G0/G1 Switch 2 Induces Cell Survival and Metastasis through Integrin-Mediated Signal Transduction in Human Invasive Breast Cancer Cells

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Abstract
Human breast cancer cell line, MDA-MB-231, is highly invasive and aggressive, compared to less invasive cell line, MCF-7. To explore the genes that might influence the malignancy of MDA-MB-231, DNA microarray analysis was performed. The results showed that G0/G1 switch 2 (G0S2) was one of the most highly expressed genes among the genes upregulated in MDA-MB-231. Although G0S2 acts as a direct inhibitor of adipose triglyceride lipase, action of G0S2 in cancer progression is not yet understood. To investigate whether G0S2 affects invasiveness of MDA-MB-231 cells, G0S2 expression was inhibited using siRNA, which led to decreased cell proliferation, migration, and invasion of MDA-MB-231 cells. Consequently, G0S2 inhibition inactivated integrin-regulated FAK-Src signaling, which promoted Hippo signaling and inactivated ERK1/2 signaling. In addition, G0S2 downregulation decreased β-catenin expression, while E-cadherin expression was increased. It was demonstrated for the first time that G0S2 mediates the Hippo pathway and induces epithelial to mesenchymal transition (EMT). Taken together, our results suggest that G0S2 is a major factor contributing to cell survival and metastasis of MDA-MB-231 cells.

Key Words: G0S2, FAK-Src signaling, Hippo pathway, EMT

INTRODUCTION

Triple-negative breast cancer is a type of breast cancer that lacks estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (Her2/neu receptor) (Mukherjee and Zhao, 2013; Yadav et al., 2015). Triple-negative breast cancer accounts for 10% to 20% of human breast cancers (Zhang et al., 2013). It shows high cell proliferation and metastasis (Thike et al., 2010; Yadav et al., 2015). Furthermore, triple-negative breast cancer patients exhibit poor prognosis and higher risk of recurrence (Pogoda et al., 2013). Since triple-negative breast cancer lacks hormone receptors, hormone therapies and drugs that target ER, PR, and Her2 may be ineffective against it. Thus, targeted therapy and chemotherapy are the main therapeutic methods against triple-negative breast cancers. Thus, it is essential to elucidate aggressive characteristics of triple-negative breast cancers for finding novel therapeutic targets and approaches.

The Hippo pathway initially was known as a regulator which controls organ size and proliferation. Recent studies have discovered that the Hippo pathway is strongly involved in cell-cell contraction, cell polarity, apoptosis, and cell cycle. In cancer, the Hippo pathway is a tumor suppressor, and its deregulation is recognized as an important feature in many cancers. The transcription coactivator YAP1, which is a major target of the Hippo pathway, is implicated with induction of EMT as well as proliferation, invasion, metastasis. YAP1 induces expression of genes such as amphiregulin, cysteine-rich angiogenic inducer 61 (CYR61), and connective tissue growth factor (CTGF) through TEA domain (TEAD) family transcription factors and they are highly activated in metastatic breast cancer cells (Lamar et al., 2012).

The G0/G1 switch gene 2 (G0S2) is a protein comprising 103 amino acids (Zandbergen et al., 2005). Expression of G0S2 is abundant in metabolically active tissues, such as adipose tissue, liver, heart, and skeletal muscle (Zhang et al., 2017). In cell organelles, G0S2 is mainly located in the endoplasmic reticulum (ER), mitochondria (Zandbergen et al.,...
Materials and Methods

Chemicals and reagents

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Hyclone (Logan, VI, USA). The bicinchoninic acid (BCA) protein assay kit, and goat-anti mouse IgM Alexa Flour 488 were purchased from Thermo Scientific (Waltham, MA, USA). The D-Plus™ CCK cell viability assay kit was purchased from Dongin LS (Seoul, Korea). Mitomycin C was obtained from Sigma (St. Louis, MO, USA). M-MLV reverse transcriptase and RNase inhibitor were purchased from Promega (Madison, WI, USA). dNTP mixture was obtained from TaKaRa Bio (Shiga, Japan). QGreen™ 2X SybrGreen qPCR Master Mix was purchased from CellSafe (Gyeonggi, Korea). Rabbit polyclonal antibodies for FAK, p-FAK (Tyr925), Src, p-Src (Tyr416), p-ERK1/2 (Thr202/Tyr204), integrin α5, integrin β1, LAT51, p-LAT51 (Ser909), and p-YAP1 (S127) were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies for E-cadherin, β-catenin, ERK1, and CYR61, mouse monoclonal antibodies for YAP1 and α-tubulin, and goat anti-rabbit IgG Texas-Red were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody for G0S2 was obtained from CUSABIO (College Park, MD, USA). All other chemicals and reagents were of highest quality commercially available.

Cell culture

Human breast cancer cell lines BT-20, HCC-70, T47D, MDA-MB-231, and MCF-7 (Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. MCF-10A human breast epithelial cells were obtained by Dr. Aree Moon (Duksuang Women’s University, Seoul, Korea) and cultured in DMEM/F12 medium (Welgene, Seoul, Korea) containing 5% horse serum, 0.5 mg/mL hydrocortisone, 10 μg/mL insulin, 100 ng/mL choleria toxin, 20 ng/mL EGF, 100 μg/mL streptomycin, and 100 U/mL penicillin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO2.

Transient transfection with siRNA

Cells were transfected with G0S2-specific siRNA (37.5 nM, target sequence: 5'-TGCACTAGGGAGGAAGGATAA-3', QIA-GEN) with Neon® Transfection System (Invitrogen, Carlsbad, CA, USA) and cultured in RPMI 1640 medium supplemented with 10% FBS without antibiotics overnight. The medium was replaced with RPMI 1640 medium containing 10% FBS with antibiotics and cultured for 24 h.

Cell viability assay

G0S2 siRNA-transfected cells (7×10^4 cells/well) were seeded in 96-well cell culture plates. After stabilization for 48 h, 10 μL of D-Plus™ CCK solution was treated to each well, followed by incubation at 37°C for 2 h. The formed formazan dyes were quantified by measuring the absorbance at 450 nm with a Sunrise™ microplate reader (Tecan, Männedorf, Switzerland).

Cell cycle analysis

Cells were transfected with G0S2 siRNA for 48 h, and then, the cells were harvested with 0.05% trypsin-EDTA and washed with PBS. The obtained cell pellets were fixed in 70% ethanol overnight at –20°C. After washed with PBS to remove ethanol, the cells were incubated in 200 μL of MUSE® cell cycle reagent (Merck Millipore, Darmstadt, Germany) for 30 min at room temperature in the dark. Cell cycle was measured by a MUSE® Cell Analyzer (Merck Millipore).

Apoptosis assay

After culturing for 48 h with G0S2 siRNA, the cells were harvested with 0.05% trypsin-EDTA and washed with PBS. The cell pellets were treated with caspase-3/7 working solution containing a DNA-binding dye at 37°C for 30 min, and then, treated with 7-AAD working solution for 5 min at room temperature. Apoptotic cells were measured by fluorescence-activated cell sorting using a MUSE® Cell Analyzer (Merck Millipore).

Colony forming assay

Cells were transfected with G0S2 siRNAs, and then, 200 cells were seeded in a well of a 6-well culture plate and cultured for 10 days. The culture medium was changed every two days. Subsequently, 4% formaldehyde solution in PBS was treated for 10 min at room temperature to fix the cells. After that, the fixed cells were washed with PBS several times and stained with 0.2% crystal violet for 10 min. After drying enough, the number of colonies containing over 50 cells was measured using a microscope (OLYMPUS, Tokyo, Japan).
In vitro scratch assay
Transfected cells (7×10^5 cells/well) were seeded in 6-well culture plates. After stabilization for 48 h, cells with over 90% confluence were washed with PBS and incubated in mitomycin C solution (10 μg/mL) at 37°C for 2 h. After washing several times, a scratch into cell monolayer was created using a 200 μL pipette tip. Scratch images were captured using a microscope at the indicated time points.

Transwell invasion assay
Measurement of cell invasion was performed using the QCM™ 24 well Invasion Assay Kit (Merck Millipore) in accordance with the manufacturer’s instructions. In brief, cells transfected with G0S2 siRNA were seeded in cell culture inserts with 8-μm pore size polycarbonate membrane coated with reconstituted basement membrane extract. Invasive cells through the membrane were stained with 4',6-diamidino-2-phenylindole (DAPI). Five fields per membrane in a cell culture insert were photographed using the ZEISS LSM700 Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany) and the number of invasive cells was counted.

Quantitative PCR
Total RNA was extracted using Ribospin™ kit (GeneALL, Seoul, Korea). 0.5 μg of total RNA was reverse-transcribed as follows: initially at 37°C for 1 h, followed by at 95°C for 5 min in 20 μL total volume containing 5x RT buffer, 10 mM dNTPs, 100 pmol oligo-dT primer, 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV), and 40 units of RNase inhibitor. qPCR was conducted using the QGreen™ Master Mix (1:250) or goat anti-mouse IgM-Alexa Fluor 488 (1:250) at 4°C overnight, washing with cold PBS three times, followed by washing with cold PBS three times. The cells were blocked with 3% bovine serum albumin in PBS at room temperature for 1 h. Incubation steps of antibodies were conducted as follows: primary antibody (1:250) incubation at 4°C overnight, washing with cold PBS three times for 3 min, subsequently, incubation with goat anti-rabbit IgG-Texas Red (1:250) or goat anti-mouse IgM-Alexa Fluor 488 (1:250) at room temperature for 4 h. After washed with cold PBS several times for 3 min, the coverslips were mounted on glass slides using Ultra Cruz™ mounting medium containing DAPI (Santa Cruz Biotechnology). Fluorescence signals were measured and captured using a ZEISS LSM 800 confocal laser scanning microscope (Carl Zeiss).

Western blot
Whole cells were harvested and solubilized with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, and 1 mM sodium orthovanadate. The total amount of protein in the lysates was measured by the BCA method in accordance with the supplier’s recommendations. Proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8 to 15% polyacrylamide gels and transferred onto 0.45 μm PVDF membranes. After that, membranes were blocked with 5% (w/v) bovine serum albumin or 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) at 4°C for 2 h, and then, incubated overnight with primary antibodies at 4°C. After washed in TBS-T 3 times 10 min at room temperature, membranes were incubated with secondary antibody for 2 h at 4°C. All these steps related to membranes were conducted on a shaker. Each target protein band was visualized by the enhanced chemiluminescence (ECL) (Bionote, Gyeonggi, Korea), and the band intensities were analyzed using a ChemiDoc XRS densitometer and quantified using Quantity One software (Bio-Rad, CA, USA).

Statistical analysis
All data are shown as mean ± standard errors of the means (SEM). Comparison of two groups with one characteristic was conducted using unpaired Student’s t-test and comparison of more than two groups with one characteristic was conducted using one-way ANOVA followed by Dunnett’s Multiple Comparison t-test on Graph-Pad Prism 7 software (GraphPad Software Inc., CA, USA). p-values of <0.05 were considered statistically significant.

Table 1. Microarray data analysis for invasive MDA-MB-231 cells and less invasive MCF-7 breast cancer cells (Cut-off ≥30-fold)

| Gene symbol | Gene name | Definition | MDA-MB-231 / MCF-7 ratio | Gene symbol |
|-------------|-----------|------------|--------------------------|-------------|
| G0S2        | G0/G1 switch 2 | 634.7 | NM_015714                 |
| ANKR1       | Cardiac ankyrin repeat protein | 574.2 | NM_014391                 |
| VIM         | Vimentin   | 411.9 | NM_003380                 |
| FOXA2       | Forkhead box A2 | 352.4 | NM_021784                 |
| ANXA1       | Annexin A1 | 348.6 | NM_000700                 |
| GPX1        | Glutathione peroxidase 1 | 319.6 | NM_201397                 |
| MMP1        | Matrix metalloproteinase 1 | 302.1 | NM_002421                 |
| LDHB        | Lactate dehydrogenase B | 290.3 | NM_002300                 |
| FOXO1       | Forkhead box Q1 | 258.6 | NM_033260                 |
| XAGE1B      | X antigen family, member 1B | 254.7 | NM_001097594               |
RESULTS

G0S2 is overexpressed in MDA-MB-231 cells

To explore which genes might affect the invasiveness of MDA-MB-231 cells, differential gene expressions were evaluated and compared between highly invasive MDA-MB-231 cells and less invasive MCF-7 cells by DNA microarray analysis. Table 1 shows the ten highly expressed genes in MDA-MB-231 cells compared with MCF-7 cells. The difference in G0S2 expression was the greatest between MDA-MB-231 and MCF-7 cells. mRNA expression level was also measured by qPCR to confirm the results of DNA microarray analysis (Fig. 1). Consistent with DNA microarray analysis, the mRNA expression level of G0S2 showed the greatest difference between the cell lines. These results indicated that G0S2 is highly expressed in MDA-MB-231 cells.

G0S2 overexpression is associated with invasiveness of triple-negative breast cancer cell lines

To investigate whether G0S2 overexpression is a characteristic of triple-negative breast cancer lines, mRNA levels of G0S2 were measured and compared among triple-negative breast cancer lines, BT-20, HCC-70, and MDA-MB-231, hormone-dependent breast cancer cell lines, MCF-7 and T47D, and breast epithelial cell line, MCF-10A. We found that G0S2 expression is considerably higher in triple-negative breast cancer cell lines compared with less invasive hormone-dependent breast cancer cells, such as MCF-7 and T47D cells (Fig. 2A). Moreover, consistent with the mRNA levels, we confirmed that G0S2 protein levels were markedly higher in MDA-MB-231 cells (Fig. 2B).

To determine the association between G0S2 expression and relapse-free survival (RFS) of triple-negative breast cancer patients, Kaplan-Meier analysis from the KM plotter database (http://kmplot.com) was used. As shown in Fig. 2C, higher G0S2 expression leads to lower RFS in patient groups who have triple-negative breast cancers. This result indicated that G0S2 overexpression is associated with local, regional, or distant metastasis (Gelman et al., 2012). Taken together, G0S2 expression is highly upregulated especially in triple-negative breast cancer, which indicates that higher G0S2 expression affects invasive phenotypes of breast cancer and poor prognosis.

Knockdown of G0S2 expression decreases tumorigenesis and enhances apoptosis

To explore the role of G0S2 in tumorigenesis in MDA-MB-231 cells, cell viability and colony formation were deter
Inhibition of G0S2 expression reduces migration and invasion

To further determine whether G0S2 is involved in the metastatic progression of MDA-MB-231 cells, wound healing and transwell invasion assays were performed. In wound healing assay, the results showed that the wound closure of the cells after treatment with G0S2 siRNA was inhibited by about 50% compared to the control at 24 h after scratching, while the wound of control cells was closed completely at 24 h after scratching (Fig. 4A). In the transwell invasion assay, the number of invaded cells across pores was significantly decreased in the group treated with G0S2 siRNA compared to the control (Fig. 4B). These results demonstrated that G0S2 promoted metastasis by inducing migration and invasion of MDA-MB-231 cells.

Knockdown of G0S2 inhibits activation of FAK-Src and ERK1/2 signaling

Integrins are well-known ECM receptors and crucial for cell migration and invasion (Hood and Cheresh, 2002). Of them,
Knockdown of G0S2 activates Hippo signaling pathway

Recent studies have revealed that Hippo signaling pathway may contribute to tumorigenesis and cancer development (Zygulska et al., 2017). Integrin-induced FAK-Src is an upstream regulator of the Hippo signaling pathway, where Src, phosphorylated at Tyr 416, induces the active form of yes-associated protein 1 (YAP1) and its translocation to the nucleus by inactivating large tumor suppressor kinase 1 (LATS1) (Si et al., 2017). Upregulation of YAP1 in the nucleus is one of the pre-metastatic events of breast cancer (Lamar et al., 2012), and it promotes transcription of YAP1 target genes, including CYR61 (Si et al., 2017). To determine the effect of G0S2 knockdown on the Hippo pathway in MDA-MB-231 cells, the mRNA levels of LATS1, YAP1, and CYR61 were measured by qPCR. Our results showed that treatment with G0S2 siRNA increased the mRNA level of LATS1, whereas the mRNA level of YAP1 was strongly suppressed. Interestingly, we also found that the expression of CYR61, which is a YAP1 target gene, was significantly suppressed by G0S2 inhibition (Fig. 6A).

To further elucidate whether the Hippo signaling pathway is activated by G0S2 knockdown, the degrees of phosphorylation of LATS1 and YAP1 were evaluated. As a result, phosphorylation of LATS1 at Ser909 and YAP1 at Ser127 were significantly increased, and YAP1 and CYR61 protein levels were decreased after G0S2 inhibition (Fig. 6B). Furthermore, confocal microscopy analysis revealed that knockdown of G0S2 expression might inhibit YAP nuclear localization by increasing the accumulation of phosphorylated YAP1 protein in the cytoplasm or inducing degradation of phosphorylated YAP1, because 14-3-3 proteins binds to the phosphorylation site, Ser127, of YAP1, or phosphorylation of YAP1 induces proteasome-mediated degradation (Fig. 6C) (Freeman and Morrison, 2011; Lamar et al., 2012). Thus, these findings suggested that G0S2 inhibition activated the Hippo pathway by activating phosphorylation of LATS1 and YAP1 and preventing YAP1 nuclear localization.

Knockdown of G0S2 expression induces E-cadherin expression and represses β-catenin expression

E-cadherin is one of the typical epithelial cell markers in EMT, and its downregulated expression is associated with highly invasive and metastatic cancers (Elisha et al., 2018). β-catenin is critical in cell-cell adhesions by linking E-cadherin to the actin cytoskeleton (Hatsell et al., 2003), and the aberrant expression of β-catenin is significantly associated with breast cancer cells (Lopez-Knowles et al., 2010). We observed that the expression of E-cadherin was increased while the expression of β-catenin was decreased by G0S2 suppression (Fig. 7A). Consistently, the data from western blot analysis also showed that G0S2 inhibition significantly increased E-cadherin protein levels, whereas it re-

**Fig. 4.** G0S2 inhibition suppresses migration and invasion of MDA-MB-231 cells. MDA-MB-231 cells were treated with G0S2 siRNA for 48 h. (A) Cell migration was assessed by the wound healing assay. The wound area was observed at the indicated time points (6, 12, and 24 h) by microscopy and photographed. The data is shown as mean ± SEM (n=3). Statistically significant differences from control when *p<0.05. (B) Invaded cells were stained with DAPI and photographed by confocal microscopy. The number of cells was counted in five randomly chosen microscope fields and the percentage of invaded cells was calculated. The quantitative data are represented as the mean ± SEM (n=3). Statistically significant differences from control when *p<0.05.
Protein 1/2  (Landskron which promote cell proliferation, invasion, and angiogenesis, have also been reported as tumor progression factors TNFA decreased. Especially, the mRNA levels of showed that mRNA expression level of those cytokines was measured by qPCR after knockdown of G0S2. The results in inflammatory cytokine expressions, their mRNA levels were measured with Quantify One software. Protein levels were normalized against α-tubulin levels. The data is shown as mean ± SEM (n=3). Statistically significant differences from control when *p<0.05.

**DISCUSSION**

The first step of metastasis is migration of cancer cells away from the primary tumor site, a process called tumor invasion (Glentis et al., 2014). Therefore, cell migration and invasion are considered hallmarks of cancer cells along with cell proliferation (Hanahan and Weinberg, 2011). Specially in triple-negative breast cancers, with fatal metastasis, suppression of migration and invasion is very important for treatment. Here, we hypothesized that G0S2 overexpression could be involved in invasive phenotypes of MDA-MB-231 cells.

The CpG site in the G0S2 gene has been demonstrated to be overwhelmingly hypomethylated in MDA-MB-231 cells compared with MCF-7 cells, and the mRNA expression of G0S2 was found to be higher in MDA-MB-231 (Cheishvili et al., 2015). Moreover, when G0S2 is reduced in MDA-MB-231 cells, tumor invasiveness was significantly decreased. In contrast, a few studies have shown that G0S2 induces apoptosis (Welch et al., 2009), and low expression of G0S2 results in high recurrence of hormone-receptor positive breast cancer cell lines (Yim et al., 2016). However, the role of G0S2 in...
breast cancer cells, especially in triple-negative breast cancer, remains controversial. Therefore, we investigated whether G0S2 plays a crucial role in invasive breast cancer cell survival and metastasis.

Our results demonstrated that G0S2 tends to be overexpressed in triple-negative breast cancer cell lines. Consistently, overexpression of G0S2 increases accumulation of LDs (Antalis et al., 2011), and higher levels of LDs are related to higher aggressiveness in estrogen receptor-negative breast cancer cell lines (Nieva et al., 2012). Taken together, the oncogenic characteristics induced by overexpressed G0S2 could be correlated with high levels of LDs in MDA-MB-231 cells.

Our studies showed that G0S2 promoted cell proliferation, migration, and invasion in MDA-MB-231 cells by activating integrin α5β1-mediated FAK-Src and ERK1/2 signaling through phosphorylation of Tyr925, Tyr416, or Thr202, and Tyr204. This is consistent with previous results that revealed FAK-Src signaling activation promotes cell survival, adhesion, angiogenesis, migration, and metastasis by upregulating integrin α5β1 expressions (Mitra and Schlaepfer, 2006; Mierke et al., 2011; Ye et al., 2017). We found that G0S2 knockdown significantly repressed phosphorylation of Src at Tyr416. Tyr416

Fig. 6. Knockdown of G0S2 activates the Hippo pathway via integrin-mediated FAK-Src. MDA-MB-231 cells were treated with G0S2 siRNA for 48 h. (A) Alteration of LATS1, YAP1, and CYR61 mRNA levels was identified using qPCR. All results were normalized to 18S rRNA. The data is shown as mean ± SEM (n=3). Statistically significant differences from control when *p<0.05. (B) Transfected cells were harvested, and total cellular proteins were extracted for western blot assay. The proteins were separated by SDS-PAGE (8%) and western blot assay was performed using indicated antibodies. The band intensities were measured with Quantity One software. Protein levels were normalized against α-tubulin levels. The data is shown as mean ± SEM (n=3). Statistically significant differences from control when *p<0.05. (C) Confocal microscopy was performed to observe alteration in expressions of YAP1 (left) and phosphorylated YAP1 (right) by G0S2 inhibition. Scale bar indicates 20 μm.
phosphorylation is strongly associated with Src activity, and the active Src can phosphorylate FAK at Tyr925, promoting recruitment of the Grb2-SH2 domain and activation of ERK pathway (Schlaepfer et al., 1994; Canel et al., 2010). Moreover, integrin β1 induces phosphorylation of Src at Tyr416 to promote Src tyrosine kinase activity in human colon cancer cells (Gupta and Vlahakis, 2009). Thus, knockdown of G0S2 may cause downregulation of Src and FAK activity through suppression of integrin α5 and β1 expression. Furthermore, ERK1/2 is also activated by induction of integrin α5β1 expression (Danen and Yamada, 2001) and induce cell-matrix adhesion (Kim and Gumbiner, 2015; Lai et al., 2018). Since p130 Crk-associated substrate (CAS) phosphorylation by integrin α5β1 enhances adhesion-mediated cell survival and migration by interaction with focal adhesion proteins, paxillin, and tensin (Huveneers and Danen, 2009), it may be possible that

Fig. 7. Reduction of G0S2 expression downregulates β-catenin and upregulates E-cadherin. MDA-MB-231 cells were treated with G0S2 siRNA for 48 h. (A) The expressions of E-cadherin (left) and β-catenin (right) were observed by confocal microscopy. Scale bar indicates 20 μm. (B) Whole cell lysate was subjected to western blot analysis for E-cadherin (left) and β-catenin (right) expression. The band intensities were measured with Quantity One software. Protein levels were normalized against α-tubulin levels. The data is shown as mean ± SEM (n=3). Statistically significant differences from control when *p<0.05.

Fig. 8. The alteration in mRNA expressions of MMPs and inflammatory cytokines by G0S2 inhibition. MDA-MB-231 cells were treated with G0S2 siRNA for 48 h (A, B) Total RNA was isolated, and qPCR was performed to confirm the changes in mRNA levels of MMPs and inflammatory cytokines by G0S2 siRNA. All results were normalized to 18S rRNA. The data is shown as mean ± SEM (n=3). Statistically significant differences from control when *p<0.05.
G0S2 activates CAS phosphorylation to promote cell motility and metastasis.

Previously, Hou et al. (2016) reported that malignant phenotypes, such as changes in cell shape and EMT markers or formation of invasive protrusion, in MDA-MB-231 cells are mainly mediated by integrin β1. Our data also demonstrated that G0S2 induced EMT because G0S2 siRNA strongly upregulated expression of E-catenin; therefore, integrin β1-mediated activation by G0S2 may play a crucial role in malignancy of MDA-MB-231 cells. However, further studies are needed to determine whether G0S2 interacts directly with integrins.

Previous studies have demonstrated that integrins directly bind to fibronectin and then stimulate FAK-Src signaling. After activation of FAK-Src signaling, YAP1 is activated in a PI3K-PDK1-dependent manner (Kim and Gumbiner, 2015). Subsequently, dephosphorylated LATS1/2 activates YAP1, resulting in YAP1 accumulation in the nucleus (Fan et al., 2013; Kim and Gumbiner, 2015). In many human cancers, the nuclear YAP1 protein expression was found to be upregulated compared with that in normal tissues (Pan, 2010; Bao et al., 2011), which results in enhancement of cell migration and invasion (Lamar et al., 2012). The expression of CYR61, which is the target gene of YAP1, is promoted by transcriptional regulation of YAP1 and TEAD (Zhao et al., 2008). CYR61 protein is linked to the cell surface and extracellular matrix (Perbal, 2004) and promotes tumorigenesis in breast cancer via integrin-mediated signaling (Tsi et al., 2002). Based on these studies, we suggested that the knockdown of G0S2 expression suppressed CYR61 expression via the transcriptional inactivation of YAP1. However, further studies would be necessary to demonstrate how G0S2 controls upstream regulators of the Hippo pathway, including fibronectin.

In addition, our results showed that G0S2 knockdown suppressed β-catenin protein levels and mRNA levels of MMP1, MMP2, MMP3, MMP7, MMP9, and MMP14 and IL6, IL8, VEGF, and TNFA. Inhibition of β-catenin not only decreased the secretion of inflammatory cytokines, such as IL6, IL8, VEGF, and TNFA (Liu et al., 2016) but also the expression of MMP2 and MMP9 involved in EMT (Son and Moon, 2010). Based on these studies, we suggested that G0S2 indirectly alters the expressions of the abovementioned molecules through integrin-mediated activation of FAK-Src and ERK1/2 signaling, leading to promotion of cell proliferation, migration, and invasion of MDA-MB-231 cells.

G0S2 is highly expressed in MDA-MB-231 cells. However, Heckmann et al. (2016) showed that G0S2 is a short-lived protein due to proteasomal degradation by polyubiquitination at Lys25. Thus, further studies would be needed to elucidate why high G0S2 expression is maintained without its degradation in MDA-MB-231 cells.

In summary, inhibition of G0S2 suppressed proliferation, migration, and invasiveness of invasive breast cancer MDA-MB-231 cells. The scheme in Fig. 9 summarizes novel findings about the mechanisms induced by G0S2. Taken together, overexpression of G0S2 in MDA-MB-231 cells plays a key role in malignant tumor progression. Thus, G0S2 might be an effective therapeutic target for triple-negative breast cancers.

**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest.

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