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Glycodelin suppresses Endometrial cells Migration and Invasion but stimulates Spheroid attachment

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ABSTRACT
Glycodelin contains four isoforms with diverse biological functions. Glycodelin-A is a potential diagnostic marker for cancer patients and receptive marker of secretory endometrium. Yet, direct evidence of glycodelin on regulating endometrial epithelial cells migration, invasion and attachment of trophoblastic spheroids (blastocyst surrogate) is lacking. In this study, the human glycodelin gene was stably transfected into the human endometrial (HEC1-B) cells. Forced-expression of glycodelin in HEC1-B cells did not affect cell proliferation, cell viability and cell cycle progression, but significantly reduced migration and invasion of the stably transfected cells. The migration rate returned to normal levels when the glycodelin stably expressing HEC1-B cells were treated with glycodelin RNAi. Furthermore, forced-expression of glycodelin in HEC1-B cells significantly increased the attachment of trophoblastic spheroids onto the endometrial epithelial cells. In sum, glycodelin suppressed endometrial cell migration/invasion but enhanced spheroids attachment.

Keywords: Glycodelin; spheroid; migration; invasion; attachment.
INTRODUCTION

The lipocalin subfamily member glycodelin has several isoforms found in the reproductive system, namely glycodelin-A (GdA), glycodelin-F (GdF), glycodelin-S (GdS), and glycodelin-C (GdC) (Seppala et al., 2007). The exact biological function of glycodelin remains unknown, but it is believed to play important roles on endometrial receptivity, maternal-fetal immunity, and gynecological cancer progression. For example, GdA inhibits sperm-oocyte interaction by binding onto the sperm head; GdS in seminal plasma maintains sperm in an uncapacitated state; GdF in follicular fluid and the Fallopian tube inhibits sperm-oocyte binding and the premature progesterone-induced acrosome reaction; and GdC in cumulus matrix displaces sperm-bound inhibitory glycodelin isoforms and enhances sperm-oocyte binding (Seppala et al., 2007; Yeung et al., 2009).

Glycodelin-A, a progesterone-regulated glycoprotein, is highly expressed during the secretory phase in the human endometrium (Seppala et al., 2002). The level of GdA gradually increases in the endometrial glands 4 to 5 days after ovulation and reaches its peak on day 10, coinciding with the window of implantation (WOI) (Seppala et al., 2002; Carson et al., 2002). Embryo implantation involves apposition and adhesion of embryo to the endometrial epithelium, penetration of the endometrial epithelium, and invasion of the extravillous trophoblast cells through the decidualized endometrial stromal cells (Bentin-Ley et al., 1999). Among various steps in implantation, attachment of embryo onto endometrial epithelial cells is essential for successful implantation and pregnancy. For example, pinopodes are formed at the apical region of the endometrium that facilitate adhesion of blastocyst to the endometrial surface (Bentin-Ley et al., 1999). Moreover, embryo implantation is regulated by various endometrial adhesive (e.g. integrins) and anti-adhesive (e.g. MUC1) factors (Chervenak and Illsley, 2000). Expressions of these adhesive and anti-adhesive factors are associated with an increase in glycodelin expression during WOI (Hohn et al., 2000; Seppala et al., 2002;). Furthermore, several microarray studies
have compared gene expression patterns of the endometrium during the menstrual cycle (Carson et al., 2002; Talbi et al., 2006). A large number of genes showed cyclical variation in expression, but only a few genes including osteopontin, apolipoprotein D, dickkopf/DKK1 and olfactomedin were found to be common in all these studies (Horcajadas et al., 2004). Abnormal levels of glycodelin are associated with unexplained infertility and early pregnancy loss (Seppala et al., 2009).

Glycodelin is also involved in the progression of gynecological cancers. An aberrant expression of glycodelin is associated with endometrial, ovarian and breast cancers (Seppala et al., 2009). Forced-expression of glycodelin in breast cancer cell lines and cancer cell xenografts reverses the malignant phenotype of the cells (Kamarainen et al., 1997; Hautala et al., 2008). How glycodelin modulates these changes has yet to be determined. In the present study, we stably transfected glycodelin gene in human endometrial (HEC1-B) cell line and studied the effects of glycodelin force-expression on cell migration, cell invasion, and trophoblastic spheroids attachment onto the endometrial cells.
MATERIALS AND METHODS

Cell lines and forced-expression of glycodeolin in HEC1-B cells

Human endometrial adenocarcinoma cells (HEC1-B, Cat no.: HTB-113) were obtained from ATCC (Manassas, VA, USA) and cultured in Minimal Essential Medium (MEM) (Sigma, St Louis, MO, USA) supplemented with 10 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen) at 37 °C under 5% CO₂ in a humidified incubator. Total RNA from human endometrial tissue was purified using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA, USA). Total RNA was reverse transcribed using the First strand cDNA synthesis kit (Amersham, Pittsburgh, PA, USA). The human glycodeolin cDNA was amplified by PCR using a glycodeolin forward primer (5’-GTCAAGCTTGATGGACATCCCCAGACCA-3’, HindIII site underlined) and a glycodeolin reverse primer (5’-GAGGAATTCTTCGAAACGGCACGGCTCTTCCAT-3’, EcoRI site underlined). The glycodeolin coding region was cloned into the pHM6 mammalian expression vector (Roche Diagnostics Co., Switzerland). This vector was then transfected into HEC1-B cells using lipofectamine 2000 (Invitrogen). The selection of the stable clones was carried out using 50 µg/ml G418 for one month. The stable clones were maintained in MEM medium containing supplements and 50 µg/ml G418. All the experiments were performed in passage 5 to 10 of the stable selected HEC1-B clones.

Quantitative PCR

Total RNA isolated from cultured cells was reverse transcribed with TaqMan reverse transcription reagent kit (Applied Biosystems, Foster City, CA, USA) and multi-script reverse transcriptase. The resulting cDNAs were analyzed using qPCR on a glycodeolin TaqMan probe (Hs00171462_m1, Applied Biosystems). Quantitative PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems). Multiplex PCR was
performed on 20 μl volumes containing 5 μl of sample cDNA, 10 μl 2X TaqMan Universal
PCR Master Mix, 1 μl 20X Gene Expression Assay for targets and 1 μl eukaryotic 18S
rRNA as the internal control. The relative levels of gene expression were detected using the
threshold cycle (C_T) method (also known as the 2^{ΔΔCT} method) using endogenous 18S as
the internal control. All the experiments were performed in triplicates and repeated at least
3 times.

Western blotting

Total proteins from HEC1-B were dissolved in RIPA solution (1X PBS, 1% Nonidet P-40,
0.5% sodium deoxycholate, 0.1 % SDS) containing protease inhibitors. The samples were
separated on 12% SDS-PAGE and the proteins were transferred to a PVDF membrane.
After thorough washing, the membrane was probed with glycodelin antibody (rabbit
antibody raised against glycodelin peptide, amino acids 69-83) at 1:1000 dilution, α-
tubulin (1:5000, Santa Cruz, Santa Cruz, CA, USA) and β-actin antibody (1:5000, Sigma).
Positive signal was detected by chemiluminescence using goat anti-rabbit or goat anti-
mouse IgG conjugated-HRP secondary antibodies.

Flow cytometry

Transfected HEC1-B cells were collected and fixed in 70 % ethanol. The cells were
washed in phosphate buffered saline twice and stained with 2.5 μg/ml propidium iodide.
DNA content was measured by flow cytometry on a BD FACSCantoII Analyzer (BD
Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser. Fluorescence
signals were measured using a 585 nm band pass filter. The results were analyzed using
WinMDI 2.9 (The Scripps Research Institute Cytometry Software, San Diego, CA, USA)
and Cylchred (Cytonet UK, Cardiff, UK).
Cell Migration Assay
The migration rate of glycodelin overexpressing HEC1-B cells was determined in a wound healing assay. Briefly, 2 x 10^5 HEC1-B cells in 2 ml MEM medium on 6-well plates were grown overnight at 37 °C. The cells were treated with 20 µg/ml mitomycin C (Sigma) for 3 h. The culture medium was changed and a wound was created on each well using the tip of a pipette. The migration rate was expressed as a percentage of the width of the wound at 48 h divided by the width of the wound at 0 h.

Cell Invasion Assay
The cell invasion of the transfected HEC1-B cells was studied using a BD Biocoat™ Matrigel™ Invasion Chamber (BD Biosciences). The invasion chambers used a 9 µm pore size polyethylene terephthalate membrane, which was chosen for this study because it could effectively block non-invasive cells from migrating through the membrane while allowing invasive cells to pass through. The HEC1-B cells (5 x 10^4 cells/ml in 0.5 ml serum-free MEM medium) were seeded on the invasion chamber and incubated at 37 °C for 24 h. The empty vector clone was used as the control. Non-invading cells were removed from the interior chamber by scrubbing with a cotton swab. The invading cells on the bottom of the membrane were stained with crystal violet stain for 25 min.

RNAi transfection
RNAi targeted against glycodelin (Human LOC649984, Thermo Fisher Scientific, Lafayette, CO, USA) was diluted to 10 nmol and transfected into glycodelin overexpressing HEC1-B cells using lipofectamine 2000 (Invitrogen).

Spheroid Attachment Assay
Spheroid Attachment Assay was performed as previously described (Kodithuwakku et al.,
Human trophoblastic JAr (blastocyst surrogate) and HEC1-B cells were used as the co-culture model. Spheroids (60-200 μm) were prepared from trypsinized JAr cells in an incubator at 37 °C and rotated at 100 rpm for 24 h. Spheroids were then transferred onto a confluent monolayer of transfected HEC1-B cells on a 12-well culture plate (in MEM with 10 % FBS and 1 % L-glutamine). After incubation at 37 °C for 24 h, unattached spheroids were removed by shaking the culture plates at 140 rpm for 10 min. The attached spheroids that remained on the HEC1-B monolayer were then counted. The attachment rate was expressed as a percentage of the number of attached spheroids divided by the total number of spheroids added onto the HEC1-B monolayer.

Statistical Analysis

All the data were analyzed by statistical softwares (SigmaPlot 11.0 and SigmaStat 2.03; Jandel Scientific, San Rafael, CA, USA) and presented as Mean ± S.E.M. The non-parametric analysis of variance on rank test for multiple comparisons followed by the Mann-Whitney U test was used when the data were not normally distributed. A probability value <0.05 was considered to be statistically significant.
RESULTS
Forced-expression of glycodelin on cell growth
The cDNA coding region of glycodelin was cloned into the pHM6 mammalian expression vector and transfected into HEC1-B cells. Gentamycin was used to select glycodelin forced-expressing clones (4-1 and 4-2), and the empty vector transfected control clone (1-5). Western blotting results confirmed the expression of glycodelin protein in clones 4-1 and 4-2, but not in clone 1-5 (Fig. 1A). Quantitative PCR results confirmed that the expression levels of glycodelin transcript in clones 4-1 and 4-2 were $10^5$-fold higher than in clone 1-5 (Fig. 1A). Forced-expression of glycodelin had no effect on cell proliferation and cell viability in all the selected clones (data not shown).

Flow cytometry was used to monitor the DNA content of stably transfected cells at different phases of the cell cycle. We analyzed the 2N (G0/G1 phase) and 4N (G2/M phase) DNA content. No changes were observed in the percentages of cells in G0/G1, S and G2/M phases in all the selected clones (Fig. 1B). This result was confirmed by qPCR of the cell cycle checkpoint transcripts including p16, p21 and p27 (data not shown).

Forced-expression of glycodelin on cell migration and invasion
Cells in the wound healing assay were treated with mitomycin C to suppress cell proliferation. Therefore, the change in area covered by cells was only caused by cell migration, but not division and proliferation. Forced-expression of glycodelin in HEC1-B cells in clones 4-1 and 4-2 suppressed the migration of the transfected HEC1-B cells when compared with the control clone 1-5 (Fig. 2A). The migration rates in clones 4-1 and 4-2 were significantly lower (23% and 35%, respectively; $p<0.05$) than in control clone 1-5 (51%). The effect of glycodelin on cell invasion was studied using a cell invasion chamber assay. The cell invasion of clones 4-1 and 4-2 was significantly decreased (30% each; $p<0.05$) compared with the control clone 1-5 (Fig. 2B).
Suppression of glycodelin in stably transfected cells increases cell migration

RNAi knockdown of the glycodelin expression in the stably transfected clones was performed to confirm the effect of glycodelin on cell migration. The expression of glycodelin protein in clone 4-1 was found to be down-regulated by glycodelin RNAi compared with the control clone 1-5 (>50% reduction) (Fig. 2C). Transfection of glycodelin RNAi did not affect migration in the control clone 1-4, but significantly increased the migration rate (43%) in clone 4-1 (Fig. 2C). No changes in migration rate were observed when the cells were treated with non-target RNAi.

Effect of glycodelin forced-expression on spheroid attachment

Forced-expression of glycodelin in HEC1-B cells increased the rate of attachment of trophoblastic spheroids onto the endometrial epithelial cells (Fig. 3A). The rate of attachment of spheroids on glycodelin forced-expressing clones 4-1 and 4-2 was significantly higher (84% and 89%, respectively; p<0.05) than on control clone 1-5 (72%) (Fig. 3B).
1 **DISCUSSION**

2 Previous studies showed that GdA inhibits NK cell activity, monocyte chemotaxis, T and B cell proliferation, and induces apoptosis in monocytes (Alok and Karande, 2009). In the present study, forced-expression of glycodelin in the HEC1-B cells did not significantly affect the cell cycle progression as confirmed by the cell proliferation assay, qPCR, and flow-cytometry analysis.

3 Glycodelin has been found in breast cancer tissues and was detected in 50% of lymph node metastases, 38% of recurrent breast cancers, and 40% of breast cancers with distant metastases (Jeschke et al., 2005). Chemotherapy-treated ovarian cancer patients with higher glycodelin expression had longer survival times and less metastases than those with glycodelin-negative ovarian cancers at the same stages and differentiation grades (Koistinen et al., 2005). Moreover, GdA expression was significantly reduced in G2 compared to G1 ovarian cancer tissue (Jeschke et al., 2005), but there was no change in cell cycle check point marker in the present study as confirmed by RT-PCR analysis. Similarly, ovarian cancer at surgical stage FIGO III-IV had a significantly lower GdA expression compared with FIGO I-II stage tumors (Tsviliana et al., 2010). From our findings, we suggest that glycodelin could be used as a biological marker for cancer progression.

4 Forced-expression of glycodelin in the HEC1-B cell line inhibited endometrial epithelial cancer cell migration and invasion in vitro. The decrease in migration ability could be reversed when these cells were transfected with glycodelin RNAi. Forced-expression of glycodelin in HEC1-B cells resulted in a reduction of carcinoma-associated gene expression, including MUC1 expression (Koistinen et al., 2005). MUC1 is an anti-adhesive molecule that is overexpressed in many cancer cells and induces invasive growth and metastasis (Koistinen et al., 2005). A low level of MUC1 is associated with the reversal of malignancy (Coronado et al., 2001; Sivridis et al., 2002).
Other than cancer, migratory and invasive properties of endometrial epithelial cells were suggested to be responsible for endometriosis and adenomyosis (Chen et al., 2010; Dentillo et al., 2010; dos Santos Hidalgo et al., 2011). The main clinical features of endometriosis are pelvic pain and infertility (Giudice and Kao, 2004). Glycodelin is down-regulated in eutopic endometrium tissue of endometriosis patients (Meola et al., 2009; Kao et al., 2003). These studies showed strong correlation between glycodelin and endometriosis. Together with role of glycodelin in other gynaecological diseases, glycodelin is responsible for number of pathways which affects steroid receptivity, growth and invasive potential in endometriotic tissues (Koistinen et al., 2005; Gaetje et al., 1995).

In the current study, increase in glycodelin expression could reduce migratory and invasive property of endometrial epithelial cells, which might cause a reduction in endometriosis. Therefore, increase in glycodelin expression might contribute to advantage for successful implantation and pregnancy in an indirect way by minimizing the effect of endometriosis and up-regulation of receptive marker (Uchida et al., 2007).

Blastocyst apposition and adhesion to the receptive endometrium are necessary steps to establish a successful implantation and pregnancy. In this study, forced-expression of glycodelin in HEC1-B was found to up-regulate the attachment rate of spheroid (blastocyst surrogate) onto the endometrial cells. Glycodelin was up-regulated in the endometrium during the receptive phase of the cycle (Liu et al., 2008). In humans, endometrial receptivity is highest at the mid-secretory phase of the menstrual cycle, in which glycodelin is highly expressed at the apical membranous protrusions called pinopodes (Stavreus-Evers et al., 2006). Pinopodes express adhesion surface molecules that are involved in embryo attachment (Lessey et al., 2000). At the adhesion phase of implantation, the embryo induces a paracrine cleavage of anti-adhesive molecule MUC1 in the endometrial epithelial cells (Meseguer et al., 2001). Other adhesive molecules such as integrins, heparin-binding epidermal growth factor-like growth factor, and leukemia
inhibitory factor are co-expressed on the pinopode during mid-secretory phase of menstrual cycle (Lessey et al., 2000; Aghajanova et al., 2003; Stavreus-Evers et al., 2002), and these promote attachment of embryo onto the receptive endometrium. Other molecules are found to suppress spheroid attachment onto endometrial cells in vitro, such as olfactomedin-1 that is down-regulated in the receptive endometrium (Kodithuwakku et al., 2011).

In conclusion, forced-expression of glycodelin in HEC1-B cells suppresses endometrial adenocarcinoma cell migration and invasion, but not cell cycle progression in vitro. Moreover, cells with forced-expression of glycodelin have higher rates of spheroid attachment than the controls, suggesting that an increased expression of glycodelin might favor embryo attachment in vivo. The use of glycodelin as a cancer marker and therapeutic target for gynecological cancer warrants further investigation. Moreover, forced-expression of glycodelin in human endometrium for fertility treatment should be explored.

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AUTHOR CONTRIBUTION

KHS, CLL, WSBY and KFL conceived the experiments. KHS, CLL and KFL carried out the experiments and all authors analyzed the data. KHS and KFL wrote the paper and all authors had final approval of the submitted and published versions.
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FIGURE LEGENDS

Figure 1  Effects of glycodelin forced-expression in HEC1-B cells on cell cycle progression. (A) Quantitative analysis of glycodelin mRNA expression in the HEC1-B cells transfected with empty vector control (Clone 1-5) and glycodelin expressing plasmid (Clones 4-1 and 4-2). Representative Western blotting of the transfected clones probed with anti-glycodelin and β-actin antibodies are shown on the right. (B) Flow cytometry analysis of the DNA content in the transfected clones (1-5, 4-1 and 4-2) at G0/G1, S and G2/M phase. The DNA content for cells in G2/M phase is higher than in S phase and G0/G1 phase (Right). All the experiments were performed in triplicates and repeated at least 3 times.

Figure 2  Forced-expression of glycodelin in HEC1-B suppresses cell migration and invasion. (A) Forced-expression of glycodelin in clones 4-1 and 4-2 suppresses cell migration at 48 h compared to the control clone 1-5. (B) Forced-expression of glycodelin suppresses cell invasion at 24 h in clones 4-1 and 4-2 but not in control clone 1-5 (Scale bar, 100μm). (C) Transfection of glycodelin RNAi (Gd) suppressed glycodelin expression (left panel) and restored the cell migration rate (right panel) in clone 4-1. No effect was observed when the cells were transfected with transfection reagent (-) or non-target (NT) RNAi control. There was no change in cell migration rate in control clone 1-5 when transfected with glycodelin or non-target RNAi. a-b denotes significant difference from each other at p<0.05. All the experiments were performed in triplicates and repeated at least 3 times.

Figure 3  Forced-expression of glycodelin in the transfected HEC1-B cells stimulates spheroid attachment. (A) Spheroids (60-200 μm) were prepared from JAr cells for the co-culture study (Scale bar, 100 μm). (B) The spheroid attachment rates of clones 4-1 and 4-2
were significantly higher \((p<0.05, a-b)\) than in the control clone 1-5. The results were obtained from three or more pooled experiments and the total number of spheroids used per group was more than 200.
Figure 1

A

Log_{10} Gd mRNA expression level (Fold-change)

Clone

1-5 4-1 4-2

B

Cell population (%)

G0/G1 S G2/M

Clone

1-5 4-1 4-2 1-5 4-1 4-2 1-5 4-1 4-2

Clone

1-5 4-1 4-2

Glycodelin

β-actin
Figure 2

(A) Migration rate (%)

(B) Invasion rate (%)

(C) RNAi treatment of Clone 4-1

- Gd      NT

Clone 4-1

Glycodelin
α-tubulin

Clone 1-5 4-1 4-2

0h 48h
Figure 3

A

B

Attachment rate (%)

1-5 4-1 4-2

Clone

160 178 199

217 211 233

a b b

bar scale