Advances in Brief

Expression and Function of CYR61, an Angiogenic Factor, in Breast Cancer Cell Lines and Tumor Biopsies[1]

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Abstract

We have previously shown that expression of heregulin (HRG) is closely correlated with breast cancer progression. We have subsequently isolated Cyr61, a ligand for the $\alpha_v\beta_3$ integrin that is differentially expressed in HRG-positive cells, and have shown that it is expressed in all of the invasive and metastatic breast cancer cell lines tested. Preliminary evaluation of Cyr61 expression in breast tumor biopsies revealed expression of Cyr61 in about 30% of invasive breast carcinomas. Significantly, we demonstrated that Cyr61 is a downstream effector of HRG action, because a Cyr61-neutralizing antibody abolished the ability of HRG-expressing cells to migrate *in vitro*. Furthermore, we have shown that HRG-expressing cells denote higher levels of $\alpha_v\beta_3$ expression, and we have established that Cyr61 action is mediated, at least in part, through its receptor $\alpha_v\beta_3$, because a functional blocking antibody of the $\alpha_v\beta_3$ blocked the Matrigel outgrowth of HRG-expressing cells. These results strongly suggest that Cyr61 is necessary for HRG-mediated chemomigration and that Cyr61 plays a functional role in breast cancer progression, possibly through its interactions with the $\alpha_v\beta_3$ receptor.

Introduction

Many E2[5]-dependent and antiestrogen-responsive breast tumors spontaneously progress to an E2-independent and antiestrogen-resistant phenotype, becoming deadly metastatic diseases. The mechanism by which breast cancer appears to progress from an E2-dependent to an E2-independent phenotype is still under investigation. We have shown that expression of HRG, an activator of *erb*B-2/-3/-4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype (1). Furthermore, we demonstrated that HRG induces breast cancer progression, as determined by loss of ER function and response, tumorigenicity (2), invasion (3), and metastasis (4). It has been hypothesized that HRG induces activation of the *erb*B signaling pathways, leading to regulation of downstream genes that regulate and control cancer progression. Therefore, to develop effective targeted therapies, it is important to identify gene(s) directly involved in HRG-induced breast cancer aggressiveness.

With this in mind, we have isolated and identified the human homologue of a mouse immediate-early response gene, *Cyr61*, differentially expressed in ER-negative, HRG-positive breast cancer cells. Cyr61 is a secreted cysteine-rich protein that is associated with the cell surface and the extracellular matrix (5). Cyr61 mediates cell adhesion, migration, and angiogenesis (6, 7). In this report, we establish that Cyr61 is coexpressed with HRG in all of the metastatic breast cancer cell lines tested, its expression is inversely correlated with ER expression, and it is associated with HRG-induced breast cancer chemomigration and metastasis, possibly through interactions with the $\alpha_v\beta_3$ integrin receptor. Furthermore, we establish that Cyr61 was expressed in about 30% of invasive breast cancer tumor biopsies, implying a possible role in breast cancer progression.

Materials and Methods

Cells and Cell Culture

Breast cancer cell lines were obtained from the American Type Culture Collection and routinely cultured in phenol red-containing improved MEM supplemented with 5% (v/v) fetal bovine serum and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2, unless otherwise specified.

Plasmids and Generation of Riboprobes

A Cyr61 riboprobe plasmid was constructed by cloning a PCR fragment of Cyr61 cDNA into the pCRII TA cloning vector (Invitrogen). The sequence of primers used to generate the Cyr61 fragments was as follows: (*a*) forward primer, 5′-TGTGGAACTGGTATCTCCACACGA-3′ (nucleotides 727–750); and (*b*) reverse primer, 5′-TCTTTTCACTGAATATAAAATTAAAA-3′ (nucleotides 1739–1764). The Cyr61 riboprobe construct was sequenced using Sequenase v.2.0 with ^{35}S-labeled dCTP. Radioactive riboprobe was prepared by linearizing the plasmid with the restriction enzyme *Dde*I, which generated a 524-bp fragment, and followed by reverse transcription *in vitro* using the SP6 RNA polymerase in the presence of [^{32}P]UTP. The riboprobe plasmid of GAPDH was kindly provided by Dr. Francis Kern (University of Alabama, Birmingham, AL). Radiolabeled GAPDH riboprobe was generated using T7 RNA polymerase as described above, except that it was linearized by the restriction enzyme *Bam*HI.

RNase Protection Assay

Total RNA was extracted by Tripure isolation solution (Roche Molecular Biochemicals) and quantified by spectrophotometry. RNA (30 µg) was hybridized with 100,000 cpm of ^{32}P-labeled Cyr61 riboprobe for 12–16 h at 50°C. ^{32}P-labeled GAPDH riboprobe (10,000 cpm) was added to each sample as an internal control. Hybridized RNA samples were digested with 25 µg of RNase A for 30 min at 28°C. The reaction was terminated by incubating with proteinase K (250 µg/ml) and 0.5% SDS for 15 min at 37°C. After phenol extraction, RNA samples were coprecipitated with 10 µg of yeast tRNA in absolute ethanol. RNA was redissolved in a denaturing loading buffer and resolved by electrophoresis on a 6% polyacrylamide-urea gel. Protected fragments of Cyr61 (305 bp) and GAPDH (100 bp) were visualized by autoradiography. [^{32}P]dCTP-end-labeled pBR322/*Msp*I (New England Biolabs) was used as a molecular weight marker.

Western Blot Analysis

Cyr61 Present in Conditioned Media. Subconfluent human breast cancer cells were maintained in serum-free media for 3–4 days. The conditioned media were collected, and the Cyr61 protein was purified by heparin affinity

chromatography. The column was washed with 0.3–0.6 M NaCl in 10 mM Tris-HCl (pH 7.5). The Cyr61 protein was eluted at 0.9 M NaCl and desalted by PD-10 Sephadex G25M columns (Amersham-Pharmacia). The eluted fractions were concentrated (10×) and resolved by 12% Tris-glycine SDS-PAGE. The separated proteins were electroblotted onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham-Pharmacia). The blotted membrane was blocked overnight at 4°C with 5% (w/v) BSA in Tris-buffered saline containing 0.5% Tween 20 (TBST) and incubated with the rabbit anti-Cyr61 polyclonal antibody (1:5,000 dilution) for 1 h at room temperature. After three washes with TBST, the blot was incubated with a 1:10,000 dilution of horseradish peroxidase-linked donkey antirabbit IgG secondary antibody. The Cyr61 protein was detected by the enhanced chemiluminescence reaction using Hyperfilm (Amersham-Pharmacia).

Cyr61 Present in Breast Cancer Tumor Specimens. Breast tumor specimens were lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM DTT] with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined by a Micro BCA detection reagent kit (Pierce). Equal amounts of proteins were loaded and separated by SDS-PAGE followed by Western blot analysis as described above.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded breast tumor sections were deparaffinized in xylene and hydrated in a graded alcohol series. Slides were quenched for endogenous peroxidase activity in the presence of 0.3% H_2O_2 for 30 min and blocked with 10% (v/v) horse serum for 30 min. Slides were then incubated with a polyclonal anti-Cyr61 antibody (1:5000) overnight at 4°C. The sections were washed in PBS before the incubation with a biotinylated antirabbit IgG secondary (1:200) antibody for 30 min. The sections were then incubated with an avidin-biotin complex (VECTASTAIN Elite ABC reagent; Vector Laboratories) for 30 min, and the reaction was developed in the presence of hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride. The slides were counterstained with hematoxylin solution and mounted with the aqueous Crystal mount media.

Chemomigration and Chemotaxis Assays

Boyden chamber chemomigration assays were performed using a 48-well chemotaxis chamber (Neuro Probe). Breast cancer cells (20,000 cells/well) were plated onto the upper chambers in triplicate or quadruplicate onto a 12 μm polycarbonate filter membrane coated with collagen (Becton Dickinson). The conditioned media derived from NIH3T3 fibroblast culture was used as a chemoattractant in the lower chambers. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 16 h. After the incubation, the membrane was removed from the chamber. The cells on the top surface were removed, and the cells on the bottom side of the membrane were fixed in methanol and stained with a Diff-Quick Stain kit. Membranes were then mounted onto glass slides, and the cells that migrated through the pores to the opposite side of the membrane (bottom side) were quantified using a light microscope.

Matrigel Outgrowth Assay

Cells (5,000 cells/well) were mixed with 150 μl of Matrigel (Becton Dickinson) and plated in triplicate onto the Matrigel-coated 12-well plates for 1 h at 37°C. Cells were then cultured in the media containing the indicated concentrations of antibodies for 7–10 days. The pattern of the cells' outgrowth in Matrigel matrix was examined and photographed using a phase-contrast microscope.

Results and Discussion

Cyr61 Is Differentially Expressed in HRG-positive *versus* HRG-negative Breast Cancer Cell Lines. We have demonstrated that expression of HRG is highly associated with aggressive progression of breast cancers to hormone independence, antiestrogen resistance, invasion, and metastasis (2–4). To identify genes that were involved in the HRG induction of breast cancer progression, a number of genes were isolated and cloned by differential expression in MDA-MB-231 HRG-expressing cells. Sequence and homology analyses indicated that one of the genes is the human homologue of a mouse immediate-early response gene, *Cyr61*. Cyr61 was highly and selectively expressed in MCF-7/HRG [MCF-7/HRG (HRG-transfected MCF-7) clones, *e.g.,* T2, T6, T7, and T8] but was nearly undetected in MCF-7/V cells (vector-transfected MCF-7 cells). A 5–25-fold increase in the Cyr61 mRNA level was observed in MCF-7/HRG cells as compared with MCF-7/V cells. HRG-positive MDA-MB-231 cells also expressed high levels of Cyr61 (Fig. 1*A*). To determine whether the protein was also selectively expressed, we performed Western blot analysis and immunohistochemistry using an anti-Cyr61 polyclonal antibody. As shown in Fig. 1*B*, Cyr61 protein expression was observed in the MCF-7/HRG cells but not in the vector control cells. These studies were performed using cells cultured under serum-depleted conditions.

In addition, immunohistochemical staining was performed on paraffin sections of MCF-7/HRG and MCF-7/V tumors formed in xenografted athymic nude mice. These tumors were observed as 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 those observed in mammary infiltrating ductal carcinoma known as no special type (Fig. 1*A*). Similar features were observed in all of the tumors examined. As can be seen in Fig. 1*C*, Cyr61 expression was very predominant in MCF-7/HRG-derived tumors (*right panel*). Expression of the Cyr61 protein was localized to the cytoplasm of the tumor cells, whereas only a weak staining of Cyr61 was observed in tumors derived from MCF-7/V cells supplemented with E2 (*left panel*). Once again, our data demonstrate a differential expression of Cyr61 in HRG-expressing cells.

We mapped the human *Cyr61* gene to chromosome 1p (data not shown), consistent with previous studies showing the localization of Cyr61 to chromosome 1p22.3 (8, 9). Abnormalities of chromosome 1p have correlated with ER negativity and a poor prognosis in breast cancer (10) and other malignancies (11–13).

It has been shown that murine Cyr61 is regulated by 12-*O*-tetradecanoylphorbol-13-acetate in the liver (14), as well as by E2 and Tam in the uterus (15). We have shown that the human homologue of Cyr61 is regulated by E2 and several antiestrogens including Tam and ICI 182,780 in ER-positive breast cancer cells.[6] The induction of Cyr61 was most significant in MCF-7 cells [up to a 10–12-fold increase by 6 h of treatment with E2 (10^{-9} M) or 3 h of treatment with ICI 182,780 (10^{-7} M)] and to about a 5–6-fold increase with Tam (10^{-7} M). On the other hand, the up-regulation of Cyr61 was not significant in HRG-expressing cells, with an increase of only 1.5–2-fold by any of the treatments (data not shown). These results are consistent with our published data demonstrating that HRG promotes an estrogen-independent phenotype and that HRG blocks ER function resulting in MCF-7/HRG cells that fail to respond to E2 and the consequential inability of E2 to induce the expression of E2-regulated genes.

Cyr61 Is Overexpressed in HRG-positive, ER-negative Breast Cancer Cell Lines. To assess whether up-regulation of Cyr61 expression was a result of HRG overexpression in MCF-7/HRG cells or whether it was a common theme occurring in breast cancer cells, we examined its expression in many human breast cancer cell lines. Basal level of Cyr61 expression was measured in cells cultured under serum-depleted conditions to prevent the influence of E2 on Cyr61

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[6] M. S. Tsai, E. Gilad, M. Cardillo, and R. Lupu. Heregulin (HRG) promotes tumor formation, manuscript in preparation.
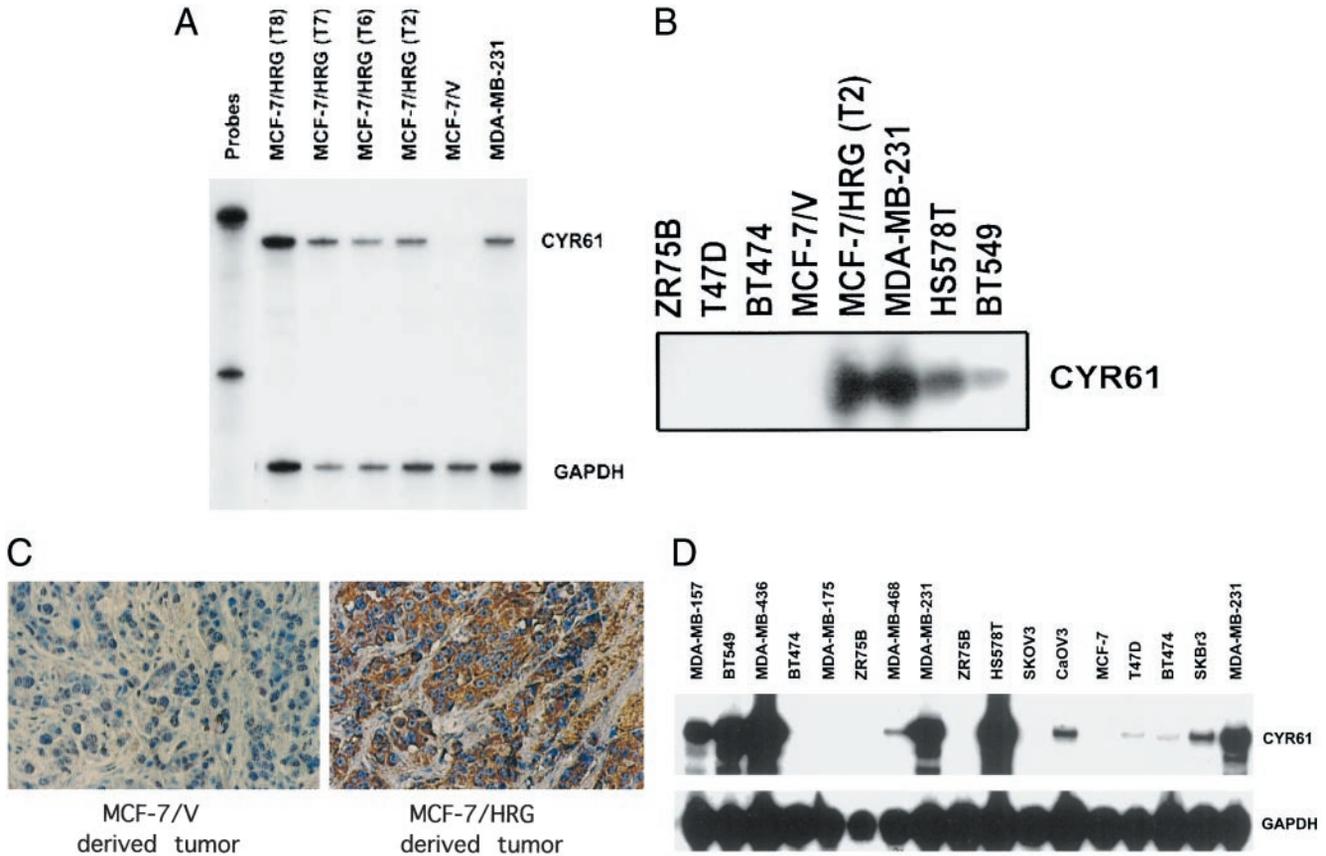
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Fig. 1. Expression of Cyr61 in breast cancer cells. *A*, subconfluent MCF-7/HRG and MCF-7/V cells were maintained in serum-depleted conditions for 3 days. Total RNA was isolated, and 30 μg of RNA were analyzed by RNase protection assay. RNA from MDA-MB-231 cells was used as a positive control for Cyr61 expression. The GAPDH probe was used as an internal control for RNA loading. *B*, subconfluent breast cancer cell lines were cultured in serum-free media. Conditioned media were collected after 3 days, and heparin column chromatography was performed. The Cyr61 protein was eluted and analyzed by Western blotting analysis as described in "Materials and Methods." *C*, immunohistochemical analysis was performed as described in "Materials and Methods" for MCF-7/V- and MCF-7/HRG-derived tumor sections. *D*, breast cancer cells were cultured as described in *A*, and total RNA was isolated and analyzed by RNase protection assay.

expression. As shown in Fig. 1, *B* and *D*, a tight correlation between Cyr61 mRNA and protein expression exists in all of the cell lines tested. Cyr61 is highly expressed in MDA-MB-231, HS578T, BT549, and MCF-7/HRG cells, all of which are HRG-expressing and ER-negative cells, but it is low or undetectable in cells that do not express HRG and are ER-positive, including MCF-7, ZR75B, T47D, and BT474 cells. These studies were performed by RNase protection assays and by Western blot analysis in which a secreted M_r 45,000 protein derived from conditioned media was detected using an anti-Cyr61 polyclonal antibody.

Our data indicate that a high level of Cyr61 expression correlates with HRG expression and inversely correlates with ER expression, response to E2, and sensitivity to antiestrogens (16). Moreover, the expression of Cyr61 strongly correlates with vimentin expression, a known marker for invasiveness (17), and is associated with the ability of breast cancer cells to invade *in vitro* and metastasize *in vivo*. On the other hand, low to undetectable levels of Cyr61 expression were seen only in the HRG-negative, ER-positive, E2-dependent, antiestrogen-sensitive breast cancer cells. These data are summarized in Table 1. Taken together, these data show that Cyr61 expression is associated with HRG expression and is apparently linked to breast cancer progression. Because *Cyr61* is an early response gene, it could be argued that its expression would be up-regulated in rapidly proliferating cells. Thus, it is critical to establish that up-regulation of Cyr61 in MCF-7/HRG cells is not attributable to a proliferative advantage of these cells. Cell cycle analysis by flow cytometry demonstrated that no differences in cell cycle distribution were observed between the MCF-7/HRG cells and the parental MCF-7 cells (18).

Cyr61 Is Expressed in about 30% of Breast Tumor Biopsies. To determine whether expression of Cyr61 may have clinical relevance in breast cancer, its expression in biopsies was determined. A pilot study was performed using Western blot analysis on proteins extracted from paraffin sections. Forty percent (4 of 10) of the tumor specimens, all of which were ER-negative invasive breast carcinomas, showed high expression of the Cyr61 protein (Fig. 2*A*). Total cell lysates of MDA-MB-231 and MCF-7 were used as positive and negative controls, respectively. It is noteworthy that Cyr61 protein expression was low in cell lysates of MDA-MB-231, because Cyr61 is mostly secreted to the cultured media. Additional studies revealed that Cyr61 was detected in about 30% of breast tumor specimens (*n* = 55) by immunohistochemistry (Fig. 2*B*). Cyr61 staining was demonstrated to be specific because it was completely blocked in the presence of excess recombinant Cyr61 protein (data not shown). No staining was observed in normal components of the biopsies. These data suggest that in at least 30% of these tumors, Cyr61 may be required for survival; therefore, it may be strongly implicated in breast cancer progression.

An Anti-Cyr61-Neutralizing Antibody Blocks Chemomigration of MCF-7/HRG Cells. To demonstrate that Cyr61 is a direct downstream regulator of HRG action, studies were performed using a Cyr61-neutralizing antibody [Refs. 5–7; kindly provided by Dr. Lester F. Lau (University of Illinois, Chicago, IL)]. For these studies, we used MCF-7/HRG cells, which have been shown to migrate through collagen in a Boyden chamber assay (as shown below). Cells were treated with increasing concentrations of the antibody, and the ability of the cells to migrate *in vitro* was assessed. The anti-Cyr61-neutralizing antibody inhibited migration of MCF-7/HRG cells in a dose-

5605

Table 1 *Expression of Cyr61 in breast cancer cell lines*[a]

Cell line	Cyr61	HRG	ER	Invasive in vitro	Metastatic in vivo	$\alpha_v\beta_3$[b]
MCF-7	−	−	+ + + +	−[c]	−	+/−
T47D	−	−	+ +	−[c]	−	−
BT474	−	−	+ +	−[c]	−	−
MDA-MB-175	−	+/−[d]	+	−[c]	−	ND
ZR75B	−	+/−[d]	+	−[c]	−	−
MDA-MB-468	+	−	−	+[e]	−	−
SKBR-3	+	−	−	+[e]	−	−
MDA-MB-157	+ +	+ +	−	+	ND	ND
MDA-MB-436	+ + +	+ + +	−	+ + +	ND	ND
BT-549	+ + +	+ + +	−	+ + +	+	ND
MDA-MB-231	+ + + +	+ + + +	−	+ + + +	+	+ + +
MDA-MB-435	+ + + +	+ + + +	−	+ + + +	+	+ + +
HS578T	+ + + +	+ + + +	−	+ + + +	+	ND
MCF-7/HRG	+ + +	+ + +	−/+	+ + +	+	+ +

[a] − indicates no expression; the number of plus signs indicates the increase in expression.

[b] The $\alpha_v\beta_3$ integrin expression is based on results from Ref. 19 and our preliminary data. ND, not determined.

[c] Cells require E2 for invasion *in vitro* and growth *in vivo* and never metastasize *in vivo*.

[d] E2 induces expression of HRG.

[e] Cells require ligand (epidermal growth factor or HRG) to invade but never metastasize *in vivo*.

dependent manner (Fig. 3A). No effect was observed when a control IgG antibody was used under the same conditions and concentrations. Similar results were observed in other invasive, HRG-expressing breast cancer cells, such as MDA-MB-231, HS578T, and BT549 (data not shown). These studies suggest, for the first time, a possible association between the increase in Cyr61 expression and the invasive potential triggered by HRG. Additional studies are required to assess the direct association between Cyr61 and HRG and their joint action resulting in breast cancer progression. Of note, the anti-Cyr61-neutralizing antibody had no effect on MCF-7 cells. It is important to note that MCF-7 cells do not migrate through collagen.

The $\alpha_v\beta_3$ Integrin Receptor Is Involved in Cyr61-Mediated Breast Cancer Progression. Because Cyr61 was shown to be a ligand for the $\alpha_v\beta_3$ integrin (19), we speculated whether Cyr61 requires expression of $\alpha_v\beta_3$ for its action. Thus, we assessed the level of $\alpha_v\beta_3$ expression in MCF-7/HRG cells and showed that the level of $\alpha_v\beta_3$ was augmented in MCF-7/HRG cells compared with the MCF-7/V cells (data not shown), as determined by immunofluorescence staining using an anti-$\alpha_v\beta_3$ antibody on cultured cells. We then speculated that if the action of Cyr61 is mediated through the $\alpha_v\beta_3$ receptor, it is plausible that blockage of the $\alpha_v\beta_3$ integrin will modulate the growth characteristics of MCF-7/HRG cells. Thus, Matrigel outgrowth and migration studies were performed in the presence and absence of an anti-$\alpha_v\beta_3$ functional blocking antibody. We determined that this antibody specifically blocked the Matrigel outgrowth of HRG-expressing cells in a dose-dependent manner (Fig. 3B). No effects were observed when control IgG was used. Similar inhibitory effects of the anti-$\alpha_v\beta_3$ antibody were seen in HRG-positive MDA-MB-231 cells (data not shown).

The results indicated that the functional $\alpha_v\beta_3$ integrin is required for maintaining the invasive capacity of HRG-expressing cells, and that the aggressive phenotypes induced by HRG are mediated, in part if not entirely, by Cyr61 and its receptor, $\alpha_v\beta_3$ integrin. Because Cyr61, an angiogenic factor, and its receptor, $\alpha_v\beta_3$, are both induced
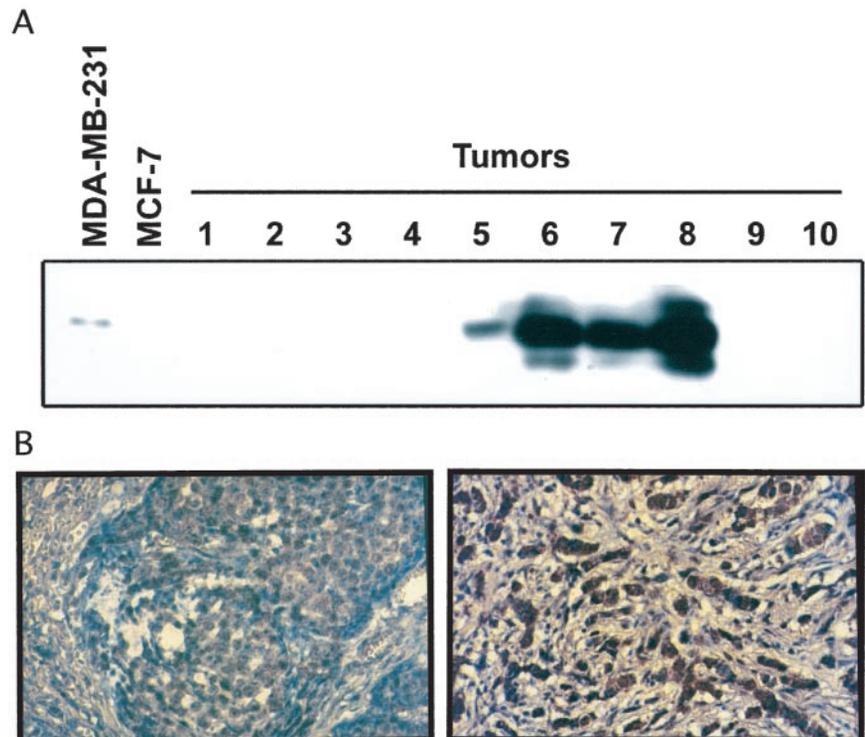

Fig. 2. Expression of Cyr61 in human breast tumor biopsies. *A*, human breast tumors were lysed in radioimmunoprecipitation assay buffer, and equal amounts of protein were resolved on a 4–20% gradient SDS-polyacrylamide gel. Western blotting analysis was performed as described in "Materials and Methods." Cell lysates of MCF-7 and MDA-MB-231 cells were used as negative and positive controls, respectively. *B*, immunohistochemical analysis of human breast carcinoma biopsies was performed as described in "Materials and Methods." Two representative microphotographs are shown for Cyr61 expression.
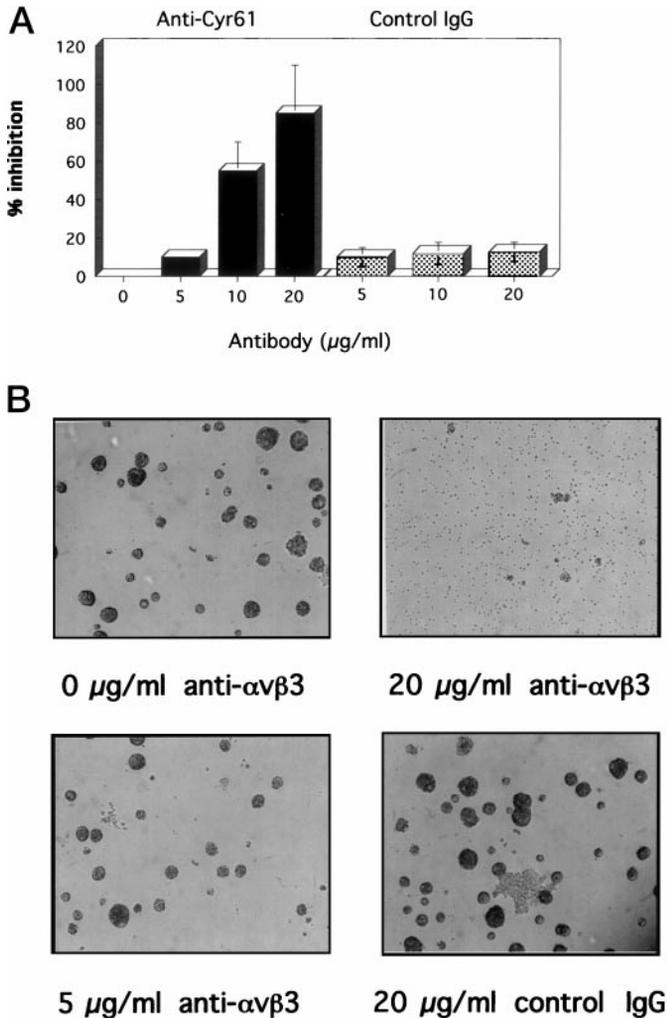
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Fig. 3. *A*, blocking the invasive phenotypes of HRG-expressing cells by a Cyr61-neutralizing antibody. MCF-7/HRG cells were treated in the absence or presence of increasing concentrations (5, 10, and 20 μg/ml) of the anti-Cyr61 antibody or a control IgG for 16 h in the Boyden chamber assay. Chemomigration was measured based on the number of cells traversing collagen-coated filters. Data are the mean of triplicates from a representative experiment. SD was calculated for each data point. *B*, inhibited outgrowth of MCF-7/HRG cells by a functional blocking antibody of $\alpha_v\beta_3$. MCF-7/HRG cells were treated in the absence or presence of increasing concentrations of the anti-$\alpha_v\beta_3$ antibody (LM609; only the 5 and 20 μg/ml concentrations of antibody are shown) or a control IgG (20 μg/ml) in Matrigel outgrowth assay for 7 days. Outgrowth pattern was examined and photographed.

in the MCF-7/HRG cells, it is tempting to postulate that these factors are involved in the increased neovascularization that we have observed in the tumors formed by MCF-7/HRG cells in athymic nude mice.[6] The exact mechanism by which HRG promotes an aggressive breast cancer phenotype is still unknown. However, the identification of Cyr61 expression in breast cancer tumor progression is of great significance, especially because its receptor, the $\alpha_v\beta_3$ integrin, was recently shown to be a good prognostic indicator in breast cancer (20, 21). Studies are under way to determine whether ectopic expression of Cyr61 alone, in HRG-negative cells, is sufficient and/or necessary to

confer some biological activities induced by HRG, such as loss of E2 response, acquisition of antiestrogen resistance, and chemomigration.

Acknowledgments

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