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<b>13. ABSTRACT (Maximum 200)</b>  During the first year of our HER4 and breast cancer grant, we have made progress in methods, cell line and reagent development. We have made MDA-MB 453 and 32D cell lines that stably express the Epidermal Growth Factor (EGF) receptor:Human EGF receptor 4 (HER4) chimera. Antisera directed against the chimera have demonstrated its expression. These 2 transfected cell lines will be used to compare HER4 and EGF receptor tyrosine phosphorylated substrates. Activation of the EGF receptor:HER4 chimera by adding EGF to transfected 32D cells stops their growth. This result suggest that our overarching hypothesis, i.e., the HER4 signal is different from that of the EGF receptor, is correct. To isolate downstream HER4 signaling elements, we are creating recombinant molecules as bait for use in the yeast 2 hybrid system. We are testing the ability of these constructs to be tyrosine phosphorylated when expressed in yeast. Lastly, generation of a HER4 antibody should allow us to do Western blot analysis of HER4 content in a variety of breast cancer samples, allowing attempts to correlate HER4 expression and breast cancer prognosis.				
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## INTRODUCTION:

The human EGF receptor 2 gene (HER2) is amplified and overexpressed in 20-30% of invasive breast cancers (1). Moreover, numerous articles indicate that poor prognosis breast cancers exhibit increases in EGF receptor content and/or an EGF receptor autocrine loop with the production of TGF $\alpha$  (2, 3). Both EGF receptor and HER2 can interact with each other or with HER3 leading to growth and proliferation (4, 5). However, the 4th member of the family, HER4, in preliminary studies appears to have a different output, differentiation rather than proliferation (6, 7). Thus, the HER4 signal may slow the growth of breast cancer. Our tasks are to obtain definitive evidence that HER4 provides a different biologic signal to breast epithelium, i.e., differentiation rather than proliferation, and to elucidate the pathway, or elements of the pathway, that differ between HER4 and the original 3 members of this receptor family (EGF receptor, HER2 and HER3). To this end, we dedicated the first year to creating molecular reagents and cell lines and devising new technology which will allow us to prove that HER4 sends a differentiation signal. These reagents should also give us the wherewithal to isolate the unique members of the HER4 pathway, substrates phosphorylated by this tyrosine kinase.

Specifically, we have created an EGF receptor:HER4 chimera recombinant cDNA and transfected it into MDA-MB 453 breast cancer cells and into the mouse 32D cell line. Stable neo-resistant transfectants have been isolated from these transfections. These cell lines are now beginning to be used to test the hypothesis that the EGF receptor signal differs from that of the EGF receptor:HER4 chimera. Secondly, we have developed GST:HER4 proteins which will be useful for mapping autophosphorylation sites and we have purified and used these proteins to raise specific anti-HER4 antisera as an experimental reagent and as a reagent that can be used to assess the levels of HER4 protein expression in breast cancers. Thirdly, we are approaching the task of isolating novel substrates by harnessing the power of the yeast 2 hybrid technology. This technique has been used to study protein:protein interactions. Our work during the first year has been aimed at creating 2 hybrid molecules that can act as bait in the yeast 2 hybrid system. These consist of the tyrosine kinase domain of HER4 with its associated autophosphorylation sites in the C terminus, the Gal 4 DNA binding domain either with or without a leucine zipper motif to enhance dimerization. This kinase is active when transfected in human 293T cells. If this HER4 tyrosine kinase is active in yeast, it will allow us to detect 2 types of substrates, those that interact with the HER4 cytoplasmic domain directly and those that interact with the phosphorylated HER4 cytoplasmic domain.

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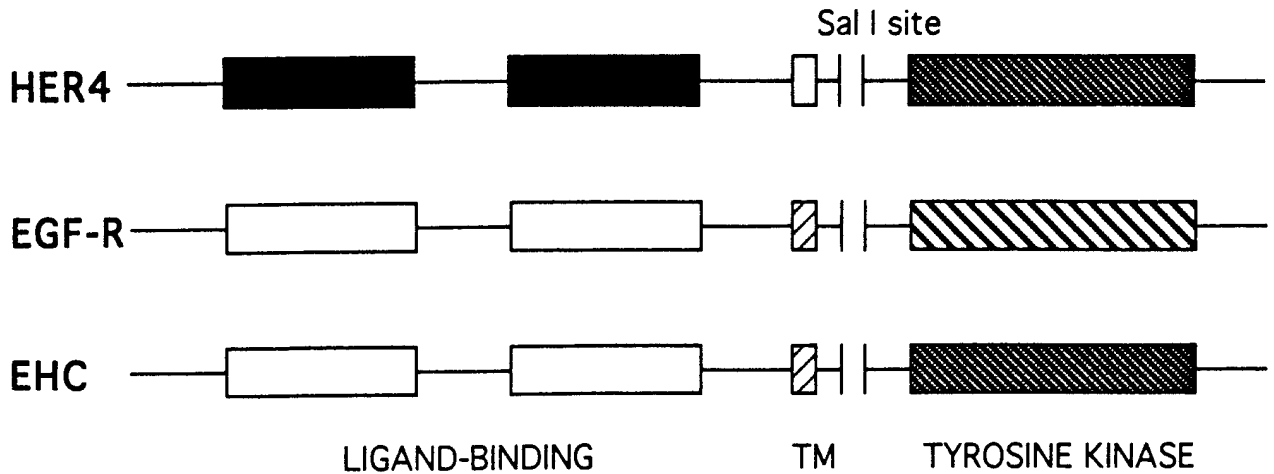
Our statement of work emphasized 2 tasks for the first 12 months of this grant. Task 1 was to devise breast cancer cell models distinguishing the growth promoting actions of the EGF receptor versus the differentiation promoting effect of HER4. Task 3 was to be initiated between months 6 and 24 of this proposal and it was to use transfected cells to determine the molecular weights of HER4 P-Tyr substrates and to map HER4 tyrosine autophosphorylation sites. We have also initiated technology development for Task 4 to identify novel HER4 substrates, even though this was slated for months 12-36 of this grant.

**A. The creation of cell lines expressing the EGF receptor:HER4 chimera:** We have constructed a chimeric receptor with the extracellular and transmembrane domains of the EGF receptor and the cytoplasmic domain, including the tyrosine kinase and autophosphorylation sites, from HER4. This has been placed in the retroviral vector pLXSN; the full length EGF receptor cDNA has also been placed in this same vector (Fig 1). These contain neo-resistant genes and therefore can be used to select stable transfectants. We have successfully created MDA-MB 453 stable transfectants expressing the EGF receptor:HER4 chimera. Both the population and several independent clones have been selected. The population and several of the clones have been shown to be both neo-resistant and to express the EGF receptor:HER4 chimera. The MDA-MB 453 cell line was reputed to be devoid of EGF receptor (6). However, in cloning this cell line, we have uncovered that several of the clones contain full length EGF receptor. This complicates the analysis because the addition of EGF would not only activate the chimeric receptor but would stimulate the endogenous EGF receptor. Not all clones of MDA-MB 453 contain the EGF receptor. Our task during the next year will be to retransfect and create a population of chimera expressing cells. Then, we will perform a larger clone isolation hoping to isolate clones which express the chimera and do not express endogenous EGF receptor. This will give us the pure experimental system that we would like to have, in which EGF will activate the HER4 signal and produce differentiation.

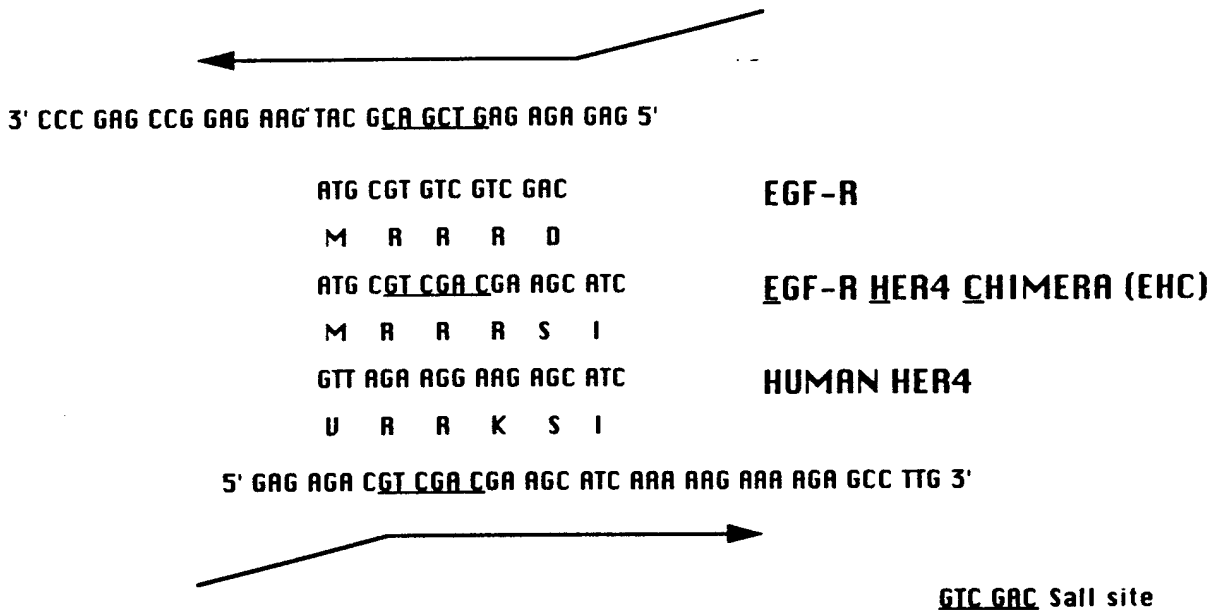
**B. The EGF receptor:HER4 chimera in 32D cells:** To obtain proof of principle we have used another cell line which we know does not contain endogenous EGF receptor, the mouse 32D cell line. This cell has been used by several groups to test the function of full length tyrosine kinase receptors and determine their ability to cause proliferation or to prevent apoptosis (8). 32D cells are dependent upon the cytokine, IL-3. When IL-3 is withdrawn, the entire population of 32D cells undergoes programmed cell death. Transfection of receptors such as the EGF receptor or an EGF receptor HER2 chimera allows EGF, in essence, to replace IL-3 (8, 9). Not only do cell lines transfected with the EGF receptor or the HER2 chimera live upon withdrawal of IL-3, but EGF-treated cells proliferate as if they are receiving the cytokine signal. We have transfected the EGF receptor:HER4 chimera into 32D cells. These data are extremely encouraging and support our hypothesis that HER4 sends a different type of signal than the EGF receptor. On withdrawal of IL-3 from the chimera containing cell lines, all cells die. If EGF is added to stimulate the HER4 chimera during IL-3 withdrawal, the cells fail to die, i.e., the HER4 chimera produces an anti-apoptotic signal. However, the cells do not grow. This is in contrast to the control cells in which we have placed the full length EGF receptor; the cells both live and proliferate in response to EGF.

Adding both IL-3 and EGF together demonstrates that the HER4 signal clearly predominates, that is, these cells do not grow even though they have the IL-3 stimulus (Fig 2). IL-3 is a very potent growth stimulus in these cells; they double in number in ~ 16 hours when treated with IL-

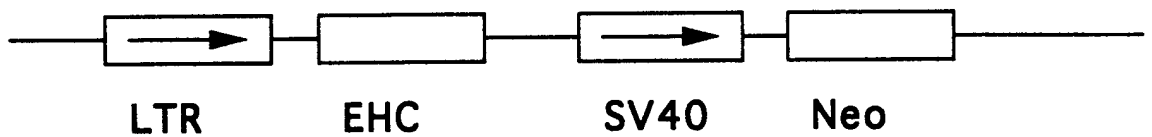
# A. Domain Structure of the EGFR, HER4 and EHC



# B. Sequence and PCR Primers for the Juxtamembrane Region of EGFR and HER4



# C. The EGFR/HER4 Chimera Expression Vector(pLXSN)



**Figure 1**

### HER4 chimera Growth Curves

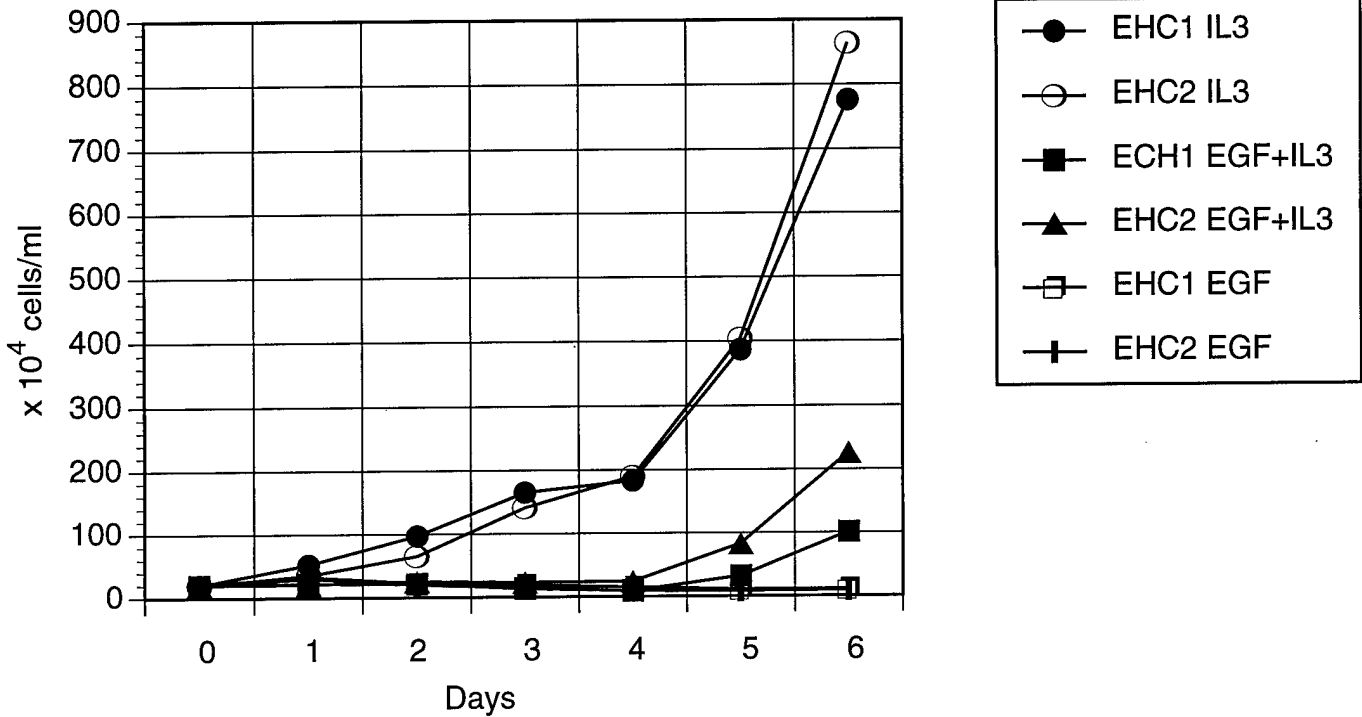


Figure 2. HER4 sends a negative growth signal. 32D cells were transfected with the HER4 chimera (pLSXN.EHC) and stable neo-resistant clones were selected. Two clones selected by FACS analysis of their surface EHC receptor content were chosen to monitor growth (EHC1 and EHC2). Cells were grown in media with no additives (cells die within 2 days, data not shown), with 100 pg/ml IL3, 100 pg/ml IL3 plus 100 ng/ml EGF, or 100 ng/ml EGF alone. When treated with IL3 both clones proliferate rapidly (see top two curves). They grow at the same rate as nontransfected or vector transfected stable 32D cell lines (data not shown). The addition of EGF sustains cell viability but does not stimulate proliferation (see bottom two curves). Interestingly, in cells treated with both EGF and IL3, the EGF signal constracts the IL3 growth stimulatory signal (see middle two curves). Therefore we suggest that activation of HER4 sends an antiproliferative signal.

3. Yet co-treatment with IL-3 and EGF (the HER4 signal) almost completely stops growth. Therefore, the HER4 signal prevents the proliferative response to IL-3. This provides us with a model for studying the anti-proliferative (perhaps differentiation) pathway triggered by the HER4 tyrosine kinase.

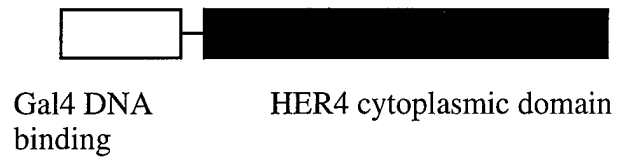
We are in the process of starting task 3, the identification of P-Tyr substrates. This will be a straightforward analysis in 32D cells which lack endogenous EGF receptor. We will compare the P-tyr substrates in cells expressing the transfected EGF receptor to those expressing the EGF receptor:HER4 chimera. This will become a better experiment in the MDA lines after we have retransfected the MDA cell lines and tried to obtain populations or clones which contain the chimera without any contaminating endogenous EGF receptor.

**C. Production of antisera:** We have created 2 GST:HER4 proteins covering regions of the C terminal portion of the molecule that contained putative autophosphorylation sites. These will be used in the future to help map the autophosphorylation sites. During year 1 of this proposal, the GST:HER4 protein was used to create antisera specific for HER4. We have just received several batches of antisera and the results are encouraging. The anti C terminal HER4 antisera recognizes HER4 expressed transiently in 293 T cells. More importantly, it will detect the HER4 chimera expressed in stable transfectants which express the chimera at a much lower level than those seen after transient transfection in 293 T cells. We hope to be able to use this antisera for Western blotting and will initiate preliminary studies during the next 12 months to determine if we can detect HER4 in samples of normal breast epithelium and in breast cancer samples. If we do, we hope to be able to create a subproject with the San Antonio Breast Cancer SPORE to study some of their sets of breast cancer samples in which the patient's prognosis is already known. We have an ongoing relationship between the UNC Breast Cancer SPORE and the San Antonio SPORE; thus, this is a realistic expectation, if our antisera are of high quality and specificity.

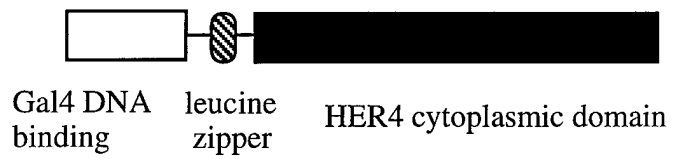
**D. Development of the yeast 2 hybrid system:** This is a powerful technology for studying protein:protein interaction. This technique has been used successfully with the insulin receptor (10, 11). It can be used just by expressing the C terminus of HER4 and should be able to isolate proteins that interact directly with the HER4 tyrosine kinase domain or C terminus. However, it will be even more useful if we are able to create a HER4 tyrosine kinase domain that is capable of autophosphorylation in the yeast. This will create binding sites for phosphotyrosine binding SH2 groups from putative downstream substrates. To this end, we have created 3 recombinant molecules. We are using the Gal 4 system; therefore, we have created a Gal 4:HER4 chimera. This does not contain the HER4 transmembrane region and will be soluble. On the other hand, the Gal 4 region which is used as the key detection element of the yeast 2 hybrid system also has a dimerization domain. It is our hypothesis that the expression of a HER4 tyrosine kinase domain will create an active kinase if there is a dimerization motif within the expressed protein. Since the Gal 4 binding element may not be sufficient, we are trying another approach which is to insert a leucine zipper from a transcription factor between the Gal 4 domain and the HER4 tyrosine kinase. This creates a leucine zipper protein:protein interaction that should activate the HER4 molecule (see Fig 3 for schematic constructs). Lastly, we have created a test molecule using PLC  $\gamma$  cDNA; this includes the SH2 and SH3 domains. This will allow us to see whether the Gal 4:HER4 construct is capable of detecting an SH2 containing protein in the context of the 2 yeast hybrid system. We have obtained a yeast 2 hybrid, target cDNA library from an epithelial cell which is suitable. We will begin to screen yeasts that are deficient in the ability to grow in histidine and which will activate

Figure 3. Gal4 DNA binding domain-Her4 constructs

**A. Gal4-Her4**



**B. Gal4-Leucine Zipper-Her4**



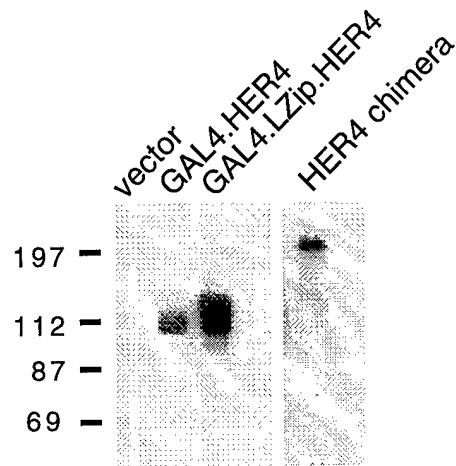


Figure 4. Mammalian expression of GAL4.HER4 fusion proteins. The human embryonic kidney epithelial cell line, 293T, was transfected with mammalian expression vectors (pcDNA3, pcDNA3.GAL4.HER4, pcDNA3.GAL4.LZIP.HER4, and pLXSN.EHC). After 24 hours the cells were lysed and immunoprecipitated with anti GAL4 or anti tyrosine phosphate antibody, followed by Western blot analysis with anti tyrosine phosphate antibody. All tyrosine phosphorylated bands are of the appropriate size, and indicate the fusion proteins maintain their kinase activity.

$\beta$  galactosidase in the presence of a positive interaction between the Gal 4 activation domain and the Gal 4 DNA binding domain. Figure 4 shows the result of expressing the Gal 4 HER4 protein, the Gal 4 leucine zipper HER4 protein, and the EGF receptor HER4 chimera in 293T cells. Clearly, all are expressed and tyrosine phosphorylated indicating that the construction process has not changed the HER4 tyrosine kinase domain.

**E. Can the HER4 signal block breast cancer cell growth:** The 32D cell result (the HER4 signal inhibited IL-3-dependent growth) has led us to try the same approach with breast cancer cell lines. Figure 5 shows the result of transfected vector clone of the HER4 chimera into BT474 cells. The cells are transfected, cultured for 2 days, and then incubated with G418. Only transfected cells survive and go on to form colonies. At the end of 2 weeks the colonies are stained with crystal violet. It is clear from Figure 5 that that chimera, even without EGF activation, is somewhat growth inhibitory suggesting that HER4 can slow the growth of breast cancer cells. The indirect aspect of this result is that cells producing TGF $\alpha$  should autoinhibit their growth if they contained this EGF receptor HER4 construct.

#### **FUTURE DIRECTIONS:**

We are beginning a collaboration with Dr. David Lee to test directly the hypothesis that a HER4 tyrosine kinase signal will inhibit breast cancer development. Our HER4 chimera will be placed into a vector in which it will be driven by a breast specific promoter, the whey acidic protein promoter. We will create a transgenic animal in collaboration with Dr. Lee's group. The transgenic animals will be crossed with Dr. Lee's TGF $\alpha$  whey acidic protein transgenic animals. The TGF $\alpha$  mice have an accelerated rate of breast cancer formation. The EGF receptor:HER4 chimera TGF $\alpha$  mice will constantly stimulate both an EGF receptor and the HER4 signal. There should be a higher level of the HER4 signal due to the expressing of the EGF receptor:HER4 chimera from a strong, breast-specific promoter. This will test for the putative anti-proliferative effect of HER4 and will enable us to test our hypothesis in vivo (Does the HER4 signal stop breast cancer development?).

#### **CONCLUSIONS:**

In summary, during the first year of this proposal we have created a number of reagents which will help us determine whether HER4 sends an anti-proliferative or differentiation signal to the breast epithelium and will help us elucidate the pathway. We have created 2 sets of cell lines (MDA-MB 453 and the 32D cells) expressing the EGF receptor:HER4 chimera. These have been stably transfected and we have selected some clones which have the desired property for our studies. We will begin to compare the HER4 and EGF receptor signals in these 2 cell lines when the full length, EGF receptor clones of these cells are obtained. We have created an antibody to the HER4 C terminus and hope to use this antibody, both experimentally and in studies, to determine whether HER4 is over or under expressed in human breast cancers. The latter will help determine whether the HER4 level is related to the prognosis of breast cancer. Lastly, we have made numerous constructs that will allow us to apply the yeast 2 hybrid system to the isolation of substrates for the HER4 tyrosine kinase. In the next year, we should be able to prove or disprove our central hypothesis, that HER4 is anti-proliferative or differentiative in cells, and should initiate programs aimed at isolating HER4 substrates.

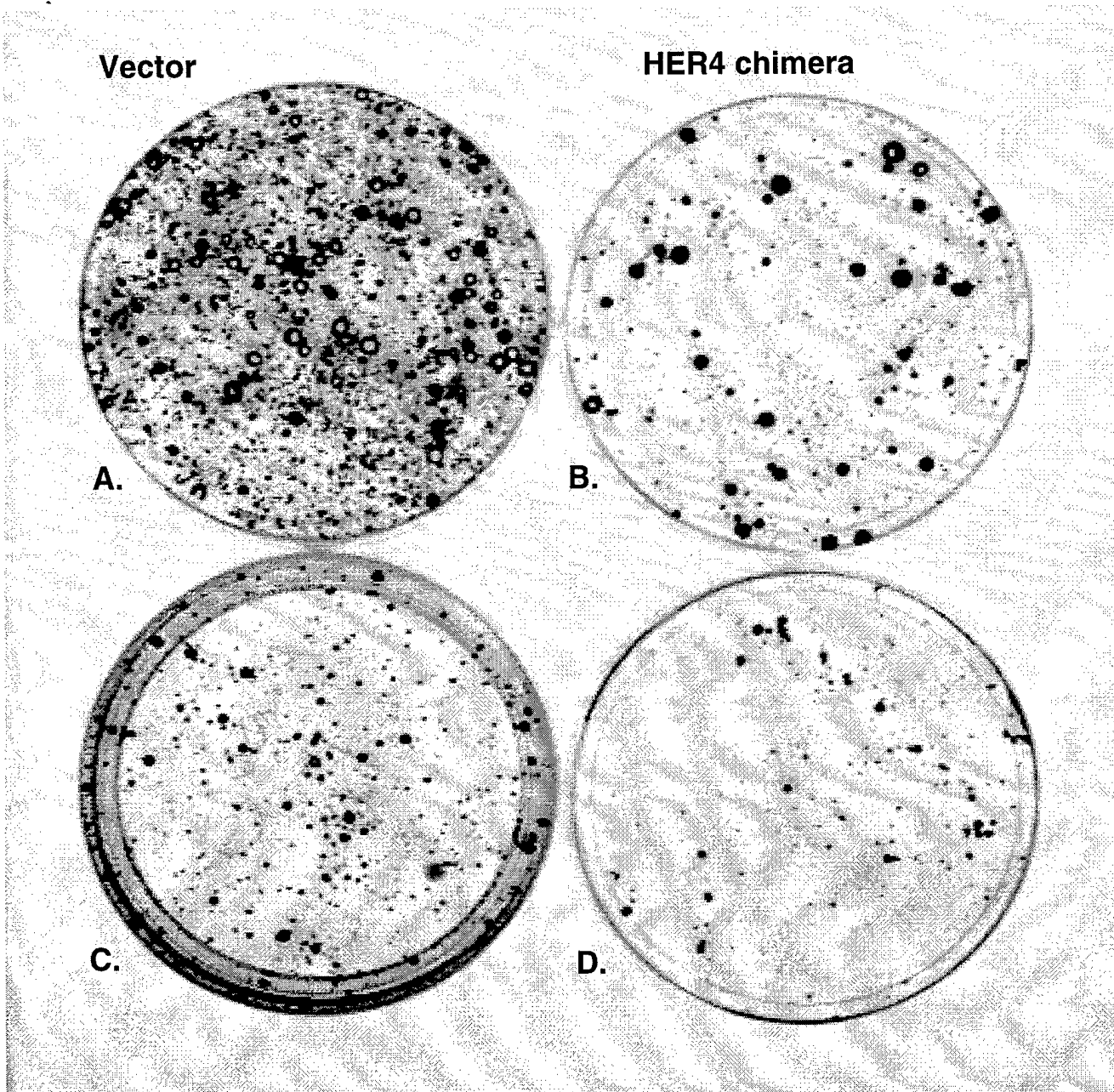


Figure 5. The breast cancer cell line BT474 was transfected with vector alone (pLXSN) (plate A and C) or the HER4 chimera (pLXSN.EHC) (plate B and D). Two days later the cells were counted and plated at a high density of  $2.5 \times 10^5$  (plate A and B) or a lower density of  $1 \times 10^5$  (plate C and D) per 100 mm plate. Transfected cells were selected for by adding 500  $\mu\text{g/ml}$  G418. At the end of two weeks the now selected neo-resistant cells colonies were fixed and stained with Crystal Violet. Even in the absence of ligand the HER4 chimera seemed to inhibit overall colony formation. These data like that in 32D cells (Fig. 2) suggest that HER4 is antiproliferative.

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