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Transcription Factors Sp1 and Sp3 Regulate Expression of Human *ABCG2* Gene and Chemoresistance Phenotype

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ABCG2 is a member of the ATP binding cassette (ABC) transmembrane proteins that plays an important role in stem cell biology and drug resistance of cancer cells. In this study, we investigated how expression of human *ABCG2* gene is regulated in lung cancer A549 cells. Binding of Sp1 and Sp3 transcription factors to the *ABCG2* promoter *in vitro* and *in vivo* was elucidated by electrophoretic mobility shift assay and chromatin immunoprecipitation assay. The *ABCG2* promoter activity was impaired when Sp1 sites were mutated but was enhanced by overexpression of Sp1 or Sp3 proteins. Knockdown of Sp1 or Sp3 expression by short interfering RNA significantly decreased the expression of *ABCG2* mRNA and protein, resulting in attenuated formation of the side population in A549 cells. In addition, Sp1 inhibition *in vivo* by mithramycin A suppressed the percentage of the side population fraction and sphere forming activities of A549 cells. Moreover, inhibiting Sp1- or Sp3-dependent *ABCG2* expression caused chemosensitization to the anticancer drug cisplatin. Collectively, our results demonstrate that Sp1 and Sp3 transcription factors are the primary determinants for activating basal transcription of the *ABCG2* gene and play an important role in maintaining the side population phenotype of lung cancer cells.

INTRODUCTION

ABCG2 is a member of the ATP binding cassette (ABC) transporters, which uses cellular ATP to drive the transport of various substrates including drugs, metabolites, and other compounds across cell membranes (Mo and Zhang, 2012). The human *ABCG2* gene was first cloned from doxorubicin-resistant human MCF-7 breast cancer cells, and its coded protein contains 655 amino acids, including a single N-terminal ATP-binding cassette, and 6 transmembrane segments (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). ABCG2 is a half-transporter, requiring dimerization or oligomerization to become functionally active (Kage et al., 2002; Xu et al., 2007). ABCG2 is widely distributed in normal tissues and highly expressed in stem cells, and identified as a molecular determinant of the side population (SP) phenotype, which is characterized by the ability to transport the fluorescent dye Hoechst 33342 (Scharenberg et al., 2002; Zhou et al., 2001). Several previous reports suggested that ABCG2 is fundamental for the maintenance of the stem cell phenotype and plays an important role in promoting proliferation and blocking differentiation of stem cells (Bunting, 2002). ABCG2 overexpression is also frequently observed in a variety of tumor cells (Hirschmann-Jax et al., 2004), and the $ABCG2^{+}$ subset of tumor cells are often enriched in cells with cancer stem-like phenotypes (Ho et al., 2007), which represent a population of drug resistant cells that can survive treatment and repopulate the tumor (Dalerba et al., 2007).

The human *ABCG2* gene is transcribed by a TATA-less promoter, with several putative Sp1, AP1, and AP2 sites and a CCAAT box (Bailey-Dell et al., 2001). Both positive and negative cis-regulatory elements have been identified in the *ABCG2* promoter, and functional hormone and hypoxia response elements upstream of the *ABCG2* gene have been reported (Ee et al., 2004; Krishnamurthy et al., 2004; Szatmari et al., 2006). DNA methylation and histone modifications were reported to play important roles in the epigenetic regulation of *ABCG2* expression in human renal carcinoma and multidrug-resistant cells, respectively (To et al., 2006; 2008; Turner et al., 2006). The *ABCG2* gene is also under posttranscriptional regulation by microRNAs and carries the binding sites for microRNAs such as hsa-miR-328, -519c, and -520h in its 3′-untranslated region (Pan et al., 2009; To et al., 2009; Wang et al., 2010).

In this study, we investigated how expression of the human *ABCG2* gene is regulated in lung cancer A549 cells and found that both Sp1 and Sp3 transcription factors are critical for activating basal transcription of the *ABCG2* gene, and play an important role in maintaining the SP phenotype of lung cancer cells.

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MATERIALS AND METHODS

Cell culture

A549 were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed essentially as described (Lee et al., 2011). Oligonucleotide sequences from the human *ABCG2* promoter used as EMSA probes are shown in Fig. 1A.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed essentially as described (Lee et al., 2011) using antibodies specific for Sp1 or Sp3 (Santa Cruz Biotechnology, Inc.). The sequences of the ABCG2 primers were as follows: ABCG2 proximal promoter, 5′-TTCAGCCGG GTCGCAGGGCGCTTAT-3′, 5′-TGGACCGCCAGAGCTGAAC GCAGTG-3′; ABCG2 distal, 5′-TTTCGTGTGTGTTTTCTCCA-3′, 5′- GCTCACCCAGATAAGTGAAAAGA-3′.

Plasmids

To generate the wild type (WT) human ABCG2 promoter luciferase reporter construct, we subcloned the *ABCG2* gene -1058 to +307 region 5′ to the luciferase gene between the *Xho*I and *Hin*dIII sites in the polylinker of the pGL3-Basic luciferase reporter vector (Promega). Site-directed mutagenesis of the ABCG2 Sp1 sites in this plasmid was performed using the QuikChange kit (Stratagene). Three mutagenic primers, 5′-TGACCCTAGCC CCGAGGGAGTTAGGTGGTACCAGTCCTGCTGG-3′, 5′-GG CCCGGCAGTCGGGGCCACTAATCACCCCCTGTCGCGAA CCCCGACCTGGGGA-3′, and 5′-TGGGGAAACCCGGGGC GCTGAAAAGGGGCCACTGCGTTCAGCT-3′ were used to introduce the indicated underlined 3-bp changes into the Sp1 sites at -212, -119, -99, and -61, respectively. The pCMV-Sp1 and pN3-Sp3FL were purchased from Addgene. The shRNA lentiviral vectors for Sp1 and Sp3 were described previously (Lee et al., 2011).

Transient transfections and luciferase assays

The transient transfections were performed using Effectene transfection reagent (QIAGEN) according to the manufacturer's instructions. In each case, 8×10^4 cells in logarithmic growth phase were transfected with 200 ng supercoiled test plasmid and 10 ng pRL-SV40 as a transfection efficiency control. The cells were allowed to recover for 24 h at 37°C, and harvested and analyzed for luciferase activity using a luminometer (TD-20/20 Luminometer; Turner Designs, Promega) and a dual luciferase assay system kit (Promega).

Lentiviral transduction

The 293T cells (2×10^6) were seeded onto 100-mm dishes, and 16 h later were transiently transfected with 1 μg lentiviral vector and 3 μg each packaging vector (Invitrogen Life Sciences) by using Effectene (QIAGEN). Supernatants were collected 48 h after transfection, and filtered through a 0.45 μM filter. A549 cells (2×10^5) were seeded into 24-well plates and spin-infected in the presence of 6 μg/ml of Polybrene® (Sigma-Aldrich). Twenty-four hours post-infection, cells were treated with 2 μg/ml puromycin or G418 for 5 days to eliminate uninfected cells.

Quantitative real time PCR

Total RNA was isolated using TRIzol (Invitrogen) and first-strand cDNAs were made using the PrimeScript RT Kit (Takara). Quantitative real time PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems). The sequences of the primers were as follows: human ABCG2; 5′-ATGG ATTTACGGCTTTGCAG-3′, 5′-TCTTCGCCAGTACATGTTGC-3′; human Sp1, 5′-GCGAGAGGCCATTTATGTGT-3′, 5′-GGC CTCCCTTCTTATTCTGG-3′; human Sp3, 5′-ATTCTGGAGA ACGCCCTTTT-3′, 5′-TATGTTTGGCAAGGTGGTCA-3′; 18S rRNA, 5′-CGGCTACCACATCCAAGGAA-3′, 5′-GCTGGAATT ACCGCGGCT-3′.

Flow cytometry

ABCG2 expression was assayed by flow cytometry using an antibody that specifically recognizes membrane-bound ABCG2 epitopes (ABCG2-PE; eBioscience). SP abundance was determined as described (Goodell et al., 2005) with minor modifications. Briefly, cells (1×10^6/ml) were incubated at 37°C for 60 min with 5 μg/ml Hoechst 33342 (Sigma-Aldrich), washed, and resuspended in ice-cold HBSS with 2% fetal calf serum (FCS) and 2 μg/ml propidium iodide (Sigma-Aldrich). Fumitremorgin C (FTC; 10 μmol/L; Enzo Life Sciences), a potent and specific inhibitor of ABCG2 activity, was used as a positive control for the assay. Labeled cells were analyzed by flow cytometry on a LSRII (BD Biosciences).

Sphere-forming assay

A single cell suspension from trypsinization was cultured in serum-free DMEM/F12 medium with growth factors (10 ng/ml of EGF and bFGF) at a density of 1×10^3 cells/ml. After 10 days, spheres were attached by adding FBS (10%), stained with Diff-Quick solution (Sysmex, Japan), and counted.

MTT assay

Approximately 2.0×10^3 cells were seeded into each well of a 96-well plate. After 24 h incubation, the cells were exposed to various concentrations of cisplatin for 72 h. After incubation, 10 μl MTT was added to each well and cells were further incubated for 4 h, followed by 100 μl solubilization buffer overnight. The mixture was gently shaken for 10 min. Cell viability was evaluated based on absorbance at 550 nm in a Sunrise microplate absorbance reader (Tecan).

RESULTS

Sp1 and Sp3 directly bind to the ABCG2 promoter region and increase ABCG2 promoter activity

Analysis of the *ABCG2* promoter region revealed several potential consensus sequences for factor binding, including four consensus Sp1 binding sites (Yuan et al., 2004). We therefore performed EMSA to evaluate binding of ^{32}P-labeled DNA probes that spanned these Sp1 sites by proteins from A549 nuclear extracts. When WT probes spanning the Sp1 sites at -212 (WT1), -119 and -99 (WT2), and -61 (WT3) (shown in Fig. 1A) were assayed, two complexes (designated as C1 and C2) were detected with extracts from A549 cells, but the mutations of the Sp1 sites eliminated the formation of these complexes (Fig. 1B). The complexes generated with WT probes were competed by a 125-fold excess of unlabeled wild type competitors, whereas mutant Sp1 oligonucleotides were not effective at inhibiting the formation of complexes (Figs. 1C, 1D, and 1E), indicating that these complexes were specific. The complexes formed with WT probes were evaluated for Sp1 and Sp3 using

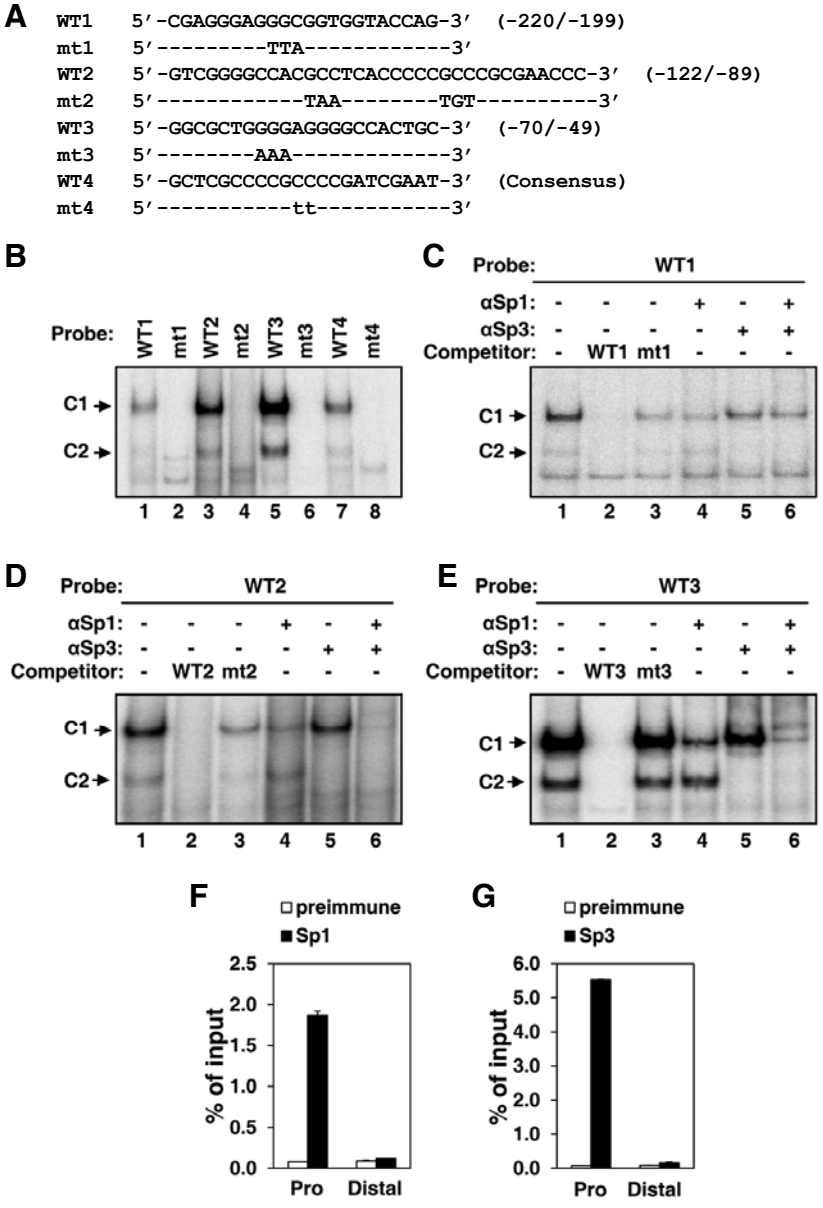

Fig. 1. Sp1 and Sp3 factors directly bind the ABCG2 promoter**.** (A) Sequences of the WT1, WT2, and WT3 probes (spanning the -220/-199, -122/-89, -70/-49, respectively), and consensus Sp1/Sp3 probe (WT4) used in (Figs. 1B-1E) are shown. For mutant (mt) probes, the mutations are shown in lower case, and hyphens indicate identity to the WT sequence. (B) Electrophoretic mobility shift assay (EMSA) using wild type (WT) and mutant (mt) probes shown in Fig. 1A and nuclear extracts from A549 cells. (C-E) EMSA using WT1 (C), WT2 (D), or WT3 (E) probes and nuclear extracts from A549 cells. In lanes 2 and 3, EMSA were performed in the presence of a 125-fold molar excess of the indicated cold competitors. In lanes 4-6, EMSA were performed in the presence of antibodies to Sp1 and/or Sp3, as indicated. (F, G) Sp1 and Sp3 bind to the *ABCG2* promoter *in vivo*. Chromatin immunoprecipitation (ChIP) assays were performed with A549 cells in the presence of preimmune serum, anti-Sp1 (F) or anti-Sp3 antibodies (G). DNA was purified and used as a template for qRT-PCR with pairs of primers that amplify the proximal promoter (pro) or distal region of the *ABCG2* gene. Data are shown as mean ± SEM of results from three experiments.

antibodies to each protein. We found that anti-Sp1 blocked the formation of the C1 complex and anti-Sp3 antibodies resulted in a supershift of the C2 complex to generate a slower mobility complex, confirming the binding of Sp1 and Sp3 to these regions (Figs. 1C, 1D, and 1E). We next performed chromatin immunoprecipitation to examine whether Sp1 and Sp3 bind to the endogenous *ABCG2* promoter in A549 cells *in vivo*. As shown in Figs. 1F and 1G, constitutive recruitment of Sp1 and Sp3 to the *ABCG2* proximal promoter, but not to the distal region, was observed in A549 cells.

To evaluate the functional significance of Sp1 sites in the *ABCG2* promoter region, we cloned the promoter region of the *ABCG2* gene, mutated each Sp1 site alone and in combination in the context of the -1058 to +307 human ABCG2-luciferase reporter construct, and assayed the promoter activity of each mutant construct in A549 cells (Fig. 2A). Whereas the wild type construct showed strong promoter activity, selective mutation of the Sp1 sites at -212 (mt1) markedly decreased promoter activity. However, selective mutations of the Sp1 sites at -119 and -99 (mt2) or -61 (mt3) modestly decreased promoter activities, although simultaneous mutations of the three Sp1 sites (mt5) significantly decreased promoter activity. These results suggest that the Sp1 site at -212 is most critical for maximal *ABCG2* promoter activity in A549 cells. To further demonstrate that the Sp family of transcription factors is involved in the transcriptional regulation of human *ABCG2*, we co-transfected A549 cells with a luciferase reporter construct in the presence and absence of expression plasmids encoding Sp1 and Sp3. The activity of the pABCG2-1058-WT construct was markedly increased in the presence of pCMV-Sp1 and pN3-Sp3FL, indicating that transcription factors Sp1 and Sp3 directly regulate the expression of *ABCG2* (Figs. 2B and 2C).

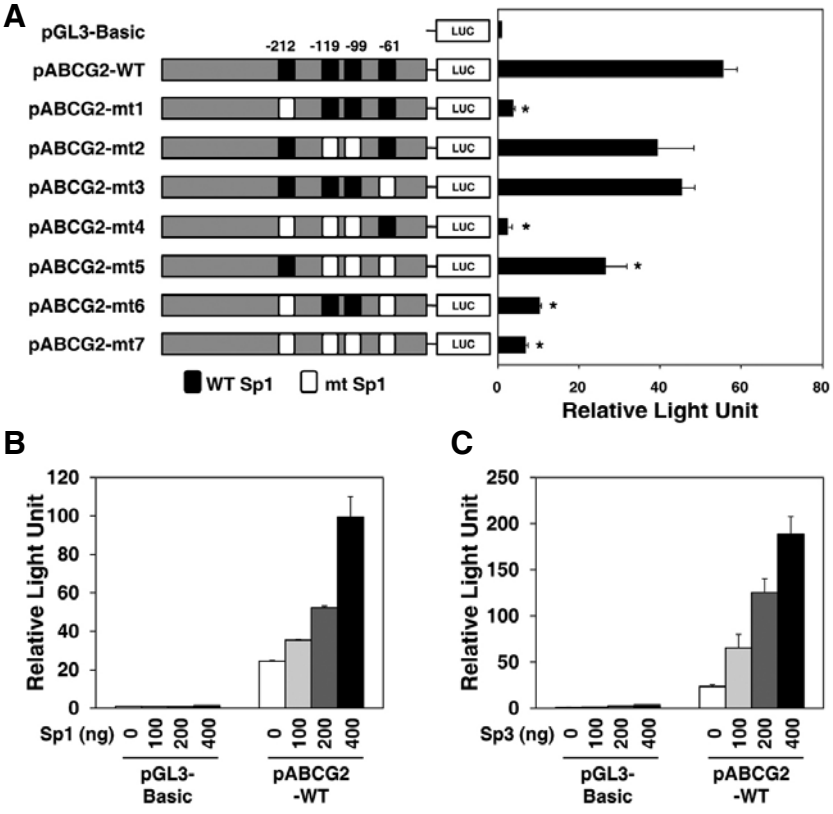

Fig. 2. Sp1 and Sp3 factors increase ABCG2 promoter activities. (A) Mutations in the Sp1/Sp3 sites were introduced into the pABCG2-WT construct, and luciferase (Luc) reporter constructs were transfected into A549 cells. *P < 0.05 compa-red with cells transfected with pABCG2- WT construct. (B, C) A549 cells were transfected with pABCG2-WT construct (100 ng) together with different amounts (0, 100, 200, and 400 ng) of Sp1 (B) or Sp3 vector (C). Data are shown as mean ± SEM of results from three experiments.

Sp1 and Sp3 are required for ABCG2 gene expression and SP phenotype

Because Sp1 and Sp3 directly bind to the *ABCG2* promoter region and increase ABCG2 promoter activities in transient transfections, we investigated whether *ABCG2* gene expression is dependent on Sp1 and Sp3 using a lentiviral vector and RNA interference to inhibit the expression of these genes. The expression of Sp1 or Sp3 at mRNA and protein levels were markedly decreased in A549 cells infected with lentivirus containing shRNA against Sp1 or Sp3, whereas expression of β-actin protein was unaffected (Figs. 3A-3D). Strikingly, ABCG2 mRNA and protein were significantly decreased in Sp1- or Sp3-diminished A549 cells compared to the control cells (Figs. 3A, 3B, 3E, and 3F). The SP phenotype is mainly mediated by ABCG2 in cancer cells. To investigate whether reduced ABCG2 expression affects SP formation in Sp1- or Sp3-diminished A549 cells, Hoechst 33342 dye exclusion assays were performed. The results show that control cells contain approximately 4% of the SP fraction which was completely blocked by FTC treatment, whereas the percentage of SP drops to less than 1% in Sp1- or Sp3-diminished A549 cells (Figs. 3G and 3H).

To further investigate the role of Sp1 and Sp3 transcription factors on *ABCG2* gene transactivation, we treated A549 cells with mithramycin A, a drug that binds to the minor grooves of the GC-rich motifs of the DNA and hence blocks Sp1 binding. As shown in Figs. 4A and 4B, the expression of ABCG2 mRNA and protein was markedly decreased in A549 cells by treatment with mithramycin A, in a dose-dependent manner. Consistently, mithramycin A treatment suppressed the percentage of SP fraction (Fig. 4C) and sphere forming activities (Figs. 4D and 4E), suggesting an essential role of Sp1 and Sp3 transcription factors in the function of ABCG2, as well as in maintaining the stemness of A549 cells.

Inhibiting Sp1- or Sp3-dependent ABCG2 expression causes chemosensitization to the anticancer drug cisplatin

To determine whether decreased expression of Sp1 or Sp3 could sensitize A549 cells to anticancer drug-induced cytotoxicity, we incubated either Sp1- or Sp3-diminished cells with cisplatin for 3 days and measured the percentage of viable cells using the MTT assay. In the absence of cisplatin, knockdown of Sp1 expression had little effect on the viability of A549 cells (Fig. 5A), while A549 cells exhibited reduction in cellular viability after knockdown of Sp3 (Fig. 5B). Interestingly, both Sp1- and Sp3-diminished A549 cells, with lower endogenous levels of ABCG2, exhibited significantly enhanced sensitivities to cisplatin compared to the control A549 cells (Figs. 5A and 5B), demonstrating that inhibiting Sp1- or Sp3-dependent *ABCG2* expression causes chemosensitization to the anticancer drug cisplatin.

DISCUSSION

In the present study, we show that Sp1 together with Sp3 directly regulated transcriptional expression of the *ABCG2* gene, and diminished expression of Sp1 and Sp3 resulted in a dramatic decrease of SP formation and enhanced chemosensitivity to anticancer drug in a lung cancer cell line. Previous studies have shown that the *ABCG2* proximal promoter region contained several putative Sp1 binding sites to which the homeobox gene *Msx2* recruits Sp1 to increase *ABCG2* gene expression (Hamada et al., 2012). In addition, another group has reported that increased occupancy of Sp1 within the *ABCG2*

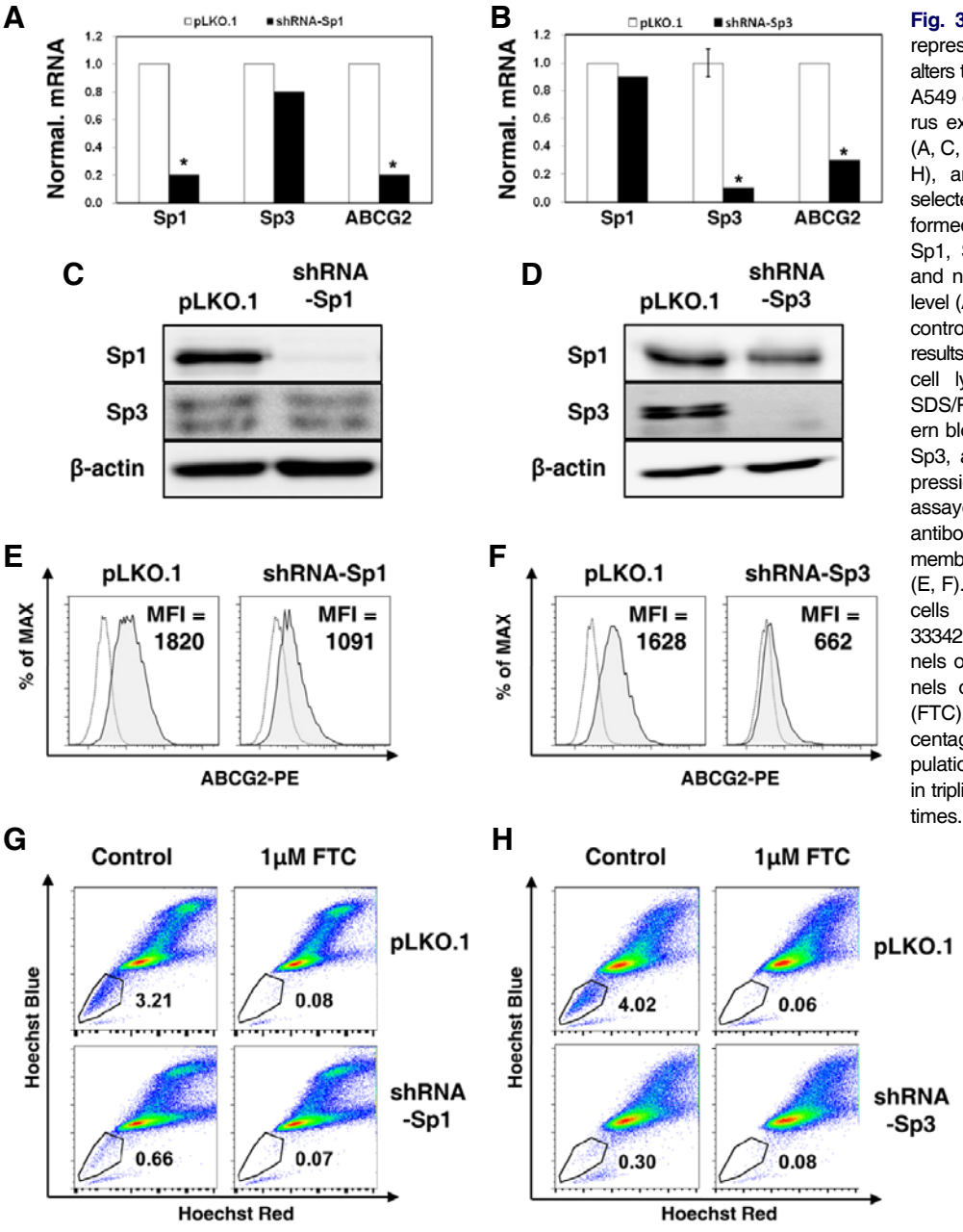

Fig. 3. Knockdown of Sp1 or Sp3 represses *ABCG2* expression and alters the SP phenotype in A549 cells. A549 cells were infected with lentivirus expressing shRNA against Sp1 (A, C, E, and G) or Sp3 (B, D, F, and H), and drug resistant cells were selected. Real-time PCR was performed to analyze mRNA levels of Sp1, Sp3, and ABCG2 expression and normalized with the 18S rRNA level (A, B). *$P < 0.05$ compared with control. Values are mean ± SEM of results from three experiments. Whole cell lysates were separated with SDS/PAGE and subjected to Western blotting using antibodies to Sp1, Sp3, and actin (C, D). ABCG2 expression on the cell surface was assayed by flow cytometry using an antibody that specifically recognizes membrane-bound ABCG2 epitopes (E, F). To determine SP abundance, cells were incubated with Hoechst 33342 dye in the absence (left panels of G, H) or presence (right panels of G, H) of fumitremorgin C (FTC). The SP is shown as a percentage of the whole viable cell population. Reactions were performed in triplicates and were repeated three times.

promoter coincided with upregulation of *ABCG2* and simultaneous mutation of five Sp1 binding sites markedly attenuated *ABCG2* promoter activities (Zhang et al., 2012). Thus far, however, no definitive data that support Sp1 transcription factor as the direct regulator of the *ABCG2* gene expression has been reported. Although it has been shown that mithramycin A, a known inhibitor of Sp1 binding, mediated dose-dependent decreases in *ABCG2* expression, treatment with mithramycin A also resulted in decreased expression of other transcription factors such as AhR and Nrf (Zhang et al., 2012). This makes it uncertain whether Sp1 directly regulates *ABCG2* gene expression.

Several results from our study support the hypothesis that Sp3 together with Sp1 are required for transactivation of the *ABCG2* gene in A549 cells. Sp3, one of the Sp1-related transcription factors, is ubiquitously expressed and recognizes the same sequence as Sp1 (Safe and Abdelrahim, 2005). First, EMSA and ChIP assays showed *in vitro* and *in vivo* binding activity of Sp1 and Sp3 to *ABCG2* promoter regions. Second, site-specific mutagenesis of Sp1/Sp3 binding sites significantly diminished *ABCG2* promoter activity and increased expression of either Sp1 or Sp3 enhanced *ABCG2* promoter activity. Third, specific knockdown of Sp1 or Sp3 using a shRNA lentivirus-based system resulted in significantly diminished ABCG2 expression at both mRNA and protein levels. Fourth, treatment with mithramycin A, an inhibitor of Sp1/Sp3 binding, significantly decreased ABCG2 expression, consistent with a previous report (Zhang et al., 2012).

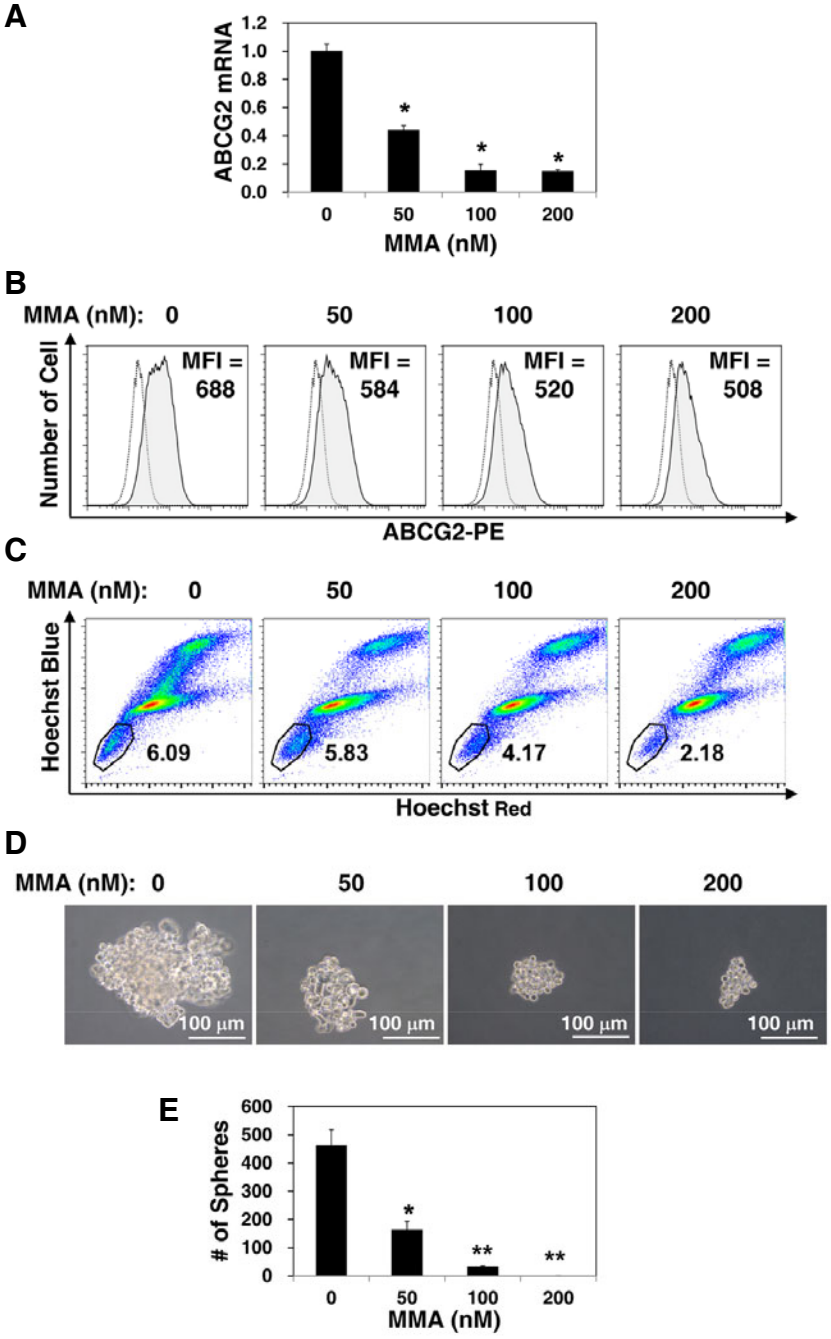

Fig. 4. Mithramycin A blocks the expression of the *ABCG2* gene and alters the SP phenotype and sphere-forming activities in A549 cells. (A) A549 cells were treated with different amounts of mithramycin A (0, 50, 100, and 200 nM) for 24 h. Real-time PCR was performed to analyze mRNA levels of ABCG2 expression and normalized with the 18S rRNA level. (B, C) A549 cells were treated with different amounts of mithramycin A before staining with anti-ABCG2 antibody (B) or Hoechst 33342 dye (C), and analyzed by flow cytometry. Reactions were done in triplicate and repeated three times. (D) A549 cells were treated with different amounts of mithramycin A and the sphere-forming assay was performed to analyze the maintenance of stemness in A549 cells. Representative images of spheres (D) and quantification of the assays (E). * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared with controls. Values are mean ± SEM of results from three experiments.

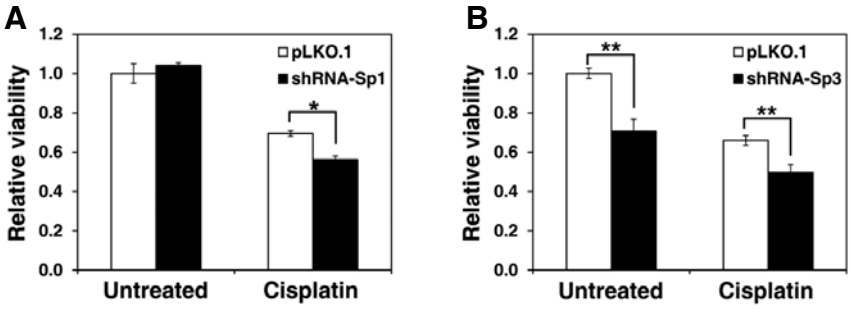

Fig. 5. The role of Sp1 and Sp3 on the chemoresistance phenotype of A549 cells. Sp1-diminished (A) or Sp3-diminished (B) A549 cells were treated with cisplatin (7 mM) for 72 h and cellular viability was measured by the MTT assay. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared with controls. Values are mean ± SEM of results from three experiments.

The expression of the *ABCG2* gene is an important determinant of the SP phenotype and the SP cells in lung cancer cell lines were an enriched source of tumor-initiating cells with stem cell properties (Ho et al., 2007). Recently, phosphoinositide-3 kinase pathway has been shown to regulate ABCG2 function in malignant pleural mesothelioma, and inhibiting the PI3K/Akt pathway strongly decreased the SP phenotype resulting in increased sensitivity to chemotherapeutic drug (Fischer et al., 2012). In line with these reports, decreased expression of *ABCG2* resulting from depletion of Sp1 and Sp3 either by shRNA approaches or treatment of mithramycin A demonstrated diminished SP phenotypes. Furthermore, sphere-forming ability of A549 cells was suppressed by mithramycin A in a dose-dependent manner, suggesting that Sp1 and Sp3 play an important role in maintenance of stemness of lung cancer cell lines through regulating *ABCG2* expression. Elevated expression of *ABCG2* is known to contribute to multidrug resistance in cancer chemotherapy (Chen et al., 2011). Depletion of Sp1 or Sp3 and a parallel decrease in *ABCG2* expression caused enhanced sensitivity to cisplatin in A549 cells.

In summary, our findings clarified the role of Sp1 and Sp3 in the regulation of the human *ABCG2* gene and demonstrated that modulating the expression of *ABCG2* by inhibiting Sp1 or Sp3 may be an alternative way to sensitize *ABCG2*-mediated drug resistance for better treatment of lung cancers.

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