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TITLE: Inhibiting Lysine-Specific Demethylase 1 Activity as a Potential Therapeutic Treatment for Castration-Resistant Prostate Cancer

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14. ABSTRACT

Persistent AR expression and activity are found in the majority of castration-resistant prostate cancer (CRPC) and CRPC resistant to enzalutamide, indicating a pressing need for further development of novel AR-targeted therapies. Lysine-Specific Demethylase 1 (LSD1) functions as a transcriptional corepressor through demethylation of histone 3 lysine 4 (H3K4) but also has a coactivator function on AR with unclear mechanism. We showed that LSD1 broadly regulates AR function by possibly affecting enhancer availability prior to androgen stimulation through interaction with pioneer factor FOXA1. LSD1 inhibition globally disrupted FOXA1 chromatin binding prior to androgen treatment and thus impaired further AR recruitment, resulting in the global inhibition of AR transcriptome. In this project, we have then discovered that methylated lysine 270 (K270) residue of FOXA1 is a direct substrate of LSD1 and its demethylation stabilized AR recruitment. Our findings provide novel mechanistic insights on the role of LSD1 in PCa development and suggest that LSD1 inhibition may be a new therapeutic strategy in treating AR-driven CRPC.

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TABLE OF CONTENTS

Page

INTRODUCTION

Androgen receptor (AR) signaling is critical for prostate cancer (PCa) development and remains important for PCa that relapse from the androgen deprivation therapy (called castration-resistant PCa or CRPC) including the more aggressive androgen deprivation therapies, such as enzalutamide. Therefore, there is a pressing need to develop novel strategies targeting AR signaling. Lysine-specific demethylase 1 (LSD1, KDM1A) is well known for its function to demethylate histone 3 lysine 4 and repress gene transcription. However, LSD1 can also function as a coactivator of AR in PCa cells with an unclear mechanism. We showed previously that LSD1 is associated with FOXA1 and activates AR-dependent enhancers to facilitate the transcription of androgen-regulated genes. Our current data suggest LSD1 functions to maintain the accessibility of AR regulated enhancers through promoting the chromatin binding of the pioneer factor FOXA1. Inhibition of LSD1 globally impairs the chromatin binding of FOXA1 and thus disrupts FOXA1-dependent AR cistrome, resulting in the suppression of PCa growth *in vitro* and *in vivo*. Mechanistically, we discovered that LSD1 can directly demethylate FOXA1 at K270 and this demethylation stabilized FOXA1 chromatin binding and opened the chromatin structure at the enhancers. Therefore, our current progress supports the hypothesis that LSD1 is a major therapeutic target to suppress AR/FOXA1 signaling in PCa.

KEYWORDS

LSD1, KDM1A, FOXA1, androgen receptor, AR, CRPC, demethylation

ACCOMPLISHMENTS

- **What were the major goals of the project?**

  Specific Aim 1*: Identify mechanisms of action by which LSD1 maintains FOXA1 chromatin binding and subsequent AR recruitment.*

  Major Task 1: Determine the role of LSD1 on chromatin structure and enhancer distributions

  We will first establish stable PCa cell clones with LSD1 knock-out using CAS9/CRISPR methods and then determine the effects of LSD1 silencing/inhibition on enhancer landscape.

  Month 1-12, Percentage of completion: 90%

  Major Task 2: Determine the role of FOXA1 as a critical LSD1 substrate

  We will validate K270-methylated FOXA1 as an LSD1 substrate and then establish stable PCa clones with FOXA1-K270R mutants to determine the effects of K270-methylated FOXA1 on chromatin bindings of full-length AR or AR-V7 splice variant.

  Month 6-30, Percentage of completion: 75%

  Major Task 3: Identify the additional components of the AR enhancer-associated LSD1/FOXA1 complex

  We will isolate the chromatin-bound LSD1/FOXA1 complex and then identify the components of this complex.

  Month 24-36, Percentage of completion: 10%

  Specific Aim 2*: Assess the therapeutic potential of LSD1 inhibitors in CRPC models.*

  Major Task 4: Major Task 4: Determine the therapeutic efficacy of LSD1 inhibitor treatment alone and in combination with enzalutamide or bromodomain inhibitor in CRPC patient-derived xenograft models.

  We will first generate xenograft models with doxycycline regulated LSD1 downregulation and then assess the efficacy of LSD1 downregulation in xenograft models and determine the mechanisms of action.

  Month 1-36, Percentage of completion: 30%

- **What was accomplished under these goals?**

  Major Activities: Through the support of this grant, I have been able to continue my proposed research in

the Center for Personalized Cancer Therapy, at University of Massachusetts Boston. For research networking, I have established collaborations with labs of Drs. Steven P. Balk (Beth Israel Deaconess Medical Center), Timothy Rebbeck (Harvard TH Chan School of Public Health), Housheng Hansen He (University of Toronto), and Eva Corey (University of Washington). I have actively participated in various seminars and meetings within the Harvard Cancer Center Program, such as the Dana-Farber Cancer Institute prostate cancer SPORE seminars. I have also attended the SBUR (Society for basic Urological Research) annual meeting (Nov 7-10, 2019, New Orleans LA) and a member from my lab has given a poster presentation related to this funded project during the meeting. A manuscript summarizing current results has been accepted and published in *Nature Genetics*.

Specific Objectives: Specific aim 1 is to identify mechanisms of action by which LSD1 maintains FOXA1 chromatin binding and subsequent AR recruitment. Specific aim 2 is to assess the therapeutic potential of LSD1 inhibitors in CRPC models.

Significant Results: For aim 1, we have performed ATAC-seq to determine the effect of LSD1 inhibition on enhancer landscape. We have also validated FOXA1-K270 as a substrate of LSD1 using in vitro demethylation assay and mass-spectrometry analysis and demonstrated that K270R mutant of FOXA1 has increased chromatin binding. For aim 2, we have tested LSD1 inhibitors in two CRPC PDX models. We now summarize the critical results below.

LSD1 silencing decreases FOXA1 binding and the subsequent AR binding. We knocked out LSD1 in CWR22-RV1 cells (LSD1-KO) using the CRISPR/Cas9 approach. The silencing of LSD1 expression was confirmed by immunoblotting LSD1 (**Fig. 1**, right panel). FOXA1 chromatin binding was significantly decreased in the LSD1-KO line versus the control line (**Fig. 1**, left panel). As a result, the chromatin binding of AR-V7 was dramatically decreased in the LSD1-KO line (**Fig. 2**). The DHT-induced AR-FL binding was also decreased by LSD1 silencing (data not shown). These data suggest that LSD1 silencing has a similar suppression effect as LSD1 inhibition on FOXA1 chromatin binding and subsequent ARFL/AR-V7 recruitment.

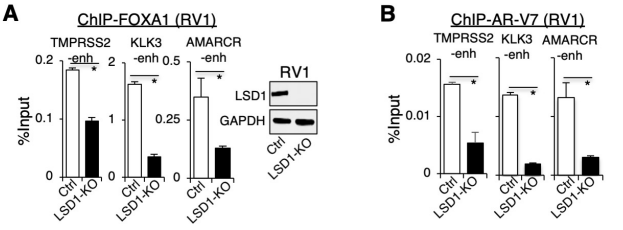

Figure 1. LSD1 silencing decreased FOXA1 and AR-V7 binding. A, LSD1-KO cell line was established in CWR22-RV1 cells using CRISPR/CAS9 approach (by sgRNA targeting LSD1). LSD1 expression was immunoblotted in LSD1-KO line versus control line. **B**, ChIP-qPCR for AR-V7 binding at a panel of known sites in LSD1-KO line versus control line.

LSD1 inhibition represses chromatin opening of FOXA1-occupied enhancers. Since FOXA1 functions to decompact chromosome at enhancers, we next determined whether LSD1 inhibition suppresses enhancer accessibility. Using ATAC-seq, we show that LSD1 inhibition markedly decreased the chromosome accessibility at FOXA1 occupied sites prior to androgen stimulation (**Fig. 2A-B**). LSD1 inhibition also led to a rapid decrease of acetylated-histone 3 lysine 27 (H3K27ac) and DNase hypersensitivity (DHS), confirming that these enhancers are less active prior to AR binding (data not shown). Overall, these data confirmed that chromatin accessibility at FOXA1-binding sites is decreased by LSD1 inhibition.

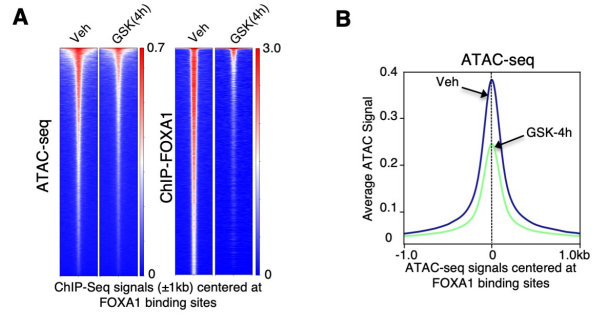

Figure 2. LSD1 inhibition decreased chromatin accessibility at FOXA1-ocupied enhancer. A,B, ATAC-Seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing) was performed in LNCaP cells treated with vehicle or GSK2879552 at 4h. (A) Heatmap view for ATAC-seq signal intensity at FOXA1-binding sites. (B) The mean of ATAC-seq signals at FOXA1-binding sites.

LSD1 regulates FOXA1 chromatin binding through directly demethylating FOXA1 at lysine 270. We hypothesized that LSD1 may regulate FOXA1 binding through direct demethylation of FOXA1. A mass-spectrometry analysis was performed on the immunopurified FOXA1, leading to the identification of methylated-lysine 270 (K270me) as a potential substrate of LSD1. This

residue resides at the carboxyl-end of the wing2 region (aa 247-269) of Forkhead DNA binding domain (FKHD), a hot spot region for recurrent FOXA1 mutations in PCa, and was recently reported as a critical residue for FOXA1 interaction with nucleosome core. The demethylase activity of LSD1 on FOXA1 peptide containing K270me was subsequently determined using a demethylation assay and validated by mass-spectrometry (**Fig. 3A-B**). We then established stable cell lines expressing doxycycline-regulated V5 tagged FOXA1-K270R mutant or FOXA1-WT (wildtype control). The level of lysine-methylated FOXA1 was noticeably reduced in cells expressing methylation-deficient mutant K270R, suggesting K270 is a major methylation site (**Fig. 3C**). We then generated a FOXA1-K270me-specific antibody to validate LSD1-mediated demethylation in PCa cells. As seen in **Fig. 3D**, K270me was only detected in immunoprecipitated FOXA1-WT but not K270R mutant and its level was significantly increased by LSD1 inhibition, confirming that K270me is demethylated by LSD1 in vivo.

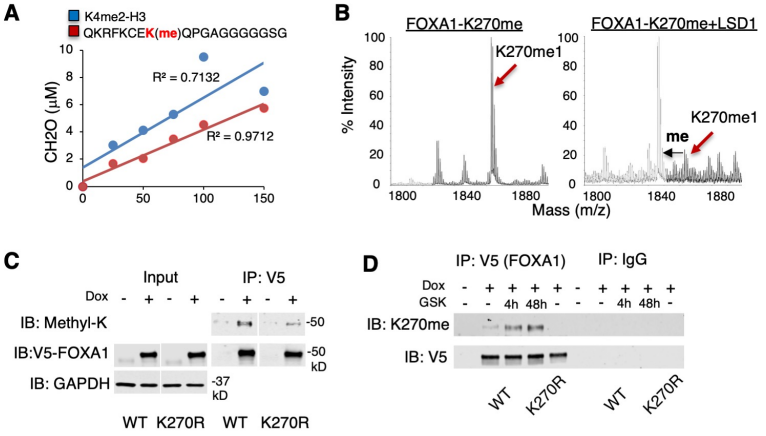

Figure 3. LSD1 regulates FOXA1 chromatin binding through directly demethylating FOXA1 at lysine 270. A, The demethylation assay (measuring formaldehyde production) using synthetic H3K4me2 peptide (1-21aa) or K270-methylated FOXA1 peptide (263-281aa) as substrates incubated with recombinant LSD1 protein. **B**, The K270-methylated FOXA1 peptide incubated with/out 75nM LSD1 (1h) was analyzed by mass spectrometry. **C**, CWR22-RV1 cells stably expressing doxycycline-inducible V5-tagged FOXA1-WT or -K270R (CWR22RV1-tetFOXA1WT or CWR22RV1-tetFOXA1K270R) were generated and methyl-lysine on immunopurified V5-FOXA1 was immunoblotted. **D**, Immunoblotting for FOXA1-K270me on immunopurified V5-FOXA1 protein.

K270 methylation disrupts FOXA1 chromatin binding and thus destabilizes further recruitment of AR. We next sought to determine the role of K270me on FOXA1 chromatin binding and recruitment of AR. Using a biochemical fractionation assay, we show that the K270R mutant was more tightly associated with chromatin (**Fig. 4A**). Moreover, global FOXA1 binding intensity was significantly higher in K270R-expressing cells than WT-expressing cells (**Fig. 4B**), indicating that K270me may disrupt FOXA1 binding to chromatin. Consistently, overexpressing the K270R mutant resulted in a global increase of AR recruitments to chromosome (**Fig. 4C**). Overall, these results indicate that K270 methylation disrupts FOXA1 binding and reduces further recruitment of AR.

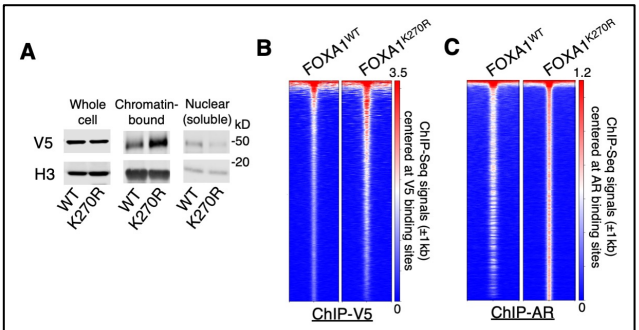

Figure 4. K270 methylation disrupts FOXA1 chromatin binding and thus destabilized AR recruitment. A, CWR22RV1-tetFOXA1^{WT} or CWR22RV1-tetFOXA1^{K270R} cells were fractionated into the soluble nuclear fraction and the insoluble chromatin-bound fraction, followed by immunoblotting for V5 and H3. **B**. V5 ChIP-seq analyses were performed in these stable cells. The heatmap view for V5-FOXA1 binding intensity were shown. **C**. AR ChIP-seq analyses were performed in these stable cells treated with DHT (10nM, 4h), and the heatmap view for AR binding intensity was shown.

LSD1 inhibitor suppressed CRPC growth in vivo. To determine the efficacy of LSD1 inhibitors *in vivo*, we established two CRPC PDX (patient-derived xenograft) models with various expression levels of FOXA1, including LuCaP35CR and LuCaP77CR. LuCaP35CR tumors express high levels of FOXA1 whereas LuCaP77CR tumors have a much weaker expression of FOXA1 (not shown). As shown in **Fig. 5A**, LuCaP35CR tumor growth was significantly repressed by an LSD1 inhibitor, GSK2879552. This treatment also suppressed FOXA1 chromatin binding and the expression of AR-FL/V7-regulated genes (not shown). However, the tumor responses to the LSD1 inhibitor in LuCaP77CR model were much weaker (**Fig. 5B**) and the suppression of AR-FL/AR-V7 signaling was not significant (not shown). LSD1 inhibition can similarly increase H3K4me2 in both models (not shown). Taken together, these *in vivo* animal studies suggest that the efficacy of LSD1 inhibitor treatment in CRPC may be correlated with

the expression levels of FOXA1.

Summary of results: These findings indicate that FOXA1 chromatin binding is regulated by LSD1 mediated demethylation at K270 and by this mechanism, LSD1 maintains the enhancer accessibility to AR or its splice variant AR-V7 and thus regulates its chromatin binding and transcriptional output. Our findings suggest that LSD1 inhibition is a promising therapeutic strategy to target FOXA1-AR signaling in CRPC. These results have been summarized in a manuscript that was recently accepted and published by *Nature Genetics*.

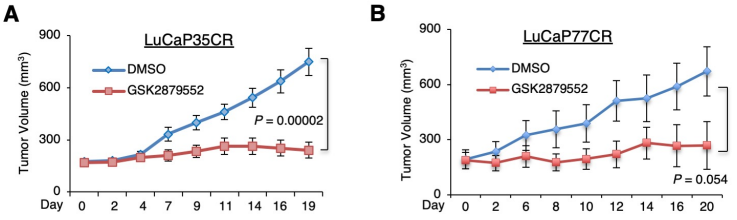

Figure 5. LSD1 inhibitor treatment suppressed CRPC tumor growth. A, **B**, Castrated SCID male mice bearing (A) LuCaP35CR or (B) 77CR xenograft tumors received daily DMSO or GSK2879552 (33mg/kg) via intraperitoneal injection. The tumor growth was recorded.

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

  Nothing to Report

- **How were the results disseminated to communities of interest?**

  Nothing to Report

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

  For Aim 1, we will focus on determining the effects of K270R mutant on the chromatin opening by ATAC-seq and AR-V7 binding by ChIP-seq. We will also start to isolate the FOXA1-LSD1 complex and identify the components of this complex. For Aim 2, we will continue to assess the effects of LSD1 downregulation/inhibitor treatment in additional CRPC PDX models to further determine if different genetic backgrounds of CRPC can alter the response of treatment. We will also start to assess the combination treatment of LSD1 inhibitor and BRD4 inhibitor in CRPC xenograft models.

IMPACT

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

  We have demonstrated that LSD1 inhibitors can globally disrupt the chromatin binding of FOXA1, close the chromatin structure of FOXA1-bound enhancers, and suppress the chromatin binding and activities of AR and AR splice variants. Recent studies also revealed a high frequency of treatment-induced structural alterations of FOXA1 in CRPC (over 25%), which lead to FOXA1 overexpression. Therefore, LSD1 inhibitors can be used to directly target FOXA1 binding, which is essential for the restored AR signaling in CRPC. Overall, the current development of the project has provided a solid foundation for the clinical development of LSD1 inhibitor in treating CRPC, particularly CRPC with high expression of FOXA1 and/or ligand-binding domain truncated AR splice variants.

- **What was the impact on other disciplines?**

  Nothing to Report

- **What was the impact on technology transfer?**

  Nothing to Report

- **What was the impact on society beyond science and technology?**

  Nothing to Report

CHANGES/PROBLEMS

Nothing to Report

PRODUCTS

- **Publications, conference papers, and presentations**

  Gao S, Chen S, Han D, Wang Z, Li M, Han W, Besschetnova A, Liu M, Zhou F, Barrett D, Luong MP, Owiredu J, Liang L, Ahmed M, Petricca J, Patalano S, Macoska JA, Corey E, Chen S, Balk SP, He HH, and **Cai C**. (**2020**) Lineage-Specific chromatin binding of FOXA1 is regulated by LSD1-mediated demethylation. ***Nature Genetics***. [Epub]

  Teng M, Zhou S, **Cai C**, Lupien M, and He HH. (**2020**) Pioneer of prostate cancer – past, present, and the future of FOXA1. ***Protein & Cell***. [Epub]

  Wang Z, Gao S, Han D, Han W, Li M, and **Cai C**. (**2019**) LSD1 activates PI3K/AKT signaling through regulating p85 expression in prostate cancer cells. ***Frontiers in Oncology***. eCollection 2019.

- **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 ORGANIZATION

- **What individuals have worked on the project?**

Name:	*Changmeng Cai*
Project Role	*Principle Investigator*
Research Identifier (e.g. ORCID ID):	*0000-0002-8701-2586*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Cai has been in charge of the overall administration and execution of this project, supervising the graduate student, and*

	coordinating the preparation of manuscripts describing the work.
Funding Support:	NIH R01CA211350

Name:	*Zifeng Wang*
Project Role	*Graduate Student*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*7.5*
Contribution to Project:	*Zifeng has been working on running molecular biology assays related to this project, including RT-PCR, immunoblotting, ChIP, flow cytometry, and high-throughput sequencing*
Funding Support:	*N/A*

Name:	*Dong Han*
Project Role	*Postdoc*
Research Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4*
Contribution to Project:	*Dong has been working on designing experiments, running molecular biological assays, and supervising graduate students.*
Funding Support:	*N/A*

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

  Nothing to Report

- **What other organizations were involved as partners?**

  Nothing to Report

SPECIAL REPORTING REQUIREMENTS

Nothing to Report

APPENDICES

N/A