HER-2/neu transcriptionally activates Jab1 expression via the AKT/ β -catenin pathway in breast cancer cells

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Abstract

Jab1 is a co-activator of activating protein-1 (AP-1) transcription factor and the fifth subunit of the constitutive photomorphogenesis 9 (COP9) signalosome, which has been shown to mediate nuclear exportation and ubiquitin-dependent degradation of the tumor suppressor p27^{Kip1}. Jab1 is overexpressed in several types of human cancer. However, de-regulation of *Jab1* gene expression in cancer cells is largely unclear. In this study, we reported that expression of Jab1 was stimulated by HER-2/neu oncogene via transcriptional activation. Promoter deletion and mutation analysis indicated that HER-2/neu stimulated Jab1 via the T cell factor (TCF) binding site located at the –380/–368 region of the human Jab1 promoter. DNA affinity precipitation assay and chromatin immunoprecipitation assay verified that binding of β -catenin and TCF-4 to this consensus site was increased by HER-2/neu. In addition, dominant-negative mutant of TCF significantly attenuated the stimulatory effect of HER-2/neu. We also demonstrated that HER-2/neu increased β -catenin/TCF-mediated Jab1 expression via the AKT signaling pathway because chemical inhibitor or dominant-negative mutant of AKT effectively attenuated the stimulatory action of HER-2/neu. IGF-I, which is a well-known AKT activator, also up-regulated the expression of Jab1 in NIH/3T3 and MCF-7 cells. Knockdown of Jab1 by small interfering RNA (siRNA) preferentially inhibited proliferation of HER-2/neu-overexpressing breast cancer cells. Taken together, our results suggest that HER-2/neu transcriptionally activates Jab1 expression to promote proliferation of breast cancer cells.

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Introduction

p27^{Kip1} was originally discovered in cells arrested by transforming growth factor- β (TGF- β ; Polyak *et al.* 1994a). Subsequent studies showed that p27^{Kip1} is a typical cyclin-dependent kinase (CDK) inhibitor and a potential tumor suppressor gene (Polyak *et al.* 1994b, Toyoshima & Hunter 1994). Recent investigations demonstrated that down-regulation of p27^{Kip1} protein is frequently found in human cancers including breast, lung, prostate, gastric, skin, colon, and ovarian cancer and is usually correlated with poor clinical outcome (Esposito *et al.* 1997, Porter *et al.* 1997, Ciaparone *et al.* 1998, Florenes *et al.* 1998, Tsihlias *et al.* 1998, Ohtani *et al.* 1999, Newcomb *et al.* 1999). The cellular

abundance of p27^{Kip1} protein is mainly controlled via post-translational regulation. At the G1 phase, p27^{Kip1} was degraded by the Kip1 ubiquitination-promoting complex (KPC) E3 complex consisting of KPC1 and KPC2 (Kamura *et al.* 2004). KPC1 contains a RING-finger domain, and KPC2 contains an ubiquitin-like domain and two ubiquitin-associated domains. KPC interacts with and ubiquitinates p27^{Kip1} and is localized to the cytoplasm. Conversely, degradation of p27^{Kip1} at the G1 to S transition was primarily regulated by the SCF complex. The SCF complex consists of the invariable components Skp1, Cul-1, Cks1, and Rbx1 as well as an F-box protein that binds to Skp1 through its F-box motif and is responsible for

substrate recognition (Podust *et al.* 2000). Phosphorylation of p27^{Kip1} by CDK2 leads to association with the F-box protein Skp2 and induces degradation of this protein via the ubiquitin/proteasome pathway. It is rational to hypothesize that de-regulation of the mediators that involved in the degradation of p27^{Kip1} may result in enhanced p27^{Kip1} proteolysis and tumor formation. Recent works supported this hypothesis and demonstrated that Skp2 is overexpressed in human cancers and is correlated with down-regulation of p27^{Kip1} (Gstaiger *et al.* 2001, Latres *et al.* 2001, Bloom & Pagano 2003).

Another important player in the mediation of p27^{Kip1} degradation is Jab1. This protein was originally identified as a co-activator of AP-1 transcription factor and is an important determiner for the specificity of activation of target genes by AP-1 proteins (Claret *et al.* 1996). Subsequent studies found that Jab1 is also the fifth component of the COP9/signalosome complex (Seeger *et al.* 1998, Wei *et al.* 1998). Recently, a new biological function of Jab1 has been suggested. Tomoda *et al.* (1999) showed that Jab1 physically interacted with p27^{Kip1} and enhanced its cytoplasmic translocation, which resulted in acceleration of p27^{Kip1} degradation via the ubiquitin/proteasome pathway. Study of primary tumor tissues indeed demonstrated that expression of p27^{Kip1} and Jab1 is inversely correlated in several types of human cancer (Shen *et al.* 2000, Sui *et al.* 2001, Kouvaraki *et al.* 2003). However, the mechanism of Jab1 de-regulation in cancer cells is largely unknown.

The HER-2/neu oncogene (also known as erbB2) encodes a trans-membrane glycoprotein, which belongs to the human epidermal growth factor receptor family (Schechter *et al.* 1984, 1985). Amplification and over-expression of HER-2/neu was found in numerous cancers and up-regulation of this oncogene was associated with increased metastasis and poor prognosis. Recent studies have uncovered several signaling pathways including the phosphatidylinositol-3-OH kinase (PI-3K)/AKT and the mitogen-activated protein kinase (MAPK) are critical mediators for the oncogenic activity of HER-2/neu. Recently, two studies demonstrated that HER-2/neu induces down-regulation of p27^{Kip1} via different mechanisms (Yang *et al.* 2000, Lenferink *et al.* 2001). First, HER-2/neu acted through AKT and glycogen synthase kinase-3 β (GSK-3 β) to reduce p27^{Kip1} protein level. GSK-3 β may phosphorylate cyclin D1 and induce degradation of this protein. Activation of AKT by HER-2/neu inhibits GSK-3 β activity and increases the formation of cyclin D1/CDK4 complex which may sequester p27^{Kip1} in the cytoplasm to enhance its turnover (Lenferink *et al.* 2001). Secondly, HER-2/neu may induce the association of Jab1 and p27^{Kip1}, thereby facilitating nuclear

exportation and degradation of p27^{Kip1} protein (Yang *et al.* 2000). In this study, we investigated whether HER-2/neu may directly regulate Jab1 expression and tried to elucidate the underlying mechanism.

Materials and methods

Cell culture and experimental reagents

NIH/3T3 and B104-1-1, a cell line derived from NIH/3T3 cells, which exhibited constitutive HER-2/neu tyrosine kinase activity due to overexpression of oncogenic HER-2/neu (Yu & Hung 1991), were kindly provided by Dr Lai MD (National Cheng Kung University, Taiwan). MCF-7 and MDA-MB-453 cells were obtained from the cell bank of the National Health Research Institute (Miaoli, Taiwan). Cells were cultured in DMEM/F12 medium containing 10% fetal calf serum (FCS) and antibiotics. Luciferase assay system, Erase-a-base system, and pGL3 reporter plasmid were obtained from Promega. Constitutively active HER-2/neu expression vector was provided by Dr Hung MC (MD Anderson Cancer Center, USA). Human β -catenin expression vector was obtained from Dr Wang CH (Kaohsiung Medical University, Taiwan). Dominant-negative ERK2 expression vector was obtained from Dr Lin WW (National Taiwan University, Taiwan). Constitutively active and dominant-negative AKT vectors were provided by Dr Kuo ML (National Taiwan University, Taiwan). Dominant-negative TCF-4 expression vector was from Dr Tsao DA (Fooyin University, Taiwan). All of these expression vectors had been checked by DNA sequencing. One-Step RT-PCR kit was from Qiagen. PD98059 and wortmannin were from Tocris (Northpoint, UK). Insulin-like growth factor-I (IGF-I) was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA).

Isolation of Jab1 promoter and construction of deletion and mutant reporter plasmids

The human Jab1 5'-untranslated region was identified by using the BLAST server at the NCBI and the human Jab1 cDNA sequence (accession number NM 006837) as a query. Prediction of putative transcription factor binding sites was performed by the Match TM program (www.gene-regulation.com/pub/programs), which uses TRANSFAC 5.0 matrices. A genomic fragment containing the 5' upstream region of the *Jab1* gene was isolated by PCR amplification from genomic DNA of A549 human lung cancer cells. The primers used are: 5'-AATGGGGAGGGGTGGTAGTG-3' (forward) and 3'-CTTGCTGCTGTTTCTTGTCG-5' (reverse). The DNA fragment was subcloned into the pGL3

basic reporter plasmid and the nucleotide sequence of the clone was sequenced. The sequence matched perfectly to the 5' upstream sequence of the human Jab1 cDNA located on chromosome 8 and contained the human Jab1 promoter region (1965 bp upstream of the transcription start site). This plasmid was named as pJab1965 and was used as a template to create a series of deletion reporter plasmids. pJab1433 and pJab405 reporter plasmids containing 1433 and 405 bp upstream of the transcription start site were generated by using the Erase-a-base system according to the procedures of manufacturer (Promega). pJab223 and pJab32 reporter plasmids were created by a PCR approach and primers that were flanked by *SacI* and *XhoI* restriction sites. The resulting fragments were then subcloned into the *SacI* and the *XhoI* sites of the pGL3 reporter plasmid. The sequence of these deletion mutants were confirmed by DNA sequencing. Mutations were introduced into the putative Sp1 and TCF binding sites by the QuikChange Site-directed mutagenesis kit according to the procedures of manufacturer (Stratagene). The sequences of the primers used for mutagenesis are: 5'-CCCACAAAA-CACTTTAT**ACCCTCCACATCCCGC**-3' for SP1 and 5'-CAAAC TTATCTC**ACCGCAGGTACCTA-TACCC**-3' for TCF. The mutated nucleotides were indicated as boldface.

Promoter activity assay

Promoter activity of Jab1 gene was analyzed as described previously (Liu *et al.* 2003). In brief, cells were plated onto six-well plates at a density of 100 000 cells per well and grown overnight. Cells were co-transfected with 2 µg full-length, deletion or mutant Jab1 promoter–luciferase constructs and 1 µg CMV-β-galactosidase plasmid. After transfection, cells were incubated in medium containing 10% FCS for 48 h and luciferase activity was determined and normalized for β-galactosidase activity. For some experiments, expression vectors of HER-2/neu, β-catenin, dominant-negative mutant of ERK2, AKT, and TCF-4 were co-transfected with Jab1 promoter–luciferase construct and promoter activity was assayed at 48 h after transfection.

RNA extraction and RT-PCR and real time quantitative PCR

Total RNA was isolated from cells and Jab1 expression was examined by using One-Step RT-PCR kit as described previously (Liu *et al.* 2003). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an

internal control to check the efficiency of cDNA synthesis and PCR amplification. cDNA synthesis was performed at 50 °C for 30 min and the condition for PCR was 25 cycles of denaturation (94 °C/1 min), annealing (60 °C/1 min), extension (72 °C/1 min), and one cycle of final extension (72 °C/10 min). The primers used were: Jab1-forward, 5'-ATGCAGGAA GCGCAGAGTAT-3'; Jab1-reverse, 5'-ACCCATCACTTCCAAGTTGC-3'; GAPDH-forward, 5'-GAGTCAACGGATTGGTC-GT-3'; and GAPDH-reverse, 5'-TGTGGTCATGAGTCCTTCCA-3'. After reaction, PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under u.v. light. In some experiments, real time PCR was performed to investigate the alteration of Jab1 expression using SYBR green PCR method. The primer sequences are: 5'-CAAGAAA-CAGCAGCAAGAAATCC-3' (forward) and 5'-AGC-ATCAGACCC ATCACTTCC-3'.

Western blot analysis

After treatments, cells were harvested in a lysis buffer and equal amount of proteins was subjected to SDS-PAGE as described previously (Hsu *et al.* 2006). Proteins were transferred to nitrocellulose membranes and the membranes were probed with different primary antibodies. Enhanced chemiluminescence reagents were used to depict the protein bands on the blots. Jab1, p27^{Kip1}, TCF-4, and β-catenin antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Anti-HER-2/neu antibody was from Calbiochem (Cambridge, MA, USA) and anti-actin antibody was obtained from Chemicon (Temecula, CA, USA). COPS3 antibody was purchased from Abcam (Cambridge, UK).

DNA affinity precipitation assay (DAPA)

We used biotinated DNA probe 5'-AAACTTATCT-CATTTAAGGTACCTATACCC-3' corresponding to the –388/–359 region of human Jab1 promoter to interact with nuclear proteins and precipitated the DNA-protein complex by streptavidin-coated beads. After centrifugation, the beads were washed and proteins were eluted by SDS-PAGE sample buffer. The binding proteins were separated by 7.5 or 12% polyacrylamide gels and analyzed by immunoblotting. Preparation of nuclear proteins and DAPA assays were performed as described previously (Hsu *et al.* 2006).

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with 1% formaldehyde at 37 °C for 10 min. Cells were washed twice with ice cold PBS with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 µg/ml aprotinin, and 1 µg/ml pepstatin A), scraped, and pelleted by centrifugation at 4 °C. Cells were resuspended in a lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), incubated for 10 min on ice, and sonicated to shear DNA. After sonication, lysate was centrifuged for 10 min at 10 000 *g* at 4 °C. The supernatant was diluted in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, and protease inhibitors). TCF-4 or β -catenin antibody was added to the supernatant and incubated overnight at 4 °C with rotation. The immunocomplex was collected by protein A/G agarose and washed sequentially with low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl, pH 8.1, and 500 mM NaCl), LiCl washing buffer (0.25 M LiCl, 1% NP40, 1% deoxycolate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1), and finally 1X TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). The immunocomplex was eluted by elution buffer (1% SDS, 0.1 M NaHCO₃, and 200 mM NaCl) and the cross-links were reversed by heating at 65 °C for 4 h. After reaction, the samples were adjusted to 10 mM EDTA, 20 mM Tris-HCl, pH 6.5, and incubated with proteinase K (40 µg/ml) at 45 °C for 1 h. DNA was recovered and subjected to PCR amplification by the primers specific for the detection of the TCF site in the *Jab1* promoter. The sequences for the primers are: sense 5'-CCGTTAGCTTTTACGCATGT-3' and anti-sense 5'-TGTGTGGGTATAGGTACCTTAAATGA-3'. The amplified sequence (–525/–347 bp from transcription start site) contained the TCF site localized at the –380/–368 bp region of the *Jab1* promoter.

siRNA treatment and cell growth assay

Jab1 siRNA 5'-AAGACCCAGCCCUUGCCUCAA-3' (sense strand) and a nonspecific RNA 5'-AAC-GUUGCGAUAGCGUAGUAC-3' (sense strand) were synthesized (Dharmacon Research Inc., Huston, TX, USA). MDA-MB-453 and MCF-7 cells were transfected with control or *Jab1* siRNA by using the LipofectAMINE reagent. Cells were received a second transfection after 12 h and maintained in 10% FCS medium for another 60 h. Effect of siRNA on cell growth was studied by trypan blue exclusion test as described previously (Hung et al. 2000).

Statistical analysis

The association between the expression of *Jab1* and *HER-2/neu* or other tumor characteristics was assessed using Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Results

Induction of *Jab1* by *HER-2/neu*

First we used NIH/3T3 and B104-1-1, a stable cell line derived from NIH/3T3 cells, which exhibited constitutive *HER-2/neu* tyrosine kinase activity due to enforced expression of oncogenic *HER-2/neu* cells as a model to investigate the effect of *HER-2/neu* on *Jab1* expression. In consistence with the data of a previous study (Yang et al. 2000), we found that p27^{Kip1} protein level was significantly reduced in B104-1-1 cells (Fig. 1A). On the contrary, protein level of *Jab1* was increased in B104-1-1 cells. To verify whether other subunits of COP9 signalosome were also affected by *HER-2/neu*, we investigated the protein level of the third subunit COPS3 and found that COPS3 was up-regulated in B104-1-1 cells. These data suggested that *HER-2/neu* simultaneously increased free *Jab1* (which involved in p27 degradation) and the entire COP9 signalosome complex (which implicated in signal transduction). We first addressed the molecular mechanism by which *HER-2/neu* controls *Jab1* expression. We performed RT-PCR analysis and found that an average 2.0-fold increase of *Jab1* mRNA level was seen in B104-1-1 cells (Fig. 1B). To investigate the mechanism by which *HER-2/neu* stimulated *Jab1* expression, we isolated the 5'-flanking region of human *Jab1* gene using PCR amplification of genomic DNA of A549 human lung cancer cells. The nucleotide sequence of the clone matched perfectly to the 5' upstream sequence of the human *Jab1* cDNA located on chromosome 8. The sequence is shown in Supplementary Fig. 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. When pJab1965 promoter-luciferase construct was transfected into NIH/3T3 and B104-1-1 cells, a 2.4-fold increase of promoter activity was detected in B104-1-1 cells (Fig. 1c). To rule out that this increase may be due to the difference of transfection efficiency between these two cell lines, pJab1965 construct was co-transfected with constitutively active *HER-2/neu* or control vector into NIH/3T3 cells. Our data demonstrated that *Jab1* promoter activity of cells co-transfected with constitutively active *HER-2/neu* was also 2.3-fold higher than that of cells

Figure 1 Up-regulation of Jab1 expression by HER-2/neu. (A) NIH/3T3 (N) and B104-1-1 (B) cells cultured in 10% FCS medium were harvested for western blot analysis. Equal amount of proteins was subjected to SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with HER-2/neu, Jab1, COPS3, and p27^{Kip1} antibodies, and enhanced chemiluminescence reagent was used to depict the protein bands on the blots. (B) Total RNA was extracted from NIH/3T3 and B104-1-1 cells. Expression of Jab1 was investigated by RT-PCR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. The signal intensity ratio of Jab1/GAPDH in NIH/3T3 was defined as 1 and the relative ratio of B104-1-1 cells was shown. (C) The pJab1965 promoter–luciferase plasmid was co-transfected with CMV- β -galactosidase plasmid into NIH/3T3 (N) or B104-1-1 (B) cells. After transfection, cells were incubated in medium containing 10% FCS for 48 h and luciferase activity was determined and normalized for β -galactosidase activity. (D) pJab1965 was co-transfected with control (C) or constitutively active HER-2/neu expression vector into NIH/3T3 cells. Luciferase activity was determined at 48 h after transfection. The expression of HER-2/neu in transfected cells was also investigated by western blot analysis.

co-transfected with control vector (Fig. 1d). Collectively, these results suggested that HER-2/neu stimulates Jab1 via transcriptional activation.

HER-2/neu activates Jab1 via the TCF binding site in the promoter

Computational analysis of the promoter indicated that several transcription factor binding sites including TCF, SP1, AP1, and STAT were found in the Jab1 promoter region. We investigated the critical elements that mediated the stimulatory effect of HER-2/neu on Jab1 promoter by deletion mutants. Our data showed that pJab1433 and pJab405 were fully responsive to HER-2/neu (Fig. 2a). Conversely, pJab223 and pJab32 were not activated by HER-2/neu. When NIH/3T3 cells were co-transfected with pJab405, pJab223, and HER-2/neu expression vector, we also found that HER-2/neu stimulated pJab405, but not pJab223, promoter activity

(Fig. 2b). Thus, we concluded that HER-2/neu activates Jab1 via the –405/–223 region of the promoter. Computational prediction showed that a potential TCF site (–380/–368) and a SP1 site (–347/–335) were found in this region. We mutated these two transcription factor binding sites to create pJab405mTCF and pJab405mSP1 mutants and tested the promoter activity of these mutants in NIH/3T3 and B104-1-1 cells. Our results showed that mutation of TCF or SP1 site significantly reduced basal Jab1 promoter activity indicating the importance of these sites in the control of basal Jab1 expression (Fig. 2c). However, mutation of SP1 site did not affect HER-2/neu-induced Jab1 promoter activity. On the contrary, HER-2/neu could not activate the promoter activity of pJab405mTCF mutant. These data suggested that up-regulation of Jab1 promoter activity by HER-2/neu is mediated via the TCF site located at the –380/–368 region of the Jab1 promoter. We next performed DAPA assay to identify

Figure 2 HER-2/neu stimulates Jab1 expression via the TCF site in the promoter region. (A) Deletion mutants of Jab1 promoter were co-transfected with CMV- β -galactosidase plasmid into NIH/3T3 (N) or B104-1-1 (B) cells. After transfection, cells were incubated in medium containing 10% FCS for 48 h. Luciferase activity was determined and normalized for β -galactosidase activity. (B) Deletion constructs pJab405 and pJab223 were also co-transfected with control (C) or constitutively active HER-2/neu (H) expression vector into NIH/3T3 cells. Luciferase activity was determined at 48 h after transfection. (C) Mutations were introduced into the SP1 (–347/–335) and TCF (–380/–368) binding sites to create the pJab405mSP1 and pJab405mTCF mutants. pJab405, pJab405mSP1, or pJab405mTCF were transfected into NIH/3T3 (N) and B104-1-1 (B) cells and luciferase activity was measured at 48 h after transfection.

the nuclear proteins that interacted with this TCF site. As shown in Fig. 3a, we found that TCF-4 and β -catenin bound to the DNA probe and the binding affinity of these two proteins was increased in B104-1-1 cells. Binding of TCF-4 and β -catenin to the DNA probe corresponding to the –388/–359 region of human Jab1 promoter is specific because these two proteins did not bind to the mutant DNA probe (Fig. 3a). We next performed ChIP assay to verify the results of DAPA assay and our data confirmed that the binding of TCF-4 and β -catenin to the Jab1 promoter was increased *in vivo* (Fig. 3b). These results suggested that HER-2/neu induced the formation of the β -catenin/TCF-4 complex and increased the

binding of this complex to the TCF site of the Jab1 promoter. To confirm the functional role of TCF-4 and β -catenin in the induction of Jab1 by HER-2/neu, we transfected dominant-negative mutant of TCF-4 into B104-1-1 cells and found that this mutant potently inhibited Jab1 promoter activity (Fig. 3c). In consistent with this result, quantitative PCR demonstrated an average 85–90% of reduction of Jab1 mRNA in cells transfected with dominant-negative TCF-4 (Fig. 3c). Also, the protein level of Jab1 was significantly attenuated. Conversely, ectoexpression of β -catenin stimulated Jab1 promoter activity and protein expression in NIH/3T3 cells (Fig. 3d). Our results suggested that HER-2/neu stimulates the Jab1 promoter via the TCF site.

AKT is involved in HER-2/neu-induced Jab1 expression

Previous studies have shown that MAPK and AKT signaling pathways were involved in the down-regulation of p27^{Kip1} by HER-2/neu (Lenferink *et al.* 2001, Yang *et al.* 2000). Therefore, we tested whether these two pathways participated in the activation of Jab1 by HER-2/neu. B104-1-1 cells were transfected with pJab405 plasmid and treated with PD98059 (10 μ M) or wortmannin (200 nM) for 48 h. Promoter activity was then assayed. As indicated in Fig. 4a, wortmannin, but not PD98059, inhibited HER-2/neu-induced Jab1 promoter activity and expression in B104-1-1 cells. Co-transfection assay also showed that dominant-negative AKT, but not dominant-negative ERK2, blocked HER-2/neu-induced Jab1 promoter activity and expression in B104-1-1 cells (Fig. 4b). These results strongly suggested that HER-2/neu stimulates Jab1 expression via the AKT signaling pathway. Since our aforementioned results indicated that TCF-4 and β -catenin were involved in the induction of Jab1 by HER-2/neu, we tested whether AKT acts via TCF-4 and β -catenin to activate Jab1. We expressed constitutively active (myristoylated form) AKT in NIH/3T3 cells and found that AKT activated Jab405 and Jab405mSP1 (Fig. 5a). On the contrary, myristoylated AKT could not stimulate Jab405mTCF. DAPA assay also showed that the binding of TCF-4 and β -catenin to the DNA probe corresponding to the TCF site in the Jab1 promoter was increased by AKT (Fig. 5b). Conversely, dominant-negative TCF-4 abolished the induction of Jab1 promoter activity by AKT indicating that β -catenin/TCF-4 complex is a downstream mediator for AKT-activated Jab1 expression (Fig. 5c). To verify that activation of AKT may up-regulate Jab1, we used IGF-I, a well-

Figure 3 Binding of β -catenin/TCF-4 complex to Jab1 promoter is stimulated by HER-2/neu. (A) One hundred microgram of nuclear proteins extracted from NIH/3T3 (N) or B104-1-1 (B) cells were incubated with the biotinylated wild type or mutant DNA probe corresponding to the -388/-359 region of human Jab1 promoter and streptavidin-coated beads. Beads were pelleted and washed thrice with cold phosphate-buffered saline (PBS). Proteins bound to the beads were eluted and separated by SDS-PAGE. Western blotting was performed to investigate the amount of TCF-4 and β -catenin binding to DNA probe. (B) ChIP assays were performed by using TCF-4 and β -catenin antibodies to precipitate the protein-DNA complex. PCRs were performed to amplify the -525/-347 region of the Jab1 promoter. (C) Control (C) or dominant-negative TCF (DN-TCF) expression vector was co-transfected with pJab405 promoter-luciferase construct into B104-1-1 cells and luciferase activity was measured at 48 h after transfection. * $P < 0.05$. The relative expression of Jab1 was investigated by real time PCR method and Jab1 protein level was also examined by western blotting. To verify the expression of DN-TCF-4, western blot analysis was performed and we detected the endogenous TCF-4 (endo) and HA-tagged DN-TCF-4. (D) NIH/3T3 cells were transfected with pJab405 and control (C) or β -catenin expression vector, and luciferase activity was measured 48 h after transfection. The Jab1 protein level was also examined by western blotting.

known AKT activator, to treat NIH/3T3 and MCF-7 cells which expressed low level of HER-2/neu and investigated the expression of Jab1. As shown in Fig. 5d, IGF-I indeed increased Jab1 expression in these two cell lines. Taken together, our data suggested that HER-2/neu up-regulates Jab1 expression via the AKT/ β -catenin/TCF-4 signaling pathway.

HER-2/neu induces Jab1 via the AKT/ β -catenin/TCF-4 signaling pathway in human breast cancer cells

We have successfully used the stable NIH/3T3 transfectants to address the molecular mechanism by which HER-2/neu stimulated Jab1 expression.

Figure 4 Involvement of AKT in HER-2/neu-induced Jab1 expression. (A) B104-1-1 cells were transfected with pJab405 reporter plasmid and incubated with vehicle (~0.05% DMSO), PD98059 (PD, 10 μ M), or wortmannin (Wor, 200 nM) for 48 h. Promoter activity was then assayed. The effect of inhibitors on Jab1 expression was also examined by western blotting. ERK2 and AKT kinase activities were studied by detecting the phospho-ERK2 (P-ERK2) and phospho-AKT (P-AKT). (B) B104-1-1 cells were co-transfected with pJab405 reporter plasmid and control (C), dominant-negative ERK2 (DN-ERK2), or AKT (DN-AKT) expression vector. Promoter activity and Jab1 protein level were assayed at 48 h after transfection. Jab1 protein level was examined by western blotting. * $P < 0.05$.

We next studied whether HER-2/neu activates Jab1 via the same signaling pathway in human breast cancer cells. As shown in Fig. 6a, MDA-MB-453 breast cancer cells which exhibited HER-2/neu overexpression had higher protein level of Jab1 than that of MCF-7 cells which expressed low level of HER-2/neu. In agreement with the results of B104-1-1 cells, our data also demonstrated that COPS3 was up-regulated in MDA-MB-453 cells (Fig. 6a). To exclude the possibility that the difference of Jab1 expression in MCF-7 and MDA-MB-453 is due to the different genetic background of these two cell lines, we transfected constitutively active HER-2/neu into MCF-7 cells and our data clearly demonstrated that Jab1 expression was up-regulated by HER-2/neu (Fig. 6a). Promoter deletion or mutation assays suggested that HER-2/neu activated Jab1 via the TCF binding site in MDA-MB-453 cells (Fig. 6b). Ectoexpression of dominant-negative TCF-4 also repressed the Jab1 promoter activity in this cell line (Fig. 6c). In addition, wortmannin also inhibited HER-2/neu-induced Jab1 promoter activity and expression in MDA-MB-453 cells (Fig. 6d). These results indicated that HER-2/neu also acts via the AKT/ β -catenin/TCF-4 signaling pathway to activate Jab1 in human breast cancer cells.

Knockdown of Jab1 preferentially inhibits proliferation of HER-2/neu-overexpressing breast cancer cells

To address the importance of Jab1 in HER-2/neu-induced cell growth, we suppressed the expression of Jab1 in MDA-MB-453 and MCF-7 cells by small interfering RNA (siRNA) and examined its effect on cell proliferation. Our results showed that our designed siRNA effectively down-regulated Jab1 protein level in both breast cancer cell lines (Fig. 7a). Knockdown of Jab1 significantly inhibited the proliferation of MDA-MB-453 cells, while Jab1 siRNA only marginally affected the proliferation of MCF-7 cells (Fig. 7b).

Discussion

Originally identified as a co-activator of AP-1 transcription factor, Jab1 has now been shown to exhibit multiple functions. One of the biological actions of Jab1 is to induce p27^{Kip1} degradation by facilitating nuclear exportation of p27^{Kip1} (Wei et al. 1998). These data suggest that Jab1 may play a critical role in the down-regulation of p27^{Kip1} in cancer cells. Pathological investigations indeed demonstrated an inverse expression of Jab1 and p27^{Kip1} in various types of human cancer (Shen et al. 2000, Sui et al. 2001,

Figure 5 AKT activates JAB1 expression via the TCF site in the promoter. (A) NIH/3T3 cells were co-transfected with various Jab1 promoter–luciferase constructs and control (C) or constitutively active AKT (A) expression vector for 48 h. Promoter activity was assayed. (B) NIH/3T3 cells were transfected with control (C) or constitutively active AKT (A) expression vector. After 48 h, nuclear extracts were harvested and DAPA assays were performed to investigate the binding of TCF-4 and β -catenin to the double DNA probe corresponding to the $-388/-359$ region of human Jab1 promoter. (C) NIH/3T3 cells were co-transfected with pJab405 and control (C), constitutively active AKT expression vector, pcDNA, or dominant-negative TCF (DN-TCF) expression vector. Promoter activity was assayed at 48 h after transfection. (D) NIH/3T3 and MCF-7 cells were treated without (–) or with (+) insulin-like growth factor-I (IGF-I, 10 ng/ml) for 24 h. RT-PCR was performed to investigate the effect of IGF-I on Jab1 expression.

Kouvaraki *et al.* 2003). However, regulation of Jab1 expression in normal and malignant cells is largely unknown. In this study, we provide the first evidence that HER-2/neu oncogene may stimulate Jab1 expression via transcriptional activation. Our conclusion is also supported by a previous study showing that inhibition of HER-2/neu by specific antibody, Herceptin, attenuated Jab1 expression (Le *et al.* 2005). Interestingly, we also found that the expression of another subunit of COP9 signalosome COPS3 was up-regulated by HER-2/neu. These results suggest that HER-2/neu may act via increase of 1) free Jab1 expression to enhance the nuclear exportation and degradation of p27, and 2) the assembly of entire COP9 signalosome to promote the activation of downstream signaling pathways. These two mechanisms may both play important roles in HER-2/neu-induced tumorigenesis.

We cloned the promoter region of human *Jab1* gene and found that a number of transcription factor binding

sites are located within this region. Indeed, we demonstrated for the first time that Jab1 expression could be regulated by HER-2/neu via the AKT/ β -catenin/TCF-4 signaling pathway. The AKT signaling pathway can activate the transcription of *Jab1* gene, which is also supported by the observation that a potent AKT activator IGF-I may stimulate Jab1 expression in NIH/3T3 and MCF-7 cells which express low level of HER-2/neu. Recent studies have identified several target genes, whose expressions were regulated by the same signaling pathway. For example, the activation of AKT increased β -catenin, a co-activator of androgen receptor, which resulted in enhanced androgen receptor activation (Sharma *et al.* 2002). Tissue-type plasminogen activator had also been shown to activate cyclin D1 via AKT/TCF/ β -catenin-dependent pathway (Maupas-Schwalm *et al.* 2005). AKT may enhance β -catenin-mediated gene transcription via two mechanisms. First, AKT can phosphorylate GSK-3 β

Figure 6 HER-2/neu stimulates Jab1 expression via the AKT/ β -catenin signaling pathway in human breast cancer cells. (A) MCF-7 (MCF) and MDA-MB-453 (453) human breast cancer cells cultured in 10% FCS medium were harvested for analysis of Jab1 by western blotting. (B) Various Jab1 promoter-luciferase construct was transfected into MDA-MB-453 cells and luciferase activity was assayed at 48 h after transfection. (C) MDA-MB-453 cells were co-transfected with pJab405 and control (C) or dominant-negative TCF (DN-TCF) expression vector for 48 h, and luciferase activity was then determined. The luciferase activity of cells transfected with control vector was defined as 100%. * $P < 0.05$. (D) MDA-MB-453 cells were transfected with pJab405 reporter plasmid and treated with 0.05% DMSO (C), PD98059 (PD, 10 μ M), or wortmannin (Wor, 200 nM) for 48 h. Promoter activity was then assayed. The Jab1 protein level in cells treated with drugs was also investigated by western blotting. * $P < 0.05$.

and inhibit its enzymatic activity (Diehl *et al.* 1998, Liang & Slingerland 2003). Since GSK-3 β may directly phosphorylate β -catenin and induce its degradation via the ubiquitin/proteasome pathway, inhibition of GSK-3 β by AKT induces nuclear accumulation of β -catenin and enhancement of TCF/ β -catenin-mediated gene transcription (Desbois-Mouthon *et al.* 2001, Skurk *et al.* 2005). Secondly, a recent study demonstrated that AKT may directly phosphorylate β -catenin to enhance its transcriptional activity (Tian *et al.* 2004). Whether HER-2/neu induces Jab1 expression via the GSK-3 β -dependent pathway is currently unknown. Future works are needed to address this issue.

Using siRNA, we clearly demonstrated that Jab1 is an important effector for HER-2/neu to promote cell growth. In addition, we found that breast cancer cells with high Jab1 expression were more sensitive to siRNA treatment although a marginal effect on cell proliferation was also observed in MCF-7 cells (Fig. 6b). In addition to induce the down-regulation of p27^{Kip1}, Jab1 also mediates the proteolysis of several intracellular molecules which play critical roles in tumorigenesis. First, Jab1 may trigger the proteolysis of the tumor suppressor p53 and exhibit tumor promoting activity in cells (Bech-Otschir *et al.* 2001). Secondly, Jab1 interacts directly with Smad4 and induces its degradation via the ubiquitin/proteasome

(A)

(B)

Figure 7 Knockdown of Jab1 preferentially inhibits proliferation of HER-2/neu-overexpressing breast cancer cells. (A) MDA-MB-453 and MCF-7 breast cancer cells were transfected with control (C) or Jab1 specific siRNA as described in Materials and Methods. After 72 h, cells were harvested for analysis of Jab1 by western blotting. (B) MDA-MB-453 and MCF-7 cells were transfected with siRNA for 72 h and viable cell number was counted by trypan blue exclusion test. The number of cells received the transfection of control siRNA was defined as 100%. * $P < 0.05$.

pathway which leads to inhibition of TGF- β -induced gene transcription (Wan *et al.* 2002). Since TGF- β exhibits anti-cancer activity in the early stages of tumorigenesis, inhibition of TGF- β signaling pathway by Jab1 may promote tumor formation. Third, Jab1 binds hypoxia inducible factor-1 (HIF-1), prevents its degradation, and enhances its transcriptional activity (Bae *et al.* 2002). Up-regulation of HIF-1 by Jab1 in turn stimulates the expression of vascular endothelial growth factor (VEGF), a major HIF-1 target, to enhance tumor angiogenesis (Bae *et al.* 2002). Taken together, our data suggest that Jab1 may be a rational target for the treatment of breast cancers with HER-2/neu overexpression.

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