A Subset of Epithelial Cells with CCSP Promoter Activity Participates in Alveolar Development

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Alveolar formation is hallmarked by the transition of distal lung saccules into gas exchange units through the emergence of secondary crests and an exponential increase in surface area. Several cell types are involved in this complex process, including families of epithelial cells that differentiate into alveolar type I and II cells. Subsets of cells expressing Clara cell secretory protein (CCSP) have been identified in both lung and bone marrow compartments, and are described as a progenitor/stem cell pool involved in airway regeneration and alveolar homeostasis. Whether these cells also participate in alveolar formation during postnatal development remains unknown. Based on their regenerative capacity, we asked whether these cells participate in alveogenesis. We used a previously described transgenic mouse model (CCSP-*tk*) in which Ganciclovir exposure selectively depletes all cells with CCSP promoter activity through intracellular generation of a toxic metabolite of thymidine kinase. Our results showed that Ganciclovir treatment in newborn CC*tk* mice depleted this cell population in lung airways and bone marrow, and was associated with alveolar hypoplasia and respiratory failure. Hypoplastic lungs had fewer alveolar type I and II cells, with impaired secondary crest formation and decreased vascular endothelial growth factor expression in distal airways. These findings are consistent with a model in which a unique population of cells with CCSP promoter activity that expresses vascular endothelial growth factor participates in alveolar development.

Keywords: alveogenesis; variant Clara cells; bone marrow; alveolar hypoplasia; vascular endothelial growth factor

Alveolar formation (alveogenesis) is a complex developmental program that describes the transition of lung architecture from terminal saccules to functional gas exchange units within the distal lung. It begins with the formation of secondary crests or “septa” that grow perpendicularly from the saccular walls, and are comprised of a central core containing connective tissue surrounded by capillaries and epithelium. Extension of septa is accompanied by differentiation of cuboidal epithelial cells into surfactant-producing alveolar type II cells and a corresponding increase in surfactant secretion. Alveolar type II cells then undergo terminal differentiation to alveolar type I cells that are largely responsible for gas exchange (reviewed in Ref. 1). Disruptions to this process result in alveolar hypoplasia hallmarked by enlarged and simplified alveoli, and can lead to respiratory

CLINICAL RELEVANCE

This research presents novel findings that a subset of cells located in bone marrow and lung compartments are necessary for alveolar development. Depletion of these cells led to alveolar hypoplasia in mice through reduced expression of the paracrine factor, vascular endothelial growth factor. Further identification and isolation of these cells may lead to new strategies to treat or prevent diseases with alveolar hypoplasia, such as bronchopulmonary dysplasia.

compromise, as seen in the chronic lung disease of premature newborns, bronchopulmonary dysplasia (2, 3).

To date, although a considerable amount of literature describes the cell types involved in maintenance of proximal lung epithelium after lung inflammation and injury (4–9), limited data exist on cell types involved in alveolar homeostasis and alveolar regeneration (10, 11). The lung is divided into distinct compartments that comprise (1) proximal and distal airways, and (2) distal lung parenchyma, including alveoli. These regions are further subdivided to include niches known to harbor lung-specific resident stem/progenitor cells that can expand and locally repopulate damaged epithelium (12). These intrapulmonary niches include the submucosal glands of the trachea and main bronchi, bronchus, and main bronchioles, and the bronchoalveolar duct junction (BADJ). A recent report also proposes an extrapulmonary niche within the bone marrow that contains epithelium-like cells that might contribute to repair after lung epithelial injury (13). Although a few studies have attempted to identify lines of cells that may specifically participate in alveolar regeneration using lineage-tracing approaches, the information remains both limited and controversial (9, 10). The current dogma proposes that each individual intrapulmonary niche contains stem/progenitor cells responsible for maintenance of its own local epithelial cell populations. The question of whether specific cell subpopulations or their precursors residing in intra- or extrapulmonary niches retain the ability to regulate alveogenesis, and thereby cross currently accepted compartmental boundaries, remains unknown.

A subset of epithelial cells with Clara cell secretory protein (CCSP) promoter activity was recently described that may function as an airway stem/progenitor cell pool that causes alveolar dysfunction when selectively depleted (5, 11). The cells are termed variant Clara cells, and were identified using a transgenic mouse line (CCSP-herpes simplex thymidine kinase [CC*tk*]), in which the mouse CCSP promoter is fitted downstream with a cDNA cassette for herpes simplex virus thymidine kinase (HSV*tk*). Exposure of this mouse line to the antiviral agent, Ganciclovir (GCV), induces an intracellular reaction with HSV*tk* to form a toxic metabolite of thymidine kinase that results in selective depletion of all cells with an activated CCSP promoter. Previous experiments comparing this

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model to the naphthalene-induced Clara cell cytotoxicity model have shown that, unlike naphthalene exposure, GCV treatment of adult *CCtk* transgenic mice leads to depletion of all cells expressing *tk*, and causes alveolar dysfunction with an inability to regenerate damaged alveoli (11). The investigators concluded that the subpopulation of cells expressing the *tk* transgene serves a stem/progenitor function in maintaining airway integrity, and their depletion results in alveolar damage as a consequence of unresolved inflammation.

The purpose of the current study was to investigate whether selective depletion of the *tk*-expressing cell population in newborn animals would impair alveolar formation during postnatal development. Given the evidence that a subset of *tk*-expressing cells may possess stem/progenitor-like qualities, we asked whether these cells participate in alveogenesis. To answer this question, we treated transgenic *CCtk* mice and their wild-type littermates with GCV in the immediate newborn period before the start of alveolar development. We found that GCV treatment in *CCtk* mice induced alveolar hypoplasia by depleting *tk*-expressing cells in both lung and bone marrow compartments, and was associated with decreased expression of vascular endothelial growth factor (VEGF) in distal airways. This is the first report to implicate a potential role for cells with CCSP promoter activity in alveogenesis during lung development.

MATERIALS AND METHODS

Animals

Transgenic *CCtk* mice (a generous gift of Dr. Barry Stripp, Duke University) were maintained and bred as a hemizygous pathogen-free colony. See the online supplement for details on animal husbandry, genotyping, dissection, and bronchoalveolar lavage.

GCV or Naphthalene Treatment

On Postnatal Day 1, all littermates within a litter received either GCV (0.625 mg/g) or saline (25 μ l equivalent volume), or naphthalene (0.3 mg/g) or oil (25 μ l equivalent vehicle volume), via intraperitoneal injection. GCV- and saline-treated animals received a corresponding repeat dose on Postnatal Day 4 to ensure adequate delivery and exposure to injection agent.

Immunohistochemistry/Immunofluorescence

Slides were cleared in CitriSolv and rehydrated through a graded series of alcohols. Slides were stained using the Histomouse Streptavidin Peroxidase kit (Zymed Laboratories Inc., South San Francisco, CA) after antigen retrieval, as described by the manufacturer (Dako Inc., Carpinteria, CA). For immunofluorescence, slides underwent antigen retrieval followed by incubation with the primary antibodies overnight. All negative control slides showed no immunostain. See the online supplement for additional details and Hart's elastin (Eln) stain.

Lung Morphometric Analysis

Four random 5- μ m paraffin-embedded tissue sections were taken from three different transgenic and wild-type lungs at specified stages, and separately stained with hematoxylin and eosin or immunostained with anti-CCSP and anti-Nkx2.1 (TTF1) antibodies, as described previously here. The sections were photographed using a SPOT Insight QE camera (Sterling Heights, MI) and software and each image file was analyzed in a blinded manner using the NIH ImageJ 1.37v program (National Institutes of Health, Bethesda, MD) to quantify the number of cells stained positively for the specified antibody. See the online supplement for lung morphometry details.

mRNA Analysis

mRNA was extracted from lungs of *CCtk* transgenic mice and wild-type littermate control animals by snap freezing immediately after dissection and transfer to -80°C . This was followed by homogenization

using a hand-held power homogenizer, and by subsequent organic solvent extraction with TRIzol and chloroform (Invitrogen Corp., Carlsbad, CA), as described by the manufacturer. See the online supplement for RT-PCR and quantitative PCR details.

Bone Marrow Harvest and Cytospin Immunofluorescence Analysis

After animals were killed, dual external hemipelvectomy was performed to isolate the femur and tibia of both legs. After removal of muscle and connective tissue, the femur and tibia were severed at the apical points, and each bone individually flushed with 150 μ l of fresh, cold bone marrow cell buffer (0.5% BSA, 2 mM EDTA, 1 $\times$ PBS) and pooled into one 1.5-ml Eppendorf tube and immediately placed on ice. A total cell count was taken, and each tube (representing one animal) was split into eight tubes (to avoid cell overlap on slides), followed by resuspension in fresh bone marrow cell buffer, and cytospun onto glass slides. Slides containing cells were incubated in 4% paraformaldehyde for 1 hour at 4°C . Paraformaldehyde was then gently rinsed off with 1 $\times$ PBS, and immunofluorescence was performed as described previously here.

Statistical Analysis

Data were analyzed using the Microsoft Excel 2003 statistical package (Microsoft Corp., Redmond, WA). Two-group comparisons were evaluated using the unpaired Student's *t* test. Data were expressed as means ($\pm$ SEM) where appropriate.

RESULTS

GCV-Induced *tk* Activation Causes Alveolar Inflammation and Injury in Adult *CCtk* Lungs

The *CCtk* transgene contains a fragment of the mouse Clara cell secretory promoter upstream of an HSV1 *tk* gene fragment (Figure 1A). Exposure of adult *CCtk* mice to GCV generates a toxic metabolite of thymidine kinase that permanently depletes all cell populations with CCSP-promoter activity and causes lung inflammation with irreversible alveolar injury (5). Data characterizing the effects of GCV treatment on *CCtk* mice are currently limited to adult mice receiving chronic GCV exposure extending over 24 hours (7–11) to several days (5). To determine whether a single dose of GCV is capable of inducing similar alveolar effects, 8-week-old adult *CCtk* mice received 10 mg GCV (0.5 mg/g) intraperitoneally, and lungs were subsequently harvested at 10 days. Results show marked leukocytic infiltrate and alveolar edema in GCV-treated transgenic lungs compared with GCV-treated wild-type control tissue (Figures 1B and 1C). GCV exposure in a survival study uniformly led to animal mortality in adult *CCtk* mice between 10–14 days as a result of alveolar compromise, as previously described (11). These data demonstrate that a single dose of GCV in adult *CCtk* mice effectively induces alveolar disruption.

GCV-Induced *tk* Activation Causes Alveolar Hypoplasia in Newborn *CCtk* Lungs

With the observation that GCV treatment of adult *CCtk* mice disrupts alveolar integrity, we investigated whether GCV treatment of newborn *CCtk* mice would impair alveolar formation. Female *CCtk* mice were bred with strain-matched wild-type males (*CCtk* males are infertile), and pups were allowed to be delivered, with day of birth identified as Postnatal Day 0. *CCtk* mice are hemizygous, and transgene inheritance follows normal Mendelian ratios to generate approximately 50% transgenic and 50% wild-type progeny, allowing for incorporation of wild-type internal controls within each litter (Figure 1D). On Postnatal Day 1, newborn pups were injected with a single dose of GCV (0.625 mg/g) intraperitoneally, which was repeated on Postnatal Day 4 to ensure adequate exposure before animals

Figure 1. Ganciclovir (GCV)-induced *tk* activation causes alveolar inflammation and injury in adult Clara cell secretory protein (CCSP)-herpes simplex thymidine kinase (Ctck) mice. (A) Schematic diagram of CCSP-*tk* transgene showing a fragment of the mouse CCSP promoter upstream of a herpes simplex virus thymidine kinase cDNA fragment flanked by bovine growth hormone polyadenylation signal. Arrow, transcription start site; chevron arrows, positions of primers for genotyping. Hematoxylin and eosin (H&E)-stained lung sections comparing GCV-treated wild-type (WT) controls animals (B) versus GCV-treated CCtk animals (C) (magnification, 40 $\times$; scale bar, 100 μ M). (D) Genotyping via PCR identifying transgenic CCtk animals. (E) Schematic timeline of GCV (G) or naphthalene (N) treatment followed by lung harvest in newborn mice.

were killed on Postnatal Day 8 (Figure 1E). Control litters received corresponding saline injections on Days 1 and 4 of life. Analysis of five independent litters showed marked alveolar hypoplasia characterized by enlarged alveolar airspaces with thinned septae in GCV-treated transgenic lungs as compared with GCV-treated wild-type and saline-treated transgenic and wild-type controls (Figures 2A–2D). Alveolar hypoplasia was quantified by morphometric measurement, showing significantly lower radial alveolar counts, representing increased alveolar airspace size in GCV-treated transgenic lungs (Figure 2E). Alveolar hypoplasia was further pronounced relative to controls at Postnatal Day 16, with radial alveolar counts decreased by nearly 70% (Figures 2F–2H) (17). Importantly, a rigorous analysis of multiple serial histological sections from each GCV-treated transgenic lung at Postnatal Days 8 and 16 showed no evidence of active or resolving inflammation, and no interstitial thickening within the airways or alveoli. Furthermore, no weight loss was noted in either the CCtk or control animals. GCV treatment was uniformly lethal in all CCtk newborns, however, with maximum survival around 16 days. By the day of expiration, animals were noted to be in respiratory distress, with gasping and cyanosis reflecting respiratory insufficiency. These data demonstrate that GCV treatment of newborn CCtk mice permanently arrests alveolar development in the absence of a detectable inflammatory response.

GCV-Induced *tk* Activation Depletes CCSP-Positive Cells in Newborn CCtk Lungs

To verify that GCV treatment depletes CCSP-positive cells in newborn CCtk mice, we examined lung CCSP protein and mRNA levels. Immunohistochemical staining showed marked depletion of CCSP-positive cells in proximal and distal airways after GCV treatment in transgenic newborn animals (Figures

Figure 2. GCV-induced *tk* activation causes alveolar hypoplasia in newborn CCtk mice. H&E-stained lung sections comparing saline-treated WT control mice (A), GCV-treated WT control mice (B), saline-treated CCtk control mice (C), and GCV-treated CCtk transgenic mice (D) at 1 week after GCV treatment. Radial alveolar counts in CCtk mice compared with controls at 1 week (E) and 2 weeks (F) after GCV treatment. Representative H&E-stained lung sections in controls (G) versus CCtk mice (H) at 2 weeks after GCV treatment (magnification, 10 $\times$; scale bar, 100 μ m; **P* < 0.05). RAC, radial alveolar count.

3A and 3B). Similarly, quantitative real-time PCR showed significant decreases in total lung mRNA levels of CCSP and cytochrome p450 (Cyp2f2, a marker of secretory Clara cells) (Figures 3C and 3D) (18). These data validate that GCV depletes CCSP-positive cells in our newborn CCtk model.

Alveolar Hypoplasia Is Hallmarked by Fewer Alveolar Type I and II Cells

With the finding that GCV treatment arrests alveolar development in newborn CCtk mice, we investigated which alveolar cell type(s) were responsible for the altered phenotype. Immunohistochemical staining for Nkx2.1 (expressed in alveolar type II cells postnatally) showed lower alveolar type II cell numbers in transgenic mice compared with control animals (Figures 4A–4C), and quantitative real-time PCR similarly showed a corresponding decrease in mRNA levels of surfactant protein (SP)-C (alveolar type II-specific protein) (Figure 4D). Quantification of alveolar type I cells was estimated through immunofluorescence for T1 α (alveolar type I-specific protein), followed by

Figure 3. GCV-induced *tk* activation depletes CCSP-positive cells in newborn CCtk mice. Immunohistochemistry for CCSP showing depleted CCSP-positive cells after GCV treatment in WT control mice (A) versus GCV-treated CCtk mice (B) (magnification, 10 $\times$; scale bar, 100 μ m). Real-time quantitative PCR analysis comparing total lung mRNA after GCV treatment in CCtk mice compared with GCV-treated WT controls for CCSP mRNA expression (C) and CYP2F2 mRNA expression (D) (* P < 0.05).

binary image conversion and summation of pixel numbers corresponding to alveolar type I cell distribution (Figures 4E and 4F). Results show proportional decreases in SP-C-positive cells and T1 α distribution after GCV treatment in CCtk mice (Figures 4G and 4H). These data demonstrate that alveolar hypoplasia after GCV treatment of newborn CCtk animals is associated with fewer alveolar type I and II cells.

Alveolar Hypoplasia Is a Result of Impaired Secondary Crest Formation

To determine the mechanisms responsible for arrested alveolar formation, we examined several genes known to be involved in alveolar secondary crest formation. Relevant gene products include α -smooth muscle actin, *Eln*, platelet-derived growth factor-A, and fibroblast growth factor (FGF) 3 and FGF4, and their receptors, *Fgfr3* and -4 (1). Immunofluorescence staining showed that α -smooth muscle actin signal was markedly decreased, and Hart's *Eln* stain similarly showed fewer secondary crests in GCV-treated transgenic lungs (Figures 5A–5D) (16). Quantitative real-time PCR analysis confirmed decreased levels of *Eln* and platelet-derived growth factor-A (necessary for elastic fiber stability during septal formation), as well as decreases in *Fgfr3* and -4 (products expressed on the surface of developing lung epithelium) (Figures 5E–5H). Taken together, these data demonstrate that GCV treatment of CCtk newborns alters the expression of genes involved in secondary crest formation.

Naphthalene-Sensitive Clara Cell Depletion Does Not Impair Alveolar Formation

Previous studies have shown that Clara cells in 7-day-old mice are sensitive to cytotoxicity induced by the volatile aromatic hydrocarbon, naphthalene. Despite low levels of cytochrome p450, differentiating Clara cells in newborn mice are more susceptible to injury by naphthalene than differentiated Clara cells in adults (19). Naphthalene injection leads to loss of

Figure 4. Alveolar hypoplasia is hallmarked by fewer alveolar type I and II cells. Immunohistochemistry for Nkx2.1 after GCV treatment in WT control (A) versus GCV-treated CCtk mice (B) (magnification, 20 $\times$; scale bar, 100 μ m). (C) Corresponding quantification of Nkx2.1-positive cells. (D) Real-time quantitative PCR analysis comparing total lung mRNA for surfactant protein (SP)-C mRNA expression after GCV treatment in CCtk mice compared with GCV-treated WT control animals (* P < 0.05). Immunofluorescence for SP-C and T1 α after GCV treatment in control (E) versus CCtk mice (F) (magnification, 20 $\times$; scale bar, 100 μ m; insets represent binary image of T1 α -positive pixels). Quantitation of SP-C-positive cells (G) and T1 α pixels (H) (* P < 0.05).

epithelial cell mass, with areas of denudement and squamation of remaining epithelial cells lining terminal bronchioles. Given our observation that GCV treatment depletes CCSP-positive cells in lung airways, we investigated whether isolated depletion of airway Clara cells via naphthalene would also impair alveolar formation. In studies paralleling GCV treatment, newborn animals received a single dose of naphthalene (0.3 mg/g) intraperitoneally on Postnatal Day 1 followed by killing at either 48 hours or 1 week later, on Postnatal Day 8 (Figure 1E). Although lungs of naphthalene-treated CCtk transgenic and wild-type animals showed evidence of epithelial cytotoxicity through loss of CCSP-positive cells at 48 hours (Figure 6A), no detectable differences were noted in alveolar morphometry or phenotype at Postnatal Day 8 (Figures 6B–6D). Levels of CCSP-positive cells had also recovered to normal by Postnatal Day 8 (data not shown). These results demonstrate that de-

Figure 5. Alveolar hypoplasia is a result of impaired secondary crest formation. Immunofluorescence for α -smooth muscle actin (α -SMA) after GCV treatment in WT control (A) versus *CcTk* mice (B). Hart's elastin (Eln) stain after GCV treatment in WT control (C) versus *CcTk* mice (D). Arrowheads indicate α -SMA ([A and B]: magnification, 40 $\times$; scale bar, 100 μ m) and Eln ([C and D]: magnification, 20 $\times$; scale bar, 100 μ m). Real-time quantitative PCR analysis comparing total lung mRNA after GCV treatment in *CcTk* mice compared with GCV-treated WT control animals for mRNA expression of platelet-derived growth factor (PDGF)-A (E), Eln (F), and fibroblast growth factor receptor (Fgfr) 3 and 4 (G and H) (* $P < 0.05$).

pletion of naphthalene-sensitive Clara cells alone does not impair alveolar formation, and suggest that alveolar impairment from GCV treatment in *CcTk* mice may be due to an impact on a more distinct cell population.

GCV-Induced tk Activation Permanently Depletes tk-Positive Cells in Newborn *CcTk* Lungs

Given the finding that alveolar hypoplasia after GCV treatment does not result from depletion of Clara cells alone, we examined the effects of GCV on the *tk*-positive cell population in the lung. Results show that *tk*-positive cells are significantly depleted in parallel with CCSP-positive cells at 1 week after GCV treatment when compared with saline-treated *CcTk* mice (Figures 7A, 7B, 7D, and 7E). Interestingly, whereas *tk*-positive cells were completely and permanently depleted by Postnatal Day 16, CCSP-positive Clara cells reappeared in proximal and distal

Figure 6. Naphthalene-sensitive Clara cell depletion does not impair alveolar formation. (A) Immunofluorescence for CCSP at 48 hours after naphthalene injection showing depletion of Clara cells (magnification, 40 $\times$; scale bar, 100 μ m). (B) Radial alveolar counts and representative H&E-stained lung sections at 1 week after GCV treatment in WT control (C) versus *CcTk* mice (D) (magnification, 10 $\times$; scale bar, 100 μ m).

lung airways (Figures 7C and 7F). These data suggest that the *tk*-positive subpopulation is permanently depleted after GCV treatment, and raise the possibility that these cells may be a key participant in alveolar formation.

Potential Mechanisms of GCV-Induced Alveolar Hypoplasia in *CcTk* Lungs

To identify the origin of *tk*-positive cells, we first examined their distribution within the lung in our naive (untreated) *CcTk* mice. Immunofluorescence staining showed that *tk*-positive cells were limited to proximal and distal airways, with no detectable expression in the distal lung. The distribution of *tk*-positive cells included the BADJ, but did not extend into the alveoli (Figure 8A). Because it is possible that low, undetectable levels of *tk* expression in alveoli might incite an early inflammatory response and lead to alveolar hypoplasia, we examined the lungs of *CcTk* newborns and their wild-type progeny via bronchoalveolar lavage and histological analysis at 48 hours

Figure 7. GCV-induced *tk* activation permanently depletes *tk*-positive cells in newborn *CcTk* mouse lungs. Immunofluorescence for CCSP (A–C) and HSVtk (D–F) at 1 week after GCV treatment (B and E) or saline treatment (A and D) and at 2 weeks after GCV treatment (C and F) in *CcTk* mice (magnification, 10 $\times$ [insets, 40 $\times$]; scale bar, 100 μ m).

after GCV injection. Results show that, although GCV-treated *CCtk* lungs already had evidence of alveolar impairment, no signs of alveolar inflammation were detectable via bronchoalveolar lavage (14, 15) or via detailed histological analysis (Figures 8B–8G). These data demonstrate that the alveolar impact of GCV treatment in *CCtk* transgenic lungs begins early, and does not result from alveolar injury. The alveolar changes may be a reflection of effects on *tk*-positive cells located outside of the alveolar compartment—namely, in the airways, BADJ, or extrapulmonary niche.

tk-Positive Cells Are also Located in the Bone Marrow

Two recent reports describe the identification of bone marrow-derived epithelium-like cells capable of repopulating injured mouse airway epithelium (13, 20). The investigators showed that CCSP-positive cells reside in the bone marrow, and can specifically home to the lung after naphthalene-induced epithelial injury. The studies provide compelling evidence that bone marrow-derived cells may contribute to lung regeneration. Based on these findings, we investigated whether the bone marrow compartment of *CCtk* mice contains *tk*-positive cells. Bone marrow and lungs from adult *CCtk* mice were harvested at 1 week after treatment with either 10 mg GCV (0.5 mg/g) or saline control intraperitoneally, and processed for cytospin, immunofluorescence, and mRNA analysis. Results from cytospins stained with immunofluorescent antibodies to detect CCSP-positive and *tk*-positive cells confirmed that CCSP-positive cells are found in bone marrow from wild-type mice treated with saline or GCV, and, more importantly, that *CCtk* mice harbor *tk*/CCSP dual-positive cells in the bone marrow (Figures 9A and 9B). Bone marrow from *CCtk* mice after

treatment with GCV showed no detectable levels of *tk*/CCSP dual-positive cells (data not shown). These findings were confirmed by mRNA extraction of cell pellets from bone marrow cytospins, showing significantly decreased CCSP expression in the GCV-treated *CCtk* mice compared with GCV-treated wild-type control animals (Figure 9C). Lung tissue analysis further verified that GCV treatment of *CCtk* mice decreased CCSP expression via quantitative real-time PCR and immunofluorescence (Figures 9D–9F). Finally, lungs of GCV-treated *CCtk* mice had a significant decrease in mitotic index, as assessed by immunofluorescence for the proliferation marker, Ki67 (Figures 9G and 9H), but showed no change in cell apoptosis via terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (data not shown). These data raise the novel possibility that bone marrow-derived epithelium-like cells may participate in lung growth and alveolar formation.

Alveolar Hypoplasia Is Associated with Decreased VEGF Expression

To determine whether alveolar hypoplasia is mediated by loss of a paracrine factor, we measured VEGF expression in lungs and bone marrow after GCV treatment of *CCtk* mice. Results show that mRNA expression of VEGF was significantly decreased in bone marrow and whole-lung homogenates (Figures 10A and 10C), and that VEGF protein expression was significantly decreased in distal airways (Figures 10D and 10E). Immunofluorescence analysis of bone marrow cytospins further demonstrated the presence of *tk*/VEGF dual-positive cells (Figure 10B). In summary, these findings imply that alveolar hypoplasia resulting from depletion of *tk*-positive cells may be due to reduced expression of the paracrine factor, VEGF.

Figure 8. Potential mechanisms of GCV-induced alveolar hypoplasia. Immunofluorescence for HSVtk in 1-week-old, naive *CCtk* mouse lung (A). Representative lung photomicrographs of H&E-staining (B, D) and immunofluorescence (A, C, E) for CCSP (green) and HSVtk (red) lungs at 48 hours after GCV treatment in WT (B and C) versus *CCtk* mice (D and E), with corresponding total bronchoalveolar lavage (BAL) cells (F) and BAL differential (G) (magnification, 4× in [A–E] [insets, 40×]; scale bar, 100 μm). L, lymphocytes; M, macrophages; N, neutrophils.

Figure 9. tk-positive cells are also located in the bone marrow. Immunofluorescence from bone marrow cytopspins showing CCSP-positive cells in WT mice (A) and tk/CCSP dual-positive cells in *Ctck* mice (B) (magnification, 40 $\times$). (C) Real-time quantitative PCR analysis after GCV treatment in *Ctck* mice compared with GCV-treated WT control mice for bone marrow CCSP mRNA and (D) lung CCSP mRNA expression (* P < 0.05). Immunofluorescence showing corresponding decrease in CCSP-positive cells in lungs after GCV treatment in WT control (E) versus *Ctck* mice (F) (magnification, 20 $\times$; scale bar, 100 μ m). Ki67-positive cells (arrowheads) after GCV treatment in newborn WT control (G) versus *Ctck* mice (H) (magnification 20 $\times$; scale bar, 100 μ m).

DISCUSSION

Alveolar formation involves the coordination of numerous cell types during lung morphogenesis. Our results show that selective depletion of a subpopulation of cells with an active CCSP promoter during alveolar development results in alveolar hypoplasia. To our knowledge, the present study is the first to propose that this unique subpopulation of cells participates in alveolar formation. These findings support a model in which manipulation of epithelial cells located within the airway and/or bone marrow compartment may affect alveolar formation in the distal lung compartment.

Results of the current study demonstrate that acute dosing of GCV in newborn *Ctck* mice results in marked alveolar hypo-

plasia through decreased alveolar type I and II cells, impaired secondary crest formation, and decreased lung VEGF expression. These phenotypic changes occurred in the absence of any detectable inflammation. This experimental strategy was compared head-to-head against the effects of naphthalene exposure showing that isolated Clara cell depletion alone does not cause alveolar hypoplasia. The naphthalene injury model in newborns has been well described by Plopper and colleagues (19), who have characterized the relationship between naphthalene-induced airway injury in neonatal and adult mice. Their data demonstrate that naphthalene increases susceptibility of Clara cells to injury in newborn mice compared with adults, despite lower levels of cytochrome P450. The naphthalene results are consistent with previous observations that CCSP^{-/-} knockout mice develop normal alveoli (21). Interestingly, although all tk-positive cells were completely and permanently deleted after GCV treatment, cells exclusively positive for CCSP (and negative for tk) soon reappeared. Although the significance of these findings remains unknown, a possible explanation for this observation may be that this cell subtype originates from local progenitors, such as basal cells that differentiate into CCSP-positive Clara cells (9, 22). Taken together, these findings imply that GCV treatment of *Ctck* mice depletes a unique subpopulation of cells, independent from naphthalene-sensitive Clara cells, that participates in alveogenesis.

Previous reports have established that the lung possesses distinct populations of progenitor cells responsible for epithelial regeneration after lung injury in adult mice (11). These cell populations reside in niches throughout the proximal–distal airway axis, and contribute to repopulation of specific cell type(s) within each locale. Although the identification of stem/progenitor populations capable of reconstituting airway epithelium is receiving increasing attention, considerable controversy still exists regarding which cells can actually participate in alveolar regeneration. Two such cell types, the bronchioalveolar stem cell (BASC) and *Oct-4*-positive stem/progenitor cell, are thought to contribute descendants to alveoli (10, 23). In addition, the subpopulation of Clara cells termed variant Clara cells, identified by CCSP promoter activity, may also possess stem/progenitor cell qualities responsible for bronchiolar regeneration, with a potential impact on alveolar homeostasis (5, 11). Finally, an epithelium-like population of bone marrow cells expressing CCSP has recently been shown to repopulate airway epithelium after injury (13, 20). Our report extends these findings and proposes that a subset of cells identified by CCSP promoter activity may play a role in alveogenesis.

The *Ctck* transgenic mouse model provides a useful tool to study the mechanisms regulating alveogenesis. Most work to date on alveolar regeneration has been limited to the description of the BASC, which is thought to coexpress CCSP and SP-C (10). Although BASCs were thought to give rise to both bronchiolar and alveolar cells based on work performed *in vitro*, the ability of these cells to contribute to alveolar descendants was recently challenged using lineage tracing analysis. Hogan and colleagues (9) recently showed that a cross between *Scgb1a1*-CreER mice (*Scgb1a1* = CCSP) and the *Rosa26*-eYFP reporter line resulted in labeling of only 1–2% of total alveolar cells in the steady state when the promoter was activated after a single injection of tamoxifen to activate the Cre recombinase–modified estrogen receptor fusion protein. A parallel experiment after hyperoxia challenge demonstrated no difference in the percentage of cells in these alveolar regions containing lineage-labeled cells in hyperoxia-exposed versus room air-exposed control mice. The investigators thus concluded that bronchiolar CCSP-positive cells do not contribute to alveolar repair. Interestingly, although studies in rabbits and

Figure 10. Alveolar hypoplasia is associated with decreased vascular endothelial growth factor (VEGF) expression. Real-time quantitative PCR analysis after GCV treatment in *CCtk* mice compared with GCV-treated WT control animals for VEGF mRNA in bone marrow (A) and lung (C) (* $P < 0.05$). (B) Immunofluorescence from bone marrow cytopins showing *tk*/VEGF dual-positive cells (arrows) in *CCtk* mice (magnification, 40 $\times$). Immunofluorescence of VEGF expression (arrowheads) after GCV treatment in WT control (D) versus *CCtk* mice (E) (magnification, 40 $\times$; scale bar, 100 μ m).

rats describe a proportion of alveolar type II cells throughout the alveoli that express low levels of CCSP (24, 25), no expression of CCSP- or *tk*-positive cells was detectable in alveoli of *CCtk* transgenic mice from our study. In addition to BASCs, the *Oct-4*-positive cell population has been introduced as a putative neonatal lung stem/progenitor cell that is capable of being cultured *in vitro* for weeks and induced to differentiate into alveolar type II and type I cells. Though a small number of *Oct-4*-expressing cells have been described at the bronchoalveolar junction of neonatal mouse lungs, no clear evidence of *Oct-4*-positive cells was detectable in our study.

Given the lack of evidence that alveolar cell precursors reside in intrapulmonary conducting airways, the possibility that an extrapulmonary niche, such as bone marrow, may harbor these precursors remains plausible. Two recent papers provide evidence that epithelium-like cells that express CCSP within the bone marrow can incorporate into airways and alveoli after transtracheal delivery after naphthalene injury (13, 20). These cells can be isolated and propagated in air-liquid interface culture to differentiate into both airway and alveolar lineages. Furthermore, lethally irradiated CCSP^{-/-} knockout animals that received tagged wild-type bone marrow showed donor-derived epithelium in both normal and naphthalene-damaged airways. It is intriguing to consider the possibility that these cells may not only contribute to lung regeneration after injury, but may also participate in alveolar formation during postnatal development. We thus asked whether *CCtk* mice harbor *tk*-positive cells in the bone marrow, and whether these cells are depleted after GCV treatment. Our results confirmed that bone marrow from *CCtk* mice contains *tk*/CCSP dual-positive cells that are depleted after GCV treatment. Although there is currently no demonstrable evidence through lineage labeling that CCSP-positive cells participate in alveolar formation by incorporation into the alveoli (9), we propose that these cells may participate in alveolar formation through indirect mechanisms.

Several recent examples in the literature support the theory that bone marrow-derived progenitor cells may exert their effects on alveolar growth indirectly through paracrine-mediated activity (26–30). Ortiz and colleagues (28) showed that conditioned media from mesenchymal stem cells inhibited TNF- α production by activated macrophages, and inhibited proliferation of IL-1 α -dependent T cells *in vitro*. Xu and colleagues (29) similarly showed that bone marrow-derived mesenchymal stem cells decreased inflammatory responses after endotoxin expo-

sure in mice, independent of lung engraftment. Balasubramanian and colleagues (27), van Haaften and colleagues (26), and Aslam and colleagues (30) have suggested that paracrine mechanisms likely account for the beneficial effects seen in rodent models of hyperoxia-induced alveolar hypoplasia after the instillation of bone marrow-derived angiogenic cells, bone marrow-derived mesenchymal stem cells, or conditioned media from bone marrow stromal cells, respectively. These observations are further supported by our data that show that VEGF expression in lungs and bone marrow is decreased after GCV treatment in *CCtk* mice, and by evidence that *tk*-positive cells coexpress VEGF. Thus, because VEGF plays an important role in lung morphogenesis (31, 32) and is expressed in bone marrow progenitor cells (33–35), our findings support a model in which depletion of *tk*-positive cells may impair alveolar formation through decreased VEGF expression.

A few alternative possibilities are important to consider. First, it is possible that, although we did not detect any evidence of acute or resolving inflammatory cell infiltrate at 48 hours, 1 week, or 2 weeks after GCV treatment, a transient inflammatory response could have been elicited between those time points. This scenario seems unlikely, however, given the kinetics of GCV-induced inflammation in adult mice, showing that alveolar leukocyte infiltration is a late phenomenon (Day 14) (11), and by our finding that alveolar changes had already begun by 48 hours. Second, we cannot rule out that there may be some low-level ectopic expression throughout the alveoli of the endogenous CCSP promoter or the 2.1-kb fragment of the mouse CCSP promoter used in the transgenic construct, as previously described in the rat CCSP promoter (36). Ablation of *tk*-positive alveolar cells induced by GCV could thus lead to fewer alveoli in transgenic mice, although no alveolar *tk*-expressing cells were detectable in our study or in the original description of the adult *CCtk* model (5). If ectopic alveolar expression of CCSP promoter were present, we would expect to see evidence of inflammation or injury, which was not detected at any time point examined. Third, it should be noted that our studies examining the effects of GCV treatment on bone marrow responses were conducted in adult mice. A complete analysis of bone marrow obtained from newborn animals treated with GCV needs further examination. Finally, it is important to recognize that the current approach to studying the potential effects of depleting *tk*-positive cells focuses exclusively on the epithelial compartment. Analysis of potential

effects on the developing endothelium, implicating a “vascular hypothesis,” also needs further examination.

In summary, this study proposes a novel concept that a unique population of *tk*-expressing cells with stem/progenitor-like properties participates in alveolar development. Current evidence suggests that these cells may contribute to alveogenesis indirectly through secretion of VEGF. These findings may be of high clinical relevance to lung disorders, such as bronchopulmonary dysplasia, where injury or ablation of this critical population of cells may be responsible for alveolar hypoplasia. Strategies to isolate and culture these cells for future rescue experiments are currently under development, and may ultimately be used to enhance or promote alveogenesis.

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