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Downregulation of *POLD4* in Calu6 cells results in G1-S blockage through suppression of the Akt-Skp2-p27 pathway

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ABSTRACT

Previously, we have shown that downregulation of *POLD4* in lung cancer cells delays progression through the G1-S cell cycle transition and leads to increased genomic instability. To date however, detailed molecular mechanisms have not been elucidated to explain how this occurs. In the present study, we found that reduction in *POLD4* by siRNA knockdown promoted downregulation of both p-Akt Ser473 and Skp2 as well as upregulation of p27. Furthermore, these protein expression levels were rescued when siRNA-resistant *POLD4* was ectopically expressed in the knockdown cells. These data suggest that the *POLD4* downregulation is associated with impaired Akt-Skp2-p27 pathway in lung cancer.

To date, despite the premise that deficits in DNA replication machinery account for multiple mutations in cancer,[1] and findings showing that laboratory-born animals and strains defective in DNA replication machineries exhibit genomic instability,[2,3] core DNA replication proteins have rarely been identified as a frequent target for alterations aberrant DNA replication in cancer.[4]

In our previous analysis of 158 lung cancer samples and 5 control samples developed by pooling tissue from 10 normal lungs, we found increased RNA expression of genes related to cell cycle and checkpoint control in the cancer samples. Among them, however, mRNA levels of *POLD4*, a subunit of DNA polymerase δ (pol δ) that participates in DNA replication and DNA repair,[5,6] were reduced in small cell lung cancer (SCLC) samples.[7,8] Furthermore, a fraction of non-small cell lung cancer patients also exhibited low levels of *POLD4* that were comparable with those found in the SCLC samples and were associated with poor prognosis. Interestingly, *POLD4* downregulation caused delays in G1-S transition, which are associated with genomic instability, a major driving force in carcinogenesis. We reconstituted an in vitro DNA replication system including PCNA, RPA, and RFC, POLD4-deficient pol δ, and showed that DNA replication activity was lower than that with intact pol δ. Furthermore, *POLD4*-low cells are associated with checkpoint activation, suggesting that *POLD4* downregulation caused impaired DNA replication/repair in cells and activate checkpoint responses.[7,8]

Recent reports suggest that the S-phase kinase-associated protein 2 (Skp2), a member of the F-box protein family, is important for regulating the G1-S transition in the cell cycle because it is required for ubiquitin-dependent degradation of the Cdk inhibitor p27.[9] Skp2 is overexpressed in various human cancers, including those of the lung, breast, gastrointestinal tract, and prostate. Skp2 expression decreases following DNA damage and induces cell cycle arrest.[10,11] Therefore, in the present study, we examined the relationship between *POLD4* downregulation and Skp2-p27 pathway in detail.

Consistent with our previous results, siPOLD4 treatment induced a significant reduction in cell proliferation (Fig. 1A), partial cell cycle blockage at the G1-S transition (Fig. 1B), and induction of p21 and p27 (Fig. 1C). Importantly, the sub-G1 population did not change with siPOLD4 treatment (Fig. 1B) and there was no difference in the number of apoptotic cells as observed by comparing TUNEL staining of the treatment populations (data not shown). Therefore, cell proliferation may be impaired by slower DNA replication and not by changes in the level of apoptosis. Cell cycle delay may be attributed to p27 activity, since knocking down p27 but not p21 could rescue siPOLD4-induced blockage of G1-S in Calu6 cells.[8] In order to determine the mechanism by which p27 is induced, we

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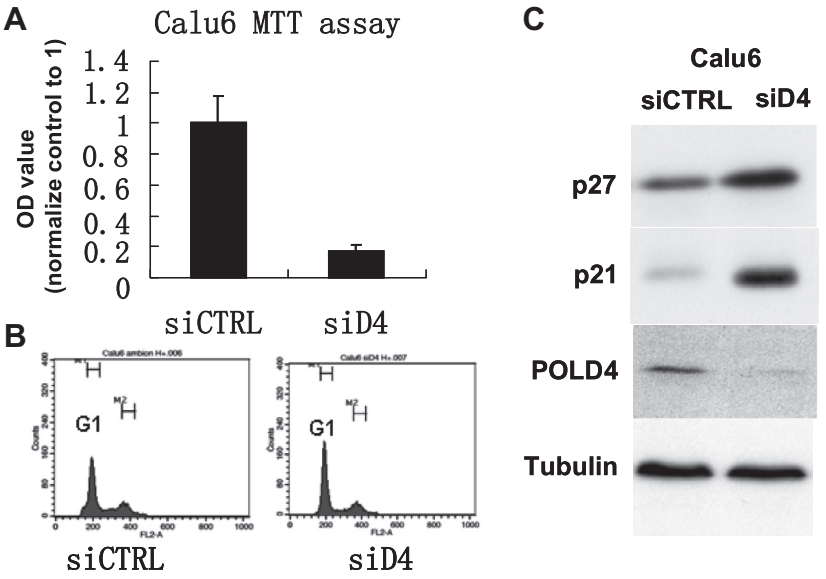

Figure 1. Downregulation of *POLD4* blocks G1-S transition in Calu-6 cells. (A) Calu6 cells were treated with 50 nmol/L of either siCTRL or siPOLD4 for 48 h. Subsequent proliferation abilities were measured using the MTT assay and analyzed by normalizing siCTRL-treated cells to an OD of 1. (B) Calu6 cells were treated with either siCTRL or siPOLD4 and then stained overnight at 4 °C with propidium iodide solution. FACS was used to determine the proportion of cells in G1 for each treatment. (C) Western blot analysis of Calu6 cells that were treated for 48 h with either siCTRL or siPOLD4 (siD4). Levels of p21, p27, and POLD4 were normalized to that of α-tubulin.

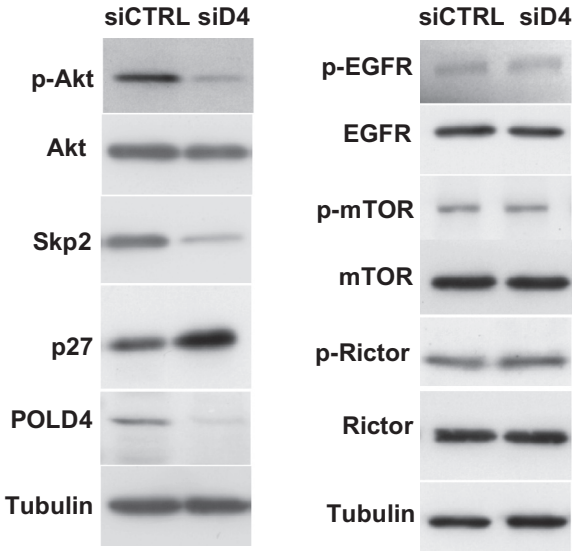

Figure 2. Downregulation of *POLD4* results in G1-S blockage through suppression of the Akt-Skp2-p27 pathway in Calu6 cells. Calu-6 cells were treated with siCTRL and siPOLD4 (siD4) for 48 h followed by western blot analysis of p-Akt (Ser473), Akt, p27, and Skp2, p-EGFR(Thy1068), EGFR, p-mTOR(Ser2448), mTOR, p-Rictor (Thr1135), Rictor, normalized to α-tubulin.

measured Skp2 expression levels. As shown in Fig. 2. Skp2 was unambiguously decreased after cells were given siPOLD4.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is a fundamental signaling pathway that mediates several cellular processes including proliferation, growth, survival, and motility.[12] DNA double stranded breaks may activate PI3K family kinases ATM, ATR, and/or DNA-PK, and activate Akt through the phosphorylation of Ser473.[13,14] Because siPOLD4 treatment activates ATM and ATR,[8] we expected that *POLD4* knockdown would result in reduced levels of p-Akt Ser473. Interestingly, we found that *POLD4* knockdown was associated with a reduced p-Akt Ser473 level (Fig. 2), while the upstream molecules, either in phosphorylation and total protein levels, were similar between CTRL and siPOLD4 treatments (Fig. 2). Ectopic expression of *POLD4* containing a siRNA-resistant silent mutation[8] rescued the Akt phosphorylation, Skp2 and p27 levels (Fig. 3A and B), indicating that alteration in these molecular signaling are indeed downstream events of POLD4.

Accumulating evidence suggests that Skp2 expression is positively regulated by the PI3K pathway.[15–17] Inhibition of the PI3K signaling pathway induces G0/G1 cell cycle arrest, upregulation of p27, and downregulation of Skp2 protein expression.[18] Therefore, one possible explanation for our results is the existence of a pathway in which high levels of POLD4 leads to Akt activation that then upregulates Skp2 and promotes cell cycle progression. In support with this hypothesis, POLD4 expression levels were near constant throughout the cell cycle progression (Fig. 4), and were not stimulated by EGF treatment, suggesting that Akt Ser473 phosphorylation status may be a bona fide outcome of POLD4 downregulation, although further experiments are needed to prove the relationships between these two molecules. It is also yet to be studied whether POLD4-Akt signaling is lung cancer specific or not (Fig. 5).

Because Akt is a survival kinase, we further asked whether reduced p-Akt Ser473 affects the drug sensitivity of Calu6 cells. As shown in Fig. 6, siPOLD4 increased the CDDP-sensitivity. CDDP-damage repair requires pol δ, thus limitation in pol δ activity may have affected the survival efficiency (Fig. 6). In contrast, sensitivities of 5-FU, a thymidylate synthase inhibitor, or VP16, a DNA topoisomerase II inhibitor, were not significantly changed by siPOLD4. These data suggest that low p-Akt Ser473 does not generally correlate with the drug sensitivities and that POLD4 down regulation and reduced p-Akt Ser473 levels may associate with the sensitivity to pol δ-related DNA repair processes. Further study to elucidate the relationships between POLD4 and Akt (Fig. 7) will help develop a novel therapeutic strategy, which targets these particular pathways in POLD4-low lung cancer.