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14. ABSTRACT Methylation at the N6 position of adenosine (m ⁶ A) is a post-transcriptional modification of mRNA. However, little is known regarding the biological meanings of this epigenetic regulation of mRNA. Recent technological advances have made it possible to detect mRNA methylation. The m ⁶ A was found near regulatory regions and its level is altered in various cancer cell lines. FTO, the fat mass and obesity associated gene, was recently shown as an m ⁶ A demethylase. FTO gene polymorphism has been associated with aggressive prostate cancer (PCA). We thus hypothesize that FTO and mRNA methylation play critical roles in PCA. To test this hypothesis, the proposed research aims to examine how FTO regulates PCA through m ⁶ A-modulated gene expression. Our data demonstrated that the levels of m ⁶ A and its modifying enzymes including FTO are de-regulated during prostate cancer progression. FTO regulates genes/pathways that play important roles in malignancy including wound response, DNA replication, inflammation, and transcription factor activities.					
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1. INTRODUCTION:

Methylation at the N6 position of adenosine (m⁶A) is a post-transcriptional modification of mRNA. However, little is known regarding the biological meanings of this epigenetic regulation of mRNA. Recent technological advances have made it possible to detect mRNA methylation. The m⁶A was found near regulatory regions and its level is altered in various cancer cell lines. FTO, the fat mass and obesity associated gene, was recently shown as an m⁶A demethylase. FTO gene polymorphism has been associated with aggressive prostate cancer (PCA). We thus hypothesize that FTO and mRNA methylation play critical roles in PCA. To test this hypothesis, the proposed research aims to examine how FTO regulates PCA through m⁶A-modulated gene expression.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*
 mRNA methylation, FTO, MeRIP-seq, RNA-seq, m⁶A, gene regulation

3. ACCOMPLISHMENTS:

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

Task 1. To determine how FTO regulates m⁶A pattern, gene expression, and PCA	Timeline	% of completion	Actual complete dates
Generate LNCaP prostate cancer cells with stable FTO knockdown	Months 1-2	100%	1/1/2014
Work out MeRIP experiment	Months 1-2	100%	8/1/2014
Perform RNA-Seq analysis of control and FTO-knockdown LNCaP cells	Months 1-3	100%	5/1/2014
Bioinformatics analysis of RNA-Seq data to identify genes differentially regulated by FTO	Months 2-5	100%	10/1/2014
Select key target genes and confirm their regulation by FTO using qRT-PCR or western blot	Months 3-6	20%	
Perform MeRIP-Seq of control and FTO-knockdown LNCaP cells	Months 2-4	50%	9/1/2014
Bioinformatics analysis of MeRIP-Seq data to identify m ⁶ A loci regulated by FTO	Months 3-6	10%	
Integrate RNA-Seq and MeRIP-Seq data to determine mRNA methylation around FTO-regulated genes	Months 6-10	10%	
Analyze top FTO-target m ⁶ A loci and related gene expression in benign and cancerous cells	Months 8-12	100%	11/30/2014

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

1) Major activities in this reporting period include:

- A. Examine the levels of m⁶A and its modifiers in prostate cancer.
- B. Determine the role of FTO on mRNA methylation and prostate cancer.
- C. RNA-seq analysis of PCA control and FTO knockdown cells.
- D. MeRIP-seq analysis of PCA control and FTO knockdown cells.

2) Specific objectives: examine the roles of FTO and m⁶A-modulated gene expression in prostate cancer.

3) Significant results or key outcomes:

3.1: Examine the levels of m⁶A and its modifiers in prostate cancer.

Methods and Results: To determine whether the levels of total m⁶A may be altered in prostate cancer, we carried out high-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (HPLC–QqQ-MS/MS) of mRNA isolated from benign PrEC, androgen-sensitive LNCaP treated with ethanol or androgen, and the AR-negative, highly aggressive Du145 cells. Our data showed that the total m⁶A level is the highest in benign cells and the lowest in Du145 prostate cancer cells (**Figure 1**). In addition, androgen stimulation inhibits m⁶A levels.

Recently, a number of proteins have been reported to be modifiers or readers of m⁶A, including FTO, METTL3, METLL14, ALKBH5, WTAP, YTHDF1, YTHDF2, and YTHDF3. To examine whether these genes may be de-regulated in PCA, we re-analyzed publically available large microarray dataset profiling primary specimens and observed de-regulation of many of these m⁶A modifiers (data not shown). In particular, FTO level is found to be significantly down-regulated in metastatic prostate cancer in several datasets (**Figure 2**).

Sample	Test	284-152 G	282-150 m6A	282268-136 A	245-113 U	244-112 C	m6A (nM)	A (uM)	m6A/A %
RM01	wx-9	1045174	46587	1585861	117704	288091	15.61698	7.911346	0.1974
RM02	wx-10	862844	30682	1880887	85746	241497	10.28527	9.383135	0.109614
RM03	wx-11	944154	33950	1496308	101092	241206	11.38078	7.464595	0.152463
RM04	wx-12	729297	23493	1271623	67395	164904	7.875365	6.343715	0.124144
RM01	wx-r-9	1069052	45957	1594314	114936	265545	15.40579	7.953516	0.193698
RM02	wx-r-10	884318	30365	1904495	85475	223693	10.17901	9.500908	0.107137
RM03	wx-r-11	936241	32997	1530025	93752	229174	11.06131	7.632799	0.144918
RM04	wx-r-12	741354	22977	1304320	66293	152269	7.70239	6.506829	0.118374
				m6A/A %					
				Average					
				STD					
		PREC		0.196		0.003			
		Du145		0.108		0.002			
		LNCaP ethl		0.149		0.005			
		LNCaP R1881		0.121		0.004			

Fig.1 m⁶A level is decreased in prostate cancer and by androgen. The mRNA was isolated from PrEC, LNCaP+ethl, LNCaP+R1881, and Du145 cells and subjected to QqQ-MS/MS analysis. The levels of A, C, G, U, and m⁶A were determined and the ratio of m⁶A/A calculated.

Conclusions: Our data thus far have suggested that (1) m⁶A level is decreased in prostate cancer and by the androgen receptor (AR) pathway; (2) modifiers of m⁶A are differentially expressed in prostate cancer; (3) FTO is down-regulated in metastatic prostate cancer.

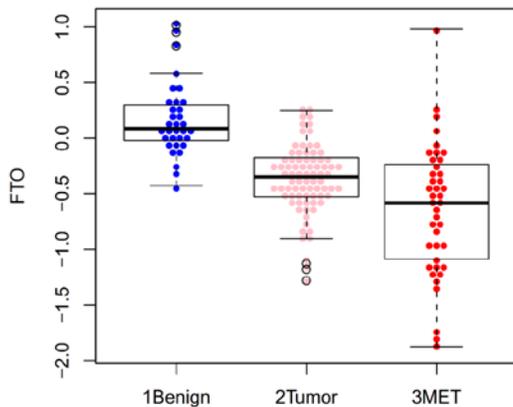


Fig.2 FTO is decreased in prostate cancer. The mRNA level of FTO is determined in a publically available microarray dataset of benign, localized and metastatic PCA tissues.

3.2: Determine the role of FTO on mRNA methylation and gene regulation in prostate cancer.

Methods and Results: In order to study FTO regulation of gene expression and function, we first examined FTO level in a series of prostate cancer cell lines and found that FTO is detectable in all cell lines tested. We next performed LNCaP and PC-3M cells with stable FTO knockdown. In both cell lines, we were able to achieve more than 70% knockdown of the FTO protein (**Figure 3**).

To determine the genes/pathways that are regulated by FTO, we first performed microarray analysis of control and FTO knockdown PC-3M cells. Bioinformatic analysis identified 468 and 162 genes that are respectively increased and decreased

following FTO knockdown. To determine the potential function of FTO in prostate cancer, we carried out gene ontology analysis of FTO downstream genes. The results revealed significant enrichment of genes involved in wound/inflammatory response, DNA replication, cell proliferation, response to stimulus such as glucocorticoid, and regulation of transcriptional regulation (**Figure 4**). Gene set

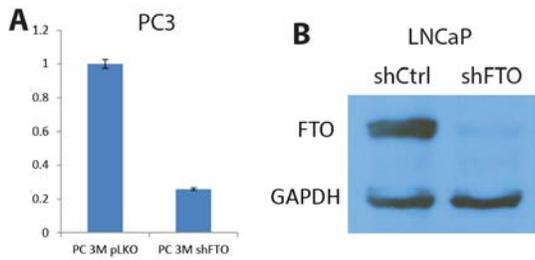


Fig.3 FTO knockdown in prostate cancer cells. The PC-3M and LNCaP prostate cancer cells were subjected to shRNA-mediated knockdown of FTO.

Term	PValue
GO:0009611~response to wounding	7.47E-06
dna replication	1.28E-05
GO:0006954~inflammatory response	3.94E-05
GO:0006260~DNA replication	1.26E-04
GO:0006952~defense response	3.01E-04
domain:Leucine-zipper	4.76E-04
GO:0051100~negative regulation of binding	7.84E-04
microtubule	8.01E-04
GO:0051384~response to glucocorticoid stimulus	8.98E-04
IPR013838:Beta tubulin, autoregulation binding site	9.45E-04
GO:0002526~acute inflammatory response	9.74E-04
GO:0043433~negative regulation of transcription factor activity	9.98E-04

Fig.4 GO terms enriched by FTO-regulated genes. GO analysis was performed using the genes that are differentially regulated by FTO knockdown.

enrichment analysis further showed that many androgen-regulated genes appear to be de-regulated by FTO (data not shown). Together with earlier observation of androgen-mediated regulation of m⁶A level, the data suggest potential cross-regulation between AR and mRNA methylation.

Conclusions: Our data suggest that (1) FTO mainly represses gene expression, which is consistent with the fact that FTO decreases mRNA methylation that stabilizes mRNA; (2) FTO regulates genes involved in critical cellular processes related to cell growth, wound healing and stimulus response; and (3) FTO function may be associated with AR signaling.

3.3: Determine the function of FTO in prostate cancer.

Methods and Results: To directly determine FTO function in prostate cancer, we carried out cell growth assay of PC-3M cells with control or FTO knockdown. Our result showed that FTO knockdown inhibited PC-3M cell proliferation (**Figure 5**). To further verify this, we in the converse overexpressed wildtype FTO in PC-3M cells. As a control, a FTO with enzymatic domain deletion was also overexpressed. Surprisingly, we did not observed significant changes of cell growth following FTO overexpression, whereas the mutant increased cell growth.

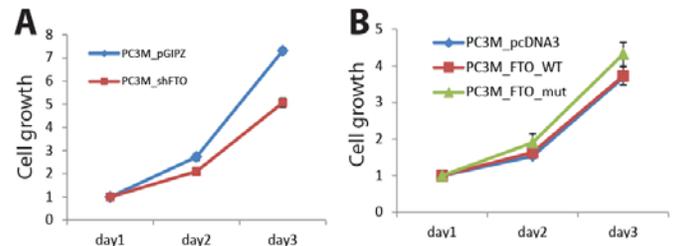


Fig.5 FTO regulation of prostate cancer cell growth. PC-3M with FTO shRNA knockdown or FTO wildtype and mutant overexpression were analyzed by WST1 cell growth assay.

Conclusions: Further investigation in additional prostate cancer cell lines will be necessary in order to reach solid conclusion regarding the role of FTO in prostate cancer. However, our preliminary data suggest that FTO might instead regulate epithelial-to-mesenchymal transition, cell migration and cell invasion.

3.4: RNA-seq analysis of PCA control and FTO knockdown cells.

Methods and Results: As our results above suggest that FTO expression may be linked to AR signaling and RNA-seq measures mRNA levels with higher sensitivity and accuracy, we performed paired-end RNA-seq of LNCaP cells with control and FTO knockdown. We obtained more than 50 million reads per sample and performed bioinformatics analysis of the sequencing reads. RNA-seq data analysis revealed over 1000 genes that are differentially regulated following FTO knockdown in LNCaP cells.

Conclusions: FTO knockdown leads to differential expression of more than 1000 genes in LNCaP cells. Further analysis is required to determine the functional terms that are enriched by FTO-regulated genes and whether they cross-talk with AR signaling.

3.5: MeRIP-seq analysis of PCA control and FTO knockdown cells.

Methods and Results: To determine whether FTO knockdown might affect m⁶A level, we performed HPLC–QqQ-MS/MS analysis of LNCaP cells with control or FTO knockdown and PC-3M cells with control and FTO overexpression. Surprisingly, we did not observe significant changes in the total level of m⁶A levels.

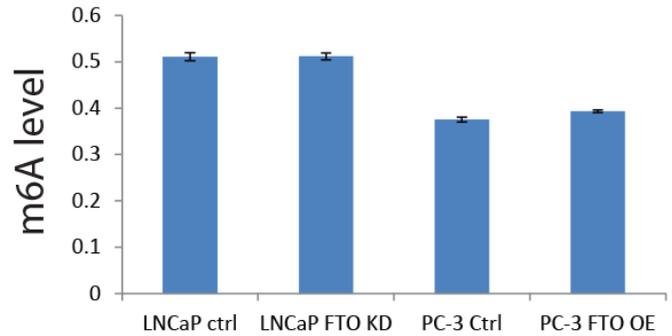


Fig.6 m⁶A level in prostate cancer cells with FTO de-regulation. The mRNA was isolated from LNCaP cells with control or FTO knockdown and PC-3M cells with control and FTO overexpression and subjected to QqQ-MS/MS analysis. The levels of A, C, G, U, and m⁶A were determined and the ratio of m⁶A/A calculated.

Conclusions: Our data suggest that FTO de-regulation does not alter the total amount of m⁶A level in a prostate cancer cell. It is still possible that m⁶A level at individual mRNA may change. We plan to carry out MeRIP experiments to examine alteration of m⁶A level in particular around the genes that are differentially expressed following FTO knockdown or overexpression.

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

“Training” activities:

At the Northwestern University, postdoctoral fellow Dr. Bing Song, Ali Zhang, and Longtao Wu have spent 2.5, 2.5, and 2.3 calendar months working on the project, respectively. They have received one-on-one training by discussion and meetings with the PI and also by working on the project. They have gained extensive training in the study of mRNA methylation and gene expression regulation.

“Professional development” activities:

The PI Dr. Yu was able to attend the 2014 Keystone Cancer Epigenetic Meeting where she interacted with many colleagues working on epigenetic regulations including mRNA methylation.

▪ **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?**

Not applicable

4. **IMPACT:**

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

Our data suggest for the first time that m⁶A levels are de-regulated in prostate cancer and that FTO may play important roles in regulating gene expression and oncogenic properties of prostate cancer. Our data in addition suggest that the FTO- m⁶A pathway may crosstalk with androgen receptor signaling. Our study forms the ground work for further investigation of a new epigenetic regulatory mechanism in prostate cancer.

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

Our study of prostate cancer sets an example for future studies of mRNA methylation, its regulation and function in other diseases including other cancer types such as breast cancer.

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

Nothing to Report

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

Nothing to Report

5. **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

6. **PRODUCTS:**

Publications, conference papers, and presentations

Nothing to Report

- **Journal publications.**

Nothing to report

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

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

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	<i>Jindan Yu</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>1071255</i>
Nearest person month worked:	<i>1.25</i>
Contribution to Project:	<i>Dr. Yu supervised all the work related to this project, designed the experiments, coordinated experiments between researchers, and write the report.</i>
Funding Support:	

▪

Name:	<i>Ali Zhang</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	<i>1079458</i>
Nearest person month worked:	<i>2.5</i>
Contribution to Project:	<i>Dr. Zhang has performed work related to FTO overexpression and knockdown in prostate cancer cells.</i>
Funding Support:	

Name:	<i>Longtao Wu</i>
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Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	<i>1074460</i>
Nearest person month worked:	<i>2.3</i>
Contribution to Project:	<i>Dr. Wu has prepared all sequencing libraries for next-generation sequencing using the Illumina sample prep protocol and performed work in arranging and receiving the sequences.</i>
Funding Support:	

Name:	<i>Bing Song</i>
Project Role:	<i>Postdoctoral Fellow</i>
Researcher Identifier (e.g. ORCID ID):	<i>1085265</i>
Nearest person month worked:	<i>2.5</i>
Contribution to Project:	<i>Dr. Song helped with cell culture, mRNA isolation, qRT-PCR analysis of gene expression, and functional assays to determine cell growth, migration and invasion.</i>
Funding Support:	

- **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

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *NA.*

QUAD CHARTS: *NA.*

9. APPENDICES:

NA