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The N-terminal domain of SV40 large T antigen represses the HER2/neu-mediated transformation and metastatic potential in breast cancers

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Abstract HER2/neu is known to be overexpressed in approximately 40% of human breast and ovarian cancers and it is associated with increased metastasis and poor prognosis. We have shown previously that the N-terminal domain of simian virus 40 large T antigen (LT425) can act as a transforming suppressor of the *HER2/neu* oncogene in human ovarian cancer. In the present study, we demonstrate that LT425 can also repress the transforming properties of HER2/neu-overexpressing human breast cancer cells. In addition, the results of a chemotaxis assay and an in vitro chemoinvasion assay further suggest that LT425 can also suppress the metastatic potential of the HER2/neu-transformed breast cancer cells. Taken together, these data clearly suggest that the inhibition of the expression of p185$^{HER2/neu}$ tyrosine kinase by LT425 is capable of suppressing the HER2/neu-mediated transformation and metastatic potential in breast cancers. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: HER2/neu; p185; Metastasis; Simian virus 40 large T antigen

1. Introduction

The *HER2/neu* protooncogene encodes a 185 kDa transmembrane glycoprotein (p185$^{HER2/neu}$) with intrinsic tyrosine kinase activity and belongs to the epidermal growth factor receptor family of four closely related receptors, termed HER1 (EGFR), HER2 (c-erbB-2; neu), HER3 and HER4 [1–5]. Amplification of the *HER2/neu* gene and overexpression of its product induce cell transformation. Extensive studies have reported that HER2/neu is overexpressed in 30–50% of human breast tumors [6–8]. Breast tumors are the most common form of malignancy found among women, but in addition to breast carcinomas, a wide variety of other human cancers are also frequently found to demonstrate amplification or overexpression of HER2/neu, including ovarian, gastric, head and neck, colon, lung, oral, cervical, and prostatic cancers [9,10]. In the past decade, it has shown that the amplification/overexpression of the *HER2/neu* gene was correlated with malignant phenomena in breast cancers, such as enhanced malignancy of the tumor, a shorter time to relapse, shorter post-relapse survival, more therapeutic resistance, and decreased overall survival rate [11]. In addition, experiments using transgenic mice indicated that introduction of the mouse *HER2/neu* gene into mice can induce mammary tumors and metastases [12], while overexpression of the *HER2/neu* gene enhances the intrinsic metastatic potential in human breast cancer cells by increasing invasion ability [13]. It appears, therefore, that HER2/neu overexpression plays an important role in human breast cancer development and metastasis and it is a promising target for the development of cancer therapies.

Several oncogene products, such as the adenovirus 5 (Ad5) E1A, c-Myc, c-Cbl, and PEA3 gene products and the simian virus 40 large T antigen (SV40LT) have been demonstrated to act as repressors of the *HER2/neu* oncogene [14–19,37]. The repression mechanisms of Ad5 E1A, c-Myc, c-Cbl and PEA3 have all been reported [15,18,21,37], but the exact molecular mechanism for the suppression of *HER2/neu* gene expression by SV40LT remains unclear. SV40LT is the major oncoprotein of the DNA tumor virus SV40. It is a multifunctional nuclear phosphoprotein and is required for the transformation and DNA replication of the virus [22–26,38–40]. It also plays a critical role in the deregulation of cellular proliferation and thus there are oncogenic concerns regarding it as a therapeutic agent. Fortunately, as we have shown in a previously study [17], when only the N-terminal domain of SV40LT (designed LT425) is used, the transforming potential of the wild type SV40LT is eliminate while its suppressing activity on HER2/neu overexpression in human ovarian cancer cells is still retained, meaning that LT425 is suitable for clinical applications in human cancer gene therapy. We also demonstrated that the enforced expression of LT425 can significantly retard the cell growth and tumorigenicity of ovarian cancer cells in vitro [17]. However, prior to the present study, it was still not known whether LT425 could also inhibit human breast cancer cell growth and suppress the transformation phenotype and metastatic potential of HER2/neu-overexpressing breast cancers. In this report, we show that expression of LT425 not only decreases the growth rate and reduces soft-agarose colony-forming ability, but also inhibits the metastasis-associated properties of human breast cancer cells. Thus, in addition to its function as a tumor suppressor of HER2/neu, LT425 may also be a metastasis suppressor of HER2/neu. Our results demonstrate that LT425 is capable of down-regulating the HER2/neu-mediated metastatic potential, suggesting that LT425 may have therapeutic potential for HER2/neu-induced metastasis.

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2. Material and methods

2.1. Construction of LT425-expressing plasmid and cell culture

The LT425-expressing and its frame-shift mutant plasmids were constructed by inserting the *LT425* gene into the *Hin*dIII/*Xho*I site of the pCMV-Tag 2A and 2B vectors (Stratagene Cloning System, USA), resulting in pCMV-Flag-LT425 and pCMV-Flag-LT425-M plasmids, respectively. A termination codon was generated right after the translation initiation codon of aLT425 due to an 'A' insertion after the Flag sequence. Thus, no LT425 peptide was formed from the plasmid pCMV-Flag-LT425-M. MDA-MB-453, a human breast cancer cell line expressing high levels of HER2/neu, was kindly provided by Dr. Hung MC (University of Texas M.D. Anderson Cancer Center, Houston, TX, USA) and cultured in L-15 (Life Technologies, Inc., USA) medium supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were transfected with pCMV-Flag-LT425 or pCMV-Flag-LT425-M using Lipofectamine plus reagent (Life Technologies, Inc.) and selected in 600 μg/ml of G418 (geneticin; Life Technologies, Inc.). Stable lines were cultured under the same conditions as the parental line with the addition of G418 (500 μg/ml) selection reagent (Life Technologies, Inc., USA).

2.2. Western blot analysis

Cell lysates were prepared and sedimented as described previously [17]. The supernatant was used for SDS–PAGE analysis. 80 μg of each cell sample were loaded onto the NuPAGE™ 4–12% Bis–Tris Gel (NP0322, Invitrogen, USA). After electrophoresis, the gel was blotted onto nitrocellulose filter membranes. The filter membranes were subjected to blocking with 5% skim milk in TBST buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The membranes were then incubated with the primary anti-neu antibody (Ab-3; 1:1000 dilution; Oncogene Science, NY, USA), anti-FLAG antibody (anti-FLAG M2; 1:1000 dilution; Stratagene) or anti-β-actin monoclonal antibody (1:5000 dilution) in fresh TBST buffer for 1 h at room temperature, followed by washing and incubation with the secondary antibody of goat anti-mouse IgG Fc-HRP (1:2000 dilution; Amersham). Visualization was by an Amersham ECL chemiluminescence system.

2.3. Cell growth rate assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) metabolic assay was performed as described by Loveland et al. [27]. In brief, the cells were seeded at a density of 3×10^3 cells into microtiter plates and incubated in 200 μl medium supplemented either with 10% or 0.1% FBS. At five 24-h intervals, viable cells were reacted with 20 μl MTT (5 mg/ml in phosphate-buffered saline (PBS); Sigma Chemical Co.) for 4–6 h at 37°C. Then, 170 μl of the remainder, which contained the MTT–formazan crystals, was dissolved in 200 μl dimethyl sulfoxide. Finally, the absorbance was measured at 545 nm.

2.4. Anchorage independence assay

For the soft-agarose growth assay used in this study, the wild type and mutant LT425 lines and their parental MDA-MB-453 cells (5×10^3 cells/well) were plated in 24-well plates in culture medium containing 0.35% agarose (Gibco-BRL, Gaithersburg, MD, USA) overlying a 0.7% agarose layer. The cells were then incubated at 37°C for 4 weeks, after which the plates were stained with *p*-iodonitrotetrazolium violet (1 mg/ml) overnight at 37°C. Colonies greater than 100 μm in diameter were counted for each dish. Each soft-agarose assay was performed in triplicate.

2.5. Chemotaxis assay

The chemotaxis assays were performed by using a transwell unit with an 8 μm pore size polycarbonate filter in a 24-well cluster plate (Costar, Cat. 3422), as described in Yu et al. [20] with slight modifications. Each lower compartment of the transwell contained 600 μl of MDA-MB-453 parental or LT425 transfected cell-conditioned medium or serum-free medium alone (as negative control). The conditioned medium was prepared from culturing cells in L-15/0.1% BSA without serum for 3 days. After harvesting cells by trypsinization, the cells were then suspended in L-15 containing 10% FBS to inactivate the enzyme, and then centrifuged and resuspended in L-15 serum-free medium. Cells (5×10^4/0.1 ml of L-15 medium) were placed in the upper compartment of the transwell unit and incubated for 6 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, filters were fixed with 3% glutaraldehyde in PBS for 2 h and then stained with 10% Giemsa for 1 h. After washing with PBS, the cells on the upper surface of the filter were removed by wiping with a cotton swab. Chemotactic activity was measured by counting the number of cells that had migrated to the lower side of the filter with a Nikon microscope (Coolpix 990) at 200× magnification.

2.6. In vitro chemoinvasion assay

To perform the in vitro invasiveness assay, we followed the methods described by Albini et al. [28], Repesh [29] and Yu et al. [20] with minor modifications. Matrigel was used as the basement membrane and purchased from Becton Dickinson Labware (MA, USA). Each filter in the transwell unit (the same as used in the chemotaxis assay) was coated with 0.1 ml of 1:60 dilution (21 μg/well) of Matrigel in cold L-15 serum-free medium to form a thin, continuous barrier on the top of the upper filter. The Matrigel was left to air-dry overnight and was reconstituted with 0.15 ml of L-15 serum-free medium at room temperature for 3 h. The lower compartment contained 0.6 ml of conditioned medium (the same as in the chemotaxis assay) as chemoattractant or L-15 medium as negative control. After removing the excess medium, each type of cell (5×10^4/0.1 ml in L-15 medium) was added on the top of the Matrigel layer, and incubated for 3 days at 37°C in a humidified 5% CO_2 atmosphere. Cells were fixed, stained and counted as described for the chemotaxis assay.

3. Results

3.1. Down-regulation of HER2/neu overexpression in breast cancer cells by LT425

To determine whether LT425 could down-regulate HER2/neu overexpression, the MDA-MB-453 human breast cancer cells were either stably transfected with LT425 cDNA (consisting of the N-terminal 178 amino acid residues of SV40LT;

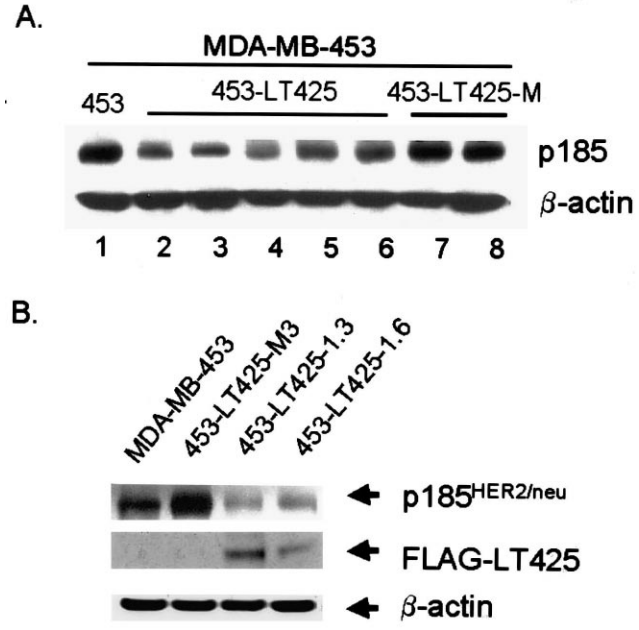

Fig. 1. Western blot analysis of the LT425-expressing MDA-MB-453 stable transfectants. A: Stable integration and expression of LT425 in MDA-MB-453 cells reduced p185 levels. All of the 453-LT425 transfectants had lower p185 expression than the frame-shift mutants (453-LT425-M) and the parental MDA-MB-453 cells. B: Comparison of the two LT425-expressing stable transfectants, 453-LT425-1.3 and -1.6, and one mutant, 453-LT453-M3, that were used in this study. The primary antibodies used in this Western blot experiment were anti-neu (upper), anti-Flag (middle), and anti-β-actin (lower) antibodies, respectively.

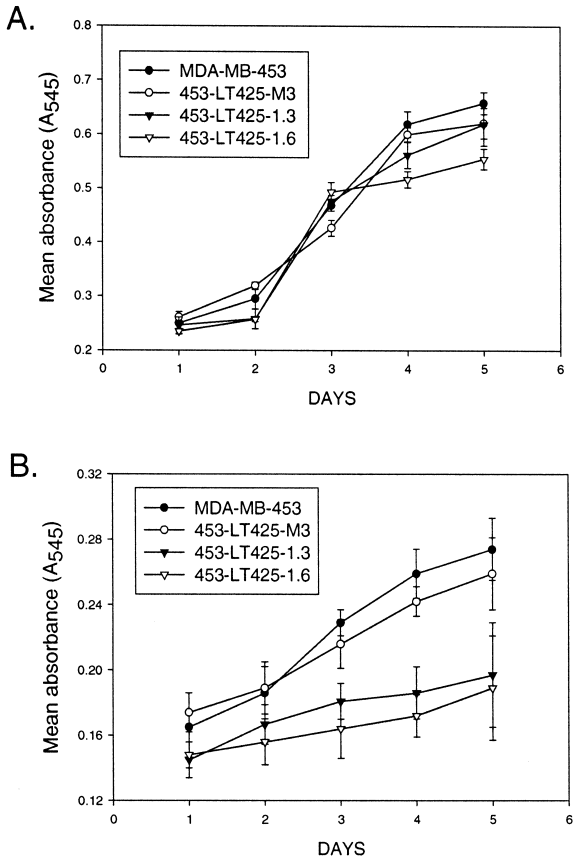

Fig. 2. Growth rate analysis of parental and LT425 stably transfected MDA-MB-453 cells. Cells were plated in 96-well microtiter plates (3×10^3 cells/well) and incubated for up to 5 days. At 24-h intervals, relative cell numbers were determined using the MTT metabolic assay. Mean values from six replicate wells were plotted against time in culture. A: 10% FBS/L-15 medium; B: serum-free (0.1% FBS) L-15 medium.

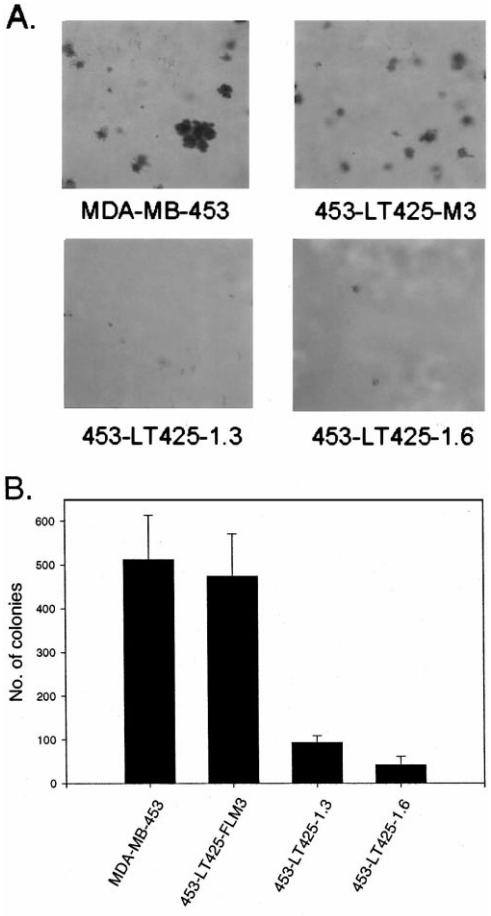

Fig. 3. LT425 inhibits the transformation phenotype of breast cancer cells. A: Soft-agarose assay of MDA-MB-453, 453-LT425-M3, 453-LT425-1.3, and 453-LT425-1.6 cell lines. All cells (5×10^3 cells/well) were subjected to anchorage-independent growth in 0.35% soft agarose for 4 weeks. B: Number of colonies formed in soft agarose as shown in (A).

the resultant cell lines are referred to as 453-LT425 transfectants) or its frame-shift mutant and then the $p185^{HER2/neu}$ expression levels were compared. As shown in Fig. 1A, all of the LT425-expressing lines had significantly reduced HER2/neu levels compared with the parental and mutant lines. In addition, the results from the densitometric analysis suggested that HER2/neu expression in the two LT425 transfected stable lines used in this study, 453-LT425-1.3 and -1.6, were dramatically lower than those in the parental line and in the 453-LT425-M3 mutant control line (Fig. 1B). The expression of LT425 in the two LT425-transfected stable lines, 453-LT425-1.3 and -1.6, but not in the parental 453 and mutant 453-LT425-M3 lines, was further identified by anti-Flag antibody (middle panel of Fig. 1B).

3.2. Effect of LT425 expression on cell growth in vitro

To examine the effect of LT425 expression on the transforming ability of HER2/neu-overexpressing human breast MDA-MB-453 cells, we assayed the two 453-LT425 transfectants, 453-LT425-1.3 and -1.6, as well as the control MDA-MB-453 (parental) and 453-LT425-M3 (mutant) lines for the known transforming parameters. Using the MTT metabolic analysis, a markedly reduced growth rate was observed in LT425-expressing MDA-MB-453 lines (Fig. 2B) in serum-free (0.1% FBS) medium compared to the parental 453 and mutant 453-LT425-M3 lines. These results showed that expression of LT425 resulted in a decrease in the cell-doubling time compared with the control and frame-shift cell lines.

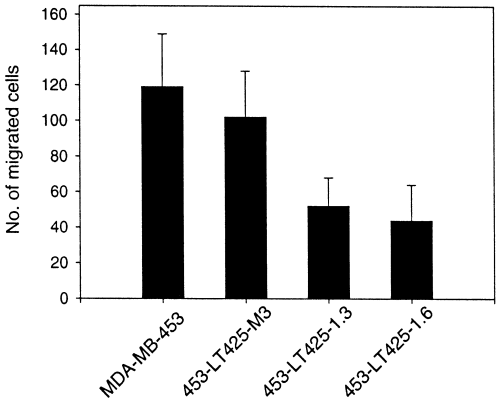

Fig. 4. Inhibition of HER2/neu-transformed MDA-MB-453 cells motility by LT425 gene products. MDA-MB-453, 453-LT425-M3, 453-LT425-1.3, and 453-LT425-1.6 cell lines were assayed for chemotactic activity. Migrated cells were counted in three random fields (200× magnification). All assays were performed in triplicate, and assays were repeated twice.

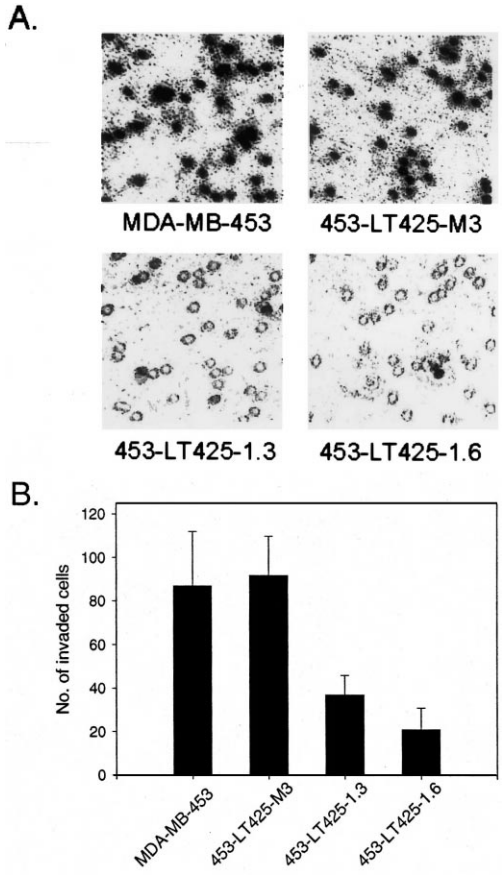

Fig. 5. Inhibition of invasion of HER2/neu-transformed MDA-MB-453 cells through a Matrigel layer by LT425 gene products. A: The lower surface of the filter from the transwell unit at the end of the chemoinvasion activity assay. B: The Matrigel-invaded cells were counted in three random fields (200× magnification). All assays were performed in duplicate, and triplicate experiments were run for each cell line.

3.3. LT425 expression can inhibit the anchorage-independent growth of human breast cancer cells

Anchorage-independent growth in semi-solid agarose is one of the indicators of transforming ability for transformed cells. To test the effect of the LT425 protein expression in breast cancer cells on anchorage-independent growth, we assayed the parental MDA-MB-453 cells, the frame-shift mutant 453-LT425-M3 and the two LT425 transfectant lines, 453-LT425-1.3 and -1.6, for their ability to grow in soft agarose. As shown in Fig. 3, HER-2/neu-overexpressing MDA-MB-453 human breast cancer cells and the mutant line were more efficient in forming soft-agarose colonies. In contrast, the colony-forming ability of the LT425-expressing lines was dramatically reduced, suggesting that LT425 expression in the MDA-MB-453 cells decreases the anchorage-independent growth ability of these tumor cells.

3.4. Abrogation of metastatic properties of HER2/neu-transformed cells by LT425

In the tumor-cell metastasis process, malignant cells must extravasate from the circulation, invade basement membrane and colonize distant sites. The metastatic phenotype involves multiple cellular events that may vary across species, and tumor-cell migration and invasion can serve as important indicators of these events. HER2/neu is likely to be involved in these processes, particularly in HER2/neu-positive breast tumors, in which HER2/neu-transformed tumor cells are reported to have higher chemotactic activities than HER2/neu negative ones [20,41]. To examine whether LT425 can inhibit chemotaxis of the HER2/neu-transformed breast cancer cells, we therefore measured the migration of 453-LT425-1.3, -1.6, -M3 and parental MDA-MB-453 cells towards a chemoattractant (cell-conditioned medium). As shown in Fig. 4, the parental and mutant lines had higher migration rates, whereas the two LT425-transfected lines showed lower chemotactic activity. These results suggest that expression of LT425 in HER2/neu-transformed cells inhibits chemotaxis in these HER2/neu-overexpressing breast cancer cells.

In addition, an in vitro invasion assays was used to compare the invasiveness of 453-LT425-1.3, -1.6, -M3 and parental MDA-MB-453 to determine whether LT425 can be used to diminish the invasive properties of HER2/neu-transformed breast cancer cells. A layer of reconstituted basement membrane (Matrigel) was used on the surface of a transwell insert filter as the invasion substrate, with the cell-conditioned medium placed in the lower chamber beneath the filter to stimulate penetration of the cells. The assay revealed marked differences in invasiveness between the LT425-transfectant lines and control lines. Both the 453-LT425-1.3 and -1.6 lines showed very low rates of invasion, whereas the parental and mutant lines showed dramatically higher abilities to invade the Matrigel layer (Fig. 5), suggesting that LT425 can inhibit the invasive ability of the HER2/neu-overexpressing MDA-MB-453 cells in vitro.

4. Discussion

The wild type SV40LT is known to affect the proliferation of HER2/neu-overexpressing cancer cell types by negatively regulating the HER2/neu promoter activity. In a previous paper, we showed that the N-terminal domain only of the SV40LT (LT425) can inhibit the expression of the *HER2/neu* oncogene at the transcriptional level and reduce the tumorigenicity of HER2/neu-transformed ovarian cancer cells [17]. In the present study, we demonstrate that the LT425 can act as a transforming suppressor of the HER2/neu-overexpressing human breast cancer cells by down-regulating the endogenous $p185^{HER2/neu}$ level and repressing the transformation properties of HER-2/neu-overexpressing breast cancer cells in vitro (Fig. 1–3).

It does not necessarily follow, however, that metastasis will likewise be suppressed by LT425 because although metastasis and transformation are related phenomena, they are also separable, and it has been suggested that they may be regulated by different molecular mechanisms [30,31]. Of itself, breast cancer metastasis represents a serious challenge to therapeutic intervention. In HER2/neu-positive breast cancer patients there is a high correlation with lymph node metastasis, and metastasis is the major cause of death for these patients [6,32–34]. The entire process of tumor cell metastasis and invasion consists of: secretion of hydrolytic enzymes to the locally degraded matrix, penetration across the basement membrane into the blood circulation, spread and adhesion to the endothelia, sequential penetration backward across the basement membrane, attachment to the matrix, secretion of hydrolytic enzymes to the invaded matrix, and locomotion into the region of the stroma [20,35,36]. Within this complex sequence of

events, both cell motility enhancement and tissue invasiveness are the two key steps for the metastatic phenotypes, and in this study, it was shown that both these two metastatic steps are inhibited by the expression of LT425 in the HER2/neu-overexpressing human breast cancer cells (Figs. 4 and 5). These results thus suggest that LT425 may also act as an effective suppressor of breast cancer metastasis.

The results of this study raise two questions. First, whether suppression of the metastatic potential of HER2/neu-overexpressing breast cancer cells by LT425 is dependent upon or independent of repression of *HER2/neu* gene expression. To clarify this issue, an irrelevant promoter, such as the RSV promoter (which cannot be regulated by LT425), should be used to drive the HER2/neu cDNA and expressed in the MDA-MB-453 breast cancer lines stably transfected with LT425. Comparison of the metastasis-associated properties between these cell lines and their parental lines should then reveal which of the possible molecular mechanisms is used by LT425 in its repression of metastasis. The second question is whether LT425 suppresses the metastatic potential of MDA-MB-453 breast cancer cells by decreasing the growth rate of these cells. As shown in Fig. 2A, there is not much difference among the growth curves of the parental MDA-MB-453 cells, the 453-LT425 transfectants, and the 453-M3 mutant line in complete medium. This suggests that LT425 can repress the intrinsic metastatic potential of MDA-MB-453 breast cancer cells without reducing their growth rates, indicating that suppression of the HER2/neu-mediated metastasis by LT425 may be through a different molecular mechanism. In conclusion, our results demonstrate that LT425 can act as both transformation and metastasis suppressor of these human breast cancer cells, suggesting a possible therapeutic benefit in cases of clinical breast cancer.

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