AWARD NUMBER: W81XWH-15-1-0650

TITLE: ALK and TGF-Beta Resistance in Breast Cancer

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REPORT DATE: October 2018

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 *(From - To)* |
|---|---|---|
| October 2018 | Annual | 30SEP2017 – 29SEP2018 |

**4. TITLE AND SUBTITLE**

ALK and TGF-Beta Resistance in Breast Cancer

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-15-1-0650

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Xin-Hua Feng & Yi Li

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

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

Baylor College of Medicine, 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**

TGF-β signaling represents a major tumor suppressor pathway. Loss of the TGF-β response is a hallmark in human cancer. However, the mechanisms underlying TGF-β resistance in breast cancer have not been elucidated. Anaplastic Lymphoma Kinase (ALK) is a tyrosine receptor kinase of insulin superfamily. IBC is relatively rare but the most lethal subtype of breast cancer. Thus, it is important to identify biomarkers, understand better current therapies and find new potential therapies for IBC. Our long-term goal is to understand the mechanisms underlying TGF-β resistance in human cancer. The short-term strategy of our research is to focus on ALK-induced inactivation of Smad4 in breast cancer. Our unifying hypothesis is that ALK causes TGF-β resistance through Smad4 inactivation and disrupts the growth constraints exerted by TGF-β signaling to promote breast tumorigenesis. To test our hypothesis, we propose the following specific aims to achieve our goals: 1. Investigate *in vivo* and clinical relevance of Smad4 tyrosine phosphorylation in breast cancer; 2. Determine the role of ALK-mediated Smad4 phosphorylation in TGF-β resistance in breast cancer; 3. Elucidate the molecular mechanisms underlying Smad4 tyrosine phosphorylation. This proposal will contribute significantly to breast cancer prevention and treatment.

**15. SUBJECT TERMS**

Smad4, ALK, breast cancer

| 16. SECURITY CLASSIFICATION OF: U | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 11 | 19b. TELEPHONE NUMBER *(include area code)* |

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**INTRODUCTION:**

TGF-β exerts its tumor suppressing function by inhibiting the growth of normal epithelial cells. Loss of the TGF-β antiproliferative response is a hallmark in human cancers [1-3]. In TGF-β signaling pathway, tumor suppressor Smad4 plays a central role in TGF-β actions. Smad4 is frequently mutated or deleted in gastrointestinal and pancreatic cancer, which counts for TGF-β resistance in these cancers. However, not all types of cancers harbor deletion or mutations in the Smad4 gene. Inactivating mutations in the Smad4 gene are rare in breast cancers [4], but TGF-β response is attenuated [5,6], indicating that the tumor suppressor activity of Smad4 is abrogated by other mechanisms.

ALK is a tyrosine receptor kinase. Abnormal expression of ALK has been reported in numerous tumors including a significant fraction of breast cancer especially triple-negative breast cancer and inflammatory breast cancer [7]. ALK activation triggers major signaling pathways (MEK/ERK, STAT3, PI3K/Akt), which promote cell proliferation while preventing cell death [8-19]. However, the effect of ALK on TGF-β action, a major anti-proliferation function in cell, has not been explored.

In our preliminary studies, we have for the first time discovered that ALK could inactivate Smad4 tumor suppressive function. In this proposal, we propose to investigate how ALK-driven inactivation of Smad4 tumor suppressor contributes to TGF-β resistance in breast cancer. We hypothesize that ALK causes TGF-β resistance through Smad4 tyrosine phosphorylation and inactivation; thus, aberrant ALK activation in breast cells disrupts Smad4-exerted growth constraints to promote tumorigenesis. Consequently, suppression of ALK activity both restores Smad4 function and blocks other oncogenic activities of ALK, thus suppressing breast tumor formation.

Specifically, in this proposal, we will determine whether aberrant activation of ALK causes TGF-β resistance by Smad4 tyrosine phosphorylation and inactivation in breast cancer cell lines. Next, we will elucidate the molecular mechanism by which ALK-mediated Smad4 tyrosine phosphorylation affects Smad4 signaling. Finally, we will determine the impact of ALK activation on Smad4 Y95 phosphorylation, mammary tumor initiation, and progression, using human tissues and mouse models (including patient-derived xenografts).

**BODY:**

In our preliminary studies, we found that ALK can phosphorylate Smad4 on a particular tyrosine residue and inactivate Smad4 activity. During the first funding year of this proposal, we have investigated whether forced activation and knockdown of ALK affect TGF-β responses in breast cancer cell lines. We have also examined whether Smad4 Y95 phosphorylation disrupts TGF-β-induced cellular responses in breast cancer cell lines. In addition, we have determined if ALK-resistant Smad4 mutant restores the TGF-β responses in ALK-activated breast cells lines. During the second funding year of this proposal, we further characterized the impact of ALK and Smad4 tyrosine phosphorylation on Smad4 signaling and transcriptional responses. For example, we have determined how specific is Smad4 tyrosine phosphorylation by ALK or other protein tyrosine kinases (PTK). We also determined the effect of ALK on other Smads and the effect of overexpressed ALK on potential tyrosine phosphorylation of Smad2/3. To reveal the molecular mechanisms underlying the action of Smad4 tyrosine phosphorylation, we determined steps in the TGF-β signaling pathway that Smad4 tyrosine phosphorylation affects. For example, we examined the effect of Smad4 tyrosine phosphorylation on Smad4 intracellular

localization. We determined the effect of Smad4 tyrosine phosphorylation on Smad4-Smad2/3 complex formation. More importantly, since the DNA-binding activity of Smad4-Smad2/3 complex is the critical step for the activation of TGF-β signal, we examined the effect of Smad4 tyrosine phosphorylation on Smad4 binding to chromatin. We used Electrophoretic Mobility Shift Assay (EMSA) to compare the DNA-binding activity among Smad4 WT, Y95E or Y95F mutants, and we found that Smad4 tyrosine phosphorylation attenuates Smad4-DNA binding ability, indicating the action mechanism of Smad4 tyrosine phosphorylation on inhibiting TGF-β signaling activity. Hence, in the third funding year of this proposal, we further analyzed the global effect of ALK on Smad4 genomic and transcriptional responses, the global effect of Smad4 Y95 phosphorylation on TGF-β mediated responses, and used animal model to prove our hypothesis that ALK activation initiates and/or promotes mammary tumor. We have completed our proposed work for year 2017-2018 as presented below:

***Major Task 1:*** *Analyzing the global effect of ALK on Smad4 transcriptional responses.*

Global mRNA-seq analysis.

Before we could work on breast cell line, we first tested the effect of ALK-mediated Smad4 phosphorylation on TGF-β transcriptional responses using HaCaT cell line, which is a human keratinocyte cell line that are commonly used to test TGF-β-responsiveness. We used CRISPR/Cas9 to knock out the endogenous SMAD4 gene in HaCaT cells and then replaced with stable expression of wild-type SMAD4, its Y95E or Y95F mutant, or GFP (as control). RNA-Seq analyses showed that 408 genes were up- or down-regulated (Fold change > 3) upon TGF-β treatment in parental HaCaT cells, whereas only 39 of them were responsive to TGF-β in the SMAD4-null cells, indicating that SMAD4-null cells profoundly lost responsiveness to TGF-β in gene transcription (**Figure 1**). Further hierarchical clustering of the global profiles of TGF-β-induced gene expression revealed that SMAD4- and Y95F-rescued SMAD4-null cells were highly similar to the parental HaCaT cells, whereas GFP- and Y95E-rescued cells formed a separate cluster. Consistently, stable expression of SMAD4 and the Y95F mutant largely rescued TGF-β responsiveness in the SMAD4-null cells, whereas the Y95E mutant failed to rescue TGF-β genome-wide responses.

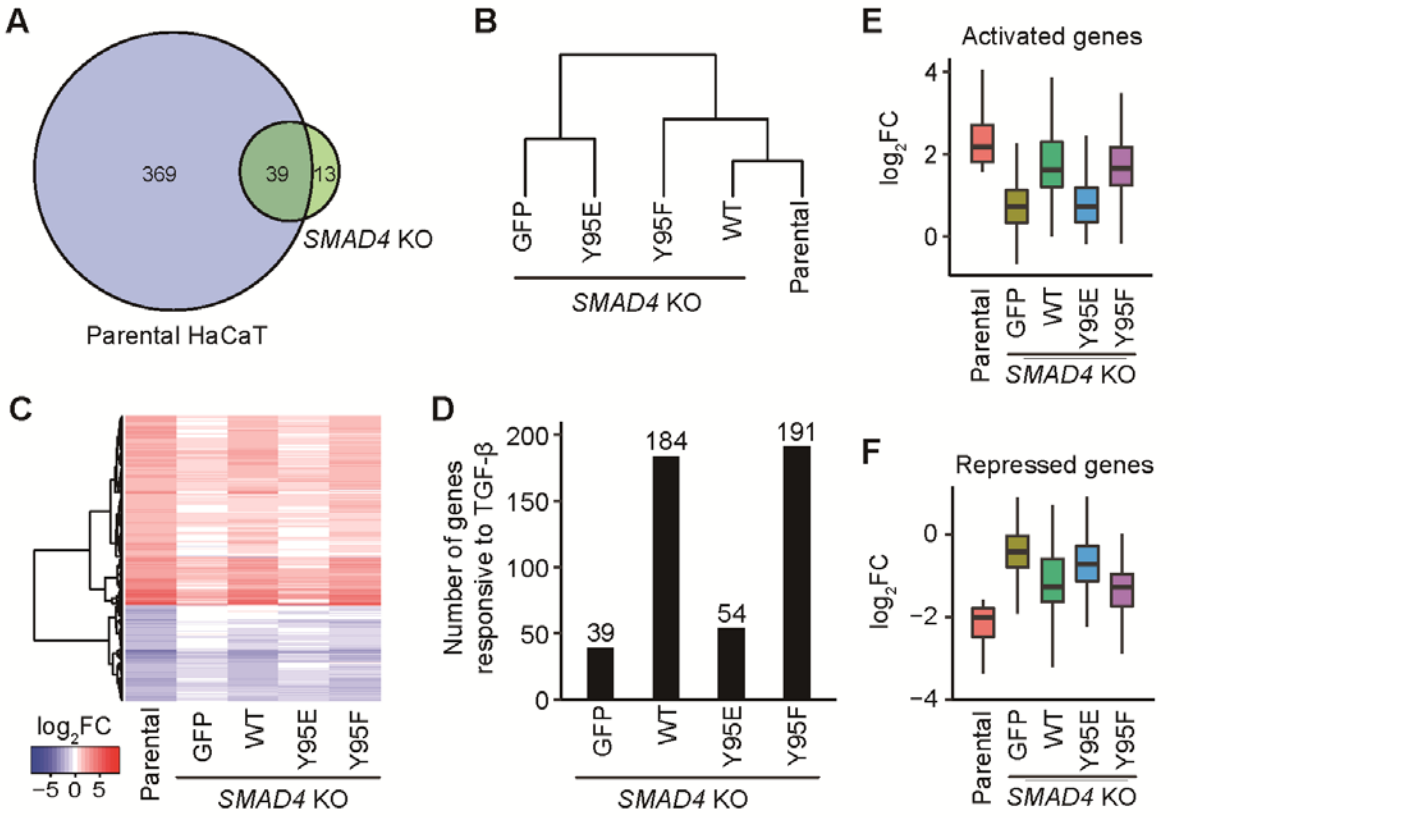


**Fig. 1.** Phosphorylation of SMAD4 on Y95 impairs genome-wide transcriptional responsiveness to TGF-β.
(**A**) SMAD4 is required for the TGF-β responsiveness of target genes. The venn diagram shows the number of TGF-β regulated genes (FC>3 upon TGF-β treatment) in parental and SMAD4-null HaCaT cells.
(**B**) The Y95F but not the Y95E mutant is functionally similar to the wild-type SMAD4 in mediating TGF-β responses. Shown is unsupervised hierarchical clustering of gene expression profiles of indicated TGF-β treated cells.
(**C-F**) SMAD4 Y95 phosphorylation plays a negative role in TGF-β transcriptional responses. (C) Heatmap shows TGF-β-induced gene expression changes in the indicated cells. Shown are the 408 TGF-β-regulated genes identified in parental cells. Colours on the heatmap represent log2FC. (D) Number of genes responsive to TGF-β in the indicated cells. Genes with > 3-fold expression change upon TGF-β treatment were considered as TGF-β responsive, and only the 408 TGF-β-regulated genes in parental cells were examined. (E and F) Box plots show the TGF-β-induced expression changes of the activated (E) and repressed (F) target genes in the indicated cells.

***Major Task 2:*** *To test whether ALK activation leads to Smad4 Y95 phosphorylation in vivo (in human tissues and mouse models).*

Note done yet.

***Major Task 3:*** *To test whether ALK activation initiates and/or promotes mammary tumor in mouse models.*

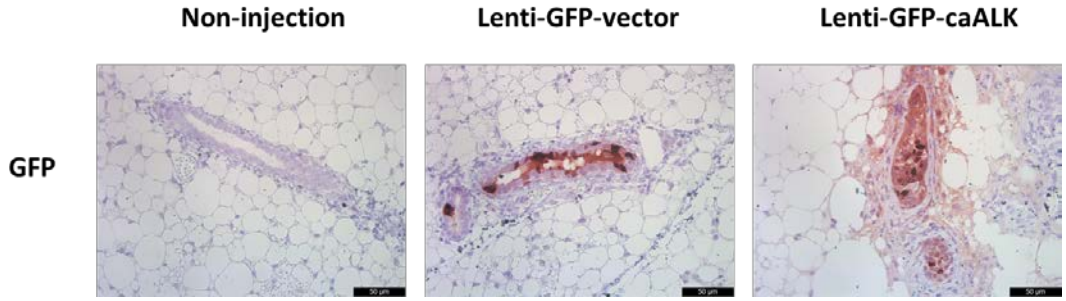


*Figure 2. IHC for GFP in non-injection mammary glands and mammary glands infected with lenti-GFP-FUCGW virus or lenti-GFP-caALK virus at Day 4 post injection.*

We first made lentivirus expressing caALK and demonstrated that it can infected mammary cells in mice via intraductal injection (Fig 2).

To test whether this virus can induce mammary tumorigenesis, we injected it intraductally into mice and collected the mammary glands at day 4 and day 21. We did not detect an increased provirus by qPCR at day 21 compared to day 4. These data suggest a weak transforming ability of ALK by itself. Next we co-injected lentivirus expressing caALK and Wnt1. At day 21, we failed to detect an accelerated expansion of early lesions in these mice compared to mice injected by either virus alone (see Fig 3). These preliminary data suggest that ALK may not accelerate Wnt1-induced mammary tumorigenesis. However, the sample size is small and the co-infection rate is uncertain. We are in the process of repeating this experiment.

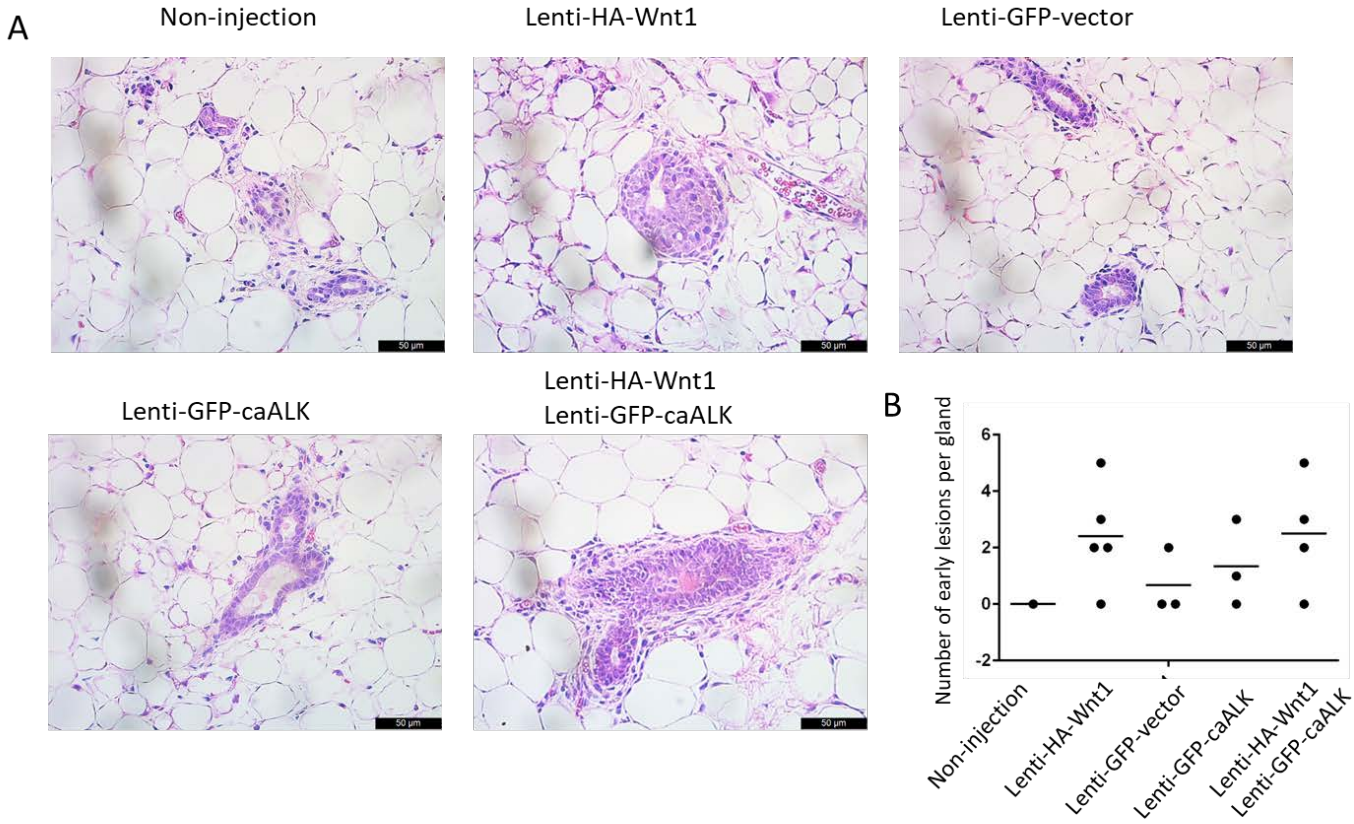


*Figure 3 A, H&E images of MG from mice injected with the viruses as indicated. B, Quantification of early lesions per gland.*

**Major Task 4:** *To investigate the impact of ALK blockade on breast cancer initiation and progression in DCIS and PDX mouse models.*

Not started yet

**KEY RESEARCH ACCOMPLISHMENTS:**

- Characterization of global effects of ALK and Smad4 phosphorylation on TGF-β genomic responses.

**REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research to include:

Cold Spring Harbor Asia meeting on Ubiquitin Family, Autophagy & Diseases, Suzhou, China, 4/9-14/2018。
"Control of canonical TGF-beta signaling by Ubiquitin and SUMO modifications"

ASCB, San Diego, 12/8-11/2008. "Mechanism of TGF-beta resistance in cancer".

Qianting Zhang, Mu Xiao, Shuchen Gu, Yongxian Xu, Ting Liu, Hao Li, YI YU, Lan Qin, Yezhang Zhu, Fenfang Chen, Yulong Wang, Chen Ding, Hongxing Wu, Hongbin Ji, Zhe Chen, Youli Zu, Stephen Malkoski, Yi Li, Tingbo Liang, Junfang Ji, Jun Qin, Pinglong Xu, Bin Zhao, Li Shen, Xia Lin, and Xin-Hua Feng. ALK phosphorylates SMAD4 on tyrosine to disable TGF-β tumour suppressor functions. Nat Cell Biology, In press.

**CONCLUSION:**

The oncogenic action of ALK has been believed to be through the signaling pathways (MEK/ERK, STAT3, PI3K/Akt), which promote cell proliferation while preventing cell death. Through our study, we for the first time revealed that ALK inhibited TGF-β signaling pathways. Furthermore, we found that ALK inhibited TGF-β signaling by tyrosine phosphorylating Smad4 at Y95. Mechanistically, Smad4 tyrosine phosphorylation completely wiped out its DNA-binding activity and transcriptional responses. Consequently, tyrosine phosphorylation disables Smad4's transcriptional factor function to inhibit tumor growth. Therefore, our findings decipher a novel crosstalk between ALK and TGF-β pathway in tumorigenesis and reveal potential TGF-β-related effects in patients with ALK treatment.

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**APPENDICES: N/A**