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FOREWORD

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INTRODUCTION

In our proposal we study the role of the class I tyrosine kinase receptor HER-2 in breast cancer. **Our studies are designed to elucidate the contribution of HER-2 to breast cancer growth and progression to hormone independence as well as the development of resistance to treatment with cytotoxic drugs, anti-hormones and HER-2 antibodies.** The HER-2/neu oncogene product is a growth factor receptor of the class I tyrosine kinase receptor family. Members of this family are frequently implicated in tumorigenesis and progression of human cancers [1]. The most common form of activating the transforming potential of HER-2 is gene amplification and receptor over-expression, which is found in breast cancer cell lines as well as in several human adenocarcinomas including breast, ovarian, lung and gastric cancer at a frequency between 20-40% [2-4]. It has been established in numerous clinical studies, that the over-expression of HER-2 in 20-30% of tumor samples of breast cancer patients correlates with a more malignant phenotype, faster progression and a poor prognosis in node-positive patients [5]. There is evidence that the poor patient outcome in HER-2 over-expressing breast cancer patients is linked to a failure of treatment with anti-hormones [6, 7] and cytotoxic drugs [8-10]. However as regards the mechanisms of action of any of these therapies it appears to be unclear and extremely difficult to work out through which target molecule the respective reagents actually act. With this in mind we will use gene specific targeting of HER-2 with ribozyme expression constructs.

OVERVIEW OF THE GOALS

- 1: we study to what extent down-regulation of spontaneous HER-2 expression by targeting with HER-2 ribozymes will affect the *in vitro* and *in vivo* phenotype of HER-2 over-expressing cancer cells.
- 2: investigate whether down-regulation of endogenous HER-2 expression affects the hormone sensitivity of breast cancer cells.
- 3: elucidate the role of HER-2 over-expression in the development of resistance to chemotherapy in cancer cells.
- 4: study whether down-regulation *in vivo* of endogenous HER-2 by targeting with tetracycline regulated HER-2 ribozymes will affect tumor growth in animals.
- 5: investigate if spontaneous expression of a truncated HER-2 receptor coding for the extracellular binding domain (ECD) is involved in the development of resistance to therapy with HER-2 antibodies.

OVERVIEW OF METHODS

For goals 1-4 cell lines are generated with depleted endogenous HER-2 expression levels. This will be achieved through stable transfections with CMV-driven anti-HER-2-ribozyme constructs or through transfections with ribozyme constructs under the control of the regulatable tetracycline promotor system. Growth is assessed by *in vitro* proliferation assays and *in vivo* tumor growth studies in athymic nude mice. Steroid hormones (goal 2) and cytotoxic agents (goal 3) are used in these assays. In goal 5 ribozyme constructs specific for a truncated HER-2 receptor are constructed and transfected into cell lines that over-express the truncated HER-2 receptor.

BODY

Specific Aim 1

Down-regulation of endogenous HER-2 levels in over-expressing cancer cells using ribozyme targeting and the effects thereof on *in vitro* proliferation and modulation of HER-2 associated signal transduction pathways.

Background

HER-2 transfection studies have shown that HER-2 can cause transformation in cultured cells [11]. On the other hand approaches using antisense technology or pharmacological targeting with HER-2 antibodies have provided only limited insight on the role of endogenous HER-2 over-expression in tumor cells. Despite the absence of a high-affinity ligand for HER-2 it is well established that HER-2 can act as an important signaling molecule as a heterodimeric partner with other members of the HER-receptor family.

Work accomplished

1. Generation of cell lines with depleted endogenous HER-2; Effect of HER-2 depletion on tumor cell proliferation and signal transduction. The work presented in the last report resulted in two peer reviewed publications in the Journal of Biological Chemistry [12] and Gene Therapy ([13], see attached reprints). Furthermore, a paper that shows the effect of a gradual HER-2 depletion in SK-OV-3 cells is accepted in Oncogene pending revisions [14].

Problems and Solutions

We tried to reduce HER-2 levels in several other cell lines (T47D, BT-474 and N87) using the tetracycline regulatable promotor system to drive ribozyme expression. In T47D cells, N87 cells and BT-474 cells we generated cell lines that stably expressed the CMV-driven VP-16-tetracycline-transactivator fusion protein. Using a luciferase reporter gene construct under the control of the tetracycline operon only T47D-600 cells showed a 10 x fold increase in luciferase expression upon tetracycline withdrawal. The other cell lines did not express luciferase at sufficiently high levels (N87) or could not be regulated (BT-474) at all. We transfected T47D-600 cells with HER-2 ribozymes under control of the tetracycline operon and detected a small decrease in HER-2 expression of about 20 % compared to control (data not shown). This small decrease had no effect of proliferation in T47D cells (data not shown). As an alternative approach we constructed an adenovirus vector that expressed CMV-driven HER-2 ribozymes. These vectors infected all cell lines tested with 100 % efficiency (using lacZ as a reporter gene; data not shown) at MOI's from 25 to 250 depending on the cell line. Furthermore, ribozymes were expressed at very high levels for 3 to 5 days after a single infection [13]. We observed HER-2 down regulation in SK-OV-3 cells and MDA-MB-361 cells [13], but not in N87 or BT-474 cells. The reasons for this differences in sensitivity are currently unclear. It could be due to different half-lives of the HER-2 protein in the various cell lines. We are currently investigating, if multiple infections can overcome this problem. Further, since adenovirus mediated ribozyme expression can down-regulate HER-2 expression efficiently in SK-OV-3 cells, we will test in animal experiments in nude mice if adenovirally delivered ribozymes can decrease HER-2 expression *in vivo* and inhibit tumor growth.

2. Effects of HER-2 depletion on Signal transduction

As a model we chose SK-OV-3 cells for the following reasons: the ribozyme-mediated down-regulation of HER-2 was most dramatic (> 90 %) in these cells and they provide a clean model since the Epidermal Growth Factor Receptor (EGFR = HER-1) is the only other HER-receptor subtype expressed in SK-OV-3 cells. They respond to EGF but not to heregulin (ligand for HER-3 and HER-4). In initial experiments we did not observe marked effects on MAP-kinase activation and c-Fos induction. We repeated those experiments with longer periods of serum-starvation (up to 48 hours), and in addition we performed these experiments under anchorage-independent conditions (cells grown in methyl cellulose). Under these conditions HER-2 reduced cells showed a decreased duration of EGF-mediated c-Fos mRNA induction (Figure 1). It is interesting that the HER-1 homodimer seems sufficient for the initial rapid c-Fos induction, but that the HER-1/HER-2 heterodimer is necessary for a sustained activation. This decrease in c-Fos induction was followed by a smaller number of HER-2 depleted cells entering S-phase (Figure 2).

Conclusions

EGF-mediated stimulation of colony formation of SK-OV-3 cells is dependent upon activation of the HER-1/HER-2 heterodimer. This can be explained by a combination of a lower percentage of cells entering S-phase and a higher fraction of cells undergoing apoptosis. These data in conjunction with other publications show that HER-2 is the preferred and limiting partner in heterodimeric HER receptor combinations. It is therefore possible that the oncogenic activity of HER-2 is mostly caused by its ability to mediate the signals of EGF-like ligands generated by tumor and stroma cells, and not so much by its constitutive over expression alone. In the light of this data much more emphasis should be laid on the detection of the various HER-receptor combinations in tumor tissues.

Specific Aim 2

To study the role of HER-2 in steroid hormone sensitivity of breast cancer cells.

Background

The proliferation of human breast epithelial cells is regulated by members of the class I receptor tyrosine kinase family as well as steroid hormones. Members of both receptor families are important prognostic factors in breast cancer [15]. Clinical data indicate that over-expression of the HER-2 gene product is associated with an estrogen receptor negative phenotype (ER⁻), a correlation which is also found in most breast cancer cell lines [6]. Additionally, in a recent publication by Slamon et al [7] estrogen dependent MCF7 cells transfected with a HER-2 expression vector became hormone independent followed by a down-regulation of estrogen receptor level and activity. Based on this, we propose that down-regulation of endogenous HER-2 expression by molecular targeting with ribozymes will alter the estrogen sensitivity of HER-2 expressing breast cancer cells.

Work accomplished

Using stable transfections and clonal selection we were able to generate MCF-7 cell lines with down-regulated HER-2 levels (Figure 3). The depletion of HER-2 expression prevented estradiol mediated stimulation of colony formation in MCF-7 breast cancer cells, demonstrating the link between the two signal-transduction pathways. To demonstrate that this effect was not a cell culture artefact, we used SK-OV-3 cells with depleted HER-2 levels, and demonstrated that as in MCF-7 cells HER-2 depletion completely abolished the estradiol-mediated growth response in soft agar (Figure 4). In order to study the molecular mechanisms of this interaction we first determined the estrogen receptor number and binding affinities in control and HER-2-depleted cells. In binding assays using tritiated estradiol we found that HER-2 depletion had no influence on the number and affinity of the estrogen receptor (Figure 5). In addition, using ER-immunostaining, we found no differences in the ER amount or cellular localization in unstimulated or estradiol stimulated cells (data not shown). Next we asked, if the ER was still functional. In transient transfection studies with luciferase as a reporter gene coupled to an estrogen response element (ERE), we could not detect any difference in luciferase induction between control and HER-2 depleted cells (Figure 6). We then tested if there were any differences in the regulation of an endogenous gene (PS2) that can be induced by estradiol in MCF-7 cells. Using Northern-blot analysis we found no difference in PS2 mRNA induction between control and HER-2 depleted cells (Figure 7).

Conclusions

The effects of HER-2 depletion on Estradiol mediated proliferation can not be explained by changes in the quantity or functionality of the estrogen receptor. There must be other indirect mechanisms, most likely changes in gene expression, responsible for the connection between the two pathways.

Next Steps

We will use unstimulated and estradiol stimulated MCF-7 control and HER-2 depleted cells and will analyze the mRNA for differences in the induction of gene expression. We will use either differential-display techniques or commercially available DNA micro array technologies.

Specific Aim 3

The role of HER-2 over-expression in the development of resistance to treatment with cytotoxic drugs.

Background

There is clinical and experimental evidence suggesting a link between HER-2 over-expression and drug resistance in human breast, gastric, lung and ovarian cancer [8-10], consistent with the association between oncogene over-expression and poor patient outcome. Experimental data so far are either correlative [8] or generated by using HER-2 antibodies in combination with cytotoxic drugs [16]. However, from these experimental approaches it is difficult or impossible to dissect the complex interplay of inhibitory and stimulatory effects on HER-2. Therefore, we propose to use molecular targeting with HER-2 specific ribozymes to down-regulate endogenous HER-2 expression in tumor cells and study the sensitivity to cytotoxic drugs at different HER-2 levels.

Work accomplished

We have generated a panel of useful cell lines with down-regulated HER-2 levels. These are the human breast cancer cell lines MDA-MB-361 and MCF-7, as well as the human ovarian cancer cell line SK-OV-3. In a first set of experiments we tested the sensitivity of SK-OV-3 cells (high levels of HER-2) and MCF-7 cells (low levels of HER-2) with normal and down-regulated HER-2 expression against cytotoxic agents representing classes of drugs with distinct mechanism. We used vinblastine, cisplatin and taxol, and established dose-response curves in 96-well plate assays. In MCF-7 cells down-modulation of HER-2 expression had no effect on the sensitivity to the different drugs (Figure 8). In contrast in SK-OV-3 cells the HER-2 depleted SK-OV-3-Rz8 cells showed a reduced sensitivity against the three cytotoxic agents used in the experiments. This difference in sensitivity can probably be explained by the lower rate of spontaneous proliferation of the HER-2 reduced cells (Figure 9).

Next steps

So far we have used only the SK-OV-3-Rz8 cells in which the HER-2 levels are most dramatically reduced (more than 95 %). We will repeat the experiments in other SK-OV-3 cell lines with different residual HER-2 expression levels, to test if the interesting effects can be repeated in more than one clone of SK-OV-3 cells. Furthermore we will use another cell line with high levels of HER-2 expression (MDA-MB-361 breast cancer cells) to test if the observed effects of HER-2 depletion on chemosensitivity are a phenomenon of general importance. We will also use more sensitive soft-agar assays to test if the effects are even more pronounced in SK-OV-3 cells, and if in MCF-7 cells differences in chemosensitivity can be demonstrated under these more rigorous conditions.

Specific Aim 4

Down-modulation of HER-2 during different phases of tumor growth.

Background

The strong association between HER-2 over-expression and poor patient outcome has already lead to clinical trials using HER-2 targeted antibody treatment in breast cancer. Yet, it is still unclear if HER-2 is only a marker for poor prognosis or a crucial growth promoting factor at different stages of tumor growth. Therefore we propose to use molecular targeting of HER-2 with ribozymes under the control of a tetracycline regulatable promoter (tet-HER-2-Rz) to study to what extent down-modulation of HER-2 at different time points of tumor development affects the tumor growth in animals of over-expressing cancer cells.

Work accomplished

In a first experiment we could demonstrate in SK-OV-3 cells that depletion of HER-2 led to a cessation of tumor growth. Preliminary data from this experiment suggest that removal of tetracycline treatment (ribozyme was activated) led to a regression of established tumors [12].

Next steps

Unfortunately the SK-OV-3 cells lost the ability to regulate HER-2 expression levels by tetracycline. This is commonly observed problem with this gene expression system. We are currently reestablishing the original SK-OV-3 cells, that are transfected with the transactivator alone, and will generate new ribozyme expressing cell lines. We will then repeat the experiment with a larger number of animals (20 per group). We will remove the tumors and test for HER-2 expression, proliferation rate (PCNA staining) apoptosis (Tunnel assay) and angiogenesis (CD31 staining). This will allow us to define the molecular mechanisms responsible for the tumor regression.

Specific Aim 5

The biological significance of a truncated HER-2/ECD receptor.

Background

Initial investigations by Dr.C.Benz (UCSF) et al [17] described the over-expression of a truncated HER-2 transcript coding for the extracellular ligand binding domain (ECD) in cell lines that are resistant to cytotoxic doses of the HER-2 antibody muAb4D5. This antibody is currently under evaluation in clinical trials and resistance to the antibody treatment in patients has been reported (Dr.C.Benz, personal communication). This prompted us to further evaluate the biological significance of the HER-2/ECD receptor. We propose to study if tetracycline regulated expression of HER-2/ECD in HER-2 over-expressing tumor cells confers resistance to treatment with the 4D5 antibody. Furthermore we will use gene specific targeting of endogenous HER-2/ECD with ribozymes to study if down-regulation of endogenous HER-2/ECD in over-expressing cells restores sensitivity to 4D5 treatment in vitro and in vivo.

Work accomplished

We have generated MCF-7 cells (ECD negative) that over-express the HER-2/ECD receptor in a tetracycline regulatable manner (Figure 10). In soft agar experiments HER-2/ECD over expression in MCF-7 cells completely abrogated heregulin-mediated stimulation of colony formation (Figure 11), whereas the estradiol-mediated growth response was unchanged (Figure 11). Since we

had experienced specificity problems with our first series of HER-ECD-targeted ribozymes, we generated another set of ribozymes directed against the HER-2/ECD mRNA, and tested their efficacy against the truncated and the full length HER-2 mRNA, which are spontaneously over expressed at high levels in MKN-7 gastric carcinoma cells. Although we detected a small effect on full length HER-2 expression (about 10 % reduction of protein expression) there was a much more dramatic effect on the expression of the truncated HER-2 receptor. In Northern- and Western blot analysis (Figure 12) the ratio of full length to truncated HER-2 dropped from almost 1:1 in control cells to 2:1 in Rz-ECD1 cells and almost 4:1 in Rz-ECD3 cells. In a next set of experiments we tested the influence of the reduction of HER-2/ECD expression on EGF-mediated stimulation of soft agar colony formation (Figure 13). While MKN7 control cells were relative insensitive to EGF (maximal 4-5 fold stimulation), the EGF-mediated stimulation was enhanced in Rz-ECD1 cells (8 fold) and Rz-ECD3 cells (15 fold), dependent on the level of HER-2/ECD depletion. To study the signal transduction mechanisms responsible for this effect, we tested for EGF-mediated MAP-Kinase and c-Fos induction. We detected a stronger and longer EGF-mediated induction of MAP-Kinase and c-Fos expression in Rz-ECD3 cells compared to control cells (Figure 14), which can explain the observed effects on proliferation. Based on this data we concluded that the truncated HER-2 receptor can act as an endogenously expressed dominant negative receptor, that protect cells against an uncontrolled proliferative response mediated by EGF-like ligands. This counter regulation led us to speculate that tumors in more advanced states should gradually lose HER-2/ECD expression. Since antibodies cannot be used to specifically detect the truncated protein, a RT-PCR method was developed to simultaneously detect full length and truncated HER-2 mRNA in tumor samples. So far we have analyzed the RNA from 20 human gastric carcinomas. HER-2 protein expression as assessed by immunostaining was detected in about 30 to 40 % of the samples and increased in advanced tumor stages, which is in line with the published literature. The more sensitive RT-PCR assay detected full length HER mRNA in 100 % of the samples. In contrast, expression of the truncated HER-2 mRNA was detected in nearly 100 % of the early tumor stages but dropped to about 40 % in late stage tumor samples, which further strengthens the notion that the truncated HER-ECD receptor can act as a tumor suppressor.

Next steps:

We will use the RT-PCR method to screen a larger number of tumor samples from gastric carcinoma and other tumors. Furthermore we are currently developing an in-situ technique that will allow to detect full length and truncated HER-2 mRNA expression in paraffin-embedded tumor and normal tissues. We will test if over expression of the truncated HER-2 protein can act as a dominant negative receptor in other HER-2 over expressing cell lines.

CONCLUSIONS

We studied the effect of down-regulation of HER-2 expression by ribozyme-targeting on in vitro and in vivo proliferation of cancer cells. HER-2 expression was found to be rate-limiting for the autocrine growth of some cell lines (SK-OV-3). Interestingly not only in HER-2 over expressing cancer cells but also in cells with low levels of expression (MCF-7) HER-2 appears to be the rate-limiting receptor to mediate the growth signals from EGF, and Heregulin-like ligands. These data demonstrate in human tumor cells that the HER-2 containing heterodimer is the rate-limiting signaling molecule, suggesting that the activity of HER-2 is due in part to its ability to increase the growth-response to stroma-derived EGF-like growth factors. Further we demonstrated that HER-2 required for the estradiol-mediated proliferative response of MCF-7 breast cancer cells through an indirect unknown mechanism. These data in conjunction with a recently published observation that HER-2 is involved in the interleukin-6 mediated proliferation of prostate carcinoma cells [18], demonstrate an increasing importance of this receptor in tumor biology. Finally, our data on the biological role of the truncated HER-2 receptor demonstrate that this protein can act as an intracellularly expressed dominant negative receptor, which may protect cells from uncontrolled growth-factor mediated proliferation and progression to more aggressive tumors.

In conclusion, we have generated a number of initial data and tools for our subsequent studies. We believe that the research has already provided new insights into cancer biology that have been published in peer-reviewed journals. Some of the new data are currently submitted for publication or prepared for publication.

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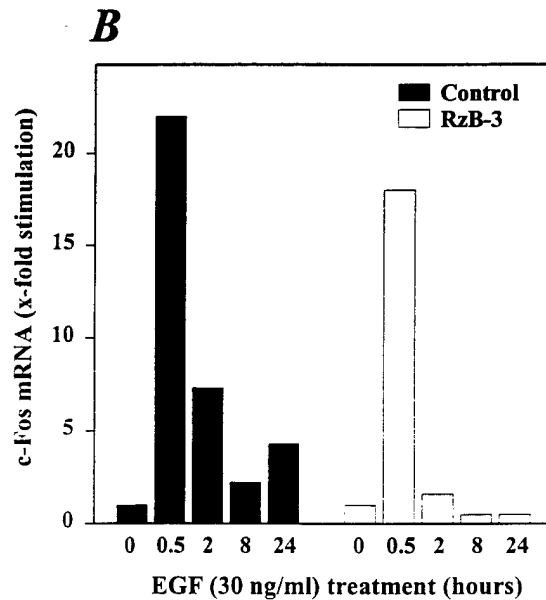
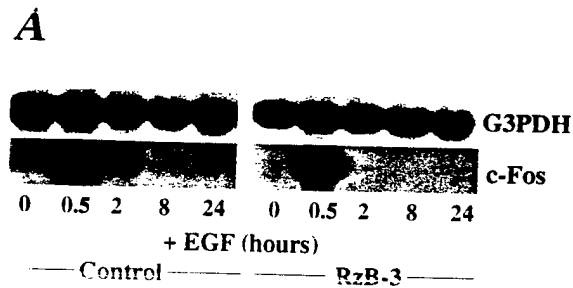
APPENDIX (FIGURES)

Fig. 1. EGF-mediated stimulation of c-Fos mRNA expression is reduced in ErbB-2 depleted SK-OV-3 cells. *A*, SK-OV-3 control cells and RzB-3 expressing cells (20 % residual ErbB-2 expression) were serum-starved for 24 hours and then stimulated with EGF (30 ng/ml) for the indicated time periods. 20 μ g of total cellular RNA were analyzed for c-Fos mRNA expression by Northern blotting. The blots were stripped and reprobed for G3PDH mRNA expression. *B*, c-Fos mRNA expression levels were quantified relative to their G3PDH mRNA levels and values are expressed as x-fold stimulation (no EGF stimulation = 1).

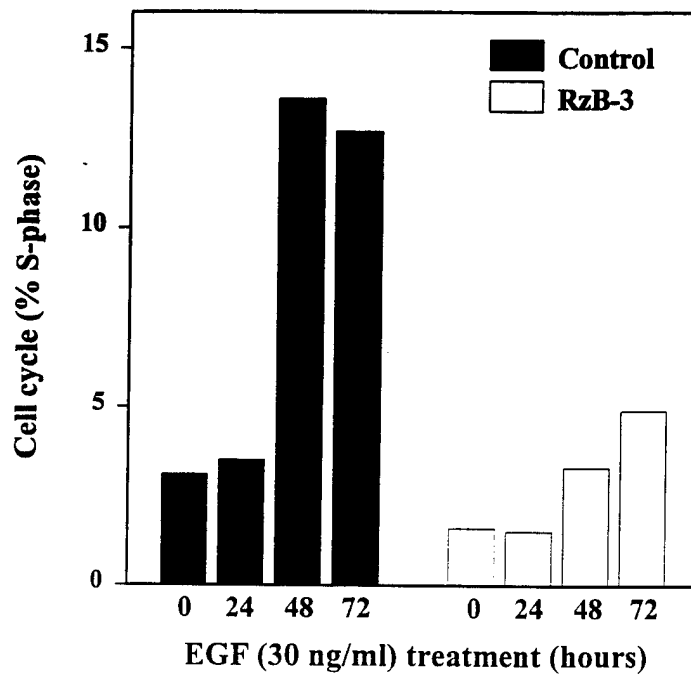


Fig. 2. EGF-mediated stimulation of cell cycle progression is reduced in ErbB-2 depleted SK-OV-3 cells. SK-OV-3 control cells and RzB-3 expressing cells (20 % residual ErbB-2 expression) were serum-starved for 24 hours and then stimulated with EGF (30 ng/ml) for the indicated time periods. Cell nuclei were stained with propidium iodide (50 μ g/ml) and the DNA content and percentage of cells in S-phase was quantified by FACS analysis.

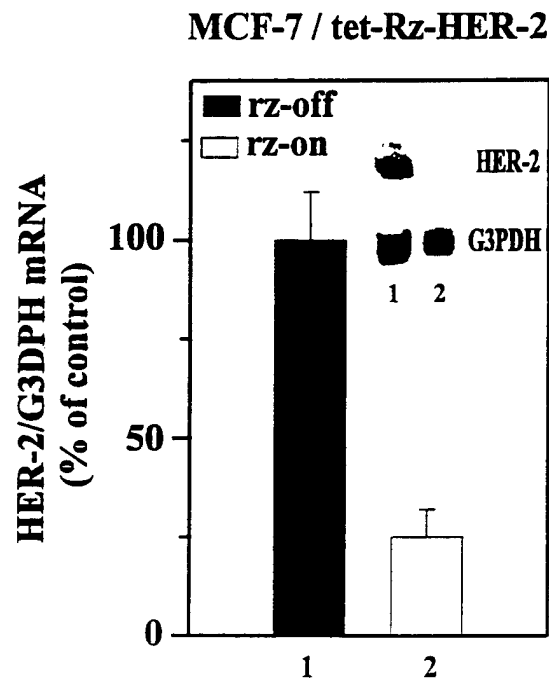


Fig. 3. Reduction of HER-2 mRNA expression in MCF-7 breast cancer cells. Total RNA (30 μ g) from MCF-7 cells stably expressing anti-HER-2 targeted ribozymes under the control of a tetracycline-regulated promoter, was analyzed for HER-2 mRNA expression in the presence (lane 1; rz-off) or absence (lane 2; rz-on) of tetracycline (1 μ g/ml). HER-2 mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA by densitometry. The inset shows a representative Northern blot. Positions of HER-2 and G3PDH specific transcripts are indicated.

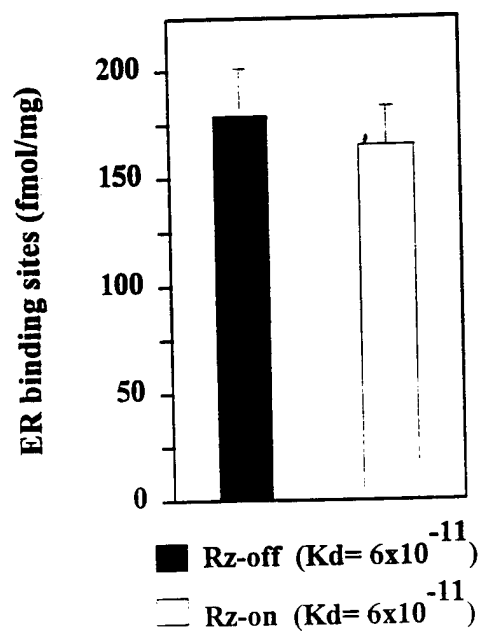


Fig. 4. Estrogen receptor binding in MCF-7/tet-Rz-HER-2 cells. MCF-7/tet-Rz-HER-2 cells were grown in the presence (rz-off) or absence (rz-on) of tetracycline (1 μ g/ml). Estrogen receptor whole cell binding assays were performed using tritiated Estradiol as described. The number of binding sites and receptor affinities were derived from Scatchard analysis (data not shown).

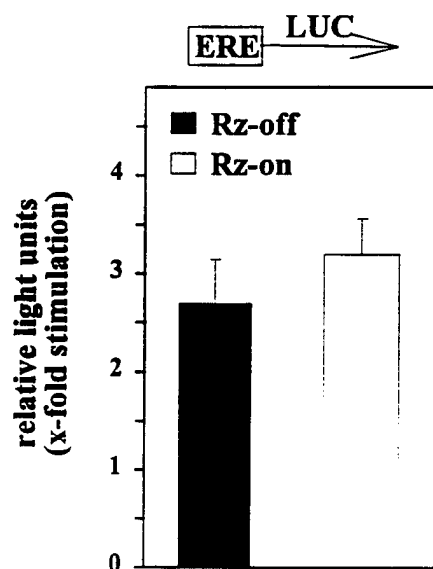


Fig. 5. Estrogen-mediated activation of a luciferase reporter construct under control of an estrogen-receptor-response element (ERE-Luc). MCF-7/tet-Rz-HER-2 cells grown in the presence (rz-off) or absence (rz-on) of tetracycline (1 μ g/ml) were transiently transfected with a ERE-Luc reporter construct and luciferase activity was quantified 48 h after the transfection in unstimulated cells and cells treated with estrogen.

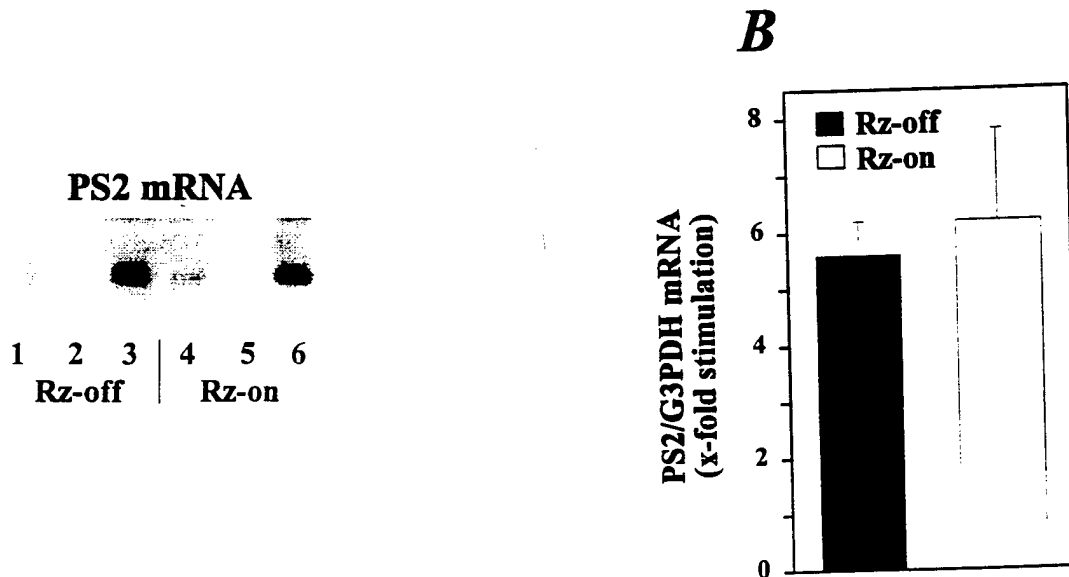


Fig. 6. Estrogen-mediated activation of endogenous PS2 mRNA expression in MCF-7/tet-Rz-HER-2 cells grown in the presence (rz-off; lanes 1-3) or absence (rz-on; lanes 4-6) of tetracycline (1 μ g/ml). Total RNA (20 μ g) from unstimulated cells (lanes 1 and 4), cells treated with a full estrogen antagonist (lanes 2 and 5), or from cells treated with estrogen (10⁻⁹ M) in the presence of the antagonist (lanes 3 and 6) was analyzed for PS2 mRNA expression by Northern analysis. PS2 mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase.

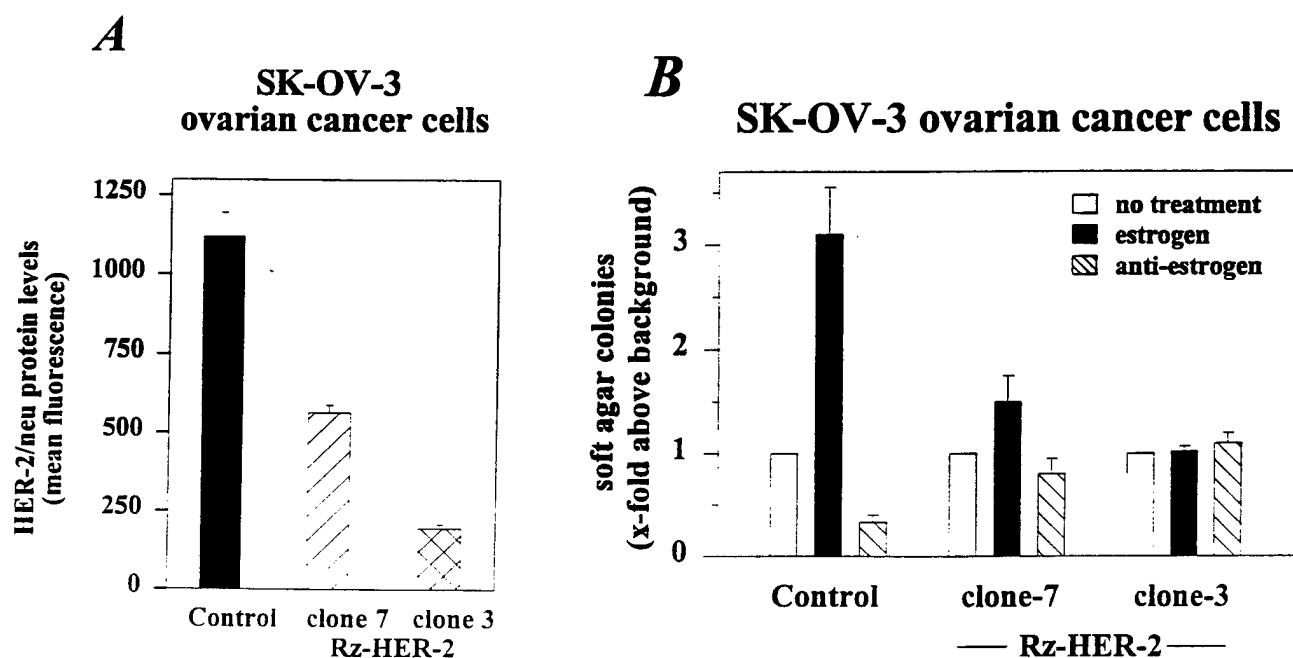


Fig. 7. Estrogen-mediated stimulation of soft agar colony formation and HER-2 expression in SK-OV-3 human ovarian cancer cells. *A*, SK-OV-3 cells were stably transfected with anti-HER-2 targeted-ribozymes. Reduction of HER-2 protein expression in control transfected cells and two clonal, ribozyme expressing cell lines, was determined by FACS analysis. *B*, soft agar colony formation of SK-OV-3 control cells and Rz-HER-2 ribozyme expressing cells (with 50 % and 20 % of control levels of HER-2 expression) in the absence or presence of estrogen (10^{-9} M) or a full estrogen antagonist (ICI 182.780 at 10^{-8} M).

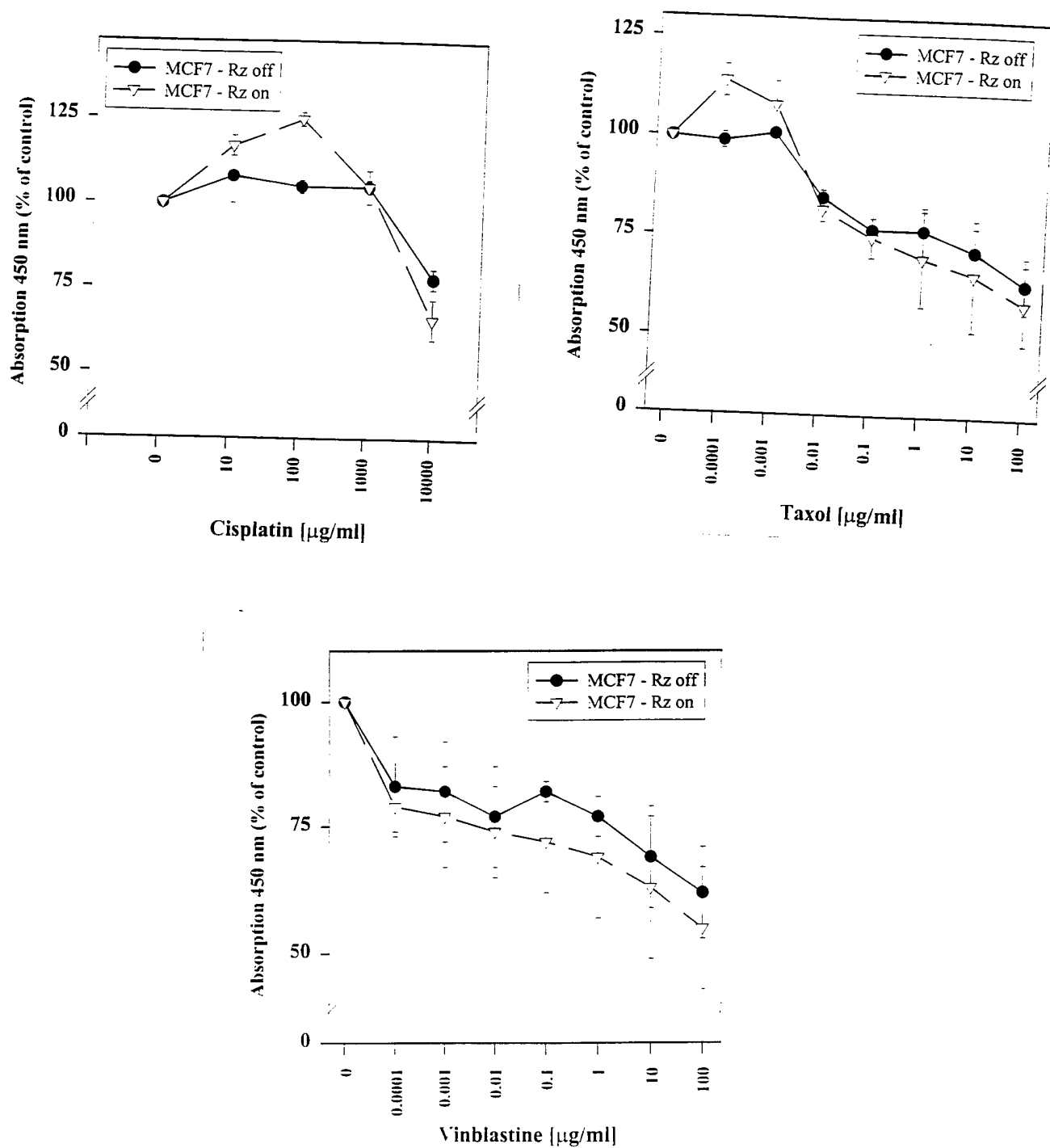


Fig. 8. Chemosensitivity is unchanged in MCF-7 cells with depleted HER-2 levels. MCF-7/tet-Rz-HER-2 cells grown in the presence (rz-off) or absence (rz-on) of tetracycline (1 $\mu\text{g/ml}$) were plated on 96 well plates. After an overnight incubation, cells were grown in the presence of Cisplatin (panel A, 0 to 10000 $\mu\text{g/ml}$), Taxol (panel B, 0 to 100 $\mu\text{g/ml}$) or vinblastine (panel C, 0 to 100 $\mu\text{g/ml}$) for up to 5 days. The cell number was determined using a modified MTT assay. The data represent mean \pm SD from three independent experiments.

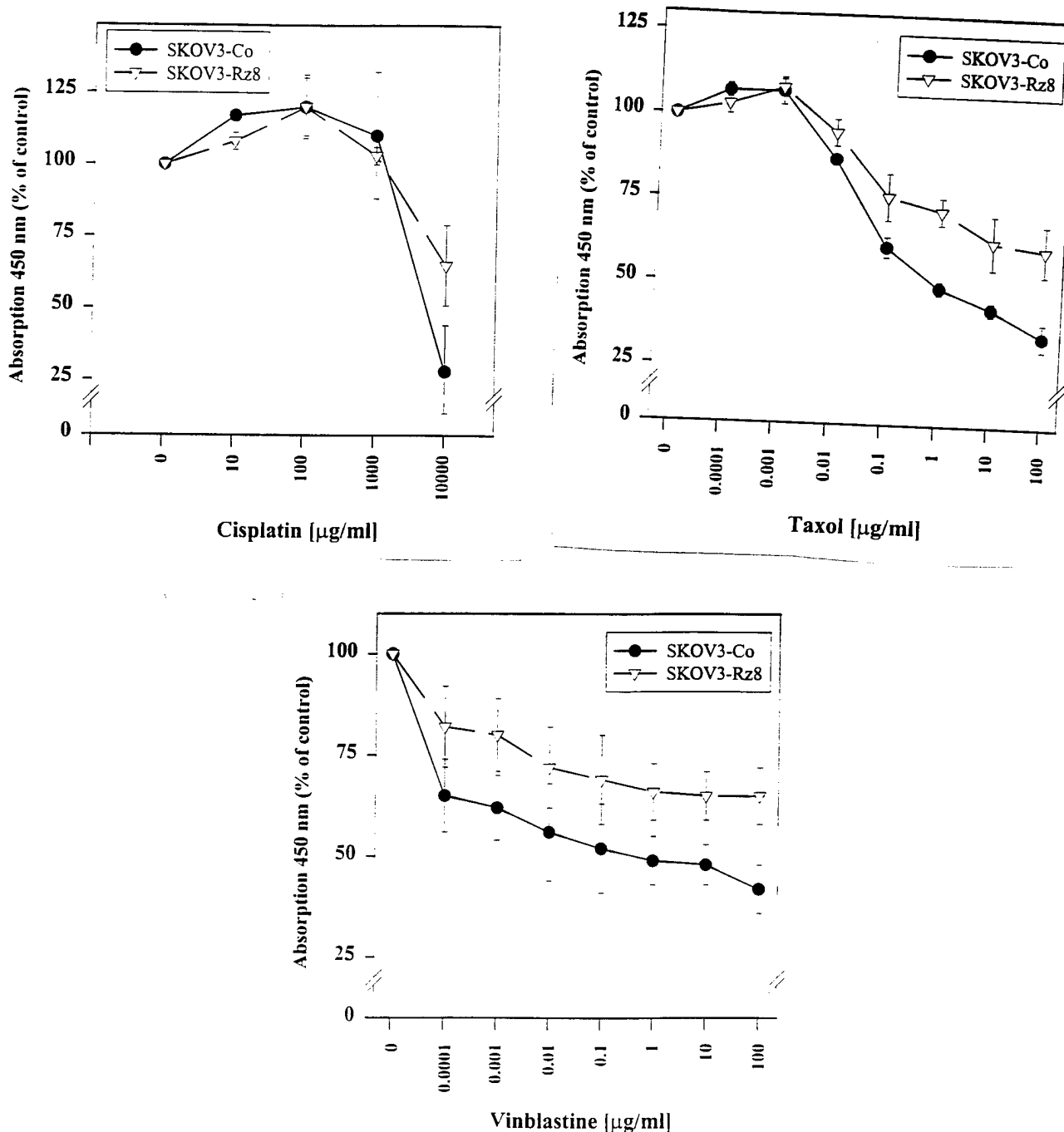


Fig. 9. Chemosensitivity is reduced in SK-OV-3 cells with depleted HER-2 levels. SK-OV-3 control cells or SK-OV-3-Rz-8 cells with reduced HER-2 levels (about 5 % residual HER-2 expression) were plated on 96 well plates. After an overnight incubation, cells were grown in the presence of Cisplatin (panel A, 0 to 10000 $\mu\text{g/ml}$), Taxol (panel B, 0 to 100 $\mu\text{g/ml}$) or vinblastine (panel C, 0 to 100 $\mu\text{g/ml}$) for up to 5 days. The cell number was determined using a modified MTT assay. The data represent mean \pm SD from three independent experiments.

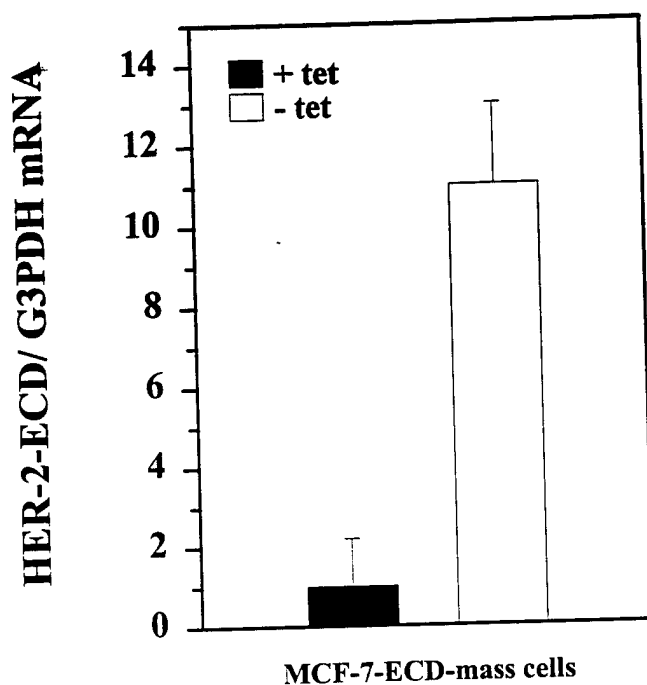


Fig. 10. Expression of a truncated HER-ECD receptor in MCF-7 breast cancer cells. Total RNA (30 μ g) from MCF-7 cells stably expressing a truncated HER-2/ECD receptor under the control of a tetracycline-regulated promoter, was analyzed for HER-2/ECD mRNA expression in the presence (lane 1; + tet) or absence (lane 2; - tet) of tetracycline (1 μ g/ml). HER-2/ECD mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA by densitometry.

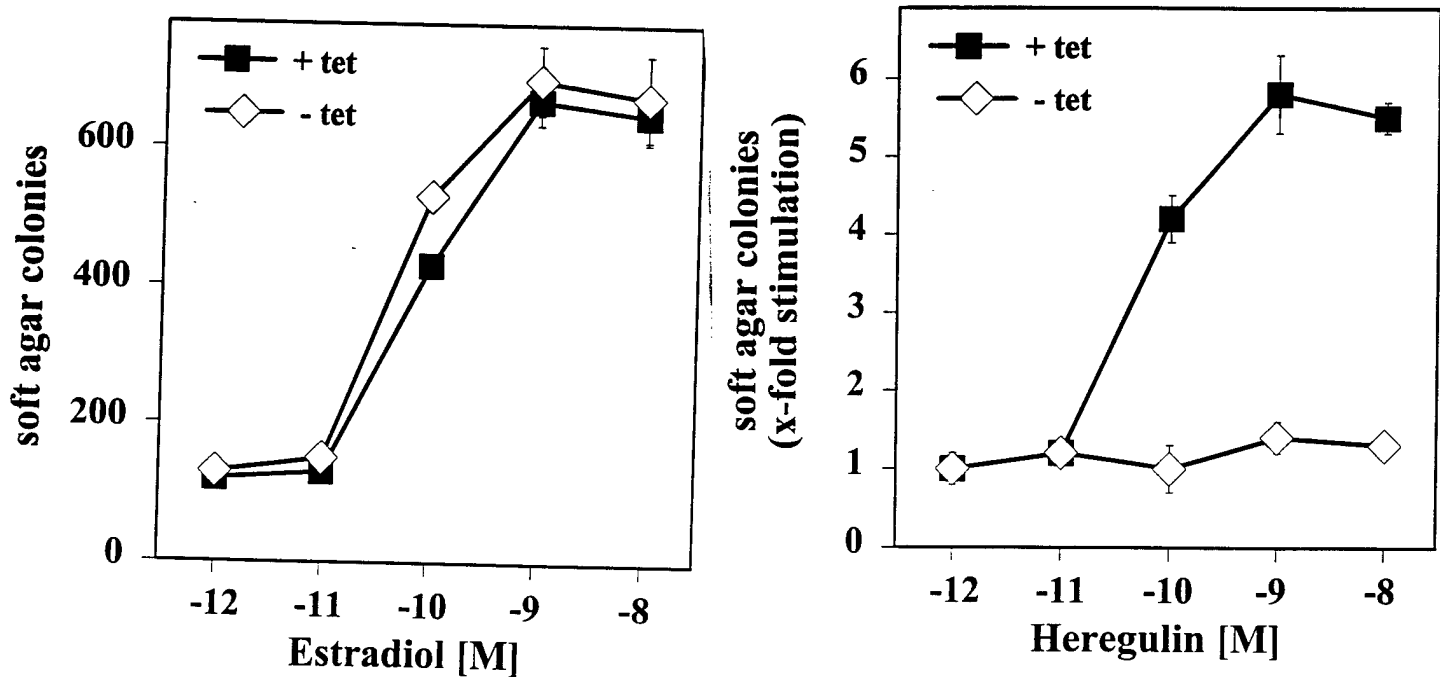


Fig. 11. Estrogen, and Heregulin-mediated stimulation of soft agar colony formation in MCF-7-HER/ECD cells. *A*, Estrogen-dose response on soft agar colony formation of MCF-7-HER/ECD cells in the presence (+ tet) or absence (- tet) of tetracycline ($1\mu\text{g/ml}$). Estrogen-induction of colony formation was determined in the presence of a full estrogen antagonist (ICI 182.780 at 10^{-8} M). *B*, Heregulin-mediated stimulation of colony formation of MCF-7-HER/ECD cells in the presence (+ tet) or absence (- tet) of tetracycline ($1\mu\text{g/ml}$). Heregulin-induction of colony formation was determined in the presence of a full estrogen antagonist (ICI 182.780 at 10^{-8} M).

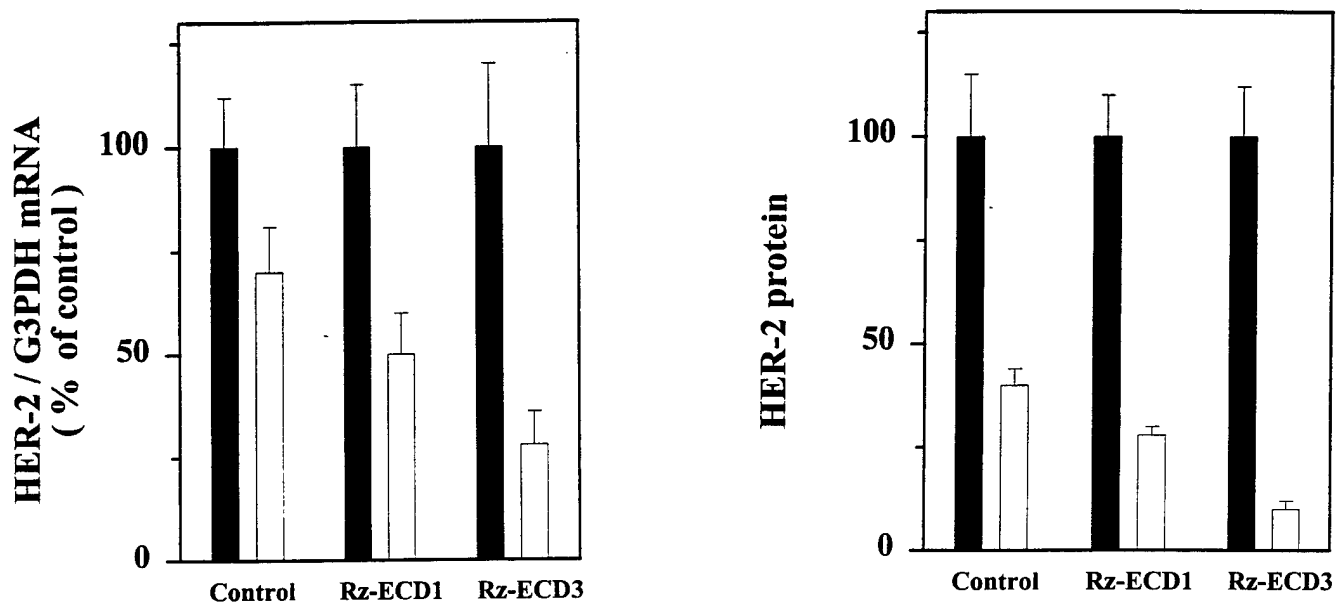


Fig. 12. Reduction of HER-2/ECD expression in MKN7 human gastric carcinoma cells. A, HER-2 full length mRNA (filled bars; 4.5 kb transcript) and HER-2/ECD mRNA (open bars; 2.3 kb transcript) were quantified by Northern analysis relative to G3PDH in MKN7 control cells and MKN7 cells stably mass-transfected with ribozymes Rz-ECD1 or Rz-ECD3. B, HER-2 full length protein (filled bars; 185 kd) and HER-2/ECD protein (open bars; 100 kd) were quantified by Western analysis in MKN7 control cells and MKN7 cells stably mass-transfected with ribozymes Rz-ECD1 or Rz-ECD3.

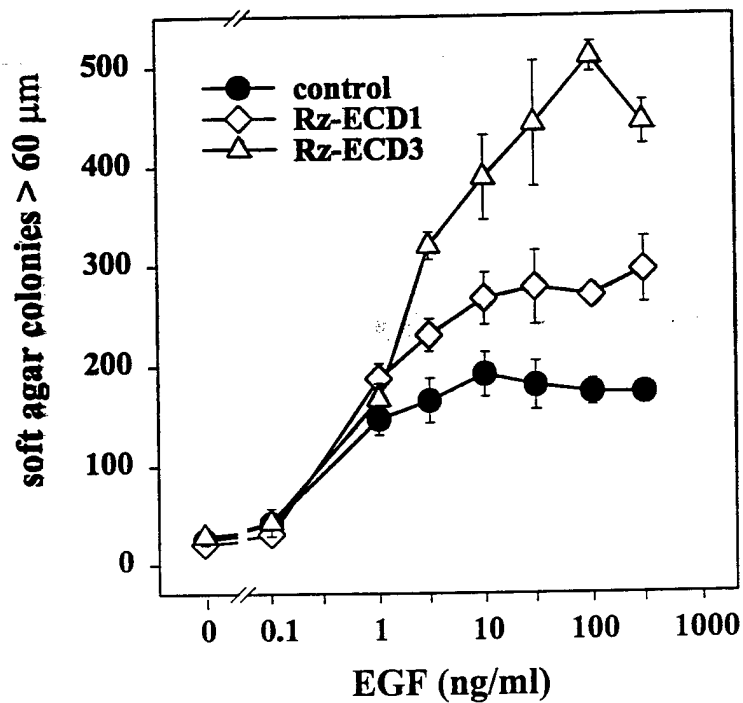


Fig. 13. Soft agar colony formation and HER-2/ECD expression. EGF-dose-response on soft agar colony formation of MKN7 control cells and MKN7 cells expressing different levels of HER-2/ECD. Soft agar colony formation of MKN7 control (filled circles),- Rz-ECD1 (open diamonds) and Rz-ECD3 cells (open triangles) (with 50 % and 25 % of control levels of HER-2/ECD expression, respectively) was determined in the absence or presence of different EGF concentrations.

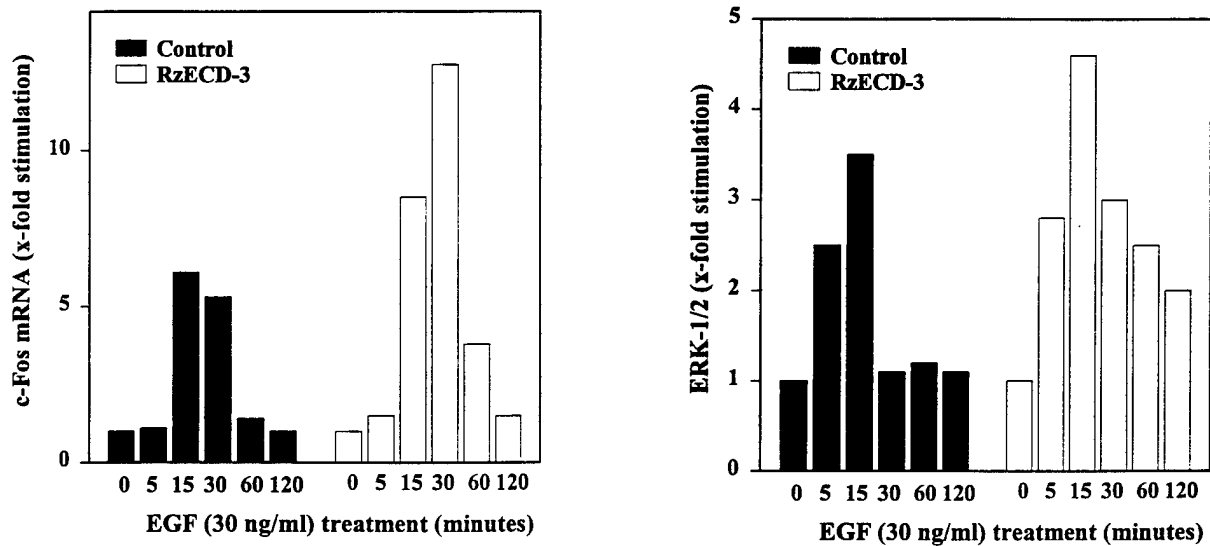


Fig. 14. EGF-mediated stimulation of MAP-Kinase protein and c-Fos mRNA expression is reduced in HER-2/ECD depleted MKN7 cells. *A*, MKN7 control cells and Rz-ECD3 expressing cells (25 % residual HER-2/ECD expression) were serum-starved for 24 hours and then stimulated with EGF (30 ng/ml) for the indicated time periods. 20 μ g of total cellular RNA were analyzed for c-Fos mRNA expression by Northern blotting. The blots were stripped and reprobbed for G3PDH mRNA expression. C-Fos mRNA expression levels were quantified relative to their G3PDH mRNA levels and values are expressed as x-fold stimulation (no EGF stimulation = 1). *B*, MKN7 control cells and Rz-ECD3 expressing cells (25 % residual HER-2/ECD expression) were serum-starved for 24 hours and then stimulated with EGF (30 ng/ml) for the indicated time periods. 10 μ g of total cellular lysate were analyzed for MAP-Kinase expression by Western blotting using an antibody that detects only the phosphorylated ERK-1/2 proteins. ERK-1/2 specific bands were quantified using densitometry.

HER-2/neu Is Rate-limiting for Ovarian Cancer Growth

CONDITIONAL DEPLETION OF HER-2/neu BY RIBOZYME TARGETING*

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Amplification and overexpression of the *HER-2/neu* proto-oncogene frequently coincide with an aggressive clinical course of certain human adenocarcinomas. To assess whether *HER-2/neu* plays a rate-limiting role in ovarian cancer, we used human SK-OV-3 ovarian cancer cells as a model. We applied a conditional mRNA depletion strategy of *HER-2/neu* with anti-*HER-2/neu*-targeted hammerhead ribozymes expressed under the control of a tetracycline-regulated promoter system. In these ovarian cancer cells, we reduced *HER-2/neu* mRNA, protein expression, and tumor growth in nude mice by transfection with *HER-2/neu*-targeted ribozymes and generated cell lines expressing different levels of *HER-2/neu*. Expression of the most effective ribozyme (Rz3) quenched *HER-2/neu* mRNA levels by >90%. Concomitantly, fluorescence-activated cell sorting analysis revealed that expression of the *HER-2/neu*-encoded surface glycoprotein was almost completely abrogated. In nude mice, tumor growth was dramatically inhibited in the *HER-2/neu*-depleted Rz3-expressing SK-OV-3 cells. Furthermore, already established tumors started to regress when Rz3 expression was activated midstream by withdrawal of the tetracycline treatment. This study supports the thesis that *HER-2/neu* can be rate-limiting for the malignant phenotype of ovarian cancer in a gene dose-dependent manner.

The *HER-2/neu* proto-oncogene belongs to the epidermal growth factor receptor family and has been implicated in malignant transformation (reviewed in Ref. 1). *HER-2/neu* can be activated by at least three different genetic mechanisms including point mutation (2), gene amplification (3), and overexpression (4). These observations are relevant to human cancer because amplification and/or overexpression has been observed in 20–30% of adenocarcinomas of the breast, ovary, lung, and stomach (reviewed in Ref. 5). Moreover, overexpression has been linked to an unfavorable prognosis in patients with breast (3) and ovarian (6) cancer.

Beyond this coincidence, it has been difficult to demonstrate directly that *HER-2/neu* is rate-limiting for tumor progression. One reason for this lack of understanding is that no ligand for *HER-2/neu* has been found, and *HER-2/neu* is now viewed

merely as a signal-transducing subunit of epidermal growth factor and new differentiation factor/heregulin receptors (7). Depending on the cellular context, *HER-2/neu*-targeted antibodies can thus cause activating and inhibitory effects, which make it difficult to dissect the precise role of *HER-2/neu*. Recently, with a novel approach using single-chain antibodies that suppress cell-surface expression of *HER-2/neu* by retention in the endoplasmic reticulum, it was shown that reduction of *HER-2/neu* reversed the transformed phenotype of *HER-2/neu*-transfected NIH/3T3 cells (8) and impaired growth factor signaling in T47D breast cancer cells (7, 9). Here we utilize an independent approach by cleaving the *HER-2/neu* mRNA with specific ribozymes and thus deplete cells of the endogenous gene product. With this approach, the effects of a functional knockout can be studied in model cell lines, and thus, the contribution of a particular gene product delineated (reviewed in Ref. 10; see also Refs. 11–14).

A major obstacle in achieving a constitutive ribozyme-mediated *HER-2/neu* depletion in stably transfected cells is that *HER-2/neu* expression may provide a growth advantage, thus making the selection of low expressing cell populations difficult. To circumvent this potential problem, we expressed hammerhead ribozymes under the control of a tetracycline-regulated promoter (15) to evaluate the effect of a conditional *HER-2/neu* depletion on *in vitro* and *in vivo* ovarian cancer cell growth. As a model, we used human SK-OV-3 ovarian cancer cells, which overexpress *HER-2/neu* due to a gene amplification event (16). We generated stably mass-transfected derivative cell lines that express anti-*HER-2/neu*-targeted ribozymes. Ribozyme expression almost completely abrogated *HER-2/neu* mRNA and protein expression, which resulted in a dramatic inhibition of tumor growth in nude mice. Furthermore, tumors that had been established in the absence of ribozyme expression started to regress when ribozyme expression was activated *in vivo*.

EXPERIMENTAL PROCEDURES

Plasmids and Generation of Constructs—Plasmids expressing the tetracycline transactivator (tTA)¹/VP16 fusion protein (pUHG15-1 (15)) and the heptamerized tetracycline operator sequence (tet-O; pUHC13-3 (15)) were obtained from Dr. Bujard (Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany). The ribozyme expression plasmid (pTET) was derived from pUHC13-3 and modified as described (17). The *HER-2/neu*-targeted hammerhead ribozymes Rz3 and Rz4 were designed and cloned as described (18). In brief, the following ribozyme coding sense and antisense oligonucleotides were annealed together and ligated into the *Hind*III restriction site of pTET: Rz3, 5'-agcttCCTGAAAGCTGATGAGTCCGTTAGGACC*AAAAAGT-CCTa-3' (sense) and 5'-agcttAGGACTTTTTTC*GTCCTAACGGACTC-ATCAGCTTTCAGGA-3' (antisense); and Rz4, 5'-agcttCAAGACCAC-CTGATGAGTCCGTTAGGACGAAACCAGCAGa-3' (sense) and 5'-

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¹ The abbreviations used are: tTA, tetracycline transactivator; FACS, fluorescence-activated cell sorting; tet-O, heptamerized tetracycline operator sequence.

agcttCTGCTGGTTTCGTCCTAACGGACTCATCAGGTGGTCTTG-a-3' (antisense) (with lower-case letters indicating *Hind*III restriction site overhangs, boldface upper-case letters showing HER-2/neu-specific antisense regions, and underlined upper-case letters indicating the hammerhead ribozyme core sequence). The resulting ribozyme expression plasmid (pTET/Rz3) contains HER-2/neu-specific antisense flanking regions of 8 nucleotides on both ends of the 22-nucleotide catalytic hammerhead ribozyme core sequence that target it to a central region in the HER-2/neu RNA just downstream of the transmembrane region of the protein. In addition, a control ribozyme was constructed (pTET/Rz3mu) that lacks catalytic activity due to a single base pair mutation in the ribozyme core region (G to T exchange; * indicates the position of the mutated G). The ribozyme pTET/Rz4 contains HER-2/neu-specific antisense flanking regions of 9 and 8 nucleotides on the 5'- and 3'-ends, respectively, of the 22-nucleotide catalytic ribozyme core that target Rz4 to its cleavage site 1991 nucleotides downstream of the translation initiation site in the HER-2/neu RNA. Correct sequences of the ribozymes were verified by DNA sequencing, and specific catalytic ribozyme activity was demonstrated in *in vitro* cleavage assays as described (18).

Cell Lines and Transfections—Human ovarian cancer cells (SK-OV-3) were obtained from American Type Culture Collection and were maintained in continuous culture at 37 °C in 5% CO₂ using Iscove's modified Eagle's medium (Life Technologies, Inc.) supplemented with glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. SK-OV-3 cells were transfected using LipofectAMINE (Life Technologies, Inc.). Briefly, cells at 50–70% confluence were incubated for 5 h with plasmid DNA mixed with LipofectAMINE (7 µl of LipofectAMINE/1 µg of plasmid DNA) in serum-free medium (Opti-MEM, Life Technologies, Inc.) at 37 °C in 5% CO₂. The transfection medium was then replaced with normal growth medium and 36 h later supplemented with the respective drugs for selection of stable cell lines. SK-OV-3 cells stably expressing tetracycline-regulated HER-2/neu-targeted ribozymes were generated in a two-step transfection protocol. In the first step, SK-OV-3 cells were transfected with 10 µg of pUHG15-1 plasmid DNA and 1 µg of pcDNA3 plasmid DNA (Invitrogen, San Diego, CA) to provide G418 resistance. After selection for stable cell lines in the presence of G418 at 0.7 mg/ml, individual tTA-expressing clones were isolated with cloning rings. To test the clones for tTA expression and tetracycline regulation, the cells were transiently transfected in the absence and presence of 1 µg/ml tetracycline (Sigma) with pUHC13-3 plasmid DNA containing a luciferase cDNA under the control of the tet-O-binding site (15). Cell lysates were prepared 36 h after the transfections, and luciferase activities were measured in a luminometer as described (17). Two clones (SK-OV-3/tTA-7 and SK-OV-3/tTA-2) that demonstrated the best tetracycline regulation of luciferase activity were used for further transfections with the ribozyme expression plasmids. SK-OV-3/tTA-7 and SK-OV-3/tTA-2 cells were then transfected with 10 µg of pTET/Rz3, pTET/Rz3mu, or pTET/Rz4 mixed with 1 µg of pZero (Invitrogen) to provide Zeocin resistance. Mass-transfected derivative cell lines (SK-OV-3/Rz3, SK-OV-3/Rz3mu, and SK-OV-3/Rz4) were obtained after selection with 0.4 mg/ml Zeocin and 1 µg/ml tetracycline.

Northern Analysis—Total cellular RNA was isolated with the RNA STAT-60 method (Tel-Test, Friendswood, TX), and 30 µg were separated and blotted as described (19). A HER-2/neu cDNA probe (1.5-kilobase pair *Eco*RI fragment) was hybridized, washed, and exposed to film for 16 h (19). To correct for variability in loading, blots were stripped, reprobed with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe (CLONTECH), and exposed to film for 1 h. Relative band intensities were measured by densitometry.

Fluorescence-activated Cell Sorting (FACS)—To quantitate HER-2/neu protein levels by FACS analysis, cells were trypsinized, washed once with growth medium containing serum and twice with phosphate-buffered saline (Sigma), and resuspended in phosphate-buffered saline at 5 × 10⁵ cells/100 µl. The cells were incubated for 30 min at 4 °C with a 1:100 dilution of a primary anti-human HER-2/neu mouse monoclonal antibody (clone 9G6.10; Neomarkers, Fremont, CA). Cells were washed twice with phosphate-buffered saline and incubated for 30 min at 4 °C in the dark with a 1:200 dilution of a fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody (Boehringer Mannheim). After two final washes with phosphate-buffered saline, the mean value of fluorescence intensity of 10,000 cells was determined by FACS (FACStar Plus, Becton Dickinson). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls.

Tumor Growth in Animals—Female athymic nude mice (NCr nu/nu; NCI, Frederick, MD) were injected subcutaneously with 1 × 10⁶ cells in 100 µl of Iscove's modified Eagle's medium (three mice/group and two

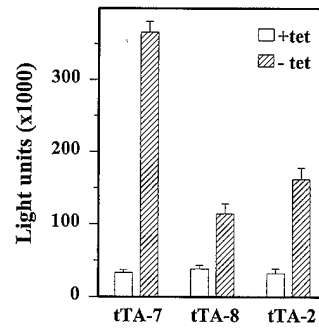


FIG. 1. Three stably transfected SK-OV-3 clones (SK-OV-3/tTA-7, SK-OV-3/tTA-8, and SK-OV-3/tTA-2) that express the tTA protein were transiently transfected with pUHC13-3 plasmid DNA, coding for luciferase under the control of the tet-O-binding site (13). Luciferase activity was measured 36 h after transfection in the absence and presence of tetracycline (*tet*).

injection sites/mouse). Mice in group 1 were injected with SK-OV-3/tTA-7 control cells, and those in groups 2 and 3 with SK-OV-3/Rz3 ribozyme-expressing cells. In the mice in group 3, slow-release tetracycline pellets (Innovative Research of America), which release 0.7 mg of tetracycline/day, were implanted subcutaneously at the day of tumor cell injection. Tumor growth was monitored for up to 2 months, and tumor sizes were estimated from the product of the perpendicular diameters of the tumors. In one mouse in group 3, the tetracycline pellet was removed after 6 weeks to activate ribozyme expression in established tumors, and tumor growth was monitored for an additional 2 weeks. In a separate study, nude mice were injected subcutaneously with 2 × 10⁶ SK-OV-3/Rz4 cells in 100 µl of Iscove's modified Eagle's medium (five mice/group and two injection sites/mouse). Tumor growth was monitored for 4 weeks in the presence (group 1) or absence (group 2) of tetracycline.

Data Analysis—Means ± S.E. are depicted unless indicated otherwise. Student's *t* test or analysis of variance for repeated measures (Statview 4.02, Abacus Concepts, Inc.) was used for comparisons between data sets, and *p* < 0.05 was considered significant.

RESULTS

Generation of tTA-expressing SK-OV-3 Cells—To avoid promoter interference and to generate cells in which tet-O-controlled transgene expression can be tightly regulated by tetracycline, a two-step transfection protocol was used as originally described (15). In the first step, SK-OV-3 cells were transfected with pUHG15-1 plasmid DNA, and individual clones were screened for tetracycline regulation of tTA-driven tet-O/luciferase expression. Individual tTA-expressing clones were transiently transfected with pUHC13-3 plasmid DNA, coding for luciferase under the control of the tet-O-binding site, and luciferase activity was measured in the absence and presence of tetracycline. In the absence of tetracycline, luciferase activity was high in all clones. The best tetracycline regulation was observed in clone 7 (SK-OV-3/tTA-7), where tTA-driven luciferase activity was repressed by >90% in the presence of tetracycline (Fig. 1). Clones SK-OV-3/tTA-7 and SK-OV-3/tTA-2 were then used for further transfections with pTET/ribozyme expression plasmids. It is important to note that luciferase activity was not completely repressed to background levels, thus demonstrating some residual promoter activity in these cells. HER-2/neu expression as measured by FACS analysis did not vary between the clones, indicating that clonal selection and/or tTA expression had no significant effects on HER-2/neu expression in SK-OV-3 cells (data not shown).

Efficacy of HER-2/neu-targeted Ribozymes in Cells in Vitro—SK-OV-3/tTA-7 cells were transfected with pTET/Rz3 or pTET/Rz3mu plasmids, and mass-transfected cell pools were selected in the presence of tetracycline to repress ribozyme expression during the selection period. After 6 weeks, stably transfected cells were grown in the absence or presence of tetracycline, and HER-2/neu mRNA levels were quantified by

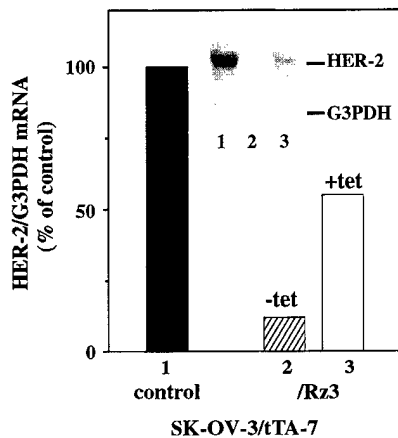


FIG. 2. Total RNA (30 μ g) from SK-OV-3/tTA-7 control cells (lane 1) and anti-HER-2/neu-targeted Rz3 ribozyme-transfected cells grown in the absence (lane 2) and presence (lane 3) of tetracycline (*tet*) was analyzed by Northern analysis. HER-2/neu mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) mRNA by densitometry. The inset shows a representative Northern blot. The positions of HER-2/neu- and glyceraldehyde-3-phosphate dehydrogenase-specific transcripts are indicated. The HER-2/neu Northern blot was exposed to film for 16 h, whereas the glyceraldehyde-3-phosphate dehydrogenase Northern blot was exposed for only 1 h.

Northern analysis (Fig. 2). HER-2/neu-targeted pTET/Rz3 ribozyme expression (Fig. 2, lane 2 (-*tet* = ribozyme on)) depleted endogenous HER-2/neu mRNA levels by 90% compared with control cells. Inactivation of ribozyme expression (Fig. 2, lane 3) was followed by a 4.5-fold increase in HER-2/neu mRNA levels, demonstrating the specificity of the ribozyme effect. Expression of the pTET/Rz4 ribozyme construct was less efficacious and reduced HER-2/neu mRNA levels in a tetracycline-reversible manner by ~50% (data not shown).

In the next experiment, ribozyme-mediated depletion of HER-2/neu protein expression was assessed by FACS analysis. Cell-surface expression of the HER-2/neu protein was suppressed by 90% when cells were grown in the absence of tetracycline (Fig. 3A, Rz3-on). HER-2/neu levels almost completely reverted to control values when tetracycline was added to the medium. In addition, a catalytically inactive mutant ribozyme (SK-OV-3/Rz3mu) had no significant effects on HER-2/neu mRNA or protein expression (data not shown), which clearly indicates that cleavage of HER-2/neu RNA and not antisense inhibition is the main mode of ribozyme action. Expression of pTET/Rz4 reduced HER-2/neu protein levels by 50% (Fig. 3B), correlating very well with the results from the Northern analysis.

In parallel experiments with other cell lines, we verified the efficacy of the anti-HER-2/neu-targeted ribozymes. Ribozyme targeting caused a reduction of HER-2/neu expression in MKN7 gastric cancer, MDA-MB-361 breast cancer, and Colo357 pancreatic cancer cells (data not shown). This strongly suggests that this targeting approach will be useful in a variety of human adenocarcinomas.

The biological significance of a HER-2/neu depletion on *in vitro* growth of SK-OV-3 cells was assessed by anchorage-dependent as well as anchorage-independent growth assays. HER-2/neu-targeted ribozyme expression did not alter cell morphology or anchorage-dependent proliferation. However, anchorage-independent growth in soft agar was inhibited by >90% in SK-OV-3/Rz3 cells and was restored to control levels by the addition of tetracycline (data not shown). This demonstrates that HER-2/neu is a rate-limiting factor for anchorage-independent growth of SK-OV-3 cells.

Down-regulation of HER-2/neu Expression Inhibits Tumor

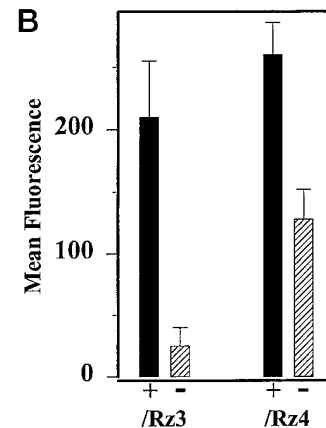
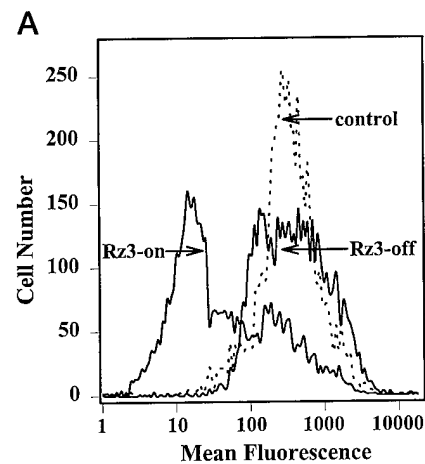


FIG. 3. A, FACS analysis of HER-2/neu protein expression in SK-OV-3/tTA-7 control cells (dotted line; mean \pm S.D. = 240 ± 25) compared with SK-OV-3/Rz3 ribozyme-expressing cells (solid lines) in the absence (Rz3-on) and presence (Rz3-off) of tetracycline. B, mean fluorescence values of FACS analysis of HER-2/neu protein expression in SK-OV-3 cells transfected with two different ribozyme constructs (pTET/Rz3 and pTET/Rz4) in the absence (-; ribozyme on) and presence (+; ribozyme off) of tetracycline.

Growth in Vivo in Nude Mice—When injected subcutaneously at 1×10^6 cells/injection site into nude mice, SK-OV-3/tTA-7 control cells grew to a mean tumor size of 100 ± 20 mm² within 44 days (Fig. 4A, ●). In contrast, tumor growth of ribozyme-expressing pTET/Rz3 cells was significantly inhibited ($p < 0.01$, Student's *t* test), and tumors did not grow beyond a very small inoculum size of 6 ± 2.6 mm² (Fig. 4A, ▽). In animals that were treated with slow-release tetracycline pellets to turn the ribozyme off *in vivo*, tumor growth started to increase after 32 days, and mean tumor sizes were 27 ± 7.8 mm² (Fig. 4A, ▲; significantly different from ribozyme-expressing tumors, $p < 0.05$, Student's *t* test). Tumor growth of SK-OV-3 cells transfected with the catalytically inactive ribozyme (pTET/Rz3mu) was not significantly different compared with control cells (data not shown).

In a subset of this study, we addressed the question of whether ribozyme-mediated abrogation of HER-2/neu expression can cause regression of established tumors. Ribozyme expression was activated in the tumor cells by removal of the tetracycline pellet after 44 days, and tumor growth was monitored for another 2 weeks. While the tumors in the tetracycline-treated animals (ribozyme off) grew continuously and reached a size of 76 ± 20 mm² (Fig. 4B, solid lines), the tumors in which the ribozyme was activated by removal of tetracycline started to regress and reached a size of 20 ± 3.5 mm² (Fig. 4B, dotted lines). This tumor regression was significant ($p < 0.05$, analysis

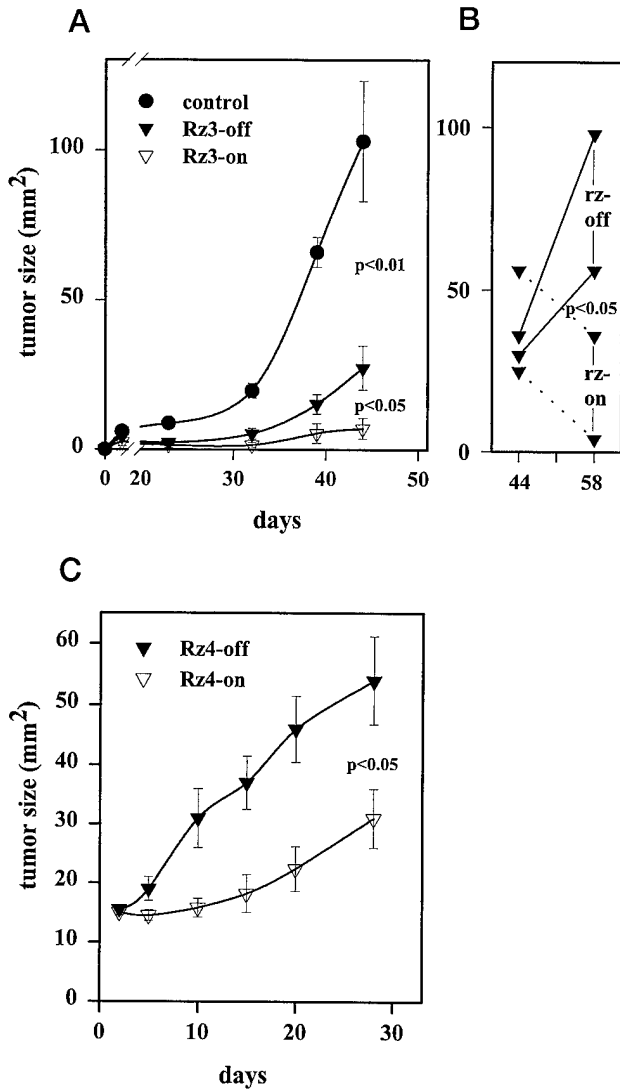


FIG. 4. Subcutaneous tumor growth in nude mice of SK-OV-3 ovarian cancer cells producing different levels of HER-2/neu. A, 1×10^6 cells were inoculated subcutaneously (three mice/group and two injection sites/mouse), and tumor growth was monitored for 6 weeks in SK-OV-3/tTA control cells (●) or pTET/Rz3 ribozyme-transfected cells in the presence (Rz3-off; ▼) and absence (Rz3-on; ▽) of tetracycline treatment. B, after 44 days, growth curves of the individual tumors in the absence (rz-on; dotted lines) and continued presence (rz-off; solid lines) of tetracycline were monitored for another 2 weeks. C, SK-OV-3 cells transfected with the pTET/Rz4 ribozyme expression construct were inoculated subcutaneously at 2×10^6 cells/injection site (five mice/group and two injection sites/mouse), and tumor growth was monitored for 4 weeks in the presence (Rz4-off; ▼) and absence (Rz4-on; ▽) of tetracycline.

of variance for repeated measures) and suggests that HER-2/neu expression is important for continuous growth of ovarian cancer cells *in vivo*. Furthermore, disruption of HER-2/neu not only arrested tumor growth, but induced regression.

To test if SK-OV-3 tumor growth correlates with the level of HER-2/neu expression, we used SK-OV-3/Rz4 cells, in which, in contrast to SK-OV-3/Rz3 cells, HER-2/neu protein levels are reduced by ~50% (see Fig. 3B). When injected subcutaneously at 2×10^6 cells/injection site in the presence of tetracycline, SK-OV-3/Rz4 cells grew to a mean tumor size of 54 ± 7.2 mm² within 4 weeks (Fig. 4C, ▼). Inactivation of ribozyme expression by tetracycline significantly inhibited tumor growth by 43% (Fig. 4C, ▽; $p < 0.05$, Student's *t* test), which correlated very well with the level of HER-2/neu reduction in these cells.

The overall faster tumor growth in this experiment compared with the previous study (see Fig. 4A) was due to the higher tumor cell inoculum (2×10^6 versus 1×10^6). This result further supports the thesis that HER-2/neu expression is rate-limiting for SK-OV-3 tumor growth in a gene dose-dependent manner.

DISCUSSION

The phenomenology of HER-2/neu overexpression in human cancer has been well studied, and particularly the easily accessible cell-surface localization made it an excellent target for antibody-based immunotherapies. HER-2/neu-specific monoclonal antibodies (20) and recombinant immunotoxins (21), both of which can inhibit *in vitro* and *in vivo* growth of transformed cells, have been described. Despite the clinical usefulness of these approaches, it remains unclear how expression of an apparently normal gene product affects tumorigenesis and tumor progression *in vivo*. Potentially more specific genetic targeting strategies such as antisense oligonucleotides have been used with limited success (22), probably due to their relatively low efficacy and specificity.

In this study, we expressed HER-2/neu-targeted hammerhead ribozymes under the control of a tetracycline-regulated promoter system in human SK-OV-3 ovarian cancer cells, which express HER-2/neu spontaneously at high levels due to gene amplification. SK-OV-3 cells provide an attractive model since the epidermal growth factor receptor is the only other human epidermal growth factor receptor expressed in these cells, which therefore proliferate in response to epidermal growth factor, but not to new differentiation factor/herregulin-like growth factors (data not shown).

To achieve a conditional HER-2/neu down-regulation, we employed a binary tetracycline-regulated gene expression system in which hammerhead ribozyme expression can be inactivated *in vitro* and *in vivo* by the addition of the nontoxic antibiotic tetracycline. The major advantage over a constitutive ribozyme expression system is that the effects of ribozyme-mediated down-regulation of HER-2/neu expression can be evaluated in one genetically identical cell line, thus preventing interferences of different genetic backgrounds between various cell lines. Furthermore, HER-2/neu can be down-regulated at different time points in tumor growth in animals, which allows the identification of the tumor stages that are most susceptible to anti-HER-2/neu-targeted therapies.

In stably mass-transfected SK-OV-3 cells, Rz3 ribozyme expression depleted HER-2/neu mRNA and protein levels by >90%, and inactivation of ribozyme expression by tetracycline reversed the effects. This inhibition is even more remarkable taking into account that mass-transfected cells (and not clonal subpopulations) were used and that HER-2/neu is expressed at very high levels in SK-OV-3 cells. This suggests that ribozymes can be more effective than antisense oligonucleotides that reduced HER-2/neu expression by only 50% in selected clones of SK-BR-3 breast cancer cells (22), which express HER-2/neu at comparable levels. This dramatic inhibition enabled us for the first time to study the effects of a functional and conditional HER-2/neu knockout on *in vivo* tumor growth of ovarian cancer cells. Tumor growth of ribozyme-expressing cells was almost completely abrogated, and inactivation of ribozyme expression by tetracycline partially reversed SK-OV-3 tumor growth. Inactivation of ribozyme expression by tetracycline was not as effective as in the cell culture experiments, which can potentially be explained by an insufficient tetracycline delivery to the tumor cells *in vivo*. Alternatively, *in vivo* tumor growth might be more susceptible to residual ribozyme activity and hence small reductions in HER-2/neu expression.

In summary, our data demonstrate that HER-2/neu has a

rate-limiting role in ovarian cancer and that ribozyme targeting can cause regression of established tumors. The conditional *HER-2/neu* depletion *in vitro* and *in vivo* will enable further elucidation of the role of *HER-2/neu* in the pathogenesis of human cancer.

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Adenovirus-mediated transduction of ribozymes abrogates HER-2/neu and pleiotrophin expression and inhibits tumor cell proliferation

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The combination of specific gene targeting technologies with efficient gene delivery systems could provide the means to evaluate the concept of anticancer strategies designed to block expression of potentially rate-limiting tumor promoting factors. Here, we constructed adenoviruses expressing hammerhead-ribozymes targeted to two of these factors, the tyrosine kinase receptor HER-2/neu or the growth factor pleiotrophin (PTN). Adenovirus-

mediated transduction of either HER-2/neu- or PTN-targeted ribozymes depleted the respective RNAs and inhibited protein expression significantly in three different human cancer cell lines. This resulted in almost complete abrogation of HER-2/neu- or PTN-dependent cancer-cell proliferation, thus demonstrating the feasibility of this approach as a future cancer gene therapy.

Keywords: gene therapy; adenovirus; ribozymes; pleiotrophin; HER-2/neu

Introduction

Tumor growth and ultimately metastasis is a complex process regulated in part by factors controlling cellular proliferation and death, as well as tumor angiogenesis.¹⁻³

One of these factors, HER-2/neu, a member of the epidermal growth factor (EGF) tyrosine kinase receptor family has been implicated in mediating growth factor signals,^{4,5} as well as in modulating hormone responsiveness of breast cancer cells.⁶ HER-2/neu is frequently overexpressed in a variety of adenocarcinomas including those of lung, breast and ovarian cancer. In these tumors, HER-2/neu overexpression serves as a marker of faster tumor progression and poor prognosis.⁷⁻⁹ Currently, targeting of HER-2/neu expression with anti-HER-2/neu antibodies is being evaluated as a potential therapeutic approach in breast cancer patients. Despite a useful role as a diagnostic marker for disease progression, it remains unclear if HER-2/neu expression is mostly a marker or a rate-limiting factor in cancer cell growth.

Tumor angiogenesis, a process whereby factors stimulating the ingrowth of blood vessels into the tumor are secreted into the local tumor milieu by cancer and stroma cells, also plays a critical role in regulating the balance between cell proliferation and cell death and by providing a route for distant spread.³ Both clinical and laboratory evidence suggests that spread of malignant cells from a localized tumor is directly related to the number of microvessels in the primary tumor.¹⁰ Of the multitude of factors secreted by tumor and stroma cells which are

potentially angiogenic,¹¹ two have been confirmed as angiogenic factors which are rate-limiting in *in vivo* tumor models. The importance of one of these, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), was demonstrated through functional knockout through use of blocking antibodies.¹² A critical role for the other factor, pleiotrophin (PTN), was shown in angiogenesis and metastasis associated with melanoma using a hammerhead-ribozyme PTN mRNA depletion strategy.¹³

The availability of ribozyme gene transfer technologies to biologically down-regulate expression of specific genes coupled with efficient gene delivery vehicles provides the means to evaluate the concept of anticancer strategies to block expression of potentially rate-limiting tumor promoting factors such as HER-2/neu or PTN. Hammerhead-ribozymes can be targeted to destroy specific mRNA transcripts by binding to and cleaving specific ribonucleotide sequences, which offers a potential over other antisense-based technologies.¹⁴ Such ribozymes targeted to specific oncogenes have been used successfully to down-regulate oncogene expression in both *in vitro* and *in vivo* models.¹⁵⁻¹⁹ To deliver ribozyme expression constructs efficiently into tumor cells *in vivo*, adenovirus vectors seem well suited as an alternative to plasmid-based gene transfer technologies. Adenovirus vectors provide highly efficient gene transfer and expression *in vitro* and *in vivo*, can be easily produced in high titers and have a broad host range. Furthermore, adenovirus vectors have been used for the efficient delivery and expression of ribozymes into tumor cells in culture^{16,20} and mouse liver cells *in vivo*.²¹

In this context, we used adenovirus-mediated ribozyme gene targeting to evaluate the potential utility of abrogating HER-2/neu or PTN gene expression as an

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anticancer strategy. Adenoviruses expressing a hammerhead ribozyme for either HER-2/neu or PTN were constructed and evaluated in four separate human cancer cell lines. Each vector led to high-level gene transfer and hammerhead ribozyme expression and to significant depletion of the respective gene products. Importantly, inhibition of either HER-2/neu or PTN gene expression in tumor cells abrogated the proliferative effects of these proteins, thus demonstrating the feasibility of these therapeutic approaches.

Results

Recombinant adenovirus-mediated ribozyme expression in human cancer cells

The overall structure of the recombinant adenovirus used in these experiments is depicted in Figure 1a. Ribozyme expressing vectors were derived from Ad-dl327 by homologous recombination as described in the Materials and methods. The deletion within the E1-region resulted in a replication-defective virus and further deletion of the E3 gene region provided space for the incorporation of transgenes. Ribozymes are expressed under the control of a viral CMV promoter and contain a SV40 polyadenylation signal to enable processing and increase transcript stability in eukaryotic cells. The HER-2/neu ribozyme (RzHER) targets the HER-2/neu mRNA 1991 nucleotides (nt) downstream of the translation initiation site. HER-2/neu-specific antisense flanking regions of 9 nt and 8 nt on the 5' and 3' ends of the ribozyme were positioned around a minimized catalytic hammerhead ribozyme core of 22 nt, a sequence which we have used successfully in previous studies.²² The sequence of the target region and its relative position in the HER-2/neu mRNA are shown in Figure 1b. The specificity of this HER-2/neu ribozyme construct was demonstrated in *in vitro* cleavage assays as well as in transient cotransfection studies in NIH/3T3 cells using the pRc/CMV plasmid as a ribozyme expression vector. A catalytically inactive mutant ribozyme and an unspecific ribozyme were used as controls in those experiments and had no effects

(unpublished data). The PTN ribozyme (RzPTN) cleaves the PTN mRNA 66 nt downstream of the translation initiation site and contains PTN-specific antisense flanking regions of 12 nt and 11 nt at the 5' and 3' ends, respectively. The specificity and efficacy of the RzPTN construct was previously demonstrated in *in vitro* cleavage assays,²² in transient cotransfection studies in SW-13 cells,²² in two human melanoma cell lines stably expressing the ribozyme in cell culture,^{13,22} and finally, in *in vivo* experiments in nude mice.¹³ In addition, we used two recombinant adenoviruses expressing either β -galactosidase (Av1LacZ4) or luciferase (Av1Luc) as controls in some of the experiments.

Recombinant adenoviruses expressing ribozymes were generated by a homologous recombination method.²³ They were plaque-purified, amplified and titrated in 293 cells as described under Materials and methods. Correct transgene integration and genomic organization of the recombinant adenoviruses was confirmed by Southern analysis (data not shown). A PCR assay for E1a with a sensitivity to detect contaminations of one copy of wild-type adenovirus in 10^7 copies of recombinant adenovirus was used to assure the absence of replication competent wild-type adenovirus (data not shown).

In a first set of experiments we compared the levels of ribozyme expression in human cancer cells after adenoviral infection to expression levels from plasmid-expression vectors stably transfected into the same cell lines. Human SW-13 adrenal carcinoma cells, human 1205 melanoma and human U87 glioblastoma cells were infected with either Av-RzPTN or Av-RzHER at MOI of 100 and total RNA was harvested 1 and 3 days after the infection. Northern analysis revealed very high expression levels of both ribozymes in SW-13 and U87 cells (Figure 2a). Ribozyme expression was easily detectable after 1 day and increased significantly after 3 days. The 50 nt difference in transcript size between the PTN and HER-ribozymes is due to a slightly modified cloning procedure. Both ribozymes were also expressed in 1205 melanoma cells although at somewhat lower levels. This was in stark contrast to the expression levels in SW-13

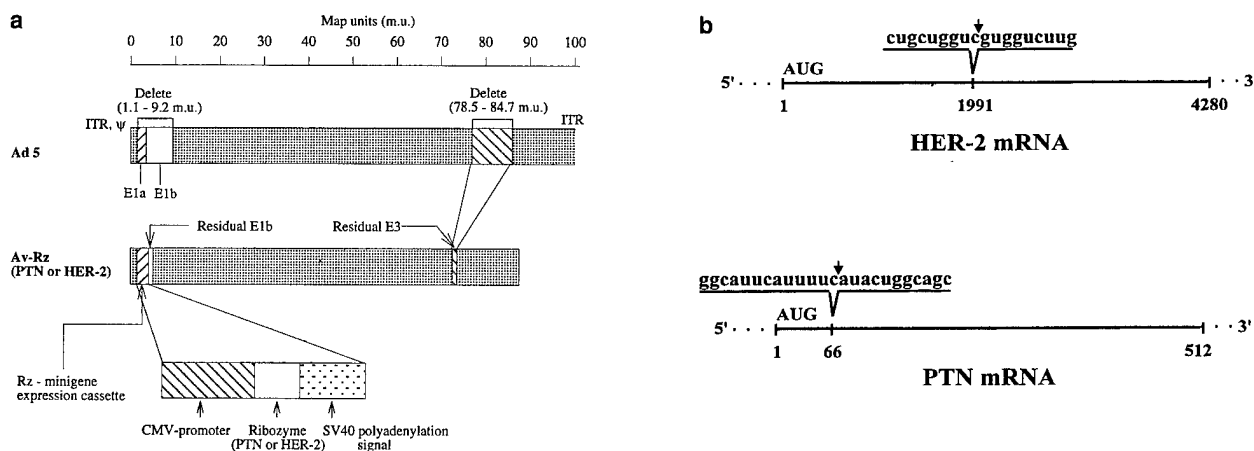


Figure 1 Genomic organization of the recombinant adenovirus ribozyme expression vectors. (a) The overall structure and functional elements of the ribozyme expression vectors, which were derived from Ad-dl327 DNA by homologous recombination. The Ad5 genome is shown for comparison along with a scale for reference (one map unit (m.u.) equals 360 bp). The HER-2/neu and PTN ribozyme minigene expression cassettes are approximately 2.6 m.u. long and consist of a CMV promoter, the ribozyme sequence and a SV40 polyadenylation signal. (b) The target sequences of the HER-2/neu and PTN ribozymes and their positions in the respective mRNAs relative to the translation initiation sites.

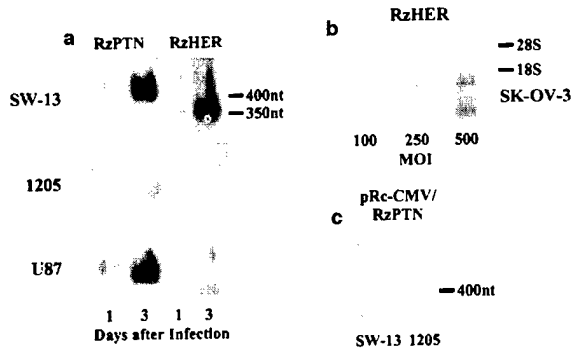


Figure 2 Expression levels of the transduced HER-2/neu and PTN ribozymes in different cell lines. (a) Human adrenal carcinoma cells (SW-13), human melanoma cells (1205) and human glioblastoma cells (U87) were infected with Av-RzHER or Av-RzPTN at an MOI of 100. Total RNA was harvested 1 and 3 days after the infection and 20 μ g were analyzed for ribozyme expression by Northern analysis. The positions of the 350 nt HER ribozyme and 400 nt PTN ribozyme transcripts are indicated. The Northern blot was exposed to film for 6 h. (b) Human ovarian carcinoma cells (SK-OV-3) were infected with Av-RzHER at increasing MOI and HER ribozyme expression was assessed by Northern analysis. The positions of 18S and 28S ribosomal RNA are shown. (c) Northern analysis of PTN ribozyme expression in SW-13 and 1205 cells which had been stably transfected with PTN ribozyme expression plasmids. The Northern blot was exposed to film for 3 days.

and 1205 cells stably transfected with RzPTN expression plasmids. Ribozyme transcripts could not be detected in 1205 cells and only at very low levels in SW-13 cells in a Northern blot that was exposed to film for 3 days (Figure 2c; versus 6 h in Figure 2a).

In a more detailed time course experiment in human MDA-MB-361 breast cancer cells, ribozyme expression peaked 3 days after adenoviral infection, decreased rapidly after day 5 and was not detectable by Northern analysis after day 10 (data not shown).

In order to evaluate the influence of different MOI on ribozyme expression, we examined RzHER levels in human SK-OV-3 ovarian cancer cells 3 days after the infection with Av-RzHER at an MOI from 100 to 500. As shown in Figure 2b, an increase in the MOI from 100 to 500 was followed by a linear, approximately five-fold increase in RzHER expression. To determine the optimum nontoxic MOI for the subsequent experiments we infected the cell lines with the β -galactosidase expressing Av1LacZ4 at different MOI and stained for β -galactosidase expression 48 h after the infection (data not shown). Since in all cell lines 100% of the cells stained positive at a nontoxic MOI of 100, this virus titer was used in the following experiments, unless indicated otherwise.

Adenovirus-mediated transduction of HER-2/neu ribozymes down-regulates HER-2/neu expression and inhibits HER-2/neu-mediated proliferation

To examine whether a transient adenovirus-mediated transduction of HER-2/neu ribozymes was sufficient to inhibit HER-2/neu expression in cancer cells, we infected SK-OV-3 cells with Av-RzHER or Av-RzPTN as control virus at MOI of 100. Total RNA was harvested 1 and 3 days after the infection and HER-2/neu mRNA levels were quantified by Northern analysis (Figure 3a). The Av-RzHER-specific ribozyme infection led to a specific

and significant depletion of HER-2/neu mRNA by approximately 75% 1 day after the infection when compared with the Av-RzPTN control infection.

In order to evaluate further the specificity and efficacy of the Av-RzHER vector, human MDA-MB-361 breast cancer cells were infected with Av-RzHER and Av1LacZ4 as control at MOI of 100. HER-2/neu protein levels were determined by FACS analysis over a period of 7 days after adenoviral infection and the mean fluorescence intensities are shown in Figure 3b. Infection with Av-RzHER led to a significant reduction of HER-2/neu protein expression compared to the control virus that had no effect on HER-2/neu levels throughout the entire experiment. HER-2/neu levels were reduced 3 days after infection and remained low until day 5, at which time the levels started to increase again. This time course correlated with the ribozyme expression levels described above.

In the next experiment, we addressed the biological significance of an Av-RzHER-mediated HER-2/neu depletion. To that end we used 32D mouse hematopoietic progenitor cells which do not endogenously express any member of the EGF receptor family and require exogenously added interleukin-3 (IL-3) for continuous proliferation in tissue culture.²⁴ We obtained 32D cells which were stably transfected with HER-2/neu and HER-3 expression plasmids (32D/H2+3). In contrast to the parental cell line, these cells survive in the absence of IL-3 when recombinant heregulin, the ligand for HER-3, is added to the media. Since only the HER-2/neu/HER-3 heterodimer becomes an active signal transducing receptor upon ligand binding,²⁵ we speculated that down-regulation of HER-2/neu would abrogate the heregulin-mediated cell survival in these cells. To test this hypothesis 32D/H2+3 cells were infected with either Av-RzHER or Av1LacZ4 and cell survival was compared with uninfected control cells. As expected, withdrawal of IL-3 led to almost complete cell death in control and infected cells (Figure 3c, lanes 4–6), whereas the cells proliferated at comparable levels in the presence of IL-3 (lanes 1–3). Heregulin promoted cell survival in the absence of IL-3 in approximately 40% of the noninfected (lane 7) and Av1LacZ4 control cells (lane 8). In contrast, heregulin was not sufficient to prevent cell death significantly in the Av-RzHER infected cells (lane 9). These results proved that the HER-2/neu ribozymes were effective in ablating the heregulin-induced cell survival. Furthermore, they showed that only the HER-2/neu/HER-3 heterodimeric complex mediates heregulin signal transduction, and that HER-2/neu is the limiting factor for the mitogenic response in these cells.

Efficacy of adenovirus-mediated PTN ribozyme expression

The specificity and efficacy of this particular PTN ribozyme was characterized earlier at the mRNA and protein level in various cell lines *in vitro* and *in vivo*.^{13,22} Here, we directly assessed the efficacy of adenovirus-mediated transduction of PTN ribozymes in a bioassay. As a model we used SW-13/PTN human adrenal carcinoma cells, which were stably transfected with a PTN expression plasmid.²⁶ In contrast to parental SW-13 cells, which do not express PTN endogenously and are not clonogenic in soft agar, SW-13/PTN cells form colonies spontaneously at a high rate.²⁶ Consequently, depletion

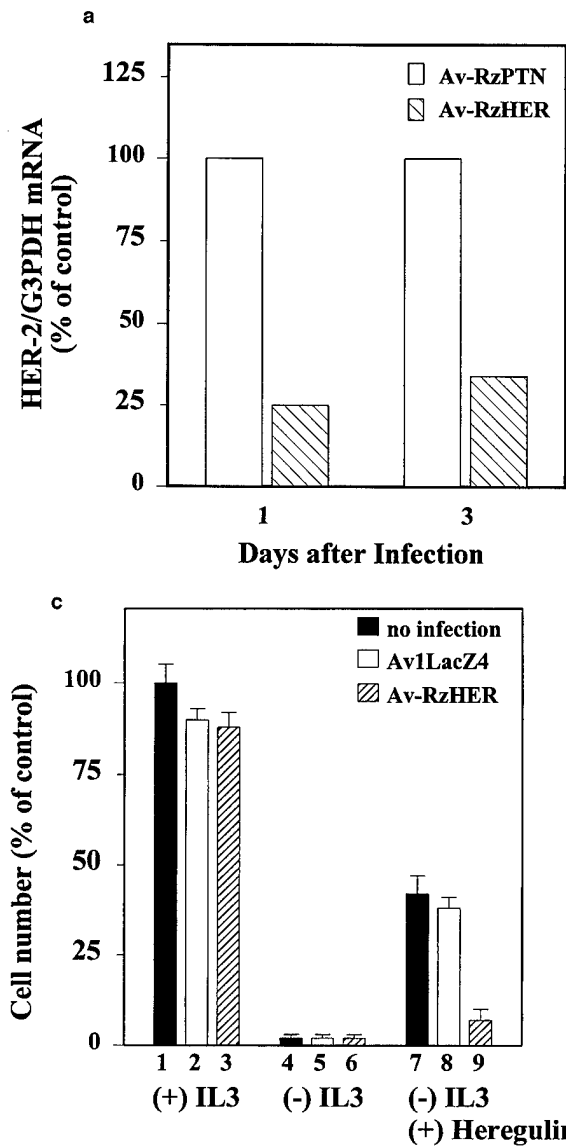


Figure 3 Av-RzHER infection efficiently down-regulates HER-2/neu expression. (a) Human ovarian carcinoma cells (SK-OV-3) were infected with Av-RzHER or Av-RzPTN at an MOI of 100 and total RNA was harvested 1 and 3 days after infection. HER-2/neu mRNA was quantified relative to G3PDH expression by Northern analysis followed by densitometry. (b) Human breast cancer cells (MDA-MB-361) were infected with Av-RzHER or Av1LacZ4 at an MOI of 100 and HER-2/neu levels were measured by FACS analysis at days 0, 1, 3, 5 and 7. (c) Mouse hematopoietic stem cells (32D/H2+3) which had been stably transfected with expression plasmids for human HER-2/neu and HER-3 receptors were infected with Av-RzHER or Av1LacZ4 at an MOI of 100. Noninfected cells served as an additional negative control. Cells were grown in the presence of interleukin-3 (IL-3; lanes 1–3), without IL-3 (lanes 4–6) or in the presence of heregulin at 10^{-9} M without IL-3 (lanes 7–9) and counted manually.

of PTN expression by adenovirus-mediated ribozyme targeting should reverse colony formation in these cells. SW13/PTN cells were infected with Av-RzPTN or Av1LacZ4 at an MOI from 1 to 100, plated in soft agar and colony formation was measured after a 10-day incubation period. Av-RzPTN infection at an MOI of 25 led to a dramatic reduction in colony formation by >85% compared with the control infection (Figure 4).

Discussion

The significance of angiogenic factors for the growth and metastasis of solid tumors has gained wide acceptance.³ Using ribozyme targeting, we recently demonstrated that pleiotrophin is a limiting factor in melanoma angiogenesis and metastasis.¹³ HER-2/neu overexpression is commonly found in human adenocarcinoma derived from tissues such as the lung, breast and ovaries. However, the biological role of HER-2/neu in tumorigenesis remains somewhat unclear. To address this question, we applied a plasmid-based ribozyme targeting strategy and success-

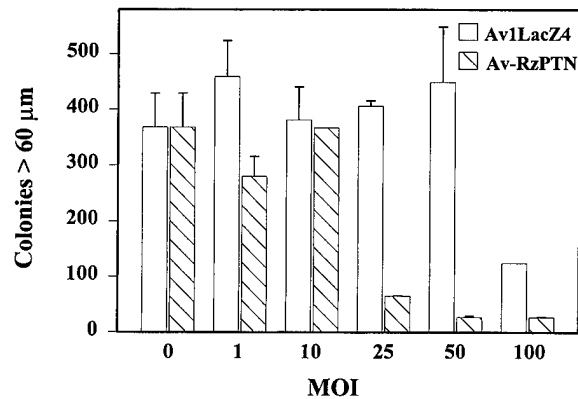


Figure 4 Av-RzPTN infection reduces growth of SW-13/PTN cells in soft agar. SW-13/PTN cells which had been stably transfected with an expression plasmid for PTN were infected with Av-RzPTN or Av1LacZ4 at increasing MOI from 1 to 100. Infected cells were plated in 35-mm soft agar dishes at 2×10^4 cells per dish and colonies >60 μm were counted after a 10-day incubation period.

fully depleted HER-2/neu levels in ovarian cancer cells, which reversed tumorigenesis in these cells (unpublished data). These results suggested that ribozyme targeting can potentially be used to achieve two important goals: (1) as diagnostic tools to elucidate the relevance of a gene product; and (2) as new adjuvant reagents in human cancer gene therapy.

In order to develop an anticancer gene therapy with ribozymes targeted against either HER-2/neu or PTN we needed to generate an efficient gene delivery vector. To that end, we selected adenovirus, which has been extensively used to obtain high levels of transduction and gene expression in cultured cells and animals.^{27,28}

In this study, we combined ribozyme gene-targeting technology with adenovirus-mediated gene delivery and were able to demonstrate a specific and efficacious down-regulation of HER-2/neu and PTN in a variety of human cancer cells. We used our previously reported PTN hammerhead-ribozyme design and applied that to generate ribozymes targeted to the HER-2/neu mRNA. The construction and testing of those plasmid-based expression vectors will be described in detail elsewhere. One of the disadvantages of our plasmid-based expression vectors is low ribozyme transcript levels. We were unable to detect ribozyme transcripts by quantitative methods such as Northern analysis or ribonuclease protection assays after transient or stable transfections. Thus it was impossible to correlate effects (or the lack thereof) to ribozyme expression levels. Fortunately, this does not appear to be the case when the adenovirus system was used. Adenovirus-mediated transduction produced high steady-state levels of ribozyme expression in four different human cancer cell lines (see Figure 2). This increase in ribozyme production is most likely due to the higher copy number of template using adenovirus as compared with stably integrated plasmid-derived transcription units, since the same ribozyme expression cassette (promoter and transcription unit) was used. The fact that we observed a viral dose-dependent inhibition of soft agar colony formation in SW13/PTN cells (see Figure 4) suggests a correlation between ribozyme expression levels and effects. The differences in expression in different cell lines are probably due to different CMV-promoter activities in the cell lines, since control infections with a β -gal expressing virus had demonstrated, that at an MOI of 100, all cells were infected and expressed similar levels of β -galactosidase activity (data not shown).

Adenovirus-mediated transduction of either HER-2/neu or PTN ribozymes yielded not only high expression levels but also led to a specific and very efficient depletion of the respective gene products in two different cell lines (see Figure 3a and b). Although the contribution of antisense effects can not be completely ruled out, it seems likely that ribozyme-mediated cleavage of the target RNA is the main mode of action, since plasmid-delivered catalytically inactive mutant ribozymes had no effects on protein expression.²² In SK-OV-3 cells, HER-2/neu mRNA levels were reduced by approximately 75% only 1 day after the infection. This suggests that low ribozyme levels can be sufficient to obtain maximal effects, taking into consideration that the peak of ribozyme expression occurs around 3 days after the infection. In addition, the ribozyme effects may have been underestimated considering the fact that the HER-

2/neu and PTN substrate mRNAs are expressed at very high levels in these cell lines.

A recently published study found that ribozymes targeted against human growth hormone had significant effects after adenovirus-mediated transduction *in vivo* in mice, and that the time course of ribozyme expression was correlated with the inhibition of hGH expression in the liver.²¹ On the other hand, we have recently obtained data from plasmid-based vectors suggesting that ribozyme expression levels do not correlate very well with their efficacy (unpublished data). It seems possible that other factors, such as target selection or ribozyme design and expression in the proper cellular compartment(s) have a much greater influence on their efficacy than ribozyme expression levels. These results could potentially be important if sufficient ribozyme levels can be achieved at lower virus titers which in turn would reduce nonspecific viral toxicity in normal cells.

One of the potential problems of a replication-defective adenovirus vector is the transient transgene expression. In rapidly proliferating MDA-MB-361 cells ribozyme-mediated inhibition of HER-2/neu protein expression started to decrease 5 to 7 days after the infection (see Figure 3b). This transient expression would mandate a need for repetitive virus administrations, which is problematic due to the strong immunogenicity of the currently available adenovirus vectors. On the other hand, a transient but very efficient depletion of crucial rate-limiting gene products such as demonstrated here (see Figures 3c and 4), could already be sufficient to alter the balance between cell proliferation and cell death and could initiate tumor regression *in vivo*.

Poor tissue penetration is one of the major obstacles in using replication-deficient adenovirus vectors in cancer gene therapy. Successful approaches rely on the development of *in vivo* tumor models, which either allow targeting of a high percentage of the tumor cells, or show efficacy already at low transduction rates. In two such models replication-deficient adenovirus vectors have been used successfully to inhibit the intracranial growth of experimental gliomas,²⁹ as well as the intraperitoneal growth of human lung cancer cells in nude mice.³⁰

We recently reported that the expression of pleiotrophin is rate-limiting for the *in vivo* growth and metastasis of human melanoma cells.¹³ Furthermore, our data indicated that targeting as few as 25% of all tumor cells was already sufficient to significantly delay tumor growth. These findings provide the experimental basis for an attractive *in vivo* model to test the antitumor/ antimetastatic efficacy of the Av-RzPTN ribozyme expressing vectors.

In conclusion, our results demonstrate that adenovirus-mediated ribozyme expression is a very efficient tool to abrogate the expression of oncogenes and growth factors in cancer cells. The efficacy of these adenoviruses in *in vivo* tumor models remains to be evaluated.

Materials and methods

Cell culture, viruses and plasmids

Human adrenal carcinoma (SW-13), glioblastoma (U87), ovarian cancer (SK-OV-3) and embryonic kidney epithelial (293) cells were obtained from American Type Culture Collection (ATCC) and were grown as adherent

cells in IMEM (Life Technologies, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS; Life Technologies); human melanoma cells (1205LU; gift from M Herlyn, Wistar Institute, Philadelphia, PA, USA) were maintained in KSFM/L15 media (Life Technologies) mixed at a ratio of 3:1 and supplemented with 5% FBS. 32D mouse hematopoietic stem cells which were stably cotransfected with HER-2/neu and HER-3 expression plasmids (32D/H2+3; gift from J Pierce, NCI, NIH, Rockville, MD, USA) were grown in IMEM with 10% FBS. Recombinant adenoviruses expressing either β -galactosidase (Av1LacZ4) or luciferase (Av1Luc) as well as Ad-dl327 virus DNA and PAVS6A plasmid DNA which were used for the subsequent adenovirus (Av)-vector constructions were described earlier.^{27,31}

Construction of recombinant adenoviral vectors

In order to generate a recombinant adenovirus that expressed hammerhead-ribozymes (Rz) targeted to pleiotrophin (PTN), we used the Rz minigene expression cassette from the pRc/CMV-Rz66 expression plasmid.²² The expression cassette was cut out as a *NruI*-*XbaI* fragment (826 nucleotides (nt)) and blunt-end ligated into the *EcoRV* side of the PAVS6A Av-shuttle plasmid. The generation of the eukaryotic expression plasmid that contains the Ribozyme targeted to HER-2/neu will be described in detail elsewhere. In brief, the following Rz-coding sense and antisense oligonucleotides (sense: 5'-agcttcaagaccactgatgagtccttaggacgaaaccagcaga-3'; antisense: 5'-agcttctgctggttctcctaaccgactcatcagggtggtcttga-3') were annealed together and ligated into the *HindIII* site of the pRc/CMV plasmid (Invitrogen, San Diego, CA, USA). This ribozyme contains HER-2/neu specific antisense flanking regions of 9 nt and 8 nt on the 5' and 3' ends of the 22 nt catalytic Rz-core, that target the Rz to its cleavage site 1991 nt downstream of the translation initiation site in the HER-2/neu mRNA (GenBank accession No. M11730). The RzHER minigene expression cassette was excised as a *NruI*-*EcoRV* fragment (778 nt) and ligated into the *EcoRV* site of PAVS6A. The correct insertion of the PTN and HER-2/neu expression cassettes into PAVS6A was verified by DNA sequencing.

Recombinant replication-deficient adenoviral vectors were constructed by a homologous recombination method²³ using PAVS6A-Rz shuttle plasmids and the 35 kb *Clal* fragment of Ad-dl327. 293 Cells were cotransfected with the respective PAVS6A plasmid and virus DNA and individual plaques containing recombinant virus were purified and further amplified in 293 cells as described.³¹ Recombinant adenoviral DNA was analyzed by restriction enzyme digestions with *EcoRI*, *NdeI* and *XbaI* followed by Southern analysis using radiolabeled oligonucleotides specific for RzPTN or RzHER as probes. Adenoviruses with the correct restriction pattern were produced in large amounts and purified in a two-step CsCl ultracentrifugation procedure.³¹ Viral titers were obtained by plaque forming assays in 293 cells and high titer virus stocks were divided into aliquots and stored at -80°C.³¹ To rule out minor contaminations with replication-competent wild-type adenovirus, PCR reactions with E1-specific primer pairs were performed using 1 μ g of purified recombinant AvRz-HER-2/neu or AvRz-PTN DNA as templates and 0.1 pg of purified Ad-dl327 DNA as positive control (sensitivity 1 in 10⁷).

Adenoviral infections of cell lines

If not indicated otherwise, cells were grown until they reached a confluency of 50 to 70%. They were then infected with the different recombinant adenoviruses at various MOI in infection medium (IMEM plus 2% FBS) for 2 h with rocking. After that, infection medium was replaced with normal growth medium and cells were used in the experiments as indicated.

Northern analysis

Total cellular RNA was isolated with the RNA STAT-60 method (Tel-Test, Friedenswood, TX, USA), separated and blotted as described.²⁶ A HER-2/neu cDNA probe (1.5 kb *EcoRI* fragment) was hybridized, washed and exposed to film for 16 h.²⁶ To correct for variability in loading, blots were stripped and reprobed with a glyceraldehyde-3 phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA, USA) cDNA probe. Relative band intensities were measured by densitometry. PTN or HER-2/neu ribozyme transcripts were detected with γ -³²P-T4-kinase labeled oligonucleotides (45 nt long) as probes. Hybridization and washing conditions were modified as follows: the hybridization buffer contained only 25% formamide and the final washing step was done at 50°C, 0.5 \times SSC/0.1% SDS for 30 min.

Fluorescence activated cell sorting (FACS)

To quantify HER-2/neu protein levels by FACS analysis, cells were trypsinized, washed once with serum containing growth medium, twice with PBS (Sigma, St Louis, MO, USA) and resuspended in PBS at 5 \times 10⁵ cells/100 μ l. The cells were incubated for 30 min at 4°C with 1:100 dilution of a primary antihuman HER-2/neu mouse monoclonal antibody (clone 9G6.10; Neomarkers, Fremont, CA, USA). Cells were washed twice with PBS and incubated for 30 min at 4°C in the dark with a 1:200 diluted FITC-labeled goat anti-mouse secondary antibody (Boehringer, Mannheim, Germany). After two final washes with PBS the mean value of fluorescence intensity of 10 000 cells was determined by FACS (FACStar plus; Becton Dickinson, San Jose, CA, USA). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls.

Growth assays

SW-13/PTN cells²⁶ were infected with Av-RzPTN or Av-RzHER at MOI of 100 for 2 h. Infected cells were then trypsinized and 2 \times 10⁴ cells were plated in 35 mm soft agar dishes in triplicates. Soft agar colony formation was evaluated after a 10-day incubation period as described previously.²⁶ 32D cell proliferation/survival assay was performed as reported.³² In brief, 32D/H2+3 cells were plated overnight at 50–70% confluence in 12-well plates and then infected with Av-RzHER or Av1LacZ4 at MOI of 100 for 2 h. Infected or noninfected cells were then grown for 48 h in the absence or presence of interleukin-3, or in the presence of recombinant heregulin (Neomarkers) at 10⁻⁹ M without addition of IL-3. Proliferation was then assessed by counting surviving cells manually using a hemocytometer.

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