Death Receptors 4 and 5 and Idiopathic Pulmonary Fibrosis

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is an irreversible lethal interstitial lung disease with an unknown cause, killing about 40,000 people per year. In IPF, lung fibroblasts become abnormally activated during tissue repair process, creating excessive scar tissue and avoiding cell death. The results from this research study suggest that the apoptotic pathway mediated by TRAIL (tumor necrosis factor-related apoptotic-inducing ligand) receptors, death receptors 4 and 5 (DR4 and DR5), is aberrant in IPF in the presence of a polymerized collagen matrix. Compared to control fibroblasts, IPF fibroblasts express lower levels of DR4 and DR5, making them more resistant to TRAIL-mediated apoptosis. Furthermore, our study suggests that DR4 may be regulated by the transcription factor FoxO3a. Previous research has shown FoxO3a to be important in conferring an apoptosis-resistant IPF fibroblast phenotype, and these results further support this idea.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is an irreversible interstitial lung disease with an unknown etiology, or cause. The disease affects between three and nine out of 100,000 people per year in Europe and North America, though this incidence appears to be increasing over time, particularly in developed nations (Hutchinson et al. 2015). This increase in prevalence is similar to cancer, heart disease, and diabetes, where chronic, long-term illnesses are becoming a greater health concern as modern medicine is better equipped to handle acute diseases. IPF is a deadly disease, claiming the lives of 40,000 patients a year (Gay et al. 1998). Most IPF patients die within 3 to 5 years, with a five-year survival rate between 30-50% (Harari and Caminati 2010; Gribbin et al. 2006; Spagnolo et al. 2015). Since symptoms are not always consistent from patient to patient, diagnosing IPF can be difficult. Furthermore, not all doctors are aware of this

disease, so many IPF patients go undiagnosed. IPF diagnosis relies on clinical features of this disease, radiography, and histopathology.

IPF patients present with symptoms similar to those of lung cancer (Hutchinson et al. 2015). Some clinical features include dry cough, shortness of breath and progressive dyspnea, or labored breathing. Another feature common in IPF patients is basilar Velcro-like rales, essentially a crackling sound originating from the base of the lungs (Harari and Caminati 2010). Around 60% of IPF patients have finger clubbing, which is a deformity marked by softening of the nailbed and thickening of the distal ends of fingers (Prasad et al. 2015).

Although the cause of IPF is unknown, some known risk factors are closely associated with the disease. The first is a person's sex. Men are more likely than women to develop IPF, with one study showing that 63% of IPF patients were men (Johnston et al. 1997). Age is also an important factor. IPF is most common in the elderly, with the average age of an IPF patient being 66 years ("Idiopathic Pulmonary Fibrosis: Diagnosis and Treatment" 2000). Additionally, smokers are more likely to develop IPF. One article stated that a majority of IPF patients are past or current cigarette users (Prasad et al. 2015). These results suggest that situations with a greater chance of lung injury also tend to lead to a higher risk of IPF. Prior studies support this concept. For example, miners exposed to silica particulates are more likely to develop IPF. Metal dust and mold spores may also make a person more likely to develop the disease (Olson and Swigris 2012).

If IPF is suspected, biopsies from multiple lung lobes are best for diagnosis since different lung lobes can have different features of IPF (Flaherty et al. 2001). The most prominent feature of IPF is the presence of fibroblastic foci, which are unusual aggregates of lung fibroblasts surrounded by collagen rich matrix (Harari and Caminati 2010). Fibroblastic foci are

the hallmark features of usual interstitial pneumonia (UIP), characterized by a high degree of lung tissue scarring. IPF is simply UIP with an unknown cause. It is unclear how IPF patients develop UIP. The current thought is that alveolar epithelial cells (AECs), the cells lining the alveoli in the lungs, suffer chronic micro-injuries of some kind. Rather than simply repair these injuries, abnormally activated fibroblasts deposit excessive amounts of type I collagen, resulting in the formation of scar tissue. By some unknown mechanism, fibroblasts continue creating scar tissue after they should have stopped (Spagnolo et al. 2015). All this evidence strongly suggests that chronic lung injury and an aberrant tissue repair process cause IPF.

Several therapeutic options have been developed in recent years. New therapies tend to focus on experimental drugs, whereas older therapies rely on corticosteroids. Doctors have been prescribing corticosteroids, for example Prednisone, for decades to manage IPF (Spagnolo et al. 2015). The idea behind corticosteroid use is facilitating breathing by decreasing inflammation. However, there is little to no evidence that corticosteroids have any efficacy against IPF (Raghu et al. 2011). Alternatively, doctors prescribe corticosteroids in conjunction with immunosuppressant drugs, particularly azathioprine. Like using corticosteroids alone, this dual therapy has had little success in randomized trials (Raghu et al. 2011). Steroids have mixed and debatable success, while the drugs nintedanib and pirfenidone have performed better in clinical trials (George et al. 2015).

Nintedanib is an intracellular inhibitor that targets multiple tyrosine kinases (Richeldi et al. 2014). This interferes with signaling pathways in IPF fibroblasts that lead to aberrant activation of fibroblasts. The FDA recently approved nintedanib as a treatment option for IPF (Spagnolo et al. 2015). Pirfenidone is an oral antifibrotic drug, meaning it blocks tissue scarring. Phase 3 trials for pirfenidone were conducted in the United States, Australia, Europe, and Japan,

and the FDA also approved pirfenidone, which may be effective in slowing down the progression of lung fibrosis (King et al. 2014), However, these drugs have not been proven to cure IPF, and a more effective treatment for the disease still needs to be developed.

Based on the pathological phenotype of IPF fibroblasts, a possible molecular target involves the induction of apoptosis. Once they have finished repairing damaged tissue, lung fibroblasts normally die via apoptosis to prevent excessive scarring. In healthy patients, this happens as a form of negative feedback; the very collagen that fibroblasts create induces a signal for the fibroblasts to undergo apoptosis. IPF fibroblasts, however, avoid growth-inhibiting mechanisms normally present in healthy patients (Nho et al. 2011). Although there are various pathways leading to apoptosis, one such possible target is the pathway mediated by the cytokine tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily of ligands (Leahomschi et al. 2013). TRAIL is the ligand for death receptors 4 and 5 (DR4 and DR5). Upon binding to these receptors, DR4 and DR5 initiate a signaling cascade that results in apoptosis, signaled by activity from caspase enzymes (Romeo et al. 2016). TRAIL can act in an autocrine, paracrine, and endocrine manner. This means a cell producing TRAIL could cause cell death in its neighbors, in cells that are relatively far away, or even in itself. Lymphocytes are the main producers of TRAIL, though all cells are capable of making the cytokine (Yang et al. 2010).

TRAIL and its pro-apoptotic receptors, DR4 and DR5, are all upregulated in alveolar epithelial cells in IPF patients (Akram et al. 2014). Alveolar epithelial cells are a different cell type from fibroblasts and form the layer of cells in the lung where gas exchange takes place. Studies showed that the increased expression of TRAIL, DR4, and DR5 leads to increased rates of apoptosis in these cells, exacerbating the already detrimental effects of excessive tissue

scarring in IPF. Since the TRAIL-mediated apoptotic pathway has already been observed in lung alveolar epithelial cells, it may also be a feature in increased viability of IPF fibroblasts. As a side note, inducing the TRAIL pathway holds promise as an experimental cancer therapy. Many research studies presently focus on preferentially targeting cancer cells using TRAIL and its apoptotic pathway (Yang et al. 2010).

When TRAIL binds to DR4 or DR5, an intracellular death domain on the receptor activates pro-caspase 8 and 10. These become caspases 8 and 10, which activate caspases 3, 6, and 7. Through signaling, DR4 and DR5 promote lysosomal, mitochondrial, and cytosolic apoptotic pathways (Bertsch 2014). Studies suggest that DR4 and DR5 signaling may regulate several transcription factors, including NFκB, MAP kinases, Akt, Src, and PKC (Bertsch 2014). Given the many pathways involving DR4 and DR5 that could result in increased cell death and in conferring IPF fibroblasts to be apoptosis-resistant on a collagen rich matrix, it is highly likely that the IPF fibroblasts have altered the expression of these receptors, protecting them from collagen matrix-induced apoptosis.

Currently, it is unclear what regulates DR4 and DR5 expression in IPF fibroblasts, though NF-κB may be a transcription factor for the *DR4* and *DR5* genes (Bertsch 2014). Another possible transcription factor controlling DR5 levels is FoxO3a, a transcription factor that regulates many genes (Sunayama 2011). Previous studies suggest that FoxO3a alteration is linked to DR4 & 5 deregulation in cancer cells (Shoeb et al. 2013), though this has yet to be shown in lung fibroblasts. Some confirmed and possible FoxO3a functions include apoptosis, cell cycle progression, DNA repair, management of oxidative stress, and muscle growth and atrophy (Huang and Tindall 2007). Some of these targets of FoxO3a present fascinating topics for research on IPF. Studies already demonstrated that FoxO3a alteration as a result of aberrant

PI3k/Akt causes IPF fibroblasts to be viable and proliferative in response to apoptosis-inducing collagen matrix (Nho and Hergert 2014). FoxO3a is a direct target of Akt and its activity regulated by the phosphorylation of a crucial serine residue. A previous study showed that FoxO3a levels are reduced in IPF fibroblasts when cultured on collagen (Nho et al. 2011). FoxO3a, in turn, regulates p27, a cell cycle inhibitor. When FoxO3a is inactive, so is p27, resulting in increased cell cycle proliferation. Consequently, IPF fibroblasts that have lower levels of FoxO3a do not halt their cycle, causing higher rates of mitosis. Furthermore, FoxO3a is known to regulate an apoptosis-inducing protein, Bim. Bim expression is reduced in fibroblasts derived from IPF lung tissues in response to a collagen-rich matrix. These findings strongly suggest that aberrantly low FoxO3a expression causes IPF fibroblasts to be resistant to cell death-inducing conditions. Therefore, given the diverse functions of FoxO3a, it is plausible that this transcription factor regulates the DR4 and 5-mediated signaling pathway.

Based on these studies, this current study seeks to determine whether IPF fibroblasts have abnormal levels of DR4 & 5, which subsequently suppress TRAIL-mediated apoptosis via FoxO3a alteration. Therefore, this study examined DR4 and DR5 expression in IPF fibroblasts and healthy lung fibroblasts using Western analysis. The samples were derived from human patients with IPF from the University of Minnesota Health hospital. These fibroblasts were cultured on a collagen matrix to simulate a scarred lung environment and compared to fibroblasts grown in non-collagen conditions as a control. Since the alteration of TRAIL-mediated apoptosis is thought to be linked to the progression of IPF, control lung and IPF lung fibroblasts cell viability were measured in response to TRAIL. To confirm results, DR4 and DR5 were silenced in healthy lung fibroblasts to see if IPF-like traits emerge. These DR4 and DR5-silenced cells were examined using caspase and cell viability assays to measure levels of apoptosis and

cell death. Additional experiments overexpressed FoxO3a to see if there were any changes in DR4 and DR5 protein expression.

It was hypothesized that reduced DR4 and DR5 expression would be present in IPF lung fibroblasts. Consequently, the lung fibroblasts would show increased resistance to cell death and lower levels of caspase activity. Given the previously shown stark decrease in FoxO3a expression in IPF fibroblasts, it also appeared likely that abnormally low FoxO3a failed to activate DR4 and DR5 expression, and this alteration causes IPF fibroblasts to be highly viable to collagen driven apoptosis in response to TRAIL.

Our results showed that IPF lung fibroblasts are more resistant to TRAIL-induced apoptosis than control lung fibroblasts when cultured on a collagen-rich matrix. We also found that IPF lung fibroblasts show decreased DR4 and DR5 expression compared to control cells when cultured on a collagen-rich matrix, but not when cultured in media lacking collagen. Using cell viability and caspase 3/7 activity assays, we found that IPF lung fibroblasts were more resistant to TRAIL-induced apoptosis when cultured on a collagen matrix. Silencing DR4 & 5 in control lung fibroblasts and overexpressing DR4 & 5 in IPF lung fibroblasts showed that the two receptors were linked to increased resistance to TRAIL-induced apoptosis. Our results also suggest that DR4 expression may be regulated by FoxO3a, though more work is needed to elucidate the DR4 and DR5 regulation mechanism.

MATERIALS AND METHODS

Primary cell culture

Lung fibroblasts were obtained from human patients with IPF and without IPF. Any information linking lung tissues to donor patients was removed at the time of patient death or

organ removal. To create primary cell culture, lung tissue was chopped into small sections of about 3-4 mm in diameter. These lung pieces were placed on tissue culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS) and 2% antibiotics for about a month. The DMEM was changed approximately every 3-4 days during this time. Fibroblasts were removed from the culture plates after the month time period and used for experiments or frozen as stock for later use. Nine cell lines each of IPF and control lung fibroblasts were used in this experiment. To control for variability due to passage number, experiments were conducted on cell lines with passage numbers between three and seven.

Three-dimensional type I polymerized collagen matrices

Three-dimensional type I polymerized collagen matrices (PC) were prepared with 80% type I collagen solution, 10% 10x DMEM, and 10% 1x DMEM. The polymerized collagen matrix was prepared with a pH of 7.2 using 0.1 M NaOH. Polymerized collagen media was added to cell culture dishes and 96-well plates and incubated at 37°C three hours before seeding cells.

Reagents, antibodies and chemicals

DMEM and FCS were purchased from Sigma-Aldrich. Type I collagen solution was purchased from Advanced BioMatrix (San Diego, CA, USA). DR4 (catalog no. 8411) and DR5 (catalog no. 15497) antibodies were obtained from Santa Cruz Biotechnologies (Dallas, TX, USA) and Proteintech (Radnor, PA, USA), respectively. FoxO3a and GAPDH antibodies were obtained from EMD Millipore (Temecula, CA, USA, catalog no. 04-1007) and Santa Cruz Biotechnologies (Dallas, TX, USA, catalog No. 32233). DR4 and DR5 siRNA were purchased

from Santa Cruz Biotechnologies (Dallas, TX, USA). Scrambled siRNA was obtained from Santa Cruz Biotechnologies (Dallas, TX, USA). DR4 and DR5 overexpression plasmids were obtained from Addgene (Cambridge, MA, USA). pcDNA overexpression plasmids were also purchased from Addgene (Cambridge, MA, USA). TRAIL was purchased from Enzo Life Sciences (Farmingdale, NY). Western blot imaging was analyzed using LabWorks Image acquisition and analysis software version 4.6 (UVP BioImage Systems).

Western blot analysis

3 x 10^5 control or IPF lung fibroblasts were cultured for 24 hours before being lysed with 1x cell lysis buffer (Cell Signaling) with a protease and phosphatase inhibitor (Sigma and Calbiochem, respectively) Cell lysates were sonicated for 10-15s and boiled for 10 minutes. SDS-PAGE was performed using 10-15% polyacrylamide gels at 120V. Separated proteins from the polyacrylamide gels were then transferred to a PVDF membrane. These membranes were blocked in 5% BSA in TTBS overnight. The membranes were incubated in primary antibody for 1-2 days with DR4 and DR5 antibodies and for 1 hour with GAPDH antibody. After incubation in primary antibody, the membranes were washed three times for 20 minutes each wash using TTBS. Membranes were incubated in secondary antibody for 1 hour then washed three times for 20 minutes each wash using TTBS. Protein expression was then measured and analyzed as described above.

Gene silencing

6-8 x 10^5 control and IPF cells were cultured in DMEM containing 10% FCS and 1% antibiotics for 24 hours at 37°C. Cells were then transfected with DR4, DR5, or scrambled

siRNA. Cells were incubated for another 24 hours, then plated onto a three-dimensional polymerized type I collagen matrix. 1 x 10^4 cells were added to each well on a 96-well plate. The cells were incubated overnight on the collagen matrix and then subjected to serum starvation by adding DMEM without FCS. After 24 hours in FCS-free DMEM, the cells were treated with TRAIL in concentrations of 0, 100, and 200 ng/mL as described below.

Gene overexpression

6-8 x 10^5 control and IPF cells were cultured in DMEM containing 10% FCS and 1% antibiotics for 24 hours at 37°C. Cells were then transfected with FoxO3a wild type adenovirus, DR4, DR5, or pcDNA overexpression plasmids as a control (Addgene, Cambridge, MA, USA). Cells were incubated for another 24 hours, then plated onto a three-dimensional polymerized type I collagen matrix. 1 x 10^4 cells were added to each well on a 96-well plate. The cells were incubated overnight on the collagen matrix and then subjected to serum starvation by adding DMEM without FCS. After 24 hours in FCS-free DMEM, the cells were treated with TRAIL in concentrations of 0, 100, and 200 ng/mL as described below. For FoxO3a overexpression, cells were not subjected to TRAIL treatment or cell viability and caspase assays.

Cell viability assay

24 hours after adding TRAIL in gene overexpression or gene silencing experiments, 20 µL of Cell Titer Blue reagent (Promega, Madison, WI, USA) was added to each well of a 96-well plate. Cell viability was measured at time intervals of 1h, 3h, 5h, 8h, and 24h after the addition of Cell Titer Blue. Between measurements, 96-well plates were incubated at 37°C. Cell

viability was measured by using a 96-well plate reader with a fluorescence filter set at 560nm (ex) and 590nm (em).

Apoptosis assay using caspase 3/7 activity

One hour after adding TRAIL in gene overexpression or gene silencing experiments, fluorescent reagent (Apo-ONE Homogeneous caspase 3/7 assay kit, Promega) was added to each well in a 96-well plate. Caspase 3/7 activity was then measured at time intervals of 2h, 4h, 7h, and 23h after the addition of fluorescent solution. Between measurements, 96-well plates were incubated at 37°C. Caspase 3/7 activity was measured by using a 96-well plate reader (BioTek, Winooski, VT, USA) with a fluorescence filter set at 499 nm (excitation) and 521 nm (emission).

RESULTS

IPF fibroblasts have higher cell viability than control cells after TRAIL treatment

We hypothesized that IPF fibroblasts would have higher cell viability when exposed to TRAIL compared to control lung fibroblasts. In order to test this, we cultured non-IPF (control) and IPF cells (n=7) on a polymerized collagen matrix overnight. The cells were then cultured in DMEM in the absence of FCS for 24 additional hours before treatment with TRAIL. Cell viability readings were conducted as described in Materials and Methods.

As seen in Fig. 1, cell viability was decreased in control lung fibroblasts at TRAIL concentrations of 100 ng/mL and 200 ng/mL. Cell viability was significantly different between IPF fibroblasts and control lung fibroblasts, with p-values <0.01. These results strongly suggest that IPF lung fibroblasts are less susceptible to TRAIL-induced cell death.

IPF fibroblasts have lower caspase 3/7 activity than control cells after TRAIL treatment

We wanted to be certain any cell death caused by TRAIL was due to apoptosis and not some other process, such as necrosis or necroptosis. To ascertain that decreased cell viability was due to apoptosis, we conducted a caspase 3/7 activity assay. This assay was conducted on 7 IPF and 7 control fibroblast cell lines as described in Materials and Methods.

Fig. 2 shows that caspase 3/7 activity increased with higher TRAIL concentrations in control lung fibroblasts. In contrast, caspase 3/7 activity remained relatively consistent in IPF lung fibroblasts. These results suggest the increased cell viability seen in IPF lung fibroblasts in the presence of TRAIL is due to lower levels of apoptosis.

DR4 and DR5 expression are lower in IPF fibroblasts under PC conditions

In normal lung fibroblasts, polymerized collagen (PC) predominantly increases levels of apoptosis. In contrast, IPF fibroblasts are resistant to collagen matrix driven apoptosis (Im 2015). Since DR4 and DR5 are proapoptotic receptors, it appears possible that IPF fibroblasts would express lower levels of DR4 and DR5 in order to avoid cell death.

To test this possibility, IPF fibroblasts (n=8) and control fibroblasts (n=8) were cultured on polymerized collagen matrices for 48 hours, after which point a lysate of these cells was collected as described in methods. Western blotting of DR4 levels (Fig. 3) and DR5 levels in these fibroblasts (Fig. 4) showed overall decreased levels of both receptors in IPF lung fibroblasts. Some cell IPF cell lines, such as 305 and 309, showed little DR5 expression, while some did not show any expression at all. Conversely, control fibroblast cell lines 222, 206 and 221 shows only marginally more DR5 expression than IPF fibroblasts. With DR4, IPF cell lines 305 and 309 did not appear to markedly differ from control cell lines. It is important to

remember that IPF fibroblasts were derived from patients with various biological backgrounds, so results may not be the same for all cells we examined.

Our results suggest that IPF fibroblasts have less of the proapoptotic receptors DR4 and DR5 when in the presence of polymerized collagen. It is therefore likely that these cells also show less TRAIL-mediated apoptosis compared to control cells.

DR4 and DR5 expression are the same in IPF and control fibroblasts under non-PC conditions

Since a polymerized collagen matrix has been shown to increase expression of DR4 and DR5 (Im 2015), we wanted to make certain that non-PC conditions did not increase DR4 and DR5 expression in both IPF and control lung fibroblasts.

To test this hypothesis, IPF fibroblasts (n=8) and control fibroblasts (n=8) were cultured in DMEM containing tissue culture plate for 48 hours, after which point a lysate of these cells was collected as described in methods. Western blotting of DR4 levels (Fig. 5) and DR5 levels in these fibroblasts (Fig. 6) showed overall equal levels of DR4 and DR5 in both control and IPF fibroblasts. These results suggest that a polymerized collagen matrix is important in triggering the IPF fibroblast phenotype in patients with IPF.

Silencing DR4 and DR5 in control lung fibroblasts increases cell viability after TRAIL treatment

DR4 and DR5 expression were shown to be higher in control lung fibroblasts when grown on a PC matrix compared to IPF cells (Figs. 3 and 4). We therefore hypothesized that silencing DR4 and DR5 in control lung fibroblasts would increase the cells' viability after TRAIL treatment.

To test this, $1x10^4$ control fibroblasts were cultured on a polymerized collagen matrix for 24 hours, followed by serum starvation. Cell viability readings were obtained as described in methods. The results suggest (Fig. 7) that cell viability increased in response to TRAIL when DR4 and DR5 were silenced, as well as when each receptor was silenced separately. These readings, however, did not have statistical significance. Western blotting confirmed successful silencing of both DR4 and DR5 in the cells (data not shown).

Silencing DR4 and DR5 in control lung fibroblasts decreases caspase 3/7 activity after TRAIL treatment

We wanted to see if the increased cell viability in response to TRAIL in control lung fibroblasts after silencing DR4 and DR5 was due to reduced apoptosis. To test this, we conducted a caspase 3/7 activity assay as described in Materials and Methods.

The results suggest that caspase 3/7 activity is reduced after silencing DR4 and DR5 as well as silencing both receptors separately (Fig. 8). Like the cell viability readings (Fig. 7), the caspase 3/7 activity assay results did not have statistical significance. Regardless, the readings suggest that silencing DR4 and DR5 potentially reduces levels of apoptosis. Western blotting confirmed successful silencing of both DR4 and DR5 in the cells (data not shown).

Overexpressing DR4 and DR5 in IPF fibroblasts decreases cell viability in response to TRAIL

To confirm our results from silencing DR4 and DR5 in control lung fibroblasts, we decided to overexpress DR4 and DR5 in IPF lung fibroblasts. We hypothesized that overexpressing DR4 and DR5 would decrease cell viability.

$1x10^4$ IPF fibroblasts were cultured on a PC matrix for 24 hours, followed by an additional 24 hours of serum starvation. Cell viability readings were obtained as described in methods. The results suggest (Fig. 9) that cell viability in response to TRAIL decreased in IPF fibroblasts when DR4 and DR5 were overexpressed together, as well as separately. Western blotting confirmed successful overexpression of the two receptors (data not shown).

Overexpressing DR4 and DR5 in IPF fibroblasts increases caspase 3/7 activity in response to TRAIL

We wanted to determine whether the decreased cell viability observed when DR4 and DR5 were overexpressed (Fig. 9) was due to higher levels of apoptosis. To test this, we conducted a caspase 3/7 activity assay as described in methods.

The results suggest that caspase 3/7 activity is higher after overexpressing DR4 and DR5 as well as overexpressing both receptors separately (Fig. 10) Western blotting confirmed successful overexpression of both DR4 and DR5 in the cells (data not shown).

Overexpressing FoxO3a in IPF fibroblasts may increase DR4 expression

Knowing that FoxO3a has been shown to regulate DR4 and DR5 expression in cancer cells (Shoeb et al. 2013), we wanted to see if we could find the same results in lung fibroblasts. To test this, we overexpressed FoxO3a as described in Materials and Methods.

We found that DR4 expression potentially increases as a result of FoxO3a overexpression (Fig. 11). Due to an unknown reason, it was not possible to see DR5 expression. Western blotting confirmed successful overexpression of FoxO3a (also Fig. 11). Densitometry confirmed

a 2.5-fold increase in DR4 expression in the cells with FoxO3a overexpression compared to the control cells (Fig. 11(b)).

DISCUSSION

Our hypothesis stated that DR4 and DR5 are underexpressed in IPF fibroblasts in a polymerized collagen matrix, and this reduced expression leads to increased resistance to apoptosis. Also, we hypothesized that the transcription factor FoxO3a, which had been previously shown to be underexpressed in IPF fibroblasts (Nho and Hergert 2014), regulated DR4 and DR5 expression. Specifically, we proposed lower levels of FoxO3a were responsible for reduced expression of DR4 and DR5. Our results largely corroborated our hypotheses.

A Western blot of cells cultured on a polymerized collagen matrix showed that both DR4 and DR5 are underexpressed in IPF fibroblasts compared to control human lung fibroblasts. In contrast, IPF fibroblasts cultured on a matrix without polymerized collagen showed the same DR4 and DR5 expression as control human lung fibroblasts. This result suggests that IPF lung fibroblasts have lower levels of two distinct pro-apoptotic receptors. Therefore, the cells would be more resistant to apoptosis. In alveoli, this would mean that IPF fibroblasts would be more likely to survive and proliferate after chronic lung injuries, leading to aberrant excessive scarring of the lung tissue.

To verify that the cells are more resistant to apoptosis, we incubated them with pro-apoptotic TRAIL on a polymerized collagen matrix and measured their cell viability over time and caspase 3/7 activity. We found that IPF lung fibroblasts have increased cell viability and lower levels of caspase 3/7 activity than control lung fibroblasts. This further supports the idea

that IPF fibroblasts have increased resistance to cell death, particularly apoptosis, as shown by caspase 3/7 activity.

Next, we silenced the genes controlling DR4 and DR5 in control lung fibroblasts to see if we could replicate an IPF fibroblast phenotype in control fibroblasts. We found that these cells had higher cell viability after being incubated with TRAIL, showing an increased resistance to cell death. Additionally, the cells had lower levels of caspase 3/7 activity, showing that this increased resistance to cell death was in the form of lower levels of apoptosis. Conversely, we also overexpressed DR4 and DR5 in IPF lung fibroblasts to see if we could increase levels of cell death, particularly apoptosis, in the cells. A cell viability assay showed that the IPF fibroblasts had decreased cell viability and increased caspase 3/7 activity. These results corroborate those from silencing DR4 and DR5 in control lung fibroblasts.

We wanted to see if FoxO3a, a transcription factor under-expressed in IPF lung fibroblasts grown on a polymerized collagen matrix, regulated DR4 and DR5. To test this, we overexpressed FoxO3a in IPF lung fibroblasts. Our results showed that, in cells where FoxO3a was overexpressed, DR4 levels were higher. Therefore, our results strongly suggest that a deficiency of FoxO3a in IPF lung fibroblasts is responsible for decreased DR4 expression, leading to increased resistance to cell death by apoptosis. The possibility that DR5 expression is also regulated by FoxO3a should be examined in the future.

While the data support our hypotheses, they have notable limitations. First, we primarily examined apoptosis. Death receptors 4 and 5 may lead to other forms of cell death as well, such as necrosis and necroptosis. Also, we performed our experiments using relatively small sample sizes. For most of the experiments, only eight cell lines were tested at a time. Particularly for caspase activity readings, standard deviation was high, so more results would add statistical

significance to the data. For all of our results, cell viability was never increased in IPF lung fibroblasts by more than around 20 percent. While this may not seem like a large amount, it must be noted that IPF lung fibroblasts appear to utilize multiple mechanisms to evade cell death. While the effects of reduced DR4 and DR5 expression may be small, they combine with other mechanisms to give IPF lung fibroblasts their unique pathological phenotype.

Despite our research here and past research, the mechanism whereby FoxO3a is underexpressed only on a polymerized matrix is still unclear. Presumably, this reduced expression of FoxO3a leads to decreased levels of DR4 and potentially DR5 as well, so it is important to understand how signaling within the fibroblasts leads to this phenotype. Future research should address this definite knowledge gap. Second, future research could attempt various therapies using our results. If it is possible to intercept or stimulate a signaling pathway to correct the expression of FoxO3a to normal levels, perhaps DR4 and DR5 expression could be restored to sensitize them to TRAIL. Future research should also examine the role of two other receptors for the pro-apoptotic ligand TRAIL, known as decoy receptors 1 and 2 (Dcr1 and Dcr2). These receptors bind TRAIL, much like DR4 and DR5, but they do not initiate a signaling pathway ending in apoptosis. Thus, they are “decoys” for TRAIL, essentially dead ends. If DR4 and DR5 are underexpressed in IPF lung fibroblasts cultured on polymerized collagen, it is also possible that Dcr1 and Dcr2 are overexpressed, causing TRAIL to not initiate a pro-apoptotic pathway when it binds to a receptor.

In conclusion, we found that DR4 and DR5, both receptors for the pro-apoptotic ligand TRAIL, are underexpressed in IPF fibroblasts when cultured on a polymerized collagen matrix. We found that increasing DR4 and DR5 expression led to lower cell viability in the presence of TRAIL and increased caspase 3/7 activity, showing that apoptosis was induced by

overexpressing these two receptors. We also showed that DR4 expression may be at least partially controlled by FoxO3a, a transcription factor previously shown to be underexpressed in IPF lung fibroblasts. These results are important, as roughly 40,000 patients with IPF die each year (Gay et al. 1998). We hope this research provides important insight into the underlying mechanism of an apoptosis-resistant IPF fibroblast phenotype, and we further hope that subsequent future studies lead to an eventual treatment for this deadly disease.

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FIGURES AND LEGENDS

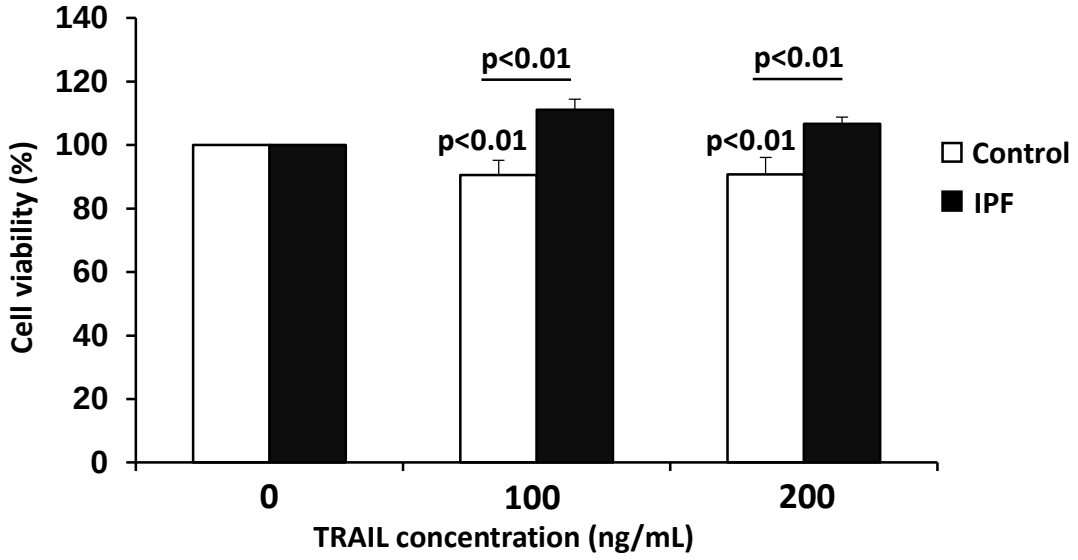

Fig 1. IPF fibroblasts have higher cell viability in response to TRAIL. $1x10^4$ lung fibroblasts for both control and IPF cells were grown overnight on a polymerized collagen matrix before serum starvation. Cell lines (n= 7) were randomly selected. The results above are shown 24 hours after treatment with TRAIL.

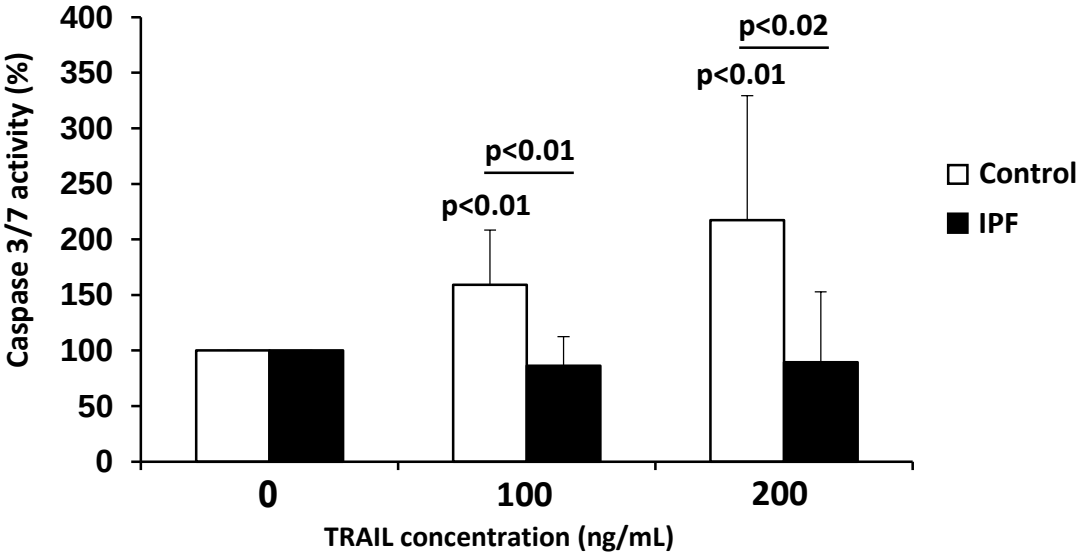

Fig 2. IPF fibroblasts have lower levels of caspase 3/7 activity in response to TRAIL. $1x10^4$ lung fibroblasts for both control and IPF cells were grown overnight on a polymerized collagen matrix before serum starvation. Cell lines (n= 7) were randomly selected. The results above are shown 24 hours after treatment with TRAIL.

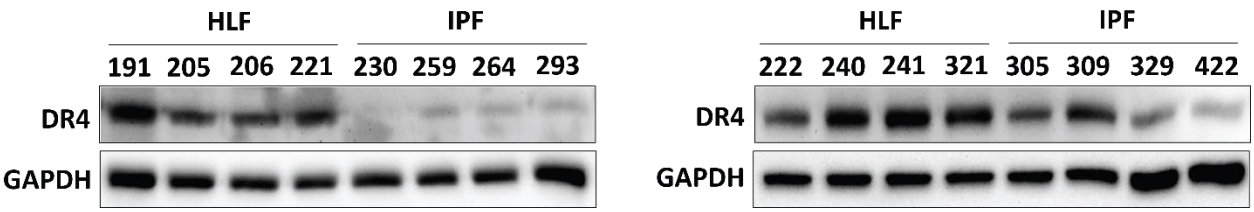

Fig 3. IPF fibroblasts express lower levels of DR4 than control fibroblasts on a polymerized collagen matrix. DR4 expression for control cells (Labeled as HLF or human lung fibroblasts, n=8) and IPF fibroblasts (n=8) was normalized to GAPDH expression. $3x10^5$ lung fibroblasts for both control and IPF cells were grown for 48 hours on a polymerized collagen matrix. Cell lines for both control and IPF fibroblasts were randomly selected.

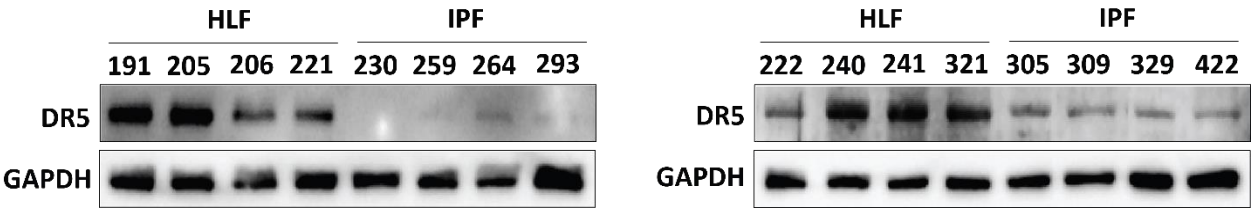

Fig 4. IPF fibroblasts express lower levels of DR5 than control fibroblasts on a polymerized collagen matrix. DR5 expression for control cells (Labeled as HLF or human lung fibroblasts, n=8) and IPF fibroblasts (n=8) was normalized to GAPDH expression. $3x10^5$ lung fibroblasts for both control and IPF cells were grown for 48 hours on a polymerized collagen matrix. Cell lines for both control and IPF fibroblasts were randomly selected.

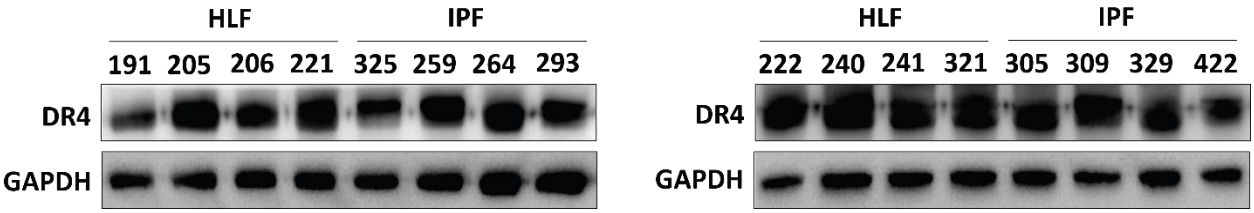

Fig 5. IPF fibroblasts express roughly equal levels of DR4 as control fibroblasts in non-PC conditions. DR4 expression for control cells (Labeled as HLF or human lung fibroblasts, n=8) and IPF fibroblasts (n=8) was normalized to GAPDH expression. $3x10^5$ lung fibroblasts for both control and IPF cells were grown for 48 hours in DMEM. The same cell lines used previously in PC conditions were used for this experiment.

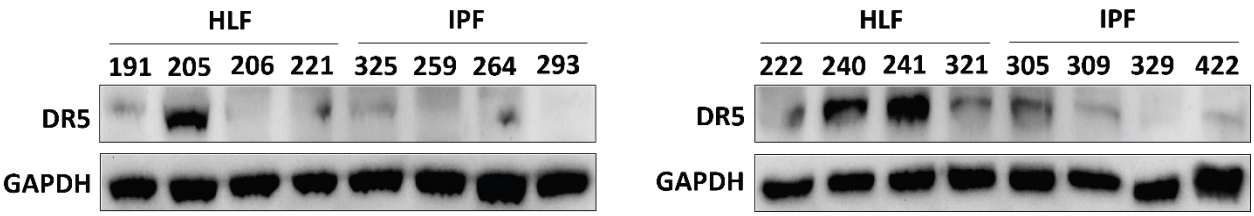

Fig 6. IPF fibroblasts express roughly equal levels of DR5 as control fibroblasts in non-PC conditions. DR5 expression for control cells (Labeled as HLF or human lung fibroblasts, n=8) and IPF fibroblasts (n=8) was normalized to GAPDH expression. $3x10^5$ lung fibroblasts for both control and IPF cells were grown for 48 hours in DMEM. The same cell lines used previously in PC conditions were used for this experiment.

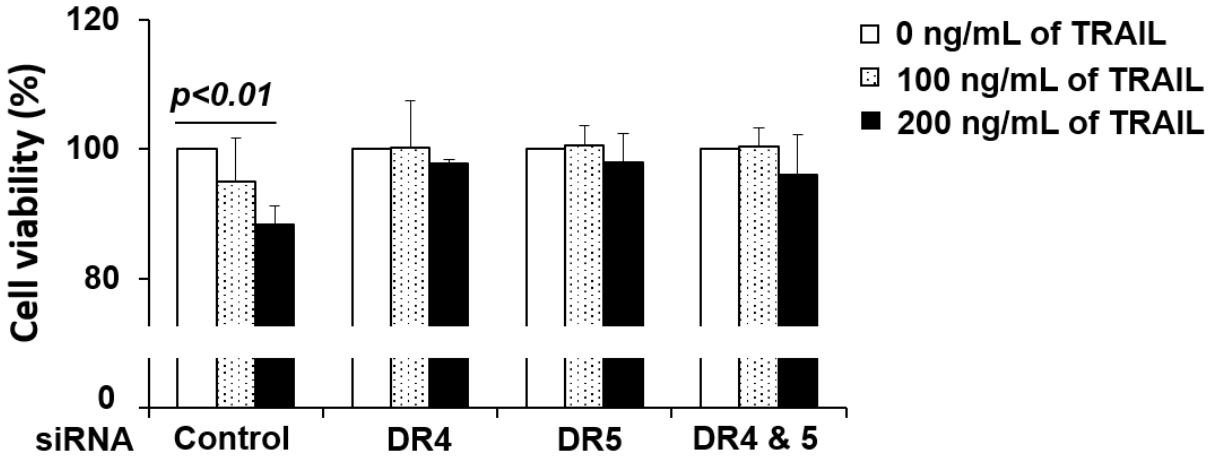

Fig 7. Silencing DR4 and DR5 increases cell viability in response to TRAIL. Control lung fibroblasts ($1x10^4$ cells, n=3) were cultured on PC for 24 hours, then subsequently cultured in DMEM without FCS for an additional 24 hours. Cell viability measurements were gathered as described in methods 24 hours after exposure to TRAIL. Cell lines were chosen at random.

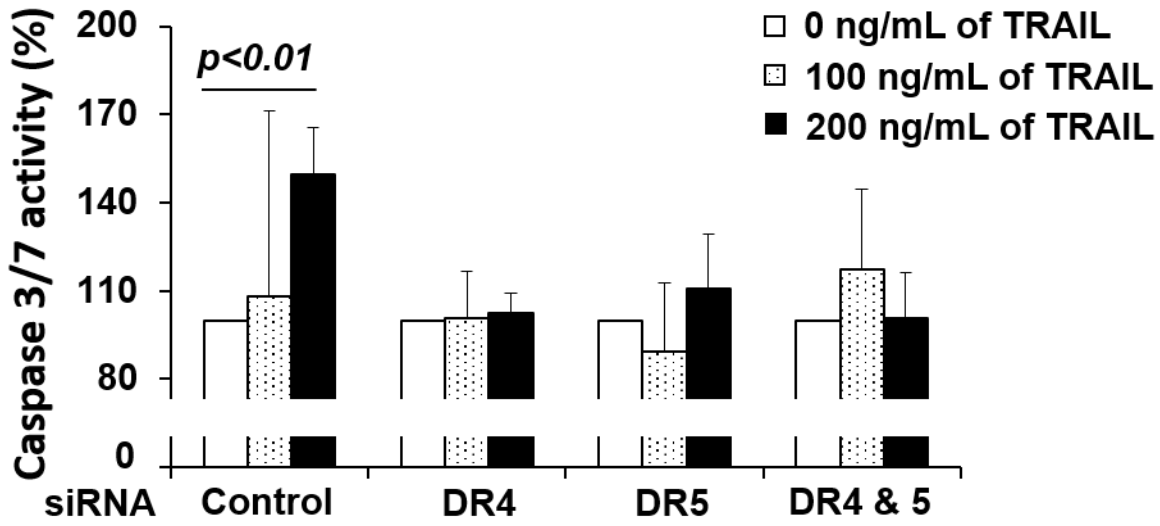

Fig 8. Silencing DR4 and DR5 decreases caspase 3/7 activity in response to TRAIL. Control lung fibroblasts ($1x10^4$ cells, n=3) were cultured on PC for 24 hours, then subsequently cultured in DMEM without FCS for an additional 24 hours. Caspase 3/7 activity results were obtained as described in methods 24 hours after exposure to TRAIL. Cell lines were chosen at random.

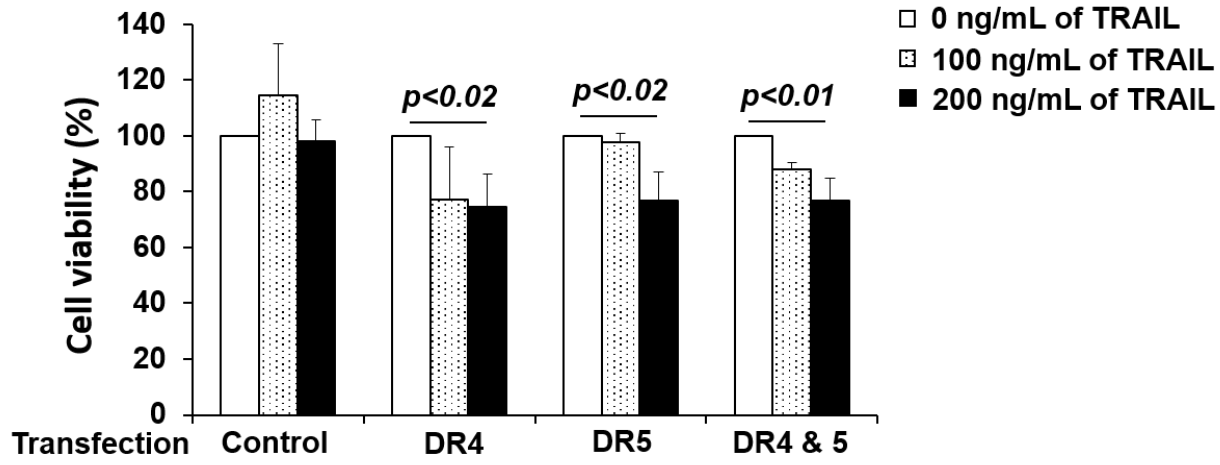

Fig 9. Overexpressing DR4 and DR5 decreases cell viability in response to TRAIL. IPF lung fibroblasts ($1x10^4$ cells, n=3) were cultured on PC for 24 hours, then subsequently cultured in DMEM without FCS for an additional 24 hours. Cell viability measurements were gathered as described in methods 24 hours after exposure to TRAIL. Cell lines were chosen at random.

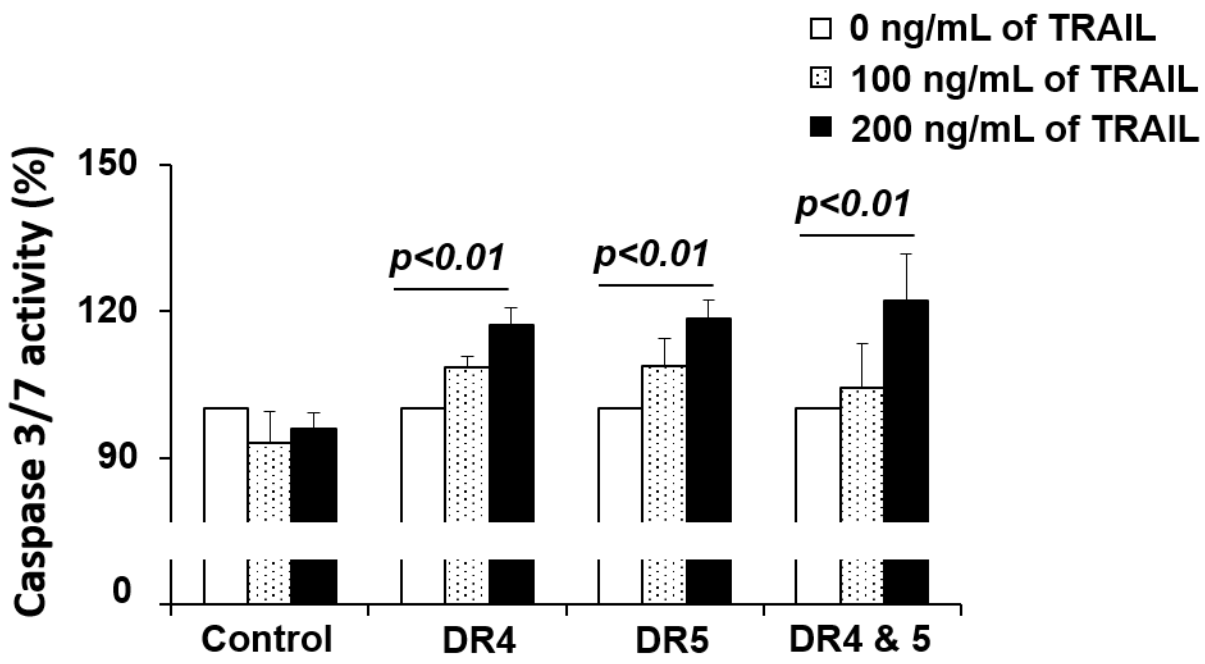

Fig 10. Overexpression of DR4 and DR5 increases caspase 3/7 activity in response to TRAIL. IPF lung fibroblasts ($1x10^4$ cells, n=3) were cultured on PC for 24 hours, then subsequently cultured in DMEM without FCS for an additional 24 hours. Caspase 3/7 activity results were obtained as described in methods at 24 hours after treatment with TRAIL. Cell lines were chosen at random.

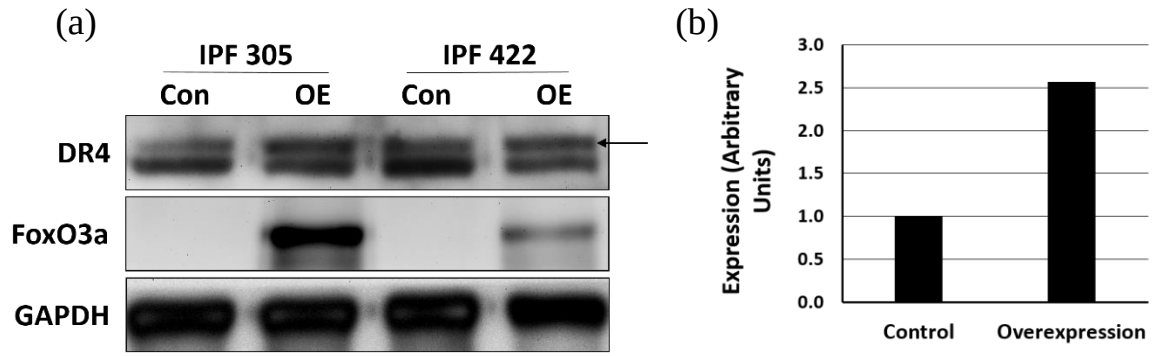

Fig 11. Overexpression of FoxO3a increases DR4 expression. (a) IPF lung fibroblasts ($3x10^5$ cells, n=2) were cultured on PC for 48 hours, then subsequently cultured in DMEM without FCS for an additional 24 hours before lysate was collected. Cell lines were chosen at random. Due to non-specific binding of the DR4 antibody, multiple bands frequently appear in Western analysis. DR4 expression corresponds to the upper band indicated by the arrow. (b) Densitometry analysis of DR4 expression, normalized to GAPDH expression, was done using the membranes from (a). No error bars or p-values are present, as n=2 did not provide strong statistical strength.