

Gob-5 Is Not Essential for Mucus Overproduction in Preclinical Murine Models of Allergic Asthma

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Overexpression of Gob-5 has previously been linked to goblet cell metaplasia and mucin overproduction in both *in vitro* and *in vivo* model systems. In this study, Gob-5 knockout mice were generated and their phenotype was evaluated in two established preclinical models of allergic asthma. We sought to determine whether the Gob-5-null animals could produce less mucus in response to allergic challenge, and whether this would have any impact on reducing goblet cell metaplasia and airway inflammation. We found that in the absence of a proinflammatory stimulus we could not detect an overt phenotypic difference between age and sex-matched knockout and wild-type animals. Allergic challenge with ovalbumin or intranasal administration of interleukin-13 produced a robust allergic response that was similar regardless of genotype. In addition, siRNA-mediated knockdown of CLCA-1 in cultured lung epithelial cells failed to reduce mucin expression *in vitro*. Thus, in contrast to previously published reports, our findings show that Gob-5 expression is not essential for mucin overproduction *in vitro* or in murine models of allergic asthma. Furthermore, we have also exploited the use of gene expression array analysis to investigate the possibility that a compensatory mechanism, involving other genes, may act to override the requirement for Gob-5-mediated mucus overproduction.

Respiratory mucus consists of a diverse family of viscoelastic glycoproteins, known as mucins, that are synthesized and secreted by epithelial goblet cells and submucosal gland cells (1, 2). Post-translational modification and assembly of mucin molecules into high molecular weight concatamers (> 30 megadaltons) has made the identification and biochemical characterization of mucins isolated from the respiratory tract difficult (3, 4). In humans, at least seven of the known mucin genes are transcriptionally expressed in the lung. Among these, MUC5AC and MUC5B are the major secreted gel-forming mucins expressed in the respiratory tract, and their overproduction has been reported in various chronic respiratory diseases (1, 3, 4). It has been suggested that an imbalance in the quality or quantity of mucus glycoproteins may impair mucociliary clearance and thereby promote airway obstruction, respiratory infection, and overall diminished lung function (1–3). In diseases such as asthma and chronic obstructive pulmonary disease (COPD), there is a significant association between mucus hypersecretion and increased morbidity and mortality (5–7). Postmortem analysis of patients who have died from an acute asthmatic attack show significant goblet cell hyperplasia, together with increased

intraluminal accumulation of mucus in the peripheral airways and mucus plugging that extends to both large and small airways (8).

The mechanisms by which goblet cells and mucus gland cells are activated to undergo expansion and differentiation to a mucin producing phenotype are poorly understood (1, 9). Possible mechanisms include activation by proinflammatory cytokines (e.g., interleukin [IL]-4, IL-9, IL-10, IL-13) or other mediators produced either by infiltrating cells (lymphocyte, leukocyte) or by resident cell (epithelial, smooth muscle) populations (1, 2, 5, 9). In accordance with this, studies with IL-10 (10) and IL-13 transgenic mice (11) or mice instilled with recombinant IL-13 (12) have demonstrated enhanced mucus production in these animal systems.

Recent studies using preclinical mouse models of asthma have shown that overexpression of the murine ortholog (Gob-5) of human CLCA-1 (calcium-activated chloride channel-1, hCLCA-1) is coupled to mucin hypersecretion, increased airway hyperresponsiveness, and infiltration of inflammatory cells into the lungs (13, 14). In these animal models, Gob-5 was found to be induced in response to ovalbumin (OVA) sensitization/challenge as well as to IL-9 overexpression. Both IL-4 and IL-13 have also been found to significantly increase the expression of CLCA-1 in cultured cell systems and/or in preclinical animal models of allergic lung inflammation (14). Furthermore, adenovirus-mediated overexpression of Gob-5 in the lung has been shown to exacerbate an allergic inflammatory response to OVA and to increase acetylcholine-induced airway hyperreactivity (13). Conversely, expression of an antisense Gob-5, in this experimental paradigm, was found to attenuate airway responsiveness, mucus production, and inflammatory cell infiltration into the lung (13).

Support for a pathophysiologic role of hCLCA-1 in disease has stemmed from two additional lines of evidence in humans. First, clinical studies have demonstrated an upregulation of hCLCA-1 expression in the lungs of patients with asthma as well as COPD and cystic fibrosis (15–17). Second, recent genetic analysis suggests a possible linkage between the hCLCA gene cluster and a known asthma locus associated with four asthma-related phenotypes (bronchial responsiveness, skin test response, total IgE, eosinophil count) (18). Furthermore, SNP analysis of the human CLCA gene has revealed associations with COPD (19) as well as childhood and adult asthma (20).

The mechanisms that regulate the activity of hCLCA-1 have not been elucidated. Its putative role as a calcium-activated chloride channel was suggested on the basis of its structural features, homology to its bovine ortholog (bCLCA-1), and preliminary electrophysiologic analysis (21). However, these studies have not ruled out the possibility that hCLCA-1 functions as an accessory or regulatory component of an existing or entirely novel channel. More definitive proof of intrinsic channel activity awaits reconstitution and electrophysiologic characterization of purified hCLCA-1 in artificial lipid bilayers. Similarly, little is known about the signal transduction pathways that couple channel activity to mucin hypersecretion. *In vitro* studies using cultured cell systems (e.g., NCI-H292, Caco-2) have demonstrated that

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overexpression of hCLCA-1 or its murine counterpart, Gob-5, results in the transcriptional induction and secretion of Muc5AC (13, 22). Consistent with its putative function as a chloride channel, the production of mucin in these cultures was shown to be inhibited by pretreating the cells with nonselective chloride channel inhibitors (e.g., niflumic acid), albeit at high concentrations ($> 10 \mu\text{M}$), which may conceivably impact other molecular pathways (22).

To assess the role of Gob-5 *in vivo*, we generated mice with a homozygous null mutation of the Gob-5 gene and tested them in two widely accepted preclinical murine models of allergic asthma. In response to both acute IL-13 instillation and OVA challenge, the phenotype of the Gob-5 null mice was indistinguishable from their wild-type (WT) littermates, regardless of the allergic end-point measured. These data demonstrate that Gob-5 expression in and of itself is not necessary for mucin overproduction mediated by proinflammatory signals in mice.

MATERIALS AND METHODS

Generation of the Gob-5 Knockout Mice

Heterozygote Gob-5 knockout (KO) mice were obtained from Deltagen (San Carlos, CA). A schematic diagram of the murine Gob-5 gene, including the disrupted region, is outlined in Figure 1. Briefly, Gob-5 KO mice were generated by homologous recombination using ES cells derived from the 129/OlaHsd mouse substrain. F1 mice were generated by breeding chimeras bearing a disruption of the Gob-5 gene with C57BL/6 females. The resultant F1N0 heterozygotes were backcrossed to C57BL/6 mice to generate F1N1 heterozygotes. F2N1 homozygous Gob-5 mutant mice were produced by intercrossing F1N1 heterozygous males and females. Genotyping of the progeny was performed by PCR of genomic tail DNA using primers spanning the LacZ-Neo insertion site and confirmed by Southern blot analysis (not shown).

Allergen or Th2 Cytokine Challenge

Gob-5 KO mice and their WT littermates (males and females, $n = 4-5/\text{group}$) were sensitized by intraperitoneal administration of 20 μg of OVA (Grade V; Sigma-Aldrich, St. Louis, MO) in presence of 2 mg of aluminum hydroxide on Days 1 and 14 (23). On Days 28, 29, and 30, they were subjected to a 20-min aerosol challenge of either phosphate-buffered saline (PBS) or 1% OVA using a nose-only inhalation system (CH Technologies Inc., Westwood, NJ). Alternatively, 5 μg of recombinant murine IL-13 (rmIL-13; Cell Sciences, Canton, MA) or vehicle (50 μl PBS) was administered intranasally for five consecutive days to groups of five mice under isoflurane anesthesia.

Determination of Cellular Infiltration and Muc5ac Levels in Lung Lavage Fluid

Bronchoalveolar lavage (BAL) and tissue harvest were performed 48 h after the last allergen challenge and 72 h after the last rmIL-13 instillation. Under sodium pentobarbital anesthesia (65 mg/kg intraperitoneally, *Somnotol*; MTC Pharmaceuticals, Cambridge, ON, Canada), the lungs were lavaged with 0.5 ml of room temperature PBS injected twice via a tracheal cannula. The total number of cells present in the BAL fluid (BALF) was determined using a Cell-Dyn 3700 hemacytometer (Abbott Laboratories, Mississauga, ON, Canada). Cytospins were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA) and stained with modified Wright-Giemsa. Manual differential cell counts were made on 100 cells/slide.

For mucin determinations BALF was centrifuged at $350 \times g$ (14,000 rpm) for 10 min at 4°C , and the supernatants were kept at -80°C until assayed. Muc5ac levels in the BALF were measured by sandwich ELISA using commercially available antibodies (Lab Vision, Fremont, CA). Briefly, Muc5ac present in the BALF was captured to a microtiter plate using an anti-Muc5ac monoclonal antibody (I-13M1). A second antibody tethered to biotin was used to detect the captured Muc5ac (45M1). Streptavidin-horseradish peroxidase (HRP) was added to the plate, and after a 30-min incubation/wash cycle it was followed with peroxidase substrate to generate a colorimetric product that was quantified by spectrometry. Data are expressed as arbitrary

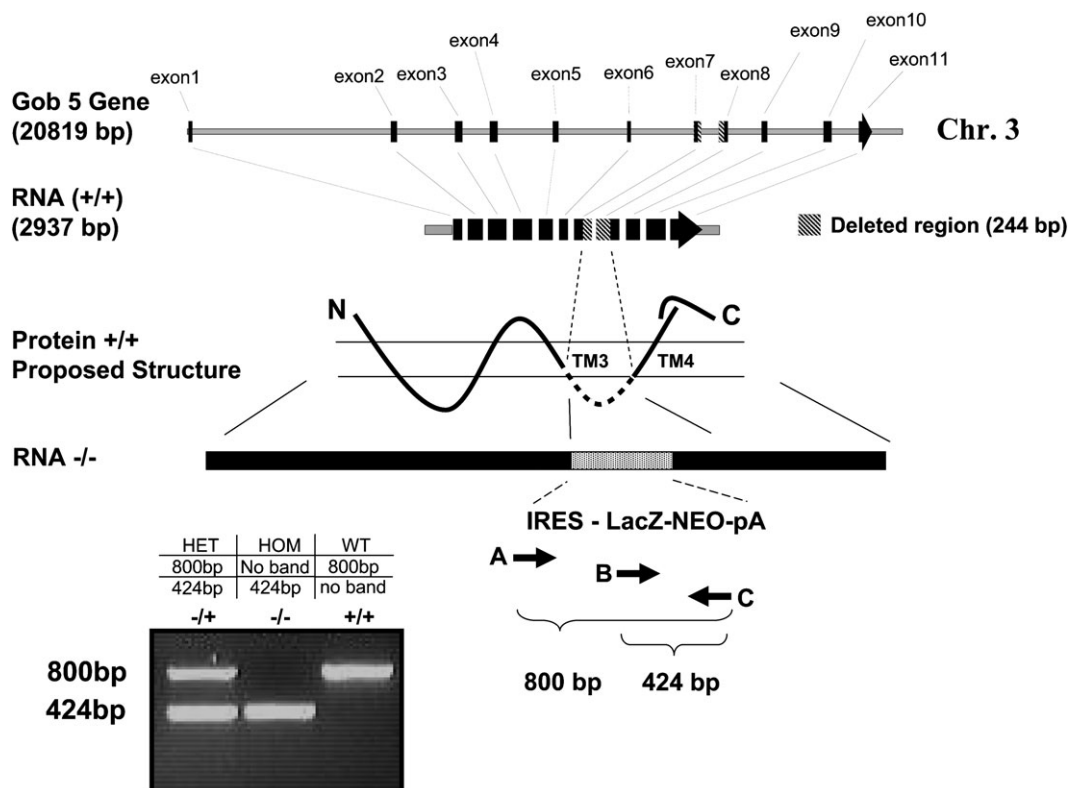


Figure 1. Schematic diagram of the knockout strategy depicting the Gob-5 gene and resulting disrupted mRNA. Gob-5 KO mice were generated by homologous recombination using a targeting construct that resulted in the deletion of a 244-bp segment of the coding region encompassed by the C-terminal end of exon 7 and the N-terminal end of exon 8. The deleted region corresponds to the C-terminal end of the putative third transmembrane domain as well as the intracellular domain between transmembrane regions 3 and 4 (21). As a consequence of recombination, the targeting construct introduced an IRES-lacZ-Neo fusion cassette at the site of disruption. The gel below shows the expected pattern of DNA fragment sizes as determined by PCR of genomic tail DNA using paired combinations of oligos A, B, and C as indicated.

units relative to a Muc5ac standard curve that was included on each plate.

Quantification of Mucus-Producing Cells

After lavage, the left lung was removed and rapidly frozen in liquid nitrogen for RNA or protein extraction. The animals were then perfused via the left cardiac ventricle with 20 ml of PBS followed by 10 ml of 4% buffered neutral formalin (BNF). The right lung was inflated via the trachea with 2 ml of 4% BNF before being removed and stored overnight in the same solution. After processing and embedding in paraffin, 5- μ m mid-trachea longitudinal sections were affixed to microscope slides and stained with periodic acid-Schiff (PAS) and Alcian blue (AB) for identification of mucus-producing goblet cells. The total amount of PAS/AB staining was quantified under light microscope and normalized to total basement length using the Northern Eclipse imaging software (Empix Imaging, Mississauga, ON, Canada).

Preparation of Murine Tissue Homogenates

Mouse tissues were stored frozen at -80°C until use. Frozen tissues were crushed with a mortar and pestle under liquid nitrogen and then placed in ice-cold lysis buffer (50 mM Tris, pH 8.0, containing 0.15 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and Complete protease inhibitor cocktail [Roche Diagnostics, Basel, Switzerland]). Tissues were disrupted twice, for 10 s each, on ice using a tissue homogenizer (Biospec Products, Bartlesville, OK), and briefly sonicated at 4°C , using a Cole Parmer 4710 series ultrasonic sonicator (Cole Parmer Instruments, Vernon Hills, IL). After a 30-min incubation on ice, debris was cleared by centrifugation at $1,000 \times g$ for 15 min at 4°C . The resultant supernatants were decanted and designated as total cell protein lysates. Protein concentrations were determined for each sample using a Bradford protein assay kit (Bio-Rad, Hercules, CA).

siRNA Knockdown in Cells

NCI-H292 cells were seeded overnight at a subconfluent density in 6-well plates and then washed once before transfection with 100 nM of a double-stranded siRNA oligonucleotide in the presence of a transfection reagent (TKO; Mirus, Madison, WI). The siRNA oligos tested were designed against either CLCA-1 or a control gene, lamin A/C, and obtained commercially (Dharmacon, Lafayette, CO). Cultures were harvested at various times after transfection (0.5–31 h) and processed for protein or RNA analysis (*see below*). Supernatants from each time point were collected and analyzed for Muc5ac production by ELISA or examined for evidence of cytotoxicity. The latter was determined using a lactate dehydrogenase (LDH) detection assay (Roche Diagnostics).

SDS-PAGE and Immunoblot Analysis

Tissue lysate fractions were mixed with 0.5 vols of SDS sample buffer (20 mM Tris-HCl, pH 6.8, containing 0.4% [wt/vol] SDS, 4% glycerol, 0.24 M β -mercaptoethanol, and 0.5% bromophenol blue), and analyzed by SDS-PAGE on a 4–20% Tris-glycine acrylamide gels (Invitrogen, Carlsbad, CA). Proteins were electrophoretically transferred to nitrocellulose membranes and blocked in Tris-buffered saline/5% nonfat milk before hybridization. Primary rabbit antiserum to hCLCA-1 (raised against amino acids 134–148 of human CLCA-1: ERIHLTPDFI AGKKL) was used at a final dilution of 1:1,200. The secondary HRP-linked donkey anti-rabbit IgG antibody (Amersham Biosciences, Piscataway, NJ) was used at a final dilution of 1:3,000. GAPDH detection was performed using a mouse anti-rabbit GAPDH antibody (1 $\mu\text{g}/\text{ml}$; Research Diagnostics, Concord, MA), followed by incubation with an HRP-linked sheep anti-mouse IgG secondary antibody (1:3,000 dilution; Amersham Biosciences). Immunodetection was performed using a standard chemiluminescent HRP substrate (Perkin Elmer, Boston, MA). Protein bands were visualized using a FUJI LAS-1000 Plus Luminescent Image Analyzer (Fuji Photo Film Co., Tokyo, Japan).

RT-PCR

Total RNA was extracted from mouse tissues using a total RNA isolation kit (RNeasy kit; Qiagen, Valencia, CA). Samples were treated with DNase (Qiagen) before reverse transcription (Roche Diagnostics). The following primers were used to amplify a 687-bp fragment spanning the site of gene disruption of mCLCA-3 by RT-PCR (Advantage cDNA

PCR kit; Clontech, Mountain View, CA): forward strand, (A') 5'-CCAT GGTCTTGTGATGCTTTCG and reverse strand, (A'') 5'-CTCTG TCTGTCTGAAGTGACTCCTC. Oligo pairs (B') forward, 5'-GGGA AAGCTGCAGGATGGAATCTT) and (B'') reverse, 5'-CCCATACT GAGTCAGCTTCTTTC CTGC) and (C') forward, 5'-GGATTGAGG ATGGTGAAGTAAG) and (C'') reverse, 5'-GCCTGAAAATTCA GTGCAAAC) were used to amplify the 5' (458 bp) and 3' (648 bp) coding regions of the Gob-5 gene, respectively. Expression of elongation factor 1- α (eF1- α) was monitored as a 250-bp RT-PCR amplicon using the following oligo primer set: forward, 5'-GCATGGTGGTTACC TTTGCT; reverse, 5'-AGCGTAGCCAGCACTGATT. The PCR reactions were denatured for 105 s at 95°C and subjected to 35 cycles of PCR (95°C for 30 s, 60°C for 30 s, 72°C for 40 s). For the analysis of Muc5ac expression by RT-PCR, a 293-bp fragment was amplified using the forward, 5'-CATCTCTACAACCCAACTA and reverse, 5'-GAG GAGGGTTTGATCTGTTT primer pair. The PCR reactions were denatured for 2 min at 94°C and subjected to 30 cycles of amplification (94°C for 30 s, 62°C for 30 s, 72°C for 1 min). Similarly, a 450-bp fragment of GAPDH was amplified using a forward, 5'-ACCACAGTC CATGCCATCAC and reverse, 5'-TCCACCACCCTGTTGCTGTA primer set. The PCR reactions were denatured for 2 min at 94°C and subjected to 30 cycles of amplification (94°C for 15 s, 58°C for 20 s, 72°C for 1 min). All PCR products were separated by agarose gel electrophoresis and visualized using the Bio-Rad gel documentation system. Expression of Muc5ac in mouse lung tissues was normalized relative to the level of GAPDH expression.

For quantitative PCR analysis, RNA samples were processed by Taqman reverse transcription followed by PCR amplification using the ABI Prism 7900 HT detection system (Applied Biosystems, Foster City, CA). Taqman primers for the internal control (18S RNA) were provided in the ABI detection kit. The PCR reactions were denatured for 2 min at 50°C followed by 10 min at 95°C and then subjected to 50 cycles of amplification (94°C for 15 s followed by 1 min at 60°C). Primers for CLCA-1, Muc5ac, and Muc5B were obtained from Biosource (Camarillo, CA). The primer combinations used were as follows: (1) CLCA-1 (forward), 5'-GATCCACCTCACTCCTGATTTC-3'; CLCA-1 (reverse), 5'-GCCCCACTCATGGACAAATGC-3'; CLCA-1 (probe), 5'-FAM-TGCAGGAAAAA GTTAGCTGAATATGGACCACA-BHQ1-3'; (2) Muc5ac (forward), 5'-GCGTGGAGATGAGAAGTATGCT-3'; Muc5ac (reverse), 5'-CAAACATGCAG TTCGAGTAGGTT-3'; Muc5ac (probe), 5'-FAM-CCGGTGCCATGCTGCCGTG-BHQ1-3'; (3) Muc5B (forward), 5'-CCATCTGCCACCTGATTCT-3'; Muc5B (reverse), 5'-GACGCAGCCCTCATAGAA-3'; Muc5B (probe), 5'-FAM-CCGTGCCACACTGT GATC-BHQ1-3'.

Gene Chip Analysis

The relative abundance of roughly 23K distinct mRNAs in the mCLCA-3 deficient (KO) and WT mice, with and without OVA challenge, was determined. From each of the following conditions (WT-PBS, WT-OVA, KO-PBS, KO-OVA), total RNA was isolated from three individually snap-frozen whole left-lobe lungs using the RNeasy midi kit and protocol (Qiagen). Two samples were processed for the WT-OVA, for a total of 11 samples. Aliquots of the samples were hybridized to proprietary two-color 60-mer oligonucleotide microarrays (Agilent, Palo Alto, CA) using a common-reference design and fluor-reversal, as follows: aliquots containing equal amounts of RNA were pooled from the three WT-PBS samples and labeled with the Cy3 fluorescent dye (Agilent) as the control channel. Aliquots of each of the other eight experimental samples (i.e., three KO-PBS, two WT-OVA, and three KO-OVA) were labeled with the Cy5 fluorescent dye (Agilent), as the experimental channel. Then the entire paradigm was repeated on separate aliquots of the same samples with the Cy3 and Cy5 labels reversed, generating a complete set of eight technical duplicates.

Image acquisition and quantification of the raw data from the 16 arrays was completed as previously described (24). As described therein, poor array features and irregularities were computationally and manually flagged and excluded from further analysis. Also described therein, the Rosetta Resolver (version 4.0) (Rosetta Biosoftware, Seattle, WA) gene expression data analysis system was used to normalize, transform, and combine the technical fluor-reversed duplicates. The technical reproducibility of the arrays was assessed by calculating the correlation for the log-ratios for each probe from the technical duplicates (the

fluor-reversed pairs). If the correlation coefficient for the probes considered to be differentially regulated was > 0.99 , the arrays were included in the analysis. The Resolver error model was used to assign measures of confidence to each biological replicate, and then to combine replicates and assign confidence measures to each of the three experimental conditions. Thus, for each probe we calculated an error-weighted measure of the relative mRNA abundance for each of three comparisons: (1) WT-PBS versus KO-PBS (3 samples, 6 chips); (2) WT-PBS versus WT-OVA (2 samples, 4 chips); and (3) WT-PBS versus KO-OVA (3 samples, 6 chips). For these direct comparisons, evidence for differential expression was only considered if the combined absolute fold change was > 2 -fold, and if the combined P value was < 0.001 . Probes with low intensities are generally excluded by the error model, but were also excluded manually if their normalized log intensity was < -1.5 .

For the indirect comparisons of WT-OVA to KO-OVA, the difference in combined log ratios for the WT-PBS versus KO-OVA comparison and the WT-PBS versus WT-OVA comparison was calculated, for each probe. A standard t test between these two groups ($n_1 = 6$ and $n_2 = 4$, respectively) was used to calculate the probability that this

difference was significant. Only probes with a 2-fold change and a P value < 0.001 were considered significantly different between the KO and WT OVA treatments.

Statistical Analysis

All data in the Figures 1–6 are expressed as the mean $\pm$ SEM, and n represents the number of animals tested. Statistical significant differences among groups were determined using an ANOVA with multiple comparisons (Tukey). Differences were considered to be statistically significant for P values of < 0.05 .

RESULTS

Generation of the Gob-5 KO Mice

Gob-5 KO mice were generated through targeted deletion of a 244-bp segment of the coding region spanning exons 7 and 8 (Figure 1). This region corresponds to nucleotides 1601–1844 of the mRNA and encompasses the putative C-terminal region of

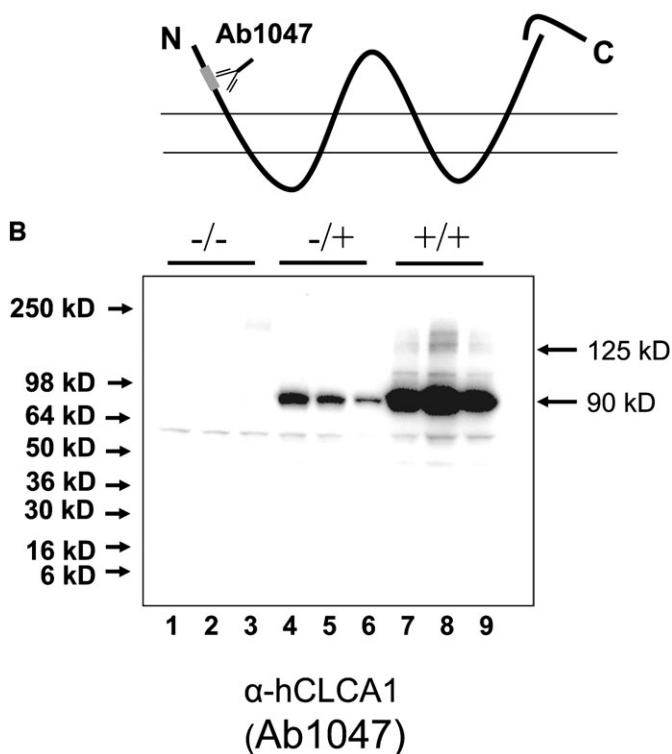
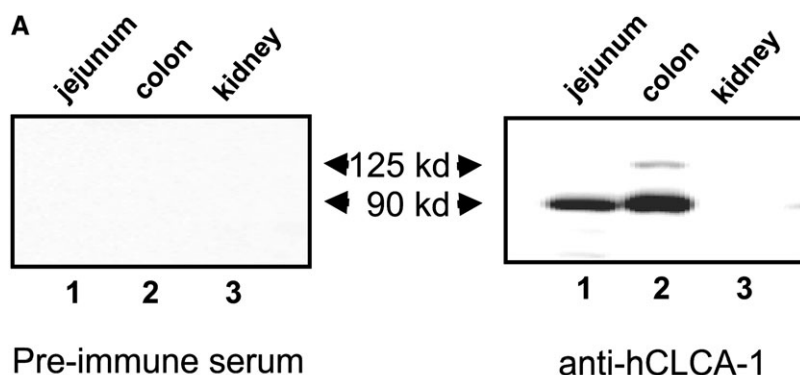


Figure 2. Expression of Gob-5 in normal mouse tissues. Immunoblot analysis of mouse tissues using anti-Gob-5 peptide antiserum. Total tissue protein lysates (53 μ g) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Gob-5 (Ab1047) or preimmune antiserum, followed by detection with chemiluminescence. (A) Analysis of Gob-5 expression in jejunum, colon, or kidney tissue of C57/BL6–129 mice. The schematic diagram depicts the putative membrane structure of hCLCA-1 and indicates the position of the N-terminal epitope used to generate the polyclonal peptide antibody (Ab1047) (see MATERIALS AND METHODS) (21). (B) Analysis of Gob-5 expression in the colon of KO mice (lanes 1–3), heterozygote mice (lanes 4–6), and WT mice (lanes 7–9). The position and apparent molecular weights of the Gob-5 subunits are indicated on each panel.

transmembrane domain-3 (TM3) as well as the second intracellular region between TM3 and TM4 (21). The genomic region deleted by the targeting vector was replaced by a LacZ-Neo reporter cassette that could be used to track Gob-5 expression *in vivo*. Genotyping of chimeric mice and subsequent progeny was performed by PCR (Figure 1) and confirmed by Southern blot analysis (not shown). Gob-5 expression was evaluated by immunoblot analysis of gastrointestinal tissue, previously shown to express high constitutive levels of Gob-5 message (Figure 2)

(21). Experiments were performed using an affinity-purified anti-body raised against the amino-terminal end of Gob-5 (amino acids 134–148), which is capable of recognizing both the mature (90-kD) and unprocessed (125-kD) form of the protein (21). A comparison of different tissues indicates that the colon and jejunum, but not the kidney or lung of normal WT mice, expressed high constitutive levels of Gob-5 protein (Figure 2 and Figure 3B, lanes 9 and 10).

Predictably, homozygous null mice failed to express detect-

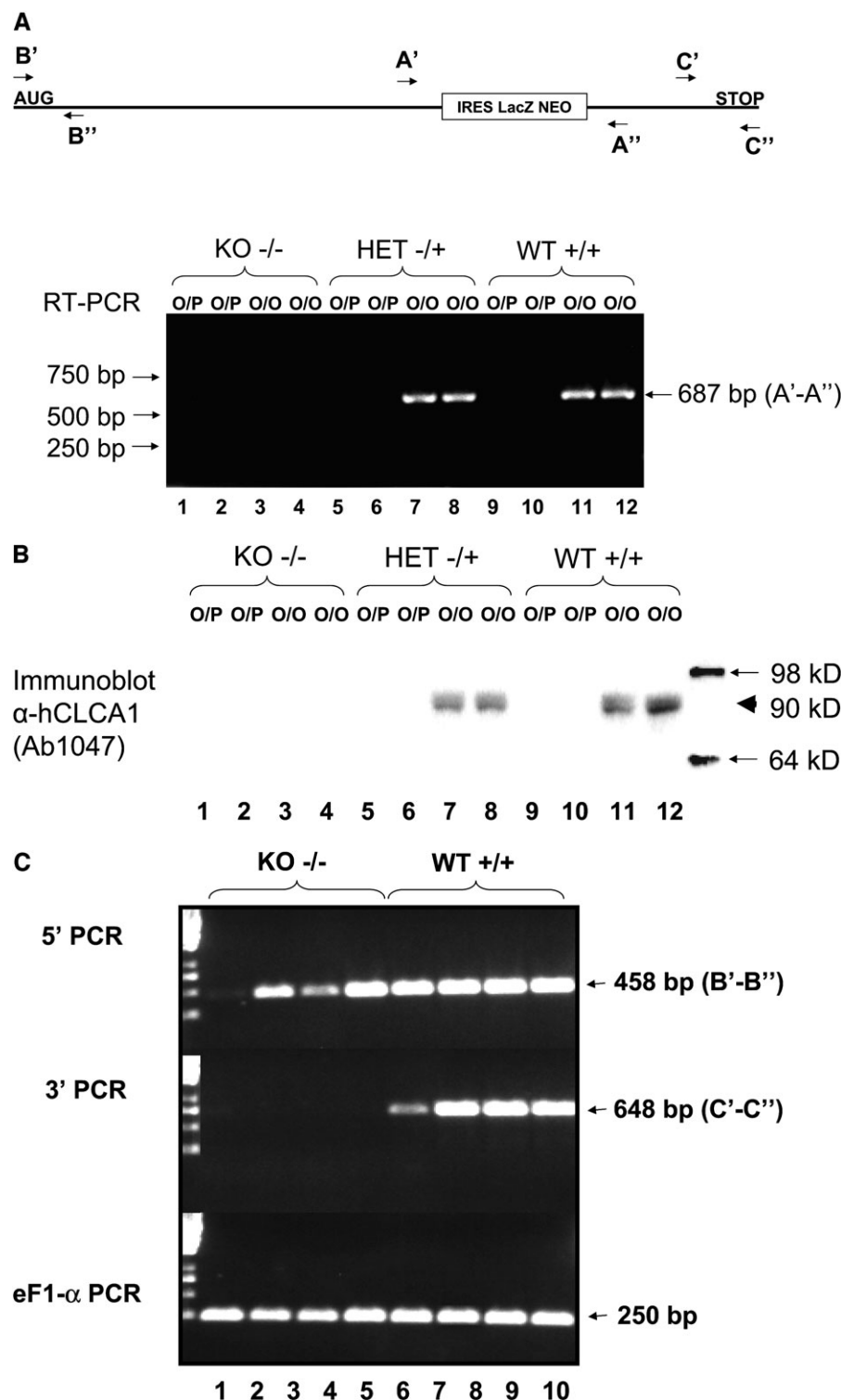


Figure 3. Analysis of Gob-5 expression and gastrointestinal histology in Gob-5 KO mice. Expression of Gob-5 mRNA (A) and protein (B) in lungs of mice subjected to OVA sensitization and PBS challenge (O/P), or OVA sensitization followed by OVA challenge (O/O). (A) A schematic of the disrupted Gob5 gene with the position of different oligo primer sets used to analyze expression by RT-PCR is shown. Total RNA was extracted and reverse-transcribed, and PCR was performed using primers that generate an amplicon spanning the region of gene disruption (A'-A'') as indicated in MATERIALS AND METHODS. (B) Lung lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Gob-5 antiserum (Ab1047), followed by detection with chemiluminescence. RNA or protein extracted from O/P-challenged mice is shown in lanes 1–2, 5–6, 9–10; RNA or protein extracted from O/O-challenged mice is shown in lanes 3–4, 7–8, 11–12. (C) Analysis of Gob5 RNA expression in the gastrointestinal tract of the Gob5 KO mice. Total RNA was isolated from colon tissue of WT, heterozygote (HET), and KO mice and analyzed by RT-PCR using primer pairs spanning either the disrupted (A'-A''), 5' (B'-B''), or 3' (C'-C'') region of the Gob-5 gene. DNA or protein molecular weight markers are indicated in A–C, as are the positions of the amplicons amplified by RT-PCR. (D) Analysis of goblet cell staining and mucin production in the gastrointestinal tract of the Gob-5 KO mice. Tissue sections (10 μ m) from the colon, jejunum, and ileum of the Gob-5 WT and KO mice were stained with PAS–Alcian blue. The various tissue layers are shown. Mu, mucosal layer; SM, submucosal layer; MS, muscularis layer. The arrows indicate mucin-containing goblet cells. Magnification: $\times 20$.

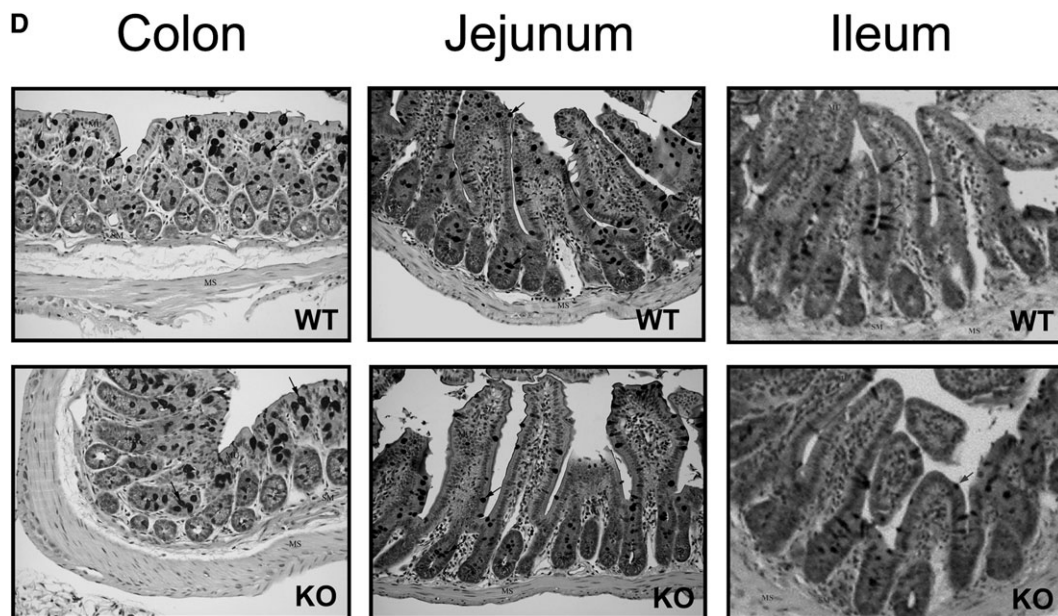


Figure 3. Continued

able levels of Gob-5 protein in the colon (Figure 2B, *lanes 1–3*), while the heterozygotes expressed less protein than what was observed with the WT animals (Figure 2B, *lanes 4–9*). A notable induction of Gob-5 mRNA and protein was observed in the lungs of both WT and Gob-5 heterozygous mice, but not homozygous KO mice, after an allergic challenge with OVA (Figures 3A and 3B). In this experiment, mRNA expression was detected by RT-PCR using primers that spanned the site of gene disruption (A'-A'', 687-bp amplicon; *see schematic in Figure 3A*). To evaluate whether a truncated transcript might be expressed from the disrupted allele, additional RT-PCR analysis was performed across the 5' and 3' coding regions of the gene (Figure 3C). The expected RT-PCR amplicon (648 bp) spanning the 3' end of the coding region was not detected in the KO mice, but was detected in all four WT littermates examined (Figure 3C, *middle panel*). Interestingly, the 458-bp RT-PCR amplicon spanning the 5'-end of the coding region (nucleotides -14 to 444) was detectable in all WT animals and three of the four KO animals examined, albeit at different levels relative to eF1- α expression (Figure 3C, *top panel*). If stable, this truncated 5' message, which is at least 458 nucleotides in length, could potentially give rise to a protein of 16,000 kD or greater. However, this 5' transcript is likely unstable and does not encode a functional protein, as we were unable to detect a truncated polypeptide of any size in either the homozygote or heterozygote Gob-5 KO animals (Figure 2B). The antibody used to detect Gob-5 protein in this experiment was raised against a peptide epitope that is comprised within the region encoded by the 458-bp 5' amplicon and as such would have detected a truncation if it was produced. Although it was not generated, the potential functional significance of such a truncation is difficult to assess. Conceivably, it might act as a dominant-negative to augment the inhibition of Gob-5 or other related family members in animals carrying one or both alleles of this mutation.

Effect of Gob-5 Gene Disruption on Mucus Production in the Gastrointestinal Tract

Despite their lack of Gob-5 expression, the homozygous KO mice did not display any overt physical or behavioral phenotype relative to their age- and sex-matched WT littermates. Progeny obtained from heterozygous matings were of normal size and

segregated according to the expected Mendelian distribution (1:2:1). Both homozygous mutant males and females were fertile and there were no genotype-related differences noted between mutant and WT control mice for any of the parameters evaluated at necropsy.

Given the constitutive level of Gob-5 expression observed in the colon of WT animals (Figure 2A) and the putative link between goblet cell metaplasia, mucin overproduction, and Gob-5 expression (13), we compared the histologic pattern of PAS-Alcian blue staining in the gastrointestinal tract between the KO and WT animals (Figure 3D). No apparent differences in tissue architecture or goblet cell staining could be detected in the ileum, jejunum, or colon (Figure 3D). Furthermore, transcriptional expression of Muc5ac in the colon was not impaired in the KO animals as compared with the WT controls (not shown).

Mucin Overproduction in the Lungs of Gob-5 KO Mice Is Not Attenuated after Allergic Challenge

Although the absence of constitutive Gob-5 expression in the colon of the KO mice failed to decrease mucin production, the possibility remained that a high level of Gob-5 expression induced in the lung in response to a proinflammatory stimulus might drive the hypersecretion of mucin observed in acute murine models of asthma. This notion is supported by studies demonstrating an exacerbation or attenuation of asthma-related phenotypes after tracheal instillation of adenoviruses encoding Gob-5 sense or Gob-5 antisense RNAs, respectively (13). To address this possibility, we subjected the Gob-5 KO mice to two distinct preclinical models of allergic asthma and compared their responses to WT littermate controls. In the first model, mice were presensitized with OVA and then subjected 2 wk later to a series of daily challenges with the same aerosolized allergen. Forty-eight hours after the final challenge, mice were killed and evaluated for the overproduction of mucin and infiltration of proinflammatory cells into the lung (Figure 4). Analysis of the BALF indicated a robust increase in both Muc5ac production and total cell counts in the OVA-treated animals relative to the vehicle controls (Figures 4A and 4B, respectively). An increase in both mucin and goblet cell numbers (Figures 4C and 4D) was also noted in the airways of OVA-treated mice as determined

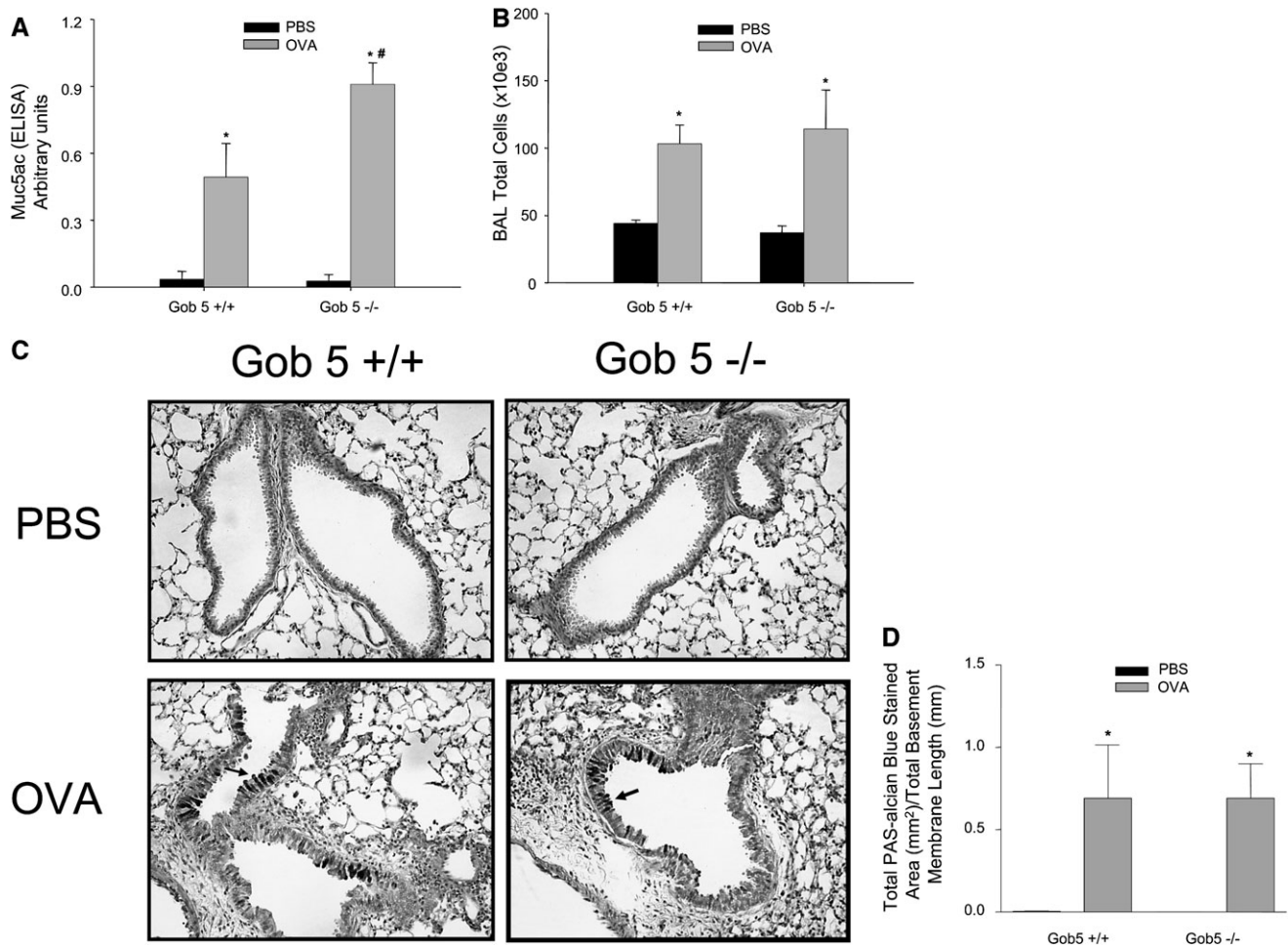


Figure 4. Deletion of Gob-5 fails to attenuate lung inflammation or mucus after antigen challenge. (A) Muc5ac levels in the BAL supernatant were elevated for both Gob-5 +/+ ($n = 4/\text{group}$) and Gob-5 -/- ($n = 5/\text{group}$) mice after antigen challenge as detected by ELISA. The increase in Muc5ac levels after antigen challenge was greater for Gob-5 -/- mice compared with Gob-5 +/+ mice. *Statistically significant from PBS-treated ($P < 0.05$); # statistically significant from Gob-5 +/+ after OVA challenge ($P < 0.05$). (B) Antigen challenge caused a similar increase in the total number of inflammatory cells in the BALF of both Gob-5 +/+ and Gob-5 -/- mice. BAL was taken 48 h after the last OVA challenge. *Statistically significant ($P < 0.05$) from PBS-treated. (C) Analysis of PAS–Alcian blue staining of mucus and mucus-secreting cells in the airways of Gob-5 +/+ and Gob-5 -/- mice after OVA challenge. No genotype-dependent morphological differences were observed between the groups. Goblet cells are indicated by the arrows. Magnification: $\times 20$. (D) Quantification of PAS–Alcian blue staining from lung tissue sections of Gob-5 +/+ and Gob-5 -/- mice after OVA challenge. *Statistically significant from PBS-treated ($P < 0.05$).

by PAS–Alcian blue staining. Surprisingly, however, the Gob-5 KO animals failed to show an attenuated inflammatory response after allergen challenge. On the contrary, our studies indicated that the KO animals developed an exacerbated mucin secretory response ($P < 0.05$) relative to their WT littermates after OVA treatment (Figures 4A and 4B). However, the relevance of this finding is unclear, given that genotype-related differences in tissue mucin levels, as determined by histologic analysis, were not statistically significant (Figures 4C and 4D).

In light of previous studies demonstrating a significant stimulation of Gob-5 expression in the lungs of mice instilled with IL-13 (14), we reasoned that perhaps Gob-5-mediated mucin overproduction might be stimulus-dependent and driven by IL-13-specific signaling pathways that are distinct from those mediated by OVA challenge. To examine this possibility, we evaluated the allergic responses of the Gob-5 KO mice after administration of recombinant IL-13 into their lungs (Figure 5). In either the Balb/C or the mixed C57/BL6–129 strain of mice, we observed a comparable and strong induction of Gob-5 protein

in response to acute IL-13 administration (Figure 6). Once again, however, we detected elevated Muc5ac levels in the BALF of IL-13-treated Gob-5 KO mice as compared with the WT controls (Figure 5A). Similarly, PAS–Alcian blue staining confirmed an increase in tissue mucin levels and goblet cell numbers in the Gob-5 KO animals (Figures 5B and 5C). However, we failed to discern any genotype-related difference with respect Muc5ac mRNA expression in the tissue (Figure 5D) or infiltration of total or individual cell populations (neutrophils, lymphocytes, macrophages/monocytes, or eosinophils) into the BALF after IL-13 treatment (Figure 5E).

Gene Array Studies of the Gob-5 KO Mice: Analysis of Gene Compensation

The lack of an attenuated inflammatory response associated with disruption of the Gob-5 gene suggested that perhaps the KO mice had circumvented the requirement for Gob-5 through a mechanism involving developmental compensation. Obvious candidates for this effect included other Gob-5 family members

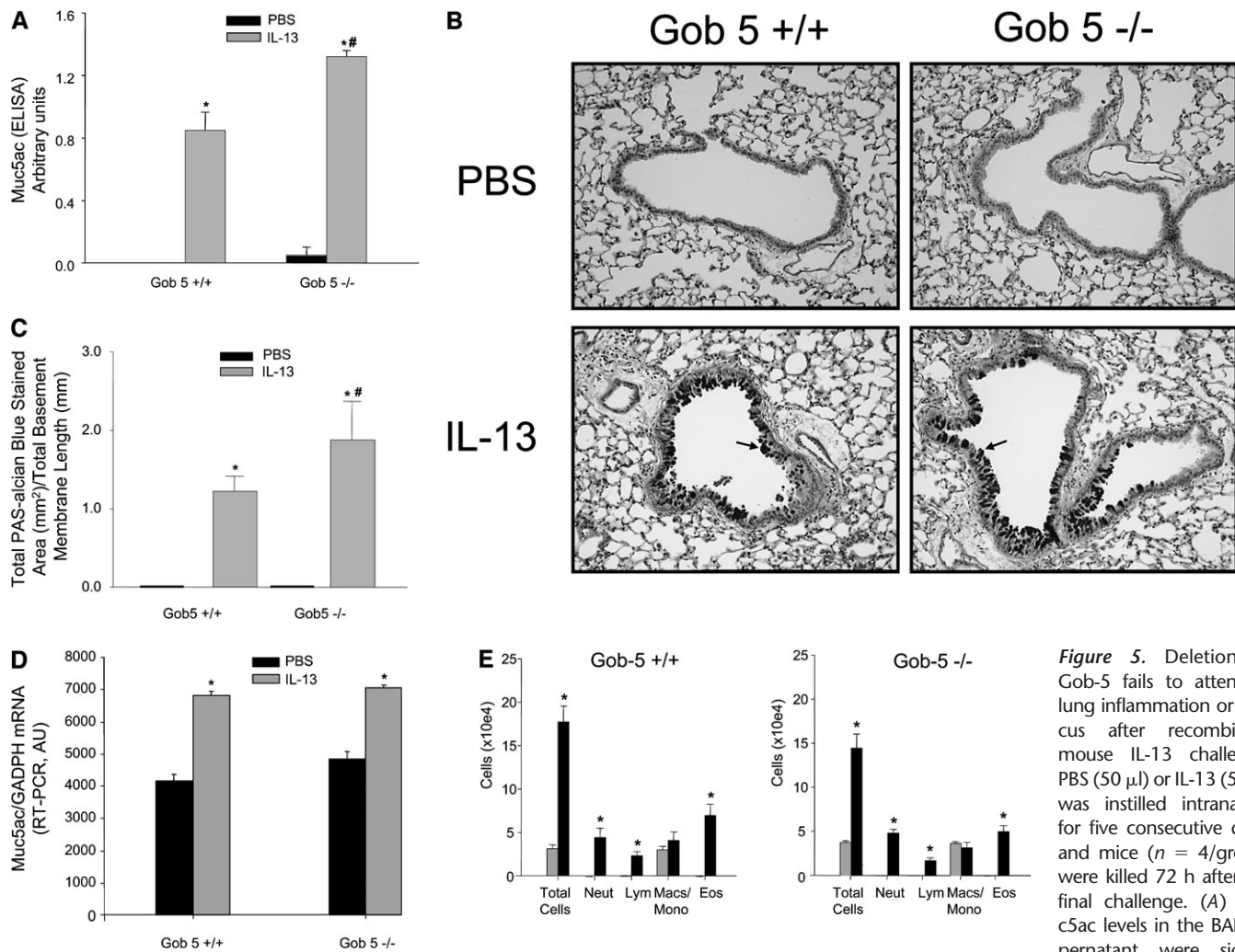


Figure 5. Deletion of Gob-5 fails to attenuate lung inflammation or mucus after recombinant mouse IL-13 challenge. PBS (50 μ l) or IL-13 (5 μ g) was instilled intranasally for five consecutive days, and mice ($n = 4$ /group) were killed 72 h after the final challenge. (A) Muc5ac levels in the BAL supernatant were significantly elevated for both Gob-5 +/+ and Gob-5 -/- mice after IL-13 challenge as detected by ELISA. The increase in muc5ac levels after IL-13 challenge was greater for Gob-5 -/- mice compared with Gob-5 +/+ mice. *Statistically significant from PBS-treated ($P < 0.05$); # statistically significant from Gob-5 +/+ after IL-13 challenge. (B) PAS-Alcian blue staining of lung tissue from Gob-5 +/+ and Gob-5 -/- mice showed an increase in the amount of mucus-secreting goblet cells in the airways after IL-13 challenge. Mucin-containing goblet cell cells are indicated by the arrows. Magnification: $\times 20$. (C) Quantification of PAS-Alcian blue staining from lung tissue sections of Gob-5 +/+ and Gob-5 -/- mice after OVA challenge. *Statistically significant from PBS-treated ($P < 0.05$); # statistically significant from Gob-5 +/+ after IL-13 challenge. (D) Muc5ac expression in lung tissues of IL-13-challenged Gob-5 KO mice. The levels of Muc5ac mRNA in lung tissues from PBS- or IL-13-challenged WT and Gob-5 knockout mice ($n = 5$ /group) were determined by RT-PCR. The Muc5ac PCR products were quantified by gel densitometry and the expression levels were normalized relative to that of GAPDH. *Statistically significant from PBS-treated ($P < 0.05$). (E) The profile of inflammatory cell infiltration in the BALF is similar in IL-13-challenged Gob-5 +/+ and Gob-5 -/- mice. Total and individual cell populations (neutrophils, lymphocytes, macrophages/monocytes, and eosinophils) are shown. Gray bars, PBS; black bars, IL-13. *Statistically significant from PBS-treated ($P < 0.05$).

as well as genes (e.g., EGF receptor) previously implicated in regulating mucin expression (2, 9, 25). Consistent with the model, several chemokine genes, including eotaxin-1, MCP-1, and TARC, were robustly upregulated in response to OVA challenge, but did not show a significant change between the WT and KO animals (Figure 7A). Also, as compared with the PBS-treated WT cohort, a dramatic increase in Gob-5 (mCLCA-3) expression was seen in the OVA-treated WT animals but, as expected, was not seen in the KO animals (Figure 7B). On the other hand, murine CLCA-1, -2, and -4 were all expressed at very low levels and did not show noteworthy changes in their expression profiles relative to the WT controls (Figure 7C). These data suggest that other CLCA family members are unlikely to be playing a compensatory role in regulating mucus

overproduction in the absence of Gob-5 expression. Analysis of additional mucin genes, including Muc1, 3, 4, 5b, and Muc6, also failed to reveal any genotype-related differences in response to OVA treatment (Figure 7A).

To investigate a possible compensatory effect by other mucoregulatory genes, we broadly compared the gene expression profiles of the Gob-5 KO and WT animals. In all cases, our assessment of technical reproducibility showed that the differentially regulated probes from the fluor-reversed pairs had a correlation greater than 0.994, so all arrays were included in the analysis. A comparison of genes expressed in the WT/OVA-challenged (WT-OVA) versus KO/OVA-challenged (KO-OVA) mice revealed only a handful of genes that were differentially regulated between the two groups. Components of the epidermal growth

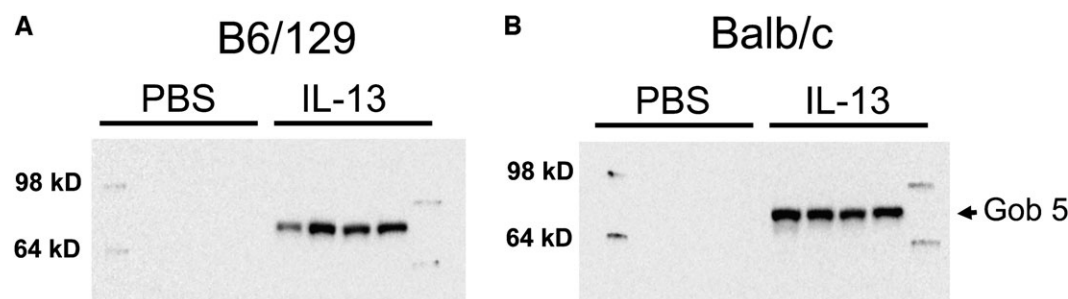


Figure 6. Expression of Gob-5 protein in the lungs of mice instilled with IL-13. Mice ($n = 4/\text{group}$) were treated by instillation with PBS or IL-13 (5 μg) for 5 d. Total lung protein lysate (72 μg) was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Gob-5 antiserum (Ab1047). Studies were performed in C57/BL6-129 (B6/129) and Balb/c mice.

factor receptor (EGFr) pathway were not identified among the genes that might be expected to serve a compensatory function given their role in mediating mucus signaling (25). Interestingly, among only nine genes that were found to be upregulated > 2 -fold as compared with the WT-OVA group, the gene dis-

playing the highest increase in expression (3.02-fold, $P < 0.001$) was arginase-1 (*arg-1*) (Figure 7A). Compared with the vehicle-challenged controls, the WT-OVA and the KO-OVA groups showed a 2.72- and 8.20-fold stimulation, respectively, in *arg-1* expression. Although the significance of the increase in *arg-1* expression is not clear, recent studies have implicated *arg-1* as a putative effector molecule involved in the tissue remodeling process that underlies the pathogenesis of asthma (26–28).

Mucin Overproduction in Cell Culture Is Not Attenuated by CLCA-1 Knockdown

The lack of effect of Gob-5 gene disruption on Muc5ac expression *in vivo* prompted us to directly examine the relationship between

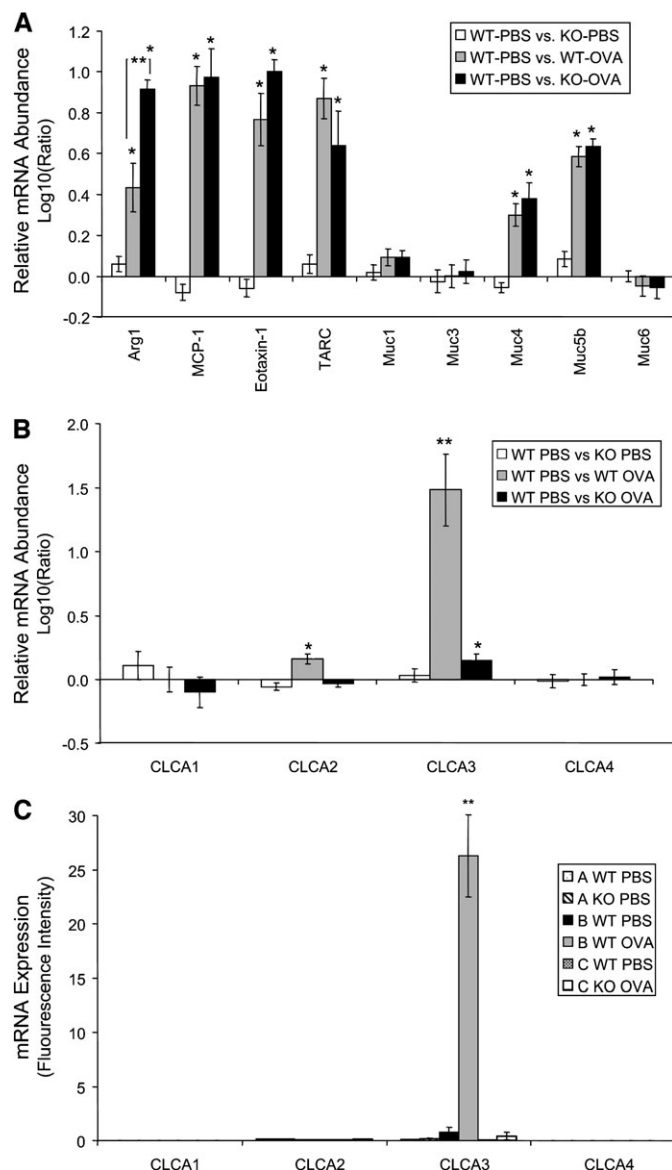


Figure 7. Gob5 (mCLCA-3) modulated in WT OVA-challenged mice but not in Gob-5 (mCLCA-3) $^{-/-}$ (KO) mice. (A) Analysis of mucin- and OVA-inducible gene expression in the Gob5 (mCLCA-3) KO mice. For each gene (MCP-1, Eotaxin-1, TARC), the relative abundance of mRNA is shown on a log base 10 scale for the following three comparisons, in order from left to right: (white bars) mCLCA-3 $^{-/-}$ (KO) mice treated with PBS compared with the WT PBS-treated baseline (three mRNA samples, six chips); (gray bars) WT treated with OVA compared with the WT PBS-treated baseline (two mRNA samples, four chips); (black bars) mCLCA-3 $^{-/-}$ (KO) treated with OVA compared with the WT PBS-treated baseline (three mRNA samples, six chips). Statistically significant results for WT-OVA versus KO-OVA, $**P \leq 0.001$ or relative to WT-PBS, $*P \leq 0.001$ are indicated. (B) For each mCLCA family member, the relative abundance of mRNA is shown on a log base 10 scale for the following three comparisons, in order from left to right: (white bars) mCLCA-3 $^{-/-}$ (KO) mice treated with PBS compared with the WT PBS-treated baseline (three mRNA samples, six chips); (gray bars) WT treated with OVA compared with the WT PBS-treated baseline (two mRNA samples, four chips); (black bars) mCLCA-3 $^{-/-}$ (KO) treated with OVA compared with the WT PBS-treated baseline (three mRNA samples, six chips). Statistically significant results for WT-OVA versus KO-OVA, $**P \leq 0.001$ or relative to WT-PBS, $*P \leq 0.001$ are indicated. A log ratio of 1.5 is roughly a 30-fold change, whereas a log ratio of 0.2 is roughly a 1.5-fold change. (C) For each mCLCA family member, the absolute abundance of mRNA in arbitrary scanner intensity units is shown on a linear scanner scale for the following six conditions, in order from left to right: Experiment set A: (dotted bars) WT PBS-treated baseline (one pool of four mRNAs, six chips); (striped bars) mCLCA-3 $^{-/-}$ (KO) PBS-treated samples (three mRNA samples, six chips). Experiment set B: (black bars) WT PBS-treated baseline (one pool of four mRNAs, four chips); (gray bars) WT OVA-treated samples from experiment set B (two mRNA samples, four chips). Experiment set C: (hatched bars) WT PBS-treated baseline (one pool of four mRNAs, six chips); (white bars) mCLCA-3 $^{-/-}$ (KO) OVA-treated samples (three mRNA samples, six chips). Statistically significant results for WT-OVA versus KO-OVA, $**P \leq 0.001$ or relative to WT-PBS, $*P \leq 0.001$ are indicated. A log ratio of 1.5 is roughly a 30-fold change, whereas a log ratio of 0.2 is roughly a 1.5-fold change.

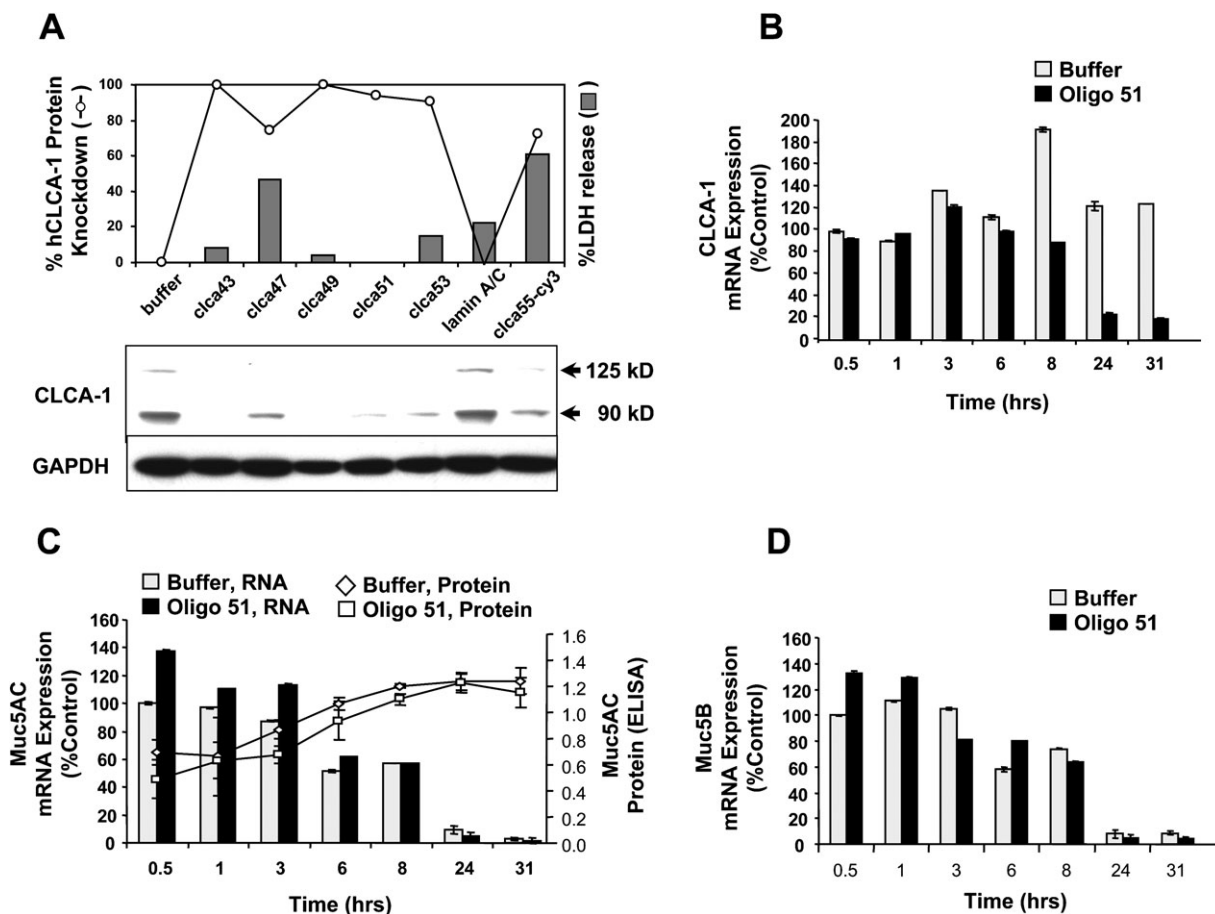


Figure 8. CLCA-1 knockdown fails to attenuate mucin production in NCI-H292 cells. Analysis of the effect of different CLCA-1 siRNAs on CLCA-1 protein expression. (A) A series of siRNAs (100 nM) directed against CLCA1 and were transfected into NCI-H292-7.9 cells and cells were cultured for 24 h. Cultures were subsequently harvested and analyzed by immunoblot for expression of CLCA-1 and GAPDH. The positions of the 90-kD and 125-kD subunits of CLCA-1 are indicated. As a measure of cytotoxicity, supernatants from the transfected cultures were examined for LDH release. (B–D) Based on a combination of low cytotoxicity and high knockdown efficiency, Oligo51 was selected to perform a time course experiment to establish the effect of CLCA-1 knockdown on mucin gene expression. Cultures were transfected with oligo51 (100 nM) and harvested at the indicated time points thereafter. Buffer (gray bars) or siRNA (black bars)-transfected cells were analyzed by quantitative PCR for the expression of CLCA-1 (B), Muc5ac (C), and Muc5B (D) message. Supernatants from the buffer (diamonds) or siRNA (white squares)-transfected cultures were also examined for the production of Muc5ac protein by ELISA (C).

mucin production and Gob-5 expression *in vitro* using a cell-based RNAi knockdown approach (Figure 8). To this end, we examined a human lung epithelial cell line (NCI-H292-7.9) that constitutively expresses Gob-5 and that overproduces and secretes Muc5ac. Knockdown of CLCA-1 in this cell system was first evaluated by comparing the capacity of a series of siRNAs to inhibit CLCA-1 protein expression. Among five siRNA oligos tested, we identified one (oligo 51) that, 24 h after transfection, could inhibit expression of CLCA-1 by ~90% in the absence of any cytotoxicity as determined by analysis of LDH release (Figure 8A). Consistent with this observation, time course studies revealed that by 8 h and 24 h, expression of CLCA mRNA was decreased by more than 50% and 80%, respectively, relative to the buffer-treated control culture (Figure 8B). As a control, an siRNA oligo directed against lamin A/C was shown to be ineffective at reducing CLCA-1 protein levels (Figure 8A).

Interestingly, analysis of mucin expression in these experiments showed an inverse relationship between the expression of Muc5ac message and production of Muc5ac protein as a function of time (Figure 8C). By 24 h post-plating protein production had peaked and mRNA levels in buffer-treated cells were al-

ready reduced by 90%. More importantly, however, treatment of the cells with the CLCA-1 siRNA (oligo 51) failed to further attenuate Muc5ac mRNA or protein expression as compared with the buffer-treated cells. A similar lack of effect on the expression of other mucins was noted when we examined Muc5B mRNA levels over the same timecourse (Figure 8D). Taken together, these data clearly demonstrate that continued expression of CLCA-1 is not an essential prerequisite for mucin mRNA and protein production in NCI-H292 cells.

DISCUSSION

Previous reports have implicated Gob-5, as well as its human ortholog (hCLCA-1), in mediating the overproduction of mucus in response to allergic lung inflammation (13, 29). In this study we sought to establish the mucoregulatory function of Gob-5 through targeted gene disruption in mice. Animals lacking Gob-5 expression maintained a robust inflammatory response after acute allergic challenge in two preclinical models of asthma. Interestingly, our analysis showed a higher level of Muc5ac production in the BALF of KO mice relative to their WT littermates,

irrespective of the allergic stimulus. These data suggest that Gob-5 expression is not necessary to drive mucin overproduction in mice after proinflammatory stimulation. In addition, we were unable to detect any genotype-dependent histologic difference in mucin or goblet cell staining in the gastrointestinal tract where Gob5 is normally constitutively expressed. Our findings are consistent with recent observations that RNAi-mediated inhibition of Gob-5 in IL-13-challenged mice fails to inhibit mucin overproduction *in vivo* (30). Similarly, we show in this study that in human lung epithelial cells, knockdown of Gob-5 protein expression (> 90%) by RNAi fails to alter the production Muc5ac as detected by RNA analysis or protein ELISA. Although this finding supports our observations *in vivo*, it is also paradoxical, in that stably selected cells overexpressing CLCA-1 produce elevated levels of Muc5ac as compared with control cell lines carrying the corresponding empty expression vector. One possibility that may explain this paradox is that CLCA-1 acts only as an initiator of the signaling process that leads to mucus production. Once the mucin signaling cascade has been triggered, it may be refractory to inhibition by modulation of CLCA-1 activity.

The lack of an attenuated mucin response noted in the Gob-5 KO mice is in sharp contrast to previous reports showing that intratracheal administration of Gob-5 antisense adenovirus results in a substantial decrease of several proinflammatory endpoints (i.e., mucus overproduction, cellular infiltration, airway hyperreactivity) after allergic challenge (13). Despite a dramatic change in the allergic responses measured, the Gob-5 antisense study by Nakanishi and coworkers did not show knockdown of Gob-5 expression in the lung tissue from OVA-challenged animals (13). The studies described by Nakanishi's group were performed using Balb/C mice, whereas in the current study the KO mice analyzed were of a mixed genotypic background (C57/BL6–129). It is possible that the lack of a mucin-related phenotype in the Gob-5 KO mice is attributable to the difference in strain background. However, our data indicate that allergic induction of Gob-5 expression in the lung is robust in Balb/C as well as in C57/BL6–129 mice.

The absence of a phenotypic effect on mucin overproduction in the Gob-5 KO mice raised the possibility of compensation by other mechanisms, including changes in gene expression. However, gene array analysis of the Gob-5 KO mice versus the WT controls ruled out a contribution by other Gob-5 family members. Indeed, with the exception of only a handful of genes, very few changes were noted between the two groups in response to OVA treatment. The most notable change in these array experiments was seen with arg-1 expression, which was elevated by 3-fold in the OVA-treated KO mice relative to their OVA-treated WT counterparts.

Although the mechanism by which arg-1 might act to compensate for the absence of Gob-5 expression is not known, one possibility, based on previous reports, is that increased arginase activity may lead to arginine metabolites that could potentially drive a number of pulmonary remodeling events, including mucus overproduction (26–28). However, whether a 3-fold increase in arginase expression is sufficient to completely compensate and perhaps overcompensate for the lack of Gob-5-mediated mucus overproduction in OVA-challenged KO mice remains to be determined.

In light of our findings, the question remains: if Gob-5 is not directly involved in mucus overproduction in mice, then what is its role and why is it overexpressed in human asthma (15, 16) and in murine models (13, 14) of the disease? Despite demonstrating overexpression of Gob-5 in human asthma (15, 16), studies have not shown a causal relationship with mucus overproduction *in vivo*. Perhaps Gob-5 has more to do with priming of the lung epithelium to help accommodate or potentiate, but not directly stimulate, mucus production. In this regard, Gob-5 may be nei-

ther sufficient nor necessary for mucus overproduction in mice. Interestingly, the studies reported by Nakanishi and coworkers showed that adenovirus-mediated overexpression of Gob-5 could accelerate mucin overproduction in mice that were challenged with OVA (13). However, these studies did not go so far as to demonstrate a direct effect of Gob-5 overexpression on mucus overproduction in the absence of an allergen challenge.

Finally, we show in this study that knockdown of Gob-5 protein expression by RNAi (> 90%) in NCI-H292 lung epithelial cells fails to alter the production of Muc5ac as detected by RNA analysis or protein ELISA. Although this finding supports our observations *in vivo*, it is also paradoxical, in that stably selected cells overexpressing CLCA-1 produce elevated levels of Muc5ac as compared with control cell lines carrying the corresponding empty vector. One possibility that may explain this paradox is that CLCA-1, at least *in vitro*, may act as a co-initiator or potentiator of the signaling process leading to mucus production. Once the mucin signaling cascade has been triggered, it may be refractory to inhibition by modulation of CLCA-1 activity. However, the extent to which such a mechanism would operate *in vivo* remains to be determined.

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