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Role of fucosyltransferase IV in epithelial–mesenchymal transition in breast cancer cells

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Epithelial–mesenchymal transition (EMT) is a crucial step in tumor progression and has an important role during cancer invasion and metastasis. Although fucosyltransferase IV (*FUT4*) has been implicated in the modulation of cell migration, invasion and cancer metastasis, its role during EMT is unclear. This study explores the molecular mechanisms of the involvement of *FUT4* in EMT in breast cancer cells. Breast cancer cell lines display increased expression of *FUT4*, which is accompanied by enhanced appearance of the mesenchymal phenotype and which can be reversed by knockdown of endogenous *FUT4*. Moreover, *FUT4* induced activation of phosphatidylinositol 3-kinase (PI3K)/Akt, and inactivation of GSK3β and nuclear translocation of NF-κB, resulting in increased Snail and MMP-9 expression and greater cell motility. Taken together, these findings indicate that *FUT4* has a role in EMT through activation of the PI3K/Akt and NF-κB signaling systems, which induce the key mediators Snail and MMP-9 and facilitate the acquisition of a mesenchymal phenotype. Our findings support the possibility that *FUT4* is a novel regulator of EMT in breast cancer cells and a promising target for cancer therapy.
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Epithelial–mesenchymal transition (EMT) is a complex process involved in embryonic development, and was first recognized as a hallmark of embryogenesis in the early 1980.[1,2] EMT is also a key event in the tumor-invasion process and has been implicated in increased cell invasion and metastatic potential of cancer cells. During this process, epithelial cells lose their defining characteristics and acquire mesenchymal properties, namely loss of cell–cell adhesion, increased motility and invasiveness, resistance to apoptosis and changes in cellular morphology. EMT has been identified as a driver of metastasis and tumor invasion, allowing cells to detach from their niche and migrate through blood and lymphatic vessels to invade different organs.[3] EMT is a vibrant, dynamic and transient process and is an important contributor to epithelial cell plasticity during tumor progression.

During the multiple step of EMT, epithelial cells lose numerous epithelial characteristics to assume the properties of mesenchymal cells. The EMT-related signaling pathways in the tumor microenvironment include TGF-β, NF-κB, Wnt, Notch and others.[4,5] Several transcription factors have been implicated in the transcriptional repression of E-cadherin, including the Snail family of zinc-finger transcription factors (Snail, Slug and Smuc), the two-handed zinc-finger members of the dEF1 family (ZEB1/dEF1 and ZEB-2/SIP1) and the basic helix–loop–helix factors Twist.[6,7] All of these transcription factors have been recognized as having a critical role in cell survival, differentiation and metastasis. Among these factors, NF-κB can directly activate the expression of potent inducers of EMT, including Snail and ZEB factors.[8] It has shown that NF-κB suppresses the expression of epithelium-specific gene E-cadherin and induces the expression of the mesenchymal-specific gene vimentin. Moreover, NF-κB induces the expression of Snail that has a central role in repressing E-cadherin expression during the loss of the epithelial phenotype.[5,9] Overexpression of Snail was recently found in both epithelial and endothelial cells of invasive breast cancer but was undetectable in normal breast.[10,11] In addition, the expression of Snail in breast carcinomas is associated with metastasis, tumor recurrence and poor prognosis. Expression of Snail is regulated at the transcriptional and post-transcriptional levels by an integrated and complex signaling network, which includes the integrin-linked kinase, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), glycogen synthase kinase 3-beta (GSK-3β) and NF-κB pathways.[12]

Glycosylation is a common post-translational regulatory event during eukaryotic gene expression. However, increasing evidence suggests that aberrant glycosylation initially induced by oncogenic transformation contributes to tumor invasion and metastasis.[13] Aberrant glycosylation is a hallmark of cancer and is associated with differential expression of enzymes, mainly glycosyltransferases. The fucosyltransferase (FUT) family of enzymes is involved in the synthesis of

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Abbreviations: EMT, epithelial–mesenchymal transition; FUT4, fucosyltransferase IV; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; GSK-3β, glycogen synthase kinase 3-beta; MET, mesenchymal–epithelial transition; DTT, dithiothreitol; PBS, phosphate-buffered saline; siRNA, small interfering RNA
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cell-surface antigens through catalyzing the transfer of GDP-fucose to the *N*-acetylglucosamines residue of glycoproteins. FUTs have been shown to be involved in various biological processes, including cell adhesion, lymphocyte homing, embryo-fetal development and tumor progression.[14–16]

The FUT family comprises four subfamilies, α1,2-, α1,3/4-, α1,6- and protein O-FUT. The α1,3/4-FUT subfamily is comprised of at least eight members: *FUT3, FUT4, FUT5, FUT6, FUT7, FUT9, FUT10,* and *FUT11*. (refs 17–20) LeY is a difucosylated oligosaccharide with the chemical structure [Fucα1$\rightarrow$2Galβ1$\rightarrow$4(Fucα1$\rightarrow$3)GlcNAcβ1$\rightarrow$R], which is overexpressed in the majority of carcinomas, including tumors of the breast, ovary, pancreas, colon and non-small cell lung cancers.[14,21] The α1,3-fucosylation of LeY is catalyzed by fucosyltransferase IV (FUT4).[22,23] In a previous study, we concluded that *FUT4* regulates cell growth through controlling cell cycle progression via the MAPK and PI3K/Akt signaling pathways.[24] We also found that FUT4 had a critical role in the MAPK pathway by mediating EGF-induced NF-κB activation and increasing expression of MMP-12.[25] Given that FUT4 may have an important role in tumor metastasis, we were prompted in this study to explore its role in the induction of EMT in breast cancer cells. Based on our results, we propose that FUT4, via activation of NF-κB and PI3K/Akt-GSK3β signaling, enhances the expression of Snail, leading to acquisition of the mesenchymal phenotype in breast cancer cells. This in turn promotes MMP-9 activity, which increases cancer cell motility and metastatic potential. Our study supports the possibility that FUT4 is a novel regulator of EMT in breast cancer cells and is a promising target for cancer therapy.

Results

***FUT4* modulation of various EMT markers in breast cancer cells.** To determine whether *FUT4* has a role in EMT, we used one normal breast epithelial cell line, MCF-10A, and two breast cancer cell lines, MCF-7 and MDA-MB-231. Analysis of *FUT4* expression in these cell lines demonstrated that *FUT4* expression was higher in the breast cancer cells than in normal breast epithelial cells, and that it was higher in MDA-MB-231 cells than in MCF-7 cells (Figure 1a). To explore the role of *FUT4* in the induction of the EMT process in breast cancer cells, we employed two

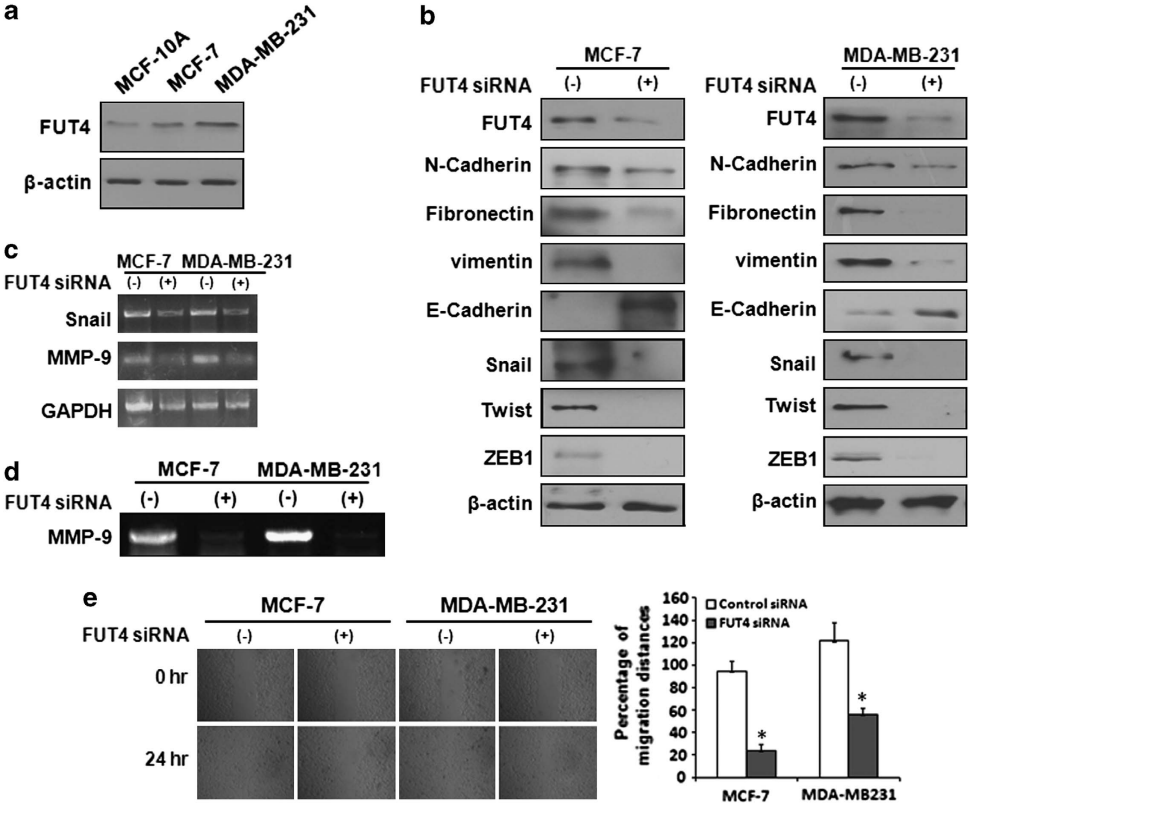

Figure 1 Effect of *FUT4* knockdown on EMT markers in breast cancer cells. (**a**) *FUT4* expression in normal mammary epithelial cells (MCF-10A) and breast cancer cells (MCF-7 and MDA-MB-231). (**b**) MCF-7 and MDA-MB-231 cells were transfected with *FUT4*-specific siRNA or control siRNA (40 nM). At 48 h post transfection, cell lysates were prepared and subjected to immunoblotting analysis for *FUT4*, N-cadherin, fibronectin, vimentin, E-cadherin, Snail, Twist, ZEB1 and β-actin served as internal controls. (**c**) Total RNA was extracted at 48 h after siRNA transfection and analyzed for Snail and MMP-9 by RT-PCR with GAPDH serving as the internal control. (**d**) MCF-7 and MDA-MB-231 cells were treated with 40 nM of *FUT4*-specific siRNA or control siRNA. After 48 h, the activity of secreted MMP-9 was measured using gelatin zymography analysis. (**e**) After *FUT4* knockdown, a wound-healing assay was performed by scratching the cell layer with a pipette tip, and phase-contrast images were taken at 0 and 24 h later to assess cell migration into the open space. Quantitative data are presented as the mean ($\pm$S.D.) percentage of migration distance ($n=10$). *Indicates a significant difference compared with the control ($P<0.05$)

experimental approaches. The first involved the transfection of *FUT4* small interfering RNA (siRNA) into MCF-7 and MDA-MB-231 cells. Knockdown of endogenous *FUT4* resulted in the increased expression of epithelial marker, E-cadherin and the reduced expression of various mesenchymal markers, namely fibronectin, vimentin, N-cadherin, Snail, Twist and ZEB1. The effect of the knockdown was more pronounced in MDA-MB-231 cells than in MCF-7 cells, as demonstrated by immunoblotting (Figure 1b). Moreover, a variety of assays demonstrated that knockdown of *FUT4* decreased the expression (RT-PCR, Figure 1c) and activity (gelatin zymography, Figure 1d) of MMP-9 and reduced cell migratory activity (*in vitro* wound-healing assay, Figure 1e).

We previously observed that MCF-7 cells had a low level of *FUT4* relative to MDA-MB-231 cells (Figure 1a). Overexpression of full-length FUT4 using pcDNA3.1-*FUT4* was accompanied by increased expression of various mesenchymal markers, including fibronectin, vimentin, N-cadherin Snail, Twist and ZEB1, and decreased expression of epithelial marker, such as E-cadherin in MCF-7 cells (Figure 2a). Moreover, cells overexpressing *FUT4* showed an increased expression of MMP-9, as well as displayed enhanced migratory potential (Figures 2b–d). Collectively, these results suggest that *FUT4* induces the acquisition of an EMT-like phenotype in MCF-7 and MDA-MB-231 cells.

Involvement of PI3K/Akt-GSK3β signaling in the *FUT4* mediation of EMT. Recent studies have suggested that activation of PI3K/Akt-GSK-3β signaling induces the EMT process. In human cancer, activation of PI3K/Akt with downregulation of E-cadherin expression and induction of EMT may be particularly important.[26–29] GSK-3β is a multifunctional serine/threonine (ser/thr) kinase that has a fundamental role in a wide variety of functions, including cell division, proliferation, differentiation and adhesion.[30–32] GSK-3β is active in resting epithelial cells, and inhibition of its activity or its expression may lead to EMT.[33] We were therefore interested in exploring the role of the PI3K/Akt-GSK-3β signaling in *FUT4*-mediated EMT in breast cancer cells. We first examined Akt and GSK-3β activity and their relationship with *FUT4*. Compared with MCF-7 cells, MDA-MB-231 cells had elevated Akt activity and decreased GSK-3β activity. Knockdown of *FUT4* resulted in decreased Akt activity and increased GSK-3β activity in MCF-7 and MDA-MB-231 cells (Figure 3a).

Next, we examined the potential involvement of PI3K/Akt-GSK3 signaling in EMT using specific inhibitors of PI3K (LY294002) and GSK-3β (SB415286). Treatment of MCF-7 and MDA-MB-231 cells with 20 μM LY294002 reduced both cellular levels of Snail and the secretion of MMP-9, and was accompanied by reduced cell motility (Figures 3b–d). In parallel, treatment of MCF-7 cells with 25 μM SB415286 increased the expression of Snail and MMP-9 and promoted cell motility (Figures 4a–c). Cyclin D was used as a positive control as it is subjected to GSK-3β-dependent proteolysis.[34–39] As anticipated, cyclin D expression increased when MCF-7 cells were treated with SB415286. To further ascertain the involvement of PI3K/Akt-GSK-3β signaling in the *FUT4*-induced acquisition of the mesenchymal phenotype, we used the alternative approach of transfecting pcDNA3.1-*FUT4* into MCF-7 cells. *FUT4* overexpression in cells resulted in increased Akt activity and attenuated GSK-3β activity, and these effects were abrogated by treatment with the PI3K inhibitor LY294002 (Figure 4d). Treatment of *FUT4*-overexpressing cells with LY294002 reduced both the cellular levels of Snail and secretion of MMP-9 and decreased cell motility (Figures 4d–f). Collectively, these results suggest that the *FUT4*-induced acquisition of an EMT-like phenotype involves activation of PI3K/Akt signaling and inactivation of GSK-3β signaling.

Involvement of NF-κB signaling in *FUT4*-facilitated EMT process. NF-κB has been identified as a central mediator of EMT in a mouse model of breast cancer progression.[40,41]

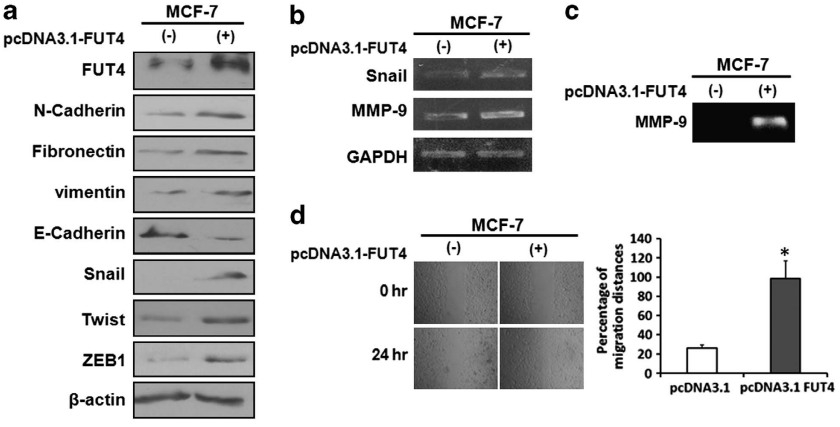

Figure 2 Effect of *FUT4* overexpression on mesenchymal-like phenotype in cells. (**a**) Cells were transfected with empty vector (pcDNA3.1) or full-length *FUT4* (pcDNA3.1-*FUT4*). At 48 h post transfection, cell lysates were prepared and subjected to immunoblotting analysis for *FUT4*, N-cadherin, Fibronectin, vimentin, E-cadherin, Snail, Twist, ZEB1 and β-actin served as internal controls. (**b**) Total mRNA was extracted and analyzed for *FUT4* Snail and MMP-9 by RT-PCR. (**c**) The culture conditioned media of *FUT4*-overexpressing cells was collected and normalized by cell number before gelatin zymography analysis. (**d**) Cell migratory activity was determined using the wound-healing assay as described in Figure 1. Quantitative data are presented as the mean ($\pm$ S.D.) percentage of migration distance ($n = 10$). *Indicates a significant difference compared with the control ($P < 0.05$)

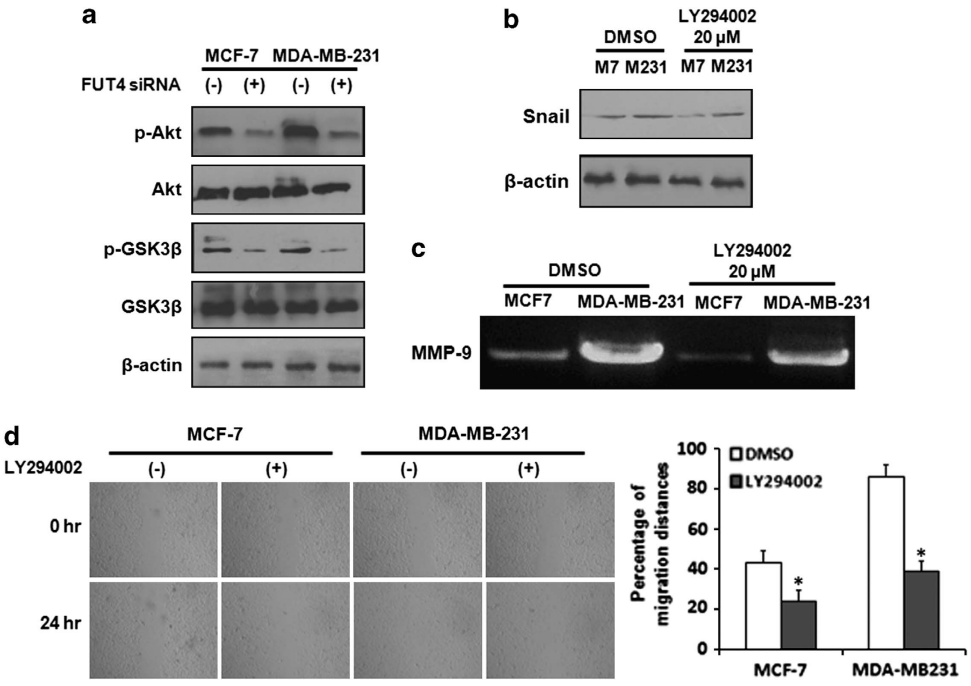

Figure 3 Involvement of PI3K/Akt-GSK-3β signaling activation during EMT in breast cancer cells. (**a**) MCF-7 and MDA-MB-231 cells were transfected with 40 nM of control or specific *FUT4* siRNA. Total proteins were subjected to western blot analysis using pAkt, Akt, pGSK-3β and GSK-3β antibodies as described in Materials and Methods. (**b**–**d**) Cells were treated with 20 μM Akt inhibitor LY294002 for 24 h. (**b**) The cellular protein levels of Snail were determined by western blot analysis. (**c**) The conditioned media were collected and MMP-9 activities were determined by gelatin zymography. (**d**) Cell migratory activity was determined by wound-healing assay. Quantitative data are presented as the mean ($\pm$ S.D.) percentage of migration distance ($n = 10$). *Indicates a significant difference compared with the control ($P < 0.05$)

To elucidate the role of NF-κB signaling in the *FUT4*-mediated EMT process in human breast cancer cells, we first examined nuclear NF-κB expression and its relationship with EMT. Compared with MCF-7 cells, the MDA-MB-231 cells, which exhibits relatively high *FUT4* expression (Figure 1a), showed markedly reduced levels of IκBα (an endogenous inhibitor of NF-κB), and an increased nuclear level of NF-κB (total cellular levels of NF-κB were comparable between the two cell lines) (Figure 5a). Moreover, treatment with the NF-κB inhibitor JSH-23 (25 μM) reduced cellular levels of Snail (Figure 5b), reduced secreted MMP-9 activity (Figure 5c) and decreased migratory activity of the MCF-7 and MDA-MB-231 cell lines (Figure 5d), suggesting the potential involvement of NF-κB in the EMT process.

We next explored the effect of *FUT4* knockdown on the MCF-7 and MDA-MB-231 cell lines. Knockdown of *FUT4* led to the decrease and increase of nuclear expression of NF-κB and IκBα, respectively (Figure 5e), suggesting that upregulation of *FUT4* may induce NF-κB activity through a reduction in cellular levels of its inhibitor, IκBα. To further confirm this, we used an alternative approach whereby we transfected pcDNA3.1-*FUT4* into MCF-7 cells. *FUT4* overexpression affected repression of IκBα levels, accompanied by elevated NF-κB activity (Figures 6a and b). Moreover, treatment of *FUT4*-overexpressing MCF-7 cells with the NF-κB inhibitor JSH-23 increased cellular levels of Snail, and increased both secreted MMP-9 activity and cellular migratory activity (Figures 6c and d). Collectively, these results indicate that the *FUT4*-induced acquisition of an EMT-like phenotype in breast cancer involves the activation of NF-κB signaling.

Discussion

Metastasis represents the end stage of a complex multistep cellular process termed as the invasion metastasis cascade, which involves dissemination of cancer cells to anatomically distant organ sites and their subsequent adaptation to foreign tissue microenvironments. The events that culminate in metastasis are driven by the acquisition of genetic and/or epigenetic alterations within tumor cells and the co-option of non-neoplastic stromal cells, which together endow incipient metastatic cells with the traits needed to generate macroscopic metastases.[42]

Complex carbohydrates, which are major components of the cell membrane, perform important functions in cell–cell and cell–extracellular matrix interactions, as well as in signal transduction. Recent studies have shown that glycan changes in malignant cells take a variety of forms and mediate key pathophysiological events during the various stages of tumor progression. Glycosylation changes are universal hallmarks of malignant transformation and tumor progression in human cancer, and can occur either across the entire cell or on specific molecules.[43,44] The molecular mechanism underlying the relationship between glycosyltransferases and cancer is unclear. Of specific interest to us is the poorly understood role of *FUT4* in the EMT process. We previously showed that *FUT4* has a role in promoting cell proliferation[45] and inhibiting the cell apoptosis.[46] In the current study, we provide strong evidence of a role for *FUT4* in metastasis and present novel mechanistic insights into how *FUT4* facilitates tumor spread. Specifically, we show that *FUT4* induces a mesenchymal

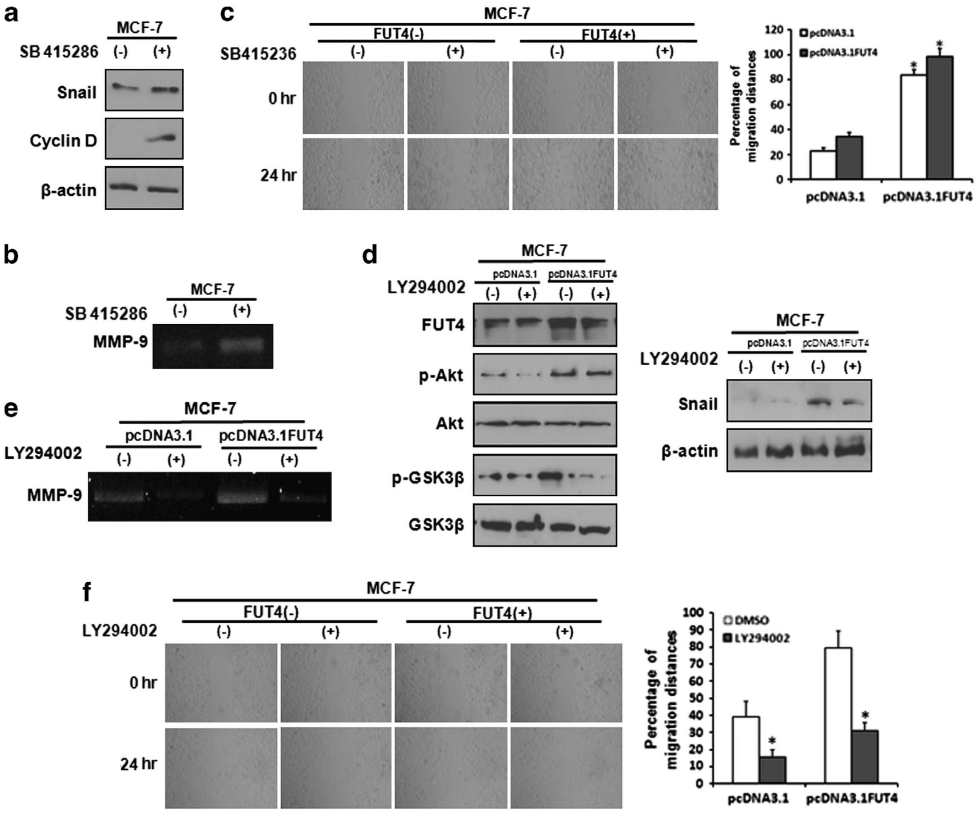

Figure 4 Effect of PI3K/Akt-GSK-3β signaling on EMT in *FUT4*-overexpressing cells. (**a**–**c**) The cells were treated with 25 μM of the specific GSK3β inhibitor SB415286 for 48 h. (**a**) The cellular protein levels of Snail and cyclin D were determined by western blot analysis. (**b**) The conditioned media were collected, and MMP-9 activities were determined by gelatin zymography. (**c**) Cell migratory activity was determined by wound-healing assay. Quantitative data are presented as the mean ($\pm$ S.D.) percentage of migration distance ($n = 10$). (**d**–**f**) Cells were transfected with empty pcDNA3.1 vector or pcDNA3.1-*FUT4,* then treated with 20 μM of PI3K inhibitor LY294002 for 24 h. (**d**) Total proteins were subjected to western blot analysis using phosphorylated Akt, GSK-3β and Snail antibodies as described in Materials and Methods. (**e**) The secreted activity of MMP-9 was detected by gelatin zymography. (**f**) Cell migratory activity was determined using the wound-healing assay. Quantitative data are presented as the mean ($\pm$ S.D.) percentage of migration distance ($n = 10$). *Indicates a significant difference compared with the respective control ($P < 0.05$). #Indicates a significant difference compared with MCF-7 cells ($P < 0.05$)

phenotype and that this, in turn, translates into increased cell invasiveness and tumor metastasis.

We found that *FUT4* positively regulates the expression of Snail in breast cancer cells (Figures 1 and 2), suggesting that *FUT4* may promote EMT, at least in part, through upregulation of Snail. The molecular mechanism of *FUT4* involvement in the acquisition of the mesenchymal-like phenotype in breast cancer cells awaits further investigation. Previous studies have documented the relationship between aberrant expression of *FUT4* in epithelial cells and constitutive activation of Akt and NF-κB,[45,46] whose pathways are involved in the regulation of EMT and the promotion of metastasis.[9,47] Aberrant activation of Akt signaling is widely implicated in many human cancers. Moreover, several studies have reported that activation of the PI3K/Akt-GSK-3β signaling pathway is also a central feature of EMT, and that this involves accumulation of Snail in the nucleus.[32,48] In our study, we found that breast cancer cells display increased Akt activity together with a concomitant reduction in GSK-3β activity, as well as have increased Snail and MMP-9 levels (Figure 3). Inhibition of PI3K activity (LY294002) attenuated the *FUT4*-induced activation of Akt and GSK-3β and suppressed the increases in expression of Snail and MMP-9 and the induction of cell motility (Figure 4). In addition, inhibition of GSK-3β activity with SB415286 resulted in increased expression of Snail and MMP-9 and enhanced cell motility (Figure 4). These results support the concept that *FUT4* acts in breast cancer cells in part via activation of PI3K/Akt signaling, resulting in inhibition of GSK-3β, upregulation of Snail/MMP-9 and, ultimately, promotion of EMT progression.

The strength of EMT is primarily dependent on the potency of EMT-inducing transcription factors that are capable of triggering cellular reprogramming.[49] Snail factors also directly regulate epithelial and mesenchymal markers.[50,51] Snails not only direct binding to the E-cadherin promoter and inhibition of its transcription but also repress other epithelial marker. Snail also could activate the expression of mesenchymal-like genes to promote cell migration. Snail1 is spontaneously induced in recurrent breast carcinomas, and high levels of Snail1 are an independent predictor for decreased relapse-free survival in breast cancer patients.[52,53] Expression of Snail factors correlates with malignancy and less-differentiated tumors, lymph node invasion and metastasis.[54] The transcription factor NF-κB is activated in a range of human cancers and is thought to promote tumorigenesis, mainly because of its ability to protect transformed cells from apoptosis. NF-κB has

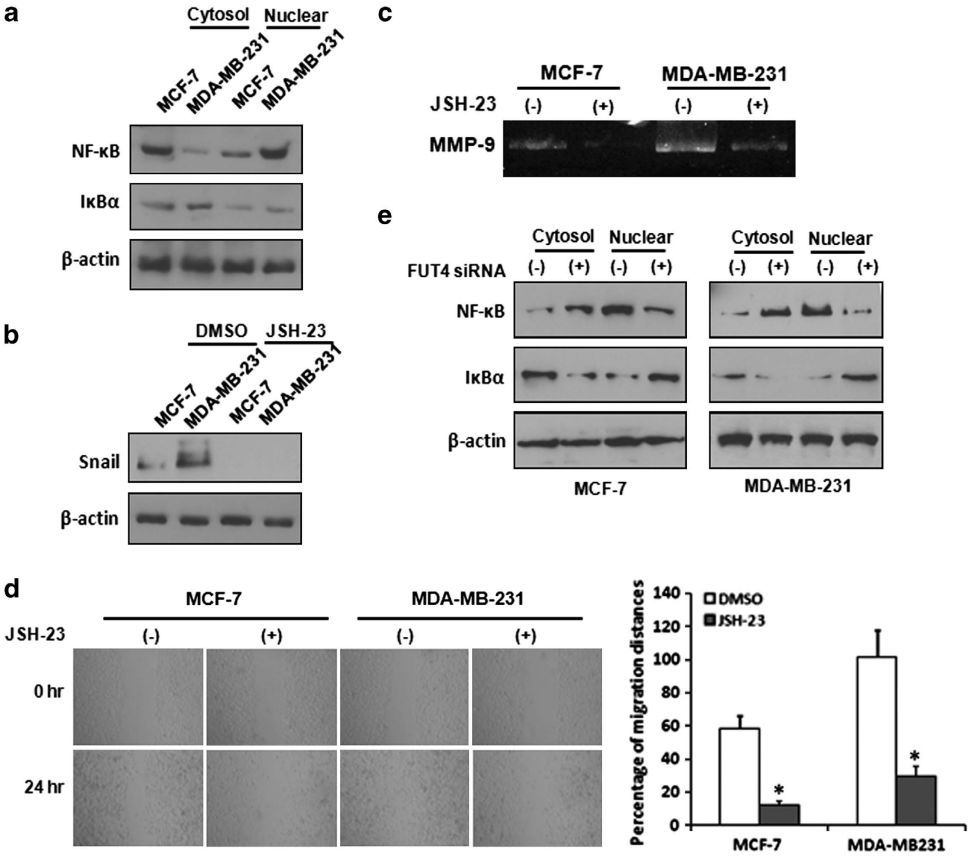

Figure 5 Effect of GSK-3β activities on EMT in MCF-7 and MDA-MB-231 cells. (**a**) Cytoplasmic and nuclear extracts from cells were immunoblotted with NF-κB and IκBα antibodies. (**b** and **c**) Cells were treated with 20 or 25 μM of JSH-23 for 24 h. Cell lysates were analyzed for Snail by immunoblotting, and the conditioned media was analyzed for MMP-9 activity by gelatin zymography. (**d**) Cells were treated with 25 μM of JSH-23 for 24 h and analyzed for migratory activity using wound-healing assay. Quantitative data are presented as the mean ($\pm$ S.D.) percentage of migration distance ($n = 10$). (**e**) Cells were transfected with FUT4 siRNA, and cytosol and nuclear NF-κB and IκBα were detected by western blot analysis

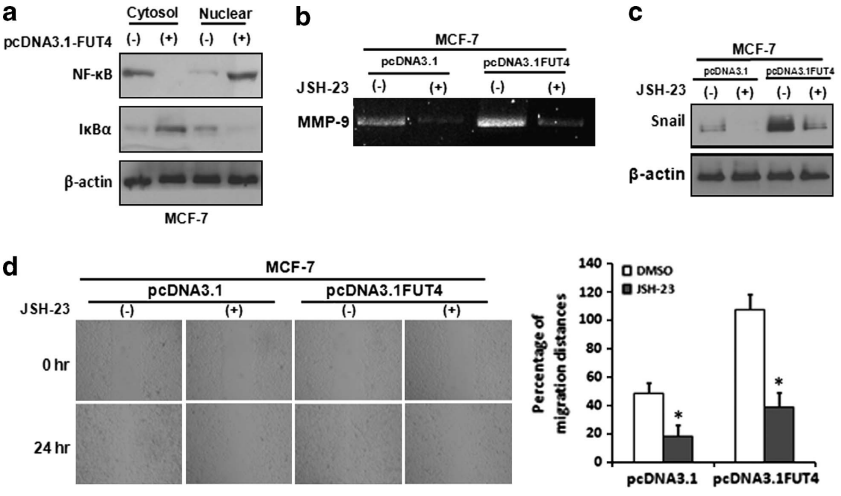

Figure 6 Effect of NF-κB activity on EMT in FUT4-overexpressing cells. (**a**) Cells were transfected with empty pcDNA3.1 vector or pcDNA3.1-*FUT4*. Cytosol and nuclear NF-κB and IκBα were detected by western blot analysis. (**b**–**e**) Cells were treated with or without 25 μM of specific NF-κB inhibitor JSH-23 for 24 h. (**b**) Conditioned media were collected, and MMP-9 activities were determined by gelatin zymography. (**c**) Cellular protein levels of Snail were detected by western blot analysis. (**d**) Cell migratory activity was determined using a wound-healing assay. Quantitative data are presented as the mean ($\pm$ S.D.) percentage of migration distance ($n = 10$). *Indicates a significant difference compared with the respective control ($P < 0.05$). $^{\#}$Indicates a significant difference compared with MCF-7 cells ($P < 0.05$)

a role in control of mesenchymal genes critical for promoting and maintaining an invasive phenotype.[8] It has also been reported that NF-κB is essential for EMT and metastasis in a model of breast cancer progression.[5] In cells that have previously undergone EMT, blocking of NF-κB activity leads to a partial reversal of the mesenchymal phenotype. Moreover, recent studies have identified NF-κB as a key modulator of TGF-β-induced EMT.[55,56] Constitutive activation of NF-κB is known to confer resistance to cell death-inducing stimuli, including chemotherapeutic agents, and promote metastasis by inducing EMT.[57] We observed that breast cancer cells have increased NF-κB activity pursuant to a reduction of IκBα, and that this was positively associated with *FUT4* expression (Figures 5 and 6). Moreover, inhibition of NF-κB activity in breast cancer cells led to a suppression of the *FUT4*-induced increases in Snail, MMP-9 and cell motility (Figures 5 and 6). We propose that *FUT4* acts at least in part through activation of NF-κB to upregulate Snail and MMP-9, culminating in the acquisition of mesenchymal characteristics by the breast cancer cells.

It is believed that cancer cells exist in intermediate states in the EMT process. Indeed, once cells have invaded the primary tumor and penetrated the surrounding tissue, they must be able to colonize at a new tissue site. To achieve this, cancer cells may undergo a mesenchymal–epithelial transition (MET), resulting in a reformation of the epithelial phenotype in terms of cell–cell adhesion.[1,4,6] Cancer cells that attain plasticity and shift through both EMT and MET may account for many of the difficulties associated with cancer clinical therapy.

In the present study, we have demonstrated that the expression of Snail is associated with enhanced NF-κB and PI3K/Akt-GSK-3β activity and that *FUT4* participates in the acquisition of a mesenchymal transition. We conjecture that the enhancement of Snail expression by *FUT4* induces the acquisition in breast cancer cells of a mesenchymal-like phenotype that then promotes the secretion of MMP-9, which enhances cancer cell motility and increases metastatic potential. These observations also support our contention that *FUT4* is a promising therapeutic target for reversing drug resistance and inhibiting the early advent of metastasis in tumor cells.

Materials and Methods

Materials. MCF-7 and MDA-MB-231 cells were obtained from the American Type Culture Collection. FUT4 siRNA and non-specific siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000 Reagent was purchased from Invitrogen (Carlsbad, California, USA). Anti-FUT4 was purchased from ProteinTech (Wuhan, Hubei, China). Anti-fibronectin and anti-β-actin were purchased from Sigma (St. Louis, MO, USA). Anti- E-cadherin, anti-N-cadherin, anti-vimentin, anti-Snail, anti-Twist, anti-ZEB1 and anti-IκBα were obtained from Santa Cruz Biotechnology. Anti-Akt, anti-p-Akt (Ser473), anti-p-GSK3β (Ser9) and anti-cyclin D1 were obtained from Cell Signaling (Boston, MA, USA). Anti-NF-κB and anti-GSK3β were obtained from BD Transduction (Franklin Lakes, NJ, USA). LY294002, JSH-23 and SB415286 were purchased from Sigma. Chemiluminescence (ECL) assay kit was purchased from Amersham. All PCR forward and reverse primers were ordered form Takara (Dalian, Liaoning, China).

Cell culture. MCF-7 cells were maintained in medium MEM supplemented with 10% calf serum, 1% Pen/Strep, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate and 10 mM HEPES. MDA-MB-231 cells were cultured in L-15 containing 10% calf serum, 1% Pen/Strep and 10 mM HEPES. All cells were incubated in a 5% CO_2 humidified atmosphere at 37 °C.

Western blot. To prepare whole-cell extracts, cells at 90% confluent were washed in phosphate-buffered saline (PBS) before incubation with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40) on ice for 10 min. The cell lysates were clarified by centrifugation at 9000 $\times$ *g* for 10 min, and the supernatants were collected. Protein concentration was determined with the Coomassie Protein Assay Reagent using bovine serum albumin as a standard. Cell lysates (50 μg) were separated by 10% SDS-PAGE min-gel. Samples were transferred electrophoretically to nitrocellulose membranes, blocked with TTBS containing 5% fat-free dry milk for 2 h and incubated for 3 h with the appropriate primary antibodies at the dilutions recommended by the suppliers. After incubation with a HRP-conjugated anti-goat secondary antibody, immunoreactive proteins were visualized with ECL detection system. Western blots shown are representative of at least three independent experiments. Densitometry of each band for the target protein was quantified by densitometry analysis with Labworks 4.6. The protein band intensity was quantified by the mean $\pm$ S.E.M. of three experiments for each group, as determined from densitometry relative to β-actin.

Preparation of nuclear and cytosolic fractions. The cells (6×10^5) were cultured on a 100-mm culture dish in 10% FBS DMEM/F12 for 24 h. These cells were incubated in serum-free DMEM/F12 for another 24 h. Cells were washed twice with cold PBS and then scraped to an eppendorf in 1 ml of PBS and centrifuged at 4 °C, 12 000 r.p.m., for 5 min. Discarding the supernatant, the pellet was homogenized in 300 μl of hypotonic lysis buffer (10 mM HEPES (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and PMSF 0.5 mM). After repeated homogenization, the homogenate was centrifuged at 4 °C, 12 000 r.p.m., for 10 min. The supernatant was the cytosolic fraction and was kept at -70 °C overnight. The pellet was washed twice in 20 μl of hypotonic lysis buffer to remove residual cytosolic proteins. After washing, the pellet was dissolved in 30 μl of hypertonic lysis buffer (20 mM HEPES (pH 7.6), 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF , 25% glycerol and 0.4 M NaCl), pipetted to homogeneity, fiercely vortexed and then stored at -70 °C overnight. Next day, the solution was centrifuged to collect the supernatant, which was the nuclear fraction. The cytosolic and nuclear fractions were quantified, and equal amounts of protein were subjected to western blot.

RT-PCR. For RT-PCR analysis, RNA was isolated from cells using Trizol according to the manufacturer's instruction, followed by first-strand cDNA synthesis using TrueScript reverse transcriptase. cDNA was amplified by PCR using the specific primer for snail, MMP-9 or β-actin as an internal control. The sequences of the upstream and downstream primers were as follows: 5′-GCTCCTTCGTCCTTCTCCTCTA-3′ (F) and 5′-GGCACTGGTACTTCTTGACA-3′ (R) for snail; 5′-TCTTCCCTGGAGACCTGAGAAC-3′ (F) and 5′-GACACCA AACTGGATGACGATG-3′(R) for MMP-9; 5′-ATCTGGCACCACACCTTCACAAT GAGCTGCG- 3′ (F) and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′ (R) for β-actin, respectively. PCR analysis was performed under the following conditions: denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation for 40 s at 94 °C, annealing for 30 s at 63 °C for MMP-9 and 58 °C for β-actin and extension for 40 s at 72 °C. The amplified products were analyzed by 1.0% agarose gel electrophoresis, followed by ethidium bromide staining. Band intensities were measured using BioImaging systems (UVP, labworksTM, ver 4.6; UVP Bio-Imaging Systems, Los Angeles, CA, USA).

Gene construction and transfection. Total RNA was isolated from healthy human uterine tissue specimens by Trizol reagent, and the full-length cDNA of FUT4 (GenBank accession number: M58596) was amplified with Takara RNA PCR Kit. The primers were 5′-CCGCTCGAGATGGGGGCACCGT GGGGCTCGC-3′ (forward) and 5′-CCGGAATTCAGTAGAGGATCAAAAAGCT GACAAC-3′ (reverse), which provided with the *Xho*I and *Eco*RI restriction site, respectively. PCR products were sequenced and then cloned into pEGFP-N1 vector through *Xho*I and *Eco*RI restriction sites to obtain recombinant pEGFP-N1-FUT4 plasmid.[24] The coding region of FUT4 was removed from the pEGFP-N1 vector using the restriction enzymes *Eco*RI and *Xho*I and then subcloned into the *Eco*RI and *Xho*I sites of the pcDNA3.1 vector. Ligation of the restriction enzyme digested FUT4, and pcDNA3.1 vector generated pcDNA3.1-FUT4. Cells were seeded into 6-cm cultured dishes and then transfected with pcDNA3.1-FUT4 using lipofectamine 2000 following the manufacturer's instructions.

Transfection of siRNA. FUT4 siRNA and non-specific siRNA were dissolved in RNase-free water provided by the manufacturer to a stock concentration of 20 μM. Cells were plated into 60-mm culture dishes and then transfected with 40 nM of siRNA using lipofectamine 2000 following the manufacturer's instructions. All assays were performed 48 h after transfection.

Gelatin zymography assay. MMP-9 activity was detected using the gelatin zymography assay. Cells (2×10^5 cells/ml) were seeded into six-well plates and treated. The supernatants were collected and used as the samples. Cells were also collected, and proteins were extracted using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN_3 and 1% NP-40) for 30 min. The protein concentration was determined using the Bradford assay. A concentration of 40 μg of total proteins of supernatants were loaded per lane and electrophoresed on 8% SDS-polyacrylamide gels copolymerized with 1% gelatin. After electrophoresis, the gels were washed five times in 2.5% Triton X-100 (20 min each) and two times in buffer without Triton X-100 to remove Triton X-100, and then incubated in 50 mmol/l Tris-Cl, pH 7.6, and 5 mmol/l CaCl2 (18 h, 37 °C). The gels were stained with 0.1% Coomassie blue R250 and destained in 10% isopropanol and 10% acetic acid in H_2O. MMP-9 was detected as transparent bands on the blue background of a Coomassie blue-stained gel.

***In vitro* wound-healing migration assay.** Cells were seeded on six-well culture plates in DMEM/F12 containing 10% FBS. After 24 h, the cell monolayers were wounded by manually scratching it with a pipette tip, and this was followed by washing with PBS. The monolayers were then incubated at 37 °C for 24 h. The monolayers were photographed at 0 and 24 h. The experiments were performed in triplicate for each treatment group.

Statistical analysis. The quantitative data derived from three independent experiments are expressed as means ($\pm$S.D.). Unpaired Student's *t*-tests were used to analyze between group differences that is repeated, and *P*-value <0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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