Expression of *ST6GalNAc1* and sialyl-Tn antigen enhances endometrial receptivity

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Research

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Abstract

Background

To characterize molecular mechanism underlying the regulation of sialylated glycan expression and its roles for endometrial receptivity and embryo implantation. Here, we characterized the role of a truncated form of sialylated *O*-glycan, sialyl-Tn, for endometrial receptivity.

Methods

The transcriptomes of human endometrium at mid-secretory phase were analyzed by Bioinformatics. Changes in gene expression, protein, and signal pathway were measured using RT-PCR and Western blot. The cell adhesion assay was visualized using a fluorescent microscope. In peri-implantation phase of mice, the expression of leukemia inhibitory factor (LIF) and sialyl-Tn were confirmed using immunohistochemistry and immnofluorescence analysis. The effect of sialy-Tn expression on embryo implantation was estimated by *in vitro* fertilization and embryo transfer using mice.

Results

In *in silico* analysis, expression of *O*-glycosylation genes, especially *ST6GalNAc1*, was significantly increased in the human uterus of mid-secretory phase. Overexpression of the *ST6GalNAc1* gene in non-receptive human endometrial AN3CA cells enhances the attachment of trophoblastic JAr cells. In an animal study, the results clearly indicated that sialyl-Tn was expressed on the surface of the mid-secretory uterus. In addition, blockade of the receptor using free sialyl-Tn epitope diminished the implantation rates of intrauterine transferred murine embryos.

Conclusion

From these results, here we suggest that sialyl-Tn expression might be a novel factor regulating the endometrial receptivity for successful embryo implantation.

Background

Embryo implantation, a key process for a successful pregnancy, is one of the remaining unmet needs to improve the success rate of assisted reproduction [1]. The endometrium is receptive to embryo only during the implantation window, a short period of time about 6–10 days after ovulation [1, 2]. A large number of molecular mediators including adhesive proteins, hormones, cytokines, and growth factors, which are involved in implantation have been identified. Among these molecules, several glycoproteins such as mucin-1 (MUC-1) and human chorionic gonadotrophin (hCG) have been focused on its possible function in relation to embryo implantation [3, 4]. However, the molecular mechanisms regulating endometrial receptivity are remained poorly understood [5].

Protein glycosylation, a highly elaborative process of post-translational modification, is tightly controlled by enzymatic regulation [6]. The expression of terminal fucose residue generated by the action of fucosyltransferases, such as an increase of sialyl Lewis X antigen synthesized by fucosyltransferase VII, was reported as a positive factor of embryo implantation [7, 8]. In spite of given importance of the terminal sialic acid residues on the cell surface for cell-cell interaction owing to its negative charge [9], the function of the sialic acid epitopes in the embryo implantation is still not elucidated. Sialyl-Tn antigen, a type of truncated O-glycosylation, has been reported as a key player in cancer progression including proliferation, invasion, and metastasis [10–12]. Although the expressions of sialyl-Tn were previously examined in the normal human reproductive organs including uterus, testis, and amniotic fluid [13, 14], there are no previous report on the expression of sialyl-Tn nor their function in receptive endometrium.

In this study, we firstly demonstrate that the expression of the sialyl-Tn antigen is increased in receptive endometrium is essential for the process of embryo implantation in both in vitro human cell lines and in vivo mouse experiment.

Materials And Methods

Materials

Anti-sialyl-Tn antibody [STn 219] (FITC) was supplied from Abcam (Cambridge, UK). The free sialyl-Tn epitope was supplied from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-β-actin antibody, β-estradiol, and progesterone were purchased from Sigma-Aldrich (St. Louis, MO). Cell Tracker™ Green CMFDA (5-chloromethylfluorescein diacetate) fluorescence dye was provided from Thermo Fisher Scientific (Waltham, MA).

Bioinformatic analyses

Gene Set Enrichment Analysis (GSEA; www.broadinstitute.org/gsea) was performed as described in previous studies [15, 16] using the transcriptomes of human proliferative and mid-secretory endometrium (NCBI GEO dataset, GSE4888) [17]. Correlation analyses were performed also using the transcriptomes of human proliferative and secretory endometrium (NCBI GEO dataset, GSE4888) as described in previous studies [18, 19]. Correlogram was built using corrgram package in RStudio (R Consortium Inc, Boston, MA), with positive and negative correlations represented in blue and red edges, respectively. Only correlations with Spearman's Rho > 0.5 or < − 0.5 ($p < 0.05$) are displayed in the interaction network.

Cell culture

Human uterine endometrial Ishikawa cell line, derived from a uterine adenocarcinoma [20], was kindly provided by Dr. Jacques Simard (CHUL Research Center, Quebec, Canada). Non-receptive uterine endometrial AN3CA cell lines [21] were purchased from American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained at 37 °C in an atmosphere containing 5% CO_2/air in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Daegu, Korea) and Roswell Park Memorial Institute medium (RPMI-1640; Welgene) containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher

Scientific) and 1% penicillin/streptomycin (PSA; Thermo Fisher Scientific), respectively. The choriocarcinoma JAr cell was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI1640 (Welgene) containing 10% FBS and 1% PSA.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using RiboEx™ (GeneAll, Seoul, Korea) and then subjected to reverse transcription with oligo-dT primers using M-MLV reverse transcriptase (Thermo Fisher Scientific). The cDNA was amplified by PCR using AccuPower®PCR PreMix (Bioneer Co., Daejeon, Korea). The primers used in this study were as follows: ST6GalNAc1, forward 5'-GTCCAGTGGTCCTTGCTTCTGGCTG-3' and reverse 5'-ATTTCCTTGGGTCGTCTTTGTGTCC-3'; β-actin, forward 5'-CAAGAGATGGCCACGGCTGCT-3' and reverse 5'-TCCTTCTGCATCCTGTCGGCA-3'.

Expression of ST6GalNAc1 gene

AN3CA cells were transfected with the ST6GalNAc1 gene in the mammalian expression vector, pcDNA3.1, which was kindly gifted from Dr. Philippe Delannoy (University of Lille, France).

Flow cytometric analysis

AN3CA cells were fixed with 3.7% formaldehyde and incubated with FITC-conjugated anti-sialyl-Tn antibody in Carbo-free blocking solution for 2 h. The cells were evaluated by flow cytometry (BD FACS CANTO II; BD Biosciences, San Jose, CA).

Adhesion assay

AN3CA cells were seeded into 6 well plates and cultured to a confluent monolayer. After the pretreatment of hLA, the cells were incubated with leukemia inhibitory factor (LIF) treatment for 48 h. The JAr cells were labeled with Cell Tracker™ Green CMFDA (Invitrogen, Carlsbad, CA) and gently added onto a monolayer of AN3CA cells. After gently shaking at 40 rpm for 30 min at 37 °C, the cells were vigorously washed to remove non-binding cells. The attached JAr cells were visualized by using a fluorescent microscope (Axio Imager M1, Carl-Zeiss, Oberkochen, Germany). Five fields of each sample were chosen randomly and the number of adhered cells was averaged after quantifying with ImageJ software (NIH; Bethesda, MD)

Animals

Female C57BL/6 mice (7–8 weeks old, weight 20–22 g) were purchased from Orient Bio, Co. (Seongnam, Korea). Mice were ovariectomized (OVEX) and recuperated 14 days later. Animals were fed on a standard diet with ad libitum access to water, and kept on a 12 h light: 12 h dark cycle. All experimental procedures were examined and approved by the Animal Research Ethics Committee at the Pusan University of Korea (no. PNU-2016-1212).

Induction of peri-implantation phase and immunochemistry

Twenty-eight female mice were randomly divided into two groups: control and PE. In the prime phase, mice were injected subcutaneously (s.c.) with 100 ng β-estradiol (in 100 μl corn oil) or vehicle for 3 days. In interphase, no hormone was treated for the next two days. In the peri-implantation phase, mice were daily injected s.c. with 6.7 ng β-estradiol and 1 mg progesterone (in 100 μl corn oil) for 6 more days. Mice were respectively sacrificed on day 6, 8 and 10, and both uterine horns were excised to determine the expression of LIF and sialyl-Tn using immunohistochemistry and immunofluorescence analysis, respectively. The uterine tissues of mice were fixed in 3.7% formalin and embedded in paraffin and were then cut into 4 μm serial sections. The sections were immunostained with LIF or sialyl-Tn (FITC) antibodies, visualized with the Dako Envision kit (Dako, Jena, Germany) and fluorescent microscope (Axio Imager M1) respectively.

Animals and embryo collection and Embryo transfer in utero

Inbred ICR female mice (6–8 weeks old) were induced to superovulate by intraperitoneal injection of 5 IU serum gonadotropin from a pregnant mare (Sigma-Aldrich) followed by injection with 5 IU human chorionic gonadotropin (hCG; Sigma-Aldrich) 46 later. Superovulated female mice were mated with fertile male mice and euthanized by cervical dislocation 46 h after hCG injection. The day of vaginal plugging was designated as 0.5 days post coitum (dpc). Mouse 2-cell embryos were collected from oviducts on 1.5 dpc and cultured in pre-warmed the Quinn's Advantage Blastocyst Medium (SAGE/Origio, Malov, Denmark) containing 10% serum protein substitute in an incubator at 37 and 5% CO_2. Implantation potential of embryos from the control and sialyl-Tn epitope groups was examined via embryo transfer as described previously [22, 23]. In the sialyl-Tn epitope group, embryos were transferred with a 50 ng/ml sialyl-Tn epitope. Five to six embryos from two different groups were transferred on 3.5 dpc to the contralateral uterine horn of pseudopregnant recipients provided by mating with a vasectomized male. Two days after transfer, implantation sites in uterine horns of pregnancy were detected by intravenous injection of Chicago blue dye. Clear blue bands in utero were considered implantation sites.

Statistical analysis

Statistical analysis of results was performed by a Student's t-test (for in vitro adhesion assay) or Chi-square test (for in vivo embryo transfer) using GraphPad Prism (GraphPad Software, San Diego, CA). Values are expressed as the mean ± standard deviation (SD) and the minimum significance level was set at a P value of 0.05. All experiments except for animal studies were independently performed at least 3 times.

Results

Bioinformatic analysis

To identify the functions of protein glycosylation in human embryo implantation, we applied a bioinformatic approach with the NCBI GEO database (GSE4888) [17]. The results from GSEA using the transcriptomes of human proliferative and mid-secretory endometrium indicated that glycosylation,

especially O-linked glycosylation is the most prominent and significant gene set (Fig. 1A and B). Among the genes involved in O-glycosylation, the expression of sialyltransferases, such as ST6GalNAc1, ST6Gal1, ST3Gal6, are significantly increased in the uterus of mid-secretory phase, which is corresponding to implantation window (Fig. 1C). To identify putative targets of LIF, a major cytokine regulating endometrial receptivity [24], we performed correlation analyses using the same GEO dataset. Interestingly, a highly significant positive correlation between LIF/gp130/STAT3 signaling pathway and ST6GalNAc1 was observed (Fig. 1D and E). Other cytokines and growth factors related to endometrial receptivity including IL-1α, IL-6, IL-8, IL-11, IL-15, and HB-EGF [25] showed a lower positive correlation with the expression of ST6GalNAc1 compared with LIF.

ST6GalNAc1 expression mediated adhesion of trophoblast to endometrial cells

Among the sialic acid-binding immunoglobulin-type lectins (Siglecs), Siglec-6 is a receptor for the sialyl-Tn epitope and expressed on the surface of trophoblast [26]. As ST6GalNAc1 is responsible for the expression of sialyl-Tn antigen and termination of O-glycan (Fig. 2A), we confirmed the expression of the ST6GalNAc1 gene in the receptive endometrial Ishikawa and non-receptive AN3CA cells [21, 27]. The results shown in Fig. 2B demonstrated that non-receptive AN3CA cells did not express ST6GalNAc1 at the presence of LIF or not. Whereas, receptive Ishikawa cells expressed ST6GalNAc1 even at the absence of LIF. Thus, we exogenously overexpressed the ST6GalNAc1 gene in non-receptive endometrial AN3CA cells. The overexpressed ST6GalNAc1 gene successfully induced both sialyl-Tn expression and receptivity to JAr cells (Fig. 2C-E). These results clearly demonstrated that the expression of ST6GalNAc1 and sialyl-Tn were sufficient for mediating the cell-cell interaction between trophoblast and endometrial cells.

Sialyl-Tn expressed at implantation window was crucial for endometrial receptivity

To examine the expression of sialyl-Tn at the period of implantation window in mice endometrium, estradiol and progesterone were serially treated to ovariectomized female mice for mimicking normal hormonal cycle (Fig. 3A), according to previous studies [28, 29]. The expression of LIF appeared in the uterus of estradiol and progesterone treated-mice (PE group) from day 8 and prominent at day 10 (Fig. 3B). The expression of sialyl-Tn was elevated from day 6 and day 8 in the uterus of the PE group than the control group. However, the expressions are fuzzily distributed in the stoma region of the uterus. On day 10, the sialyl-Tn was expressed highest and prominent in the luminal region of the endometrium (Fig. 3C). To confirm the function of ST6GalNAc1 expression in embryo implantation in vivo, the genetic abrogation of its expression is required. However, there are no available knockout mice in previous research papers or mouse phenotyping centers. It is possible that knockdown of ST6GalNAc1 may be

embryonic lethal. Thus, to overcome the absence of knockout mice, we confirm the in vivo effect of sialyl-Tn on endometrial receptivity by blocking its receptor using free sialyl-Tn epitopes in murine IVF model. The result clearly demonstrates that the pre-incubation of the murine embryo with free sialyl-Tn for blocking its receptor can reduce the implantation rate of the transferred murine embryo (Table 1). These results collectively suggested that the sialyl-Tn antigen expressed on the endometrium induced by LIF in the period of implantation window had a key role in the adhesion between trophoblast and endometrium.

Table 1. Effect of sTn-Ser on embryos implantation rate in mice.

	Recipients	No. of embryos transferred	Implanted embryos	Implantation rate*	Average
Con	#1	6	5	83.3	64.2 ± 9.4^{a} (29/44)
	#2	6	3	50.0	
	#3	5	2	40.0	
	#4	6	6	100.0	
	#5	5	2	40.0	
	#6	6	6	100.0	
	#7	5	2	40.0	
	#8	5	3	60.0	
Free sialyl-Tn	#1	6	4	66.7	32.1 ± 8.0^{b} (14/44)
	#2	6	0	0.0	
	#3	5	1	20.0	
	#4	6	1	16.7	
	#5	5	3	60.0	
	#6	6	2	33.3	
	#7	5	1	20.0	
	#8	5	2	40.0	

* Implantation rate = Implanted embryos / Transferred embryos

[ab] Different letters indicate statistically significance by Chi-square test ($p < 0.001$)

Discussion

Previously, several sialic acid-containing glycan epitopes, such as α2,3, α2,6, and α2,8 sialic acids linked to glycosphingolipids or glycoproteins, are expressed on the surface of uterine endometrium [30, 31]. The sialic acid epitopes expressed on the surface of the uterine endometrium was affected by hormonal regulation and aging [31–35]. In addition, injection of LIF could modulate glycoconjugates on the apical

surface of the murine uterine epithelium including terminal sialic acid [36]. However, the expression and roles of sialic acid epitopes in the embryo implantation site are still controversial [34, 35, 37]. In this study, we firstly found that the expression of a unique α2,6 sialic acid epitopes, sialyl-Tn antigen on the surface of endometrial cells might play a crucial role in receptivity toward trophoblast during the implantation window period.

The sialyl-Tn, which is a truncated form of O-glycan synthesized by ST6GalNAc1, is related to the development of various epithelial tumors including gastric, colon, breast, lung, prostate, cervical, endometrial cancer, and endometriosis [38–40]. Its receptor, Siglec-6 was reported that highly expressed on the immune cells and placental trophoblast [41]. The roles of Siglec-6 in the leptin-related regulation of gestational trophoblast disease [42] and preterm preeclampsia [43] were suggested, its function in embryo implantation are still not reported. In this study, we blocked the receptor-ligand binding by using free sialyl-Tn epitopes, and they successfully abrogated the adhesion of trophoblast to endometrial cells in murine IVF model. However, the precise mechanism underlying the LIF/sialyl-Tn/Siglec-6 axis should be illuminated by further extensive experiments.

From our recent study, the expression of integrin αVβ3 and αVβ5 induced by LIF treatment are required for endometrial receptivity [44]. However, the pattern of integrin expression in AN3CA is very similar to receptive endometrial cells, such as HEC-1A and Ishikawa [45]. To explain the phenomena, an element added to the integrins are required. Previous studies reported that several integrins including α2, α2b, α5, β1, and β3, are O-glycosylated proteins [46, 47]. In addition, other adhesive molecules such as CD44 and trophinin are also highly glycosylated proteins [48]. Thus, the precise proteins, which added by sialyl-Tn and mediating embryo implantation, should be elucidated by further extensive studies. In this study, we shed a light on the understanding of the role of protein sialylation in endometrial receptivity.

Conclusions

LIF increases the cell surface sialyl-Tn antigen in uterine endometrial cells through the induction of ST6GalNAc1 expression. LIF-stimulated sialyl-Tn expression enhances the endometrial receptivity by increasing cell-cell interaction between trophoblast and endometrial cells by means of in vitro and in vivo model. From these results, we suggest that sialyl-Tn expression might be a key factor regulating the endometrial receptivity for successful embryo implantation.

Abbreviations

MUC-1: mucin-1

hCG: human chorionic gonadotrophin

LIF: leukemia inhibitory factor

GSEA: Gene Set Enrichment Analysis

RT-PCR: Reverse transcription-polymerase chain reaction

OVEX: Mice were ovariectomized

s.c: subcutaneously

dpc: days post coitum

Siglecs: sialic acid-binding immunoglobulin-type lectins

Declarations

Ethics approval and consent to participate

All experimental procedures were examined and approved by the Animal Research Ethics Committee at the Pusan University of Korea (no. PNU-2016-1212).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HJC and TWC designed the experiments and performed the major molecular experiment. JK and JHJ performed *in vitro* fertilization and embryo transfer. BSK and MJP assisted the molecular experiments. KJK perfoermed the immunohistochemistry and immunoflurescent staining. JKJ and CHK analyzed the data. DR performed the *in silico* analysis. DR and KTH prepared manuscript. KTH designed the experiments and supervised the study. All authors have gone through and approved the manuscript. All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work.

Acknowledrements

Not Applicable.

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Figures

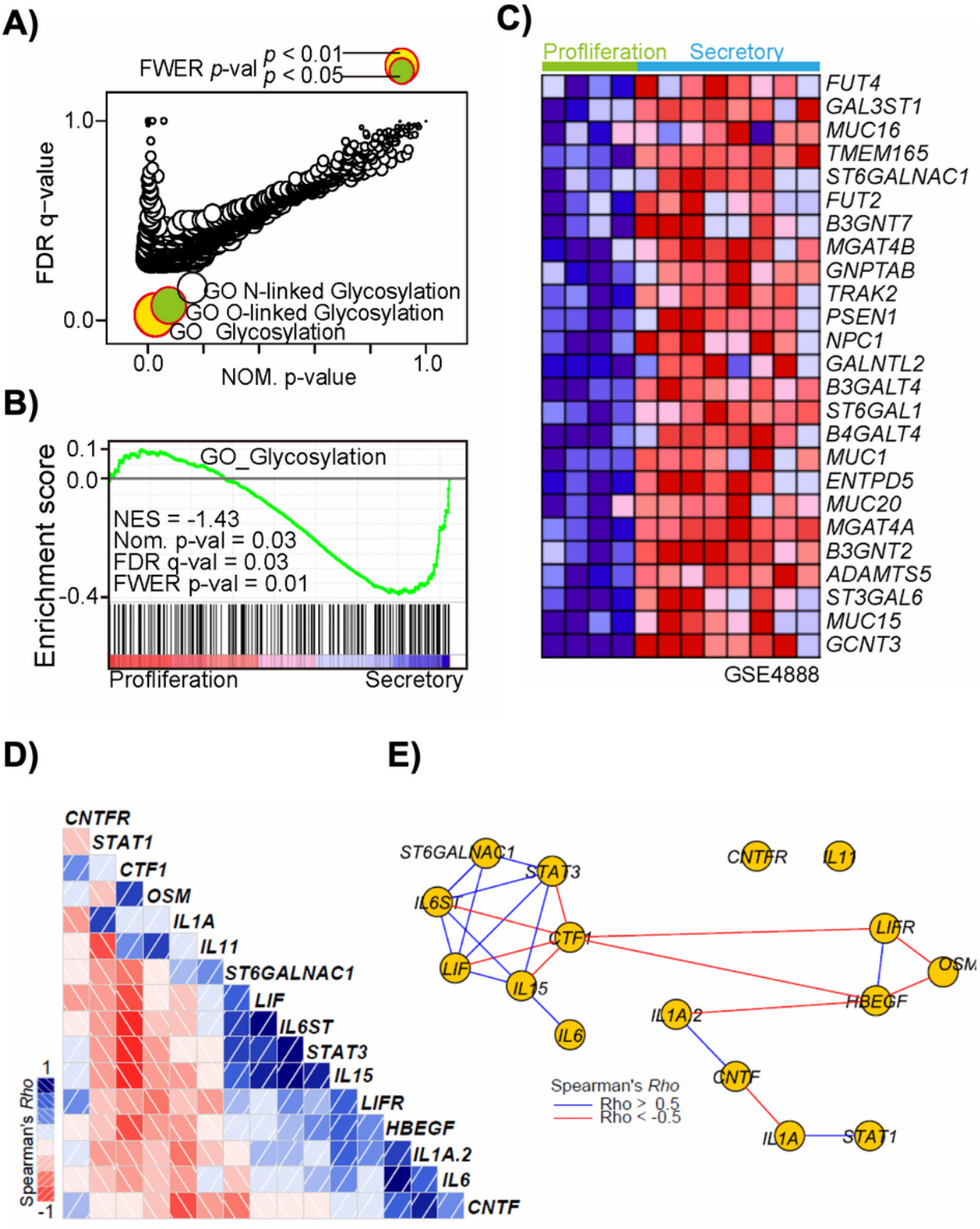

Figure 1

Bioinformatic analysis using the transcriptomes of human normal endometrium at distinct phases of the menstrual cycle. (A-C) Bubble plot, enrichment plot, and heat map summarizing the results of Gene Set Enrichment Analysis (GSEA) using the transcriptomes of human normal endometrium at distinct phases of the menstrual cycle (NCBI GEO dataset, GSE4888). (A) Bubble plot showing nominal p-value (x-axis), family-wise error rate (FWER) p-value (y-axis), and false discovery rate (FDR) q-value (size of the bubbles,

-log10) of GSEA. (B) GSEA enrichment plot showing the enrichment scores (ES) of the GO_Glycosylation gene set. (C) Corresponding heat map displaying the expression values for the top genes of GO_Glycosylation gene set in human proliferative and secretory endometrium. (D, E) Correlogram and interaction network showing correlations among ST6GALNAC1, cytokines and their down-streams in using the transcriptomes of human normal endometrium (NCBI GEO dataset, GSE4888). (D) The depth of the shading at the correlogram displays the magnitude of the correlation (Spearman's Rho). Positive and negative correlations are represented in blue and red, respectively. (E) Interaction network showing correlations of indicated genes. Positive and negative Spearman's Rank correlation coefficients are represented by blue or red edges (Rho = |0.5-1.0|, $p < 0.05$), respectively.

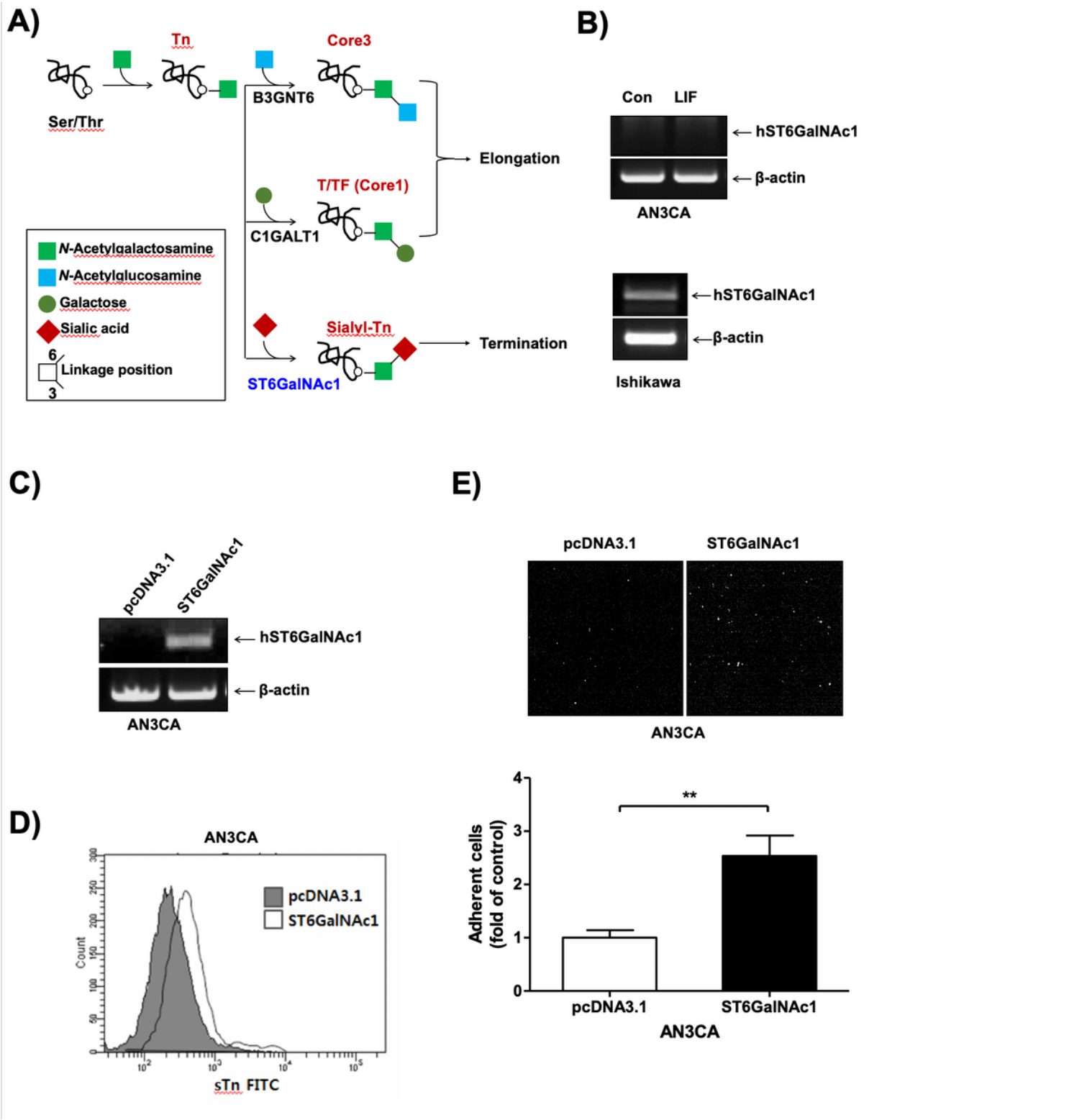

Figure 2

The adhesion of JAr cells to non-receptive AN3CA cells was reversed by ST6GalNAc1 expression. (A) Schematic diagram of O-glycan synthesis. (B) AN3CA cells were treated with LIF for 24 h. The expression of ST6GalNAc1 was determined by RT-PCR. The expression of ST6GalNAc1 in Ishikawa cells was used for positive control. (C) AN3CA cells were transfected with the ST6GalNAc1 gene in the mammalian expression vector pcDNA3.1. ST6GalNAc1 mRNA levels in total RNA obtained from each cell were

detected by RT-PCR (D). In pcDNA3.1- or ST6GalNAc1-transfected AN3CA cells, the sialyl-Tn expression and the number of adherent JAr cells were measured by FACS analysis with FITC-labeled sialyl-Tn antibody. (E) The fluorescent labeled-JAr cells attached to pcDNA3.1 or ST6GalNAc1-transfected AN3CA cells were pictured and calculated. Five different sites per well were pictured and the number of adherent cells was calculated as mean ± SD of three independent experiments. **P < 0.01 and ***P < 0.001 compared to each group.

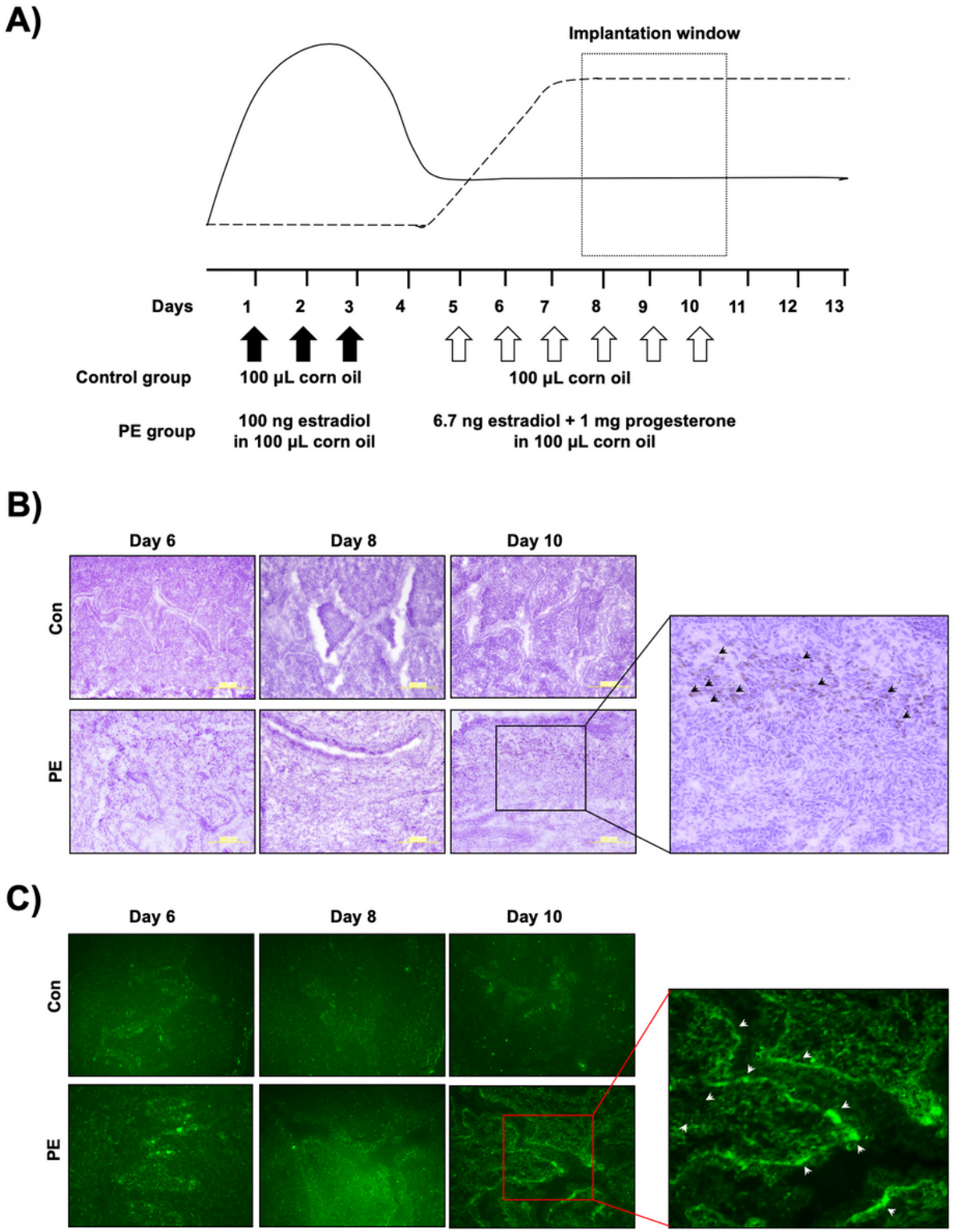

Figure 3

The expression of LIF and sialyl-Tn in the implantation window. (A) Schematic diagram of induction of peri-implantation phase. The prime phase was induced on day 0. Control and PE groups were injected s.c. with 100 ng of β-estradiol or vehicle for 3 days. The interphase was not treated on day 4. In the peri-implantation phase, mice were daily injected s.c. with 6.7 ng of β-estradiol and 1 mg of progesterone for 6 more days. Mice were sacrificed on days 6, 8 and 10, and both uterine horns were excised. The uterine tissues of mice were embedded in paraffin. The serial sections were immunostained with LIF (B) and FITC-labeled sialyl-Tn antibodies (C). The expression of LIF and sialyl-Tn were indicated by arrows.