A high throughput screening platform targeting PDLIM5 for pulmonary hypertension

Han Cheng[1*], Tianji Chen[2*], Merve Tor[2], Deborah Park[2], Qiyuan Zhou[2], Jason B. Huang[2], Nour Khatib[2], Lijun Rong[1#], Guofei Zhou[2, 3 #]

[*] These authors contributes to this manuscript equally

[1]Department of Microbiology and Immunology, [2]Department of Pediatrics, College of Medicine University of Illinois at Chicago, Chicago, Illinois, [3]University of Illinois Cancer Center, Chicago, IL.

[#] Address correspondence to:

Guofei Zhou, PhD
Department of Pediatrics,
College of Medicine University of Illinois at Chicago,
840 S. Wood Street, M/C 856, Ste 1206, Chicago, IL, 60612
guofei@uic.edu, 312-355-0073 (office)

or

Lijun Rong, PhD
Department of Microbiology and Immunology
College of Medicine University of Illinois at Chicago
8133 COMRB, 909 S Wolcott Ave, Chicago, IL 60612
312-355-0203 (office)
lijun@uic.edu

Abstract

Pulmonary arterial hypertension (PAH) is a complex disease with multiple etiologic factors. PDLIM5, a member of the Enigma subfamily of PDZ and LIM domain protein family, contains an N-terminal PDZ domain and 3 LIM domains at its C-terminus. We have previously shown that overexpression of PDLIM5 prevents hypoxia-induced pulmonary hypertension (PH), and deletion of PDLIM5 in smooth muscle cells enhances hypoxia-induced PH *in vivo*. These results suggest that PDLIM5 may be a novel therapeutic target of PH. In this study, we aim to establish a high throughput screening (HTS) platform for PDLIM5-targeted drug discovery. We generated a stable mink lung epithelial cell line (MLEC) containing a TGF-β/Smad luciferase reporter with lentivirus-mediated suppression of PDLIM5 (MLEC-shPDLIM5) and measured levels of Smad2/3 and pSmad2/3. We found that in MLEC, suppression of PDLIM5 decreased Smad-dependent luciferase activity, Smad3, and pSmad3. We used MLEC-shPDLIM5 and a control cell line (MLEC-shCTL) to screen the Prestwick library (1,200 compounds) and identified and validated paclitaxel as a PDLIM5 inhibitor in MLEC. Furthermore, we showed that paclitaxel inhibited Smad2 expression and Smad3 phosphorylation in A549 cells. Our study suggests that this system is robust and suitable for PDLIM5-targeted drug discovery.

Key words: PDLIM5, high throughput screening, TGF/Smad, luciferase reporter assay

Introduction

Pulmonary arterial hypertension (PAH) is a complex disease with multiple etiologic factors without effective treatment [1, 2]. Although there are many subcategories of PAH, including including idiopathic PAH (IPAH), heritable PAH (HPAH), and PAH associated with other diseases (APAH) such as connective tissue diseases[3], they all share common pathological changes in pulmonary artery, such as proliferation of pulmonary artery endothelial cell (PAEC) and pulmonary artery smooth muscle cell (PASMC), PASMC migration and contraction, inflammation, as well as fibroblast proliferation, activation, and migration. Numerous factors contribute to the pathogenesis of PAH, including genetic, epigenetic, and environmental factors [4]. In our previous report, we have adopted a quantitative mass spectrometry analysis/proteomics approach for global screening of miR-17~92 targets in human PASMC and have identified PDZ and LIM domain 5 (PDLIM5) as a novel miR-17~92 target [5]. We have shown that knockout of PDLIM5 enhances hypoxia-induced pulmonary hypertension (PH), whereas overexpression of PDLIM5 inhibits hypoxia-induced PH [5]. Thus, PDLIM5 protein plays a role in the development of PH [5], suggesting a potential to target PDLIM5 for the treatment of PH.

PDLIM5, also named Enigma Homologue protein (ENH), is a member of the Enigma subfamily of PDZ and LIM domain protein family and contains an N-terminal PDZ domain and 3 LIM domains at its C-terminus. PDLIM5 is highly expressed in heart and skeletal muscle [6, 7] and acts as a signal modulator to influence organ development and disease [8, 9]. PDLIM5 can interact with α-actinin, protein kinase C, protein kinase D, and ID2, a DNA transcription inhibitor, and has been implicated in heart disease and mental disorders [10]. We have shown that in PASMC, suppression of PDLIM5 induces TGF-β3, TβR1, TGF-β activity, Smad2, and phosphorylated Smad2/3 as well as nuclear localization of Smad2/3 [5]. PDLIM5 also negatively regulates the expression of SMC markers, a feature of TGF-β signaling. SMC-specific knockout of PDLIM5 enhances hypoxia-mediated vascular remodeling, and the overexpression of PDLIM5 inhibits TGF-β/Smad signaling and prevents hypoxia-induced PH *in vivo* [5]. These results indicate that PDLIM5 regulates the development of human disease by modulating TGF-β/Smad signaling. Therefore, PDLIM5 may be a novel therapeutic target for human diseases.

We sought to establish a high throughput screening (HTS) platform for PDLIM5 modulators, utilizing a TGF-β/Smad-based luciferase reporting system. Mink lung epithelial cells (MLEC) were stably transfected with a luciferase reporter plasmid driven by a truncated plasminogen activator inhibitor type I (PAI-1) promoter, which contains Smad-binding elements [11]. We generated a stable MLEC-Luc cell line with lentivirus-mediated suppression of PDLIM5. We found that suppression of PDLIM5 decreased Smad-dependent luciferase activity without affecting cell proliferation. By screening the Prestwick library (1,200 compounds) in the presence or absence of TGF-β1, we identified and validated paclitaxel as a PDLIM5 inhibitor in MLEC. Thus, this assay system can be used to discover novel PDLIM5 inhibitors via HTS.

Materials and Methods

Materials

TGF-β1 (Calbiochem, La Jolla, CA), paclitaxel (Sigma-Aldrich, St. Louis, MO), and The Luciferase Assay System and reporter lysis buffer (Promega, Madison, WI) were used in this study. The Prestwick Collection, a library of 1,200 FDA-approved drugs was purchased through the UIC Collaborative Engagement in Novel Therapeutic Research and Enterprise (UICentre) High Throughput Screen facility.

Cell Culture

Mink lung epithelial cells (MLEC) were stably transfected with a luciferase reporter plasmid containing a truncated plasminogen activator inhibitor type I (PAI-1) promoter, which contains Smad-binding elements [11 12]. MLEC were maintained in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Manassas, VA) supplemented with 10% FBS and 1% penicillin-streptomycin and were used for TGF-β reporter assay. Human non-small cell lung adenocarcinoma cell line A549 was purchased from American Type Culture Collection (ATCC) (Manassas, VA) and was cultured in DMEM media as described above. All cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

Establishment of stable cell lines with suppression of PDLIM5 (A549-shPDLIM5 and MLEC-shPDLIM5)

PDLIM5 shRNA lentiviral particles and control shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We transduced A549 and MLEC cells with a mixture of complete medium with polybrene (final concentration 5 μg/ml) and lentiviral particles as we reported previously [13]. We cultured cells in media containing puromycin dihydrochloride (1 μg/ml) to select for stable single clones, which were collected and expanded. The expression of PDLIM5 was analyzed by Western blot analysis.

TGF-β reporter assay

MLEC, MLEC-shCTL, and MLEC-shPDLIM5 were plated into 24-well plates at a density of 10^5 cells/ml/well and cultured overnight. After 48-hour incubation with TGF-β1 (5 ng/ml) or PBS, MLECs were rinsed with PBS and lysed in passive lysis buffer at 120 μl/well (Promega). Cell lysates were cleared at top speed for 2 min at 4˚C. 100-μl supernatants of each sample were transferred to new polypropylene tubes and read with 100 μl luciferase assay buffer (Promega) on a GloMax®-96 Microplate luminometer (Promega). The protein concentrations were determined using Bio-Rad protein assay solution (Bio-Rad, Hercules, CA). The relative luciferase activity was calculated by normalizing total luciferase activity to the amount of protein. Each assay was done in triplicates and repeated at least three times.

Western blotting

We measure protein levels by Western blot analysis as we described previously [14]. Briefly, after three washes with phosphate-buffered saline (PBS), cells were lysed in mRIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% deoxycholate, 150 mM NaCl, and protease inhibitors) on ice for 30 minutes. The cell lysates were cleared by centrifugation at 13,000 g for 10 minutes, and protein concentrations of the supernatants were determined using Bio-Rad protein assay solution (Bio-Rad). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to BA85 nitrocellulose membranes (PROTRAN, Whatman, Dassel,

Germany), and probed with primary and secondary antibodies. Proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, Waltham, MA). The following primary antibodies were used in this study: α-tubulin, β-actin, SMAD2/3, pSMAD2 (ser465/467), pSMAD3 (ser423/425), PDLIM5 (Sigma-Aldrich and Protein Technologies, Inc.). Anti-mouse, anti-rabbit, and anti-goat IgG-HRP conjugates were purchase from Bio-Rad. The density of the protein bands was quantified with ImageJ software [15, 16].

Bromodeoxyuridine (BrdU) cell proliferation assay

MLEC and A549 cells were plated in 96-well plates at a density of 5×10^3 cells/100 µl/well and incubated overnight. BrdU was added to the culture medium at a dilution of 1:10,000, followed with another 16-hour incubation. Cell proliferation activities were detected using a BrdU cell proliferation assay kit (Calbiochem, Gibbstown, NJ) on a GloMax®-96 Microplate luminometer (Promega) at the wavelength of 450 nm according to the user manual.

High throughput screen

This pilot screening was carried out in white 384-well plates and assembled on a PerkinElmer (Illinois) Automated Workstation (JANUS/Envision). Briefly, we seeded MLEC-shCTL and MLEC-shPDLIM5 at the density of 2,000 cells/well and incubated them overnight. The following day, we aspirated old media and added 20 µl of fresh media with or without TGF-β1 (10 ng/ml) to each well, then 20 µl of fresh media containing 25 µM of compounds. The final compound concentration was 12.5 µM and TGF-β1 was 5 ng/ml. Each 384-well plate contained 320 test wells that received compounds and 32 control wells that received DMSO vehicle. Half of the control wells were cultured in the absence of TGF-β1 while the other half was cultured in the presence of TGF-β1 for the calculation of z' factors. Following a 48-hour period of incubation, the luciferase activity was measured following the manufacturer's instructions (Neolite luciferase substrate, PerkinElmer). The coefficient of variation was calculated with R programming (R Development Core Team).

The z'-factor was determined from experimental data that were derived from the measurements of the assay signal in the presence and absence of TGF-β1 using the following equation: $z' = 1 - \frac{(3\sigma s + 3\sigma c)}{|\mu s - \mu c|}$, where: σ_s is the standard deviation of signal (with TGF-β1); σ_c is the standard deviation of control (without TGF-β1); μ_s is the mean of signal (with TGF-β1), and μ_c is the mean of control (without TGF-β1) [17, 18].

Statistical analysis

All experiments were repeated at least three times independently (most were five experiments). t-tests were performed using GraphPad Prism 4 (GraphPad, San Diego, CA) when applicable. The significance level was set at 0.05 and 0.01.

Results

PDLIM5 is required for TGF-β/Smad signaling in alveolar epithelial cells. Previously, we have shown that PDLIM5 inhibits TGF-β/Smad in pulmonary artery smooth muscle cells [5]. To investigate the role of PDLIM5 on TGF-β/Smad in alveolar epithelial cells, we transduced a luciferase reporter cell line derived from mink lung epithelial cells (MLEC-Luc) (**Fig 1A and 1B**) with control lentivirus and lentiviral particles containing small hairpin RNA against PDLIM5. After selection with puromycin, we established two stable cell lines MLEC-shCTL and MLEC-shPDLIM5 and confirmed the silencing of PDLIM5 (**Fig 1A-1C**). We found that suppression of PDLIM5 did not alter cell proliferation (**Fig 1D**), but it decreased Smad-dependent luciferase activity (**Fig 1E**) and levels of total and phosphorylated Smad3 (**Fig 1F-1G**). These results suggest that PDLIM5 is a positive regulator of TGF-β/Smad in alveolar epithelial cells,

The concept of using MLEC-shCTL and MLEC-shPDLIM5 for high throughput screening to identify PDLIM5 inhibitors. Since PDLIM5 is required for TGF-β/Smad signaling (**Fig 1**), we hypothesize that a TGF-β/Smad-based luciferase reporter system can be used to identify PDLIM5 inhibitors via high throughput screening (HTS) (**supplemental Fig S1A**). To minimize the non-specificity, we used both MLEC-shCTL and MLEC-shPDLIM5 in the absence or presence of TGF-β1 (**supplemental Fig S1B**). Theoretically, PDLIM5 inhibitors should decrease the TGF-β1-induced luciferase activity in MLEC-shCTL cells while having little effect on the TGF-β1-induced luciferase activity in MLEC-shPDLIM5 cells. Thus, in the presence of PDLIM5 inhibitors, the ratio of TGF-β1 responsiveness (fold induction of TGF-β1-mediated luciferase by normalizing the luciferase activity in the TGF+ to that of the TGF- group) should increase in MLEC-shPDLIM5 compared to that in MLEC-shCTL (MLEC-shPDLIM5/MLEC-shCTL).

A high throughput screening platform for PDLIM5 inhibitors. To examine the suitability of this system for HTS, we screened the Prestwick Collection, a library of 1,200 FDA-approved drugs. We determined the z' value in this 1,200-compound screening as described previously [17, 18] and found that the z' factors of all 16 plates exceeded 0.5, confirming the suitability of this assay system for HTS (**Fig 2A**).

We examined the distribution of the overall ratio of the TGF-β1 responsiveness between two cell lines (MLEC-shPDLIM5/MLEC-shCTL). As shown in **Fig 2B**, the distribution of the overall ratios of the TGF-β1 responsiveness exhibited a bell-shape curve that was shifted left, confirming our assumption that MLEC-shPDLIM5 cells are less responsive to TGF-β1 (**Fig 2**), therefore indicating that this system is useful for the discovery of PDLIM5 inhibitors. We calculated the coefficient of variation for 16 wells of control replicates on each plate and found that they are well below 10% in TGF+ group or 20% in the TGF- group (**Supplemental Table S1**).

Identification of paclitaxel as a PDLIM5 inhibitor by screening the Prestwick Collection (1,200 compounds). We then set up the cutoff threshold of the overall ratios of the TGF-β1 responsiveness (MLEC-shPDLIM5/MLEC-shCTL) as 1.5 for the selection of PDLIM5 inhibitors. There were 11 putative PDLIM5 inhibitors (**Table 1**). We cherry-picked these 11 hits and retested them in the second round of the HTS. In this round, we added 20 µl of media containing 25 µM of compound first, then 20 µl of media with or without TGF-β1 (final compound

concentration was 12.5 μM, TGF-β1 was 5 ng/ml) to eliminate the variation due to the order of treatment. Paclitaxel and disulfiram were confirmed in the second round screening (**Table 1**). The facts that PDLIM5 is known to regulate microtubule dynamics [19] and that paclitaxel is a well-known cancer drug that interferes with microtubule stability demonstrate the quality of our HTS system.

Paclitaxel inhibits TGF-β/Smad signaling in non-small cell lung carcinoma (NSCLC) cell line. Since PDLIM5 is required for TGF-β/Smad signaling (**Fig 1**), we reasoned that a PDLIM5 inhibitor would likely inhibit the TGF-β/Smad signaling. To further validate the screening results, we tested whether paclitaxel inhibits TGF-β/Smad signaling. We treated A549 cells with 50 nM paclitaxel for two days and measured the protein levels of PDLIM5, Smad2/3, and pSmad2/3 in the cell lysates. We found that treatment with paclitaxel (Taxol) inhibited the total Smad2 and pSmad2 and decreased the ratio of pSmad3 over total Smad3 while inducing total Smad3 (**Fig 3**). However, the levels of PDLIM5 and pSmad3 remained unchanged (**Fig 3**). These results demonstrated that paclitaxel indeed inhibits TGF-β/Smad signaling, as we predicted, validating our HTS platform as a suitable approach to identify PDLIM5 inhibitors as anti-cancer drug candidates.

Paclitaxel-mediated inhibition of TGF-β/Smad3 signaling depends on PDLIM5 in NSCLC. To address whether paclitaxel inhibits TGF-β/Smad signaling via PDLIM5 in NSCLC cells, we treated A549-shCTL and A549-shPDLIM5 with paclitaxel or vehicle and incubated for 48 hours followed with measurement of total and phosphorylated Smad2/3. We found that suppression of PDLIM5 had little effect on total Smad2, pSmad2, or the ratio of pSmad2/Smad2 (**Fig 4**). Although suppression of PDLIM5 had little effect on the ratio of pSmad3/Smad3, it decreased the amount of pSmad3 (**Fig 4**). Thus, paclitaxel inhibits TGF-β/Smad3 via PDLIM5.

Discussion

In this pilot study of PDLIM5-targeted drug discovery, we have discovered that: 1) PDLIM5 differentially regulates TGF-β1/Smad signaling in PASMC and alveolar epithelial cells; 2) we have established an HTS platform to screen for PDLIM5 inhibitors and performed a pilot HTS with the 1,200-compound Prestwick library; 4) we have identified and confirmed paclitaxel as a PDLIM5 inhibitor. Thus, our results indicate that PDLIM5 is a novel therapeutic target for pulmonary hypertension and this assay is suitable for HTS for PDLIM5 inhibitors.

Interestingly, our findings suggest that PDLIM5 has opposing effects on TGF-β/Smad signaling, depending on the cell type: in PASMC, PDLIM5 inhibits TGF-β/Smad signaling, whereas in alveolar epithelial cells, PDLIM5 promotes TGF-β/Smad signaling (**Fig 5**). In the context of TGF-β/Smad in human lung diseases, we postulate that PDLIM5 may be a factor that is anti-hypertensive yet pro-fibrosis (TGF-β/Smad induces epithelial-mesenchymal transition (EMT) and contributes to pulmonary fibrosis). Therefore, a PDLIM5 activator is required for the treatment of PH, while a PDLIM5 inhibitor is required for pulmonary fibrosis. Since PDLIM5 has opposing effects on TGF-β/Smad signaling in PASMC and alveolar epithelial cells, a PDLIM5 inhibitor in alveolar epithelial cells will likely act as a PDLIM5 activator in PASMC. Thus, a PDLIM5 inhibitor indentified from this system can be used as PDLIM5 activator for the treatment of PH. Consistently, paclitaxel was reported to prevent and reverse experimental PH [20].

TGF-β is a potent and pleiotropic cytokine that participates in the pathogenesis of numerous human diseases [21, 22]. The pleiotropy of TGF-β has been demonstrated by its capability to produce multiple effects: canonical Smad signaling, direct signaling by type II receptor kinases to regulate tight junctions and stabilization of actin filaments, and regulate MAPK and PI3K outputs[21, 23]. In cancer, TGF-β has paradoxical dual functions: tumor suppression at the early stage and tumor promotion at the later stage [21, 22]. In the early stage of cancer, TGF-β inhibits cell proliferation and induces apoptosis to inhibit tumorigenesis, whereas in the late stage, TGF-β promotes tumor spreading in part by inducing EMT [24-26]. The dual function of TGF-β suggests that the TGF-β signaling action is highly context-dependent and is influenced by spacio-temporal environments, therefore making TGF-β-targeting drug development particularly challenging [24]. Thus, the role of PDLIM5 inhibitors in lung cancer may have undesired effects. The key to this issue may lie in the fact that PDLIM5 has differential roles on Smad2 and Smad3. Consistently, we have shown that paclitaxel inhibits Smad2 expression and Smad3 phosphorylation (**Fig 3-4**), which is consistent with the findings that Smad2, but not Smad3, is mutated in lung cancer [27]. These results demonstrate the feasibility of this strategy for the lung cancer drug discovery, and it will be interesting to investigate the role of PDLIM5 in lung cancer.

We found that paclitaxel does not alter expression levels of PDLIM5; rather, it inhibits the PDLIM5-mediated cell signaling (**Fig 3**). Therefore, we anticipate that PDLIM5 inhibitors will have limited effects on PDLIM5 protein levels but inhibit molecular signaling underlining cell proliferation, survival, and spreading. PDLIM5 is known to sequester ID2 in the cytoplasm to exert its inhibitory effects on transcription [28, 29]. PDLIM5 can also recruit activated PKC and its substrates to promote phosphorylation [6, 8, 30]. This approach may minimize the adverse effect of loss of PDLIM5 on cell phenotype or function during screening.

Although suppression of PDLIM5 has little effect on cell proliferation of PASMC [5] or alveolar epithelial cells (**Fig 1**), we have discovered that paclitaxel, a potent anti-proliferative drug, is a PDLIM5 inhibitor. This may be a concern that anti-proliferative hits may dominate a screen like ours. However, this seemingly undesired outcome may be explained by the difference between the IC50 and the dose we have used for our screen: the median IC50 values of paclitaxel for the NSCLC cell lines is typically higher than 23 μM or 0.38 μM for one or five days of treatment, respectively [31]; in our screening, we used 12.5 μM at two days of treatment (**Fig 2 and supplemental Fig S1**). Thus, the inhibition of PDLIM5 by paclitaxel requires a lower dose than what causes cytotoxicity. Indeed, when using 50 nM, we found that paclitaxel is sufficient to inhibit PDLIM5-mediated Smad signaling without significant cell death (**Fig 3-4**). These results reflect the sensitivity of our assay and will eliminate predominant anti-proliferative hits. Another advantage of our screening strategy is to use the ratio of TGF-β1 responsive as a cutoff, which eliminates the bias of cytotoxicity and proliferation of compounds. However, it is worth to point out that in our strategy we used a 2x2 matrix which is a rather complicate assay and may limit its use in a large- scale robust HTS.

From our pilot screening, the majority of the compounds may not exert any effect on either TGF-β1 activity or PDLIM5 function; however, we have identified 50 compounds in the Prestwick Library that enhance PDLIM5 activity by at least 2 fold (data not shown). These compounds can also be valuable since PDLIM5 regulates TGF-β/Smad signaling in a tissue- or disease-specific manner and will be further investigated.

In summary, with the ultimate goal to identify novel small molecules to treat PH, we have established a PDLIM5-targeting drug discovery platform for high throughput screening. Future studies to screen larger libraries to expand our knowledge of drug-like chemicals that target PDLIM5 signaling are warranted. Given the limited knowledge of PDLIM5 function, this system and identified probes will be very useful tools to elucidate the molecular mechanisms underlying PDLIM5 function.

Acknowledgements

The work is partly supported by an American Lung Association Biomedical Research Grant, a Pulmonary Hypertension Association/Pfizer Proof-of-Concept award (in which American Thoracic Society provides administrative support), an Inception Grant Arm 1 from UIC CCTS and UICentre, a Gilead Sciences Research Scholars Program in Pulmonary Arterial Hypertension award (G Zhou), and NIH R01HL123804 (G Zhou). We thank Dr. Gregory R. Thatcher, Ph.D., Director of UICentre for the suggestions and Miranda Sun for proof reading.

References:

1. Humbert, M., Sitbon, O., Simonneau, G. Treatment of pulmonary arterial hypertension. *N Engl J Med* **2004,** *351* (14), 1425-36.
2. McLaughlin, V. V., Archer, S. L., Badesch, D. B., et al. ACCF/AHA 2009 expert consensus document on pulmonary hypertension: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association: developed in collaboration with the American College of Chest Physicians, American Thoracic Society, Inc., and the Pulmonary Hypertension Association. *Circulation* **2009,** *119* (16), 2250-94.
3. Simonneau, G., Gatzoulis, M. A., Adatia, I., et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* **2013,** *62* (25 Suppl), D34-41.
4. Zhou, G., Chen, T., Raj, J. U. MicroRNAs in pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* **2015,** *52* (2), 139-51.
5. Chen, T., Zhou, G., Zhou, Q., et al. Loss of MicroRNA-1792 in Smooth Muscle Cells Attenuates Experimental Pulmonary Hypertension via Induction of PDZ and LIM Domain 5. *American journal of respiratory and critical care medicine* **2015,** *191* (6), 678-92.
6. Nakagawa, N., Hoshijima, M., Oyasu, M., et al. ENH, containing PDZ and LIM domains, heart/skeletal muscle-specific protein, associates with cytoskeletal proteins through the PDZ domain. *Biochem Biophys Res Commun* **2000,** *272* (2), 505-12.
7. Ueki, N., Seki, N., Yano, K., et al. Isolation, tissue expression, and chromosomal assignment of a human LIM protein gene, showing homology to rat enigma homologue (ENH). *J Hum Genet* **1999,** *44* (4), 256-60.
8. Krcmery, J., Camarata, T., Kulisz, A., et al. Nucleocytoplasmic functions of the PDZ-LIM protein family: new insights into organ development. *Bioessays* **2010,** *32* (2), 100-8.
9. Te Velthuis, A. J. W., Isogai, T., Gerrits, L., et al. Insights into the molecular evolution of the PDZ/LIM family and identification of a novel conserved protein motif. *PLoS ONE* **2007,** *2* (2), e189.
10. Cheng, H., Kimura, K., Peter, A. K., et al. Loss of enigma homolog protein results in dilated cardiomyopathy. *Circ Res* **2010,** *107* (3), 348-56.
11. Abe, M., Harpel, J. G., Metz, C. N., et al. An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Analytical Biochemistry* **1994,** *216* (2), 276-84.
12. Zhou, Q., Pardo, A., Konigshoff, M., et al. Role of von Hippel-Lindau protein in fibroblast proliferation and fibrosis. *Faseb J* **2011,** *25* (9), 3032-44.
13. Shaikh, D., Zhou, Q., Chen, T., et al. cAMP-dependent protein kinase is essential for hypoxia-mediated epithelial-mesenchymal transition, migration, and invasion in lung cancer cells. *Cell Signal* **2012,** *24* (12), 2396-406.
14. Zhou, Q., Chen, T., Bozkanat, M., et al. Intratracheal instillation of high dose adenoviral vectors is sufficient to induce lung injury and fibrosis in mice. *PLoS ONE* **2014,** *9* (12), e116142.
15. Schneider, C. A., asband, W. S., Eliceiri, K. W., et al. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **2012,** *9* (7), 671-5.
16. Collins, T. J. ImageJ for microscopy. *Biotechniques* **2007,** *43* (1 Suppl), 25-30.
17. Zhang, Q., Liu, Z., Mi, Z., et al. High-throughput assay to identify inhibitors of Vpu-mediated down-regulation of cell surface BST-2. *Antiviral Res* **2011,** *91* (3), 321-9.
18. Zhang JH, C. T., Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.* **1999,** *4* (2), 67-73.

19. Yan, Y., Tsukamoto, O., Nakano, A., et al. Augmented AMPK activity inhibits cell migration by phosphorylating the novel substrate Pdlim5. *Nat Commun* **2015,** *6*, 6137.
20. Savai, R., Al-Tamari, H. M., Sedding, D., et al. Pro-proliferative and inflammatory signaling converge on FoxO1 transcription factor in pulmonary hypertension. *Nat Med* **2014,** *20* (11), 1289-300.
21. Akhurst, R. J.; Hata, A. Targeting the TGFbeta signalling pathway in disease. *Nat Rev Drug Discov* **2012,** *11* (10), 790-811.
22. Yingling, J. M., Blanchard, K. L., Sawyer, J. S. Development of TGF-beta signalling inhibitors for cancer therapy. *Nat Rev Drug Discov* **2004,** *3* (12), 1011-22.
23. Massague, J. TGFbeta signalling in context. *Nat Rev Mol Cell Biol* **2012,** *13* (10), 616-30.
24. Nagaraj, N. S.; Datta, P. K. Targeting the transforming growth factor-beta signaling pathway in human cancer. *Expert Opin Investig Drugs* **2010,** *19* (1), 77-91.
25. Chambers, A. F., Groom, A. C., MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* **2002,** *2* (8), 563-72.
26. MacDonald, I. C., Groom, A. C., Chambers, A. F. Cancer spread and micrometastasis development: quantitative approaches for in vivo models. *BioEssays : news and reviews in molecular, cellular and developmental biology* **2002,** *24* (10), 885-93.
27. Levy, L.; Hill, C. S. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* **2006,** *17* (1-2), 41-58.
28. Kurooka, H.; Yokota, Y. Nucleo-cytoplasmic shuttling of Id2, a negative regulator of basic helix-loop-helix transcription factors. *The Journal of biological chemistry* **2005,** *280* (6), 4313-20.
29. Lasorella, A.; Iavarone, A. The protein ENH is a cytoplasmic sequestration factor for Id2 in normal and tumor cells from the nervous system. *Proc Natl Acad Sci U S A* **2006,** *103* (13), 4976-81.
30. Kuroda, S., Tokunaga, C., Kiyohara, Y., et al. Protein-protein interaction of zinc finger LIM domains with protein kinase C. *The Journal of biological chemistry* **1996,** *271* (49), 31029-32.
31. Georgiadis, M. S., Russell, E. K., Gazdar, A. F., et al. Paclitaxel cytotoxicity against human lung cancer cell lines increases with prolonged exposure durations. *Clin Cancer Res* **1997,** *3* (3), 449-54.

Table 1. List of small molecules that inhibit PDLIM5 in MLEC.

Drug name	% of control				shPDLIM5 to shCTL TGFβ response ratio	Targeted diseases	Mechanism
	shPDLIM5 cell		shCTL cell				
	-TGF-β1	+TGF-β1	-TGF-β1	+TGF-β1			
Idebenone	71%	216%	167%	166%	3.12	Alzheimer's disease, neuromuscular diseases	increases ATP production
Disulfiram	72%	51%	236%	51%	2.53	chronic alcoholism, Lung and liver cancer	inhibits acetaldehyde dehydrogenase, proteasome inhibitor
Norgestrel-(-)-D	100%	212%	128%	100%	2.05	pregnancy	a form of progestin
N-Acetyl-DL-homocysteine Thiolactone (citiolone)	86%	111%	215%	112%	1.86	liver therapy	a derivative of the amino acid cysteine
Hydroxyzine dihydrochloride	53%	126%	94%	96%	1.78	anxiety and tension, anesthesia, nausea and vomiting, skin allergy	reduces activity in the central nervous system, also antihistamine
Calcipotriene	125%	155%	131%	95%	1.72	psoriasis	a form of vitamin D. It works by slowing down the growth of skin cells.
Paclitaxel	38%	12%	51%	8%	1.59	cancer	interference with the normal breakdown of microtubules during cell division.
Zopiclone	75%	106%	131%	122%	1.54	insomnia	increases GABA transmission
Estrone	62%	159%	80%	101%	1.53	estrogenic hormone	pregnancy and carcinogen
Tranilast	94%	135%	98%	93%	1.53	allergy, asthma	reduces collagen synthesis, inhibits growth of neurofibroma cells, inhibits interleukin-6 production
SR-95639A dihydrochloride	78%	122%	118%	123%	1.52	depression	a selective M1 muscarinic agonist

Molecules highlighted in red were confirmed in the secondary screening.

Figure legends:

Fig 1. Suppression of PDLIM5 decreases TGF-β/Smad signaling in alveolar epithelial cells. A, B) Diagrams of control lentiviral particle-transduced MLEC-Luc cells and cells transduced with lentiviral particles encoding small hairpin RNA against PDLIM5. The suppression of PDLIM5 was validated by Western blotting as shown in C. These cells were subjected to a BrdU incorporation assay for cell proliferation (D) and relative luciferase activity as measured by relative light units (RLU) (E). The effect of PDLIM5 on total and phosphorylated Smad2 and Smad3 were shown in F and G, respectively. ** $p < 0.01$.

Fig 2. Feasibility of PDLIM5-targeted HTS screening. A) The z' factor of the plates in the Prestwick collection screening. B) Distribution of ratios of the TGF-β1 responsiveness (MLEC-shPDLIM5/MLEC-shCTL).

Fig 3. Treatment of paclitaxel inhibits TGF-β/Smad signaling. A549 cells were treated with 50 nM paclitaxel (Taxol) for two days followed by Western blot analysis for PDLIM5, Smad2, Smad3, pSmad2, and pSmad3. The quantification shown in panel D is from at least three experiments. * $p < 0.05$, ** $p < 0.01$.

Fig 4. Paclitaxel-mediated inhibition of TGF-β/Smad3 signaling depends on PDLIM5 in NSCLC. A549-shCTL and A549-shPDLIM5 cells were treated with 50 nM paclitaxel (Taxol) for two days followed by Western blot analysis for PDLIM5, Smad2, Smad3, pSmad2, and pSmad3. Actin was used as control for equal loading. The quantification shown in panel B is from at least three experiments. * $p < 0.05$, ** $p < 0.01$.

Fig 5. **Schematic diagrams depicting differentiated PDLIM5-mediated regulation of the TGF-β/Smad signaling in different cell types.** A) A diagram depicting the mechanism by which PDLIM5 inhibitors modulate TGF-β/Smad signaling in alveolar epithelial cells. B) An overview of PDLIM5-mediated modulation of TGF-β/Smad signaling in pulmonary artery smooth muscle cells.