

The contribution of mitochondrial thymidylate synthesis in preventing the nuclear genome stress

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ABSTRACT

In quiescent fibroblasts, the expression levels of cytosolic enzymes for thymidine triphosphate (dTTP) synthesis are down-regulated, causing a marked reduction in the dTTP pool. In this study, we provide evidence that mitochondrial thymidylate synthesis via thymidine kinase 2 (TK2) is a limiting factor for the repair of ultraviolet (UV) damage in the nuclear compartment in quiescent fibroblasts. We found that TK2 deficiency causes secondary DNA double-strand breaks formation in the nuclear genome of quiescent cells at the late stage of recovery from UV damage. Despite slower repair of quiescent fibroblast deficient in TK2, DNA damage signals eventually disappeared, and these cells were capable of re-entering the S phase after serum stimulation. However, these cells displayed severe genome stress as revealed by the dramatic increase in 53BP1 nuclear body in the G1 phase of the successive cell cycle. Here, we conclude that mitochondrial thymidylate synthesis via TK2 plays a role in facilitating the quality repair of UV damage for the maintenance of genome integrity in the cells that are temporarily arrested in the quiescent state.

INTRODUCTION

Ultraviolet (UV) irradiation causes DNA lesions resulting from cyclobutane pyrimidine dimer (CPD) and (6–4) photoproduct formation. These lesions in genomic DNA are recognized and repaired by nucleotide excision repair (NER) pathway in mammalian cells. There are two sub-pathways of NER including global genomic NER and transcription coupled NER (1). These two pathways differ in recognizing DNA lesion sites, which is mediated by XPC-RAD23B complex in global genomic NER (2,3)

and RNA polymerase II in transcription coupled NER (4). The damaged oligonucleotide are removed by XPG and XPF-ERCC1 endonucleases (5,6), resulting in single-stranded DNA gap that requires 24–32 deoxynucleotides incorporation to complete the repair process dependent on DNA Polymerases Pol ϵ , Pol δ or Polk with DNA clamping protein proliferating cell nuclear antigen (PCNA) (7–9). Finally, the DNA nick is sealed by DNA ligase I in proliferating cells or by DNA ligase III/XRCC1 throughout the cell cycle (9,10).

To fill the gaps after DNA lesion excision in NER, sufficient amount of cellular dNTP is needed. Ribonucleotide reductase (RNR), which converts ADP, GDP, CDP and UDP to the respective dNDP, is a rate-limiting enzyme in generating a balanced pool of dNTPs. In mammalian cells, RNR is composed of two pairs of R1 and R2 subunits (11). The expression of R2 subunit is cell cycle-dependent, while R1 subunit is constitutively expressed in cycling cells. Therefore, the amounts of dNTPs are higher in proliferating than that of non-dividing cells. A homolog of R2, p53-inducible R2, can also form an active enzyme complex with R1 to have ribonucleotide reduction function (12–14). Distinct from R2 subunit, the expression of p53R2 is not cell cycle-regulated. The expression of p53R2 is, therefore, important in dNTP supply for DNA repair in G0/G1 cells (12,15,16). In accordance, a recent study has shown that RNR activity makes a major contribution to the maintenance of dCTP and dGTP pool in quiescent fibroblasts, critical for repairing UV-irradiated DNA damage (16).

As RNR does not form dTDP directly, the *de novo* synthesis of thymidine triphosphate (dTTP) relies on thymidylate synthase (TS), which catalyses the methylation of deoxyuridine monophosphate (dUMP) to form thymidine monophosphate (dTMP). dTMP is then converted to thymidine diphosphate (dTDP) by thymidylate kinase. The formation of dTMP can also be derived from the salvage pathway via cytosolic thymidine kinase 1 (TK1). The expressions of TS and TK1 are cell cycle-dependent, being maximal in the S phase and low in G0/G1

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phase (17,18). Given the lack of TS and TK1 expression, quiescent cells contain low level of dTTP. Mitochondrial thymidine kinase 2 (TK2) is another salvage enzyme for dTTP supply. Although the catalytic efficiency of TK2 is much lower than that of TK1 (19), it plays a pivotal role in dTTP synthesis for mitochondrial DNA (mtDNA) replication in non-dividing cells.

Deficiency in TK2 activity due to genetic alterations such as point mutations causes devastating mtDNA depletion syndrome in humans with death at young age (20). As such, the physiological importance of TK2 has been emphasized in mitochondrial genome integrity. Meanwhile, TK2 inhibitor has been developed to prevent mitochondrial toxicity due to misincorporation of antiviral and anticancer nucleoside analog-based drugs to mtDNA via TK2 (21). However, the possible role of TK2 in repair of nuclear genome DNA has not been explored. In this study, we found that increase in mitochondrial thymidylate synthesis via TK2 facilitated NER in the nuclear compartment. We further investigated how cells deficient of TK2 recover from UV damage in their quiescent state, and observed their re-entrance of the cell cycle progression with genome scars.

MATERIALS AND METHODS

Materials and antibodies

Anti-human TK1 and TMPK polyclonal antibody was described previously (22,23). Anti-human TS antibody (clone 4H4B1) was obtained from Zymed laboratories Inc. Anti-R1 (T16), anti-R2 (N18), anti-p53R2 (N16), anti-PCNA (PC10), anti-53BP1 (H-300), horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, donkey anti-goat antibodies and NU7441 (8-Dibenzo [*b,d*]thiophen-4-yl-2-morpholin-4-yl-4*H*-chromen-4-one) were from Santa Cruz. Anti- γ -H2AX (phospho-H2AX at Ser139) and anti-53BP1 (clone BP13) was from Millipore. Anti-(6-4) photoproduct (64 M-2) and CPD (TDM-2) were from Cosmo Bio Co., Ltd (Tokyo, Japan). Anti-cyclin A (E23.1) was from GeneTex Inc. (Irvine, CA, USA). Anti- β -tubulin, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse and TRITC-conjugated anti-rabbit antibodies, deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine, were from Sigma-Aldrich. Lipofectamine 2000 transfection reagent was purchased from Invitrogen Life Technologies.

Cell culture

Human diploid lung fibroblast, IMR-90 cells were purchased from Coriell Cell Repositories (Camden, NJ, USA). Cells with population doubling level between 25 and 35 were used for all experiments. 293FT cells for lentivirus production were purchased from Invitrogen. IMR-90 and 293FT cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) at 37°C under 5% CO₂.

UV irradiation

Cells grown on coverslip were UV-C irradiated by UVP CL-1000 UV cross-linker. For whole-cell irradiation, cells in phosphate buffered saline were subjected to 10 J/M² UV irradiation. For localized UV-irradiation, polycarbonate isopore membrane with 3 μ m pore size filters (Millipore) was placed on top of cells in Hank's balanced salt solution, and irradiated with 120 J/M² UV. Following removal of the filters, the cells were recovered with fresh medium.

Immunofluorescence staining

Cells grown on coverslip were fixed by 4% paraformaldehyde at room temperature or methanol at -20°C for 5 min. Cells fixed by paraformaldehyde were permeabilized with 0.3% Triton X-100. After blocking with 5.5% normal goat serum, cells were incubated with primary antibodies overnight at 4°C. Cells were stained with FITC- and TRITC-conjugated secondary antibodies and Hoechst33342 for 1 h at room temperature. The conditions for PCNA, CPD (24) and (6-4) photoproduct (25) were as described previously. Mounted slides were examined by using Olympus BX51 microscope. For quantification of immunofluorescence (IF) intensity, images acquired from the same exposure setting were analysed by ImageJ software. IF intensities were normalized by the background value for analysis.

Immunoblotting

Cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (100 mM Tris-HCl, pH 7.6, 250 mM NaCl, 0.1% sodium dodecyl sulphate, 0.2% deoxycholic acid, 0.5 mM dithiothreitol, 1 mM EDTA, 0.5% NP-40 and 1% Triton X-100), and proteins in each cell lysate were analysed by immunoblot using specific antibodies and horseradish peroxidase-conjugated secondary antibodies. The enhanced chemiluminescent substrate (Millipore) for reaction was added, and the signal was detected by UVP bioimaging system.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used for cDNA synthesis, which was amplified by polymerase chain reaction (PCR) using TK2 sense primer 5'-AATCG GGATCGAATATTA ACT-3', TK2 antisense primer 5'-T GAACACCGGGCTCCAGCCAA-3'.

Comet assay

For detection of both single-stranded and double-stranded DNA breaks, alkaline comet assay was used. For double-stranded DNA breaks detection, neutral comet assay was carried out. Both assays were performed using a reagent kit of single cell gel electrophoresis assay kit (Trevigen, Inc). Image data were analysed by Sicon image software (26).

TK activity assay

The assay was performed as previously described using the DE-81 filter paper (Whatman) technique (27). Briefly, cells

were harvested in the buffer containing 50 mM Tris-HCl (pH 7.9), 0.5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 1 mM dithiothreitol (DTT) for homogenization and centrifugation. The supernatants were applied to the activity assay in a final concentration of 50 mM Tris-HCl (pH 7.9), 5 mM ATP, 5 mM MgCl₂, 0.1% bovine serum albumin, 5 mM NaF, 2 mM DTT, 180 μM thymidine and [³H]-thymidine at 37°C for 30 min. After stopping the reactions, aliquots of reaction mixture were spotted onto DE-81 disc. The amount of radioactivity retained on the disc following washings by 5 mM ammonium formate was determined by scintillation counting.

dNTP determination

Cells were extracted with 1 ml of ice-cold 60% methanol at -20°C overnight, followed by centrifugation at 16 000 g for 30 min. The supernatant was boiled for 3 min and dried under vacuum. The pellets were dissolved in nuclease-free water for dNTP measurement based on the method previously described (28).

shRNA lentivirus preparation and infection

293FT cells were cotransfected with pCMVdeltaR8.91, pMD.G and pLKO.1 TK2 small hairpin RNA (shRNA) or LacZ shRNA plasmids. After transfection for 54 h, supernatants containing lentivirus were filtered through polyvinylidene fluoride (PVDF) membrane (pore, 0.45 μm, Millipore) and concentrated using Amicon Ultra column (100 k dalton molecular weight cut-off, Millipore). IMR-90 cells were infected by the virus soup containing 8 μg/ml polybrene (Sigma) for 6 h. Cells were washed twice by phosphate buffered saline and cultured in a complete medium.

S phase cell quantification

EdU (5-ethynyl-2'-deoxyuridine) incorporation assay was carried out by Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen). Briefly, cells on coverslips were incubated with 10 μM EdU for 30 min. After fixation and permeabilization, cells were incubated in Click-iT reaction cocktail containing Alexa Fluor azide for 30 min at room temperature. Following the click reaction, cell nuclei were counterstained by Hoechst 33342.

Cell growth assay

Cell growth was determined by WST1 reagent (Roche Applied Science) according to the manufacturer's manual. Briefly, cells in 96-well plates were incubated with 100 μl of mixture of WST1 reagent and culture medium in a ratio of 1:10 (V/V) per well at 37°C for 1 h. The absorbance at 450 nm of samples normalized by a background control was measured by microplate ELISA reader.

RESULTS

NER and dNTPs pools in quiescent cells after serum deprivation

IMR-90 human diploid fibroblasts were serum-deprived for 2, 4 and 10 days. These cells were UV-irradiated through isopore polycarbonate membrane filters (pore, 3 μm) to create localized UV damage in the genome. NER process can be divided into two stages, lesion recognition followed by incision and post-incision by gap-filling synthesis (29). We tested whether serum deprivation influenced the efficiency of lesion excision by (6-4) photo-product IF staining. UV-induced foci were found to disappear within 1 h in these cells regardless of serum deprivation (Figure 1A). We also performed the IF staining of PCNA, which is localized in the lesion site for the gap-filling step. NER foci revealed by the PCNA IF staining were pronounced after recovery from UV exposure at 0.5 h in both asynchronous and serum-deprived cells, all of which contained four to six prominent PCNA foci of ~3 μm in the nucleus, and the fluorescence intensity was increased with longer serum deprivation (Figure 1B). In asynchronous cells, these foci disappeared within 1 h. For cells serum-deprived for 2, 4 and 10 days, PCNA foci persisted at 1 h and diminished at 3 h, suggesting a slower rate in gap-filling (Figure 1B). Notably, the longer serum deprivation the higher intensities of PCNA foci were sustained during recovery. Taken together, these data suggest that it is the gap-filling synthesis rather than the DNA lesion removal step affected by serum deprivation.

Previously, it has been proposed that the expression level of PCNA is reduced after serum deprivation, which affects the gap-filling step of NER (30). However, we found the intensity of PCNA foci increased in the local UV damage site, although the cellular level of PCNA protein was decreased after serum deprivation (Figure 1C). The question is what causes PCNA sustained at the lesion sites? We then measured the levels of four dNTP pools in cells after serum deprivation and found that the level of dTTP in serum-deprived 2-, 4- and 10-day cells was dropped to 9, 3 and 0.2% of that in asynchronous cells, respectively (Table 1). The reduction in dCTP, dATP and dGTP pools in serum-deprived cells were also reduced but to lesser extent as compared with the dTTP pool. Western blot analyses showed severe reduction of R2, TS and TK1 proteins from 2 to 10 days of serum deprivation, while the levels of TMPK and R1 were moderately decreased and p53R2 remained unchanged (Figure 1C). The lack of TS and TK1 in serum-deprived cells most likely accounts for the drastic reduction in dTTP pool, while R1 and R2 down-regulations are responsible for the decreases in the other three dNTPs pools during serum deprivation. Therefore, deficiency of dNTP supply in quiescent fibroblasts is probably the cause of prolonged gap-filling step in NER, thereby sustained PCNA foci. To verify this, we then treated asynchronous cells with hydroxyurea, which reduces dNTP supply by blocking RNR, and found that these treated cells also displayed sustained PCNA foci after UV micro-irradiation (Figure 1D), suggesting that the shortage of dNTP

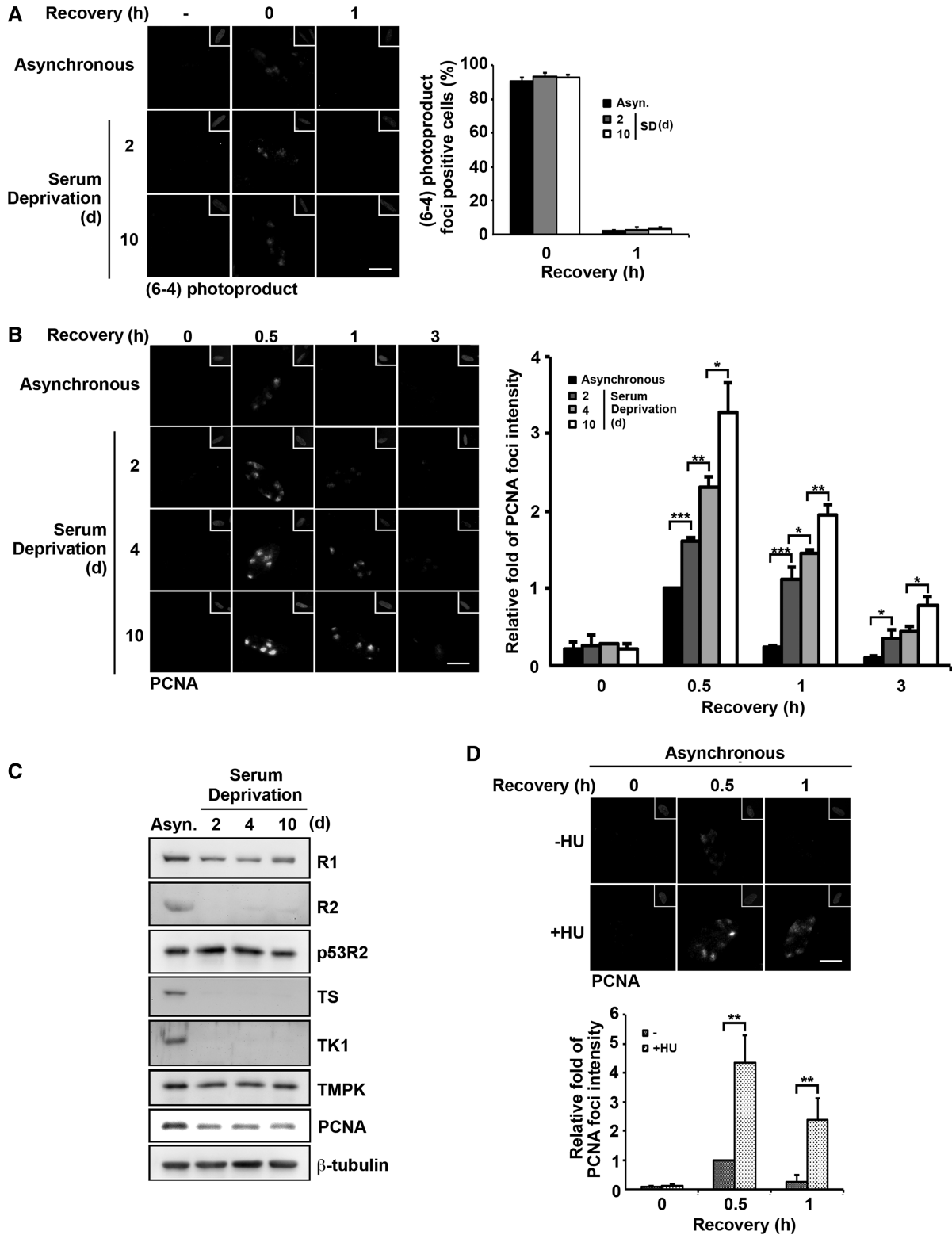


Figure 1. Serum deprivation and dNTP supply affects the gap-filling step in NER. IMR-90 fibroblasts were incubated in medium containing 10% serum (asynchronous), serum-free for 2 and 4 days or 0.1% serum for 10 days. Asynchronous and serum-deprived cells were irradiated with 120 J/M² UVC through micropore membrane (pore, 3 μ m) and were recovered, after which cells on coverslips were subjected to (A) (6-4) photoproduct and (B) PCNA IF staining (Scale bar, 10 μ m). The percentages of (6-4) photoproduct foci positive cell were counted. More than 100 cells were counted for each experiment ($n = 3$). PCNA fluorescence intensity was expressed relative to that in asynchronous cells recovered at 0.5h. Fifty cells were analysed for each experiment ($n = 3$). (C) Western blot analysis of cell lysates. (D) Asynchronous cells were pretreated with or without 2 mM hydroxyurea for 3h before local UV irradiation and during recovery. Cells were fixed for PCNA IF staining (Scale bar, 10 μ m). Fluorescence intensity was expressed relative to that in cells recovered at 0.5h without hydroxyurea treatment. Fifty cells were analysed for each experiment. Insets show Hoechst33342 staining in the corresponding cells. ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on Student's t -test.

Table 1. dNTP pools in asynchronous and serum-deprived IMR-90 cells

day	dNTP	dTTP	dCTP	dATP	dGTP
Asyn.		64.1 ± 3.08	15.3 ± 0.46	3.5 ± 0.13	5.5 ± 0.21
2		5.6 ± 3.20 (8.8%)	3.2 ± 0.82 (20.9%)	2.5 ± 0.50 (71.4%)	0.9 ± 0.29 (16.4%)
4		2.1 ± 0.50 (3.3%)	2.1 ± 1.20 (13.7%)	2.0 ± 0.50 (57.1%)	0.7 ± 0.37 (12.7%)
10		0.1 ± 0.06 (0.2%)	0.6 ± 0.29 (3.9%)	0.1 ± 0.01 (2.9%)	0.1 ± 0.02 (1.8%)

dNTP, pmole/10⁶ cells.

%, percentage of asynchronous cells.

IMR-90 cells were incubated in medium containing 10% serum (Asyn.), serum free for 2 and 4 days or 0.1% serum for 10 days.

Asynchronous and serum-deprived cells were harvested for cellular dNTP determination. The number in parentheses indicates the percentage of pool in that of asynchronous cells.

supply in quiescent cells prolongs the gap-filling step. Thus, serum deprivation causes dNTPs depletion, which delays the gap-filling step in NER.

The increase in dTTP synthesis via TK2 facilitates the gap-filling step of NER in quiescent cells

Despite limiting level of dNTP pools after 10 days of serum deprivation, these quiescent fibroblasts are still capable of completing the NER process under the micro-irradiated condition. To assess the role of mitochondrial TK2 in repairing UV damage in the nuclear genome of quiescent cells, we silenced TK2 expression by lentivirus-based shRNA infection. After serum deprivation for 2 and 10 days, we found that TK2 knock-down did not affect the levels of R1, R2, TS, TK1, TMPK or p53R2 as compared with control cells (Figure 2A). Due to the limitation of TK2 antibody in western blot, TK activity was measured in total cell lysates. For cells with serum deprivation for 2 days, an appreciated level of TK activity was detected in control cells, and TK2 knock-down reduced 30% of TK activity (Figure 2B). By BrdU incorporation experiment, it was found that ~1.5% of cells were still in S phase after 2 days of serum deprivation (data not shown). Because of the high catalytic efficiency of TK1 protein (19), the presence of TK1 in this small fraction of S phase cells may account for 70% of the TK activity measured in the 2-day serum-deprived cell lysates. After 10-day serum deprivation, the S phase fraction was reduced to <0.4% (data not shown). In these cells, >60% of cellular TK activity was decreased by TK2 knock-down (Figure 2B). We then performed micro-irradiation experiments in 10-day-serum-deprived cells. Following irradiation, (6–4) photoproduct foci were removed within 1 h in both control and TK2 knock-down cells (Figure 2C). The removal of CPD foci was slow and sustained at 16 h after recovery. The decayed rate of CPD foci was also similar in control and TK2 knock-down cells (Figure 2D). However, the intensity of PCNA foci was significantly higher and prolonged in TK2 knock-down cells than those in control cells during 0.5–3 h post-recovery period (Figure 2E). We also compared the role of TK2 in repairing UV-induced nuclear DNA lesions in asynchronous cells. It appeared that asynchronous LacZ and TK2 knock-down cells were similar in the changes of the fluorescence intensity of PCNA foci, which reached maximum at 0.5 h and declined at 1 h after recovery (Supplementary Figure S1), suggesting

that TK2 is dispensable for repairing UV-induced genomic lesions in asynchronous cells that have higher level of dTTP. Therefore, it is in the quiescent cells that TK2 is important in the gap-filling step of NER.

Under the physiological condition, low level of thymidine is supplied by the extracellular fluid and intracellular degradation of DNA. We then incubated serum-deprived cells with 5 μM thymidine. This treatment effectively decreased the fluorescence intensity of PCNA foci at 1 h post-recovery in LacZ control but not TK2 knock-down cells (Figure 2F). Addition of thymidine led to significantly elevated dTTP level in control but not in TK2 knock-down cells (Figure 2G). Thus, TK2-mediated salvage synthesis of dTTP is sufficient and necessary for facilitating DNA repair in quiescent cells. The effect of addition of four doxynucleosides was similar to thymidine addition alone. Therefore, dTTP is the major limiting factor for the repair process under this cellular condition. These results are consistent with a previous study reporting that thymidine supplement during recovery from UV irradiation promotes DNA repair in quiescent lymphocytes (31).

TK2 deficiency increases DNA double-strand breaks in quiescent cells during recovery from UV damage

It has been reported that following UV exposure, histone H2AX phosphorylation at Ser-139 (γ-H2AX) takes place in quiescent fibroblasts due to the delayed gap-filling at the single-strand break (SSB) site after removal of the DNA lesions (30). In micropore-UV-irradiated lesions, we were unable to detect γ-H2AX foci; probably, the amounts of dNTPs are still sufficient for repairing few localized lesions (each pore, 3 μm). Therefore, we then directly applied UV irradiation to the quiescent cells and found that whole-cell irradiation also caused γ-H2AX foci formation, with intensities reached maximum at 4 h and declined thereafter (Figure 3A). In TK2 knock-down cells, the intensity of γ-H2AX foci was higher during recovery from irradiation. Expression of shRNA-resistant TK2r-GFP into TK2 knock-down cells restored the repair efficiency of UV irradiation (Supplementary Figure S2), confirming the role of TK2 in repair. In control cells, addition of thymidine (5 μM) to the culture medium significantly decreased the fluorescence intensity of γ-H2AX foci, and the reduction in γ-H2AX foci intensity by thymidine addition was similar to that in cells supplied with all four deoxynucleosides. However, In TK2 knock-down

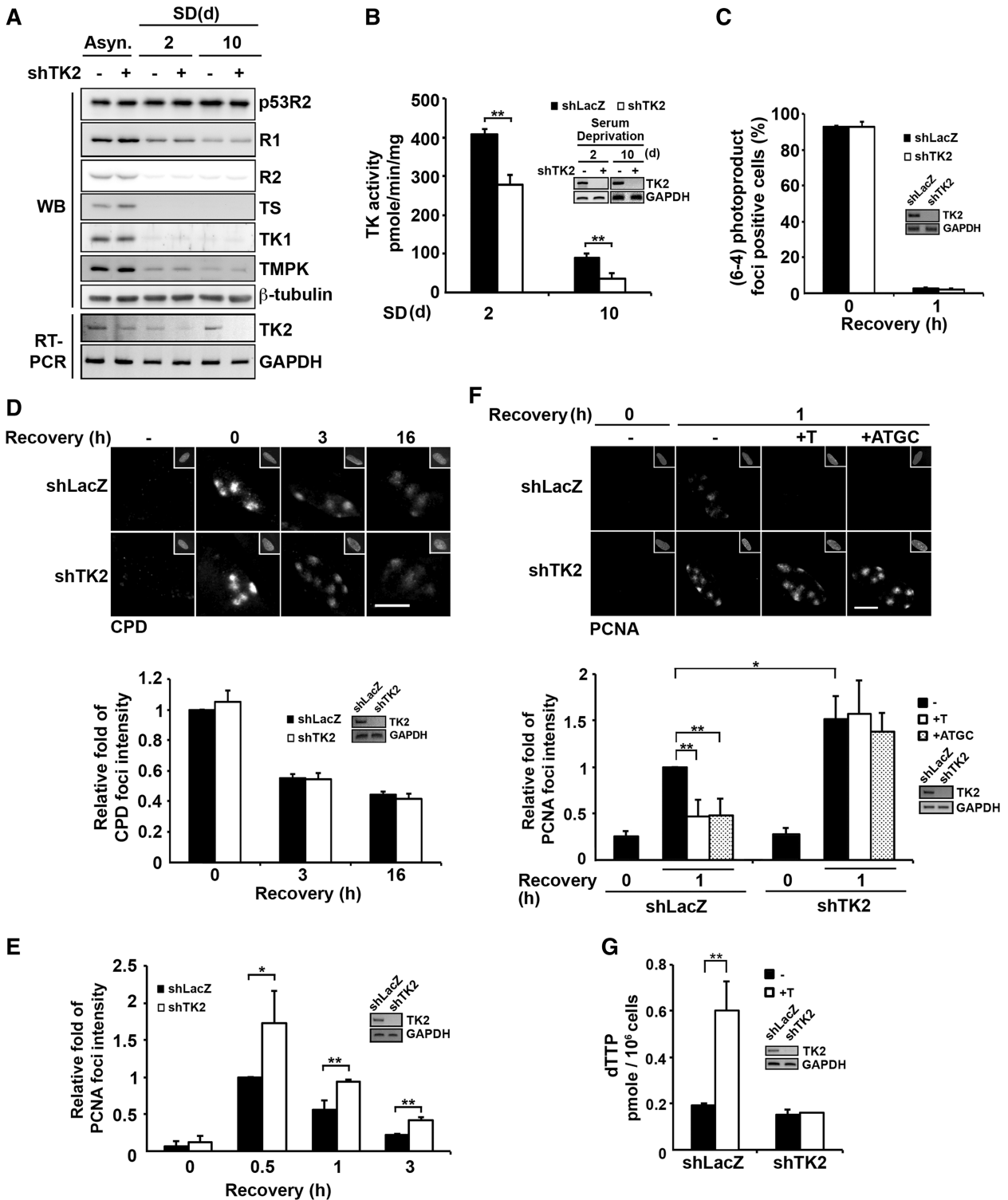


Figure 2. The contribution of TK2-mediated synthesis of dTTP to the rate of the gap-filling step in quiescent cells. IMR-90 cells were infected by LacZ or TK2 shRNA lentivirus. These cells were incubated in 10% serum containing or serum-deprived condition for (A) western blot and (B) thymidine kinase activity assay. (C) 10-day serum-deprived control and TK2 knock-down cells were irradiated by 120 J/M² UVC through micropore membrane. After recovery with 0.1% serum at indicated time, cells were fixed for (6-4) photoproduct, (D) CPD and (E) PCNA IF staining (Scale bar, 10 μm). The quantification of (6-4) photoproduct and PCNA IF staining was as described in the legend to Figure 1. CPD fluorescence intensity was expressed relative to that in control cells recovered at 0 h. Fifty cells were analysed in each experiment (*n* = 3). (F) Cells were supplemented with 5 μM thymidine or four deoxynucleosides in 0.1% dialysed serum for 8 h before UV micro-irradiation. Cells were recovered in the same medium for 1 h, and fixed for PCNA IF staining analysis (Scale bar, 10 μm). Fluorescence intensity was expressed relative to that in LacZ control cells recovered at 1 h without thymidine supplement. Fifty cells were analysed for each experiment (*n* = 3). Insets show Hoechst33342 staining of the corresponding cells. (G) After thymidine incubation for 8 h, cells were harvested for dTTP pool analysis. Reverse transcriptase-PCR (RT-PCR) of RNA extracted from indicated cells were shown. **P* < 0.05, ***P* < 0.01 based on Student's *t*-test.

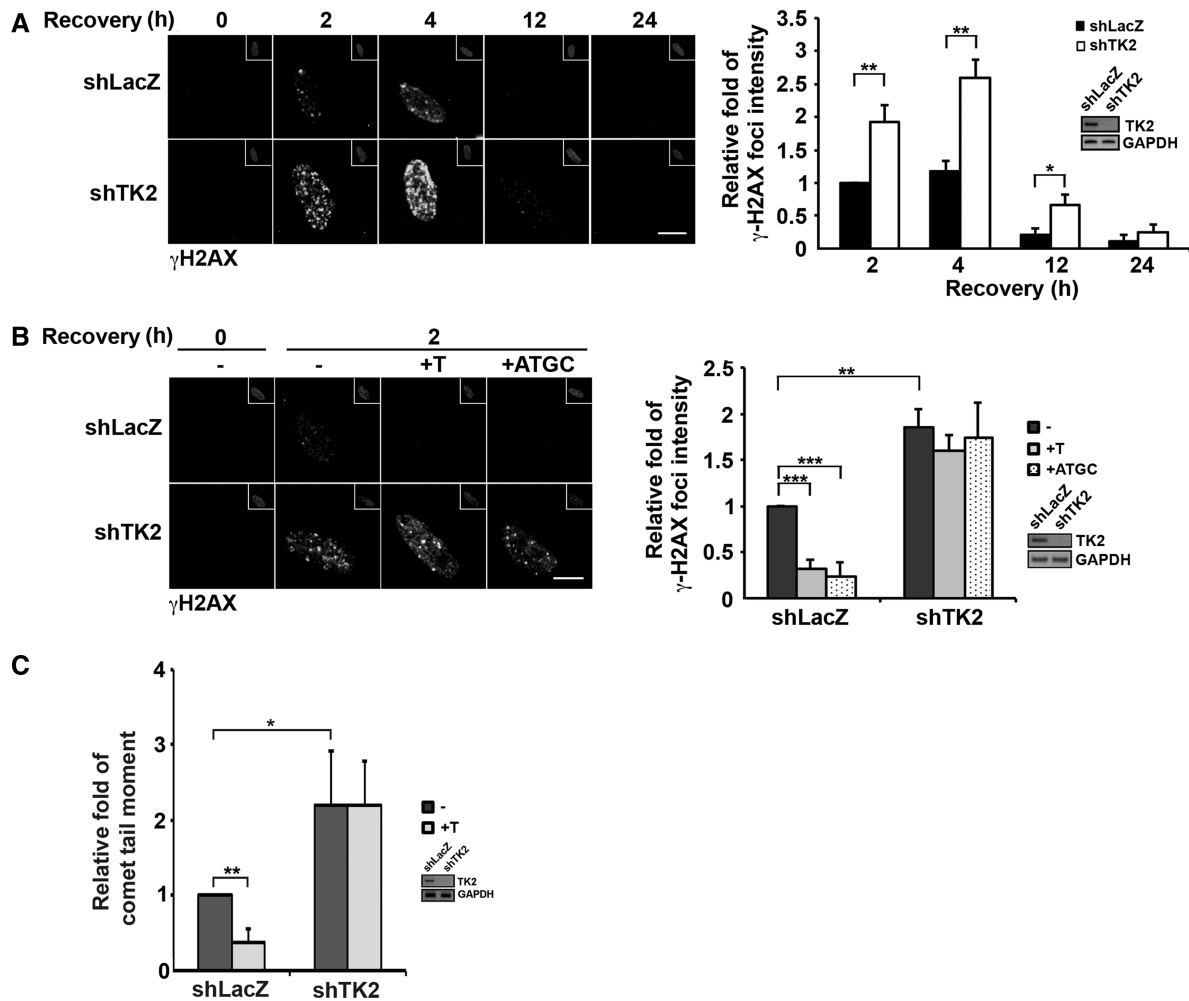


Figure 3. The effect of TK2 knock-down on DNA damage response after whole-cell UV irradiation. LacZ control and TK2 knock-down cells were serum deprived for 10 days and exposed to $10\text{J}/\text{M}^2$ whole-cell UV irradiation. (A) After recovery with 0.1% serum at the indicated time, cells were fixed for γ -H2AX IF staining analysis (Scale bar, $10\ \mu\text{m}$). Fluorescence intensity was expressed relative to that in LacZ control cells recovered at 2 h. Fifty cells were analysed for each experiment ($n = 3$). (B) Before UV irradiation, cells were preincubated with $5\ \mu\text{M}$ thymidine or four deoxynucleosides in 0.1% dialysed serum for 8 h. After recovery with or without $5\ \mu\text{M}$ deoxynucleosides, cells were fixed for γ -H2AX IF staining analysis (Scale bar, $10\ \mu\text{m}$). Fluorescence intensity was expressed relative to that in LacZ control cells recovered without thymidine supplement. Fifty cells were analysed for each experiment ($n = 3$). Insets indicate Hoechst33342 staining of the corresponding cells. (C) At 2-h recovery, cells recovered with or without thymidine were subjected to alkaline comet assay. Fifty cells were analysed for each experiment ($n = 3$). RT-PCR of RNA extracted from indicated cells were shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on Student's *t*-test.

cells, either addition of four deoxynucleosides or thymidine alone had any rescue effect (Figure 3B). The comet tail moment analysis also showed that addition of thymidine diminished DNA breaks in the nuclear genome of control cells, while TK2 knock-down cells had higher tail moment value regardless of exogenous addition of thymidine (Figure 3C). Thus, in case of whole-cell UV irradiation, thymidylate synthesis via TK2 also enhances the repair, thereby decreasing γ -H2AX foci formation.

Despite the differences in repair efficiency, we noticed that γ -H2AX foci eventually disappeared after recovery for 24 h in TK2 knock-down cells (Figure 3A). Given the low dNTP levels in quiescent cells, we speculated that gaps generated from DNA lesion excision during NER might be converted to double-strand breaks (DSBs), which can be repaired by non-homologous end joining (NHEJ) (32), a DSB repair process requires much less dNTP incorporation and mainly takes place in G1 and

G0 phase (32). Therefore, we then measured DNA DSBs by assessing foci formation of 53BP1, which binds histone H4 with Lys20 dimethylation at the DSB site to promote NHEJ (33,34), and neutral comet assay (35,36). After whole-cell irradiation, 53BP1 foci were increased and reached maximum at 12 h in both control and TK2 knock-down cells; however, a significant increase in the number of 53BP1 foci in TK2 knock-down cells was observed during recovery (Figure 4A). Neutral comet assay showed higher tail moment value in TK2 knock-down cells as compared with the controls after 12 h of recovery, indicating the presence of higher levels of DSBs in TK2 knock-down cells (Figure 4B). It is noteworthy that the timing for maximum levels of γ -H2AX and 53BP1 foci was different, highest at 4 and 12 h, respectively, in TK2 knock-down cells (Figure 4C). At 4 h, most of γ -H2AX foci were not colocalized with 53BP1 foci. At 12 h, the intensity and size of γ -H2AX foci

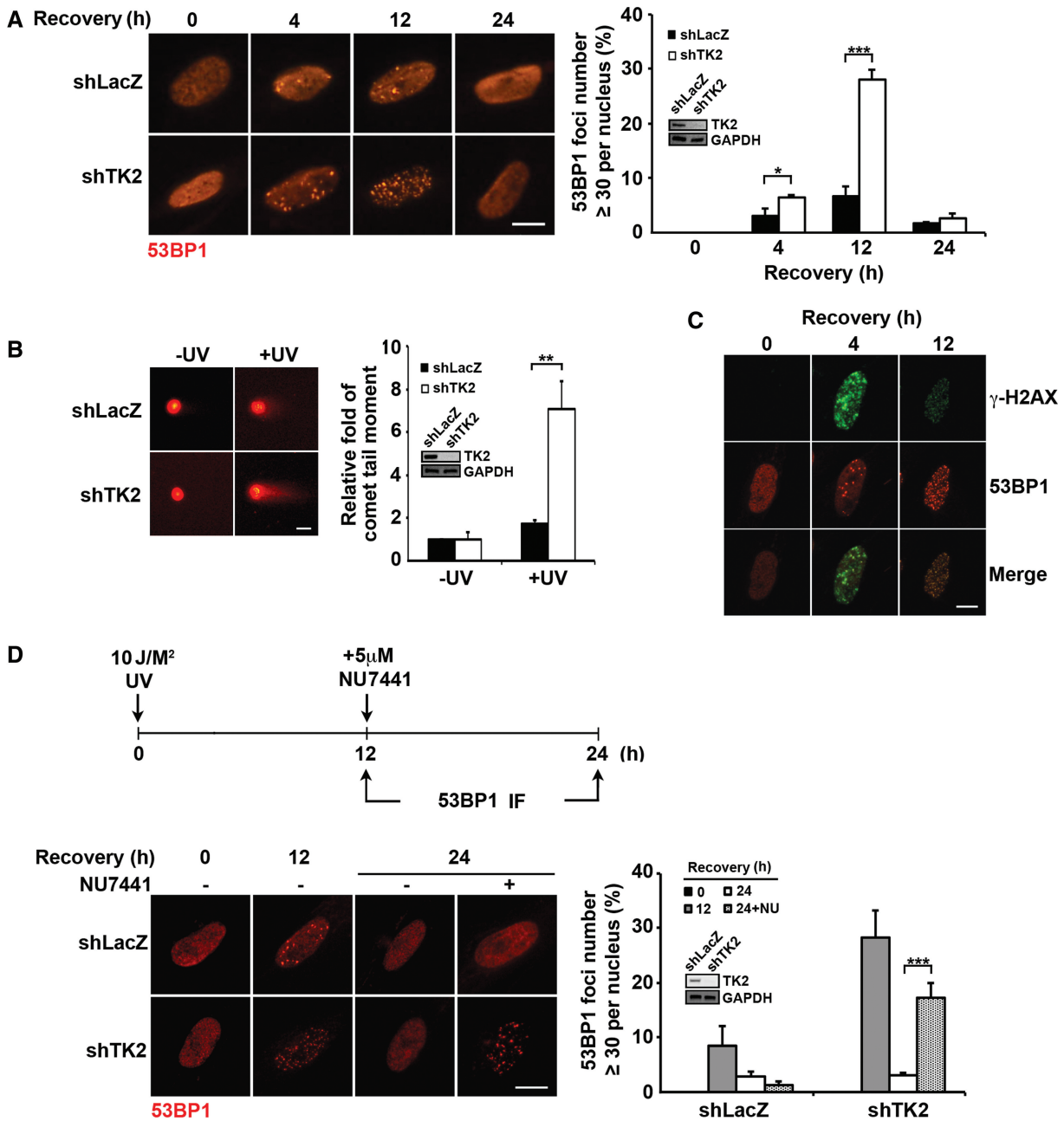


Figure 4. Analysis of DNA DSBs during recovery from UV irradiation in quiescent control and TK2 knock-down cells. LacZ control and TK2 knock-down cells were serum deprived for 10 days. After 10 J/M² whole-cell UV irradiation and recovery at the indicated time, cells were (A) fixed for 53BP1 IF staining (Scale bar, 10 μ m) and (B) analysed by neutral comet assay. Each cell containing 53BP1 foci ≥ 30 was counted and expressed as percentage. More than 100 cells were analysed for each 53BP1 foci experiment ($n = 3$) and 50 cells for each comet assay ($n = 3$). (C) Co-IF staining of 53BP1 and γ -H2AX was performed at the indicated recovery time in TK2 knock-down cells (Scale bar, 10 μ m). (D) At 12-h recovery, 5 μ M NU7441 was added in the culture medium. Cells were fixed for 53BP1 IF staining at indicated time and analysed as described in (A) (Scale bar, 10 μ m). RT-PCR of RNA extracted from indicated cells was shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on Student's *t*-test.

became much weaker and smaller, but colocalized with prominent 53BP1 foci (Figure 4C). It is possible that certain amounts of early-induced SSB gaps due to inefficient gap-filling were turned to secondary DSBs later during the recovery process and these lesions signaled 53BP1 recruitment. Nevertheless, both γ -H2AX and 53BP1 foci eventually diminished after 24 h of recovery. To investigate whether NHEJ process is involved in the repair of these DSBs, cells at 12 h after recovery from UV

irradiation were treated with NU7441, an inhibitor of DNA-dependent protein kinase (DNA-PK) that is required for NHEJ (Figure 4D). The results showed that inhibition of NHEJ sustained 53BP1 foci in TK2 knock-down cells at 24 h of recovery. In conclusion, in TK2 knock-down cells, DSBs are generated in the late stage during recovery from UV damage and NHEJ is involved in the repair of nuclear genome.

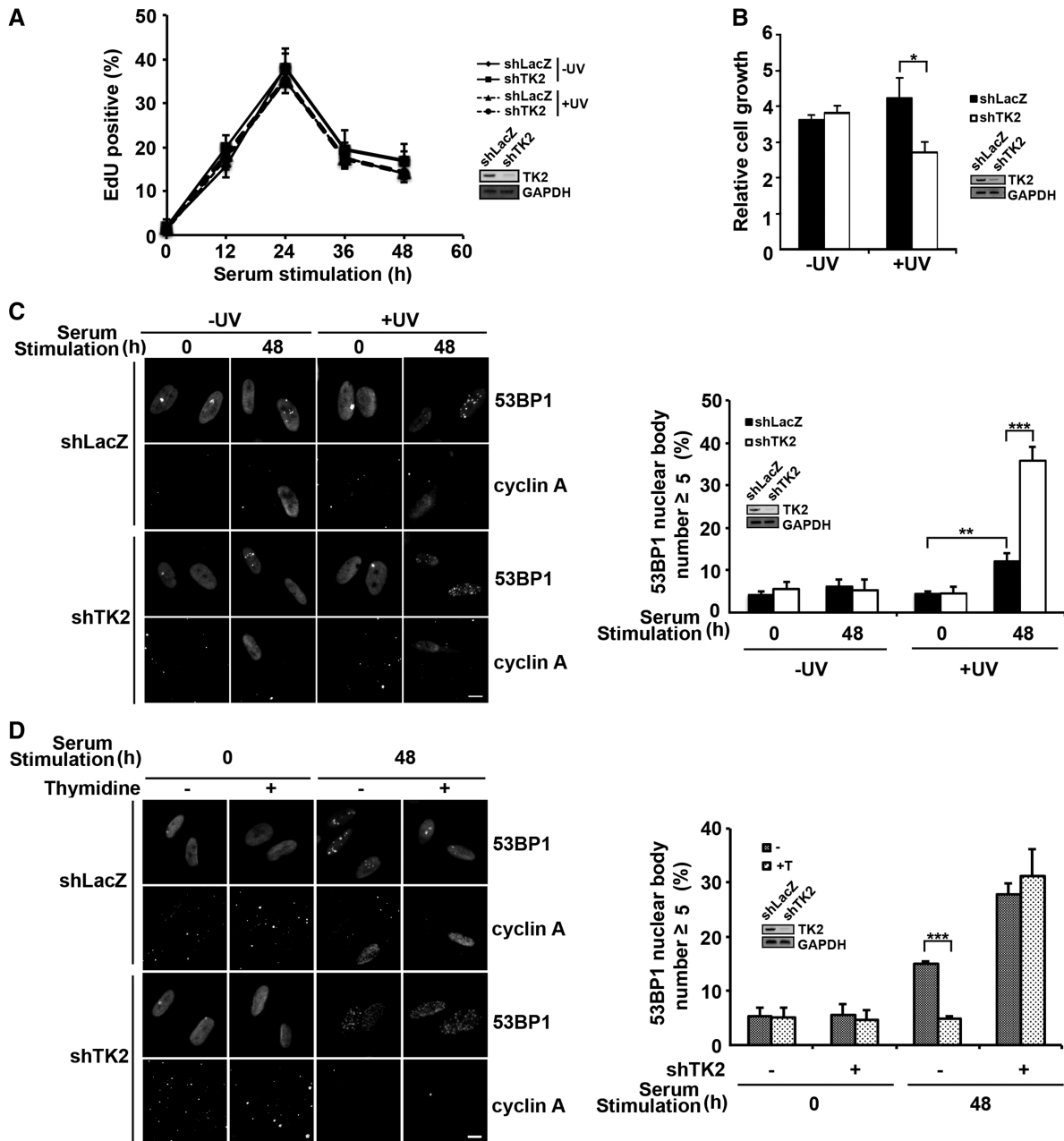


Figure 5. The correlation of the efficiency of NER during the quiescent state with the occurrence of genome stress through re-entry of cell cycle progression. Control and TK2 knock-down cells were serum deprived for 2 days before $10\text{J}/\text{M}^2$ UV irradiation. After 24-h recovery without serum, fresh medium containing 10% serum was added to stimulate the cell cycle entry. (A) S phase cell percentage at indicated time was analysed by EdU incorporation. The percentages of EdU positive cells were determined by counting >200 cells in each experiment ($n = 3$). (B) After 48-h serum stimulation, cell growth was determined by WST1 reagent. (C) These cells were fixed for 53BP1 and cyclin A IF staining (Scale bar, $10\ \mu\text{m}$). Each cell negative in cyclin A staining with 53BP1 nuclear bodies ≥ 5 was counted and expressed as percentage. More than 100 cells were counted for each experiment ($n = 3$). (D) Cells serum-deprived for 2 days were supplemented with $5\ \mu\text{M}$ thymidine for 8 h and during recovery from UV-irradiation. After 24 h, cells were serum stimulated for 48 h and fixed for 53BP1 and cyclin A IF staining analysis for quantification as described above (Scale bar, $10\ \mu\text{m}$). RT-PCR of RNA extracted from indicated cells were shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ based on Student's *t*-test.

Cell cycle progression significantly increases 53BP1 nuclear bodies in TK2-deficient cells that have recovered from UV damage in the quiescent state

Next, we asked the question whether the alteration of DNA repair by TK2 deficiency in the quiescent state could affect cell cycle progression. As 2 days of serum

deprivation already affected NER efficiency, we refreshed these cells with medium containing 10% serum to stimulate cell cycle progression. S phase entry was revealed by EdU (5-ethynyl-2'-deoxyuridine) incorporation. Regardless of UV irradiation in the quiescent state, control and TK2 knock-down cells were similar in the percentage of S phase cells, reaching maximum at 24 h

after serum stimulation (Figure 5A). This indicates that the repair of nuclear genome in these quiescent cells permitted normal re-entry of cell cycle progression. Control and TK2 knock-down cells that had not been UV-irradiated in quiescent state showed no difference in cell growth following serum stimulation for 2 days. However, for those cells that had experienced UV damage in quiescent state, TK2 knock-down caused 40% reduction in growth as compared with the control cells (Figure 5B). This reduction did not seem to result from apoptosis because both control and TK2 knock-down cells were negative in annexin-V staining regardless of UV irradiation (Supplementary Figure S3A), nor did these cells have difference in mitochondrial membrane potential as revealed by similar intensity of red fluorescence of aggregated JC1 dye in mitochondria (Supplementary Figure S3B).

It has been reported that low dose of aphidicolin treatment causes replication stress, which leads to the formation of 53BP1 nuclear bodies in the G1 phase cells in the successive cell cycle (37,38). It was proposed that DNA lesions, such as fragile sites originated from replication stress, are transmitted through M phase, and 53BP1 nuclear body has a function in shielding these DNA lesions from resection. Along this line of replication stress evidence, we then measured the formation of 53BP1 nuclear body formation in serum-stimulated cells that had UV damage and repair in the quiescent state. After 48 h of serum stimulation, cells presumably completed at least one turn of cell cycle progression. The number of 53BP1 nuclear bodies in G1 cells, which were indicated by negative in cyclin A IF staining, was significantly increased (Figure 5C). We observed that there were only a few 53BP1 nuclear bodies present in these cells before serum stimulation and therefore, the increase in 53BP1 nuclear body was a result of the cell cycle progression. In TK2 knock-down cells, the number of 53BP1 nuclear bodies was two times higher than that in control cells that had been UV-irradiated in the quiescent state (Figure 5C). Similar results were also obtained in cells with 10 days of serum deprivation (Supplementary Figure S4). Addition of thymidine in the culture medium during recovery from damage before serum stimulation consistently decreased 53BP1 nuclear bodies formation after cell cycle re-entry in control but not TK2 knock-down cells (Figure 5D). Thus, mitochondrial thymidylate synthesis via TK2 has a profound influence on the quality of repairing UV damage in the nuclear genome of the quiescent cells. The alteration of DNA repair in quiescent state due to TK2 deficiency confers nuclear genome stress during re-stimulation of cell cycle progression.

DISCUSSION

The importance of TK2 in life has been highlighted by the association of TK2 mutations with mtDNA depletion in humans (20) and in either TK2 knockout or mutated TK2 knock-in mice (39,40). In this study, our result adds another potential role of TK2-mediated dTTP formation in facilitating NER in nuclear genome of the cells in the

quiescent state. Despite the delayed repair by TK2 deficiency, these quiescent cells are capable of recovering from UV damage to re-enter the cell cycle in response to serum stimulation. However, after the subsequent cell-cycle progression, cells deficient of TK2 exhibit the nuclear genome stress. Thus, salvage synthesis of thymidylate in the mitochondrial compartment of the quiescent fibroblast has a functional contribution to protect the integrity of nuclear genome from UV damage.

Among four dNTP pools, the size of dTTP pool is reduced the most during serum deprivation. Possibly, this is because quiescent cells still contain RNR composed of p53R2 and R1 for the other three dNTPs formation, while the lack of TS in the quiescent state limits dTTP formation via the *de novo* pathway. Håkansson *et al.* have shown that inhibition of RNR in elutriated G0/G1-synchronized Balb/3T3 cells dramatically reduces levels of dATP and dGTP pools, but not dTTP or dCTP (15). Pontarin *et al.* also found the level of dTTP pool unchanged in quiescent human lung fibroblast with hydroxyurea treatment (13). A recent study has further shown similar dTTP pool in wild type and p53R2 missense mutant quiescent fibroblasts (16,41). These reports consistently indicate that the *de novo* pathway via RNR does not contribute to the major dTTP supply in the quiescent cells and suggest the importance of the salvage synthesis via TK2. In accordance, we showed that exogenous addition of thymidine increased the cellular level of dTTP in quiescent cells dependent on TK2.

It has been demonstrated that the amount of excised (6–4) photoproduct oligomer released from genome after UV irradiation in human cells was rapidly increased in 30 min and reached maximum in 1 h post-UV irradiation followed by abrupt decline in 4 h, while the amount of excised CPD was slowly increased in 4 h and stayed steadily in 8 h post-UV irradiation (42,43). Consistently, we observed that the rate of (6–4) photoproduct removal was much faster than that of CPD removal during recovery. Accordingly, the prominent PCNA staining at the micro-irradiated site in 1 h post-UV indicates DNA polymerization in the gap resulting from (6–4) photoproduct excision. The slower CPD removal might limit the gap generation for observing such PCNA foci. NER demands 24–32 dNTPs incorporation for filling each DNA repair patch. Therefore, this gap-filling process in quiescent cells needs dTTP supplied from TK2 reaction. In this study, we compared the effect of adding four deoxynucleosides and thymidine alone in the medium of quiescent cells on the gap-filling step. The rescue effects appeared to be similar, indicating that dTTP formation is the limiting factor for the gap-filling step in quiescent cells. The lack of rescue effect by thymidine supplement in TK2 knock-down cells confirmed the functional contribution of TK2 in the mitochondrial compartment to facilitating NER in nuclear genome of resting cells. It has been reported that thymidine mono-, di- and tri-phosphate are exported from mitochondria in *in vitro* experiments, and dTDP was indicated as the main phosphorylation state of thymidine for export (44). Therefore, dTTP in the mitochondrial and nuclear compartments are exchangeable (45). Our findings

suggest that this exchangeable process benefits nuclear genome repair in the quiescent state.

It has been shown that the reduction in the levels of PCNA and DNA polymerase δ and ϵ in quiescent cells delays the gap-filling step, in turn initiating ATR-dependent γ -H2AX formation (30). In this study, we found that the exogenous addition of thymidine significantly promoted the repair and decreased γ -H2AX foci after whole-cell UV irradiation. Considering that addition of thymidine should not affect the expression level of these proteins in the gap-filling step, our data clearly indicate that the increase in dTTP supply via TK2 in quiescent cells is sufficient to enhance the rate of gap-filling step in NER, thereby reducing SSB-induced γ -H2AX formation. However, it should be emphasized that TK2 deficiency did increase DSBs formation as revealed by the appearance of prominent 53BP1 foci and the increase in the comet tail moment by the neutral comet assay during the late stage of recovery from UV damage. Herein, we proposed that lack of dTTP supply in quiescent TK2 knock-down cells leads to incomplete gap-filling in SSBs, which could be converted to secondary DSBs.

The fact that the prominent 53BP1 foci induced by UV damage disappeared after 24 h of recovery indicates that these TK2 knock-down cells are capable of eliminating the DNA damage response (DDR) signals. Two possible mechanisms might be involved. One is the NER with dNTP misincorporation in the place of dTTP. Three DNA polymerases Pol ϵ , Pol δ and Pol κ were reported to participate in DNA gap-filling process of NER. Of note, DNA Pol κ , with low fidelity and processivity, is the polymerase for NER in quiescent cells with low dNTP pools (8,9). If so, the probability of misincorporation in NER in TK2 knock-down cells is higher. Alternatively, the unfilled gaps are turned to DSBs that are repaired by NHEJ, a process that requires few deoxynucleotides but is error-prone (46). Our data showing that NHEJ inhibition sustained 53BP1 foci in quiescent TK2-deficient cells support this possibility. Therefore, the lack of dTTP supply via TK2 in quiescent cells causes inefficient gap-filling in SSB patches, resulting in DSBs that are repaired through NHEJ.

Finally, we addressed the question whether either erroneous NER or NHEJ repair in quiescent TK2-deficient cells could lead to generation of nuclear genome scars. After stimulation of cell cycle re-entry, we found that 53BP1 nuclear body was significantly increased in the TK2-deficient cells in the next G1 phase of the cell cycle progression. Given that 53BP1 nuclear bodies in the G1 phase cells have been considered as markers of genome aberration resulting from replication stress, our results implied that the nuclear genome stress has been induced due to aberrant repair of UV damage in the quiescence state. A previous report has demonstrated that depletion of mtDNA decreases dTTP pool accompanied by chromosome instability in human cell lines (47). Given the central role of mitochondria in energy metabolism and cellular fate, it was unclear whether chromosome stability in the long-term culture of mitochondria-deficient cells is solely due to the decrease in the dTTP pool. Our findings in this study provide a more specific evidence for the contribution

of dTTP synthesis via mitochondrial TK2 in the quiescent state to the quality repair of NER, thus reducing the genome scars transmitted through the cell cycle progression. Several recent reports have shown that dNTP deficiency leads to replication stress and chromosome instability by oncogene expression, which can be rescued by addition of four deoxynucleosides in proliferating cells, indicating that the dNTPs from salvage pathway prevents genome stress (48,49). Our findings, on the other hand, illuminate the role of mitochondrial thymidylate synthesis via TK2 in the quiescent state for the maintenance of nuclear genome integrity, and suggest the importance of keeping optimal supply of thymidine in the cells that are temporally arrested in the quiescent state to ensure the quality of gap-filling step in NER.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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