INHIBITION OF HEREGULIN EXPRESSION BLOCKS TUMORIGENICITY AND METASTASIS OF BREAST CANCER

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ABSTRACT

The growth factor Heregulin (HRG) is expressed in 30% of breast cancer tumors. HRG induces tumorigenicity and metastasis of breast cancer cells. Our investigation into whether blockage of HRG reduces the aggressiveness of breast cancer cells demonstrated that transfection of MDA-MB-231 with an HRG antisense cDNA suppressed proliferation, tumorigenicity, and metastasis. Blockage of the aggressive phenotype is mediated possibly through inactivation of the *erbB* signaling pathways and a decrease in MMP-9 activity. Our study is the first to report that HRG is a key promoter of breast cancer progression and should be deemed as a potential target in developing therapies for the treatment of breast carcinomas.

INTRODUCTION

About 60% of human breast carcinomas express estrogen receptor (ER) and are responsive to treatment with an ER antagonist, tamoxifen (Tam). Many breast carcinomas, however, over time become less sensitive to estrogen (E2), and thus more resistant to the endocrine treatment, developing into more aggressive tumors. The molecular mechanism for this progression is still poorly understood (1).

Growth factors and their receptors have been implicated in playing an important role in the development and progression of cancer. Signaling from the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors (HER1/EGFR, HER2/*erbB*-2, HER3/*erbB*-3, and HER4/*erbB*-4) is known to be involved in growth regulation of breast cancer cells (2). The role of EGFR in certain types of cancer is well established. Amplification of the *erbB*-2 gene is found in 20-30% of breast cancer patients and correlates with a poor prognosis (3, 4), but the clinical relevance of *erbB*-3 and *erbB*-4 has yet to be determined. Unlike other members of the EGFR family, the ligand for *erbB*-2 has not yet been identified. However, *erbB*-2 can be activated by its own overexpression and homodimerization (5), or can be transactivated by heregulin (HRG) (6, 7). The HRG/*neu* differentiating factor (NDF) family of polypeptide growth factors binds to *erbB*-3 or *erbB*-4 receptors and indirectly induces activation of *erb*B-2 through the formation of *erbB*-2: *erbB*-3 or *erbB*-2: *erbB*-4 heterodimers (8-10).

The effects of HRG are mediated primarily through *erbB*-2, because functional blocking of *erbB*-2 inhibits HRG-induced cellular proliferation and transformation (11, 12). Numerous studies from others and from our laboratory have shown that biological response to HRG depends apparently upon the level of *erbB*-2 expression in breast cancer cells. In cells that overexpress *erbB*-2, low concentrations of HRG exhibit mitogenic stimulation, whereas high levels of HRG or constitutive expression of HRG induce growth arrest, cellular differentiation, or apoptosis (6, 7, 13-15). In contrast, HRG at all concentrations stimulates proliferation in breast cancer cells that express low

levels of the *erbB*-2 receptor (16, 17). Our laboratory has further shown that transfection of a low *erbB*-2-expressing cell line (MCF-7) with HRG led to the acquisition of aggressive phenotypes both *in vitro* and *in vivo*, accompanied by loss of hormone-dependency and antiestrogen sensitivity, mimicking the common progression of human breast tumors (17, 18).

Several studies indicate an inverse correlation between *erbB*-2 and estrogen receptor (ER) pathways, that is, overexpression of *erbB*-2 is associated with loss of functional ER. It has been shown that E2 inhibits and Tam induces *erbB*-2 expression in ER-positive cells (19). Expression of *erbB*-2 in ER-positive cells confers resistance to endocrine therapy (20). HRG is able to interfere with the negative cross-talk regulation between *erbB*-2 and ER by stimulating *erbB*-2 expression and inhibiting the expression and function of ER (21, 22). Recent data have shown that HRG induces repression of E2-response elements (ERE) via upregulation of metastasis-associated protein 1 (MTA-1), a component of the histone-deacetylase complex (HDAC) involved in repression of gene expression. Molecular interactions between MTA-1, ER, and HDAC1/2 have been reported, suggesting MTA-1 as a downstream effector of HRG (23). However, the detailed molecular mechanism has not yet been elucidated completely.

We have shown that HRG expression in breast cancer cell lines and in breast specimens derived from tumor biopsies is inversely correlated with overexpression of *erbB*-2 and expression of ER. HRG-expressing cell lines grow rapidly *in vitro* and display invasive characteristics. In addition, these cell lines are tumorigenic and often metastatic *in vivo* (24). Although overexpression of *erbB*-2 is a marker of poor prognosis in breast, ovarian, stomach, and lung cancer, 70% of breast cancers overexpressing *erbB*-2 are characterized as non-invasive intraductal carcinoma (25). This indicates that *erbB*-2 alone may not be sufficient for developing metastatic phenotypes and may require additional regulators for tumor progression.

Significantly, our data have shown that HRG is overexpressed in nearly 30% of breast cancer tumor biopsies that do not overexpress *erbB*-2 (26). We also have evidence that the population of tumors which overexpresses HRG is distinct from the population overexpressing *erbB*-2, and does not respond to anti-*erbB*-2 treatments (26, and data not shown). One such *in vitro* model resembling this clinical observation is the MDA-MB-231 breast cancer cell line. These cells overexpress HRG, are tumorigenic and metastatic *in vivo*, express low levels of *erbB*-2 and *erbB*-3, and do not respond to Herceptin (26, and data not shown). We have also shown that antibodies generated against HRG markedly reduce *in vitro* growth, motility, and invasion of breast cancer cells that have overexpression of HRG, indicating that HRG is essential for breast cancer cell proliferation, motility and invasion *in vitro* (24). Furthermore, we have shown that HRG promotes tumorigenicity and metastasis of breast cancer cells that do not overexpress any of the *erbB* receptors (17).

In light of these observations, we propose that HRG is a tumor-promoting factor that might be plausible as a target for breast cancer treatment. In the present study, we demonstrate that blocking HRG expression does result in inhibition of cell proliferation, invasion and motility *in vitro*, and further results in a tremendous inhibition of tumor formation in athymic nude mice and the prevention of metastasis *in vivo*. Thus, our study demonstrates indisputably that HRG is a critical marker in breast tumor growth and, even further, in the acquisition of a metastatic phenotype. Our results further provide evidence into the possible blocking mechanism of HRG action in halting breast cancer progression through decrease in *erbB*-2 and *erbB*-3 tyrosine phosphorylation, leading to decreased receptors signaling, including the *Ras*-dependent mitogen-activating protein kinase (MAPK) and reduced activation of a matrix-degrading enzyme Matrix Metalloprotease-9 (MMP-9).

RESULTS AND DISCUSSION

Construction and transfection of heregulin-β2 (HRG-β2) antisense cDNA into MDA-MB-231 breast cancer cells. The ER-negative MDA-MB-231 human breast cancer cell line is highly invasive *in vitro*, and tumorigenic and metastatic *in vivo*. This cell line is known to express four isoforms of HRG, moderate levels of EGFR, low levels of *erbB*-2 and *erbB*-3, and no *erbB*-4 receptors (data not shown). A eukaryotic expression vector (pRC/CMV) was constructed with the HRG-β2 cDNA (amino acids 1 to 426) oriented from 3' to 5' end, that is, in an antisense direction, and subsequently transfected into MDA-MB-231 cells (28). Several HRG antisense (HRG/AS) clones were isolated and the presence of antisense HRG mRNA was confirmed by the RNAse protection assay (data not shown). Multiple clones of vector-transfected MDA-MB-231 (231/V) cells were generated, all of which behaved similarly to wild-type cells.

HRG expression and its biological activity are diminished in the HRG antisense (HRG/AS)-expressing cells. After initial characterization, two representative MDA-MB-231 HRG/AS clones (C6 and C31) and one vector clone (231/V) are described in this manuscript. All other clones behaved similarly to those presented here. HRG protein expression was determined by Western blot analysis using an anti-HRG rabbit polyclonal antibody generated in our laboratory (15, 17). This antibody recognizes an epitope in the EGF-like domain of HRG that is common to all the known HRG isoforms (17). Conditioned media from C6, C31 and 231/V were collected and the HRG protein was purified by heparin chromatography as previously described (13, 29). Expression of the 45 kDa HRG protein was significantly reduced by 25- to 30-fold in the C6 cells and was extremely low to undetectable in the C31 cells, as compared to the 231/V cells (Fig. 1A). The biological activity of the remaining HRG expressed in clones C6 and C31 was next examined by their ability to induce p185 tyrosine phosphorylation in MDA-MB-453 cells, and detected by Western blot analysis using an anti-phosphotyrosine antibody (15, 17). MDA-MB-453 cells overexpress *erb*B-2 and express low levels of *erbB*-3 and *erbB*-4 receptors. Our results

demonstrate that the ability of the remaining HRG in the HRG/AS cells to induce p185 tyrosine phosphorylation was extremely low to undetectable, comparable to the untreated (C-) cells (Fig. 1B). As expected, the decreased level of HRG expression in the HRG/AS clones correlates with their inability to induce *erbB* activation in MDA-MB-453 cells. These results demonstrate that the expression of HRG/AS DNA specifically and effectively blocks translation of the HRG mRNA into protein, and therefore, significantly reduces HRG expression and its biological activity.

Blockage of HRG expression inhibits anchorage-dependent and -independent growth of the MDA-MB-231 cells *in vitro*. MDA-MB-231 is one of the most aggressive breast cancer cell lines and these cells grow rapidly *in vitro*. Thus, the effect of blocking HRG expression on the anchorage-dependent growth of the cells was first examined. The proliferation rate of HRG/AS clones C6 and C31 was decreased 35-50%, as compared to the 231/V cells (Fig. 2A). It is known that HRG-overexpressing cells, such as MDA-MB-231, grow in an anchorage-independent manner. To determine whether inhibition of HRG expression had any effect on the ability of cells to grow as anchorage-independent cells, the HRG/AS and 231/V cells were tested in the soft agar assay (17). Colonies with size between 60 and 100 μm were quantified using a soft agar colony counter (Fig. 2B). Although the total number of colonies of the C6 cells was not significantly different from that of the 231/V cells, the C6 colonies were generally at the smaller end of the size range (about 60 μm). On the other hand, the C31 cells showed a 50% reduction in anchorage-independent growth, as compared to the 231/V cells. Therefore, the decrease in HRG expression in MDA-MB-231 cells not only inhibits the anchorage-dependent growth, but also diminishes their anchorage-independent growing capabilities. These results are of great importance since MDA-MB-231 cells are extremely aggressive and rarely respond to chemotherapeutical agents. It appears that HRG is a critical factor that promotes their growth.

Obstruction of HRG expression inhibits Matrigel outgrowth and decreases chemomigration and chemoinvasion of MDA-MB-231 cells *in vitro*. MDA-MB-231 cells are

featured as stellar-like growth patterns in the Matrigel outgrowth assay (30). We have previously shown that a HRG-neutralizing antibody prevents MDA-MB-231 cells from developing stellar-like patterns in these assays (24). Thus, we predicted that the HRG/AS clones would no longer reveal stellar-like patterns as seen in MDA-MB-231 cells. In this study, cells were plated on a Matrigel layer and grown for 7 days as previously described (24). Neither of the HRG/AS clones was able to grow with stellar-like patterns as compared with the 231/V cells. The C6 cells formed small foci, and migrated through the surrounding matrix. The C31 cells were not able to form proliferative foci, nor did they migrate through the Matrigel. In contrast, the 231/V cells formed large foci with stellar-like patterns, moving outward across the Matrigel as they replicated, and forming wide pathways of multiple cells through the surrounding matrix extensively (Fig. 2C).

Since these results imply that HRG is necessary for breast cancer growth and invasiveness, we then tested the ability of the HRG/AS cells to migrate and invade using the Boyden chamber assay (31). Chemomigration is tested using a collagen matrix, and chemoinvasion is assessed with a Matrigel matrix. Both chemomigration and chemoinvasion of the C6 cells was reduced by 50%; in the C31 cells, chemoinvasion was reduced by 75% and chemomigration was completely abolished (Fig. 2D). As expected, the 231/V cells migrated and invaded rapidly through the collagen and Matrigel matrices (Fig. 2D). Our data clearly demonstrate that blockage of HRG expression reduces cellular proliferation, inhibits anchorage-independent growth, decreases chemoinvasive and chemomigration behavior, and blocks Matrigel outgrowth. All together, these data demonstrate that HRG is necessary to induce the aggressive phenotype of MDA-MB-231 cells.

Obstruction of HRG expression inhibits the ability of MDA-MB-231 cells to form tumors in athymic nude mice. To assess the effect that blockage of HRG would have *in vivo*, HRG/AS cells were inoculated into the mammary fat pad of 3- to 4-week old athymic nude mice. Four weeks after cell inoculation, we observed that the C6 and C31 cells showed a significant decrease in tumor intake, in tumor size and in total weight, as compared with the 231/V, which developed large and

vascularized tumors (Fig. 3A). The variability in tumor size among each group was not significant. However, the HRG/AS-derived tumors were extremely small (Fig. 3B) and did not appear vascularized (Fig. 3A). The mice containing the 231/V-derived tumors were sacrificed immediately 4 weeks after cell inoculation, since at that point the tumors were at the largest size allowable by the animal care and use facility. Those mice that contained the HRG/AS tumors were kept for an additional 8 weeks, during which time they did not show a significant change in the tumor intake and tumor size. It is known that MDA-MB-231 cells are not only tumorigenic but also metastatic. As expected, the 231/V cells that developed large tumors were readily metastatic, and the metastatic foci appeared in the liver and the lung (Fig. 3C). On the contrary, neither the C6- nor the C31-derived tumors metastasized even after 12 weeks from the day of the initial cell inoculation. Our *in vivo* results are in agreement with the *in vitro* data. We thus demonstrate that HRG expression is essential for the tumorigenic and metastatic phenotype of MDA-MB-231 breast cancer cells, because its blockage tremendously decreased tumor formation and abolished metastasis.

Our studies lead us to conclude that HRG expression is critical for breast cancer progression both *in vitro* and *in vivo*. Interestingly, the data presented here support the notion that the behavior of breast cancer cells in culture correlates to varying levels of HRG expression (24). It appears that the different threshold levels of HRG expression in clones C6 and C31 promote slightly different *in vitro* phenotypes (Fig. 1 & Fig. 2). Although the threshold HRG expression in the HRG/AS cells was slightly different in each clone, the *in vivo* behavior of the HRG/AS cells was similar (Fig. 3). Our results are of significant importance for the development of therapies that will be aimed at targeting HRG for use in breast cancer patients, in whom increased expression of HRG was observed.

Reduction of HRG expression decreases the level of *erbB*-2 and *erbB*-3 receptor phosphorylation. To understand the mechanism by which blockage of HRG expression reverts the aggressive phenotype of MDA-MB-231 cells, we examined whether HRG-mediated signaling

pathways are altered in the HRG/AS cells. Expression of the *erbB*-2 and *erbB*-3 receptors was assessed by performing immunoprecipitation under non-reducing conditions, followed by immunoblotting for *erbB*-2 and/or *erbB*-3 receptors using specific receptor antibodies. To evaluate the level of *erbB*-2 and *erbB*-3 tyrosine phosphorylation, immunoprecipitations as described above were followed by immunoblotting for phosphotyrosine using an anti-phosphotyrosine antibody. C6, C31, and 231/V cells were used in these studies. We demonstrated that the level of the *erbB*-2 protein, although low, was unchanged in all of the cells tested (Fig. 4A, top panel). In contrast, the level of *erbB*-2 autophosphorylation was decreased in the C6 and C31 cells, as compared to the 231/V cells (Fig. 4A, middle panel). The basal level of *erbB*-3 protein was low to undetectable (data not shown) in all of the cells, and the level of *erbB*-3 tyrosine phosphorylation was markedly decreased in the C6 and C31 cells in comparison with the 231/V cells (Fig. 4A, bottom panel).

Our data clearly suggest that by blocking HRG expression, a cascade of events leads to a decrease in both *erbB*-2 and *erbB*-3 tyrosine phosphorylation. These are signaling events that modulate cell proliferation, tumor formation and metastatic behavior of breast cancer cells. This is a very important observation supported by the previous data that the primary *erbB* heterodimer in human breast carcinomas is *erbB*-2: *erbB*-3, which is correlated with an aggressive phenotype (32). MDA-MB-231 cells do not overexpress *erbB*-2 and *erbB*-3. From our data, it is clear that the growth and the receptor signaling events in these cells are not dependent upon *erbB*-2 overexpression, but depend upon HRG expression. Obstruction of HRG expression in MDA-MB-231 cells disrupted HRG-induced heterodimerization between *erbB*-2 and *erbB*-3, leading to lowered *erbB*-2 activation. This is an extremely important finding, because it is the first indication that a growth factor such as HRG acts as a tumor-promoting agent, activating a cascade of events leading to tumor growth and metastasis.

Blocking HRG expression reduced MAP kinase activation in MDA-MB-231. Next, we examined MAPK, a downstream effector of the *erbB* receptor tyrosine phosphorylation. It has been

shown that activation of *erbB*-2 by HRG leads to breast cancer proliferation, presumably by inducing the activation of the MAPK and PI3K (phosphotidylinositol-3 kinase)/Akt pathways (33-35). It is therefore possible to hypothesize that by inhibiting HRG expression and decreasing the level of *erbB* receptor activation, a downstream effect that would be observed is through the *Ras*-mediated pathway. To test this hypothesis, the levels of the phosphorylated MAPK (ppMAPK) were determined in these cells. ppMAPK was greatly decreased in the C6 and C31 cells by a more than 80% reduction as compared with the 231/V cells cultured under the same conditions (Fig. 4B, top panel). To demonstrate that a decrease in ppMAPK was specific, and not due to a decrease in total MAPK protein, we also assessed the levels of the MAPK protein. Total MAPK protein levels in the C6 and C31 cells were not altered as compared to 231/V cells (Fig. 4B, middle panel). Protein loading control was shown by using an anti-actin antibody (Fig. 4B, bottom panel).

Here we demonstrate that HRG-induced tumorigenicity and invasiveness is, at least in part, regulated through *erbB* receptors-mediated *Ras*-dependent MAPK pathways. These events have previously been shown *in vitro* and in other model systems, which have demonstrated that HRG promotes cellular proliferation through the *Ras*-dependent MAPK (33). It has also been shown that upon activation by HRG, *erbB*-2 becomes phosphorylated and bound to the SH2 domain of the Grb2 (35), which in turn leads to activation of MEK and MAPK (34). Blockage of HRG expression promotes a decrease in *erbB*-3 and *erbB*-2 activation as well as a decrease in activation of the downstream signaling molecules. This is the first report demonstrating that in fact HRG is the key regulator of these events in breast carcinoma, and that blockage of HRG expression leads to a reversion of a very aggressive and metastatic phenotype to a non-aggressive and non-metastatic phenotype. The changes in the phenotype were probably mediated by inhibition of the HRG/*erbB* signaling pathway with a decrease in *Ras*-dependent MAPK activation.

Inhibition of HRG expression promotes decreased MMP-9 gelatinase activity. Matrix metalloproteases (MMPs) have been associated with tumor cell invasion and metastasis (36).

Invasion-associated processes include reduced cell adhesion, and an increase in migration, chemotaxis and membrane ruffling, in addition to increased secretion of matrix-degrading proteases. To determine the mechanism by which HRG promotes metastasis of breast cancer cells, we investigated whether blockage of HRG resulted in modulation of MMP activities. Assessment of MMP activity was performed using a reverse zymography as previously described (27, 37). We found a striking difference in the MMP-9 activity, which was low or undetectable in the C6 and C31 cells, as compared with 231/V cells, which secreted high levels of MMP-9 enzymatic activity (Fig. 4C).

MMP-9 is a metalloprotease that plays a role in degradation of type IV collagen (gelatin), and it is highly expressed in breast carcinoma (38). Our results strongly suggest that enzymatic activity of MMP-9 is associated with HRG expression, and that both are involved in the invasive and metastatic phenotype of MDA-MB-231 cells. Moreover, our results are consistent with previous observations, in which increased production of pro-MMP-9 and secretion of MMP-9 are associated with metastasis induced by activated *Ras*-transformed breast cancer cells (39). MMP-9 is required for this process, because a ribozyme directed against MMP-9 abolishes the ability of the cells to metastasize (27). Moreover, it has been shown recently that the *erbB*-2-mediated *Ras*-dependent MAPK pathway is involved in upregulation of metalloproteases (39). We have demonstrated that HRG promotes tumorigenicity via upregulation of an angiogenic factor Cyr61 (31), and also via receptor tyrosine phosphorylation and MAPK activation (18). However, this is the first report to demonstrate the importance of MMP-9 activity driven by HRG expression and the function of MMP-9 in promoting metastasis.

In summary, the present study demonstrates without question the crucial role that HRG plays in acquiring an aggressive phenotype of the human breast cancer cells MDA-MB-231. By effective blockage of HRG expression using stable transfection with an antisense RNA expression vector for HRG, we show nearly complete reversion of the tumorigenic phenotype of the MDA-MB-231

cells. That is, we demonstrate that obstruction of HRG expression reduces proliferation, invasion and metastasis both *in vitro* and *in vivo*. We further demonstrate that the effects resulting from inhibiting HRG expression is mediated, at least in part, through the decrease in *erbB*-2 and *erbB*-3 tyrosine phosphorylation, leading to a decrease in activated MAPK and MMP-9.

This is the first report addressing the possibility of targeting HRG in breast cancer and demonstrating the importance of such intervention. We show clearly here that blockage of HRG expression results in nearly complete inhibition of tumor formation and obstruction of metastasis *in vivo*. Our unique and novel findings provide new insights into the development of potential therapies targeted to block HRG expression and thereby to halt the progression of breast cancer, which will benefit a large breast cancer population.

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28. The heregulin-β2 (1278-bp) cDNA fragment (amino acids 1-426) was ligated in an inverted, that is, an antisense orientation into the pRC/CMV expression vector (Invitrogen) at the HindIII and XbaI sites. The insert and orientation were confirmed by DNA sequencing. The HRG/AS construct was transfected into MDA-MB-231 cells using the calcium phosphate precipitation method. Stable transfectants were selected by culturing the cells in improved minimal essential media (IMEM) supplemented with 5% fetal bovine serum (FBS) and 600 μg/ml G418 for 4-6 weeks. Clonal cell lines were generated by selecting, isolating, and plating individual G418-resistant clones.

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FIGURE LEGENDS

Fig. 1. HRG expression and its ability to induce *erbB*-2 tyrosine phosphorylation are diminished in the HRG-AS clones. (A) Partially purified HRG from the conditioned media (CM) collected from vector-transfected cells (231/V) and HRG/AS-transfected cells (C6 and C31) was detected by Western blot analysis with an antibody against HRG that recognizes a 45-kDa protein. Recombinant HRG-β1 protein (32 kDa) was used as a positive control. (B) Induction of p185 tyrosine phosphorylation of the *erb*B receptors in MDA-MB-453 cells. Partially purified HRG derived from the CM (Fig. 1A) collected from 231/V, C6, and C31 cells were used to determine p185 tyrosine phosphorylation by Western blot analysis using an anti-phosphotyrosine antibody. MDA-MB-453 cells were treated in the presence or absence of HRG-β1 protein, denoted as positive (C+) and negative (C-) controls, respectively.

Fig. 2. Obstruction of HRG expression results in a significant decline in the aggressiveness of MDA-MB-231 *in vitro*. (A) Anchorage-dependent growth was decreased in HRG/AS cells. Cells (1000/well), 231/V (●), C6 (❍), and C31 (▲), were seeded in 96-well plates. Growth was evaluated over a period of 7 days by fixing cells with trichloroacetic acid, staining with sulforhodamine B, and measuring optical density at 515 nm. (B) HRG/AS cells were significantly less clonogenic than the 231/V cells in soft agar. Cells (5,000/well) were plated in triplicate in agar layer and grew for two weeks at 37°C. Colonies of 60-100 μm were stained with *p*-iodonitrotetrazolium purple and quantified with an AccuCount 2000 automatic colony counter (22). (C) The pattern of Matrigel outgrowth of the HRG/AS clones was markedly changed as compared with the 231/V cells, from a stellar-like pattern with invasive components in the 231/V to small foci with limited invasive components, if any, in the HRG/AS cells. Cells (25,000/well) were plated in triplicate in Matrigel in a 12-well plate, and microphotographs were taken at day 7 (24). (D) The HRG/AS showed decreased chemoinvasion and chemomigration activities. Boyden chamber assay of HRG/AS clones was performed as previously described (24). In brief, cells (20,000 cells/well)

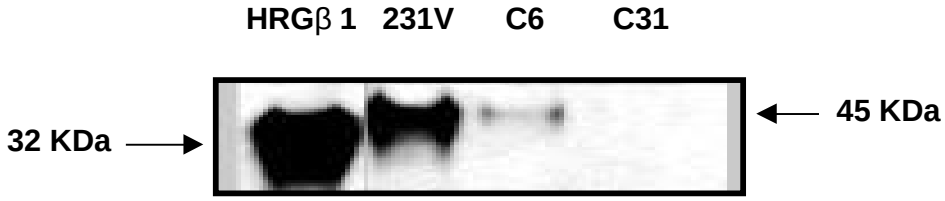
were plated in quadruplicate in the upper chamber of a 48-well Boyden chamber onto polycarbonate filters coated with either collagen IV or Matrigel in serum-free media. CM from NIH3T3 fibroblast was used as a chemoattractant in the lower chambers. After incubation for 6 hr at 37°C, cells on the top surface were removed, and filters were then fixed, and stained with crystal violet. The number of cells that migrated through the pores was assessed by microscopy.

Fig. 3. Blockage of HRG expression suppresses tumorigenicity and metastasis of MDA-MB-231 cells *in vivo.* (A) Photographs of athymic nude mice bearing human breast tumors developed by implanting 231/V, C6 and C31 cells ($5x10^5$ per site) into the mammary fat pads of 3-4 week old athymic nude mice (17,18). (B) Tumor size was calculated by three-dimensional measurements. The tumors produced by the 231/V cells ranged from 500 to 1,600 mm^3, and no significant tumors were seen in mice inoculated with the HRG/AS cells (C6 and C31). Studies were performed for 12 weeks; however, all the measurements were performed 4 weeks after inoculation. No significant change in tumor development was observed in the HRG/AS-inoculated mice. Control 231/V mice were sacrificed after 4 weeks because of the large appearance of the tumors. (C) Metastases derived from the primary tumors were observed by H&E staining in the sections of liver (a) and lungs (b) from mice inoculated with the 231/V cells. No metastases were observed in the HRG/AS-inoculated mice. Arrows indicate the detection of human breast cancer epithelial cells.

Fig. 4. Inhibition of HRG expression causes decreased activation of *erbB*-2, MAPK, and MMP-9 activity in MDA-MB-231 cells. (A) Expression and activation of *erbB* receptors were downregulated in HRG/AS cells. The *erbB* receptors were immunoprecipitated from lysates prepared from 231/V, C6, and C31 cells, and immunoblotted for *erbB*-2 (top panel), *erbB*-2 tyrosine phosphorylation (middle panel), and *erbB*-3 tyrosine phosphorylation (bottom panel). (B) Activation of MAPK was decreased in HRG/AS clones. Cells (231/V, C6, and C31) were serum-starved for 24 hr. Cell lysates were collected, resolved by SDS-PAGE, and immunoblotted with antibodies against phosphorylated MAPK (top panel), MAPK (middle panel), and actin (bottom

panel). (C) MMP-9 enzymatic activity was diminished in HRG/AS cells. Conditioned media were collected and concentrated 100X from 231/V, C6, and C31 cells after 72-hr serum starvation. Equal amounts of protein were loaded onto a SDS-Gelatin-PAGE and analyzed for MMP-9 activity using reverse zymography as described previously (27, 31).

A



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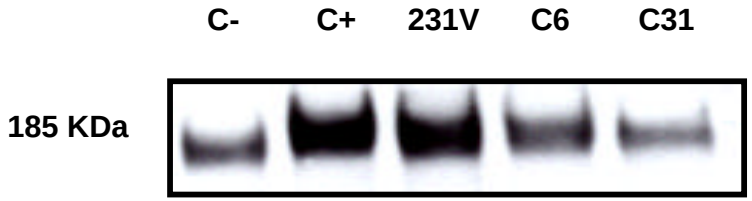

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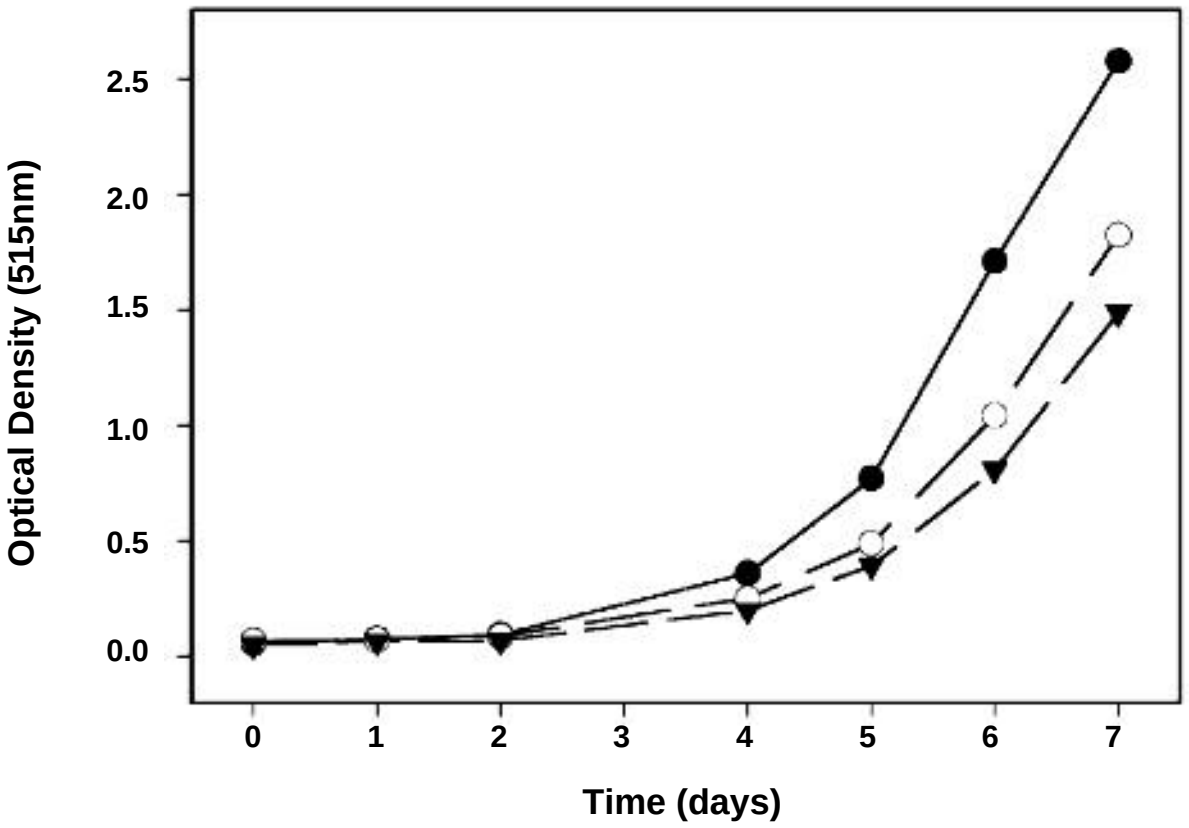

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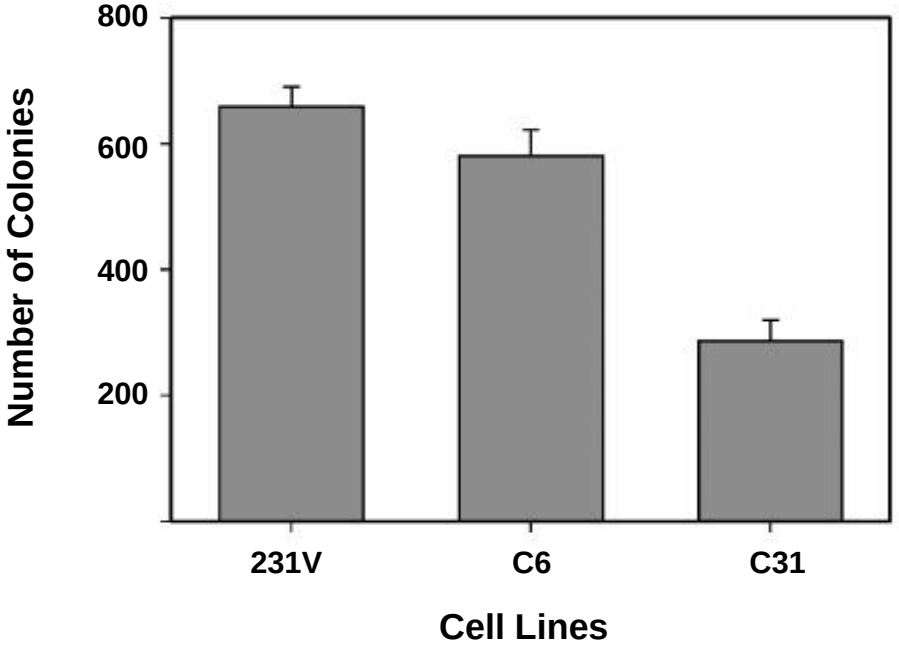
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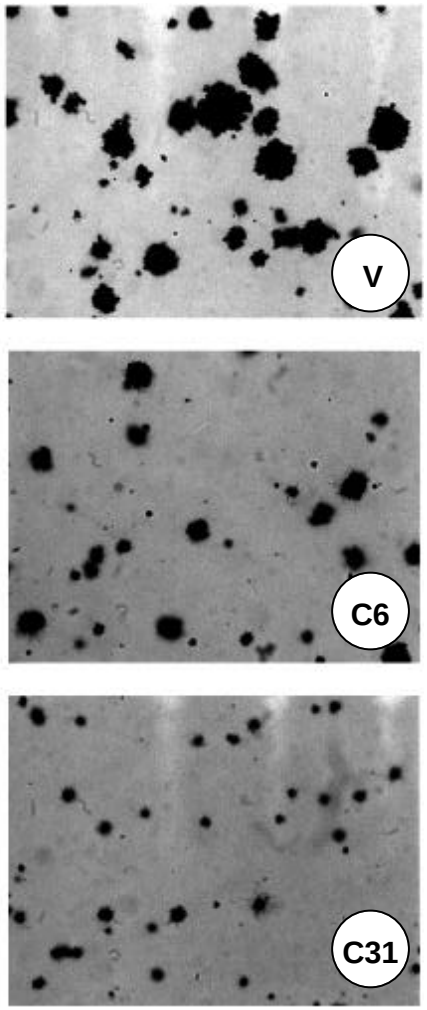
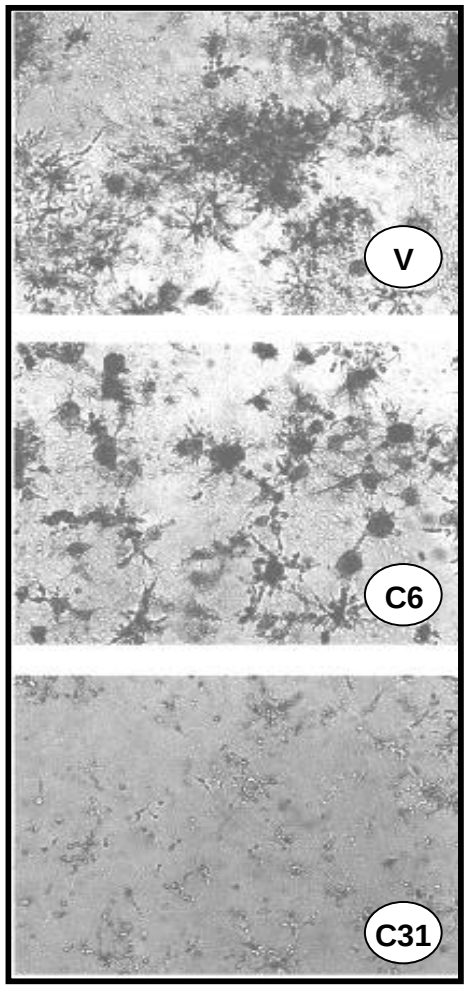
600
400
200
Number of Colonies
231V
C6
C31
Cell Lines


V
C6
C31

C



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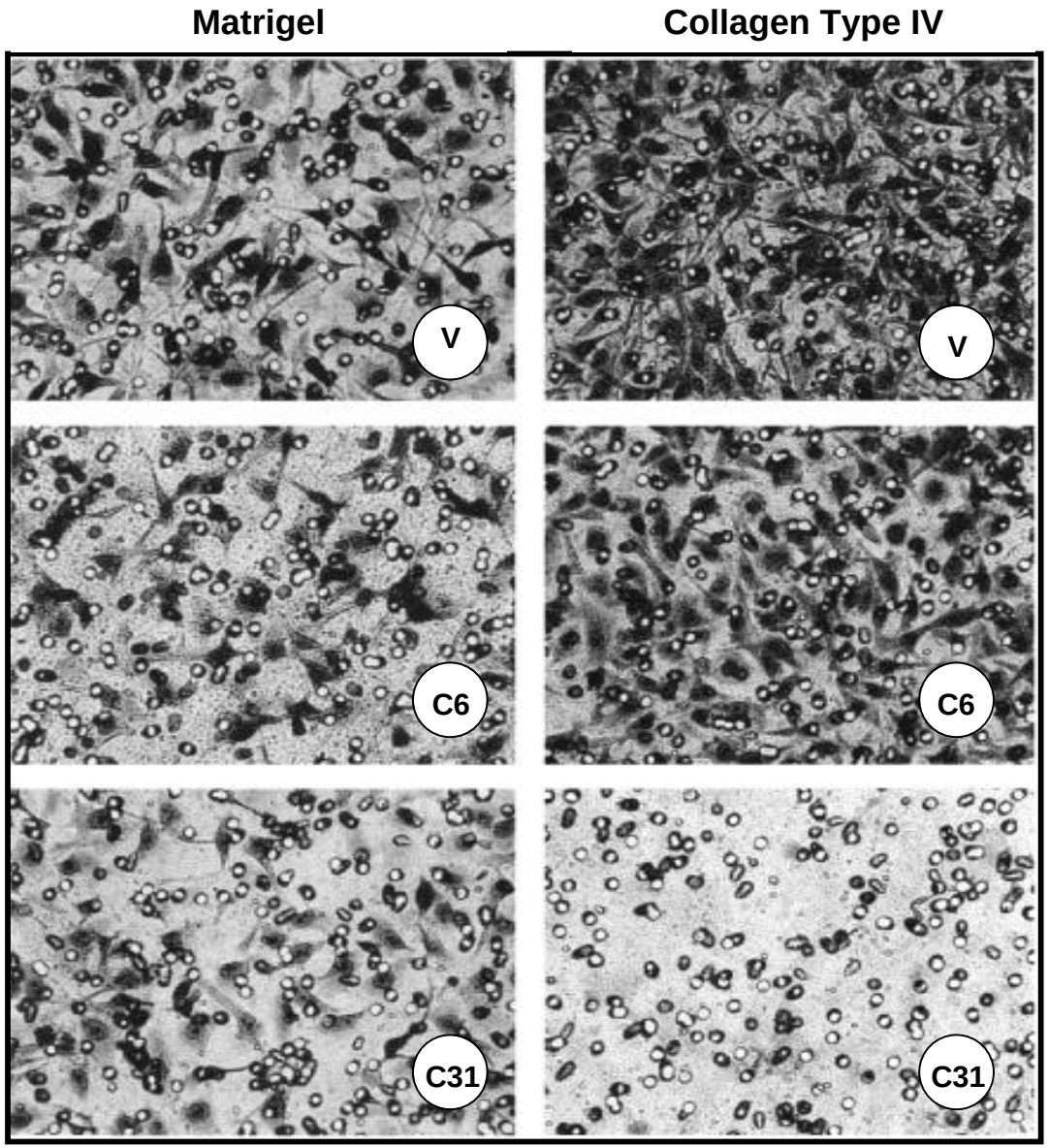

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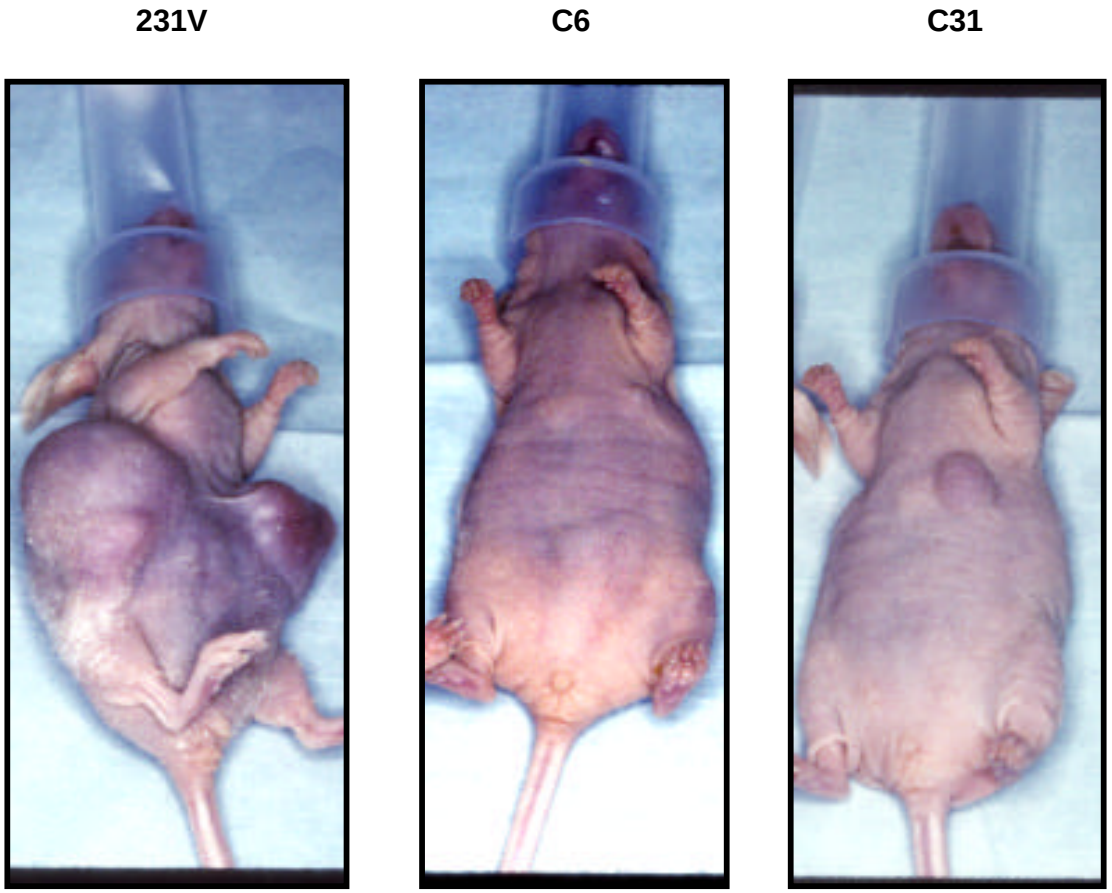

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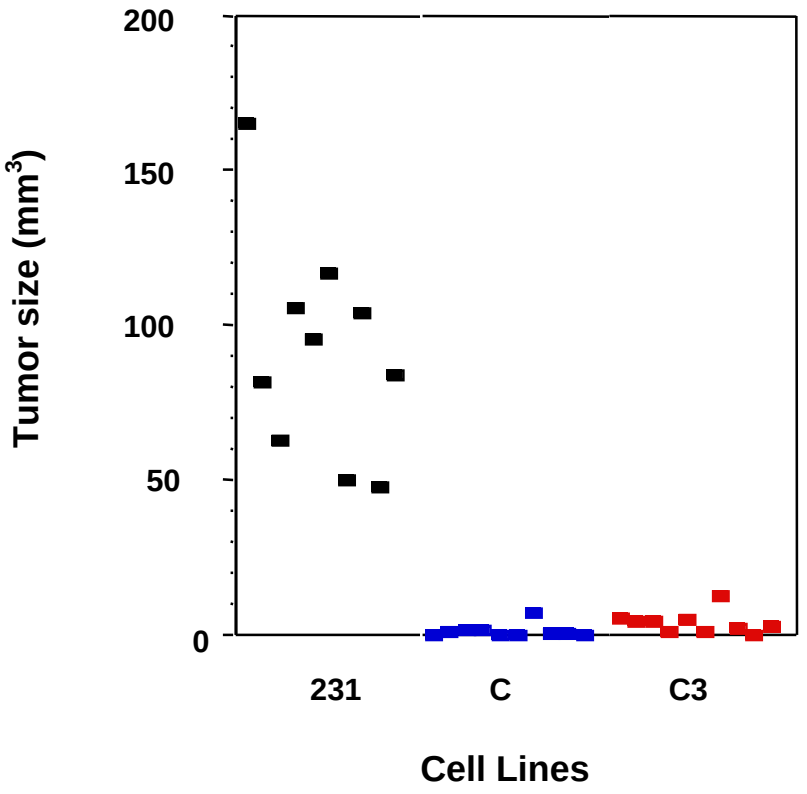

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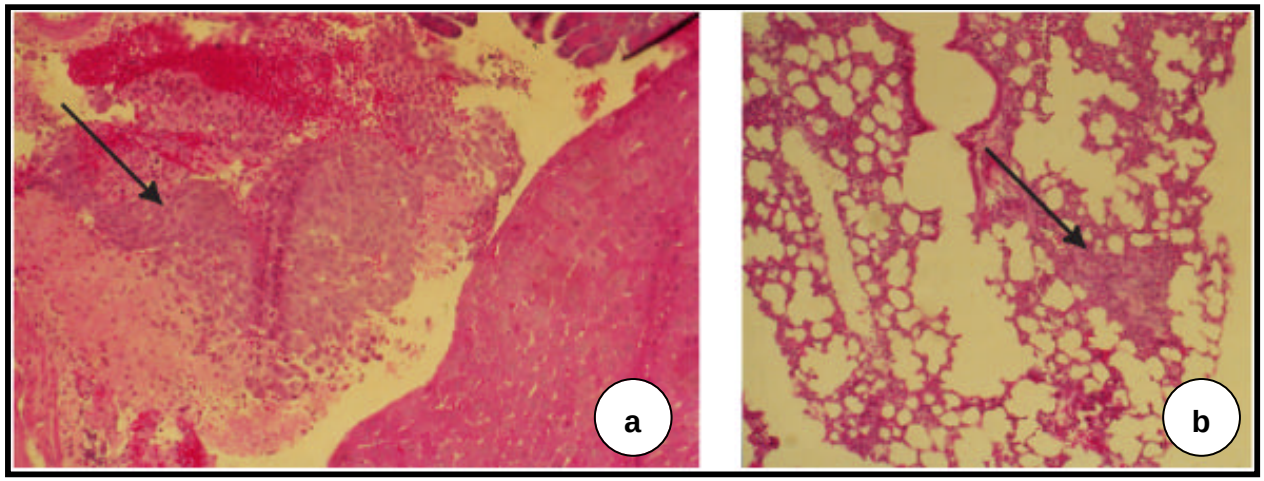

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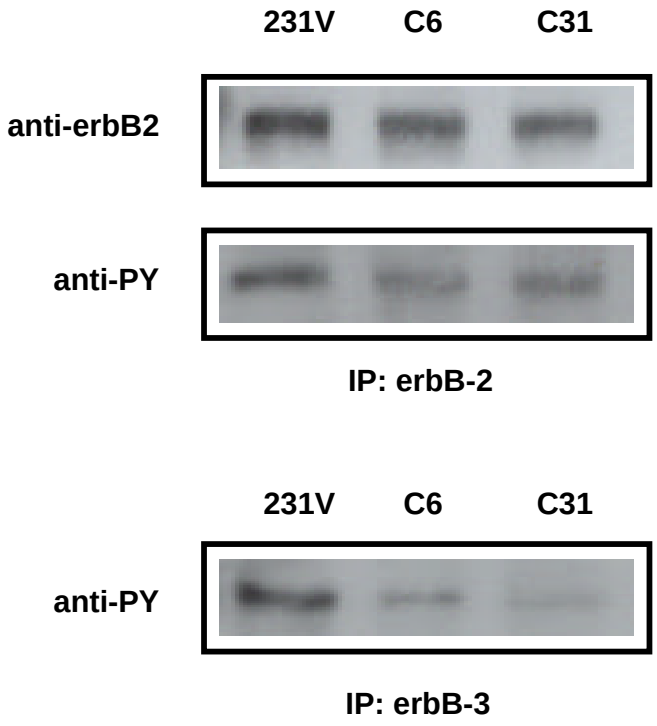
231V
C6
C31
anti-erbB2
anti-PY
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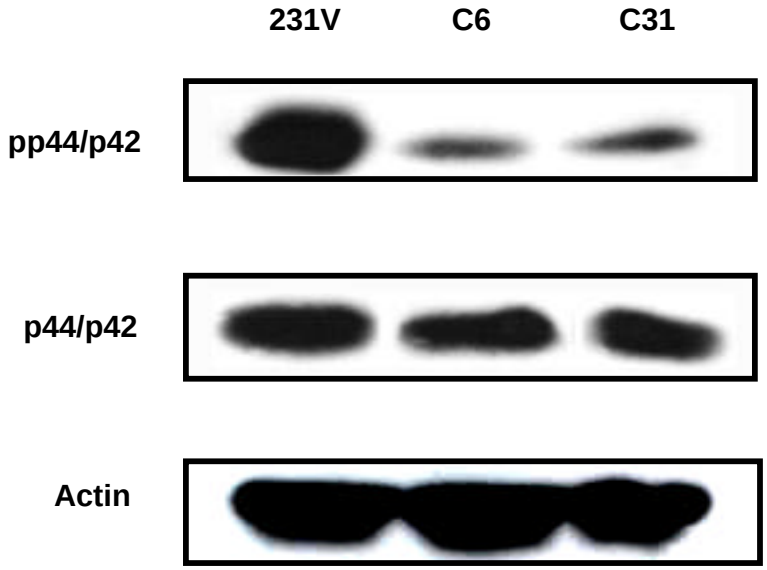
231V
C6
C31
anti-PY
IP: erbB-3

B



C