ABCG8-mediated sterol efflux increases cancer cell progression via the LRP6/Wnt/β-catenin signaling pathway in radiotherapy-resistant MDA-MB-231 triple-negative breast cancer cells

YOUNG SHIN KO[1,2], JU YEONG WON[1,2], HANA JIN[1], NAM BINH NGUYEN[1,2], YAERAM WON[1,2], VEDASTE NSANZIMANA[1,2], SEUNG PIL YUN[1,2], SANG WON PARK[1,2] and HYE JUNG KIM[1,2]

[1]Department of Pharmacology, College of Medicine, Institute of Medical Sciences, Gyeongsang National University, Jinju, Gyeongsangnam-do 52727, Republic of Korea; [2]Department of Convergence Medical Science, Gyeongsang National University, Jinju, Gyeongsangnam-do 52727, Republic of Korea

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Abstract. Expression levels of ATP-binding cassette (ABC) transporters are known to be increased in various tumor cells, including in breast cancer, and they are responsible for mediating drug resistance, leading to treatment failure. In the present study, gene expression array analysis revealed that among ABC transporter subtypes, ABC subfamily G member 8 (ABCG8) was one of the most increased in radiotherapy-resistant triple-negative breast cancer (RT-R-TNBC) cells compared with in TNBC cells. ABCG8 is involved in sterol efflux; however, its role in cancer is not well known. Therefore, the present study investigated the effect of ABCG8 on tumor progression in RT-R-TNBC cells. Gene expression profiling was conducted using the QuantiSeq 3' mRNA-Seq Service, followed by western blotting to confirm protein levels. Loss-of-function assays using small interfering RNA (si) transfection were performed to assess the roles of ABCG8 and its regulatory signaling pathways. RT-R-MDA-MB-231 cells exhibited increased cholesterol levels in both cells and the surrounding media via induction of sterol regulatory element binding protein 1 (mature form) and fatty acid synthase. siABCG8 transfection increased intracellular cholesterol levels but decreased cholesterol levels in the media, indicating an accumulation of cholesterol inside cells. Additionally, RT-R-MDA-MB-231 cells exhibited increased levels of β-catenin compared with MDA-MB-231 cells, which was significantly reduced by ABCG8 knockdown. Furthermore, ABCG8 knockdown led to cell cycle arrest in the G_2/M phase in RT-R-MDA-MB-231 cells by reducing Polo-like kinase 1 (PLK1) and Cyclin B1 expression. RT-R-MDA-MB-231 cells also exhibited increased phosphorylated-low-density lipoprotein (LDL) receptor-related protein 6 (LRP6) levels compared with MDA-MB-231 cells, and these were decreased by siABCG8 transfection. LRP6 siRNA transfection decreased β-catenin, PLK1 and Cyclin B1 expression. In addition, feedback mechanisms such as liver X receptor and inducible degrader of LDL were decreased in RT-R-MDA-MB-231 cells under normal conditions compared with in MDA-MB-231 cells. To the best of our knowledge, the present study was the first to suggest that the cholesterol exported by ABCG8, not inside the cells, may affect cancer progression via the LRP6/Wnt/β-catenin signaling pathway in RT-R-TNBC. The regulation of this pathway may offer a potential therapeutic strategy for the treatment of RT-R-TNBC.

Correspondence to: Professor Hye Jung Kim, Department of Pharmacology, College of Medicine, Institute of Medical Sciences, Gyeongsang National University, Jinjudaero 816 Bungil 15, Jinju, Gyeongsangnam-do 52727, Republic of Korea
E-mail: hyejungkim@gnu.ac.kr

Abbreviations: ABC, ATP-binding cassette; CTRL, control; EC, endothelial cell; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; FASN, fatty acid synthase; HIF-1α, hypoxia-inducible factor 1α; IDOL, inducible degrader of LDL; LDL, low-density lipoprotein; LDLR, LDL receptor; LRP6, LDLR-related protein 6; LXRα/β, liver X receptor α/β; PLK1, Polo-like kinase 1; PR, progesterone receptor; RT-R, radiotherapy-resistant; siRNA, small interfering RNA; SREBP1, sterol regulatory element binding protein 1; TNBC, triple-negative breast cancer

Key words: ABC subfamily G member 8, cholesterol, β-catenin, LRP6, radiotherapy resistance, TNBC

Introduction

Breast cancer is the leading cause of cancer death for women worldwide. In addition, the incidence rates of breast cancer have markedly increased over the past few years. The incident cases of BC increased from 876,990 in 1990 to 2,002,350 in 2019, and the estimated annual percentage change for incidence increased by an average of 0.33% per year worldwide (1). The cure rate is high when the cancer is diagnosed early and treated comprehensively with surgical resection,

radiation and chemotherapy. However, an increase in mortality has been reported due to treatment failure caused by cancer recurrence after an apparent cure or metastasis to distant sites from the original tumor. Furthermore, 25-30% of all patients with breast cancer experience disease recurrence, which often leads to mortality (2). Expression of receptors such as estrogen receptor (ER), progesterone receptor (PR) and HER2 is vital for the evaluation of treatments. Triple-negative breast cancer (TNBC) is characterized by the absence of ER, PR and HER2, and has the worst prognosis among aggressive breast cancer subtypes due to the lack of treatment targets (3). General anticancer therapy and radiation therapy are used after surgery for patients with TNBC if there is no metastasis when TNBC is diagnosed. However, if treatment resistance is acquired, anticancer drugs and radiation do not exhibit therapeutic efficacy, ultimately increasing tumor recurrence and metastasis (4,5). Due to the acquisition of therapy resistance, patients with TNBC have a short survival period and a mortality rate of 40% within 5 years from diagnosis (6). Therefore, innovative treatment strategies for patients with TNBC with acquired treatment resistance are urgently needed.

In our previous study, radiotherapy-resistant (RT-R)-TNBC cells (RT-R-MDA-MB-231 cells) were established and their characteristics compared with MDA-MB-231 cells were clarified (7). RT-R-MDA-MB-231 cells exhibited increased migration, adhesion to endothelial cells (ECs), and invasion through ECs-Matrigel-coated membranes by upregulating adhesion molecules (such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1) and epithelial-mesenchymal transition (EMT)-associated proteins (such as MMP-9, Snail-1 and β-catenin) (7). Furthermore, RT-R-MDA-MB-231 cells may contribute to tumor progression by enhancing premetastatic niche formation through the hypoxia-inducible factor 1α (HIF-1α)-lysyl oxidase axis (8). ATP-binding cassette (ABC) transporters, the largest family of transmembrane proteins, are divided into seven subfamilies (ABCA-ABCG). The role of ABC transporters in multidrug resistance is well recognized because they are responsible for ATP-dependent export of various xenobiotics, including drugs (9,10). However, the drug efflux-independent roles of ABC transporters in cancer biology are not well known. ABC transporters are involved in the export of lipids and metabolic products across plasma and intracellular membranes (11). These substances can act as signaling substances for cells and surrounding other cancer cells. For example, ABCC4 can export prostaglandins; ABCC1 can export leukotriene C4, sphingosine-1-phosphate and lysophosphatidyl inositol; ABCB5 can export IL1β; and ABCG2 can export androgens (12). ABCB1/2/5, ABCC1/2/3/4/5/6/10 and ABCG2 are known to be directly related to resistance to chemotherapy by mediating the efflux of chemotherapy agents (13,14). However, ABCG5 and ABC subfamily G member 8 (ABCG8) are known to be partially involved in the outflow of sterols and there is little research on their function in cancer by mediating sterol efflux (15,16). Therefore, the present study aimed to identify the differences in gene expression levels of ABC transporters between RT-R-MDA-MB-231 cells and MDA-MB-231 cells. The present study also aimed to investigate the role of the ABC transporter subtype that shows the most significant change between these two cell lines.

Materials and methods

Cell culture and treatment. The MCF10A breast epithelial cell line was obtained from American Type Culture Collection, and the MCF7 (non-TNBC), and MDA-MB-231 and MDA-MB-453 (TNBC) human breast cancer cell lines were obtained from the Korean Cell Line Bank; Korean Cell Line Research Foundation. RT-R breast cancer cells (RT-R-MCF7 and RT-R-MDA-MB-231 cells) were generated by repeatedly applying 2 Gy X-ray irradiation until a final dose of 50 Gy (2 Gy; 25 times) was achieved, as described previously (7). MCF10A cells were cultured in DMEM/F-12 (cat. no. 11320-033; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5% horse serum (cat. no. 16050122; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, 100 g/ml streptomycin, 0.5 g/ml hydrocortisone, 10 g/ml insulin, 20 ng/ml epidermal growth factor, 2 mM L-glutamine and 0.5 μg/ml amphotericin B. All cancer cell lines were cultured in RPMI 1640 medium (cat. no. SH30027.01; Cytiva) supplemented with 10% fetal bovine serum (cat. no. 160000-044; Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin (cat. no. SV30010; Cytiva). The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. The RT-R breast cancer cells were used through five passages. MCF10A, MCF7, RT-R-MCF7, MDA-MB-231, RT-R-MDA-MB-231 and MDA-MB-453 cells were used for the detection of ABC transporter subtypes (ABCA9, ABCB4, ABCG5 and ABCG8) by western blot analysis. In other assays, MDA-MB-231 and RT-R-MDA-MB-231 cells were used. RT-R-MDA-MB-231 and MDA-MB-231 cells were treated with digoxin (cat. no. D6003; Supelco; Merck KGaA; dissolved in DMSO) at a dose of 400 nM for 24 h at 37˚C or cholesterol (C4951; Sigma-Aldrich; Merck KGaA; dissolved in sterile water) at various concentrations (0, 10 and 20 μM) for 24 h at 37˚C for the indicated experiments.

Cell viability assay. MDA-MB-231 and RT-R-MDA-MB-231 cells were seeded at a density of 10^4 cells/well in 24-well plates. The cells were treated with different concentrations (0.01, 0.05, 0.1, 0.5 and 1 μM) of doxorubicin (DOXO; cat. no. D1515; Sigma-Aldrich; Merck KGaA; dissolved in sterile water) or paclitaxel (PTX; cat. no. T7402; Sigma-Aldrich; Merck KGaA; dissolved in DMSO) for 24-72 h at 37˚C. Cell viability was assessed using the D-Plus™ CCK cell viability assay kit (cat. no. CCK-3000; Donginbiotech Co., Ltd.). After treatment, 20 μl CCK-8 solution was added to 200 μl serum-free medium in each well, and the cells were incubated for 30 min at 37˚C in the dark. The absorbance at 450 nm was measured using a microplate reader (Molecular Devices VersaMax; Molecular Devices, LLC).

Gene expression array analysis. Gene expression profiling was performed using the QuantiSeq 3' mRNA-Seq Service (ebiogen, Inc.). Briefly, total RNA was extracted from MDA-MB-231 and RT-R-MDA-MB-231 cells using TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The quality of the RNA was evaluated using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Inc.). Libraries were prepared from the total RNA (500 ng) using the Lexogen

Quant-Seq 96 prep kit (cat. no. 015.2X96; Lexogen GmbH) according to the provided instructions. The library concentration was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) and a Qubit™ Flex Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.), and the library concentration per sample was ~4 nM, which was diluted to a concentration of 20 pM. The final library concentration was adjusted to 1.5 pM after quantitative PCR-based quantification (StepOnePlus™; Applied Biosystems; Thermo Fisher Scientific, Inc.) before sequencing. Reverse transcription was performed using an oligo-dT primer containing an Illumina-compatible sequence. The reaction conditions were 85˚C for 3 min (primer hybridization) and 42˚C for 15 min (cDNA synthesis). The Lexogen Quant-Seq 96 prep kit (cat. no. 015.2X96; Lexogen GmbH) contained reverse transcriptase and master mix, including buffer, dNTPs and oligo-dT primer. The RNA template was then degraded, and second strand synthesis was initiated using a random primer with an Illumina-compatible linker sequence at its 5' end by incubation at 98˚C for 1 min and 25˚C for 30 min. Subsequently, the double-stranded cDNA library was amplified as follows: Initial denaturation at 98˚C for 30 sec, 12 cycles of 98˚C for 10 sec, 65˚C for 20 sec and 72˚C for 30 sec, and a final extension at 72˚C for 1 min. This procedure was also performed using the Lexogen Quant-Seq 96 prep kit. Purified libraries were sequenced using an Illumina NextSeq500 system with Single-End 75 bp (cat. no. 20024906; Illumina, Inc.). The raw sequencing data underwent quality control checks using FastQC (version 0.11.5; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Data mining was carried out using ExDEGA (v5.2.1; ebiogen, Inc.). The corresponding datasets have been submitted to a public database (Gene Expression Omnibus; www.ncbi.nlm.nih.gov/geo). ABC transporters were selected from the dataset (GSE287883) and analyzed further.

Gene silencing with small interfering (si)RNA. MDA-MB-231 cells and RT-R-MDA-MB-231 cells were transfected with 100 nM negative control siRNA (siCTRL; cat. no. SN-1003; Bioneer Corporation), ABCG8 siRNA (siABCG8; cat. no. 64241-1; Bioneer Corporation) and LDL receptor (LDLR)-related protein 6 (LRP6) siRNA (siLRP6; cat. nos. 4040-1; Bioneer Corporation) using Lipofectamine® 3000 (cat. no. L3000015; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cells were incubated in complete medium containing transfection reagent for 24 h at 36˚C, and the medium was then replaced with fresh medium and cells were used for experiments after 24 h of incubation. The siRNA sequences were as follows: siCTRL forward, 5'-CCUACGCCACCAAUUUCGU-3' and reverse, 5'-ACGAAAUUGGUGGCGUAGG-3'; siABCG8 forward, 5'-CGUCAGAUUUCCAACGACU-3' and reverse, 5'-AGUCGUUGGAAAUCUGACG-3'; siLRP6 1 forward, 5'-CUGCUUUGGAUUUUGAUGU-3' and reverse, 5'-ACAUCAAAAUCCAAAGCAG-3'; aiLRP6 2 forward, 5'-UGAGAACACCUAUUCUACA-3' and reverse, 5'-UGUAGAGGUGUUCUCA-3'; and siLRP6 3 forward, 5'-UGUUGACCAGUUAUCAGUA-3' and reverse, 5'-UACUGAUAACUGGUCAACA-3'. The silencing efficiency was determined by western blot analysis.

Western blot analysis. MDA-MB-231 cells and RT-R-MDA-MB-231 cells were harvested, and lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitors. The samples were centrifuged at 16,000 x g for 20 min at 4˚C and the supernatants were collected. The protein concentration was determined using the Bradford method. Equal amounts of protein (20-50 μg/lane) were subjected to 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Hybond-P+ polyvinylidene fluoride membranes (Amersham; Cytiva). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at room temperature, and then incubated with the following primary antibodies overnight at 4˚C: Anti-ABCA9 (cat. no. PA5-75606; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.), anti-ABCB4 (cat. no. PA5-78692; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.), anti-ABCG5 (cat. no. bs-5013R; 1:1,000; BIOSS), anti-ABCG8 (cat. no. ab223056; 1:1,000; Abcam), anti-GAPDH (cat. no. MA5-15738; 1:5,000; Invitrogen; Thermo Fisher Scientific, Inc.), anti-Cyclin D1 (cat. no. ab16663; 1:1,000; Abcam), anti-Cyclin B1 (cat. no. ab32053; 1:1,000; Abcam), anti-OCT4 (cat. no. ab18976; 1:1,000; Abcam), anti-β-catenin (cat. no. sc-7963; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-AKT (cat. no. sc-8312; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-GSK3β (cat. no. sc-9166; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-p-GSK3β (cat. no. sc-11358; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-p-AKT (cat. no. 9271; 1:1,000; Cell Signaling Technology, Inc.), anti-fatty acid synthase (FASN; cat. no. 3180; 1:1,000; Cell Signaling Technology, Inc.), anti-STAT3 (cat. no. 4904; 1:1,000; Cell Signaling Technology, Inc.), anti-p-STAT3 (cat. no. 9131; 1:1,000; Cell Signaling Technology, Inc.), anti-N-cadherin (cat. no. 4061; 1:1,000; Cell Signaling Technology, Inc.), anti-HIF-1α (cat. no. ab179483; 1:1,000; Abcam), anti-inducible degrader of LDL (IDOL; cat. no. ab74562; 1:1,000; Abcam), anti-LDLR (cat. no. ab52818; 1:1,000; Abcam), anti-LRP6 (cat. no. 3395; 1:1,000; Cell Signaling Technology, Inc.), anti-phosphorylated-(p-)LRP6 (cat. no. 2568; 1:1,000; Cell Signaling Technology, Inc.), anti-liver X receptor (LXR) α/β (cat. no. sc-377260; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-Polo-like kinase 1 (PLK1; cat. no. 4513; 1:1,000; Cell Signaling Technology, Inc.) and anti-sterol regulatory element binding protein 1 (SREBP1; cat. no. sc-13551; 1:1,000; Santa Cruz Biotechnology, Inc.) (precursor form 125 kDa, mature form 68 kDa). The expression of the proteins was detected using horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, including anti-rabbit IgG (cat. no. 12-348; 1:5,000; Sigma-Aldrich; Merck KGaA) and anti-mouse IgG (cat. no. A90-116P; 1:5,000; BETHYL; Fortis Life Sciences, LLC), and western ECL substrates (cat. no. 170-5061; Bio-Rad Laboratories, Inc.) for western blotting detection. The protein levels were normalized to β-actin (cat. no. MA5-15739; 1:5,000; Thermo Fisher Scientific, Inc.). The density of protein was assessed using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Inc.), and relative protein levels were semi-quantified using ImageJ software (version 1.53e; National Institutes of Health).

Total cholesterol quantification. For quantification of intracellular total cholesterol, $1x10^6$ cells (MDA-MB-231 and RT-R-MDA-MB-231 cells) were collected in 100 μl PBS (Ph 7.2-7.4). Cells were frozen for a few seconds using liquid nitrogen, and thawed at room temperature, which was repeated five times to release intracellular components. Subsequently, the supernatant was collected after centrifuging for 20 min at 4˚C at 400-800 x g. For quantification of total cholesterol in the cell media, cell culture media were collected into aseptic tubes and centrifuged for 1 min at 4˚C at 400-800 x g, and the supernatant was transferred into new tubes. Total cholesterol was quantified using the Human Total Cholesterol ELISA Kit (cat. no. ab287836; Abcam) according to the manufacturer's instructions. The absorbance at 450 nm was measured with a microplate reader (Molecular Devices VersaMax; Molecular Devices, LLC).

Cell cycle analysis. MDA-MB-231 or RT-R-MDA-MB-231 cells, which were transfected with siCTRL or siABCG8, were fixed with 75% ethanol at 4˚C overnight, and stained with PI solution (50 μg/ml PI; cat. no. 537059; Sigma-Aldrich; Merck KGaA; 0.7 μg/ml RNase A; cat. no. R6513; Sigma-Aldrich; Merck KGaA) at room temperature for 30 min in the dark. DNA content was analyzed using flow cytometry (FACSverse; BD Biosciences) and the data were analyzed using FlowJo software (version 10; Tree Star, Inc.).

Statistical analysis. All data were statistically analyzed using GraphPad Prism 8 software (Dotmatics). Statistical comparisons were performed using an unpaired Student's t-test for two-group comparisons or one-way ANOVA followed by Tukey's post hoc test for comparisons of three or more groups. The results of the cell viability assay were compared using two-way ANOVA followed by Sidak's post hoc test. The data are presented as the mean ± SD of three to five independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ABCG8 expression is increased in TNBC cells compared with in non-TNBC cells, and is significantly increased in RT-R-TNBC cells compared with in TNBC cells. Gene expression array analysis of MDA-MB-231 and RT-R-MDA-MB-231 cells revealed that ABCA9, ABCB4 and ABCG8 were upregulated by >2-fold in RT-R-MDA-MB-231 cells (RT-R-TNBC) compared with in MDA-MB-231 cells (TNBC) (Fig. 1A). The protein levels of ABCA9, ABCB4 and ABCG8 in MCF10A (normal epithelial cells), MCF7 (non-TNBC), RT-R-MCF-7, MDA-MB-231 (TNBC) and RT-R-MDA-MB-231 cells were subsequently determined by western blot analysis. Because ABCG5 can form a heterodimer with ABCG8, and this heterodimer of ABCG5/G8 serves as a major sterol transporter in liver and intestinal cholesterol efflux (16), the expression levels of ABCG5 were also detected. The results showed that ABCG5 and ABCG8 protein levels were higher in MDA-MB-231 cells than in MCF7 cells. Furthermore, the expression levels of ABCG8, which were upregulated in MDA-MB-231 cells, were significantly increased in RT-R-MDA-MB-231 cells (Fig. 1B and F), although the increase of ABCG8 expression at the protein level in RT-R-MDA-MB-231 cells (1.6-fold compared with MDA-MB-231 cells) was slightly lower than that at the RNA level (~2.88-fold compared with MDA-MB-231 cells), which may be due to methodological differences. In addition, it was confirmed that ABCG8 protein expression was increased in other TNBC cell lines, such as MDA-MB-453 cells compared with in MCF7 cells, a non-TNBC cell line (Fig. 1G and H). However, ABCG5 protein expression did not significantly differ between MDA-MB-231 and RT-R-MDA-MB-231 cells. Therefore, in subsequent experiments, the role of ABCG8 in cancer progression was examined in RT-R-MDA-MB-231 cells.

ABCG8 knockdown enhances chemotherapy-mediated cytotoxicity in TNBC and RT-R-TNBC cells. ABC transporters are known to mediate chemotherapy resistance by pumping out chemotherapeutic drugs (14,17). Therefore, the present study examined the effect of ABCG8 knockdown on chemotherapy-mediated cytotoxicity in TNBC and RT-R-TNBC cells. After confirming the effectiveness of ABCG8 knockdown using siABCG8 by western blot analysis (Fig. 2A and B), the effect of ABCG8 knockdown on the viability of MDA-MB-231 and RT-R-MDA-MB-231 cells treated with Doxo or PTX (0.01, 0.05, 0.1, 0.5 and 1 μM) for 24-72 h was determined (Table SI). As shown in Table SI and Fig. 2C and D, Doxo and PTX reduced the viability of MDA-MB-231 and RT-R-MDA-MB-231 cells in a dose-dependent manner over 24-72 h, and the reduction in cell viability caused by Doxo or PTX was even greater when cells were transfected with siABCG8. Specifically, for 1 μM Doxo treatment, knockdown of ABCG8 significantly decreased cell viability from 86, 67 and 51% to 65, 57 and 43% at 24, 48 and 72 h, respectively, in MDA-MB-231 cells, and from 96, 67 and 46% to 72, 61 and 36% at 24, 48 and 72 h, respectively, in RT-R-MDA-MB-231 cells (Fig. 2C). For 1 μM PTX, siABCG8 transfection led to a significant reduction from 60 to 46% at 72 h in MDA-MB-231 cells, and from 70 and 67% to 54 and 43% at 48 and 72 h in RT-R-MDA-MB-231 cells (Fig. 2D). Notably, both MDA-MB-231 and RT-R-MDA-MB-231 cells transfected with siABCG8 exhibited substantial reductions in viability after 48 and 72 h of incubation (Fig. 2E). These results indicated that the presence of ABCG8 affected cell viability, and thus, ABCG8 may have additional functions beyond its role as a drug pump.

ABCG8 knockdown is associated with accumulation of intracellular cholesterol but reduces the expression levels of β-catenin and EMT proteins. The ABCG5/ABCG8 protein is a major sterol transporter, and tumor cells need lipids for their rapid proliferation (18). The present study examined the cholesterol production in RT-R-TNBC and TNBC cells. Both intracellular and medium cholesterol levels were significantly higher in the RT-R-MDA-MB-231 group compared with those in the MDA-MB-231 group (Fig. 3A). Subsequently, to investigate the role of ABCG8 in the cholesterol efflux from the cytosol into the media, total cholesterol levels in the cell lysates and culture media were measured. The level of total cholesterol in the lysate of RT-R-MDA-MB-231 cells was higher compared with that in MDA-MB-231 cells, and this

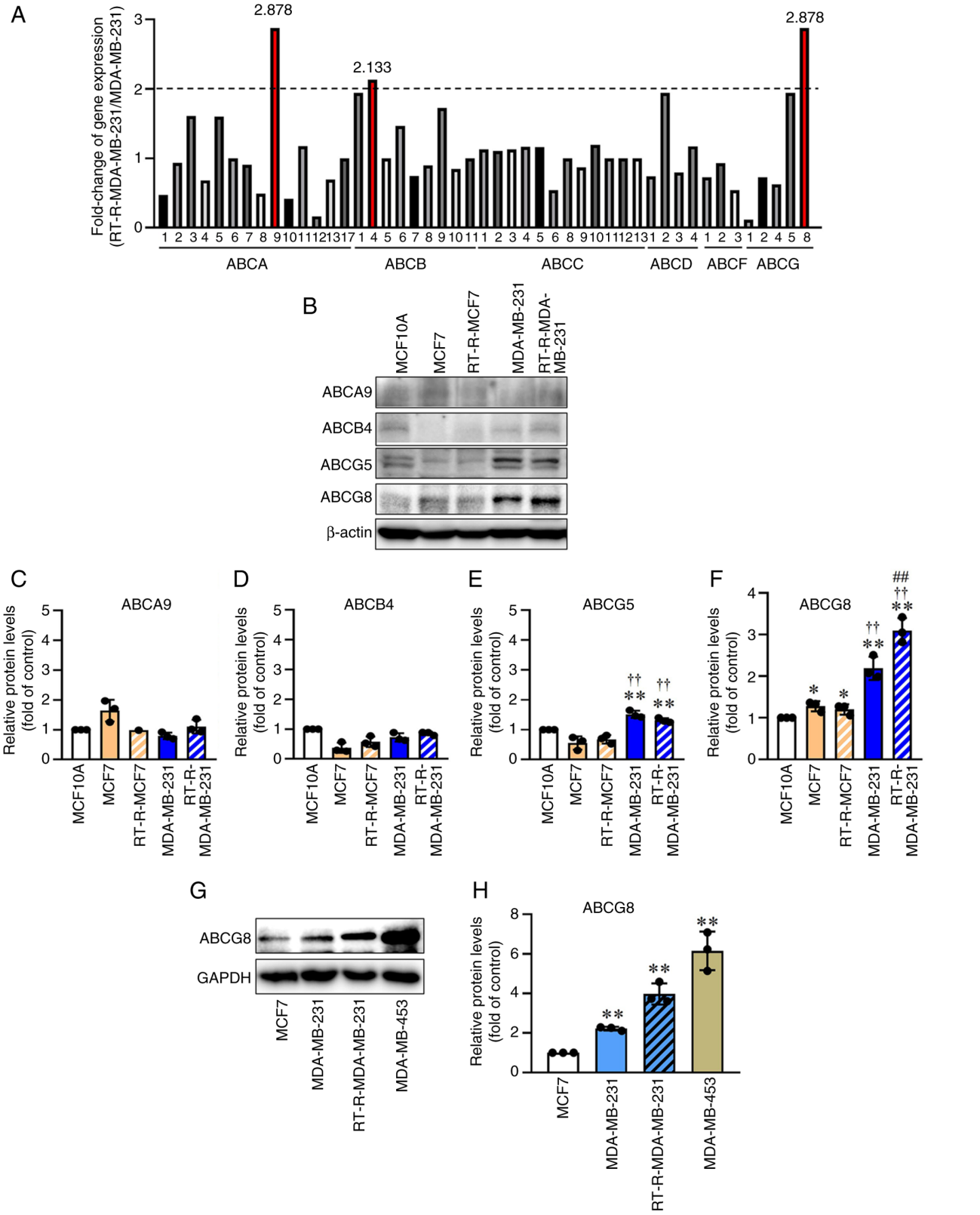

Figure 1. ABCG8 expression is increased in TNBC cells compared with that in non-TNBC cells, with this increase being more pronounced in RT-R-TNBC cells. (A) Differential expression of ABC transporters in RT-R-MDA-MB-231 and MDA-MB-231 cells was determined by gene expression array analysis. ABC transporter isotypes that were upregulated >2-fold in RT-R-MDA-MB-231 cells compared with MDA-MB-231 cells are shown in red. (B) ABCA9, ABCB4, ABCG5 and ABCG8 were selected, and the expression levels of these proteins were confirmed in MCF10A normal epithelial cells, MCF7 and RT-R-MCF7 non-TNBC cells, and MDA-MB-231 and RT-R-MDA-MB-231 TNBC cells by western blot analysis (n=3). The levels of (C) ABCA9, (D) ABCB4, (E) ABCG5 and (F) ABCG8 were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 compared with MCF10A cells; ††P<0.01 compared with MCF7 cells; $^{\#\#}$P<0.01 compared with MDA-MB-231 cells. (G) Protein levels of ABCG8 were compared between non-TNBC (MCF7) and TNBC (MDA-MB-231, MDA-MB-453 and RT-R-MDA-MB-231) cells by western blot analysis. (H) ABCG8 protein levels were compared among TNBC cell lines, including MDA-MB-231, RT-R-MDA-MB-231 and MDA-MB-453 cells, and non-TNBC cells such as MCF7 cells. Data are presented as the mean ± SD of three independent experiments. **P<0.01 compared with MCF7 cells. ABC, ATP-binding cassette; ABCG8, ABC subfamily G member 8; RT-R, radiotherapy-resistant; TNBC, triple-negative breast cancer.

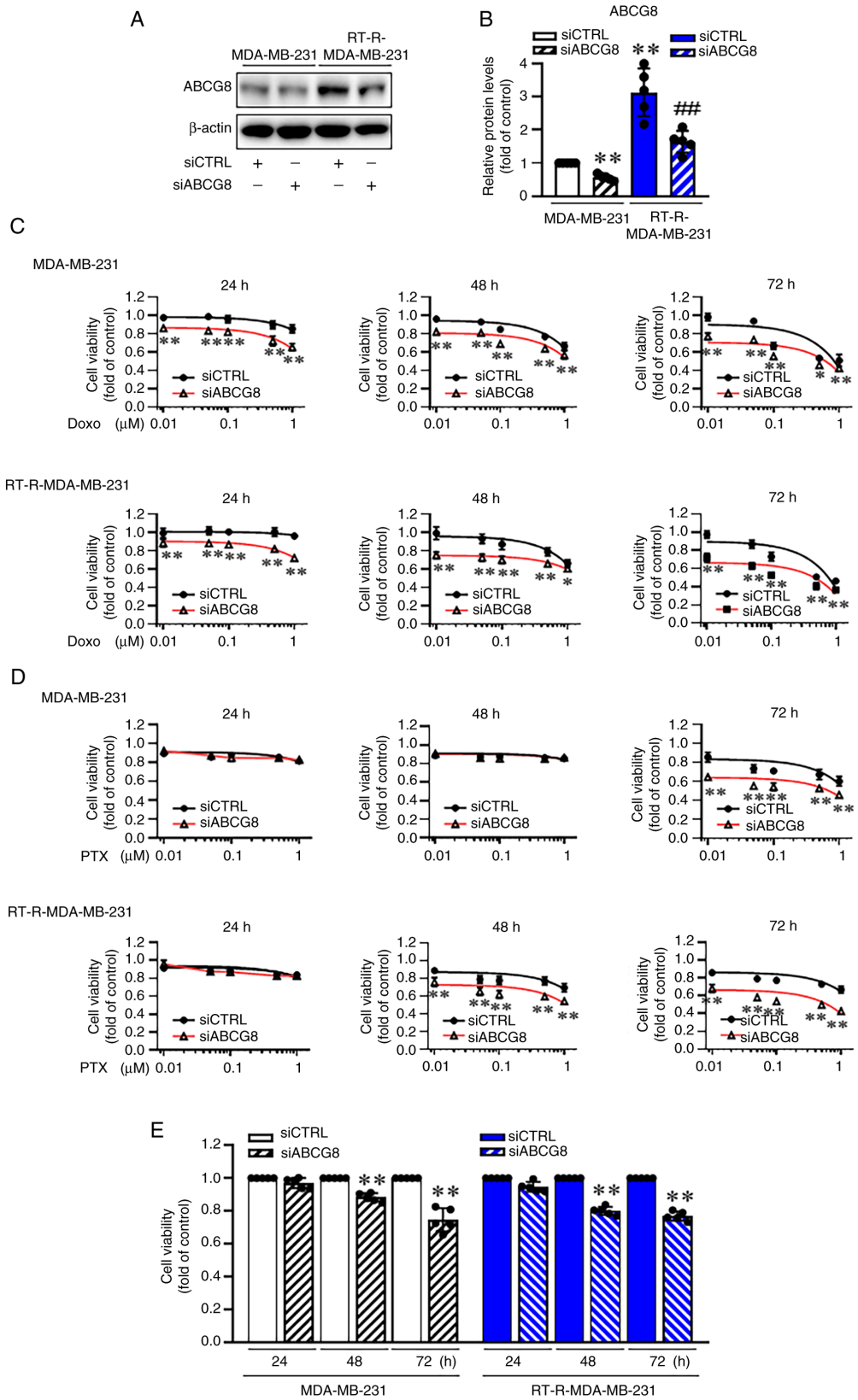

Figure 2. siABCG8 transfection enhances chemotherapy (Doxo and PTX)-mediated cytotoxicity in RT-R-MDA-MB-231 and MDA-MB-231 cells, with siABCG8 itself decreasing cell viability. (A) Cells were transfected with siCTRL or siABCG8 (100 nM) for 24 h and the transfection efficiency of siABCG8 was confirmed by western blot analysis. (B) ABCG8 expression was semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with siCTRL-transfected MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with siCTRL-transfected RT-R-MDA-MB-231 cells. Cells transfected with siCTRL or siABCG8 were treated with different concentrations (0.01, 0.05, 0.1, 0.5 and 1 μM) of (C) Doxo or (D) PTX for 24-72 h. Cell viability was assessed using a CCK-8 cell viability assay kit. Data are presented as the mean ± SD of five independent experiments. *P<0.05; **P<0.01 compared between the siCTRL group and the siABCG8 group. (E) Cells transfected with siCTRL or siABCG8 were cultured in fresh media for 24-72 h. Cell viability was determined using the CCK-8 cell viability assay kit. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with the siCTRL group for each time point. ABCG8, ATP-binding cassette subfamily G member 8; CCK-8, Cell Counting Kit-8; CTRL, control; Doxo, doxorubicin; PTX, paclitaxel; RT-R, radiotherapy-resistant; si, small interfering RNA.

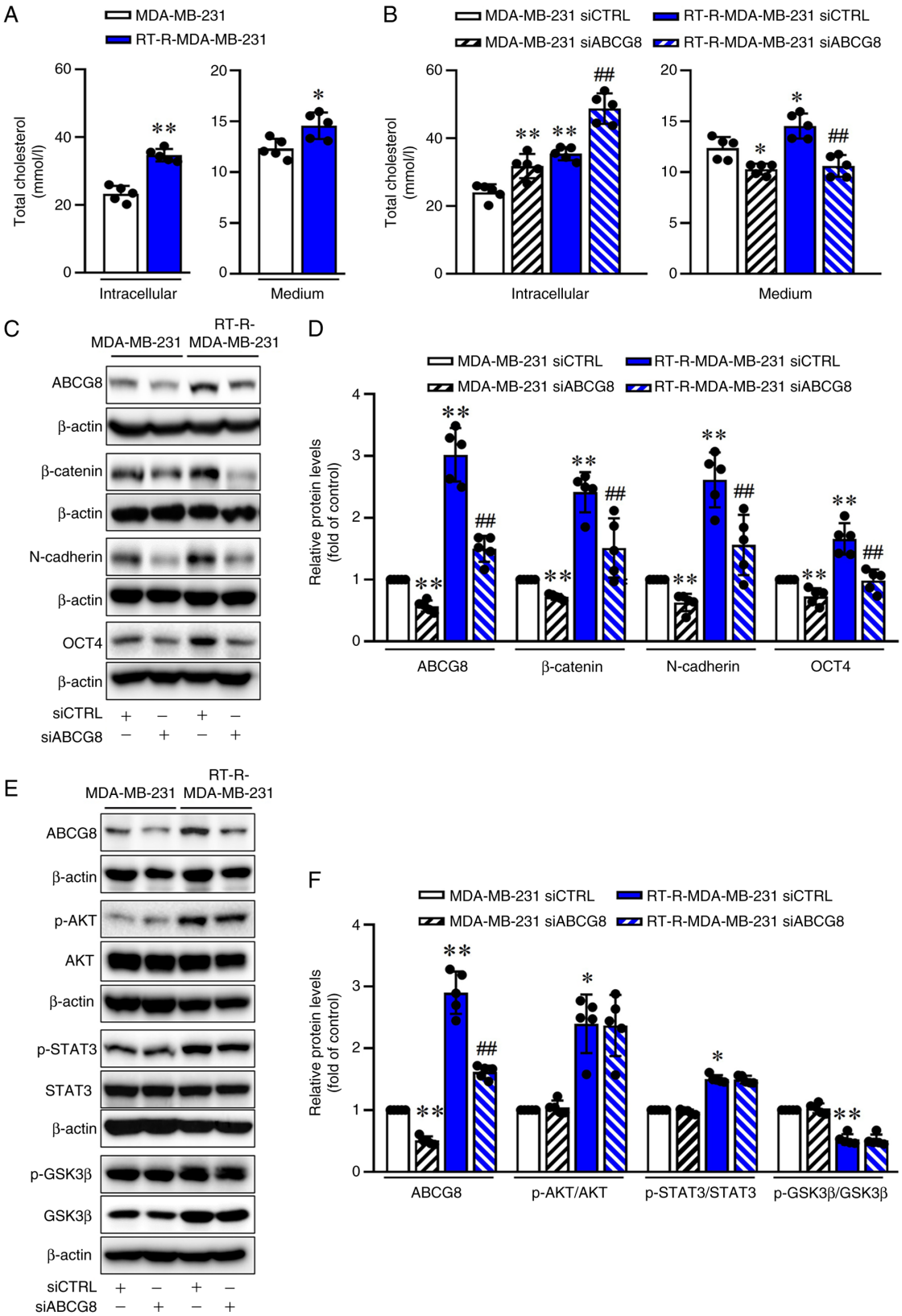

Figure 3. siABCG8 transfection leads to intracellular cholesterol accumulation and reduces epithelial-mesenchymal transition signaling. (A) Total cholesterol levels were quantified in cell lysates and culture media from MDA-MB-231 and RT-R-MDA-MB-231 cells using the Human Total Cholesterol ELISA Kit. Data are presented as the mean ± SD of five independent experiments. *P<0.05; **P<0.01 compared with the MDA-MB-231 cells. (B) Cells were transfected with siCTRL or siABCG8 (100 nM) for 24 h. Total cholesterol levels in both cell lysates and culture media were then quantified using a Human Total Cholesterol ELISA Kit. Data are presented as the mean ± SD of five independent experiments. *P<0.05; **P<0.01 compared with the siCTRL-transfected MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with the siCTRL-transfected RT-R-MDA-MB-231 cells. Protein levels of (C) ABCG8, β-catenin, N-cadherin and OCT4, or (E) ABCG8, p-AKT/AKT, p-STAT3/STAT3 and p-GSK3β/GSK3β were determined by western blot analysis. (D) ABCG8, β-catenin, N-cadherin and OCT4, or (F) ABCG8, p-AKT/AKT, p-STAT3/STAT3 and p-GSK3β/GSK3β protein levels were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. *P<0.05; **P<0.01 compared with the siCTRL-transfected MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with the siCTRL-transfected RT-R-MDA-MB-231 cells. ABCG8, ATP-binding cassette subfamily G member 8; CTRL, control; p-, phosphorylated; RT-R, radiotherapy-resistant; si, small interfering RNA.

increase was further enhanced by siABCG8 transfection. By contrast, the increased cholesterol levels in the culture media from RT-R-MDA-MB-231 cells, compared with those from MDA-MB-213 cells, were significantly decreased by siABCG8 transfection (Fig. 3B). These findings suggested that siABCG8 transfection decreased cholesterol efflux from the cytosol into the media. To investigate whether ABCG8 may be involved in regulating EMT-related proteins by expelling cholesterol, the levels of EMT-related proteins, β-catenin, N-cadherin and OCT4, were measured in MDA-MB-231 and RT-R-MDA-MB-231 cells transfected with siABCG8. As shown in Fig. 3C and D, β-catenin, N-cadherin and OCT4 were more highly expressed in RT-R-MDA-MB-213 cells than in MDA-MB-231 cells. However, their expression levels were significantly reduced by siABCG8 transfection. These results suggested that cholesterol pumped out of cells by ABCG8, rather than cholesterol inside cells, may affect the induction of EMT-related proteins. However, the levels of induced p-AKT, p-STAT3 and p-GSK3β were not influenced by siABCG8 transfection (Fig. 3E and F), suggesting that ABCG8-mediated cholesterol efflux did not affect the induction of EMT-related proteins through the p-AKT, p-STAT3 or p-GSK3β pathways.

ABCG8 knockdown induces cell cycle arrest at the G_2/M phase in RT-R-MDA-MB-231 cells by inhibiting PLK1 and Cyclin B1. Dysregulation of the cell cycle is a hallmark of cancer that enables limitless cell division (19). The Wnt/β-catenin pathway can modulate the cell cycle. Conversely, the cell cycle also influences the Wnt/β-catenin pathway (20). Therefore, the present study subsequently determined the role of ABCG8 in the cell cycle. Flow cytometry data showed that there were a higher proportion of RT-R-MDA-MB-231 cells in the S and G_2/M phases compared with MDA-MB-231 cells (Fig. 4A). Furthermore, when ABCG8 was knocked down, a 20% increase in cells in the S phase and a 17% increase in cells in the G_2/M phase in MDA-MB-231 cells, and a 20% increase in cells in the S phase and a 40% increase in cells in the G_2/M phase in RT-R-MDA-MB-231 cells were observed (Fig. 4B). This result suggested that ABCG8 knockdown affected the G_2/M phase. However, the changes observed in the G_2/M phase resulting from ABCG8 knockdown were not significant in the MDA-MB-231 cells. The increase in the G_2/M cell population due to ABCG8 knockdown was more pronounced in RT-R-MDA-MB-231 cells than in MDA-MB-231 cells. PLKs serve an essential role as key mitotic kinases and cell cycle regulators, and they also serve key roles in proliferation and cell growth (21). Among the PLKs, PLK1 has been shown to be upregulated in various types of cancer and to be associated with poor patient prognosis (22). PLK1 is a central actor during mitosis (23), which promotes entry into mitosis in the cell cycle by activating Cyclin B1 (24). Furthermore, β-catenin can regulate transcriptional induction of cell cycle regulators such as PLKs, particularly PLK1 (25,26). Therefore, the present study subsequently determined the effect of ABCG8 knockdown on PLK1 and Cyclin B1 expression. RT-R-MDA-MB-231 cells exhibited significantly increased protein expression levels of PLK1 and Cyclin B1 compared with those in MDA-MB-231 cells, which was suppressed by siABCG8 transfection. However, Cyclin D1 protein expression was significantly increased in RT-R-MDA-MB-231 cells, but was not affected by aiABCG8 transfection (Fig. 4C and D). These results indicated that ABCG8 knockdown led to down-regulation of PLK1 and Cyclin B1, which resulted in cell cycle arrest in the G_2/M phase by inhibiting cells from entering mitosis.

LRP6, the Wnt co-receptor, is activated more in RT-R-TNBC cells than in TNBC cells, and is involved in the induction of β-catenin, PLK1 and Cyclin B1. It has been reported that LDLR mediates the entry of cholesterol into cells (27), while LRP6, the Wnt co-receptor, activates the Wnt/β-catenin pathway upon cholesterol binding, resulting in the proliferation of cancer cells (28,29). If cholesterol imported into cells by LDLR activates Wnt/β-catenin signaling, increased intracellular cholesterol levels observed in siABCG8-transfected cells are expected to increase β-catenin. However, siABCG8-transfected cells exhibited increased intracellular cholesterol levels but reduced β-catenin (Fig. 3B-D). Therefore, the possibility that increased intracellular cholesterol via LDLR-mediated endocytosis could activate Wnt/β-catenin signaling in cells was excluded. Instead, the present study investigated whether the cholesterol-binding receptor LRP6, a Wnt co-receptor, could activate β-catenin and the associated EMT-related gene expression. RT-R-MDA-MB-231 cells exhibited increased levels of p-LRP6 compared with those in MDA-MB-231 cells (Fig. 5A and B). Knockdown of ABCG8 using siRNA decreased both LRP6 expression and LRP6 phosphorylation (Fig. 5C and D). Additionally, silencing of LRP6 resulted in reduced levels of β-catenin, PLK1 and Cyclin B1 compared with the siCTRL-transfected group (Fig. 5E-G). Furthermore, extracellular cholesterol treatment (10 and 20 μM; 24 h) did not significantly affect LRP6 phosphorylation and expression, and β-catenin signaling in RT-R-MDA-MB-231 cells at 10 μM, but did at 20 μM (Fig. S1A and B). When cholesterol was administered to MDA-MB-231 cells, which secreted less cholesterol than RT-R-TNBC cells, LRP6 phosphorylation and β-catenin induction were more prominent compared with those in RT-R-MDA-MB-231 cells from 10 μM (Fig. S1C and D). These findings indicated that increased LRP6 activation in RT-R-MDA-MB-231 cells was dependent on ABCG8 expression, and was associated with the induction of β-catenin, PLK1 and Cyclin B1 expression, suggesting that extracellular cholesterol exported by ABCG8 may affect LRP6 to activate the Wnt/β-catenin pathway.

RT-R-MDA-MB-231 cells exhibit increased SREBP1 (mature) and FASN expression and reduced LXRα/β and IDOL expression compared with MDA-MB-231 cells. SREBPs are well-known key transcription factors that control the expression of genes important for the uptake and synthesis of cholesterol, fatty acids and phospholipids (30,31), and it has been reported that FASN and cholesterol synthesis are linked under specific conditions (32,33). In particular, the SREBP1/FASN/cholesterol axis facilitates radioresistance in colorectal cancer (34); therefore, the present study investigated the SREBP1/FASN signaling pathways. RT-R-MDA-MB-231 cells exhibited a significant increase in the mature form of SREBP1 and FASN compared with MDA-MB-231 cells (Fig. 6A and B). Consistent with a report that hypoxia induces changes in lipid metabolism (35), RT-R-MDA-MB-231 cells

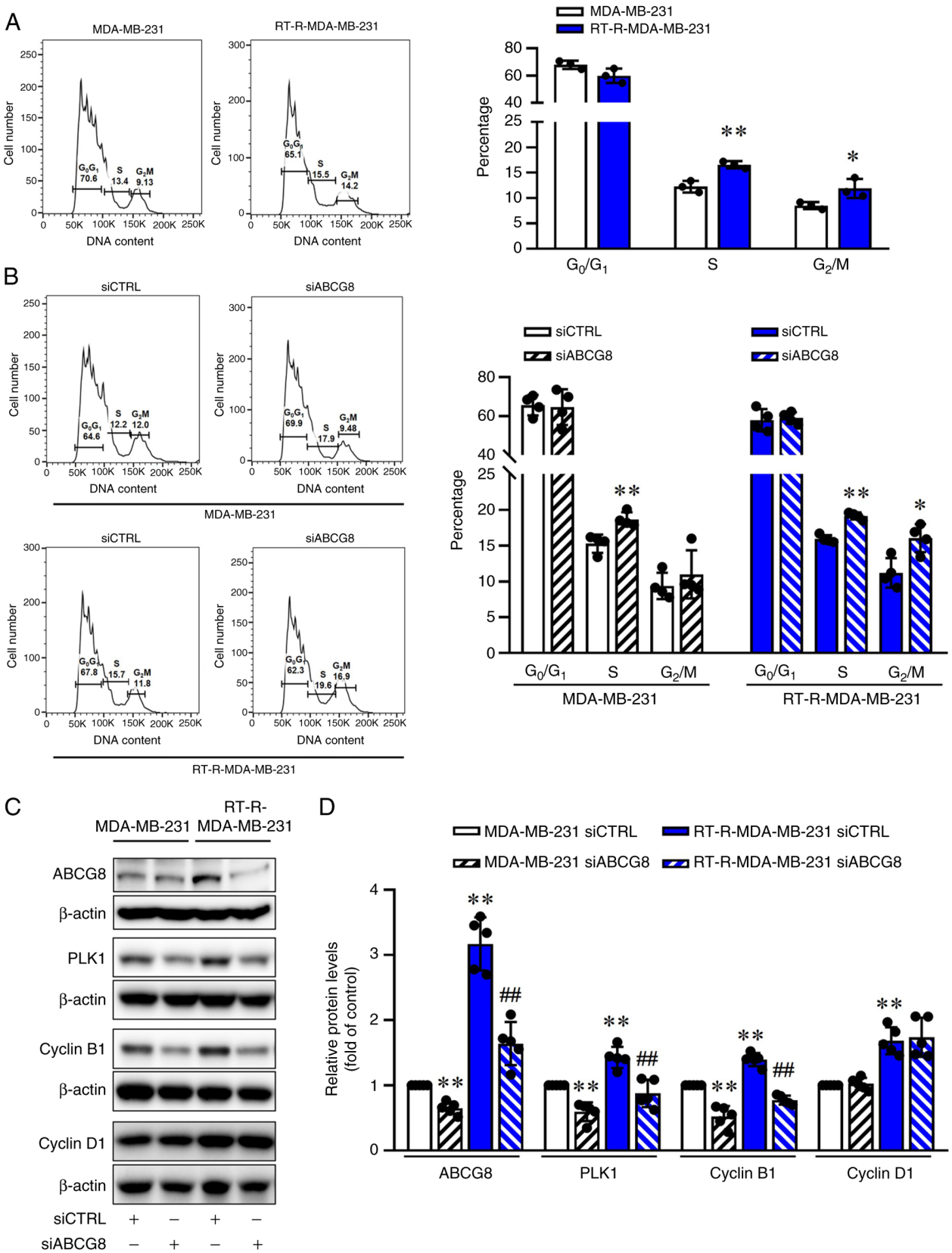

Figure 4. ABCG8 knockdown induces cell cycle arrest in the G_2/M phase in RT-R-MDA-MB-231 cells by inhibiting PLK1 and Cyclin B1 but not Cyclin D1. (A) Cell cycle distribution of MDA-MB-231 and RT-R-MDA-MB-231 cells determined using flow cytometry. Data are presented as the mean ± SD of three independent experiments. *P<0.05; **P<0.01 compared with the MDA-MB-231 cells for each phase. (B) Cells were transfected with siCTRL or siABCG8 for 24 h and the cell cycle distribution was then determined. Data are presented as the mean ± SD of four independent experiments. *P<0.05; **P<0.01 compared with the siCTRL group for each phase. (C) Protein expression levels of ABCG8, PLK1, Cyclin B1 and Cyclin D1 in cells transfected with siCTRL or siABCG8 were determined by western blot analysis. (D) Levels of proteins were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with the siCTRL-transfected MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with the siCTRL-transfected RT-R-MDA-MB-231 cells. ABCG8, ATP-binding cassette subfamily G member 8; CTRL, control; PLK1, Polo-like kinase 1; RT-R, radiotherapy-resistant; si, small interfering RNA.

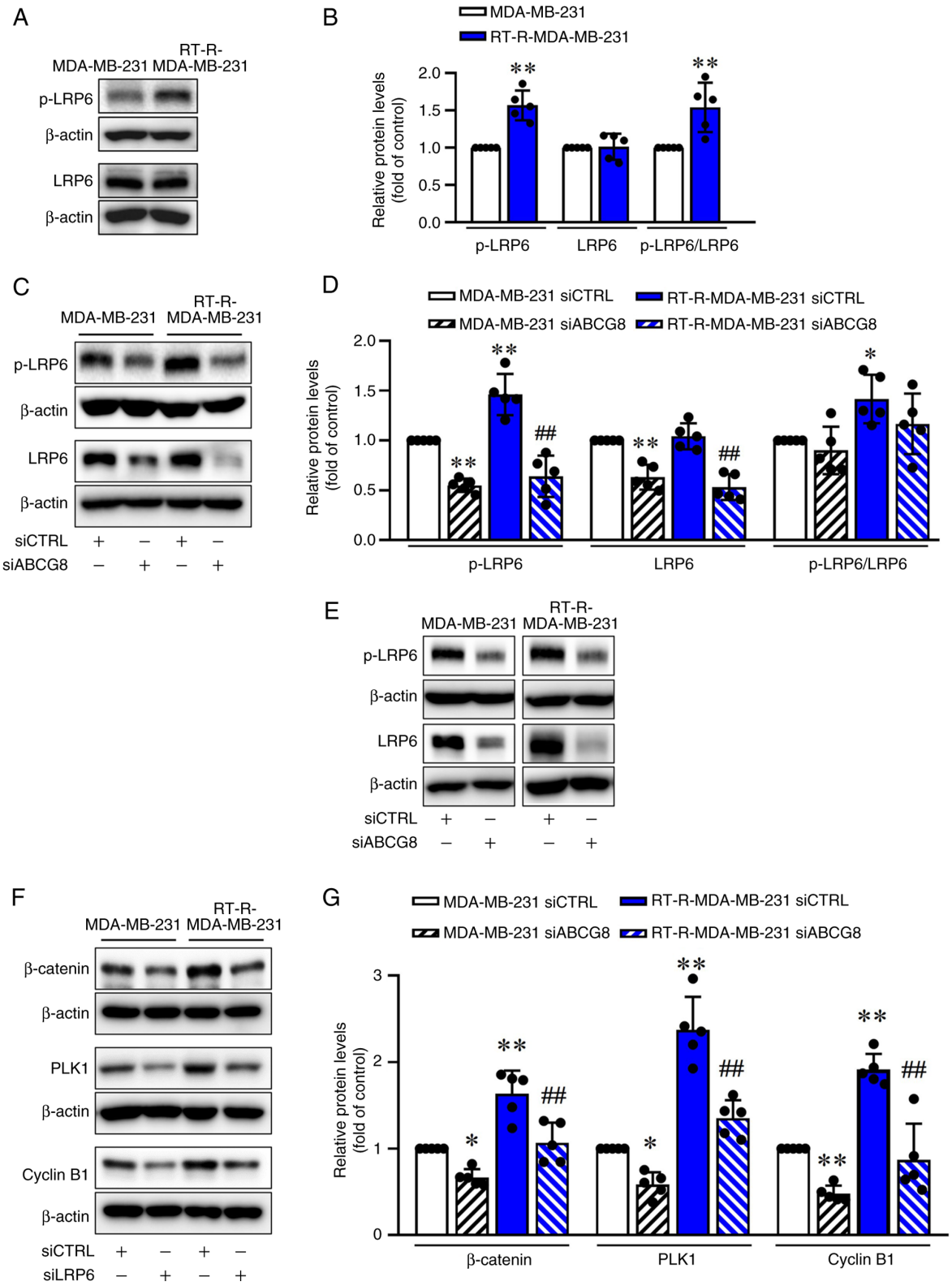

Figure 5. LRP6 is activated in RT-R-MDA-MB-231 cells, with its activation mediating cholesterol-dependent activation of the Wnt/β-catenin pathway. (A) Levels of LRP6 and p-LRP6 in MDA-MB-231 and RT-R-MDA-MB-231 cells were determined using western blot analysis. (B) Levels of LRP6 and p-LRP6 were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with MDA-MB-231 cells. (C) Protein levels of LRP6 and p-LRP6 in cells transfected with siCTRL or siABCG8 were determined by western blot analysis. (D) Levels of LRP6, p-LRP6 and p-LRP6/LRP6 were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. *P<0.05, **P<0.01 compared with the siCTRL-transfected MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with the siCTRL-transfected RT-R-MDA-MB-231 cells. (E) Cells were transfected with siCTRL or siLRP6 (100 nM) for 24 h and the transfection efficiency of siLRP6 was determined by western blot analysis. (F) Cells were transfected with siCTRL and siLRP6 for 24 h. Levels of β-catenin, PLK1 and Cyclin B1 were then determined by western blotting. (G) Levels of proteins were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. *P<0.05; **P<0.01 compared with the siCTRL-transfected MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with the siCTRL-transfected RT-R-MDA-MB-231 cells. ABCG8, ATP-binding cassette subfamily G member 8; CTRL, control; LRP6, low-density lipoprotein receptor-related protein 6; p-, phosphorylated; PLK1, Polo-like kinase 1; RT-R, radiotherapy-resistant; si, small interfering RNA.

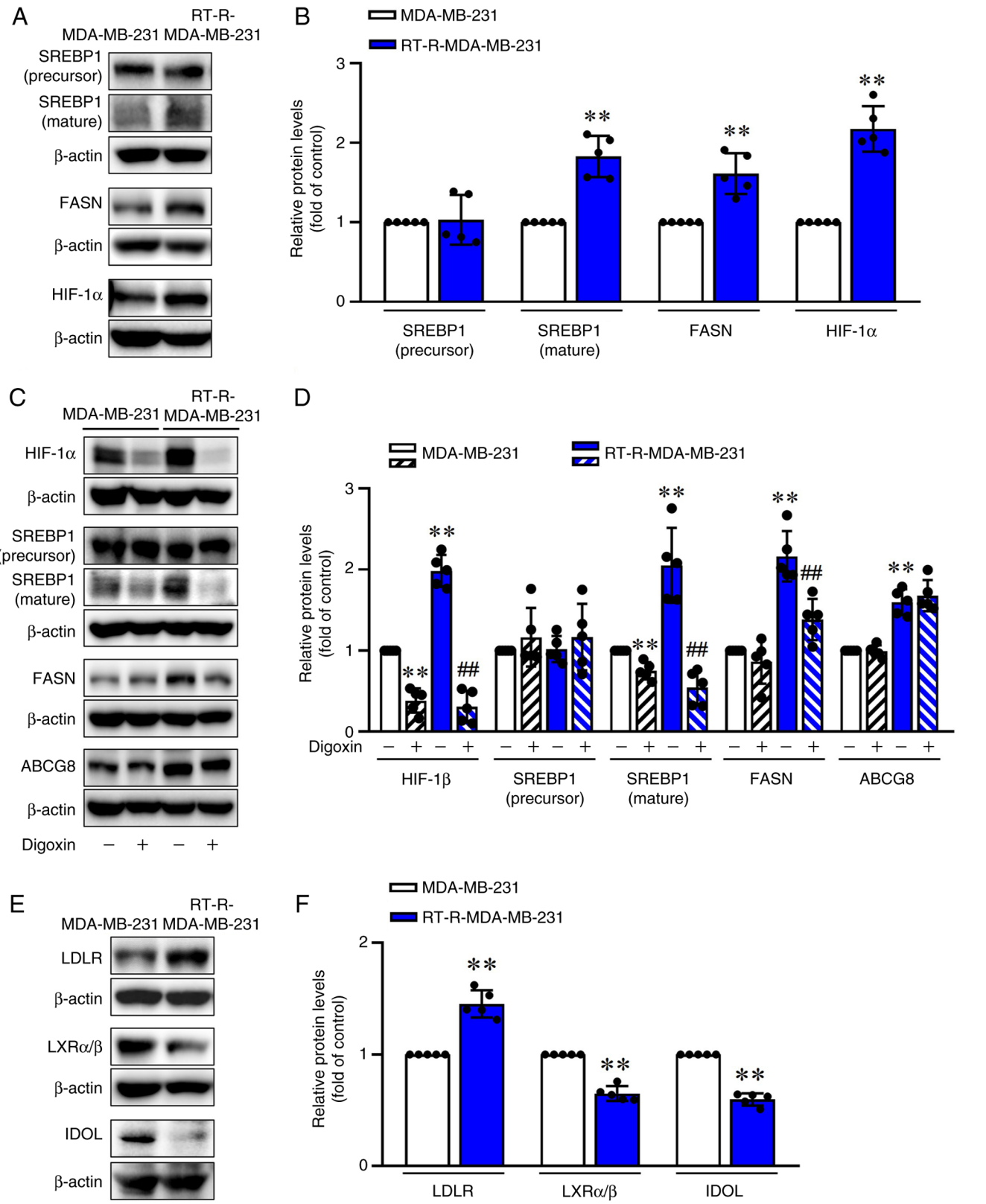

Figure 6. RT-R-MDA-MB-231 cells exhibit increased SREBP1 (mature) and FASN expression compared with MDA-MB-231 cells. (A) Protein levels of SREBP1 (precursor and mature form), FASN and HIF-1α in MDA-MB-231 and RT-R-MDA-MB-231 cells were analyzed by western blotting. (B) Levels of proteins were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with MDA-MB-231 cells. (C) Cells were treated with 400 nM digoxin, an inhibitor of HIF-1α, for 24 h, and protein levels of HIF-1α, SREBP1, FASN and ABCG8 were then analyzed by western blotting. (D) Levels of proteins were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with the untreated MDA-MB-231 cells; $^{\#\#}$P<0.01 compared with the untreated RT-R-MDA-MB-231 cells. (E) LDLR, LXRα/β and IDOL protein levels in MDA-MB-231 and RT-R-MDA-MB-231 cells were analyzed by western blotting. (F) Levels of proteins were semi-quantified and normalized to β-actin. Data are presented as the mean ± SD of five independent experiments. **P<0.01 compared with MDA-MB-231 cells. ABCG8, ATP-binding cassette subfamily G member 8; FASN, fatty acid synthase; HIF-1α, hypoxia-inducible factor 1α; IDOL, inducible degrader of LDL; LDLR, LDL receptor; LDL, low-density lipoprotein; LXRα/β, liver X receptor α/β; RT-R, radiotherapy-resistant; SREBP1, sterol regulatory element binding protein 1.

exhibited increased HIF-1α levels (Fig. 6A and B), and treatment with digoxin, a HIF-1α inhibitor (at 400 nM for 24 h), led to a marked reduction in SREBP1 (mature form), and FASN protein expression in RT-R-MDA-MB-231 cells (Fig. 6C and D). However, ABCG8 expression was not affected by digoxin (Fig. 6C and D). In addition, RT-R-MDA-MB-231 cells exhibited increased levels of LDLR and decreased levels of LXRα/β and IDOL compared with those in MDA-MB-231 cells (Fig. 6E and F). These results suggested that RT-R-TNBC increased intracellular cholesterol levels by inducing synthesis of cholesterol and by dysregulating feedback mechanisms such as the LXRα/β and IDOL feedback mechanisms to reduce intracellular cholesterol by decreasing LDLR expression.

Discussion

ABC transporters are ATP-dependent transporters composed of 48 genes. They are subdivided into seven subfamilies (ABCA-ABCG) (36), and transport xenobiotics, metabolites and signaling molecules across intracellular and extracellular membranes (11). ABC transporters are well known for mediating multidrug resistance in cancer, which can lead to chemotherapy failure (37). Studies have suggested the important roles of ABC transporters in cancer beyond their established function as a multidrug efflux pump (11); they have been reported to influence tumor cells in various cancer types by regulating their malignant potential, including tumor cell proliferation, differentiation, migration and invasion (38,39). However, there is still a lack of understanding of how ABC transporters affect cancer progression.

In the present study, RT-R-MDA-MB-231 cells exhibited a significant increase in ABCG8 expression compared with MDA-MB-231 cells. Furthermore, knockdown of ABCG8 not only increased Doxo- and PTX-mediated cytotoxicity, but also significantly reduced cell viability on its own after 48 and 72 h of incubation, suggesting the additional function of ABCG8 beyond its role as a drug pump. Since ABCG8 is involved in sterol efflux (16), the present study investigated the levels of cholesterol inside cells and in the surrounding medium. The levels of cholesterol in cells and surrounding medium were significantly higher in the RT-R-MDA-MB-231 group than in the MDA-MB-231 group. Additionally, when ABCG8 was knocked down, there was a further increase in intracellular cholesterol levels but a reduction in medium cholesterol levels. This result demonstrated that RT-R-MDA-MB-231 cells exhibited higher levels of cholesterol, and that ABCG8 in RT-R-MDA-MB-231 cells increases cholesterol efflux.

Tumor cells have an increased need for lipids to grow and proliferate quickly. Lipid synthesis and uptake are elevated in various types of cancer, including breast cancer, and represent a novel characteristic of human cancer (18,40). Cholesterol is a major component of cell membranes; it controls cellular processes such as cell proliferation, differentiation, survival and apoptosis, and is also considered a risk factor and prognostic factor in cancer (41). Studies have shown that cholesterol serves a crucial role as a signaling molecule, regulating several pathways such as the PI3K, Hedgehog and Wnt/β-catenin pathways (42,43). Therefore, the present study investigated whether cholesterol exported by ABCG8 could function as a signaling molecule and whether it might be involved in cancer progression. The present results demonstrated that the expression levels of EMT-related proteins (β-catenin, N-cadherin and OCT4) were significantly higher in RT-R-MDA-MB-231 cells than in MDA-MB-231 cells. However, these increases were reduced by siABCG8 transfection. EMT-related proteins are known to be regulated by the PI3K/AKT signaling pathway in cancer (44,45). Additionally, the STAT3 pathway is a key regulator of breast cancer metastasis, promoting cancer progression, drug resistance, inhibition of apoptosis and EMT (46,47). However, although intracellular cholesterol levels were increased by siABCG8 transfection, the increased levels of p-AKT and p-STAT3 in RT-R-MDA-MB-231 cells were not affected by siABCG8 transfection. Therefore, this result suggested that cholesterol exported by ABCG8 affected the induction of EMT-related proteins but not via the p-AKT or p-STAT3 pathways.

It has been reported that cholesterol may serve a role in cancer biology by activating the Wnt pathway through specific interactions with Dishevelled and LRP6 (48). LRP6 is a crucial Wnt co-receptor in the Wnt/β-catenin signaling pathway. LRP6 expression is frequently upregulated in human breast cancer, and LRP6 is more frequently upregulated in TNBC (49). LRP6 promotes TNBC cell migration and invasion by interacting with frizzled (a transmembrane receptor family) and by activating the Wnt/β-catenin signaling pathway (50,51) involved in various physiological processes, such as proliferation, apoptosis, migration, invasion and homeostasis. Emerging reports have suggested that abnormal activation of the Wnt/β-catenin signaling pathway can promote cancer stem cell progression and cancer metastasis (52,53), and patients with TNBC with upregulated Wnt signaling often have a poor prognosis (54). Based on the significance of LRP6 in binding cholesterol and activating the Wnt/β-catenin pathway, which ultimately contributes to the maintenance and proliferation of cancer cells, the present study investigated the expression levels of LRP6 in RT-R-MDA-MB-231 cells, and revealed that the levels of p-LRP6 were increased in RT-R-MDA-MB-231 cells compared with MDA-MB-231 cells. siLRP6 transfection reduced the expression levels of β-catenin, PLK1 and Cyclin B1. Based on these results, it was hypothesized that increased cholesterol in RT-R-MDA-MB-231 cells may be exported by ABCG8 and that the elevated cholesterol could subsequently influence LRP6/Wnt/β-catenin signaling to activate the expression of β-catenin and other EMT-related genes. ABCG8 protein levels were higher in TNBC cells such as MDA-MB-231 and MDA-MB-453 cells than in MCF7 cells, a non-TNBC cell line. Furthermore, siABCG8 transfection also affected cholesterol efflux and the LRP6/Wnt/β-catenin signaling pathway in MDA-MB-231 cells. Therefore, ABCG8 may mediate cholesterol efflux and activate LRP6/Wnt/β-catenin signaling in TNBC. The present study emphasized that ABCG8 expression, cholesterol efflux from cytosol to media, and activation of the LRP6/Wnt/β-catenin signaling were enhanced in RT-R-MDA-MB-231 cells compared with MDA-MB-231 cells. In addition, knockdown of ABCG8 using siRNA reduced the levels of p-LRP6/LRP6, β-catenin and EMT-related proteins without reducing p-GSKβ or GSKβ. This result indicated that the levels of β-catenin may be controlled by factors other than GSKβ in the Wnt signaling pathway.

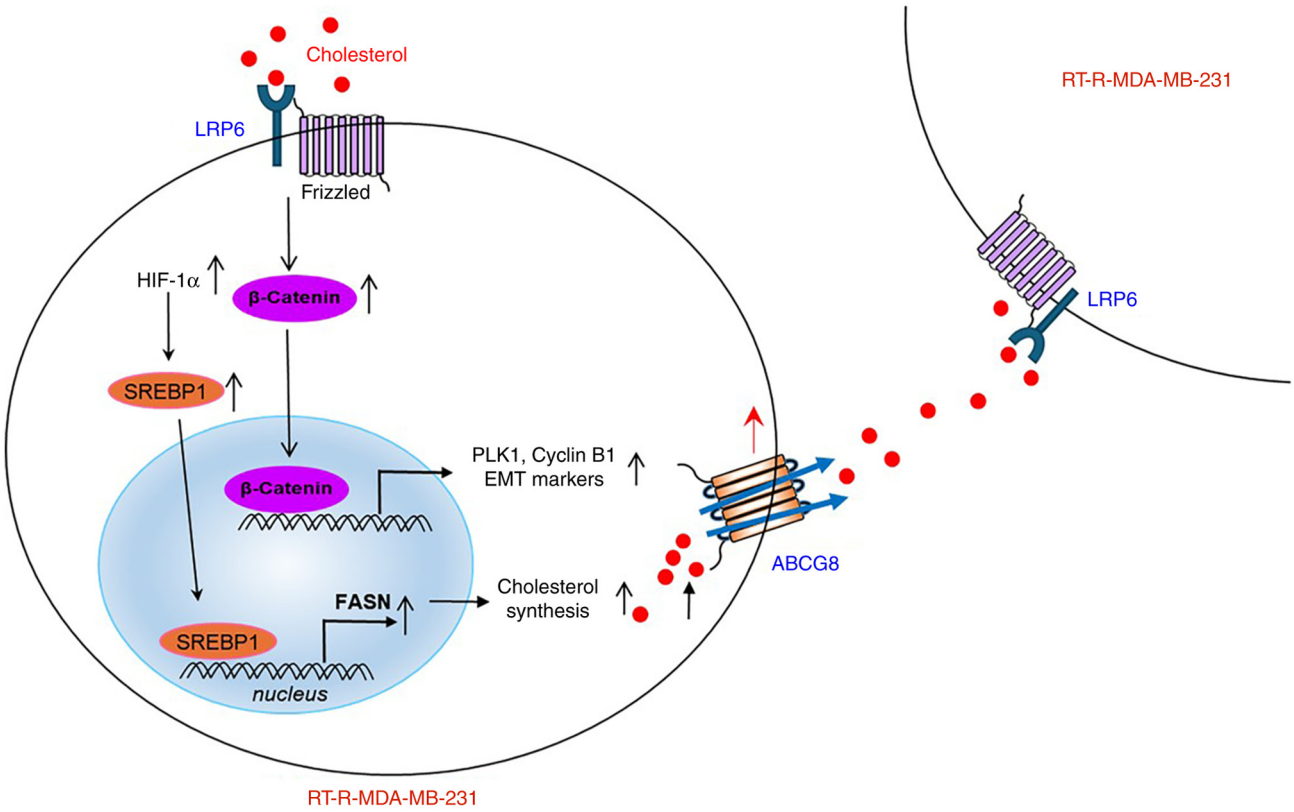

Figure 7. A proposed mechanism suggests that ABCG8 upregulation in RT-R-MDA-MB-231 cells mediates cholesterol efflux, leading to increased cancer cell progression through the LRP6/Wnt/β-catenin signaling pathway in RT-R-MDA-MB-231 cells. Induced HIF-1α in RT-R-MDA-MB-231 cells increases SREBP1 (mature form) and FASN, resulting in the production of cholesterol. Produced cholesterol is exported by ABCG8 to activate Wnt/β-catenin signaling through LRP6 receptors. Activated β-catenin can induce the expression of target genes such as PLK1 and Cyclin B1, ultimately leading to increased proliferation of cancer cells and tumor growth. ABCG8, ATP-binding cassette subfamily G member 8; EMT, epithelial-mesenchymal transition; FASN, fatty acid synthase; HIF-1α, hypoxia-inducible factor 1α; LRP6, low-density lipoprotein receptor-related protein 6; PLK1, Polo-like kinase 1; RT-R, radiotherapy-resistant; SREBP1, sterol regulatory element binding protein 1.

PLKs serve an essential role as key mitotic kinases and cell cycle regulators, and they also serve a role in proliferation and cell growth (21). PLKs are upregulated in various types of cancer, including ovarian cancer (22), breast cancer (55) and colorectal cancer (56), and promote entry into mitosis in the cell cycle by activating Cyclin B1 (23). Additionally, Wnt signaling-mediated β-catenin can activate cJUN, which results in increased expression levels of PLK1 (26). c-Jun is known as a transcriptional target and an interacting partner of β-catenin (57) and is also considered a possible transcriptional factor regulating PLK1 expression (58). Kim *et al* (26) demonstrated that TGFβ-activated PLK1 can phosphorylate β-catenin at Ser311, leading to increased stability, and promotes its nuclear translocation. Consequently, this process leads to elevated c-JUN expression, which, as part of the AP-1 complex, upregulates PLK1 expression, forming a positive feedback loop that facilitates EMT in metastatic non-small cell lung cancer. In the present study, ABCG8 knockdown significantly reduced the levels of PLK1 and Cyclin B1 in RT-R-MDA-MB-231 cells, resulting in cell cycle arrest in the G_2/M phase. LRP6 siRNA transfection also reduced β-catenin, PLK1 and Cyclin B1 levels. These results suggested that cholesterol exported by ABCG8 could activate the LRP6/Wnt/β-catenin signaling pathway, which in turn could induce the expression of PLK1 and Cyclin B1, ultimately leading to cell cycle progression.

SREBP is a crucial transcription factor that controls the expression of enzymes involved in lipid metabolism and synthesis (30), and its expression is increased in various types of cancer, including colorectal cancer (59), breast cancer (60) and hepatocellular carcinoma (61). HIF-1α is a transcription factor that is induced under hypoxia, which is involved in tumor progression, including metastasis, drug resistance and cell cycle progression. HIF-1α is related to poor prognosis in various types of cancer (62-64), including colon cancer, prostate cancer and breast cancer (65). Under hypoxic conditions, tumor cells can activate HIF-1α. Activated HIF-1α then upregulates SREBP1 and activates FASN, which can increase hypoxia-induced chemotherapy resistance and promote tumor cell survival (66,67). Our previous study revealed that increased HIF-1α expression in RT-R-MDA-MB-231 cells contributed to tumor progression (8). In the present study, the expression levels of the mature form of SREBP1 and FASN were significantly increased in RT-R-MDA-MB-231 cells compared with in MDA-MB-231 cells, and increased SREBP1 and FASN levels in RT-R-MDA-MB-231 cells were significantly reduced by treatment with digoxin, an inhibitor of HIF-1α. These results suggested that the cholesterol levels in RT-R-MDA-MB-231 cells were increased via HIF-1α-mediated SREBP1-FASN induction. However, ABCG8 induction in RT-R-MDA-MB-231 cells was not HIF-1α-dependent, as digoxin, a HIF-1α inhibitor, failed

to inhibit ABCG8 induction in RT-R-MDA-MB-231 cells. Further investigation is needed to understand the mechanism by which RT-R-MDA-MB-231 cells induce ABCG8. In normal cells, a high level of cholesterol triggers regulatory mechanisms that control the synthesis and uptake of cholesterol, leading to downregulation of SREBP and degradation of β-hydroxy β-methylglutaryl-CoA reductase. In addition, LXRα/β and IDOL are increased to reduce intracellular cholesterol by decreasing LDLR expression (68). However, cancer cells may be able to evade these feedback mechanisms in a cholesterol-rich environment. The present results showed that the expression of LXRα/β and IDOL, a feedback mechanism for the downregulation of intracellular cholesterol levels, was reduced in RT-R-MDA-MB-231 cells, resulting in increased LDLR expression. Dysregulation of feedback mechanisms as well as induction of cholesterol synthesis might maintain a high level of cholesterol within cells. The cholesterol was exported by ABCG8, which was upregulated in RT-R-MDA-MB-231 cells. The exported cholesterol in turn activated LRP6, the Wnt co-receptor, leading to the mediation of Wnt/β-catenin signaling. This activation induced the expression of PLK1, Cyclin B1 and EMT-related proteins, ultimately contributing to cancer progression (Fig. 7).

The increased cholesterol levels in highly metastatic TNBC that has acquired radiotherapy resistance may affect the maintenance of cell morphology because cholesterol is a component of cell membranes and vital for cell membrane integrity (41,69). However, to the best of our knowledge, the present study was the first to suggest the role of ABCG8 and that the cholesterol exported by ABCG8, not inside the cells, affects cancer progression through the LRP6/Wnt/β-catenin signaling pathway in RT-R-TNBC cells. The present results demonstrated that intracellular and medium cholesterol levels were significantly higher in the RT-R-MDA-MB-231 group compared with in the MDA-MB-231 group, and LRP6 was highly phosphorylated and β-catenin signaling was upregulated at baseline without any external stimulation in RT-R-MDA-MB-231 cells. This may explain why the effect of externally administered cholesterol was minimal in RT-R-MDA-MB-231 cells. Therefore, it may be hypothesized that high levels of intracellular cholesterol exported by ABCG8 may serve an important role in cancer progression by maintaining high levels of pericellular cholesterol and continuously stimulating cells. This needs to be further investigated. In conclusion, targeting the LRP6-Wnt/β-catenin signaling pathway may provide a potential therapeutic strategy for the treatment of RT-R-TNBC.

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Availability of data and materials

The sequencing data generated in the present study may be found in the Gene Expression Omnibus database under accession number GSE287883 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE287883. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

YSK performed the experiments, analyzed the data and wrote the manuscript. JYW, HJ, NBN, YW and VN conducted the experiments and analyzed the data. SPY and SWP interpreted the results, developed the methodology and revised the manuscript. HJK conceived and designed the study, directed the project, and revised the manuscript. YSK, HJ and HJK confirmed the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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