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KCTD10 Interacts With Proliferating Cell Nuclear Antigen and Its Down-Regulation Could Inhibit Cell Proliferation

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ABSTRACT

A novel gene (GenBank accession No. AF113208) named *KCTD10* (potassium channel tetramerisation domain-containing 10) was cloned from our 5300 EST database of human aorta cDNA library. Computational analysis showed that *KCTD10* cDNA is 2,638 bp long, encoding 313 amino acids with a proliferating cell nuclear antigen binding motif, mapped to chromosome 12q24.11 with 7 exons, ubiquitously expressed in all 12 tested normal tissues and 7 of 8 tested tumor cell lines from MTN membranes by Northern blot. Nuclear localization of KCTD10 was observed in A549 cells. Yeast two-hybrid analysis and immunoprecipitation assay showed that KCTD10 can interact with PCNA. In A549 cells, KCTD10 down-regulation could inhibit cell proliferation, but its over-expression could not influence cell proliferation. The results suggest that KCTD10 may be associated with DNA synthesis and cell proliferation. J. Cell. Biochem. 106: 409–413, 2009.

KEY WORDS: KCTD10; PROLIFERATING CELL NUCLEAR ANTIGEN; CELL PROLIFERATION

Proliferating cell nuclear antigen (PCNA) is a multifunctional protein that plays roles in a variety of cellular processes, including DNA replication, DNA repair, and cell cycle control, by interacting with proteins involved in these processes [Maga and Hubscher, 2003]. Especially, PCNA plays a co-ordinating role for numerous proteins involved in many processes involving DNA. As a consequence of their interaction with PCNA, several enzymes show an increase in catalytic efficiency [Maga and Hubscher, 2003]. KCTD13 (potassium channel tetramerisation domain containing 13), also named PDIP1 (polymerase δ-interacting protein 1), is a TNF-α inducible protein. KCTD13 interacts with PCNA and the small subunit (p50) of DNA polymerase δ, and stimulates polymerase δ activity in the presence of PCNA [He et al., 2001]. KCTD13 shares high similarity to one of the early response genes induced by TNF-α, TNFAIP1 (tumor necrosis factor, alpha-induced protein 1 (endothelial)) [Wolf et al., 1992]. The PDIP1 family, including KCTD13, TNFAIP1, possibly plays roles in TNF-α induced DNA replication/repair by interacting with PCNA and some enzymes (e.g., polymerase δ) involved in DNA replication/repair pathways [He et al., 2001; Zhou et al., 2005]. In 2001, we have cloned a novel gene–KCTD10 (potassium channel tetramerisation domain-containing 10) from human aorta cDNA library (GenBank Accession No. AF113208), encoding 313 amino acids. Amino acid sequence alignment shows that human KCTD10 shares 97.8% identity with mouse and rat KCTD10, 64.7% identity with human KCTD13, and 68.6% identity with human TNFAIP1, and has a PCNA binding motif "QTKVEFP". Recently, Zhou et al. [2005] analyzed the expression of *KCTD10* mRNA in rat tissues and reported that rat KCTD10 can interact with PCNA. In the present study, we characterized the biological functions of KCTD10, and studied the expression of *KCTD10* in human normal tissues, the interaction of human KCTD10 with PCNA and the association with cell proliferation.

MATERIALS AND METHODS

NORTHERN BLOT ANALYSIS

Tissue distribution of human *KCTD10* mRNA was analyzed by Northern blot on normal and tumor MTN blots purchased from Clontech (Mountain View, CA). The ^{32}P-labeled probe of 1,099 bp was synthesized by using a Prime-a-Gene Labeling System (Promega, Nepean, ON, Canada) based on supplier's protocol. The

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membranes were subjected to autoradiography after hybridization with ^{32}P-labeled *KCTD10* probe and a sequential washing.

CONSTRUCTING PLASMIDS AND THE RECOMBINANT ADENOVIRUS EXPRESSION VECTORS

The bait plasmid pGBKT7-KCTD10 was used to screen the pretransformed heart cDNA library by yeast mating. The coding region of *KCTD10* was fused to the human influenza hemagglutinin (HA) amino acid residues 98–106 and used for immunoprecipitation. The open reading frame of *KCTD10* was amplified by PCR using the aorta cDNA library as template with sense primer (TGTGAATTCCGACTTATCATGGAAGAGA) and antisense primer (TGTCGACCAATACCAAAATAATCATCT). The resultant PCR products were digested with ligated *Eco*RI and *Sal*I, and then ligated into *Eco*RI and *Sal*I sites of pGBKT7 vector and pEGFP-C2 vector, sequenced. The coding region of *KCTD10* was subcloned from pGBKT7-KCTD10 to pCMV-HA through *Sfi*I and *Sal*I sites to get pCMV-HA-KCTD10; from pEGFP-C2-KCTD10 to pAdTrack-CMV through *Bgl*II and *Sal*I sites to get pAdTrack-CMV-KCTD10, which was used for constructing the recombinant adenovirus expression vectors (Ad-KCTD10) as protocol [He et al., 1998]. The adenoviral vector for only expressing the green fluorescent protein (Ad-GFP) constructed in our lab was used as a control [Wang et al., 2005].

SUBCELLULAR LOCALIZATION

Subcellular localization of KCTD10 was determined by using immunofluorescence with anti-KCTD10 antibody (Aviva System Biology, San Diego, CA). The A549 cells, plated on glass coverslips at ~30–50% confluency, were fixed in 4% paraformaldehyde in phosphate-buffered saline, permeabilized in 0.25% Triton X-100 in phosphate-buffered saline for 10 min at room temperature, and incubated for 1 h in blocking buffer supplemented with 0.1% sodium azide. Following incubation with anti-KCTD10 (10 μg/ml) for 1 h, cells were washed with phosphate-buffered saline, incubated in blocking buffer containing FITC-labeled IgG (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100) for 45 min, then washed with phosphate-buffered saline, stained with DAPI (10 μg/ml), then analyzed on a Nikon Eclipse TC2000-E microscope.

YEAST TWO-HYBRID ASSAY

The Gal4-based Matchmaker two-hybrid System 3 (Clontech) was used for yeast two-hybrid analysis. The bait plasmid pGBKT7-KCTD10 was used to screen the pretransformed heart cDNA library by yeast mating. The diploid yeast cells were selected on the quadruple dropout selection (SD/-Ade/-His/-Trp/-Leu). The surviving yeast colonies were subjected to β-Gal analysis. The resultant putative positive prey plasmids together with bait plasmid were reintroduced into yeast by-yeast mating to further confirm protein–protein interaction. The candidate library inserts were transformed into *Escherichia coli* DH5 using electroporation. Finally, the positive clones were sequenced. The detailed procedures were carried out according to Clontech's manuals with a few modifications.

COIMMUNOPRECIPITATION ANALYSIS

To confirm a direct physical interaction between KCTD10 and PCNA in vivo, we detected the interaction both in over-expression state and in physiological concentration of KCTD10 in A549 cells. The coding region of KCTD10 was fused to the human influenza hemagglutinin (HA) amino acid residues 98–106 and used for immunoprecipitation. The 2×10^7 cells (approximately 70% confluent) were transfected with 15 μg pCMV-HA-KCTD10 using the Lipofectamine 2000. The cells were harvested 72 h after transfection. The lysates were immunoprecipitated with anti-HA Immunoprecipitation Kit (Sigma–Aldrich, St. Louis, MI). The precipitates were resolved on 8% SDS–polyacrylamide gel electrophoresis and subjected to Western blot analysis by using anti-PCNA monoclonal antibody (Lab Vision, Fremont, CA). Coimmunoprecipitation using anti-PCNA antibody and then detecting KCTD10 in physiological concentration was performed to confirm the result. Lysate of A549 cells was incubated with 5–8 μg anti-PCNA at 4°C for 1.5–2 h. The immunocomplexes were subsequently precipitated with 50 μl (bed volume) of protein A-agarose beads (Sigma, St. Louis, MO). After extensive washing with lysis buffer, bound proteins were eluted by boiling in SDS sample buffer. The proteins in the eluates (immunoprecipitated fractions) were separated on SDS–polyacrylamide gel electrophoresis and subjected to Western blot analysis by using anti-KCTD10 antibody.

siRNA-MEDIATED DOWN-REGULATION OF KCTD10

The down-regulation of KCTD10 was performed by transfection of the A549 cells with either of two siRNA duplex oligonucleotides (KCTD10-RNAi#1: UUGAGUAUCGUACCAAAGUGCUUCC; or KCTD10-RNAi#2: UCACCACACUUUCUCCUGACAUCUC (synthesized by Invitrogen)), by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Stealth RNAi Negative Control Duplexes (Invitrogen)) were used for a control siRNA.

MTT AND REAL-TIME PCR ASSAYS

The MTT assay was performed according to the supplier's procedures with some modifications (Sigma). Briefly, the cells (5,000 cells/well) were cultured in 96-well plates with 100 μl of media/well. MTT (20 μl, 5 mg/ml) solution was added to each well, and incubated at 37°C for additional 4 h in a CO_2 incubator. Each experiment was done in 12 wells and repeated thrice. The absorbance at 570 nm was recorded with a microtiter plate reader (Bio-Rad, Hercules, CA). Total RNA was isolated using RNAgent Isolation System (Promega, Madison, WI), and the mRNA was reverse transcribed to cDNA by AMV transcriptase XL (Takara Biotechnology, Dalian, Liaoning, China). Real-time PCR was performed on an opticon MJ DNA Engine (MJ research, MA). Aliquots of cDNA were used as template for real-time PCR reactions containing primers for *KCTD10*, *PCNA*, or for β-Actin. Four replicates of each reaction were performed. β-Actin was used to evaluate the amount of mRNA in each sample. We used following primers: *KCTD10*, (AATAAGCCAGCCGTGAA, ACAGGACCCTTCCGTTA); *PCNA*, (CTCCTTCCCGCCTGCCTGTA, TGAGTGCCTCCAACACCTTC); β-Actin (CAGCAAGCAGGAGTATGACGAG, AAGAAAGGGTGTAACGCAACTA). Cells infected with adenovirus or transfected with siRNA at 72 h after plating were collected for the assays.

RESULTS

UBIQUITOUS TISSUE DISTRIBUTION OF KCTD10

Two transcripts were detected in all tissues examined with particular high expression in heart, skeletal muscle, and placenta for the two transcripts, and relatively high expression of the large transcript. (Fig. 1A). These two transcripts were also detected in seven tumor cell lines except for HL-60, relatively high expression in MOLT-4, Burkitt's lymphoma Raji, SW480, A549, and G-361 (Fig. 1B).

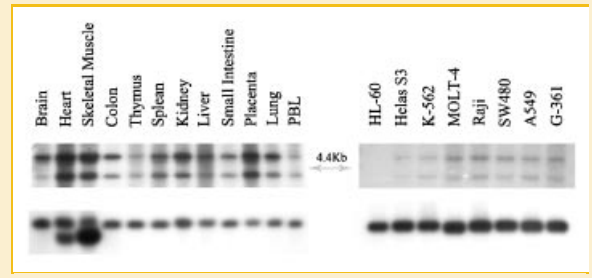

Fig. 1. Tissue distribution of *KCTD10* mRNA. A: Comparison of *KCTD10* expression in 12 normal tissues: brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung, and peripheral blood leukocytes (PBL). Two transcripts were detected in all tissues examined with particular high expression in heart, skeletal muscle, and placenta for the two transcripts and relatively high expression of the large transcript. B: *KCTD10* expression level in eight tumor cell lines which indicate HL-60: promyelocytic leukemia HL-60, K-562: chronic myelogenous leukemia K-562, MOLT-4: lymphoblastic leukemia MOLT-4, Raji: Burkitt's lymphoma Raji, SW480: colorectal adenocarcinoma SW480, A549: lung carcinoma A549, G-361: melanoma G-361. Two transcripts were detected in seven tumor cell lines except for HL-60, relatively high expression in MOLT-4, Burkitt's lymphoma Raji, SW480, A549, and G-361. The lower panel shows the expression of β-actin, which was used to evaluate the loading of mRNA.

NUCLEAR LOCALIZATION OF KCTD10 IN A549 CELLS

The subcellular distribution of endogenous KCTD10 was examined by immunofluorescence with anti-KCTD10 antibody. DAPI staining was as a reference. Nuclear localization of KCTD10 was found in A549 cells (Fig. 2).

KCTD10 INTERACTS WITH PCNA

Compute analysis suggested a PCNA binding motif at the C-terminus of KCTD10. So we thought that KCTD10 may be associated with PCNA. Yeast two-hybrid assay showed that PCNA interacts with KCTD10. The library contained a total of 3.5×10^6 clones (provided by Clontech), total three positive clones were identified: PCNA (GenBank Accession Number is NM_002592.2), EEF1G (NM_001404.4), and C11orf17 (NM_020642.3). While the yeast two-hybrid system clearly indicated that KCTD10 and PCNA could interact in a cellular context, it was necessary to confirm this interaction by coimmunoprecipitation. Coimmunoprecipitation was performed in A549 cells, and we found that KCTD10 did associate with PCNA in immunoprecipitate. To avoid the confusing of the over-expressed state of KCTD10, we also performed immunoprecipitation using anti-PCNA antibody and then detected KCTD10 by anti-KCTD10 antibody in A549 cells without transfection with KCTD10. (Fig. 3) The results were consistent. They were also in accordance with the interaction of rat KCTD10 and PCNA.

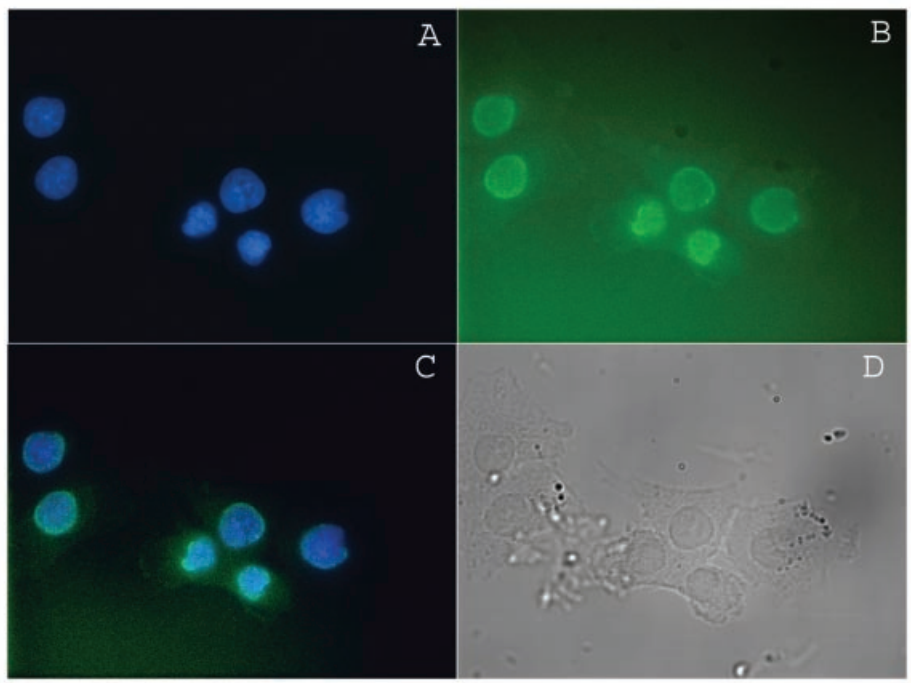

Fig. 2. Nuclear localization of KCTD10 in A549 cells. Immunofluorescence by anti-KCTD10 antibody was used to determine the subcellular localization of endogenous KCTD10. A: DAPI-fluorescence showed a nuclear staining; (B) The green FITC-fluorescence showed the KCTD10 localization; (C) The merged fluorescence showed nuclear localization of KCTD10; (D) The phase of the cells (all 600×).

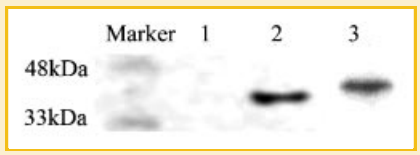

Fig. 3. Interaction of KCTD10 with PCNA in coimmunoprecipitation assay. 1: To immunoprecipitate using anti-HA antibody and then detect PCNA using anti-PCNA antibody in lysates of A549 cells; (2) To immunoprecipitate using anti-HA antibody and then detect PCNA using anti-PCNA antibody in lysates of A549 cells transfected with pCMV-HA-KCTD10; (3) To immunoprecipitate using anti-PCNA antibody and then detect KCTD10 using anti-KCTD10 antibody in lysates of A549 cells.

KCTD10 OVER-EXPRESSION COULD NOT PROMOTE CELL PROLIFERATION, BUT ITS DOWN-REGULATION COULD INHIBIT CELL PROLIFERATION IN A549 CELLS

The PDIP1 family, including KCTD13, TNFAIP1, possibly plays roles in TNF-α induced DNA replication/repair by interacting with PCNA and some enzymes (e.g., polymerase δ) involved in DNA replication/repair pathways, we speculated whether KCTD10 expression could influence cell proliferation. MTT assay and PCNA expression were used to determine cell proliferation; and real-time PCR assays were used to determine KCTD10 and PCNA expression. Real-time PCR results showed that the expression level of KCTD10 could be up-regulated to 1,000-fold by infection with Ad-KCTD10 and down-regulated to 20% by transfection with siRNA to KCTD10. A549 cells were selected for the assay due to the medium expression of KCTD10 in the cell line. To test the hypothesis, A549 cells were infected with Ad-KCTD10 or Ad-GFP, no promoted cell proliferation or higher PCNA expression was identified in cells infected with Ad-KCTD10 compared with those infected with Ad-GFP (data not shown). While in A549 cells treated with siRNA duplex oligonucleotides to KCTD10, lower PCNA expression and inhibited cell proliferation were observed (Table I). We thought KCTD10 down-regulation could inhibit cell proliferation.

DISCUSSION

In this study, we reported that the distribution of KCTD10 in normal human tissues and tumor cell lines, the interaction of KCTD10 with PCNA, and the impact of KCTD10 up-regulation or down-regulation on cell proliferation.

We found KCTD10 up-regulation could not promote cell proliferation, while its down-regulation could inhibit cell proliferation in A549 cells. KCTD10 protein shares a high degree of amino acid sequence similarity with TNFAIP1 and KCTD13. They are all TNF-α-inducible proteins. KCTD13 and TNFAIP1 are capable of stimulating the activity of DNA polymerase δ [Wolf et al., 1992]. He et al. [2001] found that in the absence of PCNA, GST-KCTD13 had little or no effect on the activity of polymerase δ. However, in the presence of PCNA, GST-KCTD13 stimulated the activity two-threefold. GST had no effect either in the presence or in the absence of PCNA. So PDIP1 might modulate the interaction of PCNA with polymerase δ. In our study, we found only up-regulation of KCTD10 could not promote cell proliferation. In the circumstance, no higher PCNA expression was observed. Being similar with KCTD13, KCTD10 may stimulate the activity of polymerase δ in the presence, but not in the absence, of PCNA. So KCTD10, like KCTD13 and TNFAIP1, may play important roles in DNA synthesis by interacting with PCNA and some enzymes (e.g., polymerase δ) involved in DNA replication/repair pathways.

KCTD10 possesses a BTB/POZ domain, which appears to play diverse roles in mediating interactions among proteins that are involved in transcription regulation, chromatin structures, and cytoskeleton organization. Yoshida et al. [1999] reported that BTB/POZ domain transcription factors regulate transcription as architectural factors. Structural changes induced by binding of architectural factors may directly or indirectly lead to activation of transcription. Some of BTB/POZ domain proteins may also participate in transcription activation by interacting with other factors through BTB/POZ domains [Kobayashi et al., 2000]. The inhibition of cell proliferation induced by down-regulation of KCTD10 may be through the function of BTB/POZ domain.

This expression of *KCTD10* mRNA in human normal tissues is not similar with that in rat tissues [Zhou et al., 2005]. In rat tissues, *KCTD10* was predominately expressed in lung, while lower expression of *KCTD10* was also detected in heart and testis. Only one transcript about 4.2 Kb was found in the expression of *KCTD10* in rat tissues, while two transcripts were detected in these human tissues. In human, a high level of KCTD10 expression was observed in heart, skeletal muscle, and placenta; while in rat that was found in lung. We think that a high level of KCTD10 in human placenta may

TABLE I. KCTD10 Down-Regulation Could Inhibit Cell Proliferation in A549 Cells

	Normalized KCTD10 amount ($2^{-\Delta\Delta C_t}$)	Normalized PCNA amount ($2^{-\Delta\Delta C_t}$)	MTT (mean ± SD)
A549 cells	1.00[a]	1.00[a]	1.44 ± 0.17
A549 cells transfected with control siRNA	1.09	1.07	1.46 ± 0.17
A549 cells transfected with KCTD10-RNAi#1	0.21	0.19	1.12 ± 0.20**
A549 cells transfected with KCTD10-RNAi#2	0.17	0.14	1.11 ± 0.19**

Cells transfected with siRNA at 72 h after plating were collected for the assays. In the real-time PCR assays, total RNA was isolated from A549 cells, A549 cells transfected with control siRNA (50 nM), or A549 cells transfected with KCTD10-RNAi (50 nM), and reverse-transcribed to cDNA. Aliquots of cDNA were used as a template for real-time PCR reactions containing primers for KCTD10, or PCNA, or for β-Actin (as an internal reference). Four replicates of each reaction were done in real-timePCR. In MTT assays, the cells (5,000 cells/well) were cultured in 96-well plates with 100 μl of media/well. MTT (20 μl, 5 mg/ml) solution was added to each well, and incubated at 37°C for additional 4 h in a CO_2 incubator. Each experiment was done in 12 wells and repeated thrice. The MTT value is a mean of 12 replicated wells from one representative experiment of the three.

[a]The relative amount was normalized to KCT10, PCNA amount in A549 cells respectively.

**$P < 0.001$.

be due to a high DNA replication and cell proliferation. Zhou et al. thought that the high level of KCTD10 expression in rat lung may suggest a role of this gene in lung development. Why KCTD10 is highly expressed in human heart and skeletal muscle? Is KCTD10 associated with human heart and skeletal muscle development? In the future, many studies are needed to elucidate the potential functions of KCTD10.

In a conclusion, our results showed that KCTD10 may interact with PCNA, and be involved in cell proliferation. Further studies are needed to elucidate the potential signal pathway and mechanisms.

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REFERENCES

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. 1998. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 95:2509–2514.

He H, Tan CK, Downey KM, So AG. 2001. A tumor necrosis factor alpha- and interleukin 6-inducible protein that interacts with the small subunit of DNA polymerase delta and proliferating cell nuclear antigen. Proc Natl Acad Sci USA 98:11979–11984.

Kobayashi A, Yamagiwa H, Hoshino H, Muto A, Sato K, Morita M, Hayashi N, Yamamoto M, Igarashi K. 2000. A combinatorial code for gene expression generated by transcription factor Bach2 and MAZR (MAZ-related factor) through the BTB/POZ domain. Mol Cell Biol 20:1733–1746.

Maga G, Hubscher U. 2003. Proliferating cell nuclear antigen (PCNA): A dancer with many partners. J Cell Sci 116:3051–3060.

Wang Y, Zhen Y, Shi Y, Chen J, Zhang C, Wang X, Yang X, Zheng Y, Liu Y, Hui R. 2005. Vitamin k epoxide reductase: A protein involved in angiogenesis. Mol Cancer Res 3:317–323.

Wolf FW, Marks RM, Sarma V, Byers MG, Katz RW, Shows TB, Dixit VM. 1992. Characterization of a novel tumor necrosis factor-alpha-induced endothelial primary response gene. J Biol Chem 267:1317–1326.

Yoshida C, Tokumasu F, Hohmura KI, Bungert J, Hayashi N, Nagasawa T, Engel JD, Yamamoto M, Takeyasu K, Igarashi K. 1999. Long-range interaction of cis-DNA elements mediated by architectural transcription factor Bach1. Genes Cells 4:643–655.

Zhou J, Ren K, Liu X, Xiong X, Hu X, Zhang J. 2005. A novel PDIP1-related protein, KCTD10, that interacts with proliferating cell nuclear antigen and DNA polymerase delta. Biochim Biophys Acta 1729:200–203.