Fucosyltransferase 8 as a functional regulator of nonsmall cell lung cancer

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The up-regulation of fucosyltransferase 8 (FUT8), the only enzyme catalyzing α1,6-fucosylation in mammals, has been observed in several malignant cancers including liver, ovarian, thyroid, and colorectal cancers. However, the pathological role and the regulatory mechanism of FUT8 in cancers remain largely unknown. In the current study, we report that the expression of FUT8 is up-regulated in nonsmall cell lung cancer (NSCLC) and correlates with tumor metastasis, disease recurrence, and poor survival in patients with NSCLC. Knocking down FUT8 in aggressive lung cancer cell lines significantly inhibits their malignant behaviors including in vitro invasion and cell proliferation, as well as in vivo metastasis and tumor growth. The results of glycoproteomic and microarray analyses show that FUT8 globally modifies surface antigens, receptors, and adhesion molecules and is involved in the regulation of dozens of genes associated with malignancy, suggesting that FUT8 contributes to tumor progression through multiple mechanisms. Moreover, we show that FUT8 is up-regulated during epithelial–mesenchymal transition (EMT), a critical process for malignant transformation of tumor, via the transactivation of β-catenin/lymphoid enhancer-binding factor-1 (LEF-1). These results provide a model to illustrate the relation between FUT8 expression and lung cancer progression and point to a promising direction for the prognosis and therapy of lung cancer.

TGF-beta | E-cadherin | fucose

Fucosylation, the transfer of fucose from GDP-fucose to glycoconjugates such as glycoproteins and glycolipids, is catalyzed by a family of enzymes called fucosyltransferases (FUTs). So far, 13 FUTs are known to be encoded by the human genome, including FUT1 to 11, protein *O*-fucosyltransferase 1 (POFUT1), and POFUT2. Through these FUTs, fucoses could be attached to N-, O-, and lipid-linked glycans through an α1,2- (by FUT1 and 2), α1,3- (by FUT3 to 7 and FUT9 to 11), α1,4- (by FUT3 and 5), or α1,6- (by FUT8) linkage, or directly link to the serine/threonine residues of EGF-like or thrombospondin repeats (by POFUT1 and 2, respectively) (1, 2). In mammals, fucosylated glycans have pivotal roles in many aspects of biological processes such as lymphocyte homing, immune responses, fertilization, and development (3). Moreover, aberrant fucosylation, which results from the deficiency or overexpression of FUTs, is associated with a variety of human diseases, including cystic fibrosis, leukocyte adhesion deficiency type II, and cancers (3, 4).

Unlike other FUTs, which are functionally redundant, FUT8 is the only enzyme responsible for the α1,6-linked (core) fucosylation by adding fucose to the innermost GlcNAc residue of an N-linked glycan. A growing body of evidence indicates that core fucosylation is important for regulating protein functions. For example, deletion of the core fucose from the Fc region of IgG1 greatly improves its binding affinity to Fcγ receptor IIIa, which in turn enhances antibody-dependent cell-mediated cytotoxicity for over 50 folds (5, 6). Core fucosylation is also crucial for the ligand binding affinity of TGF-β1 receptor (7), EGF receptor (8), and integrin α3β1 (9). Lacking the core fucose on these receptors leads to a marked reductions in ligand binding ability and downstream signaling. Furthermore, the increase in core fucosylation on E-cadherin has been shown to strengthen cell–cell adhesion (10).

Both transgenic and knockout mouse models have been generated to study the physiological role of FUT8 (7, 11, 12). Ectopic expression of FUT8 in mice results in an accumulation of lipid droplets in hepatocytes and proximal renal tubular cells. This steatosis-like phenotype observed in transgenic mice is linked to the activity of liver lysosomal acid lipase, which becomes inactive when over core-fucosylated (11), suggesting that excess core fucosylation may lead to a breakdown of normal lipid metabolism. On the other hand, knocking out FUT8 in mice is reported to dramatically decrease the postnatal survival: 70% of the mice died within 3 d of age, whereas the 30% that survived showed severe growth retardation and an emphysema-like phenotype (7). FUT8-null mice also exhibit multiple abnormal behaviors associated with schizophrenia such as increased locomotion and strenuous hopping behavior (12). These findings suggest that FUT8 is not only necessary for normal development, especially in lung and brain, but also critical for maintaining homeostasis.

The up-regulation of FUT8 mRNA, protein, and activity has been observed in several malignant tumors including liver, ovarian, thyroid, and colorectal cancers, and linked to the severity of cancers (13–16). For example, in papillary thyroid carcinoma, higher expression of FUT8 is linked to bigger tumor sizes and more metastases in lymph nodes; in colorectal carcinoma, the expression of FUT8 is associated with poor prognosis. We previously also found that FUT8 expression is strikingly up-regulated in a highly invasive lung cancer cell line, CL1-5, compared to its less aggressive parental cell line CL1-0 (17). However, the pathological role and the regulatory mechanism of FUT8 in cancer progression remain largely unknown. In the current study, we report the clinical relevance of FUT8 in nonsmall cell lung cancer (NSCLC) and show that FUT8 is required to maintain the aggressiveness and malignant properties of lung cancer cells. In addition, we found that β-catenin/lymphoid enhancer-binding factor-1 (LEF-1) signaling, a major event occurring in epithelial–mesenchymal transition (EMT), could up-regulate FUT8 expression during tumor progression. Together with the data from glycoproteomic and gene microarray analyses, we propose a model to illustrate how FUT8 promotes lung cancer progression.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE42407 and GSE42405).

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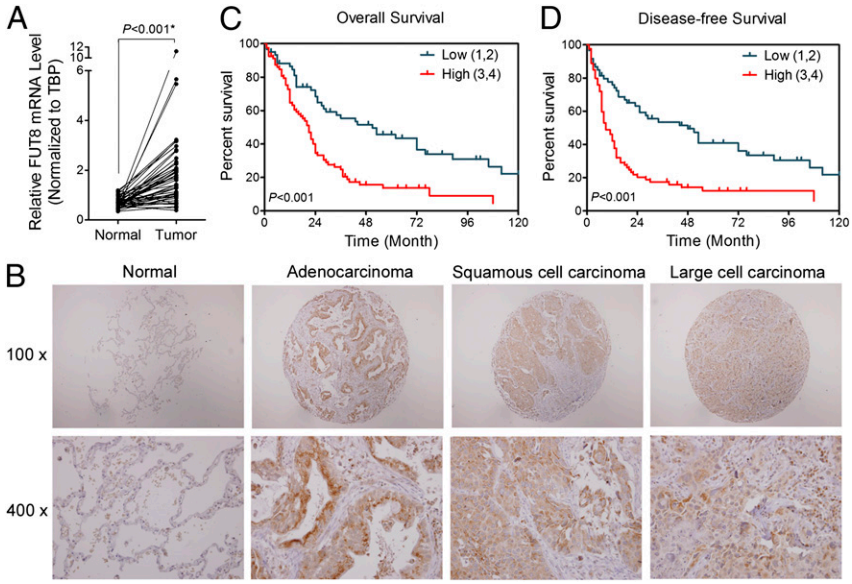

Fig. 1. Up-regulation of FUT8 in NSCLC is associated with poor prognosis. (*A*) Higher expression of FUT8 mRNA in tumor lesions than in normal tissue. FUT8 expression in lung tumor and paired normal tissue from 47 patients with NSCLC was measured using Q-PCR. For comparison, the mRNA level of FUT8 was normalized to TATA box binding protein expression in each sample. *P* value was obtained by Student *t* test. (*B*) Specimens from patients with NSCLC show high FUT8 protein expression. Representative images of FUT8 IHC staining in normal lung tissue and different types of primary NSCLC are shown. (*C* and *D*) Higher expression of FUT8 in NSCLC correlates with poor overall survival and disease-free survival of patients. Kaplan–Meier plots of overall (*C*) and disease-free (*D*) survival in 140 patients with NSCLC stratified by the expression level of FUT8. The specimens with the FUT8 staining intensity scores of 1 or 2 were categorized in a "low" expression group, whereas the intensity scores of 3 or 4 were placed in a "high" expression group. All *P* values were calculated by log-rank (Mantel–Cox) test.

Results

Up-Regulation of FUT8 in NSCLC Significantly Correlated with Tumor Metastasis, Recurrence, and the Survival of Patients. To investigate the clinical relevance of FUT8 expression in lung cancer, the mRNA level of FUT8 in tumor lesions of patients with NSCLC was determined by quantitative RT-PCR (Q-PCR). Forty-two of 47 tumor samples had a higher expression level of FUT8 compared with their respective adjacent normal tissues (Fig. 1*A*). The expression of FUT8 was examined further by immunohistochemistry (IHC), which was performed on a set of human NSCLC tissue arrays containing 86 adenocarcinomas, 46 squamous cell carcinomas, 8 large cell carcinomas, and normal lung tissue controls. The result showed that FUT8 was up-regulated in all of these types of NSCLC, especially in adenocarcinoma, but not in normal lung tissues (Fig. 1*B*). The correlation between FUT8 expression (based on IHC) and pathological features of NSCLC, shown in Table 1, reveals that the up-regulation of FUT8 is associated with distal metastasis and disease recurrence. Moreover, Kaplan–Meier survival curves revealed that FUT8 expression inversely correlated to overall survival (Fig. 1*C*) and disease-free survival (Fig. 1*D*) of NSCLC patients. Univariate Cox proportional hazards regression analysis pointed out that the expression level of FUT8, and the T (tumor size), N (lymph node) or M (metastasis) status affected overall and disease-free survival. After accounting for the effects of T, N, and M factors, multivariate Cox regression analysis showed that FUT8 expression was significantly associated with lower overall and disease-free survival (Table S1). These results suggested that the up-regulation of FUT8 correlated with poor prognosis in patients with NSCLC.

Up-Regulation of FUT8 Enhanced Tumor Metastasis and Growth. To understand if FUT8 has biological significance in the aggressiveness of lung cancer cells, CL1-5 and PC14, which had a higher level of FUT8 expression and invasiveness compared to CL1-0 and A549 (Fig. 2*A*), were subjected to FUT8 knockdown, and CL1-0 and A549 were stably transfected with FUT8 (Fig. S1*A*) and examined for their aggressiveness in vitro and in vivo. As expected, knockdown of FUT8 significantly decreased the invasion ability of CL1-5 and PC14 cells (Fig. 2*B*). However, overexpressing FUT8 in CL1-0 and A549 only slightly enhanced cell invasion (Fig. S1*B*). To further evaluate the effect of FUT8 on invasiveness, two stable FUT8 knockdown clones of CL1-5 cells, CL1-5/shFUT8-1 and CL1-5/shFUT8-2 (Fig. S2), were injected i.v. into nonobese diabetic (NOD)-SCID mice, and the numbers of metastatic nodules in lungs were counted after 7 wk. As shown in Fig. 2*C*, silencing FUT8 expression resulted in decreased lung metastasis compared to control, suggesting that FUT8 is a positive regulator of cancer invasion and metastasis.

Table 1. Correlation between FUT8 expression and clinicopathological factors in 140 patients with NSCLC

Characteristics	FUT8 expression		*P* value*
	Low (0, 1)	High (2, 3)	
Age			
Years, mean ± SD	63.0 ± 9.6	61.2 ± 10.7	0.3079
Sex			
Male	34	39	0.3951
Female	26	41	
Smoking status			
Yes	25	33	1.000
No	35	47	
Histological type			
Adenocarcinoma	29	57	0.0105
SCC	28	18	
Large cell carcinoma	3	5	
Stage			
I+II	30	28	0.0849
III+IV	30	52	
Tumor status (T)			
T1–T2	39	59	0.2711
T3–T4	21	21	
Lymph node metastasis (N)			
N0	28	25	0.0787
N1–N3	32	55	
Distal metastasis status (M)			
M0	47	48	0.0280
M1	13	32	
Recurrence status			
Yes	27	59	0.0008
No	33	21	

SSC, squamous cell carcinoma.
*Statistical significance of differences between groups in all the characteristics except for "Histological type" was analyzed by two-sided Fisher's exact test. The *P* value for "Histological type" was analyzed by χ^2 test.

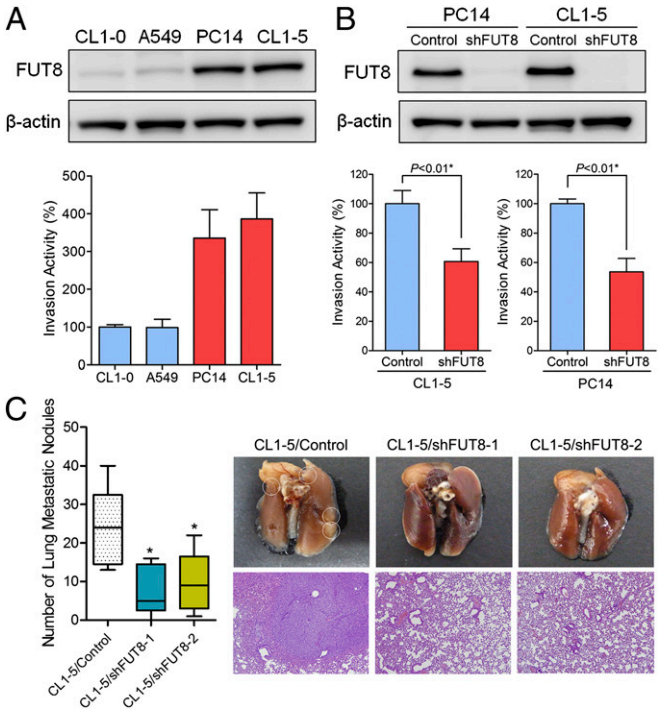

Fig. 2. Knockdown of FUT8 significantly inhibits in vitro cell invasion and in vivo lung metastasis. (*A*) Expression level of FUT8 positively correlated with the invasion ability of lung adenocarcinoma cell lines. (*B*) Silencing FUT8 expression in CL1-5 and PC14 cells suppressed their invasion ability. The invasion ability (*A* and *B*, *Lower*) of lung adenocarcinoma cell lines and their derivatives was determined by Transwell assay. Data are shown as mean ± SEM ($n = 3$), and all *P* values were obtained by Student *t* test. (*C*) Knockdown of FUT8 inhibited the metastatic ability of lung cancer cells in vivo. The number of lung metastatic nodules in NOD-SCID mice with i.v. injection of CL1-5/control or CL1-5/shFUT8 stable clones was counted and displayed by box-whisker plots (*Left*). Results shown are mean ± SEM ($n = 5$), and the upper and lower extremes indicate the largest and smallest value of each dataset, respectively. $*P < 0.05$ compared with CL1-5/control by Student *t* test. Representative images of isolated lungs and H&E-stained lung sections in each group are shown (*Right*).

We next assessed the effect of FUT8 on cell proliferation. Results of in vitro proliferation assay showed that knockdown of FUT8 caused significant inhibition of cell proliferation in CL1-5 and PC14 cells, compared with control (Fig. 3 *A* and *B*). Interestingly, loss of FUT8 expression did not change the growth rate of normal lung epithelial cells, Nuli-1 and BEAS-2B (Fig. 3 *C* and *D*), suggesting that knocking down FUT8 influenced the proliferation of cells only with a high expression level of FUT8, i.e., aggressive cancer cells. To further investigate if FUT8 silencing affects tumor growth in vivo, CL1-5/control or CL1-5/shFUT8 stable clones were s.c. injected into the ventral region of NOD-SCID mice, and the tumor volume was measured weekly. As shown in Fig. 3*E*, knocking down FUT8 in CL1-5 dramatically suppressed its growth in vivo (sixfold smaller). Taken together, our data demonstrated that FUT8 was necessary for cancer cells to maintain their malignancy, in terms of tumor metastasis and growth.

β-Catenin/LEF-1 Transactivated FUT8 Expression During EMT. EMT, the conversion from epithelial to mesenchymal phenotype, is an essential developmental process and is recently recognized as a major player in tumor progression (18). Intriguingly, a correlation between FUT8 expression and cell morphology was observed in the cell lines we used. In contrast to CL1-0 and A549 (low FUT8 expression), which exhibited an epithelial morphology, CL1-5 and PC14 (high FUT8 expression) showed an elongated and spindle-like mesenchymal morphology (Fig. 4*A*). To further study if the expression of FUT8 is associated with EMT, EMT in A549 cells was induced by TGF-β1 (19), and the expression level of FUT8 was determined. After 72 h of treatment with TGF-β1, FUT8 expression was elevated in a dose-dependent manner. Furthermore, the long-term exposure to TGF-β1, which has been shown to completely convert A549 cells from epithelial to mesenchymal phenotype (20), induced a higher expression level of FUT8 than the short-term exposure (Fig. 4*B*). This suggests that the molecular events involved in TGF-β1–induced EMT can up-regulate FUT8 expression. Emerging evidence points out that the E-cadherin/β-catenin signaling pathway plays a key role in regulating EMT via cross-talk with the TGF-β1 signaling pathway. Loss of E-cadherin expression caused by the TGF-β1 signaling pathway leads to the translocation of β-catenin from plasma membrane to nucleus, where it complexes with LEF-1 to promote EMT (21–23). *In silico* promoter analysis of FUT8 5′-UTR showed that the region of promoter 1 (0–3 kb upstream from the first exon, Fig. S3) contains multiple LEF-1/T-cell factor binding sites (Transcription Element Search System, ref. 24). To examine whether the β-catenin/LEF-1 pathway is involved in regulating FUT8 expression, lithium chloride (LiCl) was used to activate β-catenin signaling in A549 cells (25, 26), and the expression level of FUT8 was monitored. As shown in Fig. 4 *C* and *D*, treatment of LiCl increased FUT8 expression at both protein and mRNA levels in a time- and dose-dependent manner. These data suggested that the E-cadherin/β-catenin/LEF-1 signaling axis regulated the expression of FUT8 during EMT.

Expression of FUT8 and E-Cadherin in Clinical Specimens and the Link to Patient Survival. In support of the involvement of EMT in FUT8 expression, we examined the expression of FUT8 and E-cadherin in serial sections of NSCLC tumor tissue array by IHC. As shown in Fig. 4*E*, the reciprocal pattern of FUT8 and E-cadherin expression was observed in clinical specimens. In tumor specimens with low E-cadherin expression, more than 70% (29/41) were accompanied by high FUT8 expression (Fig. 4*F*). In addition, we found that the patients with low E-cadherin and high FUT8 expression had the worst overall survival (Fig. 4*G*), suggesting that the expression levels of E-cadherin and FUT8 could be a prognostic index for NSCLC patients.

Core Fucosylation Modulates the Expression of Genes Involved in Multiple Biological Functions. To understand the downstream molecular events in response to the global alteration of core fucosylation, microarray analysis was used to profile the changes in gene expression following FUT8 silencing. Another microarray dataset, CL1-0 vs. CL1-5, was used as a reference here. The genes significantly (twofold, $P < 0.01$) up- or down-regulated in both CL1-0 and CL1-5/shFUT8 cells (Table S2) were selected for functional annotations using a Gene Ontology (GO) database (27, 28). The result revealed that many genes involved in cell adhesion, motility, growth, angiogenesis, and inflammation were under the control of core fucosylation (Fig. 5*A* and Table S3), providing not only a positive role for FUT8 in modulating cell behaviors, but also the molecular basis to explain how FUT8 supports malignancy in many aspects.

Discussion

In the current study, we report that the expression of FUT8 is significantly up-regulated in tumor tissues of patients with NSCLC, and the up-regulation of FUT8 is associated with more tumor metastases, higher recurrence, and poorer survival in patients with NSCLC. According to these findings, we speculate that FUT8 is a key factor in promoting malignant progression of tumors. The clinical relevance of FUT8 overexpression in hepatocellular carcinoma (HCC) has been elucidated. However, there is a report showing that ectopic overexpression of FUT8 in

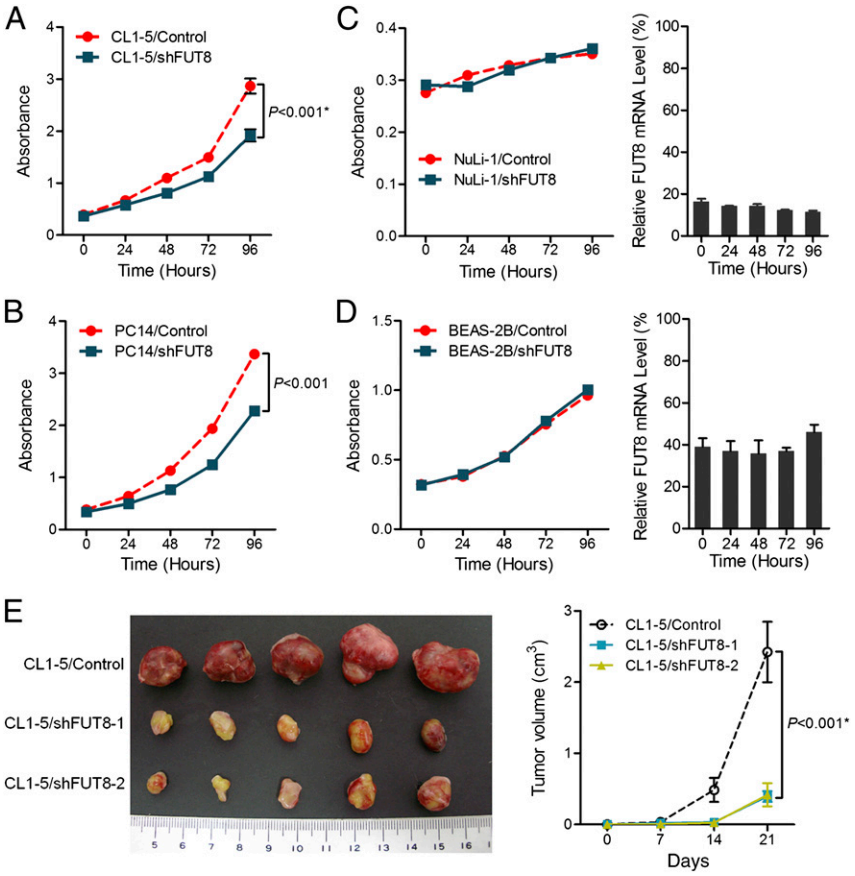

Fig. 3. Knocking down FUT8 in lung cancer cells suppresses cell proliferation in vitro and tumor growth in vivo. (*A* and *B*) Silencing FUT8 expression in CL1-5 and PC14 slowed down their proliferation rate. Cell proliferation of CL1-5 cells (*A*) and PC14 cells (*B*) with or without FUT8 knockdown was measured by WST-1 assay at different time intervals ranging from 0 to 96 h. Mean ± SEM of absorbance from each group (n = 8) is shown. All P values were obtained by two-way ANOVA. (*C* and *D*) Knockdown of FUT8 did not affect normal lung epithelial cell proliferation. Briefly, NuLi-1 (*C*) and BEAS-2B (*D*) were transduced with shControl- or shFUT8-bearing lentivirus. After 2 d postinfection, the proliferation rate of NuLi-1 and BEAS-2B with or without FUT8 knockdown was then determined using WST-1 assay at different time intervals ranging from 0 to 96 h (*Left*). Results are shown as mean ± SEM (n = 8). Meanwhile, the expression level of FUT8 was examined at each time point using Q-PCR. For comparison, FUT8 expression was normalized to GAPDH expression, and the relative mRNA level of FUT8 was calculated using the equation: $(2^{-(\Delta Ct,\ shFUT8\ -\ \Delta Ct,\ shControl)}) \times 100\%$ (*Right*). (*E*) Knockdown of FUT8 remarkably reduced the volumes of tumor mass. Volumes of tumors formed by CL1-5/control or CL1-5/shFUT8 stable clones in NOD-SCID mice were measured using a vernier caliper weekly. *Left* shows the gross view of isolated tumors. *Right* shows the mean ± SEM (n = 5) from each group at each time point. P value was obtained by two-way ANOVA.

a hepatocellular carcinoma cell line, Hep3B, suppresses tumor metastasis (29). Our results that suppressing FUT8 expression significantly inhibited tumor metastasis and growth seem to conflict with the observation in hepatocellular carcinoma. This discrepancy can be due to the expression of E-cadherin, which is detected in Hep3B but not these aggressive lung cancer cell lines we used. E-cadherin is a calcium-dependent cell–cell adhesion molecule with a pivotal role in tumor suppression (30), and it has been reported that core fucosylation of E-cadherin can enhance cell–cell adhesion in human colon carcinoma cells (10). Therefore, overexpressing FUT8 in Hep3B cell, an epithelial-type cell, which is positive in E-cadherin expression, may strengthen E-cadherin–mediated cell–cell interaction and reduce the metastatic abilities of FUT8 transfectants. In contrast, in the highly aggressive CL1-5 and PC14 lung cancer cells, which have already gone through EMT and lack E-cadherin expression (the mesenchymal-type cells), the tumor-promoting ability of FUT8/core fucosylation is no longer limited by E-cadherin. This hypothesis also explains why the effect of FUT8 overexpression on cancer invasion in the epithelial-type lung cancer cells, CL1-0 and A549, were marginal (Fig. S1*B*).

As mentioned, core fucosylation is critical for the function of TGF-β receptor by affecting the binding affinity toward TGF-β (7). Recently, knocking down FUT8 is reported to attenuate TGF-β–induced EMT in human renal proximal tubular epithelial cells (31), suggesting an essential role for core fucosylation in EMT. Our result of the up-regulation of FUT8 during EMT in cancers further points out that there is a positive feedback loop between FUT8 expression and TGF-β receptor signaling to promote EMT and tumor development. Notably, a population of patients with NSCLC (80% are adenocarcinomas) shows a high expression level of FUT8 despite the presence of E-cadherin, implying that there are unknown mechanisms other than EMT involved in regulating FUT8 expression.

The results of microarray analysis indicate that core fucosylation could modulate the expression of genes involved in a wide range of cellular functions, providing the molecular basis to explain the higher malignancy in cancer cells with FUT8 overexpression. However, microarray analysis only reflects the overall outcome in response to the global changes in core fucosylation. To figure out how core fucosylation regulates a specific glycoprotein to participate in a particular phenotype, such as tumor growth, we used a stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomic strategy to explore the dynamic changes of core fucosylated glycoproteins during lung cancer progression (Fig. 5*B*). A total of 215 N-linked glycopeptides within 163 glycoproteins were identified in *Lens culinaris* agglutinin (LCA, a lectin prefers to bind α1,6-fucosylation)-enriched fraction from CL1-0 and CL1-5 cells (Table S4). Some of the glycoproteins identified with high SILAC ratios (CL1-5/CL1-0), including EGFR and integrins, are known to regulate the malignancy of cancers. It has been reported that core fucosylation on EGFR and integrins can potentiate their ligand binding capability (8, 9), and therefore may enhance the downstream signals to support tumor growth and metastasis. Together with microarray data, understanding the effect of core fucosylation on many other glycoproteins with high SILAC ratios and the impacts on the respective biological functions would help decipher the detailed molecular mechanism of FUT8 in tumor progression.

Although it is well known that FUT8 catalyzes α1,6-fucosylation at the innermost GlcNAc of the N-linked glycan core, it is recently reported that FUT8 possesses a very different specificity to transfer fucoses to chito-oligosaccharides (32). Therefore, it is interesting to explore the additional specificity of FUT8,

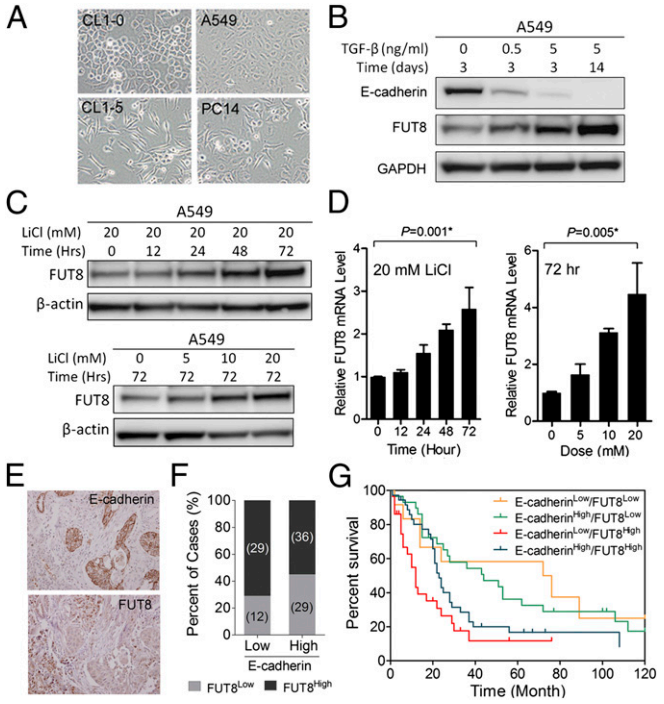

Fig. 4. Expression of FUT8 is elevated during EMT via the transactivation of β-catenin signaling. (*A*) Cell morphology of A549, CL1-0, CL1-5, and PC14. Compared to CL1-0 and A549 cells, which have typical epithelial morphology, CL1-5 and PC14 cells show elongated, spindle-like mesenchymal morphology. (*B*) FUT8 expression was up-regulated during TGF-β1–indcued EMT. E-cadherin is a marker of epithelial cell and used as an indicator of EMT here. Lanes 1–3, short-term exposure to different doses of TGF-β1 (0–5 ng/mL). Lane 4, long-term exposure to 5 ng/mL of TGF-β1. (*C* and *D*) FUT8 expression was elevated in a time- and dose-dependent manner upon LiCl treatment at both protein (*C*) and mRNA (*D*) levels. After treating with different concentrations of LiCl at the indicated time intervals, A549 cells were harvested and the expression level of FUT8 was determined by Western blot (*C*) and Q-PCR (*D*). For comparison, the expression level of FUT8 mRNA was normalized to β-actin, and the mean ± SEM of relative folds of FUT8 expression (against untreated) from three independent experiments is shown. All *P* values were calculated by one-way ANOVA. (*E* and *F*) IHC staining showed an inverse correlation between FUT8 and E-cadherin expression. To show the histological colocalization of E-cadherin and FUT8, serial sections of NSCLC tumor tissue array were used. Representative images of the inverse expression of E-cadherin and FUT8 within the same specimen (*E*) and the statistics of results (*F*) are shown. (*G*) The Kaplan–Meier plot shows that the expression level of E-cadherin could be an index to further distinguish a population of patients with worse overall survival from the patients with high FUT8 expression ($P = 0.01$). One hundred and six patients with NSCLC were grouped into four populations based on their expression pattern of FUT8 and E-cadherin. *P* value was calculated by log-rank (Mantel–Cox) test.

and to understand if FUT8 can contribute to cancer progression through mechanisms beyond core fucosylation.

The association of FUT8 overexpression with distal metastasis, tumor recurrence, and lower patient survival in NSCLC indicates that FUT8 expression can be a promising prognosis indicator for NSCLC. Moreover, because silencing FUT8 could inhibit tumor growth and metastasis without affecting the proliferation of normal lung epithelial cells, FUT8 can also be a potential therapeutic target for NSCLC.

Here, we propose a model based on our results to demonstrate the molecular mechanism of FUT8 in lung cancer progression (Fig. 5*C*). During the process of EMT, cancer cells lose the expression of E-cadherin, leading to the nuclear accumulation of β-catenin. The nuclear β-catenin then cooperates with LEF-1 to activate FUT8 expression. The global alteration of core fucosylation on cell surface molecules following FUT8 up-regulation changes the response of cancer cells to their microenvironment including extracellular matrix and growth factors, which in turn promote the progression of cancer cells via activating the malignancy-associated genes. Our study not only reveals the pathological role and the regulatory mechanism of FUT8 in NSCLC, but also sheds light on discovering promising prognostic factors and therapeutic leads to target NSCLC, and possibly other cancers.

Materials and Methods

Cell Lines and Clinical Specimen. Human lung adenocarcinoma cell lines CL1-0 and CL1-5 were described previously (33). Human lung adenocarcinoma cell line A549 and lung epithelial cell line NuLi-1 and BEAS2B were from ATCC. Human PC14 was kindly provided by Y. Hayata (Tokyo Medical College, Tokyo). Clinical samples used for Q-PCR and IHC were obtained from

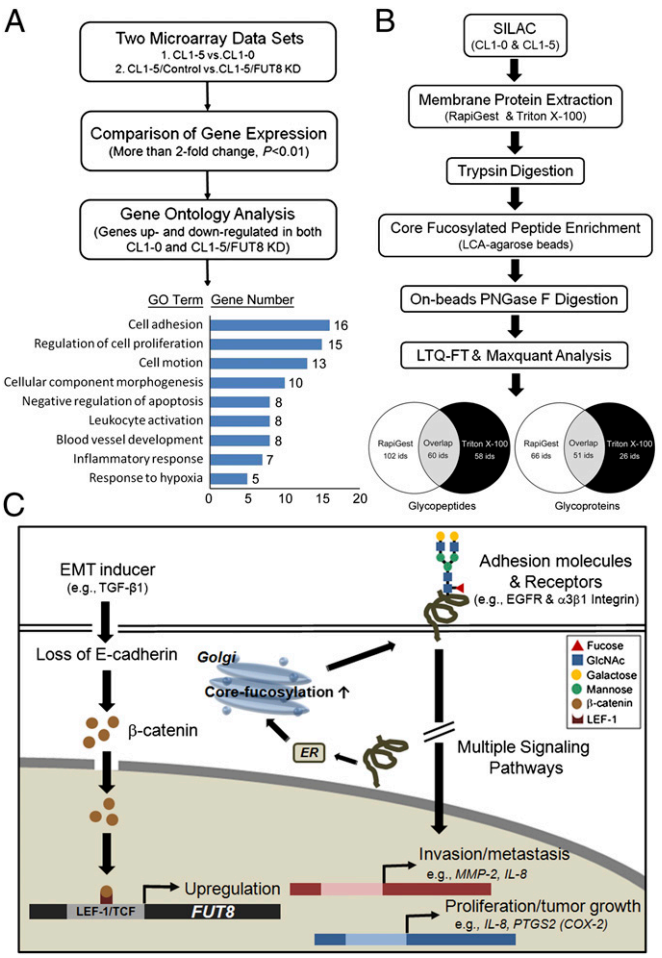

Fig. 5. Discovering the underlying mechanisms for FUT8 activity in lung cancer progression. (*A*) Strategy for microarray analysis. Expression level of 152 genes was significantly changed in both CL1-0 and CL1-5/shFUT8 cells. These genes were then subjected to an online database for functional annotation. The bar chart shows the predominant GO terms. (*B*) Flowchart of glycoproteomic analysis. A total of 215 N-linked glycopeptides within 163 glycoproteins were identified, as summarized in Table S2. (*C*) Proposed positive feedback route of FUT8 to promote lung cancer progression. During EMT, the expression of E-cadherin is suppressed, resulting in the accumulation of β-catenin in nucleus, where it complexes with LEF-1 to transactivate FUT8 expression. Following the up-regulation of FUT8, the core fucosylation of surface molecules is increased, which in turn alters the response of cancer cells to their microenvironment, leading to malignant changes in cell behaviors through activating the expression of genes involved in cell adhesion, motility, growth, angiogenesis, and inflammation.

National Taiwan University Hospital (NTUH, Taipei, Taiwan) and Kaohsiung Medical University Hospital (KMUH, Kaohsiung, Taiwan), respectively, with informed consent and approval of institutional review board (NTUH Research Ethics Committee, and KMUH IRB).

Cell Invasion Assay. The invasion assays was performed in 24-well FluoroBlok cell culture inserts (BD Biosciences) with 8-μm pore-size PET membrane. The insert was coated with 100 μL of 1 μg/μL Matrigel matrix (BD Biosciences) at 4 °C overnight. Following starvation for 6 h in serum-free RPMI 1640, cells were harvested from one subconfluent 10-cm dish by cell dissociation buffer (Life Technologies), spun at 500 × *g* for 3 min, and resuspended in RPMI 1640. Cells (4×10^4) in 500 μL of RPMI 1640 were seeded onto the insert and 750 μL of RPMI 1640 with 10% (vol/vol) FBS was added into the lower chamber of the transwells. After incubation for 18 h at 37 °C, the medium inside the insert was removed and the insert was then placed in a new 24-well plate. The invaded cells at the reverse side of the insert were labeled with a fluorescent dye Calcein AM (4 μM in Dulbecco's PBS) (BD Biosciences) for 1 h at 37 °C. The fluorescence was measured with 494 nm/517 nm (excitation/emission wavelength) by a SpectraMax M5 microplate reader (Molecular Devices).

Cell Proliferation Assay. Cell proliferation was assayed using a water soluble tetrazolium salt, WST-1 [4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate], according to the manufacturer's instruction (Roche). Briefly, 2×10^3 cells per well were seeded in 96-well plates. After 24, 48, 72, or 96 h of cell growth, WST-1 (20 μL per well for 200 μL culture medium) was added and allowed to incubate for 2 h at 37 °C. The absorbance at 450 nm and 690 nm (as reference) were read by a SpectraMax M5 microplate reader (Molecular Devices).

Xenograft Models. To assess the effect of FUT8 on tumor growth, five NOD-SCID mice (6 wk old, female) were s.c. injected with CL1-5/control, CL1-5/shFUT8-1, and CL1-5/shFUT8-2 (2×10^6 cells in 100 μL of PBS) in the ventral region of mice. Tumor growth was monitored weekly by measuring the perpendicular tumor diameters, length (*L*) and width (*W*), with a vernier caliper. The volume of tumor (*V*) was calculated by the formula $V = LW^2/2$. For experimental metastasis, CL1-5/control, CL1-5/shFUT8-1, and CL1-5/shFUT8-2 (1×10^6 cells in 100 μL of PBS) were i.v. injected into lateral tail vein of five NOD-SCID mice (6 wk old, female). Mice were killed under anesthesia 7 wk after injection. Metastatic nodules in lungs were quantified using a dissecting microscope. After counting, lungs were fixed, embedded, sectioned, and stained with hematoxylin and eosin.

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