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Histone 3 Trimethylation of IGFBP-7 Gene Promoter by Expression of D5 Stat5a in Breast Epithelial Cells

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Abstract

As a very important transcription factor, signal transducer and activator of transcription 5a (Stat5a) has been reported to be involved in human reproductive cancers such as breast, prostate and ovarian cancer. However, up to date, the exact role of Stat5a in breast cancer is still not clear. The data reported are conflicting. D5 Stat5a is a variant of Stat5a we cloned recently. This newly cloned variant behave like its full length counterpart in terms of dimerization, being activated by prolactin and nuclear translocation, however it also behave differently in terms of effect on cell proliferation and interaction with other transcription factors. In the present study, we examined its effect on cell proliferation of cultured breast cancer cells (MCF-10A and MCF-7) by using adenovirus-mediated gene transfer and MTS technology. Also, quantitative real time polymerase chain reaction (qRT-PCR), chromatin immunoprecipitation assay (ChIP) and Western blot were used to probe the possible changes of insulin-like growth factor binding protein-7 (IGFBP-7) expression including mRNA and protein, and the epigenetic changes with overexpression of this newly cloned variant. The results clarified that D5 Stat5a (1) behaves as a promoting factor to the cell proliferation of MCF-10A and MCF-7, (2) induces enhancer of zeste homology 2 (EZH2) expression in breast epithelial cells, as well as histone 3 trimethylation of IGFBP-7 promoter region, and (3) lower IGFBP-7 expression was detected in breast cancer tissue. Taking together, we concluded that the mitogenic effect of D5 Stat5a on breast cells is, at least partly, through up-regulation of histone methyltransferase, EZH2, and therefore inhibiting IGFBP-7 expression by increasing H3K27Me3 of IGFBP-7 promoter region.

Key Words: breast cancer, chromatin immunoprecipitation assay (ChIP), D5 Stat5a, H3K27Me3, histone modification, IGFBP-7

Introduction

The Stat proteins are a family of transcription factors activated by multiple cytokines, and hormones. Stats have unique and overlapping functions. The two closely related Stat5 proteins (Stat5a and b) are induced by a variety of hematopoietic cytokines, growth factors and hormones, the latter including prolactin and growth hormone (30, 31). Knockout of Stat5a interferes with prolactin-mediated functions in the mammary gland (1, 21), whereas knockout of Stat5b interferes with growth hormone-regulated functions in the liver and causes liver fibrosis and cancer development (12, 37). Studies of mice in which both

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Stat5a and b have been knocked out show a key role for these molecules in prolactin-regulated function in the ovary, the potential of hematopoietic stem cells to repopulate the bone marrow (5), the ability of interleukin-2 (IL-2) to induce T-cell proliferation (20), and the ability of IL-3 to mediate mast cell development and survival (13, 23). Also, a sizable body of data from cell lines, mouse models, and human tissues now implicates the roles of these transcription factors in oncogenesis including breast cancer (9, 33) and prostate cancer (10, 32). Mammary tumorigenesis was delayed when the Stat5a gene was deleted from transgenic mice with mammary-directed expression of transforming growth factor-α (TGF-α) or SV40 large-T (25). Significant elevations in the DNA-binding activity of both Stat3 and Stat5 were noted in malignantly transformed breast tissues when compared to normal tissues (11, 18, 36). Constitutive Stat5a/b and Stat3 activation has been detected in a mouse model of mammary adenocarcinoma. Also, mammary cancer progression can be delayed by loss of Stat5a (25). These data strongly suggest a tumor-promoting role for Stat5a. Interestingly, several recent reports have found higher Stat5 activation to be associated with a less invasive phenotype and a better prognosis (22, 27). In this regard, Stat5 has been shown to promote homotypic adhesion and inhibit invasive characteristics of human breast cancer cells (27). In prostate cancer cells, Stat5 is a key survival factor (Ali1998). Activation of Stat5 in human prostate cancer is strongly associated with high histologic grade and cell death is induced in prostate cancer cells by inhibition of Stat5 (16). Thus, there are conflicting data on the role of Stat5 in breast cancer.

D5 Stat5a is a variant of Stat5a we cloned and reported recently, which is missing thirty amino acids of the N-terminus corresponding to the whole of exon 5 (29, 31) and that is why it has been named as Delta 5 or D5 Stat5a. Although as an alternative product splicing messenger of Stat5a gene, it has been proved to have the same characteristics of its full partner, Stat5a, in terms of dimerization, being activated by prolactin and nuclear translocation, our preliminary data have also shown it behave differently in bioactivities such as interaction with different transcription factors from its full partner Stat5a, and more importantly, D5 Stat5a overexpression increased viable cell number of prostate or breast cancer epithelial cells. However, the molecular mechanisms underlying the mitogenic effect was still unknown. Insulin-like growth factor binding protein 7 (IGFBP-7) has been proven to be interfering the insulin-like growth factor (IGF) signal pathway by binding to IGF with high affinity thereby limiting IGF access to IGF receptor (7). Investigators have accepted IGFBP-7 as a potential tumor suppressor gene for a variety of cancers. Also, in a RNA-sequencing study, alteration of IGFBP-7 expression induced by D5 Stat5a was serendipitously observed (unpublished data).

The purpose of the present study is to establish the possible relationship between D5 Stat5a and IGFBP-7. We examined if D5 Stat5a induced proliferation of breast epithelial cell is associated with down-regulation of IGFBP-7 using quantitative real time PCR and Western blot technology. And the associated epigenetic modification involved in the process has also been investigated accordingly.

Materials and Methods

Chemicals and Reagents

Breast cancer cells (MCF-10A, MCF-7) were obtained from American Type Culture Collection (ATCC). Reverse transcriptase, Taq DNA polymerase, enhanced chemiluminescent western blotting substrate (ECL) and MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) are product of promega (Promega, Madison, WI, USA). Primary antibodies against EZH2, against trimethylation of histone 3 at lysine 27 (H3K27Me3) were purchased from EMD Millipore (Billerica, MA, USA). Eukaryotic expression vector pGFP^{2}N1 was from PerkinElmer (Waltham, MA, USA). TRIzol reagent was purchased from Invitrogen (Thousand Oak, CA, USA).

Construction of D5 Stat5a Eukaryotic Expression Vectors

Plasmid carrying D5 Stat5a cDNA was made as described previously (2). Briefly, human D5 Stat5a cDNA was achieved by reverse transcription of MCF-7 mRNA. The entire coding sequences of human D5 Stat5a (2934 bp) were amplified by PCR using *Taq* DNA polymerase and sense/antisense primers harboring unique *Mlu*I (at sense) and *Kpn*I (at antisense) restriction sites. The extra DNA base pairs corresponding to *Mlu*I (ACGCGT) and *Kpn*I (GGTACC) were designed to be upstream of the initiator codon on the sense and to be downstream of the stop codon on the antisense. An extra triplet ACC was also designed to be immediately before the initiator codon, ATG, to facilitate and therefore increase the transcription rate in transfected mammalian cells. The primers (sense/antisense) were as follows (cleavage sites for *Mlu*I and *Kpn*I are shown in boldface): 5'-GAC **ACG CGT** ACC ATG GCG GGC TGG ATC CAG-3'-3(sense)/5'-AAC **GGT ACC** A TCA GAG GGA GCC TCT GGC AGA-3'-3(antisense). The linearized PCR product of D5 Stat5a cDNA, as well as pGFP^{2}N1, which have a cytomegalovirus early

promoter and a polyadenylation site, were then digested with *Mlu*I and *Kpn*I and purified from an agarose preparative gel. The fragments then were subcloned in-frame into the *Mlu*I/*Kpn*I site of the pGFP^{2}N1 vectors to produce the plasmid we needed to use in the present study, D5 Stat5a-pGFP^{2}N1. Sequence analyses were performed to verify the correct orientation and open reading frame of the newly made constructs.

Cell Culture and Recombinant Adenovirus Infection

Breast epithelial cells, MCF-10a and MCF-7, were maintained in RPMI 1640 containing 10% FBS and 100 U/ml of streptomycin/penicillin. When the cells were 75%-80% confluent, they were infected with the recombinant adenovirus at a M.O.I of 10, 20 or 30 for 120 minutes and then changed the medium to normal RPMI1640 and containing prolactin (50 ng/ml) as indicated in the figures to initiate the signal transduction. After a further 48-hour incubation, infected cells were subjected to total RNA extraction for transcription and protein extraction for translation examination, or fixation with 1% formaldehyde for chromatin immunoprecipitation (ChIP) assay.

Viable Cell Number Determination by MTS

The cells were raised and infected with adenovirus containing or not containing D5 Stat5a cDNA as described, then subjected MTS assay. This assay was performed under stringent conditions previously described (22). Briefly, 42 mg of MTS reagent powder was dissolved in 21 ml of DPBS and filter-sterilized using a 0.2 μM filter. To this was added 100 μl of PMS/2 ml MTS solution under light-protected conditions. Twenty microliters of the freshly combined MTS/PMS solution was added to each well of a 96-well assay plate containing 100 μl of cells in culture medium. The cells plus reagents were then incubated at 37°C, 5% CO_2 for 3 h, after which time the absorbance at 490 nm was recorded using a microplate spectrophotometer. The number of cells was in the linear range of the assay.

Western Blot

Whole-cell lysates were prepared by scraping cells into ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3 VO_4, 10 mM NaF, 1 mM PMSF) containing additional protease and phosphatase inhibitors 6 (cocktail from Promega, Madison, WI, USA). The lysate was rotated 360° for 1 h at 4°C followed by centrifugation at 12,000 × *g* for 10 min at 4°C to remove cellular debris. Proteins were quantified using a Bio-Rad assay kit (Hercules, CA, USA). Protein samples were subjected to electrophoresis on reducing SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Western blot analyses were performed using antibodies against human IGFBP-7 and β-actin (Santa Cruz Biotech, Santa Cruz, CA, USA). Detection was with peroxidase-labeled second antibodies and enhanced chemiluminescence (ECL system, Santa Cruz). The final IGFBP-7 protein expression results were normalized with their specific β-actin levels.

RNA Isolation, Reverse Transcription and SYBR Green Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

RNA extractions were performed using TRIzol reagent according to the vendor's protocol. Briefly, Breast tissues were homogenized in a glass-Teflon homogenizer in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) per 100 mg of tissue. Monolayer cultured cells were lysed by adding 1 ml of TRIzol per 10 cm^2 and passing the cell lysate several times through a pipette. The homogenized samples were incubated for 5 min at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Zero point two ml of chloroform was added per 1 ml of TRIzol reagent and the sample tubes were capped securely. The tubes were then shaken vigorously by hand for 15 sec and incubated at 15 to 30°C for 2 to 3 min. The samples were centrifuged at 12,000 × *g* for 15 min at 2 to 8°C. The aqueous phase was then transferred to a fresh tube, and the organic phase was saved. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol, incubating samples at 15 to 30°C for 10 min and then centrifuging at 12,000 × *g* for 10 min at 2 to 8°C. The RNA precipitate was washed with 75% ethanol, air-dried, dissolved in DEPC-treated water and stored at -80°C for later use. First-strand synthesis and double strand cDNA were achieved using reverse transcriptase and pfu polymerase (Stratagene, La Jolla, CA, USA). SYBR Green Quantitative Real Time PCR was performed on an Illumina ECO real time PCR machine. The relative mRNA amounts were determined according to their Ct number (Cycle threshhold) and normalized to that of 18S rRNA. The primers used in this study were shown in Table 1 below.

ChIP Assay

Breast epithelial cells (MCF-10A and MCF-7) were maintained and infected with adenovirus carrying or not carrying D5 Stat5a cDNA as described above. Cells were then fixed using formaldehyde (1% in PBS, freshly prepared) for 12 min and washed by ice-cold PBS solution three times. The collected cells

Table 1. Primers used in the present study

Purpose	Primer sequences (S/A)*
Probe IGFBP-7 promoter:	5'-TCTTGTGGCAGCTGGAACAT-3'/ 5'-TTCTCTCCTTCATCGTTATC-3'
Probe IGFBP-7 mRNA:	5'-CGTGCGGCTGCTGCCCTATG-3'/ 5'-CCGCCGGCTGCTGCCCCGGC-3'
Probe 18S rRNA:	5'-CGCCGCTAGAGGTGAAATTC-3'/ 5'-CCAGTCGGCATCGTTTATGG-3'
Probe EZH2 mRNA:	5'-TAATGTGCTGGAATCAAAGG-3'/ 5'-TGGCTTCATCTTTATTGGTG-3'
Non-specific control primer:	5'-GGAGTGACTATAGTTAGCAA-3'/ 5'-GCCTGACCAGTATTTATCAT-3'

*S, Sense primer; A, Antisense primer.

(centrifuge at 2000 rpm for 5 min) were subjected to sonication to produce 200-500 bp DNA fragments (size was clarified by agarose gel electrophoresis). The sonicated chromatin samples were 1:10 diluted in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-Cl, pH 8.1, 1 × protease inhibitor cocktail). The DNA fragments were then immunoprecipitated with specific antibody (Ab) against EZH2 or H3K27Me3. A control immunoprecipitation using IgG was set up in parallel to distinguish non-specific precipitation. The DNA-EZH2-Ab or DNA-H3K27Me3-Ab complexes bound to protein G beads were then eluted with elution buffer (1% SDS in TE). The resultant DNA were quantified using NanoDrop (ND2000), or subjected to protein digestion (protease H) and the relative amount of certain genomic DNA sequences was examined using qRT-PCR.

Tissue Sample Collection

The tissues (0.5 cm × 0.5 cm × 0.5 cm) were collected immediately after surgery from the patients diagnosed with breast cancer by Jishou University Affiliated Hospital. The procedures were reviewed and approved by the Hospital Research Office Ethics Commission. Normal tissue adjacent to the removed cancer was also taken for comparison between normal and cancerous tissues for the same patient.

Data Analysis

All experiments were conducted a minimum of three times. Within each experiment, a minimum of three replicates was used. Data are presented as the mean ± SEM, frequently normalized to control values (such as β-actin in RT-PCR and western blot experiment) to combine experiments and to normalize among cell lines. Statistical significance was determined by analysis of variance with post tests. Results were considered significant at $P < 0.05$.

Results

Overexpression of D5 Stat5a Increased Viable Cell Number of Breast Epithelial Cells

As shown in Fig. 1B, overexpression of D5 Stat5a for 48 h increased the viable cell number of both MCF-10A and MCF-7 in a dose dependent manner. Compared to that of control cells (Con + PRL), the viable cell number increased 6.21%, 19.5% and 34.66% in cells infected with adenovirus carrying D5 Stat5a cDNA at M.O.I 10, 20 and 30 respectively in MCF-10A cells, and increased 4.70%, 24.71% and 40.10% respectively in MCF-7 cells. This result is in a well agreement with our previous work (29).

Overexpression of D5 Stat5a Decreased the Expression of IGFBP-7 in Breast Epithelial Cells

As a regulator of bioavailability of IGF, IGFBP-7 has been accepted as a tumor suppressor by lots of investigators (7). Cross talk between IGF-1 and Stat5 signal pathway has been also suggested by some authors (14). Is there any relationship between IGFBP-7 and D5 Stat5a signal pathway? MCF-10A and MCF-7 cells were infected with adenovirus carrying D5 Stat5a cDNA for 48 h as described above and total RNA extraction was performed using TRIzol reagent. IGFBP-7 mRNA was examined by quantitative RT-PCR. As shown in Fig. 2, IGFBP-7 mRNA was decreased in both MCF-10A and MCF-7 cells. Taking MCF-10A cells as an example, the relative mRNA were decreased from 0.995% of control cells to 0.770%, 0.679% and 0.158% in cells infected with adenovirus carrying D5 Stat5a cDNA at M.O.I 10, 20 and 30 respectively (Fig. 2A). Correspondingly, Western blot showed that overexpression of D5 Stat5a decreased the IGFBP-7 protein level in MCF-7 cells (Fig. 2B).

Higher H3K27 Trimethylation of IGFBP-7 Gene Promoter Region Was Detected in D5 Stat5a Overexpressing Breast Epithelial Cells

The epigenetic modification of histone has been regarded as the key step in the regulation of gene expression. The above results led us to hypothesize that D5 Stat5a might induce the change of epigenetic signature. MCF-10A and MCF-7 cells infected with recombinant adenovirus were then subjected to ChIP assay. DNA fragments were immunoprecipitated with an anti-

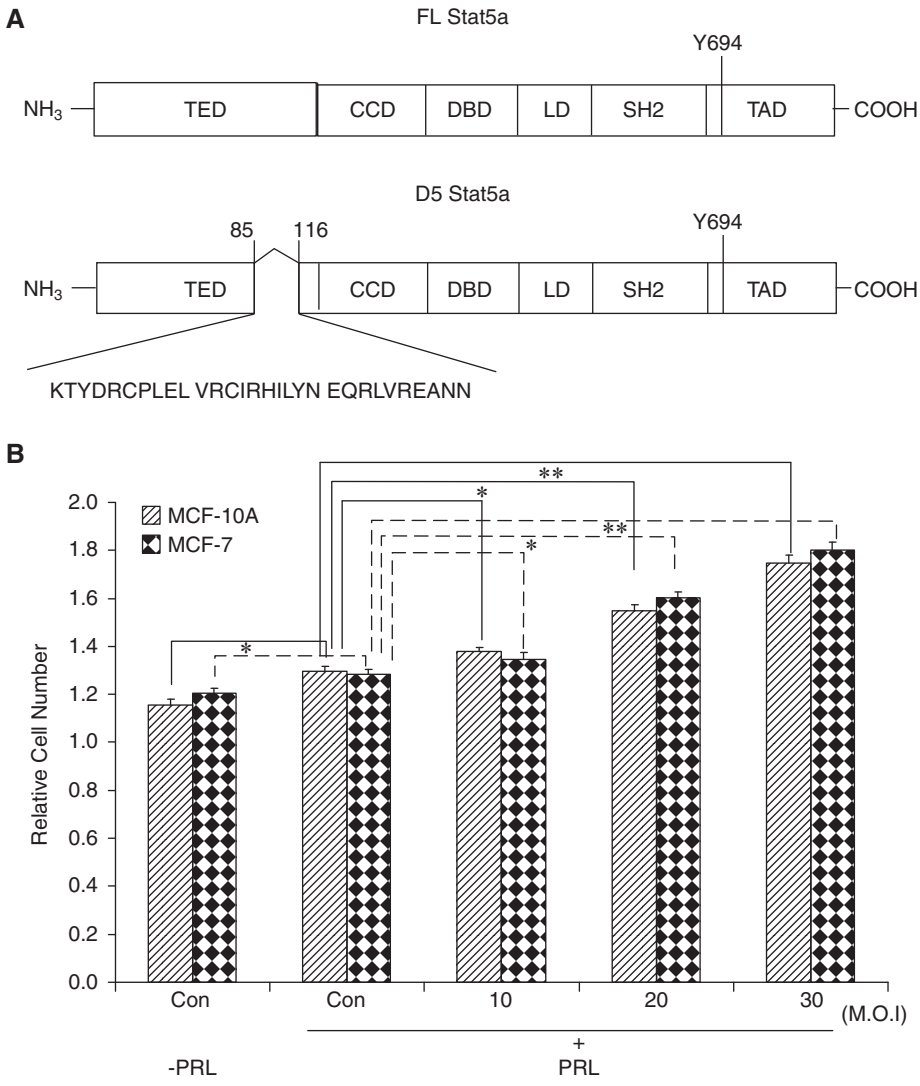

Fig. 1. Overexpression of D5 Stat5a increased the viable cell number of breast epithelial cells. A. Cartoon showing the difference between Stat5a and D5 Stat5a. B. Breast epithelial cells (MCF-10A and MCF-7) raised in RPMI1640 were arranged into five groups: Con without PRL, Con+PRL, and the other three groups of cells were infected with adenovirus carrying D5 Stat5a cDNA at M.O.I 10, 20 and 30 respectively. *$P < 0.05$, **$P < 0.01$. TED, tetramer domain; CCD, coiled coil domain; DBD, DNA binding domain; SH2, src homology 2; LD, link domain; TAD, transcription activation domain; Y694, tyrosine 694; PRL, prolactin; M.O.I, Multiplicity of infection; NS, non-significant difference.

body against H3K27Me3 and DNA sequences in the resultant DNA were quantified by qRT-PCR using primers designed to amplify the promoter region of IGFBP-7 gene (Table 1). As shown in Fig. 3, the precipitated fragments were 0.0850 ± 0.0193 (% of input) and 0.0062 ± 0.0020 (% of input) in D5 Stat5a overexpressing and control MCF-10A cells respectively (Fig. 3A), and 0.13 ± 0.0199 (% of input) and 0.0206 ± 0.0020 (% of input) in D5 Stat5a overexpressing and control MCF-7 cells respectively (Fig. 3B) indicating D5 Stat5a induced trimethylation of H3K27. Further, a set of parallel ChIP assays were performed using non-specific control primers which were designed from the corresponding genomic sequence of 250,000 bp upstream of transcription start site of IGFBP-7 gene. As shown in right part of Fig. 3A and B, all the relative immunoprecipitated DNA amounts were much less than 0.005 (% of input) implying that the precipitated DNA is specific to IGFBP-7 gene.

Overexpression of D5 Stat5a Lead to Up-Regulation of EZH2 mRNA Expression

As an important histone methyltransferase, EZH2 is responsible for the H3K27Me3. The fact that D5 Stat5a induced H3K27Me3 of IGFBP-7 promoter region as described above, logically, raised a question to answer: whether D5 Stat5a overexpression

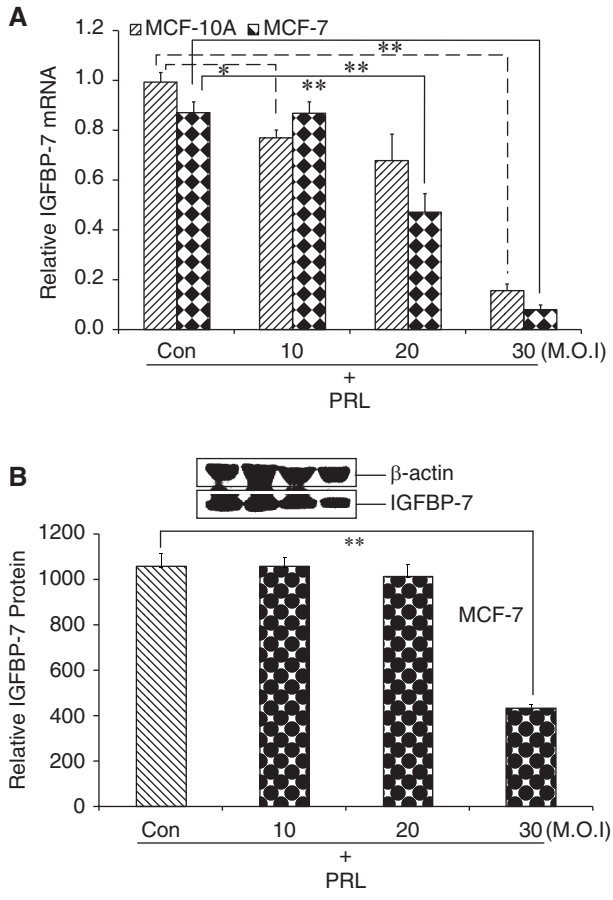

Fig. 2. Overexpression of D5 Stat5a induced down-regulation of IGFBP-7 expression. Breast epithelial cells (MCF-10A and MCF-7) were raised and infected as described above. Relative mRNA of IGFBP in MCF-10A and MCF-7 cells and IGFBP-7 protein in MCF-7 cells were examined using qRT-PCR (A) and Western blot (B) respectively. *$P < 0.05$, **$P < 0.01$. Con, control; M.O.I, Multiplicity of infection.

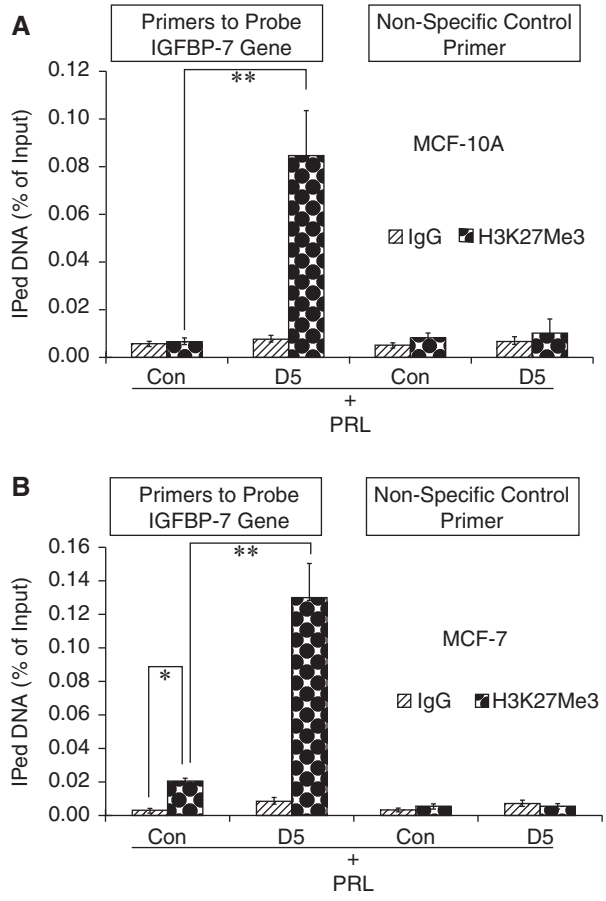

Fig. 3. Overexpression of D5 Stat5a increased H3K27Me3 of IGFBP-7 gene promoter region in breast epithelial cells. MCF-10A (A) and MCF-7 cells (B) were infected with adenovirus carrying or not carrying (Con) D5 Stat5 cDNA as in Fig. 1. Control cells and cells infected with virus at M.O.I 30 were subjected to ChIP assay using antibody against H3K27Me3. Additionally, another antibody against IgG was also used to rule out the background noise. Further, in order to subtract unspecific primer sequence binding to genomic DNA, an additional qRT-PCR was conducted using unspecific control primers which were designed from 250,000 bp upstream of IGFBP-7 gene transcription start site (right panel of A and B). *$P < 0.05$, **$P < 0.01$.

induced EZH2 expression? We, therefore, examined the effect of D5 Stat5a on expression of EZH2 using qRT-PCR. As shown in Fig. 4, overexpression of D5 Stat5a increased EZH2 expression by 4.72 and 3.12 folds in MCF-10A and MCF-7 cells respectively (Fig. 4). Further, the relative EZH2 mRNA level in MCF-7 control cells is higher (significantly) than in MCF-10A control cells implying aberration of EZH2 expression in cancerous breast epithelial cells.

Low Expression of IGFBP-7 in Breast Cancer Tissue Was Detected

IGFBP-7 has been reported to be a tumor suppressor and to be inversely correlated with progression of breast cancer. Also, the fact that higher EZH2 expression in MCF-7 control cells than in MCF-10A control cells as shown in Fig. 4 implicated the higher histone methylation of some critical tumor suppressor genes including IGFBP-7 gene. We therefore examined the expression IGFBP-7 in breast cancer tissues from ten patients. As shown in Fig. 5, the IGFBP-7 mRNA was down-regulated obviously in most of the ten patients.

Discussion

Breast cancer is the leading cause of death among women between 40 and 55 years of age and is the second overall cause of death among women. Many factors including transcription factors have been sug-

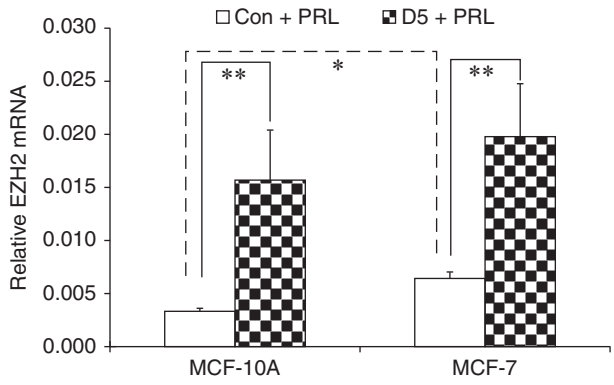

Fig. 4. Overexpression of D5 Stat5a lead to up-regulation of EZH2 expression in breast epithelial cells. Cells were infected with adenovirus carrying or not carrying (control) D5 Stat5a cDNA and EZH2 mRNA detection using qRT-PCR. $*P < 0.05$, $**P < 0.01$.

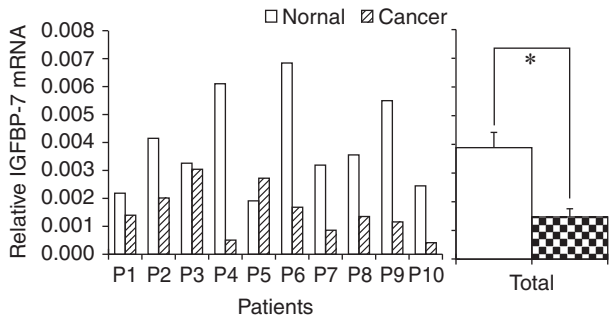

Fig. 5. Low expression of IGFBP-7 mRNA was detected in breast cancer tissue and normal adjacent breast tissue. Human breast tissues including ductal carcinoma histologically and normal adjacent breast tissue were sampled as described in methods. Relative IGFBP-7 mRNAs were detected by real-time PCR using primers: 5'-CGTGCGGCTGCTGCCCTATG -3' (sense) / 5'-CCGCCGGCTGCTGCCCCGGC-3' (antisense). The amplicon size was 145 bp. Eighteen S rRNA was also detected for each sample at the same time as the internal control. The primers used for 18S rRNA were 5'-CGCCGCTAGAGGTGAAATTC-3' (sense)/5'-CCA-GTCGGCATCGTTTATGG-3' (antisense). The left and right panels show the relative mRNA for each patient and statistical summary, respectively. $*P < 0.05$.

gested to be involved in the disease (17, 35, 39). We have reported a variant of human Stat5a in which 30 amino acid sequences corresponding to the exon 5 of human Stat5a gene is missing (Fig. 1A). As a new variant of human Stat5a, D5 Stat5a has been certified to be a dominant form in breast cancer tissue and to have some different characteristics from its full length counterpart, for example, promoting cell growth, interacting with other transcription factors such as NFκ-B, SP-1 and AP1-2 (29). The data we reported previously lead us to conclude that D5 Stat5a plays a pathogenic role in the pathogenesis, progression and prognosis of breast cancer. However, as to the molecular event downstream of D5 Stat5a, little has been known. The IGF system components have been implicated in breast cancer progression. IGF-I and IGF-II are mitogenic and anti-apoptotic peptides that influence the proliferation of various cell types including normal and transformed breast epithelial cells (34). The IGF system is a key growth regulatory pathway in breast cancer. IGFBP-7 has been shown to be an effective regulator of the bioactivity of IGF which plays a key role in the growth, differentiation, and proliferation of mammalian cells. IGFBP-7 regulates the bioavailability of IGF by binding to IGFs with high affinity thereby limiting IGF access to IGF receptor (IGFR) thereby inhibiting IGF activity (34). Importantly, IGFBP-7 has been reported to be inversely correlated to the pathogenesis, progression and prognosis in breast cancer (4). In our RNA-sequencing experiment, we found that overexpression of D5 Stat5a in breast cancer cells led to significant decrease of IGFBP-7 gene expression (unpublished data) and this phenomenon led us hypothesis that there would be a relationship between D5 Stat5a and IGFBP-7.

In the present study, we tried to answer if there is any cross talk between our newly cloned D5 Stat5a and IGFBP-7 in breast epithelial cells and probe the possible epigenetic changes of IGFBP-7 gene promoter region caused by D5 Stat5a.

In order to examine the effect on breast epithelial cells induced by D5 Stat5a, we overexpressed D5 Stat5a in breast epithelial cells including MCF-10A (relatively normal) and MCF-7 (cancerous) by using adenovirus transfer technology instead of chemical transfer protocol, since the cultured human breast cancer cells such as T48D and MCF-7, to our experience, are reluctant to accept exogenous DNA, the transfer efficiency of these cells using chemical reagent is relatively low (less than 50%). Theoretically, the transfer efficiency by adenovirus can be more than 90% and this has been confirmed in our case by using green fluorescence signal (data not shown).

As shown in Fig. 1, overexpression of D5 Stat5a increased the viable cell number of both relatively normal (MCF-10A) and cancerous (MCF-7) epithelial cells. Compared to control cells, the viable cell number increased 6.21%, 19.50% and 34.66% in cells infected with adenovirus carrying D5 Stat5a cDNA at M.O.I 10, 20 and 30 respectively in MCF-10A cells, and increased 4.70%, 24.71% and 40.10% respectively in MCF-7 cells. This result is well agreement with our previous observation (29). One would be asking here: why we needed to examine the viable cell number in the present study because we have already reported the similar data (29). The answer

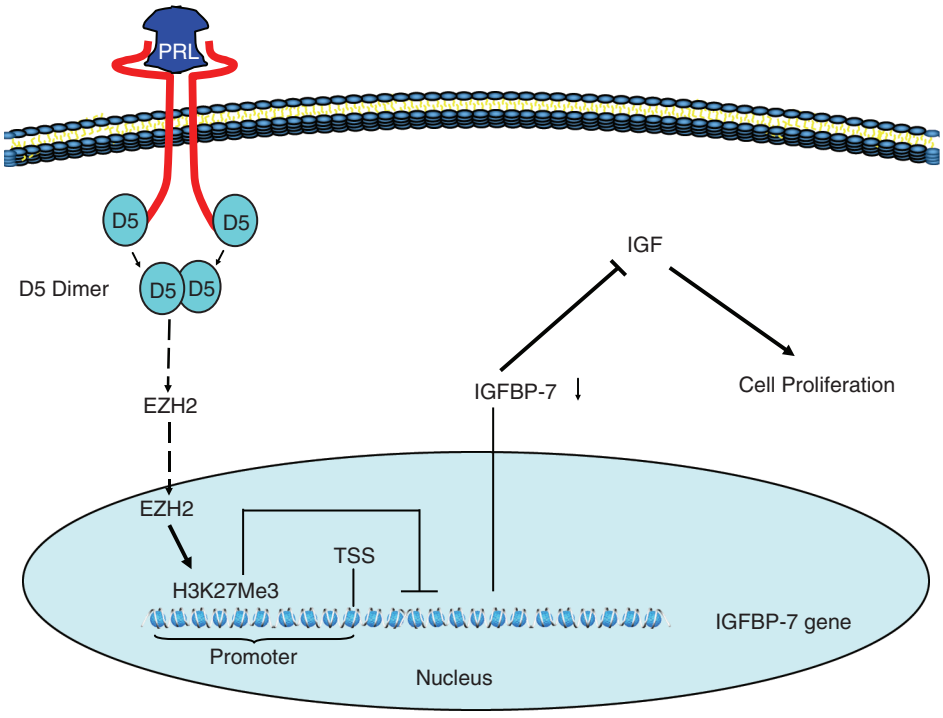

Fig. 6. Schematic signal pathway of D5 Stat5a in regulation of IGFBP-7 expression and breast epithelial cell growth D5, D5 Stat5a; PRL, prolactin; EZH2, enhancer of zeste homology 2; TSS, transcription start site; IGF, insulin-like growth factor.

should be: (i) although our previous work has already proved the mitogenic effect of D5 Stat5a on the breast epithelial, the dose-response has not been performed yet, and (ii) in the previous study, the relative normal cell type MCF-10A was not included in the previous experiment.

In order to elucidate the possible cross talk between D5 Stat5a and IGFBP-7, we examined the effect of overexpression of D5 Stat5a on IGFBP-7 mRNA and protein level. As shown in Fig. 2, both IGFBP-7 mRNA (Fig. 2A) and protein (Fig. 2B) was down-regulated with the exogenous D5 Stat5a expression in the breast epithelial cells. As an important epigenetic covalent modification by so-called Polycomb repressive complex 2 protein (PRC 2), H3K27Me3 plays a critical role in regulation of gene expression (8, 14, 26, 28) and it has been widely accepted that H3K27Me3 acts as a repressive mark (2, 6). Therefore, H3K27Me3 modification is generally associated with low expression, or silencing of a certain gene. The above results lead us to hypothesis that low expression of EZH2 might be related to an epigenetic modification in IGFBP-7 gene. EZH2 has been suggested by several authors to be a marker for cancers including breast cancer (19, 38). We therefore examined the effect of D5 Stat5a overexpression on the H3K27Me3 of IGFBP-7 gene promoter region. As we expected, high trimethylation of IGFBP-7 promoter region was observed (Fig. 3) in both MCF-10A (Fig. 3A) and MCF-7 (Fig. 3B) cells. The DNA fragments immunoprecipitated by antibody against H3K27Me3 is 0.0850 ± 0.0193% and 0.13 ± 0.0199% in endogenous D5 Stat5a expressing MCF-10A and MCF-7 cells respectively, while in the corresponding control cells (no exogenous D5 Stat5a expressed), the numbers are 0.0062 ± 0.002% and 0.0206 ± 0.0020% respectively. To filtrate out the background noise, two measures were taken: (i) in addition to the antibody against H3K27Me3, antibody against IgG was also used to rule out the non-specific binding of antibody protein to the DNA fragment, and (ii) in real time PCR, a pair of primers ("non-specific control primers", see the right part of the Fig. 3, A and B) designed to amplify unrelated genomic DNA from more than 250,000 bp upstream of transcription start site (TSS) of IGFBP-7 gene to rule out the non-specific amplification. Our results clarified the antibody-DNA complex is specific to H3K27Me3 (Fig. 3) and the resultant DNA is specific to the promoter region of IGFBP-7 gene. Interestingly, in control MCF-7 cells, the DNA fragments precipitated with antibody against H3K27Me3 is significantly higher than that with against IgG (Fig. 3B). We interpret this phenomenon as the higher methylation of IGFBP-7 promoter region existing in cancerous epithelial breast cancer cells, and this account for why this phenomenon was not observed in the relatively normal MCF-10A control cells (Fig. 3A). Further, this result stimulated our interest to examine the IGFBP-7 expression in

breast cancer tissue (Fig. 5). In an examination of paired samples of breast cancer tissue and adjacent normal tissue from the same patients, we observed lower IGFBP-7 expression in most of cancer tissues (9/10) versus the paired normal tissues. Our result certified that IGFBP-7 gene promoter might be locked by hypermethylation of histone 3 thereby inhibiting the IGFBP-7 expression. This result is good agreement with what Östlund reported (24).

EZH2 is a silencer of tumor suppressor genes and, therefore, has been accepted as a biomarker of breast cancer (15, 31). Lot of reports including ours suggested EZH2, a methyltransferase of H3K27, is responsible for the H3K27 trimethylation (31). Using real time PCR, we examined the effect of D5 Stat5a on the expression of EZH2 in the cells and higher expression was found in D5 Stat5a overexpressing cells (Fig. 4). And the fact that the higher EZH2 in MCF-control cells than in MCF-10A control cells told us aberration of EZH2 is a characteristics of cancer breast cells and this is good agreement with the result shown in Fig. 3B. D5 Stat5a is an accelerator of EZH2/H3K27Me3.

Taking together, we concluded that D5 Stat5a plays a pathogenic role in breast cancer through, at least partly, increase trimethylation of IGFBP-7 promoter region thereby inhibiting IGFBP-7 expression (Fig. 6). Our data shed light of D5 Stat5a as a potential therapeutic target for basic research and clinic treatment of breast cancer. A lot of work is on the waiting list and lots of questions need to be answered: what is the alternative switch between D5 and full length Stat5a in the transition from normal to cancerous breast epithelial cells? What is the authentic mechanism underlying the expression induced by D5 Stat5a?

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