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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 <i>in vivo</i> 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.					
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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 expression or activity both blocks other oncogenic activities of ALK and restores Smad4 function, 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:

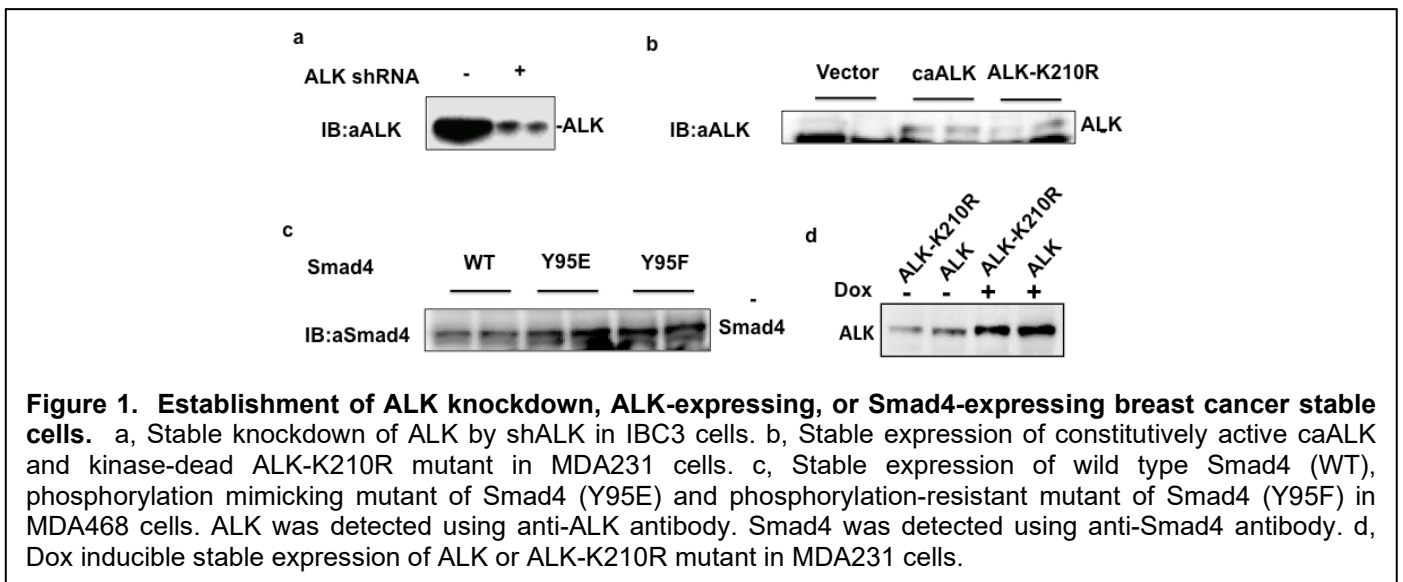
In our preliminary studies, we found that ALK can phosphorylate Smad4 on a particular tyrosine residue and inactivate Smad4 activity. During the first funding year of this proposal, we have investigated whether forced activation and knockdown of ALK affect TGF- β responses in breast cancer cell lines. 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. Thus, during the second funding year of this proposal, we further characterize the impact of ALK and Smad4 tyrosine phosphorylation on Smad4 signaling and transcriptional responses. For example, we determine how specific is Smad4 tyrosine phosphorylation by ALK or other tyrosine kinases? What steps in the TGF- β signaling pathway does Smad4 tyrosine phosphorylation affect? What is the global

effect of Smad4 Y95 phosphorylation on TGF- β mediated responses? We have completed our proposed work for year 2016-2017 as presented below:

Task 1. Examination of cellular responses to TGF- β .

As planned in our proposal, the major effort in our first funding year was to produce stable cell lines expressing caALK, knockdown of ALK or Smad4 or its variants in breast cancer cells. These cell lines will be our study system to facility our characterization of ALK and Smad4 phosphorylation.

1. We used various cloning strategies such as PCR, restriction enzyme digestion et al., and have successfully generated expression constructs in lentiviral vectors encoding shALK (ALK-specific shRNA for ALK knockdown), caALK (a constitutively active form of ALK), kdALK (an kinase dead, inactive form of ALK by point mutation: K $\rightarrow$ R at amino acid 210), Smad4, shSmad4 (Smad4-specific shRNA for Smad4 knockdown), Smad4-Y95E (a Y95 phosphorylation mimicking mutant of Smad4), and Smad-Y95F (a Y95 phosphorylation-resistant mutant of Smad4).
2. We have transfected 293FT cells with these lentiviral constructs together with lenti virus packaging plasmids, and have produced lentiviral particles expressing shALK, shSmad4, caALK, ALK-K210R, Smad4, Smad4-Y95E, and Smad-Y95F.
3. By infecting cells with these lentiviral particles and using puromycin drug as selecting marker, we have successfully generated the following stable cells:
 - MDA-IBC3 cells that stably expressing shALK and shRNA control;
 - MCF10A and MDA-MB-231 cells stably expressing caALK or ALK-K210R;
 - Smad4-deficient MDA-MB-468, MCF10A, MDA-MB-231 and IBC3 cells stably expressing Smad4 WT, Smad4-Y95E, or Smad4-Y95F;
 - More importantly, we have generated a Dox inducible expression of caALK or ALK-K210R under the control of tet-on promoter in MDA231 cells, hence, we control the intensity and duration for the expression of ectopic genes.



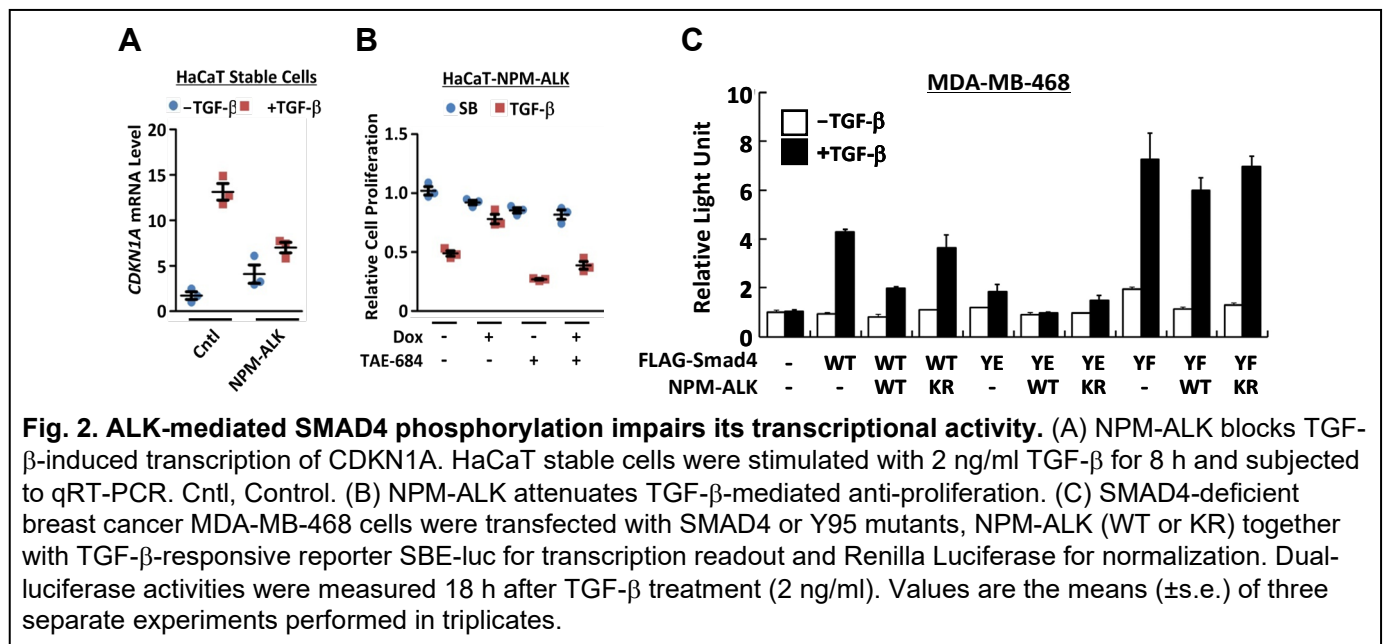
The successful establishment of these stable cells were confirmed by Western blot with specific antibodies and gene specific Q-PCR. Figure 1 is the representative data showing the successful establishment of stable cell lines.

4. We have examined the cellular responses of cells with manipulated levels of caALK (e.g. NPK-ALK).

We have examined expression of TGF- β target genes such as those regulating cell cycle, extracellular matrix proteins, and EMT markers. As shown in Figure 2A, TGF- β induced CDKN1A expression, which could be abrogated by ectopic expression of caALK. We have also monitored cell proliferation of the stable cell lines expressing Dox-inducible caALK (Figure 2B). Apparently, TGF- β -mediated suppression on cell proliferation could be blocked by Dox-induced caALK expression. Addition of ALK inhibitor TAE-684 clearly rendered caALK-expressing cells to be inhibitable by TGF- β .

5. We examined the cellular responses of cells with manipulated levels of caALK.

Like in ALK-activated lymphoma and lung cancer cells, activated ALK markedly attenuated SMAD4 responses in MDA-MB-468 cells. As shown in Figure 2C, putting back of SMAD4 into breast cancer MDA-MB-468 cells, which are SMAD4-deficient, could restore TGF- β transcriptional responses. In contrast, expression of ALK could significantly block SMAD4-mediated TGF- β response. Furthermore, as we know the tyrosine phosphorylation on SMAD4 (by using mass spectrometry), we created tyrosine-to-glutamate (YE) and tyrosine-to-phenylalanine (YF) substitutions and tested their ability to restore TGF- β response in MDA-MB-468 cells. The YE mutant completely failed to restore the TGF- β response. The YF mutant, however, not only induced the highest TGF- β response, but not became resistant to the blockade by ALK.

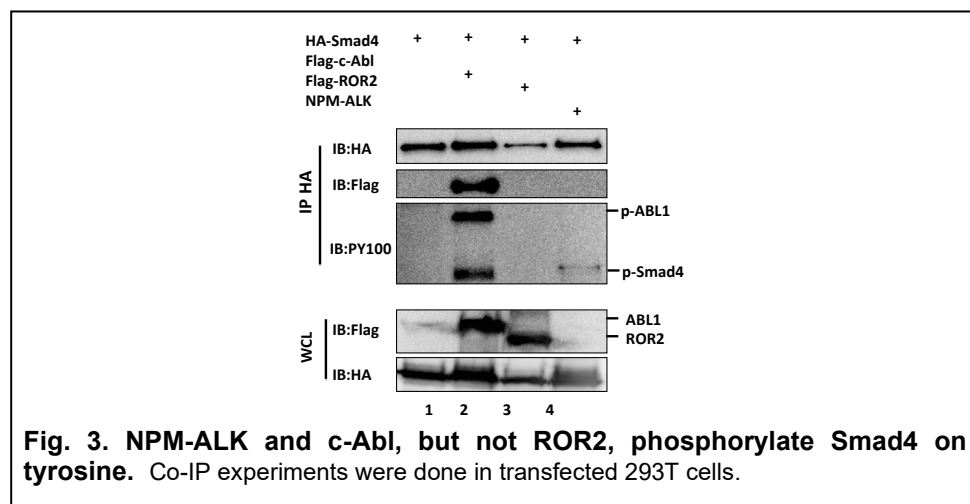


Task 2: Examining the effect of ALK on Smad signaling activities.

1. Examining Smad4 phosphorylation by different protein tyrosine kinases (PTK).

The human genome encodes 90 protein tyrosine kinases (PTK), including 58 receptor tyrosine kinases and 32 non-receptor tyrosine kinases. Activation of many of these kinases (e.g. EGFR, Her2, BCR-Abl, c-Src, just name a few) has been associated with tumorigenesis, and thus may also interplay with Smad proteins. Hence, we were wondering if Smad4 is phosphorylated and its activity is regulated by different protein tyrosine kinases. This investigation will provide insights into the mechanisms underlying TGF- β resistance in many cancer types.

Specifically, in our study, we have constructed an expression library of all PTKs. Out of 90 PTKs, 80 PTKs were expressed in transfection experiments. We thus screened the effect of these PTKs and examined their ability to phosphorylate Smad4 using phosphor-Tyr Western blotting analysis. We found a few of PTKs can phosphorylate Smad4, including c-Abl (Fig. 3). More importantly, using PY95 antibody, we could detect Smad4 phosphorylation in breast cancer tissues, where EGFR family members are also activated. We are now testing whether EGFR family members can phosphorylate other sites of Smad4 or

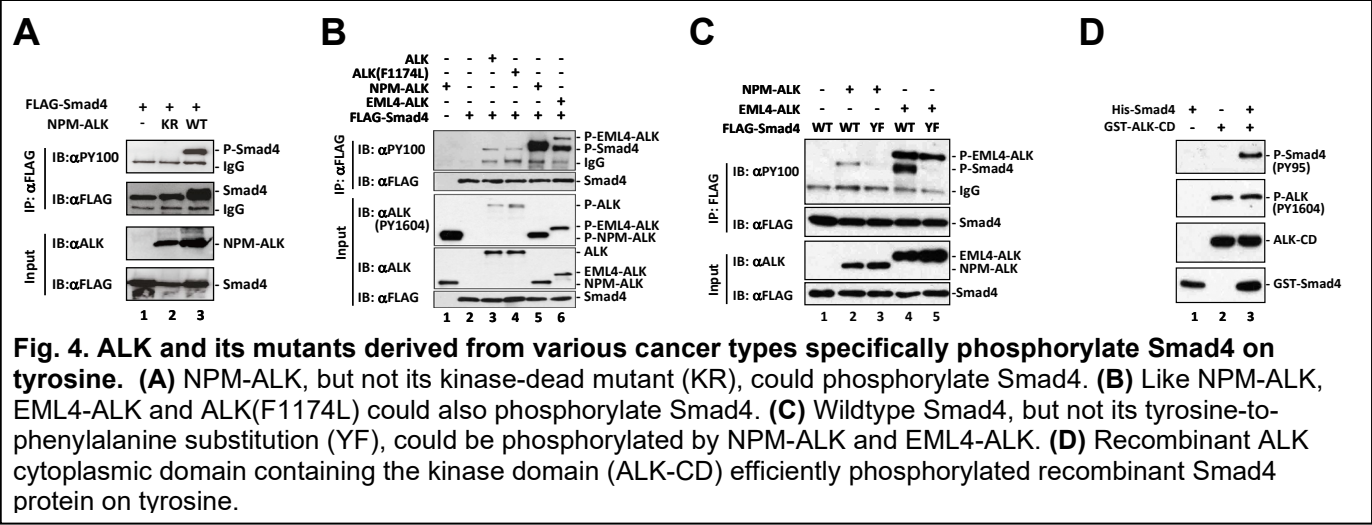


other Smads.

- Examining the effect of ALK on other Smads and the effect of overexpressed ALK on potential tyrosine phosphorylation of Smad2/3.

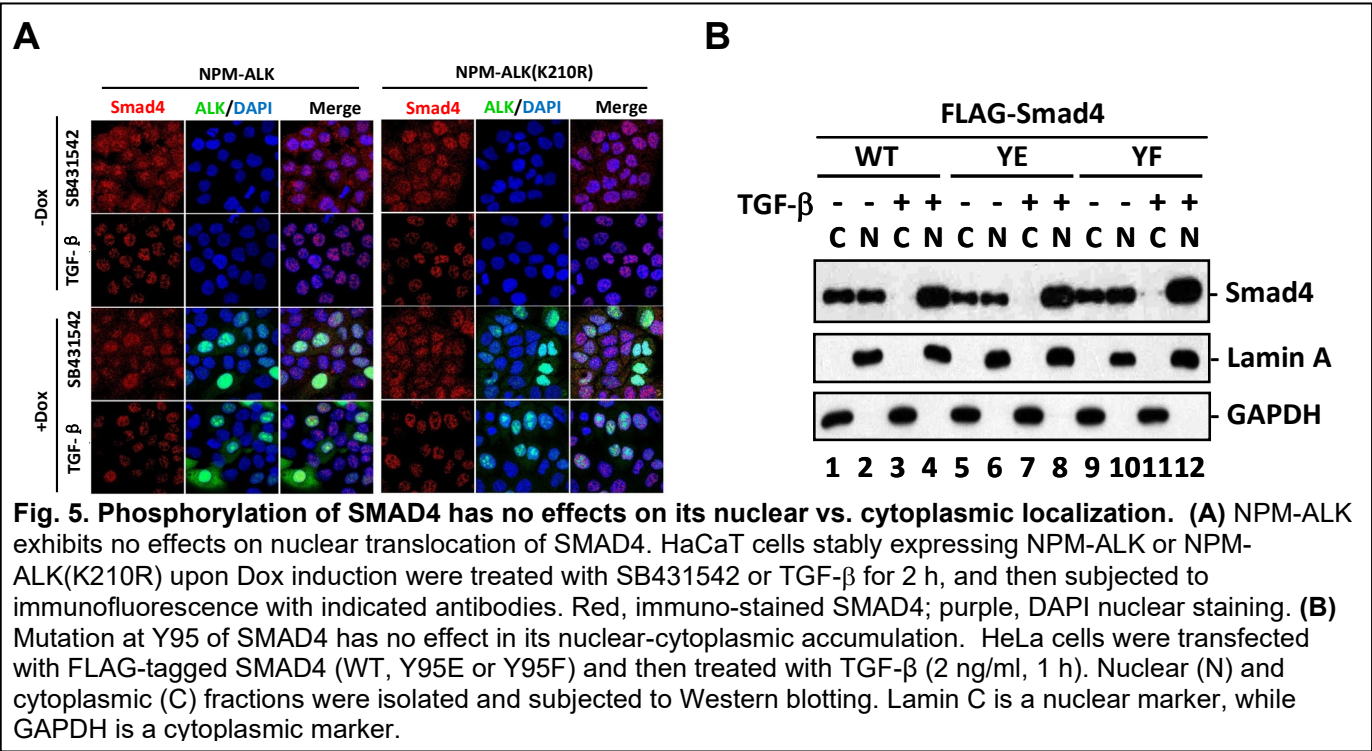
Smad4 belongs to the structurally related Smad family of intracellular signal transducers for TGF- β superfamily. The human genome encodes eight Smad proteins. Besides co-Smad Smad4, there are five receptor-activated Smads (R-Smads), which are serine phosphorylated by specific type I receptors upon ligand stimulation (i.e. TGF- β /activin-specific Smad2/3 and BMP-specific Smad1/5/8). After identifying the phosphorylation of Smad4 by ALK, we were interested in determining if ALK phosphorylates Smad4 specifically or also targets other Smad proteins.

We used cell transfection, immunoprecipitation and Western blot with specific antibodies, endogenous Smad tyrosine phosphorylation, and *In vitro* kinase assay to examine the effect of ALK on other Smads and the effect of overexpressed ALK on potential tyrosine phosphorylation of Smad2/3. We found that ALK specifically tyrosine-phosphorylated Smad4 but not other intracellular mediators of TGF- β signaling such as Smad2 and Smad3, suggesting the specific regulation of Smad4 activity by ALK (Fig. 4).



3. Examine the effect of Smad4 tyrosine phosphorylation on Smad4 intracellular localization.

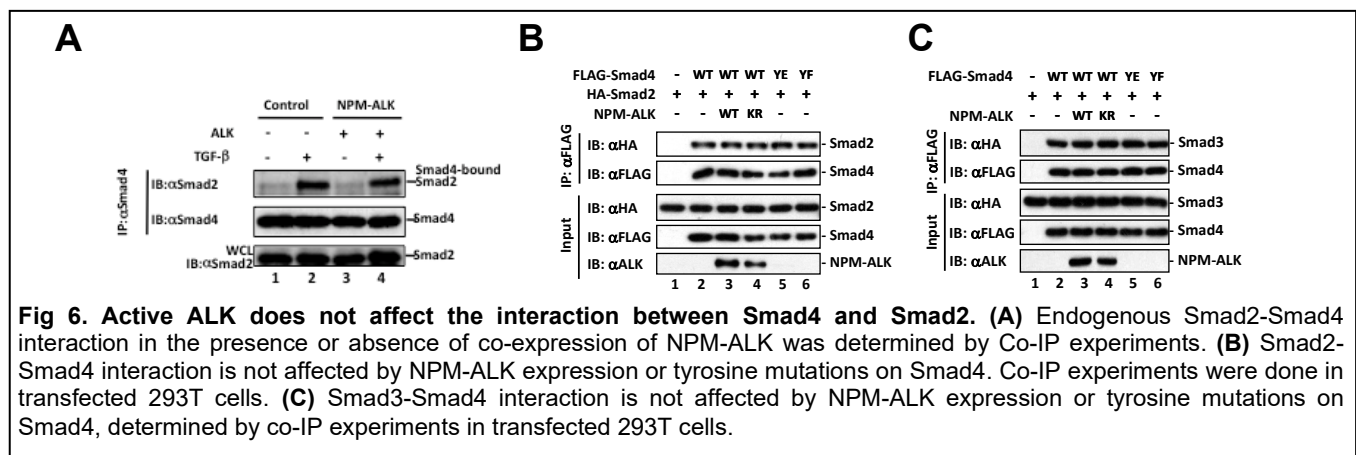
TGF- β treatment promotes nuclear accumulation of Smad4 and the subsequent transcriptional activation of target genes. To further characterize in detail how ALK regulates Smad4 activity, here we examined if ALK and resulted Smad4 tyrosine phosphorylation changes the subcellular distribution of



Smad4 using conventional methods such as immunofluorescence staining (Fig. 5A) or cell fractionation assay (Fig. 5B). We found that overexpression of ALK had no effect on Smad4 nuclear-cytoplasmic partitioning. Furthermore, we made mutation of Y95 into acidic E (i.e. Y95E to mimic phosphorylation) or F (Y95F to cause phosphorylation-defective) on Smad4, and found that these mutations did not impact the intracellular localization of Smad4.

4. Examine the effect of Smad4 tyrosine phosphorylation on Smad4-Smad2/3 complex formation.

Since the function of Smad4 in mediating TGF- β signaling relies largely on its association with Smad2 and Smad3 to form a large transcriptional factor complex, we determined the effect of ALK on TGF- β -induced complex formation between Smad4-Smad2/3 in BC cell lines with/without ALK knockdown or with ALK overexpression. For this purpose, we immunoprecipitated Smad4 with Smad4-specific antibody from a

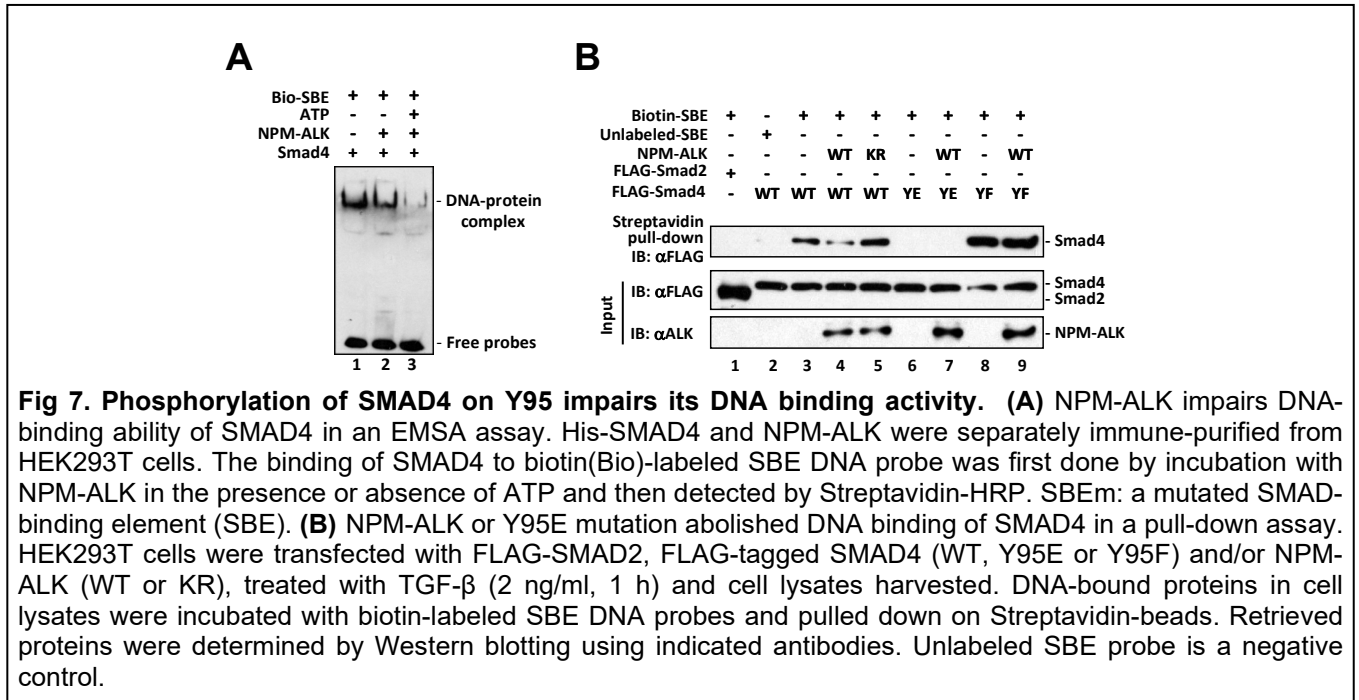


variety of cell lines (e.g. MDA231 cells), and the presence of Smad2 in the precipitated complex was determined by Western blot with Smad2-specific antibody. We found that overexpression of active ALK (NPM-ALK) did not alter the interaction between Smad4 and Smad2 at endogenous levels (Fig. 6A) or in transfected cells (Fig. 6B). Likewise, overexpression of active ALK did not alter the interaction of Smad4 with Smad3 (Fig. 6C). Furthermore, the YE or YF substitution had no effect on the Smad2/3-Smad4 interactions (Fig. 6B and 6C).

5. Analysis of Smad4 WT, Y95E or Y95F DNA-binding activity using Electrophoretic Mobility Shift Assay (EMSA).

We have examined the effect of Smad4 tyrosine phosphorylation on its binding to DNA by using two DNA-binding assays: EMSA and DNA pull-down. Specifically, EMSA detects the retarded mobility of DNA-protein complexes in comparison to free DNA probes. As expected, SMAD4 effectively binds to Smad-binding element (SBE). Notably, this gel-shifting ability of SMAD4 was nearly abolished by NPM-ALK in the presence of ATP (Fig. 7A). In DNA-pulldown assays, wild-type SMAD4 efficiently bound to biotin-labeled SBE, which was inhibited by NPM-ALK, but not kinase-dead NPM-ALK(K210R). The Y95E mutant was

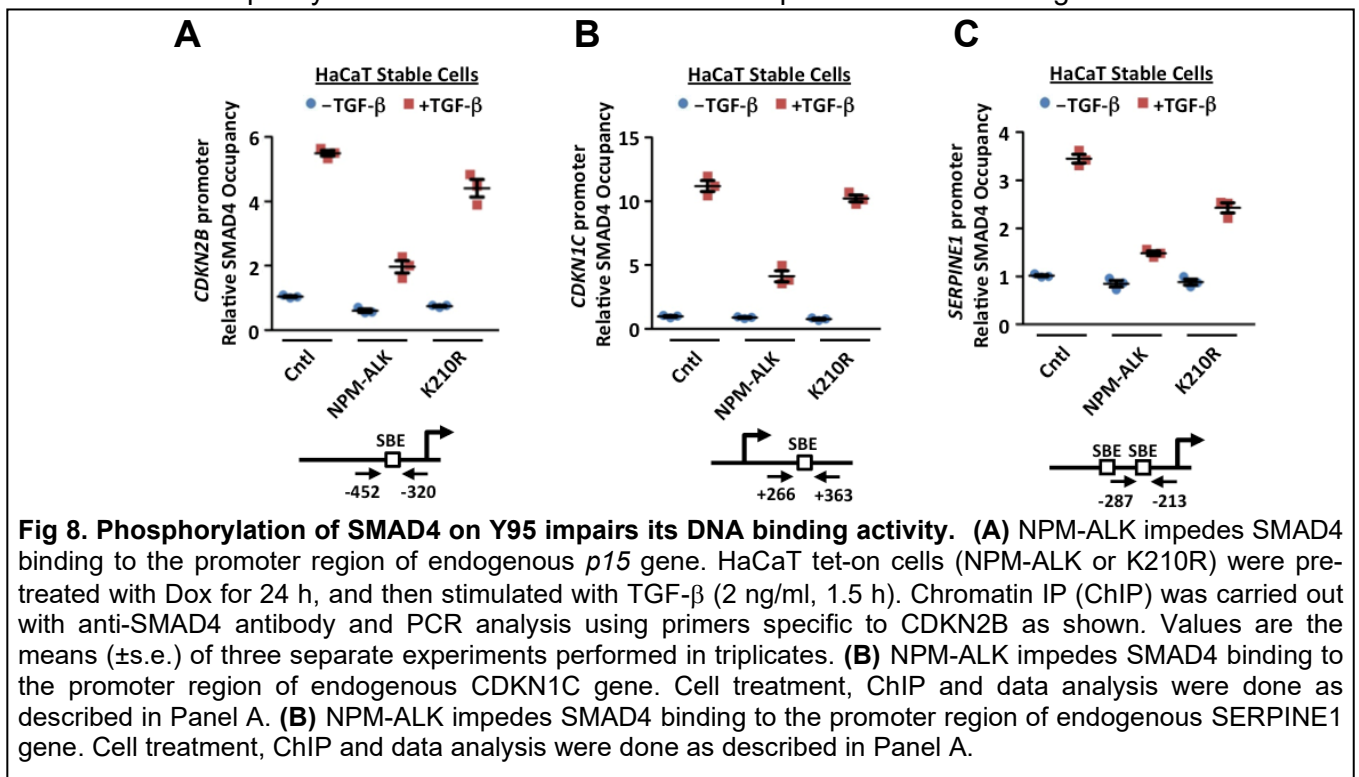
unable to bind to SBE (Fig. 7B, lane 6). Interestingly, the Y95F mutant exhibited a higher level of SBE-binding activity than wild-type SMAD4, which is not affected by NPM-ALK.



Task 3: Analysis of the global effect of ALK on Smad4 genomic and transcriptional responses

1. Examine the effect of Smad4 tyrosine phosphorylation on Smad4 binding to chromatin.

Since SMAD4 and its cognate partner SMAD3 can bind directly to the promoters of TGF-β target genes such as CDKN1A, CDKN1C and SERPINE1 and induce their transcription, we then assessed whether ALK influences the occupancy of SMAD4 and SMAD3 on the promoters of these genes. We have further

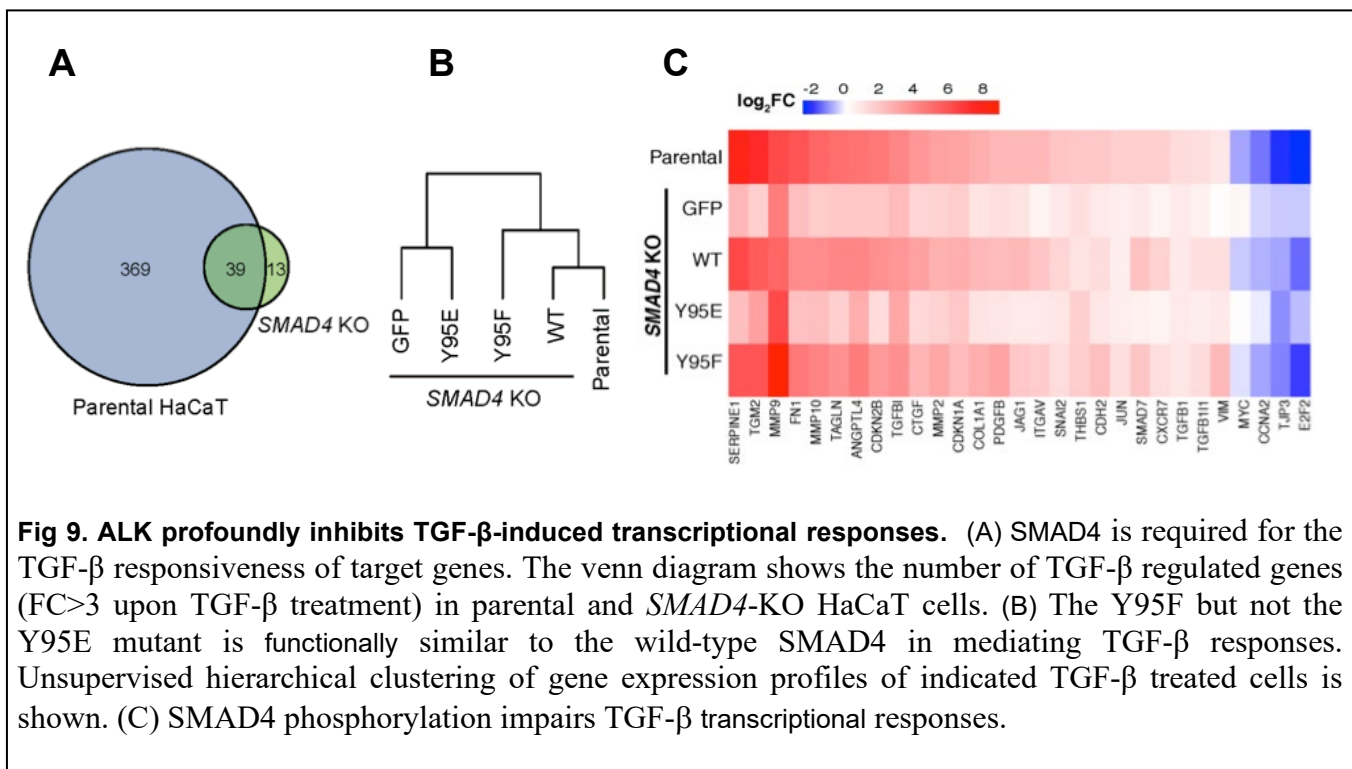


examined the effect of Smad4 tyrosine phosphorylation on its binding to chromatin by using a more physiological relevant approach: Chromatin Immunoprecipitation (ChIP). Specifically, the effect of phosphorylation on Smad4 binding to endogenous TGF- β target gene promoters was examined. Cell lysates was collected and sonicated to generate chromosomal fragments. Anti-Smad4 or anti-Smad4(pY95) antibody was used to immunoprecipitate total Smad4 or Smad4(pY95), and the recruitment of Smad4 or Smad4(pY95) to chromatin was quantitatively analyzed by qPCR with primers covering a specific promoter region of TGF- β target genes of p15, p57, PAI-1 and p21. Immunoprecipitation with control IgG, qPCR with primers covering a distal region or the promoter region of GAPDH was included as negative controls. In HaCaT tet-on cells, inducible expression of oncogenic ALK, but not its K210R mutant, markedly reduced TGF- β -induced occupancy of SMAD4 on the promoters of CDKN2B (Fig. 8A), CDKN1C (Fig. 8B) and SERPINE1 (Fig. 8C). On the contrary, ALK depletion profoundly increased the TGF- β -induced occupancy of SMAD4 on the promoters of these genes (data not shown), and similarly affected the occupancy of SMAD3 on the same gene promoters in SUDHL-1 cells (F data not shown). These results support the conclusion that ALK potentially blocks the SMAD3/4-chromatin association on the TGF- β target promoters.

2. SMAD4 tyrosine phosphorylation abolishes TGF- β -induced genomic responses

To examine the effects of Y95 phosphorylation on SMAD4-mediated transcription, 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). These HaCaT cells were treated with or without TGF- β and subjected to RNA-Seq analyses. RNA-seq experiment in Figure 9 was performed twice with similar results and the data shown was collected from one representative experiment.

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 (Fig. 9A), indicating that SMAD4-null cells profoundly lost responsiveness to TGF- β in gene transcription. 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 (Fig. 9B). 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. Next, we specifically examined a group of known TGF- β target genes, including SERPINE1, ANGTL4, CTGF, CDKN2B, CDKN1A, JAG1, SMAD7, MYC, CCNA2 and E2F2. As shown in Fig. 6I, TGF- β induced upregulation or repression of most of the selected target genes in both parental cells and SMAD4-null cells stably expressing wild-type SMAD4 or its Y95F mutant, but not or to a lesser extent in the GFP- or Y95E-rescued SMAD4-null cells. Together, our genome-wide transcriptional analyses supported the negative role of SMAD4 Y95 phosphorylation in TGF- β responses.



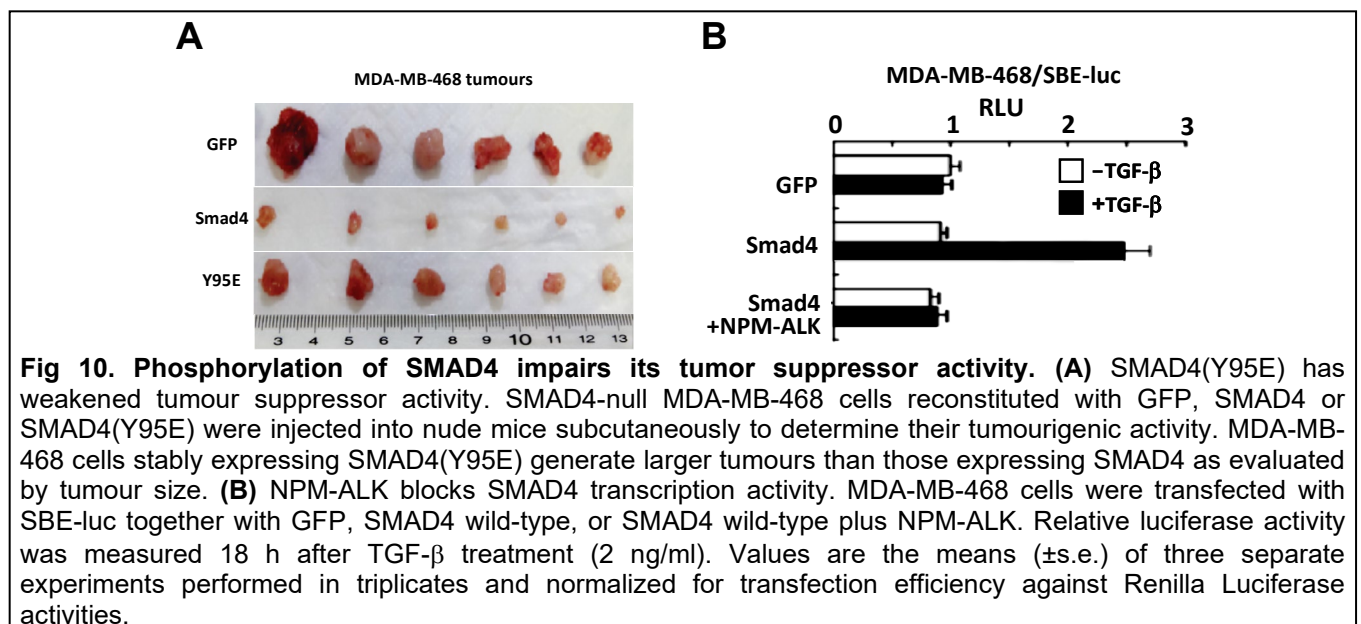
Task 4: To test whether ALK activation leads to Smad4 Y95 phosphorylation in vivo

1. Examining the levels of ALK and Smad4 phosphorylation in a large number of human breast tumor samples, including IBC

Not pursued

2. Performing lentivirus-mediated delivery of caALK into mammary glands and examining Smad4 Y95 phosphorylation.

TGF-β signalling plays paradoxical roles in tumorigenesis, promoting both tumor suppression and metastasis. The current paradigm is that tumor suppression is the dominant activity of TGF-β in normal tissues and tumors at early stages. As a tumor suppressor, SMAD4 can potently suppress proliferation and promote apoptosis. We established cell lines stably expressing wild-type SMAD4, its Y95E mutant, or GFP (as control) in breast cancer MDA-MB-468 cells. These cells were injected subcutaneously into nude mice to induce tumors. About 40 days later, the nude mice were executed and tumors were dissected and weighed. Results show that parental or control GFP cells led to obvious early tumor growth. Expression of wild-type SMAD4 moderately inhibited the growth of tumors, consistent with its tumor suppressor function. In contrast, the ability of SMAD4 to inhibit tumor growth was greatly compromised by the phospho-mimicking Y95E mutation (Fig. 10A). In MDA-MB-468 cells, while SMAD4 restored TGF-β response, NPM-ALK blocked SMAD4 transcriptional response (Fig. 10B).



Task 5: To test whether ALK activation initiates and/or promotes mammary tumor in mouse models.

Performing lentivirus-mediated delivery of caALK into mammary glands of WT mice and MMTV-Wnt mice.

No progress made

Task 6: To investigate the impact of ALK blockade on breast cancer initiation and progression in DCIS and PDX mouse models.

No progress made

KEY RESEARCH ACCOMPLISHMENTS:

- Determined the specific regulation of ALK on Smad4.
- Determined that Smad4 is phosphorylated by other protein tyrosine kinases establishing a general regulatory mechanism of TGF-β activity by protein tyrosine kinases.
- Determined that how Smad4 Y95 phosphorylation regulates Smad4 activity. Tyrosine phosphorylation of Smad4 does not affect its binding to other Smad partners such as Smad2 and Smad3; nor does it affect nuclear accumulation of Smad4 upon TGF-β stimulation.
- Determined that how Smad4 Y95 phosphorylation blocks the DNA-binding activity of Smad4.
- Determined the effect of ALK and Smad4 Y95 phosphorylation on TGF-β tumor suppressing and transcriptional responses.

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

- TGF- β Meeting, Leiden, Netherlands, 8/21-23/2016. “Smad Signaling”
- 5th International Breast Cancer Stem Cell Symposium, Shanghai, 10/9-10/2016.
- TGF- β Meeting, Uppsala, August 31-September 2, 2017
- TGF- β Meeting, Xiamen, September 26, 2017.
- The 2018 San Antonio Breast Cancer Symposium, San Antonio, Texas, from December 4-8, 2018.
- American Society of Cell Biology (ASCB)-EMBO 2018 Meeting, San Diego, California, December 8-12, 2018
- Zhang Q et al., 2019. ALK phosphorylates SMAD4 on tyrosine to disable TGF- β tumour suppressor functions. *Nat Cell Biol.* 21(2):179-189.

CONCLUSION:

TGF- β signaling exerts its tumor suppressive actions through Smad-dependent transcriptional responses. 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 Tyrosine-95. Mechanistically, Smad4 tyrosine phosphorylation completely wiped out its DNA-binding activity and thus TGF- β -mediated canonical transcriptional responses. Consequently, tyrosine phosphorylation disables Smad4 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