The core fucosylation (α1,6-fucosylation) of glycoproteins is widely distributed in mammalian tissues, and is altered under pathological conditions. To investigate physiological functions of the core fucosyltransferase (Fut8)-null mice and found that disruption of Fut8 induces severe growth retardation and death during postnatal development. Histopathological analysis revealed that Fut8+/− mice showed emphysema-like changes in the lung, verified by a physiological compliance analysis. Biochemical studies indicated that lungs from Fut8−/− mice exhibit a marked overexpression of matrix metalloproteinases (MMPs), such as MMP-12 and MMP-13, highly associated with lung-destructive phenotypes, and a down-regulation of extracellular matrix (ECM) proteins such as elastin, as well as retarded alveolar epithelia cell differentiation. These changes should be consistent with a deficiency in TGF-β1 signaling, a pleiotropic factor that controls ECM homeostasis by down-regulating MMP expression and inducing ECM protein components. In fact, Fut8−/− mice have a marked dysregulation of TGF-β1 receptor activation and signaling, as assessed by TGF-β1 binding assays and Smad2 phosphorylation analysis. We also show that these TGF-β1 receptor defects found in Fut8−/− cells can be rescued by reintroducing Fut8 into Fut8−/− cells. Furthermore, exogenous TGF-β1 potentially rescued emphysema-like phenotype and concomitantly reduced MMP expression in Fut8−/− lungs. We propose that the lack of core fucosylation of TGF-β1 receptors is crucial for a developmental and progressive/destructive emphysema, suggesting that perturbation of this function could underlie certain cases of human emphysema.

the physiological importance of fucose modifications on proteins has been highlighted recently by the description of human congenital disorders of glycosylation (CDG). The disease CDG-IIc is due to lack of the GDP-fucose transporter activity (1, 2), α1,2-, α1,3-, α1,4-, and α1,6-fucosyltransferases have been described; α1,6-fucose is found linked to the Asn-linked GlcNAc in the N-glycan core. All of these fucosylations are terminal capping reactions, and the fucose residues have no substituent. In contrast, O-fucosylation, in which fucose is attached directly to a serine or threonine residue in a particular protein context such as an EGF repeat, undergoes the elongation of the oligosaccharide into a tetrasaccharide (NeuAcα2−3Galβ1−4GlcNAcβ1−3Fuc-Ser/Thr), in which Fringe has been found to be the N-acetylgalactosaminyltransferase acting on O-linked fucose of the Notch receptor (3). Because Notch receptors play key roles in numerous developmental events, several features of the phenotype in CDG-IIc could be explained by defects in Notch function. However, Sturla et al. (4, 5) reported that reduced fucosylation is mainly confined to terminal fucosylation of N-glycans, and that protein O-fucosylation levels such as those that occur in Notch are unaffected in CDG-IIc; therefore, we speculate that core fucosylation may be responsible for the phenotype of CDG-IIc.

GDP-L-Fuc:N-acetyl-β-D-glucosaminide α1,6-fucosyltransferase (Fut8, EC2.4.1.152) catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of hybrid and complex types of N-linked oligosaccharides on glycoproteins (6). Core Fut8 is the only core FucT in mammals, but there are core α1,3-Fuc residues in plants, insects, and probably other species. The Fut8 gene is expressed in most rat organs with a relatively high level of expression in brain and small intestine (7). In good agreement with the Fut8 gene expression, α1,6-fucosylated glycoproteins are widely distributed in mammalian tissues (8).

Furthermore, the expression of Fut8 and the extent of core fucosylation are altered under pathological conditions such as hepatocellular carcinoma and liver cirrhosis (8, 9).

The molecular cloning of the Fut8 gene (10) enabled us to manipulate it and to remodel the N-glycans in animal models. Overexpression of the Fut8 gene caused steatosis in the liver and kidney due to a decreased lysosomal acid lipase activity accompanied by its over-fucosylation (11). Recently, it was reported that the core fucose-deficient IgG1 (produced in a fucose-deficient Chinese hamster ovary cell line) showed improved binding to FcγRIII. As a consequence, antibody-dependent cellular cytotoxicity activity mediated by their interaction was enhanced (12, 13). These findings strongly suggested that core fucosylation of N-glycans modifies the function of the glycoproteins.

To define the physiological roles of a particular glycosylation, gene targeting technology to disrupt the relevant glycosyltrans-
Fucosyltransferase gene function is considered as the best approach currently available. In fact, accumulating evidence on gene targeting for glycosyltransferases has elucidated a variety of novel functions of carbohydrates and provided new insights into their roles in vivo (14).

Here we report the generation of Fut8-null mice and describe critical roles of core fucosylation in vivo.

Materials and Methods

Gene Targeting. A part of the mouse Fut8 gene spanning 13.9 kb, which includes the exon containing the translation-initiation site, was isolated by screening a mouse 129Sv/Ei genomic library (Stratagene), using a SacI–SacI fragment of porcine Fut8 cDNA (nt 39 to 373) (10) as a probe. A targeting vector was constructed by replacing the 184-bp SacI–HindIII fragment containing the translation-initiation site with a 4.9-kb SacI–SalI fragment of the plasmid pGKL8RseBgeo (15) that contains an internal ribosome entry site (IRES)-LacZ-Neo cassette, flanked with a 1.5-kb XhoI–NotI fragment of the plasmid pMC1DTPA (16), which encodes diphtheria toxin A chain (DT-A) for negative screening (see Fig. 4, which is published as supporting information on the PNAS web site). The targeting vector was transfected into D3 embryonic stem cells, and clones were selected with G418. Southern blot analysis of selected clones with 5′- (A) and 3′- (B) probes (Fig. 4) revealed that 2.5% (4 of 343) of the embryonic stem clones had undergone correct homologous recombination. Targeted cell clones were then injected into blastocysts from B6C3F1 male chimeras derived from two lines. Northern blot analysis of lung and brain RNA revealed transcripts were detected as a single 3.5-kb band in wild-type mice (Fig. 4). Consistent with this finding, Fut8 activity could not be detected in tissues from animals after age indicated were fixed in 0.1 M PBS containing 4% paraformaldehyde and embedded in paraffin. For immunohistochemical analysis, the dewaxed sections were pretreated with avidin-biotin blocking and hydrogen blocking for 10 min at 37°C, and then incubated with rabbit anti-P-Smad2 antibody (Cell Signaling Technology, Beverly, MA), anti-MMP-12 antibody, or anti-SP-C antibody (Santa Cruz Biotechnology) for 16 h at 4°C. Localization of the first antibody was visualized by an avidin-biotin coupling (ABC) immunoperoxidase technique, using a commercial kit (Vector Laboratories) according to the manufacturer’s instructions.

Therapeutic Administration of Exogenous TGF-β to Fut8−/− Mice. We performed i.p. injection of recombinant TGF-β1, which contains the translation initiation site, with an IRES-LacZ-Neo-pA cassette (Fig. 4). Genotypes of pups from intercrosses between heterozygous mice were determined by Southern blotting (Fig. 4). The phenotype described here was identical in two lines. Northern blot analysis of lung and brain RNA revealed that expression of full-length Fut8 mRNA was abolished in homozygous mutant mice, whereas Fut8 transcripts were detected as a single 3.5-kb band in wild-type mice (Fig. 4). Consistent with this finding, Fut8 activity could not be detected in Fut8−/− tissues including brain or lung, even with six times longer incubation than that used for Fut8+/+ specimens (Fig. 4D and data not shown). Furthermore, the analysis of N-glycan structures showed that the elution profile of Fut8−/− lung lacked the peaks of oligosaccharides with core fucose eluting at 30–35 min, as described in refs. 19 and 20 (Fig. 4E). These oligosaccharides were also confirmed by mass spectrometric analysis (Fig. 5, which is published as supporting information on the PNAS web site). This finding agrees with previous reports indicating that there are no additional genes homologous to Fut8 in mammals and lower organisms (21, 22).

Thus, the Fut8 gene is the only one responsible for the core fucosylation of N-glycans in mouse tissues.
Core Fucosylation Is Essential for Mice Survival and Growth. *Fut8*−/− mice were born apparently healthy with almost the expected Mendelian inheritance: Of 277 pups, there were 59 (21.3%) *Fut8*+/−, 147 (53.1%) *Fut8*−/−, and 71 (25.6%) *Fut8*+/+ mice. At embryonic day 19, frequencies of *Fut8*−/−, *Fut8*+/, and *Fut8*+/+ mice were 26, 52, and 22 of 100 embryos, and there were no apparent anomalies in *Fut8*−/− mice. In contrast to *Fut8*−/− mice, embryonic lethality was observed in mutant mice deficient in the FX gene, which encodes an enzyme in the de novo pathway for GDP-fucose synthesis and is responsible for all cellular fucosylation, e.g., α1,2; α1,3; α1,6; etc. (23). The appearance of *Fut8*−/− mice could not be distinguished from *Fut8*+/- and *Fut8*+/+ mice within 3 days of age, but ≈70% of them died during this period (Fig. 1L). Most of the survivors manifested severe growth retardation (Fig. 1B). This pattern observed in the *Fut8*−/− mice is quite different from other N-linked medial- and trans-Golgi glycosyltransferases, such as GnT-III, GnT-V, or ST6Gal1-null mice (14), suggesting that core fucosylase has a unique role in the regulation of proliferation and differentiation after birth.

Progressive Emphysema-Like Changes in *Fut8*−/− Lungs. Histological analyses by hematoxylin/eosin staining of 3-, 7-, 10-, and 18-day-old and 8-week-old *Fut8*−/− mice showed a symmetrical reduction in the size of most organs, which otherwise appeared devoid of pathological signs. The lungs of *Fut8*−/− mice, however, apparently displayed generalized air-space enlargement and dilated alveolar ducts, compared with those of *Fut8*+/- and *Fut8*+/+ mice (Fig. 2A). The mean linear intercept was calculated at the ages indicated. From postnatal day 7, diameters of the pulmonary alveoli of *Fut8*−/− mice were increased significantly, compared with those in *Fut8*+/- mice (Fig. 2B). To evaluate the functional relevance of this morphological abnormality, lung compliance was evaluated by a static air deflation curve (Fig. 2C). The *Fut8*−/− lungs had larger total lung capacities and increased lung compliance compared with *Fut8*+/- and *Fut8*+/+ lungs. These results indicate that the altered architecture of the enlarged *Fut8*−/− mouse airspace contributes to the increased compliance. We also measured pressure–volume relationships in body-weight-matched young wild types (≈7 days old) to cancel the difference in total lung capacity. Again, static lung compliance in *Fut8*−/− mice was greater than that in wild-type mice (data not shown). When *Fut8*−/− mice breathed room air under resting conditions, respiratory minute volume and rate, as determined by body plethysmography, were higher than in wild-type mice. Ventilatory responses to systemic hypoxia (12% O2 or hypercapnia (5% CO2/21% O2), or increases in the respiratory minute volume, were significantly attenuated in *Fut8*−/− mice, compared with *Fut8*+/- and *Fut8*+/+ mice (Fig. 2D). These findings, although not specific markers of emphysema, suggest that *Fut8* is involved in the physiological control of ventilation. However, we do not believe that the main reason that *Fut8*−/− mice die is lung disorder. Interestingly, besides TGF-β1 receptor as described below, we also found that loss of core fucosylation resulted in modest down-regulation of several other receptors-mediated signaling, such as EGF receptor and integrins (unpublished data), which are responsible for cell growth and differentiation. Therefore, we would like to take the hypothesis that the growth retardation and early death of *Fut8*−/− mice can be attributed to dysregulation of many receptors-mediated signaling.

Enhanced Expression Levels of MMPs in *Fut8*−/− Lungs and *Fut8*−/− Cells. Pulmonary emphysema is believed to result from decreased structural integrity of connective tissues due to a defect in their formation or to an abnormal proteolysis. Elastin and fibrillar collagen are major components of the extracellular matrix (ECM), which sustains the normal lung architecture. On the other hand, MMPs are a group of zinc- and calcium-dependent proteinases that have an important role in the normal turnover of ECM components. Abnormal production of MMPs is implicated in the induction of emphysema. Thus, transgenic mice expressing human MMP-1 develop emphysema through destruction of collagen fibrils (24). Furthermore, MMP-2, MMP-9, and MMP-12 lead to emphysema by degradation of elastin fibers (25, 26). Therefore, expression levels of collagens, elastin, and a number of MMPs of putative relevance in lung pathology, including McolB (a mouse orthologue of human MMP-1) (27) and MMP-2, -8, -9, -12, -13, and -14, were examined in the lungs of Fut8-mutant mice. RT-PCR analysis showed that there were no significant changes in the expression levels of collagens, MMP-2, -8, and -14 in lung tissues from *Fut8*−/− mice and *Fut8*+/- mice (Fig. 3A). However, expression levels of McolB, MMP-12, and MMP-13 were greatly enhanced (Fig. 3A); in addition, a slight increase in MMP-9 levels was detected in lungs from *Fut8*−/− by using real-time RT-PCR (Fig. 6A and Table 2, which are published as supporting information on the PNAS web site). Conversely, elastin expression was down-regulated in lungs from Fut8-deficient mice. On the other hand, fragmentation and a significantly reduced number of elastic fibers were observed by elastin staining in *Fut8*−/− mice (Fig. 6B), supporting the view that the degrading phenotype, i.e., emphysematous changes, occurs in *Fut8*−/− lung. Actually, emphysema-like changes were coincident with increased expression levels of MMP-12 from postnatal day 7 of *Fut8*−/− mice (Fig. 6C). Furthermore, only in the bronchoalveolar lavage fluid of *Fut8*−/− mice did macrophages look vacuolated, which, along with MMP-12 expression, indicates activation (Fig. 6D). In addition, an enhanced expression of CD68, a marker of macrophage, was clearly observed in *Fut8*−/− lung (data not shown). Recently, Morris et al. (28) reported that the loss of the epithelial integrin αvβ6, which causes a local deficiency in active TGF-β1, results in the increased expression of MMP-12 and leads to a slowly progressive, age-related emphysema. Likewise, McolB is a murine orthologue of human MMP-1, an enzyme that has been associated repeatedly with lung pathology, including emphysema (24), whereas MMP-13 or collagenase-3 is a very potent enzyme with wide substrate specificity, which is also associated with pulmonary diseases. It will also be interesting to examine whether core

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**Fig. 1.** Semilethality and growth retardation in *Fut8*−/− mice. (A) Survival ratio of *Fut8*−/− (−/−, solid line), *Fut8*+/- (+/−, broken line), and *Fut8*+/+ (+/+, dotted line) mice after birth. (B) A 16-day-old *Fut8*−/− pup (−/−) with a *Fut8*+/- littermate (+/+).
fucosylation affects MMP proteolytic function, because some MMPs such as MMP-9 contain N-glycans with core fucose. Taken together, these results suggest that overexpression of a set of MMPs might be causally linked to the development of emphysema in Fut8−/− mice.

To gain insight into the mechanism of induction of these MMPs in Fut8−/− mice, we established embryonic fibroblasts from Fut8+/+ and Fut8−/− mice. Consistent with the data from the lungs shown in Fig. 3A, under resting conditions, McolB mRNA transcripts (Fig. 3B) and MMP-12 (Fig. 3C) and MMP-13 (data not shown) proteins secreted into the media were barely detected in wild-type embryonic fibroblasts, whereas they were clearly detected in Fut8−/− cells. To examine whether these changes in MMP expression levels were also manifested in the respective MMP proteolytic activities, gelatin and casein zymographies were performed by using the conditioned media from these embryonic cells. A differential band of ~45 kDa, likely corresponding to the gelatinolytic activity of MMP-12, was observed in the gelatin zymogram of Fut8−/− cells. Likewise, a band of ~55 kDa was detected in the casein zymogram of Fut8−/− cells (Fig. 6D and E). In fact, the increased expression levels of both MMP-12 and MMP-13 secreted into media in the Fut8−/− cells were also confirmed by a Western blot (Fig. 6F). Consistent with these results, the enhancement of MMP-12 expression was also observed in lung tissues of Fut8−/− mice (Fig. 6G). These results demonstrate that the increased RNA and protein expression levels of some MMPs are accompanied by an increase in the proteolytic potential of cells from Fut8-deficient mice.

Lack of Core Fucose in TGF-β Receptor Leads to Inhibiting Its Function. Recently, it became clear that modification by N-glycosylation can affect the biological functions of many glycoprotein receptors. As described above, we found that loss of core fucosylation resulted in down-regulation of several receptor-mediated signaling pathways, such as TGF-β1 receptor, EGF receptor, and integrins, which are responsible for cell growth and differentiation, and also emphysema. The TGF-β1 receptor-mediated signaling pathway is a key pathway for regulating expression of ECM proteins, including suppression of MMPs to produce a “synthetic” phenotype (29). When the embryonic fibroblasts were treated with IL-1β, which enhances MMP expression, McolB mRNA levels were elevated markedly in both Fut8+/+ cells and Fut8−/− cells (Fig. 3B). The enhancement of McolB expression stimulated by IL-1β was blocked by TGF-β1 treatment in Fut8+/+ cells but not in Fut8−/− cells (Fig. 3B), indicating that the deletion of Fut8 does not alter IL-1β-receptor-mediated function but diminishes TGF-β1-mediated signaling. The decreased response of TGF-β1 stimulation was also observed in protein expression levels of MMP-12 secreted into the media (Fig. 3C). To examine how core fucose affects TGF-β1-mediated signaling, we measured the binding activity of TGF-β1 cells (Fig. 3D). The binding ability of 125I-TGF-β1 was reduced significantly in Fut8−/− cells compared with Fut8+/+ cells, which could be rescued by reintroducing Fut8 to Fut8−/− cells (Fig. 3D). Consistent with this, the amount of TGF-β1 bound and cross-linked to type I, II, and III receptors was suppressed dramatically in Fut8−/− cells; these features were recovered by reintroducing Fut8 to Fut8−/− cells (Fig. 3E). Actually, the levels of core fucosylation...
detected by AAL lectin in the TGF-β type II receptor, which is the primary binding subunit for TGF-β1 (30–32), were abolished in Fut8−/− cells, whereas they were recovered by restoring Fut8 (Fig. 3F). The TGF-β signaling via receptors to intracellular mediators of the Smad family was suppressed significantly in Fut8−/− cells (Fig. 3G). Smad2 is a direct substrate for the activated TGF-β1 signaling via receptors to intracellular mediators.
I receptor. In addition, Smad2 phosphorylation at C-terminal serine residues is required for its nucleolar translocation (33). The down-regulation of Smad2 phosphorylation levels in Fut8−/− mice was rescued by reintroducing Fut8 (Fig. 3G), consistently imputing a role for core fucosylation of Smad2 in lung tissues revealed that P-Smad2 levels were greatly suppressed in Fut8−/− mice, compared with that in Fut8+/+ mice (Fig. 3H). Taken together, these results demonstrate that core fucosylation plays an important role in the regulation of TGF-β1 receptor function. Therefore, we assume that Fut8−/− lungs are committed to overexpressing MMPs, probably because they escape from the TGF-β1 suppressor mechanism, which operates in wild-type lungs, although other functions of core fucosylation of N-glycan-bearing glycoproteins might also be involved in the development of emphysema.

Exogenous TGF-β1 Treatment Rescued Emphysema-Like Changes in Fut8−/− Mice. We have performed rescue experiment with ip. injection of exogenous TGF-β1 to postnatal-day-18 Fut8 knockout mice. Importantly, exogenous TGF-β1 resulted in a significant rescue of the emphysema-like phenotype (Fig. 3I and J), stimulated the formation of elastin fiber (data not shown), and concomitantly reduced MMP-12 expression (Fig. 6I) in Fut8−/− lung. These data strongly support the hypothesis that the TGF-β1-mediated signaling pathway is down-regulated in Fut8−/− lungs. We do not exclude the possibility that aberrant regulation of other receptors may contribute partly to the emphysema-like changes.

In contrast to the mild and gradual development of emphysema in integrin β6 knockout mice, which causes a local deficiency in active TGF-β1, the Fut8 deficiency as well as the induction of cytokines such as IL-13, TNF-α, and IFN-γ (25, 34, 35) results in the severe and rapidly progressive development of emphysema. Interestingly, the absence of β6 integrin leads mainly to MMP-12 overexpression in the lungs of mutant mice, whereas in Fut8−/− lungs, MMP-13 is also induced at even larger amounts than MMP12, as assessed by real-time PCR quantitative analysis (Fig. 6A). This fact, together with the wide substrate specificity of MMP-13, could contribute to the explanation of the differences in the severity of emphysema phenotypes between integrin β6- and Fut8-deficient tissues. It has been reported that disruption of the latent TGF-β1-binding protein 4 (LTBP-4), which regulates TGF-β targeting to ECM and TGF-β-mediated signaling, causes abnormal lung development (36).

Using antibodies specific for surfactant protein C (SP-C), a marker of differentiated type-II alveolar epithelial cells, we found that expression levels of SP-C protein at each stage were slightly weaker in Fut8−/− lungs than in Fut8+/+ lungs (see Fig. 7, which is published as supporting information on the PNAS website), suggesting that alveolar development was also disturbed by the lack of Fut8 expression. The retarded alveolar epithelial cell differentiation may also contribute partly to emphysema-like changes of Fut8−/− lung from postnatal day 7 (Fig. 2B). Indeed, given the fact that alveolarization continues past day 7, at least part of the phenotype is related to abnormal lung development. Nevertheless, because it continues to increase after lung development, we conclude that both alveolar development and progressive (destructive) emphysema occur in the absence of Fut8.

TGF-β activation also leads to emphysema formation. Marfan syndrome is a human autosomal dominant disorder of connective tissue caused by mutations in fibrillin-1. Fibrillin-1 usually functions to limit the activation of TGF-β, although the precise mechanism by which fibrillin-1 controls TGF-β activation is still unknown. Studies with mutant mice have revealed that fibrillin deficiency causes a pronounced TGF-β activation that triggers the developmental inhibition of alveolarization, induces apoptosis in the developing lung, and finally results in destructive emphysema (37). Nevertheless, very recent results have demonstrated that Marfan syndrome can also be caused by loss of TGF-β signaling function due to TGF-β receptor type II mutations in a group of patients lacking mutations in fibrillin (38). These findings emphasize the idea that the TGF-β signaling pathway plays an important role in lung integrity, and consequently, there is an absolute need to maintain carefully the precise levels of all components of this complex pathway. Our finding that defects in core fucosylation profoundly dysregulate TGF-β activation and signaling in Fut8−/− mice adds a level of control to this pathway and opens the possibility that similar defects could be found in some cases of human emphysema.

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