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ABCA9, an ER cholesterol transporter, inhibits breast cancer cell proliferation via SREBP-2 signaling

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Abstract

The association between cholesterol metabolism and cancer development and progression has been recently highlighted. However, the role and function of many cholesterol transporters remain largely unknown. Here, we focused on the ATP-binding cassette subfamily A member 9 (ABCA9) transporter given that its expression is significantly downregulated in both canine mammary tumors and human breast cancers, which in breast cancer patients correlates with poor prognosis. We found that ABCA9 is mainly present in the endoplasmic reticulum (ER) and is responsible for promoting cholesterol accumulation in this structure. Accordingly, ABCA9 inhibited sterol-regulatory element binding protein-2 (SREBP-2) translocation from the ER to the nucleus, a crucial step for cholesterol synthesis, resulting in the downregulation of cholesterol synthesis gene expression. ABCA9 expression in breast cancer cells attenuated cell proliferation and reduced their colony-forming abilities. We identified ABCA9 expression to be regulated by Forkhead box O1 (FOXO1). Inhibition of PI3K induced enhanced ABCA9 expression through the activation of the PI3K–Akt–FOXO1 pathway in breast cancer cells. Altogether, our study suggests that ABCA9 functions as an ER cholesterol transporter that suppresses cholesterol synthesis via the inhibition of SREBP-2 signaling and that its restoration halts breast cancer cell proliferation. Our findings provide novel insight into the vital role of ABCA9 in breast cancer progression.

KEYWORDS
ABCA9, breast cancer, cholesterol metabolism, FOXO, SREBP-2

Abbreviations: ABC, ATP-binding cassette; ABCA, ATP-binding cassette subfamily A; CMT, canine mammary tumor; COPII, coat protein complex II; ER, endoplasmic reticulum; FOXO1, forkhead box O1; HBC, human breast cancer; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS-1, 3-hydroxy-3-methylglutaryl-CoA synthase 1; INSIG, insulin-induced gene; LDLR, low-density lipoprotein receptor; LXR, liver X receptor; MβCD, methyl-β-cyclodextrin; PDI, protein disulfide isomerase; PI, propidium iodide; S1P, site-1 protease; S2P, site-2 protease; SCAP, SREBP-cleavage activating protein; SREBP, sterol-regulatory element binding protein; TAZ, tarfazzin; TCF4, transcription factor 4; TGF-β, transforming growth factor β; YAP, yes-associated protein.

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1 | INTRODUCTION

Cholesterol is an essential lipid of cell membranes that affects their biophysical properties, membrane trafficking, and signal transduction.[1] In addition, cholesterol is synthesized in almost all mammalian cells, being a precursor of steroid hormones, cholesterol esters, vitamin D, and bile acid.[2] Cholesterol synthesis takes place in the ER, where it then moves to other cell membranes.[3] Cholesterol is mainly located in the plasma membrane. Although the ER exhibits particularly lower cholesterol levels compared with other cellular organelles, most of the cell machinery regulating cholesterol homeostasis resides in the ER.[4] Therefore, the balance and maintenance of cholesterol levels in the ER are critical.

Cholesterol homeostasis is tightly regulated using complex processes such as biosynthesis, uptake, export, and storage.[5] The key players involved in the regulation of cellular cholesterol homeostasis are SREBP and LXR. SCAP senses ER sterol levels, and when ER membrane cholesterol levels are low, the INSIG protein is dissociated, SCAP binds to COPII proteins, escorts SREBP from the ER to the Golgi, and SREBP is then translocated to the nucleus through cleavage by S1P and S2P. Finally, the translocation of SREBP results in the activation of genes related to cholesterol synthesis. In contrast, when ER membrane cholesterol levels are high, SREBP forms a complex with INSIG and SCAP and is retained in the ER, and LXR activation is induced to regulate intracellular cholesterol homeostasis.[6]

Dysregulation of cholesterol homeostasis has been frequently reported in multiple cancer types.[7] Highly proliferative cancer cells maintain elevated cholesterol levels through a sufficient cholesterol supply obtained via the aberrant expression of cholesterol metabolism-related genes.[8] Moreover, current evidence shows that intracellular cholesterol accumulation affects cancer progression, namely its proliferation and migration rates.[9] Therefore, a better understanding of the mechanisms regulating cholesterol levels in cancer cells is needed. In this regard, several ABC transporters are known to play an essential role in maintaining intracellular cholesterol homeostasis.

ABC transporters are a superfamily of transport proteins, responsible for the translocation of various substrates into and out of cells through the energy of ATP hydrolysis. The ABCA, mainly responsible for lipid transport, is composed of 12 members.[10] Specifically, the ABCA1 transporter is known to mediate the transport of cholesterol and phospholipids. ABCA1 deficiency endows cancer cells with defenses against apoptotic stimuli characterized by increased mitochondrial cholesterol levels, thereby increasing cancer cell survival.[11] The ABCA7 transporter also regulates intracellular cholesterol homeostasis and promotes the epithelial-to-mesenchymal transition process in ovarian cancer through transforming growth factor β (TGF-β) signaling.[12] Regarding the ABCA9 transporter, its expression was first reported in monocyte differentiation.[13] Downregulation of ABCA9 has been demonstrated in both breast and colorectal cancers.[10] Recently, a study relying on RNA-seq analysis suggested ABCA9 as a novel essential gene for triple-negative breast cancer development and progression.[14] Although preliminary data have suggested the participation of ABCA9 in different types of cancer, its functional role in cancer cells is still unknown. Therefore, this study aimed to specifically investigate the molecular functions of ABCA9 in breast cancer.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Breast cancer cell lines were purchased from the Korean Cell Line Bank (Seoul). MCF-10A was cultured in mammary epithelial cell basal medium with mammary epithelial growth medium supplements and growth factors (Lonza). SKBR-3 and T-47D were cultured in RPMI 1640 medium (HyClone), and HEK-293T, MCF-7, MDA-MB-231, and MDA-MB-453 cells were cultured in DMEM/high glucose medium (HyClone). All culture media were supplemented with 10% fetal bovine serum (HyClone) and 1% antibiotic–antimycotic (Gibco), and cultures were performed at 37°C in a 5% CO_2 atmosphere.

2.2 | RNA isolation and quantitative RT-PCR

RNA isolation and quantitative RT-PCR were carried out as described previously.[15] Briefly, reverse transcription was performed using the CellScript All-in-One 5× First Strand cDNA Synthesis Master Mix (CellSafe). The list of primers used for qRT-PCR is presented in Table S1. Relative mRNA quantity was measured using the ΔΔCt comparative method. The *GNB2L1* gene was used as an endogenous reference.

2.3 | Statistical analysis

Statistical analysis was performed using GraphPad Prism v8.0 software and data were expressed as mean ± standard error of the mean (SEM) of three independent experiments. Statistical significance was evaluated using Student's *t* test, and one-way or two-way ANOVA. A p-value < 0.05 was considered statistically significant.

The detailed Materials and Methods are described in Appendix S1.

3 | RESULTS

3.1 | ABCA9 exhibits functional and structural features similar to other ABCA subfamilies

Different ABCA subfamily members have high homology in the nucleotide sequence and peptide structures (Figure 1A, B). Specifically, the phylogenic analysis revealed that ABCA5, ABCA6, ABCA8, ABCA9, and ABCA10, which have not been well studied yet, are highly conserved and clustered into a separate branch

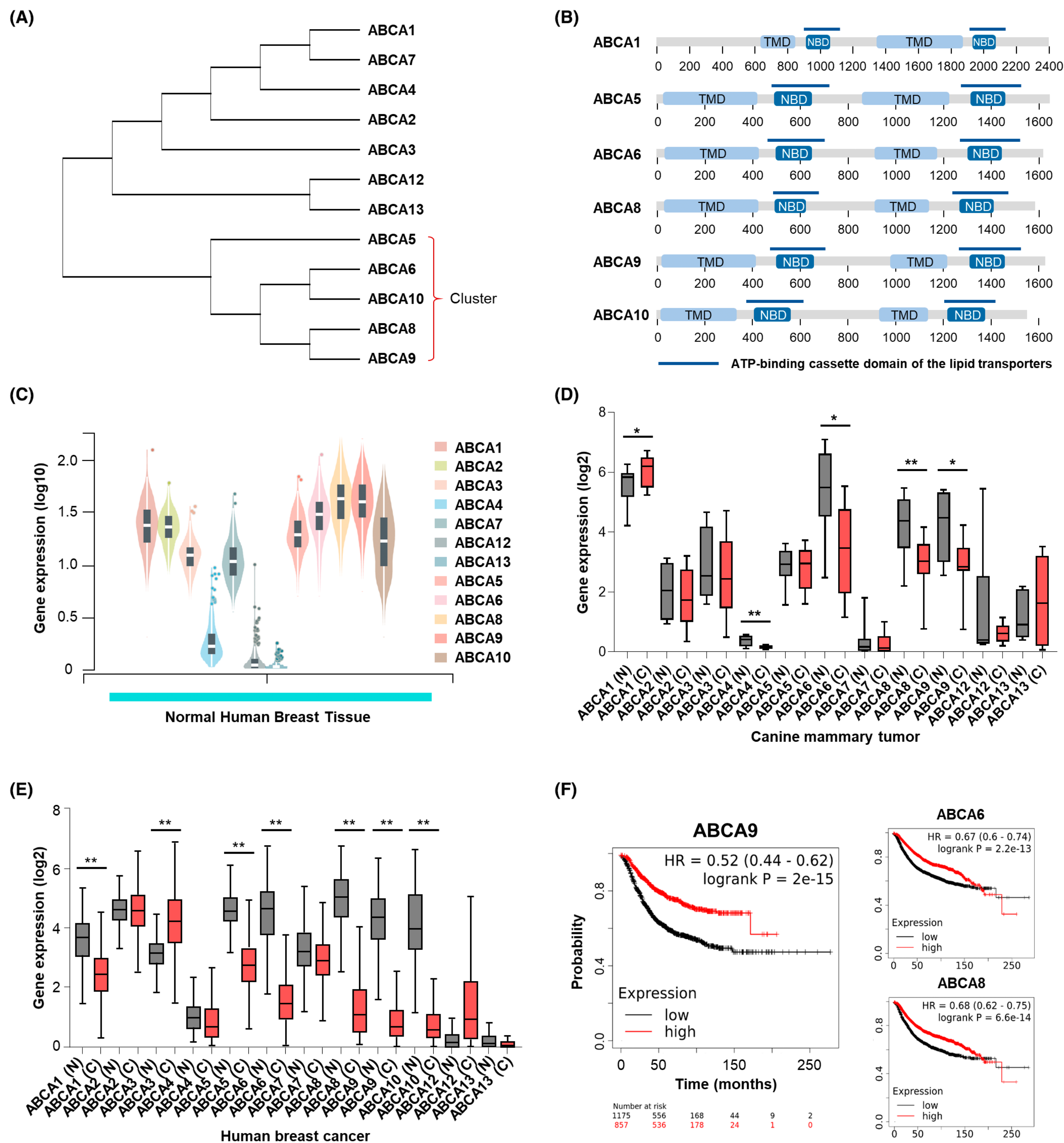


FIGURE 1 ABCA9 has functional and structural features similar to ABCA subfamily. (A) Sequence homology was analyzed through the phylogenetic tree between ABCA transporters. (B) Domain analysis of ABCA1 and cluster showed the functional similarity between ABCA1 and ABCA5-10. (C) The expression of the ABCA transporters was confirmed in normal human breast tissue using the GTEx database. (D, E) ABCA transporters are differentially expressed in HBC patients, CMT samples and adjacent normal transcriptome data. Only the expression of ABCA6, ABCA8, and ABCA9 showed a significant decrease in both HBC and CMT. (F) A significant correlation between the high expression levels of *ABCA6*, *ABCA8*, and *ABCA9* genes and longer RFS was verified by the Kaplan–Meier plot. Data are presented as the mean ± SEM and *, **, ***, and NS denote $p < 0.05$, $p < 0.01$, $p < 0.001$, and nonsignificance, respectively.

(Figure 1A). Similar to ABCA1, they also have common structures, such as two transmembrane domains and two nucleotide domains, representative of a cholesterol transporter feature for regulating the cholesterol efflux process (Figure 1B). Various levels of ABCA gene expressions in the human breast tissue are suggestive of their diverse roles in cellular physiological conditions (Figure 1C). Notably, ABCAs belonging to the cluster, including ABCA9, exhibited an exceptionally high expression in normal human breast

tissue, with the exception of ABCA12 and ABCA13 that were expressed at low levels.

We explored the supporting data related to cancer from our own and from public human transcriptome datasets.[16] As part of comparative medicine, naturally occurring CMTs have been suggested as a good animal model for studying HBC, given their similarities.[17] Indeed, as CMTs have a transcriptome signature similar to that of HBCs, we investigated the expression of the canine orthologous ABCA transporter genes in the CMT transcriptome data. By comparing the gene expression of the tumor and adjacent normal tissue, we found that ABCA6, ABCA8, and ABCA9 genes were significantly downregulated in CMTs (Figure 1D). These genes were also commonly downregulated in HBCs, showing cross-species evidence (Figure 1E). Additionally, a low mRNA expression of ABCA6, ABCA8, and ABCA9 was correlated with aggravated relapse-free survival (RFS) in breast cancer patients (Figure 1F). Given that Kaplan–Meier plots showed the most significant differences and that studies on the relationship between ABCA9 and breast cancer have not been conducted yet, in this study we focused on the role of ABCA9 in breast cancer.

3.2 | ABCA9 functions as a cholesterol transporter and contributes to cholesterol accumulation in the ER

Assessing the subcellular localization of ABCA9 is essential for understanding its function as a cholesterol transporter. Given that ABCA transporters have distinct subcellular localizations, we transfected Flag-tagged ABCA9 into HEK-293T cells to confirm its subcellular localization. ABCA9 was detected in the ER through co-staining with PDI, an ER marker. Further co-localization analysis confirmed this finding, demonstrated by a high Pearson correlation between ABCA9 and PDI (Figure 2A). We validated that ABCA9 was localized to the ER by staining with calnexin, an additional ER marker (Figure S1). In addition, using Golgin-97 as a Golgi apparatus marker and MitoTracker for mitochondria, we additionally confirmed that ABCA9 was distinct from the Golgi apparatus and mitochondria, which are other cytoplasmic components (Figure 2B, C). To determine its potential as a cholesterol transporter, we tested a cholesterol-loading condition using cholesterol-methyl-β-cyclodextrin and a cholesterol depletion condition using MβCD, a cholesterol depletion reagent. When cells presented high cholesterol levels, ABCA9 expression remained unaltered (Figure 2D) whereas in the absence of cholesterol, ABCA9 expression was decreased (Figure 2E). These expression patterns indicated that ABCA9 levels could be regulated based on the availability of cholesterol, suggesting that ABCA9 is responsive to cholesterol levels. We next tested the functional role of ABCA9 in mediating the transportation, and putative accumulation of cholesterol into the ER. Filipin staining allowed the visualization of cholesterol and its ER accumulation was verified using confocal microscopy. Interestingly, ABCA9-overexpressing cells showed a higher mean fluorescence intensity of Filipin in the ER compared with control cells (Figure 2F). These results suggested that ABCA9 is a cholesterol transporter working in the ER with the ability to accumulate cholesterol within the ER.

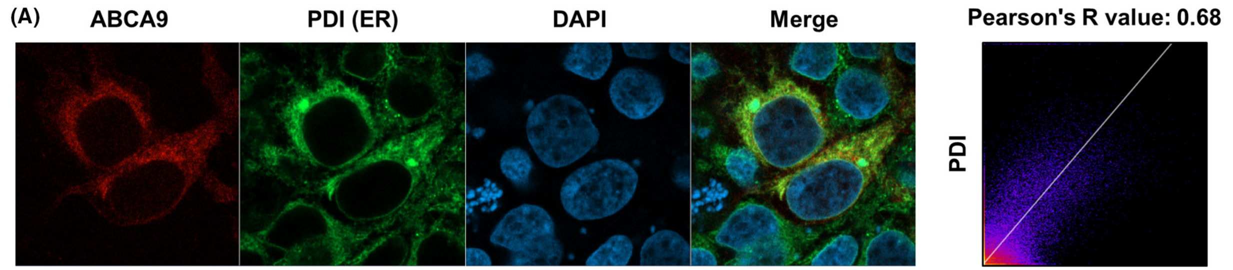
3.3 | ABCA9 inhibits the progression of breast cancer cells

Cholesterol can be used as a nutrient in tumor development and progression.[8] It has been reported that cholesterol levels, which can be linked to cell proliferation, invasion and migration, increase during breast cancer development.[18] Therefore, we aimed to investigate the role of ABCA9 in breast cancer cells by assessing the basal expression levels of ABCA9 in various breast-related normal and cancer cell lines. ABCA9 expression was drastically downregulated in all breast cancer cell lines (Figure 3A). To determine the effects of ABCA9 restoration in breast cancer cells, we transfected ABCA9 into MCF-7 and SKBR-3 HBC cell lines and analyzed cell proliferation and colony formation (Figure 3B). Forced expression of ABCA9 significantly inhibited breast cancer cell proliferation in both breast cancer cell lines (Figure 3C). Similarly, colony formation was also significantly attenuated ABCA9 overexpression in both breast cancer cell lines compared with control cells (Figure 3D). Flow cytometry analysis using annexin-V and PI markers revealed that ABCA9 transfection increased the number of apoptotic cells in both cancer cell lines (Figure 3E; for quantitative data see Table S2). However, ABCA9 overexpression in MCF-7, but not in SKBR-3, significantly reduced the number of proliferative cells stained by Ki-67 (Figure 3F). These data suggested that restriction of cellular cholesterol affected cancer cell growth through various mechanisms, differently impacting proliferation or apoptotic pathways. These results indicated that ABCA9 expression inhibited breast cancer cell progression.

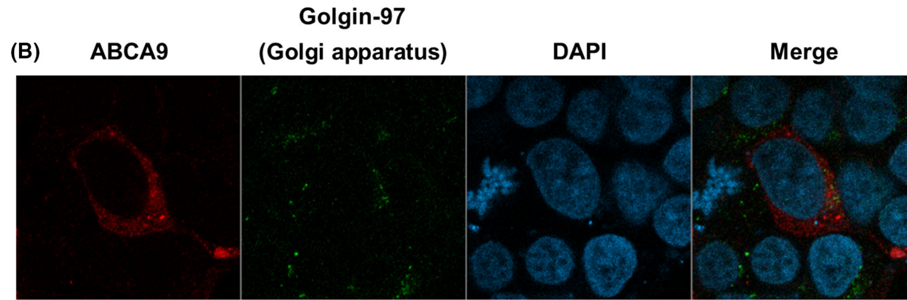
3.4 | ABCA9 inhibits SREBP-2 nuclear translocation in breast cancer cells

Importantly, ER is an essential organelle that regulates intracellular cholesterol levels, with SREBP-2 being the master transcription

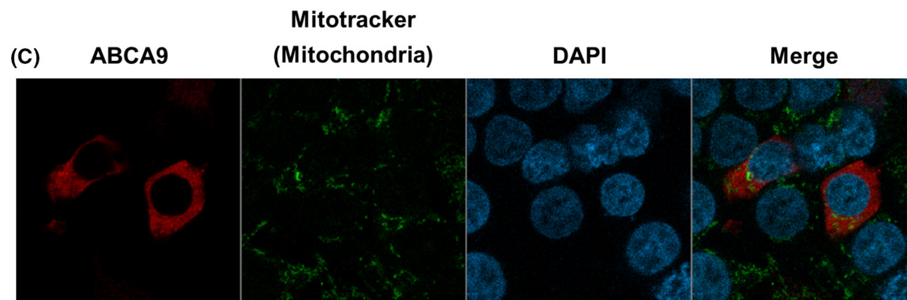
FIGURE 2 ABCA9 is cholesterol responsive, localizes in the ER and affects ER cholesterol levels. (A) Subcellular localization of ABCA9 was identified using PDI as an ER marker. The co-localization of ABCA9 and PDI showed a high Pearson correlation. (B) Subcellular localization of ABCA9 was distinguished from Golgi apparatus marker Golgin-97 and (C) mitochondria using MitoTracker. (D) ABCA9 expression did not altered after the cholesterol-loading condition with cholesterol-methyl-β-cyclodextrin, but (E) decreased in cholesterol depletion conditions with MβCD treatment. (F) ABCA9 significantly accumulated cholesterol in the ER, which was confirmed by measuring the intensity of Filipin as a cholesterol probe in 73 cells expressing ABCA9 and 194 cells not expressing ABCA9. Filipin staining experiments were independently repeated three times.



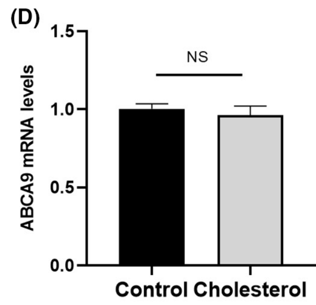
(A)
ABCA9
PDI (ER)
DAPI
Merge
Pearson's R value: 0.68
PDI
ABCA9



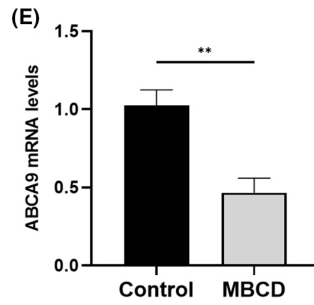
(B)
ABCA9
Golgin-97
(Golgi apparatus)
DAPI
Merge



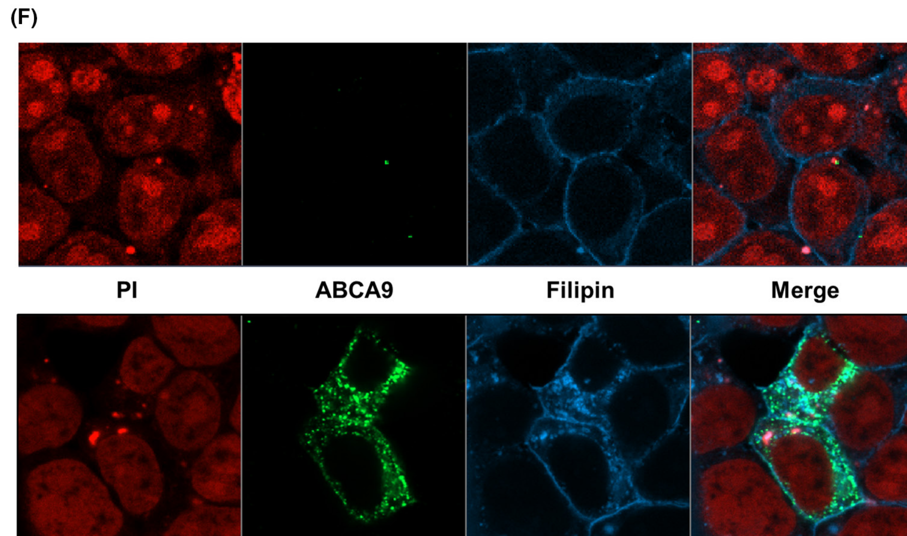
(C)
ABCA9
Mitotracker
(Mitochondria)
DAPI
Merge



(D)
ABCA9 mRNA levels
1.5
1.0
0.5
0.0
NS
Control
Cholesterol



(E)
ABCA9 mRNA levels
1.5
1.0
0.5
0.0
**
Control
MBCD



(F)
PI
ABCA9
Filipin
Merge
Mean fluorescence intensity (x1000)
15
10
5
0
***
Control
ABCA9

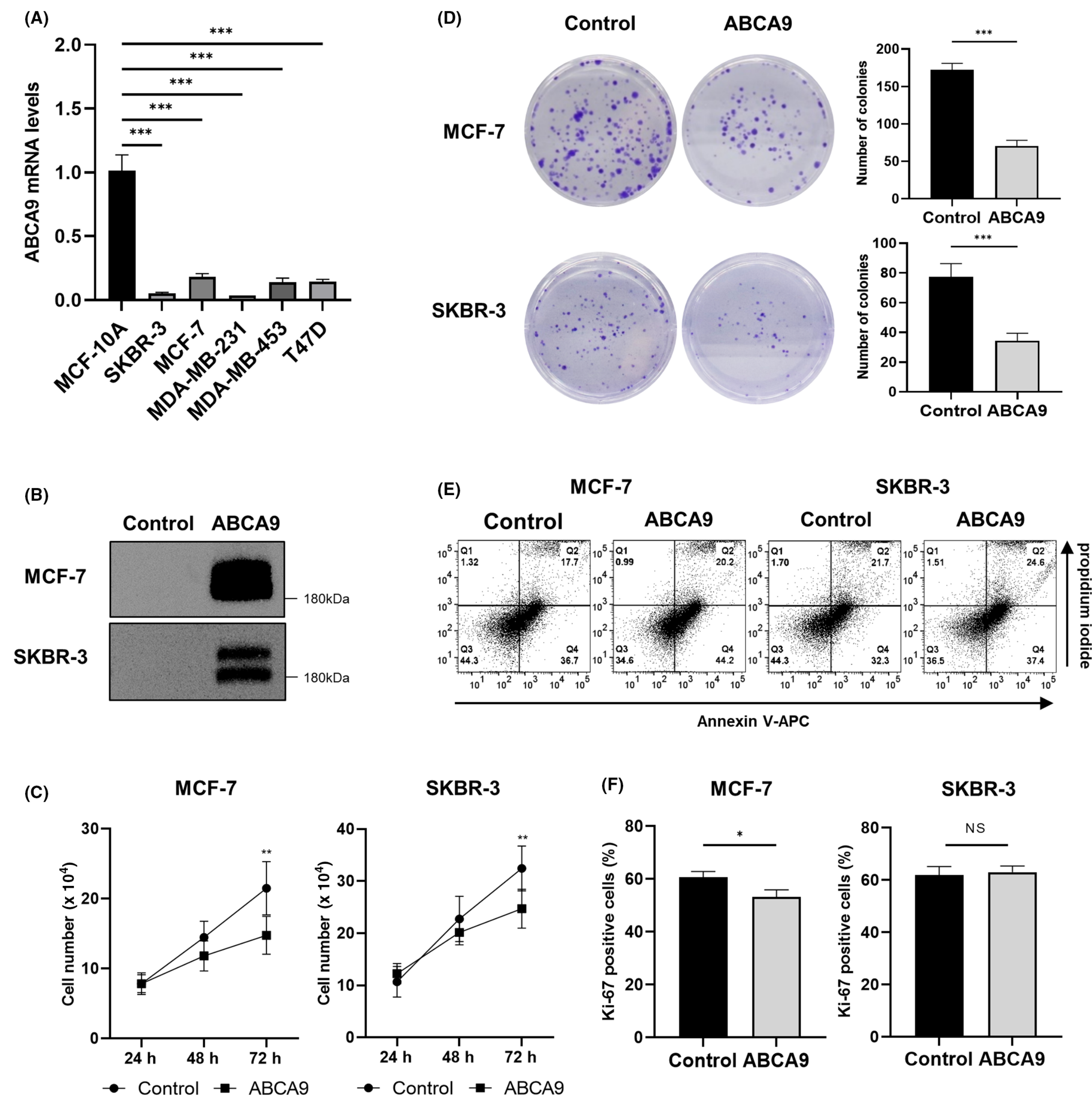


FIGURE 3 ABCA9 expression is suppressed in breast cancer cells and its restoration inhibits breast cancer cell progression. (A) The mRNA expression level of ABCA9 was decreased in breast cancer cell lines compared with the normal breast cell line MCF-10A. (B) The transient ABCA9 protein expression was detected by western blot using anti-Flag. (C) Cell numbers were counted at 24, 48, and 72 h after ABCA9 transfection for cell proliferation assay and significantly decreased at 72 h in both cell lines. (D) After ABCA9 restoration, colony-forming ability was inhibited in both cell lines. (E) ABCA9 restoration increased the number of apoptotic cell populations (Q2 + Q4, Q2: late-apoptosis, Q4: early-apoptosis) by ~10% in both cell lines. (F) ABCA9 significantly reduced the expression of the cell proliferation marker Ki-67 in MCF-7, but not in SKBR-3 cells. pcDNA 3.1 empty vectors were used as a negative control.

factor for cholesterol synthesis. SREBP-2 and its translocation from the ER to the nucleus depend on the concentration of cellular cholesterol and has been known as the main mechanism responsible for maintaining cholesterol homeostasis.[19] Given that ABCA9 contributes to cholesterol accumulation in the ER (Figure 2), we next aimed to investigate whether ABCA9-induced cholesterol accumulation in the ER was dependent on SREBP-2 signaling and whether it affected breast cancer cell progression. First of all, we confirmed that ABCA9 was localized to the ER by staining the ER markers PDI and calnexin in MCF-7 and SKBR-3 cell lines (Figure 4A, Figure S2). After that, nuclear fractionation was performed 24 h and 48 h after ABCA9 transfection to measure

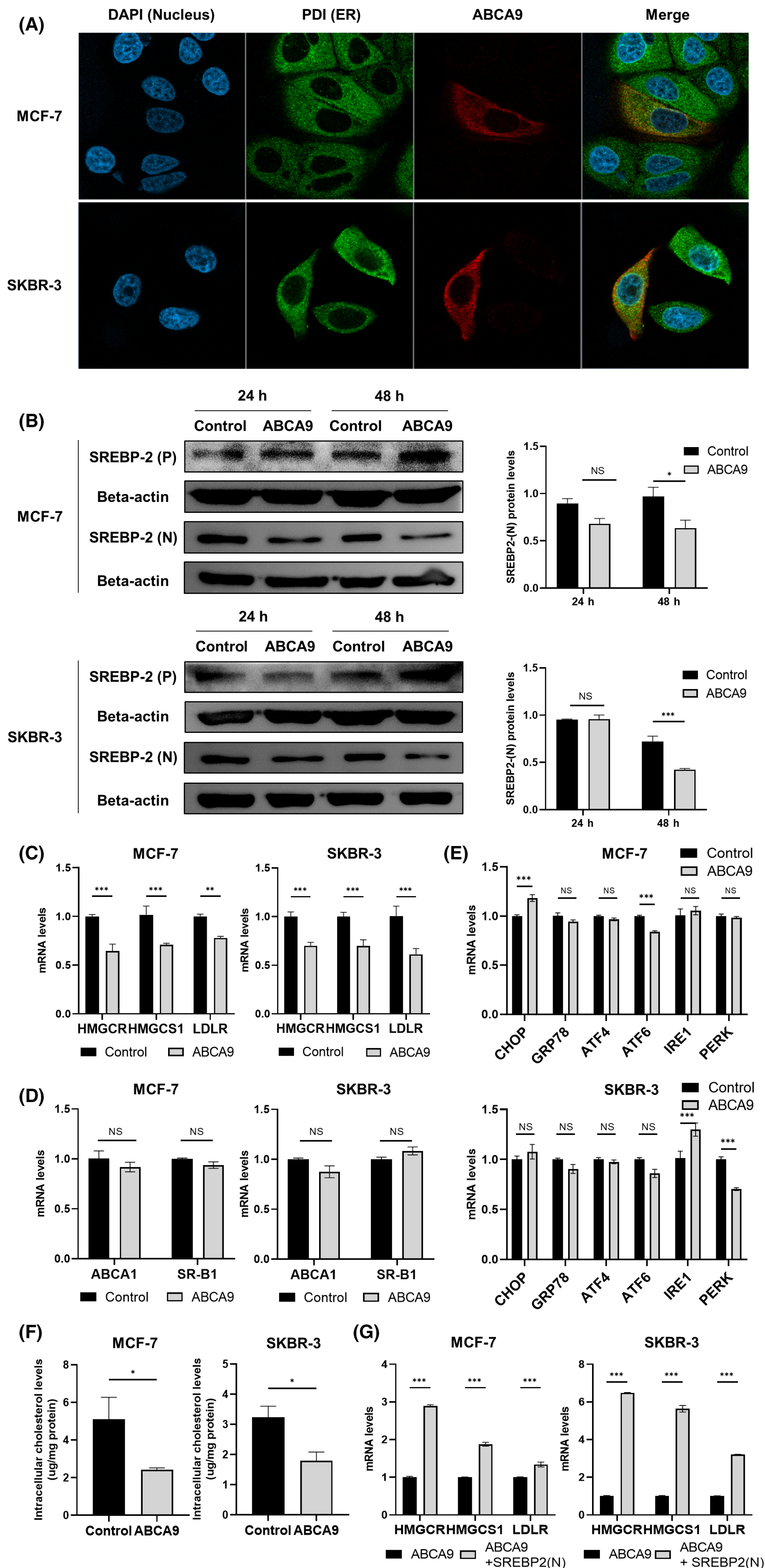


FIGURE 4 ABCA9 suppresses cancer cell proliferation through inhibition of SREBP-2 signaling. (A) ER localization of ABCA9 was confirmed using PDI as an ER marker. (B) Mature SREBP-2 (SREBP-2(N)) levels were decreased in the nuclear fractions of MCF-7 and SKBR-3 cells 48 h after ABCA9 transfection, accordingly, the precursor SREBP-2 levels were increased in the cytoplasmic fraction. (C) The expression of cholesterol synthesis genes was reduced in both cell lines after ABCA9 transfection. (D) The expression of cholesterol efflux genes and (E) ER stress response genes remained unchanged after ABCA9 transfection in both cell lines, except for *CHOP* and *ATF6* in MCF-7 cells, and *IRE1* and *PERK* genes in SKBR-3 cells. (F) ABCA9 overexpression decreased intracellular total cholesterol levels in both cell lines. (G) Active SREBP-2 (N) introduction under ABCA9 overexpressing conditions restored and further elevated the expression of genes related to cholesterol synthesis.

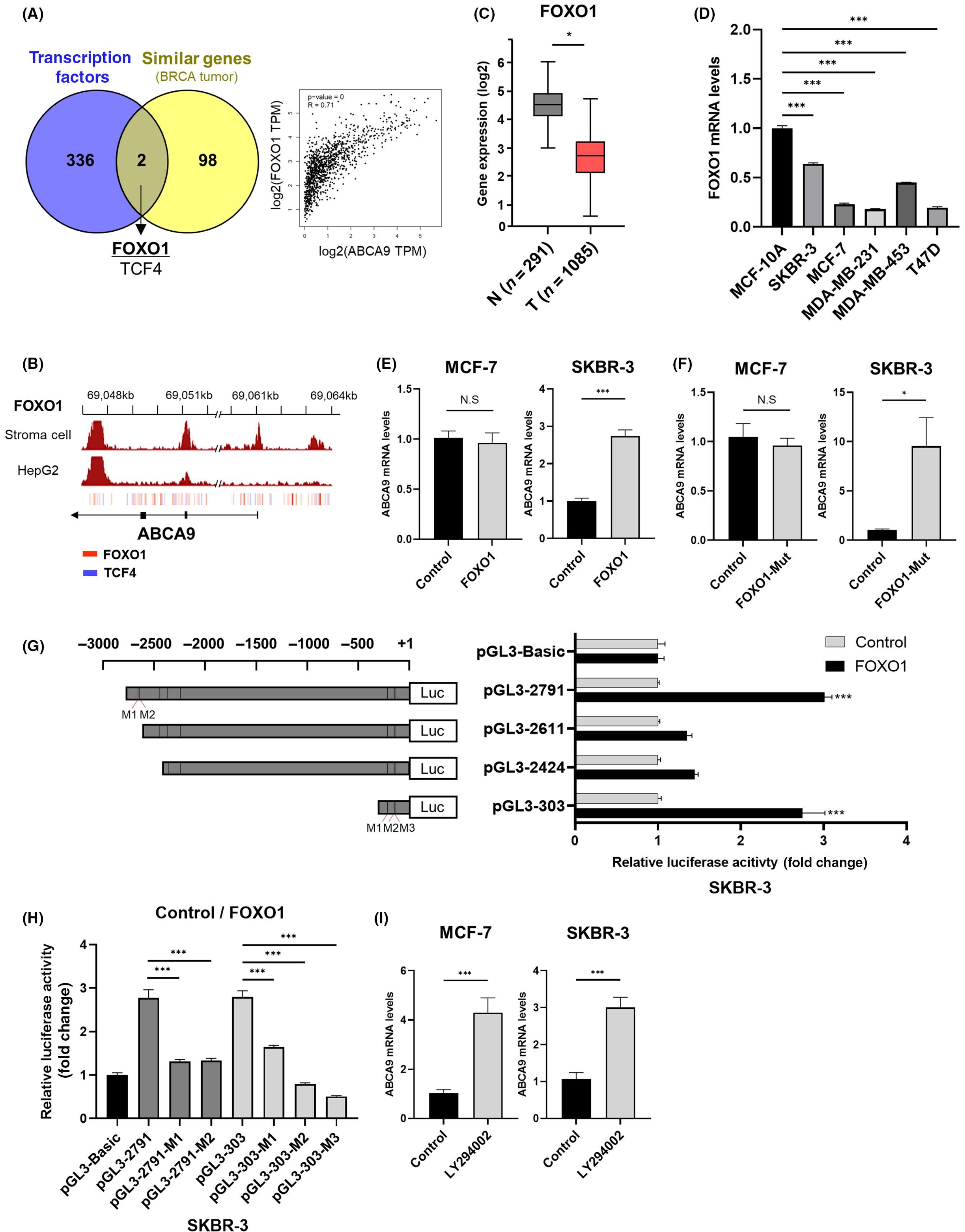
the activity of SREBP-2. Mature SREBP-2 protein levels in the nuclear fraction were significantly decreased in ABCA9-transfected MCF-7 and SKBR-3 breast cancer cells. Conversely, more SREBP-2 was retained in the cytoplasmic fraction upon ABCA9 overexpression (Figure 4B). To test the effects of ABCA9 on the expression of cholesterol synthesis-related genes, mRNA levels of the genes encoding HMGCR, HMGCS-1, and LDLR were measured. Accordingly, HMGCR, HMGCS-1, and LDLR relative expression levels were decreased after ABCA9 overexpression in both breast cancer cell lines (Figure 4C). However, there were no significant alterations in the expression of genes involved in cholesterol efflux (Figure 4D) and ER stress response (Figure 4E). To determine whether the downregulation of the cholesterol synthesis enzymes impacted total cholesterol levels, we measured cholesterol levels. As expected, the intracellular cholesterol levels were decreased upon ABCA9 overexpression (Figure 4F). Last, we assessed whether the reduction in cholesterol synthesis genes caused by ABCA9 overexpression could be rescued by mature SREBP-2 introduction. As expected, mature N-term SREBP-2 transfection not only blocked the reduction of cholesterol synthesis gene expression promoted by ABCA9 overexpression, but also further increased their expression (Figure 4G). The ABCA9 knockdown effect in MCF-10A cells showed the opposite tendency with cancer, but there were also some differences (Figure S3). These results demonstrated that the reduced levels of the mature SREBP-2 caused by ABCA9 overexpression led to decreased cellular cholesterol levels and breast cancer cell proliferation.

3.5 | FOXO1 is a transcription factor involved in the regulation of ABCA9 expression

We sought to identify that transcription factors regulate ABCA9 expression. The sequences of the ABCA9 promoter and enhancer regions were determined from the Ensembl database (https://www.ensembl.org/). We examined 338 transcription factors capable of binding to this region using RVista 2.0.[20] Additionally, 100 genes displaying an expression pattern similar to that of ABCA9 expression in breast cancer were extracted using the GEPIA2 database (http://gepia2.cancer-pku.cn/). First, we identified FOXO1 and TCF4 as potential transcription factors regulating ABCA9 expression (Figure 5A). A Pearson correlation between the expression of ABCA9 and both transcription factors showed that FOXO1 had a higher correlation (0.71) with ABCA9 compared with that of TCF4 (0.55). To further ascertain the transcriptional regulation of ABCA9, we surveyed the public database for a FOXO1 and TCF4 ChIP-sequencing dataset.[21,22] Unfortunately, there were no conclusive findings regarding the existence of FOXO1 or TCF4 ChIP-seq data related to breast cancer cells. Nonetheless, the binding of FOXO1 to the ABCA9 promoter and enhancer regions was confirmed in other cell lines such as stromal and HepG2 cells (Figure 5B). However, no peaks for TCF4 binding on ABCA9 were found in other cell lines (data not shown).

FOXO1 has been known as a tumor suppressor, often downregulated in various cancers. It is critically involved in multiple cellular processes, such as oxidative stress, DNA repair, cell cycle, apoptosis, and autophagy.[23] In our study, the downregulation of FOXO1 mRNA was confirmed in samples of breast cancer patients (Figure 5C). We also validated this finding in breast cancer cell lines compared with normal breast cell line MCF-10A (Figure 5D). To demonstrate whether FOXO1 has the ability to regulate ABCA9 expression, we transiently transfected Flag-tagged FOXO1 into SKBR-3 and MCF-7 cell lines. FOXO1 overexpression was found to promote a three-fold increase in ABCA9 expression in SKBR-3 cells, but not in MCF-7 cells (Figure 5E). FOXO1 phosphorylation and cytoplasmic localization are mechanisms known to inactivate its function.[24] As FOXO1 is phosphorylated by Akt, which may lead to its inactivation and subsequent inhibited nuclear translocation, we further investigated the role of constitutively active FOXO1 in regulating ABCA9 expression through a triple mutant (T24A/S256A/S319A) of the site phosphorylated by Akt in FOXO1. As a result, SKBR-3 cells showed a significant increase in ABCA9 expression, but there was no difference in MCF-7 (Figure 5F). To determine the capacity of FOXO1 to bind to the ABCA9 promoter and regulate ABCA9 expression in cancer cells, we cloned the ABCA9 promoter regions containing putative FOXO1 binding sites. A dual-luciferase reporter assay demonstrated that FOXO1 differentially bound to ABCA9 promoter regions in both cell lines. pGL3-ABCA9 (−2791) and pGL3-ABCA9 (−2424) constructs harboring (−2791 to +1) and (−2424 to +1) ABCA9 promoter regions showed increased luciferase activity in MCF-7 cells (Figure S4). Conversely, pGL3-ABCA9 (−2791) and pGL3-ABCA9 (−303) showed significantly higher luciferase activity compared with the control in SKBR-3 cells. (Figure 5G). To confirm that the increase in luciferase activity in the pGL3-ABCA9 (−2791)

FIGURE 5 ABCA9 is regulated by FOXO1. (A) *FOXO1* and *TCF4* are depicted as common genes in the transcription factor prediction for ABCA9 and the gene expression profiling similar to ABCA9 in breast cancer (left panel). Correlation between ABCA9 and FOXO1 expression in breast cancers (right panel). (B) FOXO1 transcription factor ChIP-seq data in stromal and HepG2 cells visualized by the Integrative Genomics Viewer. FOXO1 and TCF4 binding sites are indicated in ABCA9 gene regions. (C) A decrease in FOXO1 expression was confirmed in breast cancer patient samples from the GEPIA2 database. (D) *FOXO1* mRNA expression was lower in breast cancer cell lines compared with MCF-10A. (E) SKBR-3, but not MCF-7, cells showed elevated expression of *ABCA9* after *FOXO1* overexpression. (F) Overexpression of *FOXO1* with mutations in the sites phosphorylated by Akt caused increased *ABCA9* expression in SKBR-3, but not in MCF-7, cells. (G) A significant increase in luciferase activity by FOXO1 was observed in the pGL3-ABCA9 (−2791) and pGL3-ABCA9 (−303) constructs in SKBR-3 cells. (H) Mutation of the predicted *FOXO1* binding sites in the *ABCA9* promoter region reduced the luciferase activity of the *ABCA9* promoter in SKBR-3 cells. Luciferase activity was calculated as the ratio between the control vector and FOXO1. (I) *ABCA9* expression in both cell lines was increased by PI3K inhibitor LY294002 (20 μM).



and pGL3-ABCA9 (−303) constructs was caused by FOXO1 binding, we mutated the predicted binding sites of FOXO1 in the ABCA9 promoter region, and luciferase activity was further confirmed. First, when two FOXO1 binding sites at the pGL3-ABCA9 (−2791) position were mutated, respectively, the luciferase activity of pGL3-ABCA9 (−2791), which was increased compared with the control,

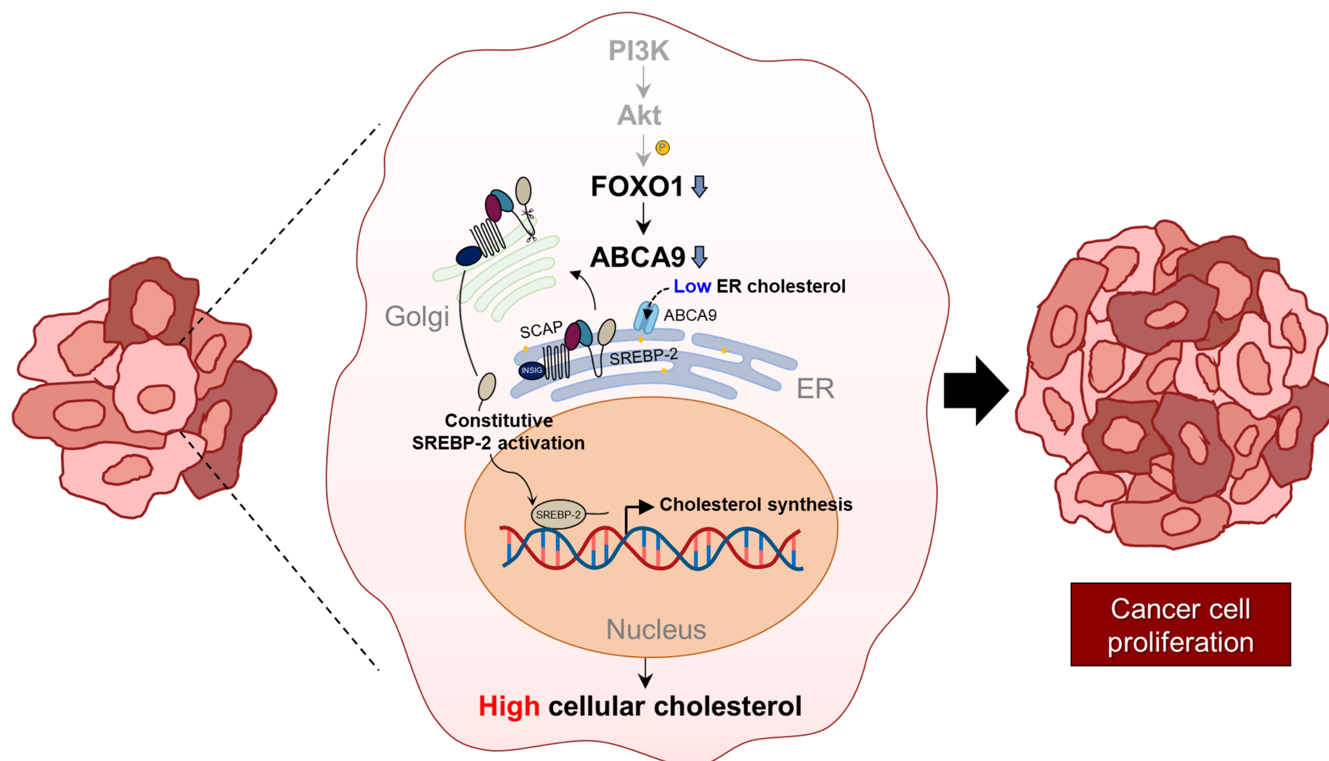


FIGURE 6 Downregulation of ABCA9 in breast cancer leads to the activation of constitutive SREBP-2, which maintains high cellular cholesterol levels and supports cancer cell proliferation.

was decreased in both mutant forms. Similarly, in the three FOXO1 binding sites at the pGL3-ABCA9 (−303) position, luciferase activity was slightly decreased when the first FOXO1 binding site was mutated and it was greatly reduced when the other two FOXO1 binding sites were mutated (Figure 5H). These results suggested that FOXO1 directly activates the ABCA9 promoter and regulates ABCA9 expression. However, the discrepancy observed between MCF-7 and SKBR-3 cell lines also suggested that other regulatory mechanisms may be at play, including epigenetic, post-translational modifications and different hormone receptor statuses. The PI3K/Akt pathway is upstream of FOXO1 and its activation leads to the phosphorylation of FOXO1, which accumulates in the cytoplasm as an inactive form.[25] Therefore, to investigate the regulatory role of FOXO1 phosphorylation in the mediation of the expression of ABCA9, we treated cells with the PI3K inhibitor. Akt inactivation induced by LY294002 treatment triggered the accumulation of dephosphorylated FOXO1 in the nucleus that triggered its gene activation. Moreover, ABCA9 mRNA expression was significantly increased in both MCF-7 and SKBR-3 cells upon treatment with LY294002 (Figure 5I). These results suggested that FOXO1 is a transcription factor that regulates the expression of ABCA9.

4 | DISCUSSION

Numerous studies have highlighted the critical role of cholesterol metabolism in cancer development and progression. Indeed, alterations in cholesterol homeostasis are considered a major hallmark of cancer.[26] Cancer cells often reprogram cholesterol metabolism and use cholesterol on demand through de novo synthesis or uptake. Dysregulation of cholesterol homeostasis leads to cholesterol accumulation, which promotes cancer cell proliferation, invasion and migration, apoptosis avoidance, and chemotherapeutic drug resistance.[27] In particular, SREBP-2 and its target genes, namely genes encoding for enzymes responsible for cholesterol synthesis, are significantly upregulated in cancer. Consequently, SREBP-2 is known to promote tumor progression in various cancer types.[28,29]

Although many studies have addressed the contribution of cholesterol metabolism to breast cancer progression, knowledge about the functions and regulatory mechanisms of cholesterol transporters remains very limited. In particular, with respect to the ABCA subfamily that acts as a lipid transporter, only a few ABCA transporters have been studied in breast cancer. For example, ABCA1 dysregulation has been shown to affect intracellular cholesterol levels and contribute to tumor progression. In breast cancer, ABCA1 expression was found to be upregulated in the mesenchymal state and promoted tumor cell migration.[30,31] Conversely, ABCA8 was frequently shown to be downregulated in cancer and to inhibit breast cancer proliferation through the AMPK/mTOR signaling pathway.[32,33] ABCA9 was first reported as a cholesterol-responsive gene, with cholesterol importation leading to a decline in ABCA9 mRNA levels. However, the exact function and role of ABCA9 in breast cancer are still undiscovered. Therefore, in this study, we analyzed the expression profiles of ABCA9 from transcriptome data sets of both HBC

and CMT. ABCA9 was significantly downregulated in both HBC and CMT and localized in the ER membrane, contrasting with ABCA1 and ABCA8, which are mainly located in the plasma membrane and involved in cholesterol efflux.[34,35]

In our hands, ABCA9 expression remained unaltered after cholesterol loading into HEK-293T cells (Figure 2D). However, and importantly, in breast cancer cells, ABCA9 mRNA expression was found to be increased upon cholesterol loading (Figure S5). Different cholesterol metabolic rates and gradients may underlie the discrepancy observed regarding ABCA9 expression between normal and cancer cells. As described, cancer cells efficiently utilize cholesterol as their energy source, stimulating cancer cell growth by converting cholesterol into 27-hydroxycholesterol. The 27-hydroxycholesterol, one of the cholesterol metabolites, is an estrogen receptor agonist capable of modulating the receptor functions and inducing tumor progression in estrogen receptor-positive breast cancers.[36]

Most of the molecular machinery that regulates cellular cholesterol homeostasis resides in the ER, therefore, understanding the role of ABCA9 present in ER membranes is of great importance. Our set of experiments successfully demonstrated how ABCA9 affects the progression of breast cancer cells. We first showed that ABCA9, similar to the classic ABCA1, retained the functional domains of a cholesterol transporter. We next showed that ABCA9 overexpression led to the accumulation of cholesterol in the ER, inhibiting the nucleus translocation of SREBP-2. Moreover, we showed that ABCA9 overexpression significantly reduced the proliferative and colony-forming abilities of breast cancer cell lines. Interestingly, our results revealed that ABCA9 overexpression could have a role in blocking the well known process of SREBP-2-associated cancer cell growth.[19] Indeed, restoring ABCA9 in breast cancer cells decreased cholesterol-dependent cancer cell growth.

The results so far prompted us to seek the molecular mechanism underlying ABCA9 downregulation in breast cancer cells. We found that transcription factor FOXO1, a critical downstream regulator of the insulin and insulin-like growth factor 1 signaling pathway, was significantly downregulated in breast cancer cells and acted as a regulator of ABCA9 expression. However, its actions were found to work differently in distinct cell lines, with FOXO1 overexpression significantly increasing ABCA9 mRNA expression in SKBR-3 but not in MCF-7 cells. These findings were further confirmed through experiments using a triple mutant form of FOXO1 (T24A/S256A/S319A), which is incapable of being phosphorylated by Akt, predominantly nuclear and constitutively active. Although further investigation is needed in the future, various FOXO1 post-translational modifications could explain this cell type-dependent discrepancy of FOXO1 regulation on ABCA9 transcription. In HBC, several studies have found that up to 40% of cancers exhibited PIK3CA mutations.[37] Indeed, we have previously shown that ~29% of PIK3CA H1047R human hot spot mutations are also present in CMT.[38] Of note, this PIK3CA gain-of-function mutation activates Akt signaling and regulates FOXO1 downstream expression. Accordingly, the PI3K/Akt pathway negatively regulates the expression of FOXO1 via its phosphorylation. Importantly, in breast cancer cells, PI3K and Akt are activated due to a gain-of-function mutation in PIK3CA, resulting in the phosphorylation of FOXO1, which then remains inactive in the cytoplasm. Therefore, one can infer that this may be a putative mechanism responsible for the decreased expression of ABCA9 in breast cancer cells along with the low expression of FOXO1 transcripts. Conversely, we have identified that treatment with the PI3K inhibitor LY294002 triggered the dephosphorylation of FOXO1, resulting in increased expression of ABCA9 in both cell lines. This suggests that PI3K inhibition can lead to the activation of FOXO1, possibly through changes in FOXO1 post-translational modifications or binding partners, thereby influencing ABCA9 expression. In addition, PI3K inhibition-mediated FOXO1 activation, and ABCA9 transcription in MCF-7 cells can be initiated through the activation of other intracellular pathways, such as the mTOR–S6K1/4EBP pathway.[39]

To determine the FOXO1 region binding to the ABCA9 promoter, we constructed ABCA9 promoters with four regions based on FOXO1 binding sites present in a public ChIP-seq database. Although promoter activity varied depending on the regions and cell types, pGL3-ABCA9 (−2791) and pGL3-ABCA9 (−303) constructs showed vigorous activity. A comparison of luciferase activity between −2791 and − 2611 constructs revealed that the FOXO1 binding site between these regions on the ABCA9 promoter was critical for the regulation of ABCA9 expression. The decrease in luciferase activity in the −2611 and − 2424 constructs and the subsequent increase in the −303 construct indicated the presence of a repressive regulatory element between −2611 and −303. Also, binding of FOXO1 to the +1 region of −303 represented the core promoter of FOXO1 activation for ABCA9, after the removal of negative regulatory elements.

The FOXO family of transcription factors is involved in the regulation of cholesterol homeostasis and hepatic HDL cholesterol clearance.[40] Specifically, liver-specific FOXO1 and FOXO3 knockout are known to cause lipid abnormalities such as increased serum triglyceride and cholesterol.[41,42] Li et al. reported that high cholesterol levels could decrease FOXO1 phosphorylation levels.[43] Taking this into account, it is possible that, in cancer cells, changes in the post-translational modification of FOXO1 by cholesterol may modulate the expression of ABCA9. However, given that ABCA9 expression can be controlled by other factors, such as the concentration and regulation of cholesterol, more detailed studies addressing its regulation are required.

Clinical studies have also hinted at the involvement of ABCA9 in cancer progression. Indeed, ABCA9 expression was reported to be upregulated after platinum-based chemotherapy in ovarian cancer.[44] Interestingly, during chemotherapy, the expression of HMGCR was upregulated, activating the lipid synthesis pathway in resistant cells.[45,46] In addition, it was shown that resistant cells in $HER2^+$ breast cancer depend on the mevalonate pathway for cell survival and proliferation mediated by mTOR, YAP, and TAZ.[47] Collectively, these studies showed that alterations in cholesterol metabolism in cancers also interfered with treatment reactions. Given that the sensitivity to chemotherapy varies according to cholesterol levels, more studies addressing the effects of ABCA9 on cholesterol regulation,

and therefore in cancer, in real clinical chemotherapy settings are needed.

Altogether, this study showed, for the first time, that ABCA9 is a cholesterol transporter mainly present in ER membranes that has a crucial role in fine tuning cholesterol homeostasis through the negative regulation of SREBP-2 nucleus translocation. Moreover, we revealed the mechanism by which ABCA9 may participate in breast cancer cell development, showing that depletion of ABCA9 caused by the decreased FOXO1 transcription factor allows breast cancer cells to keep turning on SREBP-2 signal transduction. This, in turn, results in the activation of cholesterol synthesis genes, which accelerates the production of cholesterol that supports cancer cell growth (Figure 6).

AUTHOR CONTRIBUTIONS

J.Y. Cho conceived and supervised the study and revised the manuscript. H.J. Hwang performed all experiments and wrote the manuscript draft. J.Y. Cho and K.H. Lee provided extensive scientific discussion and revised the manuscript.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional Reviewer Board: N/A.

Informed consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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