Role of Secretoglobin 3A2 in Lung Development

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Rationale: Secretoglobin 3A2 (SCGB3A2) was originally identified as a downstream target in lung for the homeodomain transcription factor NKX2-1, whose null mutation resulted in severely hypoplastic lungs. A very low level of SCGB3A2 is expressed in lungs at Embryonic Day (E) 11.5 during mouse development, which markedly increases by E16.5, the time when lung undergoes dramatic morphologic changes, suggesting that SCGB3A2 may be involved in lung development in addition to a known role in lung inflammation.

Objectives: To determine whether SCGB3A2 plays a role in lung development.

Methods: To assess a potential role for SCGB3A2 during early lung development, wild-type and *Nkx2-1*-null fetal lungs of early developmental stages were subjected to *ex vivo* organ culture in the presence of SCGB3A2. *Nkx2-1*-null fetuses were exposed to SCGB3A2 during early organogenesis period through intravenous administration of this protein to *Nkx2-1*-heterozygous pregnant females carrying these null fetuses. Cultured lungs and fetal lungs were subjected to histologic and immunohistochemical analyses. To assess a role for SCGB3A2 in late lung development, SCGB3A2 was administered to pregnant wild-type females during mid- to late organogenesis stages, and the preterm pups and/or their lungs were evaluated for extent of maturity using breathing motion, gross morphology and histology of lungs, expression of gestational stage-specific genes, and phospholipid profiles.

Measurements and Main Results: SCGB3A2 significantly promoted both early and late stages of lung development.

Conclusions: SCGB3A2 is a novel growth factor in lung.

Keywords: secretoglobin 3A2; uteroglobin-related protein 1; NKX2-1; fetal lung development; growth factor

Lung arises by budding from the ventral foregut at approximately Embryonic Day (E) 9.5 in mouse gestation (1). Mouse lung development is classified as four stages; pseudoglandular (E9.5–16.5), canalicular (E16.5–17.5), terminal saccular (E17.5–perinatal day [P] 5), and alveolar (P5–30) (2, 3). This classification is a representative of the complexity of morphologic and functional changes that occur during lung development. It is known that this temporal and spatial lung development is controlled by various transcription factors and growth factors (2, 4–6). Among them, NK homeobox 1 (NKX2-1; also called TITF1, TTF1, or T/EBP) is expressed in lung, thyroid, and ventral forebrain

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Secretoglobin 3A2 (SCGB3A2) is predominantly expressed in the airways and functions as an antiinflammatory agent. Whether SCGB3A2 plays any physiologic role other than inflammation is not known.

What This Study Adds to the Field

SCGB3A2 is a novel growth factor, promoting both early and late stages of fetal lung development.

during early embryogenesis (7, 8), and plays a critical role in the genesis of these organs (8). NKX2-1 expression appears in the ventral wall of the anterior foregut, with the emergence of the lung primordium at E9.5 (9). NKX2-1 expression continues in the epithelial cells during lung development and throughout adulthood, at which time expression is confined to epithelial type II cells (10). In the *Nkx2-1*-null fetal lung, rudimentary bronchi with lean mesenchymal layers are formed, which do not develop beyond the stage of main bronchi (8–10). The downstream targets for NKX2-1 that cause this defect are not known.

Secretoglobin 3A2 (SCGB3A2), previously named as uteroglobin-related protein 1 (UGRP1), was originally identified as a downstream target of NKX2-1 through a suppressive subtractive library screening of mRNAs isolated from the lungs of *Nkx2-1*-null versus wild-type mouse fetuses (11). SCGB3A2 is a member of the SCGB gene superfamily composed of secretory proteins with small molecular weight (12). SCGB1A1, the prototypical protein of this superfamily, also called uteroglobin or Clara cell secretory protein, was proposed as a novel cytokine (13). On the basis of several lines of evidence, SCGB3A2 was proposed to play a role in lung inflammation (11, 14–18). In fact, we have reported that intranasal administration of recombinant adenovirus expressing SCGB3A2 suppresses the allergen-induced lung inflammation in a mouse model for allergic airway inflammation (19). The expression of SCGB3A2 in fetal mouse lung becomes detectable at E11.5, markedly increases by E16.5, and remains high throughout adulthood (11). The expression pattern suggests the possibility that SCGB3A2 may be involved in a role other than inflammation. However, the role of SCGB3A2 in the NKX2-1-mediated lung development still remains to be determined.

This study was initiated based on the hypothesis that SCGB3A2 plays a role in fetal lung development. The results demonstrate that SCGB3A2 is a novel growth factor accelerating lung development during both early and late developmental stages.

METHODS

SCGB3A2 Protein and Animal Studies

Two kinds of recombinant mouse SCGB3A2 protein were used in this study, which were obtained using bacterial expression plasmids pET32a–Trx (thioredoxin)–His (histidine)–SCGB3A2 (pET32a from Novagen, San

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Diego, CA) and pDest-544-His6-NusA (N utilization substance protein A)-TEV (tobacco etch virus)-SCGB3A2. In these plasmids, a mouse SCGB3A2 cDNA sequence excluding the region encoding the signal peptide (from +155 to +443) was used. After extensive purification, the Trx-His-tagged recombinant SCGB3A2 protein was used for all *ex vivo* and cell culture studies, whereas highly purified tag-free, endotoxin-free (endotoxin level, 0.2 EU/mg) SCGB3A2 derived from pDest-544-His6-NusA-TEV expression plasmid was used for all animal studies (detailed purification procedures available on request). Mice received an intravenous injection of up to a total of 200 μ g of SCGB3A2 via the tail vein (less than 5 EU/kg of the maximum endotoxin allowed). All animal studies were performed after approval by the National Cancer Institute (NCI) Animal Care and Use Committee. Breathing scores were determined by two independent investigators by observing for 2 minutes pups placed on moistened filter paper at 37°C immediately after removal from the mother. Scoring assignment was performed according to the criteria described by Ozdemir and colleagues (20).

Lung Analysis

For *ex vivo* organ culture studies, fetal lungs were cultured in Dulbecco's modified Eagle medium/F12 containing 10% fetal bovine serum on a 0.4- μ m pore membrane (Millipore Corp., Billerica, MA), which was placed on the top of steel wire mesh in an organ culture dish (Becton Dickinson, Franklin Lakes, NJ). The lungs were incubated with various additives as indicated, or transfected using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA) or electroporated with scrambled control or SCGB3A2-specific siRNA, and cultured for 4 days at 37°C in a 5% humidified CO₂ incubator. Electroporation was performed using a whole lung in Dulbecco's modified Eagle medium and a condition of 25 milliseconds, 50 V, in a 1-mm cuvette (21). The sequence of SCGB3A2 siRNA was as follows: sense 5'-r(CCCUGUUGUUGACAAAUA)d(T*T)-3' and antisense 5'-r(UAAUUUGUCAACAACAGG-2'OMeG)d(A*G)-3'. For primary culture studies, fetal lung epithelial and mesenchymal cells were isolated as described (22). Mesenchymal cells treated with 5-bromo-2'-deoxyuridine (BrdU) were subjected to fluorescence-activated cell sorter analysis (details are provided in the online supplement). Histologic analysis was performed as described in the online supplement.

DNA Microarray

DNA microarray was performed using RNAs isolated from 4-day organ-cultured *Nkx2-1*-null lungs with and without SCGB3A2 treatment. RNAs were amplified using a MessageAmp aRNA kit (Ambion, Austin, TX) before being reverse-transcribed to label with Cy3 and Cy5 (GE Healthcare Life Sciences, Piscataway, NJ) using a FairPlay microarray labeling kit (Stratagene, La Jolla, CA). Microarray analysis was performed using four mouse arrays (22.3K) obtained from the NCI Microarray Facility according to the instructions of the NCI (<http://nciarray.nci.nih.gov/>) and the manufacturer; lowness normalized, background subtracted VALUE data were obtained from log 2 of processed red signal/processed green signal with GeneSpringGX 7.3.1 software (Agilent Technologies, Palo Alto, CA). Gene ontology (GO) analysis (<http://www.geneontology.org>) (23) and pathway analysis were performed using mouse gene data (Mm-Std_20060628.gdb) and mouse pathway data (Mm_Contributed_20070917) from MAPPFinder (<http://www.genmapp.org>) (24). The MAPPFinder analysis was performed on the dataset using two criteria, either an increase (log ratio ≥ 1 , red) or decrease (log ratio ≤ -1 , green) in gene expression. Because log ratio was calculated based on Cy5/Cy3 and SCGB3A2⁺ and SCGB3A2⁻ samples were respectively labeled with Cy3 and Cy5, genes with a negative log ratio in green are up-regulated by SCGB3A2, whereas genes with a positive log ratio in red are down-regulated. All effective genes were submitted to the Gene Expression Omnibus (GEO; ID GSE11226; <http://www.ncbi.nlm.nih.gov/geo/>).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed by an SYBR Green master mixture and analyzed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primer sequences and PCR conditions are provided in the online supplement.

Statistical Analysis

The Student unpaired *t* test was used to analyze the difference between two groups. Values were regarded as significant at *P* < 0.05.

Liquid Chromatography-Mass Spectrometry (LC-MS)-based Metabolomic Analysis of Lipids in Amniotic Fluid

Lipids in amniotic fluid were extracted by a modified Folch method (25). Briefly, 40 μ l of combined amniotic fluid from fetuses of each pregnant mouse was mixed with 600 μ l of chloroform-methanol (3:1) and 110 μ l of water. After centrifugation, the lower organic phase was transferred to a new glass tube and dried by nitrogen flow. The prepared lipid fraction was reconstituted in a chloroform-methanol mixture and injected into an ultra-performance liquid chromatography-quadrupole time-of-flight (UPLC-QTOF) Premier LC-MS system (Waters, Millford, MA). An Acquity UPLC bridged ethylsiloxane/silica hybrid (BEH) C₈ column (Waters) was used to separate lipid species at 60°C. The flow rate of mobile phase (solvent A: 20 mM ammonium acetate, pH 5.5; solvent B: 90% acetonitrile and 10% acetone) was set as 0.5 ml/minute with a gradient ranging from 50 to 99% B over a 10-minute run. The mass spectrometer was operated in the positive electrospray ionization mode. Capillary voltage and cone voltage were maintained at 3 kV and 20 V, respectively. Source temperature and desolvation temperature were set at 120°C and 350°C, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (600 L/h), and argon as collision gas. Mass chromatograms and mass spectral data were acquired by MassLynx software in centroided format, and then deconvoluted by MarkerLynx software (Waters) to generate a multivariate data matrix. The intensity of each ion was calculated as the percentage of total ion counts in the whole chromatogram. The data matrix was further exported into SIMCA-P+ software (Umetrics, Kinnelon, NJ), and transformed by mean-centering and Pareto scaling, a technique that increases the importance of low-abundance ions without significant amplification of noise. Principal components were generated by multivariate data analysis to represent the major latent variables in the data matrix and were described in a scores scatter plot.

RESULTS

Effect of SCGB3A2 on Fetal Lung Development

SCGB3A2 expression was detected by immunohistochemistry (IHC) in the epithelial cells of E11.5 and E13.5 normal fetal lungs; in the latter, the expression was particularly evident around the growing tips of bronchi (Figure 1A). To determine a possible role for SCGB3A2 in lung development, lungs from E11.5–E12.0 fetuses were subjected to *ex vivo* organ culture with and without purified recombinant SCGB3A2 protein. E11.5–E12.0 fetal lungs are at early stages of branching (see Figure 1B) and thus are suited for studying lung development in *ex vivo* organ culture (6). The recombinant SCGB3A2 contained Trx and His tags as a fusion protein at the N-terminus of SCGB3A2. This recombinant fusion protein was used for *ex vivo* and cell culture studies, whereas for *in vivo* studies highly purified, tag-free, and endotoxin-free SCGB3A2 was used.

After 4 days of culture, the branching of normal fetal lung harvested at E11.5 was facilitated by the addition of SCGB3A2, with approximately three additional branching events in comparison to control (Figures 1B and 1C). The addition of 2% SCGB3A2-specific antiserum in the culture media together with SCGB3A2 protein counteracted the effect of SCGB3A2. Preimmune serum did not have a significant influence on lung branching morphogenesis, whereas anti-SCGB3A2 antiserum delayed lung branching morphogenesis about 0.5 times and was statistically significant as compared with control. In general, branching proceeds dichotomously and each branching point where the conducting airways meet can be serially numbered from the upper airways along the bronchial tree, thus demonstrating the degree of branching. Note that trifurcation branching events rather than bifurcation were sometimes observed in SCGB3A2-treated lungs (Figure 1B;

marked by an *arrows*). Branching of SCGB3A2-treated lungs proceeded 8.3 ± 1.2 times, which was significantly greater than control lungs (5.6 ± 0.55 times), lungs treated with fractions derived from the vector only that was subjected to the same expression and His-tag affinity purification steps as SCGB3A2 (5.3 ± 0.58 times), lungs treated with anti-SCGB3A2 antibody (4.9 ± 0.25 times), and lungs treated with SCGB3A2 and anti-SCGB3A2 antibody together (5.8 ± 0.84 times) (Figure 1C, *left*). To exclude the possibility that the effect of SCGB3A2 observed on branching morphogenesis was due to a small amount of bacteria-derived molecules in the Trx/His-tagged SCGB3A2 preparation, highly purified, tag-free, endotoxin-free SCGB3A2 and conditioned media from mammalian cultures expressing GFP-SCGB3A2 were used in *ex vivo* experiments (data not shown, and *see* Figures E1A and E1B). In both cases, identical results were obtained. Furthermore, when lungs were treated with SCGB3A2 siRNA, they exhibited approximately 1.5 times more delayed branching morphogenesis as compared with control siRNA (5.0 ± 0.71 vs. 6.5 ± 0.84 , respectively) (Figure 1C, *right*). Collectively, these results demonstrate that the promoted branching morphogenesis is due to SCGB3A2.

Because SCGB3A2 is a downstream target for NKX2-1, we next examined whether SCGB3A2 has any effect on the morphology of *Nkx2-1*-null fetal lungs. After culturing for 4 days *ex vivo*, *Nkx2-1*-null lungs harvested at E16.5 displayed distended morphology, consisting of one layer each of epithelia and mesenchyme (Figure 1D, *ex vivo*, and *see* Figure E1C). Upon the addition of SCGB3A2, these lungs underwent drastic morphologic changes, including pleated and/or dentate epithelia and ductlike structures appearing with increased layers of mesenchyme. Furthermore, when SCGB3A2 was administered to *Nkx2-1*-heterozygous females carrying null fetuses, stratified columnar cells with cilia, phenotypes normally found in wild-type mice, appeared throughout the epithelia of the lungs of *Nkx2-1*-null fetuses, which otherwise were composed of one to two layers of columnar epithelial cells covered with flattened epithelial and few ciliated cells (Figure 1D, *in vivo*). These data demonstrate that SCGB3A2 promotes fetal lung development.

Localization of an SCGB3A2-specific Receptor-like Molecule in Fetal Lung

To determine whether a receptor or a receptor-like molecule specific to SCGB3A2 is present in fetal lung, and where it might be localized, wild-type fetal lung primary epithelial and mesenchymal cells were treated with Trx/His tag-containing SCGB3A2, and were then subjected to immunocytochemistry (ICC) using anti-His and anti-Trx antibodies (Figure 2). With these antibodies, strong signals were detected after the addition of SCGB3A2, only on the surface of mesenchymal, but not epithelial, cells. The addition of fractions containing only the Trx/His tag without SCGB3A2 did not produce any positive signals (data not shown). Because MARCO, a macrophage scavenger receptor with collagenous structure, was previously demonstrated as a receptor for SCGB3A2 (15), ICC was also performed for MARCO. MARCO is expressed in lung alveolar macrophages and is involved in pulmonary inflammation (15). Unlike anti-His and anti-Trx antibodies, anti-MARCO antibody did not produce any positive immunoreactivity in either epithelial or mesenchymal cells of mouse fetal lung primary cultures. *In situ* hybridization and IHC further confirmed that there was no expression of MARCO in E13.5 fetal lungs, whereas the expression was found in alveolar macrophages of adult mouse lung by IHC as expected (data not shown). These data suggest that an SCGB3A2-specific receptor or a receptor-like molecule, distinct from MARCO, may exist on the mesenchymal cells of fetal lung.

SCGB3A2 Induces Cell Proliferation

To examine whether SCGB3A2 induces cell proliferation, the expression of phosphorylated histone H3 as a mitosis marker and Ki-67 as a proliferation marker, was examined by IHC using *Nkx2-1*-null mice with and without SCGB3A2. In *ex vivo* cultured *Nkx2-1*-null lungs and lungs of *Nkx2-1*-null mice, phosphorylated histone H3 and Ki-67 were marginally expressed in epithelial and mesenchymal cells without SCGB3A2 (Figures 3A and 3B). Upon administration of SCGB3A2, the expression of these markers was markedly enhanced in both epithelial and mesenchymal cells, with statistical significance as determined by positive cell numbers (Figure 3B). The effect of SCGB3A2 on wild-type fetal lungs was next examined by pulse labeling cultured lungs with BrdU (Figure 3C). SCGB3A2-treated lungs produced approximately 15% higher BrdU incorporation with statistical significance as compared with lungs not treated with SCGB3A2. The effect of SCGB3A2 on cell proliferation was further determined by BrdU incorporation into primary mesenchymal cells prepared from wild-type mouse fetal lungs, assuming that an SCGB3A2 receptor-like molecule may be present on mesenchymal cells (Figure 3D). These results revealed a statistically significant increase in BrdU incorporation upon SCGB3A2 treatment, indicating that SCGB3A2 enhances fetal lung cell proliferation.

Microarray Analysis of SCGB3A2-treated *Nkx2-1*-null Lungs

To gain insight into genes potentially controlled by SCGB3A2 that might be responsible for lung development, microarray analysis was performed using *Nkx2-1*-null lungs that were cultured for 4 days with and without SCGB3A2. We chose lungs of *Nkx2-1*-null fetuses instead of wild-type for this analysis because it is likely that genes that are involved in lung development and directly downstream of SCGB3A2 may be better represented in *Nkx2-1*-null lungs. In the presence of SCGB3A2, 32 and 60 genes were up- and down-regulated more than twofold, respectively (*see* Tables E1 and E2). GO term analysis revealed that many down-regulated genes are intracellular and/or membrane bound, or cytoplasmic by cellular component terms, and are involved in metabolic processes by biological process terms (Table 1). In contrast, none of the up-regulated genes were specifically categorized in terms of cellular component, whereas with biological process and molecular function genes, iron ion transport/homeostasis, and ferric iron binding, respectively, were identified as a significantly altered biological process, although the total frequency was very low (total frequency of 0.2 and 0.1%, respectively). Furthermore, MAPPFinder pathway analysis revealed several pathways, in which many genes are simultaneously up- or down-regulated by SCGB3A2. These include cytoplasmic ribosomal proteins, IL-1 and IL-9 signaling pathways, oxidative stress, mRNA processing, proteasome degradation, tricarboxylic (TCA) cycle, translation factor, and cell cycle (Figures E2A–E2J).

SCGB3A2 Promotes Lung Development *In Vivo*

We observed that SCGB3A2 has a role during early lung development. Because SCGB3A2 expression markedly increases after E16.5 (11), it may also have a role in later stages of lung development. To address this question, the effect of SCGB3A2 on the late gestational stages of fetal lung development was studied *in vivo*. First, to demonstrate that SCGB3A2 crosses the placenta, Flag-tagged, highly purified SCGB3A2 was injected into the tail vein of a mother of E15.5 fetuses, and a whole fetal body was collected 30 minutes later. Tissue extracts from six fetuses were combined and subjected to immunoprecipitation with anti-Flag antibody, followed by ELISA using SCGB3A2-

Figure 1. Morphologic changes of mouse fetal lungs by secretoglobin 3A2 (SCGB3A2). (A) Immunohistochemistry for SCGB3A2 in Embryonic Day (E) 11.5 and E13.5 wild-type fetal lungs. Arrows indicate representative positive signals. (B) Ex vivo organ culture of wild-type fetal lung harvested at E11.5 before culture (Day 0), after 4 days of culture in control media (Day 4 Cont), in the presence of 250 ng/ml SCGB3A2 (+SCGB3A2), 250 ng/ml SCGB3A2 and 2% SCGB3A2-specific antiserum (+SCGB3A2, Anti-SCGB3A2), 2% preimmune serum (+Preimmune serum), and 2% SCGB3A2-specific antiserum (+Anti-SCGB3A2). Fetal lungs were also transfected or electroporated with control (+Cont siRNA) and SCGB3A2-specific siRNA (+SCGB3A2 siRNA). In both methods, the same results were obtained. Cultured lung in each condition is representative of experiments repeated 3 to 10 times, each performed using more than three lungs per condition. Trifurcation found in SCGB3A2-treated lungs is shown by arrows. (C) Degree of branching was counted for fetal lungs harvested at E11.5 and cultured for 4 days: (left) in control media (Cont, n = 5), in the presence of fractions that were obtained using a Trx/His tag-containing expression plasmid without SCGB3A2 insert (His, n = 3), anti-SCGB3A2 antibody

(Ab, n = 4), Trx/His-tagged purified SCGB3A2 (SCGB, n = 6), and Trx/His-tagged purified SCGB3A2 and anti-SCGB3A2 antibody together (SCGB + Ab, n = 5); and (right) with control siRNA (Cont, n = 6) and SCGB3A2-specific siRNA (SCGB, n = 5). The results are the mean $\pm$ SD. NS = not significant. * P < 0.05, *** P < 0.005. (D) Effect of SCGB3A2 on *Nkx2-1*-null fetal lungs. For ex vivo studies, *Nkx2-1*-null lungs harvested at E16.5 (GI [gross image]) were cultured with and without SCGB3A2 for 4 days and were subjected to hematoxylin-and-eosin (H&E) staining. For in vivo studies, pregnant mothers carrying *Nkx2-1*-null fetuses were injected with 10 μ g SCGB3A2 daily for a week before fetuses were collected at E16.5. Insets in the in vivo *Nkx2-1*-null lungs show ciliated cells that are infrequently seen. Representative ciliated cells are shown by arrows.

Figure 2. Immunocytochemistry (ICC) for the presence of a secretoglobin 3A2 (SCGB3A2) receptor-like molecule. Primary fetal lung epithelial and mesenchymal cells were treated with (10 μ g/ml) and without SCGB3A2, and were subjected to ICC for MARCO, antihistidine and antithioredoxin antibodies. Positive signals (green fluorescence) are seen only on the surface of SCGB3A2-treated mesenchymal cells.

Figure 3. Secretoglobin 3A2 (SCGB3A2) induces proliferation. (A) Phosphorylated histone H3 and Ki-67 immunostaining of *ex vivo* cultured or *in vivo* lungs of *Nkx2-1*-null fetuses with and without SCGB3A2. *Insets* in *ex vivo* studies show positive cells that are rarely seen. *Insets* in *in vivo* studies are magnified images of the small squared areas. *Black* and *red* arrows show representative positive signals in epithelia and mesenchyme, respectively. (B) Numbers of immunopositive cells for phosphorylated histone H3 and Ki-67 are counted in epithelial (Epi) and mesenchymal (Mes) cells separately or as a whole (E+M) from *ex vivo* cultured and *in vivo* lungs of *Nkx2-1*-null fetuses with and without SCGB3A2, and are expressed in a bar graph as the mean $\pm$ SD. Counting was conducted by two blinded independent observers using five areas, each randomly chosen, from three *in vivo* tissues and five *ex vivo* tissues. The number obtained was expressed as a percentage of the total cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$. (C) Wild-type fetal lungs were *ex vivo* organ cultured in the absence (Cont) and presence (SCGB3A2) of SCGB3A2 for 3 days and were pulse labeled with 5-bromo-2'-deoxyuridine (BrdU) for 15 minutes before harvest. Sections were stained for BrdU (*upper panels*), and positive cells were counted and plotted (*lower panel*). Counting was conducted as described above using four lungs in each group. The number obtained was expressed as a percentage of BrdU incorporated cells. * $P < 0.05$. (D) Primary fetal lung mesenchymal cells treated with and without SCGB3A2 (10 μ g/ml) for 48 hours were labeled with 10 μ M BrdU for 1 hour and were subjected to fluorescence-activated cell sorter analysis. The distribution of BrdU incorporation versus DNA content is shown as a percentage of the total population exhibiting the fluorescence signal of interest in a squared area denoted gate 5 (g5). Values are the mean $\pm$ SD ($P < 0.05$) from three separate experiments. For each sample, 10,000 cells were analyzed.

specific antibody. Tissue extracts from fetuses of Flag-tagged SCGB3A2-injected mice had more than 30-fold higher SCGB3A2 levels (~ 800 ng/ml) as compared with fetuses from phosphate-buffered saline (PBS)-injected mice (~ 25 ng/ml), suggesting that SCGB3A2 most likely crosses the placenta. Next, highly purified, tag-free, endotoxin-free SCGB3A2 was injected through the tail vein daily to pregnant female mice from E13.5 through E16.5,

followed by removal of pups from the mother at E17.5, which otherwise would be born at E19.0–E20.0.

Pups removed at E17.5 from mothers receiving a total of 100 and 200 μ g SCGB3A2 displayed similar body length and body and lung weights compared with those of PBS-treated E19.0 pups, which were statistically significantly larger than PBS-treated E17.5 pups (Figure 4A). Breathing scores (20) were also

TABLE 1. OVERREPRESENTED GENE ONTOLOGY TERMS FOUND IN DIFFERENTIALLY EXPRESSED GENES REGULATED BY SECRETOGLOBIN 3A2

Gene Ontology Term	Cluster Frequency (%)	Total Frequency (%)	Corrected P Value
Cellular component			
Down-regulated			
Intracellular part	55.90	48.90	0.00714
Intracellular membrane-bound organelle	44.90	38.60	0.02934
Membrane-bound organelle	44.90	38.60	0.03296
Cytoplasm	34.90	25.70	3.85×10^{-7}
Cytoplasmic part	28.10	19.00	1.22×10^{-8}
Mitochondrion	11.20	5.90	6.00×10^{-7}
Organelle membrane	7.50	4.40	0.0113
Organelle envelope	5.80	2.90	0.00139
Envelope	5.80	2.90	0.00139
Mitochondrial part	5.50	2.50	0.00018
Mitochondrial envelope	4.30	2.10	0.01709
Mitochondrial membrane	4.10	2.00	0.02622
Organelle inner membrane	4.10	1.90	0.00753
Mitochondrial inner membrane	3.90	1.80	0.00774
Biological process			
Up-regulated			
Iron ion transport	2.30	0.20	0.00765
Iron ion homeostasis	1.90	0.20	0.02728
Down-regulated			
Protein metabolic process	25.40	19.50	0.02145
Cellular macromolecule metabolic process	25.20	19.10	0.01035
Cellular protein metabolic process	24.50	18.90	0.03209
Molecular function			
Up-regulated			
Ferric iron binding	1.90	0.10	0.00028

higher and were statistically significant in SCGB3A2 100 and 200 μg -treated E17.5 and PBS-treated E19.0 pups as compared with PBS-treated E17.5 control animals in the following order: SCGB3A2 100- μg -treated E17.5 < SCGB3A2 200- μg -treated E17.5 < PBS-treated E19.0 pups. More than one-half of SCGB3A2 200 μg -treated E17.5 pups had breathing scores of 2 or 3, the number that PBS-treated E19.0 control pups exhibited. In agreement with the breathing scores, SCGB3A2 200- μg -treated E17.5 lungs appeared to be well air-inflated, which is similar to PBS-treated Day 0 lungs, as compared with PBS-treated E17.5 lungs (Figure 4B). Furthermore, histologic examination revealed that red blood cells were found inside immature alveolar walls of PBS-treated E17.5 lungs, an observation normally obtained with this gestational age of mouse fetal lungs (Figure 4C). In contrast, in SCGB3A2-treated lungs, red blood cells were already in contact with airways, indicative of the lung's ability to exchange air, a phenotype typically found in E19.0 normal fetal lungs. When a percentage of airspace was compared among four groups of lungs, SCGB3A2-treated lungs and PBS Day 0 lungs had statistically significantly larger airspace than lungs of PBS-treated E17.5 control pups. The airspace was in the order of SCGB3A2 100- μg -treated E17.5 < SCGB3A2 200- μg -treated E17.5 < PBS-treated Day 0, which was in inverse relation to breathing scores (Figure 4D).

Next, the expression of several genes known to have markedly increased expression toward the end of gestation was examined by quantitative RT-PCR (Figure 4E). Expression of surfactant protein (SP)-A and SP-D (26), aquaporin 1 (27), and leptin receptor (28, 29) genes were all significantly enhanced in E17.5 lungs upon SCGB3A2 treatment, regardless of the amount, as compared with PBS, and the levels were similar to E19.0 control pups. Finally, using a novel LC-MS-based metabolomic analysis,

amniotic fluid lipidomes of the SCGB3A2 100- and 200- μg -treated mice were compared with those of immature E17.5 and mature E19.0 control animals (Figure 4F). Examining the lipid composition of amniotic lipids has been widely used to predict fetal lung maturity (30). A two-component model from a partial least squares discriminant analysis showed that the lipid species in the amniotic fluid of mice treated with 200 μg SCGB3A2 were similar to that of mature control mice and significantly different from immature control animals. Interestingly, the amniotic fluid lipidome of mice treated with 100 μg SCGB3A2 was different from both immature E17.5 and mature E19.5 control animals, suggesting that the lipid components of amniotic fluid evolve after fetal development and maturation.

DISCUSSION

This study demonstrates that SCGB3A2 promotes the development of fetal lungs. This is the first report describing SCGB3A2 as a growth factor, in addition to its antiinflammatory activity as recently demonstrated using a mouse model for allergic airway inflammation (19). The growth factor activity of SCGB3A2 correlates with the expression of SCGB3A2 that was found at E11.5 and greatly increased by E16.5, during the period when the lung undergoes dramatic morphologic changes (2, 3). An SCGB3A2-specific receptor-like molecule or a molecule that SCGB3A2 can bind to may be present on the surface of mesenchymal cells, suggesting a possible role for this molecule in the SCGB3A2 signaling.

Upon SCGB3A2 treatment, *Nkx2-1*-null lung *ex vivo* cultures exhibited dramatic morphologic changes, such as appearance of pleated/dentate epithelial layers with increased mesenchyme, indicating that SCGB3A2 induced proliferation and invagination, characteristic processes for branching (3, 31). In contrast, no invagination was observed *in vivo*. Because of this incomplete rescue of lung phenotypes *in vivo*, SCGB3A2-treated, *Nkx2-1*-null fetuses cannot survive beyond birth as originally described for *Nkx2-1*-null mice (8). The absence of invagination might be due to the low concentrations of SCGB3A2 protein that can ultimately reach the lung and/or due to space constraints in the thorax. Alternatively, other factors that can be supplied from serum in *ex vivo* cultures are required to cooperate with SCGB3A2 to correct many downstream target genes for NKX2-1 that are missing in the *Nkx2-1*-null lungs. Note that known direct NKX2-1 target genes, such as SP-A (32), SP-B (33), SP-C (34), and Clara cell secretory protein (also called SCGB1A1) (35) are critical for lung function, but are not required for lung development *per se* as judged by their respective knockout mouse studies (36–40). Because an SCGB3A2-specific receptor-like molecule might be present on mesenchymal cells, SCGB3A2 may first promote cell proliferation in mesenchymal cells directly or indirectly, which induces other factors, which in turn induce epithelial cell proliferation through the epithelial-mesenchymal interaction (3, 6, 22, 41). Lung development is a complicated process with a series of temporal and spatial changes in morphology and gene expression, regulated by various transcription factors and growth factors (2–6). The NKX2-1–SCGB3A2 pathway may be only a fraction of a myriad of pathways downstream of NKX2-1 that cooperate to allow lungs to properly develop. It is important to note that caludin-18, a tight junction protein, was also identified as a downstream target for NKX2-1 by a suppressive subtractive hybridization between *Nkx2-1*-null versus wild-type mouse lungs (42). Studies on the NKX2-1–SCGB3A2 signaling pathway, including identification of an SCGB3A2-specific receptor-like molecule, are in progress.

In *ex vivo* organ culture studies, addition of SCGB3A2 promoted approximately three rounds of additional branching

Figure 4. Lung maturity assessment. (A) Embryonic Day (E) 17.5 and E19.5 pups were removed from mothers who had received phosphate-buffered saline (PBS), or 100 or 200 μ g secretoglobin 3A2 (SCGB3A2), and were subjected to breathing score assessment, body length, and body and lung weight measurements (see movie data for breathing score assessment in the online supplement). The number of cohorts is as follows: 30 pups from four mothers for PBS-treated E17.5; 32 pups from four mothers for 100- μ g SCGB3A2-treated E17.5; 37 pups from five mothers for 200- μ g SCGB3A2-treated E17.5; and 7 pups from one mother for PBS-treated E19.0. The results are the mean $\pm$ SD. * P < 0.05, ** P < 0.01. NS = not significant. (B) Gross image of lungs from PBS- and 200- μ g SCGB3A2-treated E17.5, and PBS-treated Day 0 pups. (C) Histology of lungs from PBS, 100- and 200- μ g SCGB3A2-treated E17.5 and PBS-treated E19.0 pups. (D) Percentage of airspace was determined from histologic samples of lungs from PBS, 100- and 200- μ g SCGB3A2-treated E17.5 and PBS-treated Day 0 pups. Measurements were performed on

four to five sections randomly chosen from three to five lungs in each group. Slides were viewed by using a $\times 20$ objective and were transferred to a computer screen. A computer-generated, 121-point lattice grid was superimposed on each field, and the number of intersections (points) falling over respiratory parenchyma or airspace was counted. Points falling over bronchioles, large vessels, and smaller arterioles and venules were excluded from the study. (E) qRT-PCR analysis for the expression of surfactant protein (SP)-A, SP-D, aquaporin 1 (AQP1), leptin receptor (OB-R) genes expressed in lungs from PBS- ($n = 9$), 100- μ g ($n = 14$) and 200- μ g ($n = 16$) SCGB3A2-treated E17.5, and PBS-treated E19.0 ($n = 4$) pups. The results are shown as the means $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.005. NS = not significant. (F) Scores scatter plot of the partial least squares discriminant analysis (PLS-DA) model on the lipidomes of amniotic fluid samples from the PBS vehicle-treated control (both E17.5 and E19.5) and the SCGB3A2-treated mice (E17.5 from both 100 and 200 μ g). Each principal component summarizes an independent latent variable detected by PLS-DA. The $t[1]$ and $t[2]$ values represent the scores of each sample in principal components 1 and 2, respectively. Fitness (R^2 value) of the model to the acquired dataset is 0.582, and the prediction power (Q^2 value) of the model is 0.375. The model was validated through the recalculation of R^2 and Q^2 values after the permutation of sample identities. Note that each point represents the average of combined amniotic fluids from seven to nine fetuses from a mother, due to volume availability. The dark gray arrow indicates a direction from immaturity to maturity. Thin gray arrows suggest a possible intermediate lipid profile between immaturity and maturity.

as compared with control, which was completely suppressed by the simultaneous addition of anti-SCGB3A2 antibody. It seems that SCGB3A2-specific antibody binds to SCGB3A2 in a way that interferes with SCGB3A2 binding to a receptor-like molecule, thus blocking downstream activities. Furthermore, branching was delayed approximately 0.5 times by the addition of anti-SCGB3A2 antibody in comparison to control, although no statistical differences were found when compared with those treated with His tag-containing fractions or those of SCGB3A2 and antibody together. These results nevertheless suggest that the endogenously produced SCGB3A2 secreted into the culture media (11) may partially be responsible for branching morphogenesis. When lungs were treated with SCGB3A2-specific siRNA, branching morphogenesis was delayed 1.5 times as compared with control siRNA. This may be due to the fact that

siRNA would take 1 to 2 days to turn off expression of SCGB3A2, which leads to the decrease of SCGB3A2 levels in the media, which in turn results in delays in branching morphogenesis. By then, the lung has already gone through some degree of branching, which makes the effect of siRNA on branching morphogenesis less effective. Alternatively, SCGB3A2 may act in a paracrine manner. Thus, if delayed branching was only due to the action of SCGB3A2 present in media, one would expect that the effect of anti-SCGB3A2 antibody when added to the media by itself could be larger than that observed. The precise mechanism as to how SCGB3A2 exerts its growth factor activity requires further studies.

GO term analysis of microarray data revealed that SCGB3A2 mainly affects genes whose protein products are located in the intracellular and/or membrane-bound, or cytoplasmic, compartments

Figure 4. (continued).

and are responsible for cellular protein/macromolecule metabolic process. In fact, many genes listed in Tables E1 and E2 can be categorized in these GO terms. The fact that most changes were found in intracellular and/or membrane-bound, or cytoplasmic, compartments supports our hypothesis that the SCGB3A2 signal goes through a SCGB3A2-specific receptor-like molecule, leading to a myriad of changes in molecules in the intracellular and/or cytoplasmic compartments. It is also interesting that the metabolic process was the most affected biological process (~19% down-regulation) by SCGB3A2 and none of the other GO terms, such as cell cycle, cell growth, cell differentiation, and transcription, were significantly altered. It is noteworthy that SCGB3A1, a homolog of SCGB3A2 (43), was among the genes that were down-regulated by SCGB3A2 (see Table E2). Furthermore, most of the pathways revealed by the MAPPFinder pathway analysis were those of intracellular and cytoplasmic compartments, which are in good agreement with the GO terms. Of interest was that several genes associated with the cell cycle were also found to be simultaneously up- or down-regulated by SCGB3A2. How SCGB3A2 regulates the expression of genes involved in these pathways, and in particular, how SCGB3A2's down-regulation of the metabolic process relates to cell proliferation remains to be understood.

The most impressive finding in this study is that administration of SCGB3A2 to a pregnant female mouse promoted lung development of preterm pups, which exhibited equivalent lung phenotypes to those of matured term pups as judged by breathing scores, morphologic and histologic observation, expression of several genes known to have increased levels toward the end of gestation, and lipid profiles. No obvious abnormality was noted with any other organs/tissues of preterm pups or mothers treated with SCGB3A2. However, body length and weight were slightly increased in SCGB3A2-treated pups, in accordance with the increase of lung weight, suggesting that SCGB3A2 might be involved in general pathways of growth

promotion. Alternatively, there might be a factor or factors produced that set the size of the body to accommodate the developing lung. Because SCGB3A2 directly affects lung development in *ex vivo* lung organ culture studies, we believe that the *in vivo* effect of SCGB3A2 on late gestational stages of lung development is a direct effect of SCGB3A2 on fetal lungs after crossing into the placental circulation. However, the possibility cannot be excluded that SCGB3A2 indirectly affects fetal lung development such that SCGB3A2 may activate a receptor located in other organs/cells in the mother, placenta, or fetus, including the fetal central nervous system, which influences the expression of hormones or factors that in turn affect fetal lung development. However, the organ culture studies would suggest a direct effect of SCGB3A2 on the lung.

A novel metabolomic approach, which has been adopted in other lipid-related research fields (44), was used to examine and compare the lipid profiles of amniotic fluid from control and SCGB3A2-treated samples. Distinctive grouping of immature and mature amniotic fluid samples as well as the samples from SCGB3A2 treatments demonstrated the phenotyping capability of metabolomics on the fetal lung development through analyzing the lipid species in the amniotic fluid, and further suggests that SCGB3A2 treatment can lead to the generation of a favorable lipid profile for lung maturation. Further structural identification of the phospholipids contributing to the sample groupings will provide more insight into lung development, especially the role of individual phospholipid surfactants.

In conclusion, the present study demonstrates that SCGB3A2 is a novel growth factor that promotes development of early and late gestational stages of lung. This study may provide a new direction in research on development and function of the lung.

Conflict of Interest Statement: R.K. is a coapplicant of the U.S. Provisional Patent Application No. 60/880134, filed on January 12, 2007, titled "SCGB3A2 as a growth factor and anti-apoptotic agent," which is generally related to methods of using the secretory protein SCGB3A2 for promoting lung development and

treating lung disease. This patent application is related to U.S. Patent Application No. 60/847747, filed on September 27, 2006. T.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Q.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.A.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K. is a coapplicant of the U.S. Provisional Patent Application No. 60/880134, filed on January 12, 2007, titled "SCGB3A2 as a growth factor and anti-apoptotic agent," which is generally related to methods of using the secretory protein SCGB3A2 for promoting lung development and treating lung disease. This patent application is related to U.S. Patent Application No. 60/847747, filed on September 27, 2006.

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