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Master's Thesis

Protein Phosphatase 5 participates in the regulation of 53BP1 after DNA damage

Graduate School of Chosun University

Department of Bio - Materials Engineering

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DNA 손상 반응과정 중 Protein Phosphatase 5 에 의한
53BP1 조절기작에 대한 연구

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Advisor: Professor Ho Jin You

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ABSTRACT

Protein Phosphatase 5 participates in the regulation of 53BP1 after DNA damage

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The DNA double-strand break (DSB) is the principle cytotoxic lesion for ionizing radiation and radio-mimetic chemicals but can also be caused by mechanical stress on chromosomes or when a replicative DNA polymerase encounters a DNA single-strand break or other type of DNA lesion. Inaccurate repair or lack of repair of a DSB can lead to mutations or to genomic instability, cancer development or cell death according to apoptosis. One of the earliest events in the damage response is phosphorylation of histone H2AX at Ser139 by members of the phosphatidylinositol-3 kinase-like family of kinases such as ataxia telangiectasia mutated(ATM), ataxia telangiectasia related(Rad 3, ATR),

ATM related kinase(ATX), DNA dependant protein kinase(DNA-PK) to create γ-H2AX. Within minutes of DNA damage, γ-H2AX appears at discrete nuclear foci that contain DNA repair factors like the MRN repair complex, 53BP1, BRCA1, and MDC1. Through yeast two-hybridization system we could find several cDNAs putative interacting with 53BP1. After DNA sequencing, we found that one is a cDNA for PP5, serine/threonine phosphatase family. The binding of PP5 with 53Bp1 was highly increased after DNA damage. To evaluate the interaction of PP5 and 53BP1, we made overexpressed- and suppressed-PP5 cell lines and confirmed the effects by Western blotting and immunofluorescense analysis after DNA injury. So, we could investigate that the dephosphorylation of 53BP1 was regulated by PP5 and also influenced the maintenance and accumulation of 53BP1 foci.

I. INTRODUCTION

The DNA double-strand break (DSB) is the principle cytotoxic lesion for ionizing radiation and radio-mimetic chemicals but can also be caused by mechanical stress on chromosomes or when a replicative DNA polymerase encounters a DNA single-strand break or other type of DNA lesion. Inaccurate repair or lack of repair of a DSB can lead to mutations or to genomic instability, cancer development or cell death according to apoptosis (42). Cellular responses to DNA damage are tightly controlled by a group of checkpoint proteins to ensure genomic integrity and stability. DSBs are repaired by two major pathways, homologous recombination (HR) and non-homologous end joining (NHEJ). During HR, the damaged chromosome enters into synapsis with, and retrieves genetic information from, an undamaged DNA molecule with which it shares extensive sequence homology. In contrast, NHEJ bring about the ligation of two DNA DSBs without the requirement for extensive sequence homology between DNA ends, does not need synapsis of the broken DNA with an undamaged partner DNA molecule, leading to imprecise joining. The repair of DSBs by NHEJ requires at least five proteins. Three are components of the DNA-dependent protein kinase (DNA-PK) complex, the two DNA end-binding subunits of Ku (Ku70 and Ku80), and the catalytic subunit termed DNA-PKcs. The remaining two are DNA ligase IV and Xrcc4.

One of the earliest events in the damage response is phosphorylation of histone

H2AX at ser139 by members of the phosphatidylinositol-3 kinase-like family of kinases such as ataxia telangiectasia mutated(ATM), ataxia telangiectasia related(Rad 3, ATR), ATM related kinase(ATX), DNA dependant protein kinase(DNA-PK) (PI3KK) to create γ-H2AX (4). Within minutes of DNA damage, γ-H2AX appears at discrete nuclear foci that contain DNA repair factors like the MRN (Mre11/Nbs1/Rad50) repair complex, p53 binding protein 1 (53BP1), BRCA1, and MDC1. 53BP1 is one of the two p53 binding proteins identified in one of the earliest implementations of the yeast two-hybrid screen (5). It contains two tightly packed tudor domains that binds methylated K79 of histone H3(6) or K20 of histone H4 (7) and a C-terminal tandem BRCT domains. BRCT domains are thought to be protein-protein interaction domains and are found in many DNA damage response proteins (8, 9). 53BP1 is phosphorylated after ionizing radiation (IR) in anataxia telangiectasia mutated (ATM)-dependent fashion and colocalizes with those of phosphorylated histone H2AX and the Mre-11/Nbs1/Rad50 complex to regions of DNA DSBs (10-12). Furthermore, moderate checkpoint defects have been reported in cells depleted of 53BP1 (13). Analysis of 53BP1 knockout (KO) mice confirmed an important role for 53BP1 in genomic stability (Morales et al., 2003; Ward et al., 2003), with null mice recapitulating some of the ATM-deficient phenotypes, although they were less severe (14, 15). The function of 53BP1 is not well known but several papers reported that 53BP1 is strongly related with NHEJ repair (16, 17).

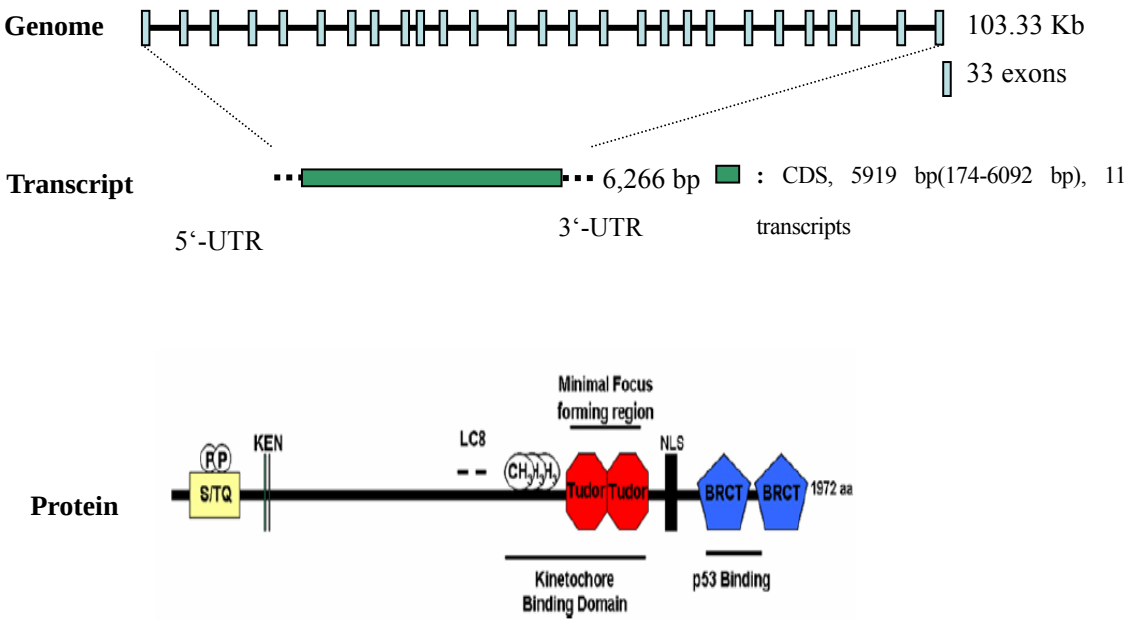

Fig. 1. **The structure of genome and 53BP1 domain**

Protein phosphatase 5 (PP5) is a member of the serine/threonine phosphatase family that also includes PP1, PP2A, and PP2B. PP5 is unique among phosphatases in that it contains a series of 34-amino acid tetratricopeptide repeat (TPR) motifs that serve as a protein-protein interaction domain (18). Through the TPR domain, PP5 interacts with a number of proteins and has been reported to be involved in regulating various biological processes such as the activity of glucorcorticoid receptor (19), apoptosis (20), and cell growth (21). Recently, it is known that PP5 plays an important role in DNA damage repair and cell cycle arrest by attenuating the activities of two closely related checkpoint kinases, ATM and ATR (22, 23). Another report shows that PP5 interacts with DNA-

PKcs and dephosphorylates with specificity at least two functional sites (24). More recent work utilizing cells from PP5-deficient mice have also confirmed that PP5 participate in ATM-mediated G2/M DNA damage checkpoint pathway (25).

As mentioned by Wechsler et al. (24), little is known about the dephosphoryltion of DNA damage response (DDR) proteins after repair process. So, we investigated the dephosphorylation and the change of DNA foci formation corresponding dephosphorylation of 53BP1, one of the important DNA damage response proteins. To evaluate this, we chose the PP5 as a candidate phosphatase responsible for the dephosphorylation of 53BP1.

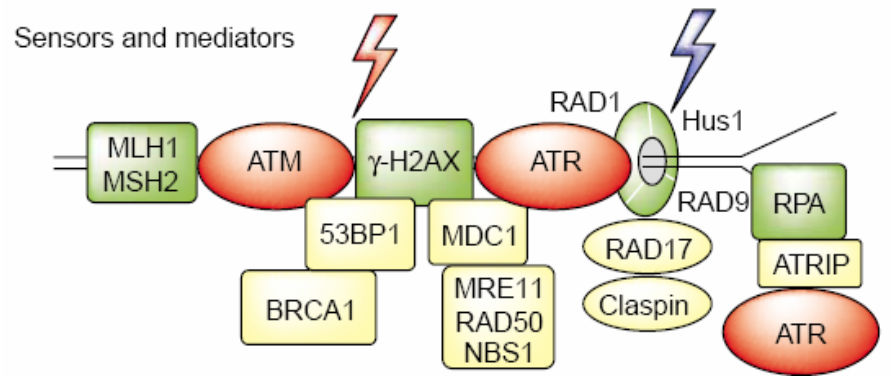

Fig. 2. The participation of 53BP1 into the DNA damage checkpoint machinery

II. MATERIALS AND METHODS

1. Cell lines and Drug Treatment

The following cell lines were used in this study: U2OS, U2OS-PO (U2OS transfected with full-length human PP5), and U2OS-PS (U2OS transfected with siPP5 construct). U2OS cell line was grown in McCoy's 5A medium supplemented with 10% fetal bovine serum, 10 ug/ml streptomycin, and 10 units/ml penicillin at 37˚C with 5% CO2 incubator and checked by microscope for everyday. U2OS-PO and -PS cells were grown under neomycin condition (800ug/ml). To cause DNA damage, exponentially growing cells were treated with 200 ng/ml neocarzinotatin (NCS, Sigma-Aldrich) and harvested at different times after treatment (time-dependent 0h, 1h, 3h, 6h, 12h and 24h).

2. Stable Cell Lines

Full-length human PP5 cDNA was directly cloned into the pcDNA 3.1 TOPO using PP5 gene-specific primers 5‘- ATG GCG ATG GCG GAG GGC GA-3´and 5‘-GAA TTC CAT TCC TAG CTG CAG CAG-3´. Synthetic siRNA duplex for PP5 (AAC AUA UUC GAG CUC AAC GGU) was purchased from Bioneer and cloned into the pSilencer TM neo (Ambion) (22). The resulted plasmids were transfected into U2OS using Lipofectamin 2000 (Invitrogen). The cells were cultured in McCoy's 5A medium supplemented with 10% FBS. After 24 h, G418 antibiotic (Sigma-Aldrich) was added to

the culture medium at a concentration of 800 ug/ml to select G418-resistant colonies. After 4 weeks of culture with the change of the G418-containing medium every 3 days, G418-resistant colonies were isolated and confirmed by western blot.

3. Western Blot

Cells were washed with phosphate-buffered saline (PBS) and lysed on ice for 5 minutes by M-PER (Mammalian Protein Extraction Reagent, Thermo-Scientific) buffer added with Protease Inhibitor Cocktail tablet EDTA free (Roche). After incubation, extracts were mixed for 5 minutes using micro tube mixer (Tomy MT360) and centrifuged at 13,000 rpm for 15 minutes. The supernatant was diluted with 5X SDS-sample buffer that contains 125mM Tris (Amresco), 960mM Glycine (Amresco), 0.5% SDS (sodium dodecyl sulfate, Amresco) and boiled in 5minutes. After cellular protein concentrations were determined using the dye-binding microassay (Bio-Rad, Hercules, CA), samples were loaded 10 μg, 20 μg or 50 μg (amount depends on target antibodies) per lane and electrophoresed on 6% or 8% SDS polyacrylamide gels. The proteins were blotted onto Polyvinylidene Fluoride transfer membranes (PVDF, BiotraceTM, Pall Corporation). After electroblotting, the membranes were blocked by 5% non-fat dry milk (skim milk) in Tris buffer saline containing 0.1% Tween-20 (TBST, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 hour. The membranes were then rinsed with TBST and then incubated with appropriate primary antibodies

against: phospho-53BP1 (S1778) (Cell signaling Technology), 53BP1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-BRCA1 (T68) (Cell signaling Technology), BRCA1 (Santa Cruz Biotechnology), PP5 (BD bioscience), and α-Tubulin (NeoMarkers) in TBST at 4℃ overnight. We followed manufacturer's protocol for dilution of all primary antibodies.

After primary antibody incubation, the membranes were washed with TBST and incubated with secondary antibodies diluted in TBST for 2 hours at room temperature, and washed again. Then the blotted proteins were visualized by developing using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

4. Immunoprecipitation

Cells were washed with phosphate-buffered saline (PBS) and lysed on ice for 20minutes in RIPA buffer (25mM Tris-HCl, pH 7.6, 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS) supplemented with Protease Inhibitor Cocktail Tablet (Roche). After incubation, total cell extracts were sonicated and centrifuged at 13,000 rpm at 4℃ for 20minutes. 2mg of extracts were incubated with either 2μg 53BP1 antibody (Santa Cruz Biotechnology) and 2μg control IgG (Santa Cruz Biotechnology) and protein A-Sepharose beads (Santa Curz Biotechnology) for overnight at 4 °C in a total volume of 1ml with shaking. The beads were then gently washed three times with PBS, and the precipitated complexes were resuspended in 2X-SDS sample loading buffer. After boiling, the samples were resolved by sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE), 50μl of total cell extracts were used as the input, then transferred to polyvinylidene difluoride membrane, and immunoblotted using an enhanced chemiluminescence detection system (iNtRON, Biotech, Seoul, Korea).

5. Immunofluorescence Analysis

Essentially the standard procedure was followed (40). All cells grown on cover slips were briefly rinsed with PBS and fixed with freshly prepared 2% paraformaldehyde in PBS for 15 minutes. Slides were either directly processed or added 70% Ethanol (EtOH) and stored at 4°C for later analysis within 1 month. After being washed with PBS, the cover slips were blocked in 5% BSA in PBS for 1 hour at RT then incubated overnight at 4°C with the following primary antibodies: mouse monoclonal g-H2AX (ser-139), 1: 200 (Upstate Biotechnology); rabbit polyclonal 53BP1 (ser 1778), 1: 100 (Cell Signaling Technology, Beverly, MA). Secondary antibodies labeled with Alexa 488 or Alexa 594 (Molecular Probes) were added at 1: 200 and were incubated at RT for 1 h. Slides were mounted with containing DAPI. Images were acquired with Nikon ELIPSE 80i microscope.

6. Nonhomologous End-Joining Assay

To document a role for 53BP1 phosphorylation in NHEJ, we used the plasmid pEGFP-Peml-Ad2 (41). Briefly, the NHEJ reporter plasmid was digested with HindIII for overnight and purified by using a Qiagen gel extraction kit. U2OS, U2OS-1, and U2OS-2 cells were transfected by using a lipofectamine 2000 (Invitrogen) according to

manufacturer's instructions. In a typical reaction, 5 X 10^5 cells were transfected with 0.5µg of predigested NHEJ reporter substrate along with 0.5µg of pDsRed2-N1 (Clontech) to serve as transfection control. Expression of GFP (Green Florescence Protein) and DsRed was monitored by fluorescence microscopy (Nikon, Eclipse TE2000-U). After transfection, cells were incubated for 48h. Cells then were harvested, resuspended in 0.5 ml of PBS, pH 7.4 (GIBCO, Invitrogen), and analyzed by FACS (Fluorescence Activated Cell Sorting) .

III. RESULTS

1. PP5 binds with 53BP1 after DNA damage

We performed a yeast two-hybrid screen with BRCT domain of the human 53BP1 as bait. Among the cDNAs encoding putative interacting with 53BP1 we could find the cDNA for human PP5 gene which was verified by DNA sequencing. To confirm the interaction between PP5 and 53BP1, we performed co-immunoprecipitation analysis using 53BP1 antibody in U2OS and over-expressing U2OS (U2OS-PO) cells. As mentioned in several reports, it is difficult that PP5 interact with other proteins. Hence we established a stably over-expressed human wild-type PP5 in U2OS cells using pcDNA 3.1 TOPO vector. As shown in Fig. 3A, V5-His tagged PP5 could be detected at the higher position than endogenous PP5 by western blotting in U2OS-PO cells. Co-immunoprecipitation assay using 53BP1 antibody in U2OS-PO cells showed that binding between PP5 and 53BP1 highly increased 3 hours after NCS treatment (Fig. 3B). The given delay of the interaction that postulated PP5 act as phosphatase in DNA damage response. To date some papers reported that PP5 participated in DDR, three mentioned that PP5 involved a regulatory proteins of ATM (22, 25)and ATR(23) not phosphatase. Only one indicated that PP5 dephosphorylated the pT2609 and pS2056 of DNA-PKcs after DNA damage in HeLa cells (24). We could not find the changes of PP5 expression after NCS treatment either in U2OS or U2OS-PO cells (Fig. 3C).

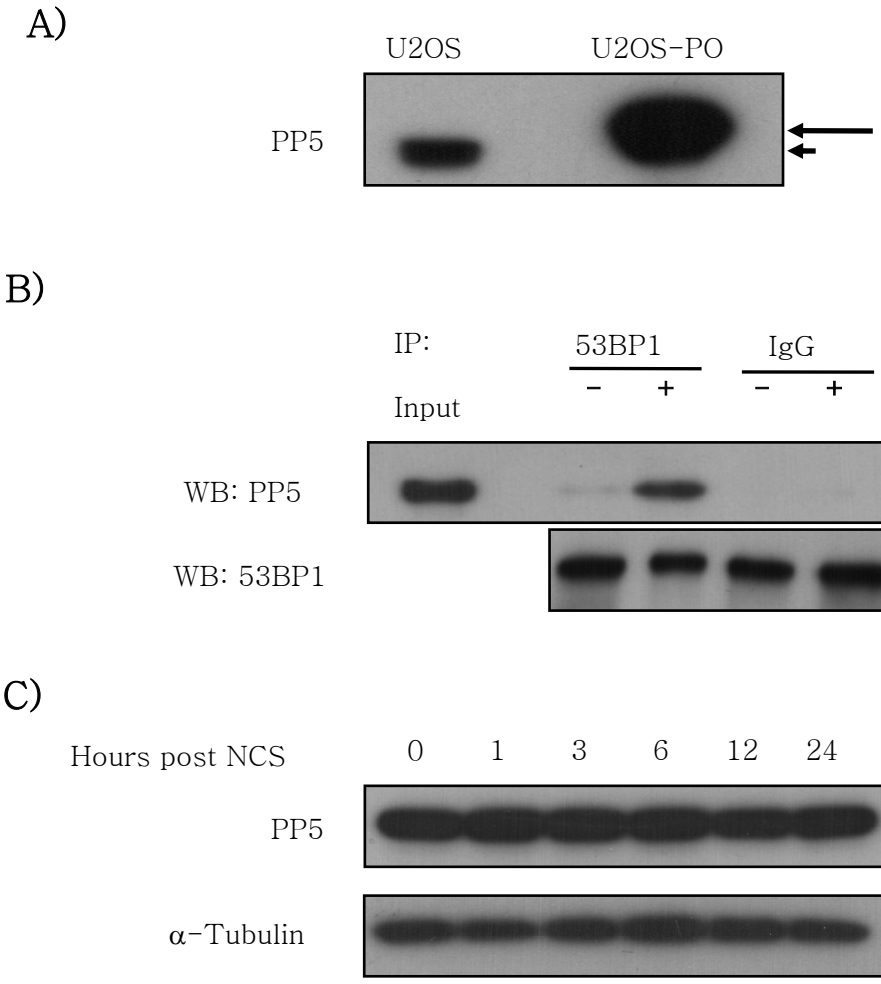

Fig. 3. **PP5 interacts with 53BP1 after NCS-induced DNA damage**. *A*, To make PP5 over-expressing cells, U2OS cells were transfected with pcDNA 3.1-PP5 plasmid. The transfected cells were selected with G418 (800 ug/ml) for 4 weeks and then screened by Western blotting using PP5 specific antibody. Arrow indicates a V5/His tagged PP5 and arrow head endogenous PP5. *B*, U2OS and U2OS-PO cells were treated with NCS (200 ng/ml) for 3 h. Proteins were extracted by RIPA buffer and immunoprecipited with 53BP1 antibody. *C*, The level of PP5 expression was analyzed by Western botting after NCS (200 ng/ml) treatment.

2. Over-expression of PP5 Induces the earlier dephosphorylation of 53BP1 specifically

53BP1 was known to has several phosphorylation sites after IR-induced DNA damage (31). The phosphorylation of 53BP1 at Ser25/29 was well characterized by numerous groups, but the phosphorylaton at Ser1778 on BRCT domain of 53BP1 was little. Thus, we focused on the phosphorylation at Ser1778 of 53BP1. As expected, the phosphorylation of 53BP1 at Ser1778 was mediated by ATM after NCS-induced DNA damage (Fig, 4A). To confirm our hypothesis that PP5 may function as a phosphatase for 53BP1 after DNA damage, we compared the phosphorylation level at Ser25 and SerS1778 of 53BP1 in U2OS and U2OS-PO cells with or without NCS treatment. We found that the dephosphorylation of 53BP1 at both sites was rapidly preceded in U2OS cell. As shown in Fig. 4B, the phosphorylation of Ser1778 of 53BP1 was highest after 1 hour treatment with NCS and then decreased by time, became significantly diminished by 24 hours in U2OS cells; in U2OS-PO cells over-expressing PP5, although early phosphorylated by ATM, was not different between the two cell types, the level of pSer1778 had significantly diminished after 12h treatment. Thus, phosphorylation of Ser25 and Ser1778 was not affected by the level of PP5, but the PP5 level was related to the dephosphorylation of 53BP1. Interestingly, the phosphorylation pattern between Ser25 and Ser1778 of 53BP1 showed significantly different. The phosphorylated 53BP1 at Ser25 was detected until 24 hours maintaining similar density, meanwhile pS1778 of

53BP1 was diminished in a time-course manner (Fig. 4B). This result suggests that there are some different roles depends on phosphorylation sites of 53BP1.

From these results, we could conclude that PP5 is the phosphatase responsible for removing the phosphate group from Ser25 and Ser1778 of 53BP1 after DNA DBSs. To confirm that the dephosphorylation of 53BP1 at pSer1778 was mediated by PP5 and not by non-specific interaction, we evaluated the phosphorylation of Ser1524 of BRCA1, which contains the same BRCT domain as 53BP1 and is also known DDR protein. As shown in Fig. 4B, neither U2OS nor U2OS-PO cells showed obvious differences in the phosphorylation pattern of BRCA1 after NCS treatment. Thus, the dephosphorylation of pSer1778 of 53BP1 appears to occur via a specific interaction mediated by PP5.

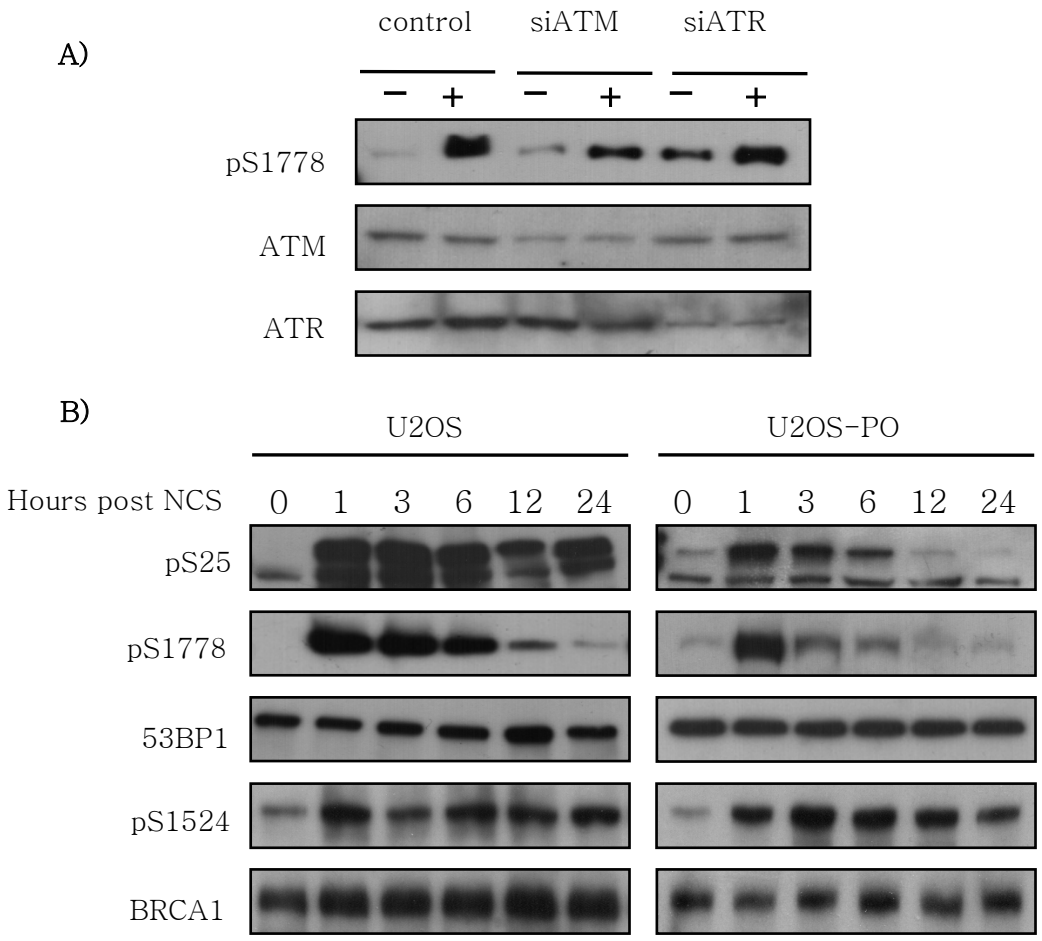

Fig. 4. **PP5 participates in the dephosphorylation of 53BP1 at Ser25 and Ser1778 after repair process.** *A*, To indentify the kinase responsible for the phosphorylation of 53BP1 at Ser1778, U2OS cells were transfected with siRNA for ATM and ATR using lipofectamin reagent. After 48 h, NCS (200 ng/ml) was treated to all cells for 1h and the phosphorylation of 53BP1 at Ser1778 was analyzed by Western blotting. *B*, The dephosphorylation of 53BP1 was mediated specifically by PP5. U2OS and U2OS-PO cells were treated with NCS (200 ng/ml) and then harvested the cells in a time-course manner (1 h, 3 h, 6 h, 12 h, and 24 h). The phosphorylation patterns of 53BP1 and BRCA1 were analyzed by Western blotting using indicated antibodies.

3. The 53BP1 foci formation is also influenced by rapid dephosphorylation at S1778

In response to ionizing radiation, 53BP1 rapidly colocalizes with γ-H2AX. H2AX, a variant of histone H2A, becomes phosphorylated and forms foci at sites of DSBs after DNA damage.The number, as well as appearance and disappearance of 53BP1 foci, matched almost completely with that of γ-H2AX (27, 28). Although the phosphorylation activity has been studied in detail, little is known about the corresponding dephosphorylation and detachment from DNA DSBs. Generally, it is regarded that either the phosphorylated protein has to be degraded or the phosphate group has to be removed. So, we tried to analyze the relationship between foci formation and dephosphorylation of 53BP1 by immunoflurescence study in U2OS and U2OS-PO cells (Fig. 5). The pattern of 53BP1 and γ-H2AX foci formation was evaluated by time-dependent manner, from 0 hour to 24 hours, after NCS-induced DNA damage. As shown in Fig.3, we could find that DNA foci pattern of pS1778 53BP1 was different in U2OS and U2OS-PO cells. In U2OS cells, DNA foci of pS1778 53BP1 was seen like numerous small and fused type at 1 hour and then changed to larger and decreased foci number at 24 hours by time-course. These patterns of foci formation were well matched with the result of Western blotting for pS1778 53BP1 (Fig. 4). However, the pattern of pS1778 53BP1 foci was changed in U2OS-PO cells (Fig. 5). We could find a little similar pattern up to 3 hours in both cells. Although the number of 53BP1 foci was smaller in U2OP-PO cells than U2OS, the foci of pS1778 53BP1 was well matched with γ-H2AX foci at 3

hours. Generally, many DDR proteins phosphorylated by PI3KK family like ATM, ATR or DNA-PKcs after DNA injury and then recruited and participated in repair processes at DNA break sites (29, 30). The phosphorylation of 53BP1 mediated by ATM and ATR after DNA damage well studied using mass spectrometer (31).

In U2OS-PO cells after NCS-induced DNA damage, both the number and size of g-H2AX foci followed a pattern almost identical to that seen in U2OS cells (Fig. 5). To again confirm the specificity of PP5 for 53BP1, we analyzed the formation of pSer1524-BRCA1 and pS1981-ATM foci after DNA damage; as for g-H2AX, there were no differences in the foci patterns between cells (data not shown). These data reconfirm that the interaction between PP5 and phosphorylated 53BP1 is specific.

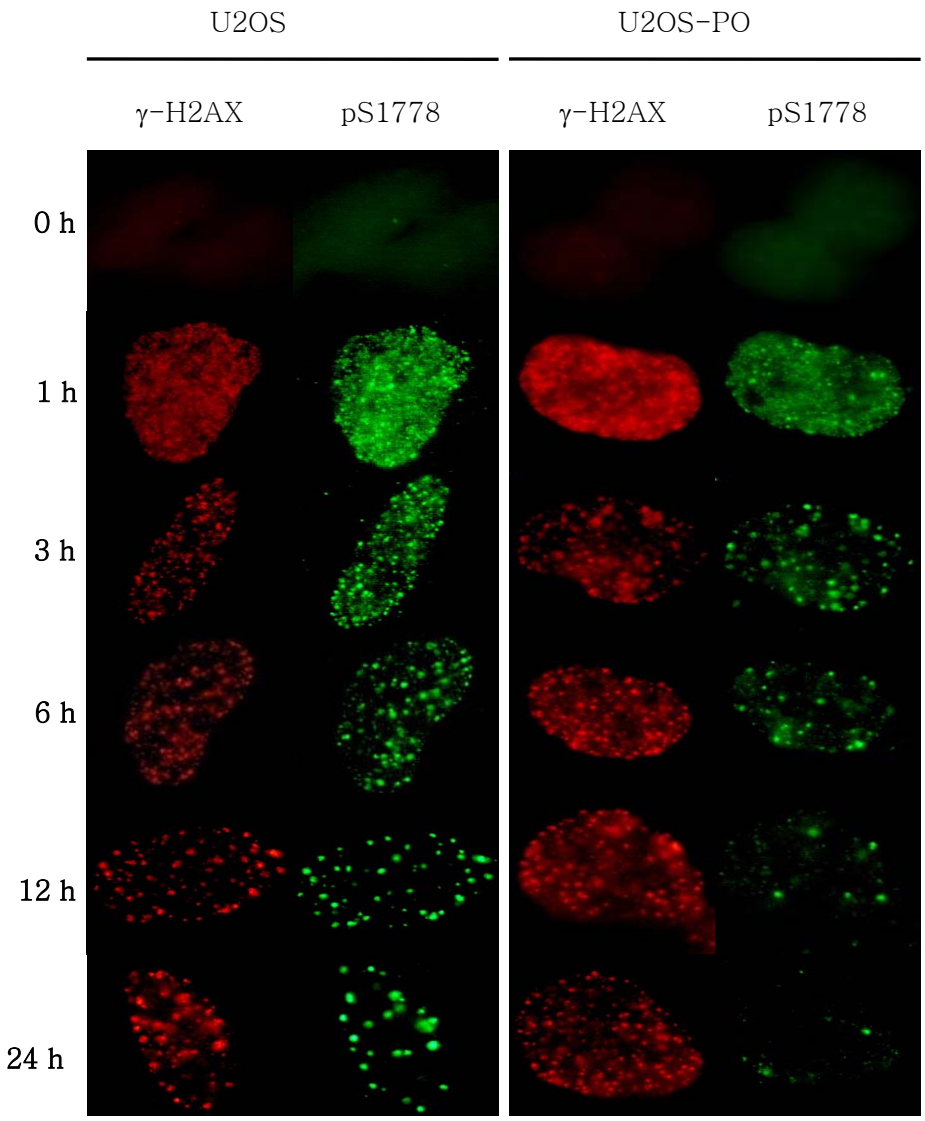

Fig. 5. **The maintenance of 53BP1 foci is affected with the phosphorylation status caused by PP5 over-expression.** U2OS and U2OS-PO cells were grown on cover-slides and then treated with NCS (200 ng/ml). 53BP1 and γ-H2AX foci were analyzed by mentioned in experimental procedure using antibodies specific for the phosphorylation-form.

4. Decreased Expression of PP5 Slightly Delays Dephosphorylation of 53BP1

To evaluate that PP5 really participate in the dephosphorylation of 53BP1 and is only phosphatase for 53BP1 after DNA damage, we established PP5-suppressed cell line using previous reported sequences (22). Firstly, we cloned siRNA duplex for PP5 into the pSilenser vector and then transfected the siPP5 plasmid into U2OS cell. Under G418 selection for 4 weeks we could obtain several PP5-suppressed U2OS clones. Through western blotting we screened those clones and chosen well suppressed one clone. The results of Western blot and IF analysis showed that PP5 expression is well suppressed (Fig. 6A and B). We tried to confirm the activity of PP5 as a phosphase at pS1778 53BP1 in PP5-suppressed U2OS cells –U2OS-PS. As expected we could find that dephosphorylation at pS1778 of 53BP1 was maintained until 24 hours after NCS treatment in U2OS-PS cells (Fig. 6C). In U2OS cells, the level of pS1778 53BP1 showed a peak at 30 min and then decreased slowly until 24 hours. At 24 hours we could see that pS1778 53BP1 was completely disappeared. However, the level of pS1778 53BP1 was high at 1 hour and then decreased slightly at 3 hours in U2OS-PS cells. The level of pS1778 53BP1 at 3 hours continuously maintained up to 12 hours and decreased again at 24 hours. However, we could not convince that PP5 is an only enzyme participating with dephosphorylation of 53BP1. Recently, Travesa and colleagues reported for the dephosphorylation of Rad53, which plays a central role in preventing genomic instability and maintaining viability in *Saccharomyces cerevisiae*. According to their data, Rad53

kinase was dephosphorylated by different enzymes, Ptc2/3 and Pph3, depends on DNA damages (34).

Next, we investigated the influence of PP5 suppression on foci formation by pSer1778-53BP1 after DNA damage in U2OS-PS cells. As shown in Fig. 5, there are some differences in pSer1778-53BP1 foci formation between U2OS and U2OS-PS cells. At 1 hour after DNA damage, the foci of pS1778-53BP1 were similar between U2OS and U2OS-PS cells; however, after 3 h, the pattern of foci formation differed between the two. In U2OS cells, the number of pSer1778-53BP1 foci decreased and the size increased in a time-dependent manner; in contrast, the number and size of pSer1778-53BP1 foci were maintained from 3 to 24 h in U2OS-PS cells. These foci patterns are almost identical to the patterns on Western Blots after DNA damage. Thus, the maintenance of 53BP1 is strongly related with the phosphorylation status, which may also influence the repair process after DNA damage.

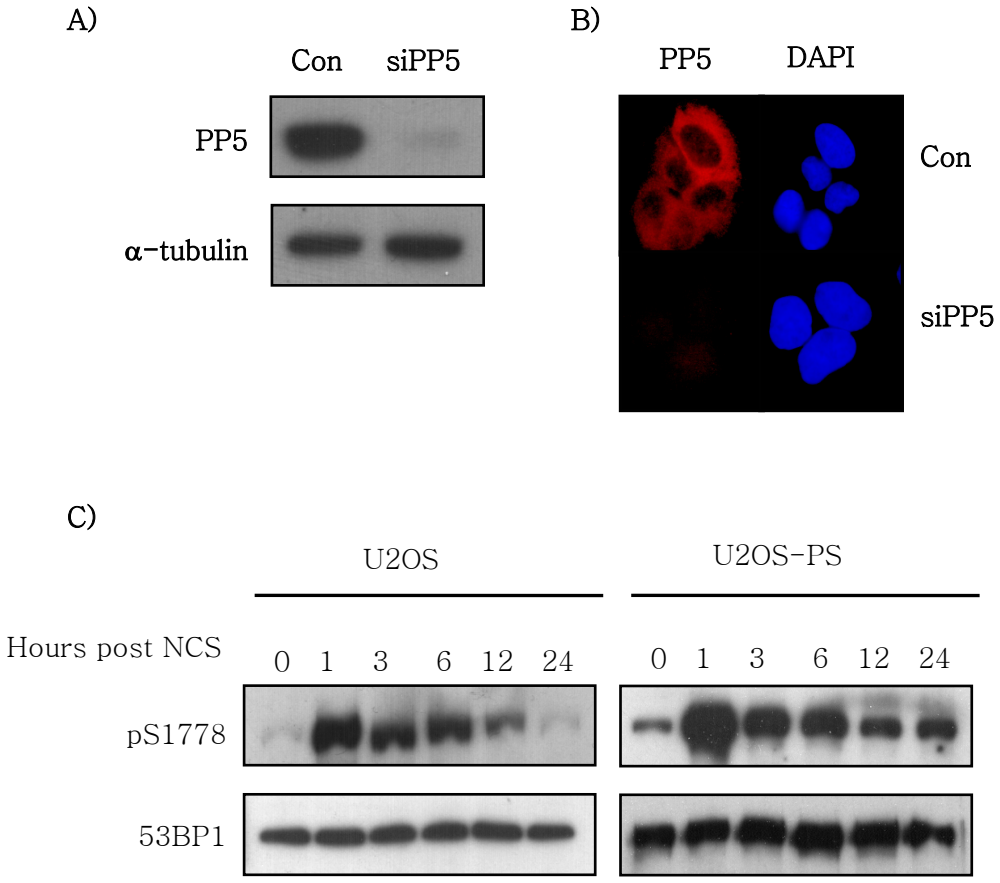

Fig. 6. **The phosphorylation level of 53BP1 at Ser1778 is prolonged in U2OS-PS cells.** *A-B*, U2OS cells were transfected with psilencer vector including siPP5 sequences to establish a PP5 suppressed cells. Selected clones under G418 condition (800 ug/ml) were screened by Western blotting (A) and IF (B) and named to U2OS-PS cells. *C*, U2OS-PS cells, PP5 suppressed cells, were treated with NCS (200 ng/ml) and harvested by time-dependent (1 h, 3 h, 6 h, 12 h, and 24 h). The phosphorylation level of 53BP1 at Ser1778 was analyzed by Western blotting.

5. Both Hypo- and Hyperphosphorylation of 53BP1 influence the NHEJ

Although it was not clearly understood about the function of 53BP1, it has been indicated that 53BP1 participated in NHEJ, not HR after DNA damage (16, 17, 35, 36). We supposed that the activity of DNA repair may be differently influenced due to the different dephosphorylation patterns of 53BP1 in U2OS-PO and U2OS-PS cells. To experimentally test this, we used three kinds of plasmids, pEGFP-Pem1-Ad2, pEGFP-Pem1, and pDsRed2-N1, and introduced the plasmid DNA for direct reporting *in vivo* over EGFP expression into the U2OS, U2OS-PO, and U2OS-PS cells. Fig. 8A shows the pEGFP-Pem1-Ad2 plasmid used in the experiments. The pEGFP-Pem1-AD2 was linearized by Hind III digestion that removes the Ad2 exon and enables upon successful intracellular circularization EGFP expression that can be detected and quantitated by FACS. Supercoiled pEGFP-Pem1 plasmid was used to evaluate the EGFP signal without requirement for rejoining, whereas the pDsRed2-N1 was used as transfection control. In Fig. 8B, panel 1 shows the autofluorescence of sham-transfected cells, and panel 2 the signal generated in cells transfected with 0.5 ug pEGFP-Pem1 and 0.5 ug pDsRed2-N1 plasmids simultaneously as a control. To evaluate the non-homologouse end joining efficiency in U2OS, U2OS-PO, and U2OS-PS cells, the linearized pEGFP-Pem1-Ad2 plasmids were transfected into the three cell lines and measured 24 hours later by FACS (Fig 8B panel 3, 4, and 5). When we compared the data from U2OS-PO and U2OS-PS cells with U2OS as a control, the NHEJ activities were decreased in both cells (Fig. 8C).

In five independent experiments, the NHEJ efficiency was shown 64.9% ± 1.7 in U2OS-PO cells and 80.3% ± 0.6 in U2OS-PS cells.

Generally, it was known that the repair of IR-induced DSBs is mainly performed by “core NHEJ” which is composed of DNA ligase IV/Xrcc4, Ku70/Ku80, and DNA-PKcs and Artemis (37). Iwabuchi and colleagues proposed a possibility that 53BP1 plays a role through Tudor domain which can bind chromatin and stimulate end-joining by DNA ligase IV/Xrcc4 in Ku70-dependent pathway (16). However, several reports showed that 53BP1 has a different pathway with Ku-dependent pathway for NHEJ (30, 35, 38). A recent study revealed that 53BP1 participates in a pathway distinct from Ku-dependent and Artemis-dependent NHEJ pathway, but still requires DNA ligase IV (39).

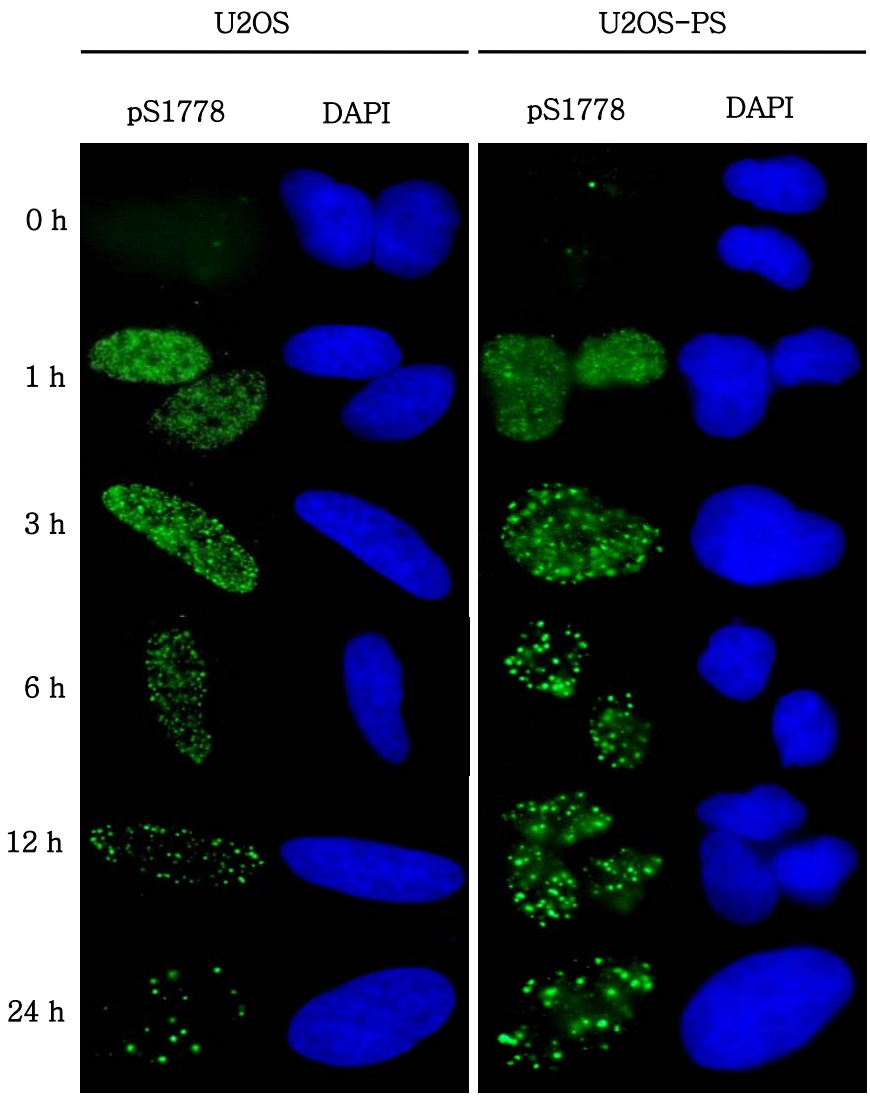

Fig. 7. 53BP1 foci is also influenced by PP5 expression level after NCS-induced DNA damage. U2OS and U2OS-PS cells were cultured on cover-slides and then induced DNA damage by NCS (200 ng/ml). Each slide was fixated with 3.7% PFA by time-dependent after NCS treatment and stained with antibody specific for pSer1778 of 53BP1.

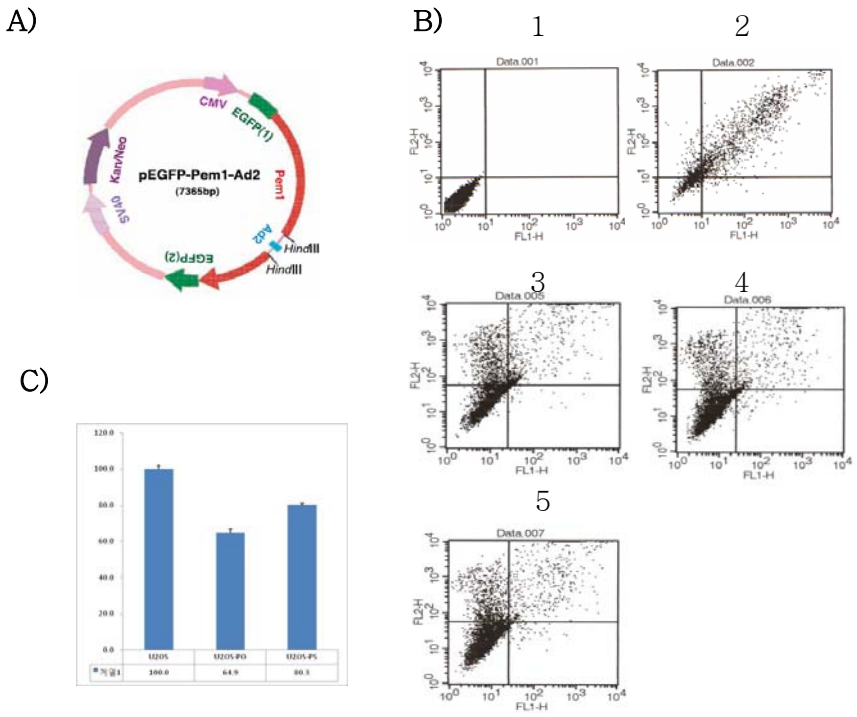

Fig. 8. NHEJ activities show lower in U2OS-PO and U2OS-PS cells than in U2OS cells. A, map of pEGFP-Pem1-Ad2 vector. Note the Pem1 intron, the Ad2 exon and the location of the HindIII cutting sites. B, dot plots of nontransfed U2OS (1), U2OS cells co-transfected with pEGFP-Pem1 and DsRed (2), U2OS cells co-transfected with HindIII linearized pEGFP-Pem1-Ad2 and DsRed (3), U2OS-PO cells co-transfected with HindIII linearized pEGFP-Pem1-Ad2 and DsRed (4), U2OS-PS cells co-transfected with HindIII linearized pEGFP-Pem1-Ad2 and DsRed (5). C, Quantitative analysis considering signals from B data. All measurements of rejoining efficiency were carried out 24 h after transfection of the corresponding plasmids

IV. DISCUSSION

After the DNA damage, there is interaction between PP5 and 53BP1, however, it was very difficult to confirm the binding after DNA damage because our immunofluorescence data showed that the PP5 was not located predominantly at nucleus region. For the location of PP5 early reports mentioned mainly at nucleus but later suggested broadly distribution and sometimes stronger in cytoplasmic region than nucleus (26). Using co-immunoprecipitation for the binding between PP5 and 53BP1 in U2OS-PO, U2OS-PS and U2OS cells, we found that PP5 may be participated in DNA damage response as a phosphatase since interaction was increased by late time after NCS treatment.

As we compared the phosphorylation levels at Ser25 and Ser1778 in both U2OS and U2OS-PO cells, we found that the phophosrylation was mediated by ATM but there is no differences between the two cell lines. But there was little different in the expression in dephophorylation due to the level of PP5 in the two cell types. This result showed that the phosphotase PP5 has somewhat role in dephosphorylating at S1778 of 53BP1 after DNA DBSs.

53BP1 rapidly colocalizes with γ-H2AX in response to ionizing radiation brought about DNA strand breaks. The number, appearance and disappearance of pSer1778 53BP1 foci in both cells U2OS and U2OS-PO, matched almost completely with that of γ-H2AX (27, 28). By time 6 hours later damage-induced, the number of pSer1778

53BP1 foci was highly decreased and not matched with γ-H2AX foci. This fact indicated that detachment from DNA break sites of DDR proteins performed by dephosphorylation. Other hand, the difference in number of foci in U2OS and U2OS-PO cell help confirm the specific interaction PP5 and phosphorylated 53BP1.

Under the DNA replication stress, Pph3 plays a role in dephosphorylation of Rad53 and Ptc2/3 after DNA methylation damage or replication stress. Like a Rad53, there is possibility that 53BP1 may be also dephosphorylated by different enzymes depends on type of DNA damage. The observation of the foci implied that PP5 might concern with the dephosphorylaton at S1778 of 53BP1. The decreased expression of PP5 slightly delays dephosphorylation of 53BP1. As mentioned, we could conclude that maintenance of 53BP1 is strongly related with the phosphorylation status and may also influence the repair process after DNA damage.

Recently, it was revealed that 53BP1 plays a role in a pathway distinct from Ku-dependent and Artemis-dependent NHEJ pathway but requires DNA ligase IV (39). In this study, the NHEJ efficiency was lower in PP5-oversexpressing cells than PP5-suppressed cells. This reason could be deduced from the function of PP5. As mentioned earlier, PP5 plays a important roles in ATM/ATR-dependent repair after DNA damage (22, 23, 25) and interact with DNA-PKcs as a phosphatase(24). Thus, the over-expression of PP5 could be higher influencer to many other proteins in checkpoint signaling pathways than in suppressed condition.

V. CONCLUSION

In summary, the numerous proteins engaged in the DNA damage response and repair process after DNA injury. Among those, 53BP1 plays an important role as a mediator. However, the function of 53BP1 has not been clear, as well as the dephosphorylation and disappearing foci of 53BP1 after DNA repair was not well studied. In this study, we demonstrated that PP5 interacts with 53BP1 and participates in the dephosphorylation of Ser1778, and Ser25 of 53BP1 after DNA damage. Furthermore, we found out the maintenance of 53BP1 foci and non-homologous end joining activity were affected according to dephosphorylation status in PP5 over- and – suppressed U2OS cells. From these results, we could suppose that PP5 has an important role in DDR.

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<국문초록>

DNA 손상 반응과정 중 Protein Phosphatase 5 에 의한 53BP1 조절기작에 대한 연구

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DNA double-strand break (DNA 이중나선절단, DSB)은 주로 방사선과 방사선 유사 물질에 의해 발생한다. 또한, 염색체에서 기계적인 스트레스와 복제과정에 DNA 중합효소가 (DNA single-strand break)DNA 단일나선절단 같은 DNA 손상을 만났을 때 발생할 수 있다. DSB의 부정확한 복구와 복구 결핍은 돌연변이 및 유전자 불안정성을 일으키고, 나아가 세포사멸사 및 암 유발을 유도한다. DNA 손상반응에서 초기현상중의 하나인 γ-H2AX의 형성은 ataxia telangiectasia mutated(ATM), ataxia telangiectasia related(Rad 3, ATR), ATM related kinase(ATX), DNA dependant protein kinase(DNA-PK)같은 phosphatidylinositol-3 kinase-like family of kinases(PIKK)에 의해 histone H2AX가 인산화된다. DNA 손상 수분 안에, γ-H2AX는 MRN complex, 53BP1, BRCA1, and MDC1같은 DNA 손상복구요소들을 포함한

뚜렷한 foci를 형성한다. Yeast two hybrid 시스템을 통하여, 53BP1과 상호작용하는 몇 개의 유전자 중 serine/threonine phosphatase family인 PP5 유전자를 확인하였다. PP5와 53BP1의 결합은 DNA 손상 후 크게 증가하였다. PP5와 53BP1의 상호작용을 확인하기 위하여, PP5 과발현 세포주와 억제된 세포주를 만들고 DNA 손상후 Western blotting and immunofluorescense analysis을 수행하였다. PP5는 53BP1의 탈 인산화를 조절하고, 또한 53BP1 foci의 축적 및 유지에 영향을 미치는 것으로 사료된다.

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

2008년 10 월 14일

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