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# Human polynucleotide phosphorylase reduces oxidative RNA damage and protects HeLa cell against oxidative stress

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## Abstract

We examined HeLa cell viability and RNA oxidative damage in response to hydrogen peroxide ($H_2O_2$) treatment. The level of damaged RNA, measured by the content of 8-hydroxyguanosine (7,8-dihydro-8-oxoguanosine, 8-oxoG), increases depending on $H_2O_2$ dosage and is inversely correlated with cell viability. The elevated level of 8-oxoG in RNA decreases after removal of oxidative challenge, suggesting the existence of surveillance mechanism(s) for cleaning up oxidized RNA. Human polynucleotide phosphorylase (hPNPase), an exoribonuclease primarily located in mitochondria, has been previously shown to bind 8-oxoG-RNA with high affinity. The role of hPNPase in HeLa cell under oxidative stress conditions is examined here. Overexpression of hPNPase reduces RNA oxidation and increases cell viability against $H_2O_2$ insult. Conversely, hPNPase knockdown decreases viability and increases 8-oxoG level in HeLa cell exposed to $H_2O_2$. Our results suggest that hPNPase plays an important role in protecting cells and limiting damaged RNA under oxidative stress.

### Keywords

Human polynucleotide phosphorylase (hPNPase); RNA damage; 8-hydroxyguanosine (7,8-dihydro-8-oxoguanosine, 8-oxoG); Oxidative stress; Cell viability

Damages of DNA, protein and lipids caused by reactive oxygen species (ROS), especially those occurring under oxidative stress, have been strongly implicated in aging and in the development of numerous age-related disorders [1]. In contrast, damage of RNA has received relatively little attention, and its impact has just started to unveil [2,3]. Recently, increased levels of oxidatively damaged RNA have been reported in patients of neurodegenerative diseases compared to age-matching normal controls [3]. Interestingly, increased levels of 8-oxoG were observed only in RNA but not in DNA when both were examined.

RNA dysfunction rendered by oxidative damage may contribute to the development of the degenerative diseases. Among the known oxidative lesions in nucleic acids, 8-hydroguanosine (7,8-dihydro-8-oxoguanosine, 8-oxoG) is abundant and appears to be most deleterious due to its high mutagenic potential [2,4]. The level of 8-oxoG in RNA is much higher than that in DNA under the same conditions [5–7]. Other oxidative damages to RNA may include modification of ribose, base excision, and strand break [2]. Oxidative damage of ribosomal

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RNA causes ribosome dysfunction *in vitro* and in tissues of Alzheimer's patients [8,9]. Oxidation of mRNA reduces translation efficiency and the production of abnormal proteins [10–12], and it is an early event preceding cell death [11].

Very little is known about how cells deal with oxidatively-damaged RNA. Presumably, oxidized RNA can be specifically targeted to degradation by ribonucleases [2]. The *E. coli* polynucleotide phosphorylase (PNPase), a 3′–5′ exoribonuclease, plays important roles in mRNA decay and degradation of defective stable RNA [13]. In a search for *E. coli* proteins interacting with oxidized RNA, PNPase was shown to bind an 8-oxoG oligoribonucleotide with much higher affinity than it does to a normal oligoribonucleotide [14]. This strongly suggests a role for PNPase in sequestering and removing oxidized RNA to control RNA quality. PNPase is evolutionarily conserved in bacteria, fly, plant, animal and human [15]. The human PNPase (hPNPase) plays roles in a number of cellular processes and is predominantly localized in mitochondria [16,17]. Interestingly, like its *E. coli* homologue, hPNPase has also been shown to specifically bind 8-oxoG RNA with high affinity [18]. These observations prompted us to examine if hPNPase participates in surveillance of oxidized RNA. Here, we report a correlation between HeLa cell viability and the level of RNA oxidation under oxidative stress, and the role of hPNPase in these processes.

## Materials and Methods

### Materials

HeLa cell was provided by Dr. Vijaya Iragavarapu-Charyulu. A plasmid pCMV-hPNP expressing hPNPase from the CMV promoter was purchased from Open Biosystems (Clone ID: 6062060). A control plasmid pCMV* was prepared by digesting pCMV-hPNP with *SalI* and *XhoI* to remove the *hPNP* DNA insert followed by self-ligation. An siRNA (5'-GAAACAGGUGUAACUAUUAdTdT-3') targeting the region of 1900–1918 of *hPNP* mRNA was chemically synthesized by Qiagen. AllStars Control siRNA provided in the RNAi Human/Mouse Starter Kit (Qiagen) was used as nonspecific control. Other materials include the plasmid DNA Miniprep and Maxiprep kits (Qiagen), restriction enzymes and T4 DNA ligase (New England Biolabs), *Pfu* DNA polymerase (Stratagene), guanosine (Sigma-Aldrich), 8-hydroxyguanosine (8-oxoG) (Calbiochem). All other chemicals are reagent grade.

### Isolation of RNA and analysis of 8-oxoG levels

Total RNA was isolated from cultured HeLa cells using TRI Reagent (Molecular Research Center). Guanosine and 8-oxoG in RNA were determined after HPLC separation according to conditions described previously [5]. The level of 8-oxoG is represented as number of 8-oxoG per $10^5$ G.

### Analysis of the level of hPNP mRNA

Total RNA samples were treated with the TURBO DNA-free Kit (Ambion) to remove residual DNA. Relative levels of *hPNP* mRNA were analyzed by semi-quantitative reverse transcription-PCR (RT-PCR) using human *GAPDH* (*hGAPDH*) mRNA as internal normalization control, as previously described [19,20]. The *hPNP* primers are 5'-TTGTTGGACCTGGTGGCTAT-3' (forward) and 5'-TCTGACCACGGTTGTAGCTG-3' (reverse), producing a 425 bp product. The *hGAPDH* primers are 5'-CGGAGTCAACGGATTTGGTC-3' (forward) and 5'-ACTGTGGTCATGAGTCCTTC-3' (reverse), producing a 516 bp DNA. PCR products from desired cycles were separated on agarose gels and quantified after staining with ethidium bromide using the Epi Chemi II Darkroom (UVP Laboratory Products).

### Treatment of HeLa cells with $H_2O_2$ and determination of cell viability

HeLa cells were cultured in a humidified atmosphere with 5% $CO_2$ at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1% penicillin and 1% streptomycin. According to a procedure described previously [19], HeLa cultures were treated with $H_2O_2$ for certain length of time prior to the MTS assay of cell viability using the CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay kit (Promega). Relative cell viability in $H_2O_2$-treated cultures was presented as the percentage of the residual MTS-reducing activity.

### Transfection of DNA or siRNA into HeLa cells

HeLa cultures were transfected with DNA constructs (2 μg/ml medium) using Lipofectamine 2000 (Invitrogen). siRNA constructs (5 nM) were introduced into HeLa cells using the RNAi Human/Mouse Starter Kit (Qiagen).

## Results

### $H_2O_2$ treatment reduces viability of HeLa cell and increases oxidation of cellular RNA

Addition of exogenous $H_2O_2$ has been widely used as a convenient method to increase cellular ROS levels. Here we show that HeLa cell viability of reduces dramatically in the presence of sub-millimolar concentrations of $H_2O_2$ (Fig. 1A). Similar responses of HeLa cell to this concentration range of $H_2O_2$ have been previously reported [21]. As expected, cell viability decreases depending on $H_2O_2$ dosage, especially between 0.2 to 0.5 mM. Viable cells drop below 20% under 0.5 mM or higher $H_2O_2$. Note there is no change, or sometimes a modest increase, in cell viability when 0.1 mM $H_2O_2$ was added (Fig. 1A and data not shown), consistent with similar mitogenic response described previously [21]. Treatment for 2 to 25 hours caused no significant difference in relative cell viability (data not shown), presumably due to degradation of $H_2O_2$ at the longer time points.

We have examined if RNA damage occurs when exposed to $H_2O_2$. We detected no difference in RNA intactness in cultures treated without or with 0.1 or 0.5 mM $H_2O_2$ (Fig. 1B, lanes 1–3). RNA degradation was observed only when a much higher concentration of $H_2O_2$ (10 mM) was applied (Fig. 1B, lane 4). Therefore, RNA fragmentation can be induced by $H_2O_2$ but it is not a detectable damage at the low $H_2O_2$ dosages enough to cause significant cell death.

We then examined if oxidized nucleotide lesions elevate in RNA upon $H_2O_2$-treatment. Interestingly, 8-oxoG level increased remarkably in RNA in response to the oxidative insult. As shown in Fig. 1A, the level of 8-oxoG increased 3–4 folds in cells treated with 0.2–0.5 mM $H_2O_2$ in a concentration-dependent manner. The increase in 8-oxoG is associated with a sharp decrease in cell viability. Treatment with 0.1 mM $H_2O_2$ caused no increase in 8-oxoG level and no decrease in cell viability. Likewise, $H_2O_2$ at 0.5 and 0.7 mM resulted in similar reductions in cell viability and increases in 8-oxoG level. Therefore, RNA can be damaged in the form of oxidatively-modified bases at lethal concentrations of $H_2O_2$ though fragmentation is undetectable. Importantly, the level of 8-oxoG is inversely correlated to the level of cell viability.

### $H_2O_2$-induced 8-oxoG is specifically removed from RNA in HeLa cells

We further examined if 8-oxoG level reduces after removal of oxidative challenge. HeLa cell cultures were treated with or without $H_2O_2$ followed by washing and incubating with $H_2O_2$-free medium, or treated with $H_2O_2$ continually. Samples were withdrawn in a time course to analyze 8-oxoG content. As shown in the left panel of Fig. 1C, 8-oxoG level remains low in control cultures. As expected, there is a quick increase in 8-oxoG content after addition of $H_2O_2$. The level of 8-oxoG remains high in the period of time tested when $H_2O_2$ was continually

present. In the cultures pulse-treated with $H_2O_2$, the increased 8-oxoG level drops shortly after $H_2O_2$ is removed, by nearly half of the initial elevation after 30 minutes. This reduced level, still higher than the level at start, remains in the later time points. The removal of 8-oxoG in RNA most likely occurs also in the control cultures and cultures continually treated with $H_2O_2$. In both cases, 8-oxoG production and removal may become close to equilibrium, resulting in relatively steady levels of this lesion in RNA in the later time points.

We then tried to see if extended incubation after a pulse $H_2O_2$ treatment helps to further remove the residual 8-oxoG in RNA. An increase in the level of 8-oxoG after adding $H_2O_2$ and a decrease shortly after removal of $H_2O_2$ were observed (Fig. 1C, right panel), similar to the result shown in the left panel of Fig. 1C. The remaining half of the $H_2O_2$-induced 8-oxoG was completely removed after 24 hours. A similar observation was described previously in human lung epithelial cell [6]. In that case, 8-oxoG in RNA returned to basal level 24 hours after removal of oxidative challenge.

## hPNP overexpression increases cell viability and reduces RNA oxidation in response to $H_2O_2$-treatment

A plausible mechanism for eliminating 8-oxoG-containing RNA may involve recognition and degradation of such RNA. Both *E. coli* PNPase and hPNPase were shown to bind 8-oxoG RNA with specificity [14,18]. We have observed that PNPase protects *E. coli* cells against oxidative stress, possibly by degrading oxidized RNA (Jinhua Wu, Xin Gong, Shaohui Wu and Zhongwei Li, unpublished observations). Human PNPase is highly homologous to *E. coli* PNPase, sharing 39.8% identity and 57.2% similarity in their sequences (based on an analysis using EMBOSS Pairwise Alignment from EBI, http://www.ebi.ac.uk/emboss/align/). These enzymes may share similar function in degrading defective or damaged RNA. Therefore, we studied the possible role of hPNPase in controlling oxidatively damaged RNA.

The plasmid pCMV-hPNP carrying *hPNP* gene was introduced into HeLa cell to examine the effect of hPNPase overexpression. Equimolar pCMV* DNA was transfected into control cultures. As shown in Fig. 2A, *hPNP* mRNA is moderately overexpressed from pCMV-hPNP. At 48 hours after transfection, the normalized level of *hPNP* mRNA in cultures with pCMV-hPNP is 2 to 3 fold of that in the controls.

$H_2O_2$-treatment was carried out at 48 hours after transfection. As shown in Fig. 2B, the viability of control cells decreases depending on the dosage of $H_2O_2$, in a manner similar to that shown in Fig. 1A. Interestingly, in the presence of 0.3–0.7 mM $H_2O_2$, cell viability was significantly improved by the introduction of *hPNP* gene (Fig.2B). Overexpression of *hPNP* causes 25–35% reduction in cell death in this range of $H_2O_2$ concentration (Table 1). This rescue effect was not observed at higher $H_2O_2$ concentration when few cells survived. These data clearly demonstrate a protective role of overexpressed hPNPase against oxidative stress.

In order to examine if 8-oxoG production and removal in RNA are affected by *hPNP* overexpression, 8-oxoG level was analyzed in transfected cells pulse-treated with $H_2O_2$. Interestingly, overexpression of hPNPase significantly reduced the level of 8-oxoG in RNA in the entire time course (Fig.2C). Before $H_2O_2$-treatment, the level of RNA 8-oxoG in cultures overexpressing *hPNP* is about 30% lower than that in control cultures. Similar differences were observed after $H_2O_2$ addition. However, the 8-oxoG curves from the transfected cells are different from those shown in Fig 1C, presumably due to the differences in culture and treatment conditions. This behavior prevented us from identifying any change in the pattern of 8-oxoG removal following a pulse $H_2O_2$ treatment. Nevertheless, these data indicated that *hPNP* overexpression decreases the level of 8-oxoG in RNA, which is coupled with a partial rescue of HeLa cell.

### Knockdown of hPNPase increases $H_2O_2$-induced RNA oxidation and cell death

To further examine the effect of hPNPase reduction, we have introduced an siRNA to knockdown this enzyme. The siRNA sequence was depicted from a construct previously shown to knockdown both *hPNP* mRNA and hPNPase's poly(A) degradation activity in HeLa cell [21]. As shown in Fig. 3A, *hPNP* mRNA is down-regulated by at least 80% at 24, 48 and 72 hours after the introduction of the *hPNP* siRNA, compared to the level in the control. In contrast, *hGAPDH* mRNA was unaffected by the siRNAs. Similar knockdown efficacy by this *hPNP* siRNA was reported previously [21].

HeLa cell viability was determined at various $H_2O_2$ concentrations at 48 hours after siRNA introduction. As shown in Fig. 3B, *hPNP* knock-down significantly decreased the viability of HeLa cells under the treatment of 0.4 and 0.5 mM $H_2O_2$. We noted that the response of the siRNA-transfected cells to various dosages of $H_2O_2$ is different from those shown in Fig. 1A and 2B, presumably due to variations caused by transfection. Nevertheless, the significant reduction in cell viability by *hPNP* siRNA further supports the notion that hPNPase is important in protecting cells against oxidative stress.

A pulse $H_2O_2$-treatment was conducted to HeLa cells at 48 hours after siRNA transfection to study the effect of *hPNP* knockdown on 8-oxoG level and its removal. In cultures with the nonspecific siRNA, the level of RNA-borne 8-oxoG first increased to nearly 2 fold after $H_2O_2$-addition, then decreased in the next 30 minutes after removal of $H_2O_2$ (Fig. 3C), a response similar to that of non-transfected cultures (Fig. 1C). Importantly, cells transfected with *hPNP* siRNA contains higher basal level of RNA-borne 8-oxoG than those with the control siRNA, suggesting that *hPNP* knockdown causes an increase in RNA oxidation even under normal aerobic growth (Fig. 3C). Upon pulse $H_2O_2$-treatment, 8-oxoG level increases dramatically in *hPNP* siRNA-transfected cells, with a delayed and elevated peak after removal of $H_2O_2$. Compared to controls, *hPNP* knockdown causes accumulation of higher 8-oxoG in RNA at all time points.

## Discussions

In this work, we have shown that HeLa cell viability is inversely correlated with RNA oxidation when exposed to $H_2O_2$. While overexpression of hPNPase reduces 8-oxoG in RNA and improves cell viability against $H_2O_2$-treatment, knockdown of hPNPase causes converse effects. Our observations suggest an important biological role for this exoribonuclease under oxidative stress.

Although hPNPase is an exoribonuclease shown to preferentially bind 8-oxoG RNA [18], its role in directly degrading oxidized RNA cannot be easily established. First of all, *E. coli* PNPase and hPNPase has been reported to bind but not to degrade oligoribonucleotide made of 8-oxoG, although both enzymes degrade normal RNA [14,18]. The *E. coli* cell-free extracts containing PNPase do not favor degrading oxidized RNA over normal RNA *in vitro* (Xin Gong and Zhongwei Li, unpublished observation), suggesting that PNPase *per se* is not sufficient for selective degradation of such RNA. The preferential removal of oxidized RNA in cells likely involves interactions missing *in vitro* [2]. Secondly, human PNPase is induced by type I interferons and contributes to cell terminal differentiation and senescence [16,17,22]. It was further shown that hPNPase plays roles in tumorigenesis [22], the cellular response to viral infection [17] and maintaining mitochondrial homeostasis [23]. hPNPase is responsible for the degradation the cytosolic c-myc mRNA and for the processing and adenylation of mitochondrial RNA [16,17,21,24]. However, a link of hPNPase's diversified roles to its RNase activity has been only suggested in limited cases [16,17]. The situation is more controversial when hPNPase, initially found mainly in mitochondria, was recently shown to localize in the intermembrane space of mitochondria [23] where no RNA is present. Therefore, certain

function of hPNPase may be indirectly related to or independent of its activities on RNA [24]. In the case of controlling RNA oxidation level, it is unlikely that hPNPase works as a general antioxidant since its overexpression increases ROS level in cell [25]. Oxidized RNA may be selectively degraded by any residual hPNPase present in the matrix of mitochondria or in the cytoplasm. Alternatively, hPNPase may regulate other activities that are directly involved in eliminating oxidatively damaged RNA. Further studies are required to better understand the role of hPNPase under oxidative stress and in other physiological processes.

After the removal of oxidative challenge, 8-oxoG-containing RNA appears to be reduced in a biphasic pattern (Fig. 1C). Our results suggested an important role for hPNPase in limiting 8-oxoG in RNA (Fig. 2C and 3C). Currently, it is not clear if hPNPase functions in any specific part of the biphasic removal of 8-oxoG. Oxidative damage is expected to occur randomly in RNA. Various damaged RNA species may be removed by different mechanisms which remain to be elucidated.

The fact that cell viability and RNA oxidation are correlated, and that hPNPase helps in both reducing RNA damage and increasing cell viability, suggests a causative relationship of RNA oxidation and cell death under oxidative stress. Damage of RNA is obviously as deleterious as the damage of other macromolecules, however, evidence of cell death caused by RNA damage has been missing. The extent of cell death caused by RNA oxidation remains to be examined by future studies. We have shown that moderate overexpression of hPNPase reduces nearly 30% of 8-oxoG in RNA (Fig. 2C) and rescues 25–35% cells at various $H_2O_2$ concentrations (Table 1). Though the effect is moderate, hPNPase must play an important role in controlling damaged RNA, a complicated process in which the involvement of multiple overlapping activities is expected.

RNA-borne 8-oxoG is much more abundant than its DNA counterpart in the urine and plasma of human and rat [26], suggesting that 8-oxoG derived from degradation of oxidized RNA may serve as a promising biomarker for cellular oxidative stress. The possible role of human PNPase in releasing 8-oxoG from RNA and in controlling its level in biological fluids remains to be elucidated.

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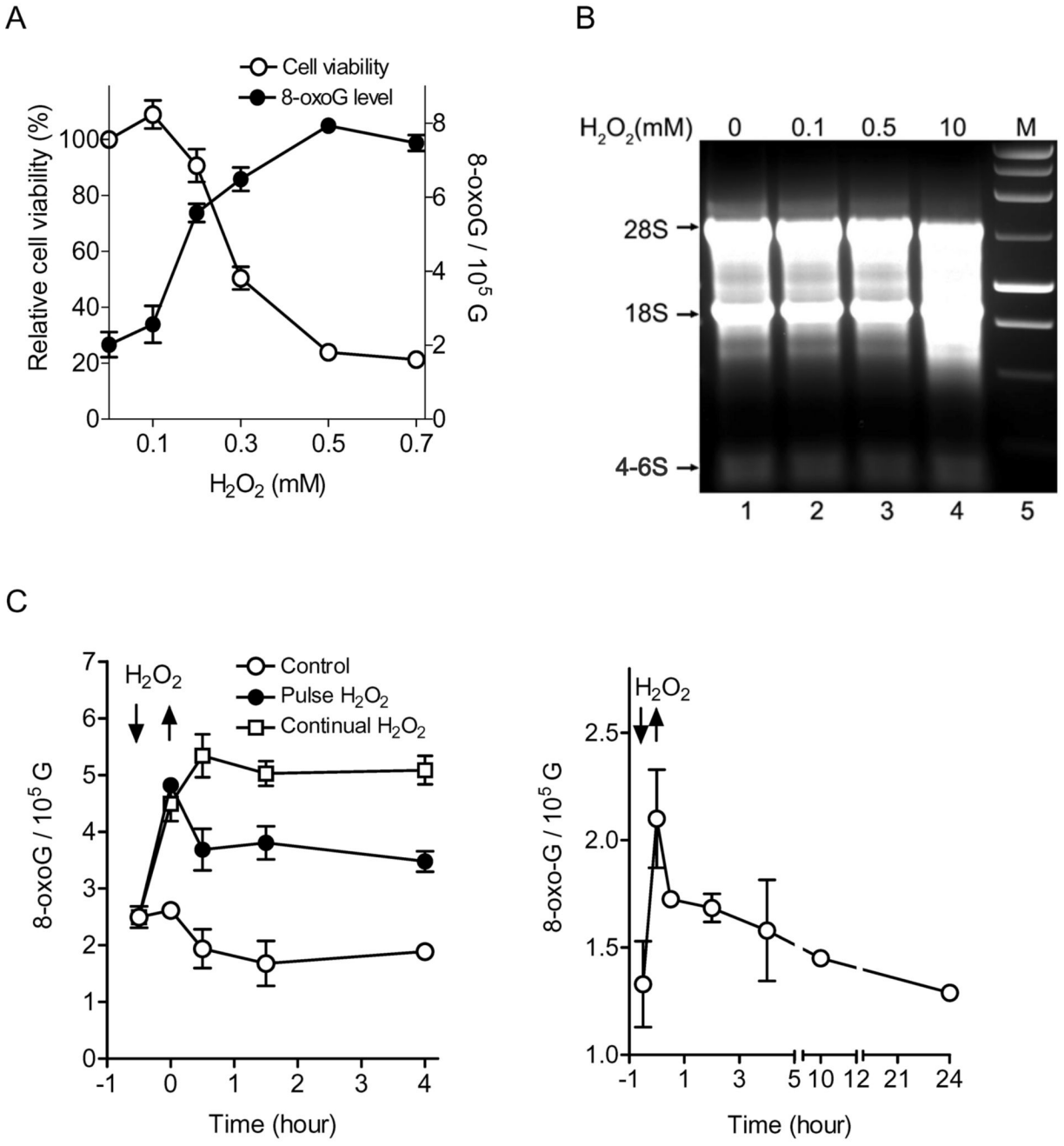


**Fig. 1.**

HeLa cell viability and RNA damage in response to $H_2O_2$ treatment. (A) Cell viability was measured in cultures grown in 96-well plates and treated by indicated concentrations of $H_2O_2$ for 2 hours. The mean ± SD of five replicates were plotted. To measure 8-oxoG level, cells grown in 6-well plates were treated with indicated concentrations of $H_2O_2$ for 2 hours before RNA isolation. The mean and SEM of triplicates were plotted. (B) Total RNAs prepared from cells underwent 2 hours $H_2O_2$-treatment were separated on 1.2% agarose gels and stained with SYBR Gold (Invitrogen). The sizes of known RNA species are marked on the left. A DNA size marker was included in Lane 5. (C) RNA 8-oxoG levels were determined in cells exposed to a pulse or a continual treatment of 0.2 mM $H_2O_2$. In the pulse treatment, $H_2O_2$ was

added and removed at the time points indicated by the arrows. Cells were washed once and incubated with pre-warmed $H_2O_2$-free medium in the remaining part of the time course. The control culture was also treated with washing and medium change. The mean ± SD from triplicates were plotted. An extended time course after pulse treatment was plotted in a separate graph on the right.

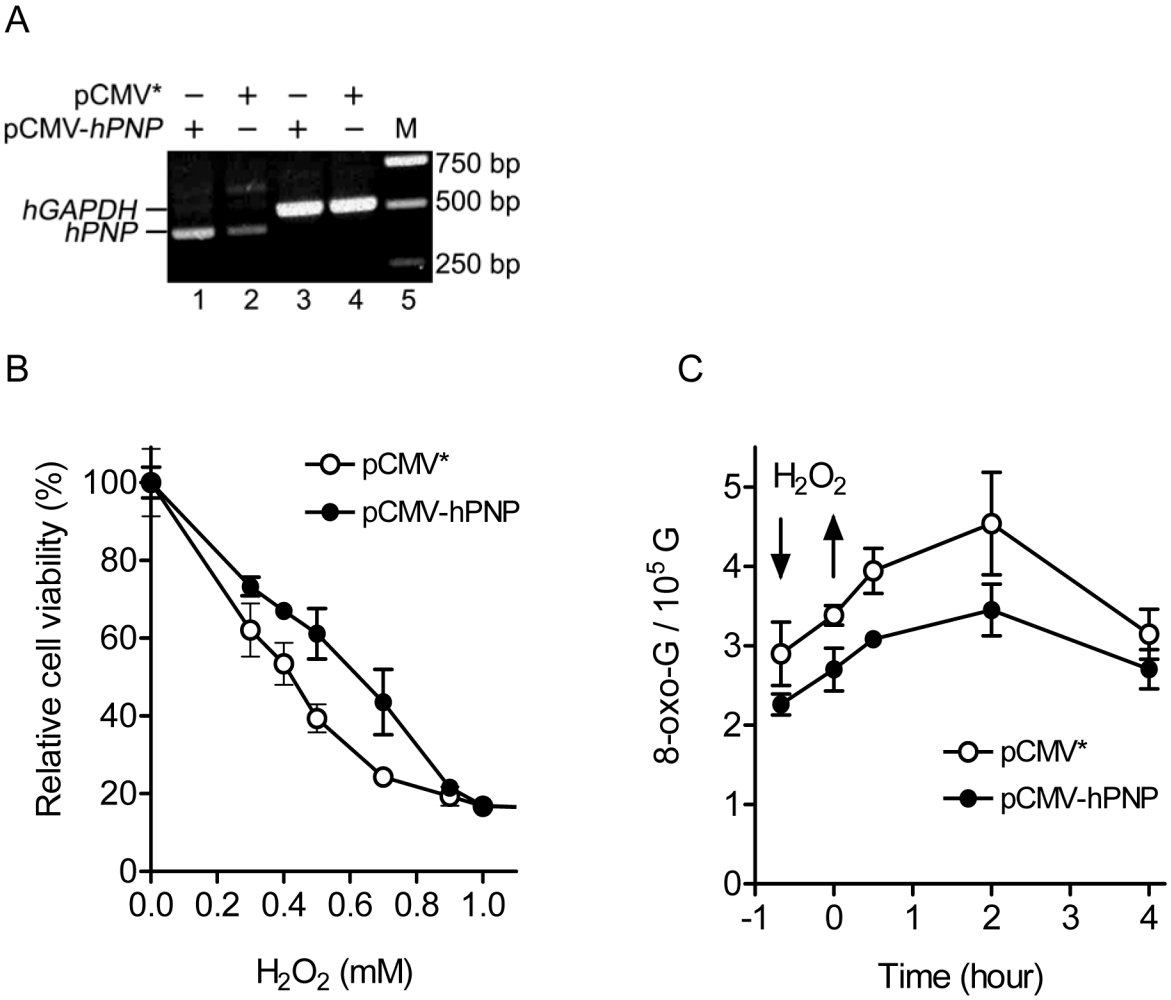


**Fig. 2.**

Effect of hPNPase overexpression on RNA oxidation and viability of HeLa cells treated with $H_2O_2$. (A) The level of over-expression of *hPNP* mRNA was determined by semi-quantitative RT-PCR after transfection of pCMV-hPNP and the control plasmid pCMV*. RT-PCR products (marked on the left) from RNA isolated 48 hours after transfection with indicated DNA are shown together with a DNA size marker ("M"). (B) Relative viability of HeLa cell in response to treatment with indicated concentrations of $H_2O_2$ at 48 hours after transfection. The mean ± SD from 4 replicates were plotted. (C) Levels of 8-oxoG were determined in RNA isolated from cells pulse-treated with 0.3 mM $H_2O_2$ at 48 hours after transfection. The mean ± SD of triplicates were plotted.

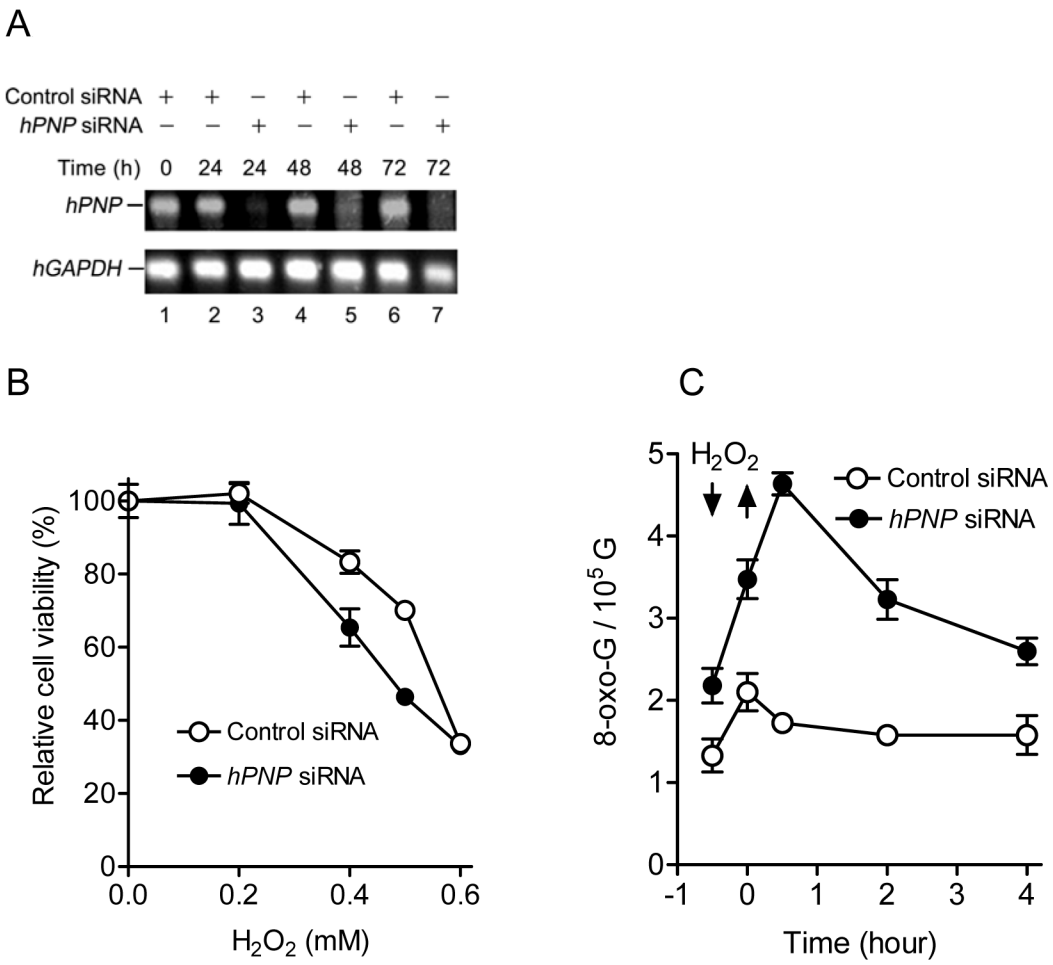


**Fig. 3.**

Effect of hPNPase knockdown on 8-oxoG content in RNA and on viability of HeLa cell exposed to $H_2O_2$. (A) At indicated time points after transfection of 5 nM *hPNP* siRNA or control siRNA, the levels of *hPNP* mRNA were determined by RT-PCR using *hGAPDH* as internal normalization control. (B) HeLa cell cultures 48 hours after transfection were exposed to indicated concentrations of $H_2O_2$ and relative viability levels was determined as in Fig. 1A. The mean ± SD from 5 replicates were plotted. (C) Cells transfected with siRNA for 48 hours were pulse-treated with $H_2O_2$ and 8-oxoG levels were determined as described in Fig. 1C. The mean ± SD of triplicates were plotted.

**Table 1**

Restoration of HeLa cell viability by *hPNP* overexpression against $H_2O_2$-treatment

| $H_2O_2$ (mM) | pCMV* | | pCMV-hPNP | | Rescue by *hPNP* overexpression | |
|---|---|---|---|---|---|---|
| | Rel. Viability (Mean ± SD) | *a.* Decrease (%) | Rel. Viability (Mean ± SD) | *b.* Decrease (%) | *c.* Rel.Viability Rescued *a*–*b*) | % Rescued ((*c*/*a*)x100) |
| 0.3 | 62.1 ± 6.9 | 37.9 | 73.3 ± 2.4 | 26.7 | 11.2 | 29.5 |
| 0.4 | 53.4 ± 5.4 | 46.6 | 67.0 ± 1.8 | 33.0 | 13.6 | 29.3 |
| 0.5 | 39.4 ± 3.6 | 60.6 | 61.1 ± 6.5 | 38.9 | 21.8 | 35.9 |
| 0.7 | 24.3 ± 2.1 | 75.7 | 43.5 ± 8.4 | 56.5 | 19.3 | 25.5 |
| 0.9 | 19.4 ± 2.5 | 80.6 | 21.5 ± 0.7 | 78.5 | 2.2 | 2.7 |

*Data from Fig. 2B were analyzed.