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Posttranslational modification of E-cadherin by core fucosylation regulates Src activation and induces epithelial–mesenchymal transition-like process in lung cancer cells

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Abstract

E-cadherin is often dysregulated in aggressive lung cancer, the mechanism of which cannot always be explained at the level of transcription. In 66 patients with lung cancer, immunohistochemical staining demonstrated that co-localization of E-cadherin and core fucose by *Lens culinaris* agglutinin was significantly less extensive in tumor than in nontumor tissue. Through gain and loss of fucosylation experiments in the giant lung carcinoma cell lines 95C and 95D, our results revealed that E-cadherin core fucosylation in 95C cells overexpressing α-1, 6-fucosyltransferase (Fut8) inhibited Fut8-95C cell migration, whereas knockdown of Fut8 in 95D cells enhanced migration of short-interfering RNA-targeting Fut8 (siFut8)-95D cells. The level of active Src (phosphorylated Src [Y416]) was significantly reduced in Fut8-95C cells, but elevated in siFut8-95D cells. In protein complexes immunoprecipitated from Fut8-95C cell lysates with anti-E-cadherin, less phosphorylated Src (Y416) and more β-catenin were observed, but immunoprecipitates from siFut8-95D cells, containing less core fucosylated E-cadherin, contained an elevated level of phospho-Src Y416. In Fut8-95C cells, phosphorylation of Akt (Y315, Y326) and GSK-3β (S9) was significantly reduced, but β-catenin (S37) phosphorylation was enhanced. Expression of N-cadherin and Snail1 was also reduced in Fut8-95C cells, but significantly increased in siFut8-95D cells. Intriguingly, when Src kinase activity was inhibited by treatment of cells with PP2 and SU6656, regulation of N-cadherin, Snail1 and cell migration by E-cadherin core fucosylation was abrogated in both Fut8-95C and siFut8-95D cells. Therefore, posttranslational modification of E-cadherin by less core fucosylation recruited and activated Src, and induced an epithelial–mesenchymal transition-like process in lung cancer cells.

Key words: cell migration, core fucose, E-cadherin, epithelial–mesenchymal transition, lung cancer

Introduction

E-cadherin is the essential component of membrane-associated protein complexes assembled in adhesion junctions and associated with cell adhesion in multiple cell types (Schmalhofer et al. 2009). Dysregulation of E-cadherin causes dysfunction of cell adhesion and thus affects cell migration or tumor invasion (Kedinger et al. 2009). E-cadherin function depends mainly on homotypic calcium-dependent interactions

through glycosylated extracellular domains, and is critical in cell–cell adhesion and recognition. *N*-Acetylglucosaminyltransferase-III induces a stabilizing effect on E-cadherin, but *N*-acetylglucosaminyltransferase-V promotes the destabilization of E-cadherin, leading to unstable adherens junctions with impairment of cell–cell adhesion (Pinho et al. 2013). The cytoplasmic tail of E-cadherin can form complexes with β-catenin. β-catenin has multiple phosphorylation sites, including either tyrosine (Y)654, which is important for binding to E-cadherin, or serine (S)37 . Phosphorylation of β-catenin (S37) is controlled by glycogen synthase kinase-3β (GSK3β), a serine/threonine kinase, which is inactivated by phosphorylation at S9 (McCubrey et al. 2014). GSK3β is also an essential component of either the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt/GSK-3β or Wnt/β-catenin pathways. Dysregulation of E-cadherin is involved in a number of cancers (Schmalhofer et al. 2009), but this cannot be explained only at the genetic or epigenetic level. Posttranslational modification might be an alternative functional regulatory mechanism of E-cadherin dysregulation in cancer cells (Pinho et al. 2011).

Posttranslational modification is considered a rapid, efficient means of regulating protein activities. Phosphorylation is important for activation/inactivation of kinases, and histone acetylation is important for control of gene expression. E-cadherin is a glycoprotein that is core-glycosylated by α-1, 6-fucosyltransferase (Fut8) (Geng et al. 2004), but the effect of this modification on E-cadherin function is not well understood. It is not clear if additional modification of the E-cadherin by core fucosylation would alter the interaction of the intracellular domain with molecules such as Src and others that would affect the process of epithelial–mesenchymal transition (EMT). Importantly, E-cadherin and β-catenin are principal markers of EMT. Whether core fucosylation of these marker proteins would affect EMT, an important process in tumor progression and metastasis, is not clear. The correlation between core fucosylation of E-cadherin and EMT remains largely unknown. During EMT, the glycosylation profile of cells, particularly that of fucosylation, may change (Chen;et al. 2013; Li et al. 2013), but the role of fucosylation modification in EMT has not yet been identified. A recent report suggested that O-fucosylation of thrombospondin type 1 repeat is essential for restricting EMT in the primitive streak (Du et al. 2010). In previous studies, we observed that core fucosylated glycan was associated with migration of hepatocellular carcinoma cells (Wu and Chen 2002; Wu et al. 2010), and silencing of the Fut8 gene affected intracellular distribution of β-catenin (Hu et al. 2008). Therefore, we hypothesize that there may be a link between modification of core fucosylation and EMT. In this study, we explored and demonstrated the regulatory effect of core fucosylation on E-cadherin signaling and the process of EMT.

Results

E-cadherin core fucosylation

Lung cancer cell lines 95C and 95D were derived from the same patient and have low and high metastatic potential, respectively. 95C cells were transfected with Fut8 expression construct, and 95D cells were transfected with plasmid encoding short-interfering RNA-targeting Fut8 (siFut8). Fut8 expression levels in transfected cells were confirmed by RT-PCR (Figure 1A). Staining with *Aleuria aurantia* lectin and *Lens culinaris* agglutinin (LCA), which selectively bind to core fucose, was enhanced in Fut8-overexpressing 95C cells (Fut8-95C) and reduced in siRNA-mediated Fut8 knockdown 95D cells (siFut8-95D) (Figure 1B). Then, we immunoprecipitated proteins from cell lysates with either anti-E-cadherin monoclonal antibody (Figure 1C) or LCA-agarose beads (Figure 1D) and noted with interest that LCA binding signal was present at the band of E-cadherin from the protein complex immunoprecipitated by anti-E-cadherin, and E-cadherin was seen in the complex precipitated by LCA-agarose. Furthermore, the LCA binding signal was significantly reduced in siFut8-95D cells compared with cells transfected with scrambled siRNA control, but enhanced in Fut8-95C cells (Figure 1D), suggesting that E-cadherin was core fucosylated by Fut8 in both 95C and 95D cells. Interestingly, after transfection, Fut8-95C cells, with elevated levels of core fucosylated E-cadherin, migrated much more slowly, and siFut8-95D cells, with low core fucosylation of E-cadherin, migrated significantly faster, than their respective controls. Similar results were achieved in both migration (Figure 1E) and wound-healing (Figure 2A) assays. These findings indicated a negative correlation between the migratory ability of lung cancer cells and E-cadherin core fucosylation. Immunohistochemical analysis showed that co-localization of anti-E-cadherin and LCA binding signals was significantly less extensive in tumor tissue than in adjacent nontumor tissue, although LCA staining was more extensive and E-cadherin staining was much weaker in tumor than nontumor tissue. Among 66 patients, 38 (58%) revealed higher LCA staining score in tumor tissue than in nontumor tissue, but 47 (71%) showed a lower double-positive staining, or co-localization, score for tumor tissue than for nontumor tissue (Figure 2B). The immunoreactivity score of co-localization was significantly lower in tumor than in nontumor tissue.

Impacts on β-catenin phosphorylation

The intracellular domain of E-cadherin binds to β-catenin; therefore, we investigated the impact of E-cadherin core fucosylation on β-catenin phosphorylation. As shown in Figure 2C, although the level of total β-catenin remained unchanged, phosphorylated β-catenin (Y654) was significantly enhanced in the Fut8 knockdown 95D cells with reduced E-cadherin fucosylation, but decreased in the Fut8-95C cells, which had a high level of fucosylated E-cadherin, suggesting that β-catenin tyrosine phosphorylation was downregulated after modification of E-cadherin by core fucosylation.

To determine the mechanism underlying regulation of phosphorylation, we investigated the protein tyrosine kinases and noted, interestingly, that c-Src (Y416) was highly phosphorylated in siFut8-95D cells than in the scrambled siRNA control cells, whereas phosphorylation of Src (Y416) was significantly reduced in Fut8-95C cells. Phosphorylation of Src (Y416), indicating activation of Src kinase, was coincident with phosphorylation of β-catenin (Y654); however, the level of Src (Y529) phosphorylation remained unchanged. Moreover, Src and α- and β-catenin were identified in the complex precipitated by anti-E-cadherin antibody (Figure 2D). Intriguingly, we found that p-Src (Y416), the activated form of c-Src, but not p-Src (Y529), was significantly enhanced in the E-cadherin complex immunoprecipitated from siFut8-95D cells, compared with that immunoprecipitated from control cells. In contrast, in Fut8-95C cells, p-Src (Y416) was present at a significantly lower level in the E-cadherin complex than in the mock-transfected controls (Figure 2D). This indicated that active Src kinase bound with lower affinity to highly core fucosylated than to low core fucosylated E-cadherin. Thus, E-cadherin core fucosylation changed its Src-binding affinity and regulated the recruitment of activated Src kinase to the complex. Importantly, α-catenin, β-catenin, and p-β-catenin (Y654), but not β-catenin binding protein, were also recruited at a higher level to the highly core fucosylated E-cadherin

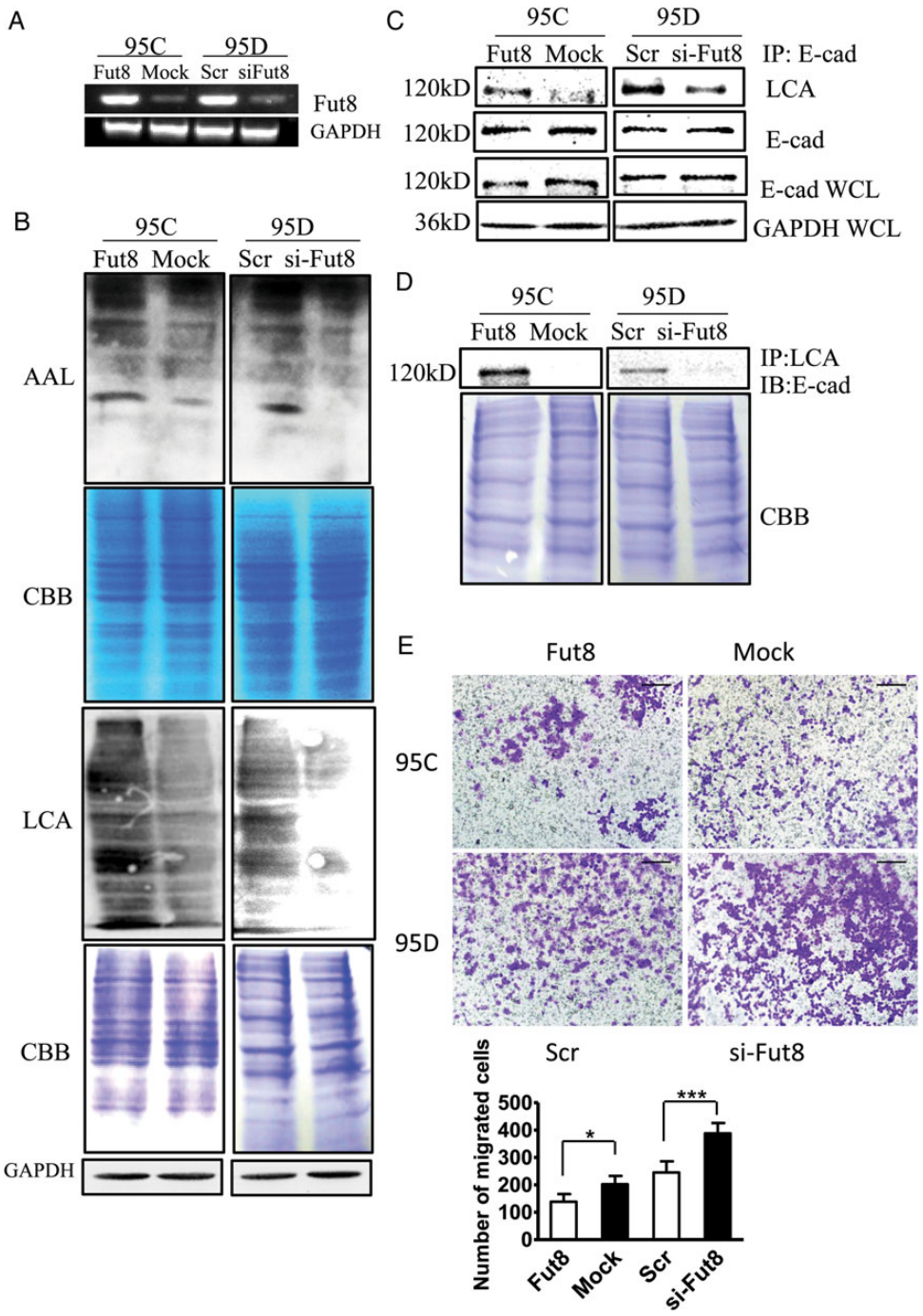


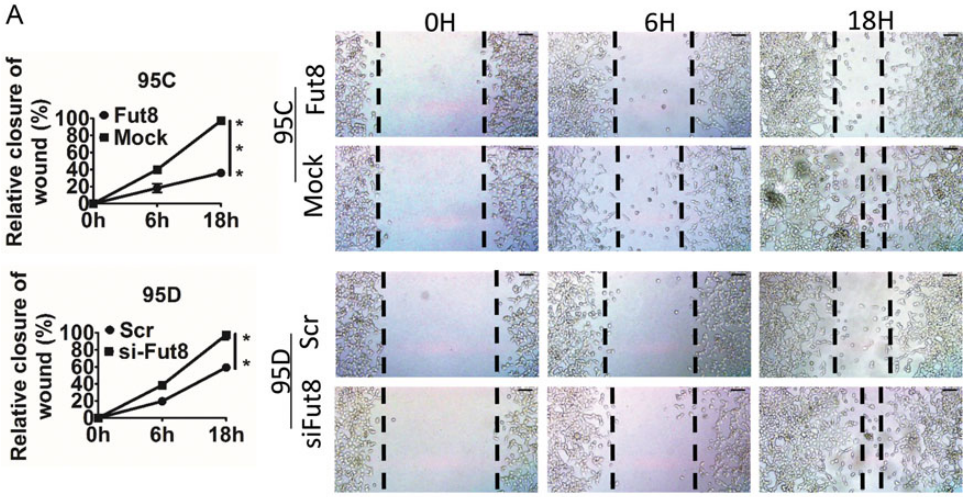
Fig. 1. E-cadherin is fucosylated in lung cancer cells. (**A**) RT-PCR analysis of Fut8 mRNA expression in 95C cells overexpressing Fut8 (Fut8) or transfected with vector alone (mock), and in 95D cells transfected with control scrambled (Scr) siRNA or siFut8. (**B**) Coomassie Brilliant Blue (CBB)-stained SDS-PAGE gel and blotting with AAL (top), LCA (middle), and anti-GAPDH antibody (bottom) of total protein in lysates of the indicated cells. (**C**) Western blot of lysates of the indicated transfected cells immunoprecipitated with anti-E-cadherin antibody (E-cad) and probed with LCA (upper panels). Western blot of E-cadherin and GAPDH (internal control) in whole cell lysates (WCL) (lower panels). (**D**) Western blot with anti-cadherin antibody (upper) and CBB stained gel (lower) of lysates of the indicated cells immunoprecipitated with biotinylated LCA. (**E**) Representative micrographs (upper) and quantitation (lower) of results of migration assays of the indicated transfected cells (size bar: 100 μm). Data are representative of at least three independent experiments. Error bars indicate SD. $^{*}P < 0.05$, $^{***}P < 0.001$. This figure is available in black and white in print and in color at *Glycobiology* online.

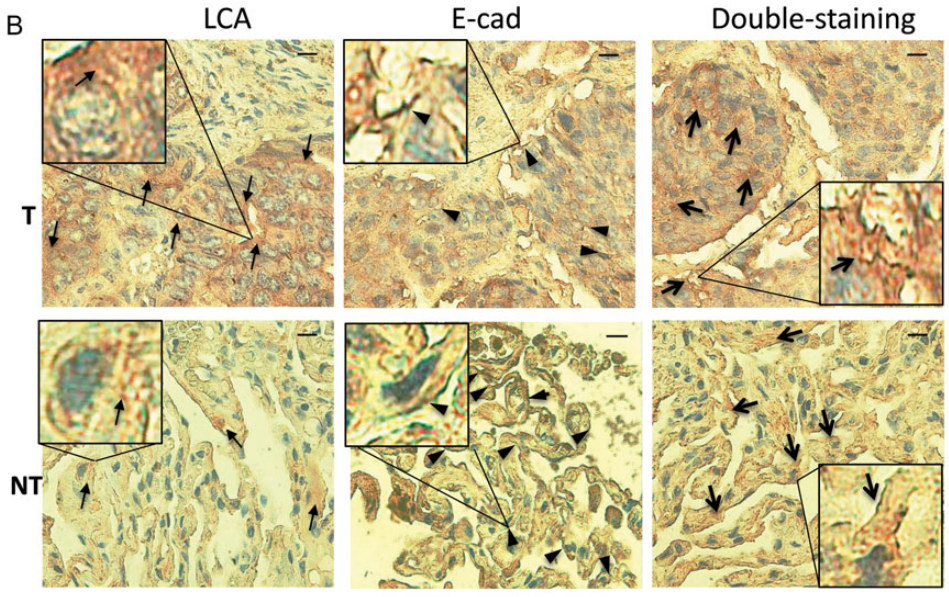
complex in Fut8-95C cells or control 95D cells than to the low core fucosylated complex in control 95C or siFut8-95D cells, suggesting that the binding ability of E-cadherin to β-catenin was enhanced after core fucosylation.

Activation of AKT and GSK3β

In addition to the phosphorylation site at Y654, β-catenin can also be phosphorylated on serine (S)37. To further understand the impact of core fucosylation on β-catenin, we assessed the level of phosphorylation

at S37 and, interestingly, observed a significant increase after E-cadherin core fucosylation with Fut8 overexpression in 95C cells, and a reduced level when E-cadherin was less core fucosylated after Fut8 knockdown in 95D cells (Figure 3A). In contrast, GSK3β was phosphorylated at a lower level on (S9) in Fut8-95C cells, but at a higher level in siFut8-95D cells. We then assessed phosphorylation of AKT and PI3K. As shown in Figure 3B, the phosphorylation levels of PI3K active subunit (P85) and AKT (S473) were essentially unchanged with Fut8 overexpression. Surprisingly, the phosphorylation levels of AKT Tyr 315 and Tyr 326 were significantly decreased when E-cadherin was highly core fucosylated in Fut8-95C cells (Figure 3C), but enhanced in siFut8-95D cells where E-cadherin was less core fucosylated. Phosphorylation of AKT





	T>NT	T=NT	T<NT	Total
LCA	38(58%)	6(9%)	22(33%)	66
E-cad	17(26%)	3(5%)	46(70%)	66
LCA-Ecad	16(24%)	3(5%)	47(71%)	66

T: tumor tissues ; NT: adjacent non-tumor tissues

Fig. 2. Core fucosylated E-cadherin regulates the phosphorylation of β-catenin and cell motility. (**A**) Representative micrographs of 95C cells overexpressing Fut8 (Fut8) and transfected with vector alone (mock) and 95D cells transfected with control scrambled (Scr) siRNA and siFut8 in wound-healing assay at indicated time points (right) and quantitation of relative wound closure (left). Size bars indicate 100 μm. (**B**) Representative micrographs (upper) of double immunochemical staining with E-cadherin antibody and biotinylated LCA in tumor (T) and adjacent nontumor tissue. Fine arrows indicate LCA staining (red). Arrow heads show E-cadherin staining (dark blue). Bold arrows indicate co-localization (brown). Original magnification: ×400. Size bar: 20 μm. Immunoreactivity scores of LCA, E-cadherin and co-localization in tumor and nontumor tissue in patients P1–P66 analyzed by Mann–Whitney *U* test (Figure 2 continued part 1, left panel). Statistical average of staining scores of E-cadherin, LCA and co-localization in tumor and adjacent nontumor tissue (Figure 2 continued part 1, right panel). (**C**) Western analysis (left) and quantitation based on densitometry (right) of β-catenin, phosphorylated (p-) β-catenin, Src and p-Src (tyrosine [Y]416, Y529) in the indicated transfected cells. (**D**) Western analysis (upper) and quantitation based on densitometry (lower) of the indicated proteins immunoprecipitated from lysates of the indicated transfected cells with anti-E-cadherin antibody. IP, immunoprecipitation; IB, immunoblotting. Data are representative of three independent experiments. Error bars indicate SD. $*P<0.05$, $**P<0.01$, $***P<0.005$, NS, not significant.

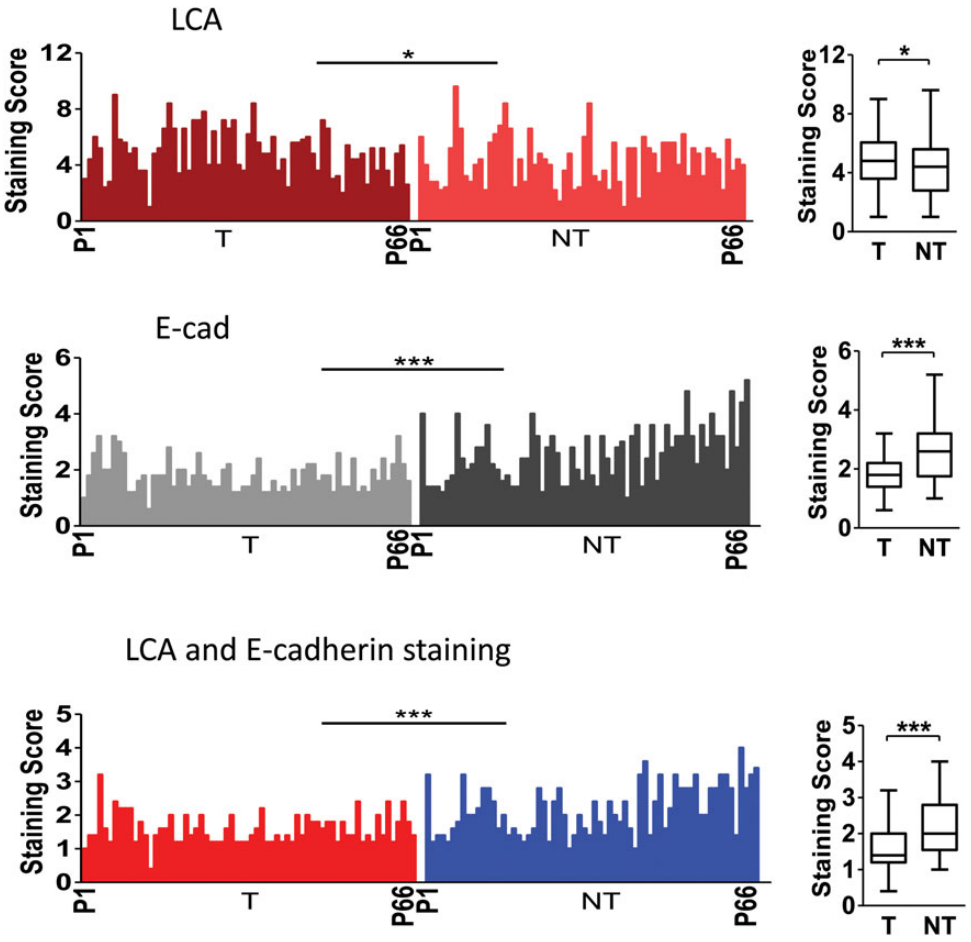


Figure 2 Continued

(Y315) and (Y326) was coincident not only with phosphorylation of GSK-3β S9 but also with activation and phosphorylation of Src (Y416). These findings suggest that E-cadherin core fucosylation hindered activation of Src and AKT, but promoted GSK-3β activation, which led to the phosphorylation of β-catenin (S37).

Roles of Src in E-cadherin core fucosylation signaling

Based on the data described in the sections of impacts on β-catenin phosphorylation and activation of AKT and GSK3β, we noted that core fucosylation of E-cadherin regulated Src activation and β-catenin phosphorylation associated with AKT and GSK-3β regulation. To further investigate the role of Src in E-cadherin core fucosylation-mediated signaling, we treated 95D cells, which had a higher level of c-Src (Y416) phosphorylation after Fut8 knockdown (Figure 2C), with Src inhibitors SU6656 and PP2 (Figure 4A and B). Phosphorylation of Src (Y416) was clearly suppressed after the inhibitor treatment. Interestingly, β-catenin (Y654) phosphorylation was significantly reduced in siFut8-95D cells after treatment with either of Src inhibitors, although not decreased significantly in the scramble 95D cells, since the basal level of Src phosphorylation was low. Simultaneously, phosphorylation of AKT (Y315) and GSK-3β (S9) was markedly decreased after SU6656 and PP2 treatment, even in siFut8-95D cells, although phosphorylated AKT (Y326) was slightly reduced compared with the control cells. Importantly, the migratory ability of 95D cells (Figure 4C) was dramatically decreased after treatment with Src inhibitors, even in siFut8-95D cells in which E-cadherin was less core fucosylated. These data indicate the importance of Src in the regulation of E-cadherin core fucosylation-mediated migration of 95D cells.

Src activation induced an EMT-like process

EMT is associated with tumor progression and is important in migration and invasive behaviors of lung and other cancer cells. Because modification of E-cadherin core fucosylation hindered phosphorylation and activation of Src, and regulated migration of 95C and 95D lung cancer cells, we next investigated whether E-cadherin core fucosylation also affected EMT. We observed no change in E-cadherin expression in either Fut8-95C or siFut8-95D cells (Figure 4D). Surprisingly, expression levels of N-cadherin and Snail1 were significantly downregulated when E-cadherin was highly core fucosylated with Fut8 overexpression, and upregulated when E-cadherin was less core fucosylated with Fut8 knockdown; however, expression of Slug, ZEB-1, CK18, and vimentin remained unchanged compared with control cells (Figure 4D). Having demonstrated the importance of Src in regulation of E-cadherin core fucosylation-mediated 95D cell migration, we treated siFut8-95D and control cells with Src inhibitors to investigate the role of Src in core fucosylated E-cadherin-mediated regulation of EMT. Surprisingly, expression of N-cadherin and Snail1 was suppressed in siFut8-95D cells, and little or no difference existed in control cells after Src inhibition (Figure 5A). These findings suggested that changes in E-cadherin core fucosylation mediated Src activation and involved an EMT-like process.

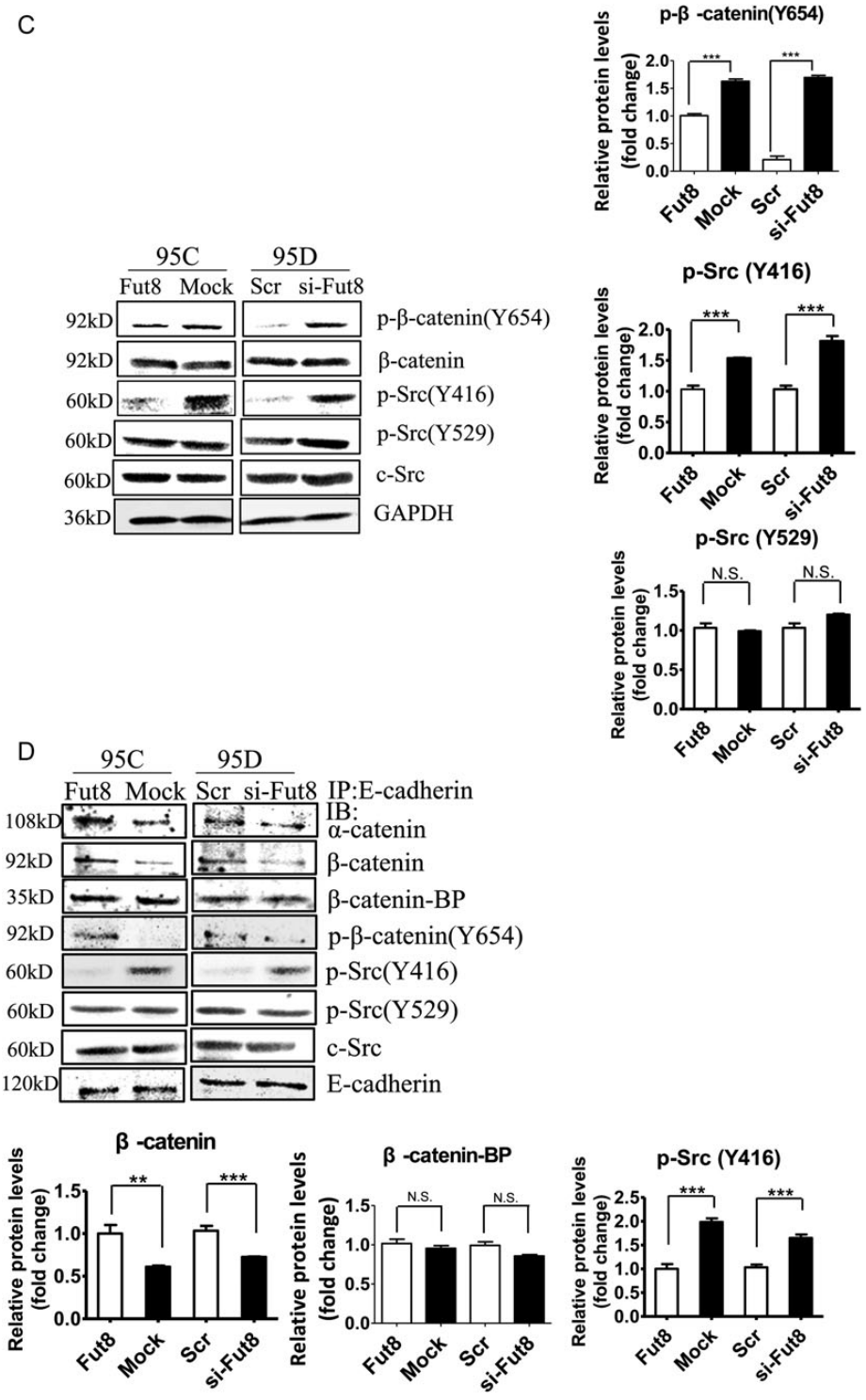


Figure 2 Continued

Core fucosylation affects galectin-3 and GSK-3β/β-catenin interaction

Galectin-3 interacts with the GSK-3β/β-catenin complex in gastric cancer cells; however, the impact of E-cadherin core fucosylation on this interaction remains to be determined. We performed immunoprecipitation with mouse monoclonal antibody against galectin-3 and observed that the levels of GSK-3β and β-catenin in the immunoprecipitated complex were greater in Fut8-95C cells, in which E-cadherin was core fucosylated to a greater extent and reduced in siFut8-95D cells where E-cadherin was core fucosylated to a lesser extent, compared with the respective control cells (Figure 5B). We also noted that levels of the phosphorylated forms of GSK-3β and β-catenin also changed in a

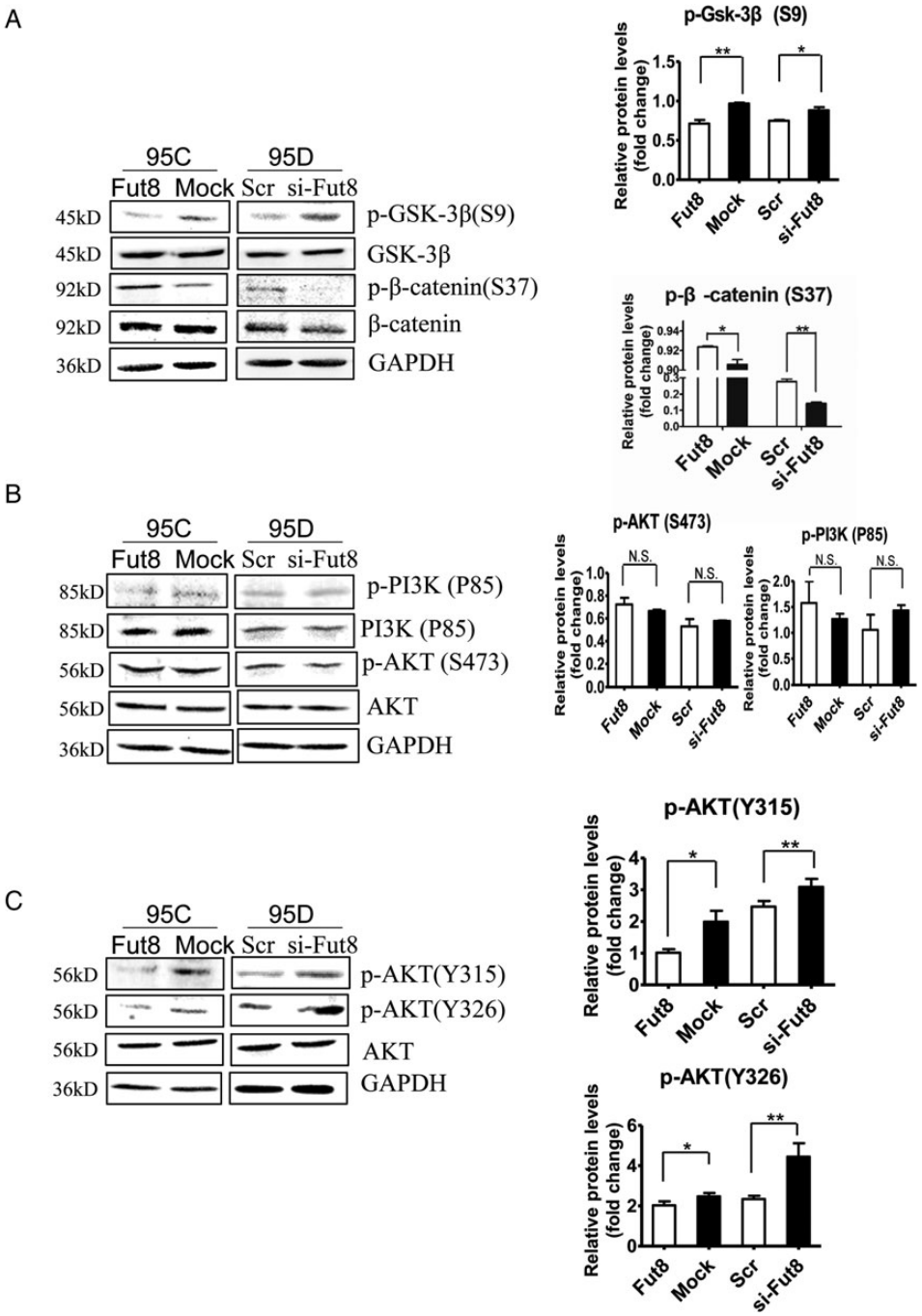


Fig. 3. Core fucosylation-mediated regulation of β-catenin is associated with AKT and GSK-3β. (**A–C**) Western blots (left) and quantitation based on densitometery (right) of indicated proteins in the indicated transfected cells. GAPDH served as internal control. Data are representative of three independent experiments. Error bars indicate SD. $*P < 0.05$, $**P < 0.01$, NS, not significant.

manner similar to that of total GSK-3β and β-catenin in both Fut8-95C and siFut-95D cells compared with controls. We mutated serine 96 of galectin-3 to alanine (S96A) and transfected 95C and 95D cells with the construct encoding the mutant. As shown in Figure 5C, the enhanced phosphorylation of GSK-3β (S9) in Fut8-95C cells and the suppressed phosphorylation of GSK-3β in siFut8-95D cells was nearly abrogated in galectin-3 mutants. Phosphorylation of β-catenin (S37) was also attenuated with galectin-3 mutation. These data demonstrate that phosphorylation of GSK-3β/β-catenin in 95C and 95D cells regulated by E-cadherin core fucosylation was associated with alteration of the galectin-3-GSK-3β interaction.

Discussion

Based on the important role of E-cadherin in cell adhesion and cell–cell contact, we investigated the effects of core fucosylation on E-cadherin regulation. Our results showed that co-localization of E-cadherin and LCA binding signals in lung cancer tissue was

significantly higher in adjacent nontumor tissue than in tumor, suggesting a biological role of core fucosylated E-cadherin in lung cancer cells. With core fucosylation, the binding properties of E-cadherin were altered, not only in the extracellular domain that is involved in cell aggregation (Geng et al. 2004; Osumi et al. 2009) but also in the cytoplasmic tail, which regulates β-catenin and Src activity. Interestingly, phosphorylation of β-catenin (Y654) was enhanced in siFut8-95D lung cancer cells with a lower level of E-cadherin core fucosylation, and attenuated in Fut8-95C cells with high core fucosylation. However, the mechanism underlying E-cadherin core fucosylation-induced phosphorylation of β-catenin (Y654) is not understood. In the present study, we noted that activation of Src kinase via phosphorylation at (Y416), was coincident with phosphorylation of β-catenin (Y654), although no change was seen in phosphorylation of Src (Y529). Furthermore, phospho-Src (Y416) was significantly enhanced in the complex with less core fucosylated E-cadherin in siFut8-95D cells, in agreement with regulation of β-catenin (Y654) phosphorylation. Phosphorylation of β-catenin (S37) was also influenced by core fucosylation because it was enhanced in Fut8-overexpressing 95C cells and reduced with Fut8 knockdown in 95D cells. Our data indicated that Src kinase was also be involved in regulation of β-catenin phosphorylation at S37 since Src kinase induced Akt phosphorylation at Y315 and Y326. The activated Akt in turn phosphorylated GSK3β and the inactivated GSK3β was then unable to phosphorylate β-catenin S37. GSK-3β is considered responsible for the phosphorylation of β-catenin (S37) because it binds β-catenin,

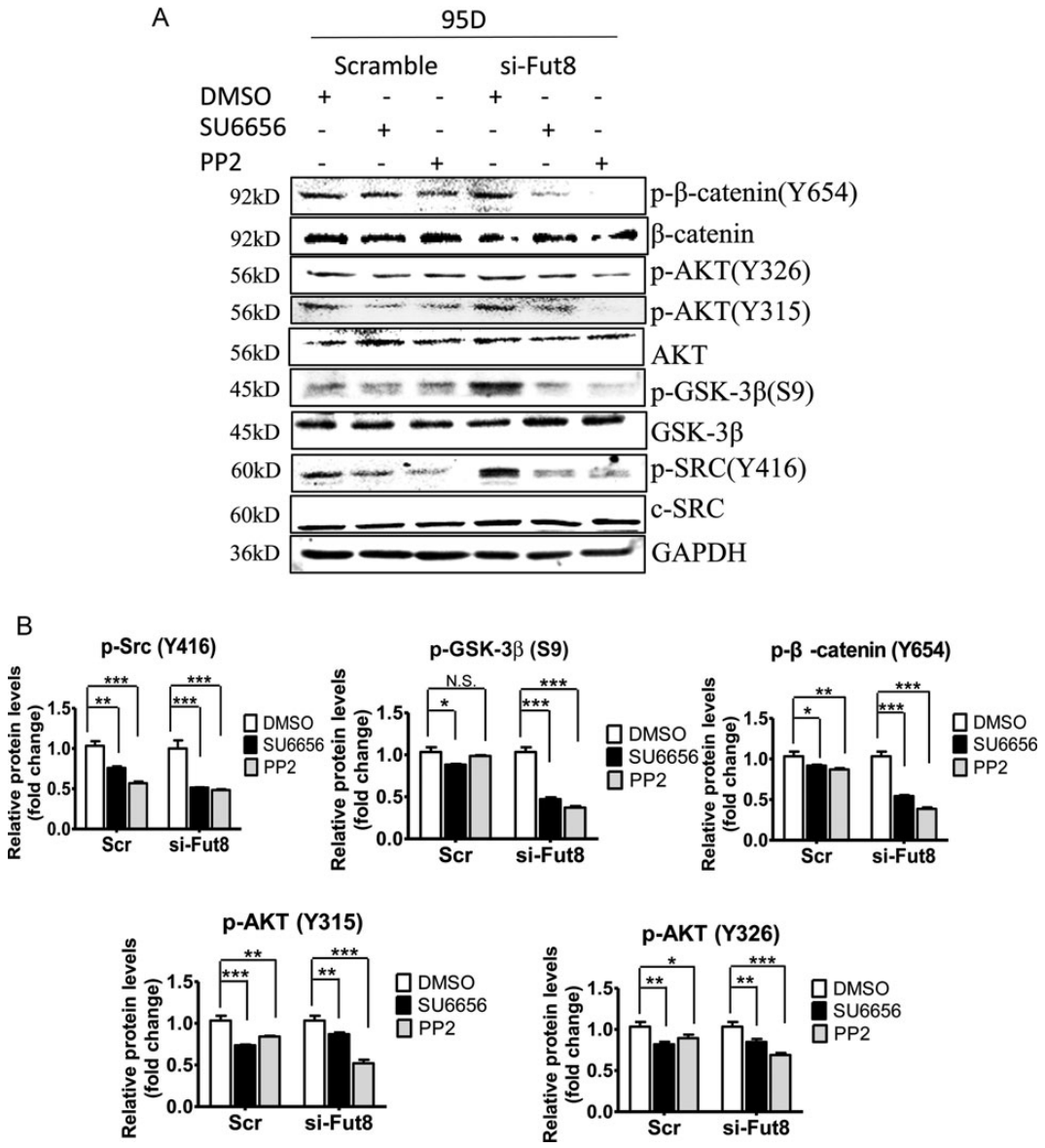


Fig. 4. The role of Src in core fucosylated E-cadherin signaling. (**A** and **B**) Western blot (A) and semi-quantitative analysis (B) of the indicated proteins in 95D cells transfected with control scrambled (Scr) siRNA and siFut8 following treatment with DMSO (control) or Src inhibitors SU6656 or PP2. (**C**) Representative micrographs (upper) and quantitation (lower) of migration of 95D cells transfected with control scrambled (Scr) siRNA or siFut8 and treated with DMSO (control) or Src inhibitors SU6656 or PP2. Size bar: 100 μm. (**D**) Western analysis (left) and quantitation based on densitometry (right) of the indicated EMT-associated proteins in the indicated transfected cells. Data are representative of three independent experiments. Error bars indicate SD. $*P<0.05$, $**P<0.01$, $***P<0.005$, NS, not significant. This figure is available in black and white in print and in color at *Glycobiology* online.

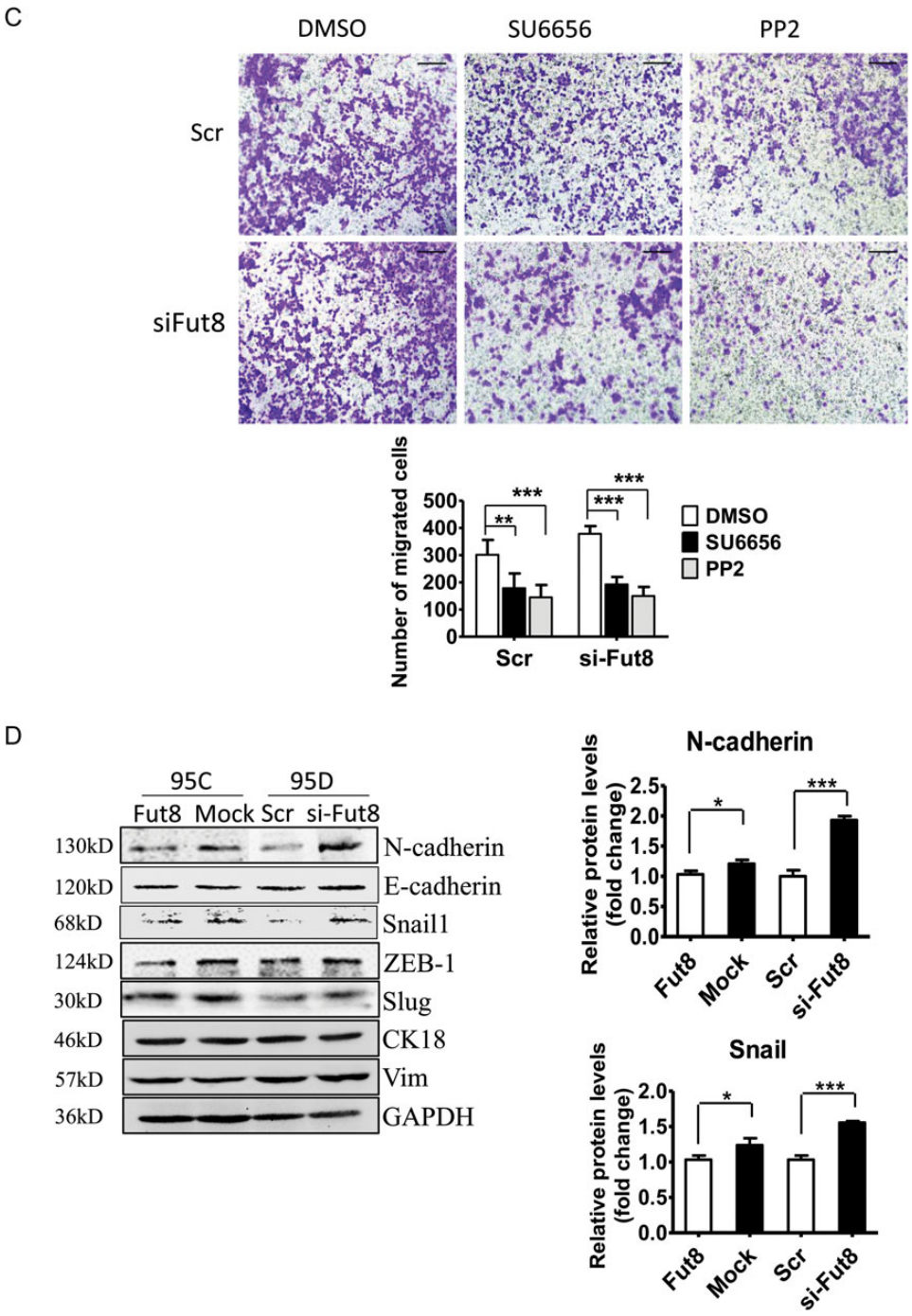


Figure 4 Continued

axin, and APC to form a β-catenin-degradation complex, facilitating phosphorylation of β-catenin (S33), (S37), (S45), and (T41) (Behrens et al. 1998; McCubrey et al. 2014), ubiquitylation and degradation. In the present study, we found that the level of phosphorylated GSK-3β was reduced in highly core fucosylated Fut8-95C cells, together with increasing levels of phosphorylated β-catenin (S37), which was consistent with our previous findings that core fucosylation led to decreasing β-catenin translocation from cytoplasm to nuclei (Hu et al. 2008). The GSK-3β/β-catenin pathway regulated by E-cadherin core fucosylation is more likely to be controlled by Src/AKT (Figure 6) than by the classic PI3K/AKT pathway, based on the finding that few if any distinguishable changes were observed in phosphorylation of the PI3K active subunit (P85) and AKT (S473) in any of the cells. On the contrary, phosphorylation of AKT (Y315) and (Y326) was clearly decreased in the highly fucosylated Fut8-95C cells, in which phosphorylated Src (Y416) was diminished. With inhibition of Src kinase, phosphorylation of AKT (Y315) and GSK-3β (S9) was markedly suppressed in siFut8-95D cells, in which Src was activated. These data suggest that the PI3K/Akt pathway might not be the main regulator of E-cadherin core fucosylation-mediated β-catenin phosphorylation, but Src/AKT

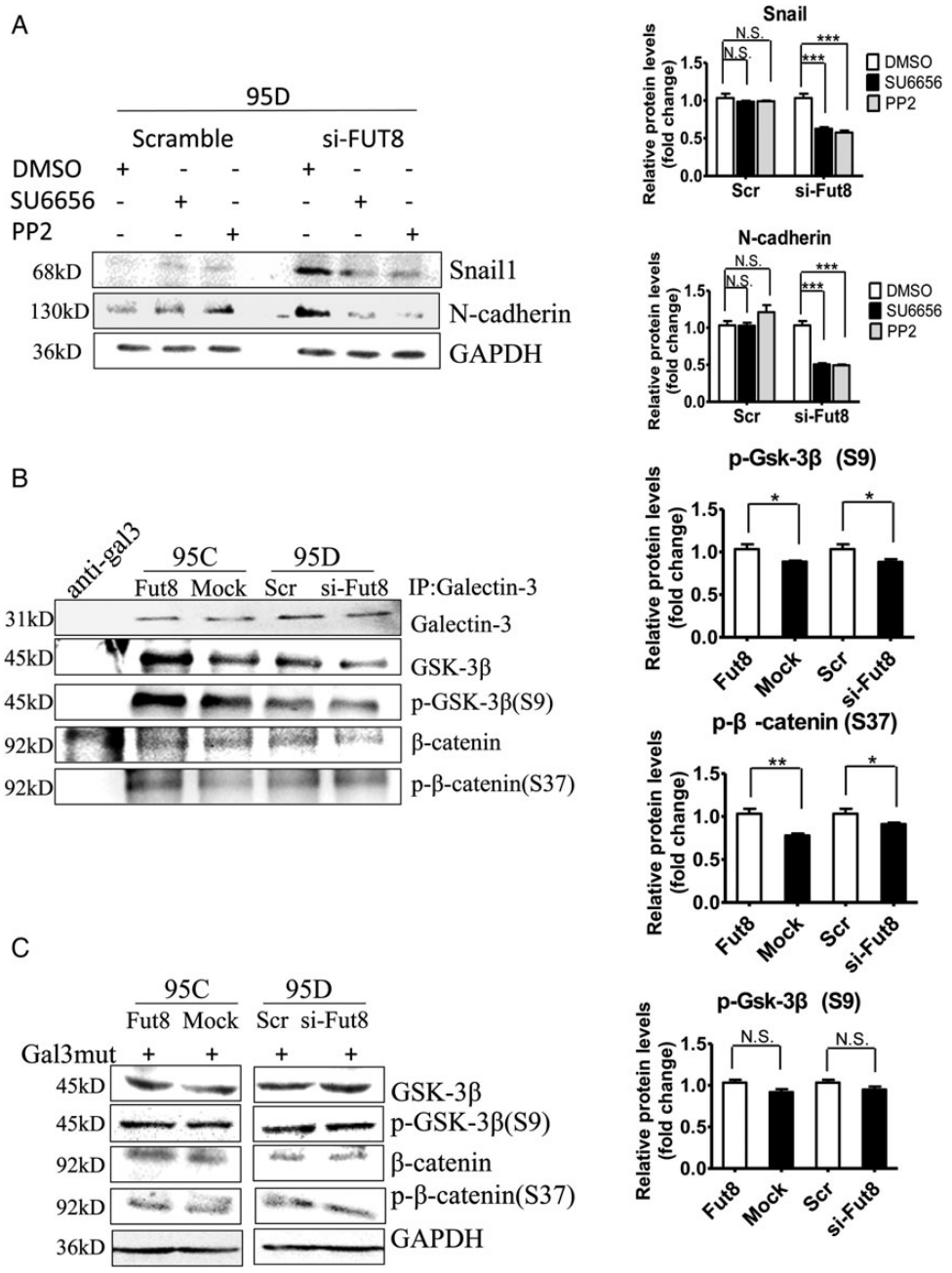


Fig. 5. Regulation by E-cadherin core fucosylation involves galectin-3 interaction. (**A**) Western blot (left) and semi-quantitative analysis (right) of the indicated proteins in 95D cells transfected with control scrambled (Scramble) siRNA and si-Fut8 following treatment with DMSO (control) or the Src inhibitors SU6656 or PP2. (**B**) Western analysis of the indicated proteins IP from lysates of the indicated transfected cells with anti-galectin-3 antibody and immunoblotted with antibodies against GSK-3β, p-GSK-3β (S9), β-catenin and p-β-catenin S37. (**C**) Western blot of the indicated proteins in 95C and 95D cells co-transfected with the indicated constructs and construct encoding galectin-3 S96A mutant. Data are representative of three independent experiments. Error bars indicate SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, NS, not significant.

signaling was the major pathway. Furthermore, our results demonstrated that E-cadherin core fucosylation altered β-catenin binding status in the complex with E-cadherin, and then the interaction of β-catenin with GSK-3β or galectin-3. Galectin-3 interacts with β-catenin and GSK-3β (Song et al. 2009). The direct interaction between galectin-3 and GSK-3β affects the stability and nuclear localization of β-catenin and the transcription activity of β-catenin/T-cell factor 4, which is associated with gastric cancer cell motility (Kim et al. 2010). The serine 96 of galectin-3 is the binding site for GSK-3β. Mutation of galectin-3 (S96A) abrogated the interaction with GSK-3β and E-cadherin core fucosylation-mediated regulation.

Importantly, E-cadherin core fucosylation hindered cell motility, and reduction of fucosylation level enhanced the migration of the lung cancer cells. With inhibition of Src kinase, cell migration facilitated by a lower level of E-cadherin fucosylation in siFut8-95D cells was nearly abolished. EMT is associated with migratory and invasive

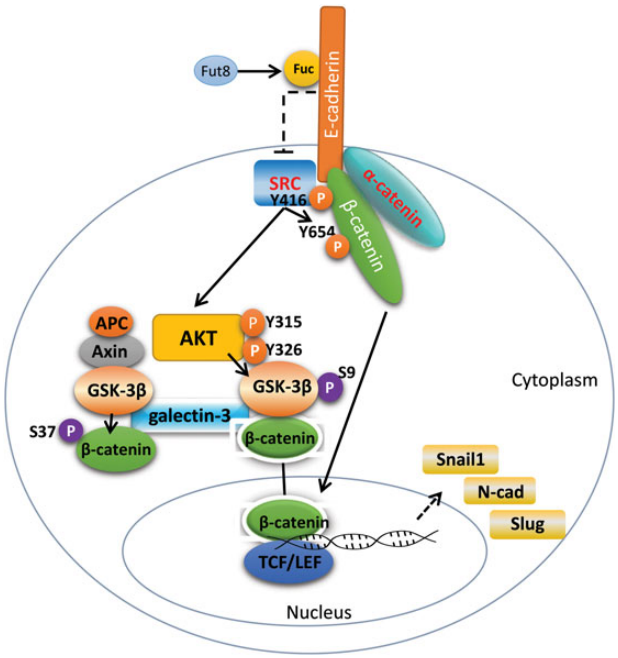


Fig. 6. Summary of E-cadherin regulation of EMT process by core fucosylation. E-cadherin protein can be modified by core fucosylation, and this affects the activation of Src kinase that is compounded with E-cadherin. When Src is activated. It phosphorylates β-catenin Y654 leading to nuclear accumulation. Src also activates Akt which subsequently inactivates GSK-3β. While non-phosphorylated GSK3β associated with APC-Axin phosphorylates β-catenin S37 leading to degradation. This figure is available in black and white in print and in color at *Glycobiology* online.

properties of tumor cells. Although E-cadherin expression level was not changed in Fut8-overexpressing or knockdown cells, expression of N-cadherin and Snail1 was significantly elevated in the siFut8-95D knockdown cells. N-Cadherin and Snail1 are usually expressed in mesenchymal cells and have been used as indicators to monitor the progress of EMT (Zeisberg and Neilson 2009). Snail1 gene expression associated with EMT is directly controlled by the β-catenin/TCF/LEF complex (Zeisberg and Neilson 2009), which particularly accumulates in the nucleus of siFut8-95D cells with less core fucosylation (Hu et al. 2008). Consistent with this, knockdown of Fut8 also induced changes similar to EMT in PC12 cells (Gu et al. 2013); however, with Src kinase inhibition, this process was abrogated because upregulation of N-cadherin and Snail1 and cell migration promoted by a lower level of fucosylation were diminished although, interestingly, N-cadherin upregulation contributed to the activation of Src kinase. Src/FAK signaling controls the fate of EMT and cell migration (Avizienyte and Frame 2005). Activation of Src can even initiate an EMT-like process in multiple cell types (Zhang et al. 2012). However, what is the upstream event for the activation of Src kinase remains largely unknown (McLachlan and Yap 2011). Our study provides the first evidence that E-cadherin modification by core fucosylation regulates Src activation. Inactive Src is in a closed conformation maintained by the interactions between the SH2 domain and C-terminal tail (Brown and Cooper 1996). Disruption of these low-affinity interactions allows Src autophosphorylation on Y416 to activate kinase activity. Altered homophilic binding of E-cadherin extracellular domain by less core fucosylation seems able to strengthen Src autophosphorylation on Y416 to activate the activity. Activated Src induces an EMT-like process and stimulates migration of lung cancer cells. Posttranslational modification of E-cadherin by core fucosylation (Figure 6) is thus an alternative mechanism of E-cadherin dysregulation in lung cancer cells.

Materials and methods

Cell culture and transfection

Giant cell lung carcinoma lines 95C and 95D, with low and high metastatic potential, respectively, were obtained from the General Hospital of PLA (Beijing, China) (Lu et al. 1989). Cells were authenticated and cultured in Gibco RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Fut8 expression vector and vector-encoding siFut8 sequence 5′-AGGCUGUGGCUAUGGCUGU-3′ were constructed as previously described (Geng et al. 2004). siRNA with scrambled sequence was used as control. Cells were transfected with vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For some experiments, Src kinase activity was inhibited by culturing cells in the presence of 10 μM PP2 or 10 μM SU6656 (Merck, Darmstadt, Germany) from stock solution prepared in DMSO (dimethyl sulfoxide).

Patients and tissue microarray

A total of 66 patients newly diagnosed with lung cancer underwent curative surgery. Diagnosis was confirmed by histological examination as adenocarcinoma type of non-small cell lung carcinoma. Tumor tissue and adjacent nontumor tissue from each patient was collected at Shanghai Chest Hospital, Shanghai Pulmonary Hospital, and Affiliated Hospital of Guilin Medical University. Paraffin-embedded tissue blocks and corresponding hematoxylin and eosin-stained sections were overlaid for tissue microarray preparation by Super-Biotek (Shanghai, China). A paraffin-embedded tissue microarray was constructed of triplicate pairs of tumor and nontumor tissue from each patient using an ATA-27 tissue arrayer (Beecher Instruments, WI).

Immunohistochemistry

Paraffin sections were stained with anti-E-cadherin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and biotin-conjugated *L. culinaris* agglutinin (Vector Laboratories, Burlingame, CA) using DouSP double-stain kit (Maxim-Bio, Fuzhou, China). The alkaline phosphatase-conjugated secondary antibody and the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium were used for E-cadherin staining (dark blue). Streptavidin peroxidase and its substrate 3-amino-9-ethylcarbozole was for LCA staining (red). Scoring of immunohistochemical staining was performed independently by two senior pathologists, blinded to all samples, using a widely accepted German semi-quantitative scoring system (Pan et al. 2011). Scoring was based on the intensity of the cytoplasmic, and/or membrane staining (none = 0, weak = 1, moderate = 2, strong = 3) and the extent of stained cells (0% = 0, 1%–24% = 1, 25%–49% = 2, 50%–74% = 3, 75%–100% = 4). The final immunoreactivity score was obtained by multiplying the intensity score by the extent score and ranged from 0 to 12.

RNA extraction and reverse-transcription–polymerase chain reaction

Total RNA was extracted from cultured cells using TRIZOL reagent (Invitrogen) and reverse transcribed to cDNA using a reverse

transcriptase M-MLV (RNase H$^-$) (Takara, Dalian, China) according to the manufacturer's instructions. GAPDH expression served as internal control. GAPDH primers were (forward) 5′-CGGATTTG GTCGTATTGGG-3′ and (reverse) 5′-cgctcctggaagatggtgat-3′; Fut8 primers were (forward) 5′-gaacttagaaggaaatgaac-3′ and (reverse) 5′-catccaccagtagcatag-3′.

Cell lysis and western blot analysis

Briefly, cells grown to ~70% confluence were washed with phosphate-buffered saline (PBS) and harvested. Cells were suspended and ultrasonicated in 0.1% SDS lysis buffer containing 10 μg/mL aprotinin, 1.5 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate ($NaVO_4$), and 1 mM sodium fluoride on ice. After centrifugation and determination of protein concentration, aliquots of lysates containing equal amounts of protein were mixed with 2 × SDS loading buffer and resolved by SDSPAGE. Proteins were then transferred onto PVDF membranes. Membranes were blocked and then incubated with primary antibodies for 4 h at 4°C. Antibodies against galectin-3, E-cadherin, and Src were from Santa Cruz Biotechnology. Antibody against phospho-Src (Tyr416) was from Cell Signaling Technology (Danvers, MA). Antibodies against GSK-3β, phospho-GSK-3β (Ser 9), phospho-β-catenin (Ser 37), Akt, phospho-Akt (Tyr 315, Tyr 326, Ser 473), PI3K (p85), phospho-p85, phospho-Src (Tyr 529), N-cadherin, Snail1, Slug, ZEB-1, CK18, and vimentin were from Bioworld (Nanjing, China). Blots were washed five times with PBS containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were detected by enhanced chemiluminescence and the signal was captured by an EMCCD Image system (Tenon, Shanghai, China). Band density was analyzed by densitometry with Tenon software.

Immunoprecipitation

Cells were washed twice with ice-cold PBS and lysed in cold IP lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM $NaVO_4$, 10 μg/mL leupeptin and 10 μg/mL aprotinin) and then incubated on ice for 20 min. Lysates were centrifuged (12 000 rpm, 20 min, 4°C) and the supernatant was precleared with normal rabbit/mouse IgG and agarose-protein A/G or LCA-agarose (Vector Laboratories), and then incubated with corresponding antibody at 4°C for 2 h on a rotator. Protein–antibody complexes were then collected with prewashed agarose-protein A/G beads or lysates were incubated with prewashed LCA-agarose at 4°C overnight. Pellets collected were washed three times with ice-cold IP buffer, and finally resuspended in SDS sample buffer (62 mM Tris–HCl, pH 6.8, 1% SDS, 100 mM DTT and 15% glycerol). Samples were boiled at 100°C for 5 min to release the immunoprecipitated complexes for western blot or lectin blot analysis.

Cell migration assay

After transfection, cells in serum-free medium were placed in the Millicell insert (8-μm pore size; Millipore, Billerica, MA) for migration assays. Medium containing 10% FBS was added to the lower chamber. After incubation, cells that had migrated through the membrane were fixed by methanol, stained with 0.1% crystal violet, and counted under an inverted microscope (Olympus, Tokyo, Japan).

Wound-healing assay was performed after scratching the monolayer with a pipet tip and washing with PBS before adding fresh medium. Wounds were then observed and analyzed under a microscope at specific time points.

Statistical analysis

All experimental data presented are mean ± SD from at least three independent experiments. Graphs were prepared and analyzed using Graph Pad (San Diego, CA) software. Differences in immunohistochemical staining scores of 66 paired samples were analyzed by Mann–Whitney *U* test. Analysis of average differences between two experimental groups was carried out using two-tailed Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

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Conflict of interest statement

None declared.

Abbreviations

EMT, epithelial–mesenchymal transition; Fut8, α-1, 6-fucosyltransferase; Fut8-95C, Fut8-overexpressing 95C cells; GSK3β, glycogen synthase kinase-3β; IP, immunoprecipitation; LCA, *Lens culinaris* agglutinin; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; siFut8-95D, Fut8 knockdown 95D cells; siFut8, short-interfering RNA-targeting Fut8.

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