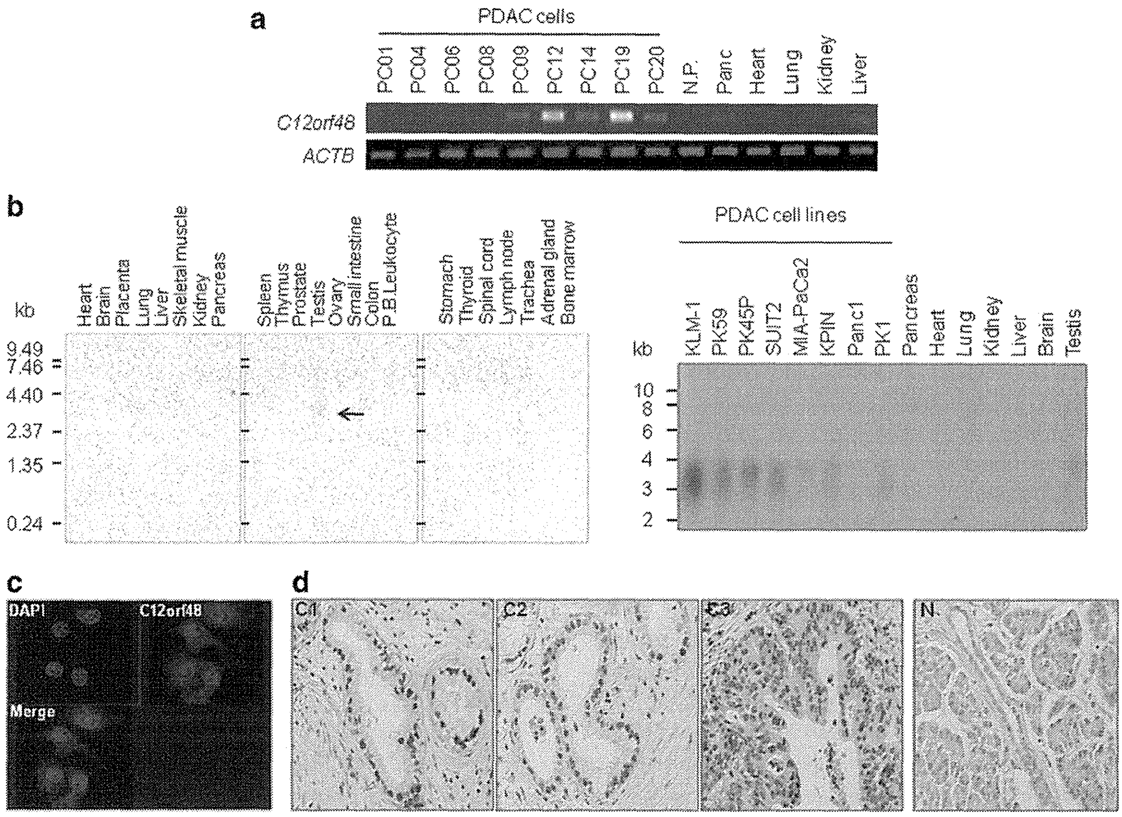


Figure 1. Overexpression of C12orf48 in PDAC cells. (a) Semi-quantitative RT-PCR validated that C12orf48 expression was upregulated in the microdissected PDAC cells (Lanes 5–9), compared with microdissected normal pancreatic ductal cells (NP), whole normal pancreatic tissue (Panc), and vital organs (heart, lung, kidney, and liver). Expression of *ACTB* served as the quantitative control. (b) Left panel; multiple tissue Northern blot analysis showed the limited expression of *C12orf48* in the testis, among the human adult organs. Right panel; Northern blot analysis for *C12orf48* expression showed that several PDAC cell lines (KLM-1, PK-59, PK-45P, and SUIT-2) strongly expressed *C12orf48*, while other normal adult organs did not. (c) Immunocytochemical analysis with anti-C12orf48 polyclonal antibody showed that C12orf48 protein (green) was localized in the nuclei of KLM-1 cells. (d) Immunohistochemical study on PDAC tissues with anti-C12orf48 antibody. *C12orf48 was strongly stained in the nuclei of PDAC cells (C1 × 200, C2 × 200, C3 × 200)*, while it was not stained in acinar cells and ductal epithelium cells of normal pancreatic tissues (N, ×200). In total, 21 of 31 (67.7%) PDAC tissues showed positive staining for C12orf48.

et al., 2004), we here focused on a novel gene *C12orf48* for this study. Semi-quantitative reverse transcription (RT)-PCR confirmed *C12orf48* overexpression in five of the nine pancreatic cancer cases examined (Fig. 1a). Northern blot analysis using the *C12orf48* cDNA fragment as a probe confirmed abundant expression of a 4-kb transcript in most of the eight PDAC cell lines we examined, but its expression was hardly detectable in any normal organs except the testis (Fig. 1b). The predicted C12orf48 protein does not contain any reported motifs or conserved domains in the database, but PSORTII program indicated C12orf48 likely to be a nuclear protein, which was confirmed by following immunocytochemical analysis using anti-C12orf48 polyclonal antibody we generated (Fig. 1c). Moreover, immunohistochemical analysis using anti-C12orf48 antibody showed positive signals in the nuclei of 21 of 31 PDAC tissues (Fig. 1d, panels C1–C3), whereas no staining was observed in any of normal pancreatic tissues (panel *n* in Fig. 1d).

### Attenuation of PDAC Cell Viability by C12orf48 Knockdown

To investigate the biological significance of C12orf48 in PDAC cells, we constructed shRNA-expression vectors specific to *C12orf48* (si1, si2, si3) as well as that to siEGFP as a negative control, and transfected each of them into KLM-1 and SUIT-2 cells. Semi-quantitative RT-PCR showed significant knockdown effects on *C12orf48* expression in the cells transfected with si1 and si3, compared with the control (Fig. 2a). MTT assay and colony formation assay revealed that depletion of C12orf48 in KLM-1 and SUIT-2 cells (Figs. 2b and 2c) caused dramatic

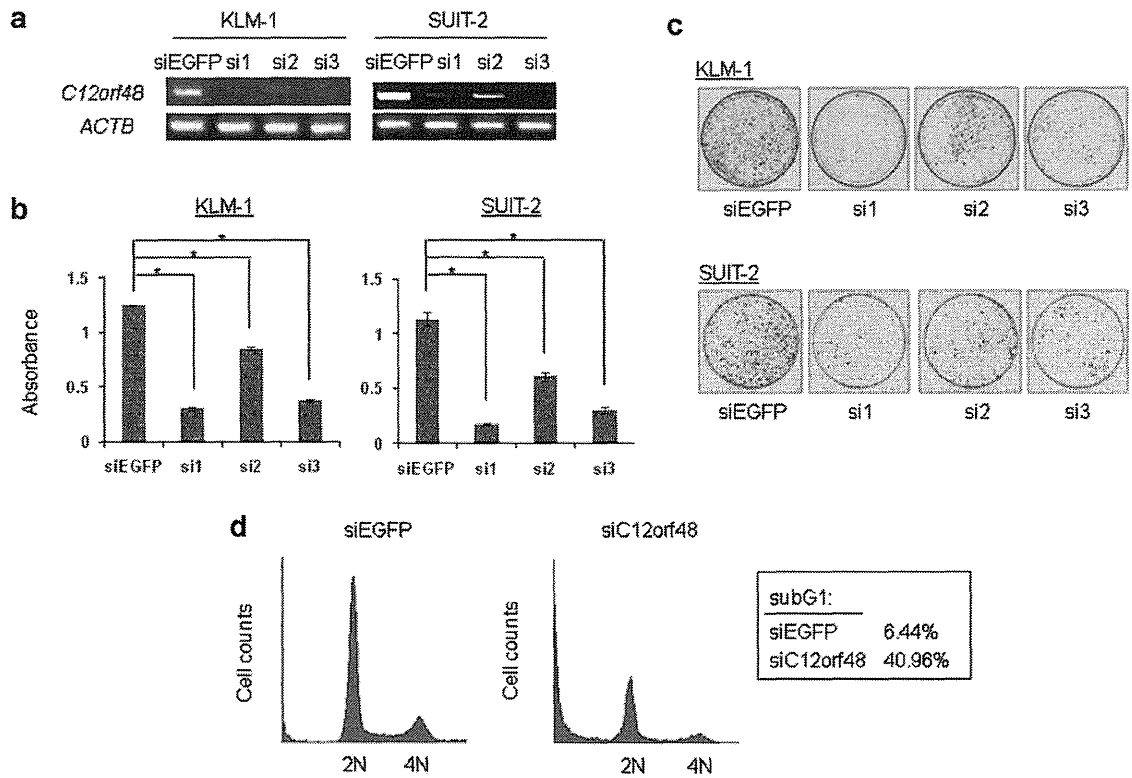


Figure 2. Effect of C12orf48-shRNA on growth of PDAC cells. (a) Semi-quantitative RT-PCR examined the knockdown effect on *C12orf48* expression in PDAC cells (KLM-1 and SUIT-2 cells) transfected with shRNA-expressing vectors specific to *C12orf48* (si1, si2, si3) or control shRNA (siEGFP). (b) PDAC cells transfected with C12orf48 si1, or si3 shRNA vectors showed a drastic reduction in their viabilities. Each average is plotted with error bars indicating the standard deviation (SD) after 6-day incubation with Geneticin. Y-axis means absorbance at 490 nm, and at 630 nm as reference, measured with a microplate reader. These experiments were carried out in triplicate (*$P < 0.005$, Student's *t* test). (c) Colony formation assays in PDAC cells after *C12orf48* knockdown. Cells were stained with 0.1% crystal violet after 14-day incubation with geneticin. (d) FACS analysis was performed 96 hr after transfection with the indicated siRNA. The percentage of cells in subG1 phase was calculated. Treatment of KLM-1 with siRNA specific to *C12orf48* caused a drastic increase in sub-G1 population (40.96%).

reduction in the number of viable cells. Furthermore, we performed FACS analysis after depletion of C12orf48 by siRNA oligonucleotide in KLM-1 cells and found a drastic increase of cells at sub-G1 population (40.96%, Fig. 2d). We also observed similar effects of siRNA oligonucleotide for C12orf48 in SUIT-2 cells (data not shown). These findings indicated that C12orf48 could play critical roles in the growth of PDAC cells.

### Interaction of C12orf48 with PARP-1

Since the biological functions of C12orf48 remain totally unknown, we attempted to isolate a protein(s) that could physically interact with C12orf48 protein. Protein complexes were immunoprecipitated by anti-Flag $M_2$ agarose from the lysates of the HEK293 cells in which Flag-tagged C12orf48 was exogenously introduced. The immunoprecipitated complexes were separated on SDS-PAGE and silver-stained. We found three bands (110, 90, and 63 kDa) in the immunoprecipitated complexes from the lysates of C12orf48-overexprerssing cells, but not in those from the mock cells (Fig. 3a). Among the three bands, the 63 kDa-band was considered to be Flag-tagged C12orf48 itself. We excised 110- and 90-kDa bands, and analyzed them by LC-MS/MS as described in Materials and Methods. As a result, the 110-kDa protein coimmunoprecipitated with C12orf48 protein was identified to be PARP-1 and the 90-kDa protein to be HSP90α (Fig. 3a). Immunoblotting by anti-PARP-1 antibody confirmed that PARP-1 was coimmunoprecipitated with Flag-tagged C12orf48 protein (Fig. 3b). Moreover, C12orf48 was also confirmed to be coimmunoprecipitated with PARP-1 protein (Fig. 3c).

### Positive Regulation of PARP-1 Activity by C12orf48

To investigate the functional significance of the interaction between PARP-1 and C12orf48, PARP-1 automodification was investigated by incorporation of $[^{32}P]NAD^{+}$ in the absence or presence of purified

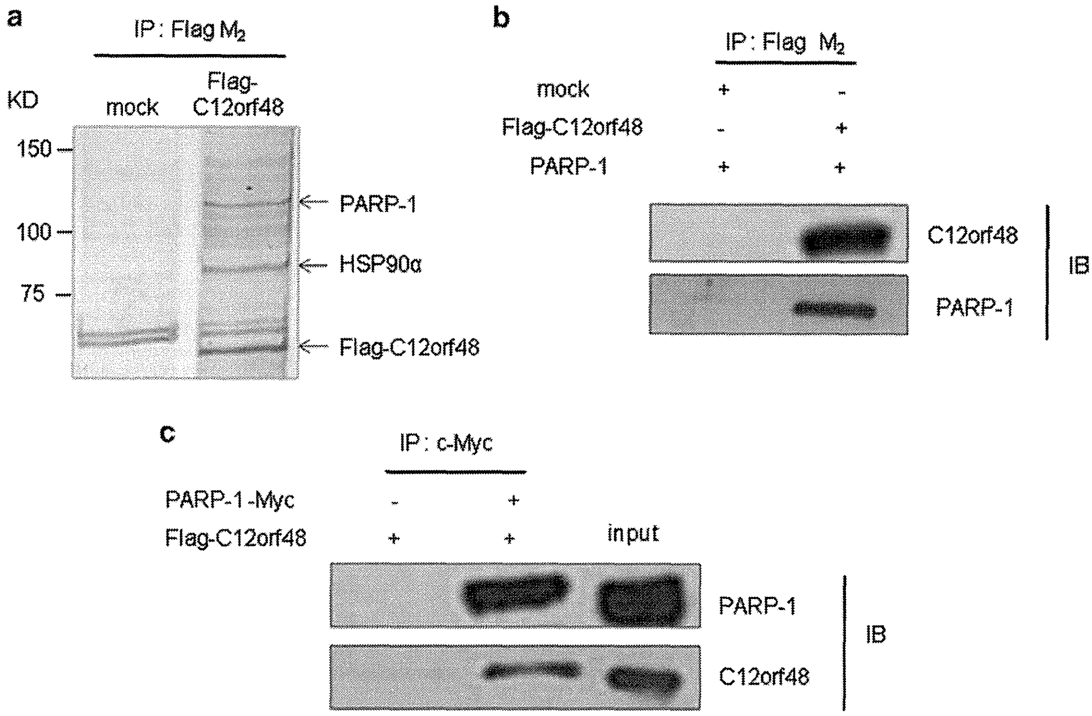


Figure 3. Identification of PARP-1 as an interacting protein of C12orf48. (a) Silver-staining of the immunoprecipitated complex separated on SDS-PAGE. Protein complexes were coimmunoprecipitated by anti-Flag $M_2$ agarose from the lysates of the HEK293 cells transfected with Flag-C12orf48 (right lane) or with mock (left lane). Two differential bands as well as Flag-C12orf48 (63 kD) were observed, and LC-MS/MS analysis identified PARP-1 (110 kD) and HSP90α (90 kD) as interacting proteins of C12orf48. (b) Western blot analysis using anti-PARP-1 antibody confirmed that PARP-1 protein was coimmunoprecipitated with Flag-C12orf48 protein. (c) Flag-C12orf48 expression vector was transfected to HEK293 cells without or with PARP-1-Myc expression vector. Cell lysates were immunoprecipitated by anti-Myc antibody. Flag-C12orf48 protein was coimmunoprecipitated with Myc-tagged PARP-1 protein.

recombinant C12orf48 protein in vitro. As shown in Figures 4a and 4b, we observed that addition of C12orf48 protein significantly enhanced the incorporation of $[^{32}P]NAD^+$ to recombinant PARP-1 protein in a dose-dependent manner when damaged DNA was coincubated, while this enhancement of PARP-1 automodification by C12orf48 was not observed in the absence of damaged DNA (data not shown). Furthermore, we transiently introduced C12orf48 into HEK293 cells and measured the PARP-1 activities in their lysates by the colorimetric PARP assay. In this experiment, PARP-1 in the cell extracts were activated by incubation with nicked DNA as described in Materials and Methods. As a result, we observed that PARP-1activities in the cell extracts were significantly enhanced by overexpression of C12orf48 (Fig. 4c).

## Reduction of PARP-1 Activity by Depletion of C12orf48 in PDAC Cells

To examine the effect of C12orf48 on the PARP-1 activity in PDAC cells, we knocked down the expression of C12orf48 or PARP-1 itself in two PDAC cell lines, KLM-1 and SUIT-2, and measured the activities of PARP-1 in their cell lysates by the colorimetric PARP assay. The knockdown effects on C12orf48 and PARP-1 expression in KLM-1 and SUIT-2 cells were confirmed with anti-C12orf48 and anti-PARP-1 antibodies (Fig. 5a). Concordant with C12orf48 expression, the PARP-1 activities to modify histone H1 were decreased to 40.8 and 34.8% in C12orf48-depleted KLM-1 and SUIT-2 cells, respectively, compared with the control cells (Fig. 5b). The magnitude of this suppressive effect of C12orf48 on PARP-1 activity was almost same as the effect when PARP-1 itself was knocked down (Fig. 5b). Furthermore, we examined the level of poly(ADP-ribosyl)ation in the C12orf48-depleted cells by Western blot analysis using anti-poly (ADP-ribose) (PAR) antibody. As shown in Figure 5c, poly(ADP-ribosyl)ated proteins were detected at high molecular weights of more than 250 kD in the siEGFP-transfected control cells, while these poly(ADP-ribosyl)ation

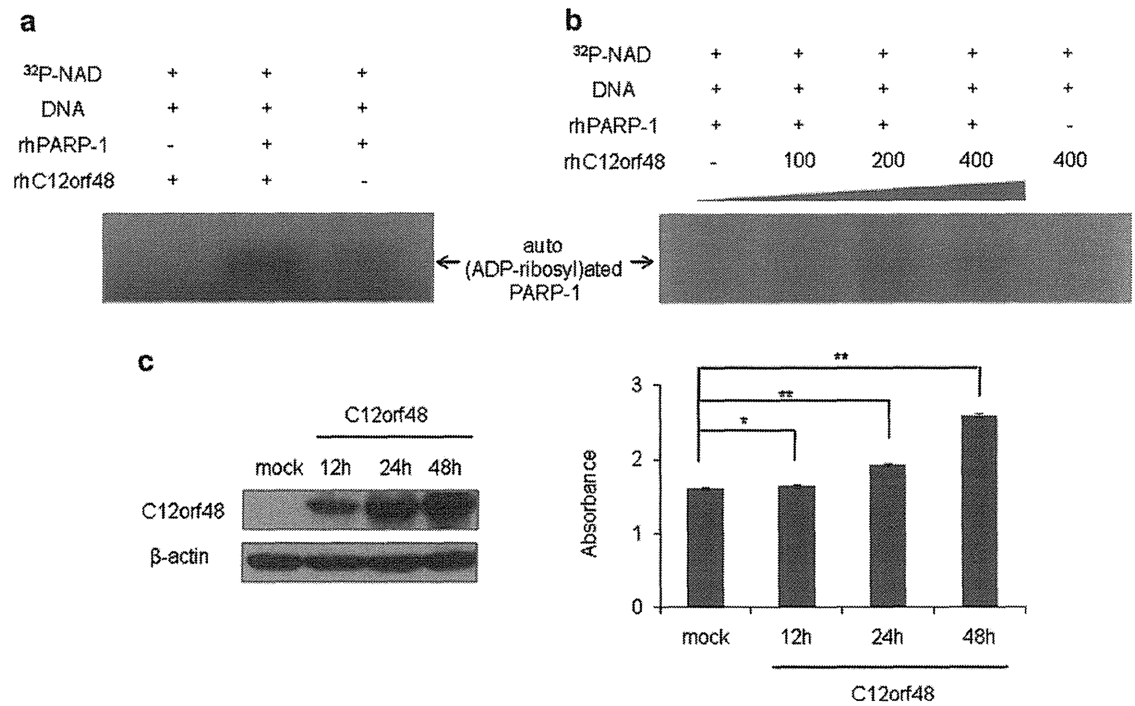


Figure 4. Regulation of PARP-1 activity by C12orf48. (a) Effect of C12orf48 on the auto(ADP-ribosyl)ation of PARP-1 in vitro. PARP-1 was automodified by incorporation of $[^{32}P]NAD^+$. Lane1, purified recombinant C12orf48 protein alone; lane2, both recombinant C12orf48 and recombinant PARP-1 protein; lane3, recombinant PARP-1 alone. (b) In vitro PARP-1 auto(ADP-ribosyl)ation was enhanced by increasing amount of recombinant C12orf48 protein. (c) HEK293 cells were transfected with C12orf48-expressing vector and harvested at indicated time points. The expression of C12orf48 was quantified by the immunoblot (left panel). The activities of PARP-1 to modify histone H1 were measured using the colorimetric PARP assays (right panel). These experiments were performed three times (*$P < 0.05$, **$P < 0.0001$, Student's *t* test).

was drastically reduced in the cells treated with C12orf48-siRNA or PARP-1-siRNA. PARP-1 is known to be the primary target for PARP-1-mediated (ADP-ribosyl)ation in vivo, with greater than 90% of PAR found on PARP-1 (Ogata et al., 1981; Huletsky et al., 1989; D'Amours et al., 1999). Automodified PARP-1 is detected clearly as a high-molecular form due to poly (ADP-riobse) formation. Therefore, it seemed that most of PAR proteins detected here were originated from auto(ADP-ribosyl)ated PARP-1. It suggested that depletion of C12orf48 could decrease PARP-1 enzymatic activity both in vivo and in vitro. In addition, depletion of PARP-1 by siRNA in KLM-1 and SUIT-2 cells (Supplementary Fig. 1) induced significant reduction in the number of viable cells. Together, these findings presumably explain that C12orf48 depletion lead to the reduction of pancreatic cancer cell viability, in part, through its direct interaction with PARP-1. However, it cannot be excluded that other C12orf48-specific and PARP1-independent effects can also affect cancer cell viability, and further study is required to clarify the roles of C12orf48 in cancer.

### Sensitization of PDAC Cells to DNA Damage by C12orf48 Depletion

PARP-1 activity is relevant for the ability of cells to repair damaged DNA. It has been reported that inhibition of PARP-1 activity could increase the susceptibility of cells to DNA damaging agents (Daniel et al., 2009; Horton et al., 2009). Given the findings that C12orf48 could regulate PARP-1 activity, we assessed that the C12orf48 depletion could sensitize cancer cells to various DNA damaging agents. As expected, C12orf48-depleted KLM-1 cells showed much higher sensitivities to Adriamycin treatment, UV irradiation, and $H_2O_2$ treatment (Fig. 6a). These findings suggested that C12orf48 might protect cancer cells from cell death following the DNA damage or cellular stresses in cancer cells through the regulation of poly(ADP-ribosyl)ation activity of PARP-1.

### Effect of C12orf48 in Cell-Cycle Checkpoint

Cell-cycle checkpoints are considered to facilitate DNA repair before entering the next cell-

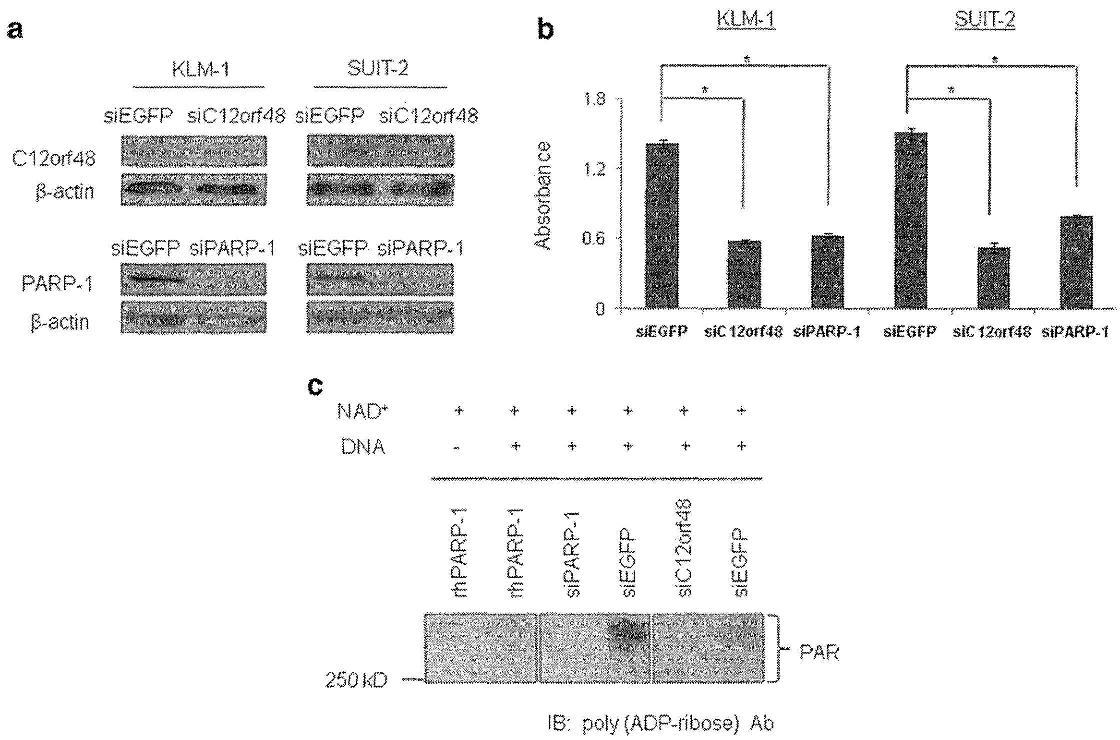


Figure 5. C12orf48-depletion reduced PARP-1 activity in PDAC cell extracts. (a) Western blot analysis using anti-C12orf48 and anti-PARP-1 antibodies confirmed knockdown effects of C12orf48 and PARP-1 expression in KLM-1 cells and SUIT-2 cells. (b) The activities of PARP-1 in the C12orf48-depleted or PARP-1-depleted cells were measured by the colorimetric PARP assays. $^{*}P < 0.005$ by Student's t test. (c) PARP-1 enzymatic activities were investigated by anti-PAR antibody. Lane 1 and 2, recombinant PARP-1 protein; lane 3-6, KLM-1 cell extracts transfected with indicated siRNA. Automodified PARP-1 was detected at high molecular weights (>250 kD) only in the mixture with nicked DNA (Lane 2), but not the mixture without nicked DNA (Lane 1). Poly(ADP-ribosyl)ated proteins were observed at higher molecular weights (>250 kD) in the lysates of the siEGFP-transfected KLM-1 cells (Lanes 4 and 6). However, these poly(ADP-ribosyl)ation was diminished in the extracts of KLM-1 cells treated with PARP-1-siRNA (Lane 3) or C12orf48-siRNA (Lane 5).

cycle phases. Loss or attenuation of the checkpoint function may increase chances to cause gene mutations and chromosomal aberrations by affecting completion of the appropriate DNA repair. To test the effect of C12orf48 depletion on the cell-cycle progression, KLM-1 cells were synchronized at the G1-phase with aphidicolin treatment. After the release from the cell-cycle arrest, the cells depleted C12orf48 or PARP-1 entered into S-phase much faster than those treated with the control siEGFP (Fig. 6b). Six hours after the release from the arrest, approximately 73.3% of C12orf48-depleted cells and 75.7% of PARP1-depleted cells were already at S-phase. On the other hand, only 26.1% of the control cells (siEGFP) entered to S-phase. These findings indicated that depletion of C12orf48 or PARP-1 in PDAC cells could have some checkpoint dysfunction and resulted in very rapid progression from G1 to S-phase. We subsequently investigated the involvement of C12orf48 in the cell-cycle checkpoints after DNA damage by measuring the cell population at each cell-cycle phase after γ-irradiation. KLM-1 cells transfected with C12orf48-siRNA or siEGFP (as a control) were exposed to 3 Gy of γ-irradiation, and collected at various time-points after irradiation. Subsequent FACS analysis showed that C12orf48 depletion in KLM-1 cells enhanced G2/M arrest, compared with the control cells (Fig. 6c). Similarly, PARP-1 inhibitors were reported to enhance the G2 arrest after γ-irradiation (Nozaki et al., 1994). Taken together, our data implied that a decrease of PARP-1 activity in C12orf48-depleted cancer cells resulted in an enhancement of G2 arrest after γ-irradiation.

## DISCUSSION

In this study, we focused on a novel gene *C12orf48*, one of the genes that were identified to be transactivated in PDAC cells through our genome-

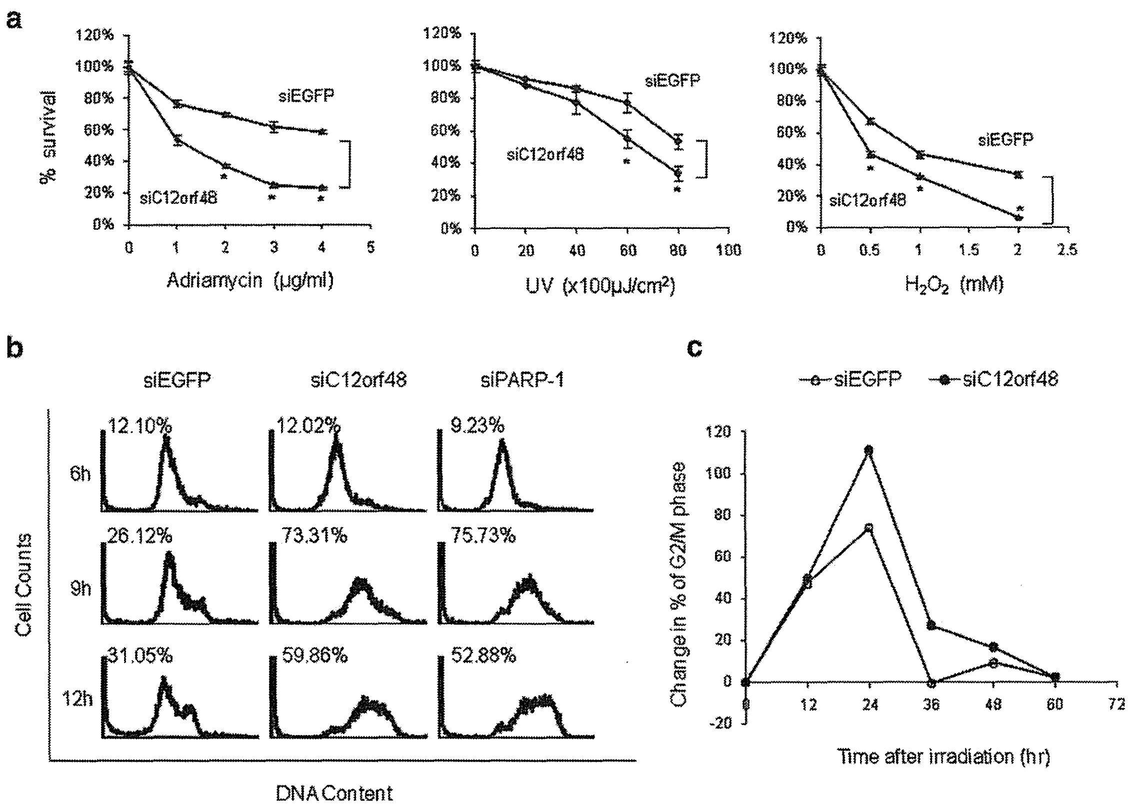


Figure 6. C12orf48-depletion sensitized PDAC cells to DNA damage. (a) The survival was reduced in the C12orf48-depleted KLM-1 cells after the exposure to DNA damaging agents. Data are shown as the averaged value from three experiments. X-axis represents the concentration of Adriamycin (left) and $H_2O_2$ (right) or the intensity of UV for DNA damaging. Y-axis represents the relative ratio of the cell numbers that was calculated in absorbance of the diameter by comparison with the absorbance value of damage-negative cells as a control. $^{*}P < 0.05$, by Student's *t* test. (b) FACS analysis demonstrated S-phase progression of synchronized KLM-1 cells. Cells were synchronized at G1-phase by aphidicolin treatment and released into S-phase by culturing in aphidicolin-free media. The population of S-phase was calculated. (c) The effects of C12orf48 depletion on the population of G2/M phase after $\gamma$-irradiation in KLM-1 cells. The KLM-1 cells with or without C12orf48 depletion were irradiated at the dose of 3 Gy, and then collected at the indicated time points for flow cytometry analysis. X-axis represents the time points after $\gamma$-radiation; Y-axis shows the relative population changes of G2/M phase by comparison with the G2/M population value of the KLM-1 cells without irradiation.

wide expression profile analysis (Nakamura et al., 2004). Its overexpression was also observed in other therapy-resistant malignancies, such as cholangiocarcinoma (Jinawath et al., 2006), castration-resistant prostate cancer (Tamura et al., 2007), and relapsed small-cell lung cancer (Taniwaki et al., 2006), indicating that it might be featured at therapy-resistant or aggresive malignancies. On the other hand, its expression was hardly detectable in any normal adult organs except the testis, implicating C12orf48 to be a cancer-testis antigen. Depletion of C12orf48 in some of PDAC cells resulted in significant reduction of cancer cell viability and survival, implying its critical roles in pancreatic carcinogenesis.

Importantly, we demonstrated that C12orf48 protein could physically interact with PARP-1 and positively regulate the enzymatic activity of PARP-1, suggesting that C12orf48, termed PARP-1 binding protein (PARPBP), might be involved in multiple cellular processes including DNA repair, chromatin modification, cell-cycle progression and genomic stability through the interaction and regulation of PARP-1. PARP-1, as a DNA nick-sensor, binds to DNA single-strand breaks (SSBs) and double-strand breaks (DSBs), and has an emerging and indispensable role in their repair. In regard to DNA damage signaling, PARP-1 is promptly stimulated and recruits the enzymes required for DNA repair to the site of DNA damage. Hence, the activity of PARP-1 plays a key role in signaling and initiating these processes. We demonstrated that C12orf48-depletion sensitized some of PDAC cells to DNA damage, suggesting that C12orf48 is likely to participate in the process of DNA repair through the regulation of PARP-1. Recent studies indicated that PARP-1 could be stimulated through its binding to nucleosomes, and modulate chromatin structures (Kim et al., 2004). Although the underlying mechanisms of PARP-1 in the modulation of chromatin structure are largely unknown, our results indicate that C12orf48 could possibly be involved in the chromatin modulation as well. Hence, development of drugs inhibiting the interaction between C12orf48/PARPBP and PARP-1 should be a good therapeutic approach to achieve very specific cytotoxicy to some of pancreatic cancer cells with minimum risk of adverse effects to normal organs.

C12orf48 has no known functional motif or conserved domain. However, a previous study on a mouse homologue of C12orf48 protein suggested its high binding affinity to single-stranded DNA and polyA homopolymers (Borsu et al., 2000). Cell-cycle checkpoints are essentially critical to ensure the fidelity of cell division in cells for verification of each of the cell-cycle processes that need to be accurately completed before going into the next phase. In our studies, we showed that knockdown of C12orf48 as well as PARP-1 caused the failure of the G1/S cell-cycle checkpoint which would usually prevent the replication of cells having defects in DNA. Hence, this G1/S checkpoint failure induced by depletion of C12orf48 or PARP-1 in cancer cells could increase a possibility of accumulation of genetic mutations and/or genomic instability, resulting in growth retardation of cancer cells. Moreover, knockdown of C12orf48 in cancer cells enhanced G2/M arrest in PDAC cells after γ-irradiation, consistent with previous reports describing that PARP-1 inhibitors enhanced the G2 arrest after γ-irradiation (Nozaki et al., 1994). However, since the underlying mechanism of PARP-1 enzymatic activity in G2-arrest regulation is unclear, additional studies will be required to clarify it.

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