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| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                  |                          |    |                                               |                                            |
| 14. ABSTRACT<br>Regulation of the stability and activity of androgen receptor by Hsp90 provides a scientific basis for current chemotherapy of prostate cancer with Hsp90 inhibitors. However, how Hsp90 activity is regulated is still not fully understood. In this current research, we have investigated the role of HDAC6 in prostate cancer development and progression through regulation of Hsp90 activity. We have found that Hsp90-ATP association, a reliable indication of Hsp90 activity, is down-regulated in HDAC6-deficient cells, and have provided evidence that loss of HDAC6 in cells also down-regulates protein levels of AR and other Hsp90 client proteins, probably through increased acetylation of Hsp90 and down-modulation of co-chaperone HOP. Requirement of HDAC6 for proper Hsp90 activity and AR activity is further substantiated by the fact that HDAC6-deficient LNCaP cells have slower growth and increased cell death (observation not yet documented) in medium with charcoal-stripped serum, which can not be reversed by androgen add-back. Another unique finding from this research is the identification of B-Raf, one of the key factors in Ras-Raf-MEK-ERK signaling axis, as a potential Hsp90 client protein. Once this finding is verified, it will have wide implications in understanding tumorigenesis as well as in cancer therapy as a whole. |                  |                          |    |                                               |                                            |
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## Table of Contents

|                                    |    |
|------------------------------------|----|
| Introduction.....                  | 4  |
| Body .....                         | 5  |
| Key Research Accomplishments ..... | 11 |
| Reportable Outcomes .....          | 12 |
| Conclusions.....                   | 13 |
| References .....                   | 14 |
| Appendices .....                   | 15 |

## INTRODUCTION

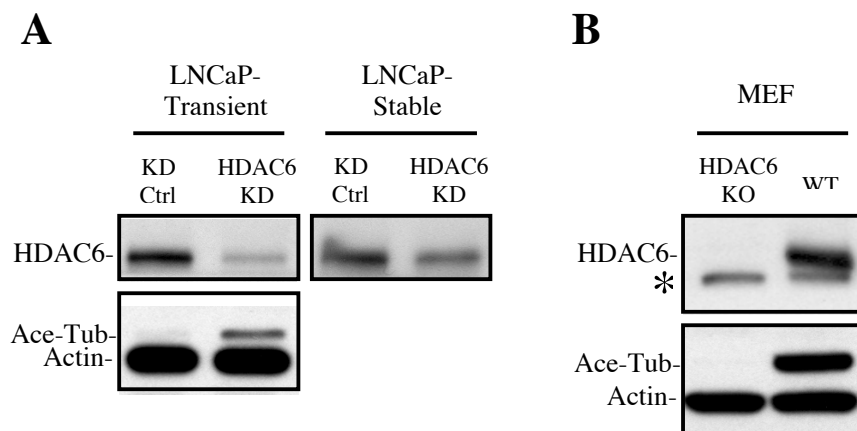
The deadly relapse of prostate cancer is highly related to the overexpression of androgen receptor (AR) and/or suprasensitivity of androgen receptor to low level androgen (1-3). Thus, regulation of AR level and its activity is important to the development of the disease as well as to the design of a better therapeutic strategy. Accumulating evidence shows that androgen receptor is in complex with heat shock protein 90 (Hsp90) that helps stabilize the receptor in cells (4, 5). In addition, ErbB2/HER2, a member of the epidermal growth factor receptor family is also a Hsp90 client protein and has been shown to contribute for efficient signaling from AR either by transactivation of AR or by contributing to AR stability (6, 7). Interestingly, the activity of Hsp90 itself is also subject to regulation through various pathways. One of them is the reversible acetylation catalyzed by yet identified acetyltransferase(s) and histone deacetylase 6 (HDAC6) (8-10). Works from our lab have also shown the activity of glucocorticoid receptor, one other HSP90 client protein, is subject to the regulation of HDAC6-mediated Hsp90 reversible acetylation (9). The purpose of this study is to investigate whether AR and ErbB2 are also regulated by HDAC6 via modulation of Hsp90 activity and how the HDAC6 regulation affect prostate cancer cell growth *in vitro* and *in vivo*.

## REPORT BODY

### 1. Generation of HDAC6 knock-down LNCaP cells and HDAC6 knock-out mouse embryonic fibroblasts (MEFs)

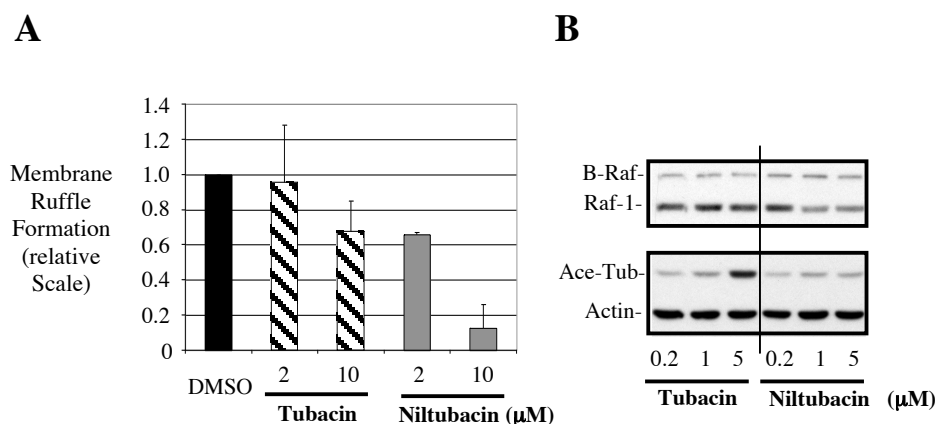
An effective way to evaluate the effect of HDAC6 on the activity of Hsp90 and its client proteins (including AR) is to down-regulated HDAC6 level in the cells. To this end, we have transiently knocked-down the protein level of HDAC6 by transfection of cells with small interfering RNA (siRNA). As shown in figure 1, there is about 20% of HDAC6 remains in the cells 3 days after transfection comparing to cells received only scrambled siRNA. Knocking-down of HDAC6 is also verified by the increased acetylation of  $\alpha$ -tubulin in cells (figure 1A) as  $\alpha$ -tubulin has been known a substrate of HDAC6 (11). To gain complete depletion of HDAC6, we also generated MEFs from both wild-type and HDAC6 knock-out mice. Immunoblotting shows the absence of HDAC6 in knock-out cells and the concomitant increase of acetylation of  $\alpha$ -tubulin (Figure 1B). To facilitate this research, we also tried to generate HDAC6 knock-out stable LNCaP cell lines using a retrovirus-mediated gene transferring. As shown in figure 1A (right panel), the HDAC6 knock-down stable line shows about 40% down-regulation of HDAC6 comparing to that in retrorival vector infected cells. Stable knock-down did not work as effective as the transient HDAC6 knock-down. Multiple infections with the virus in future study may improve the efficiency of HDAC6 knock-down in these stable cells.

In our proposal, we also planned to utilize a small molecule Tubacin as an optional approach to inhibit HDAC6 (12) though it has not been fully characterized. As HDAC6 is also involved in membrane ruffle formation (manuscript submitted), we decided to test



**Figure 1: Establishment of HDAC6 Knock-down and Knock-out Cells.** (A) Left Panel, LNCaP cells were transfected with siRNA targeting HDAC6 or with scrambled siRNA as control. Three days after transfection, cells were processed for immunoblotting for HDAC6, acetylated  $\alpha$ -tubulin or actin. Right Panel, HDAC6 was stably knocked down in LNCaP cells through a retroviral vector-mediated siRNA transfer method. Control cells received only vector. Cell lysates were evaluated for HDAC6 expression. (B) Mouse embryonic fibroblasts were prepared from wild-type and HDAC6 knock-out mice. Immunoblotting analysis was performed for HDAC6, acetylated  $\alpha$ -tubulin and actin. “\*” indicates non-specific band.

Tubacin and its non-effective derivative Niltubacin in membrane ruffle formation assay in MEFs. As shown in figure 2A, Tubacin will inhibit membrane ruffling induced by PDGF, and surprisingly enough Niltubacin has even stronger effect than Tubacin. In another set of experiment, we also treated LNCaP cells with both Tubacin and Niltubacin and examined the level of some of known client proteins of Hsp90. We found that Niltubacin down-regulates the protein level of Raf-1, though it does not increase tubulin acetylation. Obviously, Niltubacin is not just a simple non-effective derivative of Tubacin, but has its own specific functions. These results also cast serious doubt about the validity of Tubacin in our research, because Tubacin, like Niltubacin, may also have “off-target” effects on molecules other than HDAC6. As a result, in the future research, we will not use Tubacin as a way to inhibit HDAC6, but will seek down-regulation of HDAC6 by either knock-down or knock out approaches which we found are effective and sufficient for the our proposed research.

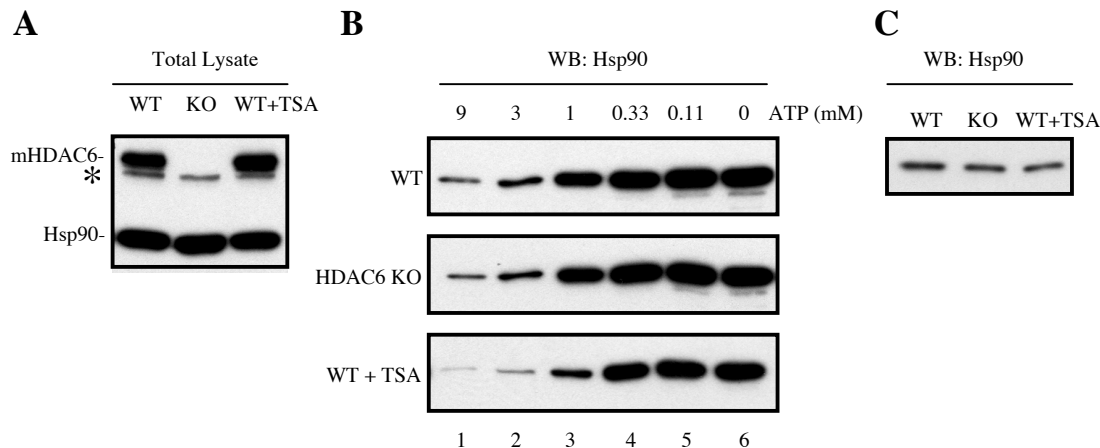


**Figure 2: Tubacin May Have “Off-target” Effect. (A)** Wild-type MEFs were either treated with Tubacin or Niltubacin at indicated concentration overnight. DMSO was used as control. Cells were then stimulated to form dorsal ruffles by incubating with PDGF-BB (50 ng/ml) for 8 minutes. Cells were stained for F-actin with phalloidin-rhodamine and ruffle formation was scored from about 500 cells in each group under microscope. Bars represent average value from two independent experiments. Niltubacin, initially used as a Tubacin control, shows even stronger effect to inhibit ruffle formation. Error bar: standard deviation. **(B)** LNCaP cells were treated with Tubacin or Niltubacin at indicated concentration overnight and processed for immunoblotting to examine the level of B-Raf, Raf-1, acetylated  $\alpha$ -tubulin and actin. Niltubacin brings down Raf-1 protein while does not increase acetyl  $\alpha$ -tubulin level.

## 2. Hsp90 activity is down-modulated in conditions inducing acetylation of Hsp90

Next, we examined the activity of Hsp90 in TSA-treated WT and HDAC6-depleted MEFs. The ability to bind ATP is essential to and a reliable indication of Hsp90 functions (13, 14). In this current study, we took two different approaches to evaluate Hsp90-ATP association. In the first set of experiment, we let Hsp90 from cell lysates of WT, HDAC6 KO and WT cells that were pretreated with TSA (a general deacetylase inhibitor, at 5  $\mu$ M for 22 hours) bind to ATP that had been immobilized on beads. Aliquots of the Hsp90-ATP beads were then incubated with different concentrations of free ATP. The later will compete with the bead-bound ATP for Hsp90 association. Hsp90 molecules with lower affinity to ATP beads were eluted. Figure 3B shows that TSA treatment not only reduces initial binding of Hsp90 to the ATP beads (compare lane 6 of all three cell groups), but also displays more elution at lower free ATP concentrations

than the other two groups (compare density changes of Hsp90 bands in lanes 3 and 4 of all groups). This result agrees very well with previous report that hyperacetylation of Hsp90 dampens its ATP binding activity (4). In the same experiment, we have seen slightly stronger binding of Hsp90 in the wild-type cells. To further confirm that Hsp90 in KO is less capable to bind ATP, we performed the binding assay in a binding condition without MgCl<sub>2</sub> as described before (4). As shown in figure 3C, binding of Hsp90 to ATP beads in WT cells exceeds that in HDAC6 KO and TSA-treated WT cells. Taken together, acetylation of Hsp90 by TSA treatment or through HDAC6 depletion inhibits Hsp90 affinity for ATP.

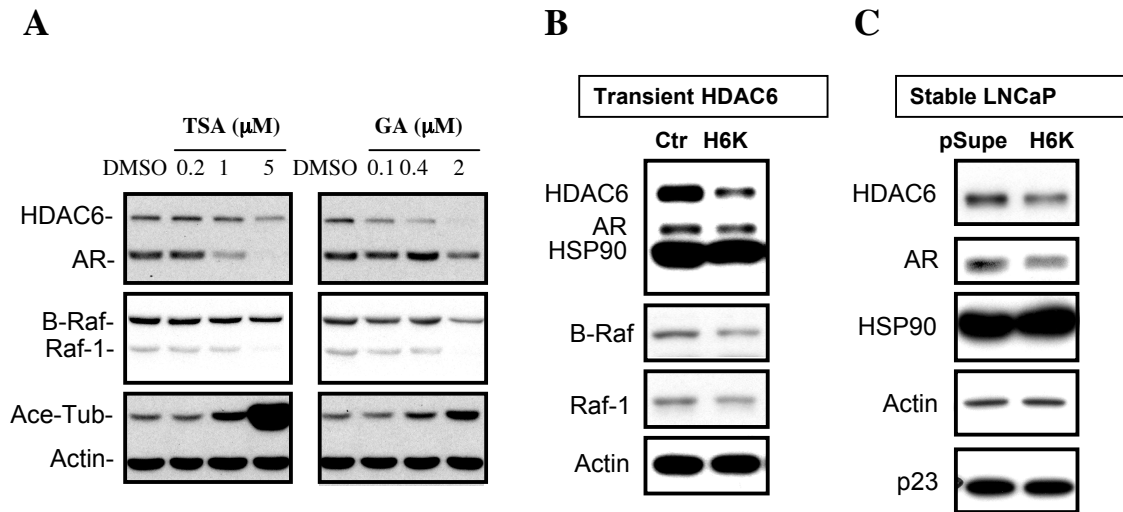


**Figure 3: Hsp90 is Inhibited in ATP Binding in Conditions Inducing Hsp90 Acetylation.** (A) MEFs from wild-type and HDAC6 KO animals and wild-type MEFs that were pre-treated with TSA were analyzed by immunoblotting using anti-mHDAC6 and anti-Hsp90 antibodies to show similar amount of Hsp90 in all groups and the absence of HDAC6 in the knock-out cells. \*: non-specific band. (B) Cell lysates as in A were incubated with ATP-beads and beads were then incubated with indicated concentration of free ATP. Hsp90 remained on the beads after incubation was evaluated by immunoblotting using anti-Hsp90 antibody. Hsp90-ATP association in TSA-treated cells is weakened. (C) The same samples as in B were allowed to incubate with ATP beads in the absence of MgCl<sub>2</sub>. Beads-bound Hsp90 was analyzed as in B to show that Hsp90 in HDAC6KO and TSA-treated cells has lower affinity for ATP.

### 3. Androgen receptor and some other Hsp90 client proteins are down-regulated in HDAC6 knock-down cells.

Acetylation of Hsp90 inhibits its intracellular functions. Very often this is reflected in the reduced stability of its client proteins. We have examined the protein levels of AR and Raf-1, both known as Hsp90 client proteins, in TSA or geldanamycin (a specific Hsp90 inhibitor)-treated cells. We found that both TSA and geldanamycin induce the reduction of AR and Raf-1 as previously reported. It has also been found that the activated B-Raf mutant (B-Raf V600E), but not the wild-type B-Raf, is a client protein of Hsp90 (15, 16). However, in our hand B-Raf from MEFs of two different preparations (data not shown) and LNCaP cells is also reduced when cells were treated with TSA or geldanamycin (figure 4A). As it is unlikely that B-Raf in both MEF preparations is V600E mutated, this observation is therefore unique. Nevertheless, further work is needed, for examples, to verify the physical association of B-Raf and Hsp90, and to provide evidence that B-Raf can be stabilized by inhibition of proteosomal activity of the cells. Raf-1 and B-Raf are essential factors in the Ras-Raf-MEK-ERK pathway, and not like

mylanoma cells that mostly harbor V600E mutation, many other cancer cells contain predominantly wild-type B-Raf. Therefore our findings will have significant implications in cancer research as general.



**Figure 4: HDAC6-deficiency Down-regulate Hsp90 Client Proteins.** (A) LNCaP cells were treated with TSA or geldanamycin at indicated concentrations for 22 hours and processed for immunoblotting using antibodies against HDAC6, AR, B-Raf, Raf-1, acetylated  $\alpha$ -tubulin and actin. AR, B-raf, Raf-1 and HDAC6 levels drop down after treatment with the drugs in a dose-dependent manner. Actin is shown as loading control. (B) LNCaP cells were transfected with siRNA to transiently knock-down HDAC6. AR, R-Raf and Raf-1 were examined to demonstrate the down-modulation of these proteins in HDAC6 knock-down cells. Hsp90 level does not change significantly. Actin is also shown as loading control. (C) HDAC6 was stably knock-down in LNCaP cells. AR level was examined as in B. Hsp90 and co-chaperone p23 were examined to show constant protein level in both cell types. Actin is shown as loading control.

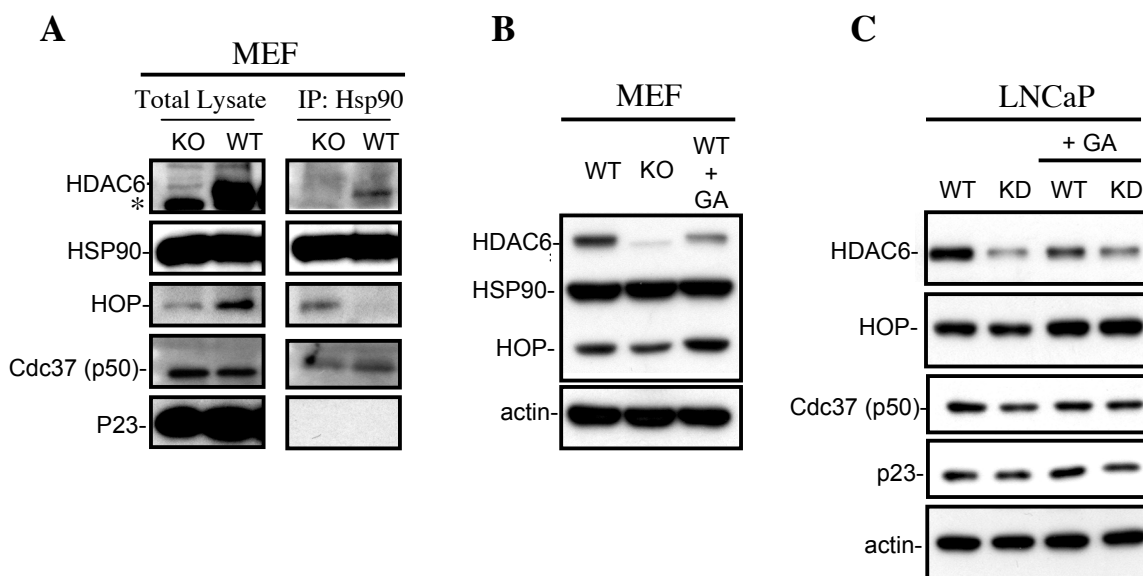
As another remarkable observation, HDAC6 level is also regulated by Hsp90. Pretreatment of cells with geldanamycin significantly reduces HDAC6 with concomitant increase of acetylation of  $\alpha$ -tubulin (figure 4A). Inhibition of Hsp90, however, does not have global effect on protein stability. As shown in figure 4A, actin level keeps constant in both TSA and GA-treated samples.

Next, we examined the level of androgen receptor in HDAC6 knock-down cells. We first transiently transfected LNCaP cells with siRNA to knock-down HDAC6 (figure 1 and figure 4B and 4C). Immunoblotting analysis of the cell lysates indicates that AR level was moderately, but clearly, reduced comparing to control group that was transfected with scrambled siRNA. This result was repeated in two more independent experiments with very similar observations. Interestingly, in HDAC6 stable knock-down cell line, although HDAC6 level keeps higher than in transient HDAC6 knock-down cells, AR level dropped even more significantly. The reason might be that the time is not long enough to have AR down-modulation to show up in the transient HDAC6 knock-down group. As loading controls, Hsp90 and actin levels are similar between HDAC6 knock-down and control cells.



#### 4. Hsp90-co-chaperone complex formation is regulated by HDAC6

Proper function of Hsp90 requires the complex formation with its co-chaperones (17-20). However, how acetylation of Hsp90 affect Hsp90 complex formation is still not clear. In this research, we have investigated this issue by co-immunoprecipitation assay using an antibody that specifically bind free form Hsp90 (21). As demonstrated in Figure 5, this antibody does not co-precipitate co-chaperones p23, cdc37 (p50) and Hsp70/Hsp90 organizing protein (HOP) in wild type cells. But surprisingly, Hsp90 co-precipitates HOP in HDAC6 knock out cells. This result suggests that HOP preferentially binds to free form Hsp90 that should be acetylated. This experiment should be done in conjunction with another antibody that recognizes complexed form of Hsp90. However, the latest supply of the antibody from the vendor was not good enough for the work (we tried but failed). We are expecting a new batch of the antibody soon.



**Figure 5: HDAC6 Regulates HOP Level and Interaction with Hsp90.** (A) **Left panel:** MEF lysates derived from wild-type and HDAC6 knock-out cells were analyzed in immunoblotting to examine the protein levels of Hsp90 and that of co-chaperones HOP, cdc37 (p50) and p23. HOP level is significantly reduced in HDAC6 KO cells. \*: non-specific band. **Right panel:** the same cell lysates as in left panel were used for immunoprecipitation using anti-Hsp90 antibody (Stressgen: SPA830). HDAC6 is co-precipitated with Hsp90 while HOP, p50 and p23 are not in wild-type cells. However, significant amount of HOP comes-down with Hsp90 in HDAC6 KO cells albeit HOP is reduced in KO cells. (B) MEFs from wild-type and HDAC6 knock-out animals were either left untreated or treated (only for WT MEFs) with geldanamycin at 2  $\mu$ M for 20 hours and cell lysates were analyzed by immunoblotting to show that HOP level drops in HDAC6 KO cells while geldanamycin up-regulates HOP. Hsp90 and actin were shown for loading control. (C) Wild-type and HDAC6 knock-down LNCaP cells were left untreated or treated with 2  $\mu$ M geldanamycin for 20 hours and processed for immunoblotting using antibodies as indicated in the figure. Geldanamycin up-regulates HOP protein level regardless of the loss of HDAC6 in cells.

We also found from this experiment that HOP level was significantly down-regulated in HDAC6 KO cells (Figure 5A and 5B) and a similar response of HOP to HDAC6 knock-down was also observed in LNCaP cells (figure 5C), indicating that HOP down-regulation in HDAC6 deficient cells is likely a general phenomenon. To determine whether HOP down-regulation

stems from the inhibition of Hsp90, we treated cells with geldanamycin and in contrast to our expectation we found that HOP is significantly upregulated (figure 5B and 5C). Geldanamycin's effect to bring up HOP is dominant because geldanamycin-treated HDAC6 KO cells also show increased HOP level, while other co-chaperones do not display similar response. Regarding the fact that both HDAC6 and geldanamycin inhibit Hsp90, the mechanism underlying these controversial facts is not available at the present time, though HOP level increases might be due to adaptive response of cells to acute inhibition of Hsp90 by geldanamycin.

## **5. Evaluation of AR activity in HDAC6-deficient LNCaP cells and inhibition of cell growth with HDAC6 deficiency.**

To evaluate AR activity in LNCaP cells, a reporter assay was adopted. The experiments were carried out by first transiently knock-down HDAC6 followed by transfection of the AR reporter gene (MMTV-luc) together with pCMV- $\beta$ gal (used to determine the transfection efficiency). Expression of the reporter gene was then induced by synthetic androgen R1881 at 10 nM for two days. Several experiments have been performed but failed due to loss of HDAC6 knock-down cells during the long (4 days) unfavorable culture condition (see explanation later). This experiment will be repeated on an improved stable HDAC6 knock-down line when it is established.

The unsuccessful reporter assay actually discloses exciting information. We have repeatedly observed slower cell growth as well as increased cell death in HDAC6 KD group during our reporter assay. We believe that using culture medium supplemented with charcoal-stripped serum during the reporter assay magnifies the defects of HDAC6 KD cells, even though in culture with ordinary serum, wild-type and HDAC6 KD cells are not distinguishable in growth rate. Importantly this defect in cell growth can not be reversed by two-day R1881 stimulation, suggesting that HDAC6 KD cells lose the ability to respond to the hormone, possibly due to the down-regulation of AR. Documentation of cell growth and apoptosis in the wild-type and HDAC6 KD cells has been planned and will establish the physiological importance of HDAC6 in prostate cancer development and progression.

## **KEY RESEARCH ACCOMPLISHMENTS:**

- o Establishment of HDAC6 stable knock-down LNCaP line
- o Provide evidence that Hsp90 activity (ATP binding) is down-regulated in HDAC6 deficient cells.
- o Provide evidence that AR and Raf-1, two known client proteins of Hsp90, are down-regulated in HDAC6 KD LNCaP cells.
- o Unique identification of B-Raf and HDAC6 as potential Hsp90 client proteins
- o Demonstrating increased Hsp90 association with co-chaperone HOP in HDAC6 KO cells, providing a mechanism as how HDAC6 catalyzed reversible acetylation affects Hsp90 complex formation.
- o Provide evidence that HOP is regulated by HDAC6, suggesting another potential angle that HDAC6 influences Hsp90 (and possibly Hsp70) activity
- o Have found (observation not yet documented) significant slower growth and increased cell death of HDAC6 KD LNCaP cells in medium with charcoal-stripped serum, which can not be reversed by R1881, indicating that HDAC6 KD cells are inhibited in growth and/or promoted in cell death.

**REPORTABLE OUTCOMES:**

none

## **CONCLUSION:**

In this current research, we have investigated the role of HDAC6 in prostate cancer development and progression through regulation of Hsp90 activity. We have found that Hsp90-ATP association, a reliable indication of Hsp90 activity, is down-regulated in HDAC6-deficient cells, and have provided evidence that loss of HDAC6 in cells also down-regulates protein levels of AR and other Hsp90 client proteins, probably through increased acetylation of Hsp90 and down-modulation of co-chaperone HOP. Requirement of HDAC6 for proper Hsp90 activity and AR activity is further substantiated by the fact that HDAC6-deficient LNCaP cells have slower growth and increased cell death (observation not yet documented) in medium with charcoal-stripped serum, which can not be reversed by androgen add-back. Another unique finding from this research is the identification of B-Raf, one of the key factors in Ras-Raf-MEK-ERK signaling axis, as a potential Hsp90 client protein. Once this finding is verified, it will have wide implications in understanding tumorigenesis as well as in cancer therapy as a whole.

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**APPENDICES:**

none

**SUPPORTING DATA:**

none