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RAPID COMMUNICATION

TTF-1 Regulates Lung Epithelial Morphogenesis

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TTF-1 is a homeodomain transcriptional factor expressed in thyroid, lung, and parts of the brain. *In vitro,* TTF-1 can activate the promoter of thyroid- and pulmonary-specific genes. We postulated that TTF-1 not only is essential for the activation of tissue-specific genes, but also may directly participate in epithelial cell morphogenesis. To test this postulate, we used an antisense oligonucleotide inhibitory strategy in an *in vitro* model of embryonic mouse lung branching morphogenesis. This strategy suppressed *TTF-1* translation and inhibited lung branching morphogenesis. The resulting abnormal phenotype was characterized by hyperplastic and unorganized proliferation of epithelial cells in the airways. The mesenchymal compartment of the lung appeared to be unaffected. These results demonstrate, for the first time, that the expression of a homeoprotein transcriptional regulator is necessary for lung epithelial morphogenesis.

The origin of the mammalian lung epithelium is the embryonic gut endoderm (Ten Have-Opbroek, 1991). The pulmonary epithelium grows into the splanchnic mesenchyme by branching morphogenesis, a process requiring epithelial–mesenchymal interactions (for a review see Minoo and King, 1994). The molecular regulatory signals and the role of transcriptional regulators in branching morphogenesis are largely unknown. The epithelial-specific thyroid transcriptional factor *TTF-1* is expressed in two of the many structures which emerge from the gut endoderm, the thyroid and the lung (Lazzaro *et al.,* 1991). *TTF-1* mRNA is detectable within the ventrally migrating edge of the lung bud, at the onset of lung morphogenesis on Embryonic Day (E) 10.5 in the rat (Lazzaro *et al.*, 1991). In E 11.5 embryos a strong signal can be detected in both branches of the primitive bronchi, and as the lung develops (from E 13.5 to E 15.5) *TTF-1* mRNA is expressed in the bronchial epithelium, while the surrounding mesenchyme and the epithelium of the upper respiratory airways remain negative. Although it has been demonstrated that TTF-1 binds to the pulmonary-specific *SP-B* and *CC10* gene promoters and regulates their expression (Bohinski *et al.*, 1994), no information exists regarding the functional role of TTF-1 in morphogenesis of the pulmonary epithelium and emergence and cytodifferentiation of the specialized epithelial cell types.

A highly specialized lung epithelial cell type, alveolar epithelial Type II cells, is the major site of synthesis of pulmonary surfactant. Using mRNA from these cells, we have cloned a number of human *TTF-1* cDNAs and determined that transcription of the *TTF-1* gene in the lung is complex (Hamdan *et al.*, unpublished). Using a full-length human lung *TTF-1* cDNA (GenBank Accession No. U33749) as probe, we found *TTF-1* mRNA and protein expression in fully differentiated alveolar epithelial Type II cells. However, in agreement with the results in rat embryos (Lazzaro *et al.*, 1991), *TTF-1* gene expression was also detectable in stages of lung development before the onset of epithelial cell differentiation. This observation suggested that *TTF-1* may play a role in epithelial cell lineage and morphogenesis during lung development.

To examine this possibility, we studied the impact of antisense oligonucleotide (ODN) inhibition of *TTF-1* mRNA translation on morphogenesis of mouse embryonic lung explants. Mouse lungs excised from E 10.5–E 11 embryos which consist of the rudimentary trachea and the primary branched major bronchi will undergo normal, although slower (compared to *in vivo*) branching morphogenesis (Fig. 1a) in a serum-free medium without the addition of exogenous factors (Jaskoll *et al.,* 1988). In this model system, antisense ODNs have been used successfully to suppress the expression of specific target genes (Seth *et al.*, 1993; Souza *et al.*, 1994). To determine the functional role of *TTF-1* in lung epithelial morphogenesis, lung rudiments from E 10.5–E 11 mouse embryos were explanted in culture

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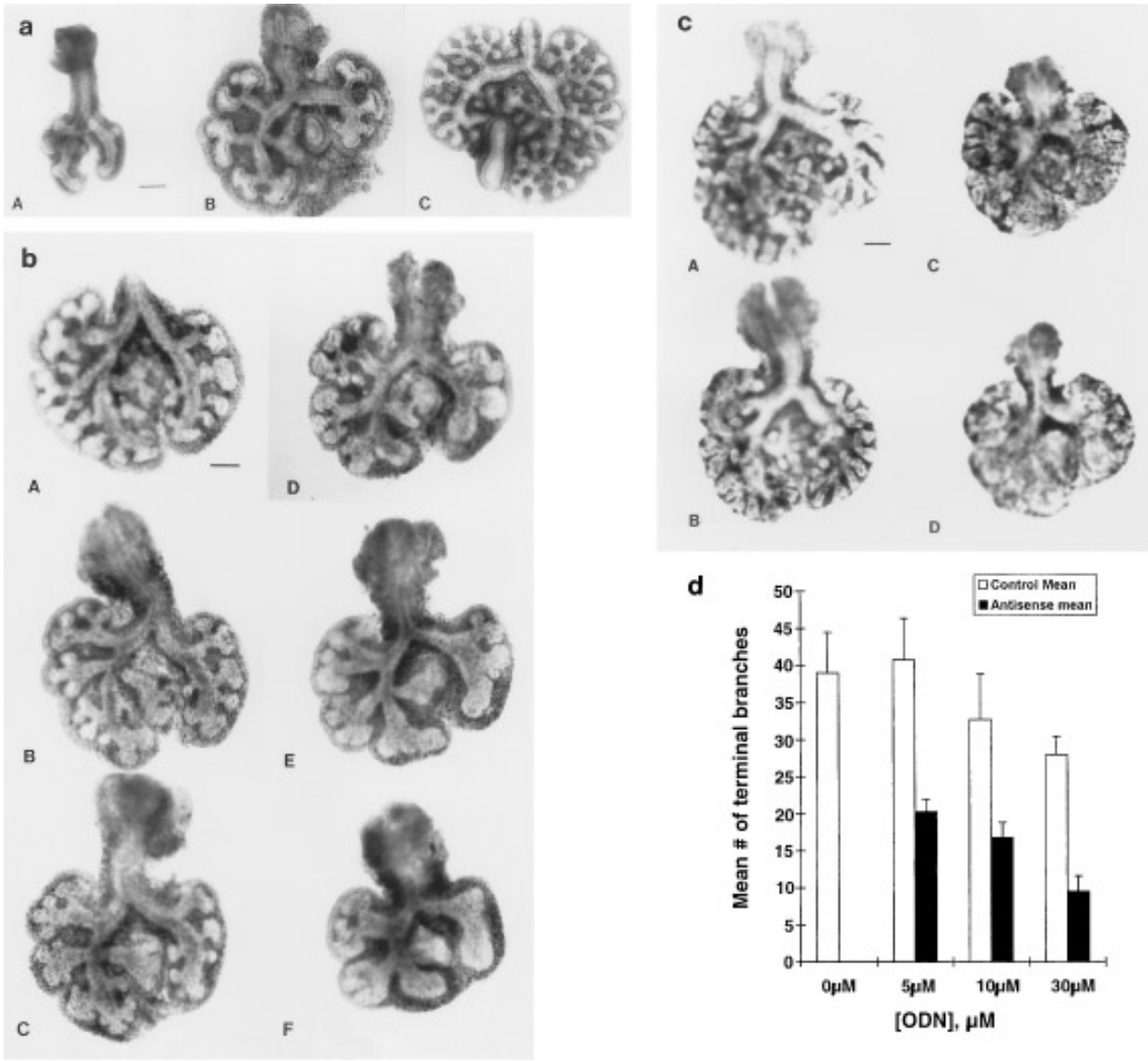

FIG. 1. (a) Morphogenesis of E 10.5–E 11 mouse embryonic lungs *in vitro.* Day 10.5–11 mouse embryonic lungs were excised and cultured *in vitro* using a chemically defined, serum-free medium. Day 10.5–11 lung immediately before (A), after 2 days (B), and after 4 days (C) in culture is shown. Scale bar, 200 μm. (b) Effect of *TTF-1* antisense ODNs on embryonic lung morphogenesis *in vitro.* E 10.5–E 11 mouse embryonic lungs were cultured in a chemically defined, serum-free medium for 2 days in the presence of either a scrambled (control, left panels) or a *TTF-1* antisense (right panels) ODN. *TTF-1* antisense and scrambled ODNs were used at 5 (A and D), 10 (B and E), and 30 (C and F) μM concentrations. Scale bar, 200 μm. (c) Effect of *TTF-1* antisense ODNs on embryonic mouse lung morphogenesis *in vitro.* Lung rudiments from E 10.5–E 11 mouse embryos were cultured in a chemically defined, serum-free medium for 4 days in the presence of either a scrambled ODN (control, left) or a *TTF-1* antisense (right) ODN. *TTF-1* antisense and scrambled ODNs were used at 10 (A and C) and 30 (B and D) μM concentrations. Scale bar, 200 μm. (d) Morphometric assessment of embryonic lung explants treated with *TTF-1* antisense and control ODNs. At the end of the 4-day culturing period, the lung explants were photographed under the microscope by transillumination and the terminal branching points were counted manually and recorded. With the exception of untreated group (0 μM), which consists of eight lungs, each point represents data collected from three to five individual lung explants. Results are expressed as mean ± SEM.

in the presence of three nontoxic doses (5 to 30 μM) of either the antisense ODN, 5′ CAT CGA CAT GAT TCG GCG GCG G 3′, designed to a highly conserved DNA motif that surrounds the human, rat, and mouse *TTF-1* translational initiation site, or the control (same nucleotide composition but scrambled sequence), 5′ AGA TCG AGC CGC

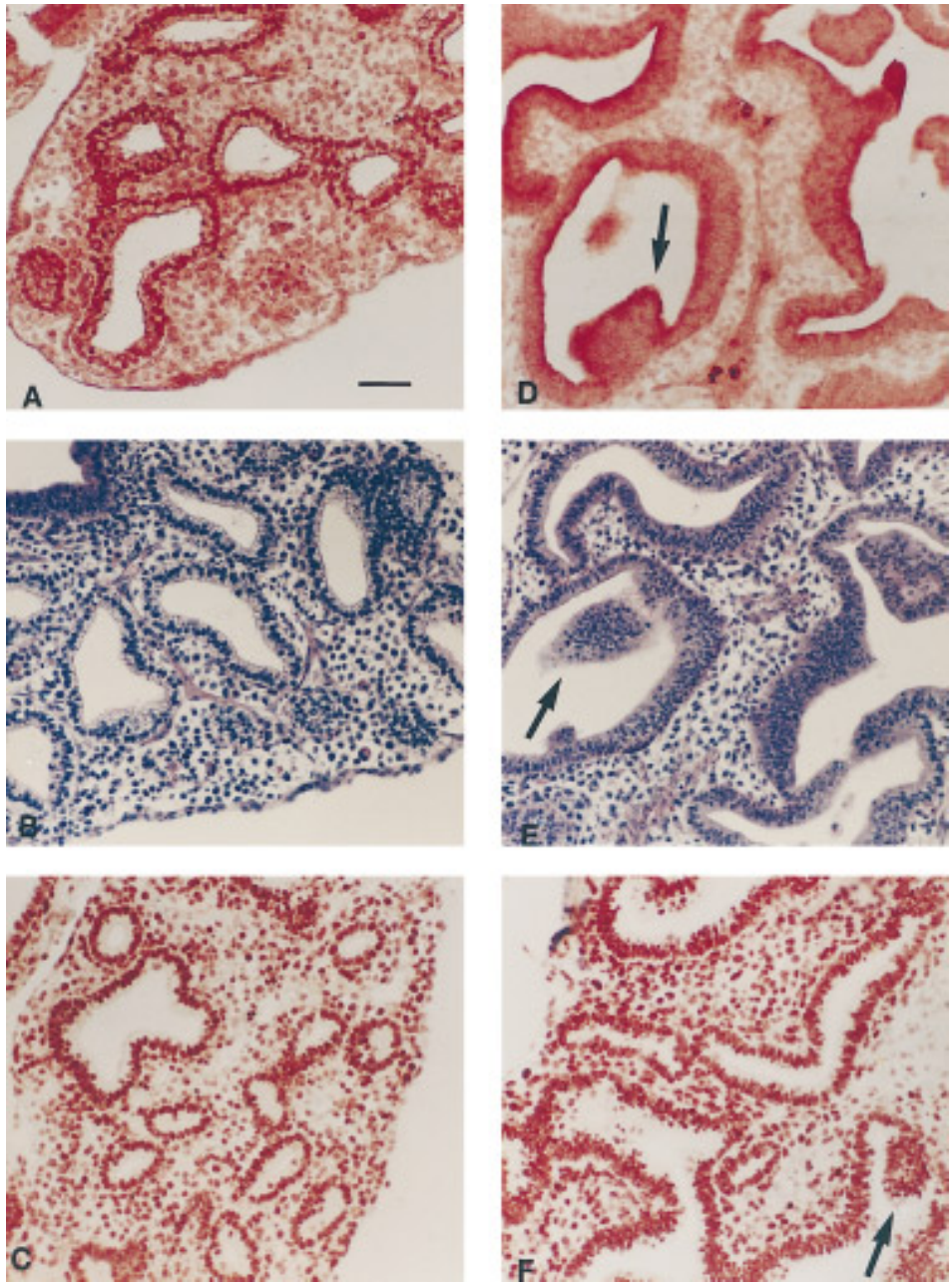

FIG. 2. Immunohistochemical and histological assessment of mouse embryonic lungs cultured in presence of either 30 μM scrambled ODN (control, left) or 30 μM *TTF-1* antisense (right) ODN for 4 days. Immunostaining with the rabbit polyclonal anti-TTF-1 antibody (A and D). Histology of the lungs visualized by hematoxylin and eosin staining (B and E). Immunostaining with a rabbit polyclonal anti-PCNA antibody (C and F). Note the epithelial thickening of the airways in D, E, and F and the dysmorphic epithelial inclusions within the airways (arrow). Scale bar, 40 μm. Morphology and pattern of staining for both TTF-1 and PCNA in untreated control lungs (no ODN) were similar to those presented in A, B, and C.

TGT AGC GTC G 3′ ODN for 2 or 4 days. At the end of the experimental period, the lungs were photographed, the extent of branching was quantified by counting the number of terminal branches, and the tissues were fixed in Carnoy's solution for histology and immunocytochemical analyses.

Upon addition of the *TTF-1* antisense ODN, lung branching and epithelial morphogenesis were inhibited in a dose-dependent manner (Figs. 1b, 1c, and 1d). The extent of inhibition was more severe in 4-day cultures, in which the *TTF-1* antisense had reduced branching by greater than

64% (Figs. 1c and 1d). In these lungs, branches consisted of large baggy structures whose histological examination revealed unorganized hyperplastic epithelial cell masses obtruding into the airway space (Figs. 2D, 2E, and 2F, arrows). Limited inhibition of branching was also observed in lungs treated with the control ODN for 2 days (Fig. 1b, panels A, B, and C). However, by 4 days in culture, both untreated lungs and those treated with the control ODN had undergone extensive branching and were clearly distinguishable from the lungs treated with the *TTF-1* antisense ODN (Fig. 1c). The staining patterns of untreated lung explants (no ODN) for PCNA and TTF-1 were also similar to those in the scrambled ODN group (Fig. 1b, panels A, B, and C). Importantly, none of the lung explants in the control groups displayed a phenotype resembling the dysmorphic epithelial features found in the *TTF-1* antisense-treated lungs.

A number of studies have used antisense ODNs to investigate the function of specific gene products during morphogenesis. Interpretations of these results must be made in the context of several technical problems that are inherent to this approach. Neither the exact mechanism of antisense ODN action nor their specificities are well understood (Boiziau *et al.*, 1991). However, small ODNs in the range used in the current study are known to be taken up by target cells and confer sufficient specificity of hybridization to target mRNA (Marcus-Sekura, 1988). We designed the *TTF-1* antisense ODN to include DNA sequences flanking the *TTF-1* translational initiation codon, an area known to be most effective in inhibiting translation of target mRNA (Marcus-Sekura, 1988). Immunostaining of lungs with an anti-TTF-1 peptide-specific antibody, developed as a part of this study, showed that TTF-1 protein is indeed extinguished or highly diminished in epithelial cells of lungs treated with *TTF-1* antisense ODN (Fig. 2D). Lungs treated with the random control ODN showed positive epithelial staining for TTF-1 (Fig. 2A). No differences were observed between thiosulfated and unmodified ODNs in their ability to extinguish TTF-1 protein nor in their ability to inhibit epithelial and branching morphogenesis. Furthermore, to determine the specificity of the *TTF-1* antisense ODNs, we investigated the expression and distribution of another nuclear protein, PCNA, which is a reliable marker of cellular proliferation. Immunostaining with an anti-PCNA antibody (Figs. 2C and 2F) suggests that treatment with *TTF-1* antisense ODN neither is cytotoxic nor results in random inhibition of nuclear proteins. We also observed no detectable morphological abnormality within the mesenchymal compartments of lungs treated with *TTF-1* antisense ODN, consistent with epithelial cell targeting by *TTF-1* antisense ODN. The abnormal phenotype resulting from treatment with the *TTF-1* antisense ODN was not seen in mouse embryonic lungs treated in additional control experiments with antisense ODN to a number of other mRNAs, including laminin A chain (5′ ACA ACA TGC GCG GCA GCG 3′) and matrix Gla protein (5′ CAC CATGAA GAG CCT GC 3′). This phenotype was consistently reproducible in 12 independent repetitions of the experiment using different mouse embryos and synthetic ODNs. Thus, taken together, these data provide a logical basis for attributing the phenotypic impacts observed in these studies to the specific suppression of TTF-1 protein rather than to a nonspecific effect of oligonucleotides. Significantly, mouse embryos carrying a homozygous null mutation in the *TTF-1* locus display defects in lung branching morphogenesis that are more severe but are similar to the phenotype described in this report (Dr. S. Kimura, personal communication).

Because TTF-1 is a transcriptional regulator, the exact mechanism by which it controls lung pattern formation and epithelial morphogenesis requires the identification of the downstream target genes. The target genes for homeoprotein transcriptional regulators are in most cases unknown. Specific mammalian cell adhesion molecules such as N-CAM and mgl-1 as well as the *Drosophila decapentaplegic* gene product are regulated by homeoproteins (Capovilla *et al.*, 1994). Although regulated by TTF-1 *in vitro*, it is difficult to envisage how the products of surfactant protein gene *SP-B* or *CC10* may be crucial to lung morphogenesis per se. One possible mechanism by which inhibition of *TTF-1* translation leads to disorganized epithelial cell proliferation and morphogenesis may be through disruption of positional information, based on which decisions regarding proliferation, migration, apoptosis, and differentiation are thought to be made by individual cells (Wolpert, 1989). Alternatively, TTF-1 could regulate the expression of factors critical for normal lung pattern formation and branching morphogenesis, such as ECM components and their receptors or various cytokines and their receptors. Because it closely mimics the *in vivo* TTF-1 loss of function phenotype obtained by homologous recombination, the antisense ODN strategy used in the current study may provide a useful and relatively simple approach for identifying the target genes and the precise mechanism by which TTF-1 controls lung branching morphogenesis.

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