c-Kit Is Essential for Alveolar Maintenance and Protection from Emphysema-like Disease in Mice

James Y. Lindsey^{1*}, Koustav Ganguly^{2,3*}, David M. Brass⁴, Zhuowei Li¹, Erin N. Potts¹, Simone Degan⁵, Huaiyong Chen¹, Brian Brockway¹, Soman N. Abraham^{6,7}, Annerose Berndt⁸, Barry R. Stripp¹, W. Michael Foster¹, George D. Leikauf^{3*}, Holger Schulz^{9*}, and John W. Hollingsworth^{1,7*}

¹Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, Duke University Medical Center, Durham, North Carolina; ²Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany; ³Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, Pennsylvania; ⁴Department of Pediatrics, Duke University Medical Center, ⁵Center for Molecular and Biomolecular Imaging, Duke University Medical Center, ⁶Department of Pathology and Department of Molecular Genetics and Microbiology, Duke University Medical Center, and ⁷Department of Immunology, Duke University Medical Center, Durham, North Carolina; ⁸Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; and ⁹Institute of Epidemiology and Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz Zentrum, München, German Research Center for Environmental Health, Munich, Germany

Rationale: Previously, we demonstrated a candidate region for susceptibility to airspace enlargement on mouse chromosome 5. However, the specific candidate genes within this region accounting for emphysema-like changes remain unrecognized. c-Kit is a receptor tyrosine kinase within this candidate gene region that has previously been recognized to contribute to the survival, proliferation, and differentiation of hematopoietic stem cells. Increases in the percentage of cells expressing c-Kit have previously been associated with protection against injury-induced emphysema.

Objectives: Determine whether genetic variants of c-Kit are associated with spontaneous airspace enlargement.

Methods: Perform single-nucleotide polymorphism association studies in the mouse strains at the extremes of airspace enlargement phenotype for variants in c-Kit tyrosine kinase. Characterize mice bearing functional variants of c-Kit compared with wild-type controls for the development of spontaneous airspace enlargement. Epithelial cell proliferation was measured in culture.

Measurements and Main Results: Upstream regulatory single-nucleotide polymorphisms in the divergent mouse strains were associated with the lung compliance difference observed between the extreme strains. c-Kit mutant mice (*Kit^{W-sh/W-sh}*), when compared with genetic controls, developed altered lung histology, increased total lung capacity, increased residual volume, and increased lung compliance that persist into adulthood. c-Kit inhibition with imatinib attenuated *in vitro* proliferation of cells expressing epithelial cell adhesion molecule.

Conclusions: Our findings indicate that c-Kit sustains and/or maintains normal alveolar architecture in the lungs of mice. *In vitro* data suggest that c-Kit can regulate epithelial cell clonal expansion. The precise mechanisms that c-Kit contributes to the development of airspace enlargement and increased lung compliance remain unclear and warrants further investigation.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Previous data suggest that bone marrow-derived cells can preserve alveolar structure in various models of lung injury. c-Kit is a receptor tyrosine kinase that contributes to the survival, proliferation, and differentiation of hematopoietic stem cells. The role of c-Kit in maintaining normal lung structure remains unknown.

What This Study Adds to the Field

Forward genetics identified a region within mouse chromosome 5 associated with the development of spontaneous airspace enlargement. *In silico* mapping of divergent mouse strains and characterization of c-Kit mutant mice indicate that genetic variation upstream of c-Kit tyrosine kinase is associated with premature emphysematous changes of the lung parenchyma of mice. Furthermore, our observations suggest a role of c-Kit in lung epithelial cell clonal expansion.

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* These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to John W. Hollingsworth, M.D., DUMC, Box 103004, Durham, NC 27710. E-mail: holli017@mc.duke.edu

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The delicate airway and parenchymal tissues of the lung follow a blueprint for structural development that relies on tightly controlled temporal and spatial interactions between many diverse cell types (1–4). In humans as in mice, the lung continues to develop after birth until the end of early adulthood (2, 3). However, because lung development proceeds more rapidly in the mouse (12–14 wk) than in the human (23–25 yr), mouse models allow for rapid testing of genetic and molecular determinants of lung structure and function. Delays and/or interruptions of normal postnatal lung growth together with normal aging of the lung may have serious functional consequences later in life (5) and can lead to reduced quality of life (6) and increased risk of mortality (7–9). Knowledge of the underlying genetic determinants of lung development could provide an unbiased view of the critical genes (10) and pathways (11) that contribute to declines in lung function during aging.

Previously we identified several quantitative trait loci (QTL) in mice that are associated with altered mouse lung function including increased static lung compliance (Cl) (12), which is a pathognomonic feature of chronic obstructive pulmonary disease (COPD). We observed a region on chromosome 5 that was associated with alterations in Cl and harbored several candidate

TABLE 1. LUNG FUNCTION MEASUREMENTS OF 10 INBRED MOUSE STRAINS

Parameter	Strain									
	AKR/J	BTBR/T ⁺ tf/J	CAST/EiJ	CZECHII/EiJ	DBA/2J	KK/HIJ	MOLF/EiJ	NOD/ShiLtJ	NZW/LacJ	WSB/EiJ
BW, g	31 (1.2)	32 (0.6)	13 (0.3)	12.0 (0.9)	24 (0.8)	35 (1.3)	13 (0.2)	22 (0.6)	27 (0.8)	14 (0.03)
Age, d	96 (0.4)	84 (0.2)	96 (0.2)	94 (0.6)	93 (0.2)	84 (0.2)	93 (2.3)	91 (0.2)	86 (0.2)	89 (0.9)
Number	8	8	6	12	8	7	14	8	8	11
Lung volume										
TLC, μ l	1,176 (32)	1,409 (26)	751 (9)	679 (9)	1,054 (17)	1,304 (29)	855 (14)	1,012 (25)	1,218 (26)	744 (27)
TLC/BW, μ l/g	38.7 (1.4)	44.4 (1.5)	57.7 (0.8)	56.6 (0.9)	43.8 (1.7)	37.5 (1.4)	64.8 (1.2)	46.4 (1.6)	44.5 (0.6)	51.5 (1.7)
FRC, μ l	304 (2)	343 (9)	272 (3)	272 (4)	289 (11)	304 (9)	281 (4)	303 (6)	310 (5)	273 (6)
FRC/TLC	0.26 (0.01)	0.24 (0.01)	0.36 (0.01)	0.40 (0.01)	0.27 (0.01)	0.23 (0.01)	0.33 (0.01)	0.30 (0.01)	0.25 (0.01)	0.37 (0.01)
V _D , μ l	231 (4)	251 (3)	179 (5)	177 (4)	232 (3)	246 (2)	217 (5)	222 (2)	250 (4)	201 (4)
V _D /TLC	0.20 (0.01)	0.18 (0.01)	0.24 (0.01)	0.26 (0.01)	0.22 (0.01)	0.19 (0.01)	0.25 (0.01)	0.22 (0.01)	0.21 (0.01)	0.27 (0.01)
Compliance										
C _L , μ l/cm H ₂ O	58.3 (2.8)	82.1 (3.0)	31.6 (2.2)	25.2 (0.9)	61.1 (1.5)	61.2 (2.7)	34.8 (1.9)	61.5 (3.1)	92.4 (7.0)	30.2 (1.4)
C _L /TLC, 1/cm H ₂ O	49.8 (2.7)	58.2 (1.7)	46.1 (3.2)	36.3 (1.1)	58.0 (1.3)	47.0 (1.9)	40.7 (2.0)	60.7 (2.4)	75.9 (5.5)	40.8 (1.6)
C _{dyn} , μ l/cm H ₂ O	41.1 (1.7)	57.7 (2.4)	22.6 (1.5)	19.9 (0.8)	38.4 (0.8)	46.5 (1.3)	24.6 (0.6)	43.5 (1.9)	58.9 (3.4)	21.1 (0.6)
Diffusion capacity										
D _{CO} , μ mol/min/hPa	13.9 (0.4)	17.4 (0.3)	4.7 (0.03)	3.0 (0.1)	9.5 (0.3)	11.4 (0.7)	5.6 (0.4)	10.3 (0.4)	11.4 (0.5)	5.1 (0.4)
D _{CO} /V _A , μ mol/min/hPa/ml	12.4 (0.03)	13.1 (0.2)	6.4 (0.04)	4.7 (0.2)	10.1 (0.1)	9.3 (0.3)	7.4 (0.3)	11.2 (0.2)	10.4 (0.2)	7.5 (0.3)

Definition of abbreviations: BW = body weight; C_{dyn} = dynamic lung compliance; C_L = static lung compliance; D_{CO} = diffusion capacity; FRC = functional residual capacity; TLC = total lung capacity; V_A = alveolar volume; V_D = dead space volume.

Values represent means (SE).

genes including v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*Kit*, also known as mast/stem cell growth factor receptor, *c-Kit* oncogene, or *CD117*) (12). The KIT protein encoded by this gene is a tyrosine kinase receptor that is expressed on the surface of hematopoietic stem cells (HSCs). Binding of KIT ligand (KITLG, also known as stem cell factor or Steel factor) to KIT activates a signaling pathway important to HSC survival, proliferation, and differentiation (13, 14).

Although KIT is strongly expressed at critical points in embryonic mouse lung development (15), the role that KIT plays in both lung development and repair has not been fully explored (16, 17). In humans, KIT-immunoreactive interstitial cells have been observed in the alveolar septa of normal newborn lungs, but were absent in lungs of patients with congenital alveolar capillary dysplasia (18). In mice, HSCs may contribute to lung regeneration after injury as demonstrated after lung injury induced by lipopolysaccharide (19), elastase (20), irradiation, and combined elastase/irradiation (21). In addition, repair after elastase-induced lung injury was enhanced by intranasal hepatocyte growth factor instillation and was associated with an increase in the percentage of KIT-expressing circulating mononuclear cells (22). In the current study we assess lung function in select genetically divergent inbred mouse strains and used a mutant strain to determine how *Kit*, a candidate gene associated with lung function, may provide novel insights into the later decline in lung function.

METHODS

Mice

Mice were obtained from commercial vendors. Lung function measurements were performed in the following 10 mouse strains: AKR/J (00648), BTBR T⁺ tf/J (002282), CAST/EiJ (000928), CZECHII/EiJ (001144), DBA/2J (000671), KK/HIJ (002106), MOLF/EiJ (000550), NOD/ShiLtJ (001976), NZW/LacJ (001058), and WSB/EiJ (001145) and supplemented by nine strains previously phenotyped: 129S1/SvImJ, A/J, BALB/cByJ, C3H/HeJ, C57BL/6J, FVB/NJ, JF1/Ms, PWD/PhJ, and SWR/J (12,23,24). Single-nucleotide polymorphisms (SNPs) in *Kit* were identified using the Mouse Phenome Database (2010) and the population was interrogated for the phenotypic difference observed in mice with either allele compared with the phenotypic difference of the extreme mouse strains (CZECHII/EiJ and NZW/LacJ).

To determine whether mice with mutant *Kit* have altered lung structure and function, B6.Cg-*Kit*^{W-sh}/HNIhrJaeBsmJ mice (referred to here as SASH mice) were compared with C57BL/6J. The SASH mouse is homozygous for a *Kit*^{W-sh} allele (25), which has been backcrossed onto C57BL/6J for more than 10 generations. The *Kit*^{W-sh} mutation prevents KIT mRNA expression in mast cells and mesenchymal cells in the lung and digestive tract on Embryonic Day 13 (E13) and in adult lung, whereas expression in other tissues is normal (15, 26).

Lung Function

In the inbred mouse strains, invasive measurements of lung function measurements of total lung capacity (TLC), static lung compliance

Figure 1. Strain distribution pattern for static lung compliance (CL) in 19 inbred mouse strains. Mouse lung CL obtained from the transpulmonary pressure-volume curve varied between strains ($n = 6-16$ mice per strain, female, 12-14 wk). The range in the difference between the lowest and highest strains was about threefold (CZECHII/EiJ CL, $25.2 \pm 0.9 \mu\text{l/cm H}_2\text{O}$; NZW/LacJ CL, $92.4 \pm 7.0 \mu\text{l/cm H}_2\text{O}$). Values represent means $\pm$ standard error.

TABLE 2. SINGLE-NUCLEOTIDE POLYMORPHISMS IN MOUSE *KIT*

SNP	Location (Mbp)	Observed SNP	Allele 1 Frequency	Percentage of Phenotype	Region	Allele 1	Allele 2
rs37546426	75.969357	C/T	0.19	51.6	5' L	IRF1/2	YY1
rs36684666	75.969567	C/T	0.38	35.7	5' L	Zeste	POU1F1a
rs37967821	75.969778	C/T	0.21	53.2	5' L	MYB1	Dfb (Hoxb4)
rs38970347	75.970285	T/A	0.21	53.2	5' L	No RAF	RAF
rs36956723	75.970368	C/T	0.38	35.7	5' L	ETF, Sp1	CACCC-binding factor
rs37998535	75.970867	C/G	0.19	51.6	5' L	NF-1	C/EBP α
rs36929749	75.971053	C/T	0.19	51.6	5' UTR	T-Ag	No T-Ag
rs36972615	76.049338	G/C	0.19	51.6	CN	Asp 927	Glu 927
rs36331951	76.050775	A/G	0.21	53.2	3' UTR	A	G
rs29630739	76.051098	A/G	0.28	45.0	3' UTR	A	G
rs33755426	76.051115	G/A	0.28	45.0	3' UTR	G	A
rs13469910	76.051946	C/T	0.28	45.0	3' UTR	C	T
rs36266306	76.052269	A/G	0.19	51.6	3' UTR	G	A
rs36248044	76.052312	A/G	0.21	53.2	3' L	G	A
rs7033324	76.052937	A/G	0.19	51.6	3' L	A	G

Notes: SNP = reference single-nucleotide polymorphism identification number; Location (Mbp) = location on mouse chromosome 5 in megabase pairs (build 37); Observed SNP = base pair substitution with minor (allele 1) listed first; Allele 1 Frequency = the ratio of mice in the total population ($n = 213$) that have allele 1; Percentage of Phenotype = a comparison of the mean static compliance (CL) of mice with allele 1 to that of allele 2 divided by the maximum difference between the polar strains (NZW/LacJ vs. CZECH/EiJ) $\times 100$, based on alleles assigned by the Mouse Phenome Database (<http://www.jax.org/phenome>); Region = location near (L) or within (CN = nonsynonymous) the gene, including the untranslated region (UTR); Allele 1 = the possible consequence of the minor allele as determined by the Transcription Element Search System (30) for the 5' region, in the coding sequence, or in the 3' UTR. Allele 2 = major allele. The predicted transcriptional binding site use the TRANSFAC abbreviation.

(CL), dead space volume (V_D), diffusion capacity (D_{CO}), alveolar volume (V_A), and specific lung function values (TLC/body weight [BW]; V_D /TLC; CL /TLC, D_{CO}/V_A) were determined and statistics performed as described previously (27). In SASH and additional C57BL/6J mice, lung function was measured with a commercial system (*flexiVent*; SCIREQ, Montreal, PQ, Canada).

Morphometric Analysis of Histology

Sections (5 μ m thick) of paraffin-embedded, formalin-fixed mouse lung were stained with hematoxylin and eosin (H&E stain; AML Laboratories, Rosedale, MD). Five digital images of parenchymal architecture from each mouse were captured at $\times 40$ magnification by an investigator blinded to the experimental groups. Images were captured from anatomically comparable regions of the lung.

Alveolar volume density (AVD) and alveolar surface density (ASD) were calculated as previously described (28). Briefly, five random fields were chosen (omitting large vascular and bronchiolar structures) and images were overlaid with an 11×11 point grid (Metamorph; Universal Imaging, West Chester, PA) for point (P) and intercept (I) counting of the alveolar septa. Alveolar volume density was calculated from $P_{alveoli}/P_{parenchyma}$ and alveolar surface density from $2I_{alveoli}/LT$, where LT was the test line length within the lung parenchyma. For mean linear intercept (MLI), images were analyzed as previously described (29). Briefly, a series of grid lines was laid over each photomicrograph to determine the number of times those lines were intercepted by alveolar tissue, with $L_m = L/L_i$, where L is the total length of the lines in the grid field and L_i is the total number of times those lines are intercepted. Data from each group are expressed as means $\pm$ SE.

tween the extreme strains. Values represent means $\pm$ standard errors and differences between alleles were assessed by analysis of variance followed by an all-pairwise multiple-comparison procedure (Holm-Sidak method) (* $P < 0.001$)

Lung Epithelial Cell Assays

Flow cytometry was used to characterize populations of airway epithelial cells. Primary lung epithelial cells expressing epithelial cell adhesion molecule (EpCAM⁺ cells) were isolated and cultured on fibroblasts $\pm$ imatinib. Number and size of colonies were quantified after culture for 28 days. Similarly, coculture experiments were performed $\pm$ stem cell factor (SCF) at doses of 0–100 ng/ml, and the number and size of colonies were quantified at 14 days.

RESULTS

Lung Function Varies among Mouse Strains

Lung function measurements varied significantly among the additional age- and sex-matched (12–14 wk, female) mouse strains tested (Table 1). For example, total lung capacity (TLC) (mean $\pm$ SE) varied from $679 \pm 9 \mu$ l for CZECHII/EiJ ($n = 12$) to $1,409 \pm 105 \mu$ l for BTBR T⁺ tf/J ($n = 8$) mouse strains. The CZECHII/EiJ strain mice have the smallest TLC we have noted in the combined 19 strains tested (12, 23, 24). In the nine strains previously phenotyped, the smallest TLC was $874 \pm 17 \mu$ l for the sex- and age-matched JF1/Ms mouse strain (12). The largest TLC was $1,457 \pm 82$ for the BALB/cByJ mouse strain (24), which is only slightly smaller than observed in the BTBR T⁺ tf/J mouse strain. Thus, the strains with extreme phenotypes (CZECHII/EiJ and the BALB/cByJ) varied about twofold in TLC. This analysis also uncovered a large strain difference (4.7-fold) in D_{CO}

Figure 2. Phenotype of mice with genetic variants in the *Kit* promoter. (A) Static compliance (CL) was significantly lower in mice that have the C allele of a promoter single-nucleotide polymorphism (SNP), rs37967821, than in mice with the T allele. The C allele had a frequency of 0.21 ($n = 46$ mice) among the 19 strains ($n = 213$ mice) genotyped and could explain 53.2% of the CL difference observed in the extreme mouse strains CZECHII/EiJ and NZW/LacJ. (B) CL was significantly lower in mice that have the C allele of a promoter SNP, rs37998535, than in mice with the G allele. The C allele had a frequency of 0.19 ($n = 40$ mice) among the 19 mouse strains phenotyped and could explain 51.6% of the CL difference observed between the extreme strains. Values represent means $\pm$ standard errors and differences between alleles were assessed by analysis of variance followed by an all-pairwise multiple-comparison procedure (Holm-Sidak method) (* $P < 0.001$)

(CZECHII/EiJ $D_{CO} = 3.0 \pm 0.1 \mu\text{mol/minute/hPa}$; BTBR $T^+ \text{tf/J } D_{CO} = 17.4 \pm 0.3 \mu\text{mol/minute/hPa}$), whereas the previous greatest difference was between JF1/Ms D_{CO} ($8.3 \pm 0.4 \mu\text{mol/minute/hPa}$) and FVB/NJ D_{CO} ($15.7 \pm 1.8 \mu\text{mol/minute/hPa}$).

Divergent Mouse Strains and Association between Polymorphisms in *Kit* and Static Lung Compliance

Previously, we identified on mouse chromosome 5 a QTL that was associated with lung compliance and that contained several candidate genes including *Kit*. We measured static lung compliance (CL) for 19 mouse strains (213 mice). The range in the difference between the lowest and highest strains was about threefold (CZECHII/EiJ $CL = 25.2 \pm 0.9 \mu\text{l/cm H}_2\text{O}$; NZW/LacJ $CL = 92.4 \pm 7.0 \mu\text{l/cm H}_2\text{O}$) (Figure 1). Using a high-density SNP map for these 19 mouse strains (including the 5' untranslated region [UTR] and 3' UTR of the gene), we identified 534 SNPs within the *Kit* locus that included eleven in the 5' UTR ("promoter"), one nonsynonymous SNP, and fifteen 3' UTR SNPs.

We interrogated possible functional consequences of the 11 promoter SNPs, using the Transcription Element Search System (30). Seven of the 11 SNPs result in a change that could alter the transcription factor DNA-binding efficiency (Table 2). Of these, two were particularly noteworthy. The first, rs37967821, is a C-to-T

conversion that would convert the conserved sequence for DNA binding of Deformed (Dfd), which has homology to homeobox B4 (HOXB4), to a DNA-binding site for v-myb myeloblastosis viral oncogene homolog (MYB1). The C allele had a frequency of 0.21 among the 19 strains phenotyped and could explain 53.2% of the CL difference observed in the extreme mouse strains (Figure 2A). The second, rs37998535, is a C-to-G conversion that would form a conserved sequence to NF1 and thereby replaces a CCAAT/enhancer-binding protein (C/EBP)- α (CEBPA) binding site. The C allele had a frequency of 0.19 among the 19 mouse strains phenotyped and could explain 51.6% of the CL difference observed between the extreme strains (Figure 2B). While the functional consequences of the genetic variations associated with CL remain unknown, the nonsynonymous SNP (rs36972615) could produce an aspartic acid-to-glutamic acid conversion at position 927. It is noteworthy that the CZECHII/EiJ and JF1/Ms mouse strains share the same genotype for these three SNPs whereas the NZW/LacJ and C3H/HeJ mouse strains share the opposing genotype for these SNPs. Of the fifteen 3' UTR SNPs, seven were present in the strains tested and six were A-to-G conversions. We did not detect differences in mRNA expression of c-Kit from adult (6 wk) whole lungs between the CZECHII/EiJ and

Figure 3. SASH (B6.Cg-*Kit*^{W-sh}/HNihrJaeBsmJ) mice develop spontaneous airspace enlargement when compared with C57BL/6j mice. SASH mice have a sequence inversion in the 5' regulatory region of *Kit*, and lack KIT mRNA expression in the lung. Hematoxylin and eosin images were captured at $\times 10$ magnification for (A) 4-week C57BL/6j, (B) 4-week SASH, (C) 10-week C57BL/6j, (D) 10-week SASH, (E) 14-week C57BL/6j, and (F) 14-week SASH mice. No difference was noted between lung histological sections obtained at 4 weeks between (A) C57BL/6j and (B) SASH mice. By 10 weeks, (C) C57BL/6j mouse lung developed normally whereas (D) SASH mouse lung began to show signs of airspace enlargement. Histology at 14 weeks revealed that (E) C57BL/6j lung had intact parenchyma and well-preserved alveoli whereas (F) SASH lung showed enlarged (emphysema-like) airspaces.

Figure 4. SASH (B6.Cg-*Kit*^{W-sh}/HNihrJaeBsmJ) mice develop delayed abnormal morphology when compared with C57BL/6j mice. Analysis of (A) alveolar volume density, (B) alveolar surface area, and (C) mean linear intercept by MetaMorph software reflects observed histological changes in SASH lung histology. Data represent means $\pm$ standard errors. *Significant difference ($P < 0.05$) as determined by Student *t* test ($n = 10$ –13).

NZW/LacJ mice. However, the timing and distribution of c-Kit expression required for normal lung maintenance remain unknown.

Mice Deficient in c-Kit Develop Spontaneous Airspace Enlargement

To investigate the role of c-Kit in lung development and changes in lung function over time, we compared the lung histology of B6.Cg-*Kit^{W-sh}*/HNihrJaeBsmJ mice (referred to here as SASH for convenience) with that of C57BL/6J (control) mice at 4, 10, and 14 weeks of age. At 4 and 10 weeks of age there was no discernible difference between histological sections based on genotype (Figure 3, compare panel A with panel B, and panel C with panel D). By 14 weeks of age we observed prominent alveolar enlargement in the lungs of SASH mice (Figure 3F) when compared with C57BL/6J control lungs (Figure 3E). We then measured alveolar volume density, alveolar surface density, and mean linear intercept to quantify the histological changes we observed (Figure 4). At 14 weeks of age the SASH mice had significant decreases in both alveolar volume density (Figure 4A) and alveolar surface density (Figure 4B). At both 10 and 14 weeks of age the SASH mice had significantly increased mean linear intercept (Figure 4C) when compared with C57BL/6J control mice.

Static Lung Compliance Increases Significantly over Time in Mice Deficient in c-Kit

To assess whether the altered histology was accompanied by changes in lung mechanics at baseline, we measured CL in both C57BL/6J and SASH mice at 4, 10, and 14 weeks of age (Figure 5). As early as 4 weeks of age, CL in SASH mice was significantly increased compared with CL of C57BL/6J mice (Figure 5). The observed differences persisted and increased through 14 weeks. We also observed that the pressure–volume curves in SASH mice were shifted upward and to the left during inflation and deflation when compared with C57BL/6J mice, indicating a more dynamically compliant lung (Figure 6). Lung volumes as determined by saline fill volume in SASH mice were also significantly greater than in C57BL/6J mice (*see* Figure E1 in the online supplement).

Residual Volume and Ex Vivo Lung Compliance Are Significantly Increased in Mice Deficient in c-Kit

Emphysema is characterized by increased lung residual volume and reduced lung compliance. Therefore, we measured lung residual volumes directly from degassed lungs in both C57BL/6J

Figure 5. SASH (B6.Cg-*Kit^{W-sh}*/HNihrJaeBsmJ) mice develop delayed increased static lung compliance when compared with C57BL/6J mice. Static lung compliance was determined from tracheal airway pressure and volume, which was generated by applying a 2-second sine wave volume perturbation to the tracheal cannula with an amplitude of 0.2 ml and a frequency of 2.5 Hz. Data represent means $\pm$ standard errors. * $P < 0.05$ versus C57BL/6J, # $P < 0.05$ versus C57BL/6J at 4 weeks, ** $P < 0.05$ versus SASH at 4 weeks and C57BL/6J at 10 and 14 weeks. Statistical differences were determined by Student *t* test ($n = 5$ –8).

and SASH mice. We did not observe differences in either residual volume or *ex vivo* lung compliance at 4 weeks of age. However, at both 10 and 14 weeks SASH mice had significantly increased residual volume when compared with age-matched C57BL/6J mice (Figure 7). Total excised lung weight was not significantly different between the strains (data not shown). Consistent with our *in vivo* pressure–volume loops (Figure 6), we observed that *ex vivo* pressure–volume loops in 10- or 14-week-old SASH mice were shifted upward and to the left, indicating a more dynamically compliant lung when compared with C57BL/6J mice (Figure 8).

c-Kit Regulates Epithelial Progenitor Cell Expansion

We did not observe differences in the number or properties of EpCAM⁺ lung epithelial cells by flow cytometric analysis of total cells isolated from lungs of SASH mice compared with C57BL/6J mice (Figures E2 and E3). However, treatment of cultured lung epithelial progenitor cells in the presence of

Figure 6. SASH (B6.Cg-*Kit^{W-sh}*/HNihrJaeBsmJ) mice develop delayed decreased airway pressure at higher volumes when compared with C57BL/6J mice. Pressure–volume loops were generated by stepwise (seven equal steps) inflation and deflation of the lungs of C57BL/6J mice and SASH mice at baseline. (A) Four weeks ($n = 12$); (B) 10 weeks ($n = 10$ –14); (C) 14 weeks ($n = 11$). Ppl = airway tracheal pressure measured at each indicated volume (Vpl) on inflation and deflation limbs. WT = wild type.

Figure 7. SASH (B6.Cg-*Kit^{W-sh}/HNhrJaeBsmJ*) mice develop delayed increase in residual volume when compared with C57BL/6J mice. Direct measurement of residual volume from degassed lungs in age-matched C57BL/6J and SASH mice was performed. Data represent means + SEM. * $P < 0.01$ ($n = 4$ or 5). RV = residual volume; WT = wild type.

imatinib, an inhibitor of c-Kit tyrosine kinase, resulted in a dose-dependent inhibition of epithelial growth as shown by significant decreases in the mean size and size distribution of colonies (Figures 9A–9D). Treatment of epithelial progenitor cells with stem cell factor (SCF), the ligand for c-Kit tyrosine kinase, increased the size distribution of epithelial colonies (Figures 9E–9G).

DISCUSSION

An emerging global health problem, COPD is rapidly becoming the third most common cause of death and is the only major disease whose contribution to morbidity and mortality continues to increase (31). Emphysema, a key component of COPD, is characterized structurally by progressive loss of alveolar architecture and increased alveolar size, and functionally by a concomitant loss of gas exchange capacity in the lung (32). Thus, airspace enlargement and reduced lung compliance constitute a major cause of declining lung function, dyspnea, hypoxemia, and decreased quality of life in patients. Because no effective therapy exists to attenuate loss of lung function in patients with emphysema, insights into the cell biology of tissue loss could contribute to novel pharmacological interventions that could improve the prognosis of patients with COPD.

In our previous genetic analysis of lung function of age-matched offspring derived from the JF1/Ms mouse strain crossed with the C3H/HeJ mouse strain, we observed a QTL on mouse chromosome 5 that was associated with lung compliance. This analysis was based on an initial assessment of nine mouse strains, of which JF1/Ms and C3H/HeJ demonstrated the greatest phenotypic difference. In the present study, we found that CZECH/EiJ and NZW/LacJ had a slightly greater difference in lung compliance, but were quite similar to JF1/Ms and C3H/HeJ, respectively. On the basis of the likely candidate genes contained in the QTL region on chromosome 5, we selected *Kit* for additional analysis. By expanding the phenotyping of mouse strains by an additional 10 strains, we developed additional information useful in associating genotype with phenotypes.

On the basis of the results with these 19 mouse strains, an analysis of *Kit* genetic variants revealed several SNPs with possible functional consequences. Two promoter SNPs (rs37967821 and rs3799853) were of special interest. The first, rs37967821, alters a conserved sequence of an HOXB4-binding site such that it is converted to an MYB1 site. In the mouse lung, HOXB4 is expressed in mesenchyme and epithelial cells throughout development (33). In HSCs, HOXB4 treatment can produce HSC

Figure 8. SASH (B6.Cg-*Kit^{W-sh}/HNhrJaeBsmJ*) mice develop delayed decreased airway pressure at higher volumes when compared with C57BL/6J mice. *Ex vivo* pressure–volume loops were generated by stepwise (seven equal steps) inflation and deflation of surgically excised lungs from C57BL/6J mice and SASH mice. (A) Four weeks ($n = 4$); (B) 10 weeks ($n = 5$); (C) 14 weeks ($n = 4$). Ppl = airway tracheal pressure measured at each indicated volume (Vpl) on inflation and deflation limbs. WT = wild type.

expansion (34), whereas HOXB4 expression in HSCs can reduce long-term engraftment (35). The other promoter SNP, rs3799853, could eliminate a putative C/EBP α -binding site. Lung-specific gene-targeted *Cepba* mice have mildly altered fetal lung development, but at 90 days these mice have abnormal alveolar histology (diminished alveolar septa, increased mean linear intercept), mucous cell hyperplasia, and inflammation, features consistent with COPD (36). Previously, Didon and colleagues (37) reported that C/EBP-binding activity is increased in the airway epithelial cells of healthy smokers compared with never-smokers, whereas C/EBP-binding activity was not increased in the epithelium of smokers with COPD. Thus, these investigators proposed that inappropriate C/EBP activity could render the epithelium incompetent for efficient regeneration.

Figure 9. Primary lung epithelial cell growth is impaired by imatinib. Cells expressing epithelial cell adhesion molecule (EpCAM⁺ cells) were isolated from the lung by flow cytometric sorting and grown in culture with (A) control or (B) imatinib at 9.1 μ M. The average radius of all colonies for each condition was determined. (C) The mean radius of colonies is reduced by imatinib (2.7 and 9.1 μ M) when compared with saline treatment (* P < 0.01). (D) Number of colonies was characterized by radius, and the number of large colonies was greater with saline treatment when compared with imatinib (9.1 μ M). EpCAM⁺ cells were isolated from the lung by flow cytometric sorting and grown in culture with (E) control or (F) stem cell factor at 50 ng/ml. (G) Number of colonies was characterized by radius, and the number of large colonies was greater with stem cell factor treatment (6.25–100 ng/ml) when compared with control.

Both of the promoter SNPs have relatively high allelic frequency (~20%), which could explain much (~50%) of the phenotypic difference noted between the polar mouse strains. It is noteworthy that the CZECHII/EiJ and JF1/Ms mouse strains share the same genotype for these SNPs whereas the NZW/LacJ and C3H/HeJ mouse strains share the opposing genotype for these SNPs. Thus, the genotypes are consistent and serve to link our previous genome-wide analysis with this candidate gene.

In addition to the promoter differences, SNPs associated with CL were identified in other regions of the *Kit* gene. A nonsynonymous SNP (rs36972615) could produce an aspartic acid-to-glutamic acid conversion at amino acid position 927. This amino acid substitution is likely to have minor consequence to protein structure because these amino acids share identical side chain polarity (polar), side chain charge (negative), and hydropathy index (−3.5). Three of the 3' UTR SNPs (rs33755426, rs36266306, and rs36248044) involved G-from-A conversions that could disrupt segments of the polyadenosine mRNA region, a region that often alters mRNA stability. Together, our SNP interrogation suggests that regulation of *Kit* gene expression (transcription or message stabilization) may be more important than amino acid substitutions in the mouse. It should be noted that as with any study of genetic variants, the association may be not be due to the exact SNP that we examined but rather to another SNP in linkage disequilibrium with the SNPs identified in *Kit*. Furthermore, we do not know the functional consequences of each individual SNP identified to be associated with changes in CL. Thus, more direct functional evidence that *Kit* has a role in determining lung compliance was needed.

Further supporting the possible role of this candidate gene, lung function was examined in SASH mice that harbor an insertion in the 5' UTR of *Kit*. Importantly, the *Kit*^{W-sh} mutation

diminishes KIT mRNA expression in mast cells and mesenchyme cells of the lung on E13 and in adult lung, whereas expression in other tissues is normal (25). We observed altered lung histology in the SASH mouse that was accompanied by alterations in lung volumes and both static and dynamic compliance, which are indicative of an emphysema-like phenotype. These observations suggest that signaling through the KIT receptor tyrosine kinase is essential for maintenance of normal lung parenchymal homeostasis in mice. The specific molecular mechanism by which the presence of KIT spares the lung from developing spontaneous airspace enlargement during normal growth is worthy of additional investigation. Nonetheless, the SASH mouse provides an additional model of emphysema in which delayed-onset alveolar enlargement occurs in the absence of differences in inflammation. This suggests that dysregulation of *Kit* expression may be a more proximal cause of emphysema and possibly an event elicited by inflammation or inflammatory mediators.

The pathogenesis of COPD remains an area of considerable interest (38,39). Two hypotheses have been proposed for the etiology of emphysema in COPD, namely the proteinase-antiproteinase imbalance hypothesis (40, 41) and the oxidant-antioxidant hypothesis (42). Accordingly, mouse models of spontaneous emphysema have features consistent with one of these two hypotheses (43, 44). In the *pallid* mouse, for example, spontaneous emphysema is associated with an antiproteinase deficiency (45) that is presumed to increase the burden of active neutrophil elastase in the lung (46). Mice deficient in Toll-like receptor-4, the receptor for bacterial LPS, develop emphysema spontaneously that is associated with an imbalance in antioxidant capacity (44). It remains controversial whether spontaneous airspace enlargement observed in murine models is homologous to human emphysematous lung disease (47).

Human studies of COPD have focused mainly on chromosomes 2 and 12 and on a distal region of chromosome 4 (~145 cM). Human *KIT* is located on chromosome 4 (55 Mbp) and near/in a region (~55–70 Mbp) found to have suggestive linkage to both moderate airflow obstruction (48) and postbronchodilator flow limitation (49, 50). In addition, *KIT* is near a region (48–56 cM) associated with the ratio of FEV₁ to FVC in the Framingham Heart Study (51). To our knowledge, there are no human genome-wide association studies of lung compliance. Our studies using divergent strains of mice provide a candidate gene on human chromosome 4, which could contribute to lung compliance. Studies in animal models could additionally provide insight into the molecular and cellular mechanisms that regulate lung homeostasis.

Here, we have reported that many pathognomonic features of emphysema are evident in the SASH mouse. c-KIT protein is a recognized distinguishing marker for HSCs and is a receptor for c-KIT ligand, also known as stem cell factor. Given the association with stem cell function, we consider the possibility that *Kit* dysregulation may limit normal stem cell function and thereby contribute to the pathogenesis of airspace enlargement due to an inappropriate maintenance of alveolar structure, rather than loss of lung tissue due to dysregulation of response to injury. The role of stem cells in the maintenance of lung architecture remains an area of substantial controversy (52), with conflicting reports regarding whether cells derived from bone marrow can function as lung epithelial progenitor cells (17, 53). However, previous work suggests a role for bone marrow-derived cells in lung repair after injury with combined radiation and elastase (19, 21), and bone marrow transplantation can salvage spontaneous emphysema observed in the *tight skin* (Tsk) mouse that harbors a spontaneous *fibrillin 1* mutation (54). Furthermore, in a murine model of elastase-induced emphysema, intranasal hepatocyte growth factor causes a reduction in airspace enlargement, a return of static lung compliance to control levels, and repair of alveolar wall destruction that was associated with an increase in the expression of bone marrow-derived c-KIT-positive cells (55). Overall, previous studies suggest a potential role for bone marrow-derived cells in protection against the development of airspace enlargement in mice in the context of lung injury. However, we did not use a model of lung injury in our studies. Our findings suggest that c-KIT derived from either epithelia or fibroblasts contributes to normal lung homeostasis.

To determine whether KIT protein expression varied in mouse lung epithelial cells, we performed flow cytometric analysis (56) on dissociated lung cells to quantify alveolar and airway epithelial cell populations. No detectable difference was noted between the SASH and C57BL/6J mouse strains among either EpCAM^{pos}/Sca-1^{neg} (alveolar epithelial cells) or EpCAM^{pos}/Sca-1^{low} (conducting airway epithelial cells) populations within total lung cell preparations (Figures E2 and E3). However, we did observe differences in the capacity of epithelial progenitor cells to undergo *in vitro* clonal expansion in a manner dependent on c-Kit. Because of the limitations of our assay, it remains unclear whether these effects are either a direct or indirect effect of fibroblast-derived c-KIT on epithelia homeostasis. Evidence supports that bone marrow stromal cells can both attenuate parenchymal injury in a paracrine manner (57) and prevent arrested alveolar growth through paracrine activity (58). Furthermore, lung stromal cells can regulate the growth of lung epithelial cells (59). Interestingly, KIT mRNA is highly expressed in the developing lungs of mice (E14.5) in stromal cells that are in close proximity to epithelia (Figure E4). In humans, KIT-immunoreactive interstitial cells have been observed in the alveolar septa of 12 normal newborn lungs, but were absent in lungs from 2 cases of congenital alveolar capillary dysplasia

(18). On the basis of these observations, we hypothesize that *Kit* expression in interstitial mesenchyme or bone marrow stromal cells facilitates an appropriate alveolar septa microenvironment, which helps maintain alveolar structure possibly through local paracrine activity.

Our study has a limitation in that at this time we cannot identify or assign the specific cell types that are required to express *Kit* to maintain adult lung structure, but rather highlights the temporal consequences of *Kit* deficiency to the development of lung structure and function. Although the specific role of *Kit* in bone marrow-derived stem cells in this model remains unknown, our study supports a role for *Kit* expression in lung development and maintenance of normal lung architecture in a monopodial branched lung, as exists in rodent models, and suggests that a better understanding of strategies to control *Kit*-dependent signaling in lung injury and repair may be of value to restore normal lung homeostasis.

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