Platelet-derived growth factor-A and sonic hedgehog signaling direct lung fibroblast precursors during alveolar septal formation

Stephen E. McGowan and Diann M. McCoy

Department of Veterans Affairs Research Service and Department of Internal Medicine, University of Iowa Carver College of Medicine, Iowa City, Iowa

Submitted 14 January 2013; accepted in final form 4 June 2013

McGowan SE, McCoy DM. Platelet-derived growth factor-A and sonic hedgehog signaling direct lung fibroblast precursors during alveolar septal formation. Am J Physiol Lung Cell Mol Physiol 305: L229–L239, 2013. First published June 7, 2013; doi:10.1152/ajplung.00011.2013.—Alveolar septal formation is required to support the respiration of growing mammals; in humans effacement of the alveolar surface and impaired gas exchange are critical features of emphysema and pulmonary fibrosis. Platelet-derived growth factor-A (PDGF-A) and its receptor PDGFR-α (PDGFRα) are required for secondary septal elongation in mice during postnatal days 4 through 12 and they regulate the proliferation and septal location of interstitial fibroblasts. We examined lung fibroblasts (LF) to learn whether PDGFRα expression distinguished a population of precursor cells, with enhanced proliferative and migratory capabilities. We identified a subpopulation of LF that expresses sonic hedgehog (Shh) and stem cell antigen-1 (Sca1). PDGF-A and Shh both increased cytokinesis and chemotaxis in vitro, but through different mechanisms. In primary LF cultures, Shh signaled exclusively through a noncanonical pathway involving generation of Rac1-GTP, whereas both the canonical and noncanonical pathways were used by the Mlg neonatal mouse LF cell line. LF preferentially oriented their primary cilia toward their anterior pole during migration. Furthermore, a larger proportion of PDGFRα-expressing LF, which are more abundant at the septal tips, bore primary cilia compared with other alveolar cells. In pulmonary emphysema, destroyed alveolar septa do not regenerate, in part because cells fail to assume a configuration that allows efficient gas exchange. Better understanding how LF are positioned during alveolar development could identify signaling pathways, which promote alveolar septal regeneration.

SECONDARY ALVEOLAR SEPTA are required for sufficient pulmonary gas exchange in growing mammals. All secondary septa develop after birth in rats and mice, and a majority does so in humans (5). Mice are born with simplified primary saccules that lack secondary septa, including those that express PDGFRα (PDGFRα-LF) proliferate and are located at the base of the elongating secondary septa at P4. At P12, when proliferation is less but the septal PDGFRα-LF are the most populous (21, 28). At P12, the PDGFRα-LF preferentially localize to the tip of the secondary septa, indicating that their position in the septum changes during septal outgrowth (28). It remains unclear what factors drive the progressive repositioning of this cell population to the distal septal tip, which comprises the alveolar entry ring and merges with the alveolar duct. PDGF-A, which exclusively signals through PDGFRα, promotes cell migration in vitro; others have hypothesized that PDGF-A promotes the migration or “spreading” of LF outward in the extending secondary septum (3, 15). However, it is not known how PDGF-A directs migration and how the process is influenced by other factors. We hypothesized that PDGF and sonic hedgehog (Shh) signal cooperatively to direct the positioning and movement of LF in the elongating secondary septum.

Both Shh and PDGFs regulate the migration of mesenchymal cells, including those that express PDGFRα during organ development (11). Disruption of Sonic hedgehog signaling during secondary alveolar septation results in enlarged air spaces and a diminished gas-exchange surface (26). Mammalian hedgehog signaling requires primary cilia, which organize and regulate several steps in the canonical hedgehog signaling pathway, including activation of smoothened (Smo) and the Gli-transcription factors and signal dampening through Gli degradation in the proteasome (20). Smoothened is a G protein-coupled receptor and regulates fibroblast migration independently of Gli through the activity of GTPases such as Rac and Rho. Primary cilia are anchored at the centriole, elongate and direct migration in growth arrested cells (40), and serve as mechanosensors that transmitted strain and pressure in many cell types including fibroblasts and smooth muscle cells (41). Primary cilia appear in postmitotic cells, and in non- or slowly dividing cells the ciliary microtubules are stabilized by acetylation, which provides a convenient marker for primary cilia.

The surface glycoprotein stem cell antigen-1 (Sca1, LY6AE) is present on a variety of progenitor cells. McQuilter and associates (31) identified a population of Sca1+, CD34+, CD45−, CD31− pulmonary mesenchymal precursor cells that expresses PDGFRα and has a gene ontology profile that is
enriched in genes involved in cell migration, cell adhesion, and extracellular matrix production. Wu and coworkers (47) described a PDGFRα-expressing population of epidermal cells which migrate into the subjacent myocardium. Epicardially derived Shh directs perivascular mesenchymal cells during the formation of the coronary vasculature (24). Sim and coworkers (42) identified PDGFRα-expressing oligodendrocyte precursor cells, which migrate along neurons and lay down a myelin sheath. In each of these instances ectodermal cells are closely applied to and direct the migration of adjacent mesenchymal cells.

MATERIALS AND METHODS

Materials. SMOOTHENED AGONISTS. SAG, N-methyl-N’-(3-pyridinylbenzyl)-N’-(3-chlorobenzyl)[b] thiophene-2-carbonyl]-1,4-diaminocyclohexane, EMD, Gibbstown, NJ. Pumiponic acid, 9-cyclohexyl-N-[4-(morpholiny1)phenyl]-2-(1-naphthalenyl)oxy]-9H-purin-6-amine, Cayman Chemical, Ann Arbor, MI.

SMOOTHED ANTAGONISTS. KAAD-cycloheximide, 3-keto-N- (aminoethyl-aminoacryl dihydrocinnamoyl)cycloheximide, EMD. SANT-1, N-[3,5-dimethyl-1-phenyl-1H-pyrazol-4yl]methylene]-4-(phenylmethyl)-1-piperazinamine, Tocris Biosciences, Minneapolis, MN.

POLYPEPTIDE STIMULANTS. Recombinant mouse Shh-NH2 terminus and recombinant human PDGF-AA, R&D Systems, Minneapolis, MN.

ANTIBODIES. Anti-α-smooth muscle actin (α-SMA) mouse monoclonal 1A4 mouse IgG2a-PE conjugate, final concentration 0.0625 μg/ml (R&D Systems, IC1420P). Anti-Sca1: rat IgG2a anti mouse A647 conjugate, final concentration 1.25 μg/ml (Invitrogen MSCA21). Ki67: goat IgG polyclonal, final concentration 1 μg/ml (Santa Cruz sc7846). Secondary antibody for Alexa Fluor 568 donkey anti-goat IgG diluted 1:2,000. CD34: brilliant violet rat anti-mouse CD34, rat IgG2a monoclonal clone RAM34, final concentration 1.25 μg/ml (BD Biosciences). Mouse monoclonal anti-cytokeratin 18 (clone Cyt90) final concentration 1 μg/ml (Sigma-Aldrich, St. Louis, MO). Shh: 5e1 anti-Shh (mouse IgG1) final concentration 0.4 μg/ml, Developmental Studies Hybridoma Bank, labeled with the A405 Zenon mouse IgG1 labeling kit (Invitrogen). Acetylated α-tubulin: mouse monoclonal antibody raised against sea urchin sperm axonemes final concentration 0.2 μg/ml (Santa Cruz Biotechnology, Santa Jose, CA, sc-23950).

CELL LINE. Mlg2980 (ATCC CCL-206) were maintained in medium containing 50% Ham’s F12, 50% DMEM, 10% FBS, 10 mM HEPES, 100 μg/ml streptomycin, and 100 U penicillin G ml per milliliter for 16 h prior to addition of SAG or purmorphamine. Total RNA was isolated by use of TRI reagent (Sigma-Aldrich) according to the manufacturer’s instructions. After centrifugation to remove cellular debris, the supernatant was combined and the film was imaged and analyzed with a Quantity-One Imaging Analysis System (Bio-Rad, Hercules, CA).

Rac1-GTP detection. Primary cultures of LF or Mlg cells were exposed to 150 nM SAG, 100 nM KAAD-cycloheximide, 500 μM SANT-1, or 10 μM purmorphamine or to a corresponding dilution of DMSO (the solvent at a final concentration of 0.1%) for 10 min and the cell layers were quickly cooled to 4°C. The cell layers were washed twice with 25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM Na2VO4. The LF were lysed in 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 5% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 20 μg/ml aprotonin (lysis buffer) combining three 100-mm culture dishes in 600 μl. After centrifugation to remove cellular debris, the protein concentration of the supernatant was analyzed using the 660-nm Protein Assay (Thermo Scientific, Rockford, IL). GST-human PAK-PBD, (protein activated-kinase protein binding domain) was used to capture the Rac-GTP. After binding to GST-agaro and washing with lysis buffer, the PK-PBD-Rac-GTP complexes were dissociated with Laemmli sample buffer. The agarose matrix was removed by centrifugation, and the samples were stored at −80°C until they were subjected to SDS-PAGE on a 12% reducing gel. Western immunoblotting was performed with equal quantities of protein, the mouse monoclonal antibody (IgG2B) in the Rac1-GTP detection kit, a peroxidase-labeled anti-mouse IgG secondary antibody, and the film was imaged and analyzed with a Quantity-One Imaging System (Bio-Rad, Hercules, CA). The densities of Rac1-GTP were normalized to the density of Rac1 for each lane, to account for inadvertent differences in the amounts of total Rac1 in the samples.

LSCM analysis of primary cilia. Lungs from mice bearing the PDGFRα-GFP insert at P4, P8, and P12 were uniformly inflated, fixed, and sectioned at 20 μm intervals (28). After permeabilization with 0.3% Triton X-100 and blocking with 2% normal goat serum, 5
mg/ml BSA, and 1 μg/ml of 2.4g2 monoclonal antibody (to block mouse Fc receptors), the sections were incubated overnight at 4°C with mouse monoclonal anti-acetylated tubulin. Following extensive washing with 0.145 M NaCl, 0.0015 M K2HPO4, 0.0027 M KCl, and 0.0086 M Na2HPO4, pH 7.4 (PBS) containing 0.1% Triton X-100, and the sections were incubated with goat anti-mouse IgG-AlexaFluor 568 (1:2,000). Nuclei were stained with 1:5,000 dilution of To-Pro3

The mixture was incubated for 16 h, then washed with PBS and 0.1% Triton X-100 and stained for acetylated tubulin and nuclear DNA, with the same contrast optics, an Olympus LUC PLAN FL ×20 objective (N.A. 0.45) with a long working distance, enclosed in an environmental chamber at 37°C with a humidified atmosphere containing 5% CO2 in air. The motorized Prior H117 microscope stage was controlled in the x- and y-dimensions by an Oasis Blue (Objective Imaging, Cambridge, UK) controller and in the z-dimension by the IX-81 controller. Both controllers were commanded by Olympus CellSens Dimension software, including the multiposition and multichannel 5D add-ons (Olympus America, Center Valley, PA). Four different positions along the interface between the monolayer and the intervening gap were imaged every 30 min in the bright-field phase-contrast and fluorescent (GFP, Chroma 49002 filter cube with excitation at 470–495 nm and a band-pass emission filter 525–550 nm) modes by use of an Olympus XM-10 camera and a motorized filter turret. The captured images were analyzed with CellSens software and the nuclei of cells that migrated into the intervening gap were enumerated, distinguishing PDGFRα-expressing (GF+ α) from nonexpressing (GF− α) cells.

Microfabrication of the gradient microfluidic device. A photolithographic mask was prepared by using Adobe Illustrator following the design of Amadi and associates (2) with modifications to increase the length of the channel for migration and the size of the gel-filling ports. A master was made by spin coating a silicon wafer with SU8–100 (Microchem, Newton, MA) to create a channel depth of ~150 μm. The microfluidic device (μFD) was prepared by using Sylgard 184 [poly(dimethyl siloxane) (PDMS), Dow Corning, Midland, MI] with a silicone-to-catalyst ratio of 10:1, following standard soft-lithography techniques. Following polymerization, PDMS oligomers were removed by refluxing with ethanol for 16 h using a Soxhlet extractor (6). The PDMS μFD was bonded to a 30-mm coverglass serving as the bottom of a 50-mm culture dish (EM Sciences, Hatfield, PA) by exposure to an oxygen plasma for 60 s in a plasma system (PlasmaTech PE-50, Carson City, NV). To restore the hydrophobic surface, the μFD and dish were heated at 80°C for 18 h prior to adding an aqueous medium.

Analysis of chemotaxis using the gradient μFD. The channels of the μFD were initially filled with 100% ethanol and then aqueous OptiMEM. A solution of 1.7% collagen (PureCol InfaMed, Fremont, CA) in Eagle’s modified essential medium with 2% FBS that had been cooled to 4°C was loaded into the filling ports of the device. The collagen was allowed to polymerize for 45 min at 37°C, and ~1.5 × 10^5 primary LF or Mlg were added to each filling port. After 2 h, when the collagen had fully polymerized, OptiMEM containing 2% FBS, 2 mg/ml BSA, 3 mM CaCl2, and 100 μg/ml streptomycin was added to the filling ports. After 24 h the FBS concentration in the medium was reduced to 1.5%. The source well of the μFD was filled with either 100 ng/ml PDGF-A or 5 μg/ml Shh and the control well was filled with fresh medium without stimulants. The culture dish was mounted in the IX81 microscope and the atmospheric humidity was maintained at 80–85% by use of an ultrasonic humidifier. Time lapse imaging was performed for 16 h using imaging using a UPLFLN ×20 0.5 N.A. Olympus objective with DIC optics. Images were captured from four fields for each condition (control or chemotaxis) and analyzed using CellSens software to annotate the x- and y-coordinates and distance traveled for each 30-min interval, and the speed and persistence were calculated as previously described (29).

Statistical methods. Data were expressed as means ± SE of the number of different mice that were used or the number of different experiments that were performed using cell cultures (38). ANOVA (either 1- or 2-way as indicated in the figure legends) was performed by using Systat (Chicago, IL) and Student’s t-test by using Microsoft Excel. Additional details describing how the data from different images or image stacks were combined appear in the method for a particular stereological procedure. Post hoc tests are described in the figure legends. Values of P < 0.05 were considered significant.
RESULTS

A subpopulation of neonatal mouse LF display Sca1. Senescence of mesenchymal progenitor cells contributes to emphysema, and others have observed fibroblastic progenitors might be strategic for alveolar septal regeneration in emphysema, we examined their presence and properties during secondary septation using flow cytometry of freshly isolated neonatal mouse LF. Fibroblasts bearing the Sca1- marker were more abundant in the subpopulation that did not express lungs of adult mice (8, 31). Reasoning that fibroblastic progenitors might be strategic for alveolar septal regeneration in emphysema, we examined their presence and properties during secondary septation using flow cytometry of freshly isolated neonatal mouse LF. Fibroblasts bearing the Sca1- marker were more abundant in the subpopulation that did not express

Fig. 1. Flow cytometry showed that lung fibroblasts (LF) displaying stem cell antigen-1 (Sca1+) exhibit sustained proliferation and delayed differentiation. A: LF isolated at postnatal days 4, 8, or 12 (P4, open bars; P8, hatched bars; or P12, solid bars) from lungs of mice expressing the PDGFRα-GFP marker were stained for the intracellular proteins Ki67 and α-smooth muscle actin (α-SMA, ACTA2) or the surface protein, Sca1, and subjected to flow cytometry. LF that express PDGFRα-SMA, ACTA2) or the surface protein, Sca1, and subjected to flow cytometry. LF that express PDGFRα are designated GFP+. LF isolations from 5 separate litters of mice were analyzed at each age (mean ± SE). *P < 0.05 compared with GFP- at same age, **P < 0.01 compared with GFP+ vs. P4 and P8 GFP+ vs. P8 GFP+: 2-way ANOVA, Student-Newman-Keuls post hoc test. B: LF isolated from 4 different litters of mice at P8 were stained for CD34 and Sca1 and analyzed by flow cytometry (mean ± SE). **P < 0.01 compared with GFP- or to CD34- LF (t-test for unpaired variables).

Fig. 2. Flow cytometry showed that more Sca1+ LF contain sonic hedgehog (Shh). LF were isolated from mice expressing the PDGFRα-GFP marker at P8, permeabilized (perm, open bars) with saponin or remained unpermeabilized (unperm, open bars), stained for Shh or Sca1, and distinguished as PDGFRα-expressing (GFP+) or nonexpressing (GFP-) LF. LF isolations from 5 separate litters of mice were analyzed (mean ± SE). A: *P < 0.05 permeabilized compared with unpermeabilized within a particular PDGFRα subpopulation. **P < 0.01 GFP+ vs. GFP- permeabilized. *P < 0.05 GFP+ vs. GFP- unpermeabilized; 2-way ANOVA, Student-Newman-Keuls post hoc test. B: *P < 0.01 Sca1+ compared with Sca1- Student’s t-test unpaired variables. C: *P = 0.02, Ki67+ in Sca1+ compared with Sca1- Student’s t-test unpaired variables. D: RNA was obtained from LF freshly isolated at P4, P8, or P12 (n = 3, 4, and 4, respectively) and the mean ± SE levels of steady-state mRNA were compared. **P < 0.05 P8 Shh vs. P4 or P12 Shh; or P8 Patched (Ptc) vs. P4 or P12 Ptc. 2-way ANOVA, Tukey post hoc test. E: Gli1 and smoothened mRNA were similarly analyzed using LF, which were freshly isolated at P4, P8, or P12 (n = 3, 4, or 3, respectively). *P < 0.05, P4 vs. P12.
evaluate the source of the intracellular Shh, we assessed Shh
expression, which is regulated by Shh (Fig. 2D). Both gli1 and
smoothened mRNA diminished significantly after P8 com-
pared with P4 (Fig. 2E).

Smoothed agonists stimulate Shh signaling through varied
pathways. Because the expression of Shh and Shh-responsive
signaling components were synchronously elevated at P8,
whereas Gli1 was not, we examined Shh signaling in cultured
LF. There are two potential Shh signaling pathways: 1) The
canonical pathway whereby Shh binds to its plasma mem-
brane receptor Patched, releasing smoothed from the inhibitory
effects of Patched, thereby enabling Smoothed translocation
to the primary cilium. Smoothened agonists can circumvent
patched and activate smoothed, resulting in a canonical
increase in the Gli transcription factors and Patched. 2) The
noncanonical pathway whereby Smoothened signals as a G
protein-coupled receptor, independently of Gli. Others have
demonstrated the importance of noncanonical Shh signaling
in fibroblasts and endothelial cells, where activation of Smooth-
ened promotes subcortical actin remodeling and polarization
of migrating cells (4).

Surprisingly, we observed that a 12-h exposure to smooth-
ened agonists SAG or purmorphamine (not shown) failed to
alter the expression of target genes gli1, gli2, patched, and
hedgehog inhibitory protein (HHip) in primary cultures of LF
(Fig. 3A). We examined a variety of concentrations of SAG
and durations of exposure up to 24 h and did not observe a
significant increase in gene expression in gli1, gli2, or ptc. We
also examined the expression of downstream Shh target genes
in the continuous neonatal mouse lung fibroblast cell line
(Mlg2980). In contrast to the primary LF cultures, purmorph-
amine strikingly increased gli1 and patched gene expression
in Mlg cells (Fig. 3B).

After observing that SAG and purmorphamine failed to alter
expression of the canonical signaling intermediates, gli or ptc,
in primary LF, we evaluated noncanonical signaling. We ex-
amined the effects of smoothed agonists on Rac1 activation,
an approach taken by others using NIH3T3 fibroblasts (35).
Primary cultures of LF were exposed to the smoothed

PDGFRα (GFP−) at P4 and P8, but there was a progressive
increase in the proportion of Sca1+ cells in the PDGFRα-
expressing (GFP+) subpopulation up to at least P12 (Fig. 1A).
Although the proportion of LF bearing the Ki67 antigen, a
marker of the potential for cellular proliferation, declined in
both the Sca1+ and Sca1− LF subpopulations after P4, Ki67+
cells occupied a larger proportion of Sca1+ than of Sca1− LF
at P8 and P12. This indicates that a subgroup of PDGFRα-
expressing Sca1+ LF maintains proliferation, whereas there is
debate in the overall proportion of proliferating LF. At P4
and P8, α-SMA was lower in Sca1+ than in Sca1− LF, but
between P8 and P12 there was an increase in the proportion of
Sca1+ LF that contained α-SMA. This suggests that the
transition of the Sca1+ LF subpopulation to a myofibroblastic
phenotype is delayed, relative to the Sca1− subpopulation. A
significantly larger fraction of the PDGFRα-expressing
(GFP+) than GFP− LF displayed CD34, and CD34+ LF were
more likely to also display Sca1 (Fig. 1B).

Sca1+ neonatal LF contain Shh. Aortic smooth muscle cells
arise in part from Shh-containing, Sca1+ mesenchymal cells
that migrate from the adventitia into the media (34). We
queried whether both Sca1 and Shh may mark PDGFRα-
expressing LF destined to form the alveolar entry rings, con-
sistent with migration away from the septal base (28). A larger
proportion of LF within the PDGFRα−GFP+ than within the
GFP− subpopulation contained Shh, and a larger fraction of
GFP+ LF bound the antibody after permeabilization, consist-
tent with an intracellular location (Fig. 2A). A larger proportion of Sca1+ than Sca1− LF contained Shh at P8 (Fig. 2B) and LF
with Ki67 were more abundant within the Shh+ subpopulation
(Fig. 2C). Although we found that LF contain Shh and express
the Shh gene, most prior studies have concluded that pulmo-
nary epithelial cells produce Shh, which acts on mesenchymal
cells (45, 32). To detect epithelial cells contaminating the
fibroblast preparation using FACS, we immunostained for
cytokeratin 18, which was present in 2.5 ± 0.2% of the isolated
cells. Cytokeratin 18-positive cells comprised 1.3 ± 0.3% of
Shh+ cells, indicating that contaminating epithelial cells did
not account for the Shh+ cells in the isolated fibroblasts. To
evaluate the source of the intracellular Shh, we assessed Shh
gene expression in freshly isolated LF at P4, P8, and P12. Shh
mRNA was most abundant in LF at P8, as was patched gene
expression, which is regulated by Shh (Fig. 2D). Both gli1 and
smoothened mRNA diminished significantly after P8 com-
pared with P4 (Fig. 2E).

Smoothened agonists increase Gli and Ptc mRNA in Mlg but not primary mouse LF cultures. Primary LF that had been isolated at P12 and cultured
were exposed to the smoothened agonist SAG for 12 h (A); Mlg cells were exposed to purmorphamine for various times or remained unexposed (control). B, mRNA
for Gli1 or Gli2, Ptc, or hedgehog inhibitory protein (HHip) was quantified. A: primary LF (mean ± SE, n = 3 separate cohorts of cultured LF). B: Mlg cells
(mean ± SE, n = 3 at 3 h, n = 4 at 6 and 12). Horizontal broken line shows control (set at 1); y-axis scale is log base 2.

AJP-Lung Cell Mol Physiol • doi:10.1152/ajplung.00011.2013 • www.ajplung.org
agonists SAG or purmorphamine (not shown) and Rac1-GTP was quantified. SAG significantly increased Rac1-GTP (Fig. 4, A and C). Blocking endogenous Shh with the smoothened antagonist KAAD-cyclopamine or SANT-1 did not significantly reduce Rac1-GTP, indicating that endogenous Shh did not significantly alter Shh signaling under these culture conditions. Purmorphamine and SAG also increased Rac1-GTP in cultured Mlg cells (Fig. 4, B and D), although the response to SAG was less robust compared with primary LF.

Shh and PDGF-A increase LF migration through different mechanisms. Observing that PDGFRα-expressing (GFP+) LF predominate at the tips of the secondary alveolar septa during elongation, suggests that PDGF-A, which only signals through PDGFRα, may promote their migration relative to LF which do not express PDGFRα (GFP−). We first studied whether PDGF-A specifically enhanced chemokinesis of PDGFRα-expressing LF. Approximately 80% of the LF that were isolated from mice bearing the PDGFRα-GFP insertion actively migrated in the presence of PDGF-A (Fig. 5, A), than in its absence (○). The migration of LF that did not express PDGFRα (GFP−, bottom) was not altered by PDGF-A.

Fig. 4. Rac1-GTP is increased by smoothened agonists in cultured primary mouse LF and Mlg. Representative immunoblot using primary LF showing active Rac1 (Rac1-GTP) and total Rac 1 (control) SAG or purmorphamine (not shown) and Rac1 after exposure to purmorphamine (purmo) and unexposed control. B: representative immunoblot using Mlg cells showing Rac1-GTP and Rac1 after exposure to purmorphamine (purmo) and unexposed control. C: combined results (mean ± SE) from 5, 4, and 3 experiments performed with primary LF and SAG, KAAD-cyclopamine, and the antagonist SANT-1, respectively. *SAG compared with control, P < 0.05 1-way ANOVA, Student-Newman-Keuls post hoc test. D: combined results from 3 experiments using Mlg. **P < 0.01 Purmo compared with control, *P < 0.05, SAG compared with control.

Fig. 5. Time-lapse microscopy demonstrated that LF migration is increased by PDGF and Shh Primary LF cultures were established on glass, and confluent cells that migrated into a central empty space were enumerated. A: top shows PDGFRα-expressing (GFP+) LF. More GFP+ LF migrated in the presence of PDGF-A (○), than in its absence (●). The migration of LF that did not express PDGFRα (GFP−, bottom) was not altered by PDGF-A. Mean ± SE of 4 separate experiments. *P < 0.05, PDGFRα-A compared with medium only with GFP+ LF. B: cumulative number of LF that migrated into the central space after exposure to PDGF-A (PDGF, ●), sonic hedgehog (Shh, ▲), PDGF-A and Shh (PDGF+Shh, ◊), or unexposed (control, ○). Mean ± SE or 4 replicate fields, for all conditions in 1 experiment that was representative of 3 other separate experiments. *P < 0.05 compared with control, **P < 0.05 compared with PDGF-A or Shh alone.
express PDGFRα (are GFP+) in culture. Therefore the chemokinetic effect of PDGF-A was compared in GFP+ and GFP− LF residing within the same monolayer. As expected, only the GFP+ LF (Fig. 5A, top) were stimulated by PDGF-A to enter empty portion of the chamber (Fig. 5A, bottom, GFP− LF). We also assessed the chemokinetic effects of Shh and PDGF-A separately, or in combination on the migration of aggregate population of both GFP+ and GFP− LF. Although GFP− LF were included in the analysis, PDGF-A still increased cell migration (Fig. 5B). Shh also promoted LF migration and there was an additional increase in migration when Shh was combined with PDGF-A (Fig. 5B).

To delineate the migration of individual cells, LF were studied by using a μFD shown in Fig. 6A. Primary LF were isolated from PDGFRα-GFP+ mice, a chemotactic gradient of PDGF-A was established, and the migration of GFP+ cells was quantified. Figure 6B shows that a protein diffusion gradient was established in the observation region of the μFD. The migration of LF or Mlg, without regard to their PDGFRα expression status, was studied by using a gradient of PDGF-A or Shh. The cellular paths traversed over 16 h are shown for three LF exposed to only medium (control) or medium containing Shh diffusing from a source located to the right of the origin (Fig. 6C). The speed of migration is based on the accumulated distance migrated and does not account for changes in direction. Directional persistence is dependent on the consistency of both speed and direction (43). PDGF-A increased the speed of PDGFRα-expressing LF but did not significantly alter the persistence (Fig. 6D). In contrast, Shh did not alter the speed but significantly increased the persistence of both primary LF and Mlg cells (Fig. 6, E and F).

Primary cilia of migrating LF orient toward the direction of migration. Primary cilia, which are required for hedgehog signaling in mammals, also enhance migration of fibroblasts that express PDGFRα (39). Mesenchymal cells display a single primary cilium that may exhibit a uniform orientation with respect to chemical or mechanical gradients (12). We compared the orientation of primary cilia in primary LF that migrated away from the orientation of the cilia of LF that remained in the confluent portion of a monolayer. The cilia of migrated LF were preferentially oriented toward the direction of migration, whereas for LF that remained in the confluent area cilia were randomly oriented (Fig. 7).

PDGFRα-GFP+ LF are preferentially located at the secondary septal tips (28). We posited that if primary cilia are required for this preferential localization, a larger fraction of PDGFRα-GFP+ LF may display primary cilia, compared with other alveolar septal cells. During septal elongation, signifi-

![Fig. 6. PDGFRα-GFP+ LF are preferentially located at the secondary septal tips (28).](http://ajplung.physiology.org/)

![Diagram of the microfluidic device (μFD) used to establish a gradient of PDGFRα or Shh.](http://ajplung.physiology.org/)
Fig. 7. Migrating LF orient primary cilia in a forward direction when exposed to Shh. Cultured primary LF that had been isolated from mice bearing the PDGFRα-GFP knockin were exposed to Shh for 24 h, and cells that had migrated into the empty space were classified on the basis of the orientation of primary cilia along the axis of migration (toward, open bars) into the central empty space (A). There was no preferential orientation for LF that did not migrate (B), in which cilia were as likely to point away (hatched bars) or perpendicular (neutral, solid bars) relative to the direction of migration. C: mean ± SE of the ratios of migrated to unmigrated LF from 3 separate experiments. A total of 204 LF that had and 562 LF that had not migrated were analyzed in the combined analysis. *P < 0.05 comparing orientation of cilia toward the leading edge in LF migrated vs. unmigrated LF.

**DISCUSSION**

During accelerated secondary septal elongation (P4 through P12), PDGFRα-expressing (GFP+) alveolar fibroblasts are most abundant and become more concentrated at the tip of the elongating septum (28). Although Sca1+ LF are present in the GFP− subpopulation, only the PDGFRα-expressing GFP+ LF, which also predominately display CD34, show a temporal enrichment in Sca1+ cells. This enrichment coincides with an overall decline in the abundance of LF containing Ki67. In contrast to the Sca1− LF, the proportion of Ki67+ cells within the Sca1+ fraction increases from P4 through P12, suggesting that Sca1+ LF selectively retain their proliferative capacity. Furthermore, this Sca1+ subpopulation is enriched in Shh+, Ki67+ LF, and some of the Shh resides intracellularly. The intracellular pool of Shh is at least partially acquired endogenously. Whereas in primary LF, Smoothed only signals as a G protein-coupled receptor, both canonical and noncanonical signaling are observed in M1g cells. When combined with PDGF, Shh increases LF chemokinesis, although they also have different effects on migration, because PDGF-A increased the speed of migration, whereas Shh increased persistence. In vitro, migrating LF preferentially orient their cilia in the direction of forward cellular movement. During secondary septal elongation, the progressive enrichment of PDGFRα-expressing (GFP+) LF at the septal tip is accompanied by an enrichment of cilia in PDGFRα-expressing LF, compared with other alveolar cells.

Sca1 is widely acknowledged as a marker of stem and progenitor cells. Sca1+ cardiac mesenchymal cells are oligopotent and can differentiate into endothelial cells or myoblasts, reduce infarct size, and increase blood vessels and myocytes in the infarcted area (37, 44). Mesenchymal Sca1+ cells are more abundant in the lung than in most other adult organs (14, 31). In the lung, Sca1+ mesenchymal cells display markers of adipocyte (oil red O) or myofibroblastic (α-SMA-containing) lineage(s), and they are more proliferative than their Sca1− counterparts ex vivo (31). The function(s) of Sca1+ LF remain(s) incompletely defined. Our findings, like those of McQualter and coworkers, indicate that the Sca1+ population contains both PDGFRα-expressing and nonexpressing cells. Our data also show that during septal outgrowth (P4 through P12) PDGFRα-GFP+, Sca1+ LF progressively increase their contribution to the Sca1+ cellular pool. At P8, compared with Sca1− LF, more Sca1+ LF sustain proliferation and contain Shh, whereas fewer contain α-SMA. At P12, the Sca1+ LF comprise an even greater proportion of proliferating cells but also comprise a greater portion of the α-SMA+ LF. Therefore neonatal Sca1+ LF retain both their plasticity and proliferative capacity.

Finding that a larger fraction of Sca1+ than of Sca1− LF contained Ki67 was contrary to our expectation. Only a small minority of stem cells, which nearly uniformly express Sca1, proliferate at any one time, although they can proliferate rapidly ex vivo in a permissive culture environment (44). Oligopotent progenitors and unipotent precursor cells may also express Sca1, and their proliferation is less restricted in tissues and ex vivo. Sca1+ cardiac progenitors proliferate more rapidly than Sca1− cells when forming spherules and retain their proliferative advantage after myocardial implantation (44, 49). When isolated from the remaining lung after unilateral pneumonectomy, Sca1+,CD34+ cells were more proliferative than their Sca1−CD34− counterparts (23). In contrast to stem cells, the Sca1+ LF we have described are probably bipotent progenitors (yielding either myofibroblasts or lipofibroblasts) or precursors, capable of expanding into more of their like kind. In damaged skeletal muscle PDGFRα-expressing, CD34+, Sca1+ progenitor cells proliferate, migrate, and...
differentiate into fibroblasts and adipocytes and promote regeneration (1, 18).

We found that Sca1+ LF contain Shh during development, and others showed that Sca1+ LF reemerged during postpneumonectomy lung growth. Pneumonectomy increased the proportion of Sca1+,CD34+ cells in the residual lung and activated Shh signaling (23). In a reciprocal fashion, constitutive expression of Shh by airway epithelial cells increased Sca1+,CD34+ mesenchymal and endothelial cells (23). More recently, Chen and associates (6) demonstrated that pneumonectomy in mice that harbored a dominant negative fibroblast growth factor-10 mutation increased Shh gene expression in the remaining lung. These findings suggest that the increase in Sca1+, Shh+ LF during postpneumonectomy growth follows the developmental pattern that we observed during septal formation.

Fibroblasts from other organs also both express Shh and respond to its signaling. Sca1+ adventitial progenitor cells in the developing aorta express Shh, which is required for their maintenance and directs their migration into the arterial media where they produce α-SMA (34). As we have observed in lung fibroblasts, hepatic stellate cells also express the Shh gene and respond to smoothened agonists by increasing Rac-GTP (9).

We observed that Shh promotes directionally persistent migration of LF in a chemotactic gradient. Others showed that Shh increases directional migration of oligodendrocyte precursors along neurons during myelin sheath formation (33, 42). During limb development, muscle progenitor cells are directed by autonomous Shh signaling to migrate distally from the somites by increasing their directional persistence (16). Using both primary LF and Mlg cells, we found that Shh increases migrational persistence but not speed and uniformly orient the...
REGULATION OF LUNG FIBROBLAST DURING ALVEOLAR SEPTATION

primary cilium. On the basis of our findings, we hypothesize that Shh promotes uniform orientation of LF (particularly PDGFrα-expressing LF, which are more likely to display primary cilia) and organizes their migration toward the distal secondary septum.

It remains unclear why activating smoothened did not elicit canonical signaling in primary LF, whereas Mlg cells exhibited a 25-fold increase in Glil1 mRNA. We used both SAG and purmorphamine at a variety of concentrations and durations of exposure, so it is unlikely that the unresponsiveness resulted from suboptimal conditions for stimulation. Our findings suggest that Shh signals through the noncanonical pathway in primary LF, which, as others have shown, is sufficient to increase fibroblast migration (35). Smoothened is regulated by other mechanisms: 1) it is dependent on intraflagellar transport proteins for entry and translocation within the primary cilium; 2) it is phosphorylated and activated by PKA, which is dependent on adequate intracellular cAMP; 3) smoothened is ubiquitinated and degraded, a process that is regulated by a ubiquitin-specific protease USP8; and 4) smoothened activity is also influenced by phosphorylation of suppressor of fused (SuFu) by PKA or glycosyn synthase kinase-3β (17, 19, 48). In primary LF, negative regulation through one or more of these pathways could dampen signaling through the canonical pathway.

Using NIH3T3 fibroblasts, others have shown that PDGFrα localizes to the base of the primary cilium, thereby focusing downstream PDGFrα signaling through Akt at the centriole (39, 10). Fibroblasts without primary cilium migrate more slowly and fail to respond to a chemotactic gradient of PDGF-A (39). Very little is known about primary cilia in pulmonary alveoli.

Primary cilia are present on mouse fetal airway epithelial cells (39, 10). Fibroblasts without primary cilia migrate more slowly and fail to respond to a chemotactic gradient of PDGF-A (39). Very little is known about primary cilia in pulmonary alveoli.

REFERENCES


