Title: Ionizing Radiation Induces Myofibroblast Differentiation via Lactate Dehydrogenase

Authors:
JL Judge¹,³
KM Owens²
SJ Pollock¹
CF Woeller¹
TH Thatcher²,³
JP Williams¹
RP Phipps¹,²,³
PJ Sime¹,²,³
RM Kottmann²,³

¹Department of Environmental Medicine, ²Department of Medicine, Pulmonary and Critical Care Medicine, ³Lung Biology and Disease Program, University of Rochester, Rochester New York

Correspondence should be addressed to R. Matthew Kottmann, MD, 601 Elmwood Ave, Box 692, Rochester, NY 14642. Email: matt_kottmann@urmc.rochester.edu.

Author Contributions
JLJ, THT, JPW, RPP, PJS and RMK conceived and designed the experiments. JLJ, KMO, SJP, CFW, and RMK performed experiments and collected data. JLJ, THT, JPW, RPP, PJS and RMK analyzed the experiments, wrote the manuscript and/or provided critical input.

Running Title:
Radiation induces lactate production in lung fibroblasts

Keywords: lactate; lactate dehydrogenase; myofibroblast; pulmonary fibrosis; ionizing radiation

Support
This research was supported in part by T32ES007026, T32HL066988, U19AI091036, the Greg Chandler and Guy F. Solimano Pulmonary Fibrosis Research Fund, and the Davis Endowment. RMK was supported in part by a Parker B. Francis Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Abstract

Pulmonary fibrosis is a common and dose-limiting side-effect of ionizing radiation used to treat cancers of the thoracic region. Few effective therapies are available for this disease. Pulmonary fibrosis is characterized by an accumulation of myofibroblasts and excess deposition of extracellular matrix proteins. Although prior studies have reported that ionizing radiation induces fibroblast to myofibroblast differentiation and collagen production, the mechanism remains unclear. Transforming growth factor-beta (TGF-β) is a key pro-fibrotic cytokine that drives myofibroblast differentiation and extracellular matrix production. However, its activation and precise role in radiation-induced fibrosis is poorly understood. Recently, we reported that lactate activates latent TGF-β through a pH-dependent mechanism. Here, we wanted to test the hypothesis that ionizing radiation leads to excessive lactate production via expression of the enzyme lactate dehydrogenase-A (LDHA) to promote myofibroblast differentiation. We found that LDHA expression is increased in human and animal lung tissue exposed to ionizing radiation. We demonstrate that ionizing radiation induces LDHA, lactate production and extracellular acidification in primary human lung fibroblasts in a dose-dependent manner. We also demonstrate that genetic and pharmacologic inhibition of LDHA protects against radiation-induced myofibroblast differentiation. Furthermore, LDHA inhibition protects from radiation-induced activation of TGF-β. We propose a pro-fibrotic feed forward loop, in which radiation induces LDHA expression and lactate production, which can lead to further activation of TGF-β to drive the fibrotic process. These studies support the concept of LDHA as an important therapeutic target in radiation-induced pulmonary fibrosis.
Introduction

Pulmonary fibrosis is characterized by progressive and often irreversible accumulation of matrix proteins that lead to impairment of lung function. Radiation-induced lung injury can result in pulmonary fibrosis, which occurs in up to 20% of patients who receive thoracic radiation therapy (5, 23). The pathophysiology surrounding radiation-induced pulmonary fibrosis is poorly understood with few effective therapies available.

One of the hallmarks of pulmonary fibrosis, regardless of the cause, is the accumulation of excess extracellular matrix proteins such as collagen. These extracellular matrix proteins are primarily produced by scar-forming cells called myofibroblasts, which are differentiated lung fibroblasts that exhibit a myocyte-like phenotype. In normal wound healing, myofibroblasts produce extracellular matrix proteins that are essential for wound contracture. During the final phases of normal wound healing, myofibroblasts undergo apoptosis. However, during the pathologic process of fibrosis, myofibroblasts persist and contribute to prolonged matrix protein generation, deposition, and accumulation (26, 27), eventually leading to loss of normal tissue architecture and function.

Transforming growth factor-beta (TGF-β) is a potent pro-fibrotic cytokine that induces myofibroblast differentiation. Latent TGF-β exists in the extracellular matrix in the lung and must be activated by dissociation of the latency associated peptide (LAP) in order to exhibit its biological functions. There are several known activation routes including proteolysis, mechanical stretch, integrin binding, and decreases in pH (1).

Recently, we reported that lactate activates TGF-β via a pH-dependent mechanism (17). Importantly, this activation occurs with physiologic levels of lactate and physiologically attainable pH. We found that lactate is increased in the lungs of patients with idiopathic pulmonary fibrosis (IPF) compared to healthy controls, and induces myofibroblast differentiation in primary human lung fibroblast cultures (17). Furthermore, the enzyme responsible for lactate production, lactate dehydrogenase-A (LDHA), is increased in IPF lung tissue, and the expression of LDHA in primary human lung fibroblast cultures is regulated by TGF-β (17). Thus, we have proposed a positive feed-forward loop in which TGF-β upregulates LDHA, leading to increased lactate production and further activation of TGF-β. Alternatively, this feed-forward loop could be initiated by increased production of lactate, leading to local or transient decreases in pH and activation of latent TGF-β in the ECM. This active TGF-β can then signal fibroblasts and myofibroblasts to differentiate, increase ECM production, and increase LDHA expression and lactate production.

We hypothesize that this feed-forward loop leads to persistent myofibroblast differentiation thereby promoting the progression of fibrosis.

Although it is well documented that thoracic radiation induces collagen expression and causes late-stage fibrosis in the lung (9, 25, 31), few studies have explored the cellular mechanisms surrounding radiation-induced myofibroblast differentiation. TGF-β has been implicated in the development of radiation-induced fibrosis in several organs including the skin, lung, and liver (24, 30, 40) and TGF-β levels in the lung increase in the weeks following thoracic radiation (9, 31, 40). However, the mechanism of TGF-β activation in radiation-induced fibrosis remains poorly understood. Given our recent findings demonstrating the role of LDHA expression in IPF lung tissue, we sought to examine whether ionizing radiation regulates LDHA and if inhibition of LDHA may represent a novel therapeutic target in radiation-induced fibrosis. We report here that LDHA is up-regulated in radiation-induced fibrosis lung tissue and in irradiated lung fibroblasts, that lactate is required for radiation-induced myofibroblast differentiation, and that inhibition of LDHA activity prevents radiation-induced myofibroblast differentiation in primary
human lung fibroblast cultures. These studies implicate LDHA as a possible therapeutic target for radiation-induced pulmonary fibrosis.

Materials and Methods:

Human Cell Culture and Tissue Samples
Primary human lung fibroblast strains were derived from tissue explants as previously described (8). All donors gave informed written consent. Tissue sections from patients with radiation-induced lung fibrosis or control (non-fibrotic) tissue sections were obtained from the Department of Pathology at the University of Rochester and were de-identified. All human subjects research was performed under the supervision of the University of Rochester Research Subjects Review Board (RSRB).

Cell Culture and Irradiations
Fibroblasts were cultured in Eagle’s minimum essential media (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Sigma Aldrich, St. Louis, MO), 2 mM L-Glutamine, antibiotic and antimycotic (Gibco, Carlsbad, CA) at 37°C with 7% CO₂. Cells were irradiated with a 137Cs γ-ray source at approximately 2.70 cGy/min dose rate at indicated doses. Human recombinant TGF-β1 (R&D systems, Minneapolis, MN) was used at 1 ng/ml. LDHA was genetically inhibited using Smart Pool ONTARGET siRNA or Smart Pool Non-targeting Control Pool (Thermo Scientific, Waltham, MA) and silimporter transfection reagent (Upstate Cell Signaling Solutions, Charlottesville, VA) according to the manufacturer’s specifications. Cells were transfected 18 hours prior to irradiation. TGF-β1 receptor 1 was inhibited using SB431542 (Sigma Aldrich) at 2.5 µM added 30 minutes prior to irradiations, and then added daily on days 2-4. Cell viability was measured using Alamar Blue reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s specifications. LDHA was pharmacologically inhibited with Gossypol (Sigma Aldrich) at indicated doses starting 1 hour prior to irradiations.

TGF-β Activity
A TGF-β bioassay was performed as previously described (17). Briefly, Mv1Lu mink lung epithelial cells were cultured with conditioned media from irradiated human lung fibroblasts for 24 hours before proliferation rates were measured using a ³H labelled thymidine incorporation assay. The inverse of proliferation rates are expressed as a fold change from 0 Gy controls. TGF-β bioactivity was also measured using a previously described Smad-dependent TGF-β luciferase reporter containing 4 tandem smad binding elements (SBE) upstream of the minimal thymidine kinase (TK) promoter (38). Primary human lung fibroblasts were transfected with pSBEx4-TK-luc and a CMV-Renilla luciferase (Promega, Madison, WI) via electroporation with an Amaxa nucleofector. Transfected cells were plated and grown for 24 hours before being irradiated or treated with TGF-β (5 ng/ml). After 36 hours, cells were lysed with Dual-glow luciferase assay buffer (Promega) and luciferase was measured using a Varioskan luminescence plate reader (Thermo Scientific). Luciferase readings are normalized to non-irradiated controls.

Western Blotting
Cell lysates were run on SDS-PAGE under reducing conditions and probed for expression of α-SMA (Sigma Aldrich), LDHA (Abcam, Cambridge, MA). Glyceraldehyde 3-phosphate dehydrogenase (Abcam) was used as a loading control. Densitometry was performed as previously described (8).
Immunohistochemistry

Paraffin embedded lung tissue sections from de-identified patients with radiation-induced fibrosis, or from C57BL/6 mice exposed to 5 Gy total body plus 10 Gy thoracic radiation as previously described (22) were stained for α-SMA (Sigma Aldrich) and LDHA (Abcam) as previously described (17).

Immunofluorescence

Primary human lung fibroblasts were irradiated in T-25 flasks, then trypsinized the next day and sub-cultured for 48 hours in glass chamber slides for immunofluorescence staining. Cells were then fixed in 4% paraformaldehyde and stained with an antibody to α-SMA (Sigma Aldrich) followed by an anti-mouse AlexaFluor 568 (Invitrogen). Slides were mounted with Prolong Gold (Invitrogen) supplemented with DAPI to visualize nuclei and imaged using a Zeiss Axio Imager Z.I Microscope.

Quantitative Real-Time Polymerase Chain Reaction

RNA was isolated from primary human lung fibroblast and mouse lungs as previously described (8). Reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time polymerase chain reaction was performed using SsoAdvanced SYBR Green (Bio-Rad). The following primer sequences were used: human 18S: Forward, GGTCGCTGCTCCTCTCCCA; Reverse, AGGGGCTGACGCGGCCATGGTT; human Col1a1: Forward, TTGAAGGAGGTGTTCCCATCT; Reverse, ACACACACATATTTGGCATGGTGTT; mouse 18S: Forward, GCTTGCTCGCGCTTCCTTACCT; Reverse, TCAGTTACGGCCGCTGCT; mouse LDHA: Forward, TGGCGACTCCAGTGTGCCTG; Reverse, AGCAGTGCCACCACCCCTACCT.

Soluble Collagen Slot Blot

Soluble collagen was measured in cell supernatants as previously described (20). Briefly, 5 µl of cell supernatants were applied to a PVDF membrane using a vacuum manifold (Schleicher and Schuell, Keene, NH) under non-denaturing conditions. Membranes were probed using an antibody to collagen 1 (Santa Cruz Biotechnology, Dallas, TX).

Seahorse Bioanalyzer Extracellular Acidification Rate (ECAR) Measurement

Primary human lung fibroblasts were irradiated in T-25 flasks, then trypsinized the next day and sub-cultured into XF96 well plates at 5,000 cells per well (Seahorse Bioscience, North Billerica, MA). The extracellular acidification rate (ECAR) was measured using a Seahorse Bioscience XF96 Flux Analyzer according to company specifications. Briefly, ECAR was measured every 5 minutes for 1 hour. Data was analyzed once cells equilibrated to calibration fluid and extracellular acidification rates reached a plateau (after about 20 minutes).

Lactate Measurements

Lactate levels were measured in cell supernatants using a Nova BioProfile Automated Analyzer (Nova Biomedical, Waltham, MA) according to company specifications. Lactate levels are expressed as fold change from 0 Gy controls.

Statistical Analysis

All data are expressed as mean ± standard deviation. T-test and one-way analysis of variance (ANOVA) with Tukey post-test were used to establish statistical significance using Graph Pad Prism software (La Jolla, CA). Results were considered significant if the p-value was less than 0.05.
Results:

**LDHA Expression is Increased in Radiation-Induced Pulmonary Fibrosis**

We previously reported that LDHA expression is increased in the lung tissue from patients with idiopathic pulmonary fibrosis (IPF) (17). To investigate whether LDHA is also increased in radiation-induced pulmonary fibrosis, immunohistochemical staining for LDHA and α-smooth muscle actin (α-SMA) was performed on serial paraffin embedded tissue sections from patients with radiation-induced pulmonary fibrosis and non-irradiated controls. (Figure 1A-C). Fibrotic lung tissue was also stained with Gomori’s Trichrome to visualize collagen deposition (Figure 1D). Lung tissue from patients with radiation-induced pulmonary fibrosis had increased cellular content in the interstitium compared to healthy lung tissue (Figure 1B&C), with increased staining for α-SMA, a marker of myofibroblast differentiation. There was increased staining for LDHA (Figure 1C) corresponding to areas of intense α-SMA expression (Figure 1B&C).

LDHA expression was also increased in the lung tissue in C57BL/6 mice 26 weeks after exposure to 5 Gy total-body plus 10 Gy thoracic irradiation (Figure 1E&F). In this model, pulmonary fibrosis is evident beginning at 20 weeks post radiation and is progressive thereafter (22, 37). Non-irradiated control mice showed some LDHA staining, which was primarily localized in the alveolar epithelium (Figure 1E). However, mice exposed to radiation had increased cellular content in the interstitium and increased LDHA staining localized to the interstitium compared to non-irradiated controls. LDHA mRNA expression was measured with quantitative real-time PCR (qRT-PCR) in total lung homogenates harvested between 12-26 weeks post radiation. Compared to non-irradiated control mice, mice exposed to radiation had increased LDHA mRNA levels starting at 16 weeks post-radiation (Figure 1G). By peak fibrosis at 26 weeks, mice exposed to radiation had a 3-fold increase in LDHA mRNA levels compared to control mice (Figure 1G).

**Ionizing Radiation Induces LDHA Expression and Lactate Production in Primary Human Lung Fibroblasts**

To investigate the mechanisms involved in radiation-induced pulmonary fibrosis, we next determined whether ionizing radiation induces the expression of LDHA in primary human lung fibroblast cultures. Lung fibroblasts were irradiated with 0, 3, 7, and 10 Gy of ionizing radiation and viability was assessed after 5 days using the Alamar Blue assay. Because 10 Gy resulted in a significant decrease in cell viability compared to 3 and 7 Gy (Figure 2A), we used 3 and 7 Gy for our remaining experiments.

Ionizing radiation induced LDHA expression in primary human lung fibroblasts in a dose-dependent manner, with a greater than 5-fold induction at 7 Gy (Figure 2B&C). Importantly, radiation exposure also significantly increased the rate at which irradiated fibroblasts released acid into the media (extracellular acidification rate, ECAR). At least some of this extracellular acid release was in the form of lactate, as cell supernatants from irradiated fibroblasts contained significantly higher levels of lactate (Figure 2E).

**Ionizing Radiation Induces Myofibroblast Differentiation**

Irradiated lung tissue expresses high levels of α-smooth muscle actin (α-SMA), a marker of myofibroblast differentiation (Figure 1). To determine whether ionizing radiation induces myofibroblast differentiation, primary human lung fibroblasts were exposed to 3 and 7 Gy ionizing radiation and cultured for 5 days. Radiation induced α-SMA expression in a dose-dependent manner (Figure 3A&B). α-SMA was also analyzed using immunofluorescence staining. 7 Gy radiation caused a significant increase in actin fiber staining following radiation in a similar manner to the induction of α-SMA by transforming growth factor beta (TGF-β) (Figure...
In addition, 7 Gy induced extracellular soluble collagen 1 protein (Figure 3D) and in collagen 1 and 3 mRNA expression (Figure 3E&F).

**Ionizing Radiation Activates TGF-β**

TGF-β has been reported to play an important role in radiation-induced tissue injury in multiple organs, including skin and lung. Furthermore, we have demonstrated that lactate can activate latent TGF-β in human lung fibroblast cultures. To test whether radiation leads to activation of latent TGF-β in human lung fibroblast cultures, a mink lung epithelial cell bioassay was performed with cell supernatants from control and irradiated cultures to determine levels of TGF-β bioactivity. 7 Gy radiation significantly increased TGF-β bioactivity in 5-day cell culture media from irradiated cells (Figure 4A). Additionally, we transfected the lung fibroblasts with a highly specific Smad-dependent TGF-β reporter construct prior to irradiation, and determined luciferase activity after 36 hours. Irradiation caused a 2.5-fold increase in TGF-β luciferase activity compared to 0 Gray controls (Figure 4B), and this was comparable to the effect of addition of exogenous active TGF-β. To demonstrate that myofibroblast differentiation is driven by this activation of TGF-β, and not some other effect of ionizing radiation, we incubated fibroblasts with a specific TGF-β receptor 1 inhibitor SB431542 (15). SB431542 significantly attenuated radiation-induced α-SMA expression (Figure 4 C&D), confirming that radiation-driven activation of latent TGF-β plays a key role in radiation-induced myofibroblast differentiation.

**LDHA is Required for Radiation-driven Myofibroblast Differentiation**

To determine whether increased lactate production is required for irradiation-driven myofibroblast differentiation, we used an siRNA approach to silence LDHA prior to irradiation. We achieved a high degree of LDHA knockdown (Figure 5A). Ionizing radiation was unable to drive myofibroblast differentiation, as determined by α-SMA expression, in LDHA knockdown fibroblasts (Figure 5A&B). LDHA siRNA also prevented radiation-induced TGF-β activation (Figure 5C). Compared to control siRNA transfected cells, cells transfected with LDHA siRNA prior to radiation produced less lactate (data not shown) and had lower ECAR than cells transfected with control siRNA (Figure 5D).

**Gossypol, an LDH Inhibitor, Inhibits Radiation-Induced Myofibroblast Differentiation**

We next determined whether pharmacologic inhibition of LDHA would also prevent radiation-induced myofibroblast differentiation. Pre-treatment of lung fibroblasts with Gossypol, an LDH inhibitor derived from cottonseed, prior to irradiation with 7 Gy strongly prevented radiation-induced myofibroblast differentiation in a dose-dependent manner (p≤0.001) (Figure 6A&B). Interestingly, Gossypol also reduced baseline levels in α-SMA expression (Figure 6A&B). Additionally, Gossypol inhibited radiation-induced collagen production (Figure 6C), and radiation-induced TGF-β bioactivity (Figure 6D).

**Discussion**

We have previously reported that lactate is increased in lung tissue from patients with idiopathic pulmonary fibrosis (IPF), and that lactate promotes fibroblast to myofibroblast differentiation via pH-dependent activation of latent TGF-β (17). Here, we show for the first time that lactate also plays a central role in radiation-induced pulmonary fibrosis. Ionizing radiation induces the expression of LDHA in lung fibroblasts, which leads to increased extracellular acidification, increased extracellular lactate, and increased TGF-β bioactivity (Figures 2-4). Expression of LDHA is required for efficient TGF-β bioactivity and fibroblast to myofibroblast differentiation, as siRNA knockdown of LDHA expression largely ablates the pro-fibrotic effect of irradiation.
LDHA preferentially converts pyruvate to lactate and is up-regulated during hypoxia when oxygen is in limited supply for oxidative phosphorylation (36). LDHA is also highly up-regulated in tumor cells regardless of oxygen tension, a phenomenon termed the Warburg effect (14, 36). The Warburg effect describes a highly glycolytic phenotype with a high production of lactate, regardless of whether oxygen is available for oxidative phosphorylation. Here, we report characteristics consistent with the Warburg effect in primary human lung fibroblasts exposed to radiation, suggesting that similar changes in metabolic pathways may be seen in radiation-induced fibrosis and cancer.

Other metabolic similarities related to tumor growth are evident in pulmonary fibrosis, including uncontrolled cell proliferation and tissue invasion (35). Interestingly, consistent with highly metabolic tumors, patients with IPF have increased $^{18}$F-FDG metabolism in lung parenchyma when visualized with high resolution PET/CT, indicating that areas of fibrosis have increased glucose uptake (12). Increased glucose uptake can contribute to accelerated growth and proliferation, a characteristic that is also consistent with fibroblasts from fibrotic lung tissue (16, 29). Taken together with our results of increased LDHA expression in myofibroblasts in radiation-induced fibrosis (Figure 1), we hypothesize that radiation causes changes in cellular metabolism in fibroblasts that is similar to changes seen in tumor cells to allow for increased proliferation to drive fibrosis.

Ionizing radiation drives activation of TGF-β through upregulation of LDH and lactate, and this active TGF-β in turn drives myofibroblast differentiation and collagen production. While the importance of myofibroblasts to lung fibrosis is well-understood, it is certainly possible that radiation-induced upregulation of lactate and TGF-β will upregulate pro-fibrotic functions of fibroblasts without inducing myofibroblast differentiation. It should also be noted that while we have examined the direct effects of ionizing radiation on lung fibroblasts, it is possible that other lung cell types contribute to lactate production, extracellular acidification, and TGF-β activation in lung fibrosis. For example, we observed some epithelial LDHA staining in lung tissue from patients with radiation-induced pulmonary fibrosis (Figure 1), as well as in IPF, sarcoidosis and nonspecific interstitial pneumonia (17). Epithelial cells may upregulate LDHA and lactate production, which then exerts a pro-fibrotic bystander effect on nearby fibroblasts. We plan to investigate other cellular sources of lactate during fibrogenesis in future studies.

We demonstrate here that ionizing radiation leads to increased TGF-β bioactivity (Figure 4) and that silencing LDHA with siRNA dampens this bioactivity and prevents radiation-induced extracellular acidification (Figure 5). We have previously shown that lactate activates TGF-β through a pH-dependent mechanism (17). It is interesting that even though irradiation strongly induces myofibroblast differentiation and collagen, there was only a relatively modest increase in active TGF-β in the culture medium at day 5 (Fig 4A). It should be noted that the amount of active TGF-β in the medium at any given point is a balance between expression, activation and uptake, and may not reflect the total amount available to the cells over time. A second bioassay...
indicated that the amount of active TGF-β available to the cells during the first 36 hours is comparable to addition of exogenous TGF-β (Figure 4B).

It is well-established that radiation causes induction of TGF-β (3, 24, 40), though the mechanisms of radiation-induced TGF-β activation are poorly understood. One suggested mechanism is oxidation dependent activation through redox related mechanisms (4). Activation through redox mechanisms would likely occur very quickly following radiation, given that reactive oxygen species induced by ionizing radiation are short lived (18). We hypothesize that radiation also causes TGF-β activation via extracellular acidification from increased lactate production, which would provide a mechanism for long-term and sustained TGF-β bioactivity during fibrosis. While our in vitro mechanistic studies were performed on a short time scale, we believe that lactate-induced acidification following radiation is a slow-rolling, feed-forward mechanism of TGF-β activation. Given that we have reported that TGF-β can induce expression of LDHA (17), we propose a positive feed-forward loop in which radiation causes extracellular acidification that activate latent TGF-β, which can further induce LDHA. Over time, this feed-forward cycle can result in amplification of fibrotic processes to give rise to tissue fibrosis. Therefore, it is critical to interrupt this fibrotic feed-forward loop when designing therapies for radiation-induced pulmonary fibrosis.

Inhibition of LDHA is an area of active research in the oncology field since LDHA overexpression is associated with cancer cell growth, poor prognosis and drug resistance (2, 7, 39, 42). Here, genetic knockdown of LDHA expression prevented radiation-induced myofibroblast differentiation (Figure 5). However, therapeutic gene silencing in patients presents several technical hurdles. Gossypol, a potent LDHA inhibitor derived from cottonseed (11, 19) has been explored as a novel pharmaceutical therapy in breast cancer (34), and has been shown to have anti-tumorigenic effects in breast and colon cancer cells (10, 33, 34). Here, we show that Gossypol potently inhibits myofibroblast differentiation, collagen production and TGF-β bioactivity with similar efficacy to genetic knockdown.

It should be noted that Gossypol may have other effects in addition to LDH inhibition including induction of apoptosis, modulation of cell cycle regulatory proteins and/or inhibition of other enzymatic activities (28). These so-called off-target effects may in fact be beneficial in the context of fibrosis. For example, resistance to apoptosis by myofibroblasts is hypothesized to contribute to the pathogenesis of fibrosis in vivo (13, 41). While Gossypol itself may not ultimately be adopted as an anti-fibrotic therapy, new and more specific LDHA inhibitors are under development. Our results are the first proof of principle that inhibition of LDHA activity may have therapeutic benefit in lung fibrosis.

Acknowledgements
We would like to acknowledge Eric Hernady and Jen-nie Miller for their technical help with mouse radiation experiments and tissue collection, Amali Epa for help with collagen slot blot assay, Dr. Hsi-Min Hsiao for help with real-time PCR, Dr. Joshua Munger for use of the Nova BioProfile Analyzer, Dr. Paul Brooks for use of the Seahorse Bioanalyzer.
**Figure Legends:**

**Figure 1:** Lactate dehydrogenase A (LDHA) expression is increased in radiation-induced fibrotic lung tissue. Lung biopsies were obtained from patients who received thoracic radiation for cancer treatment and from non-irradiated controls, immunostained for α-SMA or LDHA, and developed with Nova Red. (A) Non-fibrotic lung sections were stained for LDHA. (B, C) Serial paraffin embedded tissue sections from radiation-induced fibrotic lung tissue were stained for LDHA and α-SMA. (D) Radiation-induced fibrotic lung tissue was stained with Gomori Trichrome. (E-G) C57BL/6 mice were exposed to 5 Gy total-body plus 10 Gy thoracic radiation and were harvested at 12-26 weeks post-radiation. Paraffin embedded lung tissue sections from control (E) and irradiated (F) mice at 26 weeks post radiation were stained for LDHA. (G) RNA was isolated from whole lung homogenates and mRNA levels of LDHA were measured by quantitative real-time PCR and normalized to GAPDH. Isotype controls for immunohistochemical staining are inset in (B) and (E). Scale bars represent 100 µm. *p≤0.05 by ANOVA compared to non-irradiated control, n=3-8 mice per group.

**Figure 2:** Ionizing radiation induces LDHA expression, lactate production, and extracellular acidification in lung fibroblasts. Primary human lung fibroblasts were exposed to 0, 3, 7, and 10 Gy radiation from a $^{133}$Cs γ-ray source and cell lysates and supernatants were collected at 5 days post-radiation. (A) Cell viability was measured using an Alamar blue assay of mitochondrial activity (n=3). (B) LDHA protein expression was analyzed by Western blot. One representative experiment is shown (n=3). (C) LDHA expression relative to GAPDH was determined by densitometry and normalized to 0 Gy control. (D) Extracellular acidification rates (ECAR) were measured using a Seahorse Bioscience XF96 Flux Analyzer (n=10-12). (E) Lactate levels were measured in the supernatants using a Nova BioProfile Automated Analyzer (n=3). * p≤0.05 compared to 0 Gy control by ANOVA. #p≤0.05 compared to 3 and 7 Gy. ns= no statistical significance.

**Figure 3:** Ionizing radiation induces myofibroblast differentiation. Primary lung fibroblasts were exposed to 0, 3 and 7Gy and cell lysates and supernatants were collected at 5 days post-radiation. (A, B) α-SMA protein expression was analyzed by Western blot and densitometry. One representative experiment is shown (*p≤0.05 by ANOVA compared to 0 Gy, n=3). (C) Immunofluorescence staining for α-SMA (red) was performed on cell cultures exposed to 7 Gy radiation or transforming growth factor-beta (TGF-β). Cell nuclei were stained with DAPI. (D) Soluble collagen was measured in supernatants from irradiated cells cultures at 5 days post radiation using a Slot Blot Assay (*p≤0.05 by ANOVA compared to 0 Gy, n=3). (E, F) Total RNA was isolated from cells exposed to 7 Gy radiation at 3 days post-radiation and quantitative real-time PCR was performed for Col1a1 and Col3a1 (*p≤0.05 by t-test, n=3).

**Figure 4:** Ionizing Radiation activates TGF-β. (A) TGF-β bioactivity was measured using a mink lung cell proliferation assay. Mink lung cells were incubated with supernatants from primary human lung fibroblast cell cultures exposed to 0 or 7 Gy radiation harvested at day 5 and proliferation was measured using $^3$H-thymidine incorporation. Inverse of proliferation rates relative to 0 Gy controls were plotted (*p≤0.05 by t-test, n=3). (B) Lung fibroblasts were transfected with a Smad-dependent TGF-β luciferase reporter assay prior to irradiation or addition of TGF-β, and harvested 36 hours after treatment (n=6). Fold changes in normalized luciferase activity from 0 Gy controls were plotted (*p≤0.05 by t-test, n=6). (C, D) Lung fibroblasts were treated daily with 2.5 µM TGF-β receptor 1 inhibitor (SB431542), starting 30 minutes prior to exposure to 0 or 7 Gy radiation. Cell lysates were collected 5 days post radiation and were analyzed for α-SMA protein expression levels by Western blot and densitometry (*p≤0.05 by ANOVA, n=3).
Figure 5: LDHA is required for radiation-driven myofibroblast differentiation. Primary human lung fibroblasts were transfected with either an LDHA siRNA pool or a non-targeting control siRNA pool 18 hours prior to exposure to 7 Gy irradiation. (A, B) Cell lysates were collected 5 days post radiation and were analyzed for LDHA and α-SMA protein expression levels by Western blot and densitometry. (n=3). (C) TGF-β bioactivity was measured in cell supernatants using a mink lung cell bioassay (n=6). (D) ECAR was measured using a Seahorse Bioscience XF96 Flux Analyzer (n=20-22). * p≤0.05 by ANOVA.

Figure 6: Gossypol, an LDH inhibitor, inhibits radiation-induced myofibroblast differentiation. (A, B) Primary human lung fibroblasts were pretreated with Gossypol at 1 and 5 µM starting 1 hr prior to irradiation with 0 or 7 Gy. Cell lysates were collected 5 days post radiation and were analyzed for α-SMA protein expression levels by Western blot and densitometry. One representative set of conditions is shown (n=3). (C) Soluble collagen 1 was measured in culture supernatants using a Slot Blot Assay (n=3). (D) TGF-β bioactivity was measured in cell supernatants using a mink lung cell bioassay (n=6). *p≤0.05 by ANOVA.

References


Figure 1: LDHA Expression in Increased in Radiation-Induced Pulmonary Fibrosis

Human Mouse

A. B. F.

LDHA

E.

G.

α-SMA

Mouse

F.

G.

Trichrome

G. LDHA mRNA

Fold Change Relative to 18S

Control 12 16 18 26

Weeks After Irradiation

*
Figure 2: Radiation induces LDHA expression and lactate production in primary human lung fibroblasts.
Figure 3: Radiation induces myofibroblast differentiation

A. 

\[ \alpha\text{-SMA} \quad \text{GAPDH} \]

\[ \text{Gray:} \quad 0 \quad 3 \quad 7 \]

B. 

\begin{align*}
\text{Fold Change in } \alpha\text{-SMA} & \text{ Relative to GAPDH} \\
\text{Gray:} & \quad 0 \quad 3 \quad 7
\end{align*}

\begin{align*}
\alpha\text{-SMA Expression} & \\
\text{Gray:} & \quad 0 \quad 3 \quad 7
\end{align*}

C. 

0 Gray \quad 7 Gray \quad \text{TGF-\text{\textbeta}}

D. 

\begin{align*}
\text{Soluble Collagen 1} & \\
\text{Intensity Units} & \quad 5000 \quad 10000 \quad 15000 \quad 20000
\end{align*}

\begin{align*}
\text{Gray:} & \quad 0 \quad 3 \quad 7
\end{align*}

E. 

\begin{align*}
\text{Fold Change in Col1a1} & \text{ Normalized to 18S} \\
\text{Gray:} & \quad 0 \quad 7
\end{align*}

F. 

\begin{align*}
\text{Fold Change in Col3a1} & \text{ Normalized to 18S} \\
\text{Gray:} & \quad 0 \quad 7
\end{align*}
Figure 4: Radiation Activates TGF-β

A. TGF-β Bioactivity

B. TGF-β Luciferase Activity

C. α-SMA Expression

D. α-SMA Expression Relative to GAPDH
Figure 5: LDHA is required for radiation-driven myofibroblast differentiation

A. 

\[ \text{Fold Change in } \alpha\text{-SMA Relative to GAPDH} \]

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>7</th>
<th>0</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDHA siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. 

\[ \text{Fold Change in } \alpha\text{-SMA Relative to GAPDH} \]

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>7</th>
<th>0</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDHA siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. 

\[ \text{TGF-}\beta\text{ Bioactivity} \]

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>7</th>
<th>0</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDHA siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D. 

\[ \text{ECAR (mpH/min)} \]

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>7</th>
<th>0</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDHA siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6: Gossypol, an LDH Inhibitor, Inhibits Radiation-Induced Myofibroblast Differentiation

A. 

\[
\begin{array}{cccccc}
\text{Radiation} & - & - & - & + & + & + \\
\text{Gossypol (μM)} & 0 & 1 & 5 & 0 & 1 & 5 \\
\end{array}
\]

B. 

\[
\begin{array}{cccc}
\text{Fold Change in } \alpha\text{-SMA} \\
\text{Relative to GAPDH} \\
\end{array}
\]

C. 

\[
\begin{array}{cccc}
\text{Soluble Collagen 1 Intensity Units} \\
\text{Gossypol (μM)} & 0 & 1 & 5 \\
\end{array}
\]

D. 

\[
\begin{array}{cccc}
\text{Fold Change in TGF-β Bioactivity} \\
\text{Gossypol (μM)} & 0 & 1 & 5 \\
\end{array}
\]