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14. ABSTRACT

Based on the results in preliminary data and in year #1 of the proposal, we hypothesize that MDH2 plays a crucial role in determining prostate cancer chemosensitivity. In year #2, we further determined the effect of inhibiting MDH2 on prostate cancer energy metabolism and response to chemotherapy. We found that MDH2 knockdown via stable shRNA significantly disrupts the metabolic efficiency and redox balance in prostate cancer cells. Further, we made a novel observation that MDH2 knockdown significantly enhances the chemotherapy induced signaling cascade that is required to exert mitochondria-based apoptosis. This provides a potential mechanism underpinning the observed clinical correlation between MDH2 and outcome of chemotherapy in prostate cancer. It also presents evidence for the first time that MDH2 can be a novel molecular target to improve prostate cancer chemotherapy.

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Docetaxel, chemosensitivity, MDH2, mitochondria

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Introduction:

Genetic and epigenetic alterations in the metabolic pathways are associated with mitochondrial dysfunction and cancer development. In most cases, however, the mechanisms by which these alterations mediate cancer cell response or resistant to therapeutics are unknown. The goal of this proposal is to investigate one of such mechanisms that mediate prostate cancer docetaxel sensitivity. Based on the results in our preliminary data and in year #1 of the proposal, we confirmed our original hypothesis that MDH2 overexpression in prostate cancer cells confers resistance to chemotherapy. During the investigation in year #2 (the current progress report), we investigated the effect of MDH2 on prostate cancer metabolism, redox and signaling transduction of chemotherapy-induced apoptosis.

Body:

Material and Methods:

1) Metabolism measurements. To measure the effect of MDH2 on prostate cancer cellular metabolism, we measured one of the key metabolite-OAA, which is both a substrate and product of the reversible reaction catalyzed by MDH2. The cellular and mitochondrial OAA measurement is performed by a commercial kit from Biovision. Briefly, whole cell lysates or mitochondria fraction were obtained from cultured cells, and OAA staining reagent was added. The concentration of OAA was read by a UV spectra meter at wavelength 570 nM. Additional metabolic products that are related with MDH2 activity include ADP, ATP, NAD+ and NADH. Therefore, these metabolites were also measured in the whole cell protein lysates and mitochondrial fractions of cells with MDH2 shRNA and control shRNA. The measurements were also performed with commercial kits from Biovision based on the manufacturer protocols.
2) Cellular oxygen consumption and reactive oxygen (ROS) production. One of the metabolic consequences of MDH2 activity is cellular oxygen consumption, which subsequently can also affect the cellular generation of ROS. To measure the oxygen consumption in prostate cancer cells, we suspended 10 million cells in fresh culture media in a sealed reaction vial and the oxygen level in the media was measured by a Clark-type electrode and recorded manually every minute over the course of 30 minutes. The decrease of oxygen concentrations in media represents the consumption by the cells. To measure the ROS level, we stained cells with the total ROS staining reagents (H2DCFDA) purchased from Enzo Life Science based on manufacturer protocol. Afterwards, the stained cells were subject to flow cytometry analysis to quantitative measure ROS level.
3) Cell viability. The cell viability with shRNA MDH2 and shRNA control in the presence and absence of docetaxel (DTX) and JNK inhibitor (SP600125) were carried out as we previously described using dye exclusion methods(1).
4) Western blots. We used western blot to measure the effect of MDH2 on JNK and apoptosis signaling pathways in the context of docetaxel treatment. Cells were treated by docetaxel and whole cell protein lysates were harvested for western blot. All the antibodies were purchased from Cell Signaling.

Results:

1) ***MDH2 shRNA inhibits OAA metabolism***. We clarified the molecular mechanism of increased docetaxel sensitivity after MDH2 knockdown (observed in year #1, progress report). Since MDH2 is an important enzyme in TCA cycle, we first examined the intracellular OAA levels after MDH2 knock down. Mitochondria OAA levels were almost unaffected in MDH2 knock down cells compared to control cells. However, intracellular OAA levels were significantly increased ($P<0.01$) in MDH2 knock down cells, which indicates accumulation of OAA in the cytosol (figure 1).

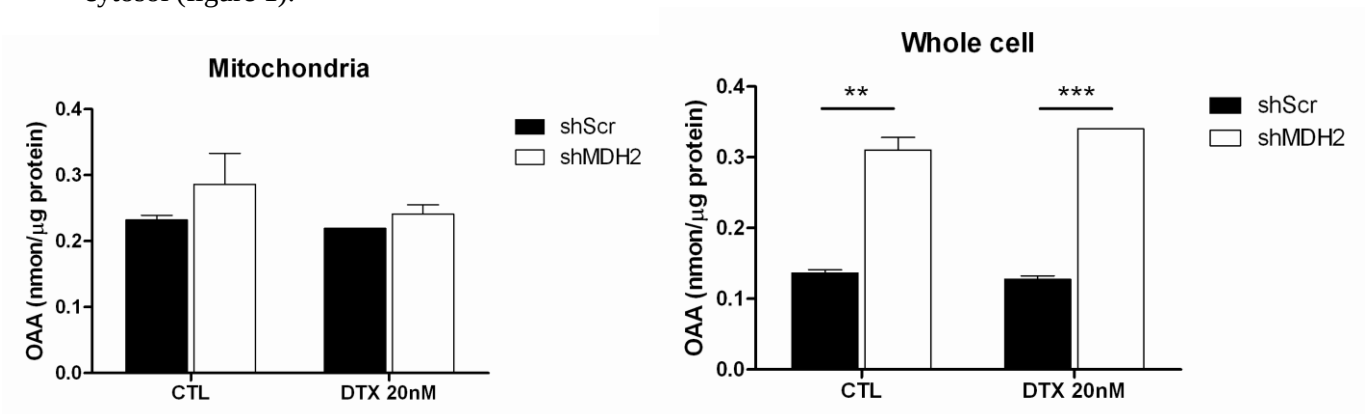

Figure 1: PC3 cells with stable shRNA against MDH2 (shMDH2) or shRNA control (shScr) were treated with vehicle or 20 nM docetaxel (DTX). 24 hours later, whole cell protein lysates (right) or mitochondria fractions (left) were harvested and measured for OAA concentrations as described in the method. Bars are mean and standard deviation of 3 experiments. **, and *** $P < 0.01$, t-test.

2) ***MDH2 shRNA decreases ATP production and increases NAD+ level***. Since the conversion of malate and OAA by MDH2 and transfer of these metabolites by malate-aspartate shuttle are accompanied by the generation of NAD+ and NADH, we next measured cellular NAD+ and NADH levels. The results showed that NAD+ as well as NAD+/NADH ratio was unaffected in whole cell lysates (data not shown) while increased in mitochondria of MDH2 knock down cells compared to control knock down PC3 cells (figure 2, left). NADH is the driving force for mitochondria respiratory chain to generate ATP. We next examined whether the increased NAD+/NADH ratio could affect cellular ATP levels. The results showed that ATP levels were significantly decreased ($P<0.001$) in MDH2 knock down cells in both PC3 and C42B cell lines compared to control knock down cells, with an increase ($P<0.001$) in ADP/ATP ratio in both cell lines (figure 2, middle and right).

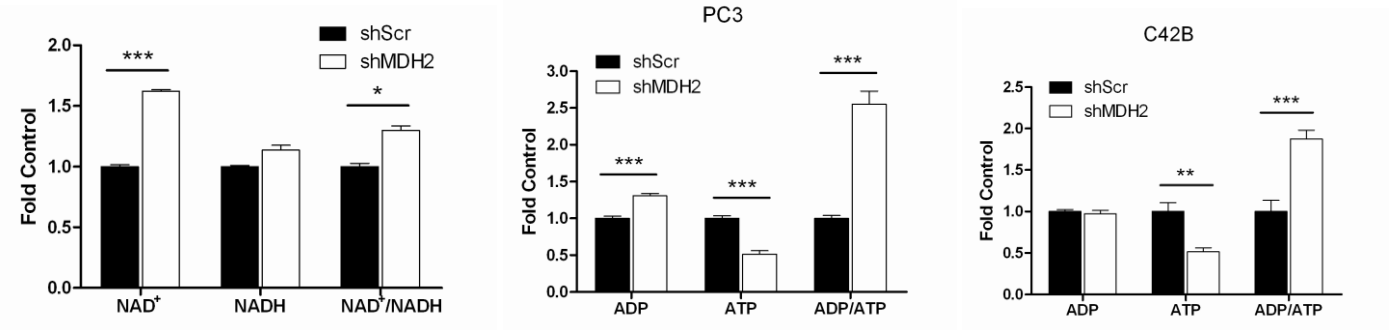

Figure 2: PC3 (left, middle) and C42B (right) prostate cancer cells were measured for cellular metabolite NAD+, NADH, ADP, and ATP levels with the context of MDH2 shRNA as described in the methods. Bars are mean and standard deviation of 3 experiments. *, **, and *** $P < 0.001$, t-test.

3) ***MDH2 in prostate cancer cells regulates ROS production***. Since oxygen is an important component for ATP generation through oxidative phosphorylation in mitochondria respiratory chain, we next evaluated whether MDH2 knock down could affect oxygen consumption in prostate cancer cells. The results indicated that cellular oxygen consumption was not affected by MDH2 (figure 3).

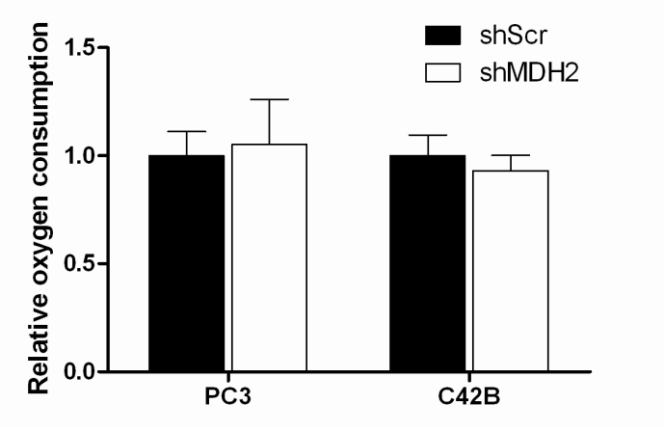

Figure 3: PC3 and C42B cells with shRNA control and MDH2 shRNA were measured for oxygen consumption as described in the methods. Bars are mean and standard deviation of 3 experiments.

Since ROS is a major by-product of oxygen consumption and ATP production, we measured cellular ROS levels by H2DCFDA staining. The results in figure 4 showed that ROS staining intensity was increased (peak shift to the right on X-axis, figure 4) in MDH2 knock down cells compared with control knock down cells with and without docetaxel. **The results in figure 1-4 indicated that shRNA knock down of critical TCA cycle enzyme MDH2 caused mitochondria inefficiency, which is phenotyped by the increase of NAD+, decrease of ATP production, and increase of ROS, while the oxygen consumption remain the same.**

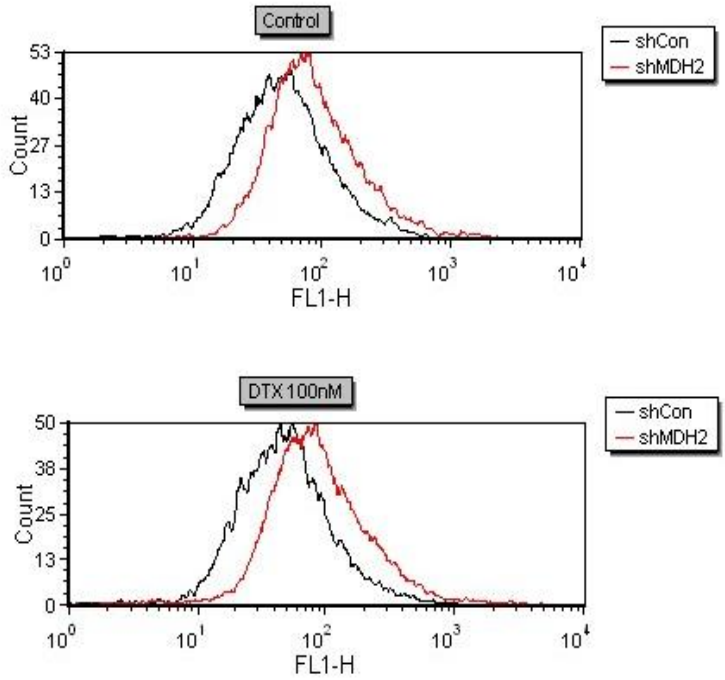

Figure 4: PC3 cells with shRNA control or shRNA against MDH2 were treated with vehicle control or docetaxel overnight. Afterwards, cells were harvested and the ROS levels were measured by flow cytometry as described in the methods. The ROS intensity shows as the peak value of FL1-H. The shift of peak value to the right as seen in the red curve (shMDH2 cells) represents an increase in ROS. A representative experiment is shown.

4) ***MDH2 regulates docetaxel response pathway***. Docetaxel was reported to induce cell apoptosis through JNK and mitochondria apoptosis signaling pathways. We examined JNK and apoptosis signaling pathway activity after docetaxel treatment. The results in figure 5 showed that docetaxel treatment increased SEK, JNK1 and ATF2 phosphorylation. More importantly, in MDH2 knock down cells, phosphorylation of these signaling molecules were further increased compared to control knock down cells. Phosphorylation and inactivation of the anti-apoptotic protein bcl-2 by JNK, and subsequent activation of apoptotic proteins is an important mechanism of docetaxel induced mitochondria apoptosis. We found that, in consistence with JNK signaling pathway, bcl-2 protein phosphorylation was further increased in MDH2 knock down cells compared to control cells. And we observed similar results in the induction of activated caspase-3 and cleaved PARP levels (figure 5).

   To further investigate the role of JNK pathway in MDH2 knock down related docetaxel sensitivity, we measured JNK pathway protein phosphorylation after removal of docetaxel treatment. The results showed that JNK, c-Jun and bcl-2 phosphorylation were rapidly decreased after docetaxel withdraw in control cells. However, in MDH2 knock down cells we observed a sustained activation of these proteins, with only a slight decrease after 24h (figure 6).

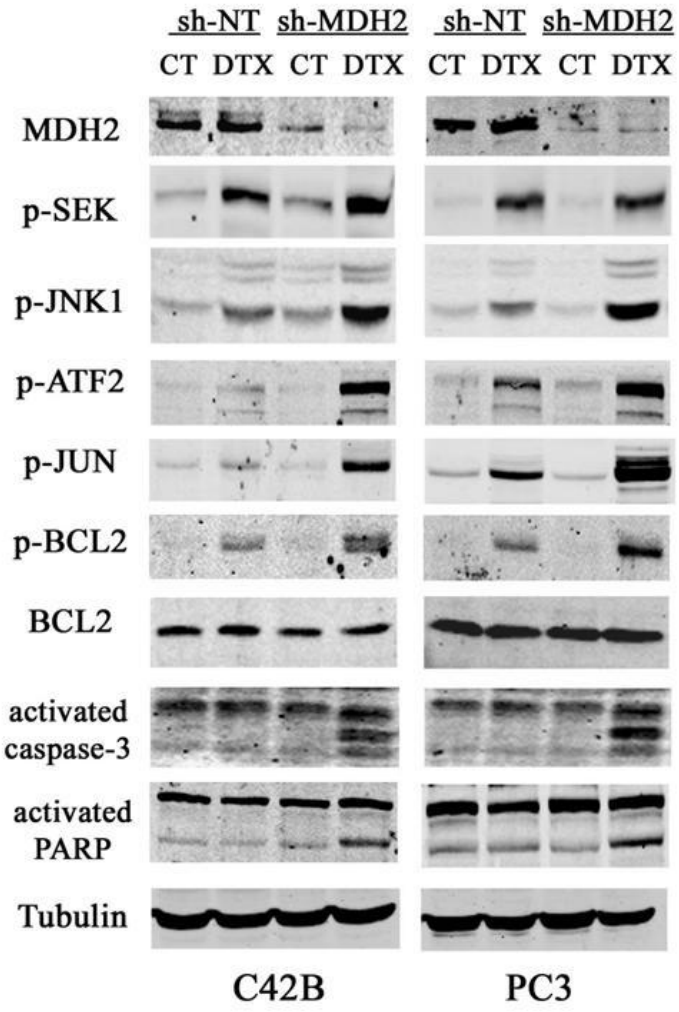

Figure 5: C42B and PC3 cells with control shRNA (sh-NT) and MDH2 shRNA (sh-MDH2) were treated with vehicle and docetaxel (DTX) overnight. Afterwards, whole cell protein lysates were harvested for western blot analysis by antibodies as indicated in the figure.

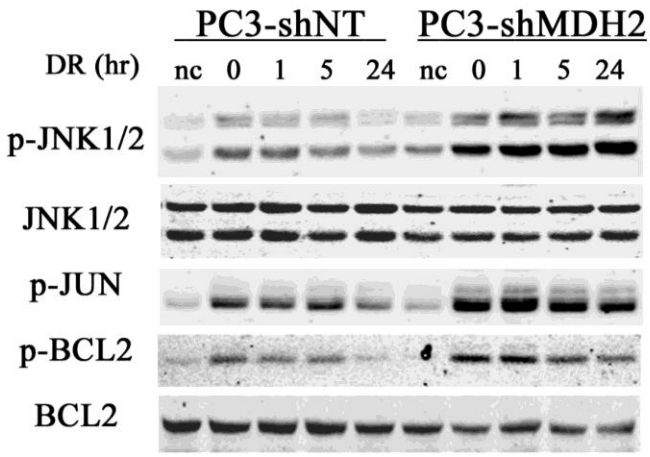

Figure 6: PC3 cells were treated with vehicle (nc) or docetaxel overnight. Afterwards, drug (docetaxel) containing media was removed, and fresh normal media was added to the cells. Whole cell protein lysates were harvested at the indicated time after drug removal (DR) for western blot analysis.

To further confirm the role of JNK pathway in MDH2 related docetaxel cellular response, we co-treated the PC3-shMDH2 cells with a JNK inhibitor SP600125 and different concentrations of docetaxel. The results showed that SP600125 rescued the shMDH2 cells from docetaxel-induced cytotoxicity (figure 7). **Taken together (figure 5-7), our data show that MDH2 regulates docetaxel response via the JNK and mitochondria apoptotic pathways. Prostate cancer with MDH2 overexpression can have dysregulated metabolic and docetaxel response to confer chemoresistance.**

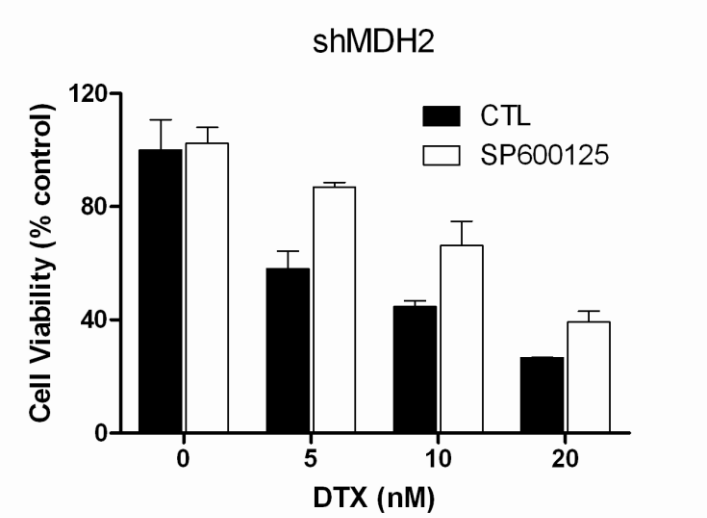

Figure 7: PC3-shMDH2 cells were treated with docetaxel and JNK inhibitor SP600125 as indicated for 24 hours. The cell viability was measured by dye exclusion as described previously. Bars are mean and standard deviation of 3 experiments. SP600125 vs. CTL at DTX 5, 10, and 20 nM were significantly different, $P < 0.01$, t-test.

Key Research Accomplishments:

a. We measured the metabolic and redox changes due to MDH2 shRNA in prostate cancer cells
b. We measured the effect of MDH2 shRNA on docetaxel-induced signaling cascade.

Reportable Outcomes:

a. MDH2 regulates prostate cancer metabolism and redox
b. MDH2 regulates the apoptotic signaling cascade induced by docetaxel chemotherapy

Conclusion:

These data confirm our hypothesis, and clarify that MDH2 regulation on prostate cancer chemoresistance is due to a novel interaction between MDH2 and JNK signaling cascade that is critical in the docetaxel induced apoptotic signaling.

1. Geng, H., Rademacher, B. L., Pittsenbarger, J., Huang, C. Y., Harvey, C. T., Lafortune, M. C., Myrthue, A., Garzotto, M., Nelson, P. S., Beer, T. M., and Qian, D. Z. (2010) *Cancer Res* **70**, 3239-3248