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The Function of Glycan Structures for the Maintenance and Differentiation of Embryonic Stem Cells

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1. Introduction

Various types of glycoprotein and glycolipid are present on the cell surface and function to regulate cell-cell interactions, cell-extracellular matrix interactions, and signals from extrinsic factors, for instance, Wnt, fibroblast growth factor (FGF), Hedgehog (Hh) and bone morphogenetic protein (BMP). The glycan structures on proteins and lipids change dramatically during differentiation. Some of these glycoproteins and glycolipids can be used as markers for the identification of embryonic stem cells (ES cells), such as stage-specific embryonic antigen-1 (SSEA-1) (Atwood et al., 2008; Muramatsu & Muramatsu, 2004), SSEA-3, TRA-1-60 antigen and TRA-1-81 antigen (Adewumi et al., 2007).

ES cells were originally isolated from the inner cell mass (ICM) of blastocysts and have the essential characteristics of pluripotency and self-renewal. Pluripotency enables the cells to differentiate into all the cell types that constitute the adult body. In 1981, the first mouse ES cell lines were established, and these have proved invaluable as a tool for gene-targeting strategies in mice (Evans & Kaufman, 1981; Martin, 1981). Since the establishment of human ES cell lines in 1998, they have been used in a large number of research studies looking at potential applications for regenerative medicine (Thomson et al., 1998). Thus, ES cells are promising tools for biotechnology and possess key features that should allow their exploitation in the development of cell replacement therapies. To exploit the potential of ES cells for these various purposes, a better understanding of the molecular mechanisms that control self-renewal and pluripotency and also direct differentiation of ES cells is required. Several signaling cascades activated by extrinsic factors such as leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988), BMP (Ying et al., 2003) and Wnt (Miyabayashi et al., 2007; Sasaki et al., 2008; Sato et al., 2004) and the expression of intrinsic factors, such as Oct3/4 and Nanog, maintain self-renewal and pluripotency in ES cells (Boiani & Scholer, 2005). Likewise, Wnt, FGF, Hh and BMP signaling also play key roles in the determination of cell fate during the differentiation of ES cells (Kunath et al., 2007; Sasaki et al., 2009).

Proteoglycans are one form of sulfated glycoprotein and consist of several different types of core protein and glycosaminoglycans (sulfated glycans). There are two types of glycosaminoglycan: heparan sulfate (HS) and chondroitin sulfate (CS). They are ubiquitously present on the surface of many different types of cell and are known to play crucial roles in regulating several signaling pathways (Bishop et al., 2007). A large number

of physiologically important molecules can bind to specific sulfated regions of HS and CS. Genetic analyses using *Drosophila* have demonstrated that HS is involved in signal regulatory pathways that respond to extrinsic factors, such as Wnt, FGF, Hh and BMP (Yan & Lin, 2009). On the other hand, self-renewal and pluripotency of ES cells are maintained by a balance among several signaling pathways, such as Wnt, FGF and BMP, and the differentiation of ES cells into a specific lineage is induced by disruption of this balance. Through these signals, HS and CS contribute to the maintenance and differentiation of ES cells (Kraushaar et al., 2010; Lanner et al., 2010; Sasaki et al., 2009; Sasaki et al., 2008). The aims of this chapter are (1) to describe the use of carbohydrate antigens as markers of ES cells, (2) to consider the function of HS in the maintenance of self-renewal and pluripotency of ES cells, and (3) to outline the function of HS and CS in the differentiation of ES cells.

2. Carbohydrate antigens can be used as markers of ES cells

Mouse ES cells express SSEA-1, also known as Lewis X carbohydrate antigen (LeX), which has the structure Galβ1,4(Fucα1,3)GlcNAc. It is found on both glycoproteins and glycolipids (Atwood et al., 2008; Muramatsu & Muramatsu, 2004). SSEA-1 is a marker of mouse ES cells and use of an anti-SSEA-1 antibody enables positive staining of ES cells and of the ICM, the origin of mouse ES cells.

However, human ES cells do not express SSEA-1. Instead, they express SSEA-3, SSEA-4, TRA-1-60 antigen and TRA-1-81 antigen, and these can be used as specific markers for human ES cells (Adewumi et al., 2007). The carbohydrate structures of SSEA-3 and SSEA-4 are R-3GalNacβ1,3Galα1,4R' and NeuAcα2,3Galβ1,3GalNacβ1,3Galα1,4R', respectively, and they are carried on globo-series glycolipids (Kannagi et al., 1983). Both TRA-1-60 antigen and TRA-1-81 antigen are present on keratan sulfate, a sulfated poly-*N*-acetyllactosamine. The TRA-1-60 epitope, but not that of TRA-1-81, includes sialic acid (Badcock et al., 1999). These carbohydrate structures are also expressed in the human ICM. Recently, a glycome analysis of *N*-linked glycans on human ES cells reported abundant expression of LeX and H type 2 antennae in sialylated complex-type *N*-linked glycans (Satomaa et al., 2009). LeX would not be recognized by an anti-SSEA-1 antibody when it is presented on a biantennary *N*-glycan antenna. However, the biological functions of these cell surface markers have not been fully elucidated.

3. Heparan sulfate (HS) is mainly expressed on mouse ES cells, and both HS and chondroitin sulfate (CS) increase during differentiation of embryoid bodies (EBs)

In comparison to other glycosaminoglycans (sulfated glycans), HS is highly expressed on mouse ES cells (Fig. 1A and B) (Nishihara, 2009; Sasaki et al., 2009; Sasaki et al., 2008). For example, the ratio of HS to CS is almost 5:1. Thus, the main glycosaminoglycan expressed on mouse ES cells is HS. However, the amounts of HS and CS on the cell surface increase more than five-fold during differentiation from ES cells to EBs (Fig. 2B) (Nairn et al., 2007). Therefore, it is expected that HS will be the principal contributor to the maintenance of mouse ES cells and that CS, as well as HS, will contribute to the differentiation of mouse ES cells.

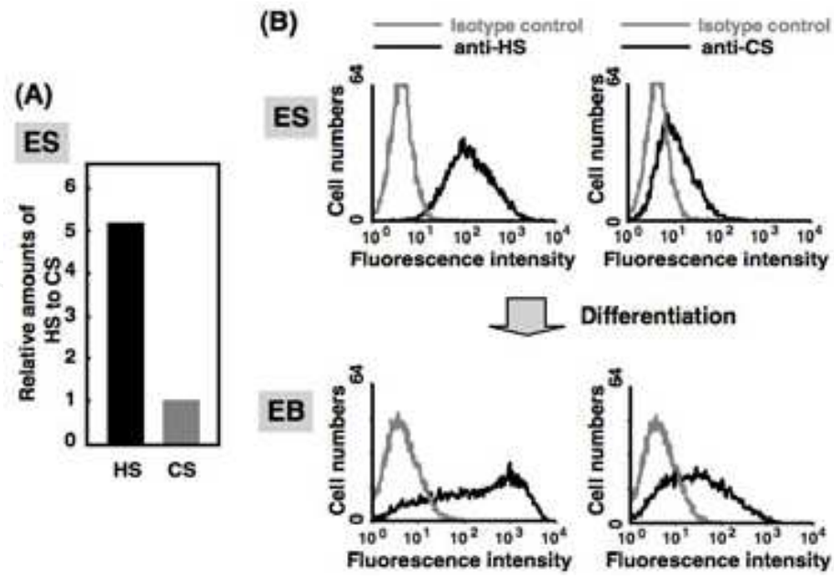

Fig. 1. The expression of heparan sulfate (HS) and chondroitin sulfate (CS) on mouse ES cells and embryoid bodies (EBs).
(A) The relative amounts of HS and CS in mouse ES cells. HS is approximately 5 times more abundant in mouse ES cells than CS. The histograms show the outcome of an HPLC analysis for unsaturated disaccharides.
(B) HS and CS on mouse ES cells and EBs. A FACS analysis of mouse ES cells and EBs using an anti-HS antibody (HepSS-1) and anti-CS antibody (2H6) shows that HS is more highly expressed on mouse ES cells and that both HS and CS increase during differentiation of EBs from ES cells.

4. Heparan sulfate (HS) and chondroitin sulfate (CS) are synthesized in the Golgi apparatus

Both HS and CS are synthesized in the Golgi apparatus by a series of glycosyltransferases and sulfotransferases (Fig. 2A). PAPS is a donor substrate for sulfotransferases**;** it is synthesized in the cytosol by PAPS synthases, and is translocated into the Golgi apparatus by the PAPS transporters, PAPST1 and PAPST2 (Goda et al., 2006; Kamiyama et al., 2006; Kamiyama et al., 2003). Therefore, if the expression of PAPS transporters is regulated, then sulfation of both HS and CS can be regulated (Sasaki et al., 2009).
HS has repeating disaccharide units of D-glucuronic acid-*N*-acetyl-D-glucosamine (GlcA-GlcNAc) that are modified differentially by epimerization and sulfation (Fig. 2B). The disaccharide repeats are synthesized by members of the EXT protein family, including EXT1, and sulfated by a series of sulfotransferases (Bishop et al., 2007). The first step in this series of sulfation reactions is catalyzed by *N*-deacetylase/*N*-sulfotransferase (NDST). Of the four known *NDSTs*, *NDST1* and *NDST2* are expressed in mouse ES cells (Nairn et al., 2007). Therefore, if we regulate the expression of *EXT1, NDST1* and *NDST2*, it should be possible to regulate elongation of the HS chain and HS-specific sulfation (Sasaki et al., 2009; Sasaki et al., 2008). A large number of physiologically important molecules can bind to specific sulfated regions of HS (Fig. 2A). As mentioned above, genetic analyses using *Drosophila* have demonstrated that HS is involved in signal regulatory pathways responding

to extrinsic factors, such as FGF, Wnt, Hh and BMP (Tabata & Takei, 2004; Ueyama et al., 2008).

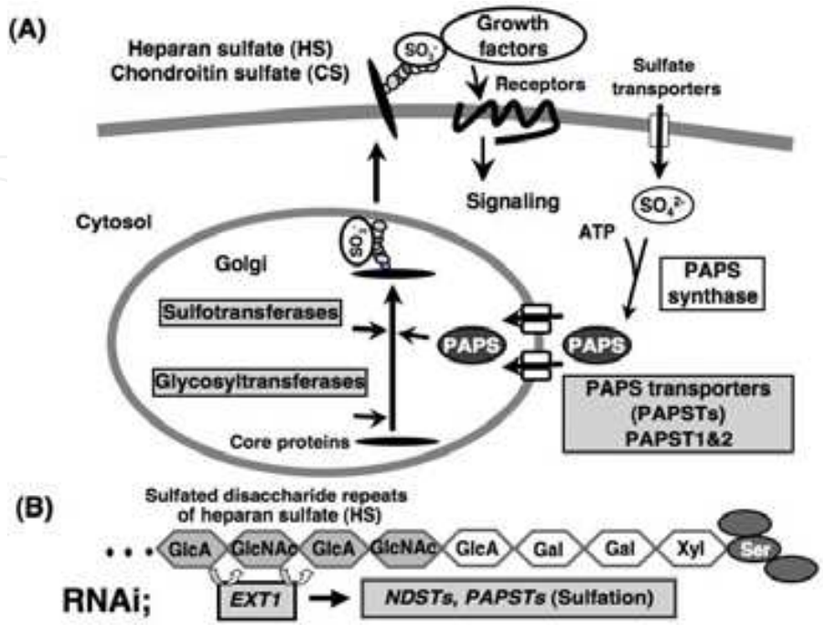

Fig. 2. Outline of the synthetic pathways for heparan sulfate (HS) and chondroitin sulfate (CS) in Golgi apparatus.
(A) HS and CS are synthesized and sulfated in the Golgi apparatus.
(B) A diagrammatic representation of the structure of HS and the role of EXT1, NDST and PAPSTs in its synthesis. The major components of HS are sulfated disaccharide repeats that are covalently bound to Ser residues of specific core proteins through the glycosaminoglycan-protein linkage region GlcAβ1,3Galβ1,3Galβ1,4Xyl-*O*-Ser. (GlcA, D-glucuronic acid; Gal, galactose; Xyl, xylose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine).

We analyzed the function of HS and sulfation of HS and CS by using RNA interference (RNAi) to knockdown (KD) *EXT1*, *NDST, PAPST1* and *PAPST2* (Fig. 2B) (Sasaki et al., 2009; Sasaki et al., 2008). Although the KD efficiency was less than 100%, we used this method rather than performing gene knockouts (KO) because, in addition to the direct effects of gene knockouts, secondary effects may also be observed that are caused by adaptation of the cells during long-term culture. For example, the expression of a novel gene might be induced that has secondary effects on the mouse ES cells. If the targets are essential for cell survival and proliferation, analysis of the knockout cells may be complicated by cell death. Thus, knockout of some genes that are related to HS sulfation, e.g., *6-O-endosulfatase*, *C5-epimerase* and *HS2ST*, leads to a number of unexpected changes in the structure of sulfated glycosaminoglycans, presumably due to secondary effects (Lamanna et al., 2006; Li et al., 2003; Merry et al., 2001). By contrast, RNAi knockdown of gene expression gave a specific effect for each gene. For instance, knockdown of *EXT1* expression in mouse ES cells resulted in the shortening of the HS chain (Sasaki et al., 2008); knockdown of *NDST1* and *NDST2* specifically reduced HS sulfation (Sasaki et al., 2009); and knockdown of *PAPST1* or *PAPST2* reduced both HS and CS sulfation (Sasaki et al., 2009).

5. Heparan sulfate (HS) contributes to the maintenance of self-renewal and pluripotency of mouse ES cells

5.1 Wnt, bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) signals work on the maintenance of self-renewal and pluripotency in mouse ES cells, while fibroblast growth factor 4 (FGF4) works on the exit from the undifferentiated ground state

As described above, self-renewal and pluripotency of mouse ES cells are maintained by several signaling cascades from both extrinsic factors, such as LIF (Smith et al., 1988; Williams et al., 1988), BMP (Ying et al., 2003) and Wnt (Miyabayashi et al., 2007; Sasaki et al., 2008; Sato et al., 2004), and intrinsic factors, such as Oct3/4 and Nanog (Boiani & Scholer, 2005).

In mouse ES cells, LIF molecules interact with the heteromeric receptor gp130 and the low affinity LIF receptor to induce activation of STAT3 (Boeuf et al., 1997; Matsuda et al., 1999; Niwa et al., 1998; Raz et al., 1999) and then upregulate the expression level of Myc (Cartwright et al., 2005). BMP induces the expression of inhibitor of differentiation (*Id*) genes, which suppress expression of genes for neural differentiation (Ying et al., 2003), through activation of Smad signaling. Thus, BMP suppresses neural differentiation and, in combination with LIF, is sufficient to maintain self-renewal of mouse ES cells without feeder cells and serum factors.

In contrast, Wnt signals play a role in the regulation of self-renewal of both mouse and human ES cells independently o**f** LIF/STAT3 signaling (Miyabayashi et al., 2007; Sasaki et al., 2008; Sato et al., 2004). The binding of Wnt to its cognate receptor, Frizzled, results in the inhibition of glycogen synthase kinase-3β (GSK3β). In turn, inhibition of GSK3β allows the stabilization and accumulation of β-catenin in the nucleus that is required for transcription of downstream genes. The canonical Wnt pathway maintains the expression of downstream Nanog, a transcription factor that is essential for the maintenance of the ICM and of ES cell pluripotency (Cole et al., 2008; Miyabayashi et al., 2007; Sasaki et al., 2008; Sato et al., 2004). The activation of Nanog sustains ES cell self-renewal without feeder cells or treatment with LIF (Sato et al., 2004).

On the other hand, FGF4 is produced in an autocrine fashion in mouse ES cells and functions in the exit from the undifferentiated ground state (Kunath et al., 2007; Ying et al., 2008). FGF4/extracellular signal-regulated kinase (ERK) signaling contributes to differentiation into neural and mesodermal lineages. However, the mechanism that regulates extrinsic signaling in ES cells has not been fully elucidated.

5.2 Heparan sulfate (HS) and its sulfation are important for self-renewal, pluripotency and proliferation of mouse ES cells

In order to analyze the function of HS and its sulfation in mouse ES cells, we constructed siRNA expression plasmids that targeted *EXT1*, *NDST1*, *NDST2*, *PSPST1* or *PAPST2* (Fig. 2A) by inserting the corresponding short hairpin RNA (shRNA) sequence into pSilencer 3.1-H1 vector, which has a PolIII promoter and a puromycin resistance gene. The construct was then transfected into mouse ES cells. After puromycin selection, we confirmed the specific knockdown of the targeted gene and the reduction of its product. Knockdown (KD) of *EXT1* expression inhibited the elongation of HS chains and resulted in a reduction in the size (~35kDa) of the HS chain compared to control cells (50~150 kDa) (Sasaki et al., 2008). Knockdown of *NDST1* or *NDST2* specifically reduced sulfation of HS. Knockdown of *PAPST1* or *PAPST2* reduced sulfation of both HS and CS (Sasaki et al., 2009; Sasaki et al., 2008).

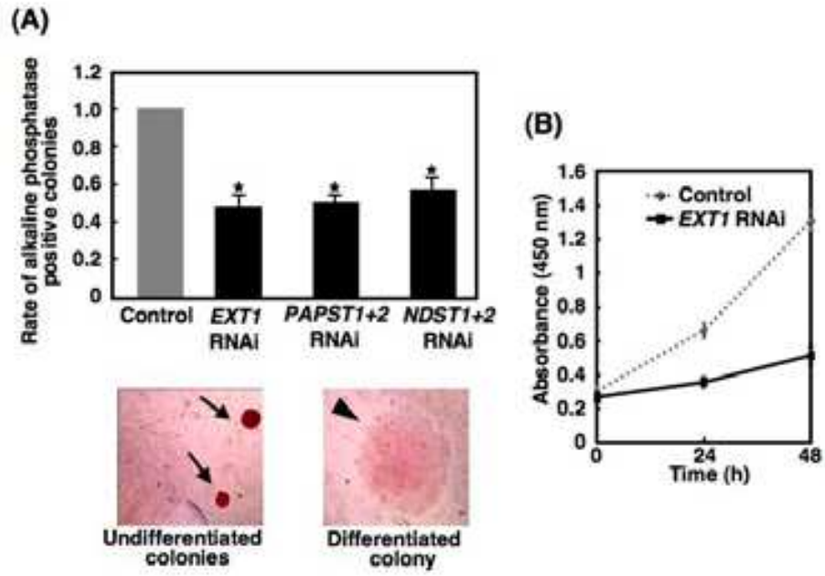

Fig. 3. The effect of HS and its sulfation on self-renewal and proliferation in mouse ES cells.
(A) Self-renewal assay. In *EXT1*-KD ES cells, the number of AP positive (undifferentiated) colonies was reduced to 50% of that of control cells, even in the presence of LIF and serum. *PAST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells also showed a reduction in the number of AP positive colonies. The rate of AP positive colonies is shown after normalization against control cells (value=1).
(B) Proliferation assay. The rate of proliferation of *EXT1*-KD cells decreased significantly compared to control cells.
The values shown are the means ± SD from three independent experiments and significant values are indicated; *P < 0.01, in comparison to control cells.

In *EXT1*-KD ES cell cultures, the number of alkaline phosphatase (AP) positive colonies, indicative of the undifferentiated state, fell to 50% of that in control cultures, even in the presence of LIF and serum (Fig. 3A). *PAST1&2*-double KD ES cells and *NDST&2*-double KD ES cell cultures also showed a reduction in the number of AP positive colonies. *EXT1-KD* ES cells showed a reduction in their rate of proliferation (Fig. 3B), as did *PAST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells (data not shown). Our analyses clearly demonstrated that HS, and in particular its sulfation, has a significant role in the self-renewal and proliferation of mouse ES cells (Sasaki et al., 2009; Sasaki et al., 2008).
Four days after *EXT1* knockdown, even in the presence of LIF, *EXT1*-KD ES cells displayed a flattened and differentiated morphology reminiscent of the stellate appearance of parietal endoderm cells (Fig. 4A). *PAPST1* or *PAPST2*-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells showed a similar morphology to the *EXT1*-KD ES cells. Real time PCR analysis for various germ layer markers showed that expression of Oct3/4 and Nanog, undifferentiated state markers, was significantly decreased in the *EXT1*-KD ES cells (Fig. 4B). All of the other types of KD-ES cells also showed reduced expression of Oct3/4 and Nanog. After withdrawal of LIF to allow further differentiation, *EXT1*-KD ES cells and the other KD-ES cells showed increased expression of markers of the extraembryonic endoderm lineage compared to control cells, and lost pluripotency (Fig. 4B).

These results indicate that HS, and in particular sulfation of HS, has a role in the maintenance of pluripotency in mouse ES cells (Sasaki et al., 2009; Sasaki et al., 2008).

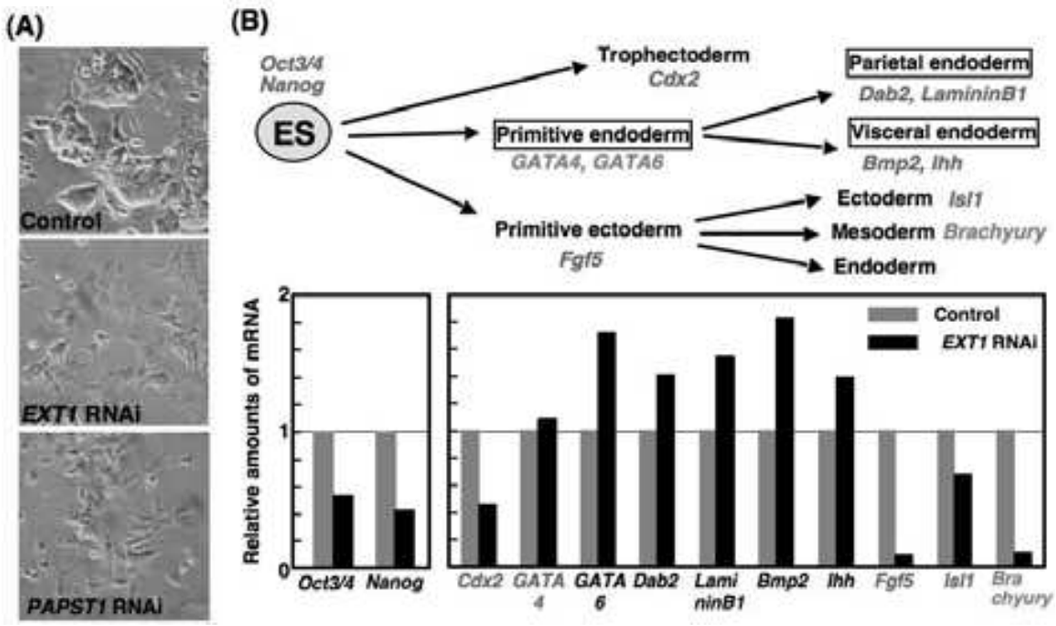

Fig. 4. Reduction in HS and in its sulfation induce mouse ES cells to spontaneously differentiate into extraembryonic endoderm cells.
(A) Photomicrographs of cells at four days after transfection with an siRNA expression plasmid vectors targeting *EXT1* and *PAPST1* in the presence of LIF. Almost all of the *EXT1*-KD ES cells and *PAPST1*-KD ES cells exhibited a flattened, differentiated morphology. Similar results were obtained by single *PAPST1* or *PAPST2* knockdown, *PAPST1&2* double knockdown and *NDST1&2* double knockdown.
(B) Real time PCR analysis of several differentiation markers four days after transfection in the presence of LIF. Oct3/4 and Nanog expression was significantly decreased in *EXT1*-KD cells compared to control cells. Increased expression of markers of the extraembryonic endoderm lineage (Gata4, Gata6, Dab2, Laminin B1, Bmp2 and Ihh) was observed in *EXT1*-KD cells. The results are shown after normalization against control cells (value=1). Similar results were obtained by *PAPST1* or *PAPST2* knockdown, *PAPST1&2* double knockdown and *NDST1&2* knockdown.

5.3 Heparan sulfate (HS) and its sulfation on mouse ES cells regulate BMP/Smad signaling

HS and its sulfation play a role in the self-renewal and pluripotency of mouse ES cells (see section 5.2 above). Various extrinsic factors, such as BMP, Wnt and LIF, affect the maintenance of self-renewal and pluripotency in mouse ES cells (Sasaki et al., 2008; Sato et al., 2004; Smith et al., 1988; Williams et al., 1988; Ying et al., 2003), (and see section 5.1 above). BMP4 acts in synergy with LIF to maintain self-renewal via the Smad-mediated induction of *Id* (inhibitor of differentiation) gene expression (Ying et al., 2003) and inhibition of p38 mitogen-activated protein kinase (Qi et al., 2004). We therefore examined the level of

phosphorylation of Smad1 in *EXT1*-KD ES cells to determine whether BMP/Smad1 signaling was altered in these KD ES cells.

No reduction in the level of phosphorylation of Smad1 could be detected in *EXT1*-KD ES cells compared to control cells (Fig. 5A), suggesting that even a short HS chain can bind to BMP4 and contribute to BMP4 signaling (Sasaki et al., 2008). However, *PAPST1* or *PAPST2*-KD ES cells, *PAPT1&2*-double KD ES cells and *NDSTT1&2*-double KD ES cells show

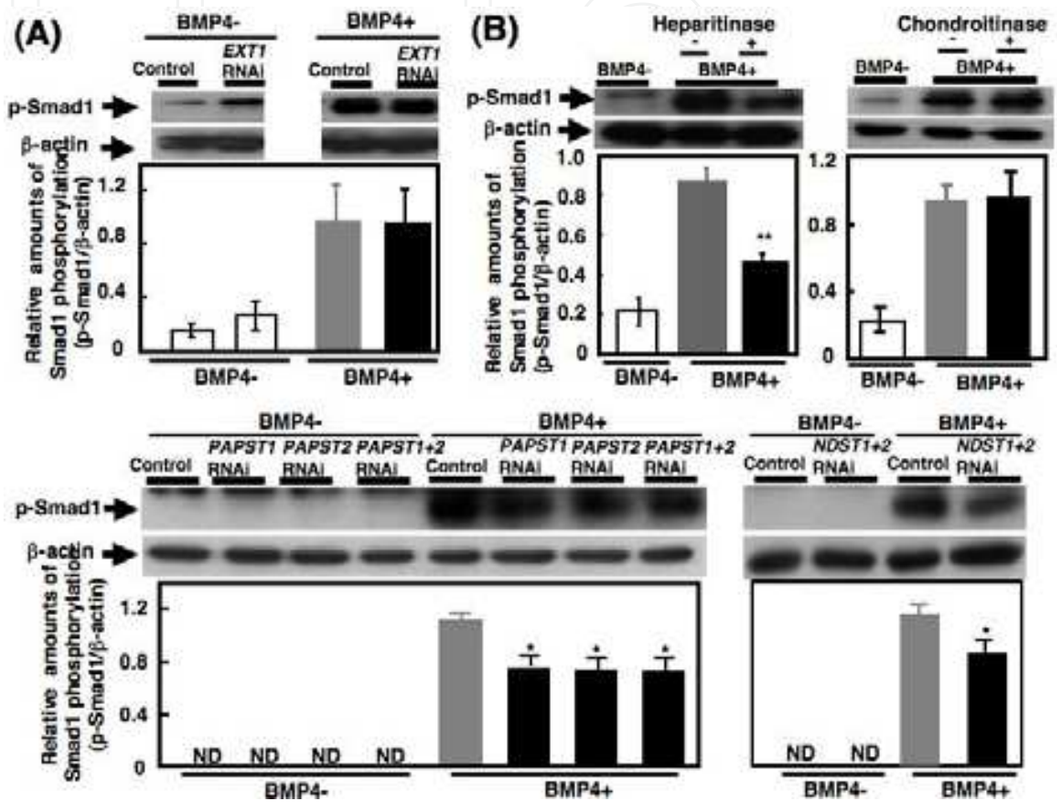

Fig. 5. HS and its sulfation regulate BMP/Smad signaling.

(A) Western blot analysis of *EXT1*-KD ES cells, *PAPST1* or *PAPST2*-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells stimulated with BMP4. The stimulation of *EXT1*-KD and control cells with BMP4 gave rise to similar increases in Smad1 phosphorylation. But *PAPST1* or *PAPST2*-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells showed reduction of phosphorylated Smad1. The histograms show mean densitometric readings ± SD of the phosphorylated Smad1/loading controls. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.05 in comparison to control cells.

(B) Western blot analysis of mouse ES cells stimulated with BMP4 after treatment with heparitinase and chondroitinase. HS-depletion by heparitinase treatment led to a reduction in BMP/Smad signaling while CS-depletion by chondroitinase treatment did not. The histograms show mean densitometric readings ± SD of the phosphorylated Smad1/loading controls. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; **P < 0.01, in comparison to untreated control cells.

significantly reduced levels of Smad1 phophorylation demonstrating that HS, and in particular sulfation of HS, is important for BMP/Smad signaling (Sasaki et al., 2009). A significantly reduced signal was also observed after heparitinase treatment while no reduction was observed after chondroitinase treatment (Fig. 5B). Thus, HS and sulfation of HS (but not of CS) on the surface of mouse ES cells regulate BMP/Smad signaling to maintain self-renewal and pluripotency.

In a similar fashion, we analyzed LIF signaling in *EXT1*-KD ES cells and in other KD ES cells, but we did not observe any reduction in signal intensity, indicating that HS and its sulfation on the cell surface does not contribute to LIF/STAT signaling (Sasaki et al., 2009; Sasaki et al., 2008).

5.4 Heparan sulfate (HS) and its sulfation on mouse ES cells regulate autocrine/paracrine Wnt/β-catenin signaling

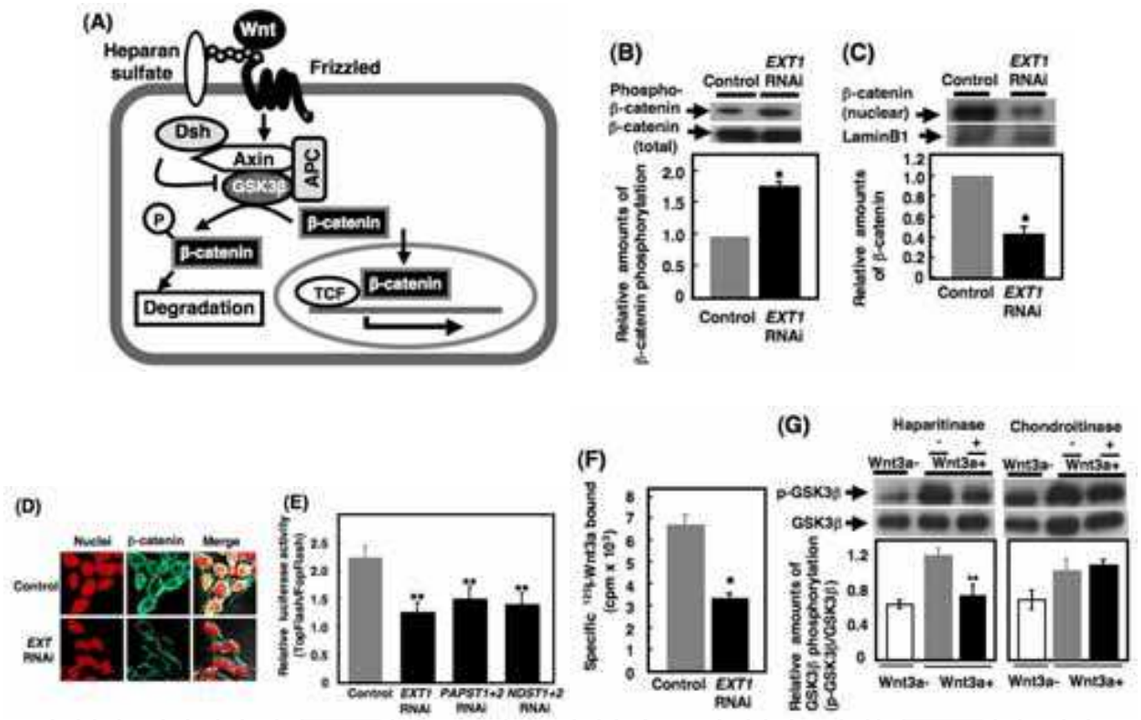

Fig. 6. HS, and its sulfation, regulates autocrine/paracrine Wnt/β-catenin signaling.
(A) Schematic diagram of the Wnt/β-catenin signaling pathway.
(B) Western blot analysis of *EXT1*-KD ES cells starved of LIF and serum for 4hr. In *EXT1*-KD cells, a significant increase in β-catenin phosphorylation was observed. The histograms show mean densitometric readings ± SD of the phospho-β-catenin/total β-catenin after normalization against control cells (value=1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.01, in comparison to control cells.
(C) Western blot analysis of nuclear extracts of *EXT1*-KD ES cells. The histograms show mean densitometric readings ± SD of the β-catenin/Lamin B_1 after normalization against control cells (value=1). Values are obtained from duplicate measurements of two

independent experiments and significant values are indicated; *P < 0.01, in comparison to control cells.
(D) Confocal images of *EXT1*-KD ES cells. Accumulation of β-catenin was significantly decreased in nuclei of *EXT1*-KD cells. (β-catenin, green; nucleus, red; merged image of β-catenin and nucleus, yellow).
(E) Luciferase reporter assay. The autocrine/paracrine luciferase activity was significantly decreased in *EXT1*-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells. Relative luciferase activities (TopFlash/FopFlash) are shown as means ± SD from three independent experiments, and significant values are indicated; **P < 0.05, in comparison to control cells.
(F) Binding assay of ^{125}I-labeled Wnt3a to *EXT1*-KD ES cells. *EXT1*-KD ES cells exhibited significantly lower specific ^{125}I-Wnt3a binding. The value of specific ^{125}I-Wnt3a binding (total cpm minus cpm bound in the presence of 100 μg/ml free heparin) was the mean ± SD of three independent experiments, and significant values are indicated; *P < 0.01, in comparison to control.
(G) Western blot analysis of mouse ES cells stimulated with Wnt3a after treatment with heparitinase and chondroitinase. HS-depletion by heparitinase treatment reduced the GSK3β in response to Wnt3a while CS-depletion by chondroitinase treatment did not. The histograms show mean densitometric readings ± SD of the phosphorylated GSK3β/GSK3β. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; **P < 0.01, in comparison to untreated control cells.

We next examined the potential influence of HS on the Wnt signaling pathway (Fig. 6A) using various gene-KD ES cells, including *EXT1*-KD ES cells. The culture medium included BMP, a serum component, and LIF, but did not include Wnt. As a first step, we performed an RT-PCR of mouse ES cells for Wnt expression and found that several Wnts, such as Wnt2, Wnt3a, Wnt4, Wnt5a, Wnt7a and Wnt10b, were expressed (Sasaki et al., 2008).
A Western blot analysis for β-catenin, which transmits Wnt signals, showed that there was a significantly higher level of phosphorylation of β-catenin in *EXT1*-KD cells than control cells in the absence of feeder cells (Fig. 6B) (Sasaki et al., 2008). We also observed significant reductions in the level of β-catenin in the nucleus (Fig. 6C and D) and of luciferase reporter activity (Fig. 6E). These results demonstrate that autocrine/paracrine Wnt/β-catenin signaling is reduced in *EXT1*-KD ES cells, indicating that HS contributes to Wnt/β-catenin signaling. In addition to *EXT1*-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells showed a reduction in luciferase reporter activity, also indicating that sulfation of HS plays a key role in Wnt/β-catenin signaling (Sasaki et al., 2009).
Next, we analyzed the binding of ^{125}I-Wnt3a and found a lower level of binding on *EXT*1-KD ES cells compared to control cells (Fig. 6F) (Sasaki et al., 2008). Thus, HS contributes to the binding of Wnt3a to the mouse ES cell surface. We also analyzed phosphorylation of GSK3β after heparitinase or chondroitinase treatment and observed a reduction in level of GSK3β phosphorylation only after HS-depletion by heparitinase treatment and not after CS-depletion by chondroitinase treatment (Fig. 6G) (Sasaki et al., 2009). This finding confirms that HS, but not CS, contributes to Wnt/β-catenin signaling. Our results demonstrate that HS and its sulfation regulates autocrine/paracrine Wnt/β-catenin signaling in mouse ES cells by enhancing the binding of Wnt to its cognate receptor, Frizzled.

5.5 Heparan sulfate (HS) and its sulfation on mouse ES cells regulate FGF4/ERK signaling

HS and its sulfation play a role in the self-renewal and pluripotency of mouse ES cells (see section 5.2). Extrinsic factors that affect the maintenance of self-renewal and pluripotency in mouse ES cells are affected by HS; this has been shown for BMP (see section 5.3) and Wnt (see section 5.4), although does not apply to LIF (see section 5.3) (Sasaki et al., 2008; Sato et al., 2004; Smith et al., 1988; Williams et al., 1988; Ying et al., 2003). In contrast to these extrinsic factors, FGF4 works on the exit from the undifferentiated ground state (Kunath et al., 2007). Mouse ES cells express FGF1, bFGF(FGF2), FGF4, FGF8, FGF9, FGF10, GHG15, FGF18 and FGFR1~4 (Sasaki et al., 2009). Thus FGF4 is produced in an autocrine fashion in mouse ES cells and is known to bind to HS (Sugaya et al., 2008). Therefore, we analyzed FGF4 signaling in gene–KD mouse ES cells showing reduction in HS and in its sulfation.

The level of phosphorylation of extracellular signal-regulated kinase (ERK) following treatment with FGF4 was significantly reduced in *PAPST1*-KD or *PAPST2*-KD ES cells, *PAPT1&2*-double KD ES cells and *NDSTT1&2*-double KD ES cells, compared to control cells (Fig.7A) (Sasaki et al., 2009). Moreover, an even larger reduction in ERK phosphorylation was observed after treating mouse ES cells with heparitinase, which digests HS on the surface of the cells, while no reduction was observed after chondroitinase treatment (Fig. 7B). These observations indicate that HS and its sulfation, but not CS, on the surface of ES cells regulate FGF4/ERK signaling.

5.6 Heparan sulfate (HS) and its sulfation on mouse ES cells regulate bFGF/ERK signaling

In addition to FGF4 (see section 5.5), bFGF (FGF2) is produced in an autocrine fashion in mouse ES cells and is known to bind to HS (Sugaya et al., 2008). Therefore, we analyzed bFGF signaling in various HS-related gene–KD mouse ES cells. The level of phosphorylation of extracellular signal-regulated kinase (ERK) following treatment with bFGF was significantly reduced in all of the HS related gene–KD mouse ES cells which were tested, compared to control cells (Fig. 8A) (Sasaki et al., 2009; Sasaki et al., 2008). Moreover, a larger reduction in ERK phosphorylation was observed after treating mouse ES cells with heparitinase, while no reduction was observed after chondroitinase treatment (Fig. 8B). These observations indicate that HS and its sulfation, but not CS, on the surface of ES cells regulate bFGF/ERK signaling.

To date, LIF, Activin/Nodal and bFGF have been reported to contribute to mouse ES cell proliferation (Dvorak et al., 1998; Ogawa et al., 2007; Smith et al., 1988; Williams et al., 1988). It is known that FGF signaling mediated by HS, contributes to the proliferation of various types of cell (Lin, 2004). Furthermore, the proliferation of mouse ES cells treated with SU5402, an inhibitor of FGFR1 tyrosine phosphorylation, was reduced compared to that of control cells (Fig. 8C), demonstrating that autocrine/paracrine FGF signaling mediated by FGFR1 contributes to mouse ES cell proliferation (Sasaki et al., 2009). Therefore, the above results suggest that the reduced proliferation of *EXT1*-KD cells, as well as other HS related gene–KD mouse ES cells (Fig. 3B), is due to a reduction in autocrine/paracrine bFGF signaling, which in turn is caused by reduced HS chain sulfation (Fig. 8A and B).

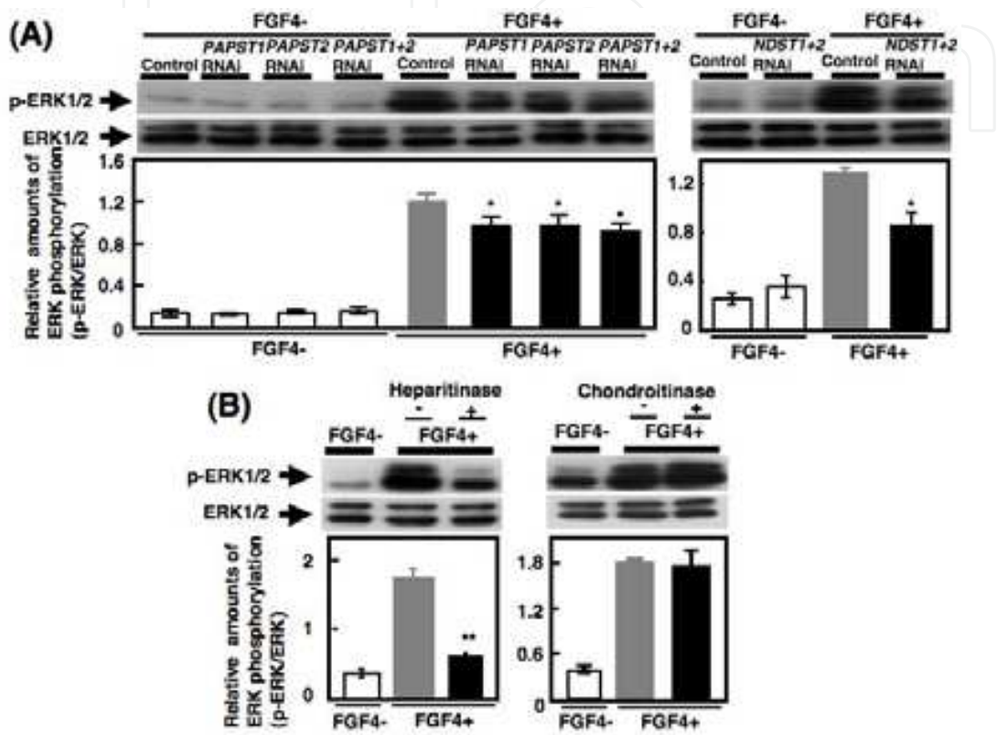

Fig. 7. HS and its sulfation regulate FGF4/ERK signaling.
(A) Western blot analysis of *PAPST1*-KD or *PAPST2*-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells stimulated with FGF4. All of the gene-KD cells showed significant reductions in ERK phosphorylation. The histograms show mean densitometric readings ± SD of phosphorylated ERK/ERK. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.05 in comparison to control cells.
(B) Western blot analysis of mouse ES cells stimulated with FGF4 after treatment with heparitinase and chondroitinase. HS-depletion by heparitinase treatment led to a reduction in FGF4/ERK signaling while CS-depletion by chondroitinase treatment did not. The histograms show mean densitometric readings ± SD of phosphorylated ERK/ERK. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; **P < 0.01, in comparison to untreated control cells.

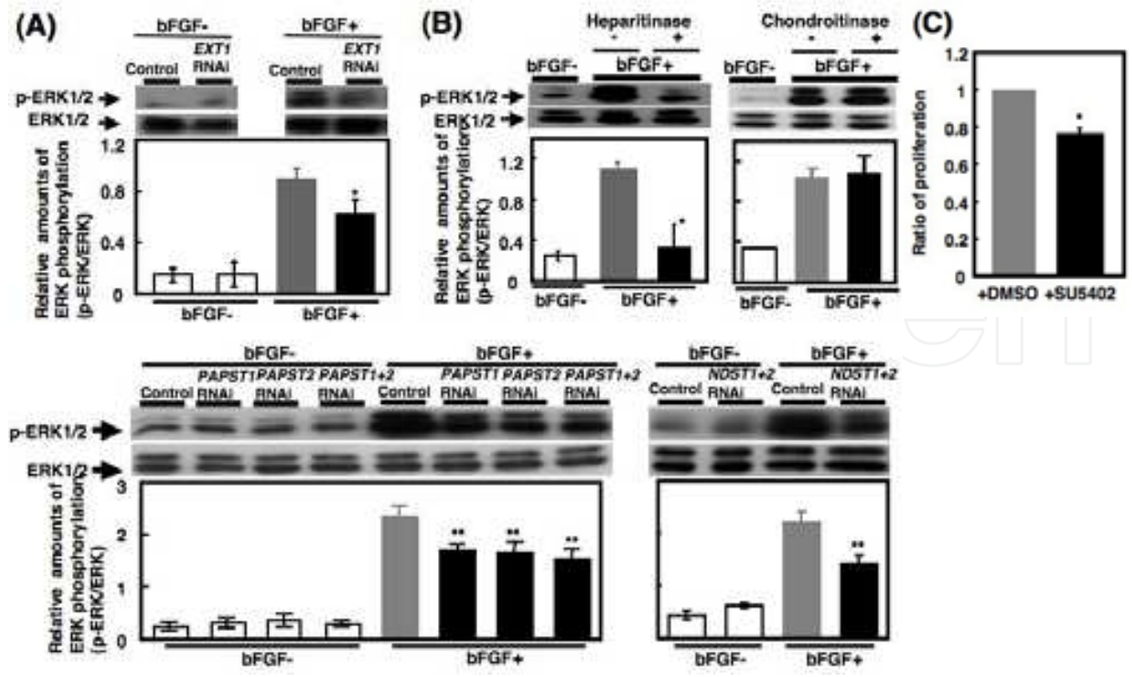

Fig. 8. HS and its sulfation regulate bFGF/ERK signaling.
(A) Western blot analysis of *EXT1*-KD ES cells, *PAPST1*-KD or *PAPST*2-KD ES cells, *PAPST1&2*-double KD ES cells and *NDST1&2*-double KD ES cells stimulated with bFGF. All of the gene-KD ES cells showed significant reduction in ERK phosphorylation. The histograms show mean densitometric readings ± SD of phosphorylated ERK/ERK. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.01 and **P < 0.05 in comparison to control cells.
(B) Western blot analysis of mouse ES cells stimulated with bFGF after treatment with heparitinase and chondroitinase. HS-depletion by heparitinase treatment led to a reduction in bFGF/ERK signaling, while CS-depletion by chondroitinase treatment did not. The histograms show mean densitometric readings ± SD of the phosphorylated ERK/ERK. Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.01, in comparison to untreated control cells.
(C) Proliferation assay of mouse ES cells treated with SU5402. The rate of proliferation after 48h of culture is shown; the values were normalized against those of DMSO-treated cells (value=1). The values shown are the means ± SD from three independent experiments and significant values are indicated; *P < 0.01, in comparison to DMSO-treated cells.

5.7 HS and its sulfation contribute to the maintenance of mouse ES cells by regulating the balance between Wnt, FGF4 and BMP4 signaling

In undifferentiated mouse ES cells, FGF4 signal, which is the key signal for the exit from the undifferentiated ground state and the initiation of differentiation (Kunath et al., 2007; Ying et al., 2008), is inhibited by BMP4 (Qi et al., 2004). Thus, differentiation is inhibited. Wnt also upregulates Nanog expression and maintains the capacity for self-renewal(Cole et al., 2008; Miyabayashi et al., 2007; Sasaki et al., 2008; Sato et al., 2004).
In HS-related gene-KD ES cells, and in cells with reduction of HS or its sulfation, the FGF4 signal is reduced but is still present. As a consequence, inhibition of the FGF4 signal by

BMP4 is reduced, and also the upregulation of Nanog is reduced (Sasaki et al., 2009; Sasaki et al., 2008). Therefore, differentiation is induced in HS-related gene-KD ES cells. In a similar manner, HS and its sulfation, but not CS, contribute to the maintenance of mouse ES cells by regulating the balance between Wnt, FGF4 and BMP4 signaling.

Recently, HS-null mouse ES cells (*EXT1-knock out* (KO) ES cells) were reported to show no defects in pluripotency (Johnson et al., 2007), but failed to transit from the self-renewal state to the initiation of differentiation following the removal of LIF (Kraushaar et al., 2010). In addition, *NDST1&2* double-KO ES cells fail to initiate differentiation but adopt a more naive pluripotent Nanog/KLF4/Tbx3 positive state due to the reduction of FGF4/ERK signaling (Lanner et al., 2010). In these cases, complete depletion of HS completely shuts down the induction of differentiation due to the total depletion of FGF4 signaling; these KO ES cells stay in the undifferentiated ground state.

6. Sulfation of heparan sulfate (HS) and chondroitin sulfate (CS) contribute to the differentiation of mouse ES cells

6.1 Reduced sulfation induces abnormal differentiation into three germ layers during embryoid body (EB) formation in mouse ES cells

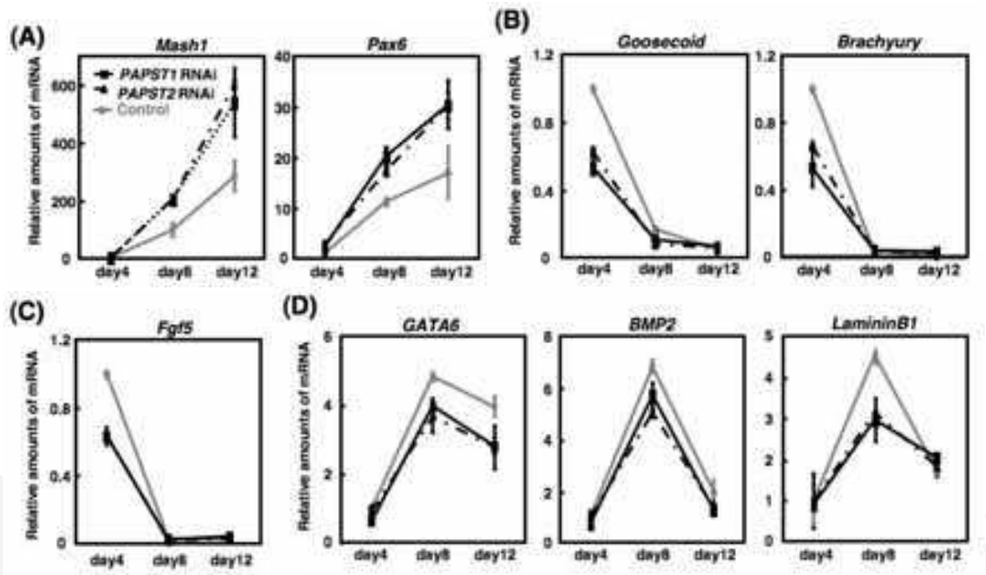

Fig. 9. Abnormal differentiation was observed in *PAPST1*-KD or *PAPST2*-KD cells during EB formation. EB formation was induced by transferring the cells to low cell binding dishes and culturing in ESC medium without LIF.

(A)-(D) Real time PCR analysis of germ layer markers at 4, 8 and 12 days after EB formation (A, neurectoderm marker; B, mesoderm marker; C, primitive ectoderm marker; D, extraembryonic endoderm (ExE) marker). The results are shown after normalization against the values obtained with control EBs on day 4 (value=1). The values shown are the means ± SD of two independent experiments.

Several extrinsic factors, such as BMP, FGF and Wnt, play important roles in the differentiation of mouse ES cells, in addition to their involvement in self-renewal (Loebel et al., 2003). BMP/Smad signaling is essential for the decision between ectodermal and

mesodermal fates. Antagonizing the BMP/Smad signal, for example, by exposure of mouse ES cells to Noggin or transfection with a Noggin-encoding plasmid, promotes neurectodermal differentiation via EB formation (Finley et al., 1999; Loebel et al., 2003). FGF4 is produced in an autocrine fashion in mouse ES cells and FGF4/ERK signaling contributes to differentiation into neural and mesodermal lineages (Kunath et al., 2007) (see section 5.1 above). Wnt/β-catenin signaling inhibits neural differentiation via EB formation. Neural differentiation in mouse ES cells can be inhibited by either inactivation of the adenomatous polyposis coli (APC) protein, which regulates the phosphorylation of β-catenin through GSK3β (Fig. 6A), or the introduction of a dominant active form of β-catenin (Haegele et al., 2003). The Wnt antagonist Sfrp2 is expressed during the neural differentiation of EBs and expression of Sfrp2 enhances neuronal differentiation (Aubert et al., 2002). During EB formation, both CS and HS are upregulated (Fig. 1). Therefore, we analyzed the influence of their simultaneous sulfation in the differentiation of EBs (which are comprised of the three germ layers, endoderm, mesoderm and ectoderm) using *PAPST1*-KD or *PAPST2*-KD ES cells.

To maintain the knockdown effects during the long culture period required for EB formation, we used stable *PAPST1*-KD or *PAPST2*-KD ES cells. Before EB formation, both *PAPST1*-KD and *PAPST2*-KD cells showed an approximately 50% reduction in the targeted mRNA compared to control cells. We examined the expression of several germ layer markers by real time PCR after EB formation (Fig. 9) (Sasaki et al., 2009). The expression of neurectoderm markers (Mash1, Pax6) increased in a time-dependent manner and the expression in *PAPST1*-KD or *PAPST2*-KD cells was higher than in control cells, indicating that neurectodermal differentiation was promoted in these KD cells. The expression of early mesoderm markers (Brachyury, Goosecoid) and a primitive ectoderm marker (Fgf5) decreased in a time-dependent manner and the expression in *PAPST1*-KD or *PAPST2*-KD cells was lower than in control cells, indicating that differentiation of primitive ectodermal and mesodermal cells was inhibited in *PAPST1*-KD or *PAPST2*-KD cells. Expression of ExE lineage markers (Gata6, Laminin B1 and Bmp2) initially increased and reached a maximum level 8 days after EB formation, after which it decreased. The expression of these genes was lower in *PAPST1*-KD or *PAPST2*-KD cells than in control cells, indicating that endodermal differentiation decreased in *PAPST1*-KD or *PAPST2*-KD cell cultures. These results indicate that *in vitro* differentiation in *PAPST1*-KD or *PAPST2*-KD cells is abnormal and that sulfation contributes to differentiation of mouse ES cell**s**.

6.2 Reduced sulfation promotes neurogenesis

The observation that *PAPST1* or *PAPST2* knockdown promoted the differentiation of mouse ES cells into neurectoderm (see section 6.1 above) prompted us to investigate neural differentiation in *PAPST1*-KD or *PAPST2*-KD cells (Sasaki et al., 2009). The expression of several neural markers, such as neural stem/progenitor cell markers (Nestin, Musashi-1) and proneural markers (Mash1, Math1, NeuroD1 and NeuroD2) was higher in *PAPST1*-KD or *PAPST2*-KD cells than control cells in the presence of all-trans retinoic acid (RA) (Fig.10A). **S**imilar results were obtained in the absence of RA. These findings indicate there is a higher frequency of neural stem/neural progenitor cells and neural precursor cells amongst the *PAPST1*- or *PAPST2*-KD -KD cells.

The ability of *PAPST1*-KD or *PAPST2*-KD cells to differentiate into neurons was then examined. At 6 days after replating EBs treated with RA, *PAPST1*-KD or *PAPST2*-KD cells

generated dense networks of neurite outgrowths as compared to control cells (Fig. 10B). Western blotting analysis showed that the level of β III-tubulin in *PAPST1*-KD or *PAPST2*-KD cells was quantitatively higher than that in control cells (Sasaki et al., 2009). FACS analysis also showed that β III-tubulin positive cells were more abundant in *PAPST1*-KD or *PAPST2*-KD cell cultures than in control cells (Fig. 10C). Similar results were obtained for EBs not treated with RA. These findings demonstrate that sulfation contributes to neurogenesis in mouse ES cells.

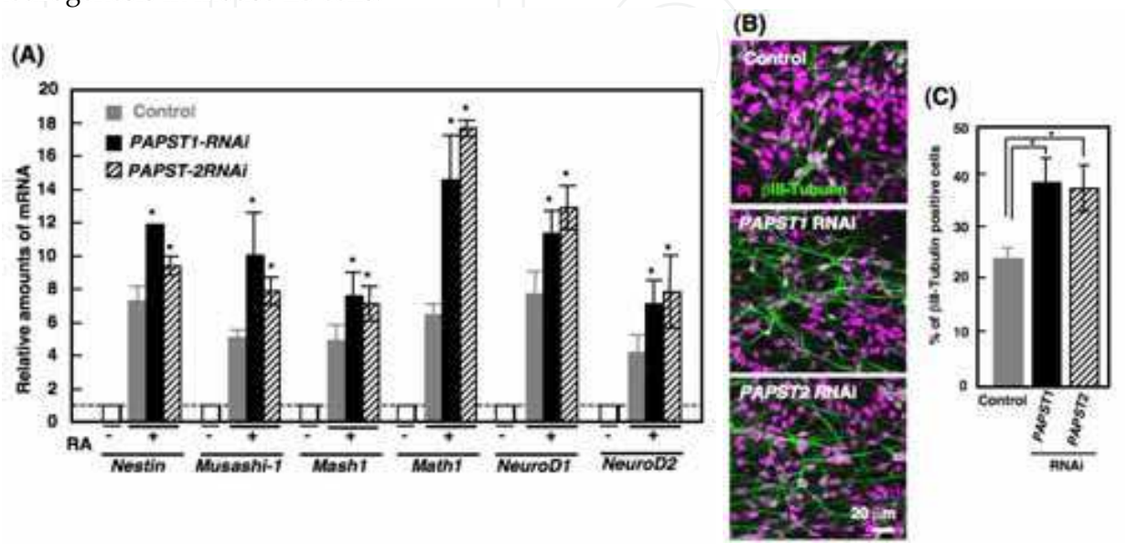

Fig. 10. Neurogenesis is promoted in *PAPST1*-KD or *PAPST2*-KD cells.
Neuronal differentiation was induced by addition of RA on days 4 and 6 after EB formation. On day 8, EBs were replated onto PDL/laminin-coated dishes in DMEM-F12 containing N2 supplement. The medium was replaced every other day and the cells were incubated for 6 days (Bain et al., 1995).
(A) Real time PCR analysis of neural differentiation markers 8 days after EB formation in the presence of RA. The results are shown after normalization against the values obtained with control cells not treated with RA (value=1). The values shown are the means ± SD of duplicate measurements from two independent experiments and significant values are indicated; *P < 0.05, in comparison to the control.
(B) Immunocytochemical staining 6 days after replating of EBs treated with RA. Representative confocal images from two independent experiments are shown. (βIII-tubulin, green; PI, purple). Scale bar, 20 μm.
(C) FACS analysis using an anti-βIII-tubulin at 6 days after replating of EBs treated with RA. Three independent experiments were performed and representative results are shown. The histograms show the ratio of the mean fluorescence intensity within the area representing βIII-tubulin positive cells to the mean fluorescence intensity over the total area ± SD of three independent experiments. Significant values are indicated: *P < 0.01, in comparison to the control

Recently it was reported that ES cells from HS-null mice show abnormal neural differentiation due to defects in FGF4 signaling (Johnson et al., 2007). The protocol used to induce neural differentiation in this study differs from the one used in our laboratory. Johnson used a Sox1-EGFP reporter cell line in an adherent cell culture; under these

conditions, neural differentiation is induced by autocrine FGF4 signaling (Johnson et al., 2007). In contrast, we used EB formation plus RA treatment; under these conditions autocrine signaling by BMP and Wnt inhibits neural differentiation (Aubert et al., 2002; Haegele et al., 2003). Thus, the variant outcomes with respect to neural differentiation might be caused by the use of dissimilar culture conditions that induce neural differentiation by different signaling pathways.

6.3 Sulfation of both HS and CS regulates several signaling pathways required for the correct differentiation of mouse ES cells during EB formation

Several signaling pathways, such as the BMP, FGF and Wnt pathways, play important roles in the mouse embryo during early embryogenesis and mouse ES cell differentiation (Loebel et al., 2003) (see section 6.1 above). We therefore examined whether defects in these signaling pathways contribute to the abnormal differentiation of *PAPST1*-KD or *PAPST2*-KD EBs (Fig. 9), especially the promotion of neurogenesis (Fig.10) (Sasaki et al., 2009).

The nuclear accumulation of β-catenin and the levels of phosphorylated ERK1/2 and Smad1 were reduced in *PAPST1*-KD or *PAPST2*-KD cells as compared to control cells (Fig.11A), indicating that Wnt/β-catenin, FGF/ERK and BMP/Smad signaling were reduced in these EBs. Furthermore, all of these signals were reduced in EBs depleted for HS and CS chains in the absence of RA (Fig. 11B). In the presence of RA, HS depletion reduced signaling via all of these pathways as compared with non-depleted EBs. By contrast, CS depletion reduced FGF/ERK and BMP/Smad signaling to a similar extent as HS depletion but promoted Wnt/β-catenin signaling (Fig. 11B).

Next, we performed a surface plasmon resonance (SPR) analysis for Wnt3a and BMP4 against heparin, a structural analogue of HS chains, and CS-E (GlcAβ1,3GalNAc(4,6SO_3)), a particular form of CS chain. Wnt3a and BMP4 bind to both heparin and CS-E (Table 1) (Sasaki et al., 2009; Sasaki et al., 2008). The KD values for the binding of bFGF and FGF4 to HS and to CS-E have been also determined (Deepa et al., 2002; Sugaya et al., 2008). The analysis clearly shows that the sulfate groups of HS and CS contribute to the binding of Wnt3a, BMP4, bFGF and FGF4 to both HS and CS.

These results demonstrate that sulfation on both HS and CS regulates BMP/Smad, FGF/ERK and Wnt/β-catenin signaling during EB formation. In addition, the reduction in such signaling contributes to the abnormal differentiation of *PAPST1*-KD or *PAPST2*-KD cells, such as the promotion of neurogenesis.

Ligand	GAG	k_a ($M^{-1}Sec^{-1}$)	k_d (Sec^{-1})	K_D (nM)
Wnt3a	Heparin	2.22×10^5	5.77×10^{-3}	26.0
Wnt3a	CS-E	8.26×10^5	2.26×10^{-2}	27.3
BMP4	Heparin	2.76×10^5	1.92×10^{-2}	69.4
BMP4	CS-E	1.44×10^5	4.33×10^{-3}	30.0

The ka, kd and KD values were determined by SPR analysis.

Table 1. The apparent association (ka), dissociation (kd) rate constants and equilibrium dissociation constants (KD) for the interaction of Wnt3a and BMP4 with immobilized heparin or CS-E.

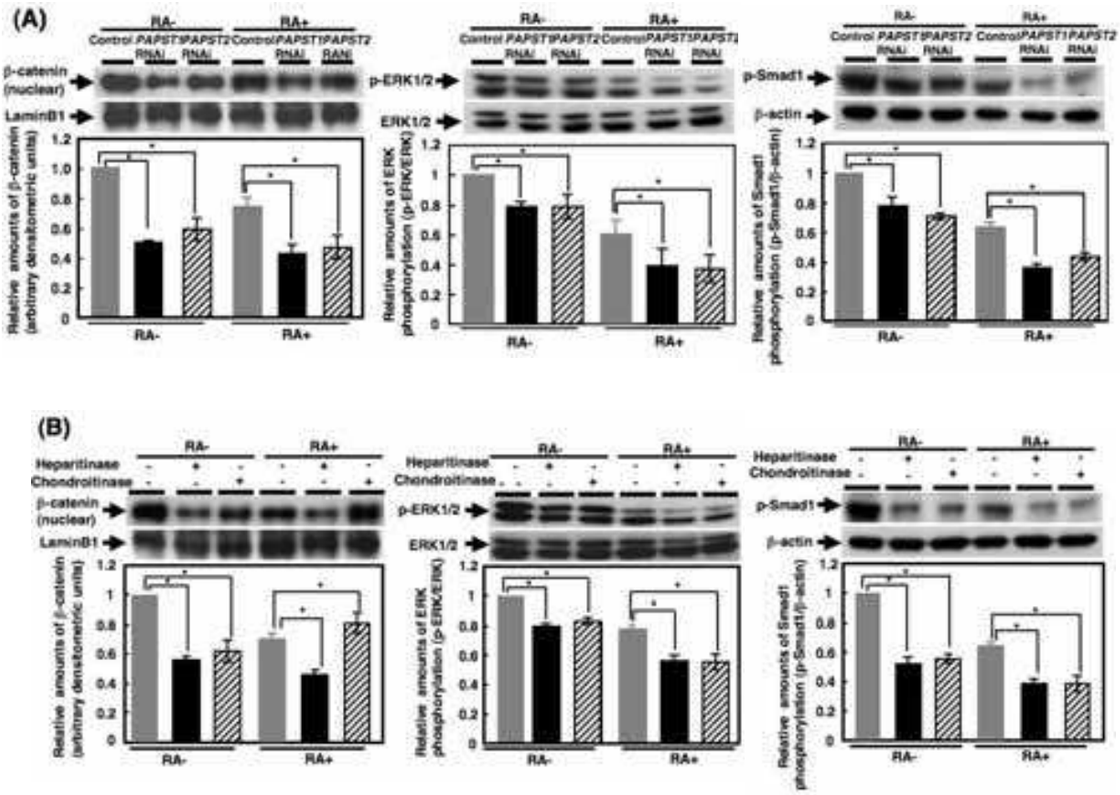

Fig. 11. *PAPST1*- or *PAPST2*-KD cells show decreased signaling in a number of pathways during EB formation.

(A) Western blot analysis of several signaling molecules in EBs on day 8. Two independent experiments were performed and representative results are shown. The histograms show mean densitometric readings ± SD of β-catenin or the phosphorylated proteins/loading controls after normalization against the values obtained with control cells in the absence of RA (value=1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.01, in comparison to the control.

(B) Western blot analysis of several signaling molecules in EBs on day 8 after heparitinase or chondroitinase treatment. Two independent experiments were performed and representative results are shown. The histograms show mean densitometric readings ± SD of β-catenin or the phosphorylated proteins/loading controls after normalization against the values obtained with cells not treated with RA and enzyme (value=1). Values were obtained from duplicate measurements of two independent experiments and significant values are indicated; *P < 0.01, in comparison to cells not treated with enzyme.

7. Conclusion

Glycan structures on mouse ES cells have roles in the maintenance and differentiation of mouse ES cells. In particular, the sulfated glycans, HS and CS, function in the biologically

important signaling pathways involving Wnt, BMP and FGF. Through these signaling pathways, glycan structures function in the maintenance of self-renewal and pluripotency and also in differentiation (Fig. 12).

The pluripotency of mouse ES cells in adherent cell culture is maintained by a balance among extrinsic signaling pathways, such as LIF, BMP, Wnt and FGF signaling, and also by a combination of extrinsic and intrinsic factors, such as Oct3/4 and Nanog (Boiani & Scholer, 2005; Chambers & Smith, 2004; Ivanova et al., 2006; Sasaki et al., 2008; Zhang & Li, 2005). In undifferentiated mouse ES cells, the sulfation of HS, but not of CS, regulates extrinsic signaling by BMP4, Wnt, bFGF and FGF4 (Fig. 12B). BMP4 and autocrine/paracrine Wnt maintain self-renewal and pluripotency by inhibiting neural differentiation (Ying et al., 2003; Zhang & Li, 2005) and maintaining the Nanog expression level, respectively (Sasaki et al., 2008; Sato et al., 2004). Autocrine/paracrine bFGF signaling contributes to the growth of mouse ES cells (Dvorak et al., 1998), while FGF4 signaling contributes to initiation of differentiation by mouse ES cells (Kunath et al., 2007).

In EBs, both HS and CS regulate signaling by BMP, FGF or Wnt and, through their interaction with these signaling pathways, they regulate the differentiation of EBs (Fig. 12C). Wnt and BMP signaling inhibit ectodermal differentiation and contribute to mesodermal and definitive endodermal differentiation (Aubert et al., 2002; Finley et al., 1999; Gratsch & O'Shea, 2002; Haegele et al., 2003; Loebel et al., 2003; Yoshikawa et al., 1997). Sulfation of both HS and CS contributes to the decision between ectodermal and mesodermal fates by regulating these signals. FGF/ERK and FGF/Akt signaling contribute to mesodermal and definitive endodermal differentiation and primitive ectodermal and visceral endodermal differentiation, respectively (Loebel et al., 2003) (Chen et al., 2000; Kimelman, 2006).

During neural differentiation of EBs after RA treatment, HS and CS regulate the extrinsic signaling by BMP, Wnt and FGF that inhibits or is required for neuronal differentiation (Fig. 12D). The transduction of extrinsic signals is dependent on the sulfation of both HS and CS and results in neuronal differentiation. Wnt and BMP signaling inhibit neurogenesis in mouse ES cells via EB formation (Aubert et al., 2002; Haegele et al., 2003). In contrast, FGF (e.g., bFGF) signaling may promote neurogenesis. On the other hand, CS has a negative effect on Wnt signaling, presumably by sequestering Wnt proteins and preventing them interacting with Wnt receptors. Thus, we propose that CS promotes the differentiation of neural stem/progenitor cells into the neuronal lineage.

In this chapter, we have outlined the functions of the sulfated glycans that are present on mouse ES cells. Some of these functions should also hold true for human ES and iPS cells. This conclusion is supported by our observation that sulfation has a similar function during neuronal differentiation in human iPS cells as in mouse ES cells (Sasaki et al., 2010). However, bFGF/ERK signaling is also regulated by HS and contributes to proliferation in mouse ES cells, whereas in human ES cells, FGF signals have a role in the maintenance of self-renewal and pluripotency (Xu et al., 2005). Indeed, human ES cells are stimulated to proliferate by addition of heparin or HS proteoglycan to the culture medium (Furue et al., 2008; Levenstein et al., 2008). In light of the currently available information, it is clear that further studies on the roles of the sulfated glycans, HS and CS, should provide greater insight into and understanding of the maintenance of self-renewal and pluripotency in human ES and iPS cells as well as in mouse ES cells and should also help to elucidate the regulation of differentiation in stem cells.

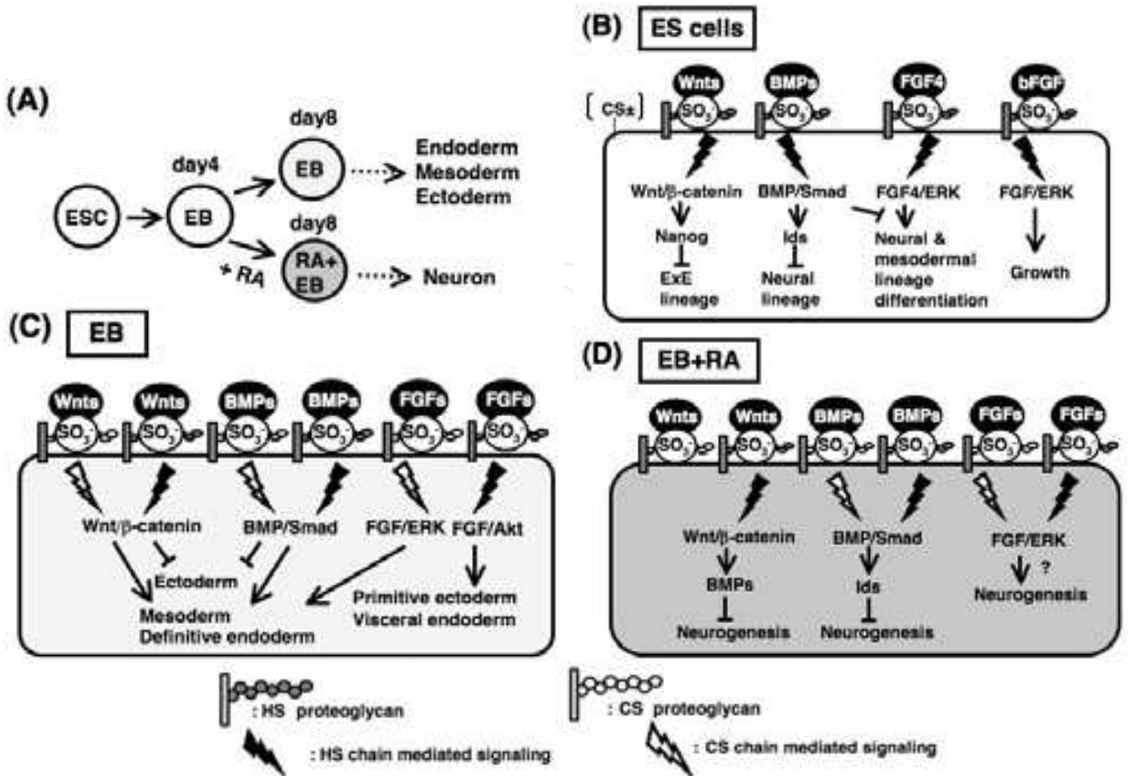

Fig. 12. Sulfated glycans contribute to the maintenance and differentiation of mouse ES cells.
(A) *In vitro* differentiation flowchart of mouse ES cells. EBs that are not treated with RA produce cells from all three germ layers (endoderm, mesoderm and ectoderm), whereas RA-treated EBs produce neurons after further adherent culture.
(B) HS regulates the extrinsic signaling (by BMP and Wnt) that is required for the pluripotency of mouse ES cells. In undifferentiated mouse ES cells, the transduction of extrinsic signals is dependent on the sulfation of HS, but not CS, and this maintains pluripotency, the undifferentiated state and growth. Autocrine/paracrine bFGF signaling contributes to the growth of mouse ES cells, while FGF4 signaling contributes to the initiation of differentiation in the mouse ES cells.
(C) Both HS and CS regulate the extrinsic signaling (by BMP, FGF and Wnt) that is required for normal differentiation of EBs. During EB differentiation into the three germ layers, the transduction of the extrinsic signals is dependent on the sulfation of both HS and CS. Wnt and BMP signaling inhibit ectodermal differentiation and contribute to mesodermal and definitive endodermal differentiation. FGF/ERK and FGF/Akt signaling contribute to mesodermal and definitive endodermal differentiation and primitive ectodermal and visceral endodermal differentiation, respectively.
(D) HS and CS regulate the extrinsic signaling (by BMP, Wnt and FGF) that inhibit**s** or is required for neuronal differentiation of RA-treated EBs. During RA-treated EB differentiation, the transduction of extrinsic signals is dependent on the sulfation of both HS and CS and results in neuronal differentiation. Wnt and BMP signaling inhibit neurogenesis and FGF (e.g., bFGF) signaling may promote neurogenesis. CS regulates Wnt signaling negatively, presumably by sequestering Wnt proteins and preventing them interacting with Wnt receptors.

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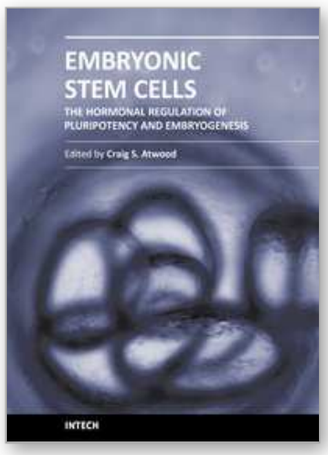

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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

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