Self-Renewal of Naïve State Mouse Embryonic Stem Cells: Role of LacdiNAc in LIF/STAT3 Signaling

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Contents

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Abstract

Recent studies have indicated that mouse and human ES/iPS cells are at different developmental stages, namely the naïve state and the primed state, respectively. They therefore require different extrinsic signals for the maintenance of self-renewal and pluripotency. LIF/STAT3 signaling is one of the important factors that distinguishes the naïve and primed states. In general, signals from extrinsic factors are regulated by the glycosylation of molecules on the cell surface and the extracellular matrix, such as ligand receptor molecules. LacdiNAc carbohydrate structures on LIFR and gp130 are required for a sufficiently strong LIFR/STAT3 signal to maintain self-renewal in naïve state cells; these structures act by controlling the localization of the receptors to cell membrane rafts/caveolae. Therefore, an important factor in the differential response of naïve and primed state pluripotent cells to LIF is the level of LacdiNAc structures on LIFR and gp130, and expression of LacdiNAc is required for the induction and maintenance of naïve state pluripotent stem cells. The characterization and functional analyses of cell surface glycans on stem cells will provide further information of value for defining and characterizing stage-specific pluripotent stem cells, and this information will be vital to the development of these cells as a resource for regenerative medicine.

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Introduction

It is now known that very large numbers of cell surface proteins are glycosylated (Kaji et al. 2006); indeed, more than 50% of secreted proteins and cell surface proteins are considered to be glycoproteins. Various types of glycoprotein and glycolipid are present on the cell surface and their patterns of glycosylation (i.e., the glycan structures on the proteins and glycolipids) change dramatically during development (Fig. 4.1). Some of these glycoproteins and glycolipids have been used as markers of embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells), for example, stage-specific embryonic antigen-1 (SSEA-1), SSEA-3, SSEA-4, TRA-1-60 antigen and TRA-1-81 antigen (Adewumi et al. 2007). Mouse ES cells express SSEA-1 (also known as Lewis X carbohydrate antigen), Galβ1,4(Fucα1,3)GlcNAc. An anti-SSEA-1 antibody gives positive staining of both ES cells and the inner cell mass (ICM), the origin of mouse ES cells. However, human ES cells do not express SSEA-1. This difference suggests that mouse and human ES cells are at different stages of development. Human ES cells express SSEA-3, TRA-1-60 antigen, and TRA-1-81 antigen (Adewumi et al. 2007). More recently, SSEA-5 has been proposed as a novel carbohydrate marker for human ES cells and iPS cells (Tang et al. 2011).

Glycan structures on the cell surface function to regulate cell-cell interactions, cell-extracellular matrix interactions, and signals from extrinsic factors, such as Wnt, Hedgehog (Hh), bone morphogenetic protein (BMP) and fibroblast growth factor (FGF). Analysis of a *Drosophila* model system showed that heparan sulfate (HS), a glycan structure, has a key role in the regulation of these basic developmental signals; these extrinsic factors are also known to act as morphogens during various stages of development (Nishihara 2010). Specific sulfated regions of HS can bind to these extrinsic factors and work as co-receptors or can stabilize the factors. In mouse ES cells, self-renewal and pluripotency are maintained by a

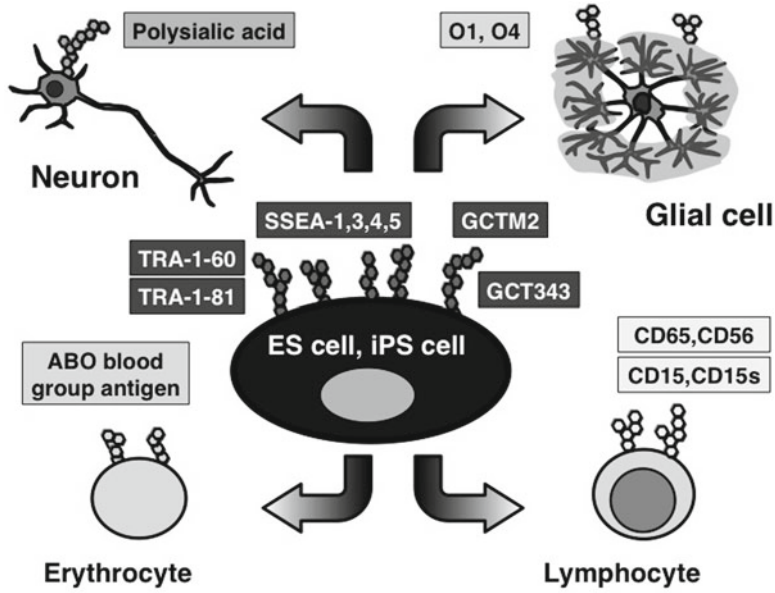

Fig. 4.1 The glycan structures on the cell surface change during differentiation. SSEA-1 is used as a marker of mouse ES cells, while SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM2 and GCT343 are used as markers for human ES/iPS cells. Polysialic acid is expressed on neural cells, while O1 and O4 are expressed on glial cells. The ABO blood group antigen is expressed on erythrocytes, while CD65, CD56, CD15 and CD15s are expressed on lymphocytes

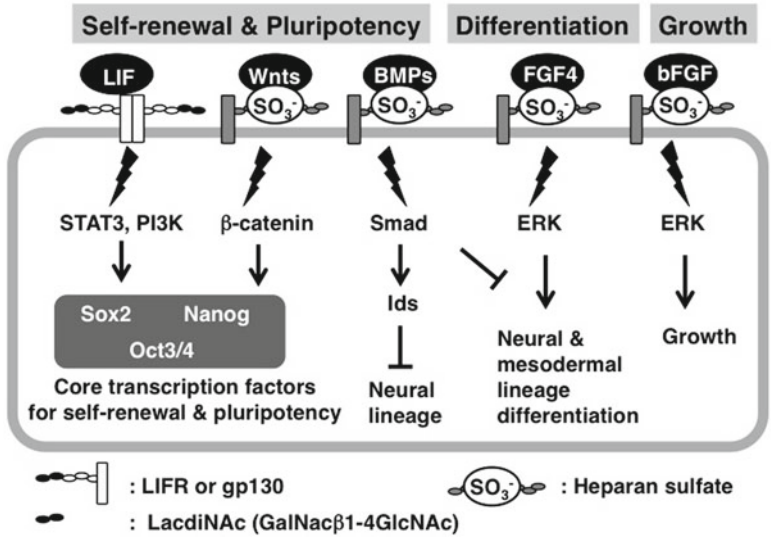

Fig. 4.2 The roles of glycan structures on mouse ES cells. LacdiNAc and heparan sulfate contribute to the self-renewal and pluripotency of mouse ES cells through LIF/STAT3 signaling, and Wnt/β-catenin and BMP/Smad signaling, respectively. Heparan sulfate also contributes to the differentiation and growth of mouse ES cells through FGF4/ERK and bFGF/ERK signaling pathways, respectively. Heparan sulfate works as the co-receptor or the stabilizer for the extrinsic factors

balance of several signaling pathways, such as Wnt, BMP, and FGF, and disruption of this balance induces differentiation of ES cells into a specific lineage. HS also contributes to the regulation of these signals and works in the maintenance and differentiation of mouse ES cells (Fig. 4.2) (Kraushaar et al. 2010; Lanner et al. 2010; Sasaki et al. 2008, 2009). However, the physiological functions of the other glycans in ES cells and iPS cells are still largely unknown. Recently, we identified a novel glycan function in naïve state mouse ES cells and primed state embryonic stem cells after an RNAi screen of more than 100 glycosyltransferases (Sasaki et al. 2011). This chapter focuses on the functions of this glycan structure, LacdiNAc, in LIF/STAT3 signaling and in self-renewal of naïve state ES cells.

LIF/STAT3 Signaling Contributes to Self-Renewal of Mouse Embryonic Stem Cells

LIF is essential for self-renewal and pluripotency of mouse ES cells, but does not have this function in human ES and iPS cells (Boeuf et al. 1997; Daheron et al. 2004; Hirai et al. 2011; Niwa et al. 2009). Therefore, LIF-responsiveness is considered to be one of the key distinguishing features of mouse and human ES cells (Fig. 4.3a).

LIF is a member of the Interleukin 6 (IL6) cytokine family and its signaling pathway is well characterized. In mouse ES cells, the binding of LIF to the LIF receptor (LIFR) induces three signaling pathways, namely, Jak/STAT3, PI3K/Akt, and SHP2/MAPK (Hirai et al. 2011; Niwa et al. 2009) (Fig. 4.2). Nanog, Sox2, and Oct3/4, which are core transcriptional factors for the maintenance of self-renewal and pluripotency, regulate each other to form the core transcriptional factors network and then collaborate to regulate downstream gene expression. LIF signaling induces the expression of these core transcriptional factors through the Jak/STAT3 and PI3K/Akt pathways to maintain this network. Thus, in mouse ES cells, LIF signaling contributes to the maintenance of self-renewal and pluripotency by inducing core transcriptional factors.

In addition to LIF, several extrinsic signaling pathways involved in self-renewal and pluripotency have been identified in mouse ES cells. Wnt/β-catenin signaling regulates the expression

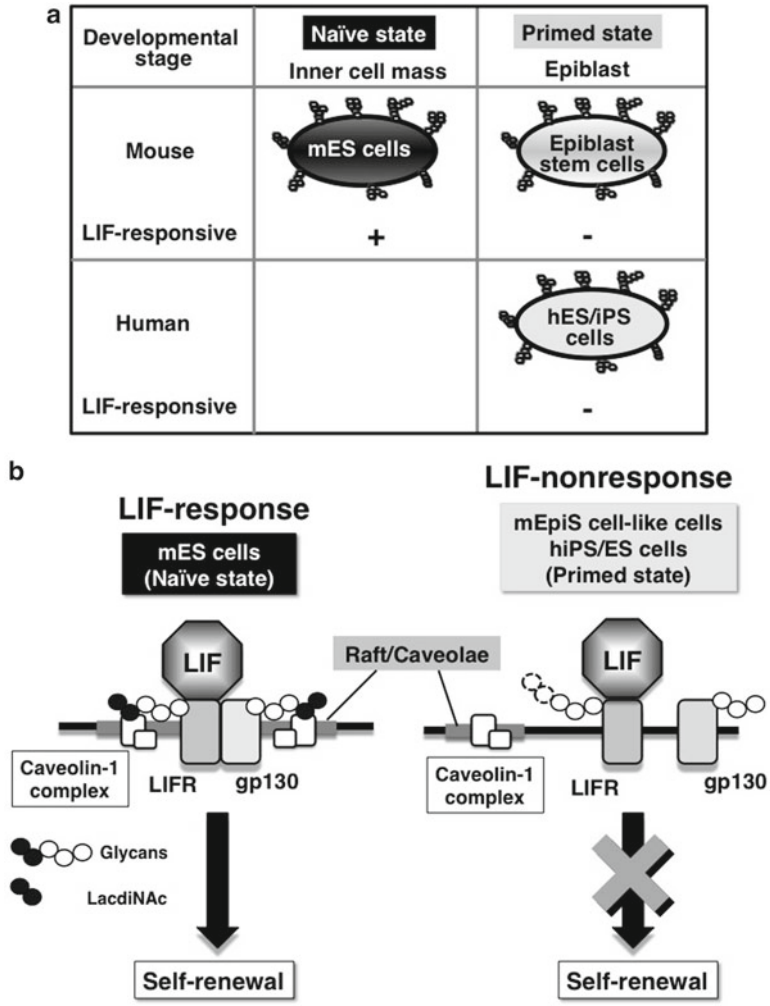

Fig. 4.3 High levels of LacdiNAc structures on LIFR and gp130 are required for maintenance of the naïve state in pluripotent stem cells. (**a**) Mouse ES cells are at a different developmental stage from human ES/iPS cells: mouse ES cells correspond to the inner cell mass (naïve state), while hES/iPS cells correspond to the epiblast (primed state). This difference is characterized by their respective levels of responsiveness to LIF. (**b**) Expression of LacdiNAc structures on LIFR and gp130 is required for the induction and maintenance of naïve state pluripotent stem cells. In naïve state mouse ES cells, the LacdiNAc structures on LIFR and gp130 contribute to the association with the caveolin-1 complex components of caveolae/rafts; this association stabilizes LIFR and gp130 localization to the rafts. This localization is necessary to induce a sufficiently strong transduction of LIF/STAT3 signaling to maintain self-renewal. In primed state pluripotent stem cells, such as mouse mEpiS cell-like cells and hiPS/ES cells, the receptors do not have LacdiNAc and cannot localize to the rafts. As a result, the cells cannot induce LIF/STAT3 signaling to maintain self-renewal

of Oct3/4 and Nanog and of their core network (Sasaki et al. 2008; Sato et al. 2004; Wray and Hartmann 2012) (Fig. 4.2). Bone morphogenic protein 4 (BMP4)/Smad signaling contributes to the maintenance of self-renewal and pluripotency in cooperation with LIF (Ying et al. 2003). By contrast, in human ES/iPS cells, BMP4 signaling induces differentiation (Xu et al. 2002), and FGF2 and activin/nodal signaling are involved in the maintenance of self-renewal and pluripotency (James et al. 2005). However, the role of Wnt/β-catenin signaling in the maintenance

of self-renewal and pluripotency in human ES cells remains uncertain, although activation of Wnt/β-catenin signaling is known to inhibit their differentiation (Sato et al. 2004; Wray and Hartmann 2012). The outcome of Wnt/β-catenin signaling might depend on other signaling factors such as FGF2 (Wray and Hartmann 2012). Thus, there is clear evidence that mouse and human ES/iPS cells maintain their pluripotency using different signaling factors.

As stated above, mouse and human ES/iPS cells are considered to be at different developmental stages (Fig. 4.3a) and, therefore, to require different extrinsic signals to maintain self-renewal and pluripotency. Pluripotent stem cells at different stages of development have been identified in the mouse, namely, epiblast stem cells (EpiS cells) and FGF/activin/BIO-stem cells (FAB-S cells) (Brons et al. 2007; Chou et al. 2008) (Fig. 4.3a). EpiS cells are derived from the post-implantation epiblast and have similar features to human ES cells. Both cell types are maintained by the same extrinsic signals, that is, activin/nodal and FGF2 signaling. Mouse EpiS cells are at a more developmentally advanced stage than mouse ES cells and can be induced to revert to mouse ES cell-like cells (Bao et al. 2009; Yang et al. 2010; Zhou et al. 2010). FAB-S cells are generated from the pre-implantation epiblast under defined culture conditions, including the presence of FGF2 and activin, and they can be induced to revert to the mouse ES cell-like state. The relative developmental stage of FAB-S cells is uncertain but they are thought to be at a developmentally more advanced stage than mouse ES cells. On the basis of the data obtained from several studies using various mouse stem cells derived from different developmental stages, human ES cells are believed to be at a later developmental stage that corresponds to the mouse post-implantation epiblast, which is now designated as the "primed state" (Fig. 4.3a). In contrast, mouse ES cells are derived from the ICM and their developmental stage is described as the "naïve state". The specific culture conditions that are required to maintain mouse ES cells have recently been shown to induce human ES cells in the primed state to revert to a naïve state corresponding to that of mouse ES cell-like cells. These naïve human ES cells can then be maintained under culture conditions containing LIF (Hanna et al. 2010; Xu et al. 2010). The findings from these studies provide support for the contention that mouse and human ES/iPS cells are at different stages of development. The difference in developmental stages of mouse and human ES/iPS cells is also reflected in their different responses to extrinsic signals. For example, LIF signaling contributes to the maintenance of self-renewal in mouse ES cells but not in human ES cells (Boeuf et al. 1997; Daheron et al. 2004; Hirai et al. 2011; Niwa et al. 2009). Therefore, LIF responsiveness is an important key factor that distinguishes the naïve and primed states of pluripotent stem cells (Fig. 4.3a).

LacdiNAc Structures Are Expressed on Mouse Embryonic Stem Cells But Not on Human Induced Pluripotent Stem Cells or Mouse Epistem-Like Cells

The LacdiNAc carbohydrate structure, GalNAcβ1-4GlcNAc, is a unique terminal structure in the outer chain moieties of *N*-glycans and *O*-glycans. It was originally identified on the human glycoprotein hormones leutropin and thyrotropin, which are secreted from the pituitary gland. Initially, LacdiNAc was thought to be restricted in vertebrates to a limited number of glycoproteins and glycolipids, such as glycoprotein hormones, although it is frequently present on glycoproteins and glycolipids in invertebrates (Manzella et al. 1996). In vertebrates, the LacNAc element Galβ1-4GlcNAc, appears to be more commonly present as a type II blood core carbohydrate structure than LacdiNAc, (Manzella et al. 1996). Various other molecules, such as glycodelins in the amnion of fetal tissue and α-dystroglycan in the extracellular matrix (Breloy et al. 2012), have recently been found to carry LacdiNAc on *N*- or *O*-glycans.

Mouse ES cells show high expression of the LacdiNAc structure on their surface (Sasaki et al. 2011). The expression of LacdiNAc decreases

during differentiation in mouse ES cells cultured without LIF. LacdiNAc expression is also reduced in mouse EpiS cell-like cells produced from mouse ES cells in EpiS cell culture conditions including activin A and FGF2 and in human iPS cells which are at developmentally more advanced stage than mouse ES cells. Thus, LacdiNAc is highly expressed in naïve state pluripotent stem cells, while it is expressed at a lower level in primed pluripotent stem cells (Fig. 4.3b) (Sasaki et al. 2011).

To date, nine types of β1,4-*N*-acetylgalactosaminyltransferase (β1,4GalNAc-T) have been cloned and their substrate specificities identified. Of these, β1,4GalNAc-T 3 and 4 show activity in the synthesis of LacdiNAc structures (Gotoh et al. 2004; Sato et al. 2003). Both recombinant enzymes have very similar substrate specificities but show very different distributions in human tissues. *β4GalNAc-T3* mRNA has been detected in the stomach, colon and testis, whereas *β4GalNAc-T4* transcripts are predominantly detected in the brain, ovary, and mammary glands. In mouse ES cells, RNAi of *β4GalNAc-T3* reduces the amount of LacdiNAc structures on the cell surface (Sasaki et al. 2011) and, therefore, β4GalNAc-T3 is considered to be the principal contributor to synthesis of the LacdiNAc structure. By comparison to mouse ES cells, expression of *β4GalNAc-T3* is lower in mouse EpiS cell-like cells and human iPS cells, reflecting the reduced expression of LacdiNAc on their cell surfaces. *β4GalNAc-T3* is expressed highly in naïve state pluripotent stem cells, but at a lower level in primed pluripotent stem cells. The role of β4GalNAc-T 4 remains under investigation.

LIFR and gp130 Localize on Lipid Rafts/Caveolae and Transmit the LIF/STAT3 Signal

Lipid rafts are defined as microdomains within the lipid bilayer of cellular membranes. They are small, heterogeneous and dynamically changeable structures enriched in glycosphingolipids, sphingomyelin and cholesterol, and subsets of transmembrane or glycosylphosphatidylinisotol-anchored proteins (Lingwood and Simons 2010). Lipid rafts are considered to be essential in cellular signaling processes because many signaling molecules, receptors and Src family kinases are assembled there. Biochemically, lipid rafts resist extraction in cold detergent and, therefore, they are described as detergent-resistant membrane fractions.

During the process of LIF binding, LIFR recruits the membrane protein gp130 to form a heterodimer (Hirai et al. 2011) (Fig. 4.3b). Heterodimerization triggers activation of associated JAK tyrosine kinases and then phosphorylation of gp130; these events result in the activation of STAT3. In mouse ES cells and embryonic neural precursor cells, gp130 and LIFR localize to one particular type of lipid raft termed a caveola (Lee et al. 2010; Sasaki et al. 2011; Yanagisawa et al. 2004). This localization is required for strong LIF/STAT3 signaling in mouse ES cells (Lee et al. 2010; Sasaki et al. 2011). However, the role of caveolin-1, a component of rafts/caveolae, in localization of the receptors is still ambiguous, although both LIFR and gp130 have caveolin binding motifs: YGTVVFAGY (amino acids 318–326) and FTFTTPKF (amino acids 604–611), respectively (Couet et al. 1997; Sasaki et al. 2011). In addition to caveolin-1, other raft/caveolar components, such as RTPF/cavin-1, caveolin-2, and glycosylphosphatidylinositol-anchored proteins might stabilize localization of the receptors.

LIFR and gp130 with LacdiNAc Structures Are Localized on Lipid Rafts/Caveolae

The LacdiNAc carbohydrate structure GalNAcβ1-4GlcNAc along with β1,4GalNAc-T 3, which synthesizes LacdiNAc (Sato et al. 2003), are highly expressed in naïve state pluripotent stem cells, such as mouse ES cells (Fig. 4.3b) (Sasaki et al. 2011). Protein analyses showed that both LIFR and gp130 are modified by LacdiNAc. Both LIFR and gp130 are localized on lipid rafts and co-immunoprecipitated by caveolin-1, a

marker for and component of lipid rafts/caveolae. LIFR and gp130 also show co-localization with caveolin-1 on the surface of mouse ES cells indicating that receptor molecules carrying LacdiNAc are localized on lipid rafts/caveolae (Sasaki et al. 2011). It has been reported that gp130 has nine *N*-glycosylated sites among the 11 potential *N*-glycosylation sites in its extracellular domain (Moritz et al. 2001). In mouse embryonic neural precursor cells, *N*-glycans on gp130 are required for heterodimerization with LIFR (Yanagisawa and Yu 2009). Potential *O*-glycosylation sites are also found in both gp130 and LIFR (Sasaki et al. 2011). However, it has not yet been determined whether there are LacdiNAc structures on *N*- or *O*-glycans that modify gp130 and LIFR on mouse ES cells.

LacdiNAc Structure Is Essential for Self-Renewal in Naïve State Pluripotent Stem Cells (Mouse Embryonic Stem Cells) Through LIF/STAT3 Signaling

Mouse ES cells were subjected to RNAi using short hairpin RNAs targeted against various glycosyltransferase genes and self-renewal of the knockdown cells was evaluated by their alkaline phosphatase activities. This analysis showed that the cell surface glycan LacdiNAc is required for self-renewal of naïve state pluripotent stem cells (Sasaki et al. 2011). Knockdown (KD) of *β4GalNAc-T3*, which synthesizes LacdiNAc, results in reduced expression of cell surface LacdiNAc, a lower proportion of alkaline phosphatase positive colonies, and a reduction in Oct3/4, Nanog and Sox2, which are markers of the undifferentiated state. In addition, the rate of proliferation of *β4GalNAc-T3*-KD cells is significantly decreased. Thus, LacdiNAc has a range of functions in undifferentiated mouse ES cells (naïve state pluripotent stem cells) and is required for self-renewal and proliferation of the cells.

LIF/STAT3 signaling is markedly reduced in *β4GalNAc-T3*-KD cells, although BMP4/Smad and FGF4/ERK signaling is not affected (Sasaki et al. 2011). Therefore, the reduction in LIF/STAT3 signaling causes the reduction in the self-renewal ability of the cells. However, no significant differences are detectable in cell surface expression of LIFR and gp130 between control and *β4GalNAc-T3*-KD cells. In contrast, in *β4GalNAc-T3*-KD cells, LIFR and gp130 are dispersed from the lipid rafts/caveolae resulting in a reduced rate of LIFR and gp130 heterodimerization in response to LIF. LacdiNAc on LIFR and gp130 is decreased in *β4GalNAc-T3*-KD cells, and the interaction and colocalization of caveolin-1 with LIFR and gp130 is also reduced. Overall, these findings demonstrate that LacdiNAc is involved in LIF/STAT3 signaling in mouse ES cells through regulation of the localization of LIFR and gp130 to rafts/caveolae and through controling their heterodimerization (Fig. 4.3b). That is, LacdiNAc structures on LIFR and gp130 are required for the localization of these receptors to the rafts/caveolae by interaction with components of the caveolin-1 complex and are necessary to maintain a sufficiently strong LIFR/STAT3 signal for maintenance of self-renewal.

Reduced Expression of LacdiNAc Structures in Primed State Pluripotent Stem Cells (Mouse Epistem-Like Cells and Human Induced Pluripotent Stem Cells) Causes the Inert State of the LIF/STAT Signal

LIF/STAT3 signaling does not function in either mouse EpiS cells or human ES cells, (Brons et al. 2007; Daheron et al. 2004), although it maintains self-renewal in mouse ES cells (Fig. 4.3a). As mentioned above, expression of LacdiNAc is reduced in primed state pluripotent stem cells such as mouse EpiS cell-like cells and human iPS cells compared to naïve state mouse ES cells (Sasaki et al. 2011). LacdiNAc structures are a key factor in determining the responsiveness to LIF/STAT3 signaling and explain the different responses of primed and naïve state pluripotent stem cells (Fig. 4.3b).

Mouse EpiS cell-like cells are similar to mouse EpiS cells and human ES/iPS cells in showing a very weak response to LIF (Sasaki et al. 2011). Expression of LacdiNAc on LIFR and gp130 is markedly lower in mouse EpiS cell-like cells and human iPS cells than mouse ES cells. Consequently the raft/caveolar localization of LIFR and gp130 is very weak in mouse EpiS cell-like cells and human iPS cells, which are primed state pluripotent stem cells. Based on these observations, the following molecular mechanism for the weak response to LIF in primed state pluripotent stem cells is proposed: LIFR and gp130 do not localize in rafts/caveolae due to the low level of LacdiNAc and, in turn, this decrease in localization causes a weak transduction of the LIF/STAT3 signal (Fig. 4.3b).

LacdiNAc Structures Are Required for the Induction of Naïve State Pluripotent Stem Cells (Mouse Embryonic Stem Cells)

Both LIF/STAT3 signaling (Bao et al. 2009; Yang et al. 2010) and LacdiNAc expression (Sasaki et al. 2011) contribute to reversion from the primed state to the naïve state.

Stable *β4GalNAc-T3*KD EpiS cell-like cells were produced from stable β*4GalNAc-T3*KD ES cells (Sasaki et al. 2011). Mouse EpiS cell-like cells can be induced to revert to mouse ES cell-like cells under defined culture conditions (Hanna et al. 2010; Zhou et al. 2010). The rate of reversion in mouse EpiS cell-like cells derived from stable *β4GalNAc-T3*KD ES cells is markedly lower than in control cells, indicating that up-regulation of LacdiNAc is required for reversion from the primed state to the naïve state (Sasaki et al. 2011).

Thus, one of the important factors in the differential response of naïve and primed state pluripotent cells to LIF is the level of LacdiNAc structures on LIFR and gp130, and the expression of LacdiNAc is required for the induction and maintenance of the naïve state in pluripotent stem cells (Fig. 4.3b).

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