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Modified p27 Kip1 is Efficient in Suppressing HER2-Mediated Tumorigenicity

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Abstract Cyclin-dependent kinase (CDK) inhibitor p27 Kip1, a haplo-insufficient tumor suppressor, is downregulated by oncogenic signal of HER2, a receptor tyrosine kinase oncogene. HER2 promotes mitogenic growth and transformation of cancer cells. HER2 signaling can enhance p27 Kip1 ubiquitination, thereby promoting p27 degradation and subsequent activation of CDK activity. p27 ubiquitination and degradation is enhanced by JAB1 binding as well as by phosphorylation on Thr187. In this study, we generated modified p27 proteins, which are mutated at Thr 187 or deleted at JAB1 binding domain. We applied these modified p27 genes as novel anticancer agents for HER2-overexpressing cells under the control of a tetracycline (tet)-regulated gene expression system. Induction of p27 T187A and p27 T187A ΔJAB inhibits HER2-activated cell growth, CDK2 activity, cell proliferation, and transformation. Significantly, a modified protein (p27 T187AΔJAB) reduced the tumor volume in a HER2-overexpressing tumor model efficiently. These findings demonstrate the applicability of employing modified p27 proteins as a therapeutic intervention in HER2-overexpressing cancers. J. Cell. Biochem. 98: 128–138, 2006.

Key words: p27; HER2; JAB1; tumor suppression; SKP2

Amplification or overexpression of the HER2/neu oncogene (also named *c-erbB2* and abbreviated as HER2) is frequently found in various types of cancer, including breast, ovarian, lung, gastric, and oral cancers [Slamon et al., 1987; Hung, 1995]. Importantly, HER2 overexpression has been associated with shorter survival in cancer patients [Slamon et al., 1987]. p27 Kip1, a CIP/KIP member, encodes a CKI (cyclin-dependent kinase inhibitor) that causes G1 arrest by inhibiting the activities of G1 cyclin-CDK [Polyak et al., 1994]. Reduced expression of p27 is frequently detected in human cancers, and has been shown to correlate with cancer development and poor survival, thus appearing as an important marker of cancer progression [Clurman and Porter, 1998]. In addition, p27 is defined as a new class of tumor suppressor and is haplo-insufficient in tumor suppression [Fero et al., 1998]. We previously showed that HER2 activity specifically causes the decrease of p27 protein level by inducing the mislocation of p27 in the cytoplasm for enhanced ubiquitin-mediated degradation [Yang et al., 2000], thus presenting potential points for therapeutic intervention in HER2-associated cancers.

JAB1, a p27-associated protein and a p27 nuclear exporter, is regulated by HER2 oncogenic activity to facilitate p27 degradation [Yang et al., 2000]. JAB1 expression is inversely correlated with p27 expression in several types

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of cancer [Esteva et al., 2003; Kouvaraki et al., 2003; Rassidakis et al., 2003; Shintani et al., 2003]. Also, JAB1 overexpression is associated with a poor prognosis for cancer patients [Sui et al., 2001]. In addition, a JAB1 knockout mouse study also clearly demonstrated that p27 is elevated in JAB1−/− embryo [Tomoda et al., 2004], providing evidence that JAB1 is a critical regulator involved in p27 degradation. These data therefore indicate a link between JAB1 overexpression and p27 downregulation in cancers. It is known that p27 is degraded through ubiquitination. Ubiquitin-mediated destruction of regulatory proteins is a frequent means of controlling cell cycle progression. The process involves E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) for the addition of ubiquitin onto a specific substrate, thereby facilitating substrate degradation by the 26S proteasome. The specific E3 involved in p27 degradation is the SCF^{SKP2}-ROC1 complex, consisting of Skp1, CUL1, ROC1, and a distinct F box protein, Skp2, which acts as substrate-recognition component. Skp2 is required for the ubiquitination and consequent degradation of p27 [Carrano et al., 1999]. It was further noted that Skp2 binds to phosphorylated p27, which is phosphorylated on Thr 187 by CDK2, and this facilitates the ubiquitination of p27 [Carrano et al., 1999]. In animal studies, Skp2 knockout mice were found to grow more slowly and have smaller organs than were controls [Nakayama et al., 2000]. Also, high levels of p27 were found in Skp2-deficient cells, which demonstrated polyploidy and centrosome over-duplication [Nakayama et al., 2000]. Thus, loss of Skp2 in the mouse leads to changes in cell proliferation. It was further shown that the loss of p27 almost completely rescued the defects observed in Skp2 (−/−) mice [Nakayama et al., 2004], suggesting that p27 is the principal target of the SCF^{Skp2} ubiquitin ligase. In addition, Skp2 is involved in several oncogenic signals [Kossatz et al., 2004; Nakayama and Nakayama, 2005]. There is inverse relation between levels of Skp2 and p27 in many types of cancer, including breast cancer [Signoretti et al., 2002], colon cancer [Hershko et al., 2001], lymphoma [Lim et al., 2002], and gastric cancer [Masuda et al., 2002]. Taken together, Skp2 overexpression leads to p27 downregulation in cancer. Here we generated modified p27 proteins, which are mutated at Thr 187 or deleted at JAB1 binding domain. We examined the effectiveness of using these modified p27 proteins as a therapeutic agent for HER2-overexpressing cancer under a gene expression system regulated by tet [Gossen and Bujard, 1992]. We found that modified p27 proteins inhibited the kinase activity of CDK2 and caused cell cycle arrest at G1 phase efficiently. Furthermore, we demonstrated that the expression of modified p27 proteins could inhibit HER2-mediated cell proliferation, anchorage-independent growth, and cell transformation. Significantly, we showed that modified p27 expression reduces tumorigenicity of HER2-overexpressing cells in nude mice. These results indicate that approaches for modulating p27 activity may be useful for intervention in HER2-overexpressing cancers.

MATERIALS AND METHODS

Cell Culture

R1B/L17, NIH3T3, and NIH3T3/HER2 cells [Zhou et al., 2000] were cultured in DMEM media containing 10% fetal bovine serum. A DEAE-dextran method was used to transiently transfect R1B/L17 cells (more than 70% of transfection efficiency) as described previously [Lee et al., 1995]. NIH3T3/HER2-tTA cell line was generated by transfecting pUHD15-1 (neo) [Gossen and Bujard, 1992] into NIH3T3/HER2 cells using Fugene 6 transfection reagent (Boehringer Mannheim) following the manufacturer's guidelines. Flag-tagged modified p27 was PCR cloned in pUHD10-3 hygromycin vector [Reynisdottir et al., 1995] and was introduced into NIH3T3/HER2-tTA cells by Fugene 6 reagent. 300 μg/ml Hygromycine B and 2 μg/ml tet were used to select for positive clones over 2–3 weeks. Positive cell clones expressing p27, p27 T187A, p27 T187AΔJAB were detected by Western blot analysis using anti-FLAG (M2) antibody (Sigma).

Western Blot

Cells were lysed in the buffer previously described [Yang et al., 2000] and the protein amounts were quantified using the Bio-Rad kit. For mouse tumor samples, tissues were homogenized in a RIPA buffer containing 1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and proteinase inhibitors using a tissue homogenizer (PowerGen 125, Fisher). Equal amounts of protein were loaded onto 12% or

8% SDS–polyacrylamide gels. Antibodies used in the study were anti-Flag (M2) (Sigma), anti-HER2 (Oncogene), anti-Cdk2, anti-tubulin, and polyclonal anti-actin antibody (Sigma).

The CDK2-Associated Histone H1 Kinase Assay

NIH3T3/HER2, NIH3T3/HER2-tTA, and NIH3T3/HER2-tTA-modified p27 cells were cultured in the media with or without 2 μg/ml tet for 16–20 h before harvesting. For transfected cells, cells were harvested 48 h post-transfection. Cells were lysed and 1 mg of total cell lysate was immunoprecipitated with polyclonal anti-Cdk2 antibody (Pharmingen). The immunoprecipitates were assayed for Histone H1 kinase activity as described previously [Yang et al., 2000].

MTT Assay

The MTT assay was done as described [Laronga et al., 2000]. Briefly, cells were seeded at 3,000 cells/well in a 96-well plate in the presence or absence of the 2 μg/ml tet for each cell line. Each sample has four replicates. Cell growth was measured for 7 days. Each day cells were incubated with 50μl of 0.2% MTT for 2 h at 37°C in the 5% CO2 incubator. Following MTT incubation, cells were lysed in 200 μl of 100% DMSO and the absorbance at 570 nm was obtained using the 96-well plate reader (Dynex Technologies).

5-Bromo-2-Deoxy-Uridine (BrdU) Incorporation Assay

For transient transfection, pCMV5-modified p27, pcDNA3-HER2, pCMV5, or a combination of pCMV5-modified p27 and pcDNA3-HER2 was transiently co-transfected into R1B/L17 cells. Cells were trypsinized and plated onto 4-well chamber slides 24 h after transfection. BrdU incorporation was performed 48 h after transfection using a BrdU labeling and detection kit (Boehringer Mannheim) following manufacturer's guidelines. For stable lines, NIH3T3/HER2-tTA and NIH3T3/HER2-tTA-p27 were cultured in the media with or without tet for 16–20 h prior to performing BrdU incorporation assay. For all staining, cells were incubated with 0.1 mg/ml of 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to stain the nuclei. Immunofluorescence was detected using Axioplan 2 fluorescent microscope (Zeiss).

FACS-Propidium Iodine Staining

Cells were cultured in DMEM complete media with or without 2 μg/ml tet for 16–20 h prior to Propidium Iodine staining. Cells were trypsinized and washed twice with PBS, then collected in 15 ml conical tubes. Following fixation of cells in cold 70% ethanol at 4°C for 1 h, cells were spinned down and incubated at 37°C for 30 min in the buffer containing 50 μg/ml propidium iodine, 5 mM MgCl2, 10 mM Tris-Cl pH7, 25 μg/ml RNaseA. Before FACS analysis, cells were continuously incubated at 4°C for overnight.

Soft Agar Colony Formation Assay

The soft agar colony formation assay was performed as described previously [Laronga et al., 2000]. In brief, 2,000 cells/well mixed in a 0.35% agarose/complete media were seeded onto 0.7% agarose/complete media bottom layer in the presence or absence of 2 μg/ml tet in a 24-well plate. A drop of media containing 2 μg/ml tet was added every 3 days for tet present group. Four weeks later, cells were stained with *p*-iodonitrotetrazolium violet (Sigma) for 16 h before being photographed.

Microfocus Formation Assay

Microfocus forming assay system was performed as previously described [Reardon and Hung, 1993]. Briefly, 400 cells of NIH3T3/HER2-tTA, NIH3T3/HER2-tTA-p27 cells were mixed with NIH3T3 cells (6.5×10^6/10-cm plate) and cultured in the media with or without 2 μg/ml tet. Medium was changed every 2 days over the 3-week period of focus formation. At the end of 3 weeks, cell monolayers were stained in crystal violet solution (0.5% crystal violet, 20% methanol) and then destained in running tap water for 30–60 min. Foci were then counted and photographed.

Tumor Growth in Nude Mice

Female 4- to 5-week-old nude mice (Charles River Laboratories, Wilmington, MA) were maintained in the animal facility at the University of Texas M.D. Anderson Cancer Center. Mice were divided into two experimental groups, four for each. NIH3T3/HER2-tTA-p27 T187AΔJAB cells (1×10^6 cells in 0.2 ml PBS per injection with 2 μg/ml tet for one group, and without tet for other) were injected subcutaneously into the flanks of mice, and each mouse was inoculated with cells at two sites. After cell inoculation, animals were fed with drinking water containing 5% sucrose in the presence or absence of 200 μg/ml doxycycline (Sigma)

[Kistner et al., 1996]. Drinking water was changed every other day for a 2-week period of tumor formation. Tumor volumes were measured and recorded three times a week from day 5 of cell inoculation. At the end of 2 weeks, the mice were sacrificed and the tumors were removed for detection of p27 T187AΔJAB gene expression.

RESULTS

Modified p27 Inhibits HER2-Mediated Mitogenic Growth

Previous results have indicated that cell lysates have ubiquitin ligase activity towards p27 phosphorylated on Thr 187. In contrast, p27T187A is resistant to ubiquitination [Podust et al., 2000]. Also p27 (aa 97–151) is the JAB1 binding domain. p27 lacking this JAB1 binding domain is resistant to Jab1-mediated p27 degradation [Tomoda et al., 1999]. Furthermore, p27 contains CDK inhibitory domain that is responsible CDK inhibition [Polyak et al., 1994]. We constructed modified p27 by mutating Thr 187 of p27 to Ala (for preventing Skp2 from binding to p27), by deleting JAB1 binding domain, and by deleting N-terminus containing CDK inhibitory domain (aa 1–92) using PCR cloning. Three types of modified p27 were generated: p27 T187A, p27 T187AΔJAB, and C-p27 (aa 92–197) (Fig. 1A), We then investigate the growth inhibitory effect of these modified p27 on cell growth by performing a CDK2-associated Histone H1 kinase assay. Cells transfected with wt p27, p27 T187A, or p27 T187AΔJAB have reduced CDK2-associated kinase activity in comparison to cells transfected with C-p27 or vector control (Fig. 1B), suggesting that modified p27 proteins, including p27 T187A and p27 T187AΔJAB, have CDK inhibitory activity.

HER2 signaling results in cytoplasmic localization of p27 and subsequent enhanced p27 degradation [Yang et al., 2000]. Because p27 T187A and p27 T187AΔJAB are designed to be resistant to degradation and still have CDK inhibitory activity, we determined whether p27 T187A and p27 T187AΔJAB expression could reduce HER2-mediated mitogenic growth using a bromodeoxyuridine (BrdU) incorporation assay. Mink lung epithelial cell R1B/L17 cells were cotransfected with p27 T187A and p27 T187AΔJAB and/or HER2-expressing plasmids and analyzed by an immunofluorescence microscope for BrdU positive cells. Wt p27 was also included in the experiment. Whereas HER2 expression leads to increased number of BrdU-positive cells, p27 T187A, p27 T187AΔJAB, or p27, causes reduction in BrdU-positive cells as compared with the CMV empty vector transfection control cells (Fig. 2A). Importantly, cotransfection of HER2 with p27 T187A, p27

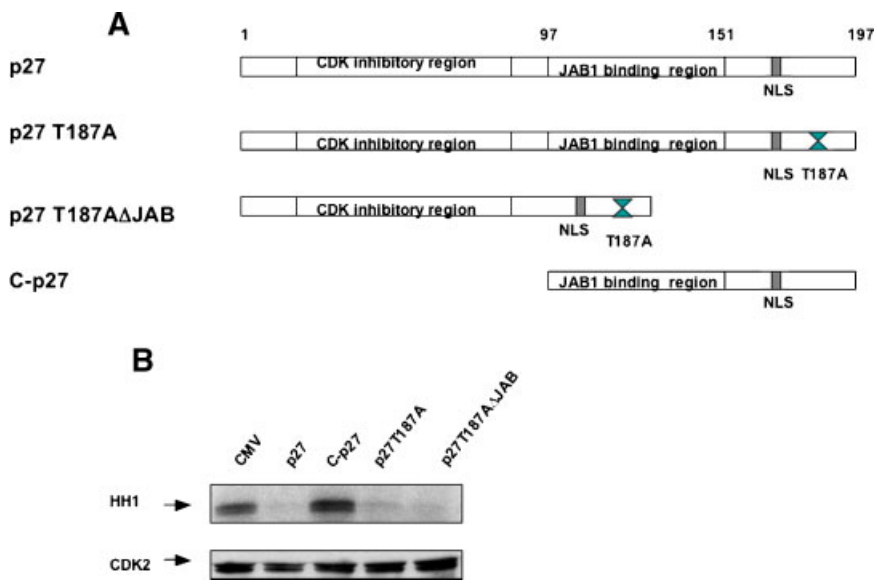

Fig. 1. Modified p27 proteins containing CDK inhibitory domain inhibit CDK2 activity. **A**: Schematic representation of modified p27 constructs. **B**: Modified p27 inhibits CDK2-associated histone H1 kinase activity efficiently. R1B/L17 cells were transfected with modified p27 or empty vector (CMV). CDK2 was immunoprecipitated and CDK2-associated histone H1 kinase activity was observed by autoradiography.

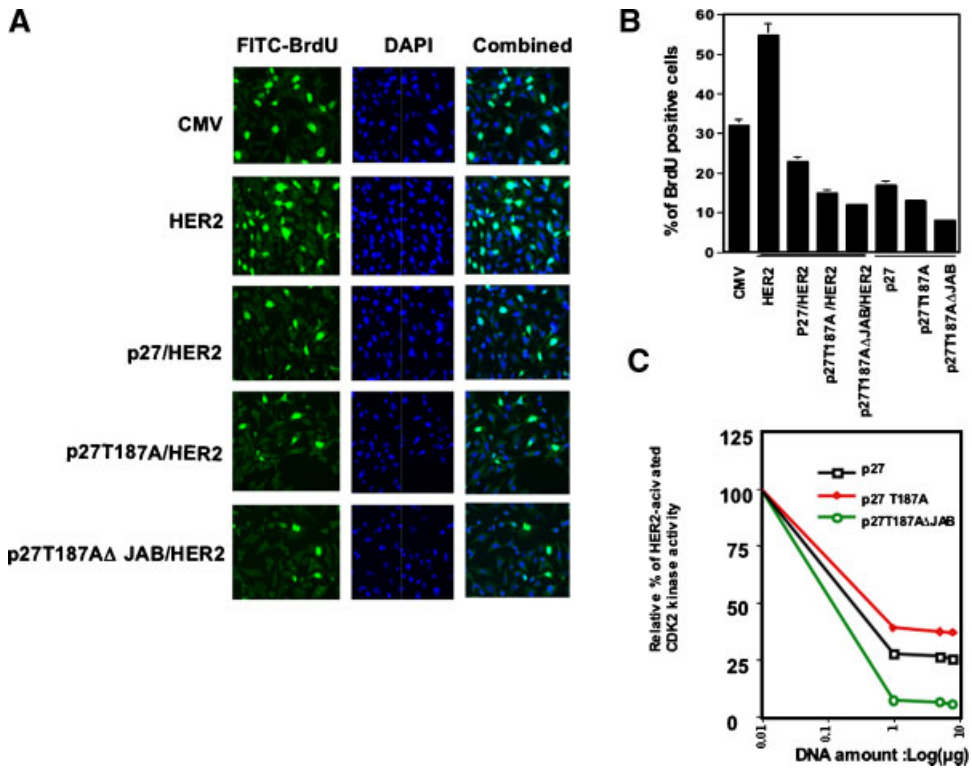

Fig. 2. Inhibition of HER2-activated BrdU incorporation by modified p27. **A**: BrdU incorporation. R1B/L17 cells were co-transfected with modified p27 and HER2. Cells were labeled with BrdU 48 h post-transfection. Empty vector transfection (CMV) was used as a control. Cells were fixed and analyzed for immunoflorescence using monoclonal anti-BrdU antibody followed by FITC-conjugated anti-mouse immunoglobulin. Nuclei were visualized by DAPI staining of DNA. Combined FITC and DAPI staining is also shown. **B**: Percentage of BrdU positive cells. Cells were labeled with BrdU as described in A. At least 300 cells were counted for BrdU staining. The percentage of BrdU-positive cells was measured. Data shown are from a typical experiment performed in triplicates. **C**: Inhibition of HER2-activated CDK2 kinase activity by modified p27. R1B/L17 cells were co-transfected with HER2 and increasing amounts of p27, modified p27, or empty vector (CMV). CDK2 was immunoprecipitated and CDK2-associated Histone H1 kinase activity was measured using Histone H1 as a substrate (HH1). The signal associated with phosphorylated Histone H1 bands was quantitated using a Phosphoimager, and the results are plotted as percentages relative to samples that received only HER2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

T187AΔJAB, or p27 causes a dramatic reduction in BrdU-positive cells as compared to the HER2 single-transfection cells (Fig. 2A,B). To investigate the growth inhibitory effect of p27 T187A, p27 T187AΔJAB on HER2-activated cell growth, we performed a CDK2-associated Histone H1 kinase assay by cotransfecting HER2 with increasing amounts of p27 T187A, p27 T187AΔJAB into mink lung epithelial cell R1B/L17 (Fig. 2C). Wt p27 was also included in the experiment. The phosphorylated Histone H1 was quantitated using a phosphoimager. A dramatic reduction of HER2-activated CDK2 kinase activity was seen in cells transfected with increasing amounts of p27 T187A, p27 T187AΔJAB as compared with the HER2 single-transfection control cells (Fig. 2C). The data indicate that ectopic expression of p27 T187A, p27 T187AΔJAB can inhibit the mitogenic growth mediated by HER2 efficiently.

Tet-regulated Modified p27 Blocks G1 Progression and the Cell Growth of HER2–Overexpressing Cells

To further characterize the biological effects of p27 T187A, p27 T187AΔJAB expression on HER2-overexpressing cells, we employed a tet-inducible system [Gossen and Bujard, 1992]. HER2-overexpressing NIH3T3 cells (NIH3T3/HER2 or 10-1 cells) [Zhou et al., 2000] were used to stably express tTA (Transcriptional activator) by transfecting with pUHD15-1 (neo) vector [Gossen and Bujard, 1992]. A cell clone (NIH3/HER2-tTA) was established. We then constructed tet-off regulated p27 T187A or p27

T187AΔJAB expressing cells by transfecting pUHD10-3 hygromycin vector [Reynisdottir et al., 1995] cloned with Flag-tagged p27 T187A, p27 T187AΔJAB into NIH3/HER2-tTA cells. We were able to identify several clones, and representative clone can have induced expression of p27T187A or p27T187AΔJAB by adjusting the concentration of tet as detected by immunoblotting with anti-Flag antibody (Fig. 3A). Wild-type p27 was included as a control. The overexpression status of HER2 was not affected in the presence or absence of tet in these clones. These results indicate that the tet-regulated system can be used to evaluate the inhibitory effect of controlled gene expression of p27 in HER2-overexpressing cells.

To assess the p27-mediated anti-proliferative effects, we measured and compared the anti-mitogenic activity of p27-expressing cell lines and parental control NIH3/HER2-tTA cell lines by performing a CDK2 kinase assay and FACS analysis. The cells expressing p27, p27 T187A, or p27 T187AΔJAB (in the absence of tet) exhibited a reduced CDK2-associated kinase activity as well as an increased G1 population as compared with their respective controls (in the presence of tet), whereas parental controls (NIH3T3/HER2-tTA) exhibited no difference in CDK2 activity and cell cycle progression in the presence or absence of tet (Fig. 3B,C). Similarly, BrdU incorporation (Fig. 4A,B), cell density (Fig. 4C), and growth rate as

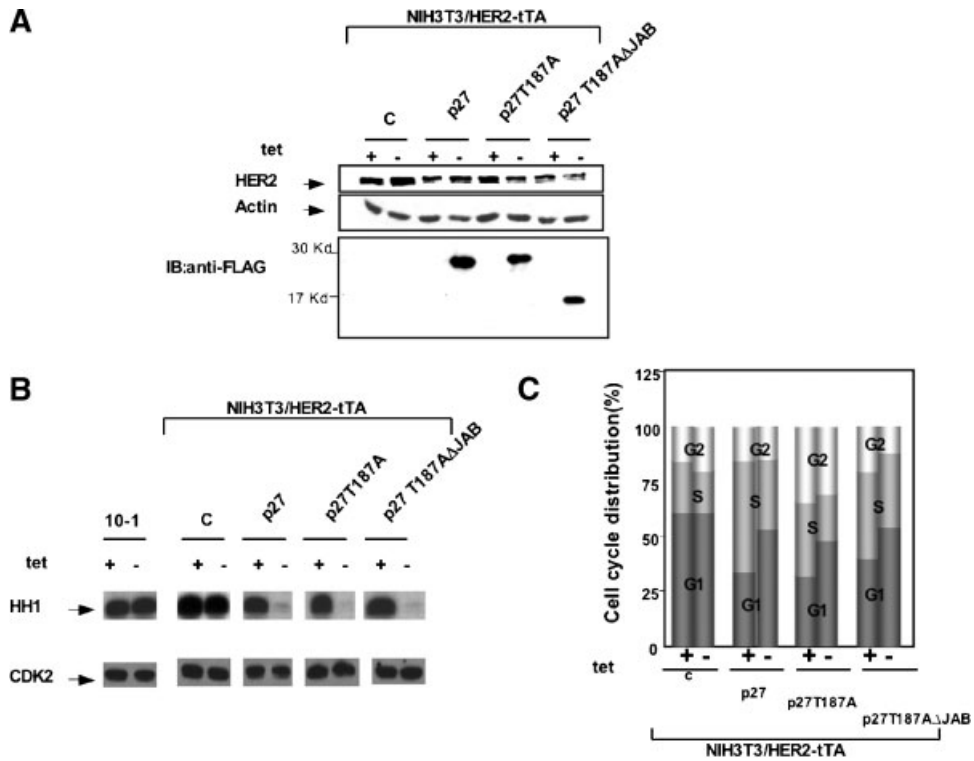

Fig. 3. Tet-off induced expression of modified p27 inhibited CDK2-associated kinase activity and causes G1 cell cycle arrest in the presence of activated HER2. **A**: Confirmed expression of modified p27 in the tet-off inducible system. pUHD10-3 hygromycin carries a multiple cloning site under the control of tet O/HCMV IE promoter. c-DNA of Flag-tagged modified p27 was cloned into pUHD10-3 hygromycin vector, and the construct was transfected into NIH3T3/HER2-tTA cells. Transfectants were selected in 600 μg/ml G-418, 300 μg/ml of hygromycin, and 2 μg/ml of tet. To examine the expression of these constructs, the culture medium was switched to medium alone or medium containing 2 μg/ml of tet. NIH3T3/HER2-tTA cells and clones that expressed Flag-tagged modified p27 in the absence of tet were probed by immunoblotting with anti-FLAG antibody. The HER2 levels were immunoblotted with anti-HER2 antibody. The levels of actin were used as an equal loading control. **B**: Inhibition of CDK2-associated Histone H1 (HH1) kinase activity by modified p27 in the presence of activated HER2. 10-1 cells (NIH3T3/HER2 cells), NIH3T3/HER2-tTA cells (**C**), and NIH3T3/HER2-t TA-modified p27 cells were used for CDK2-associated Histone H1 kinase assay. Cells were cultured in medium alone (−) or medium containing 2μg/ml of tet (+). C: Induction of G1 arrest by tet-regulated modified p27 in HER2-overexpressing cells. NIH3T3/HER2-tTA cells and NIH3T3/HER2-tTA-modified p27 cells were used for FACS analysis. Cells were cultured in the presence (+) or absence (−) of tet for 16-20 h. Cells were collected in a sorter and subjected to flow cytometry to analyze the cell cycle distribution according to DNA content. The percentage distribution in different cell cycle compartments is indicated in the stacked column graph.

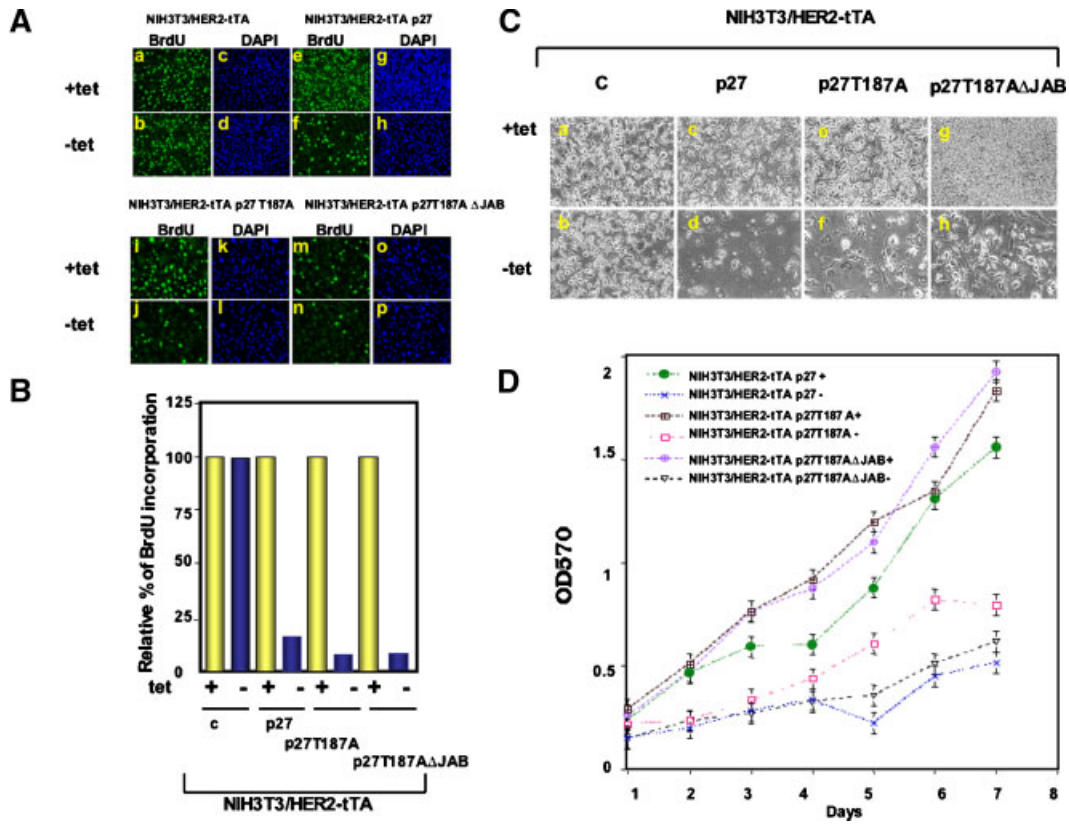

Fig. 4. Tet-regulated expression of modified p27 inhibits HER2-mediated mitogenic growth. **A**: tet-regulated modified p27 expression blocks cell cycle entry into S-phase in HER2-overexpressing cells. BrdU incorporation assays were conducted after cells were cultured in the presence or absence of tet for 20 h. Incorporation of BrdU was examined under a fluorescence microscope using FITC-conjugated anti-BrdU (a, b, e, f, i, j, m, and n). The location of nuclei is indicated by DAPI staining (c, d, g, d, h, k, l, o, and p). **B**: Relative percentage of BrdU incorporation. 300 cells were counted for BrdU staining in each condition. The number of BrdU-positive cells from each cell cultured in the tet containing medium was set as 100%. The relative % of BrdU positive cells in cells cultured in the absence of tet is presented. Data shown are from a typical experiment conducted in triplicates. **C**: Effects of modified p27 on cell density. The cells cultured in the presence or absence of tet were observed under microscope to check the cell density. **D**: MTT assays. The cells cultured in the presence or absence of tet were estimated by MTT assay everyday for 7 days. The results were expressed as the value of A570 reading. The absorbency is directly proportional to the number of cells. Bars, SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

determined by 3-(4,-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT assay) [Laronga et al., 2000] (Fig. 4D) were dramatically reduced in the cells expressing p27, p27 T187A, or p27 T187AΔJAB (in the absence of tet). Thus, our data strongly suggest that overexpression of modified p27 can function as a growth inhibitor in HER2-overexpressing cells.

Tet-Regulated Modified p27 Reverses the Transformation Phenotypes of HER2–Overexpressing Cells

The HER2 oncogene can induce transformation by causing microfocus formation and enabling cells to grow in soft agar. To determine whether p27 expression in HER2-overexpressing cells can inhibit HER2-induced cell transformation, we measured the number of microfoci formed and the number of colonies formed in soft agar for transformation phenotype. Overexpression of p27 T187A or p27 T187AΔJAB induced through tet regulation reduced the number of HER2-induced microfoci more than 95% (Fig. 5A) as compared with respective controls. NIH3T3 cells served as negative controls. In a soft agar colony assay to determine anchorage-independent growth, tet-regulated overexpression of p27 T187A or p27 T187AΔJAB reduced the HER2-induced formation of colonies by a mean of 70% (Fig. 5B and C). These results demonstrated that overexpression of p27 T187A or p27 T187AΔJAB

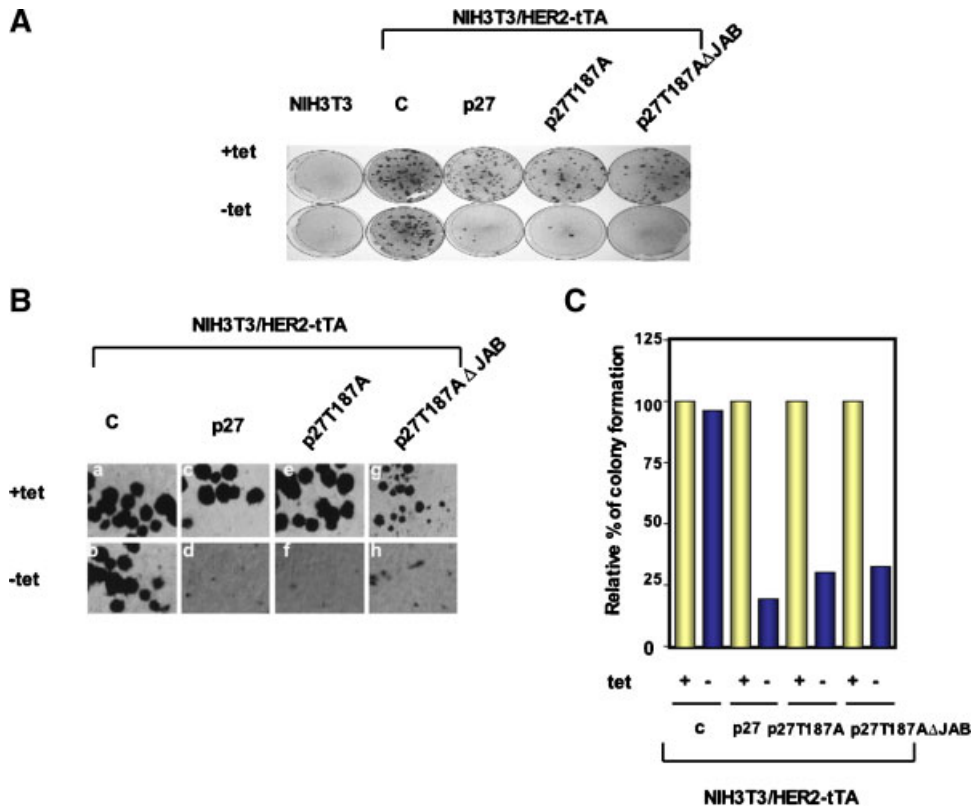

Fig. 5. Tet-regulated expression of modified p27 inhibits HER2-mediated transformation. **A**: Microfocus-formation assay. NIH3T3 cells, NIH3T3/HER2-tTA cells (**C**), NIH3T3/HER2-tTA-p27, NIH3T3/HER2-tTA-p27T187A cells, and NIH3T3/HER2-tTA-p27T187AΔJAB cells were subjected to microfoci formation assay in the presence (+) or absence (−) of tet. **B**: Soft-agar colony formation assay. NIH3T3/HER2-tTA cells (C), NIH3T3/HER2-tTA-p27, NIH3T3/HER2-tTA-p27T187A cells, and NIH3T3/HER2-tTA-p27T187AΔJAB cells were measured for anchorage-independent growth in soft agar in the presence (+) or absence (−) of tet. C, Relative % of colony formation in soft agar as shown in B. Colonies were counted in the presence or absence of tet. The number of colonies from each cell treated with tet was set as 100%. The relative % of colony formation from cells cultured in the absence of tet is shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

through tet regulation can suppress in vitro transformation phenotype of HER2−overexpressing cells.

Dox-Regulated p27 T187AΔJAB Suppresses Tumor Growth of HER2-Overexpressing Cells in Nude Mice

As modified p27 blocked HER2-induced cell transformation, we choose p27T187AΔJAB for further study and assessed its effect on HER2-mediated tumorigenicity in vivo. To verify the possible anti-tumor activity of p27T187AΔJAB, we performed a tumorigenicity assay in immunodeficient strains of mice by doxycycline (dox, a derivative of tetracycline) induction. NIH3T3/HER2-tTA-p27T187AΔJAB cells were treated with tet or without tet for 18 h and injected subcutaneously into the flanks of nude mice, and tumor formation was assayed. HER2-mediated tumorigenesis was dramatically lower in p27T187AΔJAB-expressing cells (NIH3T3/HER2-tTA-p27T187AΔJAB in the absence of dox) than in control cells (NIH3T3/HER2-tTA-p27T187AΔJAB in the presence of dox) (Fig. 6A and B). Same results were obtained when two other independent clones were used for tumorigenesis assay (data not shown). Tumor tissues from the sites of implantation were assessed for p27T187AΔJAB levels by immunoblotting. As expected, transduced Flag-p27T187AΔJAB is absent in tumors of NIH3T3/HER2-tTA-injected control mice regardless the presence or absence of doxycycline in drinking water. p27T187AΔJAB was also not detected in tumors of NIH3T3/HER2-tTA-p27T187AΔJAB-injected mice given drinking water containing doxycycline, whereas transduced Flag-p27T187AΔJAB was detected in the small tumors of NIH3T3/HER2-tTA-p27-injected

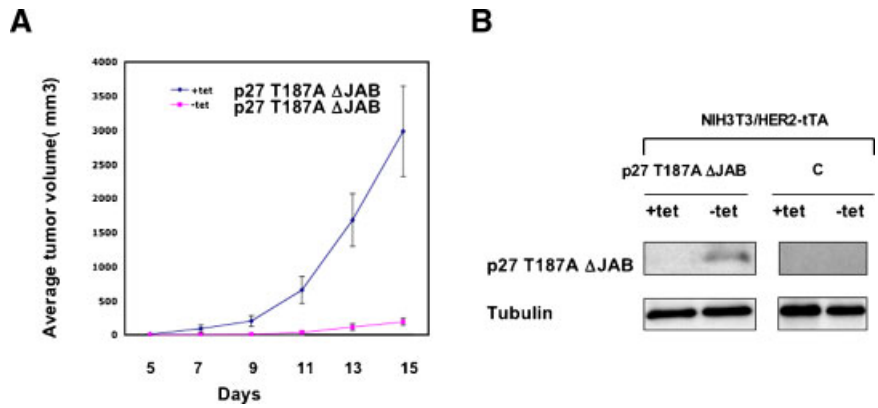

Fig. 6. Dox-regulated expression of modified p27 inhibits HER2-mediated tumorigenesis in a xenograft model. **A**: Overexpression of p27 T187AΔJAB inhibits HER2-mediated tumorigenesis in nude mice. 3T3/HER2-tTA-p27 T187AΔJAB cells were cultured in the presence (+) or absence (−) of dox for 24 h. 1×10^6 cells were harvested and s.c. injected into the flank regions of female nude mice. The mice were fed water with (+) or without (−) 200 μg/ml dox. Tumor volumes were monitored for 15 days. The change in tumor volume over a 15-day period is shown in the graph. Bars represent standard deviations. **B**: Gene expression of p27 T187AΔJAB in tumor. Tumor tissues from the sites of implantation were assessed for expressed Flag-p27 T187AΔJAB levels by immunoblotting with M2 antibodies. Representative tumors are shown. Tubulin served as a loading control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mice given drinking water without doxycycline (Fig. 6B), suggesting that the expression of p27 is directly involved in inhibiting tumor growth. These results demonstrate that overexpression of p27T187AΔJAB is efficient to abolish tumorigenicity in cells transformed by HER2.

DISCUSSION

HER2 oncogenic signals promote the induction and progression of human cancers, including breast and ovarian cancers. We previously identified p27 as a downstream target of HER2 oncogenic signals and found that p27 abundance was decreased in HER2-overexpressing cells [Yang et al., 2000]. Clinical studies of breast cancer tumor samples also indicated that overexpression of HER2 correlates with low levels of p27 [Newman et al., 2001], suggesting a critical role of p27 in suppressing proliferative and transforming signals of oncogenic HER2. The low-level expression of p27 caused by HER2 is mediated through enhanced ubiquitin-mediated degradation of p27 via Grb2/MAPK pathway [Yang et al., 2000]. Mitogenic signals of HER2 can activate MAPK signaling pathway, elevate CDK2 activity, induced cell transformation, and increase tumorigenesis while decreasing the expression of p27. As a downstream target of the HER2 oncogenic signaling pathway in cancer cells, p27 represents a logical target for intervention using gene therapy. Given the frequent deregulation of the HER2-MAPK-p27 signaling pathway, strategic overexpression of p27 should prove useful in the treatment of HER2-associated tumors.

To counteract the HER2 oncogenic signaling that degrades p27 through enhancing ubiquitin-mediated proteasome degradation, we generated modified p27 proteins (p27 T187A and p27T187AΔJAB) with a goal to design a p27 protein demonstrating a much more potent antitumor effect. As pointed out earlier, JAB1 expression is inversely correlated with p27 expression in several types of cancer. Therefore, deletion of the JAB1 binding domain on p27 is a good strategy to prevent p27 from degradation, thus presenting a potential therapeutic application in JAB1 overexpressing cancers. Following the cell cycle progression from G1 into S phase, p27 is degraded. It is regulated through ubiquitin-mediated proteasome degradation. Skp2, the F-box protein component of SCF^{Skp2} ubiquitin ligase complex, binds to p27 phosphorylated at Thr187 [Carrano et al., 1999] to facilitate p27 ubiquitination and subsequent degradation. Again, it is shown that there is inverse relation between levels of Skp2 and its p27 target in many types of cancer. Thus mutating the phosphorylation site on Thr 187 to prevent p27 from Skp2 binding is another tool to improve the half-life of p27 and potentiate p27 growth inhibitory activity. In this study, we successfully demonstrated that modified p27 proteins can inhibit HER2-activated CDK2

activity, cell transformation, and tumorigenesis, indicating that these modified p27 proteins are potential anticancer agents for HER2-overexpressing cancers. Recently, Akt pathway, which is frequently activated by HER2 oncogenic signal as well, also plays important role in phosphorylating p27 and subsequent degradation. In cancer cells, Akt-mediated human p27 phosphorylation at Thr 157 (within the nuclear localization signal (NLS) sequence of p27, aa153–166) results in impairing the nuclear import of p27 [Shin et al., 2002]. Thus, Akt activation causes cytoplasmic location of p27 in primary breast cancers [Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002]. In addition, Akt also phosphorylates human p27 at Thr 198 [Fujita et al., 2002], which also results in the nuclear export and degradation of p27. Thus, future goal will be generating another modified p27 (on the basis of p27 T187AΔJAB) containing mutated Akt phosphorylation sites to improve the stability of p27.

HER2 overexpression has been found with high frequency in many kinds of carcinoma, suggesting that HER2 overexpression plays a critical role in the development of malignancy. Therefore, the HER2 receptor is a target for the development of therapeutic agents for HER2-overexpressing human cancers. Many strategies are focused on targeting the receptor including employing antibody (Herceptin) [Baselga et al., 1998; Pegram et al., 1999]. Recently, the recombinant humanized anti-HER2 monoclonal antibody (trastuzumab) has been used for treatment of women with metastatic breast cancer overexpressing HER2. It was found that HER2 monoclonal antibody has an effect in downregulating Skp2 [Lane et al., 2000], reducing p27 phosphorylation level on Thr 187 [Le et al., 2003], upregulating p27 [Lane et al., 2000; Le et al., 2000], thereby inhibiting cell growth in HER2-overexpressing cancer cells, suggesting that our strategy to employ modified p27 proteins with Thr 187 mutation for the treatment of HER2-associated tumors is on the right track. It is not clear whether HER2-overexpressing leads to dysregulated JAB1 function in cancer. If yes, employing modified p27 proteins without the JAB1 binding domain should prove useful in the treatment of HER2-overexpressing tumors with elevated JAB1 activity and mislocated p27.

In conclusion, our studies show that overexpression of modified p27 proteins can inhibit HER2-mediated tumor growth, suggesting that employing modified p27 proteins that are resistant to degradation is a useful new strategy of anticancer gene therapy for HER2-associated cancers.

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