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Award Number: DAMD17-00-1-0490

TITLE: Blocking HER-2-Mediated Transformation With a Dominant Negative Form of HER-3

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REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Blocking HER-2-Mediated Transformation With a Dominant Negative Form of HER-3

5. FUNDING NUMBERS
DAMD17-00-1-0490

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) *(abstract should contain no proprietary or confidential information)*
Amplification of the HER-2 gene often leads to breast cancer by causing cells to make abnormally high levels of the wild-type HER-2 protein. Evidence now shows that the interaction between HER-2 and HER-3 leads to the constitutive activation of HER-2/HER-3 heterodimers in breast cancer cells with HER-2 gene amplification, and HER-2/HER-3 potently activates multiple signal transduction pathways involved in mitogenesis. This indicates that inhibition of the interaction between HER-2 and HER-3 may be an especially effective and unique strategy for blocking the effects of HER-2 in human breast cancer cells. Therefore, we constructed a bicistronic retroviral expression vector that codes for a dominant negative form of HER-3 that can inactivate the function of HER-2/HER-3. Dominant negative HER-3 also specifically inhibited proliferation induced by heregulin (the ligand for HER-2/HER-3) as well as the growth factor-independent (i.e. autonomous) proliferation and anchorage-independent growth of breast cancer cells with HER-2 gene amplification. We now propose to use dominant negative HER-3 vectors in experiments to determine the effectiveness of dominant negative HER-3 for blocking HER-2/HER-3 activation, signaling and growth in culture and *in vivo* for different breast cancer cell lines with HER-2 gene amplification.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, cancer biology, receptor tyrosine kinases, cell signaling, oncogenes gene therapy, retroviral expression vectors	28
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The HER-2 (*neu*/*erb*B-2) gene encodes a 185 kDa protein tyrosine kinase that is highly homologous to the epidermal growth factor (EGF) receptor (EGFR/HER-1/*erb*B- 1), HER-3 (*erb*B-3), and HER-4 (*erb*B-4) (1-3), which together, comprise the type 1 family of receptor tyrosine kinases (4, 5). These receptors differ in their ligand specificities (4), and while HER-1 binds several ligands closely related to EGF, HER-3 and HER-4 are the receptors for a number of different isoforms of *neu* differentiation factor/heregulin (HRG) (6-8). While no direct ligand for HER-2 has yet been cloned, it is now clear that HER-2 is capable of heterodimerization with HER-1 (9, 10), HER-3 (11, 12), or HER-4 (8), and these HER-2-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). HER-2 is amplified in 28% of primary breast carcinomas *in vivo* (13), and another 10% overexpress HER-2 without amplification of the gene (14-16). HER-2 gene amplification, concordant with high-level overexpression, is associated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17-19). Experimentally elevated HER-2 gene expression in various cell lines induces tumorigenesis in nude mice (24-27), and the potent oncogenic potential of HER-2 is generally thought to be due to its ability to constitutively activate key signaling pathways that are involved in the regulation of cell growth (28). Although HER-2 was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the HER-2 gene found in human breast cancer cells has never shown such mutations (30). The level of tyrosine-phosphorylated HER-2 in primary breast cancer *in vivo* always shows a direct correspondence with the HER-2 overexpression (31), and while the wild-type HER-2 protein possess constitutive tyrosine kinase activity when overexpressed in cell lines in the absence of any identifiable ligand (24-27, 32, 33), the HER-2 tyrosine kinase domain is also constitutively active in EGFR-HER-2 chimeric receptors in the absence of EGF (32, 33). Therefore, the overexpression of wild-type HER-2 alone is sufficient to constitutively activate its tyrosine kinase function. Additionally, heterodimeric interactions are now known to occur between the different HER proteins, including HER-1 and HER-2 (9, 10), HER-2 and HER-3 (11, 12), HER-2 and HER-4 (8), and HER-1 and HER-3 (34, 35). Our own work and that of others has now shown that heterodimer interactions between HER-2 and HER-3 are constitutively active in breast cancer cells with HER-2 gene amplification (20-23), and co-transfection of HER-3 with HER-2 greatly augments the transforming capability of HER-2 in genetically engineered cell lines (21). Therefore, we are particularly interested in how the cooperative effects of HER-2/HER-3 heterodimers activate key mitogenic signaling pathways which facilitate cell growth. Our own work and that of others has also now shown that the constitutive activation of HER-2/HER-3 in breast cancer cells is associated with the constitutive activation of phosphatidylinositol (PI) 3-kinase and mitogen-activated protein (MAP)-kinase (20-22). One strategy that has been used successfully to block the function of other receptor tyrosine kinases employs dominant negative expression vectors in which a region coding for the cytoplasmic domain of the receptor is almost completely removed. While the truncated receptor still contains the extracellular and transmembrane domains and can dimerize within the cell membrane, it lacks tyrosine kinase activity and inhibits the signal transduction docking function of both mutant/mutant homodimers and mutant/wild-type heterodimers. This strategy has been used effectively to block EGFR (36), platelet-derived growth factor receptor (37), and fibroblast growth factor receptor (38) in biochemistry studies. Recently, a dominant negative HER-2 vector was also used successfully to block HER-2 function

in normal mouse development (40). The use of such dominant negative HER-2 vectors has not yet been reported to block HER-2 in cancer cells with HER-2 gene amplification, and this is likely due to the stoichiometric problems associated with the inability to generate mutant/wild-type levels high enough for effective inhibition in human breast cancer cells with HER-2 gene amplification. However, the fact that HER-3 is not highly overexpressed in these cells, and that activated HER/HER-3 has a particularly high-affinity interaction (42-44), suggests that dominant negative HER-3 may be especially effective in blocking HER-2/HER-3 function. Therefore, we have introduced a dominant negative form of HER-3 into cells in an attempt to block the activation of HER-2/HER-3 (45).

BODY

We sought to determine the effectiveness of dominant negative HER-3 in blocking HER-2/HER-3 activation, signaling and the transformed growth of breast cancer cells with HER-2 gene amplification. As discussed in detail in the grant proposal, we routinely use the H16N-2 normal breast epithelial cells and the 21MT-1 breast carcinoma cells for our studies because they were derived from the same patient and can be grown under precisely defined serum-free conditions in culture. This well defined system allows us to study growth factor-independent (i.e. autonomous) proliferation and responses to exogenous growth factors in a manner that is not yet possible for other cell lines derived in medium containing high levels of serum. Therefore, this serum-free system was well suited to study receptor activation and signaling and to distinguish constitutive from externally mediated growth factor responses in culture (22, 41, 45). H16N-2 and 21MT-1 cells were infected with the pCMV control vector or pCMV-dn3, selected on G418, and then used to determine the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture (45). Interestingly, dominant negative HER-3 had no apparent effect on the insulin/EGF-induced proliferation of either H16N-2-dn3 or 21MT-1-dn3 cells after they had been infected with pCMV-dn3 and selected on G418. This suggested that dominant negative HER-3 preferentially inhibited HER-2/HER-3-mediated growth responses over those mediated by exogenous EGF in cells that express EGFR and are responsive to EGF-induced proliferation. Therefore, the effects of pCMV-dn3 in H16N-2 and 21MT-1 cells showed selectivity by preferentially inhibiting growth responses involving HER-2/HER-3, such as those that are constitutively activated by HER-2 or induced by HRG, and indicated that HER-1/HER-3 is not as effectively inhibited by dominant negative HER-3 as is HER-2/HER-3 at a given level of dominant negative HER-3 gene expression. While EGFR and HER-3 do interact in these and other cell lines (46), clearly the EGFR/HER-3 heterodimer interaction is weak compared to the HER-2/HER-3 heterodimer interaction (42-44). In order to further investigate the preferential effects of dominant negative HER-3 on HER-2/HER-3-mediated growth responses in 2MT-1 cells, we also compared the levels of receptor tyrosine phosphorylation in cells induced with either HRG or EGF (see Fig. 3 in 2001 annual report). While dominant negative HER-3 inhibited the constitutive tyrosine phosphorylation of HER-2/HER-3 as well as that seen with HRG stimulation, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was apparently not effected by dominant negative HER-3 in the 21MT-1 cells. <u>These results are consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 gene expression</u>.

In vivo studies were then performed using the 21MT-1-derived cell lines for injections into immunodeficient mice as described in the grant proposal. However, the 21MT-1 cell line was not tumorigenic in our studies and thus would not allow us to test the effects of dominant negative HER-3 *in vivo*. The limited growth seen for 21MT-1 cells in nude mice is apparently a common problem for a significant number of malignant and metastatic breast carcinoma cell lines (47), and earlier studies also suggested some difficulty in using 21MT-1 cells for tumor studies at later passages (48). Experiments were also done this last year comparing injections of 21MT-1 cells in both Nu/Nu and Nu/Nu CD-1 strains of nude mice as well as in scid mice that lack both T-cell and B-cell function. To date, we have not been able to detect tumors in mice injected with up to 10^7 21MT-1 cells per site in any immunodeficient strain of mouse yet tested. We also noticed on

further review of the literature that most previous studies employed fibroblasts genetically engineered to overexpress HER-2 for their *in vivo* experiments, but not spontaneously derived breast carcinoma cell lines with HER-2 gene amplification. We have also now done experiments using BT-474, MDA-MB-453 and SK-BR-3 cells for injection into immunodeficient mice. BT-474, MDA-MB-453 and SK-BR-3 cells have all been reported to contain HER-2 gene amplification and activation of HER-2/HER-3, so we have proposed to use these cell lines as well. After obtaining all three cell lines directly from the ATTC, experiments were conducted to measure the level of HER-2/HER-3 activation in culture as well as tumorigenicity *in vivo*. None of these cell lines has yet formed detectable tumors in either Nu/Nu or Nu/Nu CD-1 strains of nude mice injected with 10^7 cells per injection. However, BT-474 cells were found to consistently form measurable tumors in scid mice within a few weeks after injection. In two groups of 8 injections each, detectable BT-474 tumors were dissected out and measured after 2 months from the time of injection of 10^7 cells per site in scid mice. The mean tumor diameter for each group of 8 injections were 4.1 +/- 1.7 mm and 6.0 +/- 2.1 mm, respectively. Preliminary experiments injecting MDA-MB-453 or SK-BR-3 cells in scid mice have also shown some very small tumors, but these tumors were detected only after autopsy more than 5 months after the time of injection.

Analysis of HER-2 and HER-3 expression was also carried out using the BT-474, MDA-MB-453 and SK-BR-3 cell lines (data not shown). All 3 of these cell lines showed very high levels of HER-2, but was highest in the BT-474 cells. HER-3 was expressed by all 3 cell lines, and was moderately elevated in the BT-474 and MDA-MB-453 cells relative to SK-BR-3 cells. The constitutive activation of HER-2, HER-3 and signaling pathways was also measured in the BT-474, MDA-MB-453 and SK-BR-3 cells (data not shown). The cells were placed in serum-free medium for 48 hours prior to protein extractions so that the levels of tyrosine-phosphorylated receptors represented the *constitutive* level of receptor activation. Immunoprecipitation/Western blot measurement of tyrosine-phosphorylated HER-2 and HER-3 showed that both HER-2 and HER-3 were highly phosphorylated in the BT-474 cells. SK-BR-3 cells showed high levels of HER-2 phosphorylation, but only very low levels of HER-3 phosphorylation. MDA-MB-453 cells showed only low levels of HER-2, and very low levels of HER-3 phosphorylation. Western blotting of PI 3-kinase and SHC proteins in anti-phosphotyrosine immunoprecipitates also showed that BT-474 cells had a high level of p85 $^{\text{PI 3 kinase}}$, p46$^{\text{SHC}}$ and p52$^{\text{SHC}}$ recruited by activated HER-2/HER-3. SK-BR-3 cells also showed high levels of p46$^{\text{SHC}}$ and p52$^{\text{SHC}}$ recruitment, but only lower levels of p85 $^{\text{PI 3 kinase}}$ recruitment in anti-phosphotyrosine immunoprecipitates. MDA-MB-453 only showed some recruitment of p46$^{\text{SHC}}$, and a much lower level of p85 $^{\text{PI 3 kinase}}$ recruitment in anti-phosphotyrosine immunoprecipitates compared to the BT-474 cells.

We have now infected BT-474, MDA-MB-453 and SK-BR-3 cells with pCMV and pCMV-dn3 as outlined in the grant proposal. As previously discussed in the 2001 annual report in detail, titrations were initially performed to determine the concentrations of G418 necessary to kill BT-474, MDA-MB-453 and SK-BR-3 cells within 2-3 weeks after addition of the antibiotic, and these concentrations (i.e. 500 ug/ml for SK-BR-3 cells, 600 ug/ml for MDA-MB-453 cells and 700 ug/ml for BT-474 cells) were then used to select these cell lines on G418 after being infected with pCMV or pCMV-dn3. The BT-474, MDA-MB-453 and SK-BR-3 cells were infected using conditioned media from the PA-317 packaging cell lines that had been transfected with either pCMV or pCMV-dn3, selected on G418 and then used for infecting BT-474, MDA-MB-453 and SK-BR-3 cells. By using the PA-317-derived packaging cell lines, we successfully generated viral titers sufficient for good colony formation of BT-474, MDA-MB-453 and SK-BR-

3 cells infected with pCMV. Interestingly, we also consistently saw a much lower number of colonies growing out for the cells infected with pCMV-dn3 under identical conditions in culture (see Fig. 4 in 2001 annual report). These earlier preliminary observations have now been extended with additional experiments where triplicate wells were infected with vectors and colonies were counted after selection on G418 for a month in culture (Fig. 1). Furthermore, we noticed that many of the colonies that started to grow out in plates infected with pCMV-dn3 were small (Fig. 2B) and many did not continue to grow, showing gradual morphological abnormalities and eventual cell death (data not shown). This suggested that the growth of many of the cells infected with pCMV-dn3 may be inhibited by the expression of dominant negative HER-3 during their selection on G418.

As discussed in the 2001 annual report in detail, we confirmed the expression of dominant negative HER-3 in cells infected with pCMV-dn3 using anti-HER-3 immunocytochemistry on cells infected with pCMV-dn3 (see Fig. 5 in 2001 annual report). The anti-HER-3 H105 antibody (Neomarkers) is known to be highly specific for HER-3, and binds to an epitope within the extracellular domain of HER-3. This antibody does not work for Western blotting, but works well for immunocytochemistry (45). While all of the cell lines express wild-type HER-3, the levels are relatively low when detected with HRP/DAB staining. Therefore, moderate or high levels of HER-3 measured in cells infected with pCMV-dn3 confirmed the ectopic expression of dominant negative HER-3 (45). However, we also noticed significant heterogeneity in the level of HER-3 staining in mass selected cell populations infected with pCMV-dn3 (see Fig. 5B in 2001 annual report), and many of the cells expressed only lower levels of dominant negative HER-3. We have previously shown that 100% of cell clones from cells infected with the pCMV bicistronic retroviral expression vector coordinately co-express the Lac Z marker gene with antibiotic resistance (45). However, heterogeneity in the level of expression of inserted genes is still seen between different cell clones. Therefore, while the different mass selected cell lines infected with pCMV-dn3 showed higher levels of staining for HER-3 compared to controls, heterogeneity in dominant negative HER-3 expression between different cell clones may lead to the preferential selection of clones that express only lower levels of dominant negative HER-3 with passaging in culture.

In order to further investigate the expression of dominant negative HER-3 in cell colonies immediately after selection on G418, additional anti-HER-3 immunocytochemistry was performed (Fig. 3). While many of the smaller and dying colonies infected with pCMV-dn3 expressed very high levels of dominant negative HER-3 (Fig. 3B), other larger colonies showed only moderate or low levels of dominant negative HER-3 expression (Fig 3C). Some slow growing cell clones from pCMV-dn3-infected cell populations were also isolated and screened for their expression of dominant negative HER-3. Furthermore, the recent aquisition of a polyclonal anti-HER-3 antiserum directed against the extracellular domain of HER-3 (Transduction Labs) has now allowed us to identify the dominant negative HER-3 protein in Western blots (Fig. 4). Interestingly, the slowest growing clones which required a much longer time to grow out were the samples that showed the highest levels of the approximately 100 kD dominant negative HER-3 protein (Fig. 4, Lanes 2, 6, 8 and 9). The dominant negative form of HER-3 was also seen as a broad band in Westerns, possibly indicative of differential glycosylation and/or other modification of the ectopic protein in genetically engineered cell lines. Some cell clones also showed a significant reduction in receptor activation in anti-phosphotyrosine Western blots (data not shown). However, further work is underway to further screen these clones to assess their growth

properties as well as the levels of dominant negative HER-3 and HER-2/HER-3 constitutive activation during subsequent passaging of the cell lines in culture. BT-474, MDA-MB-453 and SK-BR-3 cells that were previously infected with either pCMV or pCMV-dn3, mass selected and passaged for further experiments were screened for the levels of HER-2, HER-3, PI 3-kinase and MAP-kinase activation. These passaged cell populations showed no significant differences in the levels of HER-2, HER-3, PI 3-kinase and MAP-kinase activation, and the BT-474-derived cells showed no significant reduction in tumor growth in scid mice *in vivo* (data not shown). However, these results are difficult to interpret because this may also indicate that the mass selected and passaged cell populations are not retaining many cells expressing very high levels of dominant negative HER-3 if they are being selected out during passaging in culture. As mentioned above, work is still in progress with the newly derived BT-474-derived cell clones. However, if high levels of the dominant negative HER-3 protein is inhibiting cell growth, we may have difficulty in generating cell lines with stable high-level dominant negative HER-3 gene expression because these cell lines may strictly require constitutive HER-2/HER-3 function for propagation in culture. If the highest level expressing cells are being selected out because of the inhibitory effects of dominant negative HER-3, this may necessitate the use of an inducible expression system to directly study the full extent of the effects of dominant negative HER-3. Therefore, we have now developed retroviral expression vectors for engineering cell lines that express regulatable dominant negative HER-3 using the Tetracycline repressor ("Tet-Off") system.

The tetracycline-repressible pRevTRE retroviral expression vector facilitates the regulated expression of ectopic genes in mammalian cells (Clontech). We constructed an inducible dominant negative HER-3 vector using the pRevTRE vector as previously outlined in the 2001 annual report. pRevTRE contains Sal I and Cla I sites that were used to insert the Sal I-Cla I dominant negative HER-3 fragment isolated from pBK-dn3 (45) into pRevTRE to generate pRevTRE-dn3 (Fig. 5B). We also constructed a control vector, pRevTRE-Lac Z, which is now being used to determine the levels of tetracycline repressor activity in cell lines stably infected with pRevTet-Off (which contains the tetracycline repressor and neomycin-resistance genes). pRevTRE-Lac Z was cloned by inserting the Hind III-Nar I Lac Z fragment isolated from pSV-β-Galactosidase (Promega) into the Hind III and Cla I sites (Nar I ends are also compatible with Cla I ends) in pRevTRE to generate pRevTRE-Lac Z (Fig. 5A). The proper construction of the vectors was verified with extensive restriction digest analysis (Fig. 6). The newly constructed pRevTRE-dn3 vector is now being used to infect BT-474pRevTet-Off, MDA-MB-453pRevTet-Off and SK-BR-3pRevTet-Off cells that we have infected with pRevTet-Off (using media from PA-17 cells transfected with pRevTet-Off and selected on G418), selected on G418, and screened for tetracycline repressor function. The pRevTRE vector utilizes a hygromycin resistance gene, and selection of these cell lines on hygromycin will then allow us to derive cell lines that express the tetracycline repressor protein as well as the dominant negative HER-3 gene driven by the CMV-TRE fusion promoter. New BT-474 cell lines expressing the tetracycline repressor protein have already been developed and screened using the pRevTRE-Lac Z vector for transfection and β-galactosidase assays to assess the tetracycline repressor function in different cell clones infected with pRevTet-Off (Fig.7). PA-317 packaging cells have also been transfected with pRevTRE or pRevTRE-dn3, selected on hygomycin and these cell lines are being used to infect tetracycline repressor-positive BT-474pRevTet-Off, MDA-MB-453pRevTet-Off and SK-BR-3pRevTet-Off cell clones with pRevTRE-dn3. The cells infected with the regulatable form of dominant negative HER-3 will be cultured in the presence of tetracycline to keep the dominant negative HER-3 gene

off during the selection on hygromycin. Subsequent to selection on hygromycin, cells with (as a control) and without tetracycline will then be used for experiments as we have outlined in the grant proposal. Cells are also being infected with pRevTRE as a control for tumor studies *in vivo*. This "Tet-Off" system should also be ideal in that we can maintain the cells in the presence of tetracycline prior to injection into mice and, with removal of tetracycline, the cells will then turn on the expression of the dominant negative HER-3 gene at the start of the experiment. Furthermore, this system also allows a wide range in the level of gene repression through the CMV-TRE fusion promoter, depending on the concentration of tetracycline employed. This will also be useful for directly studying the stoichiometric aspect of receptor inhibition in future experiments.

In summary, the specific objectives of the work have been addressed as follows: Tasks 1, 2 and 3 have been completed for the different cell lines as originally outlined in the grant proposal. H16N-2, 21MT-1, BT-474, MDA-MB-453 and SK-BR-3 cells have all been infected with pCMV and pCMV-dn3 expression vectors, selected on G418 and screened for the expression of ectopic dominant negative HER-3. In addition, the results attained from these experiments using BT-474, MDA-MD-453 and SK-BR-3 cells indicated the importance of doing more extensive analysis of different cell clones, and that work is still in progress. Furthermore, construction of the pRevTRE-Lac Z and pRevTRE-dn3 retroviral expression vectors has been completed as outlined in the 2001 annual report, and work is still in progress to develop additional cell lines expressing tetracycline-repressible dominant negative HER-3. Task 4 has been completed for the 21MT-1-derived cells as originally outlined in the grant proposal, and work is still in progress to test the effects of dominant negative HER-3 on the growth and survival of BT-474-, MDA-MB-453- and SK-BR-3-derived cell lines in culture. Tasks 5 and 6 are also still in progress. Since the 21MT-1 cell line does not provide a viable model for studying growth *in vivo*, most of the remaining experiments will employ BT-474 cells, which we have now confirmed have a high-level constitutive activation of HER-2/HER-3 and effectively form tumors in scid mice *in vivo*.

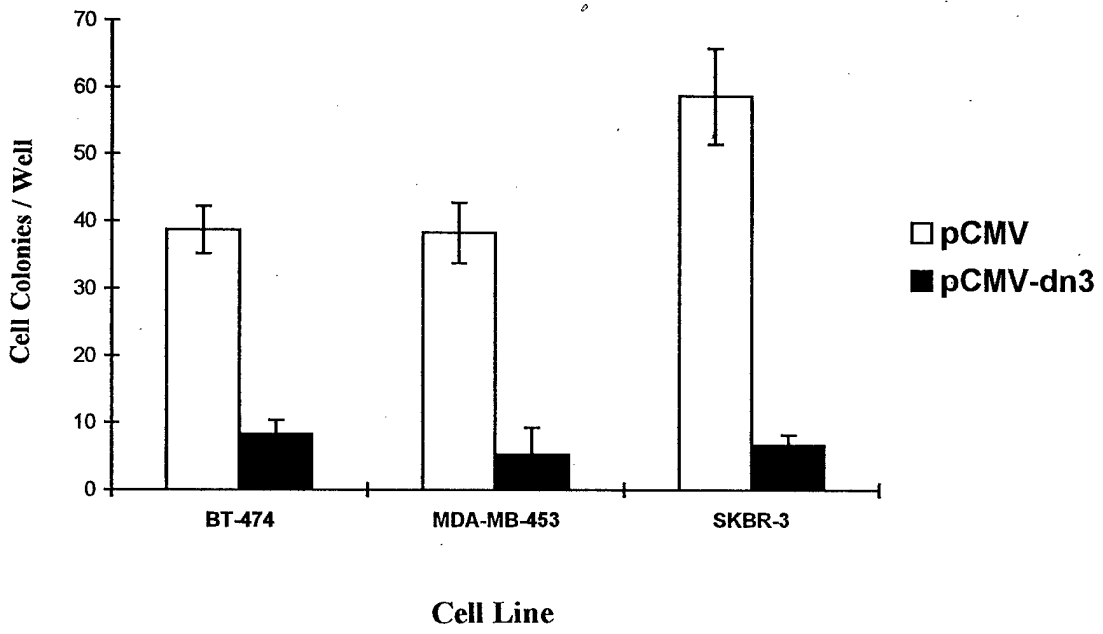
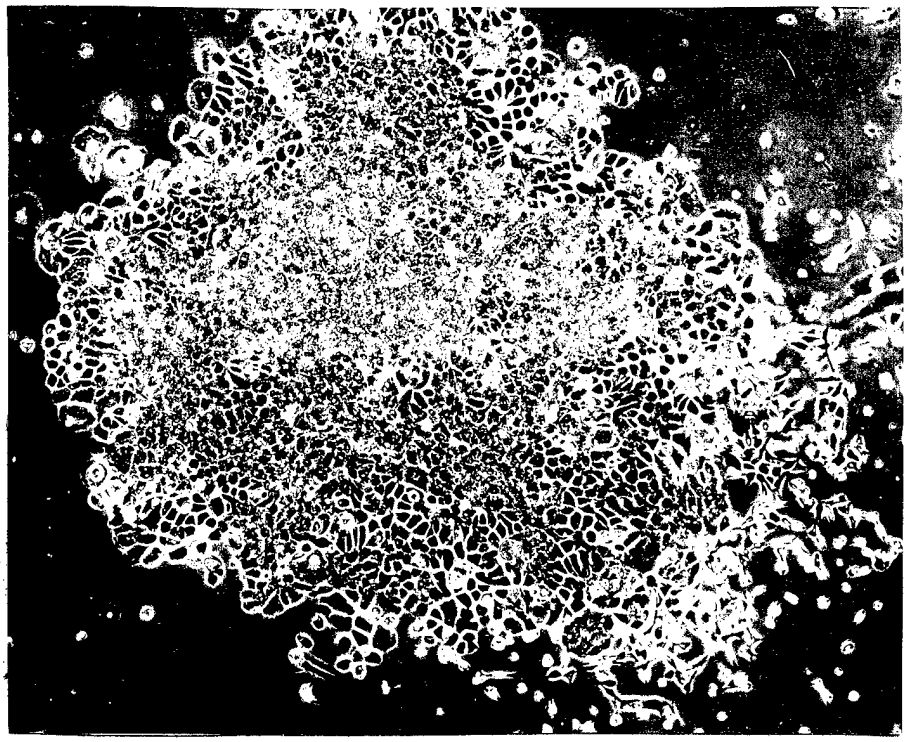


Fig. 1. *Clonal outgrowth of BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3.* BT-474, MDA-MB-453 and SK-BR-3 cells were infected for 3 days with conditioned medium collected from PA-317 packaging cells stably transfected with pCMV (□) or pCMV-dn3 (■). The infected cells were then incubated with fresh medium for 2 days and selected on G418 for a month before counting colonies. The mean average and standard deviation for triplicate samples is shown.

A



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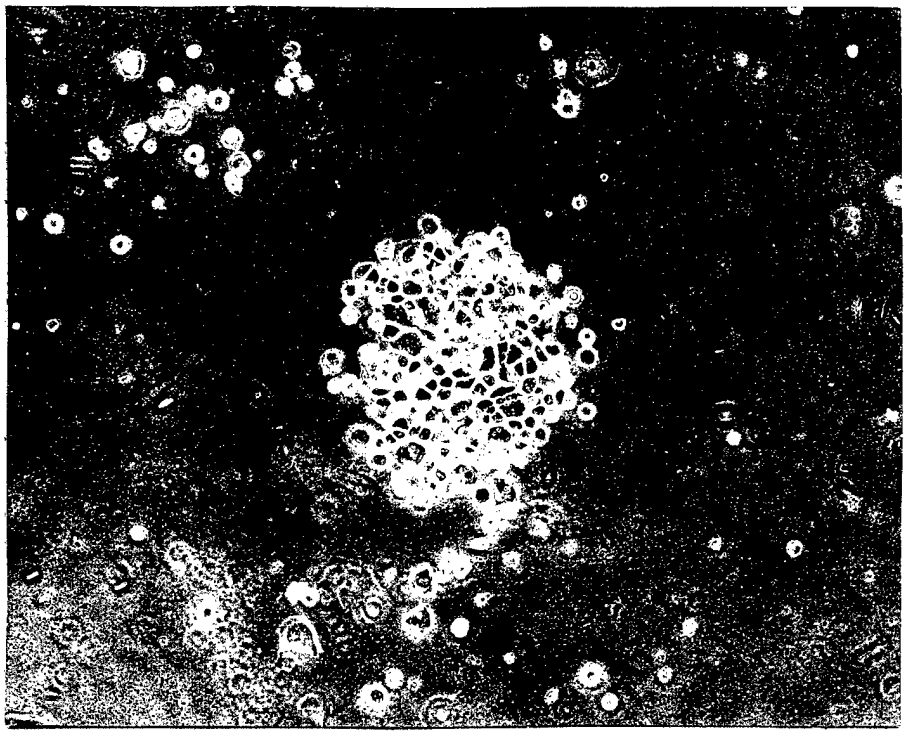


Fig. 2. *Morphology of BT-474 cell colonies infected with pCMV or pCMV-dn3.* Phase contrast microscopy of BT-474 cells infected with either pCMV (A) or pCMV-dn3 (B) and selected on G418 for a month in culture.

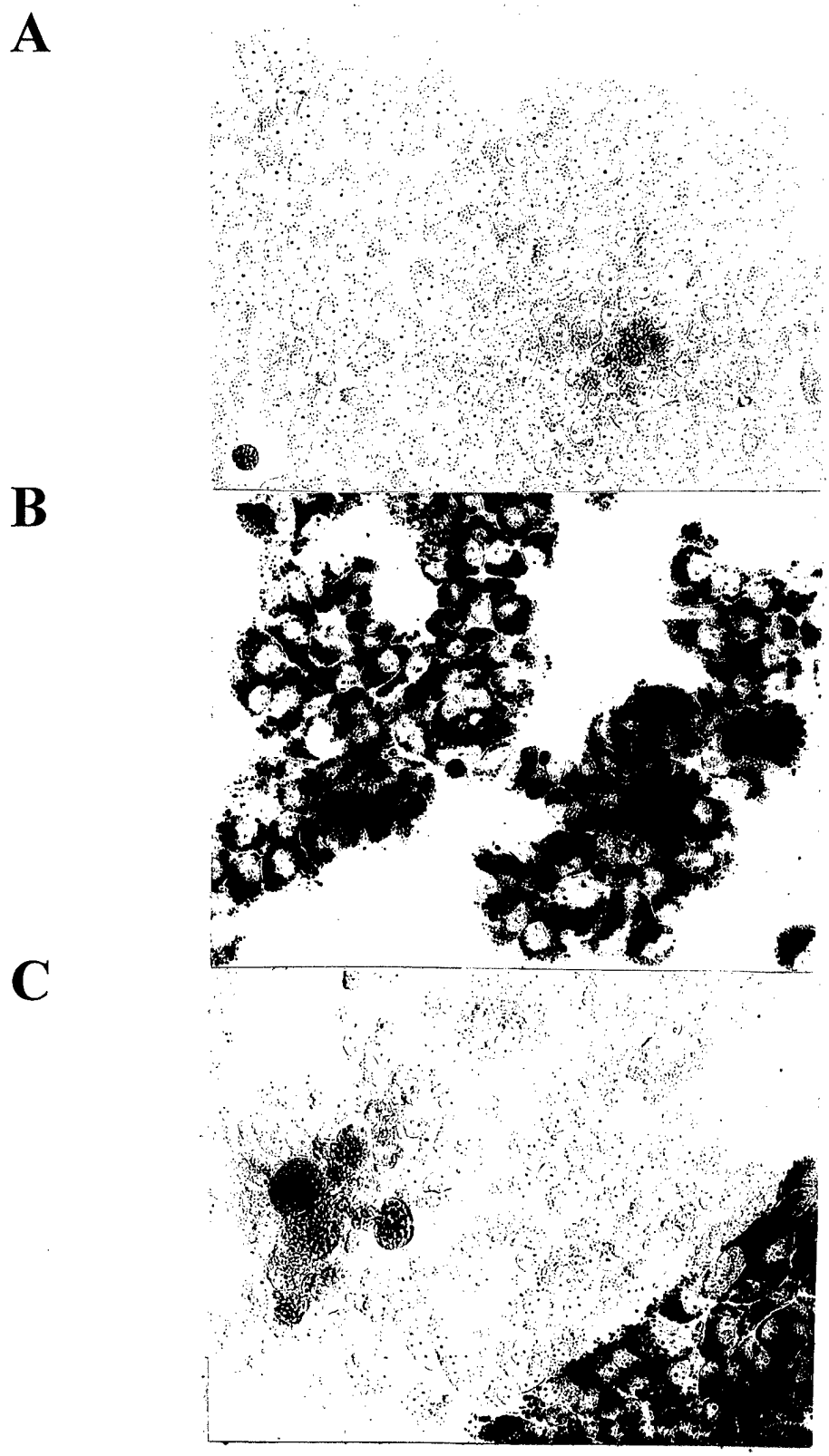


Fig. 3. *Expression of dominant negative HER-3 in cells infected with pCMV-dn3.* Bright field microscopy of anti-HER-3 immunocytochemistry in BT-474 cells infected with either pCMV (A) or pCMV-dn3 (B and C). High levels of ectopic HER-3 was seen in a number of smaller slow growing (B) and dying (not shown) cell colonies. Also notice the heterogeneity in staining between different cell colonies infected with pCMV-dn3 (C), where some colonies express low levels (C, upper left) while others express high levels (C, lower right) dominant negative HER-3.

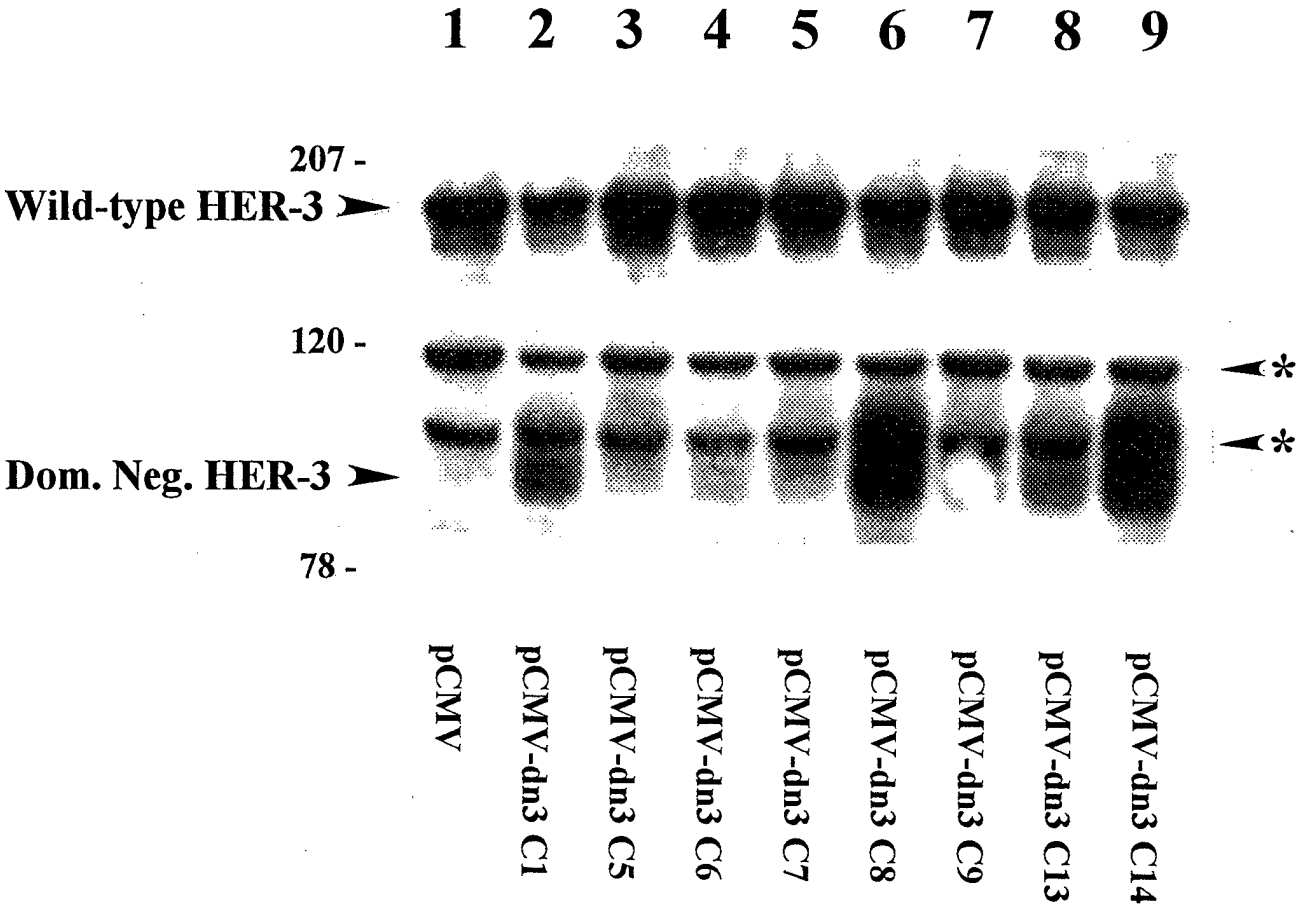
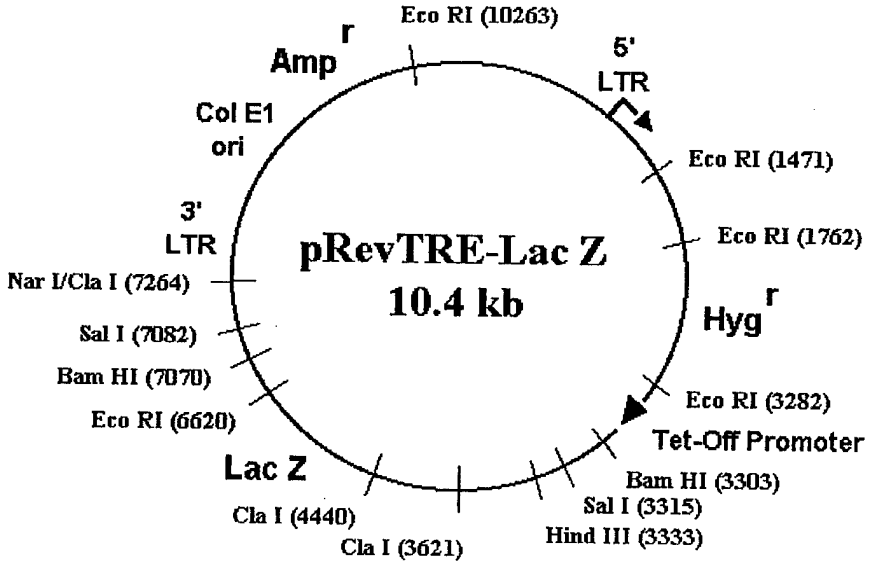


Fig. 4. *Expression of dominant negative HER-3 in cells infected with pCMV-dn3.* Western blot analysis of HER-3 expression in BT-474 cells infected with pCMV (Lane 1) or different BT-474 cell clones infected with pCMV-dn3 (Lanes 2 -9). A polyclonal anti-HER-3 antibody raised to the extracellular region of HER-3 was used to directly identify the approximately 100 kD dominant negative HER-3 protein in various clones of BT-474 cells infected with pCMV-dn3. The clones shown in Lanes 2, 6, 8 and 9 expressed the highest levels of dominant negative HER-3. High molecular weight markers are shown (kD) on the left and non-specific bands are marked (*) on the right.

A



B

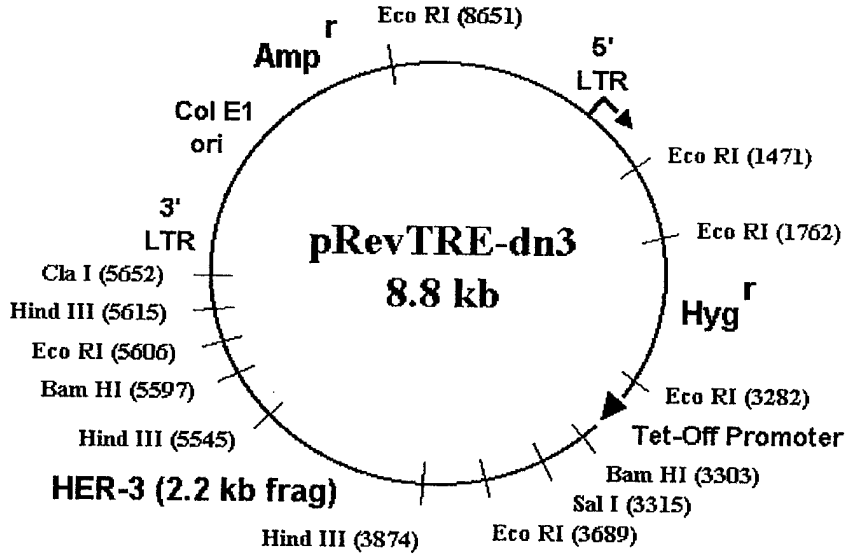


Fig. 5. *Tetracycline-repressible vectors for the expression of β-Galactosidase or dominant negative HER-3.* The pRevTRE tetracycline-repressible retroviral expression vector (Clonetech) was used to construct pRevTRE-Lac Z (A) and pRevTRE-dn3 (B). pRevTRE-Lac Z is used to transfect cell clones previously infected with pRevTet-Off (which contains the tetracycline repressor gene) and screen for tetracycline repressor activity. pRevTRE-dn3 is then introduced into positive cell clones which are then cultured in the presence of tetracycline to keep the ectopic gene off during selection on hygromycin.

		Predicted Fragment Length (kb)	
Lane	**Enzyme**	**pRevTRE-Lac Z**	**pRevTRE-dn3**
1)	Bam HI	6.6, 3.8	6.5, 2.3
2)	Cla I	9.6, 0.8	8.8
3)	Eco RI	3.6, 3.3, 1.6, 1.5...	3.1, 1.9, 1.6, 1.5...
4)	Hind III	10.4	7.1, 1.7...
5)	Sal I	6.6, 3.8	8.8

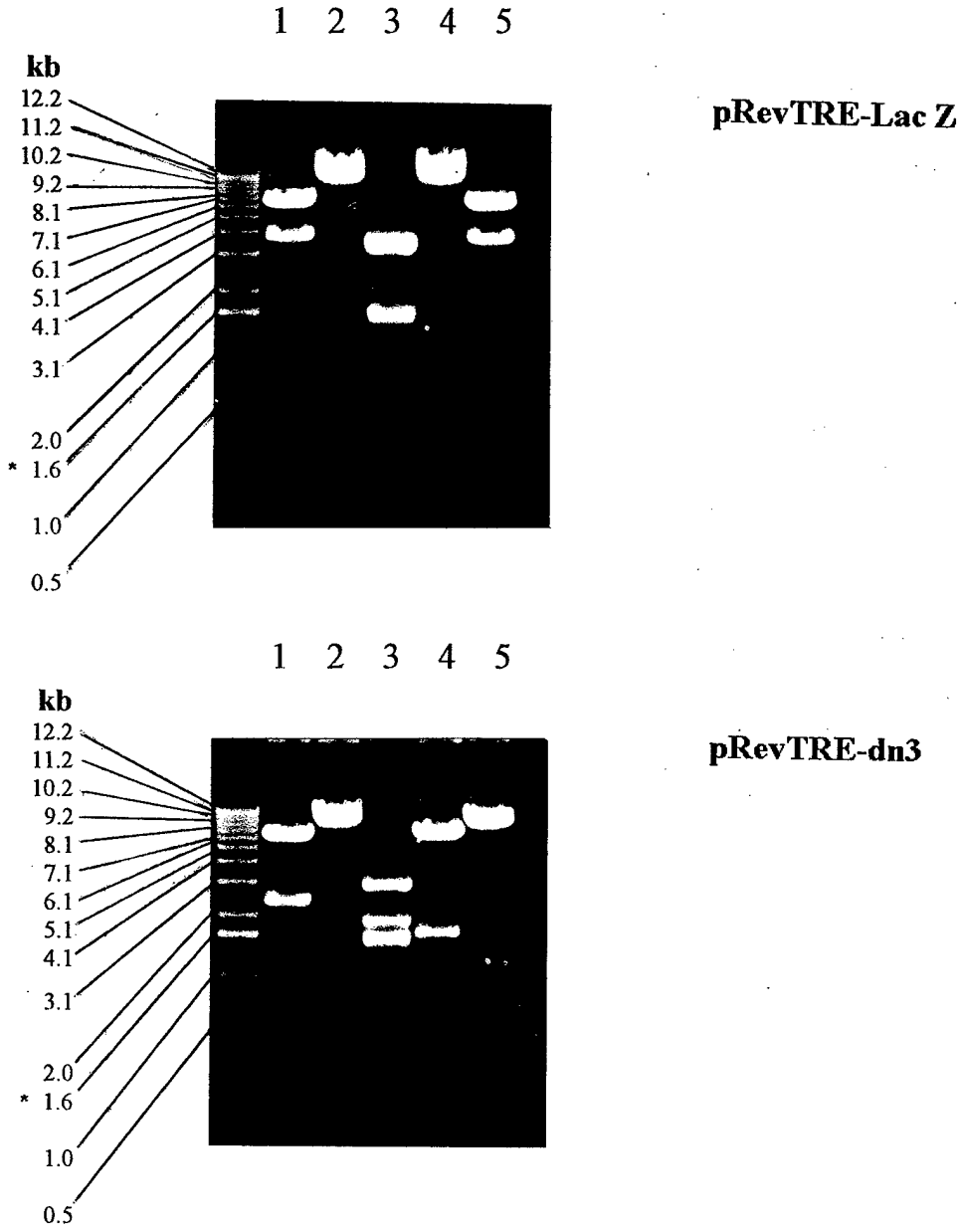


Fig. 6. *Restriction digest analysis of pRevTRE-LacZ and pRevTRE-dn3 retroviral expression vectors.* Restriction digests of plasmid DNA were electrophoresed in 0.8% agarose gels containing 0.5% ethidium bromide and photographed using UV light. A 1 kb DNA ladder is shown and the 1.6 kb marker (*) is the brightest band in the ladder.

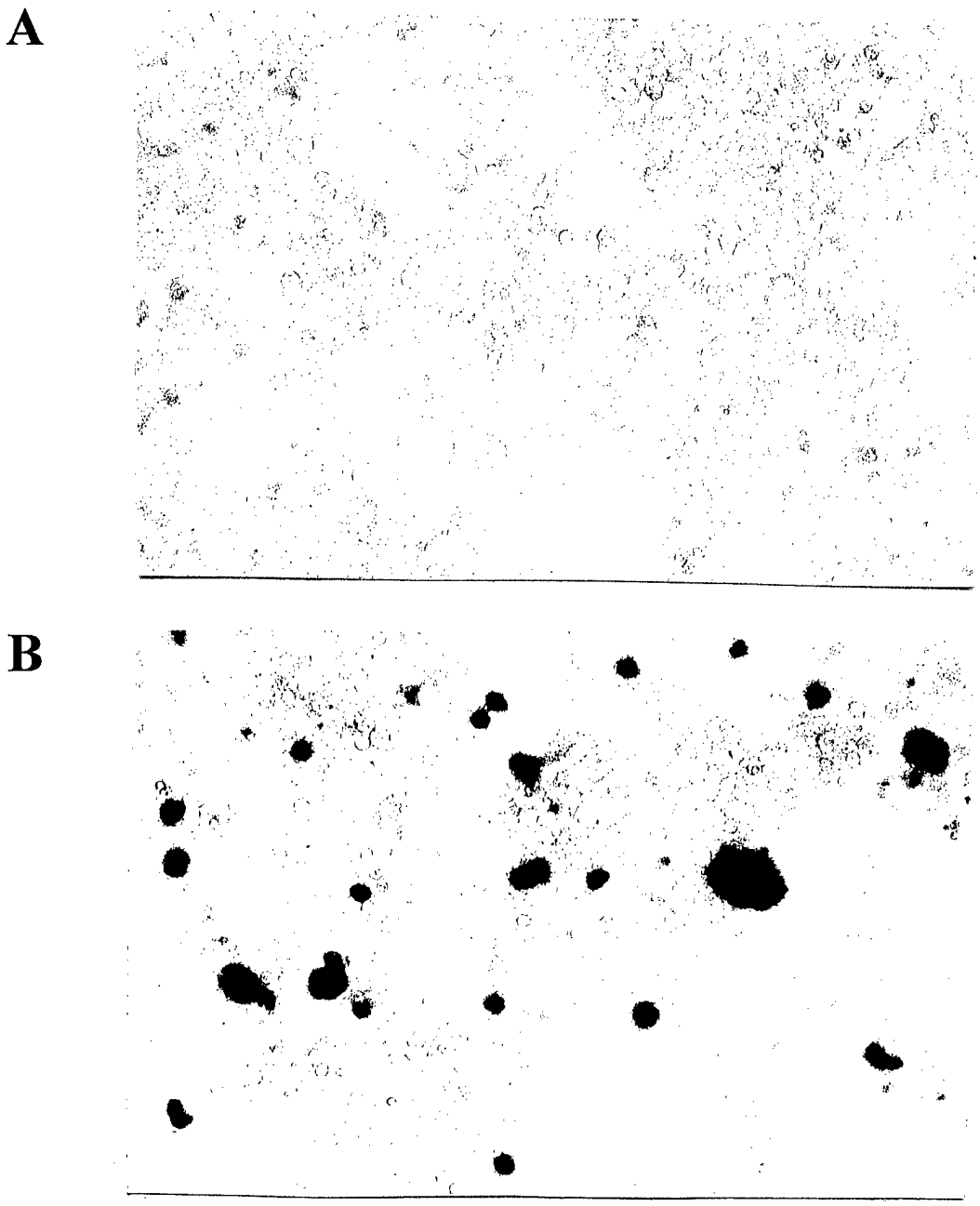


Fig. 7. *β-galactosidase activity in BT-474pRevTet-Off cells with or without tetracycline*. In order to screen for tetracycline repressor activity in different BT-474pRevTet-Off cell clones, cells were transiently transfected with pRevTRE-Lac Z and cultured either with (A) or without (B) tetracycline prior to staining for β-galactosidase activity. Bright field microscopy of cell clones stained for β-Galactosidase activity showed positive tetracycline repressor activity when the marker gene was not expressed in the presence of tetracycline. This cell clone is presently being used for further infection with pRevTRE-dn3 in order to generate BT-474 cells that express a regulatable dominant negative HER-3 gene.

KEY RESEARCH ACCOMPLISHMENTS

- The 21MT-1-derived cell lines were used for additional studies of the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*.

- BT-474, MDA-MB-453 and SK-BR-3 cells were all infected with pCMV and pCMV-dn3, and a number of cell clones infected with pCMV-dn3 were also isolated for further characterization.

- BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV and pCMV-dn3 were characterized for the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*.

- Additional vectors and cell lines were developed to study the effects of regulatable dominant negative HER-3 gene expression in BT-474, MDA-MB-453 and SK-BR-3 cells using the "Tet-Off" promoter system.

REPORTABLE OUTCOMES

A manuscript describing most of the work done with the H16N-2 and 21MT-1 cell lines was published earlier (45). Another manuscript containing additional data and work using the BT-474, MDA-MB-453 and SK-BR-3 cell lines is presently in preparation.

CONCLUSIONS

Amplification and overexpression of the HER-2 gene is involved in the oncogenic transformation of mammary epithelial cells in approximately a third of breast cancer patients. In those individuals with HER-2 gene amplification, this dominant genetic event is likely to be the principle change which drives malignancy because HER-2 is such a potent oncogene when highly overexpressed in experimental systems. However, our understanding is still fragmentary concerning the exact mechanisms by which signals for cell growth are constitutively activated in breast cancer cells with HER-2 gene amplification, and methods for permanently inhibiting the constitutive activation of signaling in such cells have not yet met with great success. Our previous work showed that the high-level overexpression of HER-2 in 21MT-1 cells was associated with the constitutive activation of PI 3-kinase and growth factor independence in culture (22). By constitutively activating key mitogenic signaling pathways to a level that is effective for autonomous growth, tumor cells escape the normal controls on cell cycle regulation. Therefore, we sought to experimentally assess the importance of the cooperative interactions between HER-2 and HER-3 during the growth factor-independent proliferation of breast cancer cells with HER-2 gene amplification, as well as in cells stimulated by exogenous HRG. Our data now confirm the importance of the HER-2/HER-3 heterodimer interaction for receptor activation and the recruiting of key signaling molecules in 21MT-1 breast cancer cells with HER-2 gene amplification, as well as in normal H16N-2 cells stimulated by HRG (45). Dominant negative HER-3 also potently inhibited the growth factor-independent and anchorage-independent growth of the 21MT-1 cells in culture (45). Therefore, our work studying the interaction between HER-2 and HER-3 offers exciting new opportunities for blocking the mechanism of autonomous growth in breast cancer cells with HER-2 gene amplification.

By itself, HER-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis showing deleterious substitutions in the enzymatic site) and is not able to activate signaling in-and-of-itself in genetically engineered cell lines that do not co-express any of the other HER kinases. However, while the other HERs have active kinase domains, HER-3 contains multiple additional docking sites for PI 3-kinase and SHC proteins not found in the other HERs. Also, as mentioned above, HER-2 is known to be an especially active tyrosine kinase that exhibits ligand-independent activation when overexpressed. These combined considerations (i.e. HER-3 docking sites combined with HER-2 kinase potential) may account for the especially potent activation of signaling induced by HER-2/HER-3 heterodimers in response to HRG in H16N-2 cells (22, 45, 46), and that is constitutively activated in 21MT-1 cells (22, 45). Interestingly, the blocking of HER-2/HER-3 function with dominant negative HER-3 showed specificity in that the H16N-2 and 21MT-1 cells infected with pCMV-dn3 and selected on G418 still proliferated in response to exogenous EGF. This suggested that HER-1/HER-3 was not as effectively inhibited by dominant negative HER-3 as was HER-2/HER-3 at a given level of dominant negative HER-3 expression. As mentioned above, HER-1 and HER-3 interact to some extent in these (46) and other cell lines (34, 35), and this interaction may be required for EGF-stimulated growth (46). However, the relative affinity of HER-1/HER-3 heterodimers is very weak compared to HER-2/HER-3 heterodimers when compared with cross-linking analysis (42-44). Therefore, in order to further investigate the preferential effect that dominant negative HER-3 had on HER-2/HER-3-mediated growth, we also compared the effects of dominant negative HER-3 on both HRG- and EGF-induced activation of HERs with anti-phosphotyrosine

immunoblotting (see Fig. 3 in 2001 annual report). While dominant negative HER-3 inhibited the levels of tyrosine phosphorylated HER-2/HER-3 in 21MT-1-dn3 cells, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was not apparently affected by dominant negative HER-3 in cells under these conditions. This result was also consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 in genetically engineered cell lines.

We have now also generated BT-474, MDA-MB-453 and SK-BR-3 cells stably infected with either pCMV or pCMV-dn3, and have screened cells infected with pCMV-dn3 for the expression of dominant negative HER-3. We are presently using these and other newly developed cell clones to further study the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses as originally outlined in the grant proposal. While we have generated BT-474, MDA-MB-453 and SK-BR-3 cells infected with these vectors, we have also seen a much lower number of colonies growing out during selection on G418 for cells infected with the pCMV-dn3 than for those infected with pCMV. Furthermore, we noticed that many of the colonies that started to grow out in plates infected with pCMV-dn3 did not continue to grow, suggesting that the proliferation and survival of many of the cell clones infected with pCMV-dn3 was inhibited by the expression of dominant negative HER-3. This is also consistent with the heterogeneity in anti-HER-3 staining seen for mass selected and passaged cells. In addition, we have now found that cells infected with pCMV-dn3 stained right after being selected on G418 showed only high-level expression of dominant negative HER-3 in smaller slow growing or dying colonies. Therefore, the low number of colonies seen for cells infected with pCMV-dn3 indicates that the growth of many of the cells which express the highest levels of dominant negative HER-3 may be inhibited and thus only form small or attenuated colonies, while clones expressing low levels of dominant negative HER-3 are selected for during subsequent passaging.

In contrast to the 21MT-1 cells, little is known about the growth factor requirements for BT-474, MDA-MB-453, and SK-BR-3 cells, which are all usually cultured under undefined conditions in medium containing 10% fetal calf serum. Therefore, it may be difficult to maintain cell populations that express the higher levels of dominant negative HER-3 under conditions where constitutive and regulative growth responses have not yet been determined as for the 21MT-1 cells. As mentioned above, if the highest level expressing cells are being selected out because of the inhibitory effects of dominant negative HER-3, this may necessitate the use of a regulatable expression system to directly study the full extent of the effects of high-level dominant negative HER-3 in these cell lines. The tetracycline-repressible pRevTRE retroviral expression vector facilitates the inhibition of ectopic genes in mammalian cells during selection on antibiotic (Clontech). Therefore, we constructed a tetracycline-repressible dominant negative HER-3 vector for further use in these cell lines. The newly constructed vector will now be used to infect BT-474, MDA-MB-453 and SK-BR-3 cells that have been infected with pRevTet-Off, selected on G418 and screened for functional tetracycline repressor activity by transfection of pRev-TRE-Lac Z vector and culture with or without tetracycline prior to assessing the level of β-galactosidase activity. The pRevTRE vector utilizes a hygromycin resistance gene and double selection on G418 and hygromycin will allow use to derive cell lines that express the Tetracycline transcriptional regulator protein and also contain the dominant negative HER-3 gene driven by the CMV-TRE fusion promoter repressed by the tetracycline repressor. The cells infected with the regulatable form of dominant negative HER-3 will then be cultured in the presence of tetracycline

to keep the dominant negative HER-3 gene off during the selection on hygromycin. Subsequent to selection on hygromycin, cells with (as a control) and without tetracycline will then be used for experiments as we have outlined in the proposal to determine the effectiveness of dominant negative HER-3 in inhibiting HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*. This "Tet-Off" system will also be ideal in that we can maintain the cells in the presence of tetracycline prior to injection into mice and, with removal of tetracycline, the cells will turn on the dominant negative HER-3 gene at the start of the experiment. This system also allows for a wide range of repression through the TRE element, depending on the concentration of tetracycline employed (Clontech). This will also be useful for directly studying the stoichiometric aspect of receptor inhibition in future experiments.

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APPENDICES

None.