AWARD NUMBER: W81XWH-13-1-0163

TITLE: Novel Therapeutic Targets to Inhibit Tumor Microenvironment-Induced Castration-Resistant Prostate Cancer

PRINCIPAL INVESTIGATOR: Feng Yang, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX 77030

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel 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 2016	Annual	15Sept2015 - 14Sept2016

4. TITLE AND SUBTITLE
Novel Therapeutic Targets to Inhibit Tumor Microenvironment-Induced Castration-Resistant Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0163

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Feng Yang, Ph.D.

E-Mail: fyang@bcm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

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

U.S. Army Medical Research and Materiel 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

We previously demonstrated that stromal TGF-beta signaling induced the expression of several AR targets as well as MAPK4 in PCa LNCaP cells, and that MAPK4 induced ligand-independent AR activation in PCa cells. Therefore, we proposed to use in vitro PCa/stroma co-culture models and in vivo xenograft models to test our hypothesis on stromal TGF-beta signaling inducing MAPK4 for androgen-independent AR activation in PCa as a direct mechanism for CRPC relapse. In this third year, we solved some previously encountered technical problems and successfully established an LNCaP cell line with Dox-inducible overexpression of MAPK4, and used it to demonstrate that MAPK4 strongly induces AR and GATA2 expression, as well as androgen-independent and -dependent activation of AR in PCa. We also demonstrated that knockdown of MAPK4 greatly inhibited AR activation in the LNCaP/HPS19I co-cultures, supporting a crucial role of MAPK4 in AR activation in these co-cultures. We are exploring several avenues to solve other technical problems, which have caused some significant delays. We expect to be able to fully finish Aim 1 and partially finish Aims 2 and 3 at the conclusion of this award.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

W81XWH-13-1-0163 " Novel Therapeutic Targets to Inhibit Tumor Microenvironment Induced Castration-resistant Prostate Cancer"

Introduction

AR signaling is essential for prostate cancer (PCa) cell growth and survival. Therefore, androgen-deprivation therapy (ADT) is a standard therapy for advanced and metastatic PCa. However, most initially responsive PCa will relapse after ADT and become lethal castration-resistant PCa (CRPC). Interestingly, most CRPCs are still AR-dependent [1, 2]. Hence, AR remains as a therapeutic target for CRPC. To develop novel therapies, it is essential to delineate the underlying mechanisms for the development of CRPC and the re-activation of AR in CRPC. Tumor microenvironment plays critical roles in regulating PCa progression [3]; however, how it affects PCa AR action remains poorly understood. By using an *in vitro* PCa/stroma co-culture model and cDNA microarray analysis, we found that TGF-β signaling in prostate stroma induces the expression of several AR targets, *i.e.* PSA, TMPRSS2, and KLK4, in PCa cells in the absence of androgen. This indicates that prostate stromal TGF-β signaling induces androgen-independent AR activation in PCa. In addition, we found that stromal TGF-β signaling strongly induces MAPK4 expression in PCa. MAPK4 is an atypical MAPK not well studied [4-6]. Its biological role in cancer remains unknown. We showed that MAPK4 overexpression in PCa strongly activates AR independent of androgen, and MAPK4 knockdown inhibits AR activation. These suggest that MAPK4 is a key factor in inducing ligand-independent AR activation in PCa. Finally, cDNA microarray on total RNA extracted from laser-captured PCa cells from human CRPC tumors revealed that MAPK4 expression is strongly correlated with AR activation (expression of PSA) in human CRPC tissues, which strongly supports the human relevance of our studies. Based on our exciting preliminary results, we propose a novel and direct mechanism for the induction of CRPC relapse: Tumor microenvironment (such as stromal TGF-β signaling) induction of MAPK4 drives androgen-independent AR activation in PCa. This pathway to CRPC raises the prospects for novel therapeutic avenues in combination with ADT, anti-TGF-β strategies directed toward the tumor microenvironment, and anti-MAPK4 therapy directed to the tumor itself. We will test our novel hypothesis by *in vitro* PCa/stroma co-culture studies, *in vivo* xenograft tumor studies, and IHC studies on human CRPC tissue arrays.

Keywords:

prostate cancer, tumor microenvironment, castration resistance, AR, TGF-β, MDV3100, prostate stroma.

Overall Project Summary

Major Task 1: Study prostate stromal TGF-β signaling induced androgen-independent AR activation in PCa and determine the biological roles of MAPK4 in mediating this action. These include Subtasks (1) Confirm that AR is required for the prostate stromal TGF-β signaling induction of PSA, KLK4, and TMPRSS2 in the TGF-β treated LNCaP/HPS19I co-cultures (1-6 months), (2) Confirm that stromal TGF-β signaling induced AR activity is ligand independent (insensitive to MDV3100 treatment) (1-6 months), and (3) Determine whether

MAPK4 is required for the prostate stromal TGF-β signaling induced AR activation in LNCaP cells (6-12 months).

For Subtask 1 and Subtask 2, we have finished the proposed studies as described in our previous annual report and in our publication (Yang et al, Oncotarget, 2014) [7].

For Subtask 3, we proposed to determine whether MAPK4 is required for the prostate stromal TGF-β signaling induced AR activation in LNCaP cells. To this end, we have previously generated the LNCaP cells with stable knockdown of MAPK4, and showed that knockdown of MAPK4 led to inhibition of AR and that GATA2, a transcription factor/pioneer factor critical for AR expression and activation[8] may mediate MAPK4 induced AR expression/activation. However, we later noticed that continuous passaging cells with stable knockdown of MAPK4 led to decreased knockdown efficiency due to the growth disadvantage of those cells with high knockdown efficiency. To solve this problem, we created LNCaP cells with Dox inducible knockdown of MAPK4 using the pInducer10 lentiviral inducible RNA interference system[9] as well as using the pLKO-tet-on system. However, the relative high concentration of Dox used in such systems induced significant toxicity to LNCaP cells (data not shown). Therefore, we are optimizing the experimental system including generating higher titer of viruses to test whether the infected cells will require lower concentration of Dox for knockdown of MAPK4. We are also in the process of generating MAPK4-knockout LNCaP cells using the CRISPR/Cas9 system. Altogether, we hope that we can generate the engineered LNCaP cells that are stable for either total loss of MAPK4 (CRISPR/Cas9 knockout) or with inducible knockdown of MAPK4 (Dox-inducible system). These stable cells will also be essential for the xenograft studies in the Subtask 3 of Major Task 2.

We have also performed limited co-culture studies using the earliest passages of the engineered LNCaP cells with confirmed knockdown of MAPK4 (LNCaP-G4) *vs.* non-targeting control (LNCaP-NT). qPCR revealed that knockdown of MAPK4 in LNCaP cells greatly inhibited PSA expression (AR activation) in these co-cultures and treatment of SD208, a TGF-β receptor 1 specific inhibitor to block basal stromal TGF-β activity in these co-cultures (without externally added TGF-β), also greatly inhibited PSA expression (Figure 1). These results are in line with our hypothesis on MAPK4 mediating stromal TGF-β signaling induced AR activation in PCa cells.

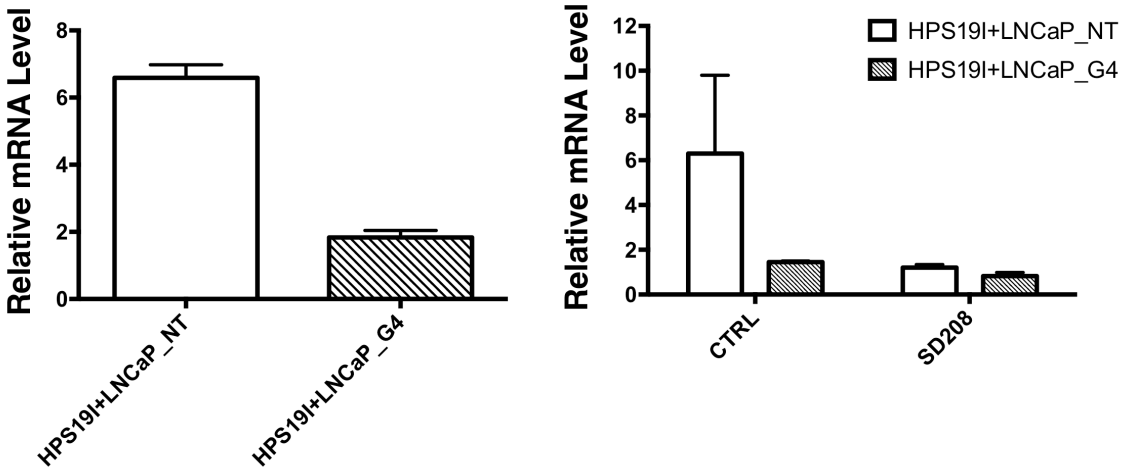

Figure 1. Knockdown of MAPK4 inhibits AR activation in the LNCaP/HPS19I co-cultures. Left Panel: PSA expression in LNCaP-NT/HPS19I vs. LNCaP-G4/HPS19I co-cultures (qPCR). Right Panel: PSA expression in LNCaP-NT/HPS19I vs. LNCaP-G4/HPS19I co-cultures treated with Control (CTRL) vs. SD208, a specific inhibitor for TGF-β receptor 1 (qPCR). Please note, more repeats, including co-cultures treated with externally added TGF-β, are needed to test our hypothesis. These repeats will be done using either LNCaP cells with stable knockdown of MAPK4 at earliest passages (cells have to be re-generated since our current stocks are in later passages with no appreciable knockdown efficiency), or LNCaP cells with Dox-inducible knockdown or CRISPR/Cas9 mediated knockout of MAPK4 (we are working on them).

Major Task 2: Determine whether inhibition of MAPK4 (in PCa) and TGF-β signaling (in stroma) enhances castration response of PCa xenografts. These include Subtasks (1). Compare LNCaP-MAPK4 *vs.* LNCaP-Ctrl xenograft growth and their response to castration (8-24 months), (2) Optimize experimental conditions for LNCaP/HPS19I xenograft studies to produce tumors with consistent HPS19I content and tumor size at the time for castration (12-24 months), and (3) Compare tumor growth in intact mice and their response to castration among xenografts made with LNCaP cells (knockdown of MAPK4 vs. control) and HPS19I cells (overexpression of dominant negative TβRII vs. control) (8-36 months).

For Subtask 1, as previously discussed in our previous Annual Report, we observed that extensive culture of LNCaP-MAPK4 cells stably overexpressing MAPK4 somehow led to significantly reduced or loss of MAPK4 overexpression in these cells. To address this problem, we have tried different approaches as described in our previously reports with limited success. In this year, we have been continuing working on this and after several rounds of optimization, we have finally successfully engineered LNCaP cells to overexpress functional levels of MAPK4 in a Dox-inducible manner. This cell line is named LNCaP-iMAPK4 and the control cell line is named LNCaP-iCtrl. Dox-induced overexpression of MAPK4 greatly induced the expression of GATA2 and AR in LNCaP-iMAPK4 cells as compared to similarly treated LNCaP-iCtrl cells (Figure 2A, Western blots). Furthermore, Dox-induced overexpression of MAPK4 greatly induced the expression of AR target gene PSA in LNCaP cells cultured in 10% charcoal-stripped serum (CSS) plus 10 μM MDV3100 for maximum androgen ligand blockade, further supporting that MAPK4 can activate AR in an androgen-independent manner. Since human CRPC tissues contain castration or higher levels of androgen, we further examined whether MAPK4 sensitizes PCa cells to low levels of androgen. Indeed, R1881 (as low as 0.01 nM) robustly induced PSA expression in LNCaP cells overexpressing MAPK4 in a dose-dependent manner (Figure 2B). Altogether, we have successfully generated the LNCaP-iMAPK4 cells with inducible overexpression of MAPK4 and have used this cell line to further document the MAPK4-induced androgen-dependent and androgen-independent activation of AR. Importantly for this Subtask, we observed that these LNCaP-iMAPK4 cells maintained their ability for Dox-induced overexpression of MAPK4 even after prolonged in vitro culturing and series of passaging. This provides a solid foundation for the proposed in vivo studies in Subtask 1.

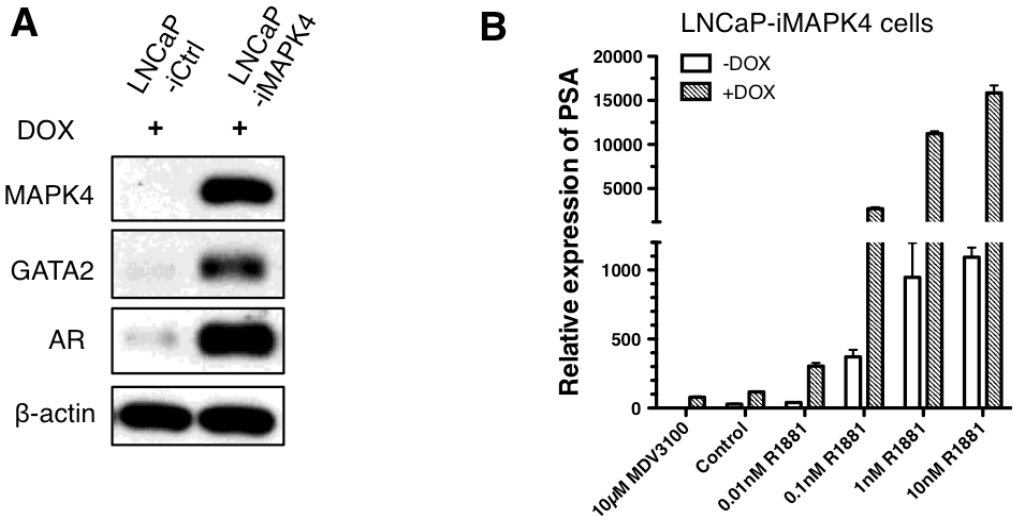

Figure 2. Generation and characterization of stable LNCaP-iMAPK4 cells with Dox-inducible overexpression of functional levels of MAPK4. (A) Dox-induced MAPK4 overexpression in LNCaP-iMAPK4 cells induces GATA2 and AR protein expression as compared to similarly treated control LNCaP-iCtrl cells (Western blots). (B) Dox-induced MAPK4 overexpression in LNCaP-iMAPK4 cells both induces androgen-independent activation of AR and greatly sensitizes them to low levels of androgen. LNCaP-iMAPK4 cells were treated with 10μM of MDV3100, control, and increasing doses (0.01, 0.1, 1, and 10 nM) of R1881 in 10% of CSS in the presence or absence of 0.5 μg/ml Dox. qPCR were used for PSA expression.

For Subtasks 2 and 3, we have performed pilot studies to test the ratio of LNCaP vs. HPS19I cells at the time of xenograft inoculation and found that a ratio ranging from 2:1 to 4:1 produces tumors with consistent HPS19I content and tumor size. As previously reported, we have generated the pCDH-dnTβRII lentiviral vector and use produced lentivirus to infect HPS19I cells for overexpression of dnTβRII. We have successfully engineered HPS19I cells for dnTβRII overexpression and 100% of the cells are RFP+ (indication for dnTβRII expression) after selection using 0.7 μg/ml of puromycin. However, we observed that the engineered HPS19I cells, which themselves are slow-growing cells, grew extremely slowly and eventually died out when being cultured in 0.7 μg/ml of puromycin. Accordingly, we have tried multiple rounds to re-engineer these cells and expanded the cells in the absence of puromycin after a short-time selection using 0.5 μg/ml of puromycin. However, these cells all went to a non-proliferating state and we could not prepare enough cells for the proposed xenograft studies *in vivo*. The slow growth nature of these human prostate stromal cells also made these gene-engineering efforts time-consuming and difficult. We are now exploring other alternative approaches. These include testing other human prostate stromal cell lines, such as HTS33B and HPS33Q. We are also exploring the opportunity to use other selection such as G418 and/or using cell sorting technique to produce the HPS19I cells with stable overexpression of dnTβRII. For the LNCaP cells, as described earlier, we are trying to produce LNCaP cells that are stable for either total loss of MAPK4 (CRISPR/Cas9 knockout) or with inducible knockdown of MAPK4 (Dox-inducible system) for the proposed xenograft studies. Due to this lack of available cell lines, we have not performed xenograft studies using the proposed engineered cell lines.

Major Task 3: Determine whether MAPK4 expression correlates with AR expression / activation in PCa cells and TGF-β signaling activation in stromal cells in human CRPC tissues. These include Subtasks (1) Perform qRT-PCR on cDNA from human CRPC tissues for the expression of MAPK4, AR, PSA, KLK4, COL1A1, FAP, and K-Alpha-1 (18-36 months), and (2) Optimize IHC protocols and perform IHC studies on human PCa tissue-microarray slides for the expression of MAPK4, AR, PSA, and phospho-Smad2 (18-36 months).

We received the final approval from HRPO for the proposed studies in April, 2016. Since then, we have made limited progress in this Task. The proposed xenograft studies *in vivo* in Task 2 are essential for providing the rationales for the studies using the extremely valuable human CRPC materials in Task 3. However, due to the technical difficulties described above, we have not been able to make significant progress in Task 2. The IHC studies are essential part of Task 3 and we have proposed to test and identify a MAPK4 antibody working for IHC. To this end, we have purchased 6 different MAPK4 antibodies from multiple vendors. We prepared cell lysates from several engineered human cell lines overexpressing MAPK4 (including PCa LNCaP cells, the PNT1A "normal" prostate epithelial cells, and other non-PCa cells), with knockdown of MAPK4 (including PCa LNCaP, VCaP, LAPC4 cells, and other non-PCa cells), and knockout of MAPK4 (only in non-PCa cells so far). We then used these cell lysates to examine the acquired 6 MAPK4 antibodies using Western blots. We confirmed that three of these MAPK4 antibodies worked to various extents in Western blots and frequently with multiple non-specific bands; the other three did not show any specificity for MAPK4. We tried to test these antibodies using some xenograft tumor tissues, but none so far has produced specific staining. Since we have successfully generated MAPK4 knockout cell lines using other non-PCa human cancer cell lines in another study, we will produce xenograft tumors using the control and MAPK4 knockout cells to further critically examine the specificity of MAPK4 antibodies in IHC. The MAPK4 antibody that only stains the cancer cells in control xenografts, but not in the MAPK4 knockout xenografts will be used for IHC on human CRPC tissues.

Key Research Accomplishments

- Prepared the paperwork and received the final approval from HRPO for the proposed studies in Tasks 3.

- Generation of LNCaP-iMAPK4 cells with Dox-inducible overexpression of functional levels of MAPK4. We have also used this experimental system and demonstrated MAPK4 induction of GATA2 and AR expression and MAPK4 induction of androgen-dependent and -independent activation of AR in PCa.

- Demonstrated that knockdown of MAPK4 in LNCaP cells greatly repressed AR activation in the LNCaP/HPS19I co-cultures.

- Identification of additional MAPK4 antibodies (altogether three) that can recognize MAPK4 in Western blot assays; their ability in specifically detecting MAPK4 in IHC still needs to be critically examined.

Conclusion

In this year, we have made progress in successfully generating the LNCaP cells with Dox-inducible overexpression of functional levels of MAPK4 and used them to demonstrate that MAPK4 induces GATA2 and AR expression as well as the androgen-independent and androgen-dependent AR activation in PCa. It is extremely interesting that MAPK4 expression greatly increased PCa cells sensitivity to low levels of androgen ligands (castration levels of androgen) since together with MAPK4 induced ligand-independent activation of AR, it may provide another novel direct mechanism for development of CRPC. We have also made progress in demonstrating that the knockdown of MAPK4 in LNCaP cells greatly inhibited AR activation in the LNCaP/HPS19I co-cultures. This partially supports our hypothesis on the potential important role of MAPK4 in mediating the stromal cell induced AR activation in the co-cultured LNCaP cells.

Due to several technical problems, including difficulties in expanding LNCaP cells with maintained efficient knockdown of MAPK4 and in growing the engineered HPS19I cells even after short-term low-concentration Puromycin selection, we have not been able to carry out the xenograft studies in Major Task 2. We will continue explore different approaches to solve these problems and expect to carry out some of these xenografts studies by the end of this grant. Since we have successfully generated the LNCaP-iMAPK4 cells, we will be able to finish the proposed xenograft studies using this cell line in Major Task 2. Since the in vivo xenograft studies in Task 2 will provide essential rationales supporting the proposed correlation studies in Major Task 3 using CRPC tissues, we are currently putting a temporary hold on this study since the CPRC tissues are extremely valuable one-time usage materials that should be used in carefully designed studies with supporting in vivo supporting data. In other case, we will fully carry out this study once we successfully acquire the supporting data from the in vivo xenograft studies. To prepare the full-scale study, we will continue testing the MAPK4 antibodies in IHC on xenografts, especially using the xenografts to be generated using the MAPK4 knockout human cancer cells (currently non-PCa cells). Alternatively, if we can successfully generate the MAPK4 knockout LNCaP cells in Task 2, we will generate xenografts using LNCaP MAPK4 knockout cells and the parental cells, and use these xenografts to test the MAPK4 antibodies on IHC. All in all, we have experienced significant technical difficulties and significant delays. We expect to be able to finish all the proposed studies in Major Task 1, and partially finish the proposed studies in Major Tasks 2 and 3 at the conclusion of the award.

Publications, Abstracts, and Presentations:

1. Wang W, Dong B, Ittmann MM, **Yang F**. A versatile gene delivery system for efficient and tumor specific gene manipulation *in vivo*. *Discoveries*. 2016;4;e58. doi: 10.15190/d.2016.5.

Inventions, patents and licenses: Nothing to report

Reportable Outcomes: Nothing to report

Other Achievements: Nothing to report

References:

1. Shafi, A.A., Yen, A.E. & Weigel, N.L. Androgen receptors in hormone-dependent and castration-resistant prostate cancer. *Pharmacol Ther* **140**, 223-38 (2013).
2. Claessens, F. et al. Emerging mechanisms of enzalutamide resistance in prostate cancer. *Nat Rev Urol* **11**, 712-716 (2014).
3. Barron, D.A. & Rowley, D.R. The reactive stroma microenvironment and prostate cancer progression. *Endocr Relat Cancer* **19**, R187-204 (2012).
4. Aberg, E. et al. Regulation of MAPK-activated protein kinase 5 activity and subcellular localization by the atypical MAPK ERK4/MAPK4. *J Biol Chem* **281**, 35499-510 (2006).
5. Kant, S. et al. Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK5. *J Biol Chem* **281**, 35511-9 (2006).
6. Coulombe, P. & Meloche, S. Atypical mitogen-activated protein kinases: structure, regulation and functions. *Biochim Biophys Acta* **1773**, 1376-87 (2007).
7. Yang, F. et al. Stromal TGF-beta signaling induces AR activation in prostate cancer. *Oncotarget* **5**, 10854-69 (2014).
8. Wu, D. et al. Three-tiered role of the pioneer factor GATA2 in promoting androgen-dependent gene expression in prostate cancer. *Nucleic Acids Res* **42**, 3607-22 (2014).
9. Meerbrey, K.L. et al. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. *Proc Natl Acad Sci U S A* **108**, 3665-70 (2011).
10. Choy, L., Skillington, J. & Derynck, R. Roles of autocrine TGF-beta receptor and Smad signaling in adipocyte differentiation. *J Cell Biol* **149**, 667-82 (2000).

Appendices: None.