Submucosal gland development in the airway is controlled by Lymphoid Enhancer Binding Factor 1 (LEF1)

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SUMMARY

Previous studies have demonstrated that transcription of the lymphoid enhancer binding factor 1 (Lef1) gene is upregulated in submucosal gland progenitor cells just prior to gland bud formation in the developing ferret trachea. In the current report, several animal models were utilized to functionally investigate the role of LEF1 in initiating and supporting gland development in the airway. Studies on Lef1-deficient mice and antisense oligonucleotides in a ferret xenograft model demonstrate that LEF1 is functionally required for submucosal gland formation in the nasal and tracheal mucosa. To determine whether LEF1 expression was sufficient for the induction of airway submucosal glands, two additional model systems were utilized. In the first, recombinant adeno-associated virus was used to overexpress the human LEF1 gene in a human bronchial xenograft model of regenerative gland development in the adult airway. In a second model, the LEF1 gene was ectopically overexpressed under the direction of the proximal airway-specific CC10 promoter in transgenic mice. In both of these models, morphometric analyses revealed no increase in the number or size of airway submucosal glands, indicating that ectopic LEF1 expression alone is insufficient to induce submucosal gland development. In summary, these studies demonstrate that LEF1 expression is required, but in and of itself is insufficient, for the initiation and continued morphogenesis of submucosal glands in the airway.

Nomenclature: Lef1 (mouse and ferret lymphoid enhancer binding factor 1 genes); LEF1 (human lymphoid enhancer binding factor 1 gene); LEF1 (lymphoid enhancer binding factor 1 protein)

Key words: LEF1, Submucosal gland, Lung development, Transgenic mice

INTRODUCTION

Submucosal glands (SMGs) secrete fluid, mucous and bacteriocidal proteins, which are important in maintaining normal lung function. SMGs are thought to play an important role in the pathogenesis of a number of hypersecretory lung diseases such as cystic fibrosis (CF), chronic bronchitis and asthma. The potential involvement of submucosal glands in the etiology of CF pathogenesis is suggested by several findings, including the high level of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) expression in SMGs (Engelhardt et al., 1992), and the severe hypertrophy and hyperplasia of SMGs characteristic of the progressing disease (Oppenheimer and Esterly, 1975; Welsh and Smith, 1995). In the normal human airway, SMGs are restricted to the cartilaginous airways (trachea and bronchi), while in CF the distribution of SMGs extends more distally into bronchioles. These findings suggest that submucosal gland progenitor cells may exist in the adult human airway, a fact that is supported by retroviral lineage analysis in reconstituted human bronchial xenografts (Engelhardt et al., 1995).

Although little is currently known about the cellular factors controlling gland developmental processes in the airway, insight can be gleaned by analogy with similar systems of organogenesis. It is likely that reciprocal inductions between epithelium and mesenchyme play crucial roles during the progressive invagination, branching and arborization of SMG tubules and ducts. These interactions may be mediated by direct cell-cell contact, by contact with the extracellular matrix, and/or by diffusible factors, as is characteristic of tubulogenesis in kidney, liver, lung, pancreas and mammary gland (Gurdon, 1992; Hay and Zuk, 1995; Hogan, 1999). To date, a number of signaling pathways and transcription factors have been implicated in controlling similar developmental processes, including the Wnt/wingless pathway, Sonic hedgehog (sh), Bone morphogenetic proteins (BMP) 2 and 4, and Hepatocyte growth factor (Beit and Maas, 1998; Birchmeier and Birchmeier, 1993; Chen et al., 1996; Dassule and McMahon, 1998; Kratochwil et al., 1996; Moon et al., 1997; Papkoff and Aikawa, 1998). In this group of developmental regulators is the sequence-specific HMG-box transcription factor LEF1.
Members of the TCF (T cell factor) family transcription factors, including LEF1, share an identical DNA recognition sequence (CCTTTCGAAC) through a conserved HMG (high mobility group) domain (Alexander-Bridges et al., 1992; Clevers and van de Wetering, 1997; Travis et al., 1991; Waterman and Jones, 1990). Functionally, the hydrophobic arm of the HMG box recognizes irregular DNA structures, such as pyridine-rich cruciform or kinked DNA, and intercalates into the minor groove. Consequently, TCF/LEF1 factors bend DNA locally, bringing distantly located DNA-binding proteins into juxtaposition, thereby facilitating the protein-protein interactions necessary for transcriptional activation (Grosschedl et al., 1994; Werner and Burley, 1997; Werner et al., 1996). Unlike other members of the TCF family, LEF1 protein also contains a context-dependent activation domain (CAD) that is dependent on the co-activator ALY in regulating expression of the T-cell receptor α (TCRα) (Okamura et al., 1998). By virtue of LEF1’s role as an ‘architectural’ transcription factor with context-dependent trans-activation characteristics, it has been suggested that LEF1 effectively coordinates multiple developmental pathways in regulating gene expression (Dassule and McMahon, 1998; Hsu et al., 1998; Riese et al., 1997).

LEF1 and the closely related TCF 1 (T cell factor 1), have been shown to be expressed in distinct but overlapping distributions in non-lymphoid tissues during murine embryogenesis (Oosterwegel et al., 1993). Further experimentation has demonstrated that homozygous germline mutation of the Lef1 gene in mice resulted in salient abnormalities in hair follicle position and orientation (van Genderen et al., 1994). These Lef1 ‘knockout’ mice exhibit developmental impairment of many additional organs that require multiple inductive epithelial-mesenchyme interactions, including kidney, teeth and mammary glands. As part of an effort to identify cellular factors and developmental pathways that control submucosal gland formation in airway, we initiated studies to examine a possible role for LEF1 in this context. Previously, we demonstrated that Lef1 gene expression is induced at a high level in submucosal gland progenitor cells at the earliest stage of gland bud formation, by in situ mRNA localization in the ferret tracheal airway (Duan et al., 1998a). During later stages of gland morphogenesis, Lef1 expression is limited to cells in the most distal invading tips of tubules. The timing and localization of Lef1 expression suggested that this transcription factor might be functionally involved in regulating airway submucosal gland development. However, since expression does not infer functional importance, we sought to directly test the requirements for LEF1 activity in airway gland development.

As described in this report, several independent model systems were utilized to modulate LEF1 expression, and the resulting effects on SMG development were quantified with morphometric analyses. Ectopic overexpression of the LEF1 gene in human bronchial xenografts by means of a recombinant adenovirus (AAV) vector, or in transgenic mice through the airway-specific promoter CC10 (Ray et al., 1997; Stripp et al., 1992), produced no detectable effect on airway submucosal gland morphogenesis. In contrast, either blocking LEF1 protein expression with antisense oligonucleotides in a ferret tracheal xenograft model or by targeted gene inactivation in a knockout mouse, resulted in complete inhibition of airway submucosal gland development. Taken together, our data suggest that LEF1 is necessary but not sufficient to promote airway submucosal gland development.

**MATERIALS AND METHODS**

**Design and evaluation of antisense oligonucleotides**

Antisense oligonucleotides (AS-ODNs) were designed from regions of the Lef1/LEF1 sequence previously demonstrated to be totally conserved between human (Waterman et al., 1991) and ferret (Duan et al., 1998a). Phosphorothioate-modified antisense (EL170 cgggagtgccgccacgg and EL172 tgaaggggatcatcgtgc) and sense (EL169 cgggatcccccacttcgct and EL171 gcggagagttggggcatcccg) ODNs were synthesized by Genosys. A 50:50 mixture of the 20-mer and 21-mer AS-ODNs against two potential translational start codons of Lef1/LEF1 (Chavany et al., 1995; Shaik-Eshleman and Liebhaber, 1988) was used for both in vitro experiments against human Lef1 and in vivo experiments against ferret Lef1. Similar mixtures of phosphorothioate-modified sense ODNs were also used as controls for the specificity of inhibition by the antisense-ODNs. To analyze the effectiveness of our antisense approach to inhibit LEF1 protein expression, 293 cells were transfected with a bicistronic plasmid vector (see below) expressing both human LEF1 and GFP using lipofectamine (BRL). Concurrently, various concentrations (0.1 to 1.0 μM) of sense or antisense ODNs and a transfection efficiency control plasmid were delivered into the cells in the same transfection cocktail. Cells were harvested 36 hours post-transfection and LEF1 expression was examined by western blot analysis (see below).

**Generation of bicistronic recombinant adenovirus expressing LEF1 and GFP**

A bicistronic pAV.LEF1/GFP proviral plasmid for the generation of recombinant adenovirus associated virus (AV.LEF1/GFP) was constructed using a pSub201 backbone [Fig. 3] (Samulski et al., 1989). The full-length human Lef1 cDNA (1.5 kb) was excised from pTF1-1 (gift of Dr K. A. Jones, The Salk Institute for Biological Studies, La Jolla, California) with BamHI, and GFP (0.9 kb) was excised with ClaI from pGreenLantern (BRL). The cDNAs were then cloned between the CMV promoter and the SV40 poly(A) signal. The total transgene cassette was brought up to 4664 bp with a stuffer sequence from human glycosylasparaginase cDNA. Virus stocks were generated by co-transfection of 293 cells with pAV.LEF1/GFP along with pRep/Cap, followed by co-infection with recombinant Ad.CMVlacZ helper virus (Duan et al., 1999b). rAAV was then purified through three rounds of CsCl density gradient centrifugation as previously described (Duan et al., 1997). Purified viral fractions were heated at 60°C for 1 hour to inactivate any residual contaminating helper adenovirus. Recombinant AAV (rAAV) titers determined by slot-blot hybridization against P12-labeled LEF1 probes were typically 5×10^{12} DNA particles/ml. Infectious titers determined by infection of 293 cells with rAAV were ~1.5×10^9 particles/ml. Co-expression of LEF1 and GFP from this vector was confirmed by immunofluorescent detection of LEF1 and fluorescent detection of GFP as described below.

**Airway xenografts models**

**Newborn ferret tracheal xenografts**

Newborn ferret tracheal xenografts were prepared as previously described (Duan et al., 1998a). In this previous study, the development of submucosal glands in the newborn ferret tracheal xenografts was shown to mirror that in age-matched littermates. In brief, newborn ferret tracheas were excised 1-12 hours after birth and directly ligated onto flexible plastic tubing. The xenograft cassettes were then transplanted subcutaneously in the flanks of athymic nu/nu mice, such that the end of the tubing was accessible for the administration of...
reagents into the airway lumen. In the current study, this model was used as an in vivo assay for SMG development by which to evaluate the effect of antisense inhibition of Lef1 expression. Immediately following engraftment of the xenograft, the tracheal lumen was infused with 30 μl of F12 medium with or without 5, 10 or 50 μM antisense or sense ODNs. The ODN solution was changed every 24 hours in the first 3 days. Starting with the 4th day of ODN exposure, the xenograft lumens were infused with 120 μl of F12/ODN solution for 36 hours and then to air for 12 hours to promote continued epithelial differentiation. Infiltration of ODNs was stopped after 4 weeks and xenografts were harvested 3 days later for morphologic evaluation of submucous gland development.

Human bronchial xenografts

Human bronchial xenografts used for AAV-mediated ectopic Lef1 overexpression experiments were produced according to previously described protocols with modifications (Engelhardt et al., 1995). Primary human airway cells were cultured in hormonally defined media as previously described (Engelhardt et al., 1993). On the second day of plating, 20% confluent cell cultures were infected with media as previously described (Engelhardt et al., 1993). On the second day of plating, 20% confluent cell cultures were infected with either the wild-type or Lef1 knockout alleles without the CC10/Lef1 transgene. For western blotting, total cellular proteins were extracted in lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS. After incubating at room temperature for 15 minutes, lysates were spun at 14,000 revs/minute, and supernatants were collected. 100 μg/lane of cellular protein lysate was electrophoresed on 7.5% SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C, Amersham). Filters were stained with 0.5% Ponceau-S, 1% acetic acid and blocked in 4% non-fat dried milk in 0.1% Tween-20 in TBS (25 mM Tris pH 8.0, 0.9% NaCl) at 4°C overnight. Blots were incubated with primary antibodies (1:500) for 1 hour and washed in blocking buffer 4°C for 10 minutes. Filters were then treated with 1:1500 HRP-conjugated secondary antibody (BMB) for 1 hour at room temperature. After final washing, signals were detected using the ECL system (Amersham).

Immunofluorescent co-localization of GFP and Lef1 protein expression in primary airway cells grown on glass coverslips was performed following fixation in 4% paraformaldehyde/PBS at room temperature for 10 minutes. After washing with PBS twice, they were permeabilized in 0.2% Triton X-100 for 10 minutes at room temperature. Samples were rinsed with PBS twice, blocked in 20% goat serum/PBS for 30 minutes and incubated in primary antibody (1:100) overnight at 4°C. After washing with 1.5% goat serum/PBS 3× 8 minutes, Texas Red-conjugated secondary antibody was applied (1:200) for 30 minutes. Coverslips were washed again for 3× 8 minutes and mounted on slides in Citifluor prior to visualization by fluorescent microscopy. Similarly, localization of GFP and Lef1 was performed on xenograft tissues infected with rAAV with the modification that tissues were first fixed in 4% paraformaldehyde/PBS for 2 hours, cryoprotected in sucrose and then sectioned at 10 μm prior to staining.

**Electrophoretic mobility shift assays for Lef1 DNA binding**

Nuclear extracts from pNAVLEf1/GFP or pAVGFP transfected 293 cells were prepared using the previously described Dignam method (Lee et al., 1988). A TCRt mini-enhancer sequence containing a Lef1 binding motif ‘TTGC(T)AAAGG’ was used for gel-shift
Fig. 1. Antisense oligonucleotide inhibition of Lef1 expression reduces submucosal gland development in newborn ferret tracheal xenografts. (A) Newborn ferret tracheas were harvested and transplanted as xenografts subcutaneously in nude mice. (A) Over the course of 4 weeks, maturation and differentiation of both the surface epithelium and submucosal glands occurs. The surface airway epithelium (SAE), gland duct openings (D) and gland tubules (T) are indicated by arrows. (B) In vitro confirmation of our antisense approach to inhibit recombinant human LEF1 expression in pAV-LEF1/GFP transfected 293 cells. Various concentrations (0.1 to 1.0 μM) of sense or antisense ODNs were co-transfected with 1.5 μg of pAVLEF1/GFP into 293 cells and LEF1 expression was analyzed at 36 hours post-transfection by western blotting with anti-human LEF1 N-terminal polyclonal antibody (UP837). The position of the LEF1 protein, which migrates at approximately 55 kDa on SDS-PAGE, is indicated by an arrow. Specific inhibition of Lef1 expression was seen at 0.5 and 1.0 μM concentrations of antisense but not sense oligonucleotides, with no discernable effect on the expression of other non-specific, cross-reactive cellular proteins. (C) Newborn ferret tracheal xenografts were treated with 5, 10 or 50 μM sense or antisense oligonucleotides and the abundance of gland duct openings and tubules was compared to that of media (F12)-treated controls as described in Materials and Methods. Morphometric analysis was performed as described in the Material and Methods. Values represent the mean (±s.e.m.) of n=4 independent xenografts analyzed for each condition (only 50 μM ODN concentration is shown). Xenografts treated with 50 μM antisense oligonucleotides exhibited an eight-fold reduction in both the number and the size of the SMGs as compared to controls treated with sense oligonucleotides or vehicle (F12 medium).

experiments (Travis et al., 1991). The sense oligonucleotide sequence was 5'-GTAGGGGCACCCCTTGAAGCTCTCC-3' and the antisense oligonucleotide was 5'-GGGAGACCGTTCAAGGTGCCTA-3'. Oligonucleotides were end-labeled with γ-32P-ATP by T4 polynucleotide kinase at 37°C for 30 minutes. Double-stranded DNA probes were made by annealing the two complementary oligonucleotides and unincorporated nucleotides were removed by gel filtration (G25 Sephadex). 20 μg of nuclear extract was incubated on ice for 20 minutes with 1 μg of poly(dIdC), 5×10^4 cts/minute probe, 3 μl of BSA (1 mg/ml), in 20 μl of binding buffer containing 25 mM Hepes pH 7.6, 60 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.75 mM DTT and 7.5% glycerol. When cold competitors (TCRα or p53) were included, they were added 5 minutes prior to the addition of radioactive probe. As an irrelevant control, unlabelled p53 oligonucleotides of the following sequence were used for competition: 5'-GAAACATGGTCTAAGCATGCTG-3' and 5'-CAGCATGCTTGACATGGTTC-3'. In supershift experiments, the antibody was added 10 minutes after the probe. Samples were electrophoresed in 5% native polyacrylamide gels for 4-5 hours. Gels were dried and exposed to X-ray film.

**Morphometric analyses**

Morphometric analyses were performed on tissue sections to determine the effects of modulating LEF1 on SMG development in the airway. Methods for quantification of SMGs are based on two assumptions: (1) there is only one duct leading to the airway surface from each gland, and (2) gland size should correlate with the number of branching tubules. Since each of the experimental models differed in gland abundance and/or distribution within tracheal/nasal samples, we have optimized morphometric analyses to meet the needs of each of these model systems. Protocols for quantifying gland abundance are outlined below for each model system.

**Nasal glands in Lef1 knockout mice**

The abundance of nasal glands in 12-day-old littermate Lef1+/+ and Lef1−/− pups were evaluated as follows. The snouts from three 12-day-old mice from each genotype (cut by cross section just in front of the eyes) were embedded with the cut face down and quick frozen in OCT medium. Serial sections were cut through the entire block and all sections encompassing the nasal septum were evaluated for the presence of submucosal glands. Since no glands were ever seen in Lef1−/− mice, the size and number of glands were not quantitated. Photomicrographs comparing the regions of glands in the nasal cavity were chosen by picking cross sections from each Lef1+/+ and Lef1−/− tissue sample that had similar size and structure of the nasal septum. This displayed a particular region rich in glands that was juxtaposed to the nasopharynx, nasal conchae and palatal shelf. It is important to point out that Lef1−/− mice have abnormalities in nasal cartilage due to the fact that Lef1 is expressed in this tissue during development. Hence, although regions were matched as best possible for structure of the septum, nasopharynx, nasal conchae and palatal shelf, it is notable that the nasal conchae had structural abnormalities.

**Tracheal glands in antisense treated ferret xenografts**

The abundance of glands within ferret xenografts is fairly high (~300 glands per xenograft). Our endpoints for quantification included both glandular size and number of glands. The number of SMGs was quantified by evaluating the number of gland duct openings into the airway lumen in each tracheal cross-section, assuming that each gland has one gland duct. Gland sizes were estimated by counting the total number of individual gland tubules per tracheal cross-section. Frozen blocks were embedded with multiple 1-2 mm rings from each 1 cm xenograft sample and sectioned at 10 μm saving every tenth section for analysis. In total, at least 100 sections were quantitated for each sample at 100 μm intervals to avoid counting the same glandular structures more than once. Four animals were evaluated for each experimental condition (i.e., sense, antisense and vehicle treated).

**Tracheal glands in human bronchial xenografts ectopically expressing LEF1**

Unlike ferret tracheal xenografts, human bronchial xenografts normally have a very low abundance of glands (typically 5-10 per xenograft). This required a more comprehensive and time consuming method of morphometric analysis, which comprehensively evaluated
occurs between 14 and 28 weeks of development stage in humans that (Leigh et al., 1986), reflecting a development occurs postnatally those in the human airway (Curtis et al., 1996) distribution of SMGs closely resemble those in mouse. The ferret is a superior animal model for tracheal xenograft airways submucosal glands in ferret branching morphogenesis of decreases the abundance and with antisense oligonucleotides

RESULTS

Tracheal gland abundance in knockout and transgenic mice

The quantification of tracheal submucosal glands in mice was by far the most challenging. This is due to the fact that tracheal submucosal glands in the mouse trachea are infrequent (typically 5-13 in adult mice). Furthermore, glands in the trachea develop postnatally. For these reasons, we chose one morphometric method for evaluating tracheal gland abundance, which was the identification of gland ducts. In adult transgenic animals, gland ducts were adjoined with large glandular masses of interconnecting tubules. In contrast, gland ducts in 3-day-old mice were identified as tubules invaginating from the surface airway epithelium that had at least 10 clearly identifiable nuclei within the interstitium. In all cases for both adult and newborn mice, tracheas were bisected longitudinally into two halves, fixed in 4% paraformaldehyde in pH 7.3 phosphate-buffered saline, and stained with PAS (Periodic Acid Schiff) prior to embedding in paraffin. For PAS staining, tracheas were incubated in 0.5% periodic acid (3 minutes), rinsed and incubated in Schiff’s reagent (3 minutes). Tracheas were then dehydrated, cleared and embedded in paraffin. Each tracheal sample was sectioned (9 μm) in its entirety and all sections stained with Hematoxylin to reveal cellular nuclei. All sections containing visible surface airway epithelium were quantitated for the presence of gland ducts. Additionally, the rostral-caudal level where gland ducts were found was recorded to give a distribution of glands relative to the cartilage ring number. Unlike human bronchial xenografts, longitudinal sections were required to obtain this information. The number of animals evaluated for each transgenic or knockout line are given in the figure legends and normally included 4-10 mice for each genotype.

Inhibition of Lef1 expression with antisense oligonucleotides decreases the abundance and branching morphogenesis of submucosal glands in ferret tracheal xenograft airways

The ferret is a superior animal model for investigation of airway SMG development, since the cell types and distribution of SMGs closely resemble those in the human airway (Curtis et al., 1987; Leigh et al., 1986; Robinson et al., 1986). In ferrets, SMG development occurs postnatally (Leigh et al., 1986), reflecting a development stage in humans that occurs between 14 and 28 weeks of gestation. This characteristic has been previously exploited to demonstrate that Lef1 mRNA expression is confined to the earliest stages of primordial bud formation in SMG progenitor cells by in situ hybridization (Duan et al., 1998a). To further investigate the functional involvement of Lef1 in submucosal gland morphogenesis, antisense inhibition experiments were conducted using a newborn ferret tracheal xenograft model.

Fig. 2. Inhibition of nasal submucosal gland development in Lef1-deficient mice. The effect of Lef1 deficiency on nasal submucosal gland development was examined in (A,B) wild-type and homozygous Lef1-deficient (C,D) mice at day 12 postnatally. Tissues were embedded in OCT and sectioned at 7 μm; H & E-stained cryosections at the mid-maxillary level. (B) Plentiful SMGs with well-developed tubular structures are evident in higher power photomicrographs of the nasal mucosa in wild-type mice. (D) In contrast, no glands are distinguishable in Lef1-deficient mice and only amorphous cellular aggregates are observed where glands are normally present. Arrows in A and C indicate areas shown at higher magnification in B and D, respectively. Arrowheads point to areas rich in SMGs of wild-type mice. N, nasopharynx; nc, nasal conchae; ps, palatal shelf; S, nasal septum.
remained at constant intensity over all doses of phosphorothioate oligonucleotide treatment, indirectly providing evidence for the specificity of antisense inhibition.

To test the physiological relevance of antisense inhibition on submucosal gland morphogenesis, F12 medium containing 5, 10 or 50 μM antisense or sense oligonucleotides was applied to the lumen of newborn ferret xenografts over a developmental period of 4 weeks (see Materials and Methods). As illustrated in Fig. 1A, SMGs develop normally in F12 control-treated newborn ferret xenografts over this time frame (Duan et al., 1998a). The numbers of glands (quantified by the number of ciliated duct openings at the lumenal surface of the airway per section) and the size of each gland (quantified by the number of tubules per tracheal section) were compared in three groups of four independent xenografts each, treated with antisense or sense oligonucleotides, or controls in which the xenograft lumen was infused with F12 medium alone (Fig. 1C). In the absence of oligonucleotides, a mean (±s.e.m.) of 0.32±0.10 gland duct openings and 2.1±0.3 gland tubules were present per tracheal cross-section. This reflected approximately 3 glands/mm² and is similar to that reported for native human bronchial airways (Engelhardt et al., 1995). Based on this abundance, we estimated that there were approximately 300 glands in control ferret xenografts infused with F12 media by the 4 week harvesting time point. In xenografts treated with 50 μM sense oligonucleotides, a mean (±s.e.m.) of 0.24±0.06 gland duct openings and 1.9±0.1 gland tubules were detected per tracheal cross-section. These numbers were not significantly different to those seen in control tracheas which did not receive any oligonucleotides. In contrast, in the presence of 50 μM antisense oligonucleotides, a greater than 8-fold decrease (P<0.001) in both SMG number and size was observed as compared to sense oligonucleotide controls. On average, only 0.035±0.01 ciliated duct openings and 0.25±0.06 gland tubules were present per tracheal cross-section in antisense treated xenografts. No significant effect on submucosal gland organogenesis was detected under lower antisense oligonucleotide concentrations of 5 and 10 μM (data not shown). These results support the hypothesis that the expression of LEF1 in SMG progenitor cells is functionally important in initiating and supporting SMG morphogenesis in the airway.

Lef1-deficient mice have impaired nasal submucosal gland development

To extend and confirm the finding that antisense inhibition of Lef1 expression blocked SMG development in newborn ferret tracheal xenografts, analyses of SMGs in the airways of Lef1 knockout mice were performed. Although the secretory cell types and abundance of SMGs in the murine airway are divergent from those of humans and ferrets, the murine nasal mucosa is especially rich in SMGs, and the function and histology of the nasal mucosa are similar to the intrapulmonary airway. We therefore hypothesized that processes controlling nasal airway gland development in the mouse may be similar to those in the cartilaginous airways of larger mammals.
Following confirmation of the Lef1-deficient genotype, SMG morphology in the nasal mucosa of 12-day-old Lef1 knockout mice and age-matched wild-type or heterozygous siblings was systemically examined. As seen in Fig. 2A,B, transverse sections at the mid-maxillary level of wild-type mice demonstrated well-defined tubular glands located within the nasal septum and beneath the nasal conchae along the lateral wall of the middle meatus. In striking contrast, Lef1−/− mice exhibit an absence of morphologically recognizable SMGs, resulting in distortion and shrinkage of the nasal submucosal tissue (Fig. 2C,D). Also of note was the fusion of two separate nasal conchae in the Lef1 mutant mice, which may reflect the loss of tissue mass from the aborted growth of submucosal glands. Under higher magnification, wild-type mice showed fully differentiated seromucous tubules and collecting ducts (Fig. 2B). However, in Lef1−/− mice, only patches of amorphous cell aggregates were seen in the same areas, and no definitive serous or mucous cells could be identified (Fig. 2D). Similarly, a comparison of the nasal turbinates of normal and mutant mice further illustrates the depletion of SMGs resulting from LEF1 deficiency (data not shown). These defects in SMG morphogenesis also extended to proximal regions of the trachea, where no SMGs could be detected in Lef-1-deficient mice (discussed later in the text).
Ectopic expression of LEF1 in human airway progenitor cells does not increase SMG development in human bronchial xenograft airways

Studies using both antisense inhibition of Lef1 expression and Lef1 knockout mice have clearly demonstrated the functional importance of LEF1 in directing SMG morphogenesis. To extend these findings, the effect of ectopic overexpression of LEF1 on SMG morphogenesis was evaluated in a bronchial xenograft model of the human airway using a recombinant AAV vector. This fully differentiated xenograft model has been previously utilized to identify progenitor cells of SMGs (Engelhardt et al., 1995). Although developing glands are somewhat infrequent in this model, we reasoned that overexpression of LEF1 might impart increased gland development. For these experiments, a bicistronic proviral plasmid (pAV.LEF1/GFP) containing cDNAs for both human LEF1 and GFP was constructed and rAAV (AV.LEF1/GFP) prepared (Fig. 3A). To determine whether this vector expressed LEF1 retaining functional DNA-binding specificity, electrophoretic mobility shift assays were performed. As shown in Fig. 3B, nuclear extracts from AV.LEF1/GFP-infected 293 cells specifically bound a TCRα mini-enhancer containing a consensus LEF1-binding motif. This binding was competitively blocked by addition of cold TCRα LEF1 DNA-binding site or anti-LEF1 antibodies, but not by an irrelevant DNA sequence (p53) or pre-immune serum. In contrast, nuclear extracts from AV.GFP (a similar viral construct encoding GFP but not the LEF1 transgene)-infected 293 cells did not exhibit binding when incubated with radiolabeled TCRα mini-enhancer probe (Fig. 3B). We next sought to evaluate whether this AV.LEF1/GFP vector could induce ectopic gland development in human bronchial xenografts by overexpressing LEF1 in airway progenitor cells.

Human bronchial xenografts were reconstituted using primary human bronchial cells infected with recombinant AV.LEF1/GFP or AV.lacZ virus. Infection efficiencies of primary cells were greater than 90% for both the lacZ and LEF1/GFP constructs (Fig. 3C). Furthermore, immunofluorescent detection of GFP and LEF1 protein demonstrated co-localization in all airway cells infected with AV.LEF1/GFP. No endogenous LEF1 was detected in AV.lacZ-infected airway epithelial cells (Fig. 3C). Denuded rat tracheas were seeded with infected primary airway epithelial cells and the xenograft cassettes were implanted subcutaneously into nude mice. By 5 weeks post-transplantation, infrequent glands

Fig. 5. Analysis of tracheal submucosal gland development in CC10/LEF1 transgenic mice. (A) The Pmel fragment of the pCC10/LEF1 plasmid was used to generate CC10/LEF1 transgenic mice by microinjection of fertilized oocytes. (B) Western blotting was used to evaluate the tissue-specific expression of CC10 regulated LEF1 in F1 generation progeny from three founder lines. The F1 genotypes derived from Southern blots (data not shown) are given above each lane for the transgenic (+) or wild-type/non-transgenic (−) alleles. LEF1 expression was detected in western blots of trachea/intralobar bronchi samples (top of B) from heterozygous progeny in two of the three founder lines illustrated (180008 and 18016). No LEF1 expression was seen in samples from these same mice in the liver (Bottom of B) or in other organs examined (data not shown). The position of the LEF1 protein is indicated by arrows. The positive and negative control lanes contain lysates from pAV.LEF1/GFP and pAV.GFP transfected 293 cells, respectively. Tracheas were harvested from 4-month-old heterozygous CC10/LEF1 transgenic mice and wild-type age-matched littermates. (C) Representative longitudinal paraffin section stained in PAS from the proximal trachea of a 4-month-old CC10/LEF1 transgenic mouse. This low-power image shows tracheal rings 1-4 with SMGs in the interstitial tissue. (D) The boxed region is enlarged in the lower panel to demonstrate gland acini and tubules. The total number of SMGs overlaying each cartilage ring was quantified using serial sections of the tracheas of ten CC10/LEF1 heterozygous mice and nine wild-type control aged matched littermates. The abundance of submucosal glands in the trachea was quantitated as described in the Materials and Methods; mean (±s.e.m.) number of glands per cartilaginous ring. No significant differences were seen between the two groups of animals.
could be seen in the submucosa of xenograft epithelium (Fig. 4A). As determined in a previous report using retroviral marking studies, these glands are derived from human airway progenitor cells (Engelhardt et al., 1995). The expression of LEF1 and GFP in xenograft epithelia was determined in fixed cryosections and demonstrated nuclear localization of LEF1 in GFP-expressing airway cells of AV.LEF1/GFP-infected xenografts (Fig. 4C,E,G). Although control AV.lacZ-infected xenografts showed no FITC GFP fluorescence in the surface airway epithelia (Fig. 4D), mesenchymal cells within the interstitium of both AV.lacZ and AV.LEF1/GFP-infected xenografts demonstrated LEF1 immunoreactive cells which were not GFP-positive (Fig. 4B-G). These cells are most likely endogenous hematopoetic cells of mouse origin which express LEF1. In most instances, GFP fluorescence in the surface airway epithelium co-localized with LEF1 expression in AV.LEF1/GFP-infected xenografts. However, infrequent LEF1-positive/GFP-negative cells in the surface airway epithelium could be seen, which may either represent migrating hematopoetic cells or differences in the plane of section showing nuclei (LEF1) and cytoplasm (GFP). On average, 12% of the reconstituted epithelium was targeted by AV.LEF1/GFP as determined by morphometric analysis of digitized fluorescent GFP images (Fig. 4H).

To evaluate whether ectopic expression of the LEF1 gene was capable of inducing gland formation, serial frozen sections were examined from xenografts that were either infected by AV.LEF1/GFP or a control AV.lacZ virus (4 independent xenografts for each vector), and submucosal gland structures (gland duct openings and tubules) were quantified. As shown in Fig. 4H, no significant differences were noted between xenografts infected with LEF1-expressing vector (3.5 gland duct openings and 12.5 gland tubular structures per 10 mm²) or the control vector (3.5 gland duct openings and 11.5 tubules per 10 mm²). These findings suggest that other factors in addition to LEF1 expression may be essential to induce gland formation in the human airway.

**Overexpression of LEF1 in the proximal airways of transgenic mice is not sufficient to increase submucosal gland abundance**

To substantiate the conclusions of the xenograft LEF1 overexpression experiments, transgenic mice expressing the human LEF1 gene under the direction of an airway-specific promoter were produced. The Clara cell 10 kDa protein promoter (CC10) has been previously used to target foreign genes to the proximal airway (Ray et al., 1997; Stripp et al., 1992) and was chosen for driving human LEF1 expression in the present study. Founder lines were established following microinjection of a linear Pme1 CC10/LEF1 fragment into murine zygote pronuclei (Fig. 5A). Western blot analysis of several organs from the progeny demonstrated tracheal/intralobar bronchial LEF1 expression in two of three founder lines (Fig. 5B). To investigate submucosal gland morphogenesis in LEF1 overexpressing transgenic mice, the abundance of SMGs was quantified in PAS-stained longitudinal tracheal sections from 4-week-old progeny as described in the Material and Methods. In total, the tracheas of 10 heterozygous CC10/LEF1 transgenic mice and 9 wild-type age-matched littermates were analyzed. Fig. 5C illustrates the morphology of submucosal glands in the proximal trachea of a transgenic mouse heterozygous for the CC10/LEF1 transgene loci, which was indistinguishable from that seen in control non-transgenic littermate mice (data not shown). Morphometric analysis of SMG abundance at various rostral-caudal levels of the trachea confirmed previous findings that submucosal glands are primarily located in the murine proximal trachea, with the highest levels (6 glands), in both wild-type and transgenic animals at tracheal ring 1 (Fig. 5D). The number of glands per cartilaginous ring decreased dramatically distally to cartilage ring one but could be seen as far as cartilage ring eight. This evaluation demonstrated no significant difference in the distribution or number of glands between LEF1 transgenic and control littermate mice. Thus, in agreement with experiments assessing the effects of virally mediated overexpression of LEF1 in human xenograft airways, these studies indicate that, although LEF1 expression is necessary for gland development, it is not a sufficient signal for initiating submucosal gland development with levels of expression achieved in these model systems.

**Rescue of submucosal gland development in Lef1 knockout mice harboring an airway-specific CC10/LEF1 transgenic allele**

Results thus far have provided strong evidence that LEF1 is required for SMG development, but also indicate that LEF1 alone is insufficient to initiate and support gland development, and suggest that other cellular factors are required as well. One potential caveat to this interpretation is that the levels of LEF1 expressed by these ectopic systems may be rate-limiting and insufficient to promote cellular commitment to SMG morphogenesis in the airway. To differentiate which of these two potential hypotheses were responsible for a lack of a dominant effect of ectopic LEF1 expression to induce airway gland development, we attempted to rescue the glandless phenotype in Lef1 knockout mice with the transgenic CC10/LEF1 allele. We reasoned that if the CC10/LEF1 transgene could rescue gland development on the Lef1 knockout background, it would be rational to conclude that expression of the LEF1 transgene was achieved in the proper compartment of gland progenitors at the correct time in development. These findings could then add further support to the notion that the lack of a ectopic gland development, in a setting of LEF1 overexpression, is due to insufficient levels of another co-factor(s) needed for gland development in the airway.

Gland rescue experiments were performed by breeding heterozygous Lef1 mutant mice (+/-) with heterozygous CC10/LEF1 transgenic mice (+/-) to produce F1 generation founders with one allele of both the mutant endogenous Lef1 gene and the CC10/LEF1 transgene (Fig. 6A). The genotype of pups of the F2 generation was determined shortly after birth by Southern blot hybridization with knockout and CC10/LEF1 probes to identify progeny homozygous for Lef1 deficiency (-/-) that also possessed at least one allele of the CC10/LEF1 gene (Fig. 6B,C). Morphometric analysis was performed as described in the Material and Methods on paraffin tracheal sections from 3-day-old littermates, since the homozygous Lef1 mutation is usually lethal by the second postnatal week (van Genderen et al., 1994). Furthermore,
hybridized with a knockout probe derived from genomic sequences BamHI and HI and out using genomic DNA from the same animals illustrated in B, Lef1 (C) Southern blot analysis of the endogenous locus was carried diagnostic for the presence of a CC10/LEF1 comprising full-length human numbers. The blot was hybridized with a radiolabeled probe Lef1 LEF1 in the endogenous gene and heterozygous for the CC10/LEF1 phenotype of CC10/LEF1 expression in the airway could rescue the glandless transgenic allele on the Lef1 CC10/LEF1 mice numbered 1-5 in A were genotyped by Southern analysis of the band is diagnostic for a knockout allele (domain. A 12.9 kb band indicates a wild-type allele (+), and a 4.9 kb (A) The breeding strategy for generating these mice. F2 generation of the CC10/LEF1 transgenic locus (B) was carried out with BamHI-digested tail genomic DNA from F2 generation progeny indicated by numbers. The blot was hybridized with a radiolabeled probe comprising full-length human LEF1 cDNA, and a 1.6 kb band is diagnostic for the presence of a CC10/LEF1 transgenic allele (+). (C) Southern blot analysis of the endogenous Lef1 locus was carried out using genomic DNA from the same animals illustrated in B, except that DNA was digested with both BamHI and EcoRI and hybridized with a knockout probe derived from genomic sequences 5' of the inserted 'neo' gene in the second exon of the Lef1 HMG domain. A 12.9 kb band indicates a wild-type allele (+), and a 4.9 kb band is diagnostic for a knockout allele (-).

evaluation of littersmates avoided any background-dependent variations in SMG abundance seen in mice. Gland abundance was analyzed from 4 pups homozygous for the Lef1 mutation in the endogenous gene and heterozygous for the CC10/LEF1 allele (Lef1+/−:CC10/LEF1+/−), 4 pups with homozygous Lef1 mutation but no CC10/LEF1 allele (Lef1+/−:CC10/LEF1+/−) and 6 wild-type control littersmates (Lef1+/+:CC10/LEF1+/−). As illustrated in Fig. 7A, newly forming gland buds, as well as more developed glands with tubules invaginating into the submucosal region, were evident in the proximal tracheas of wild-type pups (Lef1+/+:CC10/LEF1+/−). At 3 days of age, developing submucosal glands were only seen at the level of tracheal cartilage rings 1 and 2, indicative of the normal postnatal development of submucosal glands that occurs in this species, and in agreement with previous results of a rostral-to-caudal gradient in the number of submucosal glands in animals with mature submucosal glands at 4 months of age (see Fig. 5). In marked contrast, no submucosal glands were detected in the tracheae of homozygous Lef1-deficient mice (Lef1+/−:CC10/LEF1+/−) (Fig. 7C). However, sections from pups homozygous for the Lef1 mutation, but also harboring one allele at the CC10/LEF1 transgenic locus (Lef1+/−:CC10/LEF1+/−), exhibited a rescue of submucosal gland development (Fig. 7B). In these animals, developing glands were evident at the level of tracheal cartilage rings 1 and 2, as was seen in wild-type littersmates. Furthermore, the distribution of glands was also similar to the wild-type pups, with glands restricted to the proximal 2 cartilage rings. Results of morphometric analysis between these three genotypes is summarized in Table 1.

**DISCUSSION**

Submucosal gland morphogenesis in the airway has been a difficult aspect of human lung development to address for several reasons. First, progenitor cells responsible for gland development are infrequent in the airway. Second, it is difficult to reconstitute all aspects of submucosal glands using in vitro model systems. Lastly, rodent models only partially resemble the native architecture and abundance of submucosal glands in the human airway. With these limitations in mind, we have undertaken a comprehensive analysis of submucosal gland development using three species (human, ferret and mouse) in independent model systems to address aspects of LEF1 involvement in submucosal gland morphogenesis. These studies have established a requirement for the transcription factor LEF1 in SMG development in the airways. Evidence supporting this conclusion includes an 8-fold decrease in SMG morphogenesis in newborn ferret xenografts following antisense oligonucleotide inhibition of Lef1 expression, as well as the absence of tracheal and nasal airway SMGs in Lef1 knockout mice. In a previous study, we demonstrated that Lef1 expression is limited in the airway to cells aggregating to form a thickening in the basal part of the epithelium at the earliest stages of airway SMG gland bud formation (Duan et al., 1998a). Lef1 expression is then maintained in cells in the tips of elongating gland buds invading the underlying interstitium and, in later stages, Lef1 remains confined to the distal ends of growing tubules as SMG morphogenesis proceeds. Extending these descriptive observations, the current studies have shown that inhibiting Lef1 expression blocks the earliest stages of gland bud formation. These experiments strongly support an obligatory role for LEF1 in the complex, reciprocal developmental processes leading to SMG morphogenesis.

Although LEF1 is required for airway gland development, other data also indicate that LEF1 protein alone is not an adequate signal for initiating SMG morphogenesis. Results
Cartilage ring CC10/LEF1 phenotype in that the CC10/LEF1 gene or IL-11 to epithelial cells throughout the proximal cartilaginous airways of transgenic mice (Ray et al., 1997; Novak et al., 1998). Notably, the development of many parallel pathways (Hsu et al., 1998; McKendry et al., 1997; McKendry et al., 1998). Notably, the development of many structures, such as submucosal gland development, is likely to involve interactions between multiple signaling pathways.

Table 1. Quantitation of developing submucosal glands in 3-day-old LEF1 transgenic and Lef1 knockout mice

<table>
<thead>
<tr>
<th>Cartilage ring number</th>
<th>Leff1+/+ : CC10/LEF1+/+ (n=6)</th>
<th>Leff1+/− : CC10/LEF1+/− (n=4)</th>
<th>Leff1−/− : CC10/LEF1−/− (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.73±0.14</td>
<td>0.58±0.08</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.58±0.10</td>
<td>0.17±0.05</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data represent the mean ± s.e.m. number of glands found in the interstitium above each cartilage ring. The entire trachea was sectioned longitudinally and gland numbers were quantitated as visible gland ducts connecting with the surface airway epithelium.

Our findings that LEF1 is not sufficient by itself to initiate SMG development is in agreement with previous studies evaluating the mechanisms of transcriptional activation by LEF1. These studies have demonstrated that LEF1, as is the case with the other TCF family HMG transcription factors, is unable to activate transcription of target genes by itself. In T-lymphocytes LEF1 stimulates the TCR-α enhancer only in context-dependent collaboration with other DNA-binding proteins (Giese and Grosschedl, 1993; Travis et al., 1991; Waterman et al., 1991). Similarly, LEF1 can mediate Wnt signaling during development through transcriptional activation of target genes in association with the protein β-catenin. In this pathway, the tight regulation of free cytoplasmic pools of β-catenin is the key factor in mediating signaling (Cavallo et al., 1997; Papkoff et al., 1996; Yost et al., 1996). As in lymphocyte differentiation, the evidence suggests that transcriptional activation of Wnt target genes by the LEF1/β-catenin complex is also dependent on the specific context of other transcription factors activated by signaling in parallel pathways (Hsu et al., 1998; McKendry et al., 1997; Novak et al., 1998). Notably, the development of many parallel pathways (Hsu et al., 1998; McKendry et al., 1997; McKendry et al., 1998). Notably, the development of many parallel pathways (Hsu et al., 1998; McKendry et al., 1997; McKendry et al., 1998). Notably, the development of many parallel pathways (Hsu et al., 1998; McKendry et al., 1997; McKendry et al., 1998). Notably, the development of many parallel pathways (Hsu et al., 1998; McKendry et al., 1997; McKendry et al., 1998). Notably, the development of many parallel pathways (Hsu et al., 1998; McKendry et al., 1997; McKendry et al., 1998). Notably, the development of many

from two model systems support this conclusion. First, ectopic overexpression of the LEF1 gene in airway progenitor cells of human bronchial xenografts with recombinant virus did not increase the extent of gland development. Similarly, CC10-directed expression of the LEF1 gene in proximal airways of transgenic mice did not increase the number of tracheal SMGs over control non-transgenic littermates. The CC10 promoter has been documented to direct the expression of a CA T reporter gene under a hair-keratin-specific promoter has a dominant effect in directing hair follicle patterning and epithelial cell fates (Dunn et al., 1998; Zhou et al., 1995). These findings suggest that although LEF1 is required in both models of organogenesis, differences in their regulatory mechanisms must exist.

Our findings that LEF1 is not sufficient by itself to initiate SMG development is in agreement with previous studies evaluating the mechanisms of transcriptional activation by LEF1. These studies have demonstrated that LEF1, as is the case with the other TCF family HMG transcription factors, is unable to activate transcription of target genes by itself. In T-lymphocytes LEF1 stimulates the TCR-α enhancer only in context-dependent collaboration with other DNA-binding proteins (Giese and Grosschedl, 1993; Travis et al., 1991; Waterman et al., 1991). Similarly, LEF1 can mediate Wnt signaling during development through transcriptional activation of target genes in association with the protein β-catenin. In this pathway, the tight regulation of free cytoplasmic pools of β-catenin is the key factor in mediating signaling (Cavallo et al., 1997; Papkoff et al., 1996; Yost et al., 1996). As in lymphocyte differentiation, the evidence suggests that transcriptional activation of Wnt target genes by the LEF1/β-catenin complex is also dependent on the specific context of other transcription factors activated by signaling in parallel pathways (Hsu et al., 1998; McKendry et al., 1997; Novak et al., 1998). Notably, the development of many
tissues including tooth, hair and mammary glands involves interactions between Wnt pathway components and signaling by other developmental regulating molecules, including Sonic hedgehog (sh). Bone morphogenetic proteins (BMP) 2 and 4, and Hepatocyte growth factor (Dassule and McMahon, 1998; Kratochwil et al., 1996; Papkoff and Aikawa, 1998). As part of a continued effort to define the mechanisms regulating airway SMG development, we have initiated studies localizing Wnt pathway components and related factors with in situ hybridization or immunocytochemistry in the postnatal ferret trachea (T. C. R. and J. F. E., unpublished observations). The preliminary results demonstrate that cells in developing gland buds exhibit a high level of β-catenin expression, as has been previously shown for Lef1 (Duan et al., 1998a), which is consistent with involvement of the Wnt pathway in the development of SMGs in the airway.

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