國立臺灣大學醫學院生物化學暨分子生物學研究所

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Doctoral Dissertation

粒腺體內胸腺嘧啶激酶 2 對於維持核內基因體完整性之貢獻

Contribution of mitochondrial thymidine kinase 2 in maintaining nuclear genome integrity

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本論文係李明祥君(F94442020)在國立台灣大學醫學院生物化學暨分子生物學研究所完成之博士學位論文，於民國一百零三年四月十六日承下列考試委員審查通過及口試及格，特此證明。

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中華民國一百零三年四月

謝誌

終於畢業了！

在這一刻，千頭萬緒湧上心頭。回首七年的日子，在張智芬老師的嚴格教導下，我成長了許多。除了基礎的實驗技術、邏輯推演與深入思考外，我更習得了許多待人接物的道理。博士的訓練對於我而言，絕對不僅僅只是對於科學研究的追求，更珍貴的是讓心裡那個我更加勇敢與堅強，並且懂得堅持與謙虛。謝謝老師，您是我一輩子的恩人。在此也感謝各位口試委員對於我研究的指導，讓我的論文可以更加完備。

在這些日子裡，謝謝學長姐們對我的厚愛，也感謝同學以及學弟妹們帶給我的無數回憶，一切盡在不言中。親愛的阿稱學姊，謝謝你陪我走過人生的低潮以及對我大小事的照顧，我會永遠謹記在心。親愛的彥鴿，因為有你與我共患難，讓我可以堅持下去，也謝謝有你對我的叮嚀，讓我不忘鞭策自己應該更加努力。親愛的阿瑩，謝謝你對於我生活上的幫忙與各式食物的分享，讓我在緊繃的實驗中，也可以因為鮮奶油而展露笑顏。你們都是我生命中的貴人。

這段是給你的，謝謝你！這段日子辛苦了，除了必須忍受我龐大壓力下的壞脾氣外，還得不停尋找美食來滿足我挑剔的嘴。這一路走來，沒有你這堅強的後盾，我一定無法取得學位。因為你，我知道世界是美好的，值得我繼續努力打拼下去。

最後謹將本論文獻給爸爸及媽媽。謝謝您們從小以來對我的栽培及付出，讓我可以在無憂慮的環境下求學並且擁有滿滿的愛。謝謝您們給予我空間及包容，讓我得以投身研究之中。平時或許不能一直陪在您們身邊，但是我希望您們知道，我們一家人的心是永遠緊緊繫在一起的。謝謝我最愛的爸媽。

中文摘要

在休眠的纖維母細胞中，細胞質裡負責胸苷三磷酸(dTTP)合成的酵素表現量下降，導致胸苷三磷酸的量也顯著地下降。本篇論文研究闡釋了在這些休眠的纖維母細胞中，由粒腺體內胸腺嘧啶激酶 2 (thymidine kinase 2) 所催化合成的胸苷酸(thymidylate)為紫外光照射後核內 DNA 修復之限制因子，並且在修復的後期，胸腺嘧啶激酶 2 缺失的細胞會有第二級的 DNA 雙股斷裂之發生。但是即便胸腺嘧啶激酶 2 缺失的細胞修復狀況較慢，最後 DNA 損傷的信號都會消失，並且這些修復後的休眠細胞在血清的刺激下也都可以再次地進入 S 細胞週期。而這些細胞在下一個 G1 細胞週期時會產生明顯的 53BP1 核小體 (53BP1 nuclear body)，意味著這些細胞有嚴重的基因體壓力。總結，在暫時休眠的纖維母細胞中，粒腺體內胸腺嘧啶激酶 2 所催化合成的胸苷酸可以幫助紫外光照射後核內 DNA 有效及正確地修復以保持基因體的完整性。

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, the data indicate that mitochondrial thymidylate synthesis via thymidine kinase 2 (TK2) is a limiting factor for the repair of UV damage in the nuclear compartment in quiescent fibroblasts. Moreover, TK2 deficiency causes secondary DNA double-strand breaks (DSBs) formation in the nuclear genome of quiescent cells at the late stage of recovery from UV damage. Despite of slower repair in quiescent fibroblast deficient of 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. In conclusion, 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.

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Chpater I - Overview and Rationale

Part I Nucleotide excision repair (NER)

A. Introduction of nucleotide excision repair

Ultraviolet (UV) irradiation causes DNA lesions at the sites of adjacent pyrimidines, including cyclobutane pyrimidine dimer (CPD) and 6-4 photoproduct (6-4 PP) (Figure I-1) (1). In prokaryotes and lower eukaryotes, the direct repair enzymes, photolyases, split the unusual covalent bindings between the pyrimidines to repair the lesions. In higher eukaryotes, these lesions are removed by nucleotide excision repair (NER) (1,2). There are two sub-pathways of NER, global genomic NER (GG-NER) and transcription-coupled NER (TC-NER) (Figure I-2) (3). These two pathways share the core NER repair machinery. They are only different at the DNA lesion recognition step, which is mainly mediated by XPC/RAD23B and RNA polymerase II complex in GG-NER and TC-NER, respectively (4-6). After the lesions recognition, the DNA structure is open with the helicase activity of TFIIH and the repair complex is stabilized with the aid of XPA and replication protein A (RPA) bound to undamaged single-stranded DNA. The dual incision is carried out by the endonuclease activity of XPG at 4-7 nucleotides downstream, and ERCC1/XPF complex at 16-21 nucleotides upstream of the DNA

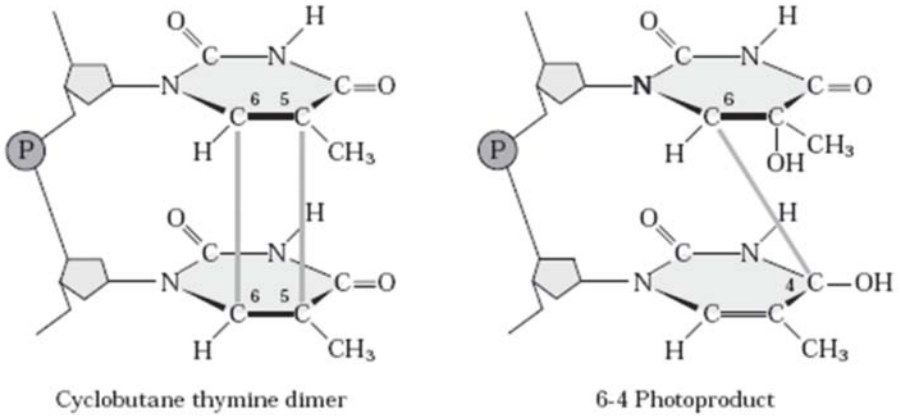


Figure I-1 UV-induced pyrimidine dimers
[Adapted from Principle of Biochemistry. Figure 8-34.]

lesions (7,8). The resulting nucleotide gaps are filled by the DNA polymerase ε, δ or κ with DNA clamping protein proliferating cell nuclear antigen (PCNA) loaded by replication factor C (RFC) (9-12). At this repair step, dNTPs are incorporated for DNA synthesis. These three DNA polymerases are preferentially used in specific cell-cycle stages. DNA polymerase ε and δ are used mainly in dividing cells and non-dividing cells, respectively (10). Under extremely low dNTP levels and stress condition, DNA polymerase κ with lower fidelity and processivity becomes the major polymerase for repair synthesis (10,11). Finally, the DNA gaps are sealed by DNA ligase I in proliferating cells and DNA ligase III/XRCC1 complex throughout cell cycle (13).

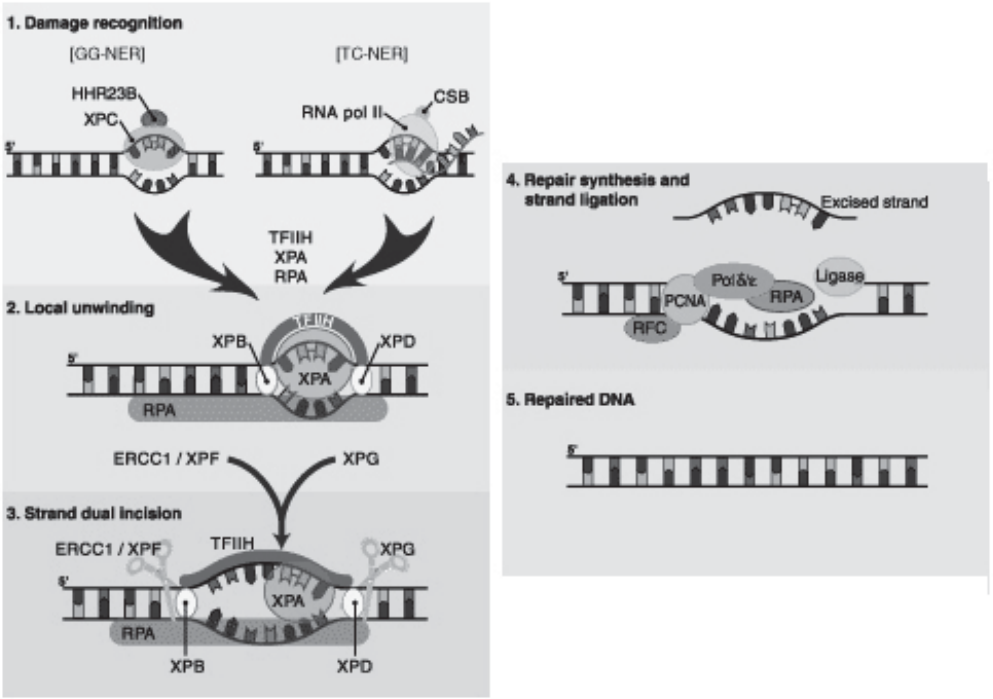


Figure I-2 Nucleotide excision repair
[Adapted from R&D system. DNA damage response.]

B. NER deficiency and human diseases

As a major repair mechanism for UV-induced DNA lesions in mammals, deficiency in NER often results in severe diseases (14). Autosomal recessive inherited diseases

such as Xeroderma Pigmentosum (mutations in XP proteins) (15), Cockayne Syndrome (mutations in TC-NER proteins CSA and CSB) (16) and Trichothiodystrophy (mutations in the subunits of TFIIH helicase proteins such as ERCC2 and ERCC3) (17) are resulted from the deficiency or mutation of NER related proteins. Patients often have high photosensitivity, high incidence of cancer, multisystem malfunction and premature aging.

C. dNTP supply and NER

In addition to the identified inherited human diseases resulted from impairment of NER proteins, the efficiency of DNA gap-filling also affects NER. Perturbing gap-filling synthesis by cytosine-β-arabinofuranoside (araC) treatment, which inhibits DNA polymerase, accumulates DNA single-stranded breaks in UV-irradiated cells (18). It was supported by enhanced DNA damage marker H2AX phosphorylation at serine 139 upon UV-C irradiation in araC treated cells (19). Moreover, hypersensitivity of non-cycling human lymphocytes to UV-B irradiation could be rescued by addition of four deoxynuclesides with the decrease in excision-induced strand breaks (20,21).

In addition, it is also reported that hydroxyurea, which inhibits ribonucleotide reductase (RNR) for dNTP supply, and araC treatment affect NER by the inhibition of further DNA lesion incision, which is resulted from the sequestrator of RPA in the gap-filling step, leading to the deficiency of RPA for functional lesion incision (Figure I-3) (22). Taken together, adequate dNTP supply is required for efficient NER.

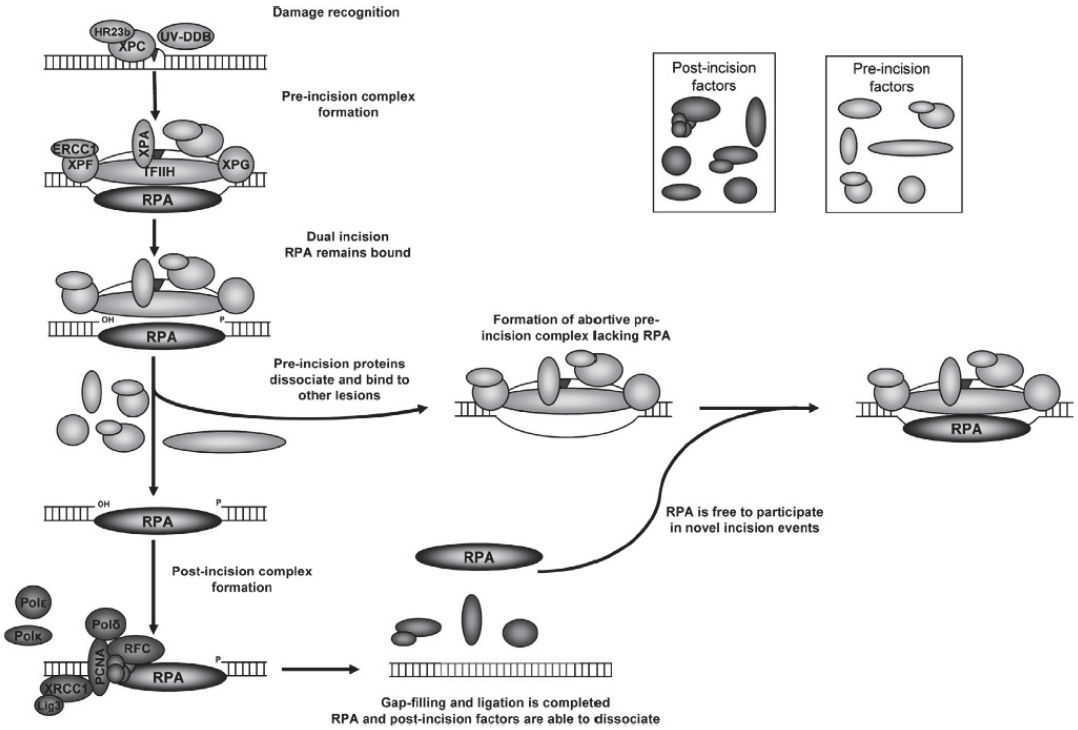


Figure I-3 Regulation of DNA lesion incision by RPA in NER
[Adapted from *Journal of cell biology*. Vol. 192, No. 3, p401-405. (2011)]

Part II dNTP pools synthesis in cycling and quiescent cells

For the balanced *de novo* synthesis of dNTP pools, RNR which converts CDP, ADP, GDP and UDP to dNDPs, is the rate-limiting enzyme for the dNTP synthesis (23). It is composed of two pairs of large R1 and small R2 subunits. Since R2 expression is tightly cell cycle-regulated, which is induced in late G1 and maximally in S phase, the dNTP synthesis for DNA repair and replication in cycling cells is dependent on R1-R2 complex (24-27). Another p53 inducible small subunit p53R2, forming complex with R1 to provide dNTPs, was identified as the role in DNA repair. Since it is not cell cycle-regulated, it provides dNTPs in quiescent cells for DNA repair and mitochondrial DNA (mtDNA) replication (28-32) (FigureI-4).

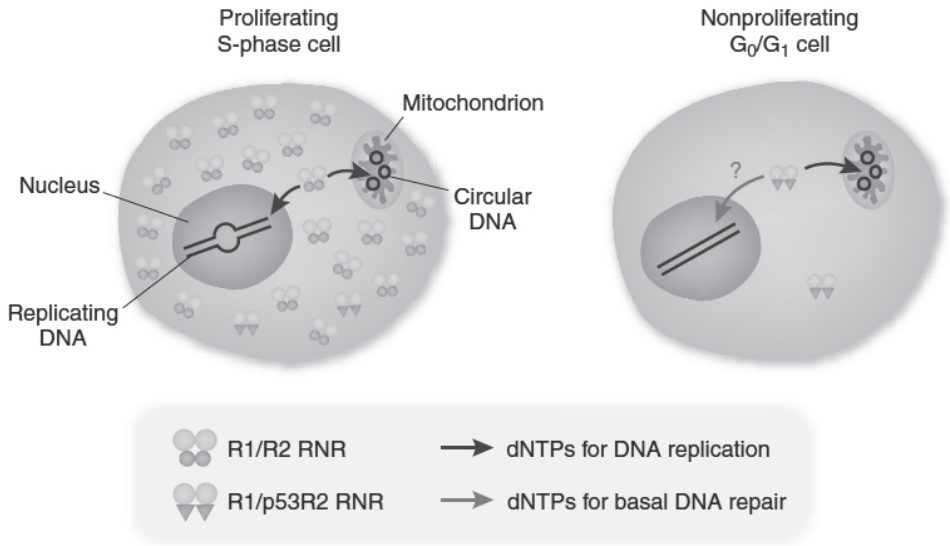


Figure I-4 *De novo* synthesis of dNTPs in S and G0/G1 cells
[Adapted from *Nature genetics*. Vol. 39, No. 6, p703-704. (2007)]

Different from the other three dNTPs, *de novo* synthesis of dTTP requires another rate-limiting enzyme thymidylate synthase (TS), which converts dUMP to dTMP (33) (Figure I-5). Since TS expression is low in G0/G1 phase, the *de novo* synthesis of

dTTP is in short in quiescent cells (34). In addition, it has been shown that dTTP pools remained unchanged in quiescent lung fibroblast with HU treatment and quiescent p53R2 missense mutant cells (29,32,35). These data suggest that the dTTP supply in quiescent cells is not majorly through the RNR mediated *de novo* pathway.

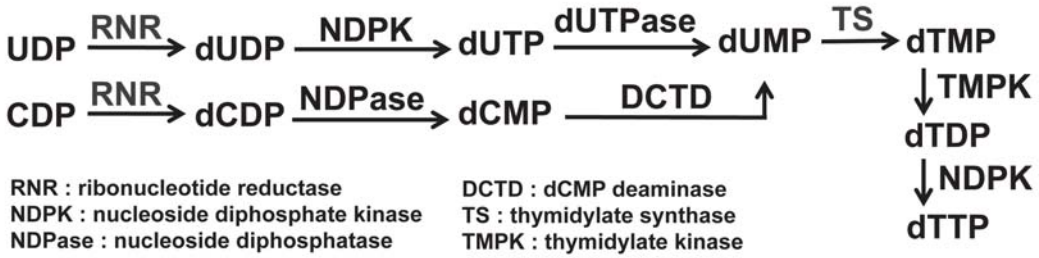


Figure I-5 *De novo* synthesis of dTTP

Part III Thymidine kinase 2 (TK2) and TK1 in dTTP metabolism

A. Role of TK2 and TK1 in dTTP synthesis

In addition to the *de novo* pathway for dTTP synthesis, the other pathway is the thymidine kinase (TK) mediated salvage pathway. There are two thymidine kinases, including cytosolic TK1 and mitochondrial TK2 in cells (36,37). They are both nuclear genes. The encoded TK2 protein with mitochondria targeting sequence is transferred into mitochondira. Both TKs transfer a phosphate group to thymidine to form dTMP, which is the limiting step for further dTTP synthesis. With broaden substrate specificity, TK2 also phosphrylates deoxycytidine to form dCMP (38). TK1 expression is tightly cell-cycle regulated at the transcription and post-translational level (39), while TK2 expression is rather stable during the cell-cycle (40). Moreover, TK2 expression was reported to be elevated in serum-deprived human fibroblasts (41). Therefore, TK2 turns to be the major source for dTTP synthesis in quiescent state. Although TK2 has less than 10% catalytic activity of TK1 to form dTMP (38), it plays an important role in mtDNA maintenance in post mitotic cells (42).

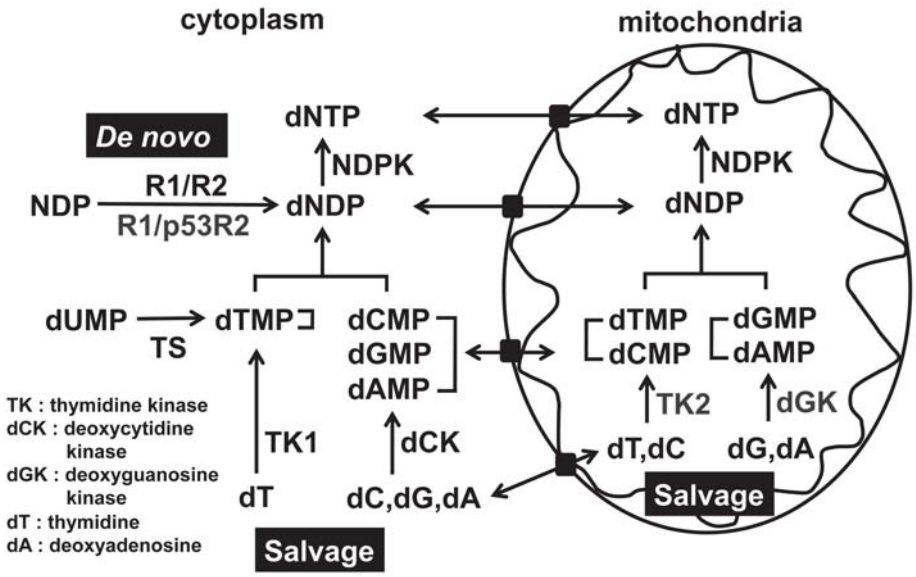
B. Thymidine kinases in DNA damage repair

The importance of TK1 in DNA damage repair has been demonstrated in our lab in cycling cancer cells (43). As the sole source of dTTP synthesis in quiescent state, the contribution of TK2 in nuclear DNA damage repair has not been investigated. One study showed that addition of thymidine during recovery from UV-irradiation facilitated DNA repair in quiescent lymphocyte support the role of salvage-mediated dTTP synthesis for nuclear DNA repair in quiescence (18).

Part IV Role of TK2 in mitochondria biogenesis

A. dNTP pools and mitochondria biogenesis

dNTPs are exchangeable between cytosol and mitochondria (44). *De novo* synthesis of dNTPs for mtDNA replication are transported from cytosol to mitonchondria (45). The other pathway of dNTPs synthesis for mtDNA maintenance is mediated by mitochondrial salvage kinases deoxyguanosine kinases (dGK), which phosphorylates deoxyguanosine and deoxyadenosine, and TK2 within mitochondria (Figure I-6) (46). With the importance of p53R2, dGK and TK2 for dNTP pools synthesis in post-mitotic cells, either gene mutation results in mitochondrial DNA depletion syndrome (MDS) (47-49).



Figrue I-6 dNTP supply in cytoplasm and mitochondria

B. Role of TK2 in mtDNA maintenance

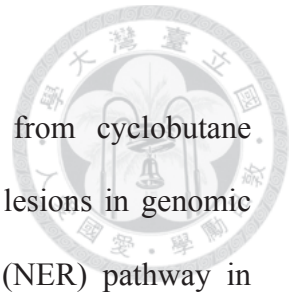
As for the importance of TK2 in mtDNA maintenance, it was reported that TK2 knock-out and mutated TK2 knock-in mice have severe mitochondrial DNA depletion syndrome (50,51). Patients with TK2 deficiency often died of myopathy and

encephlomyopathy with low mitochondria copy number at their young age (47). All these reports indicate the contribution of TK2 in mtDNA maintenance. Furthermore, with broad nucleoside analogues specificity of TK2, its inhibitors were developed for preventing the mito-toxicity of nucleoside analogues-based anti-virus and anti-cancer drugs in chemotherapy study (52). It reinforces that TK2 can phosphorylate the substrate from cytosol and make them available for use by mitochondrial DNA polymerase. However, the relationship between mitochondrial TK2 and nuclear genome is still unknown.

Rationale

In cycling cells, high expression levels of TS and TK1 support the synthesis of dTTP. In the quiescent state, the expressions of TS and TK1 are down-regulated accompanied by a drastic decrease in cellular level of dTTP pool. This evokes a question: “ How is dTTP pool supplied in quiescent cells for repair? ” A previous study has shown that RNR function is dispensable for the maintenance of dTTP pool in quiescent p53R2 mutant cells. In addition, supplement of thymidine facilitated DNA repair in quiescent lymphocytes. In this study, the contribution of mitochondrial TK2 expression in repair of UV-irradiation induced DNA damage was investigated.

Chapter II - The Contribution of Mitochondrial Thymidylate Synthesis in Preventing the Nuclear Genome Stress



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 (GG-NER) and transcription coupled NER (TC-NER) (3). These two pathways differ in recognizing DNA lesion sites, which is mediated by XPC-RAD23B complex in GG-NER (4,5) and RNA polymerase II in TC-NER (6). The damaged oligonucleotide are removed by XPG and XPF-ERCC1 endonucleases (7,8), resulting in single-stranded DNA (ssDNA) gap that requires 24-32 deoxynucleotides incorporation to complete the repair process dependent on DNA Polymerases Polε, Polδ or Polκ with DNA clamping protein proliferating cell nuclear antigen (PCNA) (9-11). Finally, the DNA nick is sealed by DNA ligase I in proliferating cells or by DNA ligase III/XRCC1 throughout the cell cycle (11,13).

After DNA lesion excision in NER, sufficient amount of cellular dNTP is required for filling the gaps. 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 (23). 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 (28-30). 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 (28,31,32). 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 (32).

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) since RNR does not form dTDP directly. 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 phase (34,39). 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 (38), 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 (47). 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 (52). 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 (53,54). 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 (HRP)-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 (64M-2) and CPD (TDM-2) were from Cosmo Bio Co., Ltd (Tokyo, Japan). Anti-cyclin A (E23.1, anti-both cyclin A1 and A2) was from GeneTex Inc. (Irvine, CA, USA). Anti-β-tubulin, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyante (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% CO_2.

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 (PBS) were subjected to 10 J/M^2 UV irradiation. For localized UV-irradiation, polycarbonate isopore membrane with 3μm pore size filters (Millipore) were placed on top of cells in Hank's balanced salt solution, and irradiated with 120 J/M^2 UV. Following removal of the filters, the cells were recovered with fresh medium.

Immunofluorescence staining

Cells grown on coverslip were fixed by 4% paraformaldehyde (PFA) at room temperature or methanol at -20°C for 5 min. Cells fixed by PFA were permeabilized with 0.3% Triton X-100 for 5 min. 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 (55) and (6-4) photoproduct (56) 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, pH7.6, 250 mM NaCl, 0.1% sodium dodecyl sulphate, 0.2% deoxycholic acid, 0.5 mM dithiothreitol, 1 mM EDTA pH8, 0.5% NP-40 and 1 % Triton X-100), and proteins in each cell lysate were analysed by immunoblot using specific antibodies and

HRP-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'- AATCGGGATCGAATATTAACT-3', TK2 antisense primer 5'-TGAACACCGGGCTCCAGCCAA-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 analyzed by Sicon image software (57).

TK activity assay

The assay was performed as previously described using the DE-81 filter paper (Whatman) technique (58). Briefly, cells were harvested in the buffer containing 50 mM Tris-HCl (pH 7.9), 0.5mM 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 $MgCl_2$, 0.1% bovine serum albumin, 5 mM NaF, 2 mM DTT, 180 μM thymidine and [^{3}H]-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 16000 g for 30min. 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 (59).

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, 0.45μm, Millipore) and concentrated using Amicon Ultra column (100k 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 PBS 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 mM 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 counter-stained by Hoechst 33342.

Cell growth assay

Cell growth was determined by WST1 (4-[3-(4-Iodophenyl)-2-[4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) reagent (Roche Applied Science). WST1 is a tetrazolium salt which can be reduced into formazan by mitochondrial dehydrogenases. The overall dehydrogenase activity is positively correlated with the number of the cells. Therefore, the number of viable cells can be quantified by the measurement of formazan produced. Briefly, cells in 96-well plates were incubated with 100 ml 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 (22). We tested whether serum deprivation influenced the efficiency of lesion excision by (6-4) photoproduct IF staining. UV-induced foci were found to disappear within 1 h in these cells regardless of serum deprivation (Figure II-1). 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 about 3 μm in the nucleus, and the fluorescence intensity was increased with longer serum deprivation (Figure II-2). 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 II-2). 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 (19). 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 II-3). 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 to10 days of serum deprivation, while the levels of TMPK and R1 were moderately decreased and p53R2 remained unchanged (Figure II-3). 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 II-4), suggesting that the shortage of dNTP 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 II-5). 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 II-6). By BrdU incorporation experiment, it was found that about 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 (38), 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 less than 0.4% (data not shown). In these cells, more than 60% of cellular TK activity was decreased by TK2 knock-down (Figure II-6). 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 II-7). 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 II-8). However, the intensity of PCNA foci was significantly higher and prolonged in TK2 knock-down cells than those in control cells during 0.5 to 3 h post-recovery period (Figure II-9). 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 (Figure II-10), 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 II-11). Addition of thymidine led to significantly elevated dTTP level in control but not in TK2 knock-down cells (Figure II-12). 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 (18).

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 (19). 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, 3μm). 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 II-13). 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 (Figure II-14), 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 cells, either addition of four deoxynucleosides or thymidine alone had any rescue effect (Figure II-15). 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 II-16). 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 II-13). 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) (60), a DSB repair process requires much less dNTP incorporation and mainly takes place in G1 and G0 phase (60). 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 (61,62), and neutral comet assay (63,64). 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 II-17). 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 II-18). 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 II-13 and Figure II-17). 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 became much weaker and smaller, but colocalized with prominent 53BP1 foci (Figure II-19). 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 II-20). 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.

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 II-21).

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 II-22). This reduction did not seem to result from apoptosis since both control and TK2 knock-down cells were negative in annexin-V staining regardless of UV irradiation (Figure II-23). Nor did these cells have difference in mitochondrial membrane potential as revealed by similar intensity of red fluorescence of aggregated JC1 dye in mitochondria (Figure II-24).

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 (65,66). 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 II-25). 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 II-25). Similar results were also obtained in cells with 10 days of

serum deprivation (Figure II-26). 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 II-27). 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 (47) and in either TK2 knockout or mutated TK2 knock-in mice (50,51). In this study, our result adds another potential role of TK2-mediated dTTP formation in facilitating the gap-filling step of NER in nuclear genome of the cells in the quiescent state. Despite the delayed gap-filling of NER by TK2 deficiency, these quiescent cells are capable of eliminating UV-induced DNA damage signal 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 provide the repair quality, ensuring the integrity of nuclear genome from UV damage (Figure II-28).

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 (31). Pontarin *et al.* also found the level of dTTP pool unchanged in quiescent human lung fibroblast with hydroxyurea treatment (29). A recent study has further shown similar dTTP pool in wild type and p53R2 misssense mutant quiescent fibroblasts (32,35). 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 (67,68). 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 gap-filling of 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 (69). Therefore, dTTP in the mitochondrial and nuclear compartments are exchangeable (44). 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 ε and δ in quiescent cells delays the gap-filling step, in turn initiating ATR-dependent γ-H2AX formation (19). In this study, we found that the increase in dTTP supply via TK2

by exogenous addition of thymidine in quiescent cells is sufficient to enhance the rate of gap-filling step in NER, thereby reducing SSB-induced γ-H2AX formation. Therefore, salvage dTTP synthesis is the key factor for gap-filling efficiency in NER during quiescence. 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 SSB-induced γ-H2AX and 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 misincoporation in the place of dTTP. Three DNA polymerases Polε, Polδ and Polκ were reported to participate in DNA gap-filling process of NER. Of note, DNA Polκ, with low fidelity and processivity, is the polymerase for NER in quiescent cells with low dNTP pools (10,11). 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 (70). Our data showing that NHEJ inhibition sustained 53BP1 foci in quiescent TK2-deficient cells support this possibility. However, whole genome sequencing is required to verify the presence of misincorporations and deletions. According to our data, 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. However, the increase in 53BP1 nuclear body is accompanied by slower cell growth in two-day serum-stimulated TK2-deficient cells. After four days of serum-stimulation, an increase in 53BP1 foci diminished (data not shown). It implied that the genome stress might be resolved in the following cycles of S-phase. Since IMR-90 cells are normal diploid cells, it is possible that these cells use alternative ways to repair the problematic DNA during S-phase with high dNTP pools. Therefore, it is interesting to understand whether cancerous stem cells also show the same phenomenon. In addition, it is also important to investigate whether this phenomenon is cell type specific.

A previous report has demonstrated that depletion of mtDNA decreases dTTP pool accompanied by chromosome instability in human cell lines (71). 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 (72,73). 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.

Perspective

In this dissertation, I demonstrated the functional role of mitochondrial TK2 in dTTP synthesis for the gap-filling step of NER in nuclear genome in quiescent cells. Despite of the delay in gap-filling of NER by TK2 knockdown, cells seem to have a secondary DNA damage and repair system to compensate the dTTP deficiency in the quiescent state. Significantly, after the cell cycle re-entry, TK2 knock-down cells exhibited replication stress and the cell growth was attenuated. Several questions still remain to be elucidated from this research.

1. **Do misincorporations and deletions occur during the gap-filling synthesis of NER in TK2 knock-down cells?** Since DNA polymerase κ with low deoxynucleotide incorporation fidelity is the enzyme for NER gap-filling synthesis in quiescent cells with extremely low dNTP pools, it is possible that misincorporations take place to get gap-filling process completed in TK2 knock-down cells without the source of dTTP supply. Moreover, DNA deletions also probably happen because DNA DSBs are induced and repaired by NHEJ in TK2 knockdown cells. In addition to the whole genome sequencing, it is easier to approach this question by sequencing UV-irradiated plasmids recovered in quiescent TK2 knockdown cells. Although the condition of repair of plasmids is different from that of the genome, this approach might provide clues to the answer of the question.
2. **Whether the replication fork indeed affected in TK2 knockdown cells?** In my study, I only used 53BP1 nuclear body to indirectly indicate the replication stress during previous S phase. In order to measure the DNA replication fork directly, I have to perform DNA combing assay. This assay is widely used for the detection of replication fork speed, symmetry, origin density, inter-origin distances, and replication restart efficiency that are parameters for DNA replication study (74).

3. **Does the early-death of TK2 mutation patients also result from nuclear genome instability?** There are already many mutations sites discovered in TK2-mutated patients. These patients were died at 2 or 3 year-old due to mitochondrial DNA depletion syndrome. MDS affects tissues that require high ATP such as muscle and neuron resulting in myopathy and encephalopathy. Since DNA damages happen everyday endogenously and exogenously, it is worthy to know whether the nuclear genome is affected in TK2 mutated patients. In addition, somatic stem cells are quiescent and required for repair of injured tissue. It is also important to pursue whether the stem cells are affected in these patients.

The importance of dTTP supply from mitochondria in nuclear genome stability has been neglected. My study implies that sufficient thymidine supply in quiescent cells is also important for the maintenance of nuclear genome stability. Understanding the above questions can provide more information linking the function of mitochondria to nuclear genome.

Table

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 / 10^6 cells
%, percentage of asynchronous cells

Table 1 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.

Figures and Legends

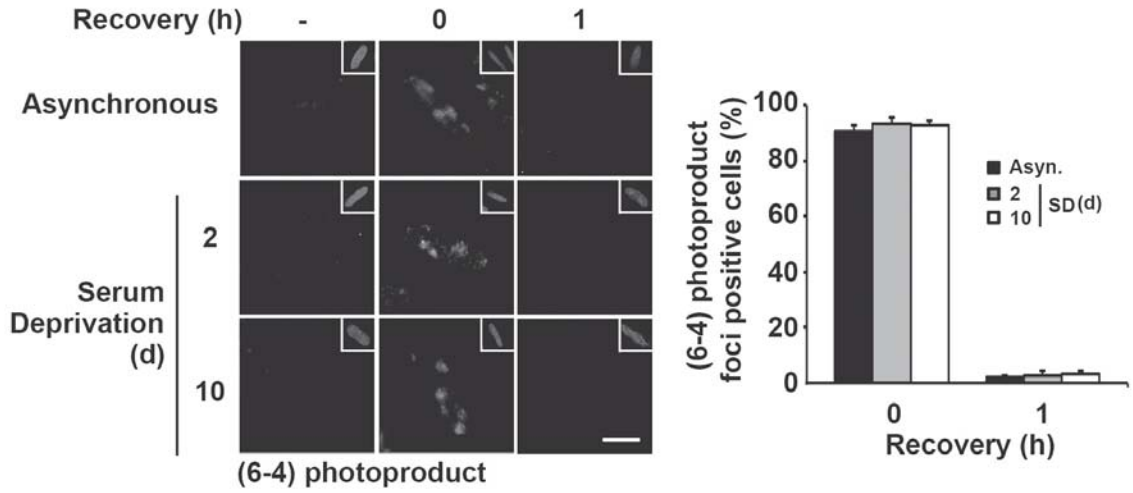


Figure II-1 IMR-90 fibroblasts were incubated in medium containing 10% serum (asynchronous), serum-free for 2 and 4 days or 0.1% serum for 10 days. Asynchronous and serum-deprived cells were irradiated with 120 J/M^2 UVC through micropore membrane (pore, 3μm) and recovered, after which cells were subjected to (6-4) photoproduct IF staining (Scale bar, 10μm). The percentages of (6-4) photoproduct foci positive cells were counted. More than 100 cells were counted for each experiment (n=3). Insects show H33342 staining in the corresponding cells (n=3).

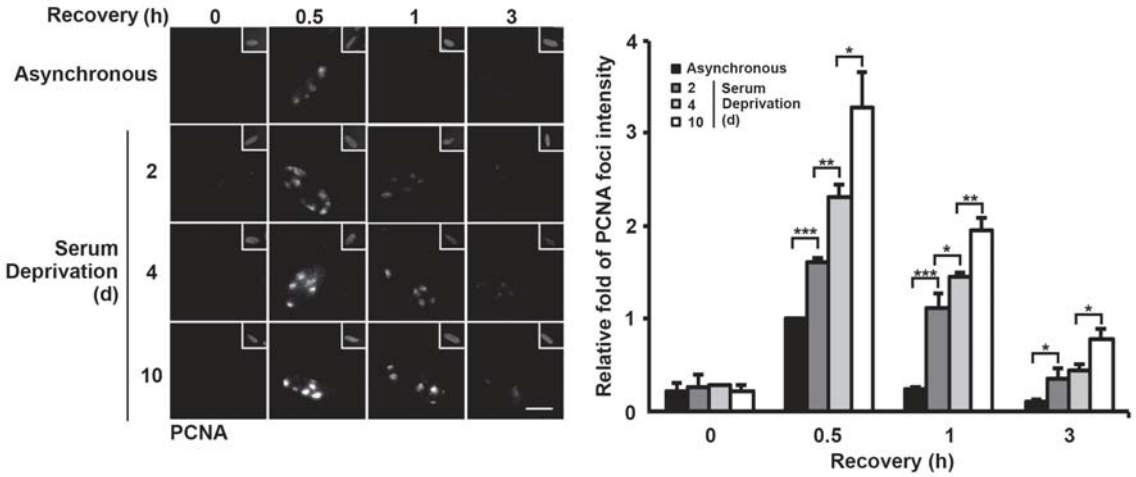


Figure II-2 Asynchronous and serum-deprived cells were local UV irradiated and recovered, after which cells were subjected to PCNA IF staining (Scale bar, 10μm). PCNA fluorescence intensity was expressed relative to that in asynchronous cells recovered at 0.5h. 50 cells were analysed for each experiment (n=3). Insects show H33342 staining in the corresponding cells (n=3). *P<0.05, **P<0.01, ***P<0.001 based on Student's *t* test.

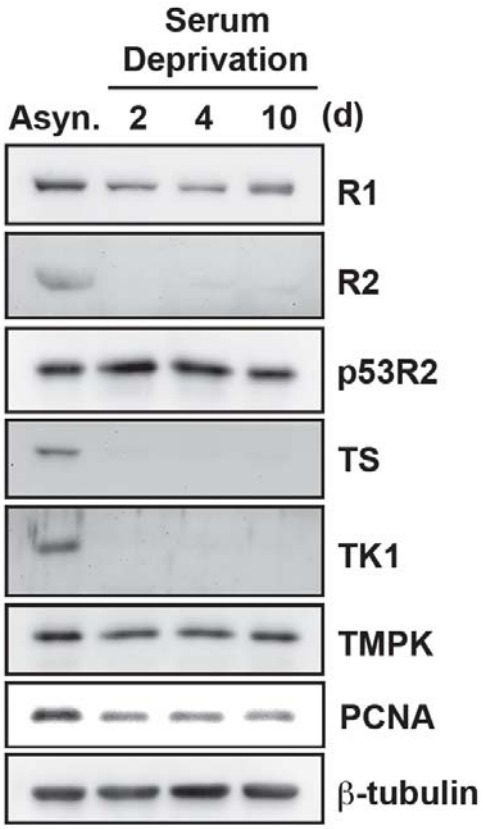


Figure II-3 Asynchronous and serum-deprived cells were harvested for western blot analysis.

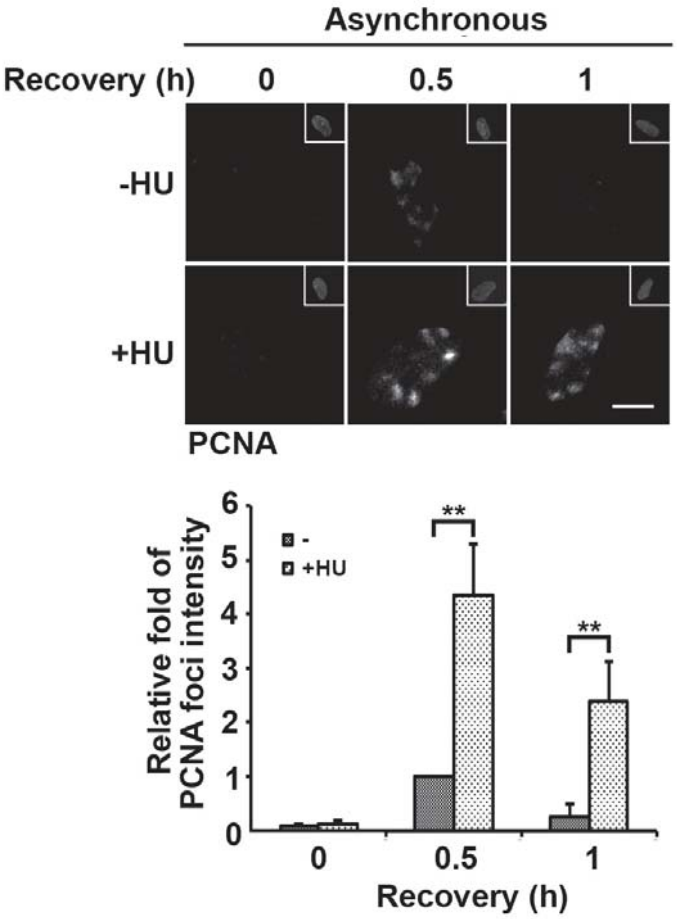


Figure II-4 Asynchronous cells were pretreated with or without 2 mM hydroxyurea for 3h before local UV irradiation and during recovery. Cells were fixed for PCNA IF staining (Scale bar, 10μm). Fluorescence intensity was expressed relative to that in cells recovered at 0.5h without hydroxyurea treatment. 50 cells were analysed for each experiment. Insects show H33342 staining in the corresponding cells (n=3). **P<0.01 based on Student's *t* test.

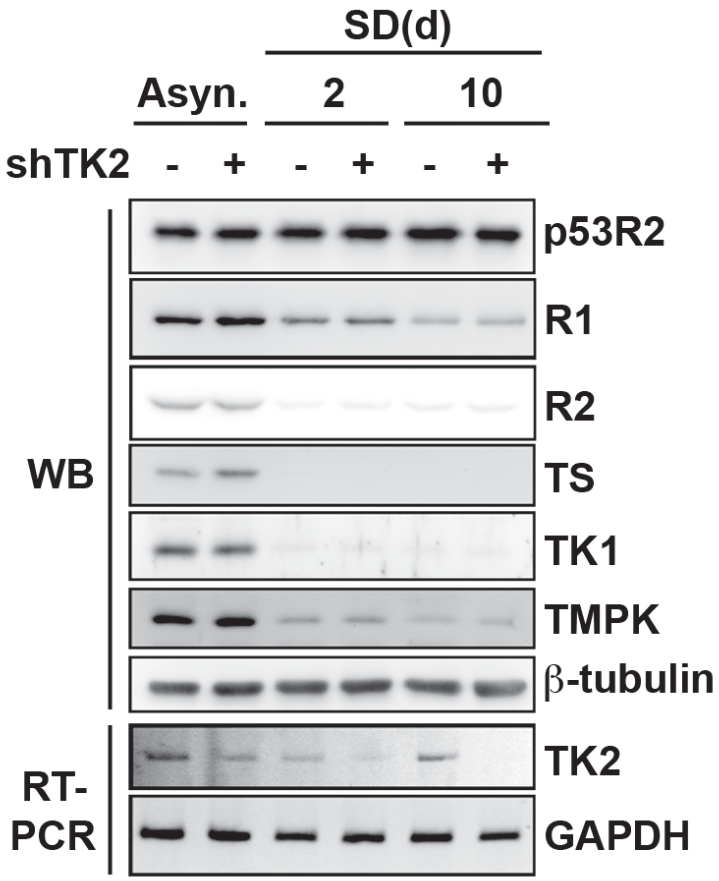


Figure II-5 IMR-90 cells were infected by LacZ or TK2 shRNA lentivirus. These cells were incubated in 10% serum containing or serum-deprived conditions for western blot analysis. RT-PCR of RNA extracted from indicated cells were shown.

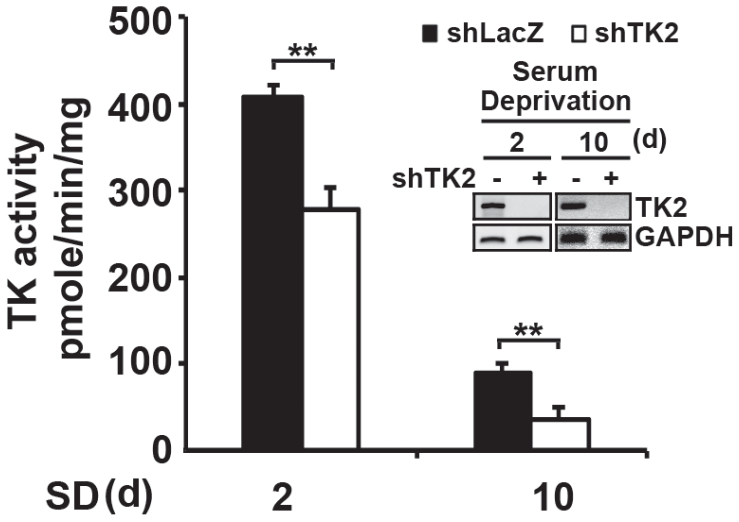


Figure II-6 Control and TK2 knockdown cells were serum-deprived and lysed for thymidine kinase activity assay. RT-PCR of RNA extracted from indicated cells were shown. **P<0.01 based on Student's *t* test.

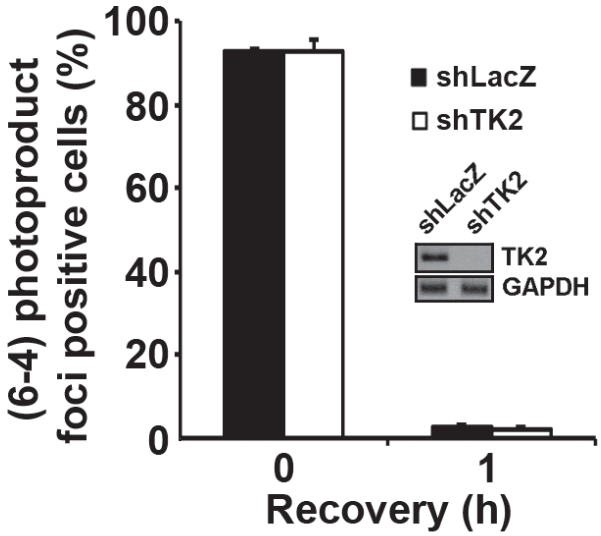


Figure II-7 10-day serum-deprived control and TK2 knock-down cells were locally irradiated. After recovery with 0.1% serum at indicated time, cells were fixed for (6-4) photoproduct IF staining. The percentages of (6-4) photoproduct foci positive cells were counted. More than 100 cells were counted for each experiment (n=3). RT-PCR of RNA extracted from indicated cells were shown.

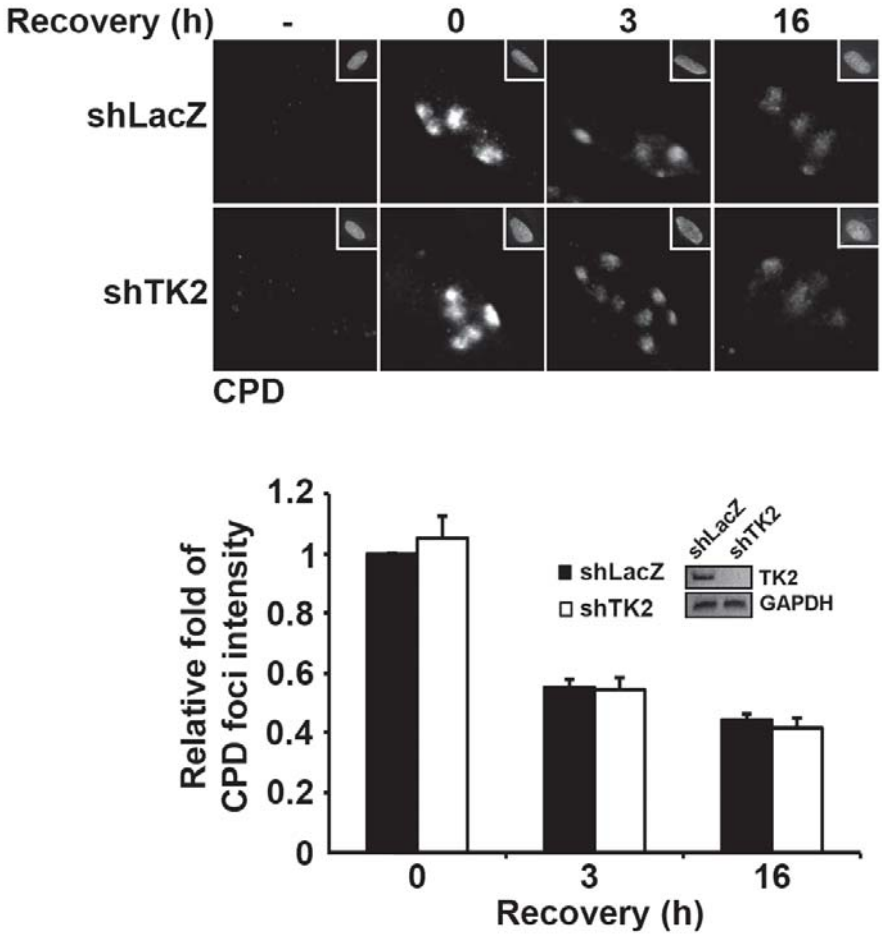


Figure II-8 10-day serum-deprived control and TK2 knock-down cells were locally irradiated. After recovery with 0.1% serum at indicated time, cells were fixed for CPD IF staining. CPD fluorescence intensity was expressed relative to that in control cells recovered at 0h. 50 cells were counted for each experiment (n=3). RT-PCR of RNA extracted from indicated cells were shown.

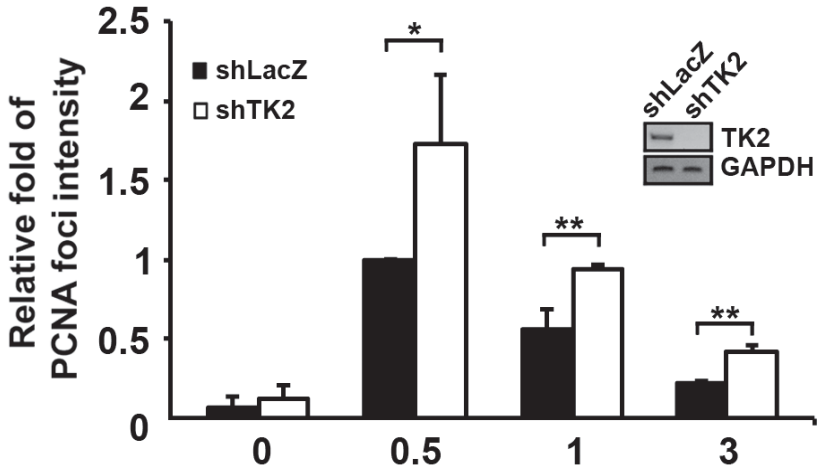


Figure II-9 10-day serum-deprived control and TK2 knock-down cells were locally irradiated. After recovery with 0.1% serum at indicated time, cells were fixed for PCNA IF staining. PCNA fluorescence intensity was expressed relative to that in cells recovered at 0.5h. 50 cells were counted for each experiment (n=3). RT-PCR of RNA extracted from indicated cells were shown. *P<0.05, **P<0.01, based on Student's *t* test.

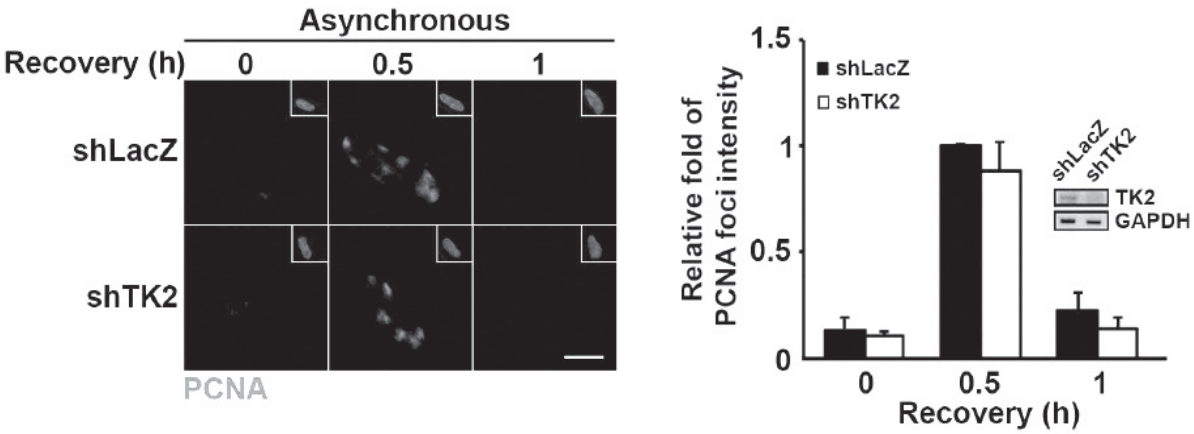


Figure II-10 Asynchronous LacZ control and TK2 knockdown cells were irradiated by 120 J/M^2 UVC through micropore membrane and were fixed for PCNA immunofluorescence staining (Scale bar, 10 μm). Fluorescence intensity was expressed relative to that in LacZ control cells recovered at 0.5 h. 50 cells were analyzed for each experiment (n=3). The Hoechst33342 staining of the corresponding cells were shown in insets. RT-PCR results of RNA extracted from indicated cells were shown.

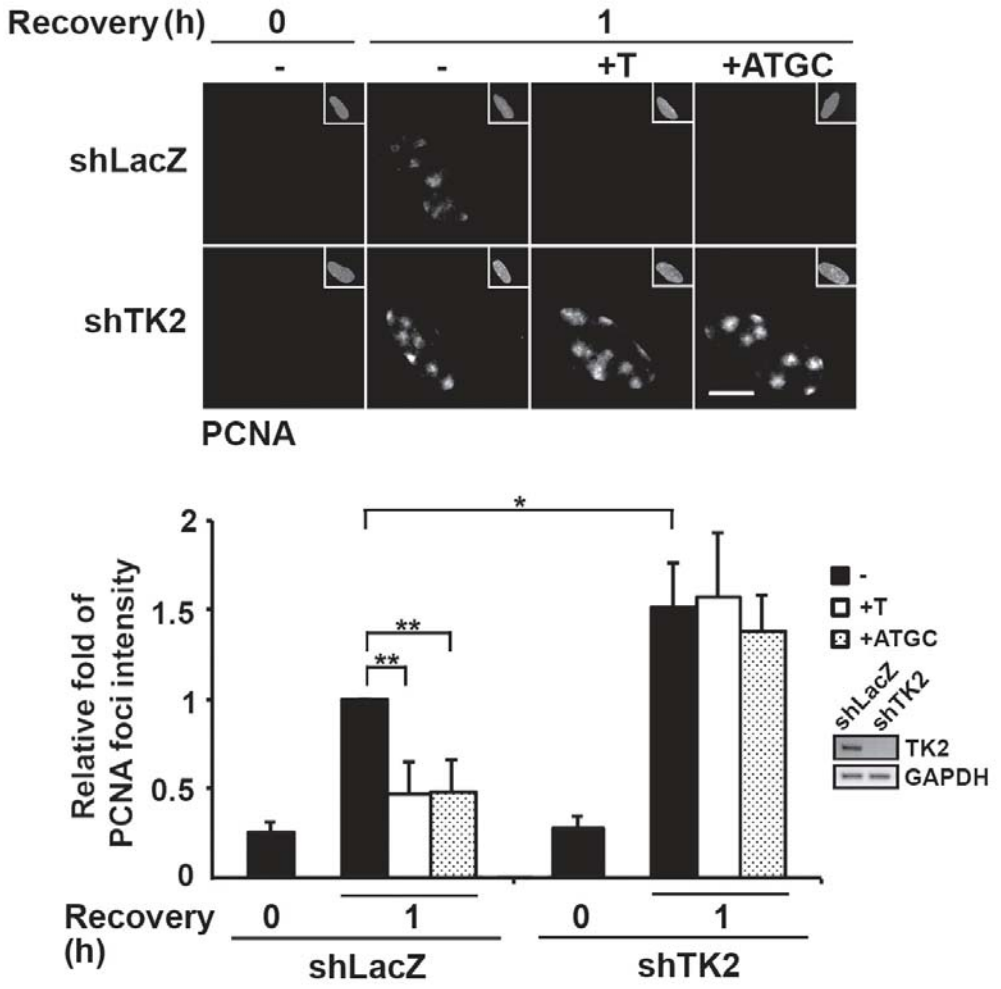


Figure II-11 10-day serum deprived control and TK2 knock-down cells were supplemented with 5μM thymidine or four deoxynucleosides in 0.1% dialyzed serum for 8h before UV micro-irradiation. Cells were recovered in the same medium for 1h, and fixed for PCNA IF staining analysis (Scale bar, 10μm). Fluorescence intensity was expressed relative to that in LacZ control cells recovered at 1h without thymidine supplement. 50 cells were analysed for each experiment (n=3). Insects show H33342 staining of the corresponding cells. RT-PCR of RNA extracted from indicated cells were shown. *P<0.05, **P<0.01, based on Student's *t* test.

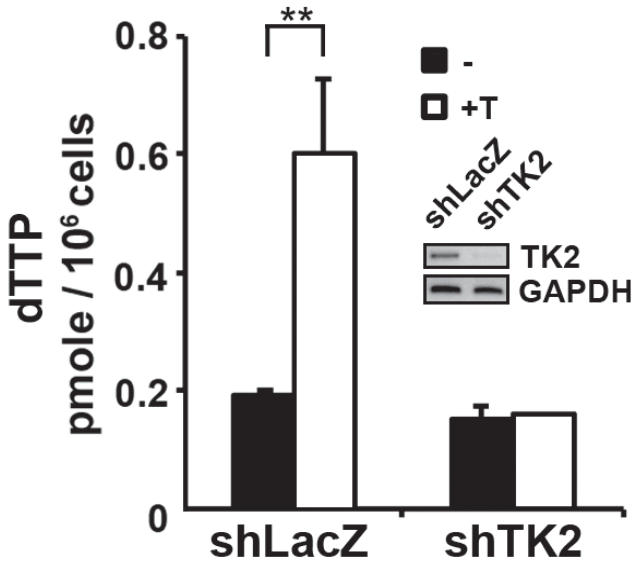


Figure II-12 After thymidine incubation for 8h, 10-day serum-deprived control and TK2 knock-down cells were harvested for dTTP pool analysis. RT-PCR of RNA extracted from indicated cells were shown. **P<0.01, based on Student's *t* test.

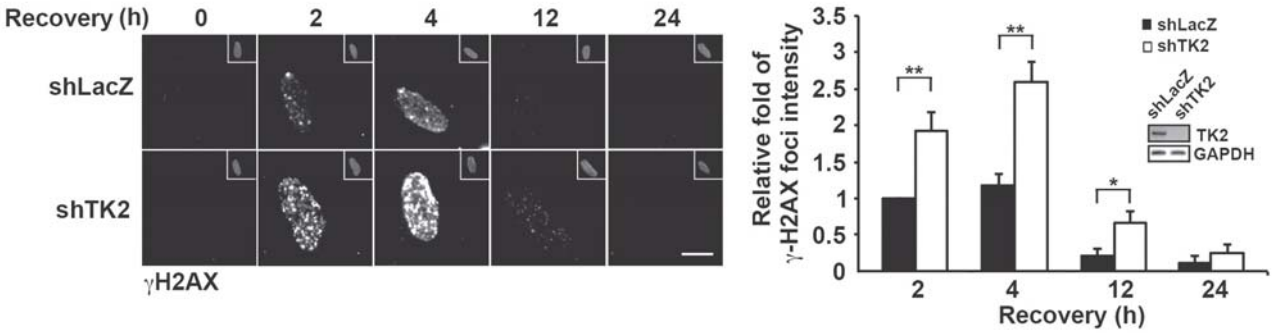


Figure II-13 Control and TK2 knock-down cells were serum-deprived for 10 days and exposed to 10J/M^2 whole-cell UV irradiation. After recovery with 0.1% serum at the indicated time, cells were fixed for γ-H2AX IF staining analysis (Scale bar, 10 mm). Fluorescence intensity was expressed relative to that in LacZ control cells recovered at 2 h. 50 cells were analyzed for each experiment (n=3). Insets indicate Hoechst33342 staining of the corresponding cells. RT-PCR of RNA extracted from indicated cells were shown. *, $p<0.05$ **, $p<0.01$ based on Student's *t* test.

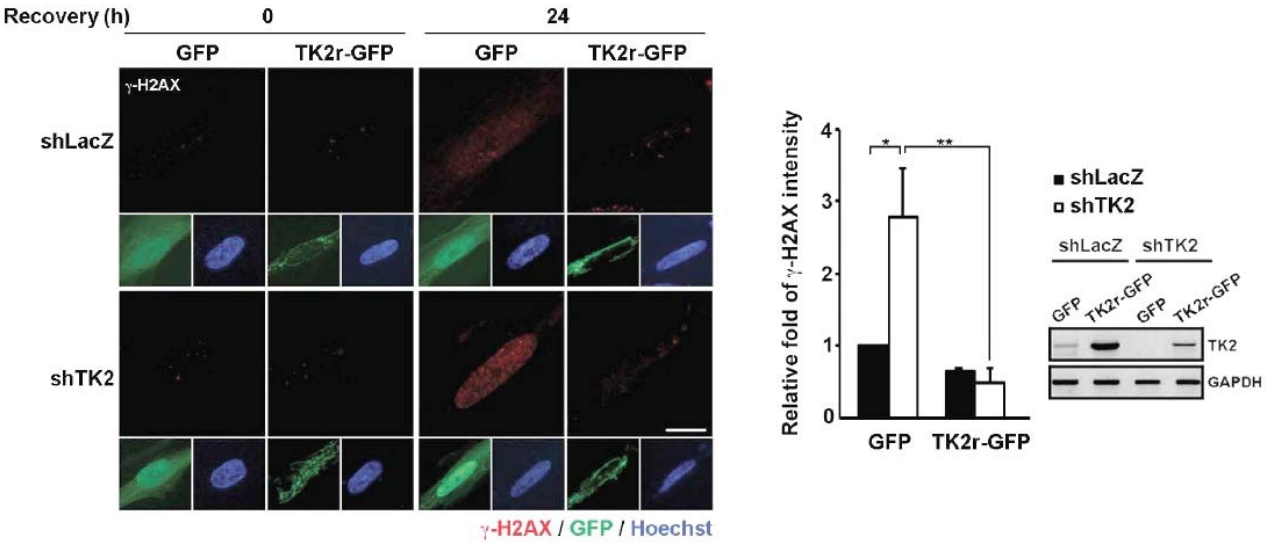


Figure II-14 LacZ control and TK2 knockdown cells were serum-deprived for 2 days. Cells transfected with GFP or TK2r-GFP (TK2 shRNA resistant) were whole-cell irradiated by 20 J/M^2 UVC. Due to the higher intensity of UV irradiation, cell death occurred during recovery in serum-free medium. In this setting, we recovered cells in 10% serum. γ-H2AX intensity was expressed relative to that in LacZ control cells with GFP expression (Scale bar, 10μm). 25 cells were analyzed (n=3). *, $p<0.05$ **, $p<0.01$ based on Student's *t* test. RT-PCR results of RNA extracted from the indicated cells were shown.

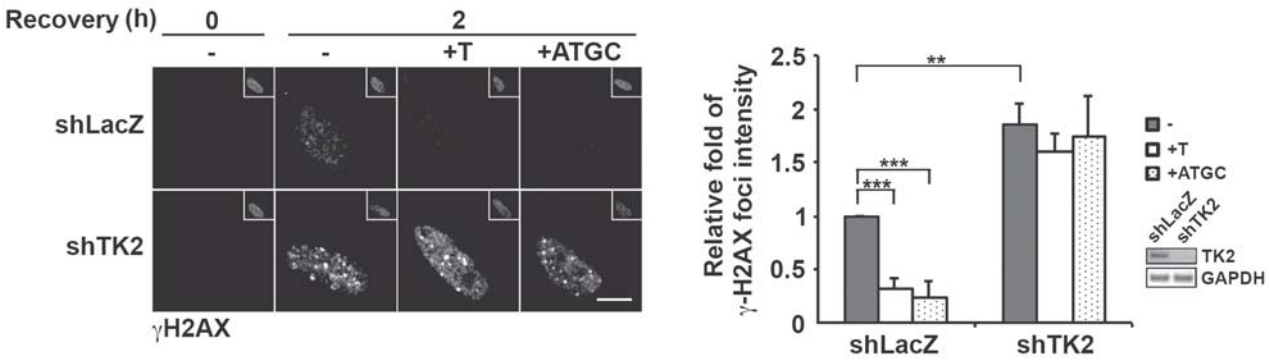


Figure II-15 Before UV irradiation, 10-day serum-deprived control and TK2 knock-down cells were preincubated with 5mM thymidine or four deoxynucleosides in 0.1% dialyzed serum for 8h. After recovery with or without 5mM deoxynucleosides, cells were fixed for γ-H2AX IF staining analysis (Scale bar, 10 μm). Fluorescence intensity was expressed relative to that in LacZ control cells recovered without thymidine supplement. 50 cells were analyzed for each experiment (n=3). Insets indicate H33342 staining of the corresponding cells. RT-PCR of RNA extracted from indicated cells were shown. **, $p<0.01$ and ***, $p<0.001$ based on Student's *t* test.

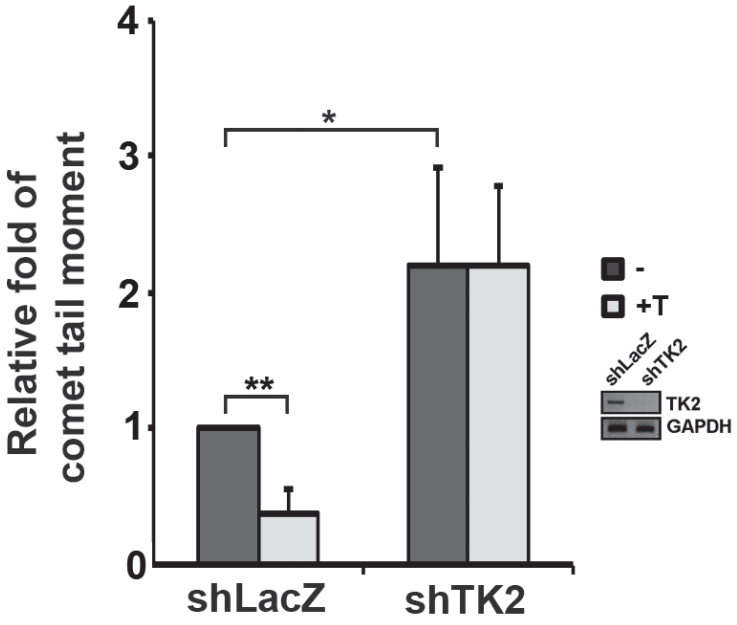


Figure II-16 Before 10-day serum-deprived control and TK2 knock-down cells were subjected to whole-cell UV irradiation. At 2-h recovery, cells recovered with or without thymidine were subjected to alkaline comet assay. 50 cells were analyzed for each experiment (n=3). RT-PCR of RNA extracted from indicated cells were shown. *, $p<0.05$ **, $p<0.01$ ***, $p<0.001$ based on Student's *t* test.

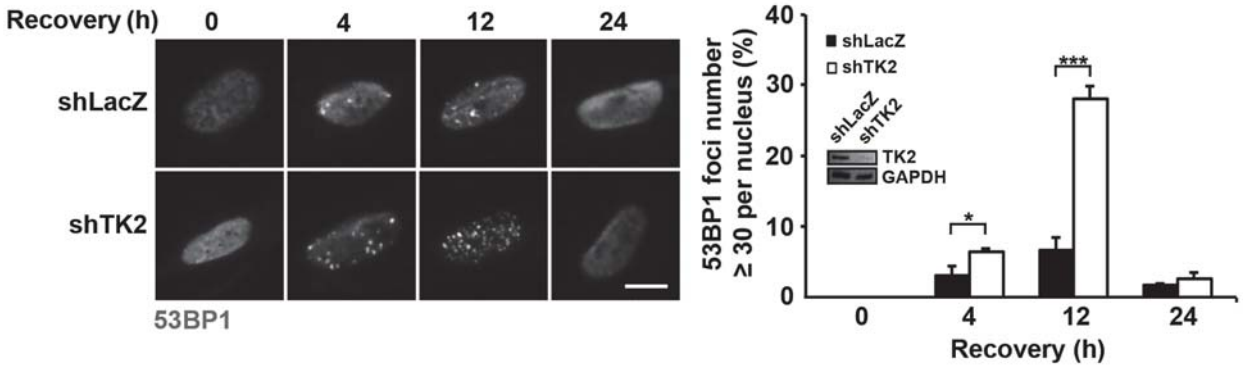


Figure II-17 LacZ control and TK2 knockdown cells were serum-deprived for 10 days. After 10 J/M^2 whole-cell UV irradiation and recovery at the indicated time, cells were fixed for 53BP1 IF staining (Scale bar, 10 μm). Each cell containing 53BP1 foci ≥ 30 was counted and expressed as percentage. More than 100 cells were analyzed for each 53BP1 foci experiment (n=3) and 50 cells for each comet assay (n=3). RT-PCR of RNA extracted from indicated cells were shown. *, $p<0.05$ ***, $p<0.001$ based on Student's *t* test.

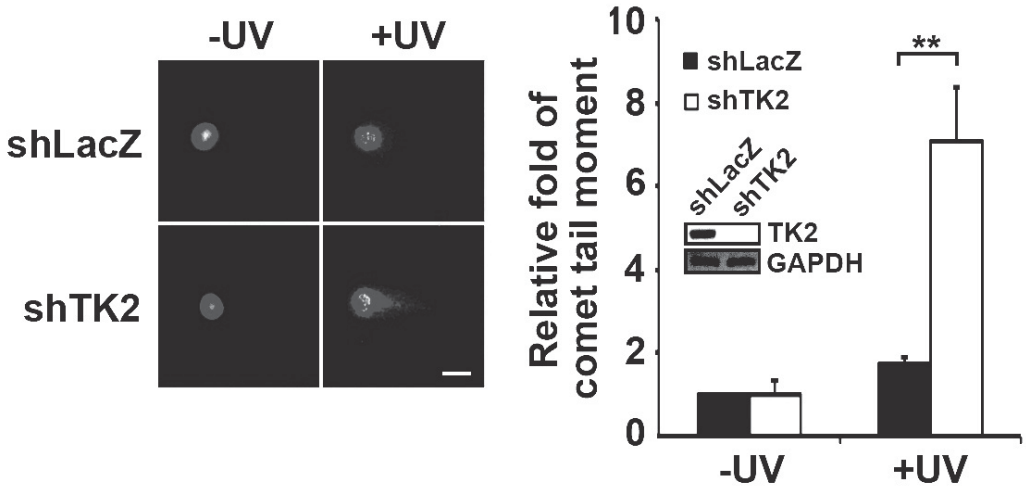


Figure II-18 LacZ control and TK2 knockdown cells were serum-deprived for 10 days. After 10 J/M^2 whole-cell UV irradiation and recovery at 12h, cells were analysed by neutral comet assay. RT-PCR of RNA extracted from indicated cells were shown. **, $p<0.01$ based on Student's *t* test.

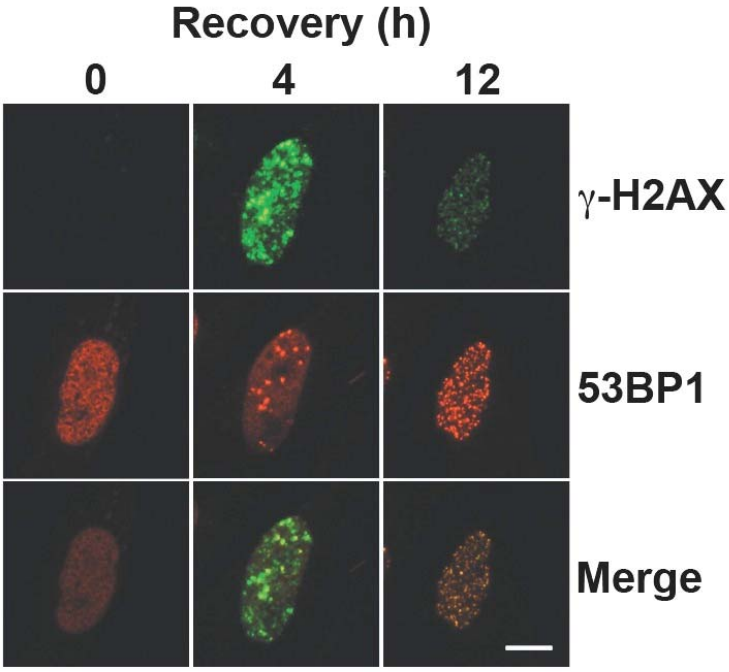


Figure II-19 Co-IF staining of 53BP1 and γ-H2AX was performed at the indicated recovery time after UV irradiation in 10-day serum-deprived TK2 knockdown cells (Scale bar, 10 μm).

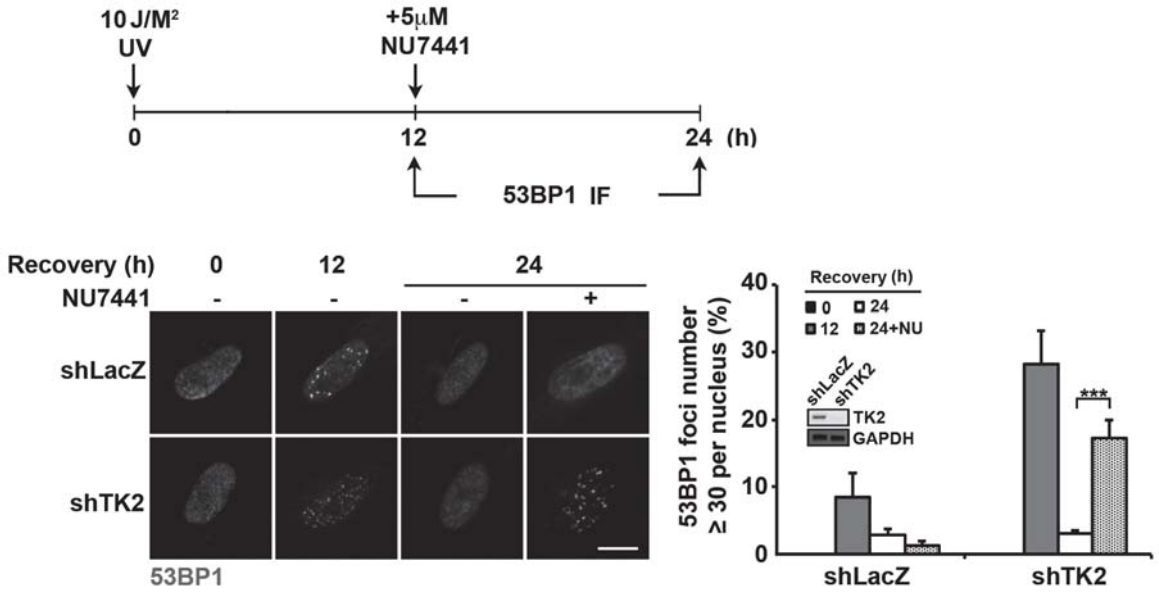


Figure II-20 After 12-h recovery from UV irradiation in 10-day serum-deprived control and TK2 knock-down cells, 5 mM NU7441 was added in the culture medium. Cells were fixed for 53BP1 IF staining at indicated time and analyzed as described in (A) (Scale bar, 10 μm). RT-PCR of RNA extracted from indicated cells were shown. ***, $p<0.001$ based on Student's *t* test.

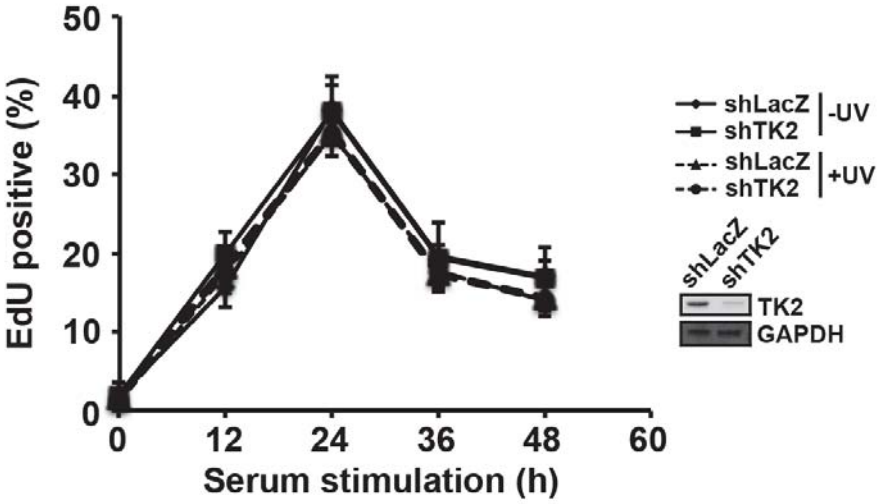


Figure II-21 Control and TK2 knockdown cells were serum-deprived for 2 days prior to 10 J/M^2 UV irradiation. After 24 h recovery without serum, fresh medium containing 10% serum was added to stimulate the cell cycle entry. S phase cell percentage at indicated time was analyzed by EdU incorporation. The percentages of EdU positive cells were determined by counting more than 200 cells in each experiment (n=3). RT-PCR of RNA extracted from indicated cells were shown.

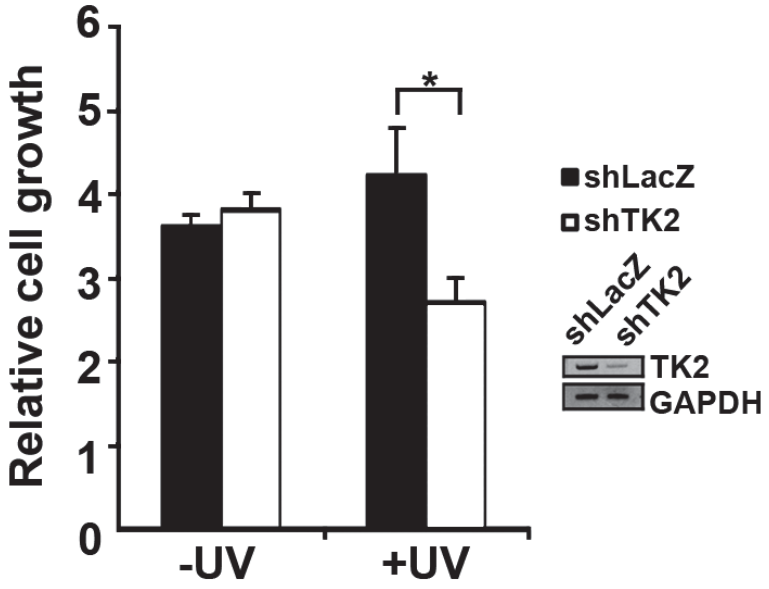


Figure II-22 Control and TK2 knockdown cells were serum-deprived for 2 days prior UV irradiation. After 24 h recovery without serum, fresh medium containing 10% serum was added back for 48 h and cell growth was determined by WST1 reagent. RT-PCR of RNA extracted from indicated cells were shown. *, $p<0.05$ based on Student's *t* test.

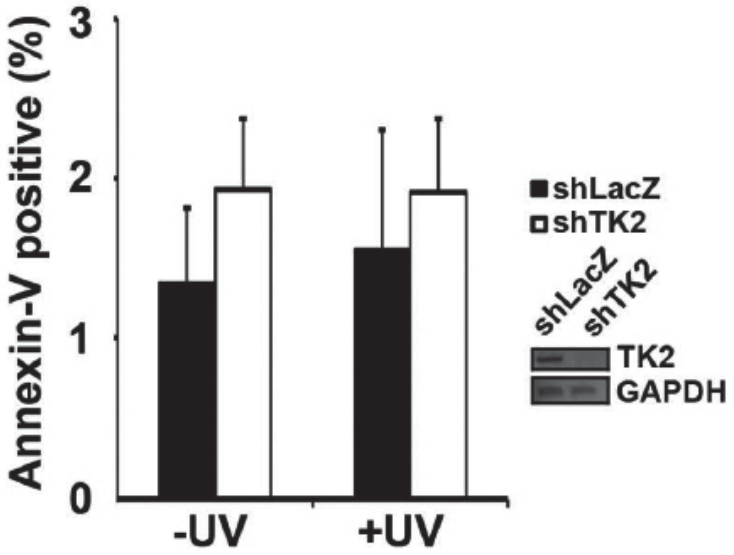


Figure II-23 LacZ control and TK2 knockdown cells were serum-deprived for 2 days and subjected to 10 J/M^2 whole-cell UV irradiation. After recovery for 24 h without serum, cells were refreshed with 10% serum containing medium for 48 h. Cells were harvested for annexin-V apoptosis assay. RT-PCR of RNA extracted from the indicated cells were shown.

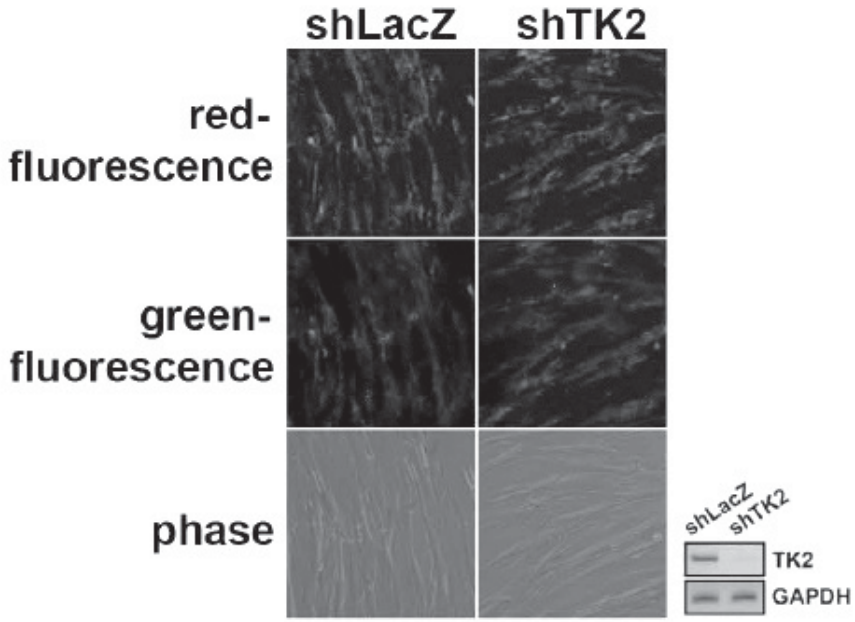


Figure II-24 LacZ control and TK2 knockdown cells were serum-deprived for 2 days and subjected to 10 J/M^2 whole-cell UV irradiation. After recovery for 24 h without serum, cells were refreshed with 10% serum containing medium for 48 h. JC-1 based mitochondrial membrane potential assay was performed. RT-PCR of RNA extracted from the indicated cells were shown.

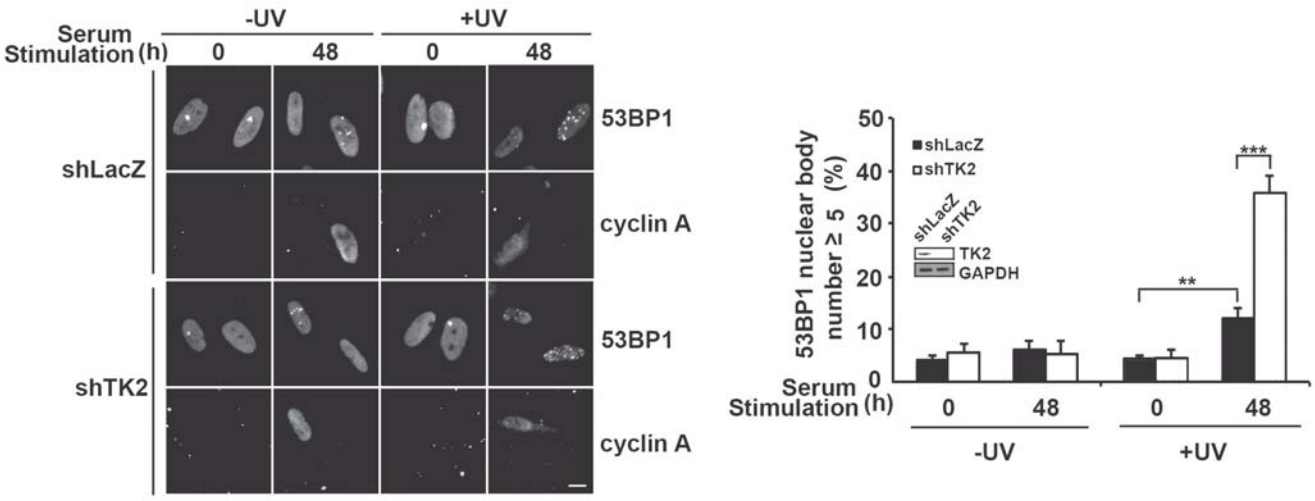


Figure II-25 2-day serum-deprived control and TK2 knock-down cells were UV irradiated and recovered without serum for 24h. After 48-h serum stimulation, cells were fixed for 53BP1 and cyclin A IF staining (Scale bar, 10 μm). Each cell negative in cyclin A staining with 53BP1 nuclear bodies ≥ 5 was counted and expressed as percentage. More than 100 cells were counted for each experiment (n=3). RT-PCR of RNA extracted from indicated cells were shown. **, $p<0.01$ ***, $p<0.001$ based on Student's *t* test.

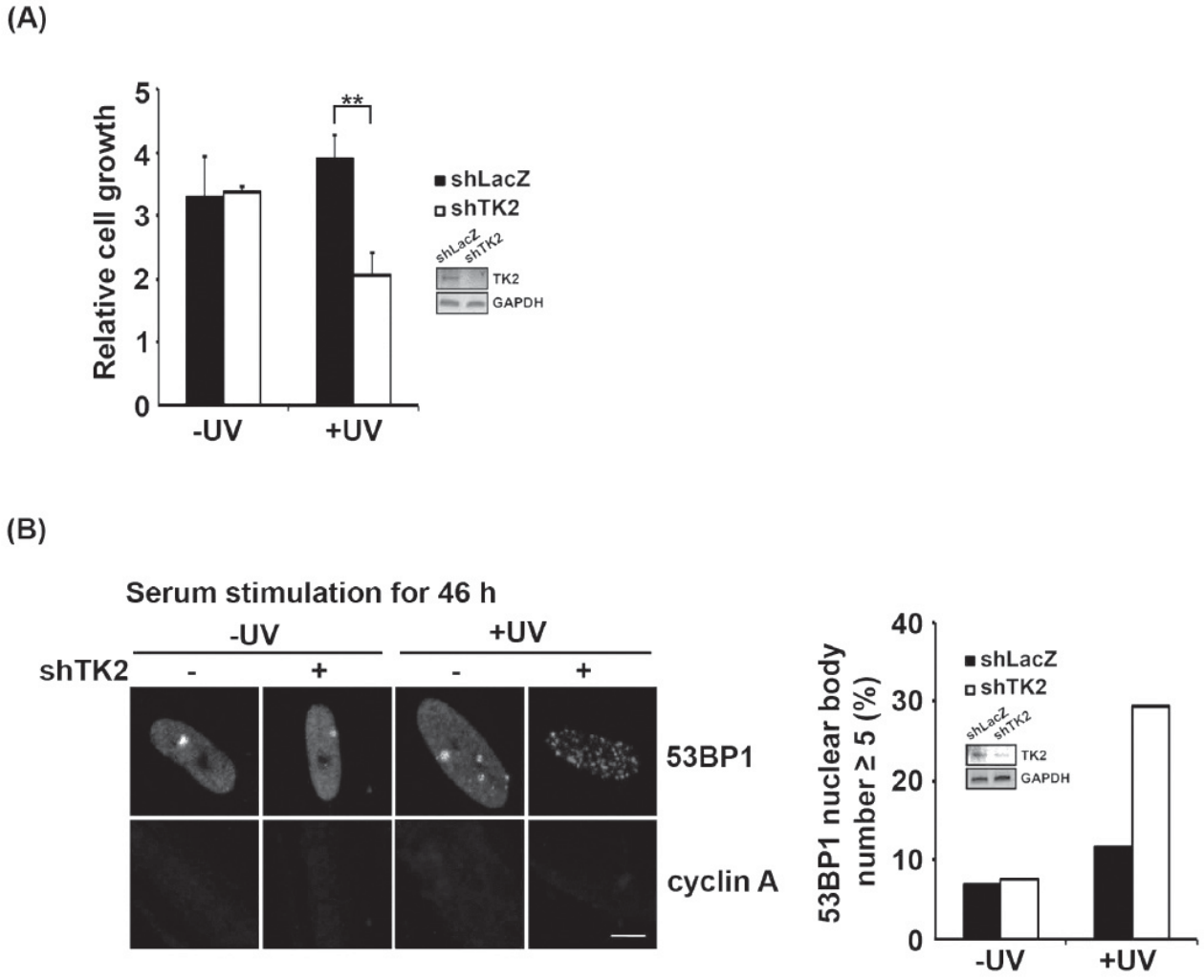


Figure II-26 LacZ control and TK2 knockdown cells were serum-deprived for 10 days prior to 10 J/M^2 whole-cell UV irradiation. After 24 h recovery with 0.1% serum, cells were refreshed with medium containing 10% serum for 2 days. (A) Cell growth was determined by WST1 reagent (n=3). **. P<0.01 based on Student's *t* test. (B) Cells were fixed for 53BP1 and cyclin A immunofluorescence staining. (Scale bar, 10μm). Cyclin A staining-negative cells were shown. Each cell containing 53BP1 nuclear bodies ≥ 5 were counted and expressed as percentage. More than 100 cells were counted. RT-PCR of RNA extracted from the indicated cells were shown.

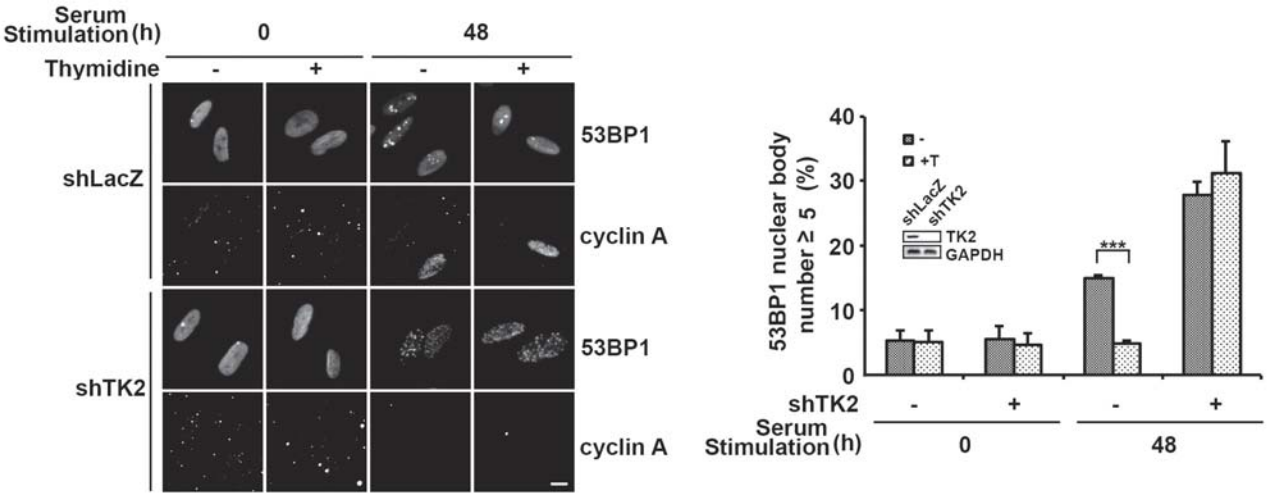


Figure II-27 Control and TK2 knock-down cells were serum-deprived for 2 days, following supplemented with 5 mM thymidine for 8 h and during recovery from UV-irradiation. After 24 h, cells were serum-stimulated for 48 h and fixed for 53BP1 and cyclin A IF staining analysis for quantification as described above (Scale bar, 10 mm). RT-PCR of RNA extracted from indicated cells were shown. *** $p<0.001$ based on Student's *t* test.

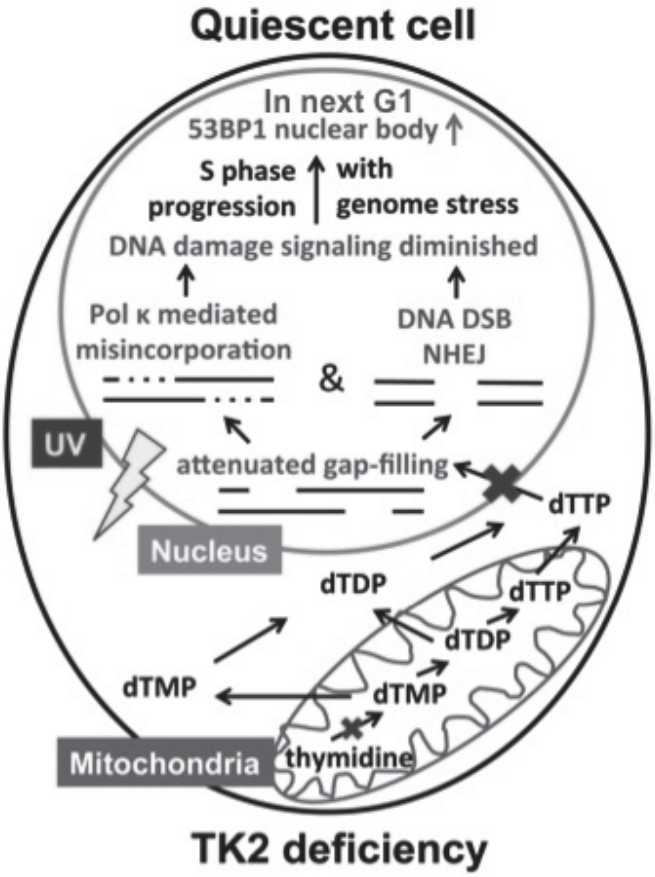


Figure II-28 Proposed model In quiescent cells, the only pathway for dTTP synthesis is depleted due to TK2 deficiency. In response to UV irradiation, insufficient dTTP for gap-filling synthesis of NER results in secondary DNA double strand breaks repaired by non-homologous end joining, representing a poor quality of repair. Although the DNA damage signal diminishes, the replication stress is induced after cell cycle entry to S phase. As indicated, 53BP1 nuclear body is induced to protect problematic DNA in the next G1 phase.

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Appendix

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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 Polε, Polδ or Polκ 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 diphosphoate (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% CO_2.

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/M^2 UV irradiation. For localized UV-irradiation, polycarbonate isopore membrane with 3 μm pore size filters (Millipore) was placed on top of cells in Hank's balanced salt solution, and irradiated with 120 J/M^2 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 GGATCGAATATTAACT-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 $MgCl_2$, 0.1% bovine serum albumin, 5 mM NaF, 2 mM DTT, 180 μM thymidine and [^{3}H]-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) photoproduct 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

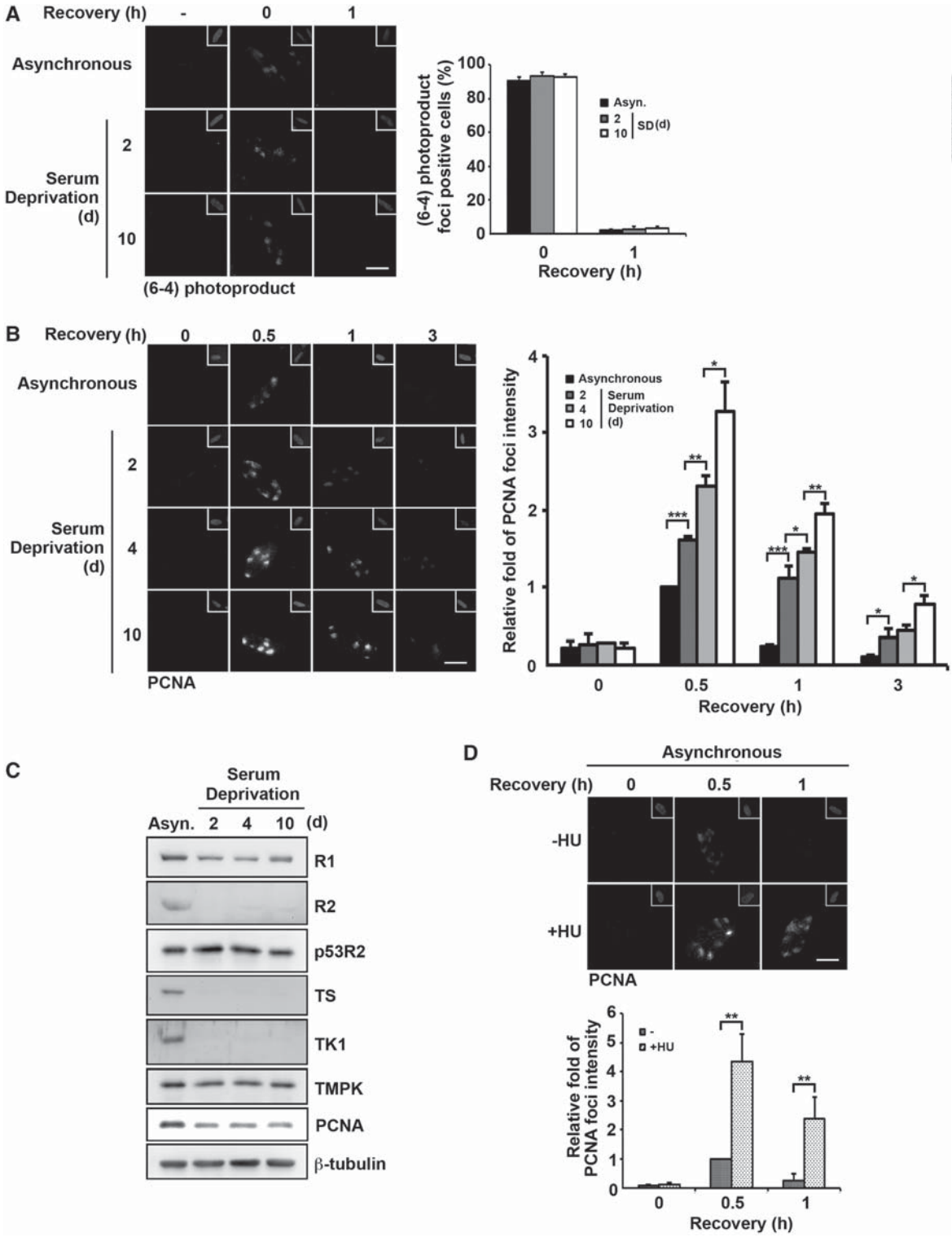

Figure. 1. Serum deprivation and dNTP supply affects the gap-filling step in NER. IMR-90 fibroblasts were incubated in medium containing 10% serum (asynchronous), serum-free for 2 and 4 days or 0.1% serum for 10 days. Asynchronous and serum-deprived cells were irradiated with 120 J/M^2 UVC through micropore membrane (pore, 3 μm) and were recovered, after which cells on coverslips were subjected to (**A**) (6–4) photoproduct and (**B**) PCNA IF staining (Scale bar, 10 μm). 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 0.5 h. 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 3 h before local UV irradiation and during recovery. Cells were fixed for PCNA IF staining (Scale bar, 10 μm). Fluorescence intensity was expressed relative to that in cells recovered at 0.5 h 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/10^6 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

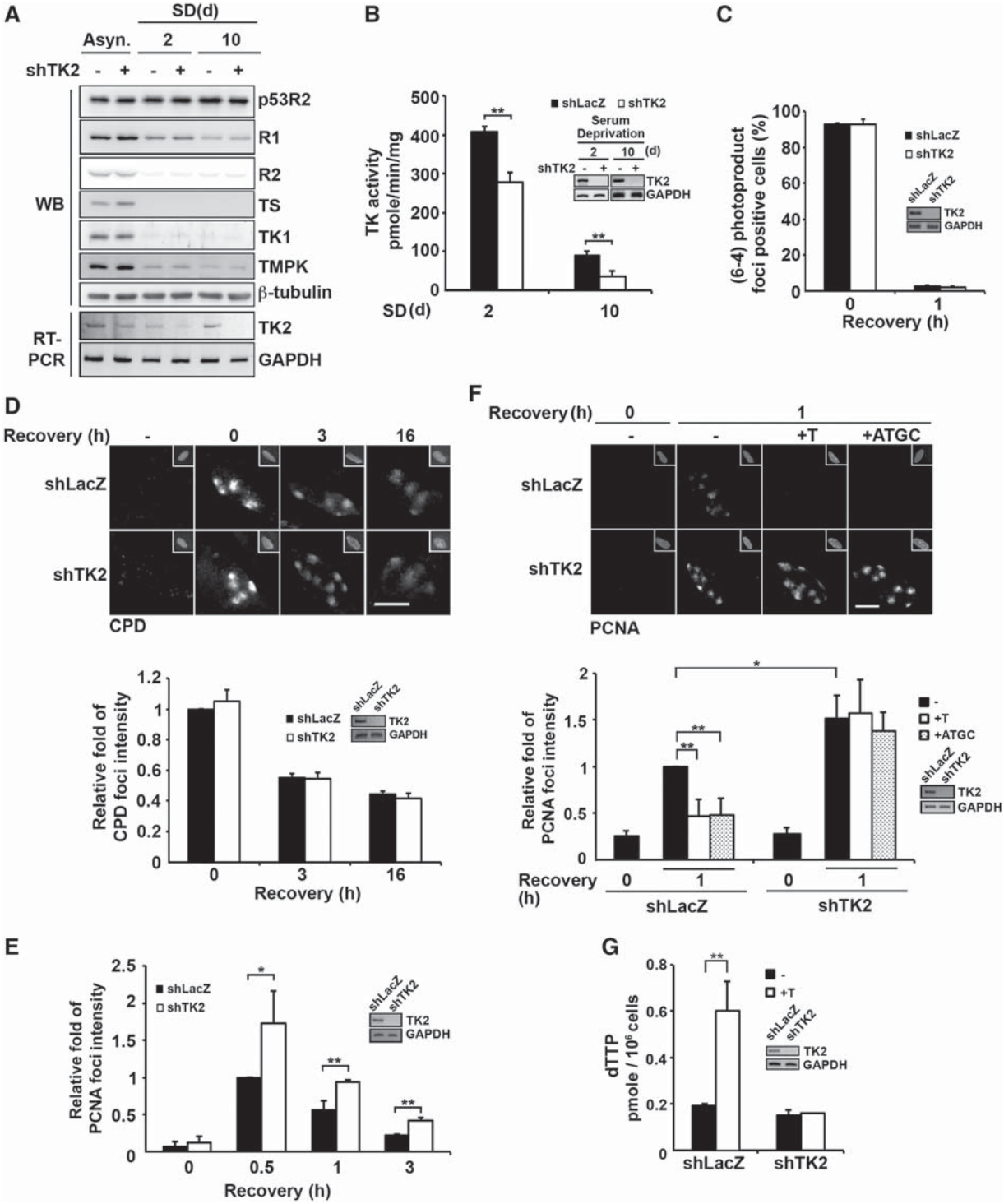

Figure 2. The contribution of TK2-mediated synthesis of dTTP to the rate of the gap-filling step in quiescent cells. IMR-90 cells were infected by LacZ or TK2 shRNA lentivirus. These cells were incubated in 10% serum containing or serum-deprived condition for (**A**) western blot and (**B**) thymidine kinase activity assay. (**C**) 10-day serum-deprived control and TK2 knock-down cells were irradiated by 120 J/M^2 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.

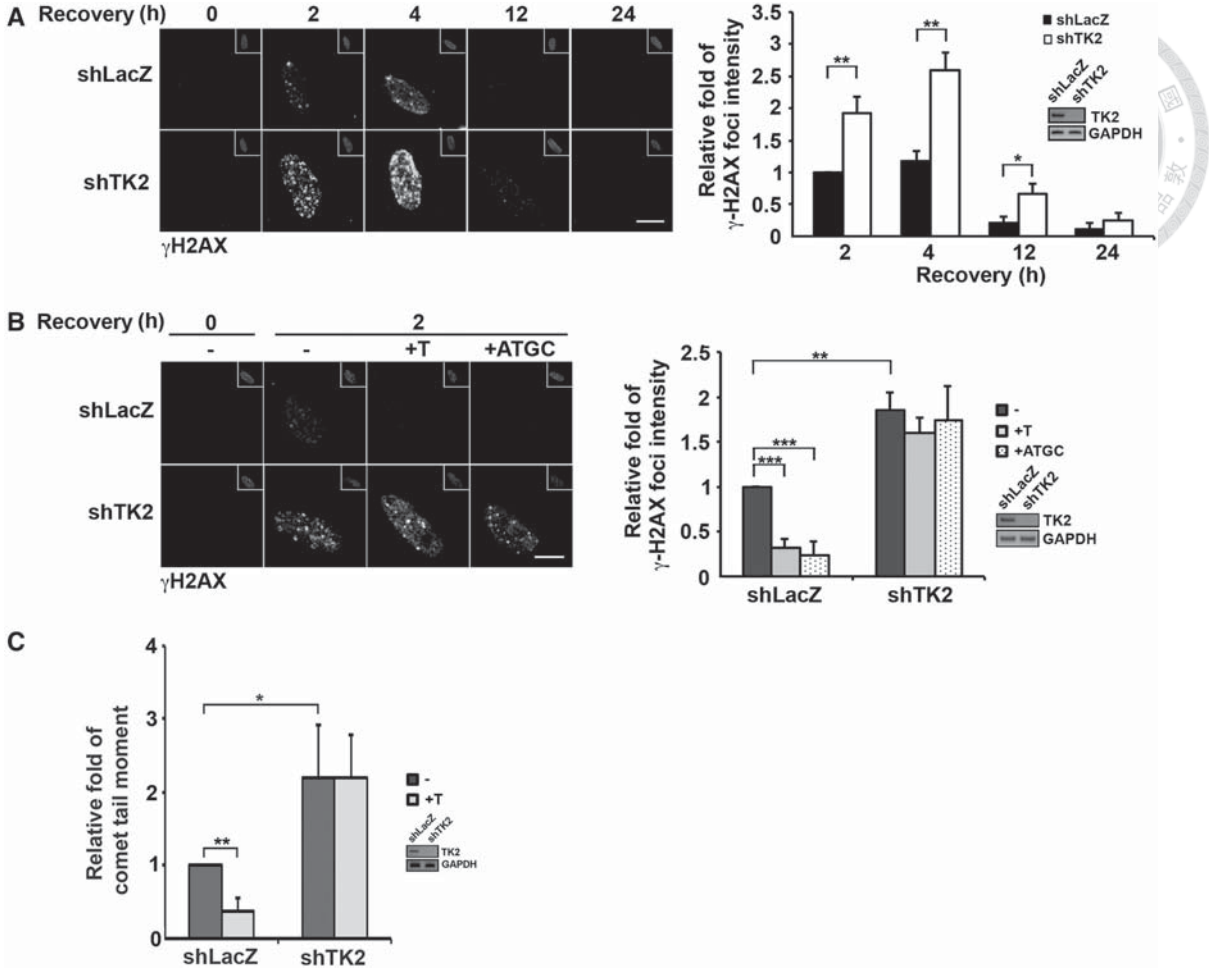

Figure 3. The effect of TK2 knock-down on DNA damage response after whole-cell UV irradiation. LacZ control and TK2 knock-down cells were serum deprived for 10 days and exposed to 10 J/M^2 whole-cell UV irradiation. (**A**) After recovery with 0.1% serum at the indicated time, cells were fixed for γ-H2AX IF staining analysis (Scale bar, 10 μm). 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 μM thymidine or four deoxynucleosides in 0.1% dialysed serum for 8 h. After recovery with or without 5 μM deoxynucleosides, cells were fixed for γ-H2AX IF staining analysis (Scale bar, 10 μm). 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

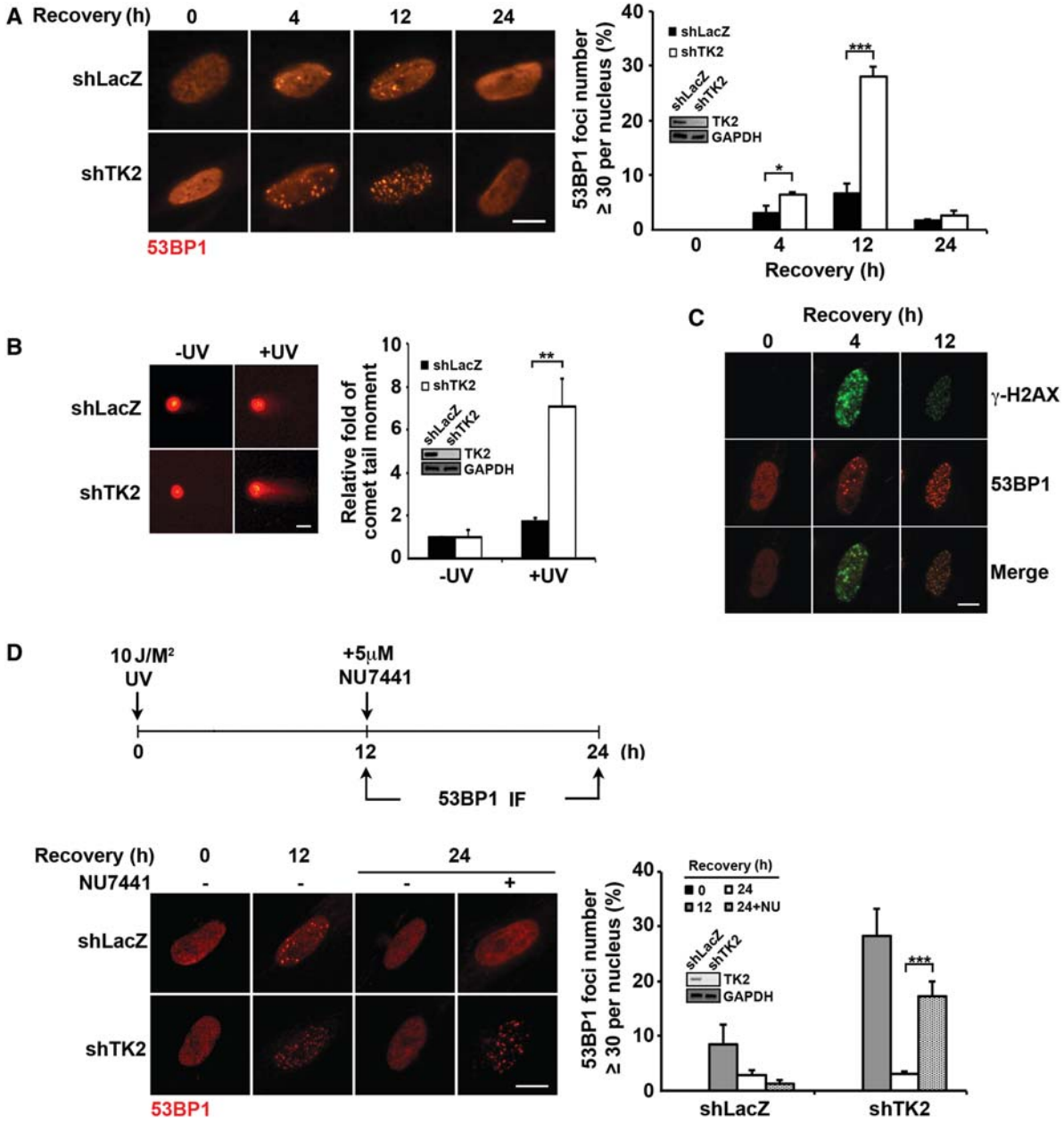

Figure 4. Analysis of DNA DSBs during recovery from UV irradiation in quiescent control and TK2 knock-down cells. LacZ control and TK2 knock-down cells were serum deprived for 10 days. After 10 J/M^2 whole-cell UV irradiation and recovery at the indicated time, cells were (**A**) fixed for 53BP1 IF staining (Scale bar, 10 μm) 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 μm). (**D**) At 12-h recovery, 5 μM 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 μm). 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.

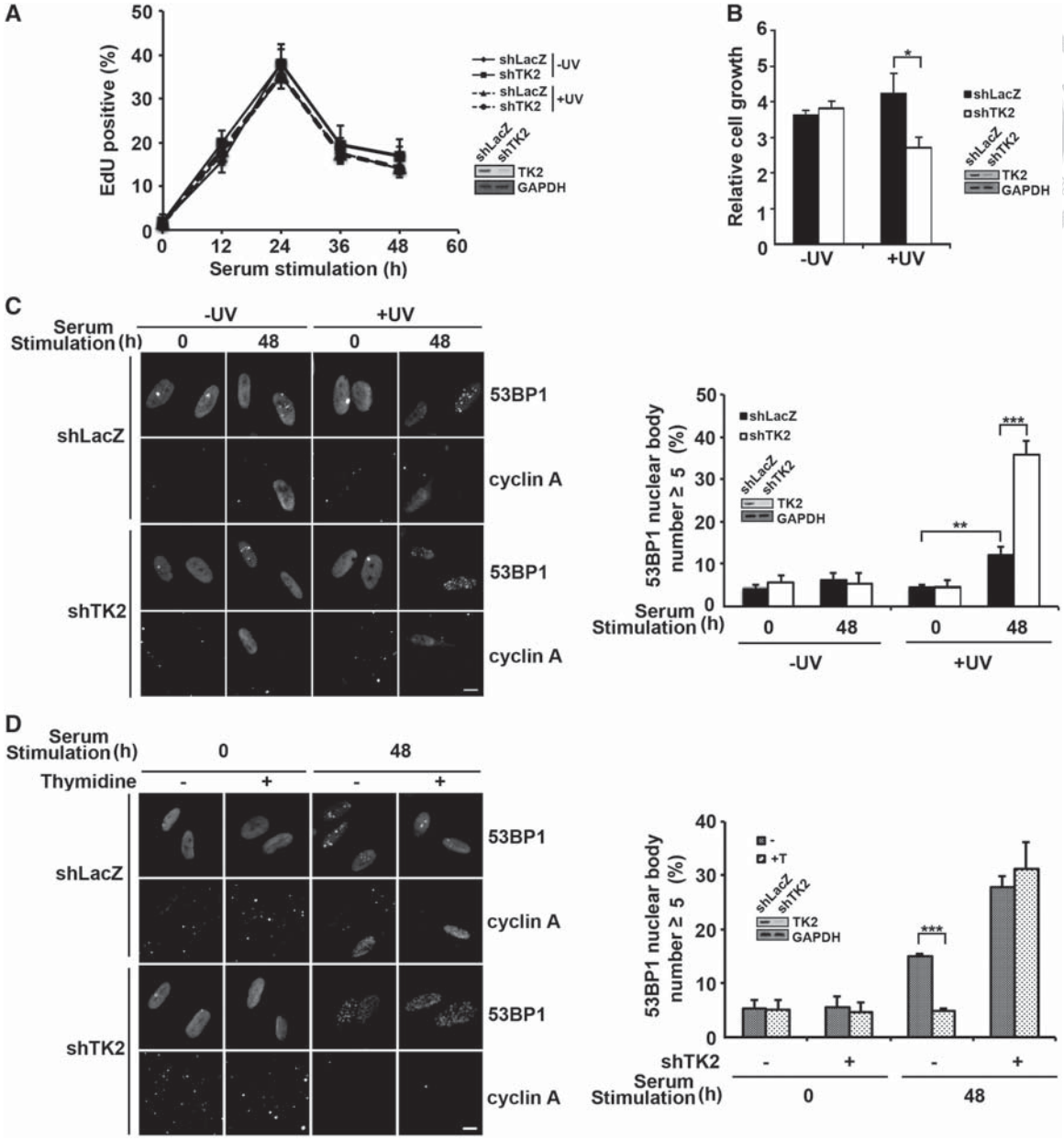

Figure. The correlation of the efficiency of NER during the quiescent state with the occurrence of genome stress through re-entry of cell cycle progression. Control and TK2 knock-down cells were serum deprived for 2 days before 10 J/M^2 UV irradiation. After 24-h recovery without serum, fresh medium 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 μm). 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 μM 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 μm). 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 misssense 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 misincoporation in the place of dTTP. Three DNA polymerases Polε, Polδ and Polκ were reported to participate in DNA gap-filling process of NER. Of note, DNA Polκ, 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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