Heregulin is sufficient for the promotion of tumorigenicity and metastasis of breast cancer cells *in vivo*.

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ABSTRACT:

The molecular mechanisms of breast cancer tumor progression from an estrogen (E2)-dependent to an E2-independent phenotype are not understood. Heregulin (HRG) binds to the *erbB* family of receptors, and induces mammary gland transformation in transgenic mice in the presence of hormonal stimulation. However, the importance of the E2-independence that is a critical event during breast cancer progression has not previously been addressed. In this study we investigated the *in vivo* role of HRG in this progression. We demonstrated that in ovariectomized nude mice HRG induced E2-independence and antiestrogen-resistance, promotes metastasis and preneoplastic transformation of the adjacent mouse mammary tissue. We show that one of the mechanisms by which HRG achieves the aggressive phenotype is mediated via an increase in activated MAPK, an increase in a matrix-degrading enzyme, MMP9, and the overexpression of VEGF. Our data provide new insights into the mechanisms of breast cancer progression *in vivo*, and reinforce the important role that HRG plays in this process.

INTRODUCTION:

About 60% of the human breast carcinomas express estrogen receptor (ER) and are characterized by a better prognosis and response to endocrine treatment [1]. Unfortunately, at some point most of the initially responsive patients will fail the endocrine treatment and will develop more aggressive tumors. ER expression is important in predicting the response to adjuvant hormone therapy, although its role as a prognostic indicator is not clear [2].

The growth and progression of breast carcinomas are regulated by a milieu of signals mediated by growth factors and steroid receptors. The more invasive phenotype of breast cancer has been correlated with upregulation of *erbB2*, a poor prognosis marker. Several studies have suggested a negative correlation between the *erbB2* and the E2 pathways. E2 inhibits *erbB2* overexpression and tamoxifen (Tam) upregulates it in ER-positive (ER+) human breast cancer cells [3]. Clinical and experimental data suggest that co-expression of the *erbB2* oncoprotein in ER+ breast cancer cells confers resistance to endocrine therapy. In fact, the response rate to tamoxifen, shown to be around 50% in ER+ patients, is reduced to 17% in the presence of *erbB2* overexpression [4]. An interesting inverse correlation has also been found between overexpression of *erbB2* and loss of functional ER [5]. The decrease in the responsiveness of breast carcinomas to E2 has been linked to the activation of the *erbB2* receptor via overexpression or via binding of Heregulin (HRG) to its receptors [6]. HRG (initially called gp30) [7] and its rat homologous Neu

Differentiation Factor (NDF) [8] are growth factors able to activate *erbB2* indirectly through their binding to *erbB3* and/or *erbB4* [9]. HRG antagonizes the E2-mediated downregulation of *erbB2* and is capable of enhancing the Tam-induced stimulation of the receptor [6]. Recent *in vitro* data from our laboratory have shown the loss of E2-dependence and the acquisition of Tam-resistance following the transfection of HRG-β2 cDNA into MCF-7 cells, an E2-dependent breast cancer cells [10]. Treatment of breast cancer cells with HRG on the background of high *erbB2* expression results in ER phosphorylation and upregulation of progesterone receptor (PR) expression [11]. This acquired phenotype depicts the passage from an initial hormonal responsive and antiestrogen-sensitive tumor cell to a later step in carcinogenesis, resembling the common progress of many human breast tumors. The molecular mechanism for this process is not entirely understood. Recent data suggest that HRG mediates active repression of E2 response elements via upregulation of metastasis-associated protein 1. Physical interaction between MTA-1, the activation factor domain of ERα, and histone-deacetylase 1 and 2 has been reported, implicating MTA-1 as a repressor of gene expression and a downstream effector of HRG [12].

The angiogenesis process is an essential requirement for tumor progression and for the survival of solid tumors [13]. Growth factors known to mediate this process are the vascular endothelial growth factor (VEGF) family. Overexpression of VEGF has been detected in tumor specimens and correlated with an advanced invasive phenotype. The expression of these factors is upregulated via *erbB2* activation and the *ras* pathway [14; 15]. Recent data from *in vitro* studies

have implicated HRG in the angiogenesis and invasion processes. HRG promoted an invasive phenotype of breast cancer cells *in vitro* [10], and upon treatment of breast cancer cells with HRG, upregulation of VEGF at the mRNA and protein levels was observed. This effect was blocked by the anti-*erbB2* receptor monoclonal antibody Herceptin and was dependent upon functional p21-activated kinase-1 [16]. An interesting finding was that this upregulation could occur independently of *erbB2* expression, suggesting an alternative mechanism for the HRG-induced increased levels of VEGF [17].

Reduced cell adhesion, increased migration, and increased secretion of both soluble and membrane-associated degrading proteinases are invasion-associated processes influenced by oncogenes, growth factors, steroid hormones, and the extracellular matrix (ECM) [18; 19]. MMPs are a family of more than eleven zinc-dependent endopeptidases [20] that increase invasiveness [21]. One such enzyme is MMP-9 or Gelatinase B, which is highly expressed in many human malignancies including breast carcinomas [22]. Metastasis induced by activated *ras*-transformed cells has been correlated with MMP-9 release [23; 24]. ProMMP-9 is upregulated in breast tumors as compared to non-cancerous tissue [25].

In this study we demonstrate that HRG is a tumor-promoting factor using a unique *in vivo* model system of breast cancer tumor progression. HRG promotes the *in vivo* progression, from an E2-dependent, antiestrogen-sensitive and non-metastatic phenotype to the E2-independent,

antiestrogen-resistant, and metastatic phenotype. We show that the possible secretion of HRG from the localized tumors induces the preneoplastic transformation of the mouse mammary gland. Our *in vivo* model offers an interesting and useful insight into the understanding of possible factors implicated in breast cancer progression and resistance to hormonal therapy. Our pathological findings also support the effect of secreted HRG itself on the surrounding tissue. This is the first time shown that constitutive expression of HRG, as opposed to cells treated with HRG; regulates the expression of VEGF *in vivo*. These findings are of great relevance to the human disease, where upregulation of VEGF has been positively correlated with the progression of breast carcinomas. We also demonstrate that the mechanism of HRG action in promoting the progression is mediated, at least in part, through the increase in mitogen activated protein kinase (MAPK) phosphorylation, implicating the *ras*-signaling pathway, and a resulting increase in activated MMP-9.

RESULTS AND DISCUSSION:

HRGβ2 induces E2-independent and antiestrogen-resistant tumors in ovariectomized athymic nude mice. The cells MCF-7 (parental), MCF-7/V (vector), and MCF-7/HRG (HRG transfected) were inoculated in the mammary fat pad of 3- to 4-week old ovariectomized athymic nude mice. Tumors formed spontaneously only in mice injected with MCF-7/HRG cells. These tumors grew independently of the E2 stimulation and were resistant to antiestrogen treatments

(Tam and ICI 172, 852) (Fig 1). In contrast, when mice were inoculated under the same conditions with MCF-7/V or the parental MCF-7 cells, the tumors appeared exclusively in the presence of E2 supplementation and in the presence of antiestrogens significantly reduce (Table 1). Interestingly, the MCF-7/HRG tumors generated in the presence of Tam were slightly larger. The data demonstrate that transfection of MCF-7 cells with HRG promotes tumor growth *in vivo* in the absence of E2 stimulation. These findings support the *in vitro* data from our laboratory, where HRG-transfected cells had a growth advantage when grown in E2-depleted medium [26]. These results are analogous to the human disease, where, in the progression process, breast carcinomas acquire antiestrogen resistance.

Our data demonstrate that HRG expression is sufficient for the progression to an E2-independent and antiestrogen resistant state, supplementing our previous results where HRG not only blocked E2 action but also ER-function [26; 27]. It is known that many breast cancer patients develop ER+ tumors that initially respond to antiestrogen treatment, but during the course of the treatment the tumors acquire antiestrogen-resistance. Although the tumors still express ER, these tumors do not respond to antiestrogen treatments. Our *in vivo* model mimics this exact scenario and shows that in the HRG-derived tumors a very similar process occurs. To the best of our knowledge, this is the first report where the upregulation of a single growth factor, such as HRG, is shown to promote such effects *in vivo*.

Anatomical pathology of the HRG-derived tumors in ovariectomized athymic nude mice: We performed both macroscopic and microscopic analysis of all derived tumors.

Macroscopic and microscopic analysis: The majority of the tumors generated by the MCF-7/HRG cells presented as firm, poorly defined masses, with extensive, centrally located areas of necrosis. The lesions measured about 2 cm in greatest dimension. In several cases, in the MCF-7/HRG derived tumors ribs involvement and fixation to the underlying soft tissue could be observed, as well as erosion of the overlying skin. In the same group, enlargement of axillary lymph nodes was detected in many cases, whereas no macroscopic metastatic foci to visceral organs were noticed. The MCF-7/HRG transfected cell group exhibited tightly cohesive areas of large, pleiomorphic cells with irregular nuclei, and numerous mitotic figures characterized the tumors. Multinucleated cells were observed. Irregular gland formation and well-formed central lumina were present. Neovascular formation and vast areas of necrotic tissue were observed. Infiltration of the adipose tissue and muscle was seen as were perineural invasion and ribs involvement. Neoplastic emboli were observed in both lymphatic vessels and blood. In a few cases lymphoplasmacytic infiltrate was present at the periphery of the tumoral masses. Tumors derived from MCF-7/HRG showed a mixture of solid, trabecular and tubular patterns. Irregular gland formation and occasional well-formed lumen were present. These features, as well as the heterogeneity and variety of histological patterns, resemble what is observed in the human mammary infiltrating ductal carcinoma termed “no special type A” (Fig 2A).

MCF-7/HRG-derived tumors metastasize to the axillary lymph node: In the majority of the cases, the metastasis to the lymph nodes was attributable to inflammatory response. However, in many mice, tumor deposits were seen in lymph node subcapsular sinuses and adjacent nodal tissue (Fig 2B). Lymph node metastases were observed only in nude mice inoculated with MCF-7/HRG cells (Fig 2C). These results demonstrate that HRG expression induces an E2-independent, antiestrogen-resistance, but also an aggressive metastatic phenotype. This observation is unique, since to the best of our knowledge no other known growth factor promotes metastasis of the MCF-7 cells, not even in the presence of estrogen. These data support the concept that HRG is able to mediate tumor promotion in more than one way. The metastatic potential of cancer cells is mediated through a variety of signals from growth factors stroma cells and from ECM receptors. HRG increases the invasive phenotype of MCF-7 cells and migration of these cells in Matrigel *in vitro*, and also in a recently developed three-dimensional model [10; 28]. However, this is the first report of increased metastatic ability of the tumor cells independently of any additional stimuli *in vivo*. TGFβ, which is another mitogenic factor for MCF-7 cells, was unable to increase the ability of MCF-7 cells to form tumors in athymic-ovariectomized mice in the absence of estradiol [29]. These reports strengthen the role of HRG not only in the proliferation of the cells but also in the metastatic process. Screening for the upregulation of HRG in breast tumors together with the currently available markers may provide a useful tool in the prediction of the beneficial effects that adjuvant therapy may provide. Our data provide critical support for

the fact that HRG overexpression could single out patients who may have a recurrence of the disease, who are thus at high risk for metastases.

HRG is highly expressed in all the MCF-7/HRG-derived tumors: To clearly demonstrate that the tumors developed from MCF-7/HRG cells expressed HRG, we performed *in situ* hybridization using a 22bp oligonucleotide corresponding to the antisense sequence of HRG. Control sense probe was used as a negative control. HRG expression was undetectable in the MCF-7/V tumors when using the antisense HRG oligonucleotide (Fig 3A). In contrast, the level of HRG expression in the tumors developed from MCF-7/HRG cells was extremely high when using the antisense oligonucleotide (Fig 3B). No expression of HRG was seen in either tumor when using the sense oligonucleotide (Fig 3C-D). Our data demonstrates that the expression of HRG is maintained *in vivo* and that the phenotypic changes are mediated through HRG action. Interestingly, the level of HRG expression in the MCF-7/HRG tumors was comparable to the levels of tumors derived from MDA-MB-231 cells that naturally express HRG (data not shown).

MCF-7/HRG cells induce *in vivo* preneoplastic transformation of the adjacent mammary gland: A fascinating finding *in vivo* was the presence of atypical mouse glands, presenting features appears reminiscent of ductal carcinoma *in situ* (Fig 4a: A-B). The appearance of the preneoplastic-transformed glands is completely distinct from the normal appearance of the glands as a thin one-cell layer of epithelial cells. Microscopic examination of the mammary

gland led us to consider that the malignant appearance is more likely attributable to the action of a paracrine factor and not to direct tumoral invasion. Since the preneoplastic transformation occurred only in the ovariectomized mice, *i.e.*, mice inoculated with the MCF-7/HRG cells, we concluded that the transformation of these tissues could be due only to the secreted HRG from the MCF-7/HRG tumors as well as possible downstream effectors of HRG that led to the transformation. Other studies substantiate the hypothesis; for example, in the studies from Leder and collaborators, which demonstrate that preneoplastic tumors were derived in HRG transgenic mice, where HRG was expressed under the control of the murine mammary tumor virus promoter (MMTV) promoter [30].

To rule out that the preneoplastic lesions were derived from the implanted MCF-7/HGR we prepared tissue sections and stained them with two human specific antibodies, an anti-epithelial mammary antigen (EMA) (Fig 4b: A-B), and an anti-keratin (KER) (Fig 4b: C-D). None of these antibodies recognize tissue of mouse origin. As can be seen, the neoplastic transformation of the mammary gland was of mouse origin. These findings are of extreme awareness, since this is the first report that an HRG-derived tumor promotes the preneoplastic transformation of the adjacent tissues in a paracrine fashion *in vivo*. The implications of these findings from a clinical point of view are of great importance placing HRG as a critical factor not only in breast cancer progression but also as a possible diagnostic and prognostic marker for the aggressiveness of the tumors and the devastating effects that the progression may have in patients in which an

upregulation of HRG expression is observed. It is also possible to postulate that the paracrine effects seen in the mammary gland are an effect of other secreted factors regulated by HRG, such as Cyr61 [32]. This is an ongoing area of investigation in our laboratory.

VEGF expression is upregulated in the MCF-7/HRG-derived tumors: Since the tumors demonstrated a clear increase in neovascularization, the tumors were stained for two known angiogenic factors: VEGF and the newly discovered angiogenic factor, Cyr61 [31]. The MCF-7/HRG tumors showed a great increase in VEGF as shown by immunohistochemistry staining with anti-VEGF antibodies (Fig 5A). These results were further confirmed by an ELISA assay, in which VEGF concentrations were measured in the conditioned media collected from MCF-7/HRG and MCF-7/V cells (Fig 5B). A 3- to 8-fold increase in VEGF expression was observed in the conditioned medium from HRG-transfected cells. This increase in the ability of the HRG-derived MCF-7 clones to secrete VEGF was in direct correlation with the levels of HRG expression (data not shown). We observed a substantial increase in Cyr61 expression in the MCF-7/HRG-derived tumors [32]. In this regard, other studies have also shown that VEGF can be unregulated in MCF-7 cells after exogenous treatment with HRG [17]. In addition, these studies have provided significant evidence that HRG mediates neovasculature in a chick embryo model system or in transgenic mice, where HRG expression was driven by the MMTV promoter [16; 17]. Our study is the first to demonstrate without any doubt that HRG regulates neovascularization of human breast cancer tumors *in vivo* in an autocrine manner. Moreover, our study provides direct

evidence that subsequent to the disregulation of HRG expression, upregulation of angiogenic factors promotes, at least in part, tumor progression, and neovascularization *in vivo***.**

MAPK is highly activated in the MCF-7/HRG cells. To understand the mechanism by which HRG promotes this aggressive *in vivo* phenotype, we examined the signaling pathways that could lead to such events. Previous studies *in vitro* support the notion that transcriptional upregulation of VEGF can be mediated by MAPK [33; 34]. In addition, the *ras* signaling pathway, and more precisely p42/p44 MAPK, are involved in the invasion process, and in upregulation of metalloproteinases as well as in the signals mediated by *erbB2* [35]. MCF-7 and MCF-7/HRG cells were assayed for MAPK activation and levels of expression. The constitutive levels of phosphorylated MAPK in MCF-7/HRG cells were at least three-fold higher than in the parental MCF-7 cells (Fig 6A). This increase was comparable to the increase obtained when the parental MCF-7 cells were exogenously treated with HRG (Fig 6A). To determine that the increase in MAPK activation was authentic, we determined the levels of MAPK protein that remained unchanged in all the cells tested (Fig 6B). Interestingly, an increase in constitutive *erbB* receptor activation was observed in the MCF-7/HRG cells (Fig 6C and [10]), implying that the phosphorylation of MAPK can be mediated via the autocrine stimulation of *erbB* without overexpression of *erbB2*, as seen in MCF-7 cells. Transformation through the *erbB2* oncogene has been proposed to involve both *ras* mediated and *ras* independent pathways [36]. Furthermore, we have shown that the Akt kinase, which is downstream of HRG, but not MAPK can be

inhibited by downregulation of Grb2, a protein associated with many tyrosine kinase receptors, including *erbB2* [37]. A recent study has shown upregulation of *ras* and MAPK in breast cancer tumor sections. This activation was achieved *in vitro* only after treatment of the cells with ligands to growth factor receptors [38]. Our model provides critical and clear evidence for the activation of MAPK via autocrine stimulation of *erbB* by HRG, suggesting that in tumors where HRG expression is upregulated, activation of MAPK can occur in the absence of other ligands.

Our finding demonstrating that MAPK is activated in these cells provides a new insight into the molecular events that may occur in an *in vivo* situation where resistance to antiestrogens is mediated by HRG via MAPK. Possible crosstalk between the E2 and the MAPK pathways is supported by studies in which ERα can be phosphorylated by MAPK. These studies however, were performed *in vitro;* they do not take into account the other stimuli such as the ECM, growth factors and cytokines, all of which may contribute to MAPK activation *in vivo*. These findings support the notion that the upregulation of HRG expression is a crucial event in the tumor progression *in vivo,* and also indicate that our *in vivo* model follows the course of the human disease most closely, where *ras* and MAPK are activated in the absence of *ras* mutations, and correlate with an aggressive and metastatic phenotype [38].

MCF-7/HRG cells secrete high levels of activated MMP-9. Since HRG induced an aggressive/metastatic behavior of MCF-7 cells, and MMP-9 is implicated in breast cancer

progression, we speculated that modulation of MMP-9 activity might be the mechanism through which HRG induces the aggressive phenotype of MCF-7 cells. Thus, conditioned media derived from MCF-7/HRG cells was analyzed by reversed zymography for expression of MMP activity. From our results, we concluded that the MCF-7/HRG cells produce significantly higher levels of MMP-9 activity (about 10-fold) as compared with control MCF-7/V cells (Fig 7). Furthermore, the MMP9 inhibitors R94138 (kindly provided by Dr. Kurakata at Sankyo, Japan) and GM6001 (Chemicon) blocked the ability of the MCF-7/HRG cells to invade through Matrigel using the Boyden Chamber assay. In addition, the inhibitors blocked the Matrigel outgrowth (data not shown). From our studies, we concluded that HRG expression associates with MMP-9 activity and correlates with the metastatic potential of a breast cancer cell. It was previously shown that metastatic potential induced by activated *ras* has been correlated with MMP-9 release [39]. MMP-9 expression was shown to be necessary for this process, since a ribozyme directed against MMP-9 completely abolished the ability of these cells to metastasize [40]. The possible mechanism by which HRG induces this aggressive phenotype is mediated by the alteration of the actin cytoskeleton morphology, by increased cellular motility, and by the invasion into the fibronectin-gelatin cross-linked matrix [7]. Now we have demonstrated that the phenotype is mediated via the modulation of MAPK and MMP9 activity.

In summary, our results provide a unique and faithful *in vivo* model for the progression of breast cancer, as well as possible mechanisms for the acquired E2-independence and antiestrogen-

resistance that is an enormous difficulty in the treatment of breast cancer. This model places HRG as a key role-player in the process, and in contrast with other studies it shows that the autocrine/paracrine effect of HRG is sufficient to promote both the E2-independence of the human derived cells *in vivo*, and full transformation of the mammary gland of mouse origin. Although similar studies have been done in HRG transgenic mice, these animals were not ovariectomized, and therefore the growth of the tumors was in the presence of E2. This is the first study to demonstrate that HRG promotes the progression of breast cancer cells *in vivo* from an E2-dependent to an E2-independent phenotype and that the mechanism by which this event is achieved is mediated via the increase in MAPK activation leading to increased expression of several angiogenic factors. Moreover, this is the first report to demonstrate that HRG promotes an *in vivo* metastatic phenotype, achieved through the increased expression of a motility factor, Cyr61 [32], and through increased activation of MMP-9. Further studies are currently underway to demonstrate that blockage of the individual signaling pathways induced by HRG could halt tumor progression and metastasis *in vivo*.

Figure Legends:

Table 1. HRG_2 induces E2-independent and antiestrogen resistant tumors in ovariectomized mice. MCF-7 cells were purchased from the American Type Culture Collection (ATCC). MCF-7 cells were transfected with HRG_2 cDNA as previously described [26]. Female athymic nude mice, 3-4 weeks old, were housed under specific pathogen-free conditions. Cell suspensions (1×10^6 cells) were inoculated in the mammary fat pad of ovariectomized NCr nu/nu athymic nude mice. Where indicated, mice received an estradiol pellet of 60-day release and/or Tam and ICI-172, 852 pellets at the time of the cell inoculation. Tumor growth was assessed by weekly examination.

Figure 1. Appearance of the MCF-7/HRG derived tumors. Mice were sacrificed when the tumors reached a size of up to 2 cm in diameter maximum or after 3-4 weeks from the inoculation time. The tumors were generated in the absence of estradiol and appear highly vascularized.

Figure 2. Anatomical appearance of the MCF-7/HRG tumor sections and development of distal metastasis. Tumors derived from both MCF-7/HRG and MCF-7 cells showed a mixture of solid, trabecular and tubular patterns. Tumors, lymph nodes and all the internal organs were removed *post mortem* for histological examination. The obtained tissues were partly frozen and

partly fixed in phosphate-buffered saline containing 10% formaldehyde, paraffin-embedded, and stained with hematoxylin-eosin (H&E) for routine examination and histopathological studies. A. The tumors generated from the MCF-7/HRG cells. B. Development of lymphatic invasion and regional lymph-node metastases. C. A well-defined collection of neoplastic cells inside the sub-capsular sinus of an axially lymph node.

Figure 3. HRG is expressed in all the MCF-7/HRG derived tumors. MCF-7 cells (upper panels) and MCF-7/HRG-transfected cells (lower panels) were fixed and embedded in paraffin. Fixed and permealized cells were treated with proteinase K for 20 and 30 min. The slides were hybridized overnight at 55°C with 1 x 10^6 DPM of ^{35}S-labeled antisense probe: with the MCF-7+ E2 derived tumors (A) with the MCF-7/HRG derived tumors (B) or sense probe with the MCF-7+ E2 derived tumors (C) with the MCF-7/HRG derived tumors (D), in a solution containing 50% formamide, 2 grams of dextran sulfate, 50 mM Tris, 500ml tRNA, and 400ml Denhard't. The slides were washed, dipped in NTB2 emulsion, and stored in light-tight boxes. After suitable exposure times, the slides were developed and stained with H&E.

Figure 4a. Preneoplastic transformation of the mammary gland *in vivo*. Tissue sections from mouse mammary glands adjacent to tumor areas derived from MCF-7/HRG cells. Sections were stained with hematoxylin and eosin at 100X.

Figure 4b. The transformed mammary gland is of mouse origin. The sections containing the transformed mammary glands were independently stained for human cytokeratin (KER) (A, B) and for an epithelial mammary antigen (EMA) (C, D). Specific staining was developed using alkaline phosphatase. The tumor sections were deparafinized, the sections were treated with methanol solution containing 3% hydrogen peroxide to block endogenous peroxidase activity, and they were incubated in horseradish peroxidase conjugated to antibodies to either EMA or KER. Afterwards the sections were stained with 3'-3'-diaminobenzidine tetrahydrochloride (DAB) (Vector, CA). All sections were counterstained with Mayer hematoxylin at 100X (top panel) and 200X (bottom panel) magnification.

Figure 5a. VEGF expression is upregulated in the HRG-derived tumors. The tissue was processed as described for Figure 4. The sections were stained using an anti-VEGF (Vascular Endothelial Growth Factor) antibody (Santa Cruz, CA). To verify that VEGF staining was specific, a VEGF peptide was used to block any possible non-specific staining. The sections incubated with an Avidin-biotin-complex (VECTASTAIN® Elite ABC reagent, Vector Laboratories) for 30 min, and the reaction was developed in the presence of H_2O_2 and DAB. The slides were counterstained with hematoxylin solution and mounted with the aqueous Crystal mount media. A. Immuhistochemical staining of MCF-7/V derived tumors from E2-treated animals. B. Peptide blocking of the VEGF antibody. C. Staining with VEGF antibody of the

MCF-7/HRG-derived tumors. D. Specific blocking of the anti-VEGF binding by a VEGF-derived peptide.

Figure 5b. VEGF expression is upregulated in the HRG-derived tumors. Quantification of VEGF in conditioned media derived from MCF-7/HRG cells an MCF-7/V cells using a specific anti-VEGF ELISA assay (R&D, MN). The assay was performed as instructed by the manufacturer. MCF-7/V and MCF-7/HRG cells were starved of serum for 24 h; conditioned medium was collected and assayed as per the manufacturer's instructions.

Figure 6. MAPK and *erbB* phosphorylation are upregulated in MCF-7/HRG cells. MCF-7/HRG cells (clones T6, T7 and T8), and MCF-7/V cells (5×10^5) were plated in IMEM containing 5% FBS, in 6-well plates. After 24 hours, cells were washed three times with serum-free media, and serum starved overnight. MCF-7 cells (5×10^5), used as control, were treated with HRG_1 (Neomarkers, CA) (30ng/ml) for 15 minutes at 37^{o}C, under serum-free conditions. All cells were lysed with lysis buffer, consisting of 50 mM HEPES 1% Triton X-100, 0.15M NaCl, 10% Glycerol, 2 mM EDTA, and 50 μM $ZnCl_2$, protein quantification was performed using the BCA reagent (Pierce, IL), and 50 μg of total protein were loaded onto a 12% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and blotted with anti-MAPK, anti-phospho-MAPK antibodies (Cell Signaling Technology, MA), or anti-phosphotyrosine 4G10 clone (Upstate Biotechnology, NY). The membranes were developed

using ECL reagents (Amersham Pharmacia Biotech, NJ) and autoradiography. A. MAPK phosphorylation in MCF-7, MCF-7 cells treated with HRG as a positive control, MCF-7/V cells, MCF/V treated with HRG, and MCF-7/HRG-transfected clones T6, T7, and T8. B. Total MAPK protein in the cells. C. Total *erbB* tyrosine phosphorylation.

Figure 7: MMP-9 expression is upregulated in MCF-7/HRG cells. MCF-7/V cells (clones V1 and V2), MCF-7/HRG cells (clones T6 and T7) cultured in serum free conditions for four days. Conditioned media was collected, concentrated and normalized for protein content. Culture supernatants of cells were concentrated and subjected to gelatin-embedded SDS-PAGE. Gelatinolytic enzymes are detected as transparent bands on the Coomassie-stained gels using Zymography. Briefly, the cultured media was run on a 7% gel containing 0.1% gelatin. After removal of the SDS with 0.1% Triton-X100, the gel was incubated overnight in the presence of 5 mM $CaCl_2$. The gel was then stained with Coomassie to demonstrate areas of gelatin degradation.

Reference List

1. J. C. Allegra, O. Korat, H. M. Do, M. Lippman, *J.Recept.Res.* 2, 17-27 (1981).

2. G. Gasparini, F. Pozza, A. L. Harris, *J.Natl.Cancer Inst.* 85, 1206-1219 (1993).

3. S. Antoniotti, P. Maggiora, C. Dati, M. De Bortoli, *Eur.J.Cancer* 28, 318-321 (1992).

4. D. Tripathy and C. C. Benz, *Cancer Treat.Res.* 63, 15-60 (1992).

5. B. P. Nicholson, *Semin.Oncol.* 27, 33-37 (2000).

6. T. W. Grunt et al., *Int.J.Cancer* 63, 560-567 (1995).

7. A. Staebler et al., *Breast Cancer Res.Treat.* 31, 175-182 (1994).

8. S. S. Bacus et al., *Cancer Res.* 53, 5251-5261 (1993).

9. K. L. Carraway, C. A. Carraway, K. L. Carraway, III, *J.Mammary.Gland.Biol.Neoplasia.* 2, 187-198 (1997).

10. M. M. Hijazi et al., *Int.J.Oncol.* 17, 629-641 (2000).

11. R. J. Pietras et al., *Oncogene* 10, 2435-2446 (1995).

12. A. Mazumdar et al., *Nat.Cell Biol.* 3, 30-37 (2001).

13. A. Saaristo, T. Karpanen, K. Alitalo, *Oncogene* 19, 6122-6129 (2000).

14. P. Charoenrat, P. Rhys-Evans, H. Modjtahedi, S. A. Eccles, *Clin.Exp.Metastasis* 18, 155-161 (2000).

15. J. Rak et al., *Cancer Res.* 55, 4575-4580 (1995).

16. R. Bagheri-Yarmand, R. K. Vadlamudi, R. A. Wang, J. Mendelsohn, R. Kumar, *J.Biol.Chem.* 275, 39451-39457 (2000).

17. L. Yen et al., *Oncogene* 19, 3460-3469 (2000).

18. B. N. Chau, K. Nandagopal, S. K. Niyogi, S. R. Campion, *Biochem.Biophys.Res.Commun.* 229, 882-886 (1996).

19. H. G. Hagedorn, B. E. Bachmeier, A. G. Nerlich, *Int.J.Oncol.* 18, 669-681 (2001).

20. S. B. Kondapaka, R. Fridman, K. B. Reddy, *Int.J.Cancer* 70, 722-726 (1997).

21. R. Hanemaaijer et al., *Int.J.Cancer* 86, 204-207 (2000).

22. B. P. Himelstein, R. Canete-Soler, E. J. Bernhard, D. W. Dilks, R. J. Muschel, *Invasion Metastasis* 14, 246-258 (1994).

23. B. P. Himelstein, E. J. Lee, H. Sato, M. Seiki, R. J. Muschel, *Oncogene* 14, 1995-1998 (1997).

24. A. Moon et al., *Int.J.Cancer* 85, 176-181 (2000).

25. K. S. Lee et al., *Clin.Exp.Metastasis* 14, 512-519 (1996).

26. C. K. Tang et al., *Cancer Res.* 56, 3350-3358 (1996).

27. M. Saceda et al., *Endocrinology* 137, 4322-4330 (1996).

28. L. Adam, A. Mazumdar, T. Sharma, T. R. Jones, R. Kumar, *Cancer Res.* 61, 81-87 (2001).

29. R. Clarke et al., *Mol.Endocrinol.* 3, 372-380 (1989).

30. E. J. Weinstein and P. Leder, *Cancer Res.* 60, 3856-3861 (2000).

31. A. M. Babic, M. L. Kireeva, T. V. Kolesnikova, L. F. Lau, *Proc.Natl.Acad.Sci.U.S.A* 95, 6355-6360 (1998).

32. M. S. Tsai, A. E. Hornby, J. Lakins, R. Lupu, *Cancer Res.* 60, 5603-5607 (2000).

33. J. Milanini, F. Vinals, J. Pouyssegur, G. Pages, *J.Biol.Chem.* 273, 18165-18172 (1998).

34. R. Yashima et al., *J.Cell Physiol* 188, 201-210 (2001).

35. K. J. Tsang and D. L. Crowe, *Int.J.Oncol.* 18, 369-374 (2001).

36. D. Harari and Y. Yarden, *Oncogene* 19, 6102-6114 (2000).

37. S. J. Lim, G. Lopez-Berestein, M. C. Hung, R. Lupu, A. M. Tari, *Oncogene* 19, 6271-6276 (2000).

38. F. C. von Lintig et al., *Breast Cancer Res.Treat.* 62, 51-62 (2000).

39. M. Ballin, D. E. Gomez, C. C. Sinha, U. P. Thorgeirsson, *Biochem.Biophys.Res.Commun.* 154, 832-838 (1988).

40.J. Hua and R. J. Muschel, *Cancer Res.* 56, 5279-5284 (1996).

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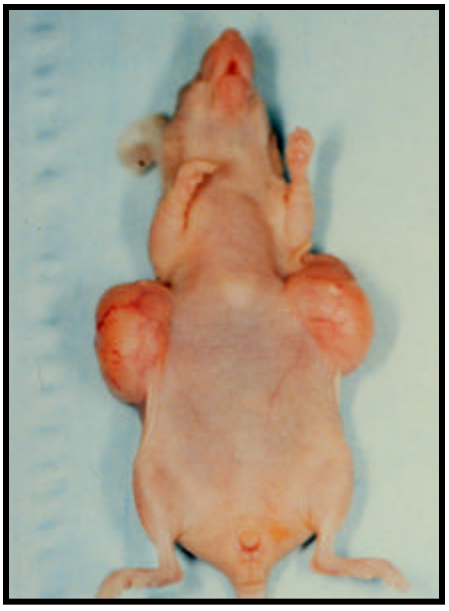

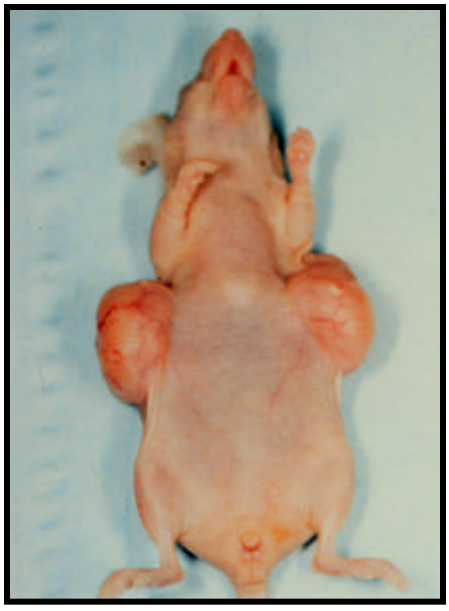

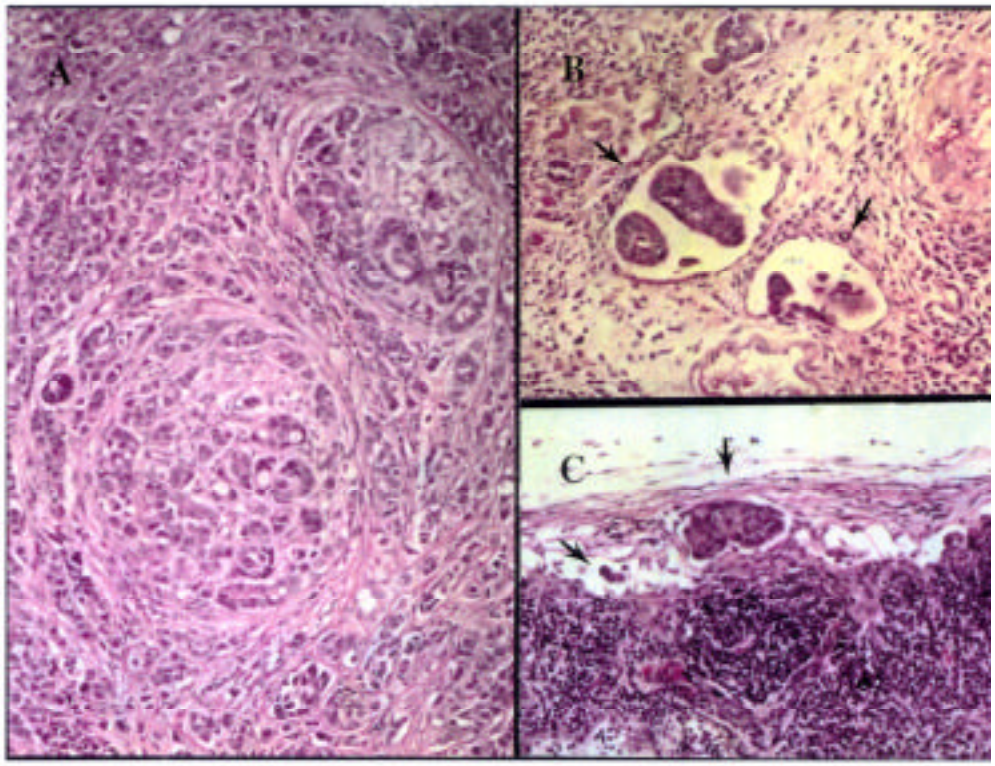
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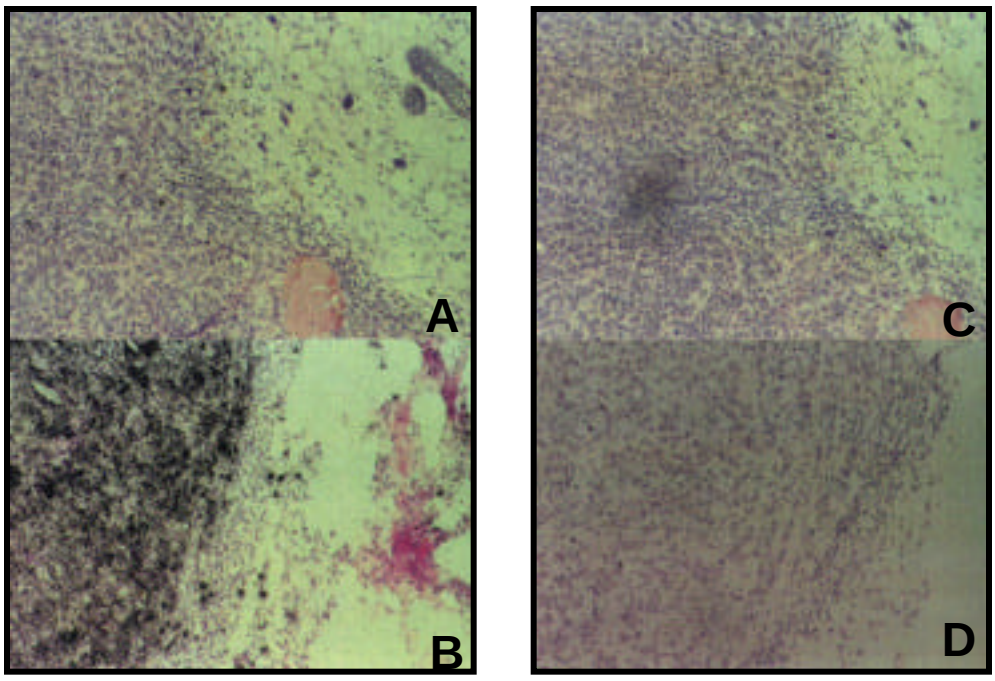
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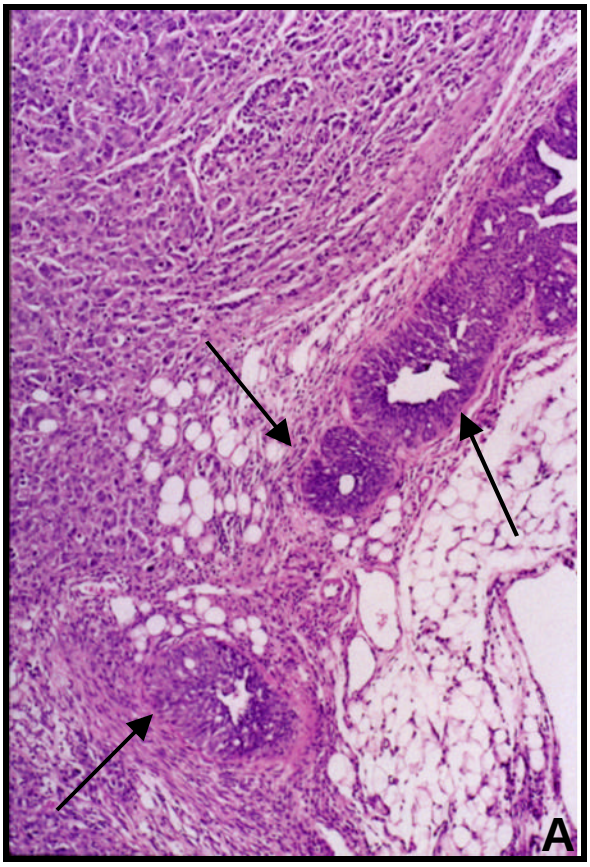

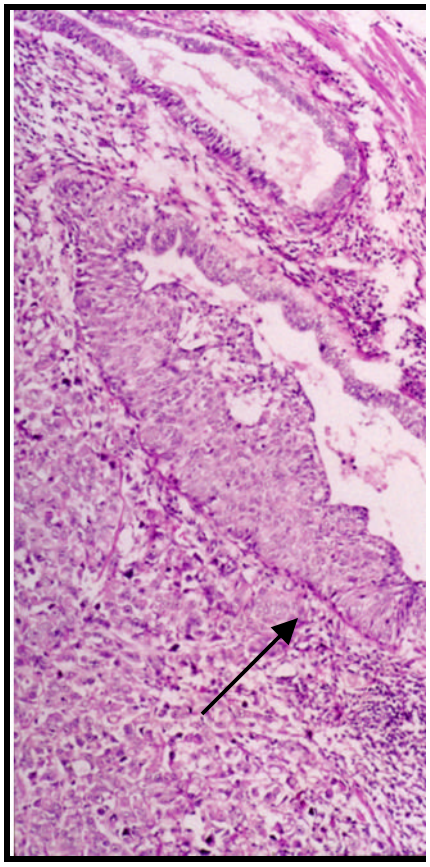

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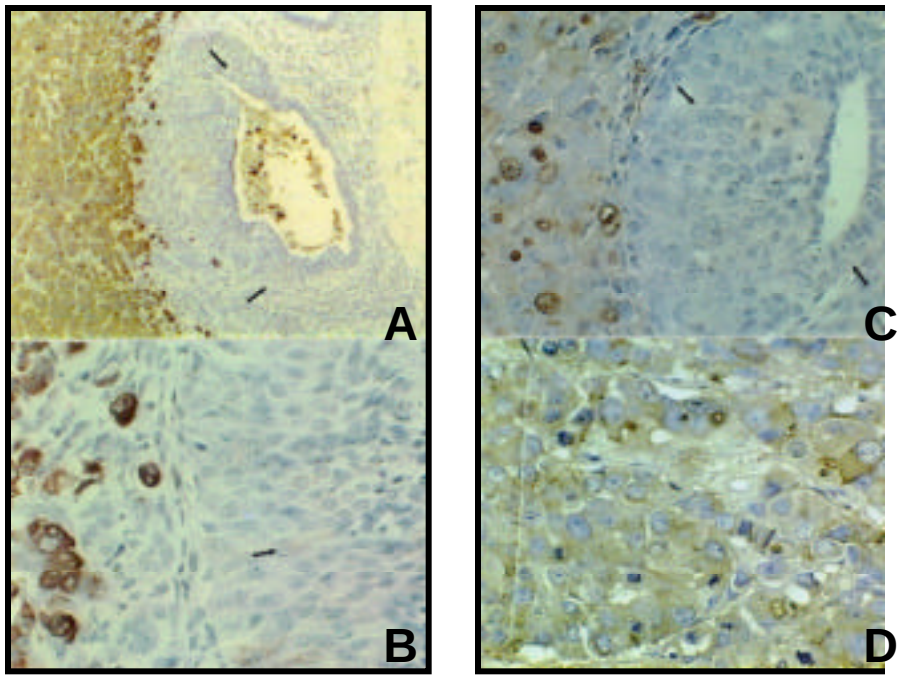
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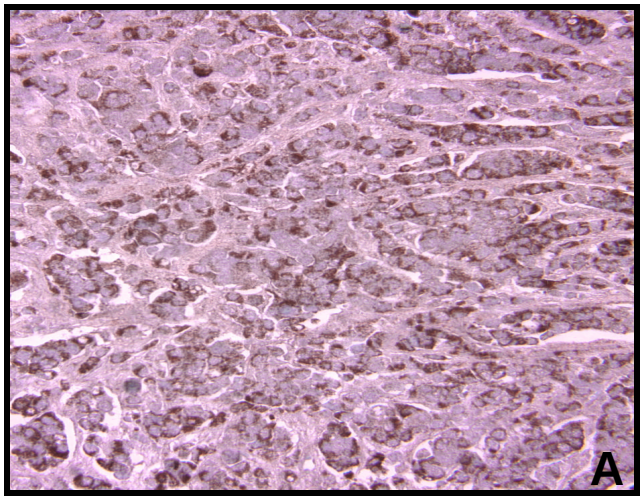
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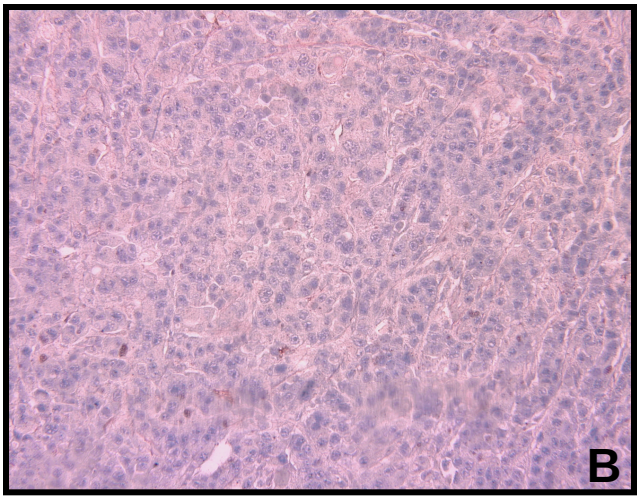
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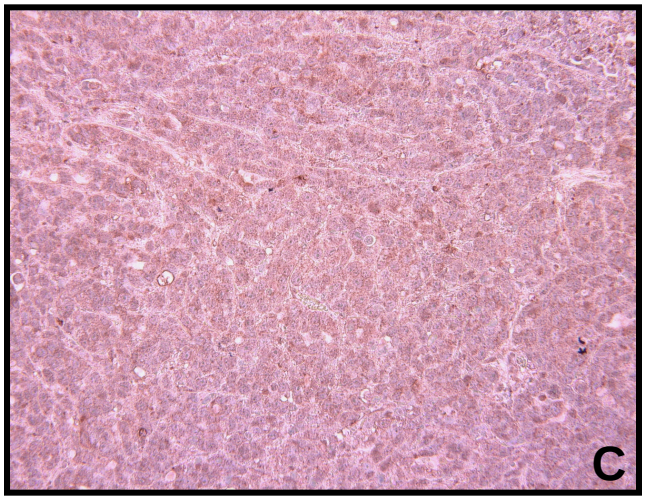
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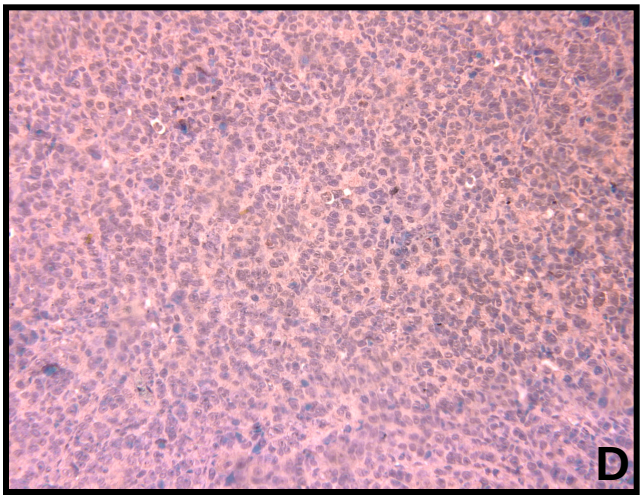
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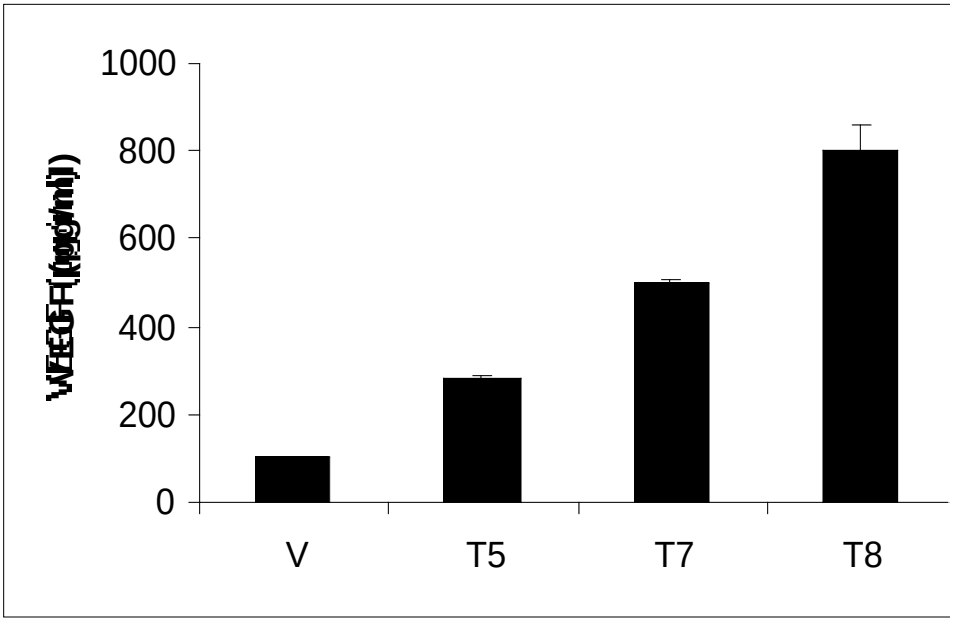
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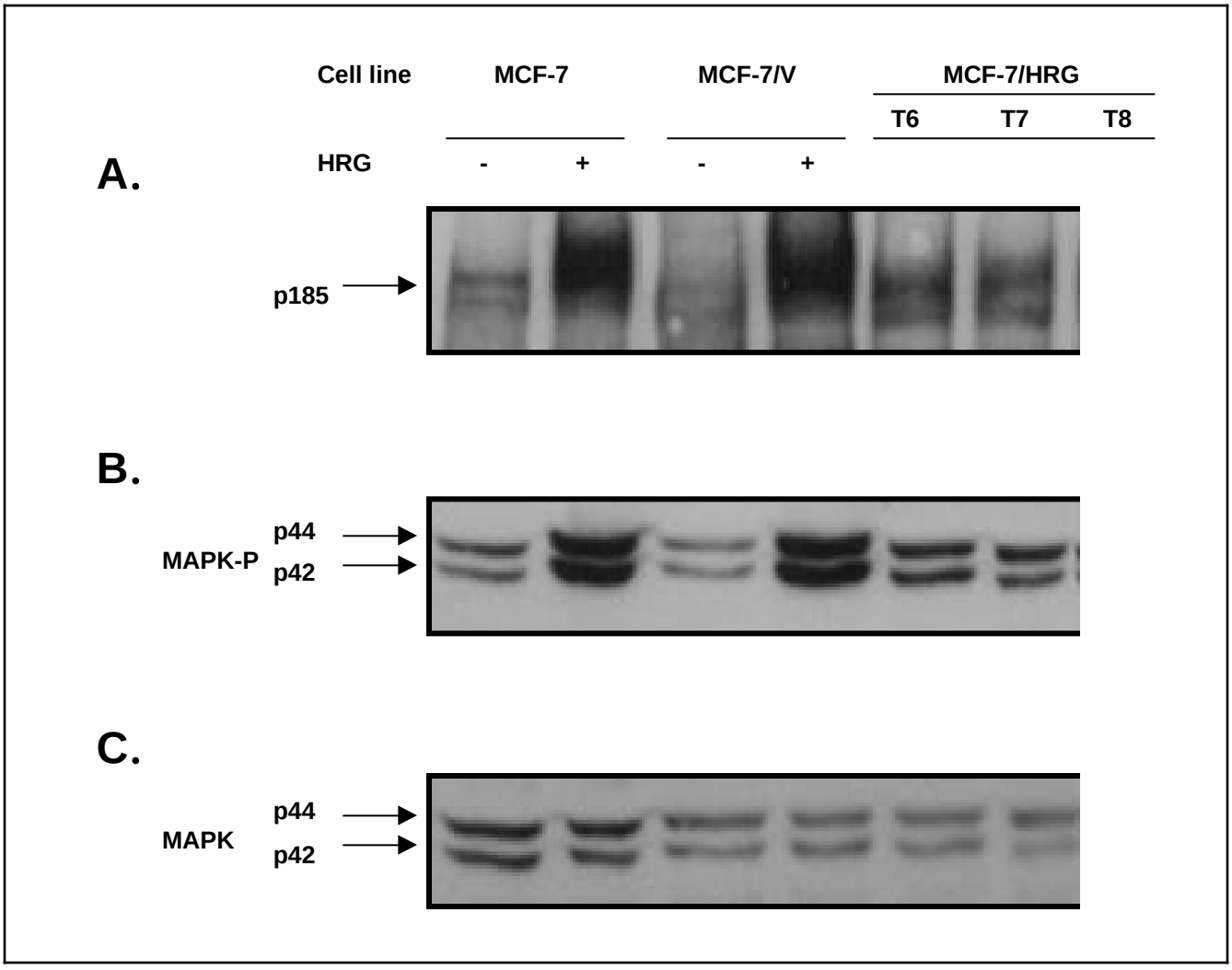
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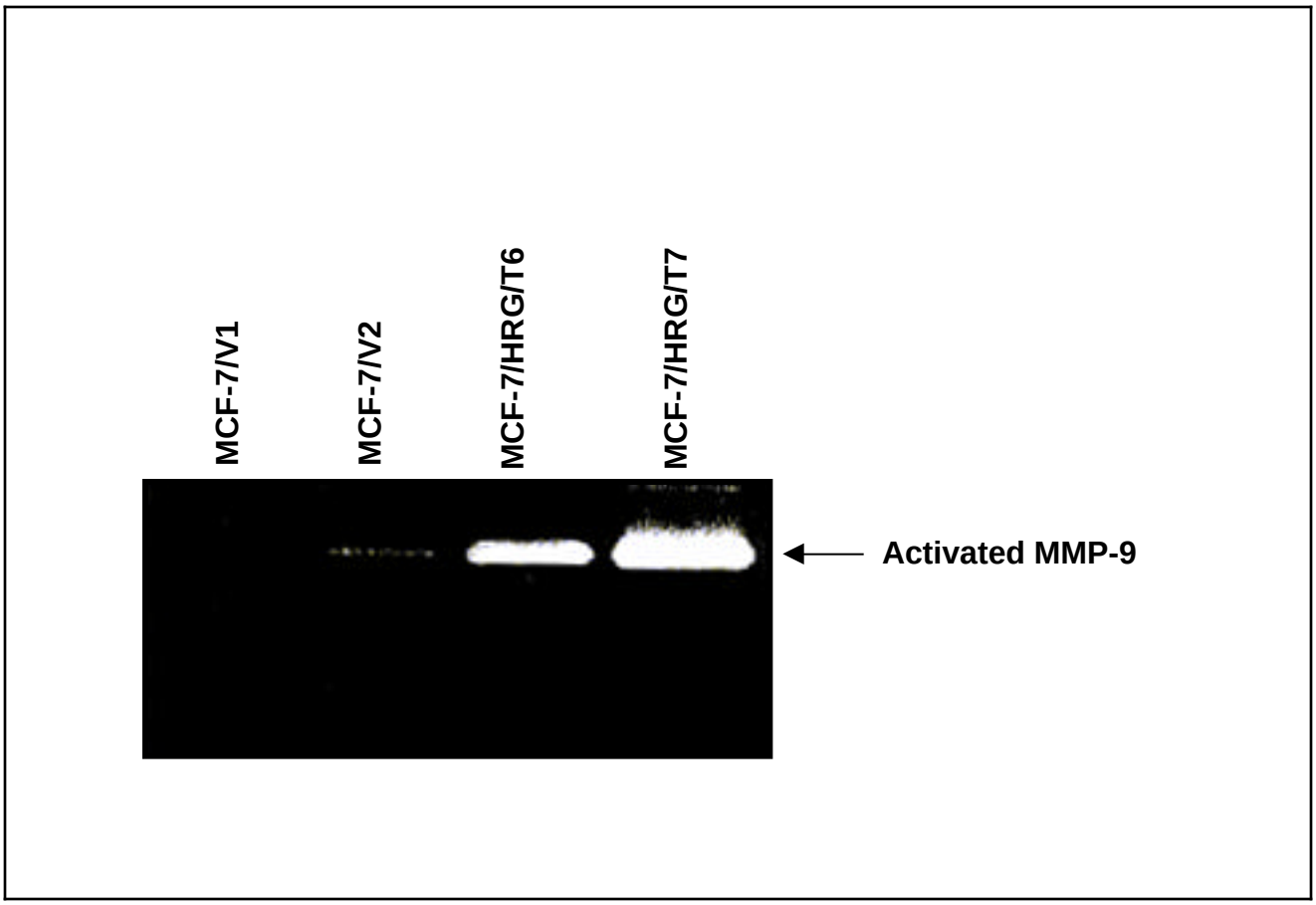
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