

1 **Glycodelin suppresses Endometrial cells Migration and Invasion but**  
2 **stimulates Spheroid attachment**

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15

1 **ABSTRACT**

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

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16 **Keywords:** Glycodelin; spheroid; migration; invasion; attachment.

17

## 1 INTRODUCTION

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

12 Glycodelin-A, a progesterone-regulated glycoprotein, is highly expressed during the  
13 secretory phase in the human endometrium (Seppala et al., 2002). The level of GdA  
14 gradually increases in the endometrial glands 4 to 5 days after ovulation and reaches its  
15 peak on day 10, coinciding with the window of implantation (WOI) (Seppala et al., 2002;  
16 Carson et al., 2002). Embryo implantation involves apposition and adhesion of embryo to  
17 the endometrial epithelium, penetration of the endometrial epithelium, and invasion of the  
18 extravillous trophoblast cells through the decidualized endometrial stromal cells (Bentin-  
19 Ley et al., 1999). Among various steps in implantation, attachment of embryo onto  
20 endometrial epithelial cells is essential for successful implantation and pregnancy. For  
21 example, pinopodes are formed at the apical region of the endometrium that facilitate  
22 adhesion of blastocyst to the endometrial surface (Bentin-Ley et al., 1999). Moreover,  
23 embryo implantation is regulated by various endometrial adhesive (e.g. integrins) and anti-  
24 adhesive (e.g. MUC1) factors (Chervenak and Illsley, 2000). Expressions of these adhesive  
25 and anti-adhesive factors are associated with an increase in glycodelin expression during  
26 WOI ( Hohn et al., 2000; Seppala et al., 2002;). Furthermore, several microarray studies

1 have compared gene expression patterns of the endometrium during the menstrual cycle  
2 (Carson et al., 2002; Talbi et al., 2006). A large number of genes showed cyclical variation  
3 in expression, but only a few genes including osteopontin, apolipoprotein D,  
4 dickkopf/DKK1 and olfactomedin were found to be common in all these studies  
5 (Horcajadas et al., 2004). Abnormal levels of glycodein are associated with unexplained  
6 infertility and early pregnancy loss (Seppala et al., 2009).

7 Glycodein is also involved in the progression of gynecological cancers. An aberrant  
8 expression of glycodein is associated with endometrial, ovarian and breast cancers  
9 (Seppala et al., 2009). Forced-expression of glycodein in breast cancer cell lines and  
10 cancer cell xenografts reverses the malignant phenotype of the cells (Kamarainen et al.,  
11 1997; Hautala et al.,2008). How glycodein modulates these changes has yet to be  
12 determined. In the present study, we stably transfected glycodein gene in human  
13 endometrial (HEC1-B) cell line and studied the effects of glycodein force-expression on  
14 cell migration, cell invasion, and trophoblastic spheroids attachment onto the endometrial  
15 cells.

16

## 1 MATERIALS AND METHODS

### 2 Cell lines and forced-expression of glycodeilin in HEC1-B cells

3 Human endometrial adenocarcinoma cells (HEC1-B, Cat no.: HTB-113) were obtained  
4 from ATCC (Manassas, VA, USA) and cultured in Minimal Essential Medium (MEM)  
5 (Sigma, St Louis, MO, USA) supplemented with 10 % fetal bovine serum (Invitrogen,  
6 Carlsbad, CA, USA), 100 units/ml penicillin (Invitrogen), and 100 µg/ml streptomycin  
7 (Invitrogen) at 37 °C under 5% CO<sub>2</sub> in a humidified incubator. Total RNA from human  
8 endometrial tissue was purified using the Absolutely RNA RT-PCR miniprep kit  
9 (Stratagene, La Jolla, CA, USA). Total RNA was reverse transcribed using the First strand  
10 cDNA synthesis kit (Amersham, Pittsburgh, PA, USA). The human glycodeilin cDNA was  
11 amplified by PCR using a glycodeilin forward primer (5'-  
12 GTCAAGCTTGATGGACATCCCCAGACCA-3', *Hind*III site underlined) and a  
13 glycodeilin reverse primer (5'-GAGGAATTCTTCGAAACGGCACGGCTCTTCCAT-3',  
14 *Eco*RI site underlined). The glycodeilin coding region was cloned into the pHM6  
15 mammalian expression vector (Roche Diagnostics Co., Switzerland). This vector was then  
16 transfected into HEC1-B cells using lipofectamine 2000 (Invitrogen). The selection of the  
17 stable clones was carried out using 50 µg/ml G418 for one month. The stable clones were  
18 maintained in MEM medium containing supplements and 50 µg/ml G418. All the  
19 experiments were performed in passage 5 to 10 of the stable selected HEC1-B clones.

20

### 21 Quantitative PCR

22 Total RNA isolated from cultured cells was reverse transcribed with TaqMan reverse  
23 transcription reagent kit (Applied Biosystems, Foster City, CA, USA) and multi-script  
24 reverse transcriptase. The resulting cDNAs were analyzed using qPCR on a glycodeilin  
25 TaqMan probe (Hs00171462\_m1, Applied Biosystems). Quantitative PCR was performed  
26 using a 7500 Real-Time PCR System (Applied Biosystems). Multiplex PCR was

1 performed on 20 µl volumes containing 5 µl of sample cDNA, 10 µl 2X TaqMan Universal  
2 PCR Master Mix, 1 µl 20X Gene Expression Assay for targets and 1 µl eukaryotic 18S  
3 rRNA as the internal control. The relative levels of gene expression were detected using the  
4 threshold cycle ( $C_T$ ) method (also known as the  $2^{-\Delta\Delta CT}$  method) using endogenous 18S as  
5 the internal control. All the experiments were performed in triplicates and repeated at least  
6 3 times.

7

### 8 **Western blotting**

9 Total proteins from HEC1-B were dissolved in RIPA solution (1X PBS, 1% Nonidet P-40,  
10 0.5% sodium deoxycholate, 0.1 % SDS) containing protease inhibitors. The samples were  
11 separated on 12% SDS-PAGE and the proteins were transferred to a PVDF membrane.  
12 After thorough washing, the membrane was probed with glycodelin antibody (rabbit  
13 antibody raised against glycodelin peptide, amino acids 69-83) at 1:1000 dilution,  $\alpha$ -  
14 tubulin (1:5000, Santa Cruz, Santa Cruz, CA, USA) and  $\beta$ -actin antibody (1:5000, Sigma).  
15 Positive signal was detected by chemiluminescence using goat anti-rabbit or goat anti-  
16 mouse IgG conjugated-HRP secondary antibodies.

17

### 18 **Flow cytometry**

19 Transfected HEC1-B cells were collected and fixed in 70 % ethanol. The cells were  
20 washed in phosphate buffered saline twice and stained with 2.5 µg/ml propidium iodide.  
21 DNA content was measured by flow cytometry on a BD FACSCantoII Analyzer (BD  
22 Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser. Fluorescence  
23 signals were measured using a 585 nm band pass filter. The results were analyzed using  
24 WinMDI 2.9 (The Scripps Research Institute Cytometry Software, San Diego, CA, USA)  
25 and Cylchred (Cytonet UK, Cardiff, UK).

26

## 1 **Cell Migration Assay**

2 The migration rate of glycodeilin overexpressing HEC1-B cells was determined in a wound  
3 healing assay. Briefly,  $2 \times 10^5$  HEC1-B cells in 2 ml MEM medium on 6-well plates were  
4 grown overnight at 37 °C. The cells were treated with 20 µg/ml mitomycin C ([Sigma](#)) for 3  
5 h. The culture medium was changed and a wound was created on each well using the tip of  
6 a pipette. The migration rate was expressed as a percentage of the width of the wound at 48  
7 h divided by the width of the wound at 0 h.

8

## 9 **Cell Invasion Assay**

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

19

## 20 **RNAi transfection**

21 RNAi targeted against glycodeilin (Human LOC649984, [Thermo Fisher Scientific,](#)  
22 [Lafayette, CO, USA](#)) was diluted to 10 nmol and transfected into glycodeilin  
23 overexpressing HEC1-B cells using lipofectamine 2000 ([Invitrogen](#)).

24

## 25 **Spheroid Attachment Assay**

26 Spheroid Attachment Assay was performed as previously described (Kodithuwakku et al.,

1 2011). Human trophoblastic JAr (blastocyst surrogate) and HEC1-B cells were used as the  
2 co-culture model. Spheroids (60-200  $\mu\text{m}$ ) were prepared from trypsinized JAr cells in an  
3 incubator at 37 °C and rotated at 100 rpm for 24 h. Spheroids were then transferred onto a  
4 confluent monolayer of transfected HEC1-B cells on a 12-well culture plate (in MEM with  
5 10 % FBS and 1 % L-glutamine). After incubation at 37 °C for 24 h, unattached spheroids  
6 were removed by shaking the culture plates at 140 rpm for 10 min. The attached spheroids  
7 that remained on the HEC1-B monolayer were then counted. The attachment rate was  
8 expressed as a percentage of the number of attached spheroids divided by the total number  
9 of spheroids added onto the HEC1-B monolayer.

10

#### 11 [Statistical Analysis](#)

12 [All the data were analyzed by statistical softwares \(SigmaPlot 11.0 and SigmaStat 2.03;](#)  
13 [Jandel Scientific, San Rafael, CA, USA\) and presented as Mean  \$\pm\$  S.E.M. The non-](#)  
14 [parametric analysis of variance on rank test for multiple comparisons followed by the](#)  
15 [Mann-Whitney U test was used when the data were not normally distributed. A probability](#)  
16 [value  \$<0.05\$  was considered to be statistically significant.](#)

17



## 1 **RESULTS**

### 2 **Forced-expression of glycodeilin on cell growth**

3 The cDNA coding region of glycodeilin was cloned into the pHM6 mammalian expression  
4 vector and transfected into HEC1-B cells. Gentamycin was used to select glycodeilin  
5 forced-expressing clones (4-1 and 4-2), and the empty vector transfected control clone (1-  
6 5). Western blotting results confirmed the expression of glycodeilin protein in clones 4-1  
7 and 4-2, but not in clone 1-5 (Fig. 1A). Quantitative PCR results confirmed that the  
8 expression levels of glycodeilin transcript in clones 4-1 and 4-2 were  $10^5$ -fold higher than  
9 in clone 1-5 (Fig. 1A). Forced-expression of glycodeilin had no effect on cell proliferation  
10 and cell viability in all the selected clones (data not shown).

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

16

### 17 **Forced-expression of glycodeilin on cell migration and invasion**

18 Cells in the wound healing assay were treated with mitomycin C to suppress cell  
19 proliferation. Therefore, the change in area covered by cells was only caused by cell  
20 migration, but not division and proliferation. Forced-expression of glycodeilin in HEC1-B  
21 cells in clones 4-1 and 4-2 suppressed the migration of the transfected HEC1-B cells when  
22 compared with the control clone 1-5 (Fig. 2A). The migration rates in clones 4-1 and 4-2  
23 were significantly lower (23% and 35%, respectively;  $p < 0.05$ ) than in control clone 1-5  
24 (51%). The effect of glycodeilin on cell invasion was studied using a cell invasion chamber  
25 assay. The cell invasion of clones 4-1 and 4-2 was significantly decreased (30% each;  
26  $p < 0.05$ ) compared with the control clone 1-5 (Fig. 2B).

1

2 **Suppression of glycodelin in stably transfected cells increases cell migration**

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

10

11 **Effect of glycodelin forced-expression on spheroid attachment**

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

17

## 1 **DISCUSSION**

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

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

19 Forced-expression of glycodeilin in the HEC1-B cell line inhibited endometrial  
20 epithelial cancer cell migration and invasion in vitro. The decrease in migration ability  
21 could be reversed when these cells were transfected with glycodeilin RNAi. Forced-  
22 expression of glycodeilin in HEC1-B cells resulted in a reduction of carcinoma-associated  
23 gene expression, including MUC1 expression (Koistinen et al., 2005). MUC1 is an anti-  
24 adhesive molecule that is overexpressed in many cancer cells and induces invasive growth  
25 and metastasis (Koistinen et al., 2005). A low level of MUC1 is associated with the  
26 reversal of malignancy (Coronado et al., 2001; Sivridis et al., 2002).

1           Other than cancer, migratory and invasive properties of endometrial epithelial cells  
2 were suggested to be responsible for endometriosis and adenomyosis (Chen et al., 2010;  
3 Dentillo et al., 2010; dos Santos Hidalgo et al., 2011). The main clinical features of  
4 endometriosis are pelvic pain and infertility (Giudice and Kao, 2004). Glycodelin is down-  
5 regulated in eutopic endometrium tissue of endometriosis patients (Meola et al., 2009; Kao  
6 et al., 2003). These studies showed strong correlation between glycodelin and  
7 endometriosis. Together with role of glycodelin in other gynaecological diseases,  
8 glycodelin is responsible for number of pathways which affects steroid receptivity, growth  
9 and invasive potential in endometriotic tissues (Koistinen et al., 2005; Gaetje et al., 1995).  
10 In the current study, increase in glycodelin expression could reduce migratory and invasive  
11 property of endometrial epithelial cells, which might cause a reduction in endometriosis.  
12 Therefore, increase in glycodelin expression might contribute to advantage for successful  
13 implantation and pregnancy in an indirect way by minimizing the effect of endometriosis  
14 and up-regulation of receptive marker (Uchida et al., 2007).

15           Blastocyst apposition and adhesion to the receptive endometrium are necessary steps  
16 to establish a successful implantation and pregnancy. In this study, forced-expression of  
17 glycodelin in HEC1-B was found to up-regulate the attachment rate of spheroid (blastocyst  
18 surrogate) onto the endometrial cells. Glycodelin was up-regulated in the endometrium  
19 during the receptive phase of the cycle (Liu et al., 2008). In humans, endometrial  
20 receptivity is highest at the mid-secretory phase of the menstrual cycle, in which  
21 glycodelin is highly expressed at the apical membranous protrusions called pinopodes  
22 (Stavreus-Evers et al., 2006). Pinopodes express adhesion surface molecules that are  
23 involved in embryo attachment (Lessey et al., 2000). At the adhesion phase of  
24 implantation, the embryo induces a paracrine cleavage of anti-adhesive molecule MUC1 in  
25 the endometrial epithelial cells (Meseguer et al., 2001). Other adhesive molecules such as  
26 integrins, heparin-binding epidermal growth factor-like growth factor, and leukemia

1 inhibitory factor are co-expressed on the pinopode during mid-secretory phase of  
2 menstrual cycle (Lessey et al., 2000; Aghajanova et al., 2003; Stavreus-Evers et al., 2002),  
3 and these promote attachment of embryo onto the receptive endometrium. Other molecules  
4 are found to suppress spheroid attachment onto endometrial cells in vitro, such as  
5 olfactomedin-1 that is down-regulated in the receptive endometrium (Kodithuwakku et al.,  
6 2011).

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

14

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19

## 20 **AUTHOR CONTRIBUTION**

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

24

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33



1 **FIGURE LEGENDS**

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

11

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

23

24 **Figure 3** Forced-expression of glycodeilin in the transfected HEC1-B cells stimulates  
25 spheroid attachment. (A) Spheroids (60-200  $\mu$ m) were prepared from JAr cells for the co-  
26 culture study (Scale bar, 100  $\mu$ m). (B) The spheroid attachment rates of clones 4-1 and 4-2

- 1 were significantly higher ( $p < 0.05$ , a-b) than in the control clone 1-5. The results were
- 2 obtained from three or more pooled experiments and the total number of spheroids used
- 3 per group was more than 200.

Figure 1

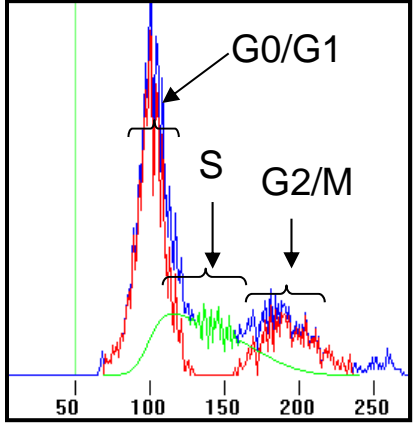
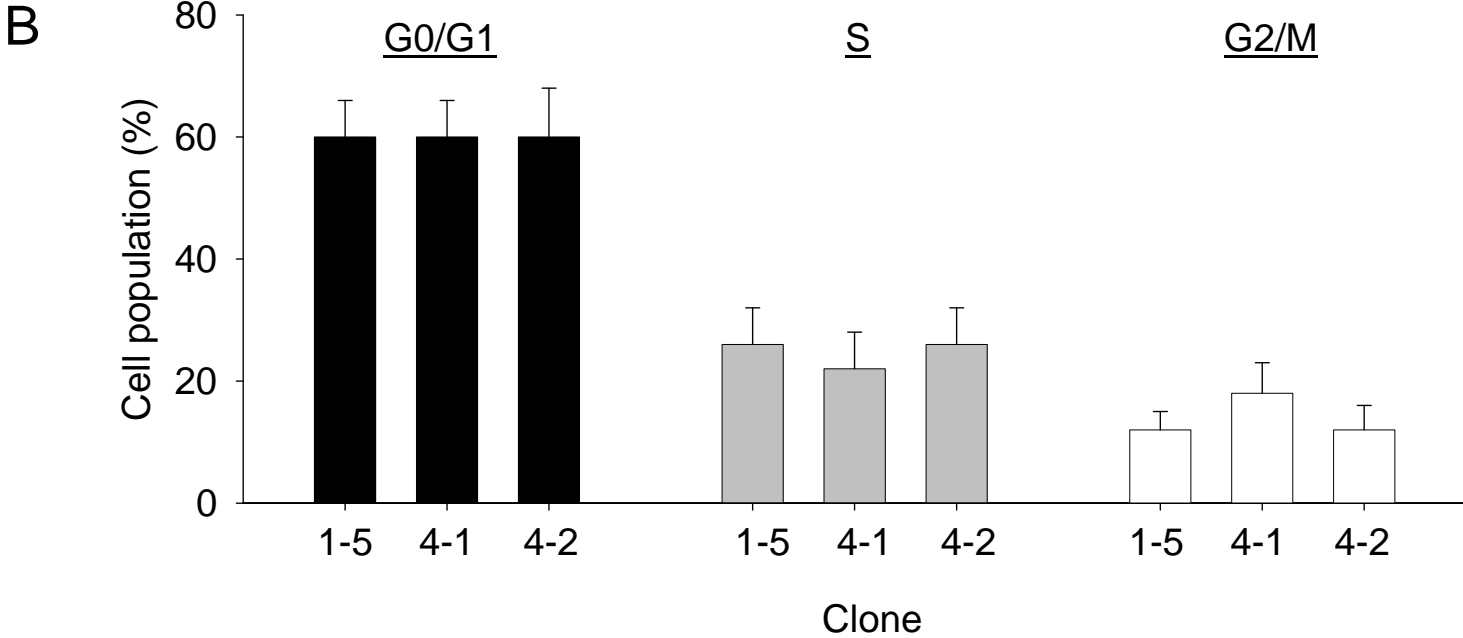
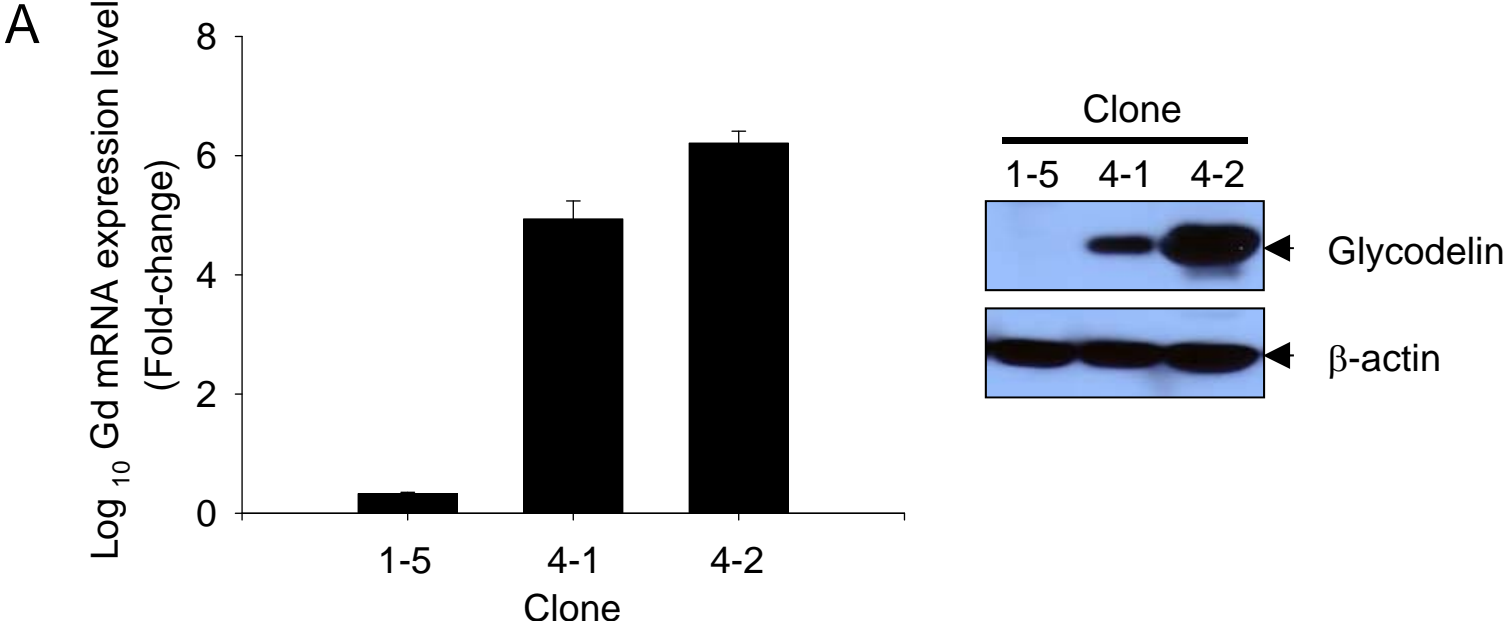


Figure 2

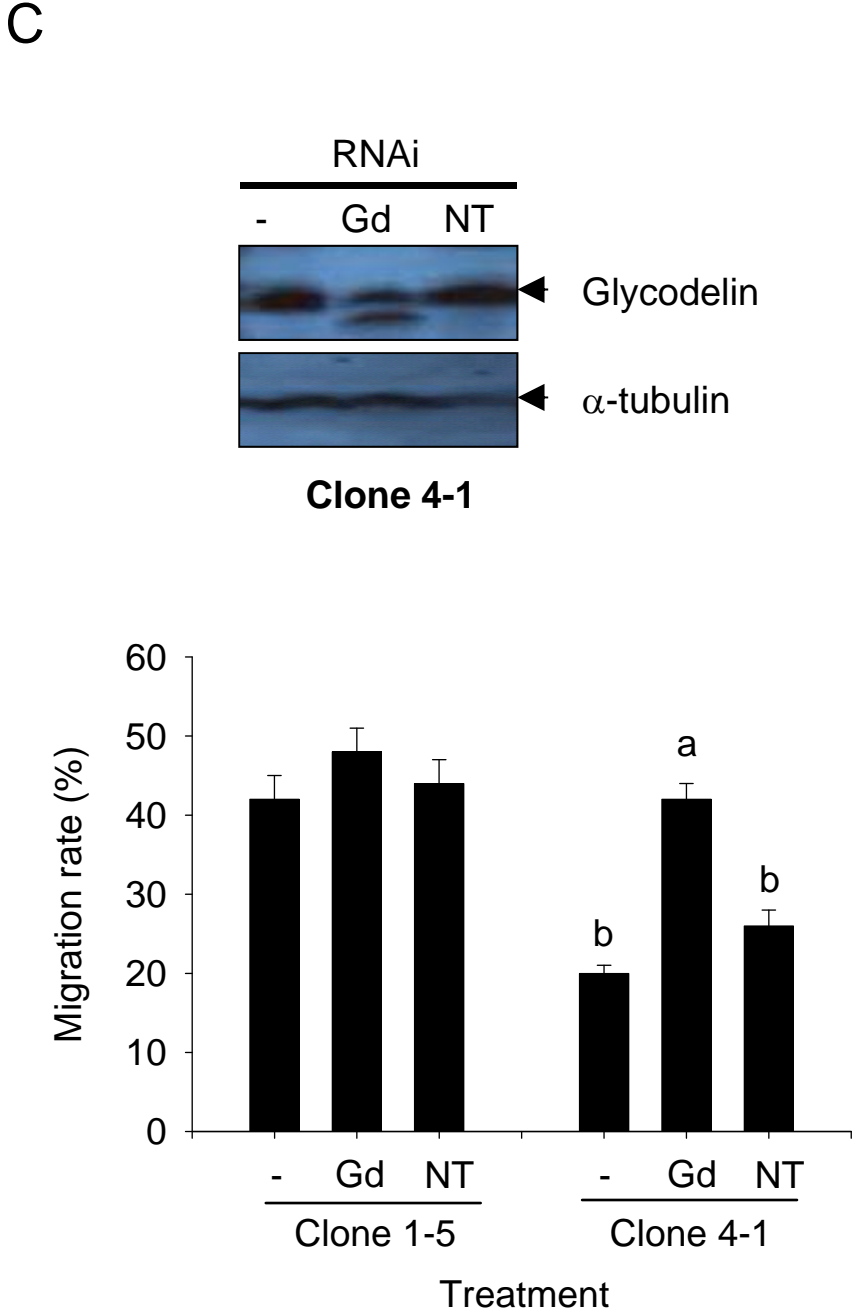
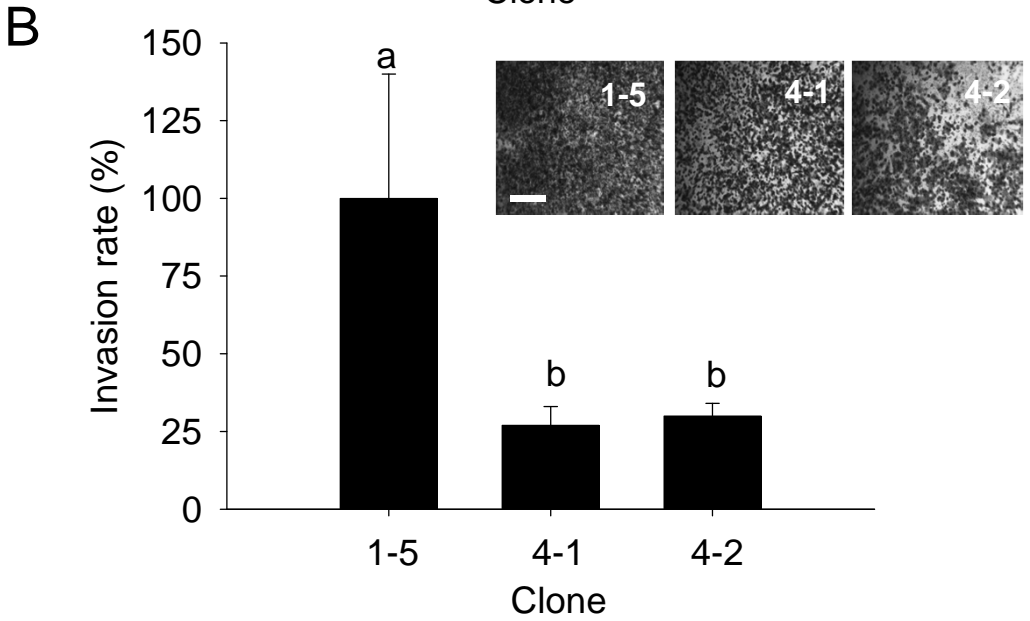
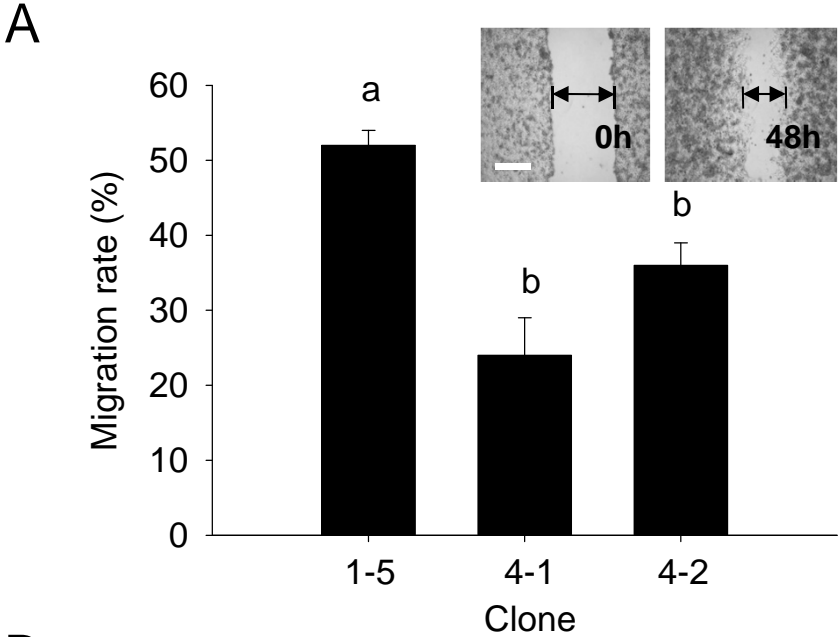
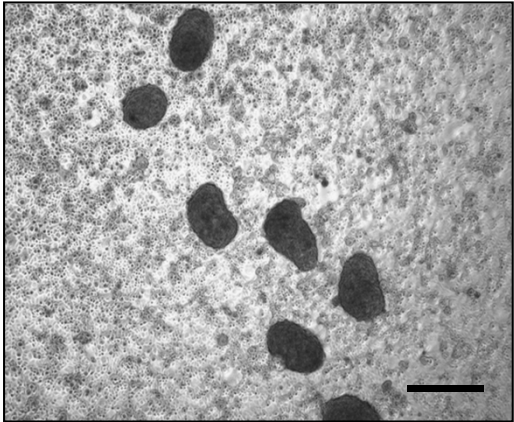


Figure 3

A



B

