Supporting Information

Miller et al. 10.1073/pnas.1201583109

SI Materials and Methods

Cell Culture and Transient Transfection Assays. For cell culture assays, a pCMV β-gal plasmid was used as a control plasmid to equalize the amount of plasmid being transfected per condition. Next, 50,000 cells were plated in each well of a 24-well plate and after 24 h, the wells were transfected in triplicate using 0.1042 μg of Wnt2-expressing plasmid, 0.0417 μg of Wnt7b-expressing plasmid, 0.2083 μg of SuperTopFlash, and 0.1042 μg of *Renilla* control luciferase expression plasmid. Variable amounts of pCMV β-gal plasmid were added to bring the total amount of DNA to 0.7 mg. Fugene 6 was used as the transfection reagent, and was added at a 3:1 ratio of Fugene:DNA. Forty-eight hours after transfection, the Dual-Luciferase Assay System (Promega) was used to perform luciferase assays. To normalize transfection efficiency, the firefly luciferase values were divided by the *Renilla* luciferase values, and the ratio was used to compare the control (pCMV β-gal), Wnt2, Wnt7b, and Wnt2+Wnt7b conditions. All assays were performed at least three to five times with consistent results and a representative assay is shown in the figures performed in triplicate ± SD. Significance in all assays was determined using two-tailed Student *t*-tests.

siRNA Knockdown Experiments. Rat On-Target Plus SMARTpool siRNAs were purchased from Dharmacon (Ctnnb1 L-100628-00-0005, Fzd5 L-095098-01-0005, Non-Targeting Pool D-001810-10-05).

Animals. Generation and genotyping of $Wnt7b^{LacZ}$ and $Wnt2^{-/-}$ mouse lines have been previously described (1, 2). Embryos were collected from embryonic day (E) 10.5–E12.5 as noted. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols.

Histology. Embryos and lung explants were collected and fixed in 4% (vol/vol) paraformaldehyde for immunohistochemistry and in situ hybridization. Following fixation, embryos and lung explants were dehydrated in an increasing gradient of ethanol washes, embedded in paraffin, and sectioned. Tissue and lung explant sections were immunostained with the following antibodies: anti-Nkx2.1 (Santa Cruz), anti-p63 (Santa Cruz), anti-Sox2 (Seven Hills Bioreagents), anti-Sox9 (Santa Cruz), and anti-SM22α (Abcam). In situ hybridization was performed as previously described (1, 2). For all histology, a minimum of three control embryos and three $Wnt2^{-/-};Wnt7b^{-/-}$ embryos were stained and visualized.

Quantitative PCR and Western Blotting. Total RNA was isolated from cells and tissues using TRIzol reagent, reverse-transcribed using SuperScript II First-Strand Synthesis Kit (Invitrogen), and used in quantitative real-time PCR analysis using SYBR green (Applied Biosystems). Lung explant mRNA was isolated using the Qiagen RNeasy Mini Kit. For Western blotting, cells were collected 48 h following transfection, lysed, and nuclear and cytoplasmic fractions were generated using a standard protocol. Briefly, 500 μL of Buffer A (10 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% Nonidet P-40) was added to transfected cells, cells were scraped, and lysates were incubated for 10 min on ice. Lysates were centrifuged at 4 °C at 625 × *g* for 10 min, and the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in Buffer B (300 mM NaCl, 5 mM Hepes, 1.4 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% (vol/vol) glycerol, and protease inhibitor mixture), homogenized, and incubated on ice for 30 min. Nuclear lysate was centrifuged at 21,130 × *g* at 4 °C for 30 min. The supernatant was collected as the nuclear fraction. Protein concentration was quantified using the Bio-Rad Protein Assay and 15 μg of protein were resolved on SDS/PAGE gels and transferred to PVDF membranes. Antibodies used include in Western blotting include: β-catenin (BD Transduction Laboratories), β-tubulin (Abcam), HN-RNPA1 (Santa Cruz Biotech).

Lung Explant Culture. Lungs were isolated from E11.5 CD1 embryos and cultured in phenol-free DMEM/F12 (Cellgro) on 0.4-μm transwells (Falcon) for either 48 or 72 h at 37 °C and 5% CO_2. Lungs for quantitative PCR (qPCR) analysis were cultured for 48 h, and lungs for immunohistochemistry were cultured for 72 h. Pdgfrα and Pdgfrß morpholinos (GeneTools) were added into the culture medium at a concentration of 15 μM. The Pdgfrα morpholino (ATGTGTGGATACATACCTGTGAGG) targeted the splice donor site of exon 2, and the Pdgfrß morpholino (ATCTGTCAAGAGCAGAGCCAAGGAA) targeted the splice acceptor site of exon 4. A standard control morpholino (CCTCTTACCTCAGTTACAATTTATA) designed by Gene-Tools was used as the control morpholino. Morpholino knockdown was assessed by qPCR using primers (TGAGGGAGAGAAACAAACGGAGGA) and (AGCTCCTGAGACCTTCTCCTTCTA) for Pdgfrα and (ACCAGCGAGGTTTCACTGGTACTT) and (ATCATTGCCCATCACAATGCACCG) for Pdgfrß. Recombinant Wnt2 (Novus Biologicals) and recombinant Wnt7a (R&D Systems) were added to the culture medium at a concentration of 0.25 μg/mL. For qPCR, a minimum of three lungs were pooled for RNA for each experimental condition. For histology, a minimum of three lungs were used for each condition.

1. Goss AM, et al. (2009) Wnt2/2b and beta-catenin signaling are necessary and sufficient to specify lung progenitors in the foregut. *Dev Cell* 17:290–298.

2. Shu W, Jiang YQ, Lu MM, Morrisey EE (2002) Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* 129:4831–4842.

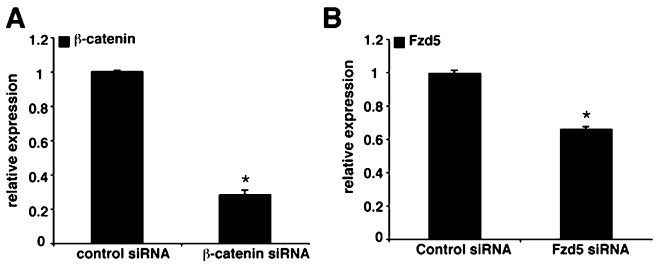

Fig. S1. Extent of siRNA knockdown for β-catenin and Frizzled receptor 5 (Fzd5). To determine the extent of β-catenin knockdown by siRNA, qPCR was performed and showed an ~75% decrease in β-catenin gene expression (*A*). To determine the extent of Fzd5 knockdown by siRNA, qPCR was performed and showed an ~35% decrease in Fzd5 gene expression (*B*). qPCR results are average of three samples ± SEM (*$P < 0.03$).

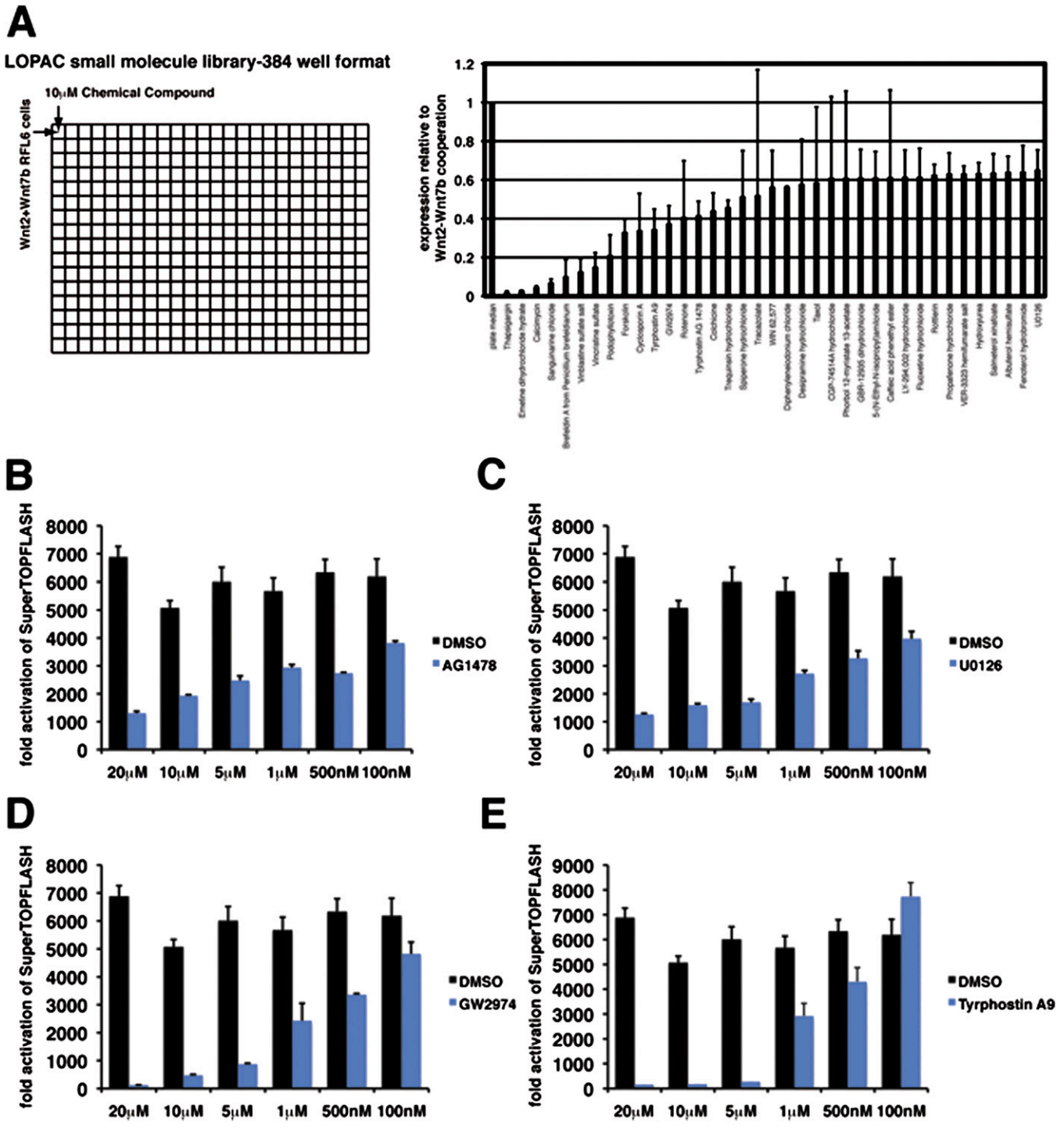

Fig. S2. Library of Pharmacologically Active Compounds (LOPAC) screen and dose-dependent response of EGF/Pdgf inhibitors. The LOPAC library was screened for compounds that inhibit the Wnt2–Wnt7b cooperative signaling. From this screen, 37 compounds were identified that inhibited the Wnt2–Wnt7b signal by at least 35% (*A*). Four of the compounds—AG1478, U0126, GW2974, and Tyrphostin A9—decreased the cooperation between Wnt2 and Wnt7b in a dose-dependent manner (*B–E*). Inhibition of Wnt2–Wnt7b cooperative signaling by the inhibitors is significant at all doses ($P < 0.05$) except for the 100-nM concentration of Tyrphostin A9.

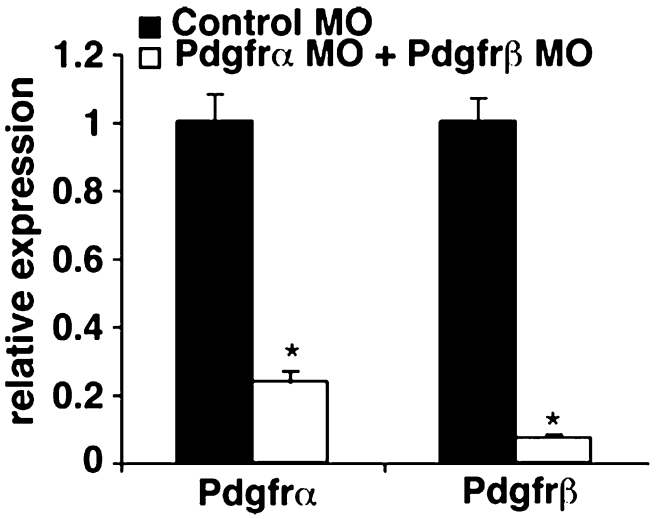

Fig. S3. Pdgfrα and Pdgrβ morpholinos resulted in knockdown of Pdgfrα and Pdgfrß gene expression in E11.5 lung explants by qPCR. qPCR results are average of three samples ± SEM (*$P < 0.01$). MO, morpholino.

Table S1. qPCR primer list

Primer	Sequence
b-catenin (rat)	F′ ACACAACCTTTCCCACCATCGAGA
	R′ TAGCAGGAGATTATGCAGCGTGGT
Fzd5 (rat)	F′ AGCGACCTTCCTCATTGACATGGA
	R′ AGAATCCCAGTGACACACACAGGT
Pdgfra (mouse)	F′ TGAGGGAGAGAAACAAACGGAGGA
	R′ AGCTCCTGAGACCTTCTCCTTCTA
Pdgfrb (mouse)	F′ ACCAGCGAGGTTTCACTGGTACTT
	R′ ATCATTGCCCATCACAATGCACCG
SMA (mouse)	F′ ATTGTGCTGGACTCTGGAGATGGT
	R′ TGATGTCACGGACAATCTCACGCT
SM22 (mouse)	F′ TCTAATGGCTTTGGGCAGTTTGGC
	R′ TTTGAAGGCCAATGACGTGCTTCC
Axin2 (mouse)	F′ AAAGAAACTGGCAAGTGTCCACGC
	R′ TTTGAGCCTTCAGCATCCTCCTGT
CD31 (mouse)	F′ ATTCCTCAGGCTCGGGTCTT
	R′ CCATGCACCTTCACCTCGTA
Flk1 (mouse)	F′ ATCATTGCCCATCACAATGCACCG
	R′ TCTCCTACAAAATTCTTCATCAATCTTG
Gapdh (mouse)	F′ AGGTTGTCTCCTGCGACTTCA
	R′ CCAGGAAATGAGCTTGACAAAGTT
Gapdh (rat)	F′ AGCATCTCCCTCACAATTCCA
	R′ TGAGGGTGCAGCGAACTTTA
Axin2 (rat)	F′ CCCGAACTATTTATTCAAAACATGAC
	R′ TCTATGGATTTCAGATCCCTAGGAA