AWARD NUMBER: W81XWH-18-1-0163

TITLE: The Role of DHEAS in Abiraterone Resistant Prostate Cancer

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CONTRACTING ORGANIZATION: Cleveland Clinic Foundation

REPORT DATE: OCTOBER 2020

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
OCTOBER 2020	Final report	07/01/2018 - 06/30/2020

4. TITLE AND SUBTITLE
The Role of DHEAS in Abiraterone Resistant Prostate Cancer

5a. CONTRACT NUMBER
W81XWH-18-1-0163

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Prostate cancer (PCa) is a leading cause of cancer deaths in American men. Androgen deprivation therapy (ADT), lowers to castrate levels the androgens that drive PCa growth through androgen receptor (AR) signaling. Despite initial success, castration-resistant PCa (CRPC) develops within 24 months of ADT and remains primarily driven by androgens. Abiraterone acetate, a CYP17A1 inhibitor, reduces circulating androgen levels by preventing the synthesis of dehydroepiandrosterone (DHEA) and its sulfated form (DHEAS), an androgen precursor. Although most patients respond well to abiraterone acetate, survival is prolonged by only about 4-5 months, and secondary resistance develops. Hypoxia is a hallmark of many types of human tumors and implicated in clinical behavior and treatment response. Preclinical study indicate that hypoxia promotes both local aggressiveness and metastasis via mediating metabolism of cancer cells to facilitate the rapid growth of tumor. Previous study suggest that hypoxia plays a critical role in prostate cancer development. Here I further determined how hypoxia affected DHEA metabolism via regulating the expression of HSD3B1 as well as co-factors required for HSD3B1 activity. I established hypoxia-resistant cell lines which can survive under hypoxia forever to simulate the hypoxic condition that exists in a tumor. My results indicate that while acute hypoxia leads to deaccelerated conversion of DHEA to downstream metabolites due to reduced co-factors, long-term hypoxia significantly increases the expression of HSD3B1 at both mRNA and protein level. HSD3B1 exhibits higher stability under hypoxia. Moreover, my study suggest that hypoxia-resistant cells exhibit faster conversion of DHEA to downstream metabolites than hypoxia-sensitive cells after reoxygenation.

15. SUBJECT TERMS
CRPC, abiraterone resistance, DHEA, hypoxia, HSD3B1

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	17	USAMRMC

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INTRODUCTION:

Prostate cancer (PCa) is a leading cause of cancer deaths in American men and is responsible for 27,000 deaths annually. Androgen deprivation therapy (ADT), the first-line treatment for advanced PCa, lowers to castrate levels the androgens that drive PCa growth through androgen receptor (AR) signaling. Despite initial treatment success, castration-resistant PCa (CRPC) eventually develops and remains primarily driven by androgens. Abiraterone acetate, a CYP17A1 inhibitor, is a second-line therapy used to treat metastatic CRPC and reduces circulating androgen levels by preventing the synthesis of dehydroepiandrosterone (DHEA) and its sulfated form (DHEAS), an androgen precursor. Although most patients respond well to abiraterone acetate, survival is prolonged by only about 4-5 months, and secondary resistance develops. Despite blockade of CYP17A, significant serum concentrations of DHEAS persist after abiraterone treatment. Our preliminary data indicate that abiraterone-resistant cells have the machinery to transport and utilize DHEAS, and loss of *HSD3B1*, which encodes 3β-hydroxysteroid dehydrogenase/Δ5-->4 isoform 1 (3βHSD1), affects DHEAS uptake, complementing our previous studies that highlight the importance of this enzyme in androgen metabolism.

Although DHEAS is the major form of DHEA, functioning as remaining androgen pool, it cannot be utilized to synthesize active androgens directly. The synthesis of potent androgens such as testosterone and DHT requires the conversion of DHEAS to DHEA in the cells. Multiple clinical studies have shown the reliance of CRPC on intratumoral androgens which are synthesized from DHEA in peripheral tissue. The conversion of DHEA to androstenedione (AD) by 3βHSD is the rate-limiting step of androgen synthesis. Human 3βHSD family has two members sharing 93.5% identity: 3βHSD1 and 3βHSD2. 3βHSD1 activity is found in peripheral tissues such as prostate, placenta, skin, and mammary glands, whereas 3βHSD2 is predominantly expressed in the adrenal gland, ovary, and testis. Therefore, understanding how 3βHSD1 is regulated is critical for deciphering the mechanism of androgen synthesis in CRPC. A gain-of-function mutation (N367T) was found on 3βHSD1 which significantly stabilizes the protein thus promoting androgen synthesis. Recent clinical studies suggested that patients with this mutation have significantly worse outcomes after ADT, as well as extended responses to steroid 17α-hydroxylase/17,20-lyase (CYP17A1) inhibition. Different from 3βHSD1 and SRD5A1 which catalyze unidirectional reactions, the conversion from 5α-dione to DHT is mediated by 17β-hydroxysteroid dehydrogenase (17βHSD), a family of bidirectional enzymes. The directionality of 17βHSD is dictated by co-factors affinity, cellular redox status and pH. Both NAD^+/NADH and $NADP^+$/NADPH can be used by 17βHSD as co-factors.

Solid tumors commonly contain hypoxic areas due to dynamic gradients of oxygen diffusion. The hypoxic microenvironment arises as a result of rapid tumor growth that results in disorganized and irregular tumor vasculature. Hypoxia is not only a feature of tumor, but also has a profound impact on tumor progression, metabolism, genomic instability, metastasis, immune responses, and treatment resistance via inducing multiple downstream molecular events. Hypoxia-inducible factor (HIF), a family of transcription factors, is a major regulator of cellular response to hypoxia. Each functional HIF is a heterodimer composed of the α-subunit and the β-subunit. The α-subunit is oxygen sensitive. In the presence of oxygen, the α-subunit is hydroxylated on the conserved proline residues by prolyl hydroxylase (PHD), which is also called egl nine homolog (EGLN). Prolyl-hydroxylated HIFα is then recognized by von Hippel–Lindau (VHL), the key component of an E3 ubiquitin ligase complex for ubiquitylation and proteasomal degradation. Hypoxia prevents the hydroxylation of HIFα, which results in its accumulation and further translocation to the nucleus where it forms heterodimer with HIF1β to induce the transcription of numerous genes. HIF1β which is also called aryl hydrocarbon receptor nuclear translocator (ARNT) is ubiquitously expressed and oxygen-insensitive. HIF1β forms heterodimers with three different α-subunit isoforms: HIF1α, HIF2α, and HIF3α, and the heterodimers bind to the consensus motif in target gene promoter regions known as hypoxia-response elements (HREs). HIF1α and HIF2α have different sets of, with some overlapping, target genes, although both of them recognize same consensus motif. The targets of HIF3α are not well elucidated. HIF transcriptional activity is also regulated by asparaginyl hydroxylation mediated by factor inhibiting HIF-1 (FIH1). The hydroxylation on the conserved asparagine residue in the C-terminal transactivation domain (C-TAD) of HIFα prevents its interaction with the p300/CBP co-activator.

Increasing evidence indicates hypoxia as a key driver in prostate cancer progression. Expression of some HIF target genes were significantly higher in prostate cancer and correlated with Gleason score and biochemical recurrence. A 28-gene hypoxia-related prognostic signature for localized prostate cancer was developed and proved to predict biochemical recurrence and metastasis. Hypoxia was also associated with early biochemical relapse after radiotherapy, as well as local recurrence in the prostate gland. Hypoxia-activated prodrug OCT1002 was shown to selectively target hypoxic tumor cells and enhance the antitumor efficacy of bicalutamide. However, although hypoxia has been shown to play a critical role in prostate cancer progression, how hypoxia affects androgen metabolism is not well understood. Here, we investigated the alteration of androgen synthesis under hypoxia in prostate cancer cells. Our results suggest that cyclic hypoxia-reoxygenation facilitates the efficient utilization of androgen precursors by prostate cancer cells to produce active androgen.

My preliminary data indicates that DHEA metabolism is affected by hypoxia. Here I further determined how hypoxia affected DHEA metabolism via regulating the expression of *HSD3B1* as well as co-factors required for 3βHSD1 activity. Although the prostate cancer cell lines I used showed adaptation to hypoxia (only modest loss of cell viability after 24h), prolonged hypoxia (48h) killed the majority. Because tumor cells experience long-term hypoxia due to inconsistent levels of oxygen and nutrient supply resulting from the disorganized tumor vasculature, I established hypoxia-resistant cell lines which can survive under hypoxia indefinitely to simulate the hypoxic condition that exists in a tumor. Different prostate cancer cell lines were grown under cyclic 12h hypoxia and 12h reoxygenation for several weeks. After several passages, the cells became resistant to hypoxia. My results indicate that although acute hypoxia leads to deaccelerated conversion of DHEA to downstream metabolites due to reduced co-factors, long-term hypoxia significantly increases the expression of *HSD3B1* at both mRNA and protein levels. Moreover, my study suggests that hypoxia-resistant cells exhibit faster conversion of DHEA to downstream metabolites than hypoxia-sensitive cells after reoxygenation.

My work also identified HIF2α as a major regulator of *HSD3B1*. Stabilization of HIF2α by knockdown of either *EGLN1* or *VHL* can increase the expression of *HSD3B1,* as well as DHEA metabolism, indicating that the EGLN1/VHL/HIF2 pathway is a major regulator of the expression of *HSD3B1*.

KEYWORDS:

CRPC, abiraterone resistance, DHEA, hypoxia, HSD3B1, HIF2

ACCOMPLISHMENTS:

What were the major goals of the project?

Training-Specific Tasks:

Milestone(s) Achieved:

1: Responsible Conduct of Research Training (24 months)

2: Monthly Prostate Cancer Working Group and Seminar Series (24 months)

3: Weekly lab meetings and journal clubs (24 months)

4: Cleveland Clinic Department of Cancer Biology weekly seminars and journal clubs (24 months)

5: Kenyon Institute's Biomedical and Scientific Writing workshop

6: AACR annual meeting

Research-Specific Tasks:

Specific Aim 1: Determine the role of DHEAS uptake and HSD3B1 expression in abiraterone (ABI) resistance.

Major Task 1: Establish long-term DHEAS- and ABI-treated cells.

Milestone(s) Achieved: Establishment of long-term treated cell lines that survive ABI treatments. (4 months)

Major Task 2: Evaluate changes in DHEAS uptake.

Milestone(s) Achieved: Link increased DHEAS uptake with ABI-resistance status. (12 months)

Specific Aim 2: Define the metabolic pathway and regulatory enzymes of DHEAS metabolism to DHT.

Major Task 3: Measure metabolism of long-term ABI-treated LNCaP and LAPC4 cells.

Milestone(s) Achieved: Determination of the effect of hypoxia on DHEA metabolism. (12 months)

Original Specific Aim 3: Identify genetic modifiers of ABI-resistant PCa using a genome-scale CRISPR knockout (GeCKO) library

New Specific Aim 3: Define the regulatory mechanism of 3βHSD1 by hypoxia.

Major Task 4: Identify the key pathways and regulators responsible for hypoxia-mediated 3βHSD1 upregulation.

Milestone(s) Achieved: Determination of EGLN1/VHL/HIF2 pathway that is responsible for hypoxia-mediated 3βHSD1 upregulation. (24 months)

I modified Specific Aim 3 for several reasons:

1. Although DHEAS is the major form of DHEA in blood, it cannot be utilized to synthesize active androgens, such as testosterone and DHT, directly in the cells. It must be converted to DHEA first.

2. Data from several studies published suggest that 3βHSD1 plays a decisive role in regulating DHEA metabolism and ABI-resistance, compared with other enzymes. Therefore, I decided to focus on this enzyme instead of high-throughput screening.

3. Our previous data suggest that hypoxia is a potent regulator of androgen synthesis, especially the conversion of DHEA to AD, by regulating the level of 3βHSD1. Hypoxia can also confer ABI-resistance to the cells. Therefore, I decided to examine the mechanism of hypoxia-mediated 3βHSD1 regulation.

4. EGLN1/VHL/HIF pathway plays a pivotal role in hypoxic response in cells. Since my previous data showed that hypoxia upregulated 3βHSD1 at mRNA level, and HIFs are transcription factors, I examined whether it is HIF that is responsible for hypoxia-mediated 3βHSD1 upregulation. Both HIF1 and HIF2 were examined.

What was accomplished under these goals?

Accomplished tasks:

Specific Aim 1: Determine the role of DHEAS uptake and HSD3B1 expression in ABI resistance.

Major Task 1: Establish long-term DHEAS- and ABI-treated cells.

Milestone(s) Achieved: Establishment of long-term treated cell lines that survive ABI treatments. (4 months)

Results:

I treated LNCaP cells with 200nM DHEAS with or without 5μM ABI for ≥ 4 months, and cells treated with both agents have greater uptake of DHEAS. In addition to LNCaP cells that harbor mutated AR, LAPC4 cells (WT AR, gift from Charles Sawyers, Howard Hughes Medical Institute) were treated with DHEAS with or without 5μM ABI for ≥ 4 months. The establishment of these ABI-resistant cells allows me to determine whether ABI-resistant CRPC utilizes DHEAS.

Major Task 2: Evaluate changes in DHEAS uptake.

Milestone(s) Achieved: Link increased DHEAS uptake with ABI-resistance status. (12 months)

Results:

I compared the uptake of [^{3}H]-DHEAS in both normal prostate cancer cells and ABI-resistant cells established by culturing the cells for ≥ 4 months in 10% fetal bovine serum (FBS) phenol red-free RPMI media containing 200nM DHEAS with 5μM ABI. 200nM DHEAS falls within the range of serum concentrations of DHEAS observed in patients after ADT and ABI combination treatment. The cells were serum starved for 48 h with 10% charcoal stripped fetal bovine serum (CSS) phenol red-free RPMI to remove residual steroid in the cells, and then incubated with 200μM DHEAS spiked with [^{3}H]-DHEAS. My results suggest that ABI treatment confer increased DHEAS uptake in the cells (Figure 1).

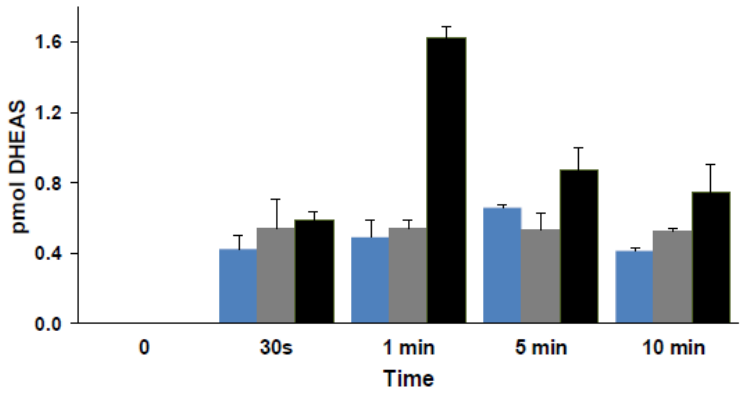

Figure 1. Uptake of [3H]-DHEAS in untreated LNCaP cells (blue), long-term DHEAS-treated LNCaP cells (gray), and long-term DHEAS-abiraterone-treated LNCaP cells (black). Cells were incubated with 200nM DHEAS spiked with [^{3}H]-DHEAS for the indicated time points upon washing with ice cold buffer. The cell samples were lysed and measured on a liquid scintillation counter. Bars represent pmol DHEAS in the cell lysate. Error bars = SD.

Specific Aim 2: Define the metabolic pathway and regulatory enzymes of DHEAS metabolism to DHT.

Major Task 3: Measure metabolism of long-term ABI-treated LNCaP and LAPC4 cells.

Milestone(s) Achieved: Determination of the effect of hypoxia on DHEA metabolism. (12 months)

Results:

Prolonged hypoxia (over 48h) led to severe cell death in my study, which prevented me from investigating the effect of chronic hypoxia on androgen metabolism. Since cancer cells experience long-term hypoxia *in vivo* resulting from disorganized vasculature, I established hypoxia-resistant cell lines which can survive under chronic hypoxia to better simulate the hypoxic condition *in vivo*. The cells were grown under cyclic hypoxia and reoxygenation for several weeks with gradually increased hypoxia exposure time. The cells became resistant to hypoxia (named as HR cells) after several passages. HR cells showed morphologic change compared with their normal counterparts which are sensitive to hypoxia (named as HS cells) and exhibited flatter and stretched shape (Figure 2A). A recent study on transgenic adenocarcinoma mouse prostate (TRAMP) model revealed three hypoxia-responsive genes: HES6, KDM3A, and SOX9. Here my results suggested that KDM3A and SOX9 were significantly upregulated in HR cells at mRNA levels, while HES6 was upregulated in HR LNCaP but not HR C4-2 (Figure 2B). In terms of the enzymes involved in androgen metabolism, dramatic upregulation was observed in HR cells, except 17βHSD4 and UGT2B15 (Figure 2C). 17βHSD4 expression was significantly decreased in HR cells (Figure 2C). Both HS and HR LNCaP exhibited same level of UGT2B15 (Figure 2C). The significant alteration of the protein level of these enzymes in HR cells may suggest their critical roles for cell survival under chronic hypoxia. The consistency was observed on the change of mRNA and protein level of 3βHSD1, 17βHSD4 and AKR1C3, while SRD5A1, 17βHSD2 and 17βHSD3 exhibited divergence on the change of their mRNA and protein levels (Figure 2C and D).

Another hallmark of HR cells was the downregulation of AR pathway (Figure 2B, C and D). Previous studies suggested that the transactivation activity of AR was increased rapidly in response to acute hypoxia (4h). However, my study revealed that chronic hypoxia dampened AR pathway by downregulating AR at mRNA and protein level. The significantly low activity of AR pathway may contribute to the slow growth of HR cells under hypoxia.

To investigate the consequence of the altered enzyme expression in HR cells, I compared the metabolism of DHEA and AD in HS cells under normoxia and HR cells under hypoxia. Surprisingly, HR cells exhibited decreased conversion of androgen precursors to downstream metabolites, and much longer preservation of androgen precursors compared with HS cells (Figure 2F and G), despite much higher level of the enzymes involved in androgen synthesis. HR cells exhibited drastically lower level of all 4 cofactors compared with HS cells, especially NADH and NADPH (Figure 2E). This may suggest that cofactors rather than the protein level of the enzymes play a decisive role in androgen synthesis. The robust upregulation of NAMPT, the enzyme catalyzing the speed-limiting step in NAD^+ biosynthesis, may help HR cells to compensate for the decreased cofactor to adapt to chronic hypoxia (Figure 2C).

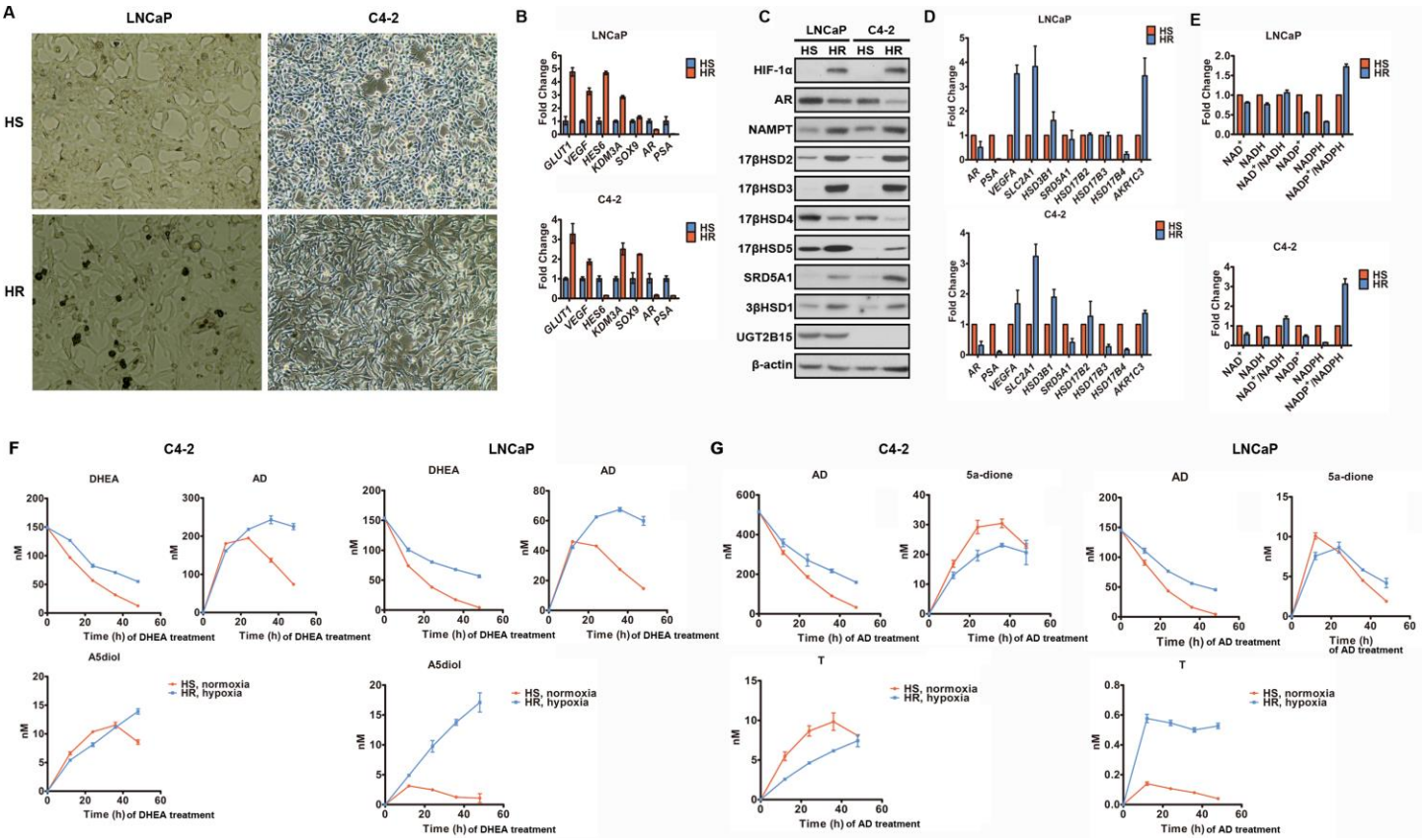

Figure 2. Hypoxia-resistant cells exhibit decreased conversion of androgen precursors to downstream metabolites, and increased preservation of androgen precursors. a. Compare the morphology of both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia. b. qPCR to determine the mRNA levels of hypoxia-responsive genes in both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia. c. Western blot to determine the protein level of the enzymes involved in androgen metabolism in both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia. d. qPCR to determine the mRNA level of the enzymes involved in androgen metabolism in both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia. e. Measurement of 4 co-factors as well as the calculation of the ratios of NAD^+/NADH and $NADP^+$/NADPH in both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia. f. Metabolism of DHEA in both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia. g. Metabolism of AD in both hypoxia-sensitive LNCaP and C4-2 cells under normoxia and hypoxia-resistant LNCaP and C4-2 cells under hypoxia.

Since HR cells exhibited decreased conversion of androgen precursors to downstream metabolites under chronic hypoxia, I examined whether reoxygenation can reverse this effect in HR cells. I compared the metabolism of DHEA and AD in HR cells under hypoxia and reoxygenation. HR cells exhibited decreased A5diol synthesis and increased 5α-dione synthesis after reoxygenation (Figure 3C and D). The consumption of DHEA and AD was also increased moderately after reoxygenation (Figure 3C and D). To explore the mechanism of the altered metabolism, I measured the level of both enzymes and cofactors involved. The protein level of the enzymes kept unchanged up to 24 hours after reoxygenation, and obvious change was only detected after 48 hours (Figure 3A). However, cofactors exhibited much more rapid response to reoxygenation that 12h reoxygenation led to significant upregulation of all 4 cofactors, especially NADH and NADPH (Figure 3B). Therefore, the rapid increase of cofactors may play the critical role in reoxygenation-mediated alteration of androgen synthesis. My study suggests that cyclic hypoxia-reoxygenation, a common feature of tumor, confers on HR cells efficient utilization of androgen precursors. During hypoxia under which the cells lack nutrient to proliferate, the androgen precursors persist much longer, and the enzyme level is upregulated, then consequent reoxygenation facilitates androgen production by rapidly increasing cofactor level without reducing protein level of the enzymes in short term.

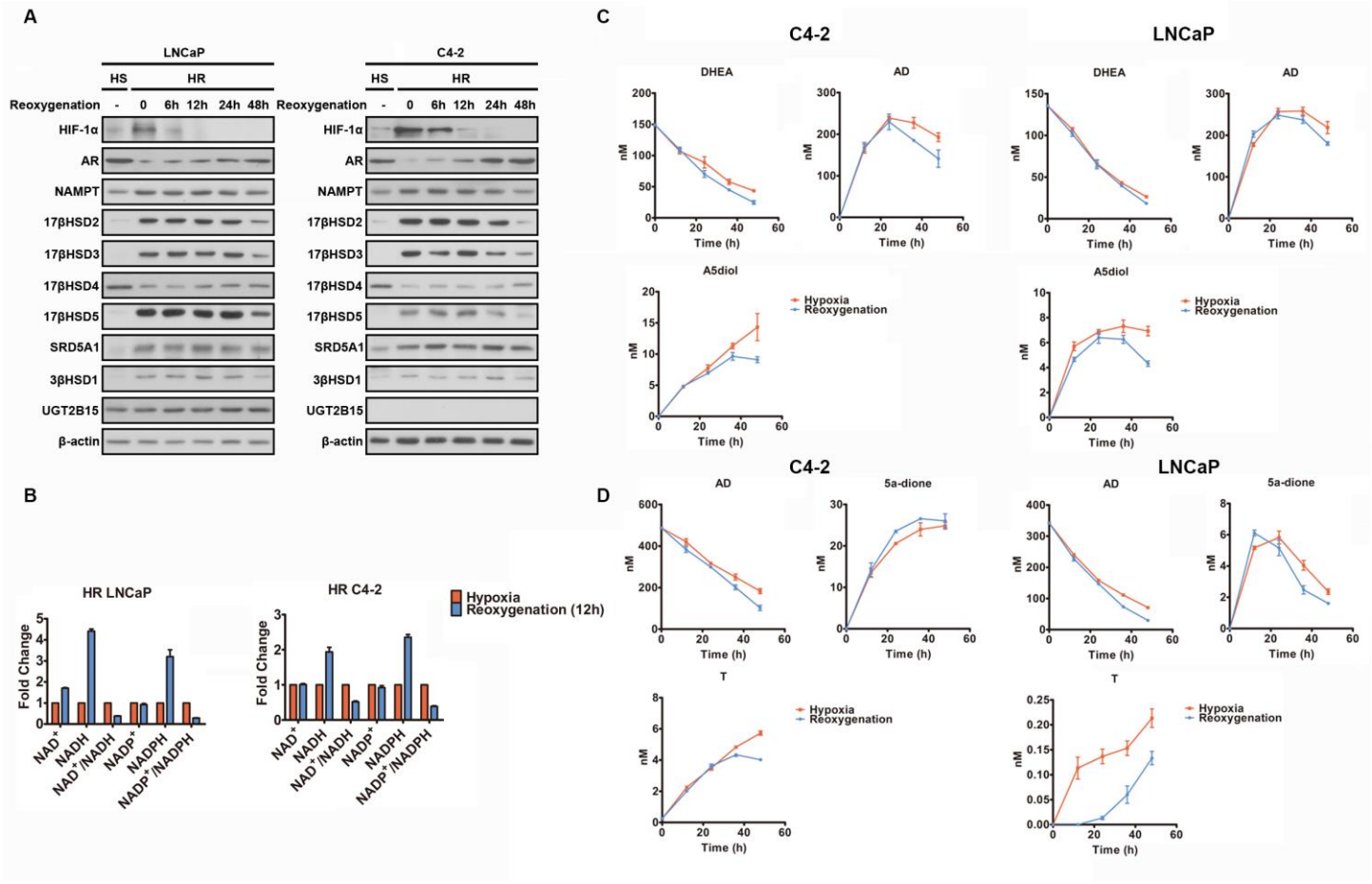

Figure 3. Survey of androgen metabolism under acute reoxygenation. a. Western blot to determine the protein levels of the enzymes involved in androgen metabolism in hypoxia-resistant LNCaP and C4-2 cells with and without short reoxygenation. b. Measurement of 4 co-factors as well as the calculation of the ratios of NAD^+/NADH and $NADP^+$/NADPH in hypoxia-resistant LNCaP and C4-2 cells with and without short reoxygenation. c. Metabolism of DHEA in hypoxia-resistant C4-2 and LNCaP cells with and without reoxygenation. d. Metabolism of AD in hypoxia-resistant C4-2 and LNCaP cells with and without reoxygenation.

I further compared the metabolism of DHEA and AD in HR cells after two-day reoxygenation and in HS cells under normoxia. HR cells exhibited even faster conversion of androgen precursors, especially DHEA, to downstream metabolites than HS cells (Figure 4A and B). HR cells also exhibited more induction of AR targets expression by DHEA than HS cells (Figure 4C). To investigate the effect of reoxygenation on protein level of the enzymes involved in androgen metabolism, I exposed HR cells under normoxia for several days and then hypoxia again. HR cells exhibited plasticity on the expression of these enzymes. The protein level of all the enzymes whose level was changed under chronic hypoxia was reverted to the that in HS cells gradually under normoxia (Figure 4D). Then I transferred these HR cells back to hypoxia again after 7-day incubation under normoxia. Interestingly, such re-incubation under hypoxia resulted in the protein level of 17βHSD2 and 17βHSD4 switched back to that before transfer to normoxia, different from other enzymes which did not show such rapid response to the re-incubation under hypoxia (Figure 4D). Compared with HS cells, HR cells exhibited similar NAD^+, $NADP^+$, NADPH but slightly higher NADH levels after two-day reoxygenation (Figure 4E). Therefore, the higher level of 3βHSD1 may contribute to the faster conversion of DHEA to downstream metabolites in HR cells.

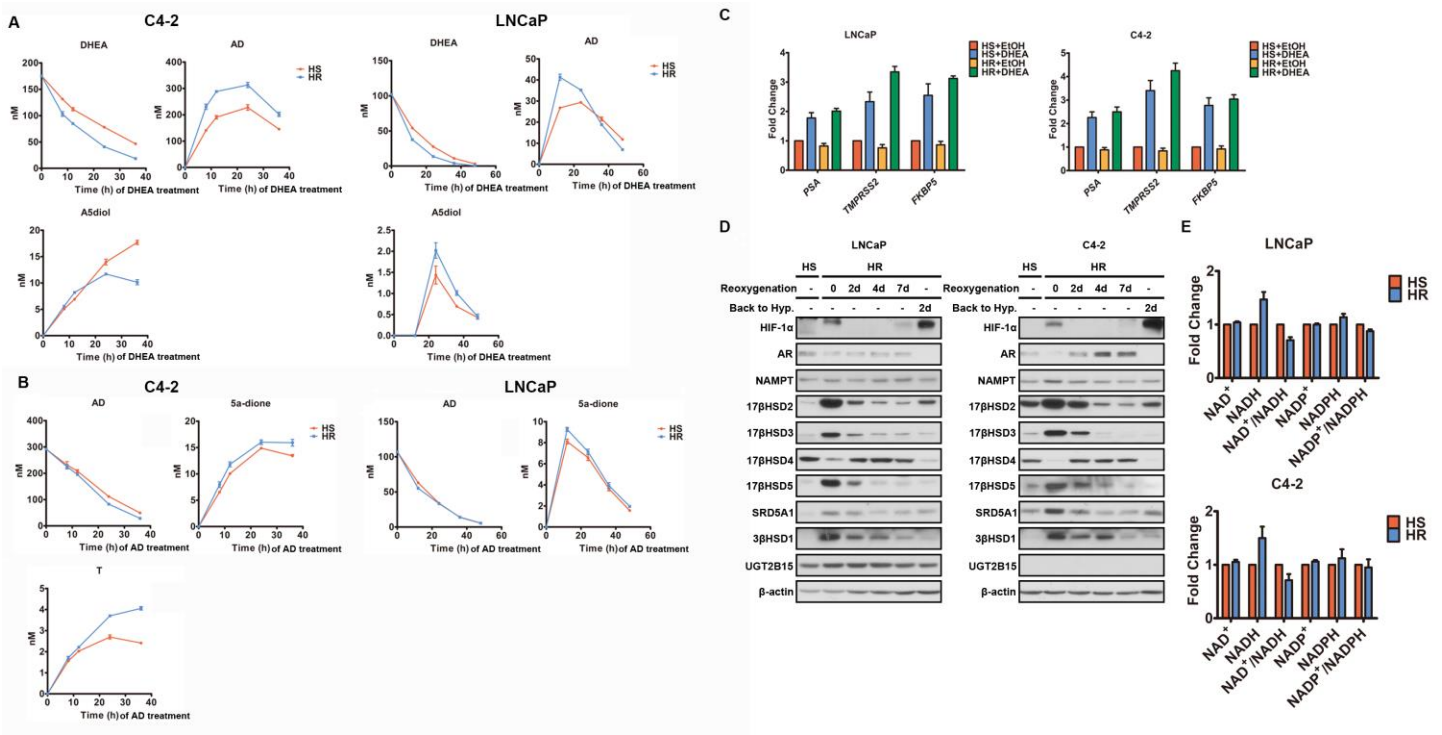

Figure 4. Chronic hypoxia reversibly upregulates 3βHSD1. a. Metabolism of DHEA in both HS and HR C4-2 and LNCaP cells under normoxia. b. Metabolism of AD in both HS and HR C4-2 and LNCaP cells under normoxia. c. qPCR to determine the mRNA levels of AR targets in both HS and HR C4-2 and LNCaP cells after DHEA treatment. d. Western blot to determine the protein levels of the enzymes involved in androgen metabolism in HR LNCaP and C4-2 cells with and without long reoxygenation. e. Measurement of 4 co-factors as well as the calculation of the ratios of NAD^+/NADH and $NADP^+$/NADPH in HS LNCaP and C4-2 cells under normoxia, and HR LNCaP and C4-2 cells with reoxygenation.

Specific Aim 3: <u>Define the regulatory mechanism of 3βHSD1 by hypoxia.</u>

Major Task 4: Identify the key pathways and regulators responsible for hypoxia-mediated 3βHSD1 upregulation.

<u>Milestone(s) Achieved</u>: Determination of EGLN1/VHL/HIF2 pathway that is responsible for hypoxia-mediated 3βHSD1 upregulation. (24 months)

Results:

3βHSD1-catalyzed conversion of DHEA to AD is a proximal step in peripheral tissues for DHT synthesis. Therefore, I focused on the mechanism of 3βHSD1 upregulation in HR cells. Inhibition of EGLN/VHL pathway plays a critical role in hypoxic response. Therefore, I determined whether EGLN/VHL pathway inhibition is responsible for 3βHSD1 upregulation. As all three isoforms of EGLN were knocked-down by siRNA to simulate hypoxia, only EGLN1 knockdown induced significant upregulation of HIF1α and HIF2α, as well as 3βHSD1 under normoxia (Figure 5A and B). While EGLN2 knockdown only increased HIF1α, HIF2α, and 3βHSD1 slightly, EGLN3 knockdown showed no effect at all (Figure 5A and B). Interestingly, EGLN2 knockdown also decreased the level of EGLN3 to a large extent by unknown mechanism (Figure 5A and B). As an E3 ubiquitin ligase, VHL is responsible for degrading the proteins hydroxylated by EGLN. Therefore, I also determined whether VHL is involved in hypoxia-induced 3βHSD1 upregulation. VHL knockdown significantly increased HIF1α and HIF2α, as well as 3βHSD1 under normoxia (Figure 5C and D). DHEA-mediated upregulation of AR targets was also enhanced by EGLN1 and VHL knockdown (Figure 5E). These data together suggested that EGLN1/VHL pathway inhibition is sufficient to induce 3βHSD1 expression.

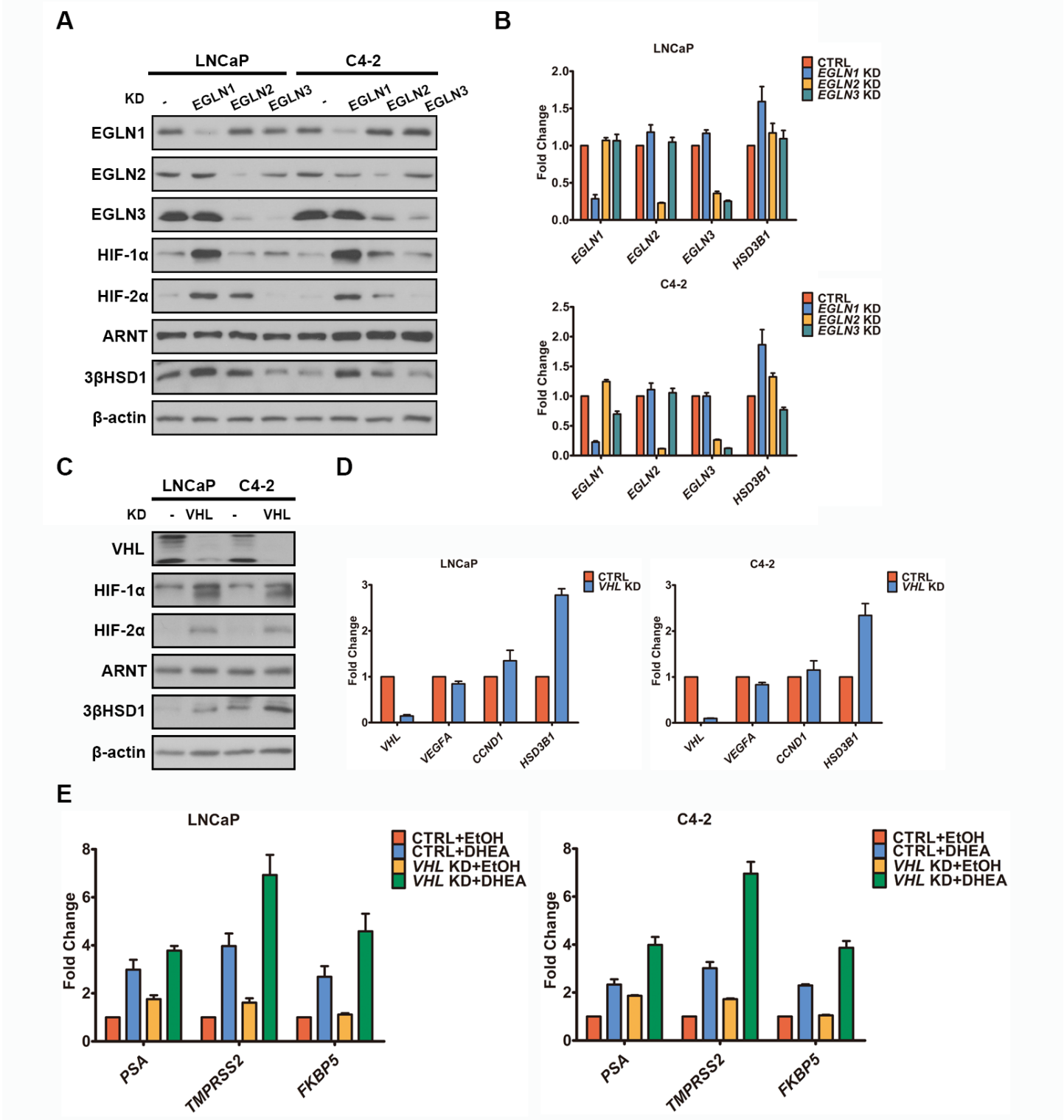

Figure 5. 3βHSD1 is upregulated by hypoxia via EGLN1/VHL pathway. a. Western blot to determine the protein levels of 3βHSD1 in the cells with and without EGLN1, 2, 3 knock-down. b. qPCR to determine the mRNA levels of 3βHSD1 in the cells with and without EGLN1, 2, 3 knock-down. c. Western blot to determine the protein levels of 3βHSD1 in the cells with and without VHL knock-down. d. qPCR to determine the mRNA levels of 3βHSD1 in the cells with and without VHL knock-down. e. qPCR to determine the mRNA levels of AR targets in LNCaP and C4-2 cells with and without VHL knock-down after DHEA treatment.

My data suggested that 3βHSD1 was elevated at mRNA level in HR cells and by EGLN1/VHL inhibition under normoxia. HIF, a transcription factor, is activated by hypoxia-induced EGLN/VHL inhibition, as a major cellular response to hypoxia. Therefore, I determined whether 3βHSD1 is a target of HIF. Dominant positive HIF1α and HIF2α were overexpressed, and both of them contain the mutations (P402A/P564A/N803A on HIF1α and P405A/P531A/N847A on

HIF2α) which abolish hydroxylation-mediated degradation and inhibition of coactivator binding. 3βHSD1 was increased at both mRNA and protein levels by the overexpression of HIF2α but not HIF1α (Figure 6A and B). This suggested that 3βHSD1 is a downstream target of HIF2α but not HIF1α. I also examined the expression of several known HIF targets as positive controls. Interestingly, they exhibited distinct regulation patterns by the overexpression of HIF1α and HIF2α (Figure 6A and B). As the association with ARNT is required for the transcriptional activity of both HIF1α and HIF2α, I also determined whether ARNT is required for HIF2α-induced 3βHSD1 upregulation. Knockdown of either HIF2α or ARNT by siRNA decreased 3βHSD1 in the cells with HIF2α overexpression (Figure 6C). HIF2α overexpression-mediated 3βHSD1 upregulation was also largely abolished in the cells with ARNT knockdown by shRNA (Figure 6D). To determine the significance of HIF2α-mediated 3βHSD1 upregulation, I compared DHEA metabolism in the cells with and without HIF2α overexpression. Conversion of DHEA to AD was increased by HIF2α overexpression (Figure 6E), but not HIF1α overexpression (Figure 7A and B). DHEA-mediated upregulation of AR targets was also enhanced by HIF2α overexpression (Figure 6F).

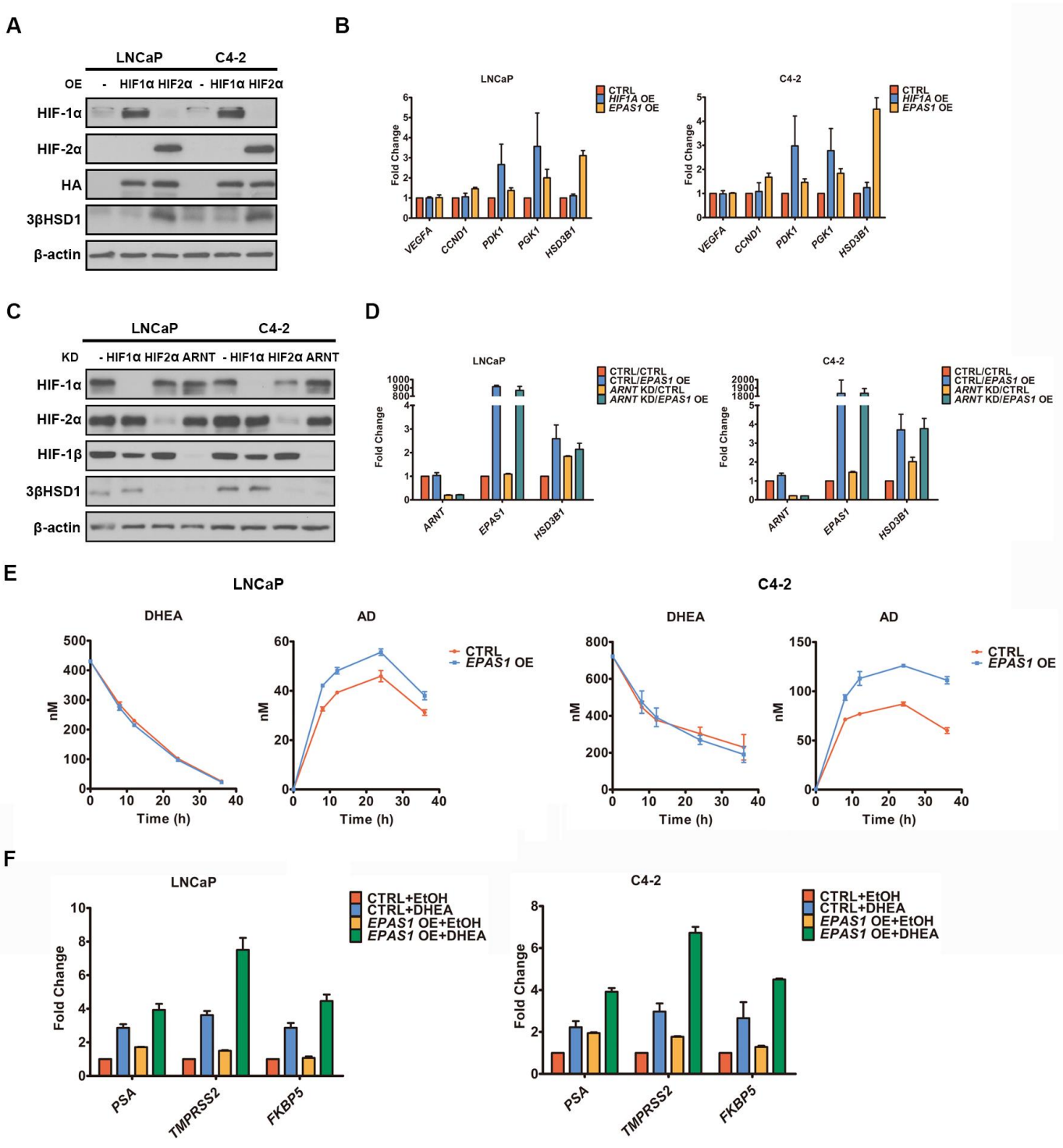

Figure 6. 3βHSD1 is upregulated by Hif2α. a. Western blot to determine the protein levels of 3βHSD1 in the cells with and without HIF1α and HIF2α overexpression. b. qPCR to determine the mRNA levels of 3βHSD1 in the cells with and without HIF1α and HIF2α overexpression. c. Western blot to determine the protein level of 3βHSD1 in HIF2α-overexpressing cells with HIF1α, HIF2α, and HIF1β knockdown. d. qPCR to determine the mRNA level of 3βHSD1 in HIF2α-overexpressing cells with and without HIF1β knockdown. e. Metabolism of DHEA in LNCaP and C4-2 cells with and without Hif2α overexpression. f. qPCR to determine the mRNA levels of AR targets in LNCaP and C4-2 cells with and without HIF2α overexpression after DHEA treatment.

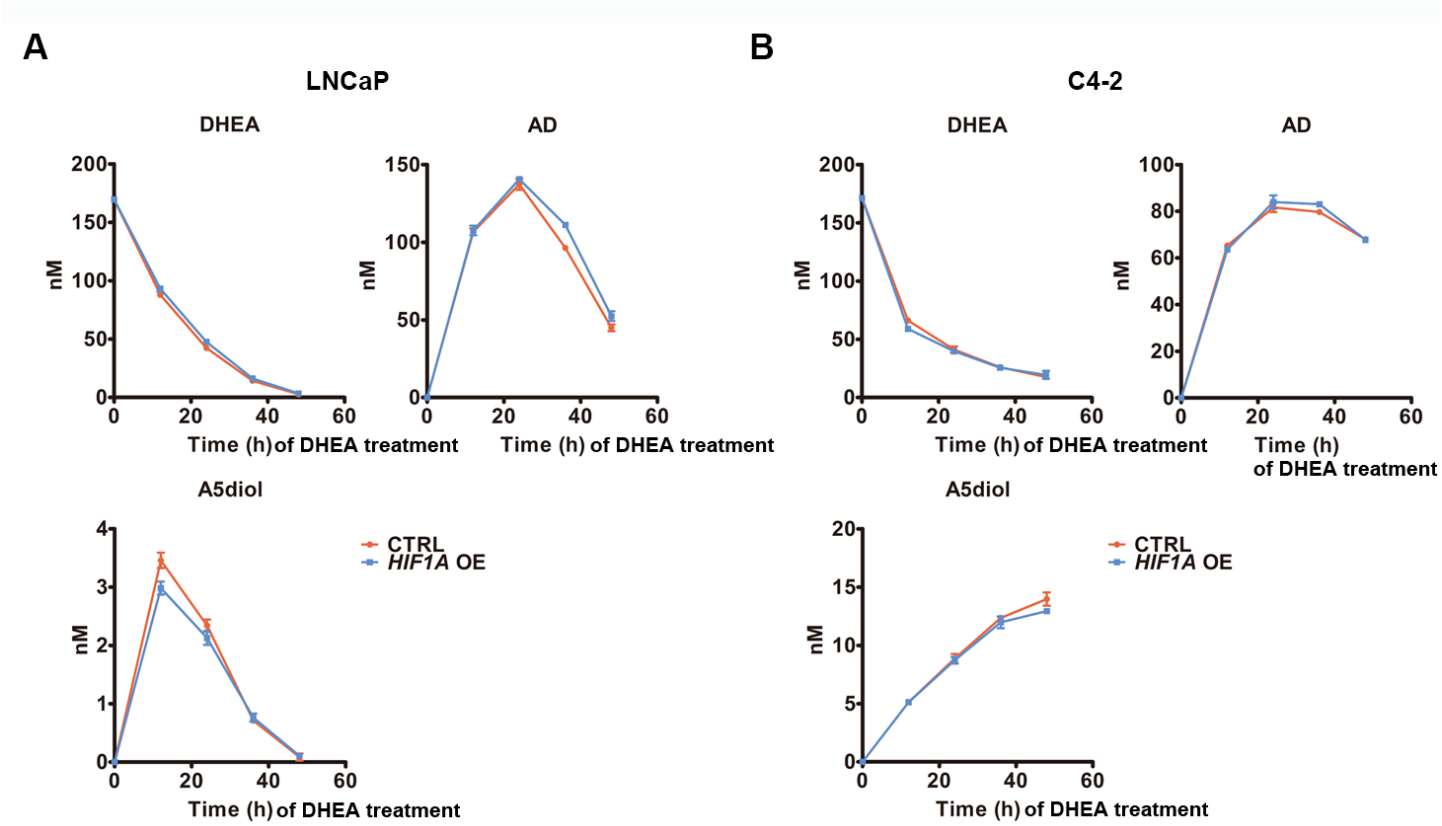

Figure 7. Overexpression of HIF1α cannot increase 3βHSD1 activity. a. Metabolism of DHEA in LNCaP cells with and without HIF1α overexpression. b. Metabolism of DHEA in C4-2 cells with and without HIF1α overexpression.

Binding to EP300, a co-activator of transcription factors, was suggested critical for transcriptional activity of HIF1α, but not HIF2α. However, my data suggested that 3βHSD1 was decreased by EP300-knockdown in HR cells (Figure 8A and B). Moreover, the mutation of N847 to A which abolished FIH-mediated HIF2α hydroxylation that inhibits EP300 binding further enhanced HIF2α-mediated 3βHSD1 upregulation (Figure 8C and D). These data together suggested that HIF2α-mediated 3βHSD1 upregulation requires EP300 binding.

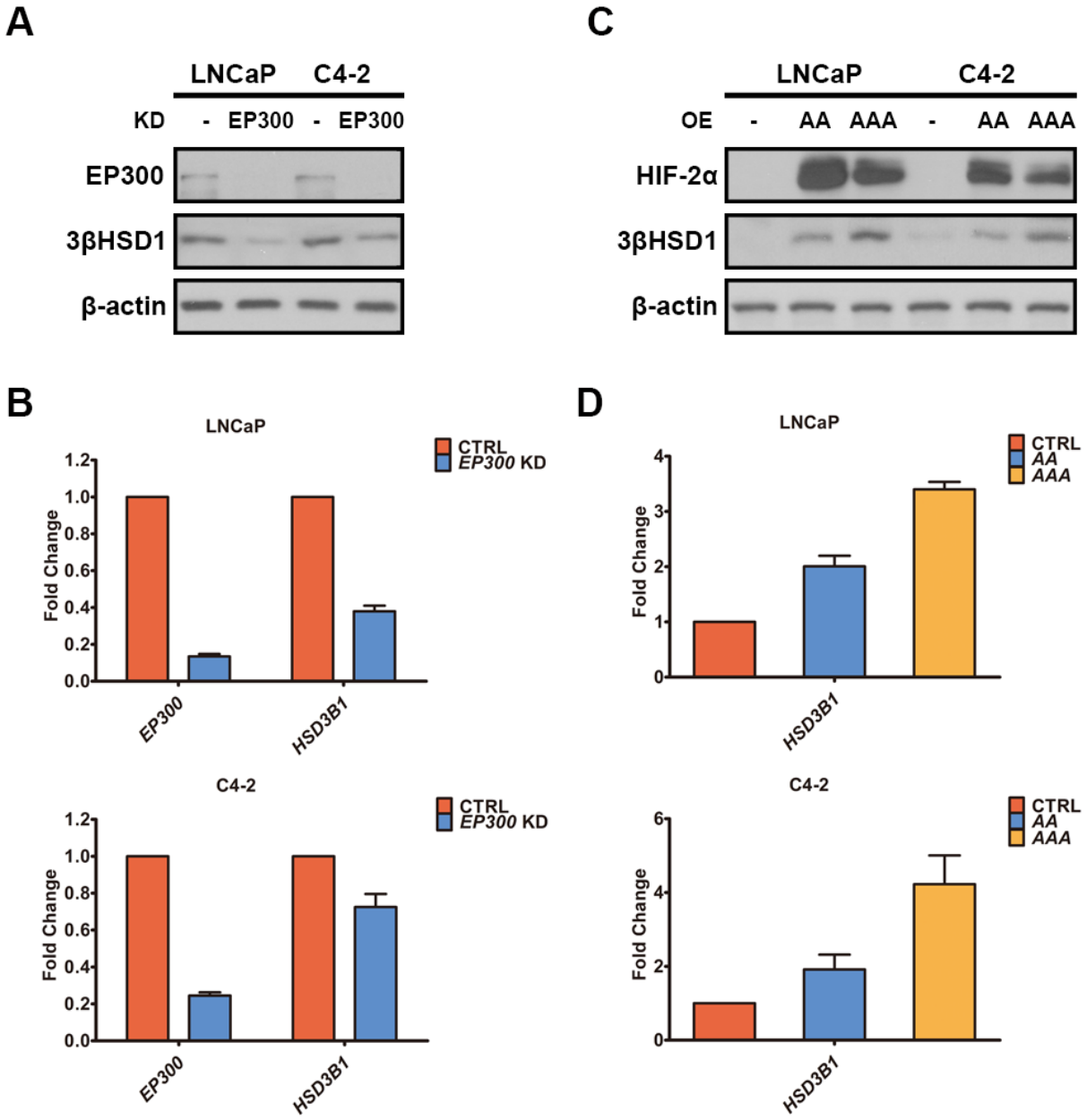

Figure 8. HIF2α-mediated 3βHSD1 upregulation requires EP300. a. Western blot to determine the protein levels of 3βHSD1 in HR cells with and without EP300 knockdown. b. qPCR to determine the mRNA levels of 3βHSD1 in HR cells with and without EP300 knockdown. c. Western blot to determine the protein levels of 3βHSD1 in the cells with and without HIF2α (AA and AAA) overexpression. d. qPCR to determine the mRNA levels of 3βHSD1 in the cells with and without HIF2α (AA and AAA) overexpression.

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

1. Attended Conduct of Research and Human Subjects training.
2. Attended monthly Prostate Cancer Working Group and Seminar Series
3. Attended and presented research at the weekly lab meetings and journal clubs
4. Attended and presented work at the weekly CCF Department of Cancer Biology seminars
5. Attended Kenyon Institute's Biomedical and Scientific Writing workshop
6. Attended AACR annual meeting

How were the results disseminated to communities of interest?

1. Presented my work at weekly lab meetings

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Although intensive study has been done, prostate cancer remains a leading cause of cancer deaths in American men. Androgen deprivation therapy as the first-line treatment for advanced PCa leads to castration-resistant prostate cancer after 24 months, despite initial success. Abiraterone acetate, a CYP17A1 inhibitor, only prolongs patient survival by about 4-5 months. Despite blockade of CYP17A, significant serum concentrations of DHEAS persist after abiraterone treatment. My study indicates that abiraterone-resistant cells uptake and utilize DHEAS to synthesize potent androgen such as testosterone and DHT in abiraterone-resistant PCa.

Hypoxia as a hallmark of many types of human tumors plays an important role in tumorigenesis. However, how hypoxia is involved in androgen synthesis in prostate cancer is largely unknown so far. My study here suggests that hypoxia alters DHEA metabolism via regulating the expression of HSD3B1 as well as co-factors required for HSD3B1 activity.

Therefore, my study laid the foundation for future studies of the clinical significance of DHEA metabolism in prostate cancer cells, with the eventual goal of developing new clinical treatment strategies, as well as a potential biomarker for personalized treatment.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

My project revealed that 3βHSD1 as well as the co-factors play critical roles in DHEA metabolism under the regulation of hypoxia. Hypoxia level could be a potential biomarker for ABI-resistant prostate cancer diagnosis.

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

PRODUCTS:

Publications, conference papers, and presentations

Manuscript in preparation

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers, and presentations.

Nothing to Report

Website(s) or other Internet site(s)

Nothing to Report

Technologies or techniques

Nothing to Report

Inventions, patent applications, and/or licenses

Nothing to Report

Other Products

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Liang Qin*
Project Role:	*Principal investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3928-8956*
Nearest person month worked:	20
Contribution to Project:	*Liang Qin is responsible for designing, performing and interpreting experiments and manuscript preparation.*
Funding Support:	**National Cancer Institute R01CA168899** Investigator: Nima Sharifi **American Cancer Society 12–038-01-CCE** Investigator: Nima Sharifi **National Cancer Institute R01CA172382** Investigator: Nima Sharifi **National Cancer Institute R01CA190289** Investigator: Nima Sharifi **Howard Hughes Medical Institute Physician-Scientist Early Career Award** Investigator: Nima Sharifi

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No Change.

What other organizations were involved as partners?

Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report.

QUAD CHARTS:

Nothing to Report.

APPENDICES: