 signaling contributes to the reversion of primed state EpiSCs to naïve state mESCs [49, 50]. Additionally, a recent study showed that weak transduction of the LIF/STAT3 signal is a possible barrier to this reversion [51]. Here, our findings indicated that expression of LacdiNAc on LIFR and gp130 was required for the reversion process. Furthermore, it has previously been shown that naïve state hESCs and metastable nonobese diabetic-iPSCs can be induced under defined culture conditions [25]; these cells can be maintained in the naïve state only under these defined culture conditions. As described in this study, LacdiNAc has a role in the reversion from the primed state to the naïve state as well as in the maintenance of the naïve state. Therefore, we propose that the defined culture conditions contribute to the transcriptional regulation of *β4GalNacT-3* followed by expression of LacdiNAc, and a sufficiently strong transduction of LIF/STAT3 signaling by LacdiNAc expression is indispensable for induction of the naïve state as well as maintenance of the naïve state.

Conclusion

We propose a molecular mechanism to explain the differential responses of naïve state and primed state cells to LIF/STAT3 signaling (Fig. 5). In naïve state mESCs, the presence of LacdiNAc on LIFR and gp130 contributes to the association of the receptors with raft/caveolar components, such as caveolin-1, and this association stabilizes localization in the rafts/caveolae. In this stable association state, LIFR and gp130 induce a sufficiently strong transduction of LIF/STAT3 signaling to maintain stem cell self-renewal. In contrast, in primed state pluripotent stem cells, such as mEpiSCs, hiPSCs, and presumably hESCs, the receptors lack LacdiNAc and show poor localization to the rafts/caveolae. The cells consequently have insufficient LIF/STAT3 signaling to maintain self-renewal. In this study, we provide the first demonstration that glycans, such as LacdiNAc, can play functionally important roles in pluripotent cells at a specific developmental stage (naïve state); the findings from these functional analyses shed light on the previously unclarified differences between mESCs (naïve state) and hESCs (primed state). Further study of the functional roles of other glycans will be useful for defining and characterizing stage-specific pluripotent stem cells with respect to stage-specific expression of glycans. Characterization of these stem cells may extend the possibilities for developing disease-specific differentiated cells for regenerative medicine.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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