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HSF1 and Sp1 Regulate FUT4 Gene Expression and Cell Proliferation in Breast Cancer Cells

Xuesong Yang,* Jiao Wang, Shuiai Liu, and Qiu Yan**

Department of Biochemistry and Molecular Biology, Dalian Medical University, Liaoning Provincial Core Lab of Glycobiology and Glycoengineering, Dalian, People's Republic of China

ABSTRACT

Lewis Y (LeY) is a carbohydrate tumor-associated antigen. The majority of cancer cells derived from epithelial tissues express LeY type difucosylated oligosaccharides. Fucosyltransferase IV (FUT4) is an essential enzyme that catalyzes the synthesis of LeY oligosaccharides. In a previous study we reported that FUT4 is associated with cell proliferation; however, despite the important role of FUT4 in cancer proliferation and apoptosis, little is known about the mechanisms underlying the regulation of FUT4 transcription. In the current study we investigated the regulation of FUT4 transcription in human breast cancer. We compared the regulation of human FUT4 gene transcription in human breast cancer cells (MCF-7 and MDA-MB-231) using promoter/luciferase analyses. Using a series of promoter deletion constructs, we identified a potential regulatory site located between 0.8 and 1.6 kb of the FUT4 promoter. As shown by EMSA and ChIP analyses, heat-shock factor 1 (HSF1) and Sp1are required for FUT4 promoter activity. In addition, we explored the role of HSF1 and Sp1 on cell proliferation, and found that the ERK1/2 MAPK and PI3K/Akt signaling pathways regulate the expression of FUT4, which play a role in cell proliferation via HSF1 and Sp1. These results suggest that FUT4 is a target gene for HSF1 and Sp1 that is required for cell cycle progression in breast cancer epithelial cells. J. Cell. Biochem. 115: 168–178, 2014.

KEY WORDS: FUCOSYLTRANSFERASE IV; HSF1; Sp1; SIGNALING PATHWAY; BREAST CANCER

Fucosyltransfease IV (FUT4, EC 2.4.1.152) is a glycotransferase that catalyzes the formation of α1,3 glycosidic linkages. Lewis Y (LeY) is a difucosylated oligosaccharide with the following chemical structure: [Fucα1 → 2Galβ1 → 4(Fucα1 → 3)GlcNAcα1 → R]. LeY belongs to the A, B, H, and Lewis blood group families. H type 2 antigen(Fucα1 → 2Galβ1 → 4GlcNAc → R) is a precursor of LeY oligosaccharides [Escrevente et al., 2006].

The α1,3 fucosylation of LeY is catalyzed by FUT4 [Taniguchi et al., 2000]. FUT4 is a critical enzyme that controls LeY oligosaccharide synthesis. FUT4 is mainly expressed in leukocytes and some epithelial cells. The expression of FUT4 is increased in different cancers, for example, gastriccarcinoma [Petretti et al., 1999], colorectal cancer [Kudo et al., 1998], pancreatic cancer [Ito et al., 1997], and lung adenocarcinoma [Martin-Satue et al., 1998]. FUT4 is also regulated by transcriptional and post-transcriptional events. The link between FUT4 and disease risk suggests that environmental factors increase or decrease enzyme activity, and may be important modulators of the risk.

The mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways govern fundamental physiologic processes, such as cell proliferation, differentiation, metabolism, cytoskeleton reorganization, and cell death and survival [Baines and Molkentin, 2005; Mullonkal and Toledo-Pereyra, 2007]. The ERK/MAPK and PI3K/Akt signaling pathways are frequently constitutively activated in many human cancers, and are associated with carcinogenesis [Rho et al., 2011].

Abbreviations: LeY, Lewis Y; FUT4, fucosyltransferase IV; MAPKs, mitogen-activated protein kinases; PI3-K, phosphatidylinositol 3-kinase; HSF1, heat-shock factor 1; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; PI, propidium iodide; siRNA, small interfering RNA.

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*Correspondence to: Xuesong Yang, Associated Professor of Biochemistry and Molecular Biology, Dalian Medical University, Dalian 114044, People's Republic of China. E-mail: yxmsnow@126.com

**Correspondence to: Qiu Yan, Professor and Director of Department of Biochemistry and Molecular Biology, Dalian Medical University, Liaoning Provincial Core Lab of Glycobiology and Glycoengineering, Dalian 114044, People's Republic of China. E-mail: yanqiu2083@126.com

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Growth and survival of many cancer cells critically depend on aberrant signaling by the ERK/MAPK and PI3K/Akt pathways, which are also involved in intensive crosstalk [Aksamitiene et al., 2012]. To date, the MAPK pathway has been implicated as an important molecular target for cancer prevention and therapy [Li et al., 2012]. In breast cancer, the PI3K/Akt and ERK/MAPK signaling pathways are involved in IGF-1-induced VEGF-C up-regulation, and have been suggested to have important roles in lymphatic metastasis [Zhu et al., 2011]. Moreover, the ERK/MAPK and PI3K/Akt pathways are important in tumorigenesis.

To date, the regulatory role of FUT4 on cancer cell survival is complex and unclear. In the current study we showed that ERK/MAPK and Akt activity is positively correlated with FUT4 expression in human breast cancer cell lines. We present a novel mechanism attributed to the potential role of ERK/MAPK and PI3K/Akt in regulating the FUT4 enzyme on tumorigenesis.

MATERIALS AND METHODS

CELL LINES AND CELL TRANSFECTION

Human breast cancer lines MCF-7, MDA-MB-231 were purchased from Institute of Biochemistry and Cell Biology Chinese Academy of Sciences (Shanghai, China). MCF-7 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air). MDA-MB-231 cells were cultured in L15 medium supplemented with 10% FBS and penicillin–streptomycin. Cell transfection was performed with Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested.

REAGENTS AND ANTIBODIES

DMEM/F12 (1:1), FBS, Lipofectamine 2000 Reagent were purchased from Invitrogen (Paisley, UK). Enhanced chemiluminescence (ECL) assay kit was purchased from Amersham (Louisville, CO). LY294002, PD98059 were obtained from Sigma Chemical Company (St. Louis, MO). The anti-p-Akt, Akt, p-ERK1/2, ERK, cyclin D1, p21, and p27 were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology. pGL6 Basic vector and Nuclear Protein Extraction Kit were purchase from Beyotime Institute of Biotechnology (Hangzhou, China). Chemiluminescent EMSA Kit was chased from Viagend-Biotech (Ningbo, China). RT-PCR products were from TAKARA (Dalian, China). HSF1 and SP1 plasmid were purchased from the Proteintech Group, Inc. (Wuhan, China).

CONSTRUCTION AND CLONING OF PLASMIDS

The human FUT4 promoter region 2.0 kb upstream from the transcription start site was amplified. Genomic DNA extracted from the normal human mammary epithelial cells was used as a template with the FUT4 F1 forward primer, 5′-ACT GGT ACC AAT AAG AAC AAT CCA GTC CCC-3′ and the FUT4 R1 reverse primer, 5′-AAT CTG GCT AGC GAG GGA GGG AGG GAG TCC-3′. PCR was performed using Ex Taq (TaKaRa) polymerase to amplify the 2.0 kb fragment. The fragment was then digested with restriction enzymes *Kpn*I and *Nhe*I and inserted into the pGL6-Basic vector. This construct was named pGL6-FUT4-2.0. Serial deletion constructs of 1.6, 1.2, 0.8, and 0.4 kb were created by PCR using pGL6-FUT4-2.0 as a template and different 5′ forward primers (5′-ACT GGT ACC TGA AGA GGG AGA AGA ATG TGA ATA-3′, 5′-ACT GGT ACC TTT TCC GAG ACG GAG TCT CGC TTT-3′, 5′-ACT GGT ACC TCT ATT TCT CCC CTA GTA TCA GTG-3′, and 5′-ACT GGT ACC TCA TTC ATC TCA CTG AAC AGG CAA-3′, respectively) and FUT4 R1 as a reverse primer. These constructs were named pGL6-FUT4-1.6, pGL6-FUT4-1.2, pGL6-FUT4-0.8, pGL6-FUT4-0.4, respectively.

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 phenylmethyl sulfonyl fluoride, 0.5% Nonidet P-40) on ice for 10 min. The cell lysates were clarified by centrifugation at 9,000*g* for 10 min and the supernatants were collected. Protein concentration was determined with the Coomassie Protein Assay Reagent using bovine serum albumin (BSA) 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 ± SEM of three experiments for each group as determined from densitometry relative to β-actin.

TRANSIENT TRANSFECTIONS AND LUCIFERASE ASSAYS

Transient transfections were performed following the manufacturer's protocol (Invitrogen). Briefly, $3–5 \times 10^5$ cells were plated on 60 mm plates the day before transfection. On the day of the transfection 9 μl of Lip was added in 191 μl of media and incubated for 5 min at room temperature. Then, 3 μg of DNA was added and the mixture was incubated for 20 min at room temperature to form DNA-lipid complexes. Firefly luciferase constructs containing the various FUT4 promoter fragments were transfected. Then the complexes were added to the cells and the cells were incubated for 6 h. Culture media were removed, and fresh culture medium was added. The cells were cultured for an additional 48 h. Cells were washed twice with cold PBS (pH 7.4) and cell lysates were prepared by adding 200 μl lysis buffer. Lysates were subjected to a freezing and thawing cycle, and the protein supernatant was collected for the dual luciferase assays. Cell extract (20–40 μl) was used for each assay in duplicate. Protein concentration was determined with the Coomassie Protein Assay Reagent using BSA as a standard. At least three independent transfections were performed, starting with fresh cell cultures, for each data point.

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

MCF-7 and MDA-MB-231cells were plated on 100 mm plates. After treated, cells were washed twice with cold PBS (pH 7.4). Trizol reagent (1 ml) was added to each plate and the cells were harvested. Total RNA was isolated following the manufacturer's protocol (Invitrogen). A total of 2 μg of RNA was reverse transcribed to cDNA by reverse transcriptase (TaKaRa). RNA was incubated at 42°C for 50 min and then incubated at 70°C for 20 min to terminate the reaction. Then, 1 μl of cDNA was used to perform PCR. The annealing temperature varied from 58 to 60°C, depending on the primers used. The primers are listed in Table I.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSAs)/GEL SHIFT ASSAYS

Nuclear protein were extracted with Nuclear Protein Extraction kit and quantified with kit mentioned above (Beyotime Institute of Biotechnology) according to the recommendation. The oligonucleotide probes were synthesized and labeled by biotin. The sequences of the oligonucleotides used in EMSA are shown below: (1) HSF1 consensus oligonucleotide: 5′-CACCTCGGCTGGAATATTCCCGACC-TGGCAGCCGA-3′; and (2) Sp1 consensus oligonucleotide: 5′-ATTCGATCGGGGCGGGGCGAG C-3′. Each binding mixture (12 μl) for EMSA contained 5.0 μg of nuclear extract, 2.0 μg of poly(dI–dC), 0.5–1.0 ng of labeled double-stranded oligonucleotide probe in binding buffer (12 mM HEPES, 12% glycerol, 0.12 mM EDTA, 0.9 mM $MgCl_2$, 0.6 mM dithiothreitol, 0.6 mM PMSF, and 2.0 μg/ml aprotinin and leupeptin (pH 7.9)). The samples were incubated at room temperature for 15 min and then electrophoresed on 4.5% polyacrylamide gels. The results were visualized with ECL detection system.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

Cells were fixed by adding formaldehyde to the medium to a final concentration of 1%. After 15 min, cells were washed with ice-cold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, shaken for 20 min at 4°C in Lysis Buffer-1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, containing protease inhibitors) and harvested using a cell scraper. After centrifugation at 3,000 rpm for 10 min the pellet was resuspended in Lysis Buffer-2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, containing protease inhibitors) and shaken at room temperature for 10 min. After centrifugation, nuclei were resuspended in Lysis Buffer-3 (50 mM Tris, pH 8.0, 1% SDS, 5 mM EDTA), and chromatin was shared by sonication. After removal of nuclear debris by centrifugation at 13,000 rpm for 5 min at 8°C, lysates were diluted 10-fold with dilution buffer (50 mM Tris pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.5% NP40) and then precleared for 3 h using 80 ml of 50% salmon sperm-DNA saturated protein A (ssproteinA)-agarose beads. Immunoprecipitation was carried out at 4°C overnight, and immune complexes were collected with ssprotein A-agarose beads. Antibodies utilized included anti-HSF1 or pre-immune rabbit serum as control for non-specific interaction. After washing three times with high salt WB buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA, 0.5 M NaCl) and twice with low salt TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), immunocomplexes were eluted with TE containing 1% SDS. Protein-DNA cross-links were reverted by incubating at 65uC overnight. After proteinase K digestion, DNA was extracted with phenol-chloroform and precipitated with ethanol using 15 mg of tRNA as carrier. PCR was performed using the following primers: Sp1, sense: 5′-CCCATTCCCAGCACTGTCTA-3′, antisense: 5′-ATCG-CACTGGCCTCTTCTAC-3′; HSF1, sense: 5′-GGCCTAGCCAGAA-CACCTAA-3′, antisense: 5′-GAAGCTGAGGCAGGAGAATG-3′. For quantification of ChIP assays, samples from at least three independent experiments were analyzed by real time PCR. Data were normalized to the input DNA and DNA from untreated cells.

TABLE I. Primer Sequences Used for PCR

Name	Sequence
HSF1	F: 5′-cctggtcaagccagagagag-3′ R: 5′-ggctatacttgggcatggaa-3′
SP1	F: 5′-tcataccaggtgcaaaccaa-3′ R: 5′-gtctgctggttttgctctcc-3′

CELL COUNTING AND CELL VIABILITY ASSAY

Cells (1×10^4 cells/well) were plated in 24-well plates. MTT assay was used to detect cell proliferation for 5 consecutive days. In brief, MTT was added to the culture medium to yield a final MTT concentration of 0.5 mg/ml and the incubation was continued for 4 h at 37°C. The cell lysates were dissolved with 200 μl dimethyl sulfoxide (DMSO) at room temperature for 10 min. Results were obtained by measuring the absorbance at a wavelength of 490 nm. The test was repeated three times.

CELL CYCLE ANALYSIS

Cells were cultured in six-well plates and allowed to grow to 75–80% confluency. Non-adherent cells were removed by washing with PBS. Cells were trypsinized, collected and washed twice with PBS. Cell pellets were resuspended in 0.5 ml of PBS and fixed in 4.5 ml of 70% ethanol overnight. Cells were collected by centrifugation and the pellets were resuspended in 0.2 mg/ml of propidium iodide (PI) containing 0.1% Triton X-100 and 0.1 mg/ml RNase A. The cell suspension was incubated in the dark for 30 min at room temperature and subsequently analyzed on FACScan flow cytometer for DNA content. The percentages of cells in different phases of the cell cycle were sorted using a ModFit 5.2 computer program. The percentages of cells at the G0/G1, S, and G2/M phases were obtained from three independent experiments.

siRNA SYNTHESIS AND TRANSFECTION

The small interfering RNA (siRNA) directed against HSF1 was designed using software developed by Ambion (Austin, TX). The sequences of HSF1 siRNA were 5′-AAGTACTTCAAGCACAACAACCCTGTCTC-3′ (sense) and 5′-AAGTTGTTGTGCTTGAAGTACCCTGTC-3′ (antisense) and those used for scrambled siRNA were 5′-AAGACCACAATATCT-GAACACCCTGCT-3′ (sense) and 5′-AAGTGTTCAGATATTGTGGTCC-CTGCTTC-3′ (antisense). The sequences of Sp1 siRNA were 5′-GATCTAAGCGCTTCATGAGGAGTGTTCAAGAGACAC TCCTCAT-GAAGCGCTTTTTTTTGGAAA-3′ (sense); 5′-AGCTTTTTCCAAAAA-AAAG CGCTTCATGAGGAGTGTCTCTTGAACACTCCTCATGAAGCG-CTT-3′ (antisense). 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.

STATISTICAL ANALYSES

At least three independent experiments were performed for each result and statistical analyses were carried out as follows. One-way ANOVA was applied to analyze the data using SPSS software, and a *P*-value of <0.05 was considered as statistically significant.

RESULTS

COMPARISON OF FUT4 PROMOTER ACTIVITIES IN A SERIES OF HUMAN FUT4 PROMOTER FRAGMENTS OF DIFFERENT LENGTHS

To understand the regulation of FUT4 transcription in various types of cells, we measured FUT4 promoter activity in a series of promoter constructs that contain different deletions (Fig. 1A). First, the 2.0 kb 5′ region upstream of the FUT4 transcription start site was amplified using PCR genomi cDNA isolated from human mammary epithelial cells as a template. Serial deletion constructs were created as described in the Materials and Methods Section. To identify important regulatory regions in the FUT4 promoter, we then determined the levels of FUT4 promoter activity in serial FUT4 promoter deletion constructs in each cell line. In breast cancer cells (MCF-7 and MDA-MB-231), the promoter activities of the pGL6-FUT4-1.2 construct were increased significantly compared with the pGL6-FUT4-0.4 ($P<0.05$) and pGL6-FUT4-0.8 ($P<0.05$) constructs. The promoter activities of the pGL6-FUT4-1.6 construct were decreased compared with the pGL6-FUT4-1.2 ($P<0.05$) construct (Fig. 1B). The FUT4 promoter activity of FUT4-1.2 was approximately threefold higher than the other constructs. We obtained the same results in the two breast cancer cell lines. In summary, these results showed that a potential positive regulatory site is located between 0.8 and 1.2 kb of the FUT4 promoter and a potential negative regulatory DNA element is present between 1.2 and 1.6 kb of the FUT4 promoter in breast cancer cells.

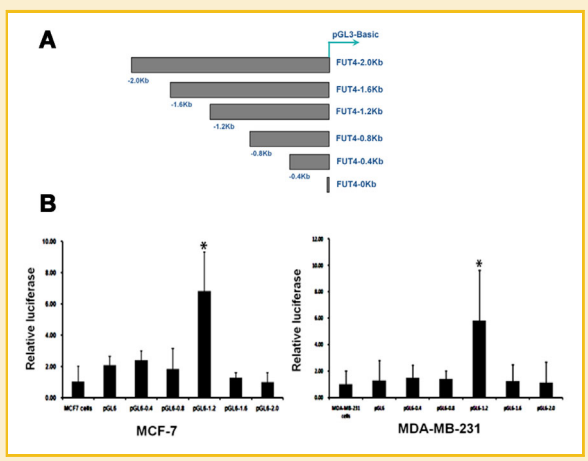

Fig. 1. A: Maps of promoter deletion constructs of the human FUT4 promoter. Genomic DNA was isolated from human blood cells and used as a template to amplify the human FUT4 promoter region of 2.0 kb. A 2.0 kb PCR product was cloned into the pGL6-basic vector and termedpGL6-FUT4-2.0 kb. The other four serial deletion constructs, FUT4-1.6 kb, FUT4-1.2 kb, FUT4-0.8 kb, and FUT4-0.4 kb, were generated from pGL6-FUT4-2.0 kb by performing PCR. The sequence of each construct was confirmed by DNA sequencing. B: FUT4 promoter activity analysis of serial FUT4 promoter deletion constructs. Six FUT4 promoter constructs were transfected into MCF-7 and MDA-MB-231 cells along with *Renilla* luciferase (pTK-RL) as an internal control. The luciferase activities of the deletion constructs were normalized to the control group cells and presented as a relative level of luciferase activity. Transfections were repeated at least three times starting with fresh cells and the data are presented as the WS.E.M ($P<0.05$).

LOCALIZATION OF THE REGULATION ELEMENT IN THE HUMAN FUT4 PROMOTER

As shown in Figure 1B, the region of the FUT4 promoter localized between −0.8 and −1.6 kb from the transcription initiation site reflects the existence of several putative factors for the regulation of FUT4 gene transcription in breast cancer cells. We analyzed the region of the FUT4 promoter between −0.8 and −1.6 kb using the MatInspector v6.2 program (core similarity, 1.0; matrix similarity, 0.75) of the Genomatix and TFSEARCH (version 1.3). Using a cut-off threshold score of 85%, TESEARCH prediction analysis identified several transcription factors that bind to this region. Several putative transcription factor binding sites exist, such as heat-shock factor 1 (HSF1), Sp1, and others (Fig. 2A). To validate the ability of endogenous Sp1 and HSF1 to bind to the FUT4 promoter, we performed EMSA and CHIP experiments. Based on the results of FUT4 promoter activity of serial deletion constructs, we chose different deletion constructs to analyze the binding of HSF1 and Sp1 with the FUT4 promoter. As shown in Figure 2B, a shifted protein-DNA complex band was observed when incubating the labeled consensus HSF1 and Sp1 probes with nuclear extracts transfected with pGL6-FUT4-1.2 and pGL6-FUT4-1.6, respectively. To determine if HSF1 and Sp1 interacted with the FUT4 promoter in vivo, chromatin immunoprecipitation (CHIP) assays were performed. The results (Fig. 2C) showed that HSF1 and Sp1 interact with the FUT4 promoter, which is in agreement with the EMSA results. The data imply that HSF1 and Sp1 interact with FUT4 promoter directly in vivo. These results demonstrated that HSF and Sp1 bind the promoter of FUT4.

HSF1 AND Sp1 REGULATION OF FUT4 EXPRESSION

To show the biological importance of HSF1 and Sp1 in regulating the FUT4 promoter, overexpression and siRNA plasmids of HSF1 and Sp1 were used. Western blot analysis showed that overexpression of HSF1 and Sp1 increased and decreased the levels of FUT4 protein in MCF-7 and MDA-MB-231 cells, respectively (Fig. 3A). Promoter activity analysis showed that overexpression of HSF1 significantly increased the activity of FUT4 promoter by approximately sevenfold, while overexpression of Sp1 decreased the activity of FUT4 promoter (Fig. 3B). There was also a 30–50% reduction in luciferase activity in the presence of HSF1-RNAi and a 300–500% increase in the presence of Sp1-RNAi compared with control (Fig. 3B). In addition, EMSA results showed that overexpression of HSF1 and Sp1 improved the shifted protein-DNA complex band, while knockdown of HSF1 and Sp1 yielded converse results (Fig. 3C). Taken together, HSF1 and Sp1 are able to regulate FUT4 promoter activity.

Heat stress is known to activate HSF1 within a few minutes. To establish the role of HSF1 in FUT4 up-regulation by heat, breast

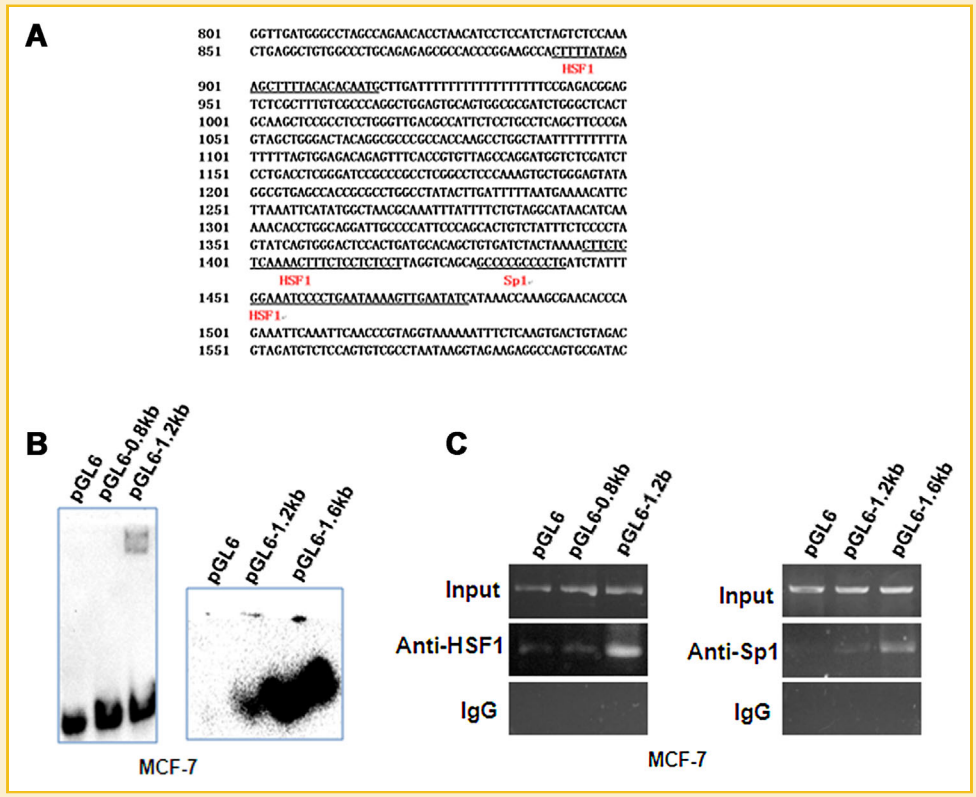

Fig. 2. Binding of HSF1 and SP1 to the FUT4 promoter. A: Nucleotide sequence of human FUT4 promoter region between 800 and 1,600 bp, as indicated in (A). Location of putative binding sites for HSF1 and Sp1 transcription factors in the FUT4 promoter identified by TFSEARCH are indicated. B: Electrophoretic mobility shift assays. Nuclear extracts of MCF-7 cells were prepared and EMSA was performed to investigate protein binding to DNA oligonucleotides representing sequences of the FUT4 promoter. C: ChIP was performed with MCF-7 and MDA-MB-231 cells to confirm the interaction of HSF1 and Sp1 with the FUT4 promoter in vivo. Chromatin incubated with antibody against IgG was used as a negative immunoprecipitation control, whereas input was used as a positive PCR control. PCR products from the ChIP assay run on an agarose gel.

cancer cells were subjected to a 40-min heat shock treatment at 43°C, then allowed to recover at 37°C. At different times during heat treatment or the recovery period, FUT4 expression was analyzed; expression of HSP-70 and FUT4 decreased after heat treatment. During the recovery, the expression of HSP-70 and FUT4 increased with time (Fig. 3D). These results further demonstrate that HSF1 regulates the transcription of FUT4.

EFFECT OF HSF1 AND Sp1 ON THE PROLIFERATION OF BREAST CANCER

In a previous study we found that FUT4 promotes cell proliferation through the MAPK and PI3K/Akt signaling pathways. In the current study we aimed to analyze the role of the transcription factors associated with the FUT4 promoter on breast cancer cell proliferation. First, we performed the cell viability assay, the results of which showed that FUT4 and HSF1 increased cell proliferation and Sp1 decreased cell proliferation (Fig. 4A). Cell cycle analysis showed that the G1/G0 phase arrest during the S phase increased after transient transfection with the FUT4 and HSF1 expression plasmid, while the S phase decreased when transfected with the SP1 expression plasmid (Fig. 4B). Cyclin D, p21, and p27 were important proteins during the G1-to-S phase. Cyclin D expression was increased and p21 and p27 were decreased after transient transfection with the FUT4 and HSF1 expression plasmid (Fig. 4C). These results demonstrated that HSF1 and SP1 may regulate cell proliferation by controlling FUT4 expression.

FUT4 REGULATES BREAST CANCER CELLULAR BIOLOGICAL FUNCTION BY ERK/MAPK AND PI3K/Akt

The ERK/MAPK and PI3K/Akt pathways play an important role in the transmission of cell signals through transduction systems to the cell nucleus, where the pathways influence the expression of genes that regulate important tumor cellular processes, such as cell growth, proliferation, and apoptosis [Brzezianska and Pastuszak-Lewandoska, 2011; Rho et al., 2011]. Our results suggested that FUT4 is one of the genes indirectly regulated by the ERK/MAPK and PI3K/Akt pathways in breast cancer cells. MCF-7 and MDA-MB-231 cells were treated with inhibitors of ERK/MAPK (PD95095) and PI3K (LY294002). Then, the proteins were collected for analysis by Western blot. The results showed that HSF1 was decreased and Sp1 was increased after treatment. FUT4 was also decreased (Fig. 5A). Cell cycle analysis showed that the G1/G0 phase arrested and the S phase decreased in MCF-7 and MDA-MB-231 cells following treatment with PD98095 and LY294002 for 48 h compared with controls (Fig. 5B), and may contribute to the decreased cell proliferation with down-regulation of FUT4. Next, we analyzed the key proteins of the G1-to-S

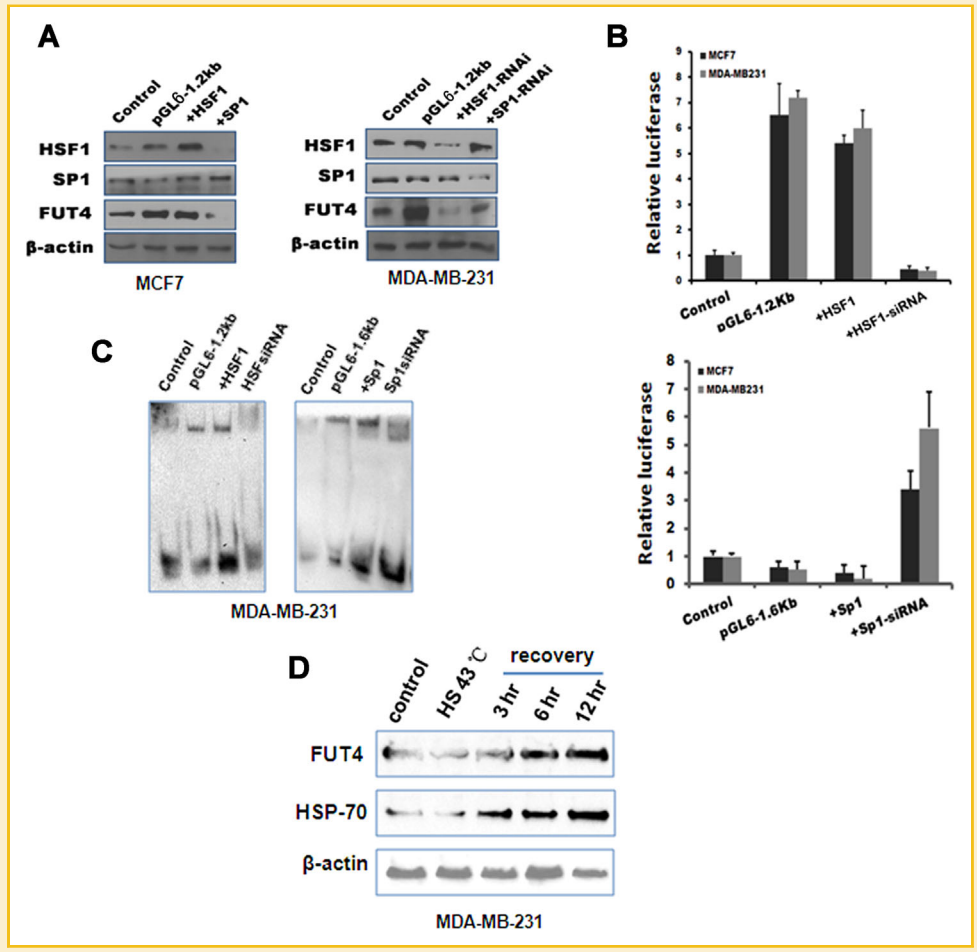

Fig. 3. The regulation of HSF1 and Sp1 on the expression of FUT4. A: pGL6–1.2 kb FUT4, and overexpression plasmid and siRNA of HSF1 and Sp1were transfected into MCF–7 and MDA–MB–231cells. Total protein from the whole cell lysates were subjected to Western blot analysis using antibodies directed against HSF, Sp1, and FUT4. β–Actin served as an internal control. B: FUT4 reporter assay analysis of the FUT4 promoter activity in MDA–MB–231 cells transfected with overexpression plasmid and siRNA of HSF1 and Sp1 compared with the untreated cells. C: Electrophoretic mobility shift assays. D: MDA–MB–231cells were subjected to heat shock at 43°C (HS) for 40 min, and recovered at 3, 6, and 12 h. Cell lysates were prepared as described in the Materials and Methods Section, and then analyzed by Western blot with antibodies against FUT4 and HSF–70.

phase checkpoint; cyclin D decreased and p21 and p27 increased after treatment with PD98095 and LY294002 (Fig. 5C). Taken together, we conclude that the ERK/MAPK and PI3K/Akt pathways may regulate the expression of FUT4 through HSF1 and Sp1, and improve breast cancer cell proliferation (Fig. 6).

DISCUSSION

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. Complex carbohydrates are comprised of three kinds of biomolecules (glycoproteins, proteoglycans, and glycosphingolipids). Recent studies have also shown that glycan changes in malignant cells take a variety of forms and mediate key pathophysiologic events during the various stages of tumor progression. Glycosylation changes are universal hallmarks of malignant transformation and tumor progression in human cancer, which take place on whole cells or some specific molecules [Kang et al., 2011]. Accordingly, such changes make glycosylated cells and molecules prominent candidates for cancer biomarkers [Li et al., 2010]. Indeed, cancer-associated carbohydrate structures play key roles in cancer progression by altering the cell–cell and cell–environment interactions [Clark and Mao, 2012; Dall'Olio et al., 2012].

Tumor-associated carbohydrate antigens result from the aberrant glycosylation which occurs with transformation to a tumor cell. Carbohydrate antigens that have been shown to be tumor-associated include the blood group Lewis-related LeY, SLeX, SLeA, and LeX [Heimburg-Molinaro et al., 2011]. LeY antigen, which is carried by glycoproteins and glycolipid cell surfaces, is predominately expressed during embryogenesis and is limited to granulocytes and epithelial lining in adults. Highly-expressed LeY is found in the majority (70–90%) of human cancers of epithelial origin, for example, breast [Madjd et al., 2005], ovarian [Takehara et al., 2002], hepatic [Liu et al., 2001], colon, and gastrointestinal cancers [Baldus et al., 2006].

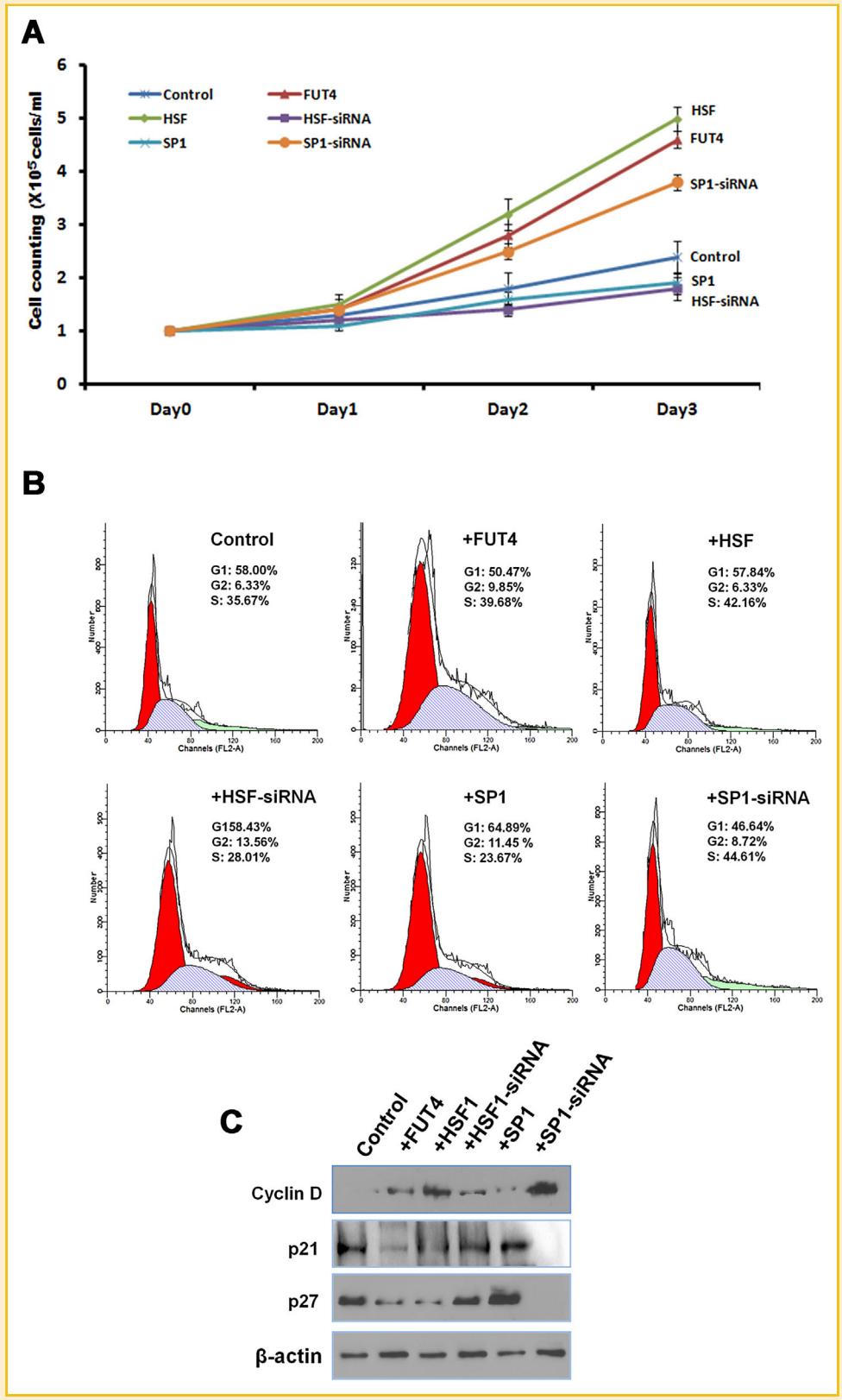

Fig. 4. Role of HSF1 and Sp1 on cell proliferation. A: Effect of HSF1 and Sp1 on survival rate of MDA-MB-231 cells. Overexpression plasmid and siRNA of HSF1 and SP1 were transfected into MDA-MB-231 cells for 24, 48, and 72 h. The cell survival rate was calculated using the MTT method. Each value represents the mean ± SD of three independent experiments. B: Cell cycle analysis. Cell cycle distributions were detected by flow cytometry analysis in MDA-MB-231 cells after transient transfection with pcDNAFUT4; siRNA directed against HSF1 and Sp1 for 48 h. C: The levels of p21, p27, and cyclin D1 expression were determined by Western blot. β-Actin was detected as a control.

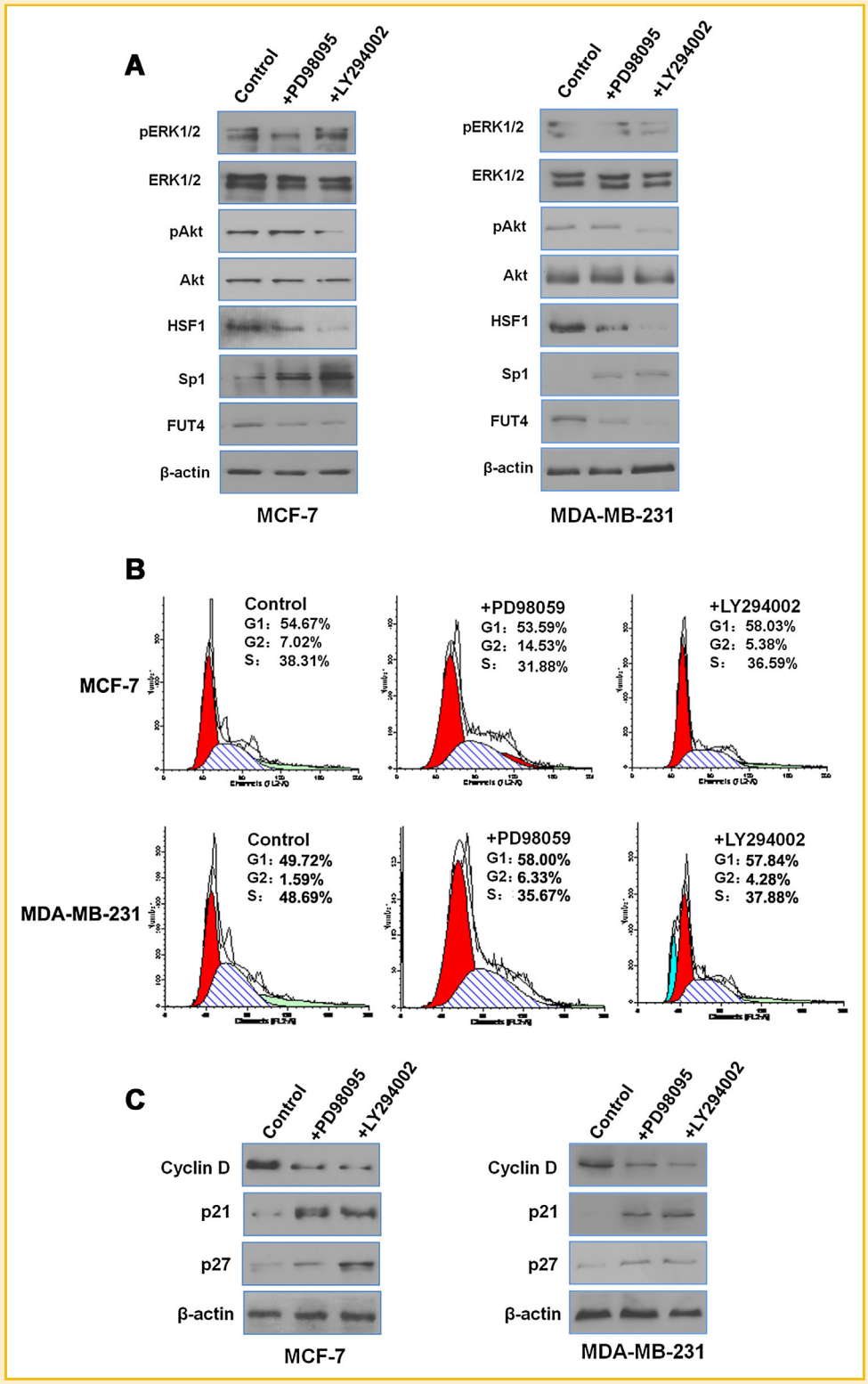

Fig. 5. FUT4 regulated cellular biological function by the ERK/MAPK and PI3K/Akt signaling pathways. A: MCF-7 and MDA-MB-231 cells were cultured for 48 h in the presence or absence of PD98095 (10 μM) and LY294002 (10 μM). The cell lysates were analyzed for total and phosphorylated levels of ERK/MAPK and Akt. B: Cell cycle analysis. Cell cycle distributions were detected by flow cytometry analysis in MDA-MB-231 cells after treatment with PD98095 (10 μM) and LY294002 (10 μM) for 48 h in MCF-7 and MDA-MB-231 cells. C: The levels of p21, p27, and cyclin D1 expression were determined by Western blot after treatment with PD98095 (10 μM) and LY294002 (10 μM) for 48 h in MCF-7 and MDA-MB-231 cells. β-Actin was detected as a control.

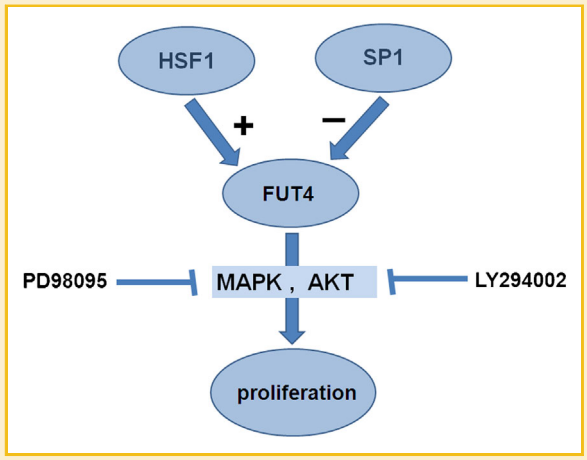

Fig. 6. Schematic diagram illustrates the mechanism underlying FUT4 regulation by ERK1/2 and PI3K/Akt signaling pathways via transcription factors (HSF1 and Sp1).

Our previous studies have shown that FUT4, which is a key enzyme involved in LeY synthesis, regulates A431 cell growth by controlling cell cycle progression via the MAPK and PI3K/Akt signaling pathways [Yang et al., 2007, 2010]; however, the mechanisms previously investigated were restricted to the upstream protein kinases, which are not direct effectors in proliferation. The upstream protein kinases need to activate downstream transcription factors implicated in the control of cell proliferation.

To determine the molecular mechanism underlying regulation of FUT4, a series of deleted FUT4 promoter luciferase plasmids were constructed. The principle of establishing FUT4 promoter vectors in this work was based on the regions that included putative cis-element binding sites at 5′-UTR upstream of the transcriptional start point. Using the dual luciferase reporter assay, the data showed that the promoter, pGL6-1.2 kb, showed the strongest activity compared with the other deleted promoters represented by the core promoter. Through analysis of transcription activity, we forecast that HSF1 and Sp1 were associated with the transcription of FUT4. We knew that the DNA binding activity of transcription factors in different cells is an important determinant of promoter activity. To date, data concerning the regulation of FUT4 transcription in breast cancer cells are limited. Our findings indicated that HSF1 enhances the activity of FUT4 promoter, while Sp1 decreases the activity of FUT4 promoter. Furthermore, HSF1 induced FUT4 expression at the protein level and Sp1 inhibited FUT4 expression. Luciferase assays also demonstrated the same results.

HSF1 is the master transcriptional regulator of the cellular response to heat and a wide variety of other stressors. Although HSF1 is best known as the master regulator of the heat shock response, it is becoming increasingly evident that HSF1 also plays an important role in supporting tumor development [Dai et al., 2007]. It has been suggested that HSF1 supports malignant transformation by coordinating a diverse array of cellular functions, including cell proliferation, survival, protein synthesis, and glucose metabolism. High HSF1 mRNA levels were also associated with an increase in ER-positive breast cancer-specific mortality. And increased HSF1 is associated with reduced breast cancer survival [Santagata et al., 2011]. HSF1 is an important transcription factor for induction of arylamine N-acetyltransferase 1 (NAT1) in human cells and is required for androgen activation of the NAT1 promoter. Human NAT1 is a widely distributed protein that has been implicated in a number of different cancers, including breast and prostate cancer [Butcher and Minchin, 2010].

Sp1 has traditionally been considered to be a ubiquitous transcription factor that is responsible for the basal/constitutive activation of a wide range of viral and mammalian genes; however, novel data strongly correlated regulated Sp1 expression with tumor development, growth, and metastasis, as it is significantly overexpressed in pancreatic, breast, thyroid, and colon tumors, and transactivates genes with a substantial role in cancer progression, proliferation, cell cycle regulation, cell differentiation, and anti-apoptotic procedures [Safe and Abdelrahim, 2005]. Studies have shown that in prostate cancer, Sp1 regulates important cancer-associated genes, such as androgen receptor, TGF-β, c-Met, fatty acid synthase, matrix metallo protein (MT1-MMP), PSA, and α-integrin [Sankpal et al., 2011]. It has been reported that Sp1 is essential for Keap1 expression and that promoter methylation blocks Sp1 binding in A549 cells. The inhibition of Sp1 binding to the hypermethylated Keap1 promoter in lung cancer cells suggests new approaches to lung cancer treatment [Guo et al., 2012]. In addition, it has been shown that Sp1 is down-regulated in lung adenocarcinoma cells with high invasiveness and in patients with stage IV lung adenocarcinoma. Moreover, Sp1 inversely regulates migration, invasion, and metastasis of lung adenocarcinoma cells in vivo [Hsu et al., 2012]. In the breast cancer, it was reported that oncoprotein HBXIP is able to activate the transcriptional coregulatory protein LMO4 through transcription factor Sp1 in promotion of proliferation [Yue et al., 2013]. It was also found that HBXIP upregulated PDGFB via activating transcription factor Sp1 to promote proliferation of breast cancer cells [Zhang et al., 2013]. As shown above, we believe that Sp1 is associated with the tumor.

We observed that FUT4 may promote breast cancer cell proliferation through HSF1 and inhibit breast cancer cell proliferation via Sp1. G1/G0 phase arrest and a decrease in the S phase in cells by FUT4 down-regulation may be the reason why LeY is highly expressed in breast cancer cells. We found that HSF1 prompted cell proliferation and altered cell-cycle distribution by regulation of p21, p27, and cyclin D1 in breast cancer cells, while the role of Sp1 is in contrast to HSF1 (Fig. 4). We used the siRNA of HSF1 and Sp1 to obtain the same results, specifically, HSF1 and Sp1 were shown to be positive and negative transcriptors, respectively. Sp1 activates the transcription of the genes encoding cyclinD, cyclinE, and p21Cip/WAK-1, which contribute to cell cycle progression [Santiago et al., 2007; Marampon et al., 2008]. Understanding the upstream signaling cascades that regulate FUT4 expression will help to clarify the biological functions of the gene. Cell proliferation, cell cycling, and apoptosis are associated with tumor development and progression. ERK is necessary for the G1-to-S phase progression and is associated with induction of positive regulators of the cell cycle and inactivation of anti-proliferative genes [Meloche and Pouyssegur, 2007]. Next, we found that inhibition of ERK and Akt

activities with inhibitors suppressed the transactivation of HSF1 and activated Sp1 (Fig. 5A). These results suggest the HSF1 and Sp1are regulated by ERK and Akt activities to control FUT4 transcription.

For the first time we have demonstrated that ERK/MAPK and PI3K/Akt regulates FUT4 expression through HSF1 and Sp1 at the transcriptional level in breast cancer cell lines. We conclude that inhibition of ERK/MAPK and PI3K/Akt down-regulates FUT4 promoter activity on transcription control, in which the transcription factors, HSF1 and Sp1, play a crucial role.

Our research highlights the rationale for developing the therapeutic potential of FUT4 for regulating breast cancer proliferation. FUT4 may play an important role in carcinogenesis; however, the complicated molecular mechanisms need further research. These data may provide support for the development of specific FUT4 inhibitors as efficient anti-cancer drugs for breast cancer.

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