CANCER GENOMICS & PROTEOMICS *21*: 158-165 (2024)
doi:10.21873/cgp.20437

POLD1 Is Required for Cell Cycle Progression by Overcoming DNA Damage in Malignant Pleural Mesothelioma

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Abstract. *Background/Aim: The prognosis of patients with malignant pleural mesothelioma (MPM) remains poor due to lack of effective therapeutic targets. DNA damage caused by long-time exposure to asbestos fibers has been associated with the development of MPM, with mutations at genes encoding DNA damage repair (DDR)-related molecules frequently expressed in patients with MPM. The present study was designed to identify novel therapeutic targets in MPM using large public databases, such as The Cancer Genome Atlas (TCGA) and Genotype Tissue Expression project (GTEx) focused on DDR pathways. Materials and Methods: The correlations between mRNA expression levels of DDR-related genes and overall survival (OS) were analyzed in mesothelioma patients in TCGA mesothelioma (TCGA-MESO) datasets. The anti-tumor effects of small interfering RNAs (siRNA) against DDR-related genes associated with OS were subsequently tested in MPM cell lines. Results: High levels of mRNA encoding DNA polymerase delta 1, catalytic subunit (POLD1) were significantly associated with reduced OS in patients with MPM ($p<0.001$, Log-rank test). In addition, siRNA targeting POLD1 (siPOLD1) caused cell cycle arrest at the G_1/S checkpoint and induced apoptosis involving accumulation of DNA damage in MPM cell lines. Conclusion: POLD1 plays essential roles in overcoming DNA damage and cell cycle progression at the G_1/S checkpoint in MPM cells. These findings suggest that POLD1 may be a novel therapeutic target in MPM.*

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Key Words: Malignant pleural mesothelioma, DNA polymerase δ, POLD1, The Cancer Genome Atlas, cell cycle, DNA damage.

Malignant pleural mesothelioma (MPM) is a highly aggressive cancer caused by exposure to asbestos fibers. Current treatments for MPM include surgery, radiation therapy, chemotherapy, and immune checkpoint inhibitors (1-3). Their clinical effectiveness, however, is limited, with 5-year overall survival (OS) rates below 10% (4). Therefore, identification of novel treatment targets for MPM are urgently needed.

Mutations in genes encoding proteins in DNA damage repair (DDR) pathways, such as the breast cancer susceptibility genes 1/2 (*BRCA1/2*) and the gene encoding BRCA1 associated protein 1 (*BAP1*), have been frequently identified in patients with MPM (5, 6). Asbestos-induced DNA damage and genome instability have been associated with the development of MPM, indicating that DDR pathways may be potential targets in these tumors (7).

DNA replication is catalyzed by the DNA polymerases α, δ and ε. The DNA Pol α–primase complex catalyzes the formation of RNA primer templates, which are extended by Pol ε on the leading strand, whereas Pol δ extends primers on the lagging strand and is heavily implicated in DNA repair (8). The human Pol δ complex consists of the catalytic subunit POLD1 and the structural subunits POLD2, POLD3, and POLD4. Although loss-of-function or amplification of Pol δ subunits has been implicated in various types of cancers (9), the roles of Pol δ in MPM development and cell survival have not yet been determined.

The present study evaluated the correlation between expression of genes encoding Pol δ components and OS in MPM patients by a pan-cancer analysis of *POLD1-4* in The Cancer Genome Atlas (TCGA) mesothelioma (MESO) databases. These analyses showed that the expression of *POLD1* was significantly associated with OS in patients with MPM. The purpose of this study was to clarify the potential of POLD1 to act as a therapeutic target in MPM by testing the effects of POLD1 inhibition in MPM cell lines.

Materials and Methods

Data collection and collation. Mesothelioma RNA sequencing (RNA-seq) data were obtained from the TCGA-MESO database (https://portal.gdc.cancer.gov/), and corresponding clinical data were obtained from the UCSC Xena website (https://xenabrowser.net/). Differences in *POLD1* mRNA expression levels between normal tissues and tumors were assessed in Genotype Tissue Expression (GTEx) project and TCGA datasets.

Cell lines and reagents. Human mesothelioma cell lines (MSTO-211H, NCI-H2052, ACC-MESO-1, NCI-H2452) and normal lung fibroblast cells (IMR-90 and MRC-5) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The ACC-MESO-4 and Y-MESO-14 cell lines ware purchased from Riken Cell Bank (Ibaraki, Japan). Cells were cultured in RPMI-1640 (Wako, Osaka, Japan) (10) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Wako) at 37°C in a humid incubator with 5% CO_2. All cell lines were negative for mycoplasma contamination, as determined using VenorGeM Classic Mycoplasma Detection Kits for Conventional PCR (Minerva Biolabs GmbH, Berlin, Germany).

Western blot analysis. The methods for preparation of cell lysates, immunoblotting, and detection of bands corresponding to immunoreactive proteins have been described elsewhere (11). Samples were blotted with primary antibodies against the following proteins: POLD1 (#ab186407, Abcam, Cambridge, UK), GAPDH (#2118, Cell Signaling Technology, Danvers, MA, USA), phospho-histone H2A.X (Ser139) (05-636, Millipore Merck, Burlington, MA, USA), phospho-ATM (ser1981) (#5883, Cell Signaling Technology), ATM (#2873, Cell Signaling Technology), vinculin (sc-73614, Santa Cruz Biotechnology, Dallas, TX, USA), phospho-Chk2 (Thr68) (#2197, Cell Signaling Technology), Chk2 (sc-5278, Santa Cruz Biotechnology), phospho-p53 (Ser20) (#9287, Cell Signaling Technology) and p53 (sc-126, Santa Cruz Biotechnology). After washing, the blots were incubated with horseradish peroxidase-coupled anti-rabbit or -mouse IgG secondary Abs (Cell Signaling Technology), as appropriate, and bands corresponding to immunoreactive proteins were visualized using an ECL Western Blotting Detection kit (Cytiva, Tokyo, Japan). To normalize for loading differences, GAPDH and vinculin were used as internal controls. We performed the western blot analysis with two independent biological replicates, each containing one technical replicate.

Small interfering RNA (siRNA) treatment. *POLD1* siRNAs (siPOLD1#1, siPOLD1#2) and negative control siRNA (siCtrl; #4390843) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). OptiMEM (#31985-07, Gibco) and 5 nM of each siRNA were added to cells using lipofectamine RNAiMAX (Invitrogen), following the manufacturer's instructions.

Quantitative real time polymerase chain reaction (qRT-PCR). Total RNA was extracted from MPM cells after 48 h of siRNA treatment using NucleoSpin RNA kits (Macherey-Nagel, Dürer, Germany) according to the manufacturer's instructions. RNA was quantified on a NanoDrop 2000 (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using Revertra Ace qPCR RT kits (Toyobo, Osaka, Japan). mRNA knockdown was measured by quantitative qRT-PCR using Brilliant III Ultra-Fast SYBR QPCR Master Mixes (Agilent, Santa Clara, CA, USA), with qRT-PCR reactions using a Thermal Cycler Dice Real Time System II (Takara, Shiga, Japan), as previously described (12). The primers used in this study included those for *POLD1* (forward, 5'-GTGAGCGCCAACTCCGTAT-3'; reverse, 5'-TCACCATACACCACCTTGGC-3') and *18S rRNA* (forward, 5'-CCGATTGGATGGTTTAGTGAG-3'; reverse, 5'-AGTTCGACCGTCTTCTCAGC-3'). *POLD1* expression was normalized to that of *18S rRNA* (internal control) and relative *POLD1* expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. We performed the qRT-PCR with three independent biological replicates, each containing three technical replicates.

Cell proliferation assays. Cell proliferation was evaluated using a WST-8 assay with the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), as previously described (13). MPM cells were transfected with siCtrl or siPOLD1 at a final concentration of 5 nM for 48 h, transferred to flat-bottomed 96-well plates (Thermo Fisher Scientific), and incubated at 37˚C for 72 h. After treatment with Cell Counting Kit-8 reagent, absorbance was measured at 450 nm using a microplate reader (GloMax: Promega, Madison, WI, USA). We performed the WST-8 assay with three independent biological replicates, each containing four technical replicates.

Cell cycle analysis. Cell cycle analysis was performed using propidium iodide (PI) and flow cytometry, as described previously (14). The cells were pulse-labeled with 10 μM 5-bromo-2'-deoxyuridine (BrdU) for 30 min, harvested, fixed in 70% ethanol in PBS and incubated at –20˚C for several days. The cells were incubated in 2 N HCl for 30 min at room temperature, treated with 0.1 M sodium tetraborate pH 8.5 for 10 min, washed once with PBS, incubated with anti-BrdU Ab conjugated to FITC (#364103; BioLegend, San Diego, CA, USA) for 30 min, washed once, and resuspended in PBS containing PE diluted 1:200 to counterstain DNA. The cells were evaluated by flow cytometry on a BD LSR Fortessa (Becton Dickinson, Franklin Lakes, NJ, USA), with the data analyzed using FlowJo software (Becton Dickinson). We performed the cell cycle analysis measurement with three independent biological replicates, each containing one technical replicate.

Analysis of apoptosis. Apoptotic cells were evaluated using an eBioscience™ Annexin-V-FITC Apoptosis Detection kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Annexin-V^+/PI^- cells indicate early apoptosis, and Annexin-V^+/PI^+ cells indicate late apoptosis. The cells were evaluated by flow cytometry using BD LSR Fortessa and BD FACSDiva software (Becton Dickinson), with the data analyzed using FlowJo software (Becton Dickinson). We performed the apoptosis measurement with three independent biological replicates, each containing one technical replicate.

The study flow-chart. We showed study flow-chart, presenting details of the experimental procedures and analyses undertaken (Figure 1).

Statistical analysis. Results in bar graphs are reported as mean±standard deviation (SD; n=three independent biological replicates) or as the mean±standard error of the mean (SEM; n=three independent biological replicates). Results in two groups were compared by unpaired *t*-tests with Welch's correction, whereas results in three or more groups were compared using one-way analysis of variance (ANOVA), followed by Bonferroni's correction.

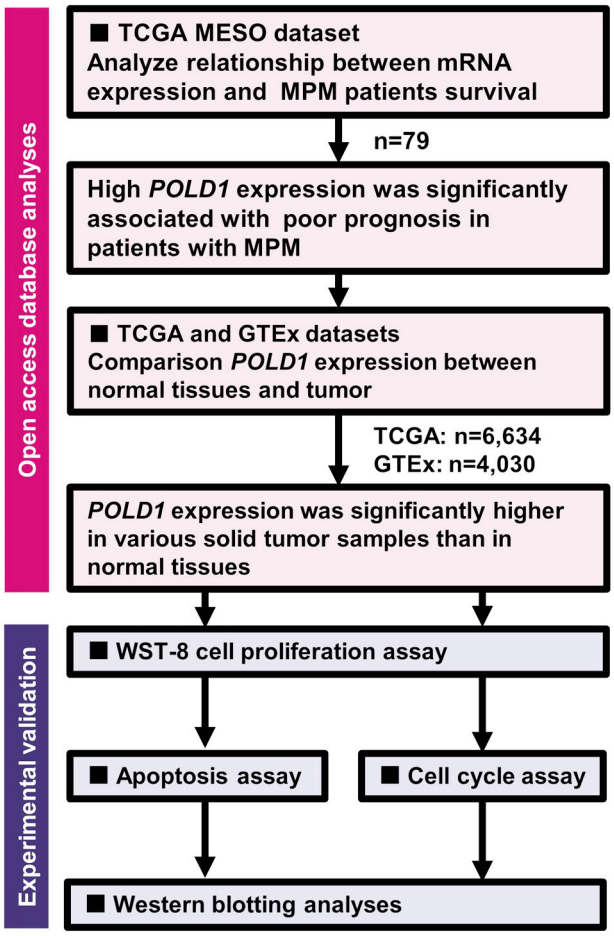

Figure 1. *The study flow-chart. First, we used TCGA and GTEx datasets to assess the possibility of pol δ for therapeutic target to MPM patients. These datasets showed that POLD1 encoding catalytic subunit of pol δ related with poor prognosis of MPM patients, and POLD1 was overexpressed in various types of tumors compared to normal tissues. Next, we performed the experimental validation to clarify the role of POLD1 in the cell cycle and apoptosis in MPM cell lines using siPOLD1. MPM: Malignant pleural mesothelioma.*

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA), with $p<0.05$ defined as statistically significant.

Results

POLD1 expression correlates with poor prognosis of mesothelioma patients. To determine the clinical relevance of Pol δ, the potential association between *POLD1-4* expression levels and prognosis of patients with mesothelioma was assessed by Kaplan-Meier analysis. OS was found to be significantly lower in patients with high than low *POLD1* expression levels ($p<0.001$, Log-rank test) (Figure 2A). A comparison of *POLD1* expression in normal tissues and tumors from TCGA and GTEx datasets showed that *POLD1* expression was significantly higher in various solid tumor samples than in normal tissues (Figure 2B). In addition, western blotting analysis showed that POLD1 levels were higher in MPM cell lines than in human normal lung fibroblasts (Figure 2C).

Knockdown of POLD1 suppressed MPM cell proliferation. The cellular role of POLD1 was assessed by *POLD1* knockdown using specific siRNAs. Treatment of cells for 48 h with siPOLD1#1 and siPOLD1#2 suppressed *POLD1* mRNA and protein expression (Figure 3A and B). Moreover, treatment of MPM cell lines with siPOLD1 suppressed their proliferation, as shown by WST-8 cell proliferation assays (Figure 3C).

POLD1 depletion induced G_1 arrest. Flow cytometry analysis of the effects of siPOLD1 on the cell cycle in PI stained MPM cell lines. PI showed that siPOLD1 induced the accumulation of 211H cells in subG_1 phase and of the other five MPM cell lines in G_1 phase (Figure 4A and B). Flow cytometry evaluation of the intra-S-phase rate of DNA synthesis after a 30 min BrdU pulse showed that BrdU positive cells were decreased after siPOLD1 treatment for 48 h, indicating that siPOLD1 suppressed intra-S-phase DNA synthesis activity (Figure 4C). Taken together, these observations indicate that POLD1 depletion induced G_1 arrest, resulting in the suppression of DNA synthesis activity.

POLD1 depletion induced apoptosis with DNA damage and replication stress in MPM cells. Evaluation of apoptosis by Annexin-V/PI staining showed that the proportions of both early (Annexin-V^+/PI^-) and late (Annexin-V^+/PI^-) apoptotic cells increased following treatment of MPM cell lines with siPOLD1 (Figure 5A and B). Analysis of the effects of POLD1 depletion on DDR showed that the numbers of foci of cells with H2A.X phosphorylated at Ser139 (γ-H2AX), which are markers of double strand breaks (DSBs), were markedly higher after treatment with siPOLD1 than with siCtrl (Figure 5C). The ATM-Chk2-p53 pathway is activated by DSBs and regulates the cell cycle at the G_1/S checkpoint (15). Evaluation of ATM-Chk2 kinase activity by western blotting showed that siPOLD1-induced activation of ATM and Chk2 induced the phosphorylation of p53 at Ser20 (Figure 5D). Taken together, these findings showed that POLD1 is an essential molecule for overcoming DNA replication stress, such as DSBs at the G_1/S checkpoint.

Discussion

Because therapeutic options for patients with advanced MPM are limited, their prognosis remains poor. Relatively little is known about the mechanisms by which MPM becomes refractory to treatment, with most patients with inoperable MPM being treated with a combination of the cytotoxic

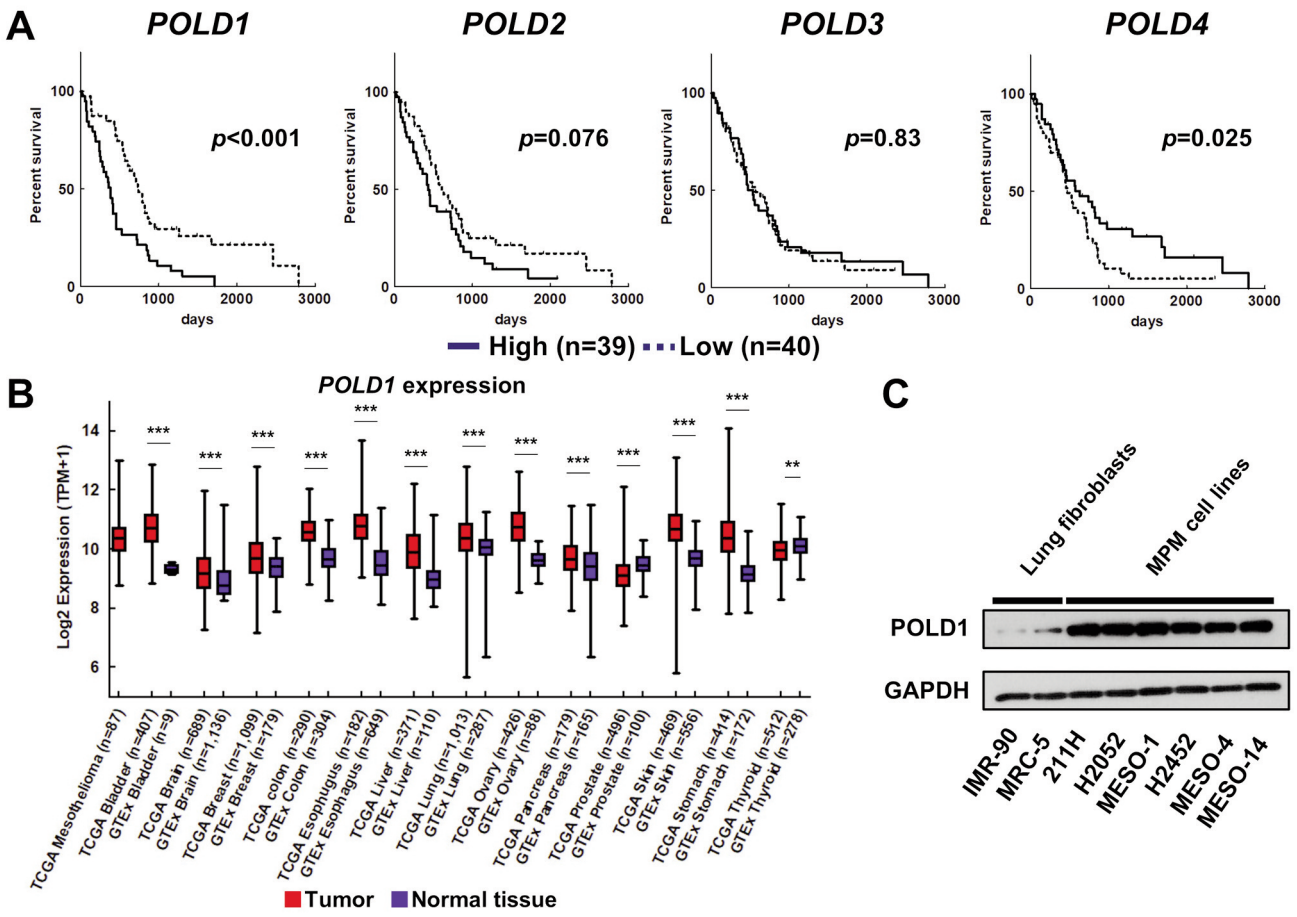

Figure 2. *Association between POLD1 mRNA expression and prognosis of mesothelioma patients. (A) Kaplan-Meier survival analysis of the associations between the levels of expression of DNA polymerase delta subunits POLD1-4 and overall survival of mesothelioma patients, based on RNA-seq analysis of DNA polymerase delta subunits POLD1-4 obtained from TCGA-MESO datasets. Comparisons by log-rank tests. (B) Comparison of POLD1 mRNA expression levels in tumor samples from TCGA and normal samples from the GTEx dataset. POLD1 expression levels were assessed using log2 (Transcript per million; TPM +1) and compared using Welch's t-test; **p<0.01, ***p<0.001. (C) Comparison of POLD1 protein expression levels in normal lung fibroblast cells and MPM cell lines using western blotting.*

anticancer drugs cisplatin and pemetrexed (16). The persistence of asbestos fibers in the pleura has been reported to be a continuous source of reactive oxygen species (ROS) and inflammation, which increase replication errors and genomic instability (7). Moreover, depletive germline variants in DNA repair genes, such as *BAP1* and *BRCA1/2*, have been found to enhance the development of asbestos-induced MPM (6, 17). These findings therefore suggested that the DDR pathway may be a therapeutic target for MPM.

Studies on the structure and function of DNA Pol δ subunits suggest that this polymerase plays a key role at replication forks following DNA damage (18). Multiple point mutations in *POLD1*, the catalytic subunit of Pol δ, have been detected in human cancers, as have amplifications of the loci encoding the accessory subunits *POLD2* and *POLD3* (9, 19). To date, however, the role of Pol δ in MPM development remains unclear. The present study therefore investigated whether Pol δ could be used as a novel therapeutic target in MPM by analyses of public database and *in vitro* experiments.

Analyses of TCGA-MESO datasets showed that high *POLD1* mRNA expression levels were significantly associated with shorter OS in patients with MPM. In addition, comparisons of TCGA and GTEx datasets showed that *POLD1* is amplified in various solid tumors. Western blotting analysis revealed that POLD1 was more highly expressed in MPM cell lines (MSTO-211H, NCI-H2052, ACC-MESO-1, NCI-H2452, ACC-MESO-4, Y-MESO-14) than in human normal lung fibroblasts (IMR-90 and MRC-5). Elevated levels of *POLD1* were found to correlate with cell cycle progression and cell survival in several types of cancer, with *POLD1* regulating genome stability through its effects on DNA replication and repair (20, 21). Taken together, these results

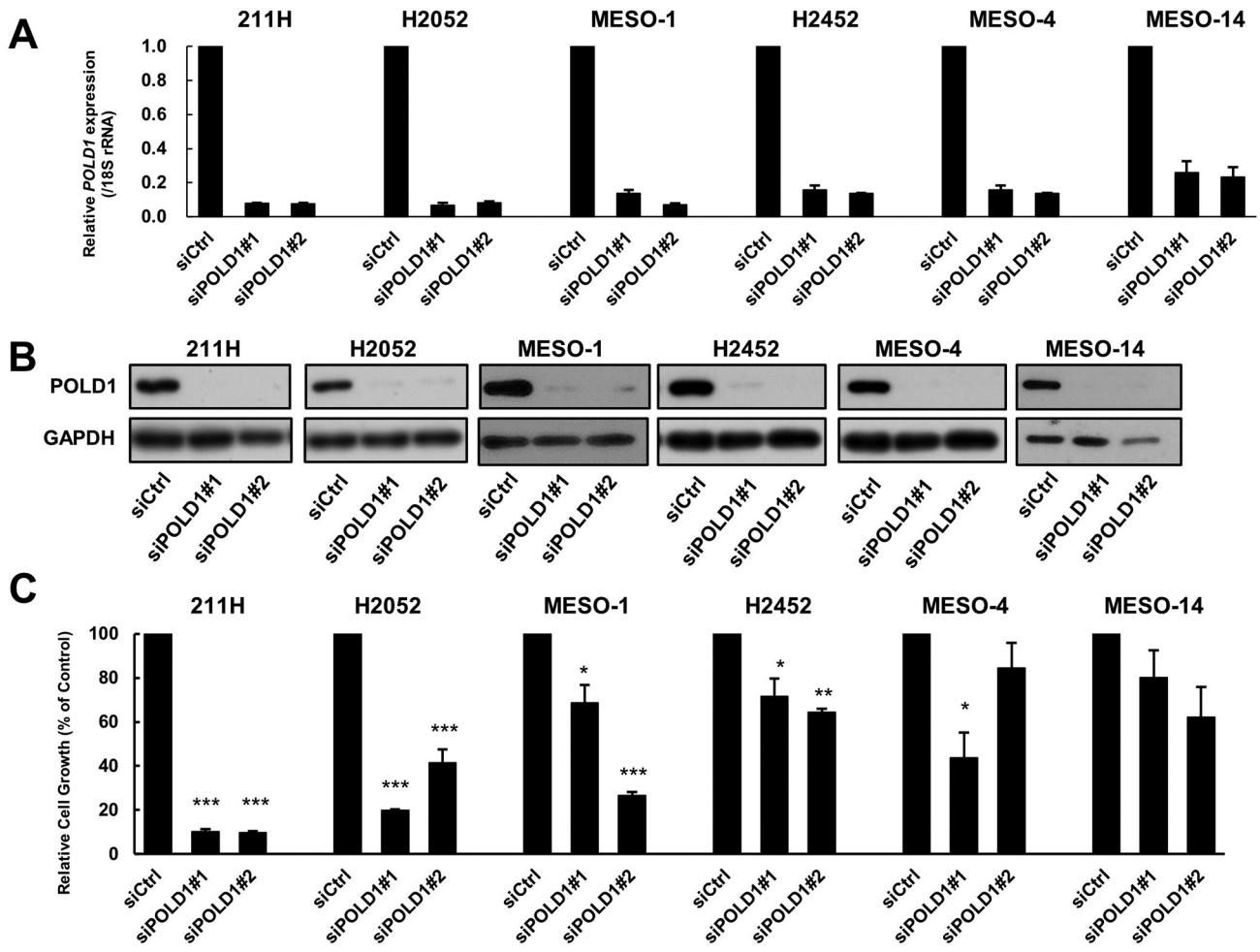

Figure 3. *POLD1 knockdown using siRNA and its effects on MPM cell proliferation. MPM cell lines were treated with 5 nM siRNA (siCtrl, siPOLD#1, or siPOLD#2) for 48 h. (A) Analysis of POLD1 mRNA levels by qRT-PCR. POLD1 expression was normalized to 18S rRNA expression as an internal control, with relative expression levels calculated using $2^{-\Delta\Delta Ct}$ methods, and data are presented as mean+SE of three independent biological experiments, each with three technical replicate samples. (B) Analysis of POLD1 protein levels by western blotting. POLD1 levels were normalized to those of GAPDH. Results shown are representative of two independent biological experiments. (C) Effect of siPOLD1 on the growth of MPM cells, as measured by WST-8 cell proliferation assays; results are presented as the growth rates of cells treated siPOLD1 relative to those of cells treated with siCtrl. WST-8 assay data are presented as mean+SE of three independent biological experiments, each with four technical replicate samples, and analyzed by one-way ANOVA, followed by Bonferroni's correction; *p<0.05, **p<0.01, ***p<0.001.*

suggested that POLD1 is required for DNA damage repair and progression of the cell cycle in MPM cells.

The anti-tumor effects of POLD1 inhibition were analyzed *in vitro* using siPOLD1 and MPM cell lines. WST-8 cell proliferation assays showed that both siPOLD1#1 and siPOLD1#2 inhibited the growth of MPM cell lines. Flow cytometry showed that siPOLD1 induced G_1 cell cycle arrest in almost MPM cell lines, whereas it induced 211H cells to accumulate in subG_1 phase, an indicator of cell death. Experiments using BrdU-pulsed 211H and H2052 MPM cells showed that siPOLD1 reduced the numbers of BrdU positive cells, suggesting that POLD1 depletion prevents DNA synthesis. These findings suggested that the accumulation of 211H cells in subG_1 phase resulted from siPOLD1-induced DNA replication stress at the intra-S-phase. POLD1 was previously reported to be important for S-phase progression under conditions of replicative stress and DNA damage (22). Taken together, these findings suggested that POLD1 is essential for overcoming DNA replication stress in intra-S-phase and for progression of the cell cycle at the G_1/S checkpoint.

The present study also assessed of the roles of POLD1 in regulating apoptosis and the replication stress pathway. Flow cytometric analysis using annexin-V/PI staining showed that siPOLD1 induced both early (Annexin-V^+/PI^-) and late

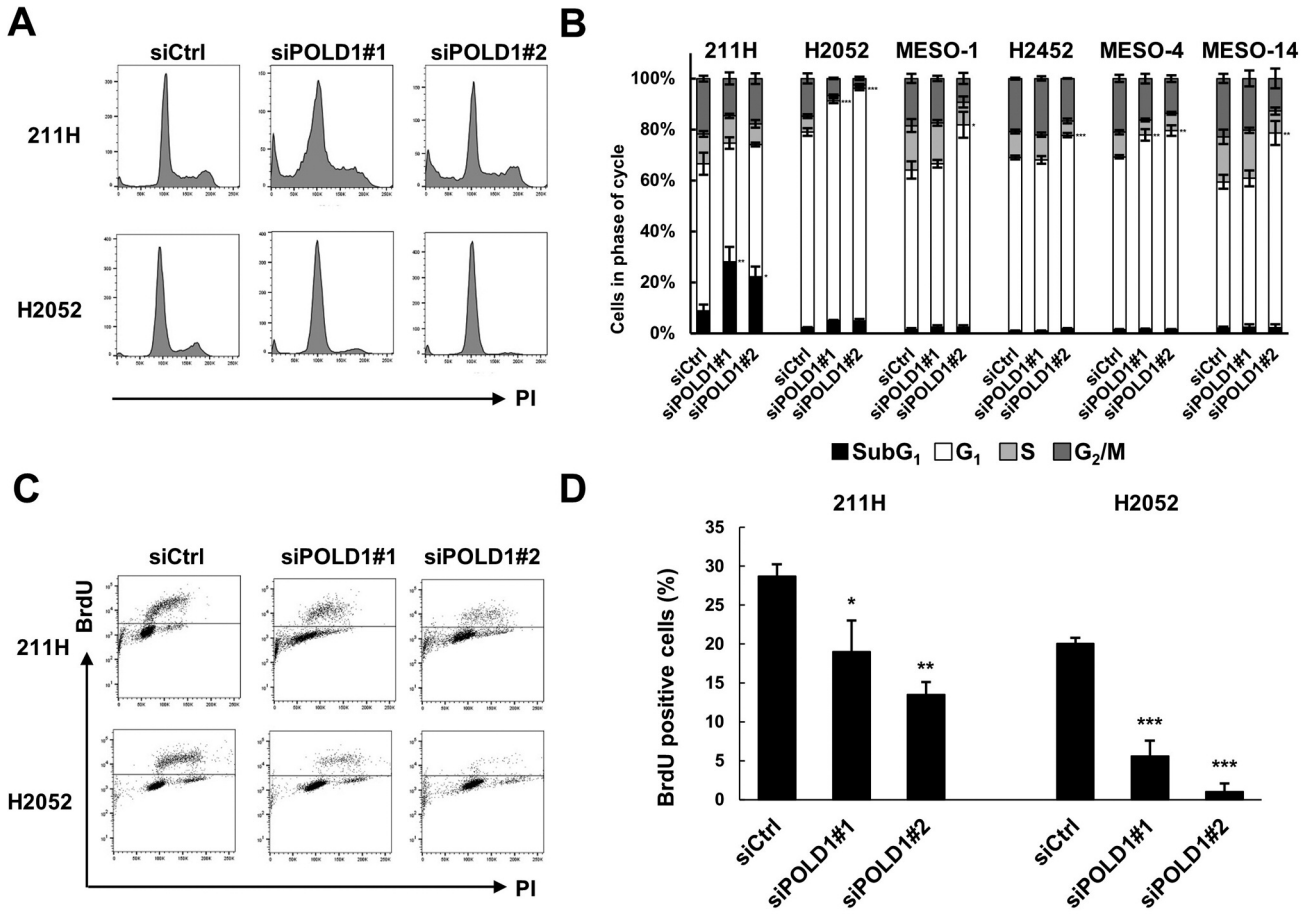

Figure 4. *POLD1 depletion induces subG$_1$ phase and cell cycle arrest at the G$_1$/S checkpoint. (A) Propidium iodide (PI) staining of MPM cells after treatment with siPOLD1 for 48 h. (B) Quantitation of the numbers of cells in subG$_1$, G$_1$, S, and G$_2$/M phases of the cell cycle. Results are presented as the mean±standard deviation (SD) of three independent biological experiments (C, D) Flow cytometry analysis of BrdU labeled cells, as determined by FITC-conjugated -BrdU mAb. (D) Quantitation of the numbers of FITC positive cells. Results are presented as the mean+SD of three independent biological experiments and analyzed by one-way ANOVA, followed by Bonferroni's correction; *p<0.05, **p<0.01, ***p<0.001.*

(Annexin-V$^+$/PI$^+$) phase apoptosis in MPM cell lines. To determine whether siPOLD1-induced apoptosis correlated with DNA damage, phosphorylation of the Ser139 residue of the histone variant H2A.X (γH2A.X) was quantitated as a marker of DSBs. Long-term (120 h) treatment of MPM cells with siPOLD1 induced apoptosis involving the accumulation of γH2A.X. Because ATM auto-phosphorylation at Ser1981 had been shown to trigger local chromatin modifications, including phosphorylation of H2A.H, as a sensor of DSBs (15, 23), the effect of siPOLD1 treatment on activation of the ATM pathway was analyzed. We found that siPOLD1 treatment of MPM cells for 24 h led to the accumulation of phosphorylated Chk2 (Thr68) and p53 (Ser20). Thus, MPM cells depend on POLD1 for DDR and cell cycle progression under conditions of DNA replication stress, with depletion of POLD1 leading to G$_1$ arrest and apoptosis involving accumulation of DNA damage.

Conclusion

Analysis based on TCGA-MESO datasets showed that high *POLD1* mRNA expression levels were significantly associated with shorter OS in patients with MPM. Further examination revealed that POLD1 depletion led to cell cycle arrest, suppression of DNA synthesis, and apoptosis caused by DNA damage in MPM. Taken together, these findings suggest that POLD1 is essential for DDRs in MPM cells and that POLD1 may be a novel therapeutic target in patients with MPM.

Conflicts of Interest

The Authors have no conflicts of interest to disclose with regards to this study.

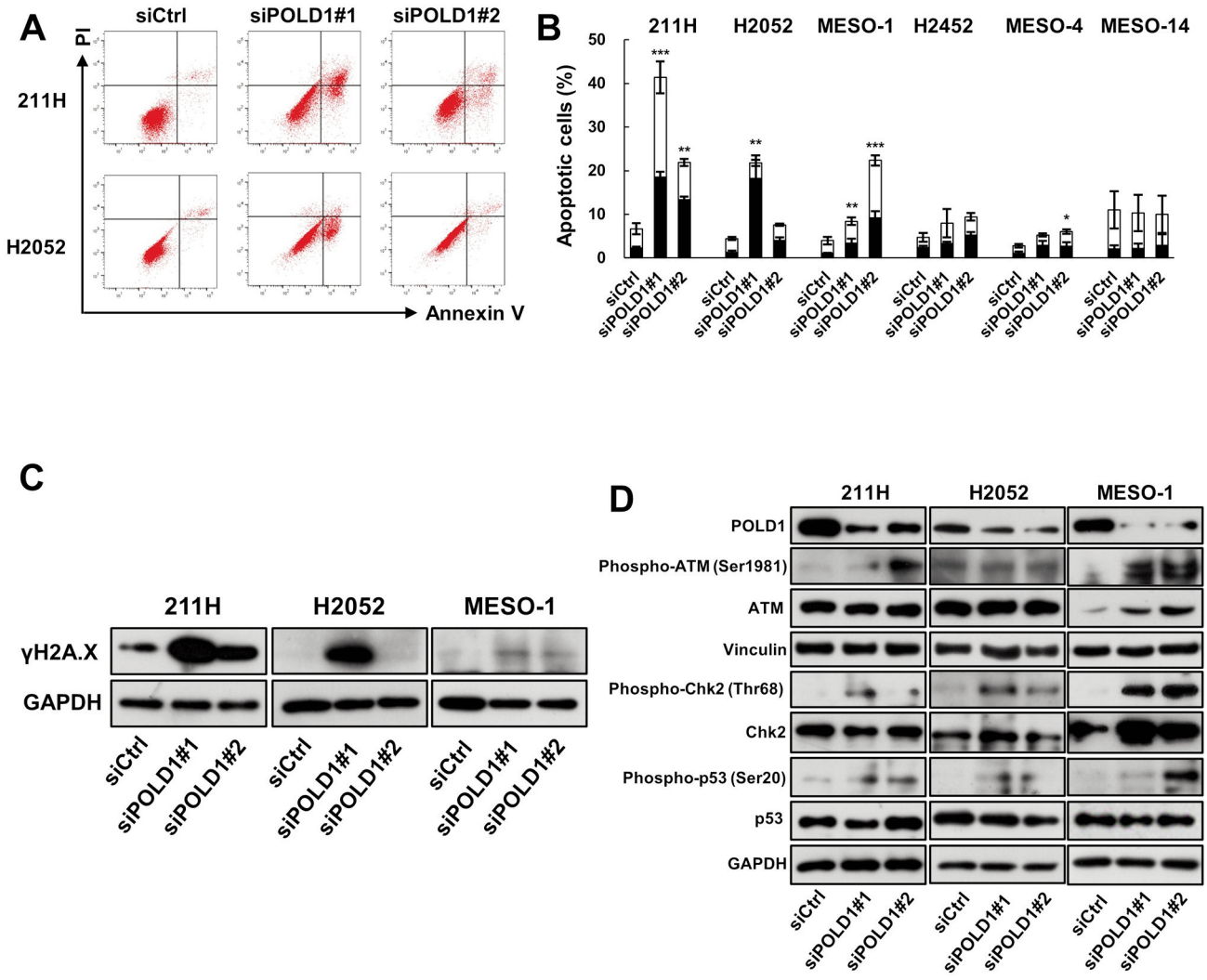

Figure 5. *Cytotoxicity of siPOLD1, as determined by its effects on cell apoptosis and the DNA replication stress pathway. (A-B) MPM cells were treated with siPOLD1 for 120 h, and cell apoptosis was determined by flow cytometry using annexin-V-FITC and propidium iodide (PI) staining. (B) Data in (A) are presented as the mean±SD of three independent biological experiments. (C-D) Western blotting analysis of (C) γH2A.X and (D) phosphorylation of the ATM-Chk2-p53 pathway as a sensor of DSBs. GAPDH and vinculin were used as loading controls. Results shown are representative of two independent biological experiments.*

Authors' Contributions

Daiki Shimizu and Miku Ishibashi performed experiments and analyzed data; Tadaaki Yamada, Yuki Toda, Shigekuni Hosogi and Eishi Ashihara provided intellectual guidance; Daiki Shimizu and Eishi Ashihara drafted the manuscript; Daiki Shimizu and Eishi Ashihara conceptualized the study.

Acknowledgements

The Authors thank Mako Tomogane, PhD, and Yusuke Sano, Ph.D., of the Laboratory of Clinical and Translational Physiology at Kyoto Pharmaceutical University, for their technical advice and assistance.

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Received October 19, 2023
Revised December 13, 2023
Accepted December 22, 2023