Argininosuccinate lyase is a potential therapeutic target in breast cancer

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Received June 13, 2015; Accepted July 23, 2015

DOI: 10.3892/or.2015.4280

Abstract. Arginine is a non-essential amino acid that modulates nitric oxide production and cancer homeostasis. In our previous study, we observed that blocking argininosuccinate lyase (ASL) attenuates tumor progression in liver cancer. However, the role of ASL in human breast cancer has been studied to a lesser degree. In the present study, we investigated the effect of targeting ASL in breast cancer. We found that ASL was induced by ER stress and was significantly upregulated in breast cancer tissues compared to that in the corresponding normal tissues. Downregulation of ASL inhibited the growth of breast cancer *in vitro* and *in vivo*. The level of cell cycle-related gene, cyclin A2, was reduced and was accompanied by a delay in G2/M transition. ASL shRNA-induced cell inhibition was rescued by exogenous cyclin A2. Furthermore, autophagy was observed in the cells expressing ASL shRNA, and inhibition of autophagy reduced cell growth, indicating that autophagy played a cell survival role in the ASL knockdown cells. Moreover, inhibition of ASL reduced NO content. Introduction of the NO donor partially restored the growth inhibition by ASL shRNA. Thus, the mechanism induced by ASL shRNA which occurred in human breast cancer may be attributed to a decrease in cyclin A2 and NO.

Introduction

Breast cancer is a common cancer in women worldwide. Many physiological conditions, including hormone secretion and metabolic homeostasis, influence breast cancer progression (1). Breast cancer patients with metabolic dysregulation are associated with poor response to current chemotherapy (2). The growth of tumor cells is coupled by metabolic reprogramming (3,4). The metabolic shift is observed during carcinogenesis and has been considered to be a reliable marker for tumors. The intermediary metabolism can also fuel cell growth. For example, cancer cells are addicted to glutamine due to its usage as a supplement. The glucose metabolites, serine and glycine, mediate one-carbon metabolism which is important in tumorigenesis (5,6). The semi-essential amino acid arginine plays an important role in nitric oxide production and the urea cycle, and is a precursor for glutamate, proline, polyamones and agmatine (7). The concentration of plasma arginine is lower in breast, colon and pancreatic cancer patients (8-10). A dietary supplement with arginine increases colonic carcinogenesis (11). Compared to breast cancer patients fed a standard diet, patients with dietary L-arginine supplementation have higher tumor protein synthesis. Furthermore, the protein synthesis rate was found to be highly correlated with Ki67 expression (12). In contrast, deprivation of dietary arginine inhibits cancer metastasis (13,14). Arginine depletion by ADI is used as an approach in cancer therapy (15,16).

Arginine is synthesized from citrulline by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). ASS catalyzes the conversion of citrulline and aspartate into argininosuccinate. Argininosuccinate is then converted into arginine and fumarate by ASL. A high level of ASS is observed in malignant lung, ovarian, gastric and colonic epithelium compared to corresponding normal epithelium (17-19). In contrast, tumors usually express reduced ASS including breast cancer, hepatocellular carcinoma, melanoma, renal cell carcinoma and pancreatic cancer, and the ASS level is inversely correlated with survival (20-23). Tumors with loss of ASS are dependent on extracellular arginine for growth, characteristic of arginine auxotrophy. Breast cancer and melanoma with deficiency of ASS are sensitive to arginine deprivation via arginine deiminase (4,24). The complex of ASS and ASL with NOS contributes to the efficient channeling for NO production (25,26). ASL-deficient mice and argininosuccinic aciduria patients have a deficiency in the production of NO (25). ASL is

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Key words: argininosuccinate lyase, nitric oxide, autophagy, endoplasmic reticulum, cyclin A, breast cancer

highly expressed in hepatocellular carcinoma and downregulation of ASL by shRNA attenuates tumor growth *in vivo* (27).

Endoplasmic reticulum (ER), which plays a major role in membrane and secretory protein synthesis, has been associated with metabolic disease (28-30). Endoplasmic reticulum stress, which emanates from the accumulation of unfolded protein, has a profound impact on the pathogenesis of many diseases, including liver disease, diabetes and cancer (31-34). In response to ER stress, the unfolded protein response (UPR) initiates signaling cascades and restores the protein-folding homeostasis. The amino acid metabolism genes are also activated under ER stress condition (35,36). Given the relationship between ER stress, metabolism and cancer progression, it is predictable that ER stress may affect metabolic enzymes in cancer cells. ASL upregulation by an ER stress inducer was observed in liver cancer cells (27). Arginine metabolic enzyme is usually expressed in liver cells. It is uncertain whether the arginine metabolic enzyme, ASL, exerts functions in other types of tissues. The present study aimed to determine the relationship between ER stress and ASL in breast cancer cells and to ascertain whether the arginine-NO complex mediates breast cancer growth.

Our results revealed that ASL is elevated by ER stress and is highly expressed in breast tumor tissues. Downregulation of ASL by ASL shRNA decreased tumor growth *in vivo* and *in vitro*. ASL knockdown induced cyclin A2 degradation and the cell growth was rescued by exogenous cyclin A2. Furthermore, ASL downregulation inhibited NO expression and induced autophagy.

Materials and methods

Reagents, chemicals and antibodies. The anti-ASL antibody was purchased from Abnova (Taipei, Taiwan). The anti-GRP78 antibody was purchased from BD (Erembodegem, Belgium). The anti-β-actin antibody was purchased from Chemicon (Pittsburgh, PA, USA). The anti-GAPDH antibody was purchased from GeneTex (Irvine, CA, USA). The anti-cyclin A2, anti-CDK4 and anti-CDK2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-cyclin B1 and anti-cyclin E1 antibodies were purchased from Epitomics (Burlingame, CA, USA). The anti-cyclin D1 antibody was purchased from Cell Signaling (Beverly, MA, USA). The anti-LC3B antibody was purchased from Sigma (St. Louis, MO, USA). The anti-HA antibody was purchased from Roche Applied Science (Mannheim, Germany). The powder of G418, thiazolyl blue tetrazolium bromide (MTT), 3-methyladenine (3-MA), bafilomycin A1, sodium nitrite, 2-amino purine and arginine were purchased from Sigma. The plasmid containing ASL was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The plasmid containing cyclin A2 was provided by Dr Ih-Jen Su (Division of Clinical Research, National Health Research Institute, Taiwan).

Cell culture. MCF-7 and MDA MB-231 cells and the stable transfectants were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit-Haemek, Israel), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Corporation, Carlsbad, CA, USA) at 37˚C in 5% CO_2.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted with TRIzol (MDBio, Taiwan). cDNA was synthesized using M-MLV transcriptase (Promega, Madison, MI, USA). PCR was performed using Pro *Taq* polymerase (PROtech Technology Enterprise Co., Ltd., Taipei, Taiwan) on a thermocycler (ABI, Foster City, CA, USA). The 5' and 3' human ASL gene-specific primers were: 5'-TGA TGC CCC AGA AGA AAA AC-3' (sense) and 5'-TTT GCG GAC CAG GTA ATA GG-3' (antisense); the 5' and 3' human GRP78 gene-specific primers were: 5'-CGC CTC ATC GGA CGC ACT TG-3' (sense) and 5'-AGG TTC CAC CGC CCA GGT CA-3' (antisense); the 5' and 3' human CCND1 gene-specific primers were: 5'-AAC TAC CTG GAC CGC TTC CT-3' (sense) and 5'-TGA GGC GGT AGT AGG ACA GG-3' (antisense); the 5' and 3' human CCNE1 gene-specific primers were: 5'-ATC CCC ACA CCT GAC AAA GA-3' (sense) and 5'-AGG GGA CTT AAA CGC CAC TT-3' (antisense); the 5' and 3' human CCNA2 gene-specific primers were: 5'-GCA CCC CTT AAG GAT CTT CC-3' (sense) and 5'-CCT CTC AGC ACT GAC ATG GA-3' (antisense); the 5' and 3' human CCNB1 gene-specific primers were: 5'-GGC CAA AAT GCC TAT GAA GA-3' (sense) and 5'-AA CAT GGC AGT GAC ACC AA-3' (antisense).

Western blotting. The cell lysates were prepared in RIPA lysis buffer. The protein concentration was determined with the Micro BCA™ protein assay kit (Millipore, Billerica, MA, USA). Cell lysates were loaded onto acrylamide gels and were then transferred onto polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, NJ, USA) after electrophoresis. The membranes were incubated with the indicated antibody for the specific protein, probed with ECL Western Blotting Detection system (Millipore) and visualized using the BioSpectrum AC imaging system.

Tissue samples. The specimens of breast cancer and corresponding normal liver were obtained from the Human Biobank within the Research Center of Clinical Medicine of the National Cheng Kung University Hospital (Tainan, Taiwan) following the approval of the Institutional Review Board.

Oncomine database analysis. The expression of ASL in clinical specimens of cancer vs. normal patients was analyzed using Oncomine database (https://www.oncomine.org/resource/login.html). We analyzed the results of fold-change, cancer subtypes and p-values with a threshold of $p<0.05$.

Kaplan-Meier plotter analysis. The overall survival of the patients with high and low ASL expression was analyzed using Kaplan-Meier plotter (http://www.kmplot.com/). We analyzed the relapse-free survival and ASL expression with probe 204608 in upper tertile patients. The number-at-risk, the hazard ratio and the log-rank p were indicated.

RNA interference and lentiviral production. The shRNA targeting ASL was obtained from the National RNAi Core

Table I. ASL expression of the normal and breast cancer tissues from the Oncomine database.

Tissue (no.)	P-value	Fold-change	Ref.
Normal breast (61) Mucinous breast carcinoma (4)	0.004	1.601	TCGA
Normal breast (61) Invasive breast carcinoma (76)	2.56E-08	1.316	
Normal breast (61) Invasive ductal and lobular carcinoma (3)	2.20E-02	1.298	
Normal breast (61) Invasive lobular breast carcinoma (36)	3.62E-05	1.359	
Normal breast (61) Invasive ductal breast carcinoma (389)	9.21E-10	1.287	

The number in the parenthesis indicates the patient number. ASL, argininosuccinate lyase; TCGA, The Cancer Genome Atlas.

Table II. ASL expression of the normal and breast cancer tissues from the Oncomine database.

Tissue (no.)	P-value	Fold-change	Ref.
Normal breast (144) Invasive lobular breast carcinoma (148)	2.36E-12	1.108	Curtis *et al* (41)
Normal breast (144) Invasive ductal breast carcinoma (1,556)	1.85E-28	1.111	
Normal breast (144) Breast carcinoma (14)	8.00E-03	1.056	
Normal breast (144) Medullary breast carcinoma (32)	9.13E-04	1.137	
Normal breast (144) Invasive ductal and invasive lobular breast carcinoma (90)	3.45E-06	1.07	
Normal breast (144) Mucinous breast carcinoma (46)	7.00E-03	1.049	
Normal breast (144) Ductal breast carcinoma *in situ* (10)	7.70E-02	1.067	

The number in the parenthesis indicates the patient number. ASL, argininosuccinate lyase.

Facility (Academia Sinica, Taipei, Taiwan). The target sequence of shRNA was 5'-AGGAGGCTGCTGTGTGTTT-3' (shASL1669). The lentiviral production was managed according to the protocol provided by the National RNAi Core Facility.

Colony formation assay. Colony formation was performed by seeding cells into 6-well plates. The colonies were stained with 2% methylene blue and counted after incubation for 10 days in 5% CO_2 and 37˚C.

Anchorage-independent growth ability. Agar (0.6%) in DMEM was prepared as an under layer in a plastic Petri dish. Five thousand cells were suspended in 0.3% agar in DMEM containing 10% FBS and added over the upper layer. The plates were placed in a 5% CO_2 atmosphere humidified incubator at 37˚C for 14 days and the colonies were quantified.

Tumorigenicity in NOD/SCID mice. NOD/SCID mice were obtained from the Animal Center of the National Cheng Kung University. All study protocols were approved by the Animal Welfare Committee of the National Cheng Kung University. MDA MB-231 cells ($5x10^5$) were subcutaneously implanted into the NOD/SCID mice. For our model of inhibiting tumor growth by lentiviral ASL shRNA, the NOD/SCID mice implanted with MDA MB-231 cells for 10 days were intratumorally injected with lentiviral particles.

Monodansylcadaverine (MDC) staining of autophagy. The monodansylcadaverine (MDC) staining was analyzed by Cayman autophagy/cytotoxicity dual staining kit (Cayman Chemical Company, Ann Arbor, MI, USA) and detected by fluorescence microscopy.

Measurement of intracellular arginine content. The intracellular arginine concentration was analyzed by HPLC analysis using Agilent ZORBAX Eclipse AAA column (Agilent PN 993400-902) (Agilent Technologies, Inc., Santa Clara, CA, USA).

Statistical analysis. All statistical analyses were performed using the Student's t-test. The error bars in the graphs represent the SEM.

Results

ASL expression is induced by ER stress and is highly expressed in breast cancer. To analyze whether ASL is induced by ER stress, the breast cancer cell lines, MCF-7 and MDA MB-231, were incubated with tunicamycin. ASL expression was increased after tunicamycin treatment as demonstrated by western blotting (Fig. 1A). We further examined whether ASL expression was increased in human breast cancer. Western blot analysis was used to detect ASL and GRP78 expression in 14 paired breast cancer and adjacent normal breast tissues. The results indicated that ASL was overexpressed in the breast cancer tissues (Fig. 1B). From the Oncomine database, ASL expression was upregulated in different subtypes of breast cancer (Fig. 1C, Tables I and II). Kaplan-Meier plotter analysis

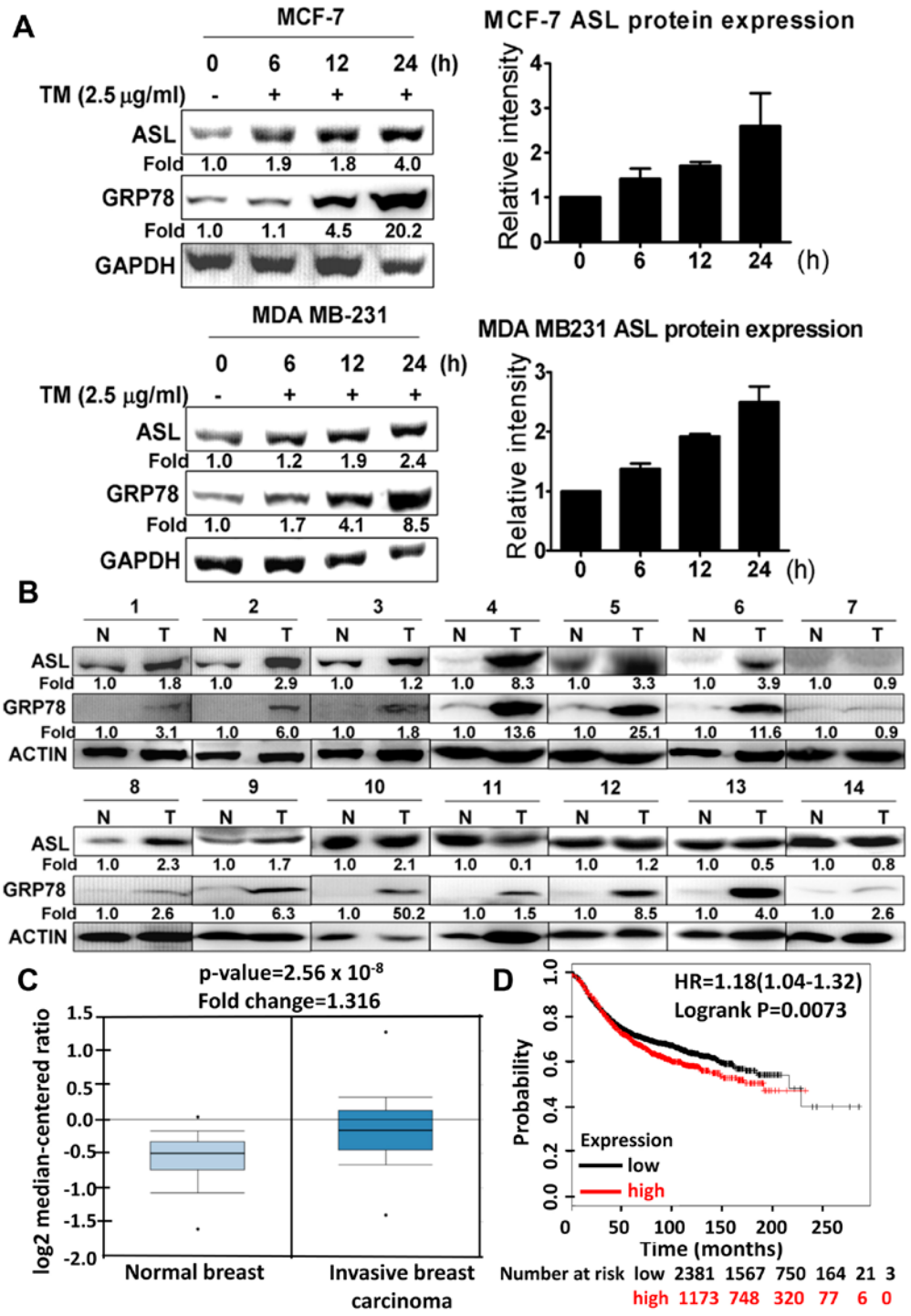

Figure 1. ASL is induced by ER stress and is overexpressed in breast cancer. (A) MCF-7 and MDA MB-231 cells were incubated with tunicamycin (TM; 2.5 μg/ml) for the indicated times. Total protein lysate was harvested and analyzed by western blotting. (B) Tumor lysates from the breast tumor tissues and corresponding normal tissues were collected and analyzed by western blotting. (C) The box plot comparing the ASL expression of normal and breast tumor tissues was derived from the Oncomine database. (D) The Kaplan-Meier plot analyzing the survival of patients with low and high expression of ASL in breast cancer tissues.

in breast cancer showed a correlation between overexpression of ASL and lower overall survival rates (Fig. 1D).

ASL shRNA inhibits breast cancer cell growth. To study the role of ASL in breast cancer, MDA MB-231 cells were transfected with ASL shRNA and stable transfectants were selected by puromycin. The mRNA and protein levels of the ASL knockdown stable transfectants were determined by RT-PCR and western blotting (Fig. 2A). The proliferation of the ASL knockdown stable transfectants was determined by colony formation assay. ASL knockdown stable transfectants exhibited a significantly reduced proliferation *in vitro* (Fig. 2B). To investigate the effect of ASL on tumorigenic ability, the anchorage-independent growth ability of the ASL knockdown

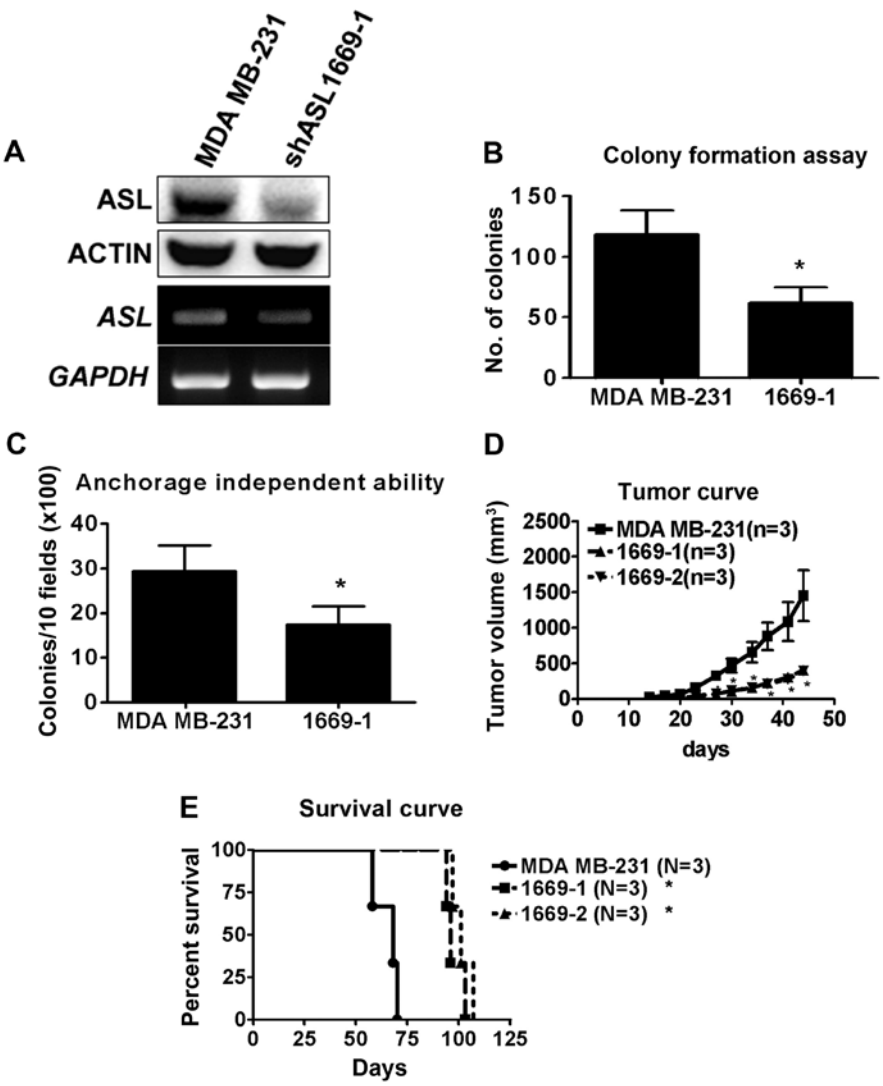

Figure 2. ASL shRNA inhibits breast cancer cell growth *in vitro* and *in vivo*. (A) ASL mRNA and protein expression in the ASL knockdown transfectants was determined by RT-PCR and western blotting. (B) The cell proliferation of the transfectants was analyzed by colony formation assay. (C) The anchorage-independent growth ability of the MDA MB-231 cells expressing ASL shRNA was analyzed by soft agar assay. The colonies with a size >100 μm were counted. The transfectants were injected subcutaneously into NOD/SCID mice, and tumor growth (D) and survival curves (E) were observed. $^{*}p<0.05$, compared with the parental cells.

stable transfectants was analyzed. Decreased anchorage-independent growth ability was observed in the ASL knockdown stable transfectants of the MDA MB-231 cells (Fig. 2C). We further investigated the tumorigenicity of ASL knockdown stable transfectants *in vivo* and found that tumor growth was decreased by ASL shRNA (Fig. 2D). Moreover, there was a significant increase in the survival rate of the ASL knockdown stable transfectants when compare to that of the parental MDA MB-231 cells (Fig. 2E). The results indicated that ASL shRNA decreased cell growth *in vivo* and *in vitro*.

ASL shRNA inhibits cyclin A2 expression and causes G2/M cell cycle delay. To further study ASL shRNA-induced growth inhibition, we examined the changes in cell cycle progression using flow cytometric methods. A delay in G2/M phase was observed in the ASL knockdown stable transfectants (Fig. 3A). We then determined whether ASL shRNA affects the changes in cell cycle-associated molecules. Cyclin A2 was significantly reduced in the ASL knockdown transfectants, while cyclin D1 E1 and B1, CDK2 and CDK4 were not decreased (Fig. 3B). The mRNA expression of cyclins was not altered (Fig. 3C). Since the cyclins are frequently regulated by protein degradation, we ascertained whether the downregulation of cyclins by ASL shRNA could be restored by a proteasome inhibitor. Addition of MG132, a proteasome inhibitor, restored cyclin A2 protein expression (Fig. 3D).

Ectopic expression of cyclin A2 restores ASL shRNA-induced growth inhibition. Since ASL shRNA reduced cyclin A2 expression and cell growth, we next examined the role of cyclin A2 in the proliferation by ectopically expressing cyclin A2 in the ASL knockdown transfectants. Ectopic cyclin A2 reversed the cell growth inhibition by ASL shRNA, indicating that cyclin A2 plays an important role in the inhibition of cell growth by ASL shRNA (Fig. 4).

ASL shRNA induces autophagy in breast cancer cells. Autophagy is required for amino acid maintenance and responds

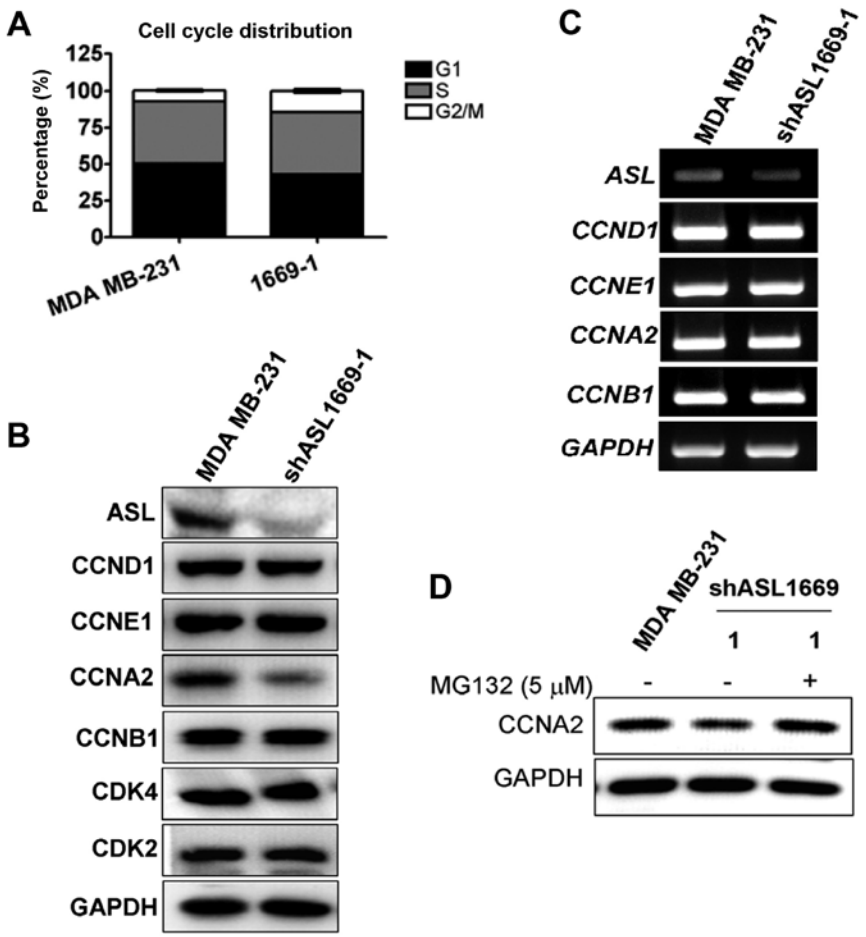

Figure 3. ASL shRNA increases cyclin A2 expression and causes a G2/M cell cycle delay. (A) Cell cycle analysis of the transfectants was analyzed with propidium iodide staining and flow cytometry. (B) Cell cycle-associated proteins, including cyclin A2, B1, D1 and E1, CDK2 and CDK4 in the transfectants were determined by western blotting. (C) Total RNA of the transfectants was harvested and analyzed by RT-PCR. (D) The transfectants were incubated with MG132 for 12 h and the protein lysate was analyzed by western blotting.

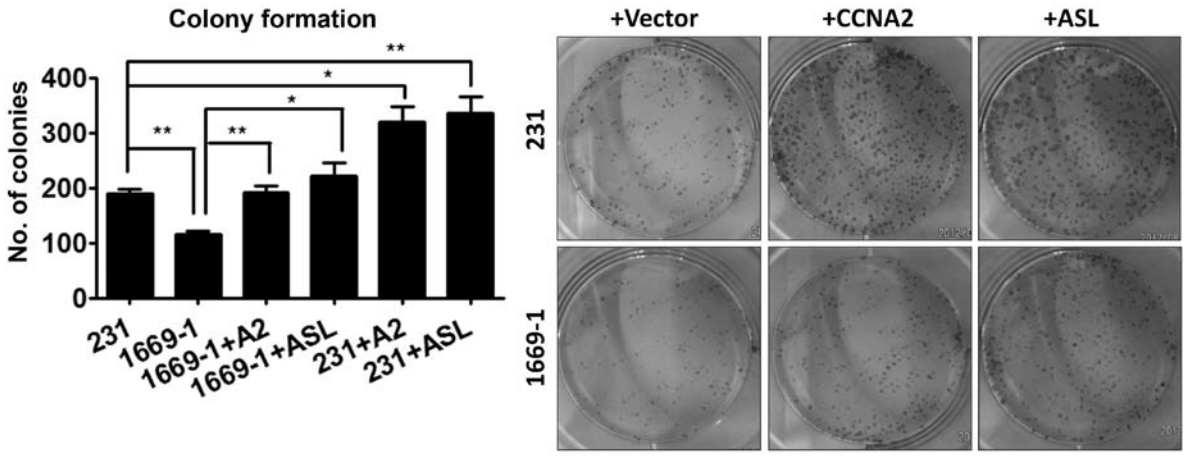

Figure 4. Ectopic cyclin A2 or ASL rescues the growth inhibition by ASL shRNA. Cyclin A2 and ASL were transiently transfected into the parental MDA MB-231 cells and ASL knockdown transfectants, respectively. The cell proliferation was examined by colony formation. $^{*}p<0.05$ and $^{**}p<0.01$.

to nitrogen deprivation in breast cancer (37). We next examined whether ASL shRNA induces autophagy. LC3B expression and autophagic vacuoles stained by MDC were increased in the ASL knockdown transfectants (Fig. 5A and B). To study the correlation between autophagy and cell growth, the autophagic inhibitors, 3-MA and bafilomycin A1, were incubated with the parental and ASL-knockdown MDA MB-231 cells.

The cell growth of the ASL knockdown transfectants was higher than that of the parental MDA MB-231 cells after treatment with the autophagic inhibitors, indicating the

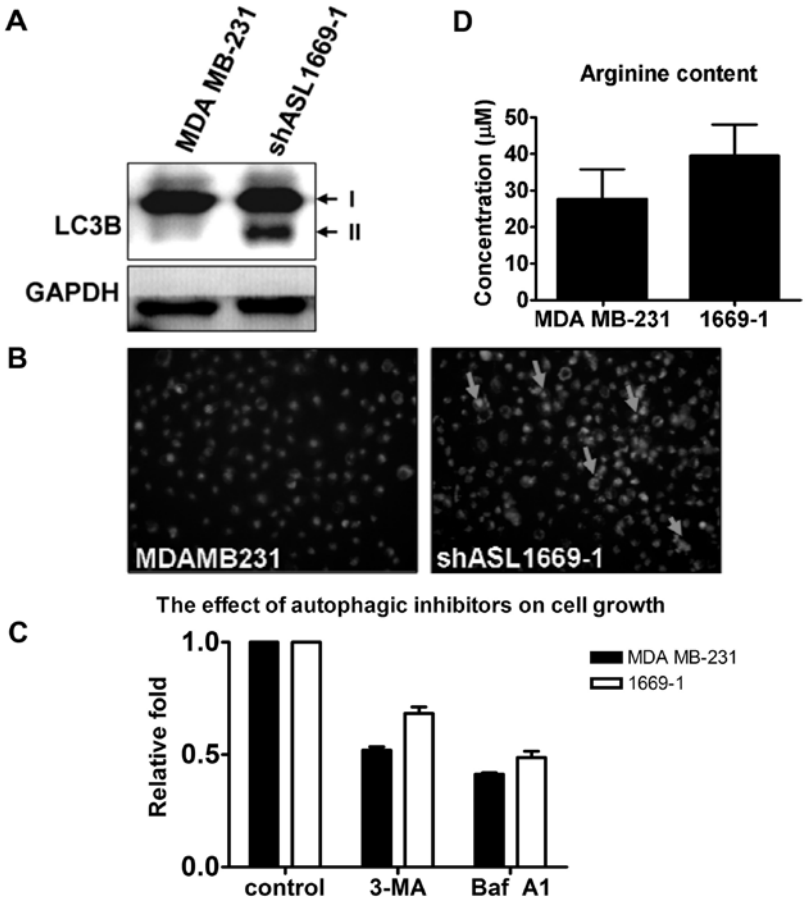

Figure 5. ASL shRNA induces autophagy. (A) The cell lysates of the MDA MB-231 cells and ASL knockdown transfectants were harvested and analyzed by western blotting with the specific anti-LC3-II antibody. (B) The autophagosomes in the MDA MB-231 cells and ASL knockdown transfectants were analyzed by mono-dansyl-cadaverine (MDC) staining. (C) Cell proliferation of the MDA MB-231 cells and ASL knockdown transfectants was analyzed by MTT assay after 3-methyladenine (3-MA) and bafilomycin A1 treatment for 24 h. The y-axis indicates the fold of absorbance values with or without inhibitor treatment. (D) Intracellular arginine in the MDA MB-231 cells and ASL knockdown transfectants was detected by HPLC.

autophagy-induced pro-survival role by ASL shRNA (Fig. 5C). The cellular arginine level was analyzed by HPLC analysis. There was no significant difference in the arginine level between parental cells and the ASL knockdown transfectants (Fig. 5D). These data indicate that autophagy is induced independent of total cellular arginine content.

ASL shRNA attenuates NO content in breast cancer cells. Nitric oxide synthase (NOS) and ASL constitute the citrulline-argininosuccinate-arginine cycle and permit nitric oxide production. Therefore, we examined the NO content in ASL knockdown transfectants. The NO content was significantly decreased by ASL shRNA (Fig. 6A). The NO inhibitor, L-NMMA, also attenuated the NO level in the MDA MB-231 cells (Fig. 6B). Since NO production is implicated in cancer progression, we analyzed whether an NOS inhibitor attenuates breast cancer growth. The proliferation was inhibited by L-NMMA in MDA-MB-231 cells as demonstrated by colony formation (Fig. 6C) and MTT assays (Fig. 6D). The NO donor, sodium nitrite ($NaNO_2$), partially restored the ASL shRNA-induced growth inhibition as demonstrated by colony formation (Fig. 6E) and MTT assays (Fig. 6F), indicating that reduction in the NO level may in part be responsible for the growth inhibition by ASL shRNA in breast cancer.

Discussion

In the present study, we demonstrated that ASL expression was induced by ER stress and was overexpressed in breast cancer. ASL shRNA attenuated cell proliferation and anchorage-independent growth. The breast cancer cells with low ASL expression had lower ability to form tumors in NOD/SCID mice. Furthermore, ASL downregulation induced autophagy. The cyclin A2 and NO levels in the ASL knockdown transfectants were decreased and ectopic cyclin A2 and NO donor restored the inhibition of cell growth by ASL shRNA. A similar effect of NO on cell proliferation was further observed using an NOS inhibitor, L-NMMA, suggesting that NO played an important role in the ASL knockdown transfectants.

ASL plays an important role in liver cancer progression (27). This finding demonstrating the effect of ASL on cancer cell growth *in vivo* and *in vitro* is consistent with the present study. Kaplan-Meier plotter database indicated that the breast cancer patients with high ASL expression were associated with a poor clinical outcome. ASL expression was also overexpressed in the breast cancer tissues in the Cancer Genome Anatomy Project (CGAP) database. Downregulation of ASL was found to contribute to tumor regression in both

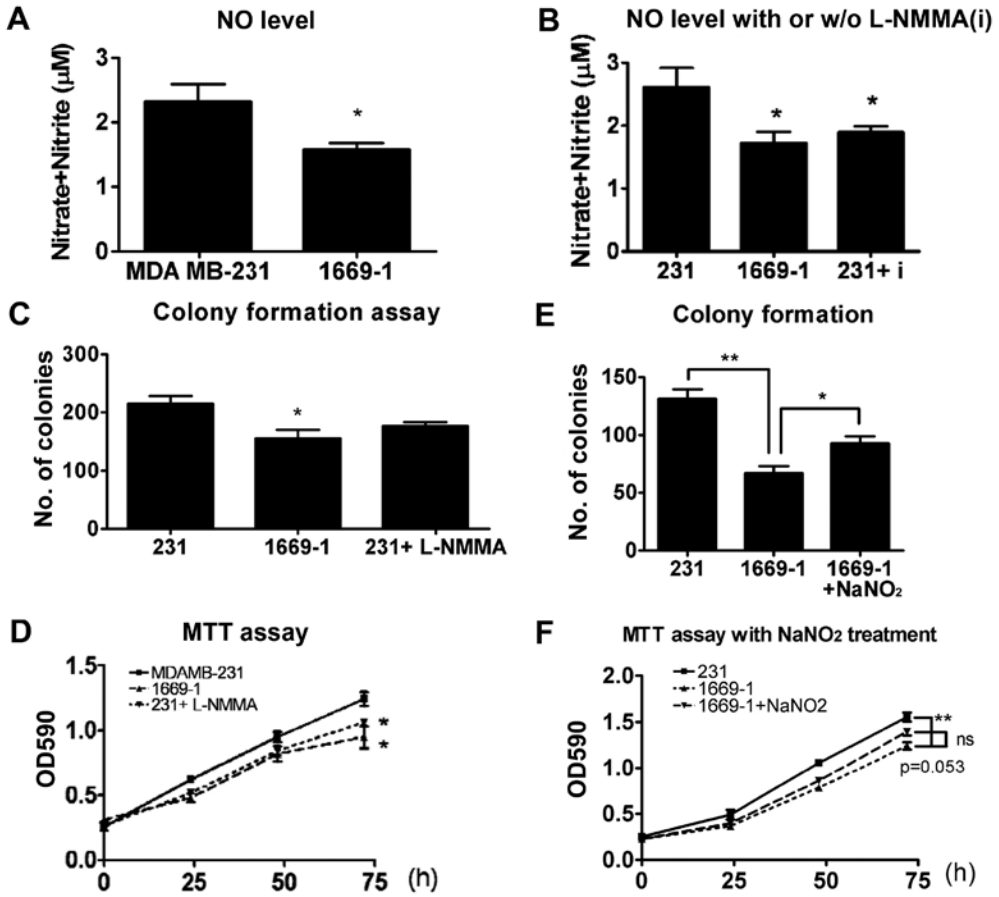

Figure 6. Nitric oxide is partially responsible for the growth attenuation by ASL shRNA. (A) The nitrate and nitrite contents in the MDA MB-231 cells and ASL knockdown transfectants were measured by a nitrate/nitrite colorimetric assay kit. (B) The nitrate and nitrite contents in the MDA MB-231 cells were measured after L-NMMA treatment. Cell proliferation of the MDA MB-231 cells was analyzed by colony formation (C) and MTT assays (D) after L-NMMA treatment. After sodium nitrite treatment, the cell proliferation of the ASL knockdown transfectants was analyzed by the colony formation (E) and MTT assays (F).

liver and breast cancer. These data indicate that ASL may play a tumorigenic role in human cancer, suggesting that ASL may serve as a therapeutic target. The role of ASL in other types of cancers warrants further study.

The mouse model with ASL deficiency has an effect on NO synthesis (25). The complex of ASL, ASS and NOS is responsible for NO recycling (38). Our data support the notion that the metabolic enzyme, ASL, contributes to NO production. Excessive NO production has been implicated in cancer progression (39). The NO donor restored the cell inhibition by ASL shRNA and the NOS inhibitor attenuated cell growth, indicating the oncogenic role of NO in cancer development. However, a previous study found that macrophage and natural killer cell-derived NO exerts an antitumor effect (40). The mechanism by which NO mediates cancer growth warrants further investigation.

In conclusion, ASL is overexpressed in breast cancer and ASL downregulation decreases tumor growth by inhibiting cyclin A2 and NO. Administration of ASL shRNA may be a novel treatment to prevent cancer cell proliferation and induce cancer cell death.

Acknowledgements

The present study was supported by a grant (to M.D. Lai) NSC-100-2325-B-006-008 from the National Science Council, Taiwan, and NHRI-EX100-9927B1 from the National Health Research Institute, Taiwan; to Establish Centers of Excellence for Cancer Research in Taiwan, DOH101-TD-C-111-003 Department of Health, Executive Yuan, Taiwan.

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