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Biologically Augmenting Radiation Therapy by Inhibiting TGF β in NSCLC from Molecular to Microenvironment

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Title: TGF β Inhibitors Enhances Radiation Sensitivity of NSCLC Cells In Vitro and In Vivo by Attenuating the DNA Damage Response

Article Type: Full Length Article

Section/Category: Biology Contribution

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Abstract: Purpose To determine whether TGF β inhibition increases the response to radiotherapy in human and mouse non-small cell lung carcinoma (NSCLC) cells in vitro and in vivo.

Methods TGF β mediated growth response and pathway activation were examined in human NSCLC NCI-H1299, NCI-H292, and A549 cell lines and murine Lewis lung cancer (LLC) cells. Cells were treated in vitro with LY364947, a small molecule inhibitor of the TGF β type I receptor kinase, or with pan-isoform TGF β neutralizing monoclonal antibody, 1D11, before radiation exposure. DNA damage response was measured by ATM or Trp53 protein phosphorylation, γ H2AX foci formation or comet assay in irradiated cells. Radiation sensitivity was determined by clonogenic assay. Mice bearing LLC syngeneic subcutaneous tumors were treated with 5 fractions of 6 Gy and/or neutralizing or control antibody.

Results NCI-H1299, A549 and LLC NSCLC cell lines pretreated with LY364947 prior to radiation exposure exhibited compromised DNA damage response indicated by decreased ATM and p53 phosphorylation, reduced γ H2AX foci, and increased radiosensitivity. NCI-H292 cells were unresponsive. TGF β signaling inhibition in irradiated LLC cells resulted in unresolved DNA damage. Subcutaneous LLC tumors in mice treated with TGF β neutralizing antibody exhibited fewer γ H2AX foci after irradiation and significantly greater tumor growth delay in combination with fractionated radiation.

Conclusions TGF β inhibition prior to radiation attenuated DNA damage recognition and increased radiosensitivity in most NSCLC in vitro and promoted radiation-induced tumor control in vivo. These data support the rationale for concurrent TGF β inhibition and radiotherapy to provide therapeutic benefit in NSCLC.

Suggested Reviewers:

Opposed Reviewers:

NYU
SCHOOL OF
MEDICINE

Mary Helen Barcellos-Hoff, Ph.D.
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Director of Radiation Biology
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September 15, 2014

Dr. Anthony L. Zietman, MD, FASTRO
Editor in Chief
International Journal of Radiation Oncology, Biology and Physics

Dear Dr. Zietman,

Thank you for the valuable comments and insights from the review of our original submission entitled “Attenuation of the DNA Damage Response by TGF β Inhibitors Enhances Radiation Sensitivity of NSCLC Cells In Vitro and In Vivo”. I am pleased to submit on the behalf of my co-authors our revised manuscript that addresses this critique of these preclinical therapeutic studies, which I hope you and the reviewers will deem appropriate for publication. I have included below a point-by-point response to the reviewers’ comments indicated in blue

To recap, these studies were based on strong mechanism-based hypothesis that TGF β inhibition can radiosensitize cancer cells based on our prior research showing its inhibition compromises ATM kinase activity. In this study we show that ATM activity is abrogated small molecule inhibition of the TGF β type I receptor kinase and indeed radiosensitizes some human non-small cell lung cancer cell lines as measured by clonogenic survival due to unrepaired DNA damage measured by comet assay. The administration of TGF β neutralizing antibodies in the syngeneic mouse model of Lewis lung cancer enhances the efficacy of fractionated radiation therapy. These data open a new venue for the use of TGF β inhibitors that are in clinical trials and show promise for efficacy in cancer therapy.

Sincerely,

Mary Helen Barcellos-Hoff, Ph.D.
Director of Radiation Biology
Departments of Radiation Oncology and Cell Biology

Reviewer #1: In this manuscript the authors describe the impact of TGF beta inhibition on radiosensitivity of NSCLC cell lines. The authors use both in vitro and in vivo experiments to demonstrate the effect of TGF beta inhibition.

While the manuscript has merit, there are a number of issues that should be addressed.

In figure 1B, the western blots have no loading control.

Loading control has been added to Figure 1A.

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Mutational inactivation of the TGF β signaling pathway is a key event during NSCLC progression; functionally, some NSCLC tumor cells whose growth can no longer be inhibited by TGF- β become more aggressive and invasive. Nonetheless, we wanted to convey that some NSCLC cell lines both maintain TGF β secretion and response to TGF β . However, given the reviewers comment and the word limit, the data on TGF β production has been removed.

Also, what does the term SMI in Figure 1C refer to.

SMI refer to "small molecular inhibitor", and has been added in Figure 1C legend.

In Figure 2. If you posit that depletion of TGFbeta signalling is impacting on DNA damage response, then why is there no change in ATM -1981 serine phosphorylation in H292 cells if they exhibit decreased p-SMAD (as shown in Figure 1). This mechanism needs to be clarified.

There may be several routes by which TGF β inhibition compromises DDR and multiple ways in which cancer cells evade TGF β control. Cancer cells are highly heterogeneous in terms of genomic alterations that include sequence changes and epigenetics. The H292 appear to have lost a intermediary between Smad and ATM. We are currently investigating what this intermediary is.

Our hypothesis that TGF β signaling is required for ATM kinase activity is well-supported by data in non-malignant human cells showing that TGF β inhibition kinase exhibit decreases phosphorylation of ATM and its downstream DDR

targets p53, Chk2 and Rad17 (Cancer Research 2006), as well as attenuating DDR in the mammary epithelium in situ (Cancer Research 2002). We went on to demonstrate that TGF β inhibition prior to radiation attenuates DNA damage recognition and enhances clonogenic cell killing in vitro and vivo in a panel of breast cancer and glioblastoma cells lines (Clinical Cancer Research 2010, Cancer Research 2011).

Notably, a new publication shows that Smad7 is important in ATM activity (Park, S., et al Smad7 enhances ATM activity by facilitating the interaction between ATM and Mre11-Rad50-Nbs1 complex in DNA double-strand break repair. Cell Mol Life Sci, 1-14, 2014.) Smad7 is downstream of Smad2/3. This has been elaborated in the discussion, e.g. “Nevertheless, the exact mechanism or pathways involving this TGF β inhibition, which attenuates IR induced DNA damage responses has not been well described”(first paragraph, page 13).

Further, gamma h2AX foci for H292 should be shown and the experiment should be repeated in A547 cells.

We quantified foci and added the results of A549 and NCI-H292 cells as suggested. This has been provided in Figure 2B.

Also there should be quantification of gamma H2AX foci as a time course experiment over a longer period until full resolution of the foci.

The objective here in assessing γ H2AX, as previously reported, was to determine the molecular recognition of DNA damage as evidenced by phosphorylation of H2AX rather than repair/recovery kinetics.

Why were only ATM and p53 examined as markers of DDR? There are other proteins activated in the DDR pathway.

We used these two endpoints to evaluate whether NSCLC cell lines behave as shown in our prior studies in breast cancer and GBM in which TGF β inhibition enhances radiation sensitivity and impairs DDR in support of translational potential. The detailed mechanism by which TGF β affects DDR is the topic of other studies.

In Figure 3, this experiment should be repeated in the other cell lines (especially H292). I would also like to see the impact of endogenous TGF beta on the resolution of comet moments as this should produce the opposite effect.

The comet data were conducted only in LLC as a prelude to conducting in vivo experiments.

We have not conducted experiments in which TGF β is added to cultures because the complex biology of TGF β often results in non-linear dose dependence and even opposite responses. For instance, it has been recently published that both TGF β inhibition and addition reduces cell migration (see “Concentration-dependent effects of transforming growth factor β 1 on corneal wound healing” Wang, et al. Mol Vis. 2011;17:2835-46. PMID: 22128231). Because of this well-

known aspect of TGF β biology, and since its addition is not clinically viable, we decided to not use this strategy in the studies reported in this manuscript

In figure 4, there needs to be statistics done on the clonogenic assays. The antibody 1D11 in LLC curve appears to overlap with no pretreatment. The DER10 in Figure F does not add to the story and should be removed.

We mistakenly omitted a statistical comparison in our original manuscript and have included it in our revision using ANOVA with Tukey test. As suggested by the reviewer and because of words limitation, we removed Figure 4F.

Again exogenous TGF beta should be added to the media and clonogenic assay repeated.

See response about adding TGF β above.

There should be quantification and statistics in Figure 5A. The delayed growth as posited by the authors is difficult to appreciate in the manner in which the data is presented in Figure 1C.

As suggested by the reviewer we added the quantification and statistics in Figure 5B. As shown in Figure 1C, NCI-H1299, A549 and LLC cells were refractory to TGF β -mediated growth regulation, only H292 cells were growth-delayed by TGF β .

Tumor mass at time of harvest should be quantified and noted.

Tumor mass at time of collection was quantified and included (Figure 5E).

Also, if histology could be examined for p-SMAD, that would greatly increase the value of the manuscript.

Data showing that radiation induces p-SMAD consistent with TGF β activation and is blocked by 1D11 are part of another study and are currently being prepared for an upcoming publication. In the manuscript submitted here, we refer to this observation as unpublished data.

The introduction is confusing. Initially the authors discuss NSCLC and then abruptly shift to GBM and breast cancer.

In the introduction, we provided data and references relative to our same area of interest - TGF β signaling and DDR- applied to different types of cancer, including breast and brain cancers. We aimed at providing the reader with sufficient background information about the topic that we have investigated in NSCLC. However, upon the reviewer suggestion, introduction has been edited for better understanding.

Please spell out TME in introduction.

This oversight is corrected.

The reference of tgf beta inhibitors in clinical trials needs to be expanded to underscore the importance of the current work.

This is an excellent point. The introduction now includes a summary of clinical trials involving TGF β inhibition in combination with radiotherapy in patients with breast cancer and GBM (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271).

The methods regarding the in vivo experiment needs clarification. How many injects of control or 1D11 antibodies were administered.

Regarding control or 1D11 antibodies injection"...receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and kept injecting every other days to termination"

How were the measurements made after two fractions, if there was such significant volume change, how is this accounted for in the growth curves?

"The measurements made after two fractions" means we measured the tumor after applying two fractions of 6Gy, i.e. the third day of irradiation. As shown in Figure 5F, we found that tumors in IR + 13C4 group presented similar tumor volume increase (compared to volume before IR) which indicated tumor growth has comparable kinetic with the two groups of sham irradiated tumors at this time point. However, the volume of half of the tumors (6/10) in IR+1D11 group decreased significantly.

Given that the experiments occurred when tumors reached 60-80 mm³ there is large margin for error on tumor measurements.

The mice were randomized when tumors reached 60-80 mm³, one day before treatment initiation, to create groups of similar comparable average volume. Each tumor was measured twice at each time-point during the experiment by two investigators to limit errors due to initial tumor small size and to increase accuracy. Add groups stats?

Reviewer #2: The authors of this study describe a study examining the effect of TGF-beta inhibition on radiosensitization of NSCLC cells using a small molecule TGBRI inhibitor and antibody against TGF-beta. The authors show data using pharmacologic experiments to suggest that TGF-beta signaling is critical for radiation response of NSCLC cells. Overall, I think this study is promising, but is somewhat derivative from work already performed with the same inhibitors in other cancers and in my mind has some deficiencies as detailed below. Finally, I would appreciate the consideration of addressing some other major and minor suggested revisions as below.

Major Revisions:

(1) How as growth inhibition measured in Figure 1C. There is no mention of the technique in the M&M, Results or Legend.

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The data of A549 and NCI-H292 have been included in Figure 2B.
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See response for Reviewer 1, questions (3) above as well concerning the repeating cells using comet assay. (I would restate the answer here. Also questions from Reviewer 1 are not numbered).
- (5) The difference in gamma-H2AX foci between 13C4 +IR and ID11+IR treated animals is difficult to appreciate. Quantification with appropriate statistical comparisons would solidify the conclusion by the authors that there is difference. As shown in Figure 5A, γ H2AX foci in vivo tissue is difficult to quantify by counting the number of foci. Here we quantify the mean intensity of γ H2AX inside the nuclear region of interest. Notably, the effect of IR and TGF β inhibition on the mean intensity is consistent with the in vitro results (Figure 5B)
- (6) Changes in proliferation (Ki-67 IHC) or apoptosis (cleaved caspase 3 IHC or similar) can be examined in the tumor xenograft studies to help refine the mechanism(s) of tumor growth delay observed in the mouse studies.
The tumors were collected 1 hr and 2 weeks after completion of IR. Neither are appropriate time points to evaluate proliferation or apoptosis. We contend that the in vitro clonogenic survival data showing that TGF β inhibition synergizes with RT is the basis for the increase in tumor growth delay.
- (7) The statistical test used to differentiate the tumor growth delay in Figure 5B and 5D is marked as ANOVA in the accompanying Legend. ANOVA is useful to compare the means of three or more unmatched groups. The depiction in Figure 5B/5D suggests a comparison of only two groups (and it appears to be over time in 5B). ANOVA is not the correct test for this comparison.
We replaced ANOVA with unpaired two-tailed Student's t-test.
Tumor volume in exceeded size limitation defined by the Institution animal protocols the two sham-IR groups so we were required to terminate these two groups one day earlier.
- (8) Page 13, 2nd paragraph is confusing and appears to be incorrect as written. There are multiple oncogenic driver sub-types most prominently in the adenocarcinoma histologic subtype. Of these oncogene driver subtypes, there is more than just EML4-ALK that has been shown to be targetable with agents that target specific oncoproteins, i.e. EGFR mutant subtype, ROS1-fusion subtype, RET-fusion subtypes, etc. Many of these have pre-clinical data and are in early phase clinical testing, but mutant EGFR oncoprotein targeting with erlotinib has been FDA approved even before crizotinib. We wished to underscore that anti-tumor agents often benefit only some NSCLC patients, due to tumor diversity

even with same histologic subtype. As we found NCI-H292 cells did not benefit from TGF β inhibition, it is crucial for future clinical translation and to target a larger component of NSCLC patients, to identify the explicit molecular biomarkers that will indicate the potential benefits from TGF β inhibition in the context of RT.

- (9) The references cited in this paragraph even given the Red J space constraints are not the best review articles that could be chosen (if possible the original literature would be ideal).

The reference 18 has been replaced by the original literature.

- (10) Page 14, 1st paragraph is also a jumble of factoids that I am not sure really apply well to radiosensitizing strategies in NSCLC. Mitomycin-C is not a drug commonly used in lung cancer. Some of the other statements in this paragraph are without references and not correct. Would help if a lung cancer clinician read and helped edit this paragraph to express what the authors wanted to convey.

In this paragraph we aimed at briefly summarizing some of the approaches used to improve NSCLC radiation therapy, which mostly involve the use of a higher radiation dose or effective radiation sensitization. Chemotherapeutic reagents were most commonly used as radiosensitizers to synergize with RT in clinical settings, however these reagents do not specifically target tumor cells, and they increase radiation toxicity to normal tissues. Because of these intrinsic limitations of chemotherapeutic reagents, we postulated that TGF β inhibitors selectively sensitize tumor response to radiation and at the same time protect normal lung from RT-induced fibrosis. In such clinical settings one could use selective escalation of the effective biologic IR dose to the tumor, and therefore improve local tumor control without increasing morbidity.

We are aware that Mitomycin-C is not used as first line in NSCLC, however it can be applied in NSCLC combination chemotherapy with concurrent radiotherapy (J Clin Oncol, 2010;28:3299-3306). In order not to misleading the reader we edited this paragraph and related references.

Minor Revisions:

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We have corrected this oversight.
- (2) Why did the authors not check the TGFB levels of A549 and LLC?
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- (4) Figure 2A - LLC panel is misaligned.
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SMI refer to "small molecular inhibitor", and has been added in Figure 1C legend.

In Figure 2. If you posit that depletion of TGFbeta signalling is impacting on DNA damage response, then why is there no change in ATM -1981 serine phosphorylation in H292 cells if they exhibit decreased p-SMAD (as shown in Figure 1). This mechanism needs to be clarified.

Our data in non-malignant cells show that TGF β inhibition kinase exhibit decreased phosphorylation of ATM and downstream DDR markers p53, Chk2 and Rad17 (Cancer Research 2006). We have demonstrated that TGF β inhibition prior to radiation attenuates DNA damage recognition and enhances clonogenic cell killing in vitro and vivo in a panel of cells lines such as mammary epithelium, breast cancer and glioblastomas (Cancer Research 2002, Clinical Cancer Research 2010, Cancer Research 2011). How this varies across cancer cell lines is an interesting question that we are actively pursuing. One avenue that we and others are investigating is whether TGF β modulation of DDR in

cancer cells occurs through SMAD-dependent or SMAD-independent pathways. A recent publication shows that Smad7 is important (Park, S., et al (2014). Smad7 enhances ATM activity by facilitating the interaction between ATM and Mre11-Rad50-Nbs1 complex in DNA double-strand break repair. Cell Mol Life Sci, 1-14). Thus there may be several routes by which TGF β inhibition compromises DDR. This has been elaborated in the discussion, e.g. "Nevertheless, the exact mechanism or pathways involving this TGF β inhibition, which attenuates IR induced DNA damage responses has not been well described"(first paragraph, page 13).

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Also there should be quantification of gamma H2AX foci as a time course experiment over a longer period until full resolution of the foci.

It is known that γ H2AX foci reflect the presence of a DSB. Our objective here in assessing γ H2AX, as previously reported, is to determine the molecular recognition of DNA damage. As suggested from the reviewer, it is excellent to quantify the γ H2AX foci to know the effect of TGF β inhibition on the DNA damage repair progression after RT. We are concerned that radiation induced γ H2AX foci doesn't equate with DSB all situations which is dose and time dependence (Mutat Res. 2010, 704: 78-87). For example, normal human fibroblasts irradiated showed persistent foci for 5 days following 4 Gy of X-rays even though by that time all DSB should be fully resolved which supported the presence of γ H2AX foci may not always signify the presence of a physical break.

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Because of manuscript size restrictions, we reported data that further confirmed prior publications in breast cancer and GBM. We believe that the comet assay in LLC cell lines may support the translational potential that then applied in the animal experiments.

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Attenuation of the DNA Damage Response by TGF β Inhibitors Enhances

Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Shisuo Du, MD, PhD,¹ Sophie Bouquet, PhD,¹ Chen-Hao Lo, MS¹, Ilenia Pellicciotta, PhD,¹ Shiva Bolourchi, PhD,¹ Renate Parry, PhD,² and Mary Helen Barcellos-Hoff, PhD^{1,3}

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² Varian Medical Systems, Inc., Palo Alto CA

³ Address reprint requests to

Mary Helen Barcellos-Hoff

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Running title: TGF β Inhibition in non small cell lung cancer (NSCLC) RT

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Dr. Sophie finished her postdoc at NYU (and so the experiments in this paper) and went to work for Roche where she's still an employee.

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Attenuation of the DNA Damage Response by TGF β Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Summary

Our study shows that TGF β inhibition synergizes with RT through control of the DNA damage response, which leads to greater tumor cell kill. There are several TGF β inhibitors in clinical trials. The excellent safety profiles demonstrated in these clinical trials, as well as the possibility of protection from late complications of lung fibrosis, provide further motivation for assessing TGF β inhibitors as an adjunct to RT for NSCLC.

Attenuation of the DNA Damage Response by TGF β Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Abstract

Purpose To determine whether inhibition of TGF β increases the response to radiotherapy in human and mouse NSCLC cancer cells *in vitro* and *in vivo*.

Methods TGF β -mediated growth response and pathway activation were examined in human, NCI-H1299, NCI-H292, and A549 NSCLC cell lines and murine Lewis lung cancer (LLC) cells. Cells were treated *in vitro* with LY364947, a small molecule inhibitor of the TGF β type I receptor kinase, prior to determination of clonogenic survival following graded radiation doses. DNA damage recognition was measured by γ H2AX and repair was assessed using a comet assay in irradiated cells pretreated with LY364947. Levels of protein involved in the DNA damage response were measured using Western blotting. —Mice bearing LLC syngeneic murine subcutaneous tumors were treated with a pan-isoform TGF β neutralizing monoclonal antibody, 1D11, and fractionated radiation therapy.

Results Irrespective of sensitivity to TGF β growth regulation, 3 of the 4 NSCLC cell lines pretreated with LY364947 prior to radiation exposure exhibited compromised DNA damage ~~recognitions-response. This was as~~ indicated by decreased ATM and p53 phosphorylation, reduced γ H2AX foci, and increased radiosensitivity, which was ~~as~~ measured by clonogenic survival. TGF β signaling inhibition in irradiated LLC cells resulted in greater residual DNA damage. Mice bearing LLC subcutaneous tumors treated with 1D11 also exhibited fewer γ H2AX

foci shortly after irradiation and greater tumor growth delay in combination with fractionated radiation exposure.

Conclusions These results indicate that TGF β inhibition prior to radiation attenuates DNA damage recognition, enhances clonogenic cell killing and promotes tumor growth delay. These results ~~, which~~ suggest that concurrent TGF β inhibition and radiotherapy will provide therapeutic benefit in NSCLC.

Key Words: ionizing radiation; non-small cell lung cancer; TGF β ; small molecule inhibitor; neutralizing antibody

Introduction

Improved treatment of non-small-cell lung cancer (NSCLC), which is one of the leading causes of deaths from cancer worldwide, is urgently needed [1]. Approximately 70% of NSCLC patients receive radiotherapy (RT), either alone or in combination with other treatment modalities such as surgery or chemotherapy. Biologically augmenting tumor cell radiosensitivity can play a crucial roles in determining treatment success [2]. TGF β ligands are enriched in the tumor microenvironment (TME), and where their production by stromal or tumor cells varies according to the tumor phenotype. In NSCLC, increased TGF β activity correlates with tumor progression and increased tumor angiogenesis [3,4]. TGF β signaling activation in Tumor microenvironment ~~the (TME)~~ has been identified as a key factor for chemotherapy resistance in NSCLC [4], in addition to its well-recognized impact on tumor promoting effects in progression and metastasis. Currently, TGF β inhibitors are in clinical trials for several types of cancer including ~~breast cancer and glioblastoma (GBM) and breast cancer~~ (reviewed in [5]). TGF β signaling blockade augments ~~glioblastoma (GBM)~~ response to chemoradiation in GBM preclinical models [6,7], and specifically inhibits GBM cancer stem cell renewal after radiation [8]. TGF β inhibition promotes clonogenic cell death in irradiated GBM and both mouse and human breast cancer cell lines and GBM cells in vitro, and ~~systemically~~ neutralizing TGF β enhances RT actions in GBM and breast cancer preclinical models [6,8,9].

Growing evidence supports a specific role for TGF β in mediating the rapid execution of the DNA damage response (DDR) [10]. *Tgfb1* null keratinocytes irradiated *in vitro* exhibit reduced ataxia telangiectasia mutated (ATM) protein auto-phosphorylation and kinase activity. This which in

~~turn~~then decreases recognition of the sites of DNA damage, which is evidenced by decreased ~~ted~~ γ H2AX foci and impairededs critical DNA damage transducers Chk2, p53, and Rad17 that ~~in turn,~~ abrogate the appropriate cell fate decisions [11]. Notably, a TGF β type I receptor kinase small molecule inhibitor phenocopies genetic depletion in human epithelial cells.

Little is known about the contribution of TGF β to the tumor response to radiation in NSCLC. TGF β activation is efficiently induced by ionizing radiation, in part due to the presence of a redox sensitive motif in the latency associated peptide (reviewed in [12]). Based on the efficacy in breast and brain tumors, it is plausible that TGF β inhibition through compromising DDR after IR-induced DNA damage could improve radiosensitivity of NSCLC irrespective of cell growth

sensitivity. TGF β signaling inhibitors are generally safe and may be efficacious in several clinical applications. Clinical trials involving TGF β inhibition in combination with radiotherapy in patients with breast cancer and —GBM —have progressed (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271). Using a small molecule inhibitor of the TGF β type I receptor kinase and a pan-neutralizing TGF β antibody currently in clinical development, ~~Where, we~~

determined the role of TGF β signaling in DDR following irradiation in NSCLC cell lines and murine Lewis lung carcinoma (LLC) tumors. We found that although most of human and murine NSCLC cell lines were refractory to TGF β -mediated growth regulation, TGF β inhibition increased radiosensitivity of NSCLC cells, compromised DDR, and the combination of TGF β neutralizing antibody with fractionated RT significantly increased tumor growth control. These results suggest that concurrent TGF β inhibition and radiotherapy will provide therapeutic benefit in clinical settings for NSCLC treatment.

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Methods

Cell culture

Human NCI-H1299, NCI-H292, A549 and murine LLC cells were purchased from American Type Culture Collection (Manassas, VA). LLC Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO).

Human NCI-H1299, NCI-H292 and A549 were cultured in RPMI-1640 Medium with 10% FBS (Sigma-Aldrich, St. Louis, MO). Cells were treated in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc., Carlsbad, CA) containing either 500 pg/ml TGF β (R&D Systems, Minneapolis, MN), 400 nM small molecule inhibitor of the TGF β type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole; Lilly designation HTS466284; Cat# 616451, Calbiochem, St. Louis, MO) or 1D11, a pan-isoform, neutralizing TGF β monoclonal antibody or 13C4, murine monoclonal isotype control antibody (kindly provided by Genzyme, Framingham, MA). [For growth studies, cells were trypsinized and counted using a Coulter counter at 24-hour and 48-hour post treatment.](#)

Clonogenic assay

To assess clonogenic survival of cells in monolayer culture, human and murine lung cancer cell lines were grown for 48 hours to about 70% confluence, at which point media was replaced with serum replacement media. Cells were treated with 400 nmol/L of LY364947 kinase inhibitor or 10 μ g/mL pan-specific TGF β -neutralizing antibody 1D11 or control antibody 13C4 for 48 h before and 3 h post radiation exposure. Cells were irradiated with graded doses of up to 8 Gy using a Varian Clinac 2300 C/D linear accelerator with 1cm bolus below (Varian, Palo

Alto, CA). Colony formation of 3 biological replicates was averaged for each treatment and corrected according to plating efficiency of respective controls. Sensitization dose enhancement ratios (DER10) were calculated as the ratio of doses required to achieve 10% surviving fraction for cells without and with TGF β inhibition.

γ H2AX foci

Tumor cryosections or cells grown on chamber slides were fixed using 2% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 100% methanol for 20 minutes at -20°C . Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 hour, and incubated with a mouse monoclonal antibody against γ H2AX (clone JBW301, Upstate Biotechnology, NY) overnight at 4°C as previously described [9]. Specimens were imaged using a 40 $\times$ objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epifluorescence. All images were acquired with a CCD Hamamatsu Photonics monochrome camera at $1,392 \times 1,040$ pixel size, 12 bits per pixel depth using the Metamorph imaging platform (Molecular Devices, Inc.).

Comet assay

LLC cells were cultured and treated with 400 nmol/L of LY364947 kinase inhibitor as described above and irradiated with 5 Gy. LLC cells were dissociated 0.5 or 4 hours after irradiation for single-cell gel electrophoresis at 19 V (300mAM, 40 min) and analysis by neutral comet assay (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. SYBR green-stained DNA comets were imaged at 400 $\times$ magnification and the extent of DNA breaks was quantified as tail moment using Comet Score software.

Western analysis

Cells were grown in complete media for 48 hour, treated with LY364947, irradiated with 5 Gy, and lysed after 1 hr. Alternatively, cells were treated with 500 pg/mL TGF β and lysed after 30 minutes. Protein estimation was carried out using the BCA protein assay kit from Pierce. One hundred micrograms of protein was electrophoresed on a 4% to 15% gradient gel from BioRad and transblotted on polyvinylidene difluoride membrane. The immunoblots were incubated with one of the following primary antibodies at 1/500 dilution: Smad2 serine 465/467 phosphorylation (Cell Signaling, Danvers, MA), Smad2/3 (BD, Franklin Lakes, NJ), p53 serine 15 phosphorylation (Cell Signaling, Danvers, MA), p53 serine 20 phosphorylation (Cell Signaling, Danvers, MA), p53 (Neomarkers, Fremont, CA), ATM serine1981 phosphorylation (Epitomics, Burlingame, CA), and ATM, clone 2C1 (GeneTex, Irvine, CA) and detected with infrared labeled antibodies using a LiCor Odyssey system.

Tumor studies

All animal experiments were carried out in accordance with guidelines specified by X X University's institutional animal care and use committee. Female C57BL/6 mice, age 6 to 8 weeks, obtained from Taconic were housed in a temperature-controlled animal care facility with a 12-hour light–dark cycle and allowed chow and water *ad libitum*. LLC cells (10^5) were injected into the right flank of mice and allowed to grow until the tumors reached an average size of 60-80 mm³. Animals were distributed randomly into groups (n=5-10) to receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and kept injecting were injected every other days to termination. Radiation was delivered at 600

cGy/min with 6MV X-rays with Varian Clinac 2300 C/D linear accelerator fitted with a 25-mm radiosurgery conical collimator (Varian, Palo Alto, CA). Superflab bolus (1.5-cm tissue equivalent material) was placed over the tumor.

TGF β 1 assay

~~Levels of total and active TGF β 1 in the supernatant of human H1299 and H292 tumor cells were assessed by a multiplex assay using a electrochemiluminescence-based ELISA (Mesoscale Discovery®). The serum free media conditioned by cells was collected after 72 hr. To measure active TGF β , the supernatant was activated by acid, as recommended by the manufacturer.~~

Results

Response of human and murine NSCLC cell lines to TGF β

Mutational inactivation of the TGF β signaling pathway in human NSCLC is associated with specific histological subtypes, more aggressive tumor behavior, and reduced patient survival [2]. NCI-H1299 and NCI-H292 are aggressive tumor cell lines established from a lymph node metastasis of a pulmonary carcinoma; A549 cell line is from a primary human adenocarcinoma; and LLC is a highly metastatic murine lung cancer cell line. ~~NCI-H1299 cells produced both 6.4 pg/mL active and 1503 pg/mL total TGF β in serum-free conditions, while NCI-H292 produced 14.4 pg/mL and 231.4 pg/mL respectively (Figure 1A).~~ Since NSCLC cells can selectively evade TGF β growth regulation while maintaining signaling, we next examined ~~whether~~ the TGF β canonical pathway, indicated by Smad2 phosphorylation (p-Smad2). TGF β treatment significantly induced p-Smad2, which was suppressed by TGF β inhibitor LY364947 (Figure ~~1B1A~~). These data indicate that receptor-mediated signaling in response to TGF β is intact. Since cell proliferation status constitutes an important component of the tumor cell radiosensitivity, we tested NSCLC tumor sensitivity to TGF β -mediated growth inhibition. Only H292 cells were growth-inhibited by TGF β , whereas NCI-H1299, A549, and LLC cells were refractory to TGF β -mediated growth regulation (Figure ~~1C1B~~).

Blocking TGF β signaling attenuates DNA damage response in NSCLC cells *in vitro*

We next assessed the radiation-induced DNA damage response. LY364947 significantly decreased phosphorylation of ATM at serine 1981, and p53 phosphorylation at serine 15 in

irradiated LLC cells (**Figure 2A**). Although p53 was undetectable in NCI-H1299 cells carrying a *Tp53* homozygous deletion, phosphorylation of ATM Ser1981 was attenuated after LY364947 treatment. No effect of LY364937 was observed in NCI-H292 cells. As shown in our prior studies [8,9], radiation-induced γ H2AX foci formation was markedly reduced by pre-treatment with LY364947 in both NCI-1299 and LLC cells *in vitro* (**Figure 2B,C**).

Next, we conducted neutral comet assays to investigate the effect of TGF β inhibition on DNA damage induction and resolution at 30 min and 4 hrs respectively after LLC cells were irradiated with 5 Gy (**Figure 3A**). The initial comet tail moment was similar for irradiated cells with or without LY364947, indicating comparable initial DNA damage. Most radiation-induced DSB are repaired in the first 1-6 hr after RT [13]. The comet tail moment decreased compared to 30 min after 5Gy IR. In contrast, the comet tail moment failed to decline in irradiated cells treated with LY364947 (**Figure 3B, C**). This persistent tail of DNA damage indicates that treatment with LY364947 impairs DNA damage repair.

Inhibition of TGF β signaling sensitizes NSCLC cell lines to irradiation

The clonogenic assay is a gold standard for radiosensitivity estimation. Human cell lines NCI-H1299 and A549 were radiosensitized by pre-treatment with LY364947, consistent with the DDR related proteins analysis, whereas radiosensitization was limited in NCI-H292 cells (**Figure 4 A-C**). Similar-These results as NCI-H292 were also detected from when TGF β was inhibited edion in NCI-H460, which is a large cell lung cancer carcinoma (data not shown). We next compared the small molecular inhibitor and a pan-isoform TGF β neutralizing antibody, 1D11, using the LLC

cell line. Clonogenic survival shows that murine LLC cells were comparably radiosensitized by pre-treatment with either 1D11 (Figure 4D) or LY364947 (Figure 4E). The DER_{40} survival is between 1.1 ± 0.14 and 1.2 ± 0.06 based triplicate determinations (Figure 4F).

Combination of TGF β inhibitor with fractionated RT increases LLC tumor growth delay

TGF β neutralizing antibodies currently in clinical development have demonstrated safety and efficacy in several studies [14,15]. To test the efficacy in combination with RT, we next examined LLC tumor bearing mice treated with 1D11 alone or in combination with fractionated RT. C57BL/6 mice bearing subcutaneous LLC tumors were treated with 5 daily fractions of 6 Gy and/or 1D11 or 13C4 (10mg/kg i.p.). Some tumors were randomly selected for harvesting 1 hr after irradiation to assess DDR by γ H2AX foci staining. Similar to the *in vitro* data from LLC cells, γ H2AX foci were reduced in irradiated tumors excised from hosts receiving treatment of 1D11 (Figure 5A and B). Notably, 1D11 alone had no significant effect on tumor growth compared with control antibody, 13C4 antibody, treated mice. However, when combined with RT, a tumor growth delay was considerably extended compared to mice receiving RT and 13C4 antibody (Figure 5B-5C and 5D). Tumor volume at the experiment termination (day 27) was significantly smaller for mice treated with 1D11 and RT when compared to RT and 13C4 antibody. Interestingly, more than half (6/11) the mice showed tumor growth rate decrease after the first two fractions of 6Gy when treated with 1D11, in which the mean volume increment was $7.9 \pm 6.9 \text{ mm}^3$ (tumor weight $0.74 \pm 0.10\text{g}$). However, tumor growth in 13C4 antibody treated mice had the same growth rate as the non-irradiated tumors, with a mean volume increase of $34.8 \pm 9.7 \text{ mm}^3$ (tumor weight $0.34 \pm 0.04\text{g}$) (Figure 5D-5E and F). These data

support the potential benefit of TGF β inhibition in the context of RT, which attenuates the DDR, radiosensitizes tumor cells, and promotes tumor control *in vivo*.

Discussion

Our study demonstrates that pharmaceutical TGF β inhibition compromises the DNA damage recognition, ~~and thus~~ repair, ~~and~~ ~~and~~ ultimately, tumor cell survival. The murine tumor cell line and most of the human NCSCL cells were radiosensitized independent of sensitivity to TGF β -mediated growth inhibition. This ~~suggesting~~ that this strategy would be effective across lung cancer subtypes. Additionally, this was further supported by our *in vivo* LLC syngeneic subcutaneous tumors, where tumor growth control was significantly improved by use of neutralizing antibodies concurrent with fractionated RT.

DNA is the main target for radiation-induced cell killing. Since there is considerable redundancy in the cell's ability to repair DNA damage, modulating the response to ionizing radiation through the inhibition of DNA repair has been a longstanding aim in translational RT research [16]. Previously, we demonstrated that TGF β is involved in ATM activation in epithelial cells [11] and chemical or genetic inhibition of TGF β signaling in these cells led to reduced ATM activation and increased tumor cell radiosensitivity in breast and brain tumor cell lines [8,9]. Consistent with our earlier studies, here we show that TGF β inhibition prior to irradiation resulted in reduced phosphorylation of H2AX and p53 in cultured NSCLC cells. Moreover, LLC tumors in mice treated with TGF β inhibitors prior to radiation exposure *in vivo* exhibited less γ H2AX foci formation, a nuclear marker of the rapid molecular radiation response. Interestingly, 1D11

neutralizing antibodies showed immediate tumor growth inhibition after two fractions. At this time point post-IR, tumor cell death results mainly from aberrant repair of radiation-induced DNA damage. This is consistent with a direct effect on radiosensitivity due to compromising the DNA damage recognition. Here, NCI-H1299 cells, which carry ing-a homozygous deletion of the p53 protein, are were still sensitized by TGF β inhibition. Nevertheless, the exact mechanism or pathways involving this TGF β inhibition that, which-attenuates eses IR induced DNA damage responses has not been well described up until now. Furthermore, the limited published data only has suggested that the sensitizing effect of TGF β inhibition is p53 independent.

Therapeutic resistance and RT induced lung damage are major challenges for NSCLC. This cancer, which-consists ss of heterogeneous histologies, are with the most common types being adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. The differential response to anti-tumor treatments among these different histologies is a general problem in treating NSCLC [17]. Since most of NSCLC harbor mutations with different clinical characteristics, it is not surprising that till now only one drug, crizotinib used in~~in~~ EML4-ALK-positive patients, has proven to be particularly effective in-for this specific subpopulation. This “tailored treatment” has resulted in an unprecedented survival benefit [18,19]. Here, we found that TGF β inhibition had little effect on IR-induced DDR related proteins in NCI-H292, which were not radiosensitized. This suggests that that benefit will be restricted to certain tumors; h. However, further studies are critical in order to identify the molecular biomarkers that will indicate the potential benefits from inhibiting TGF β ~~inhibition~~ in RT.

Hypofractionated stereotactic body radiation therapy (SBRT) allows for escalation of the fractional dose, ~~which~~ This is important ~~for~~ not only ~~improving~~ the local control rate of tumors, but ~~and the~~ overall survival for medically inoperable patients with early-stage NSCLC [20]. However, there are still many issues to be elucidated. Radiation dose and fractionation vary between institutions and protocols because there is no biologically effective dose guideline. Importantly, radiation-induced normal tissue damage is still the biggest obstacle, which limits the dose escalation of SBRT in NSCLC [~~21-23~~21,22]. Another approach with an enormous appeal involves the development of nontoxic, yet effective, molecularly targeted radiosensitizers. If a drug selectively sensitizes the tumor response to radiation, one could use selective escalation of the effective biological dose to the tumor, therefore, improving local tumor control without increasing morbidity. Drugs such as gemcitabine, cisplatin, and docetaxel ~~cisplatin and mitomycin C~~ have been demonstrated to radiosensitize tumors in NSCLC ~~and head and neck cancers~~ [23, 24]. ~~PI3K-AKT [12] and nuclear factor- κ B (NF- κ B)[25] are also regarded as the most well-studied survival pathways, which have a clear role in the response and sensitivity to RT.~~ Concurrently, these sensitizers increase the rates of local tumor control and in some cases overall survival [25]. As these radiosensitizers are not specific for tumor cells, they also increase radiation toxicity to normal tissues. This nonspecific mechanism of action is one of the major limiting factors of many radiation modulators. It is expected that chemotherapy-mediated inhibition of DNA repair mechanisms would synergize the TGF β inhibition, as shown in combination with Temozolomide and IR in GBM preclinical models [6].

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Unfortunately, radiation-induced lung injury is a major obstacle in NSCLC treatment. Since the lung represents the most RT sensitive tissue, this results in fatal complications. Among the many cytokines involved in this process, TGF β is thought to play a pivotal role [26,27]. TGF β is overexpressed at sites of injury, which contributes to delayed tissue fibrosis after radiation and chemotherapy. Various interventional therapies that aim to block TGF β signaling or ~~reduce~~ decrease tissue levels of TGF β have proven to be effective in reducing the development of fibrosis in the lung, liver, and intestines [26-28]. Reducing normal tissue injury by TGF β inhibition is undoubtedly significant in RT dose-escalation for lung cancer and will increase tumor control probability. In conclusion, targeting the TGF β pathway is a promising novel treatment candidate for NSCLC patients, as this s-is-a promising candidate for NSCLC patients, as this will ~~selectively increases~~ tumor cell killing and local tumor control, while potentially limiting ~~-late~~ effects in the surrounding normal tissue. -

Conclusions

Our data provides a strong preclinical rationale that TGF β inhibitors have therapeutic benefit ~~from TGF β inhibitors exploited~~ in the context of RT, via control of the DNA damage response by ~~that leads to greater~~ increasing radiation sensitivity.

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Figure Legends

Figure 1 TGF β signaling and growth regulation in NSCLC cell lines.

(A) ~~TGF β production was assessed in 72 hr supernatants from H1299 and H292 cell lines. Total and active TGF β was determined and normalized to number of cells.~~ (B) Western detection of p-smad2 and total Smad2 in NCI-H1299, NCI-H292, LLC and A549 cells. TGF β treatment induced phosphorylation of Smad2, which was blocked by LY364947 pre-treatment. Quantitation of the ratios of phosphorylated protein/total protein normalized to TGF β -treated alone is indicated below each lane. All cell lines were competent to signal via TGF β receptors. (CB) Growth of NSCLC cell lines 24 and 48 hr after treatment with small molecular inhibitor LY364947 (SMI), TGF β , or TGF β +SMI+LY364947. Values represent ratios of viable cells in untreated control to treatment groups (n=4). Significance was obtained using unpaired, two-tailed Student's t-test; *p<0.05, **p<0.01.

Figure 2 TGF β inhibition suppresses radiation-induced DNA damage response.

(A) Protein phosphorylation of ATM at serine 1981, Trp53 at serine 15 was assessed by Western* for human NCI-H1299, ~~and~~ NCI-H292 ~~and~~ A549 cells ~~and~~ murine LLC cells. Cells were treated with TGFβ inhibitor LY364947 for 24hr, irradiated with 2 Gy and lysed 30 mins post-IR.

(B, C) TGFβ inhibition with LY364947 significantly decreased γH2AX foci (green) in human NCI-H1299, A549 and NCI-H292 and murine LLC. Nuclei are counterstained with DAPI (blue).

Quantification of γH2AX foci shown below each panel reveal a significant reduction in the number of radiationinduced γH2AX foci with LY354947 in NCSLC (p< 0.0001; ANOVA).

~~Radiation-induced γH2AX (green) foci were immunostained in monolayers of NCI-H1299 (B) and LLC (C) cells treated with LY364947 for 48hr prior to irradiation with 2 Gy. Nuclear are counterstained with DAPI (blue). Magnification: 40X.~~

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Figure 3 Unrepaired DNA damage assessed by neutral comet assay.

(A) A representative image is shown for each treatment from the neutral comet assay assessed in LLC cells 4hr after 5 Gy and/or TGFβ inhibitor LY364947 (40x). (B, C) The comet tail moment was measured in LLC cells 30 mins (B) and 4 hr (C) after 5 Gy. Significance was determined using unpaired two-tailed Student's t-test; *p<0.05, **p<0.01. NS, non-significant

Figure 4 TGFβ inhibitors increase radiosensitivity *in vitro*.

Colony forming efficiency curves are reported for untreated irradiated controls (black squares line) and irradiated cells pretreated with the TGFβ inhibitors (grey circles). Cell lines were treated for 48 hr with LY364947 or TGFβ pan-specific neutralizing monoclonal antibody 1D11 prior to exposure to graded doses of radiation and plated 3 hr post-irradiation for clonogenic

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survival analysis. (A) Human NCI-H1299 (B) Human A549 (C) Human NCI-H292 (D) Murine LLC cells treated with LY364947 (E) Murine LLC cells treated 1D11. ~~and (F)~~ The DER₁₀ survival is indicated on the graphs. Mean $\pm$ S.E. values of triplicate determinations are shown. NCI-H1299, p=0.04; A549, p=0.03; NCI-H292, p=0.33; LLC+1D11, p=0.04; LLC+ LY364947 p=0.03, ANOVA with Tukey post test.

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Figure 5 TGF β inhibition combined with fractionated radiotherapy increases LLC tumor growth delay.

(A) γ H2AX (green) foci 1 hr after IR in tumor cells (blue, DAPI) of mice receiving 1D11 TGF β neutralizing antibody or control antibody 13C4 administered 24 hr prior to tumor irradiation with 2Gy. Fewer foci are evident following RT and 1D11 compared to RT and 13C4. (B) Quantification Mean intensity of γ H2AX inside nuclear (B) Tumor growth following 5 x 6 Gy daily fractions Averaged tumor growth curves (n=7-8) of in mice treated with 1D11 or 13C4 (mean $\pm$ S.D.). (C) Tumor growth curves for individual mice are shown. Note delayed regrowth of tumors treated with RT and 1D11. (E) Individual tumor weight at termination. (D) Tumor volume change after the first two fractions of 6 Gy RT. *p<0.05 denotes significant differences using analysis of variance (unpaired two-tailed Student's t-test ANOVA).

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Attenuation of the DNA Damage Response by TGF β Inhibitors Enhances Radiation Sensitivity of NSCLC Cells *In Vitro* and *In Vivo*

Abstract

Purpose To determine whether TGF β inhibition increases the response to radiotherapy in human and mouse non-small cell lung carcinoma (NSCLC) cells *in vitro* and *in vivo*.

Methods TGF β mediated growth response and pathway activation were examined in human NSCLC NCI-H1299, NCI-H292, and A549 cell lines and murine Lewis lung cancer (LLC) cells. Cells were treated *in vitro* with LY364947, a small molecule inhibitor of the TGF β type I receptor kinase, or with pan-isoform TGF β neutralizing monoclonal antibody, 1D11, before radiation exposure. DNA damage response was measured by ATM or *Trp53* protein phosphorylation, γ H2AX foci formation or comet assay in irradiated cells. Radiation sensitivity was determined by clonogenic assay. Mice bearing LLC syngeneic subcutaneous tumors were treated with 5 fractions of 6 Gy and/or neutralizing or control antibody.

Results NCI-H1299, A549 and LLC NSCLC cell lines pretreated with LY364947 prior to radiation exposure exhibited compromised DNA damage response indicated by decreased ATM and p53 phosphorylation, reduced γ H2AX foci, and increased radiosensitivity. NCI-H292 cells were unresponsive. TGF β signaling inhibition in irradiated LLC cells resulted in unresolved DNA damage. Subcutaneous LLC tumors in mice treated with TGF β neutralizing antibody exhibited fewer γ H2AX foci after irradiation and significantly greater tumor growth delay in combination with fractionated radiation.

Conclusions TGF β inhibition prior to radiation attenuated DNA damage recognition and increased radiosensitivity in most NSCLC *in vitro* and promoted radiation-induced tumor control *in vivo*. These data support the rationale for concurrent TGF β inhibition and radiotherapy to provide therapeutic benefit in NSCLC.

Key Words: ionizing radiation; non-small cell lung cancer; TGF β ; small molecule inhibitor; neutralizing antibody

Introduction

Improved treatment of non-small cell lung cancer (NSCLC), which is one of the leading causes of deaths from cancer worldwide, is urgently needed [1]. Approximately 70% of NSCLC patients receive radiotherapy (RT), either alone or in combination with other treatment modalities such as surgery or chemotherapy. Biologically augmenting tumor cell radiosensitivity can play a crucial role in determining treatment success [2]. TGF β ligands are enriched in the tumor microenvironment, where their production by stromal or tumor cells varies according to the tumor phenotype. In NSCLC, increased TGF β activity correlates with tumor progression and increased tumor angiogenesis [3,4]. Active TGF β signaling has been identified as a key factor for chemotherapy resistance in NSCLC [4], in addition to its well-recognized impact promoting tumor progression and metastasis.

TGF β activation is efficiently induced by ionizing radiation, in part due to the presence of a redox sensitive motif in the latency associated peptide (reviewed in [5]). Growing evidence supports a specific role for TGF β in mediating the rapid execution of the DNA damage response (DDR) [6]. Normal *Tgfb1* null keratinocyte cell lines irradiated *in vitro* exhibit reduced auto-phosphorylation of ataxia telangiectasia mutated (ATM) protein and its kinase activity [7]. The latter then results in decreased phosphorylation of substrate proteins upon DNA damage, including DNA damage transducers Chk2, p53, and Rad17, and by decreased γ H2AX foci, which is a chromatin modification near DNA double strand breaks; together these events impair cell survival. Notably, a TGF β type I receptor kinase small molecule inhibitor treated human epithelial cells phenocopies murine genetic depletion.

TGF β signaling blockade augments response to chemoradiation in GBM preclinical models [8,9], and specifically inhibits GBM cancer stem cell renewal after radiation [10]. TGF β inhibition promotes clonogenic cell death in irradiated mouse and human GBM and breast cancer cell lines in vitro. Systemically neutralizing TGF β enhances RT actions in GBM and breast cancer preclinical models [8,10,11]. Little is known about the contribution of TGF β to NSCLC response to radiation. Based on the efficacy in breast and brain tumors, it is plausible that TGF β inhibition could also improve radiosensitivity of NSCLC.

Currently, TGF β inhibitors are in clinical trials for several types of cancer including glioblastoma (GBM) and breast cancer (reviewed in [12]). Currently clinically viable inhibitors of TGF β signaling include small molecule inhibitors of TGF β type I receptor kinase and neutralizing antibodies that have low toxicity. Clinical trials involving TGF β inhibition in combination with radiotherapy in patients with breast cancer and GBM are underway (ClinicalTrials.gov identifiers: NCT01401062, NCT01220271). Here, we determined the effect of TGF β signaling blockade following irradiation in human NSCLC cell lines and murine Lewis lung carcinoma (LLC) tumors. TGF β inhibition increased radiosensitivity and compromised DDR in 3 of 4 NSCLC cell lines in vitro, and the combination of TGF β neutralizing antibody with fractionated RT significantly increased LLC tumor growth control in vivo. These results suggest that concurrent TGF β inhibition and radiotherapy may provide therapeutic benefit in the clinical setting for NSCLC.

Methods

Cell culture

Human NCI-H1299, NCI-H292, A549 and murine LLC cells were purchased from American Type Culture Collection (Manassas, VA). LLC Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (Gibco) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO). Human NCI-H1299, NCI-H292 and A549 were cultured in RPMI-1640 Medium with 10% FBS (Sigma-Aldrich, St. Louis, MO). Cells were treated in 10% serum replacement medium (SRM; Knockout SR, Life Technologies, Inc., Carlsbad, CA) containing either 500 pg/ml TGF β 1 (R&D Systems, Minneapolis, MN), 400 nM small molecule inhibitor of the TGF β type I receptor kinase, LY364947 ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole; Lilly designation HTS466284; Cat# 616451, Calbiochem, St. Louis, MO) or 10 μ g/ml 1D11, a pan-isoform neutralizing TGF β monoclonal antibody, or 13C4, a murine monoclonal isotype control antibody (kindly provided by Genzyme, Framingham, MA). For growth studies, cells were trypsinized and counted using a Coulter counter at 24-hour and 48-hour post plating.

Clonogenic assay

To assess clonogenic survival of cells in monolayer culture, human and murine lung cancer cell lines were grown for 48 hours to about 70% confluence, at which point media was replaced with serum replacement media. Cells were treated with 400 nmol/L of LY364947 kinase inhibitor or 10 μ g/mL pan-specific TGF β -neutralizing antibody 1D11 or control antibody 13C4 for 48 h before and 3 h post radiation exposure. Cells were irradiated with graded doses of up to 8 Gy using a Varian Clinac 2300 C/D linear accelerator with 1cm bolus below (Varian, Palo Alto, CA). Colony formation of 3 biological replicates was averaged for each treatment and corrected according to plating efficiency of respective controls. Sensitization dose

enhancement ratios (DER10) were calculated as the ratio of doses required to achieve 10% surviving fraction for cells without and with TGF β inhibition.

γ H2AX foci

Tumor cryosections or cells grown on chamber slides were fixed using 2% paraformaldehyde for 20 minutes at room temperature followed by permeabilization with 100% methanol for 20 minutes at -20°C . Then specimens were blocked with the supernatant of 0.5% casein/PBS, stirred for 1 hour, and incubated with a mouse monoclonal antibody against γ H2AX (clone JBW301, Upstate Biotechnology, NY) overnight at 4°C as previously described [11]. Specimens were imaged using a 40 $\times$ objective with 0.95 numerical aperture Zeiss Plan-Apochromat objective on a Zeiss Axiovert (Zeiss) equipped with epifluorescence. All images were acquired with a CCD Hamamatsu Photonics monochrome camera at $1,392 \times 1,040$ pixel size, 12 bits per pixel depth using the Metamorph imaging platform (Molecular Devices, Inc.).

Comet assay

LLC cells were cultured and treated with 400 nmol/L of LY364947 kinase inhibitor as described above and irradiated with 5 Gy. LLC cells were dissociated 0.5 or 4 hours after irradiation for single-cell gel electrophoresis at 19 V (300mA, 40 min) and analysis by neutral comet assay (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. SYBR green-stained DNA comets were imaged at 400 $\times$ magnification and the extent of DNA breaks was quantified as tail moment using Comet Score software.

Western analysis

Cells were grown in complete media for 48 hour, treated with LY364947, irradiated with 5 Gy, and lysed after 1 hr. Protein estimation was carried out using the BCA protein assay kit

from Pierce. One hundred micrograms of protein was electrophoresed on a 4% to 15% gradient gel from BioRad and transblotted on polyvinylidene difluoride membrane. The immunoblots were incubated with one of the following primary antibodies at 1/500 dilution: Smad2 serine 465/467 phosphorylation (Cell Signaling, Danvers, MA), Smad2/3 (BD, Franklin Lakes, NJ), p53 serine 15 phosphorylation (Cell Signaling, Danvers, MA), p53 (Neomarkers, Fremont, CA), ATM serine1981 phosphorylation (Epitomics, Burlingame, CA), and ATM, clone 2C1 (GeneTex, Irvine, CA) and detected with infrared labeled antibodies using a LiCor Odyssey system.

Tumor studies

All animal experiments were carried out in accordance with guidelines specified by NYU institutional animal care and use committee. Female 6-8 week old C57BL/6 mice obtained from Taconic were housed in a temperature-controlled animal care facility with a 12-hour light–dark cycle and allowed chow and water *ad libitum*. LLC cells (10^5) were injected into the right flank of mice and allowed to grow until the tumors reached an average size of 60-80 mm³. Animals were distributed randomly into groups (n=5-10) to receive 1D11 or 13C4 control antibody (10 mg/kg, intraperitoneal injection) 24 hr before localized irradiation and were injected every other day to termination. Radiation was delivered at 600 cGy/min with 6MV X-rays with Varian Clinac 2300 C/D linear accelerator fitted with a 25-mm radiosurgery conical collimator (Varian, Palo Alto, CA). Superflab bolus (1.5-cm tissue equivalent material) was placed over the tumor.

Statistics

Data are presented as mean plus or minus SEM. Statistical comparison between two groups was performed using the Student t test. One-way ANOVA was used to test for

differences among three or more groups. Difference was considered statistically significant, when the p value was less than 0.05.

Results

Response of human and murine NSCLC cell lines to TGF β

Mutational inactivation of the TGF β signaling pathway in human NSCLC is associated with specific histological subtypes, more aggressive tumor behavior, and reduced patient survival [2]. NCI-H1299 and NCI-H292 are aggressive tumor cell lines established from a lymph node metastasis of a pulmonary carcinoma, A549 cell line is from a primary human adenocarcinoma and LLC is a highly metastatic murine lung cancer cell line. Since NSCLC cells can selectively evade TGF β growth regulation while maintaining signaling, we first examined the TGF β canonical pathway was intact as indicated by Smad2 phosphorylation (p-Smad2) upon exposure to TGF β . Induction of p-Smad2 by a short TGF β treatment was suppressed by 50-75% by TGF β small molecule inhibitor, LY364947 (**Figure 1A**). These data indicate that receptor-mediated signaling in response to TGF β is intact in these 4 NSCLC cell lines. Since cell proliferation status confers an important component of the tumor cell radiosensitivity, we tested NSCLC tumor sensitivity to TGF β -mediated growth inhibition. Only H292 cells were growth-inhibited by TGF β , whereas NCI-H1299, A549, and LLC cells were refractory to TGF β -mediated growth regulation (**Figure 1B**).

Blocking TGF β signaling attenuates DNA damage response in NSCLC cells *in vitro*

We next assessed components of the DDR pathway previously determined to be affected by TGF β in non-malignant human cells and cancer cell lines [7,10,11]. Phosphorylation of ATM Ser1981 was attenuated after LY364947 SMI treatment in 3 of 4 cell lines. No effect of LY364937 was observed in NCI-H292 cells. Although p53 was undetectable in NCI-H1299 cells

carrying a *Tp53* homozygous deletion, LY364947 significantly decreased phosphorylation of ATM at serine 1981. Both ATM autophosphorylation and p53 phosphorylation at serine 15 was decreased by SMI treatment of LLC cells before irradiation (**Figure 2A**). As found in our prior studies [10,11], radiation-induced γ H2AX foci formation was markedly reduced by pre-treatment with LY364947 in NCI-1299, A549 and LLC cells *in vitro* (**Figure 2B**). Quantitation confirmed the absence of response in NCI-H292 cells (**Figure 2C**).

A compromised DDR could either delay or prevent resolution of DNA double strand breaks [13]. Here we tested the consequences of TGF β inhibition in LLC cells using neutral comet assays to investigate the effect of TGF β inhibition on DNA damage induction at 30 min and resolution at 4 hrs following irradiation with 5 Gy (**Figure 3A**). The initial comet tail moment was similar for irradiated cells with or without LY364947, indicating comparable initial DNA damage. Most radiation-induced DSB are repaired in the first 1-6 hr after RT [14]. The comet tail moment decreased compared to 30 min after 5Gy IR. In contrast, the comet tail moment failed to decline in irradiated cells treated with LY364947 (**Figure 3B, C**). This persistent DNA damage indicates that treatment with LY364947 impairs repair of DNA damage.

Inhibition of TGF β signaling sensitizes NSCLC cell lines to irradiation

The persistence of DNA damage should compromise cell survival. The clonogenic assay is a gold standard for estimating radiosensitivity. Human cell lines NCI-H1299 and A549 were radiosensitized by pre-treatment with LY364947, consistent with the DDR related proteins analysis. Consistent with the lack of SMI effect on DDR, radiosensitization was not evidenced in NCI-H292 cells (**Figure 4 A-C**). Radiosensitization was evident in a large cell lung cancer carcinoma cell line, NCI-H460, when TGF β was inhibited (data not shown). We next compared

the SMI to a pan-isoform TGF β neutralizing antibody, 1D11, using the LLC cell line. Murine LLC cells were comparably radiosensitized by pre-treatment with either LY364947 (**Figure 4D**) or 1D11 (**Figure 4E**).

Combination of TGF β inhibitor with fractionated RT increases LLC tumor growth delay

TGF β neutralizing antibodies currently in clinical development have demonstrated safety and efficacy in several studies [15,16]. We first determined whether the antibody achieved a biologically effective distribution to affect DDR in tumors by assessing γ H2AX foci staining in tumors harvested 1 hr after irradiation with 2 Gy. Similar to the *in vitro* data from LLC cells, induction of γ H2AX foci by radiation was reduced in irradiated tumors excised from mice treated with 1D11 compared to control antibody (**Figure 5A and B**). To test the efficacy in combination with RT, we next examined mice bearing LLC subcutaneous tumors treated with TGF β neutralizing antibodies or in combination with fractionated RT. Subcutaneous LLC tumors were treated with 5 daily fractions of 6 Gy and/or 1D11 or 13C4 (10mg/kg i.p.). Mice treated with 1D11 alone exhibited no significant change in tumor growth compared with control 13C4 antibody treated mice. However, tumor growth delay was significantly enhanced by 1D11 when combined with RT compared to mice receiving RT and 13C4 antibody (**Figure 5C**). Note that tumor regrowth initiated about 5 days following RT was considerably reduced in 1D11 treated irradiated tumors (**Figure 5D**). Tumor volume at the experiment termination (day 27) was significantly smaller for mice treated with 1D11 and RT when compared to RT and 13C4 antibody (**Figure 5E**). Interestingly, more than half (6/11) the mice showed tumor growth rate decrease after the first two fractions of 6Gy when treated with 1D11, in which the mean volume increment was $7.9 \pm 6.9 \text{ mm}^3$ (tumor weight $0.74 \pm 0.10\text{g}$). However, tumor growth in

13C4 antibody treated mice had the same growth rate as the non-irradiated tumors, with a mean volume increase of $34.8 \pm 9.7 \text{ mm}^3$ (tumor weight $0.34 \pm 0.04\text{g}$) (**Figure 5F**). These data support the potential benefit of TGF β inhibition in the context of RT, which attenuates the DDR, radiosensitizes tumor cells, and promotes tumor control *in vivo*.

Discussion

Our study demonstrates that TGF β inhibition by either pharmaceutical or biological means compromises the DNA damage recognition, repair, and ultimately tumor cell survival in the majority of NSCLC preclinical cell lines. The murine tumor cell line and 2 of 3 the human NSCLC cells were radiosensitized independent of sensitivity to TGF β -mediated growth inhibition. NCI-H1299 cells, which carry a homozygous deletion of the p53 protein, were still sensitized by TGF β inhibition, while TGF β inhibition did not alter DDR or radiosensitivity of NCI-H292, despite the apparent activity of TGF β signaling. *In vivo* efficacy was demonstrated using LLC syngeneic subcutaneous tumors, where tumor growth control was significantly improved by use of neutralizing antibodies concurrent with fractionated RT. These data suggest that this strategy might be effective in many lung cancer patients.

DNA is the main target for radiation-induced cell killing. Since there is considerable redundancy in the cell's ability to repair DNA damage, modulating the response to ionizing radiation through the inhibition of DNA repair has been a longstanding aim in translational RT research [17]. Previously, we demonstrated that TGF β is required for efficient ATM activation in epithelial cells [7], that either chemical or genetic inhibition of TGF β signaling in cells led to reduced ATM activation, and that TGF β inhibition increased tumor cell radiosensitivity in breast and brain tumor cell lines [10,11]. Consistent with our earlier studies, here we show that TGF β

inhibition prior to irradiation resulted in reduced phosphorylation of ATM, H2AX and p53 in most cultured NSCLC cells. Moreover, LLC tumors in mice treated with TGF β inhibitors prior to radiation exposure *in vivo* exhibited less γ H2AX foci formation, a nuclear marker of the rapid molecular radiation response and significantly greater growth control following fractionated RT. Interestingly, 1D11 neutralizing antibodies showed immediate tumor growth inhibition after two fractions. At this time point post-IR, tumor cell death results mainly from aberrant repair of radiation-induced DNA damage. This is consistent with a direct effect on radiosensitivity due to compromising the DNA damage recognition. Nevertheless, the exact mechanism or pathways involving this TGF β inhibition that attenuates IR induced DNA damage responses has not been determined but appears to be p53 independent [11].

NSCLC consists of heterogeneous histologies, with the most common types being adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. The differential response to anti-tumor treatments among these different histologies is a general problem in treating NSCLC [18]. Since most of NSCLC harbor mutations with different clinical characteristics, it is not surprising that till now only one drug, crizotinib used in EML4-ALK-positive patients, has proven to be particularly effective for this specific subpopulation. This “tailored treatment” has resulted in an unprecedented survival benefit [19,20]. Here, we found that TGF β inhibition had little effect on IR-induced DDR related proteins in NCI-H292, which were not radiosensitized. This suggests that that benefit will be restricted to certain tumors; however, further studies are critical in order to identify the molecular biomarkers that will indicate the potential benefits from inhibiting TGF β in RT.

Therapeutic resistance and normal lung damage are major challenges for effective RT in NSCLC patients. Hypofractionated stereotactic body radiation therapy (SBRT) allows for escalation of the fractional dose. This is important for not only improving the local control rate of tumors, but the overall survival for medically inoperable patients with early-stage NSCLC [21]. Radiation-induced normal tissue damage is still the biggest obstacle, which limits the dose escalation of SBRT in NSCLC [22-24]. An approach with an enormous appeal involves the development of nontoxic, yet effective, molecularly targeted radiosensitizers that could reduce normal tissue toxicity without affecting control. It is expected that chemotherapy-mediated inhibition of DNA repair mechanisms would synergize the TGF β inhibition, as shown in combination with Temozolomide and IR in GBM preclinical models [8]. If a drug selectively sensitizes the tumor response to radiation, one could use selective escalation of the effective biological dose to the tumor, therefore, improving local tumor control without increasing morbidity. Drugs such as gemcitabine, cisplatin, and docetaxel have been demonstrated to radiosensitize tumors in NSCLC [25]. Although these sensitizers increase the rates of local tumor control and in some cases overall survival [26], they are not specific for tumor cells, and can also increase radiation toxicity to normal tissues. This nonspecific mechanism of action is one of the major limiting factors of many radiation modulators.

Since the lung represents the most RT sensitive tissue, this results in fatal complications. Among the many cytokines involved in this process, TGF β is thought to play a pivotal role [27,28]. TGF β is overexpressed at sites of injury, which contributes to delayed tissue fibrosis after radiation and chemotherapy. Various interventional therapies that aim to block TGF β signaling or decrease tissue levels of TGF β have proven to be effective in reducing the

development of fibrosis in the lung, liver, and intestines [27-29]. Reduced normal tissue injury and increased tumor control probability by TGF β inhibition has significant appeal in RT dose-escalation for lung cancer.

Conclusions

Achieving effective targeting the TGF β pathway is a promising novel treatment option for NSCLC patients, as this selectively increases tumor cell kill and local tumor control, while potentially limiting late effects in the surrounding normal tissue.

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29. XXX

Figure Legends

Figure 1 TGF β signaling and growth regulation in NSCLC cell lines.

(A) Western detection of p-smad2 and total Smad2 in NCI-H1299, NCI-H292, LLC and A549 cells. TGF β treatment induced phosphorylation of Smad2, which was blocked by LY364947 pre-treatment. Quantitation of the ratios of phosphorylated protein/total protein normalized to TGF β -treated alone is indicated below each lane. All cell lines were competent to signal via TGF β receptors. (B) Growth of NSCLC cell lines 24 and 48 hr after treatment with small molecular inhibitor LY364947 (SMI), TGF β , or TGF β +SMI. Values represent ratios of viable cells in untreated control to treatment groups (n=4). Significance was obtained using unpaired, two-tailed Student's t-test; *p<0.05, **p<0.01.

Figure 2 TGF β inhibition impedes radiation-induced DNA damage response.

(A) Protein phosphorylation of ATM at serine 1981 and Trp53 at serine 15 was assessed by Western for human NCI-H1299, NCI-H292 and A549 cells and murine LLC cells. Cells were treated with TGF β inhibitor LY364947 for 24hr, irradiated with 2 Gy and lysed 30 mins post-IR. Phosphorylation was reduced by at least 25% in 3 of 4 cell lines. NCI-H292 did not show a reduction of phosphorylation. (B) TGF β inhibition with LY364947 significantly decreased γ H2AX foci (green) in human NCI-H1299, A549 and NCI-H292 and murine LLC. Nuclei are counterstained with DAPI (blue). (C) Quantitation of γ H2AX foci demonstrates a significant reduction in the number of radiation-induced γ H2AX foci with LY354947 in 3 of 4 irradiated NSCLC (p< 0.0001; ANOVA). NCI-H292 did not show a reduction of foci. Magnification: 40X.

Figure 3 Unrepaired DNA damage assessed by neutral comet assay.

(A) A representative image is shown for each treatment from the neutral comet assay in LLC cells 4hr after 5 Gy and/or TGF β inhibitor LY364947 (40x). (B, C) The comet tail moment was measured in LLC cells 30 mins (B) and 4 hr (C) after 5 Gy. Significance was determined using unpaired two-tailed Student's t-test; *p<0.05, **p<0.01. NS, non-significant

Figure 4 TGF β inhibition increases radiosensitivity *in vitro*.

Radiation survival curves are shown for untreated cells (black squares line) and cells pretreated with a TGF β inhibitor (grey circles) for 48 hr prior to exposure to graded doses of radiation. Cells were plated 3 hr post-irradiation for clonogenic survival analysis. (A) NCI-H1299 (B) A549 (C) NCI-H292 (D) LLC cells treated with LY364947. (E) LLC cells treated 1D11. The DER₁₀ survival is indicated on the graphs. Mean $\pm$ S.E. values of triplicate determinations are shown. NCI-H292 was not radiosensitized. Significance was determined using ANOVA with Tukey post test; *p<0.05, NS, not significant.

Figure 5 TGF β inhibition combined with fractionated radiotherapy increases LLC tumor growth delay.

(A) Immunostaining of γ H2AX (green; blue, DAPI) foci 1 hr after 2Gy in tumors of mice receiving 1D11 TGF β neutralizing antibody or control antibody 13C4 administered 24 hr prior to tumor irradiation. Fewer foci are evident following RT and 1D11 compared to RT and 13C4. (B) Quantification of mean intensity of γ H2AX inside mask defined by nuclear DAPI staining. (C) Averaged tumor growth curves (n=7-8) for mice following 5 x 6 Gy daily fractions and/or treated with 1D11 or 13C4 (mean $\pm$ S.E.). (D) Individual tumor growth curves for each treatment are shown. Note delayed regrowth of tumors treated with RT and 1D11 compared to RT alone. (E) Individual tumor weights at termination. (F) Tumor volume change after the first two fractions

of 6 Gy RT was evaluated using unpaired two-tailed Student's t-test. Asterisks denote significant differences (* <0.05 , ** <0.01).

Figure-1
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B

C

Figure-3
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Figure 3

Figure 4
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Figure-5

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A **B** **C** **D** **E** **F** **Figure 5**

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