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BHC80 is critical in suppression of Snail-LSD1 interaction and breast cancer metastasis

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14. ABSTRACT

As mentioned in the previous annual report, in addition to BHC80, we identified PARP1 as a component of Snail/LSD1. According to our preliminary data, PARP1 is critical in regulating the protein stability of Snail and LSD1, as well as Snail/LSD1 binding to the target gene promoter. In the current report, we further showed that doxorubicin treatment can enhance Snail-LSD1 interaction in a PARP1-dependent manner. In addition, Snail contains a potential pADPr-binding motif and is subject to poly(ADP-ribosyl)ation. Our data also suggested that the enzymatic activity of PARP1 is required for Snail-LSD1 binding to the PTEN promoter; upon binding, LSD1 demethylates histone H3 lysine 4 at the promoter region in favor of PTEN transcription suppression and the downstream Akt phosphorylation. Furthermore, we found that PARP1 inhibitor AZD2281 can enhance the killing effect of doxorubicin on selective breast and colon cancer cells. Together, we proposed a new mechanism adopted by cancer cells to defend themselves against DNA damage-induced apoptosis, which gives us new implications on the design of efficient cancer treatment strategies. We will continue to characterize other Snail-interacting proteins to get a clearer picture of Snail-mediated cancer progression.

15. SUBJECT TERMS
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Introduction

Cancer cells distinguish themselves from their normal siblings with the capability of evading apoptosis and presenting uncontrolled cell division, along with acquiring malignant characteristics such as invasion and metastasis. The most common chemotherapeutic drugs function by introducing DNA damage to impair cell division. Since most cancer cells outgrow their normal counterparts, the property of rapid DNA-replication makes them more vulnerable to the DNA lesions. While the conventional DNA damage-inducing drugs are used in the treatment of a wild range of cancers, unfortunately they are not smart in pinpointing cancer cells; rather they also attack normal cells with rapid dividing property and can cause a series of unamiable cytotoxic effects. On the other side, cancer cells would develop strategies to defense themselves against these drugs. For example, it has been documented that upon doxorubicin treatment, Akt became phosphorylated and activated, which then triggers a series of cellular events to eventually confer cancer cells resistant to drug-induced apoptosis. Accordingly, combinations of doxorubicin and Akt inhibitors appear as promising treatment strategies.

Shaking things up on a broader scale, appropriate drug combinations not only allow for lower dosage of every single drug in order to reduce the cytotoxic effect and discourage the development of drug resistance, but also target cancer cells with more efficiency and selectivity. Typically, rational combinations of DNA damage-inducing drugs and DNA repair inhibitors tends to be an ideal treatment option, given that several types of cancers are defective in DNA damage repair pathways. For example, many breast cancers have defects in the BRCA1/BRCA2 homologous recombination (HR) repair pathway and rely on poly(ADP-ribose) polymerases (PARP) to repair DNA lesions; these cancer cells are hypothesized to be highly sensitive to PARP inhibitors under this cellular stress. Indeed, PARP inhibitors have shown more toxicity in cancer cell lines as well as human tumors with BRCA1/BRCA2 deficiency. Currently different PARP inhibitors combined with the DNA alkylating agent Temozolomide are under investigation in several clinical trials.

Due to the variance in cell type and tumor stage, as well as the complexity of environmental context, among different cancer cells there is huge discrepancy in regard to the sensitivity to a specific drug. The development of efficient treatment strategies would heavily rely on the understanding of the mechanisms of signal transduction in response to DNA damage. In regard to the transcription factor Snail, it not only serves as a master regulator of the epithelial-mesenchymal transition (EMT), but also participates in many other cellular events, including the mediation of cell cycle and survival. The fact that Snail expression confers drug resistancy on cancer cells indicates that Snail can function as a survival factor. Recently we performed a sequential protein purification-mass spectrometry coupled analysis and identified Snail-interacting proteins, among which are Lysine Specific Demethylase 1 (LSD1) and PARP1 (Figure 1A). While PARP1 is well known as a key factor in DNA repair pathways, recent studies have also demonstrated that LSD1 can either render tumor cells resistant to DNA damage or reversely prompt cells to undergo apoptosis in different biological settings, indicating that LSD1 plays a role in cell survival.

Body

In the renewed Statement of Work (SOW) we focus on the regulative role of PARP1 in Snail/LSD1 complex. In the past year, we have substantially completed the studies as proposed in the SOW. In the following we list the renewed SOW and our accomplishments:

SOW – Study 1: How does PARP1 potentially regulate Snail/LSD1 complex? (month 13-18)

1a To confirm the physical interaction of PARP1 and Snail (month 13)

Accomplishments:

We performed co-immunoprecipitation experiments using HEK293 cells over-expressing Snail-HA and Flag-PARP1, as well as breast cancer cell line MDA-MB-157 and colon cancer cell line HCT116. As shown in Figure 1B and 1C, Snail and PARP1 proteins showed relatively modest interaction in all of the three cell lines. Interestingly, the protein interaction was significantly enhanced when the cells were treated with doxorubicin, indicating that upon activation, PARP1 becomes tightly associated with Snail.

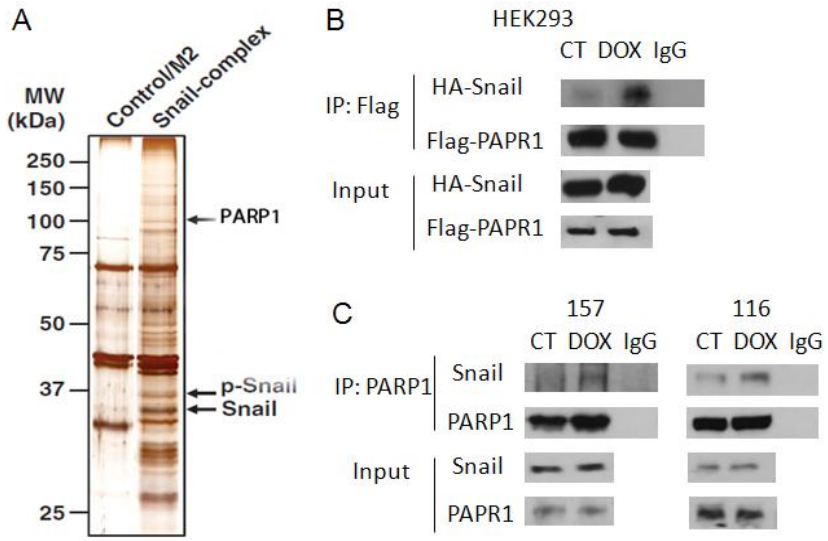

Figure 1 *Doxorubicin enhances PAPR1-Snail interaction.*

(A) The Snail complex was isolated from the stable HEK293 cells overexpressing dual-tagged Snail (HEK293-SN) by two-step immunopurification. The complex were separated on SDS–PAGE and visualized by silver staining. LSD1 and PARP1 were identified by mass spectrometry. (B) Flag-tagged PARP1 and HA-tagged Snail were co-expressed in HEK293 cells. After immunoprecipitation of PARP1, bound Snail was examined by western blotting. 1 μM of doxorubicin (DOX) was treated 6 hours before harvesting cells. (C) Endogenous PARP1 was immunoprecipitated from MDA-MB157 and HCT116 cells and bound endogenous Snail was examined by western blotting. The same doxorubicin treatment condition was used.

1b Can PARP1 mediate Snail-LSD1 interaction? (month 14-15)

Accomplishments:

In HEK293 cells overexpressing Snail and LSD1, doxorubicin treatment significantly enhanced Snail-LSD1 binding, and similar results could be obtained by co-expressing PARP1 in the cell (Figure 2A). In MDA-MB-157 and HCT116 cells, while doxorubicin consistently had positive effect, either PARP1 knockdown or treatment of PARP1 inhibitor AZD2281 significantly reduced Snail-LSD1 affinity (Figure 2B). These results indicated that PARP1 promotes the formation of the Snail-LSD1 complex.

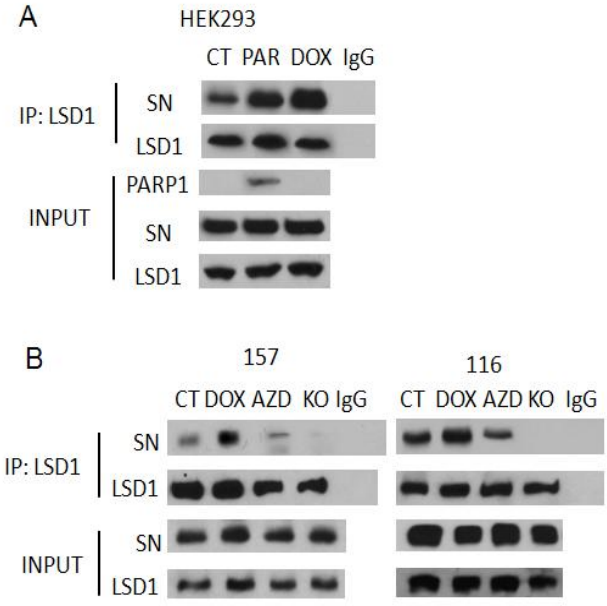

Figure 2 *PAPR1 positively regulates Snail-LSD1 interaction.*

(A) Flag-tagged LSD1 and HA-tagged Snail were co-expressed in HEK293 cells. After immunoprecipitation of LSD1, bound Snail was examined by western blotting. For comparison, cells were either co-expressed with Flag-tagged PAPR1 (lane 2) or treated with 1 μM of doxorubicin 6 hours before harvesting cells (lane 3). (B) Endogenous LSD1 was immunoprecipitated from MDA-MB157 and HCT116 cells and bound endogenous Snail was examined by western blotting. For comparison, cells were treated with doxorubicin (1 μM for 6 hours, lane 2), AZD2281 (2μM for 24 hours, lane 3), or transfected with PARP1 siRNA (lane 4).

1c To identify the specific mechanism of how PAPR1 mediate Snail-LSD1 interaction (month 15-18)

Accomplishments:

Through sequence alignment we identified three highly conserved residues Arg151, Lys152 and Ala153 of Snail protein to be in concert with the corresponding residues of the previously established pADPr binding motif, in which the positively charged lysine and arginine are strictly followed by either one of alanine, isoleucine, leucine and valine (Figure 3A). While the sequence surrounding Arg151, Lys152 and Ala153 does not exactly follow the rule for the composition of pADPr-binding motif as refined by Gagne and colleagues, the presence of the most essential residues (Arg151, Lys152) indicates the potential pADPr docking site on Snail protein. Considering that PARP1 became activated and tightly bound to Snail upon DNA damage, we went on to investigate whether Snail can interact with PARP1 through its potential pADPr-binding motif. First we generated Snail point mutant R151A/K152A and examined its interaction with PARP1. As shown in Figure 3B, the mutant significantly lost PARP1 binding affinity compared to wild-type Snail, indicating that R151, K152 are critical for PARP1 association. Interestingly, the Snail mutant also significantly lost the binding affinity for LSD1, further confirming that the presence of PARP1 is required for Snail-LSD1 association (Figure 3C). Consistently, when the cells were treated with gallotannin, an inhibitor of poly(ADP-ribose) glycohydrolase (PARG) which catalyzes the degradation of pADPr, the association of Snail-LSD1 was significantly enhanced (Figure 3D). Furthermore, the Snail mutant became less stable compared to the wild-type protein (Figure 3E), which was in accord with our previous finding that formation of Snail-LSD1 complex was required for maintaining the stability of each component.

Upon activation, PARP1 functions by attaching pADPr chain on specific glutamate, aspartate or lysine residues of its target proteins. To investigate whether Snail can undergo poly(ADP-ribosyl)ation upon association with PARP1, we immunoprecipitated Snail protein from the abovementioned stable HEK293 cells, and performed western-blot using antibody against pADPr. As shown in Figure 3F, Snail protein was poly(ADP-ribosyl)ated, the effect of which could be enhanced by doxorubicin and suppressed by AZD2281. There was no significant difference in regard to the level of poly(ADP-ribosyl)ation on wild type and the R151A/K152A mutant Snail, suggesting the existence of multiple modification sites on Snail protein. Together, we demonstrated that (1) PARP1 positively mediates Snail-LSD1 association as well as their protein stability through interacting with a potential pADPr-binding motif of Snail; and (2) Snail protein is subject to PARP1-mediated poly(ADP-ribosyl)ation on multiple residues.

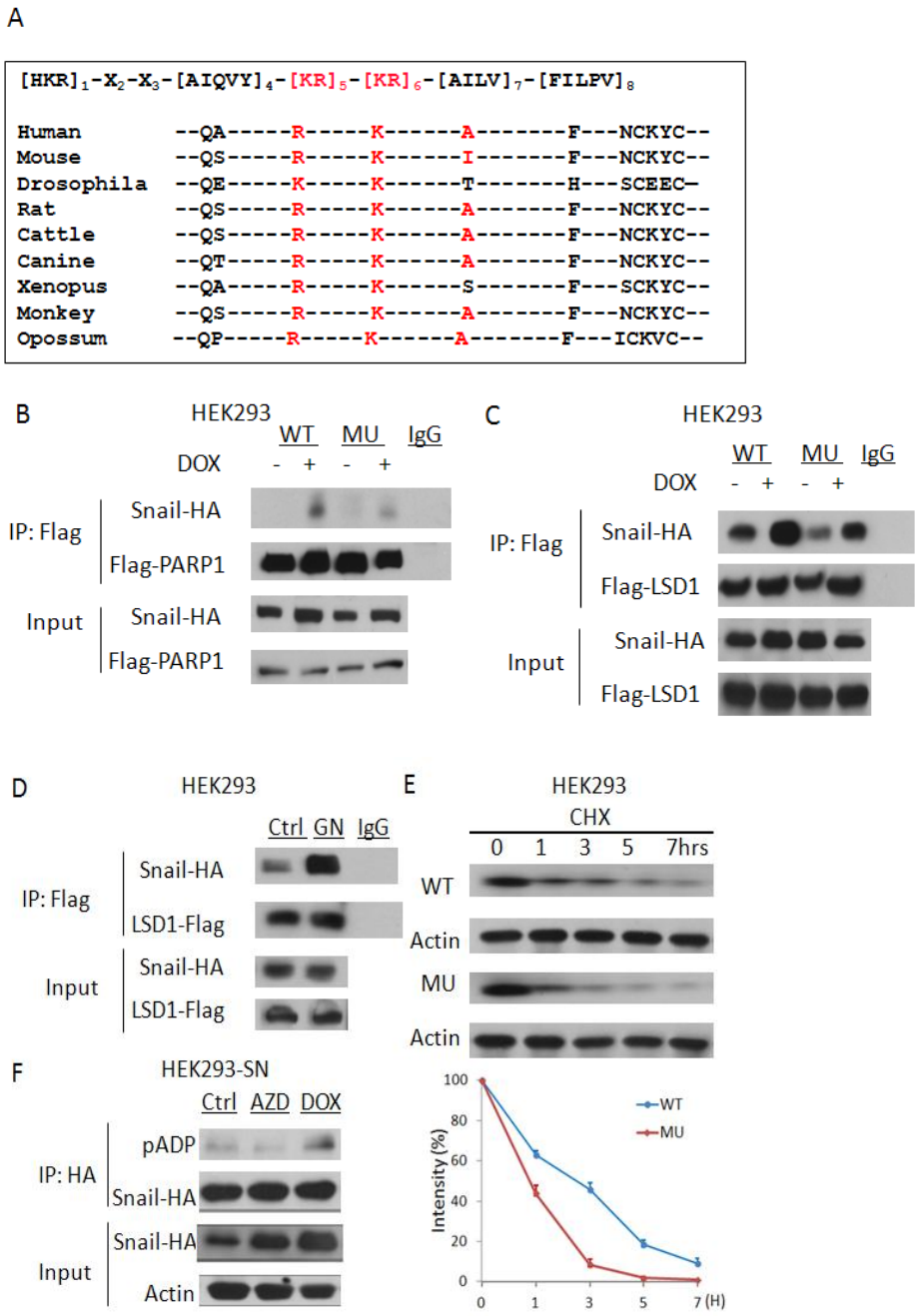

Figure 3 *Snail contains a potential pADPr-binding motif and is subject to poly(ADP-ribosyl)ation.*

(A) Sequence alignment of Snail protein with previously established pADPr-binding motif. The concert residues were highlighted with red color. (B) Flag-tagged PARP1 was co-expressed with HA-tagged wild-type or mutant (151 R151A/K152A) Snail in HEK293 cells. After immunoprecipitation of PARP1, the bound Snail was examined. For comparison, cells were treated with doxorubicin as indicated. (C) Flag-tagged LSD1 was co-expressed with HA-tagged wild-type or mutant Snail. After immunoprecipitation of LSD1, the bound Snail was examined. (D) Flag-tagged LSD1 was co-expressed with HA-tagged wild-type Snail. After immunoprecipitation of PARP1, the bound Snail was examined. For comparison, cells were treated with 10 μM of gallotannin (GN) for 6 hours (lane 2). (E) Wild-type or mutant Snail was respectively expressed in HEK293 cells and treated with 10 mg/ml of cycloheximide (CHX) for different time intervals. The level of Snail was analyzed by western blotting. Densitometry results were statistically analyzed and plotted (bottom panel, mean ± SD from 3 separate experiments). A representative western blotting experiment is shown in the top panel. (F) Snail protein was immunoprecipitated from HKE293-SN, and western blotting was performed using antibody against pADPr. For comparison, cells were treated with doxorubicin and AZD2281.

<u>SOW – Study 2: Does PARP1 mediated Snail-LSD1 interaction have any biological significance? (month 19-23)</u>

2a Can PARP1 mediate Snail/LSD1 binding to PTEN promter? (month 19-21)

Accomplishments:

Previous studies have demonstrated that Snail can bind to PTEN promoter to repress its transcription. The formation of Snail-LSD1-PARP1 complex under DNA damage condition prompted us to investigate how these proteins potentially cooperate to downregulate PTEN in favor of cancer cell survival. Since Snail interacts with LSD1 through its SNAG domain, we reasoned that Snail can recruit LSD1 to PTEN promoter for H3K4 demethylation and gene suppression. We then performed chromatin immunoprecipitation (ChIP) assays to test this hypothesis. Indeed, both Snail and LSD1 could interact with PTEN promoter in MDA-MB-157 and HCT116 cells (Figure 4A). Interestingly, the binding affinity was significantly increased upon doxorubicin treatment, indicating that PARP1 becomes activated in response to DNA-damaging agent and promotes the interaction of Snail/LSD1 with PTEN promoter. Also as expected, AZD2281 treatment or PARP1 knockdown negatively regulated the complex-promoter binding. Consistently, the level of H3K4 methylation on PTEN promoter was significantly increased upon AZD2281 treatment or PARP1 knockdown, and was decreased upon doxorubicin treatment, further confirming that PAPR1 facilitates the access of LSD1 to PTEN promoter (Figure 4B). The ChIP samples were also analyzed by quantitative real-time PCR and similar results were obtained (Figure 4C). These results are not only supported by our earlier data showing that upon poly(ADP-ribosyl)ation of Snail, the complex becomes stabilized (Figure 3E), but also in line with the notion that Snail works together with corepressors to downregulate PTEN in response to DNA damage, in such way that Snail fulfils its function as a survival factor.

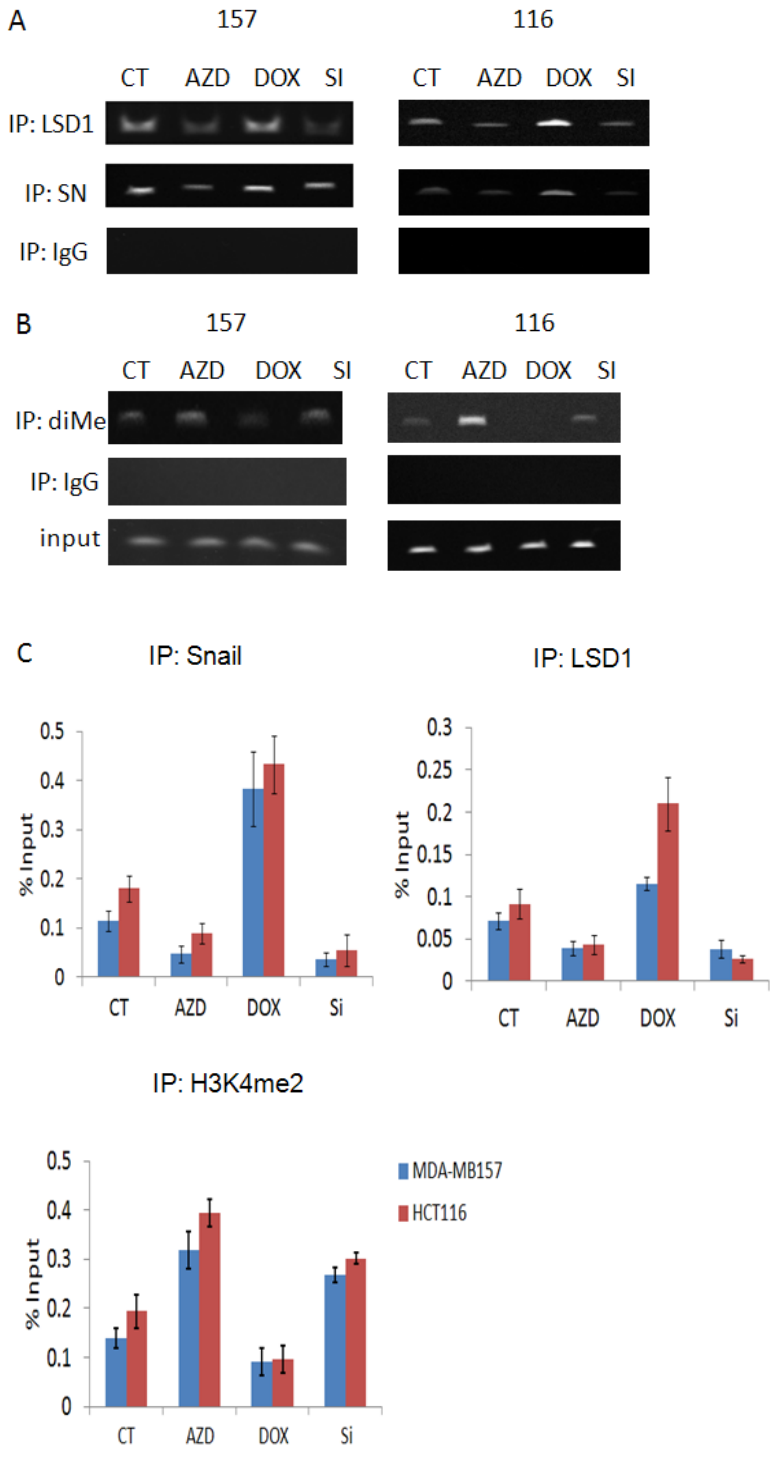

Figure 4 *The enzymatic activity of PARP1 is required for Snail-LSD1 binding to PTEN promoter.*

(A) The association of endogenous Snail and LSD1 with the PTEN promoter was analyzed by chromatin immunoprecipitation (ChIP) assay in MDA-MB157 and HCT116. For comparison, cells were treated with doxorubicin or AZD2281, or transfected with PARP1 siRNA. (B) Methylation of H3K4 on the PTEN promoter was analyzed by ChIP assay using antibody against H3K4me2. For comparison, cells were treated with doxorubicin or AZD2281, or transfected with PARP1 siRNA. (C) The ChIP samples were analyzed by quantitative real-time PCR (mean ± SD from three separate experiments).

2b To identify the biological function of Snail/LSD1/PARP1 complex (month 22-23)

Accomplishments:

Consistent with the results that doxorubicin enhanced the binding of the Snail-LSD1 repressor complex to PTEN promoter, we found that the protein level of PTEN was decreased in MDA-MB-157 and HCT116 cells upon doxorubicin treatment (Figure 5A, lane 3). Also as expected, the level of Akt phosphorylation was increased by doxorubicin. In contrast, AZD2281 treatment had the opposite effect on PTEN expression as well as Akt phosphorylation (Figure 5A, lane 2). Strikingly, when cells were treated with the two drugs simultaneously, the effect of doxorubicin on PTEN suppression as well as Akt phosphorylation was compromised by AZD2281 (Figure 5A, lane 4). To further test the idea that cancer cells apply a Snail complex-mediated defensive mechanism to evade DNA damage-induced apoptosis, we applied doxorubicin in combination with AZD2281 to cancer cells and examined their viability. As seen in Figure 5B, either doxorubicin or AZD2281 treatment can reduce the overall viability of MDA-MB-157 and HCT116 cells; the number of living cells was further decreased upon treatment of both drugs, indicating that the drug combination has enhanced cell killing effect. Taken together, our results suggest that blocking the activity of PARP1 can overcome the effect of doxorubicin on PTEN suppression and Akt activation, and sensitize cancer cells to the cytotoxic effect of doxorubicin.

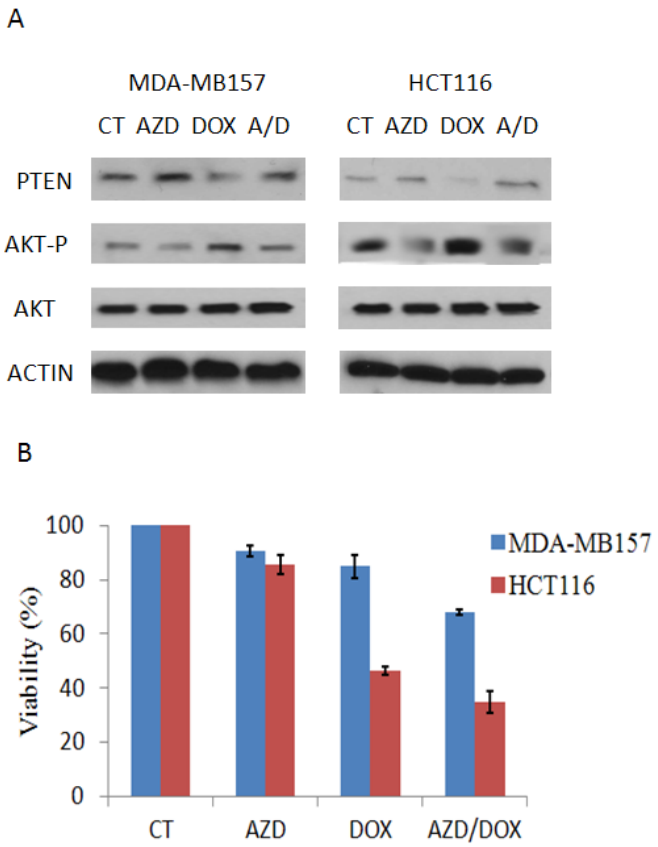

Figure 5 *AZD2281 enhances the killing effect of doxorubicin on cancer cells.*

(A) MDA-MB157 and HCT116 cells were treated with AZD2281, doxorubicin, or these two drugs together (A/D), and endogenous levels of PTEN, Akt and phosphorylated Akt (Akt-P) were examined by western blotting. (B) MTT assays were performed using MDA-MB157 and HCT116 cells and the overall cell viability was determined (mean ± SD from 3 separate experiments).

2c Manuscript preparation and submission (month 24-26)

Accomplishments:

We are currently preparing manuscript for submission.

<u>SOW – Study 3: Functional characterization of other Snail-interacting proteins (month 21-36)</u>

3a Identification of SNAG-interacting proteins (month 21-23)

Accomplishments:

To further identify SNAG-associated proteins besides LSD1, we applied peptide pulldown-mass spectrometry-coupled analysis as described above. The gel was subject to silver staining as shown in Figure 6. The protein identified include LSD1, CoREST, BHC80, HDAC1/2, EZH2, KDM5B (lysine (K)-specific demethylase 5B, which is a H3K4me3-specific demethylase) and NSD2 (Nuclear receptor-binding SET domain protein 2, which harbors histone lysine methyltransferases activity), among others.

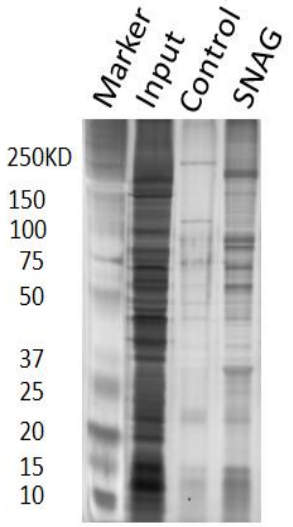

Figure 6 *Identification of SNAG peptide-interacting proteins.*

Peptide pulldown samples were separated on SDS-PAGE and subjected to silver staining before mass-spectrometry analysis. Peptide-absent sample was used as negative control.

3b Characterization of SNAG-interacting proteins (month 24-34)

We are currently searching literatures on the newly identified candidates and looking to select promising molecules for the continual study of Snail-mediated epigenetic regulation network.

3c Manuscript preparation and submission (month 35-36)

Key research accomplishments

We demonstrated that PARP1-mediated poly(ADP-ribosyl)ation of Snail is critical for Snail-LSD1 complex formation and the downstream PTEN suppression. Due to the highly heterogeneous and instable nature of cancer cells, as well as the complexity of the surrounding context, among different cancer cells there is huge discrepancy in regard to the sensitivity to a specific drug, making it impractical to find a one-cure-fits-all therapy. The development of efficient treatment strategies would heavily rely on the understanding of the signaling mechanisms adopted by cancer cells to overcome the adverse environment for survival. Our study not only provides a new insight into the working mechanism of the Snail transcriptional machinery, but also explores the potential application of PARP inhibitors in conjunction with DNA damage-inducing agents in targeting cancer cells. As PARP inhibitors are thrust into the limelight by the encouraging results of early clinical trials, our study would provide extra impetus for future drug development and help to diversify cancer treatment strategies.

In addition, through application of SNAG-peptide pulldown assay, we identified several interesting SNAG-interacting proteins. Functional characterization of these proteins will hopefully provide us with a clearer picture of Snail-mediated cancer progression.

Reportable outcomes

The manuscript entitled “Doxorubicin enhances Snail-LSD1 mediated PTEN suppression in a PARP1 dependent manner, and synergizes with PARP1 inhibitor AZD2281 in the killing effect of cancer cells” is ready for submission.

Ph.D. degree was obtained in December of 2012.

Conclusion

We followed the renewed SOW and have substantially completed the proposed studies so far. As the founding member of the PARP superfamily, PARP1 is a multifunctional protein that not only plays a role in DNA repair, but also participates in gene transcription regulation. The effect of PARP1 could either be stimulatory or inhibitory, depending on the specific environmental context and cellular signals. In the very case discussed here, PAPR1 functions as a co-inhibitor of the Snail-LSD1 complex under DNA damage condition. Upon activation by doxorubicin, PARP1 uses its pADPr for association with the pADPr-binding motif of Snail, and furthermore promotes the interaction of Snail with LSD1. Disruption of the pADPr-binding motif by point mutation not only resulted in loss of Snail-PARP1 association, but also strikingly compromised Snail-LSD1 complex formation. Consistently, blocking the degradation of pADPr by inhibiting PARG could enhance Snail-LSD1 interaction. In addition, we found that Snail could undergo poly(ADP-ribosyl)ation on DNA damage condition. Based on these results, together with previous finding that Snail interacts with LSD1 through its SNAG domain, we reasoned that binding and modification of Snail by PARP1 could change the conformation of Snail and potentially expose its LSD1-binding motif on the SNAG domain to facilitate Snail-LSD1 interaction. Therefore, LSD1 can be recruited by Snail to the target gene (PTEN in this case) promoter, where it demethylates histone H3 lysine 4 in favor of transcription repression. A detailed computer-based structure analysis would hopefully further illustrate this dynamic regulatory process and will be done in the near future. We also tried to explore our findings by specifying the residues on Snail protein that are subject to poly(ADP-ribosyl)ation. Mutation of the lysine residue on the pADPr-binding motif of Snail did not significantly compromise the level of poly(ADP-ribosyl)ation, neither did mutations on Lys9, Asp12 or Lys16 of SNAG domain, indicating that Snail can undergo poly(ADP-ribosyl)ation on multiple residues, which remain to be defined in the future. Together, our study illustrated the cooperation of Snail, LSD1 and PARP1 in PTEN transcription suppression under DNA damage condition.

The second insight provided by our study lies in the finding that PARP inhibitors in conjunction with DNA-damaging agents may represent an effective treatment strategy against a much wider range of cancers. While the conventional chemotherapeutic drugs such as doxorubicin function by targeting DNA synthesis and cell division, unfortunately they are not smart in pinpointing cancer cells; rather they also do harm to normal cells with rapid dividing property. Even worse, many solid tumors continually undergoing chemotherapy will ultimately acquire drug resistance. On the other hand, the targeted therapy including small molecule inhibitors and monoclonal antibodies may circumvent the unamiable cytotoxic effects and attack tumor cells with more accuracy and efficiency. Many cancer cells have defective DNA repair pathways. In this regard, targeting DNA repair machineries is a promising strategy for cancer treatments. We have shown in our study the enhanced killing effect of doxorubicin-AZD2281 combination on BRCA1/2 and PTEN intact MDA-MB-157 and HCT116 cells. Based on our results, we argue that in addition to the induction of DNA damage, doxorubicin treatment also enhances Snail-LSD1 mediated PTEN suppression in a PARP1-dependent manner, which results in phosphorylation and activation of pro-survival Akt. Inhibition of PARP1 can compromise this undesirable effect while synergizing the DNA-damaging effect of doxorubicin to efficiently kill cancer cells. While *in vivo* experiments are required to consolidate our results as well as to evaluate the long-term effect of PARP1 inhibition, our data expands potential therapeutic benefits of PARP1 inhibitors, especially on tumors with high levels of Snail and LSD1 expression. Furthermore, it is interesting to see if PARP1 inhibitors can synergize with LSD1 inhibitors and novel SNAG domain-mimicking compounds that block Snail-LSD1 interaction to treat these kinds of cancers. Overall, our study not only provides a new insight into the working mechanism of the Snail transcriptional machinery, but also explores the potential application of PARP inhibitors in conjunction with DNA damage-inducing agents in targeting cancer cells.

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Appendices N/A