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Effects of LeY glycan expression on embryo implantation

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Abstract. – OBJECTIVE: To investigate the correlation between LeY glycan expression and embryo implantation.

MATERIALS AND METHODS: Uterine epithelial cells before implantation were transfected with *FUT1siRNA* to inhibit *FUT1* (the gene encoding the key enzyme of LeY synthesis) expression and treated with 10 ng/ml leukemia inhibitory factor (LIF). Murine embryo implantation model in vitro was prepared by late blastocysts with identical morphology and treated uterine epithelial cells co-culture. Using RT-PCR, dot blot and observation of embryo attachment to analyze *FUT1* gene expression and LeY synthesis of uterine epithelial cells and studied further the correlation of LeY expression level and embryo implantation.

RESULTS: *FUT1* gene expression and LeY synthesis declined after cells were transfected with *FUT1siRNA*, and LIF promoted *FUT1* expression and LeY synthesis. After expression of *FUT1* gene was inhibited, attachment rate of embryos lowered, but LIF up-regulated *FUT1* expression and increased the attachment rate of embryos.

CONCLUSIONS: These results indicated regulating *FUT1* expression affected LeY synthesis, and then LeY regulated the recognition and attachment of uterus-embryo and participates in embryo implantation further.

Key Words:
LeY glycan, LIF, Uterine epithelial cell, Embryo implantation, RNA interference.

Introduction

Ovum would gradually develop into "invasive blastocyst" in the process of migrating into the uterus through oviduct after fertilization and would interact with the endometria that has already developed into "receptive" state to start up embryo implantation. Embryo implantation is divided into three processes: location, adhesion and invasion. The specific expression of cytokine (CK), matrix metalloproteinases (MMPs) and its inhibition factor (TIMPs), cell adhesion molecules (CAMS), extracellular matrix (ECM) and other implantation related factors in peri-implantation would interact with each other under the regulation of hormone and form a "regulation network" to regulate the microenvironment of the embryo and the uterus, and create conditions for successful implantation[1].

Blastocyst would firstly contact mutually with the glycan chain on the surface of cells while approaching endometrium. The LeY glycan on the cell surface is involved in the recognition and adhesion between the embryo and the uterine epithelium. To obstruct the expression of LeY glycan before implantation by LeY monoclonal antibody could significantly inhibit embryo implantation[2,3], and also inhibit the secretion and gene expression of leukemia inhibitory factor (LIF), MMPs, epidermal growth factor (EGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) in embryo and endometrial cells before implantation, and meanwhile promote the secretion of fibronectin (FN) and laminin (LN). The FN and LN increase as the expression of proteolytic enzymes decreased[4-6] indicating that LeY glycan was involved in the implantation regulation as an information molecule. The expression of fucosyltransferase (FUTI) that catalyzed the synthesis of LeY glycan in embryo and endometrium during peri-implantation was consistent with LeY glycan[7], which indicated that the expression level of glycan was regulated by its synthesis key enzyme gene.

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LIF is a kind of secreted glycoprotein with extensive biological function with a regular expression in the embryo and endometrium of human, mouse, and other mammal and high expression in "implantation window phase", which could affect the blastocyst development in morula and blastula stage, and start up blastocyst implantation[8-10]. LIF gene knockout mice could fertilize normally and produce blastocysts, but the blastocysts could not support implantation[11]. Therefore, the present view holds that LIF is the most important cytokine that would mediate blastocyst implantation[12] and that the uterus lacking of LIF is not responsive to the implantation stimuli.

To investigate the correlation between LeY glycan expression and embryo implantation, we have prepared endometrial epithelial cells on the 3rd day of pregnancy (D3) and grouped them, meanwhile transfected the uterine epithelial cells before implantation with *FUT1siRNA* to inhibit *FUT1* expression. Then, we treated them with 10 ng/ml of LIF to upregulate the *FUT1* expression. Murine embryo implantation model *in vitro* was prepared by late blastocysts with identical morphology and treated uterine epithelial cells co-culture. We used RT-PCR, Dot-blot and observation of embryo attachment to analyze *FUT1* gene expression and LeY synthesis of uterine epithelial cells, to study the correlation of LeY expression level and embryo implantation, and to understand further the effect of LeY in embryo implantation.

Materials and Methods

Experimental Animals

Adult female Kunming mice of 6-8 weeks age (22-24 g) were provided by the experimental animal center of Dalian Medical University and were fed for 12 h in the light cycle and 12 h in dark cycle. 5-10 IU pregnant mare serum gonadotropin (PMSG, Tianjin animal experiment center) were injected into the mice for superovulation. 48 h later, we injected 5-10 IU of human chorionic gonadotropin (hCG, Shandong Penglai Huatai Pharmaceutical co. Ltd), after which we mated them with adult male mice in the ratio of 1:1. Examined their vagina the next day. The ones with vaginal plug were confirmed as the first day of pregnancy (D1).

Materials

FUT1 interference reagent kit was from Qiagen, Hilden, Germany. LIF was introduced from America Chemical Company, AH6 monoclonal antibody (anti-LeY) was given away by Professor Hakomori from University of Washington, USA, RT-PCR kit was from Dalian Takara Company, β-actin antibody from Wuhan Boster Company.

Preparation and Treatment of Monolayered Endometrial Epithelial Cells

The uterus of mice was dissected in the third day of pregnancy (D3). The uterus tissue was digested with trypsin solution (Sigma-Aldrich, St. Louis, MO, USA), followed by centrifugation at low speed (500 rpm). The endometrial epithelial cells were collected, and allowed to grow in 24-well culture plate (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, 0.5 ml Ham's F-10 (Gibco, Rockville, MD, USA)+10% FBS (fetal bovine serum, Institute of Hematology, Chinese Academy of Medical Sciences) was added to the each well. The culture medium was composed of 1×10^5 cells, and cultured in incubator of 37°C and 5% CO_2[13]. The well on culture plate were divided into control and treatment groups, and were transfected with HiPerFect transfection reagent (Qiagen, Hilden, Germany). The cells in interference group were transfected with 20 μmol/L *FUT1 siRNA* 0.15 μl, cells in interference control group were transfected with 20 μmol/L control *siRNA* 0.15 μl, cells in LIF group were treated with 10 ng/ml LIF, and cells cultured in pure culture medium were grouped into a blank group. Subsequent experiments were conducted 24 h after transfection and 12 h after LIF.

Detection of FUT1 Gene Expression in Endometrial Cells

The cells from all groups were collected and washed with 75% ethanol after Trizol split (Gibco, Rockville, MD, USA), chloroform extraction and isopropanol precipitation. Finally, the precipitants were completely dissolved in 10 μl RNase Free ddH_2O. The absorbance was measured at OD_{260}, OD_{280} (UV_{754}) (TianMei Biotechnology Company, Beijing, China) and RNA purity and quality was analyzed after 1% formaldehyde denaturing gel electrophoresis, estimation of RNA content. Reverse transcription was carried out in 20 μl reaction system composed of 2 μg RNA, 4 μl Mg^{2+} (25 mmol/L), 2 μl 10× reverse transcription buffer, 2 μl dNTP (10 mmol/L), 0.5 μl

Table I. Primers used for this study.

Genes	Sequences (5′to3′)	Size (bp)
FUT1	(F)5'-CCTGGCATTTCTGTTGGTCTGT-3' (R)5'-ACCTTCCCGTAGATGGTTATGC-3'	559 bp
β-actin	(F)5'-GTGGGCCGCCCTAGGCACCAA-3' (R)5'-CTCTTTGATGTCACGCACGATTTC-3'	540 bp

(F) Stands for forward primer, (R) reverse primer, primers designed by primer 5.0.

RNase inhibitor (40 u/μl), 1 μl AMV reverse transcriptase (5 ug/μl), 1 μl random primer (Oligo dT), and complemented the system by DEPC water. The PCR conditions were annealing at 50°C for 50 min, extension 99°C for 5 min, finally at 4°C for 5 min. 2 μl of cDNA was taken for *FUT1* and *β-actin* gene amplification, 2 μl Mg^{2+} (25 mmol/L), 2 μl dNTP (2.5 mmol/L), 2.5 μl 10×Ex Taq Buffer, 0.5 μl Taq DNA polymerase, 0.5 μl (30 pmol/L) forward primer and 0.5 μl (30 pmol/L) reverse primer, complemented with ddH_2O till the volume of 25 μl was used. PCR reaction condition was pre-denaturation at 94°C for 5 min, denaturation at 94°C for 50 s, annealing at 56°C for 50 s, extension at 72°C for 1 min, then, a final extension at 72°C for 5 min after 35 cycles. The primers sequence is shown in Table I.

The PCR products were loaded on to 1% agarose gel for electrophoresis and were subsequently processed by Lab works 4.60 software. The relative intensity of each band was normalized with β-actin.

Protein Extraction and Dot-blot Analysis of Endometrial Cells

100 μl protein extract (1000 μl PBS contained 10 μl NP-40 and 10 μl PMSF) was added to each well and was transferred into Eppendorf tube after blowing, beating and binding at 4°C for 2 h. Subsequently, the tube contents were centrifuged at 4°C for 15 min at 10,000 rpm and the supernatant was transferred into a new tube and extracted proteins were quantified by Kaunas Bradford method. 20 μg of the extracted proteins in each group was attached to NC membrane by Dot blot instrument (MBI Company), incubated with 5% BSA at 37°C for 2 h. The membrane was washed with TBS and allowed to incubate with LeY primary antibody at 37°C for 2 h. The membrane was washed again with TBS and then placed into alkaline phosphatase labeled anti-IgM second antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to incubate at 37°C for 40 min. The image was developed in a dark room after exposing membrane to NBT/BCIP (Gibco, Rockville, MD, USA). The image was processed by Lab works 4.60 software and the relative intensity of each sample was calculated on the basis of β-actin, then protein content of LeY were compared and analyzed.

Blastocyst Collection and Co-Culture with Endometrial Epithelial Cells

Female mice were decapitated in the fourth day of pregnancy (D4) to rapidly collect their uterus to remove blastocysts from the uterus before implantation. The well-developed and uniform Level A advanced blastula was selected[14] and allowed to migrate into 35 mm culture dish. Afterwards, it was placed into 50 μl of pre-warmed Ham's F-10 culture medium droplet at 37°C in 5% CO_2 incubator for culture. After 2 h, blastula was transferred into 24-well culture plate and co-cultured with endometrial epithelial cells from all groups. Each well with 10 blastocysts was cultured for 12 h, 24 h, and 36 h individually in 5% CO_2 incubator at 37°C and then the attachment of embryo was observed and the ratio was calculated. The attachment rate of each group was presented by percentage between the number of attachment embryos and that of total embryos. The identification of attachment of blastocyst on the surface of uterine epithelial cell monolayer was subject to the methods of Masahide et al[15].

Statistical Analysis

SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. One-Way ANOVA was applied to make comparisons among the three groups and independent-sample *t*-test was applied to make a comparison between the groups. Repeated Measure ANOVA was applied to evaluate the outcomes of co-culture and $p<0.05$ was considered to be statistically significant.

Table II. Correlation of LeY expression of uterine epithelial cells with attachment and outgrowth rate in co-culture system.

Group		Co-culture time (h)		
		12	24	36
Attachment rate (%)	Blank group	12.0±2.0	23.3±1.2	40.7±3.1
	Interference control group	12.7±3.1	23.3±2.3	40.0±2.0
	Interference group	6.7±2.3	12.0±2.0	32.7±3.1

(F) Stands for forward primer, (R) reverse primer, primers designed by primer 5.0.

Results

RT-PCR detection on Gene Expression of Endometrial cell FUT1 After Transfection

Figure 1 shows the results of RT-PCR in comparison with the blank group and interference control group, the expression of endometrial cell LeY synthesis key enzyme gene was significantly reduced after being interfered by *FUT1siRNA* for 24 h.

Dot-blot Detection on Endometrial Cell LeY glycan Expression After Transfection

Figure 2 shows results of Dot-blot, in comparison with the blank group, the interference control group showed a significant reduction in synthesis of LeY glycan after the interference of endometrial cell *FUT1* gene with *FUT1siRNA* for 24 h.

Effects of FUT1 Gene Interference on Embryo Attachment Growth

Figure 3 shows the observed attachment and outgrowth of embryos in each group at different co-culture time points. The results of co-culture in Table II (Figure 3) at different time points, the adhesion rate of the blank group was 12.0, 23.3, 40.7%; of the interference control group was 12.7, 23.3, 40.0 % of the interference group was 6.7, 12.0, 32.7 %. The result was the average value of three repeated experiments and was presented by ±1.96S.

Effects of LIF on FUT1 Gene Expression of Endometrial Epithelial cells by RT-PCR

As was shown in Figure 4, in comparison to the blank group, *FUT1* gene expression increased after LIF (10 ng/ml) was added to endometrial epithelial cells.

Effects of LIF on LeY glycan Expression of Endometrial Epithelial cell by Dot Blot

Results of Dot-blot (Figure 5): compared with the blank group, the synthesis of LeY glycan expression was significantly increased after LIF (10 ng/ml) was applied to treat endometrial epithelial cells.

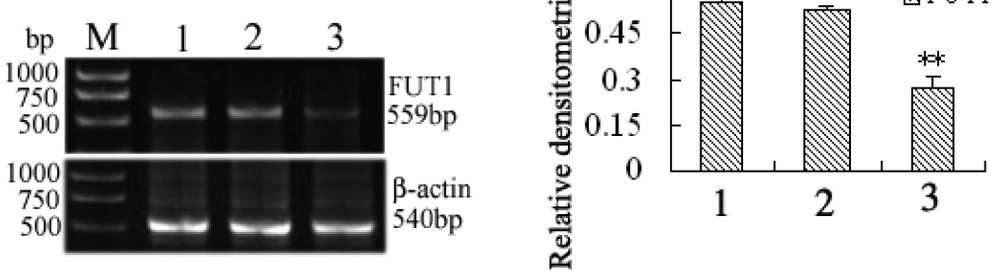

Figure 1. RT-PCR analysis of FUT1 and β-actin gene expression of uterine epithelial cells after transfection of FUT1siRNA ***(A)*** Electrophoresis of RT-PCR products ***(B)*** Relative densitometric analysis of FUT1 and β-actin gene amplification products. Lane 1: Cells were cultured with only medium, Lane 2: Cells transfected with control siRNA, Lane 3: Cells transfected with FUT1siRNA, M is DNA marker, DL2000. $^{**}p<0.01$

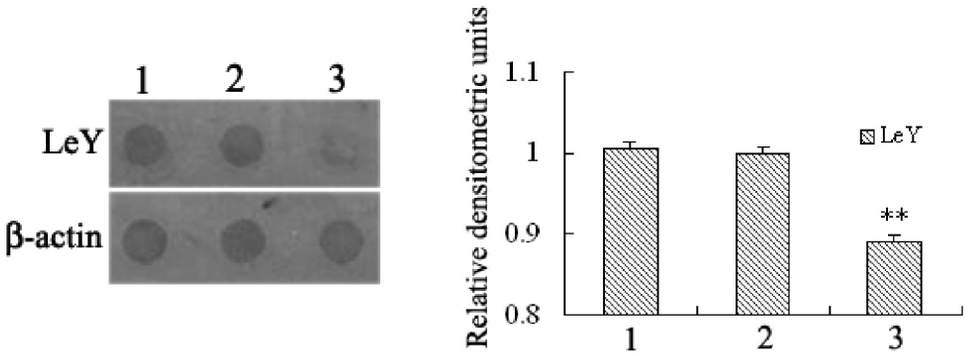

Figure 2. Dot-blot analysis of LeY and β-actin expression of uterine epithelial cells after transfection of FUT1siRNA. ***(A)*** Dot-blot analysis of LeY expression ***(B)*** Relative densitometric analysis of LeY/β-actin. Lane 1: Cells were cultured with only medium, Lane 2: Cells were transfected with control siRNA, Lane 3: Cells were transfected with FUT1siRNA. $^{**}p<0.01$.

Effects of Exogenous LIF on in-vitro Embryo Attachment and Outgrowth in co-culture System

The *in vitro* embryos and exogenous LIT treated monolayer endometrial epithelium cells were co-cultured for 12 h, 24 h and 36 h, then observed the attachment rate of embryos. Co-culture results are shown in Table I (Figure 6), the attachment rate of the blank group at different time points were 14.0, 24.7, 37.3 %; of LIF group was 18.7, 34.0, 52.0%. The result was the average value of three repeated experiments and was presented by $\bar{X} \pm 1.96S$.

Discussion

Studies have shown that during the mammal's embryo implantation period, a large amount of fucosylated antigen expressions would appear on the surface of endometrial epithelial cells and embryos[16,17], with fucosylated lactose series glycan (Le^X, LeY, H1) being the leading one. LeY glycan antigen showed specific expressions in different phases of the animals' reproductive cycle and was regulated by estrogen or progesterone. For example, during the implantation period, endometria of mice would show specific LeY glycoprotein and be up-regulated by estrogen[18,19]. Neutralizing the LeY glycan in blastocyst or endometrial surface by the specific monoclonal antibody (AH6) could prevent the blastocyst and endometrium from recognizing and attaching to each other, thus significantly inhibiting embryo implantation[2,3]. That LeY was involved in the implantation has already been preliminary verified in uterine samples of rhesus, a primate,

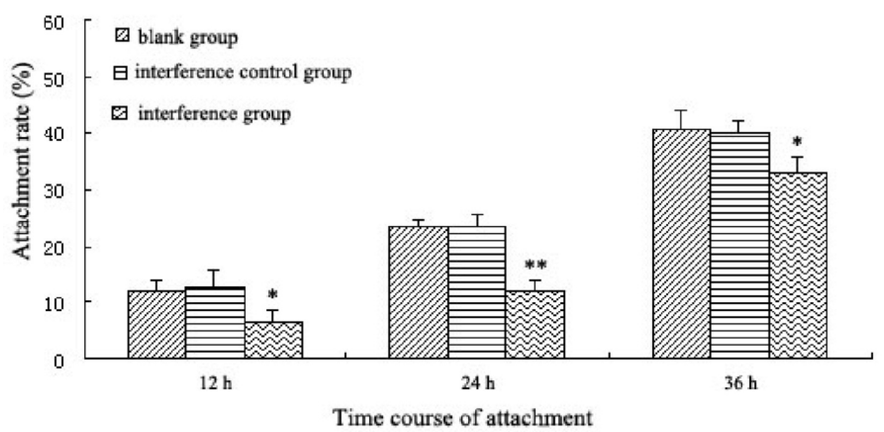

Figure 3. Effects of LeY expression on attachment rate in co-culture system. $^{*}p<0.05$ $^{**}p<0.01$Effects of LeY expression on attachment rate in co-culture system. $^{*}p<0.05$ $^{**}p<0.01$

Table III. Effects of LIF on attachment and outgrowth rate in co-culture system.

		Co-culture time course (h)		
	Group	**12**	**24**	**36**
Attachment rate (%)	Blank group	14.0±2.0	24.7±2.3	37.3±2.3
	LIF group	18.7±1.2	34.0±4.0	52.0±2.0

and human beings[20,21]. It has been found that the expression of 11 kinds of key fucosyltransferase (FUT1-11) that could catalyze the fucose synthesis and the key enzyme gene *FUT1* that was synthesized by LeY glycan in endometrium during the peri-implantation period was consistent with the expression of LeY[7], which indicated that the synthesis of glycan was regulated by its synthesis key enzyme genes. Therefore, regulating the expression of *FUT1* gene could influence the synthesis of LeY glycan, thus making the studies on related biological functions of glycan possible.

LIF was an important regulatory factor for embryo development and implantation. *LIF* mRNA was located in endometrial luminal epithelium and glandular epithelium. Its expression alters consistently with the changes of protein levels and its expression peak is nicely consistent with the starting time of blastocyst implantation. Neutralizing the LIF on blastocyst surface could not only lead to implantation failure[10], but also significantly inhibit the implantation factors that had stage-specific expression including MMPs, EGF, and so on. LIF also had dose-dependent while nourishing the embryos. Too high concentration of LIF can inhibit the growth of embryos[22]. Implantation was impossible for mice with LIF gene expression defect. LIF was the only factor that could prevent the embryo from implantation after gene knockout[11].

In our study, we have prepared endometrial epithelial cells in the third day of pregnancy (D3), applied RNAi technology and *FUT1siRNA* to inhibit the key enzyme gene synthesized by LeY glycan. Meanwhile, we also added 10 ng/ml LIF to promote the synthesis and expression of LeY glycan. We showed that in comparison with the blank group and interference control group, the expression of endometrial cell *FUT1* gene was significantly reduced after being transfected with *FUT1siRNA*. Results of Dot-blot detection showed that the expression of endometrial epithelial cell LeY was significantly reduced, which indicated that LeY expression was regulated by the expression of *FUT1* gene. *In vitro* embryo implantation models discovered the after *FUT1* gene expression was inhibited, the attachment rate and outgrowth rate of embryos at different time points were significantly lower than those of blank group and interference control group. As time went on, the inhibiting effect on attachment and growth at 36 h were not as strong as that at 12 h

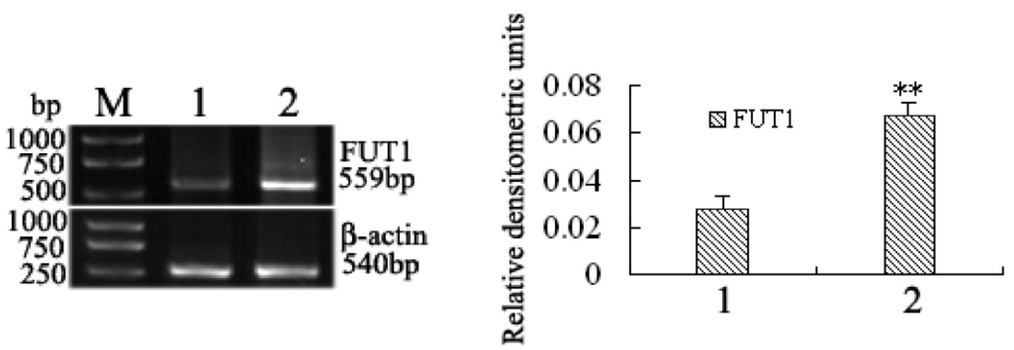

Figure 4. RT-PCR analysis of FUT1 and β-actin gene expression after LIF treated cells. *(A)* Electrophoresis of RT-PCR products; ***(B)*** Relative densitometric analysis of FUT1 and β-actin gene amplification products. Lane 1: Cells were cultured with only medium, Lane 2: Cells were treated with LIF (10 ng/ml), M: DNA marker, DL2000. $^{**}p<0.01$

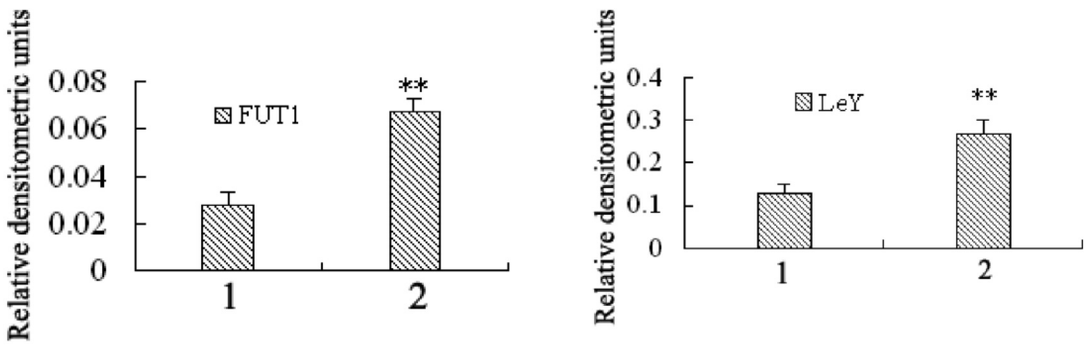

Figure 5. Dot-blot analysis of LeY and β-actin expression after LIF treated cells. ***(A)*** Dot-blot analysis of LeY expression; ***(B)*** Relative densitometric analysis of LeY/β-actin. Lane 1: Cells were cultured with only medium, Lane 2: Cells were treated with LIF (10 ng/ml). $^{**}p<0.01$

and 24 h; it might be because the inhibited *FUT1* gene function was gradually recovered. However, after LIF was added to endometrial epithelial cells, RT-PCR detection results showed that *FUT1* gene expression was greatly promoted. Dot-blot detection result showed that LeY expression was also greatly improved and LIF also improved the attachment rate of embryos (52.0%) (compared with the 37.3% of the blank group). These results indicated that regulating *FUT1* expression affected LeY synthesis, and then LeY regulated the recognition and attachment of uterus-embryo and participated in embryo implantation further.

There were two possible ways for LeY to regulate implantation, firstly, LeY might regulate the receptivity of endometrium and the invasiveness of embryo and, secondly, LeY might act as a kind of immunosuppressive molecule preventing the immunological reaction when embryos divorced from the parent body[23]. Glycoconjugates could promote angiogenesis[24]. LeY antigen had a rich expression on the surface of tumor cells, which was closely related to the proliferation, differentiation and transfer of tumor cells. Embryo implantation process was quite similar to tumor transfer process, which indicated that LeY glycan might be involved in embryo implantation through promoting angiogenesis.

Conclusions

The results of our study showed that after the expression of key glycosyl transferase gene synthesized by LeY was inhibited, the expres-

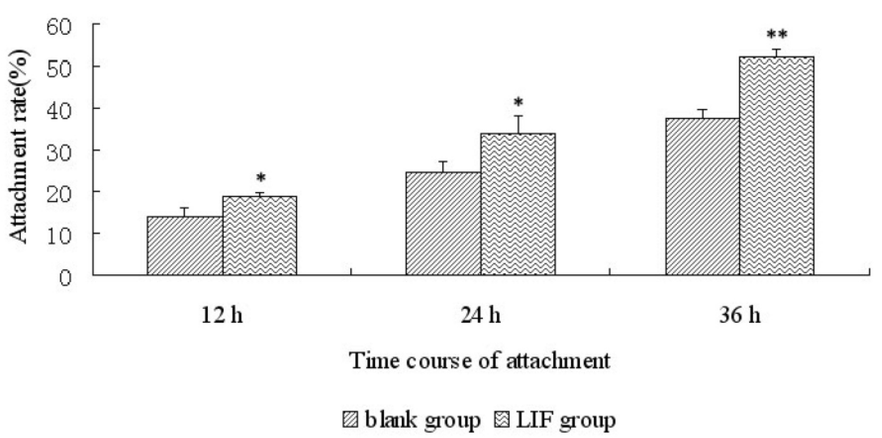

Figure 6. Effects of LIF on embryo attachment and outgrowth rate in co-culture system. $^{*}p<0.05$ $^{**}p<0.01$

sion of LeY was lowered, and the attachment rate as well as the outgrowth rate of embryos, were also lowered. The acknowledged implantation essential factor, LIF played quite an important role in promoting the LeY expression on the surface of the endometrium during peri-implantation period while facilitating embryo development and implantation process. Although some studies have reported that embryos, after gene knockout, could still achieve implantation[25]. It might be because the complexity and precision of blastocyst implantation required multiple influencing factors to coordinate expression and co-regulate to achieve implantation. After an in-depth study on the LeY glycan on the surface of endometrial cells, we have come to that, to develop a better understanding of the intrauterine environment during implantation period and the molecular markers of endometrium in different states are quite significant in reproductive medicine, including the etiologic study and treatment of infertility, and the improvement of pregnancy rate of assisted reproductive technique. Also, it also provides a new clue and method for clinical contraception.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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