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14. ABSTRACT

The major goals of this project were to generate p12 knockout cell lines and use these, as well as the wild-type cell lines, to demonstrate that loss of p12 leads to heightened sensitivity to chemotherapeutic agents and PARP inhibitors. In parallel, the sensitivities of small cell lung cancer cell (SCLC) lines that lack p12 would be studied. The goals of the study were to generate p12 knockout cells, and to analyze their sensitivities to the PARP inhibitors, The results showed that depletion of p12 in cultured cells leads to a defect in homologous recombination, and also increased sensitivity to the PARP inhibitors Olaparib, Niraparib, Rucaparib and Talazoparib. SCLC cells exhibit high sensitivity to PARP inhibitors, equal or greater than that of the p12 knockout cells. The significance of these findings is that they identify p12 expression as being required for homologous recombination. The findings also provide a basis for the prediction that cancers low in p12 expression should exhibit enhanced sensitivity to PARP inhibitors. The findings also provide a greater understanding of the specific roles of the two forms of DNA polymerase delta in human cells. The p12 is a subunit of the heteroetrameric Pol δ4 form, but is absent in the trimeric Pol δ3 form. Our studies support the concept that Pol δ3 is required for cellular DNA replication, while Pol δ4 is dispensible but is required for homologous recombination.

15. SUBJECT TERMS

Homologous recombination; PARP inhibitors; DNA Polymerase δ□□□small cell lung cancer; p12 subunit; Pol δ□, Pol δ□□

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TABLE OF CONTENTS

1. INTRODUCTION:

The subject of this proposal is to develop a novel concept, that loss of the p12 subunit of the DNA polymerase δ complex leads to loss of Pol δ functions in homologous recombination (HR). This generation of this HR defect leads to the further predicts that cells lacking p12 will exhibit high sensitivity to PARP inhibitors. This is relevant to SCLC (small cell lung cancer) tumors and SCLC (small cell lung cancer)cell lines that exhibit very low expression levels of p12. In general, this idea extends the concept that cells that have lost p12 expression should exhibit an HR defect and therefore be sensitized to chemotherapeutic agents that lead to DNA damage requiring HR repair, that including the PARP inhibitors. These PARP inhibitors have become of very significant clinical importance in the treatment of ovarian cancers with mutations in breast cancer type 1 and 2 susceptibility genes (*BRCA1* and/or *BRCA2*). The purpose and scope of this project was to generate p12 knockout (p12KO) cell lines and use these, as well as the wild-type cell lines, to demonstrate that loss of p12 leads to heightened sensitivity to chemotherapeutic agents and PARP inhibitors. In parallel the sensitivities of SCLC cell lines will be compared to assess and compare their sensitivities to the PARP inhibitors, which would be predicted to be comparable to that of the p12KO cells. These The studies will provide key findings that will highlight the potential for the use of PARP inhibitors in SCLC and stimulate research into the clinical application of PARP inhibitors guided by the use of p12 as a marker to identify potentially responsive patients.

2. KEYWORDS: Homologous recombination; PARP inhibitors; DNA Polymerase δ; small cell lung cancer; p12 subunit; Pol δ4, Pol δ3.

3. ACCOMPLISHMENTS:

Major goals of the project.

The major goals of this project were to generate p12 knockout cell lines and use these, as well as the wild-type cell lines, to demonstrate that loss of p12 leads to heightened sensitivity to chemotherapeutic agents and PARP inhibitors. In parallel, the sensitivities of SLCL cell lines will be compared to assess and compare their sensitivities to the PARP inhibitors, which would be predicted to be comparable to that of the p12 KO cells.

Task 1 as listed in the SOW was to generate p12 knockout cells in cultured cells, and then to establish the loss of p12 by western blotting and immunofluorescence, followed by analysis of their sensitivities to PARP inhibitors.

Task 2 was to perform a similar analysis of the sensitivities of SCLC cell lines to PARP inhibitors. This was performed in tandem with analysis of the sensitivities of wt and p12KO cells, and of SCLC cell lines, to PARP inhibitors. Thus, A549 wt, A549-p12KO, H1299 wt and H1299-p12KO cell lines as well as the SCLC cell lines H446, DMS114, H69AR and H1688 were analyzed. The PARP inhibitors tested were Olaparib, Niraparib, Rucaparib and Talazoparib. Once preliminary experiments had been performed, they were repeated in triplicate so that statistical analysis could be obtained.

All objectives, including preparation and submission for publication, were achieved by the end of the 12 months. These objectives were all met within the time frames of the SOW.

Major Findings and Significant Results.

The major findings and results are presented briefly here, as the work was published [1]. A copy of the paper is attached as Appendix I. Our findings are also included in an invited review [2] as Appendix II in which our studies are discussed.

We accomplished the CRISPR knockout of the *POLD4* gene that encodes p12 in A549 cells, and in H1299 cells. The latter lack p53 function, as do many cancer cells. As will be noted in the data shown below, p53 status did not affect the outcome of the experiments. We had expected that there might be difficulties in maintaining the p12KO cell lines, as the knockout of p12 leaves the cells with only the trimeric form of Pol δ, which we have called Pol δ3, so that the four subunit form of Pol δ (Pol δ4) cannot be formed, i.e., the p12 knockout cells would be null for Pol δ4. We found that p12KO cells could be generated by the CRISPR/Cas9 method with ease, and this facilitated our rate of progress significantly. We were able to assess the effects of this on cell viability and behavior. To our surprise, we found that at least within the first 20-30 cell divisions, these cells apparently grew normally and were viable. This established that the Pol δ3 form of Pol δ was sufficient to sustain cellular DNA replication, consistent with its proposed role in Okazaki fragment processing. In addition, we found that CRISPR knockout of *POLD4* was a facile process, and that isolates of knockout cells could be readily obtained in a number of different cell types. These findings demonstrated that Pol δ4 was NOT required for cell viability, and did not grossly affect cell growth. These established that Pol δ3 was sufficient for DNA replication, firmly placing it as the form that is involved in DNA replication. Together with our earlier studies (see Appendix II), this allowed assignment of Pol δ3 as the key polymerase involved in Okazaki fragment processing, i.e.. as the lagging strand polymerase.

The analysis of the sensitivities of the wt A549 and H1299 cells and their respective p12KO cells are shown in Fig. 1.

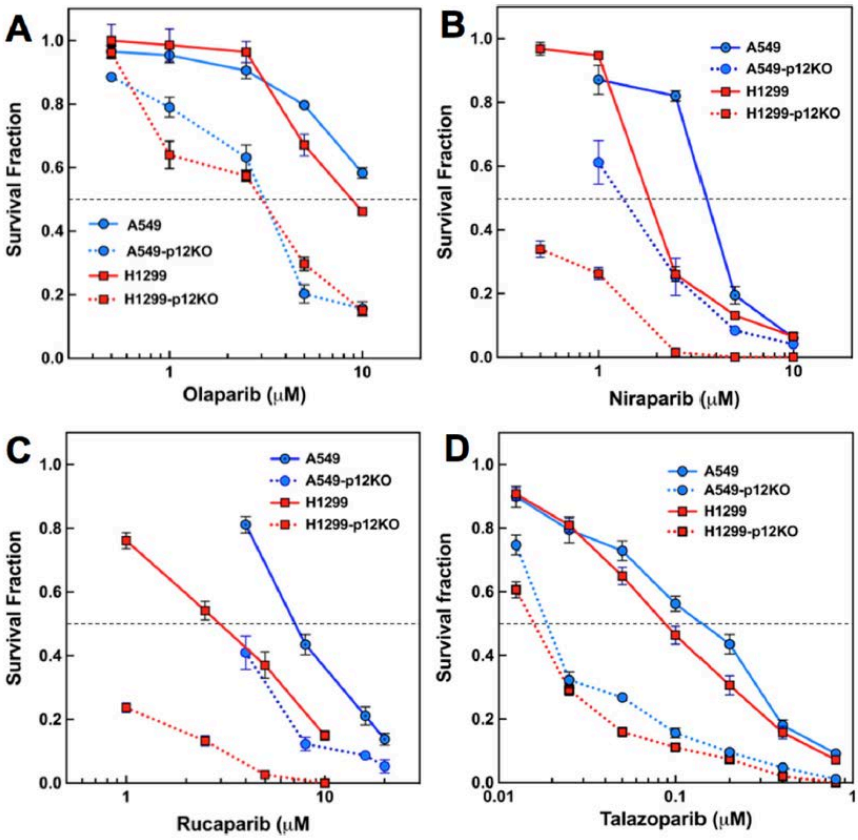

Fig. 1. Cytotoxicity of PARP inhibitors toward wt and p12KO cells. (A–D) Cytotoxicity of the PARP inhibitors Olaparib, Niraparib, Rucaparib and Talazoparib respectively, as determined by clonogenic survival assays. Data are shown as survival fraction (mean ± SD, n=3) and plotted against the log of the drug concentrations. Taken from Fig. 4 (Appendix I)

These results show that in the A549 and H1299 cells, ablation of p12 (and loss of the Pol δ4 form of Pol δ) leads to significant increase in sensitivity to all four of the PARP inhibitors tested. These results support the original concept that was the basis of the proposal. Since PARP sensitivity has been associated with the loss of HR capability as established by their use in treatment of BRACA1/2 associated cancers, they lead to the conclusion that loss of p12 (and consequently Pol δ4) leads of a defect in HR. Since Pol δ activity has been firmly established to be involved in a key step in HR, that of D-loop extension, our results show that Pol δ4 is the form of Pol δ that is involved in HR, and that Pol δ3 is unable to perform the D-loop extension step.

` Next, we tested the SCLC cell lines, after first confirming that these had negligible expression of p12 protein. In this experiment we show data for just Olaparib and Talaroparib as well as analysis for the A549 and H1299 cell lines (Fig. 2).

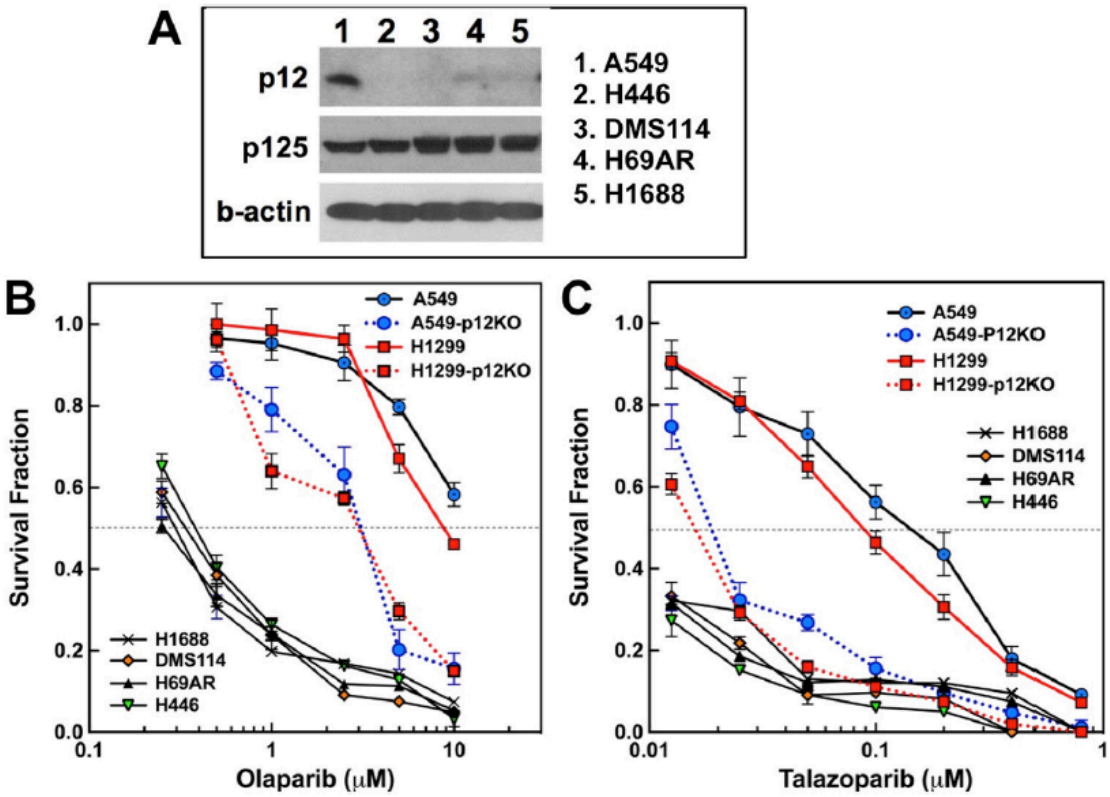

Fig. 2. Cytotoxicity of Olaparib and Talazoparib toward Small Cell Lung Cancer cell lines. (A) Western blots for p12 and p125 levelsin A549 and SCLC cell lines H446, DMS114, H69AR and H1688. (B, C) Cytotoxicity of Olaparib and Talazoparib, respectively. Taken from Fig. 6 (Appendix I).

These findings support the idea that SCLC cell lines, which lack expression of p12, are hypersensitive to PARP inhibitors. Taken together, these results support our proposal that p12 loss leads to sensitivity to PARP inhibitors. These findings are significant, and support the additional proposal that these p12KO cells are HR defective. This seems a logical deduction of our results.

In order to submit these important findings for publication, we anticipated that the reviewers might ask for more direct evidence for an involvement of p12 (and thus of Pol δ4) in homologous recombination (HR). Therefore, we performed experiments in which HR repair was studied using a plasmid based assay in H1299 and H1299-p12KO cells, as well as in H1299 –p12KO cells in which p12 was ectopically expressed as Flag-p12 (Fig. 3). The results show that loss of p12 leads to

reduction in HR activity, which can be restored by expression of Flag-p12. These results confirm that the presence or absence of p12 dictates HR capability (Fig. 3).

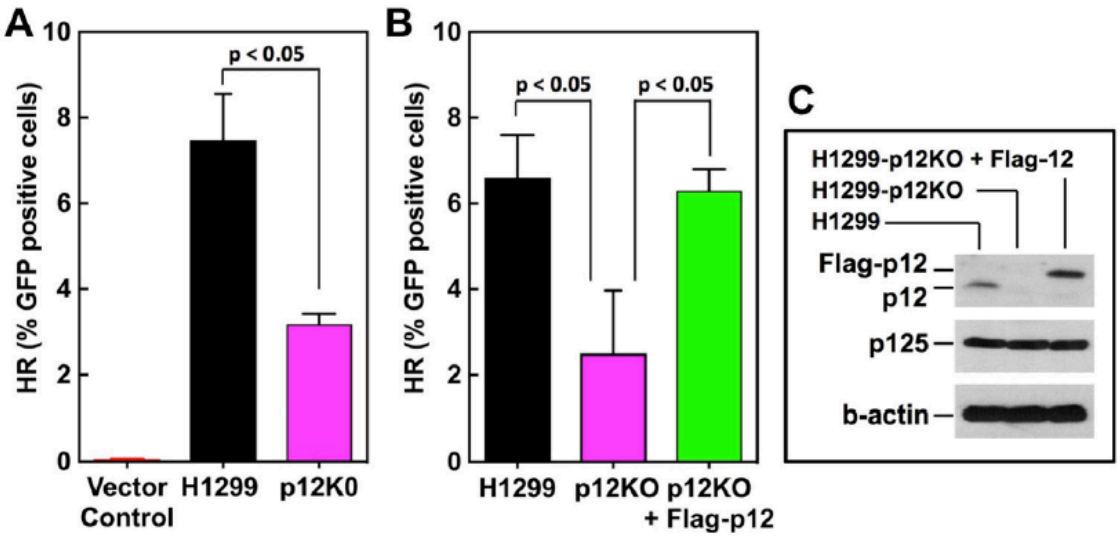

Fig. 3. GFP reporter assays for HR in p12KO cells. Cells were analyzed for HR activity using the DR-GFP based reporter assay. Data show the HR activity expressed as % GFP positive cells (n=3). Statistical analysis was performed using Graphpad Prism software. (A) H1299 and H1299-p12KO cells. (B) H1299, H1299-p12KO and H1299-p12KO cells into which Flag-tagged p12 was stably expressed. (C) H1299, H1299-p12KO and H1299-p12KO+Flag-p12 cells were Western blotted for p12, Flag-p12 and the p125 subunit of Pol δ. B-actin was used as a loading control. Adapted from Fig. 2 (Appendix I).

For further confirmation that p12 loss was associated with PARP inhibitor sensitivity, the same three cell lines were analyzed for sensitivity to the PARP inhibitors Olaparib and Talazoparib (Fig. 4). Here it is observed that ectopic expression of Flag-p12 leads to resistance to the PARP inhibitors.

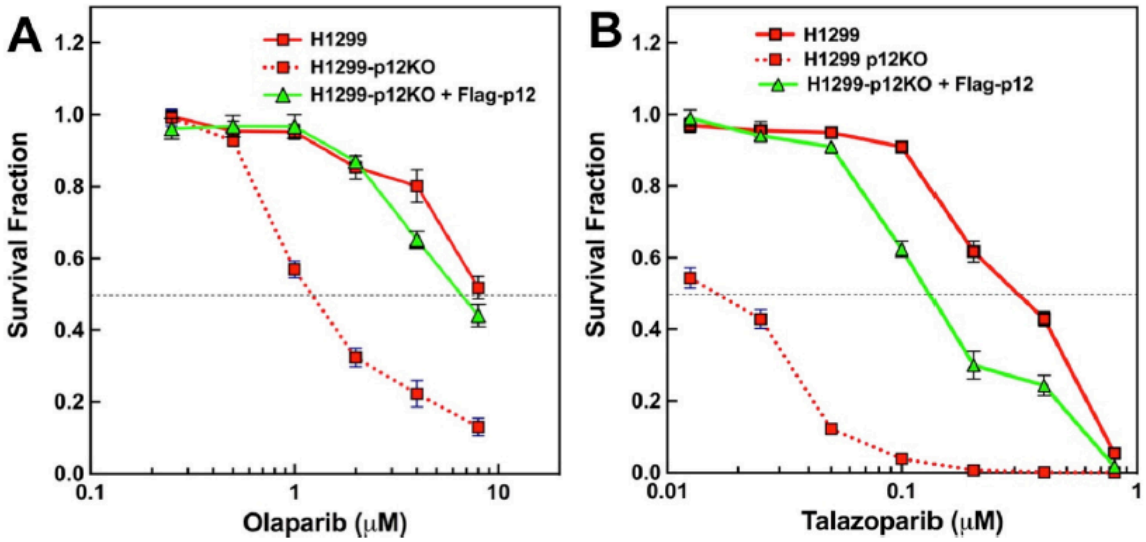

Fig. 4. Cytoxicity of Olaparib and Talazoparib toward H1299, H1299-p12KO and H1299-p12KO+Flag12 cells. Data from Fig. 5 (Appendix I).

What opportunities for training and professional development has the project provided?

"Nothing to Report."

How were the results disseminated to communities of interest?

In order to disseminate the findings to the broader scientific community we have also included our findings in an invited review in a special issue of DNA Repair which is appended as Appendix II.

Next reporting period to accomplish the goals?

"Nothing to Report." This is a final report.

4. IMPACT:

The findings that were made are novel, and are of major significance from a number of points of view. These are discussed in Appendix I and II and are briefly summarized below.

Importance to understanding the role of Pol δ in DNA replication and Repair. These studies show that the Pol δ3 form of Pol δ is sufficient to sustain DNA replication, and therefore support its role as the enzyme that is involved in DNA repair. On the other hand, these findings identify Pol δ4 as the form that is nonessential for viability but is essential for HR DNA repair. Thus, our studies reveal that the two forms of Pol δ serve different functions in DNA replication and repair (see Fig. 5, also Appendices I and II). Pol δ3 is sufficient for sustaining DNA replication, and Pol δ4 is dispensable; Pol δ4 is required for HR repair, and has a function that cannot be provided by Pol δ3. This new finding provides an answer to the question of the specific roles of the two forms of Pol δ3 that are present in human cells.

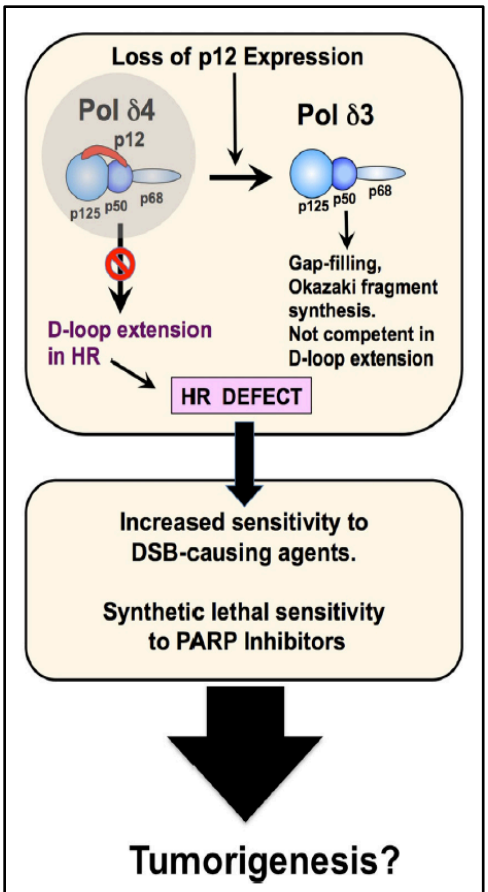

Fig. 5. Consequences of the loss of expression of p12: generation of a HR deficient phenotype. Adapted from Fig. 7 (Appendix I).

Importance to understanding sensitivity to chemotherapeutic drugs that target HR defective tumors. These studies identify a potential new cellular route to generation of HR defective cells, viz, by the loss of expression of p12, either by mutation, gene loss, or epigenetic regulation. Thus one might predict that tumors which lack p12 expression will be more sensitive to PARP inhibitors (Fig. 5).

Importance to understanding the behavior of SCLC tumors, which are initially highly sensitive to chemotherapy but rapidly acquire resistance. Our findings lead to the hypothesis that SCLC cancers which lack p12 expression are initially sensitive to chemotherapy, because of epigenetic changes which repress the *POLD4* gene. This leads to an HR defect. On exposure to chemotherapy, our findings point to the hypothesis that this leads to further epigenetic changes would result in the re-

activation of the *POLD4* gene expression. This leads to reacquisition of HR capability, accounting for the acquired resistance. Future research along these lines could lead to the exploitation of drugs that modulate epigenetic events in combined therapy

What was the impact on other disciplines?

Nothing to report, although we believe that as our findings are disseminated in the literature that the ideas we have developed will be tested in a more translational setting.

What was the impact on technology transfer?

“Nothing to report”

What was the impact on society beyond science and technology?

“Nothing to report”

5. CHANGES/PROBLEMS:

"Nothing to Report,"

6. PRODUCTS: Publications, conference papers, and presentations

Two publications to report.

S. Zhang, H.H. Chao, X. Wang, Z. Zhang, E.Y.C. Lee, M.Y.W.T. Lee, **Loss of the p12 subunit of DNA polymerase delta leads to a defect in HR and sensitization to PARP inhibito**rs. DNA Repair (Amst), 73 (2019) 64-70. DOI: 10.1016/j.dnarep.2018.11.003. (Federal support acknowledged.)

M.Y.W.T. Lee, S. Zhang, X. Wang, H.H. Chao, H. Zhao, Z. Darzynkiewicz, Z. Zhang, E.Y.C. Lee, **Two forms of human DNA polymerase delta: Who does what and why**. DNA Repair (Amst), 81 (Special Issue “Cutting-edge Perspectives in Genomic Maintenance VI” Editors Philip C. Hanawalt, Samuel H. Wilson.) (2019) Article 102656. DOI: 10.1016/j.dnarep.2019.102656. (Federal support acknowledged.)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS What individuals have worked on the project?

Participicants

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Project role	P.I.
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Nearest Person month worked	9
Contribution to project	Dr. Zhang was responsible for the overall conduct of the work and designed and performed most of the experiments
Funding Support	

Name	Xiaoxiao Wang, Ph.D.
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Contribution to project	Dr Wang performed HR assays
Funding Support	

Name	Marietta Y.W.T. Lee
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Researcher ID: ORCID	0000-0001-8696-6962
Nearest Person month worked	2
Contribution to project	Dr. Lee provided collaborative support in design and in the preparation of the manuscripts, as well as lab. reagents
Funding Support	NIEHS

Funding Support: (Complete only if the funding support is provided from other than this award).

NIEHS ES014737 to MYWTL and ZZ

What other organizations were involved as partners?

None

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES:

Appendix I

S. Zhang, H.H. Chao, X. Wang, Z. Zhang, E.Y.C. Lee, M.Y.W.T. Lee, **Loss of the p12 subunit of DNA polymerase delta leads to a defect in HR and sensitization to PARP inhibitors**. DNA Repair (Amst), 73 (2019) 64-70. DOI: 10.1016/j.dnarep.2018.11.003. (Federal support acknowledged.)

Appendix II

M.Y.W.T. Lee, S. Zhang, X. Wang, H.H. Chao, H. Zhao, Z. Darzynkiewicz, Z. Zhang, E.Y.C. Lee, **Two forms of human DNA polymerase delta: Who does what and why,** DNA Repair (Amst), 81 (Special Issue “Cutting-edge Perspectives in Genomic Maintenance VI” Editors Philip C. Hanawalt, Samuel H. Wilson.) (2019) Article 102656. DOI: 10.1016/j.dnarep.2019.102656. (Federal support acknowledged.)