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The Angiogenic Factor Cyr61 Is Induced by the Progestin R5020 and Is Necessary for Mammary Adenocarcinoma Cell Growth

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Cyr61 is a secreted pro-angiogenic factor that belongs to an emerging family of growth regulators classified as CCN (CTGF/Cyr61/NOV). Work in our laboratory has focused on sex steroid regulation of Cyr61 and its role in hormonal carcinogenesis. In this study, both Cyr61 mRNA and protein were induced by the progestin, R5020, in T47D mammary adenocarcinoma cells in a dose- and time-dependent fashion. Cyr61 gene induction by R5020 was transcriptionally regulated by progesterone receptor (PR) as the antiprogestin, RU486, and actinomycin D blocked induction completely. Moreover, Cyr61 was upregulated by epidermal growth factor (EGF) but not by R5020 in the PR– MDA-MB-431 mammary adenocarcinoma cell line, underscoring the necessity of PR. The functional significance of progestin induction of Cyr61 in breast cancer cell growth was demonstrated by anti-Cyr61 neutralizing antibodies, which diminished R5020 and EGF-dependent DNA synthesis by 30%. Moreover, anti-Cyr61 neutralizing antibodies reduced the synergistic effects of R5020 and EGF on T47D cell growth by 30%. Accordingly, protein lysates generated from stage II invasive ductal carcinomas (*n* = 20) were analyzed in order to determine the relevance of Cyr61 expression in the context of breast tumorigenesis. Remarkably, increased Cyr61 protein expression was observed in greater than 50% of primary breast tumor lysates that were progesterone receptor (PR)+ but estrogen receptor negative. Taken together, our data suggest that in addition to its proangiogenic activity, Cyr61 may be a novel mediator of progesterone activity in enhancing growth-factor-driven tumor growth in breast cancer.

Key Words: Progesterone; progestin; Cyr61; growth factor; breast cancer.

Introduction

Presently, breast cancer is the leading cause of cancer death in nonsmoking women and with increasing morbidity continues to be a major health issue in the United States (reviewed in ref. *1*). Given that the mammary gland is a physiological target of estrogen and progesterone, it appears that ovarian sex steroids also regulate both tumorigenesis and progression (reviewed in ref. *2*). This is, in part, the result of the actions of estrogen on the induction of growth factors, transcription factors, and cell cycle regulators, which, along with genetic alterations, contribute to the development of breast malignancies (reviewed in ref. *3*). In accordance with estrogen-driven carcinogenesis, approx 70–80% of primary breast tumors overexpress estrogen receptor (ER), and selective estrogen receptor modulators, such as tamoxifene, that antagonize the effects of estrogen are recommended as a first-line treatment during the earlier stages of malignancy.

Although the role of progesterone in normal mammary epithelial cell proliferation, development, and differentiation is well established, its contribution to breast tumorigenesis remains controversial. For example, despite the observation that 50–70% of primary breast tumors overexpress progesterone receptors, high doses of progestins are often used as second-line therapy during disease management as a result of their antiproliferative activities *(4)*. However, it has been demonstrated that, at the molecular level, progesterone and progestins can prime and sensitize breast cancer cells to the mitogenic effects of growth factors such as epidermal growth factor (EGF) and insulin-like growth factor (IGF) (*5,6*; reviewed in ref. *7*). This is, in part, the result of the induction or activation of cell cycle regulatory genes, growth factors, extracellular matrix proteins, signaling molecules, and growth factor receptors (reviewed in ref. *7*). Therefore, gene targets that prime tumor cell growth by augmenting the activity of growth factors may indirectly mediate the proliferative effects of progesterone during the earlier phases of tumorigenesis. Accordingly, T47D mammary adenocarcinoma cells that overexpress progesterone receptor (PR) are typically used to study progesterone and progestin target genes that regulate tumor cell growth.

An emerging group of serum-inducible immediate–early genes that coordinate diverse roles in development, cell pro-

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liferation, and tumorigenesis collectively belong to the CCN (CTGF/Cyr61/Cef10/NOVH) family. A prototypical member of this family, Cyr61, was originally identified by differential hybridization screening of a cDNA library of serum-stimulated BALB/c 3T3 fibroblasts *(8)*. In addition to serum, Cyr61 is rapidly activated within minutes at the transcriptional level in fibroblasts after treatment with EGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) *(8–11)*. Rapid and transient induction of Cyr61 by vitamin D_3, tumor necrosis factor-α (TNF-α), as well as interleukin-1 (IL-1) has also been reported in human fetal osteoblasts *(12)*. Other known inducers of Cyr61 expression are carbachol-activated muscarinic acetylcholine receptors 1 and 3 (m1 and m3), thrombin, and factor V11a *(13,14)*.

Cyr61 is a secreted, cysteine-rich heparin-binding protein that associates with the cell surface and the extracellular matrix, biochemical features that resemble the Wnt-1 proto-oncogene and a number of known growth factors *(15)*. The human Cyr61 cDNA encodes a protein of 379 a.a. (amino acid) in length with a molecular mass of 42 kDa, and the gene is located on the short arm of chromosome 1 (1p22–31) *(16,17)*. All CCN proteins (1) display a high degree of conservation among family members and across species, (2) are cysteine rich and structurally similar to extracellular matrix-associated molecules, (3) are composed of multifunctional modular domains, and (4) have been shown to mediate functions as diverse as cell adhesion, cell migration, mitogenesis, cell survival, and differentiation (reviewed in ref. *18*). Although Cyr61 has been shown to be involved in a number of physiological processes such as chondrogenesis *(19,20)*, neuronal differentiation *(21)*, wound healing, and fracture repair *(22,23)*, it was originally characterized functionally as a proangiogenic factor *(24–26)*.

Work in our laboratory has focused on sex steroid regulation of human Cyr61 and its role in hormonal carcinogenesis. For example, E_2 induces Cyr61 in ovariectomized rodent uteri in vivo *(27)*, human myometrium ex vivo *(28)*, and in the ER+ mammary adenocarcinoma cell line, MCF-7, in vitro *(29–31)*. Given the high degree of homology between murine and human orthologs, cursory analysis of the murine promoter identified a progesterone response element from –1592 to –1617, suggesting that progesterone may also regulate human Cyr61. Therefore, the goal of this study was to determine if Cyr61 is a progesterone target gene in T47D cells and, more importantly, to determine its role in mammary tumor epithelial cell growth by assessing the effects of anti-Cyr61 neutralizing antibodies on progestin-mediated DNA synthesis and cell proliferation. We provide evidence in this report that Cyr61 is induced by the progestin R5020 and may serve as a cofactor in enhancing the effects of EGF on tumor cell growth.

Results

Cyr61 is Selectively Upregulated by R5020 in T47D Mammary Adenocarcinoma Cell Lines

In order to further extend our analysis of hormonal regulation of Cyr61, the expression pattern of Cyr61 in P_4-responsive T47D cells that overexpress the progesterone receptor (PR) was assessed. These cells have been characterized previously and expressed 2.3×10^6 sites/cell compared to MCF-7 (4.5×10^5 sites/cell), BT 474 (9.1×10^4 sites/cell), and MDA-MB-231 (PR–) based on whole-cell radiolabel–ligand-binding assays *(32)*. Treatment of T47D cells with R5020 (1.0 n*M*) resulted in a modest induction of Cyr61 mRNA and protein levels, which increased to maximum levels within 8 h and sustained expression beyond 24 h (Fig. 1A,B). Moreover, P_4 treatment of T47D cells resulted in a similar kinetic profile of Cyr61 induction observed with R5020 (data not shown). The upregulation of Cyr61 by R5020 in T47D cells was dose dependent, with maximum expression occurring at 1.0 n*M* (EC_{50} = 0.3 n*M*) (Fig. 1C,D). In addition to R5020, Cyr61 was also upregulated by 10 n*M* 5α-dihydrotestosterone (DHT) in mammary adenocarcinoma cell lines but not by dexamethasone or E_2 (Fig. 2A,B). Analysis of Cyr61 message levels in T47D cells at 0, 0.5, 1.0, 2.0, 4.0, and 8.0 h of either dexamethasone or E_2 treatment did not result in increased expression (data not shown), indicating that even at earlier time-points, Cyr61 was not expressed. The DHT induction occurred in an immediate–early fashion, with maximal expression occurring within 1 h, and was completely inhibited by 2-OH-flutamide, indicating that the upregulation of Cyr61 was dependent on AR activity (data not shown). However, the maximum levels of Cyr61 protein upregulated by 10 n*M* DHT was less than half of that observed with 1 n*M* R5020, suggesting that in T47D cells, progesterone is the primary inducer of this gene (Fig. 2B).

PR Regulates Cyr61 at the Transcriptional Level

Induction of Cyr61 by R5020 in T47D cells is primarily mediated through PR because the antiprogestin RU486 abrogated expression by greater than 90% (Fig. 3A,B). Furthermore, in PR– MDA-MB-231, adenocarcinoma cells expression of Cyr61 was not upregulated by R5020, thereby demonstrating that one of the requirements for Cyr61 induction by R5020, may be the presence of PR (data not shown). The lack of induction in MDA-MB-231 cells was not the result of the inability to induce Cyr61 because EGF upregulated Cyr61 to maximum levels (10-fold above basal levels) within 30 min (data not shown). The lack of induction of Cyr61 in MDA-MB-231 cells by R5020 but not by EGF also suggests that PR signaling pathways that crosstalk with growth factor pathways and drive Cyr61 expression may be altered (reviewed in ref. *7*). Inhibition of Cyr61 upregulation was not observed in T47D cells cotreated with

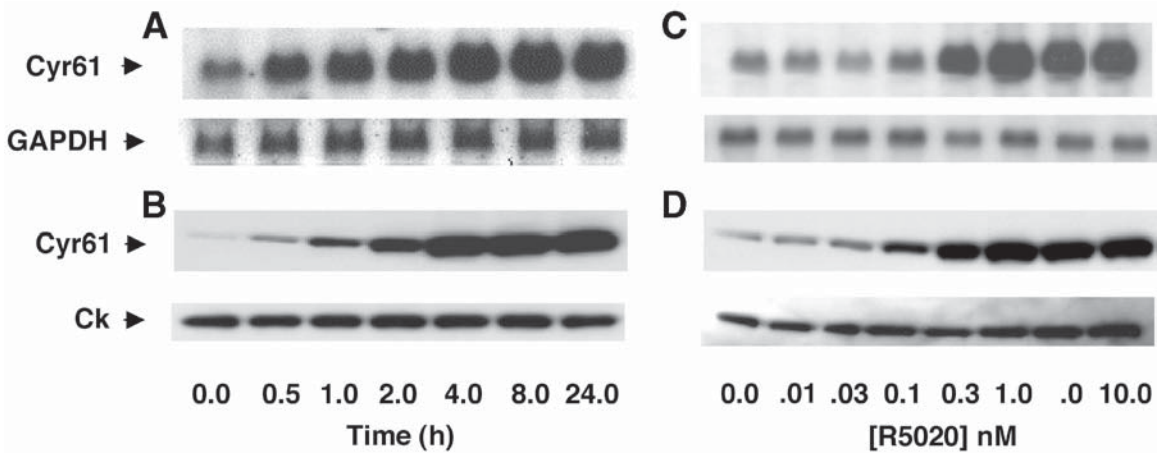

Fig. 1. Induction of Cyr61 mRNA and protein in T47D cells by R5020. Representative membrane blots of total RNA (**A,C**) and protein (**B,D**) isolated from T47D cells after treatment with 1.0 n*M* R5020 at the indicated time-points and analyzed by Northern and Western analysis, respectively, as described in the Materials and Methods section. Detection of Cyr61 proteins was accomplished by using an affinity-purified anti-Cyr61 polyclonal antibody and enhanced chemilumenescent, as described in the Materials and Methods section. Membranes were reprobed with GAPDH or cytokeratin (Ck) to monitor equal sample loading for Northern and Western blots, respectively. Each experiment was repeated at least three times.

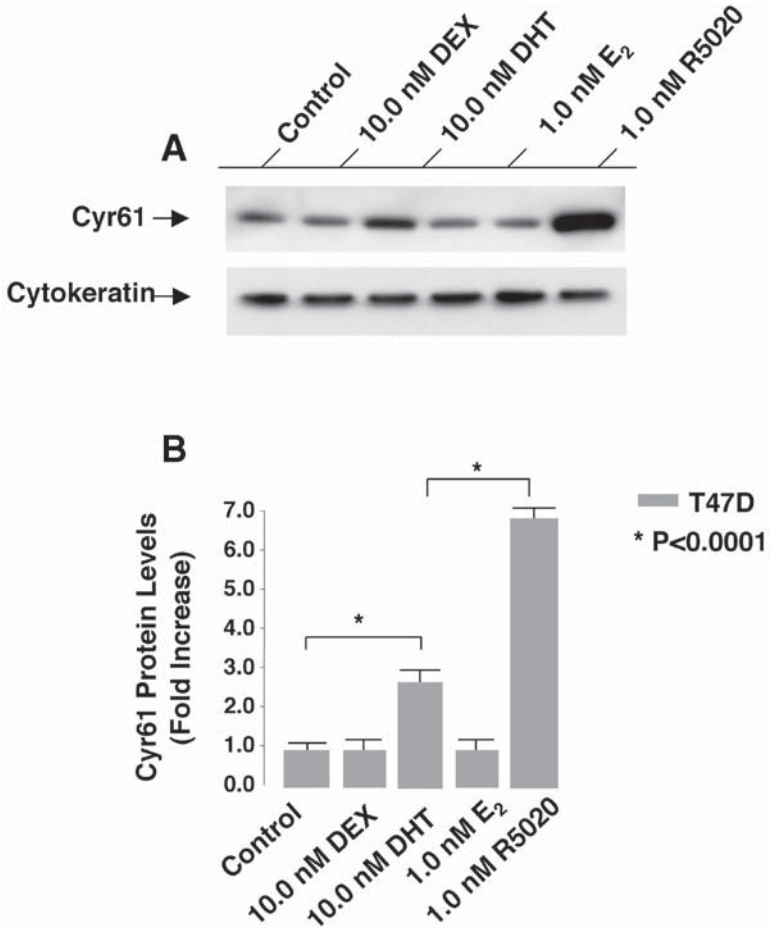

Fig. 2. Steroid hormone specificity for Cyr61 induction in T47D cells. (**A**) Representative Western blots of protein extracts from T47D cells treated for 1.0 h with 10 n*M* dexamethasone (DEX), 10 n*M* DHT, and 1 n*M* R5020 for 4.0 h. Detection of Cyr61 proteins was accomplished by using an affinity-purified anti-Cyr61 polyclonal antibody and enhanced chemilumenescent as described in the Materials and Methods section. (**B**) Cyr61 protein levels were quantitated by densitometric analysis utilizing a Bio-Rad molecular imager. Numerical values are based on the relative optical density (OD) of the band size and the total amount of Cyr61 was normalized to the level of cytokeratin. The fold expression of Cyr61 was calculated by dividing the ratio Cyr61/cytokeratin in treated cells by untreated cells. Values represent the mean ± SEM for three experiments. *Significant increase in levels compared to untreated controls.

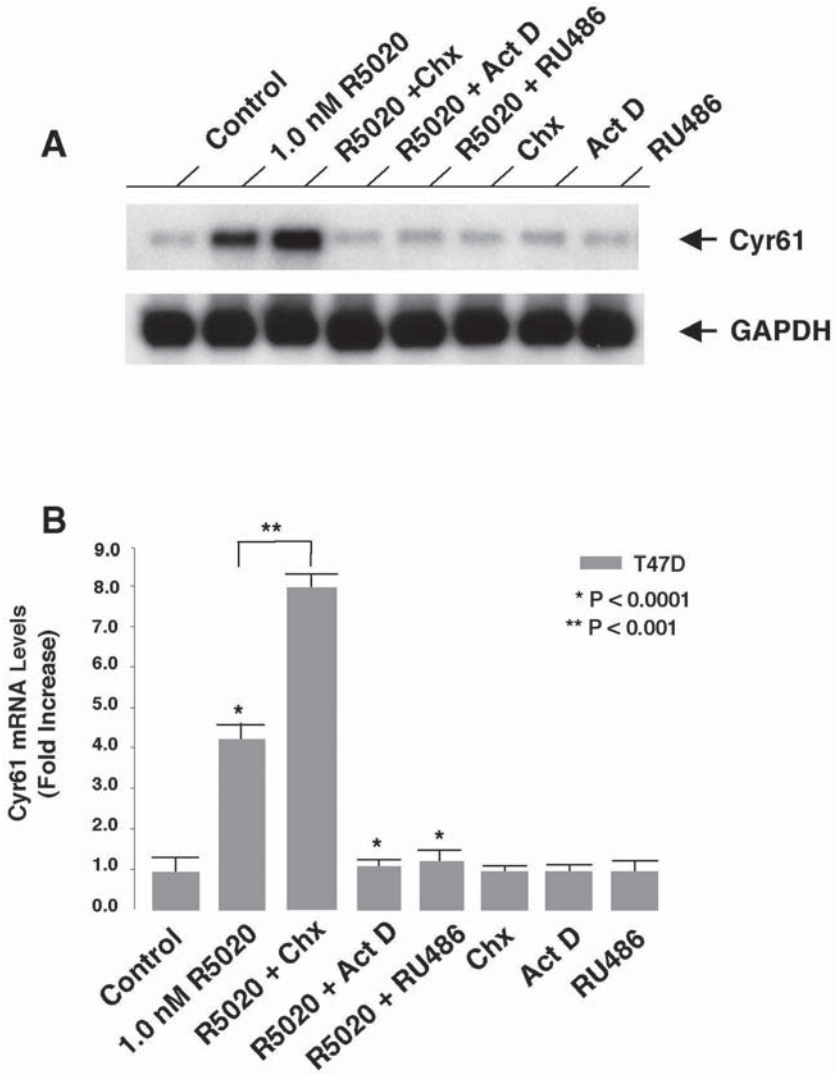

Fig. 3. Progesterone receptor specificity and transcriptional regulation of Cyr61 in T47D cells. (**A**) Representative Northern blot of total RNA isolated from T47D cells following treatments with 1.0 n*M* R5020, 10 µg/mL cyclohexamide (Chx), 1 µg/mL actinomycin D (Act D), or 100 n*M* RU486 for 4.0 h. Membranes were subsequently reprobed for GAPDH in order to verify equivalent sample loading. (**B**) The fold expression of Cyr61 mRNA was calculated by dividing the ratio Cyr61/GAPDH in treated samples to untreated control samples; values represent the mean ± SEM from three independent experiments. *Significant increase in levels compared to untreated controls. **Significant increase in Cyr61 overexpression compared to R5020 treatment alone.

10 µg/mL of the protein synthesis inhibitor cycloheximide (Chx) and 1 n*M* R5020, suggesting that de novo protein synthesis was not required for steroid induction (Fig. 3A,B). In contrast, significant overexpression of Cyr61 was observed upon cotreatment with Chx and R5020 when compared to R5020 treatment alone (Fig. 3B). The increase in Cyr61 mRNA levels after Chx cotreatment may be the result of the enhanced expression of transcription factors or augmentation of signal transduction factor activities such as JNK that have been shown to effect the expression of enzymes such as cycloxogenase 2 (COX-2) *(32)*. Alternatively, increased Cyr61 expression upon Chx and R5020 treatment may be the result of the lack of synthesis of labile proteins that regulated transcription or mRNA turnover (reviewed in ref. *33*). However, cotreatment of R5020 with 1 µg/mL actinomycin D completely blocked the steroid induction, implying that PR mediates Cyr61 expression at the transcriptional level (Fig. 3A,B). Therefore, Cyr61 is primarily induced by progesterone and progestins through PR in T47D cells at the transcriptional level.

Cyr61 Expression in PR+ Breast Tumors

Given that Cyr61 is induced in breast carcinoma cell lines in vitro, we investigated whether overexpression is observed in primary breast tumors in vivo. For this study, tumor biopsies were extracted from female patients ($n = 20$) diagnosed with stage II invasive ductal carcinoma of the breast following internal review board approval at Berkshire Hospital

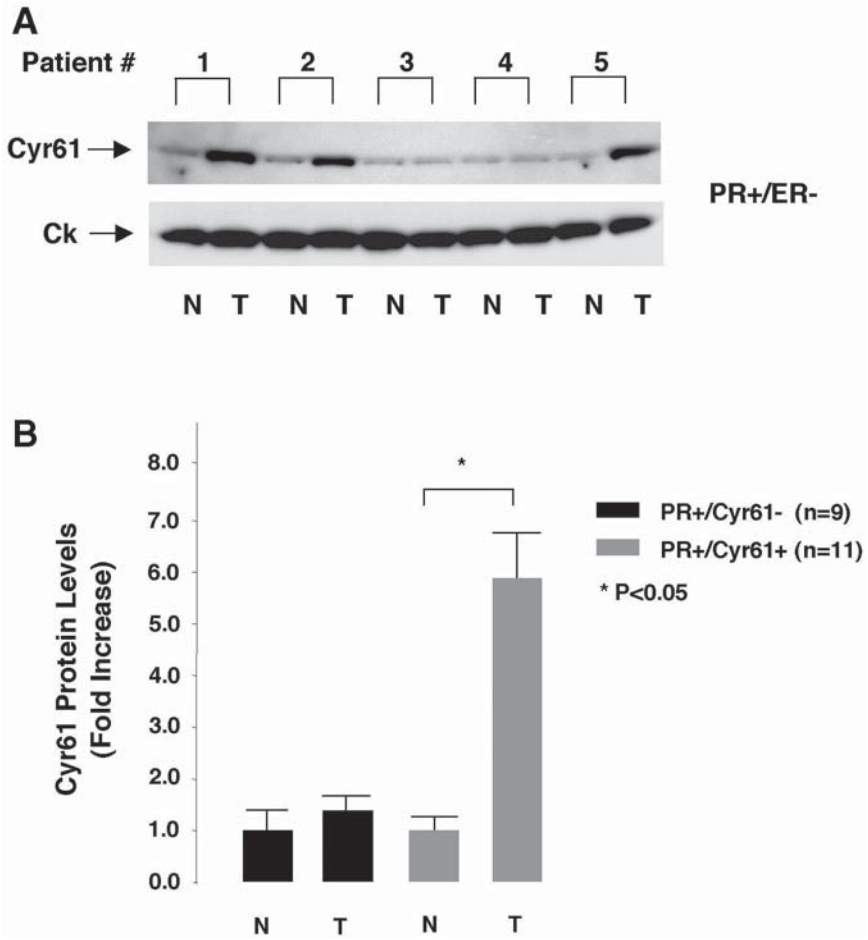

Fig. 4. Analysis of Cyr61 protein expression in human breast tumors. (**A**) Representative Western blot of protein lysates extracted from breast tumors (T) and autologous normal mammary controls (N) from five patients (#1–#5) that were PR+/ER– (n = 20). Detection of Cyr61 proteins was accomplished by using an affinity-purified anti-Cyr61 polyclonal antibody and enhanced chemilumenescent, as described in the Materials and Methods section. (**B**) The fold expression of Cyr61 was calculated by dividing the relative OD levels of Cyr61 normalized to cytokeratin in breast tumor lysates by the levels in matched healthy mammary controls also normalized to cytokeratin. Values represent the mean ± SD for 20 patients. PR+/Cyr61–: breast tumors in which Cyr61 levels are not significantly elevated compared to autologous normal mammary tissue; PR+/Cyr61+: breast tumors in which Cyr61 levels are significantly elevated compared to autologous normal mammary tissue. *Significant increase in levels compared to normal matched tissue controls, $p < 0.05$.

(Berkshire, MA). Patients were between the ages of 42 and 68 yr with a median age of 54. Matched healthy mammary tissues surrounding the breast tumors were also extracted from the same breast to serve as autologous normal controls. Healthy tissue status was confirmed by lack of positive immunostaining for proliferation markers such as PCNA and by morphological examination confirming the presence of normal intact mammary glands. In addition, tumors were classified as PR+ and ER– by immunohistochemical staining of formalin-fixed tumor biopsies using mono-specific anti-PR and anti-ER antibodies that did not crossreact with either receptor. Elevated Cyr61 protein levels were detected in 11 out of 20 PR+/ER– breast tumors compared to autologous normal healthy breast tissue. (Fig. 4A and Table 1).

Although 4 out of 20 patients were postmenopausal, none were presently administered hormone replacement therapy nor were they treated with medroxyprogesterone acetate (MPA) or other progestins. Overall, increases in Cyr61 protein levels in 11 out of 20 breast tumors were 2.5-fold to 7.7-fold higher than that observed in matched normal controls (Fig. 4B and Table 1). The observation that Cyr61 is overexpressed in early-phase invasive ductal tumors that are PR+ but ER– suggests that its expression may be involved in progesterone-mediated tumor growth in vivo.

Cyr61 Is Necessary for Steroid and Growth-Factor-Dependent DNA Synthesis in Breast Carcinoma Cells

In order to determine the function of Cyr61 in the context of steroid and growth-factor-dependent tumor cell growth, DNA synthesis was monitored in the presence of anti-Cyr61 neutralizing antibodies. These antibodies were affinity purified and detected a single band at 42 kDa, which is the molecular weight of Cyr61. Treatment of T47D cells with 10 μg/mL of anti-Cyr61 for 18 h reduced 1 n*M* R5050 and 20 ng/mL

Table 1
Summary of Cyr61 Expression in Stage II Invasive Ductal Carcinoma Breast Tumors

					Relative Cry61 levels (OD)			
Patient #	Age	Stage	PR status	ER status	Matched tissue control	Breast tumor	expression	*p*-Value
1	42	II	Positive	Negative	0.86	5.61	6.52	<0.05
2	50	II	Positive	Negative	0.95	3.32	3.49	<0.05
3	61	II	Positive	Negative	0.56	0.57	1.02	ns
4	47	II	Positive	Negative	0.43	0.49	1.14	ns
5	52	II	Positive	Negative	0.76	4.1	5.39	<0.05
6	46	II	Positive	Negative	0.89	2.12	2.38	<0.05
7	66	II	Positive	Negative	1.1	2.01	1.83	ns
8	62	II	Positive	Negative	0.35	0.43	1.23	ns
9	58	II	Positive	Negative	0.56	0.61	1.09	ns
10	64	II	Positive	Negative	0.98	3.2	3.27	<0.05
11	53	II	Positive	Negative	0.63	3.56	5.65	<0.05
12	61	II	Positive	Negative	0.88	0.89	1.01	ns
13	43	II	Positive	Negative	0.84	2.12	2.52	<0.05
14	58	II	Positive	Negative	0.93	1.02	1.10	ns
15	64	II	Positive	Negative	0.76	0.67	0.88	ns
16	53	II	Positive	Negative	0.87	3.89	4.47	ns
17	52	II	Positive	Negative	0.73	5.6	7.67	<0.05
18	49	II	Positive	Negative	0.92	4.87	5.29	<0.05
19	68	II	Positive	Negative	0.69	0.72	1.04	ns
20	49	II	Positive	Negative	0.91	3.98	4.37	<0.05

Note: PR and ER status was determined immunohistochemically as described in the Materials and Methods section. Cyr61 protein levels detected by Western analysis were quantitated by densitometric analysis utilizing a Bio-Rad molecular imager. Numerical values are based on the optical density (OD) of the band and the relative levels of Cyr61 were calculated after normalization to cytokeratin. Values derived from densitometric measurements of protein levels in breast tumor lysates and matched controls were analyzed using SAS statistical software for significance using the paired *t*-test method for two groups. The fold changes in the levels of Cyr61 protein and mRNA were considered significant if *p*-values were less than 0.05. ns = not significant.

EGF induced DNA synthesis by 30% and 35%, respectively (Fig. 5). Controls for the bromodeoxyuridine (BrdU) assay included cotreatment with 10 µg/mL nonimmune IgG (NI IgG), which had no effect, and 10 µg/mL blocking peptide, which completely reversed the neutralizing effects of anti-Cyr61 (Fig. 5). Therefore, Cyr61 appears to be necessary for initiation of the cell cycle by effecting progestin and growth-factor-mediated entry into S-phase.

Cyr61 Is Necessary for the Synergistic Actions of R5020 and EGF in T47D Cell Proliferation

In T47D cells, progestins and P_4 have biphasic effects on proliferation; a single dose of P_4 results in increased proliferation within the first 24 h, with a return to basal levels by 48 h. However, consecutive P_4 treatments following the initial pulse does not increase T47D cell proliferation, resulting in a state of cellular refractiveness *(7)*. However, cotreatment with EGF after R5020 stimulation does result in a synergistic increase in proliferation, indicating that progestins sensitize T47D cells for EGF-driven growth *(7)*. Note that EGF alone has minimal effects on growth in a 5-d continuous-treatment assay underscoring the need for P_4 or progestins to sensitize T47D cells for EGF-driven growth *(7)*. Given that Cyr61 mRNA and protein levels remain elevated beyond 48 h, we hypothesized that prolonged expression may play a role in priming T47D cell growth after R5020 stimulation. To test this hypothesis, T47D cells were cultured for 5 consecutive days with 1 n*M* R5020, 20 ng/mL EGF, or a combination of R5020 and EGF in the presence or absence of anti-Cyr61 neutralizing antibodies. Within 24 h of R5020 treatment, a 10% decrease in T47D cell proliferation was observed in the presence of anti-Cyr61 neutralizing antibodies (Fig. 6A). However, the effects of anti-Cyr61 neutralizing antibodies did not have any effect on EGF-treated T47D cells (Fig. 6B). Interestingly, anti-Cyr61 neutralizing antibodies diminished T47D cell proliferation significantly by 15% within 48–72 h of R5020 and EGF cotreatment (Fig. 6C). Furthermore, a 30% reduction in cell proliferation was observed between 96 and 120 h in the presence of anti-Cyr61 neutralizing antibodies upon cotreatment

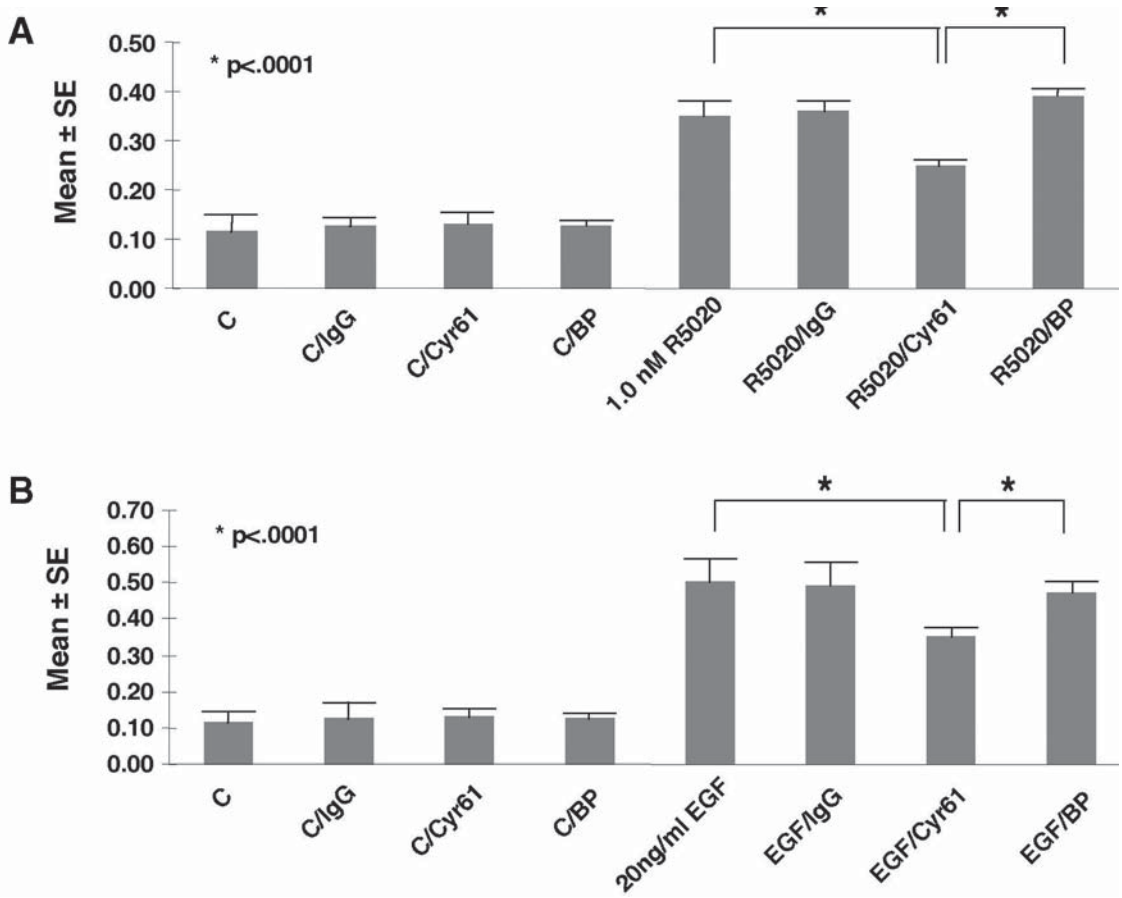

Fig. 5. Effects of anti-Cyr61 neutralizing antibodies on R5020 and EGF-dependent DNA synthesis in T47D cells. DNA synthesis in the T47D cell was measured by BrdU incorporation after treatment with 1.0 n*M* R5020 (**A**) or 20 ng/mL EGF (**B**) for 18 h. In addition to R5020 and growth factors alone, cells were either untreated (**C**), coincubated with 10 µg/mL anti-Cyr61 polyclonal antibodies (α-Cyr61), 10 µg/mL NI IgG, or a combination of α-Cyr61 and 10 µg/mL peptide epitope (α-Cyr61/P) as an antibody blocking reagent. Numerical values represent the OD at an absorbance of 450 nm ± SEM. Experiments were performed in quadruplicates and repeated three times. Absorbance values are represented as the mean ± standard error (SE). *Significant decrease in DNA synthesis compared to untreated controls. $p < 0.0001$.

with R5020 and EGF. The latter effects were PR dependent because cotreatment with 100 n*M* RU486 completely abolished the synergism between R5020 and EGF (Fig. 6D). More importantly, the neutralization activity of anti-Cyr61 antibodies were completely reversed in the presence of 10 µg/mL blocking peptide, suggesting that the reduced proliferation was Cyr61 dependent. Thus, Cyr61 may act as a cofactor in sensitizing T47D cells to the growth-promoting effects of EGF when induced by progestins or P_4 in vitro.

R5020 and EGF Synergistically Increase Cyr61 Protein Levels in T47D Cell Lines

Because neutralization of Cyr61 resulted in decreased proliferation, we sought to determine whether R5020 synergized biochemically with EGF to augment Cyr61 expression in T47D cell lines. A 5-d continuous treatment with R5020 resulted in a seven-fold increase in Cyr61 protein levels within 24 h and a 10-fold elevation was sustained over a 4-d period (Fig. 7A). Although EGF treatment results in a rapid increase in Cyr61 protein levels within 30 min, downregulation is observed within 24 h and consecutive treatments of EGF after 5 d does not elevate Cyr61 protein levels further *(21)* (Fig. 7B). However, cotreatment of T47D cells with 1 n*M* R5020 and 20 ng/mL EGF resulted in a fourfold synergistic increase in Cyr61 protein levels by 24 h and continued to rise over a 4-d period (Fig. 7C). This synergistic effect was PR mediated because cotreatment with 100 n*M* RU486 abolished augmentation of Cyr61 expression by R5020 and EGF (data not shown). Taken together, the synergistic actions of EGF and R5020 on elevating and sustaining Cyr61 expression may, in part, contribute to the priming effects of progesterone on T47D cells and growth-factor-driven proliferation. Thus, Cyr61 may represent a novel progesterone-regulated gene that serves as a potential cofactor for EGF activity in T47D cells.

Discussion

The development and progression of breast cancer occurs in a methodical process orchestrated by (1) the activation

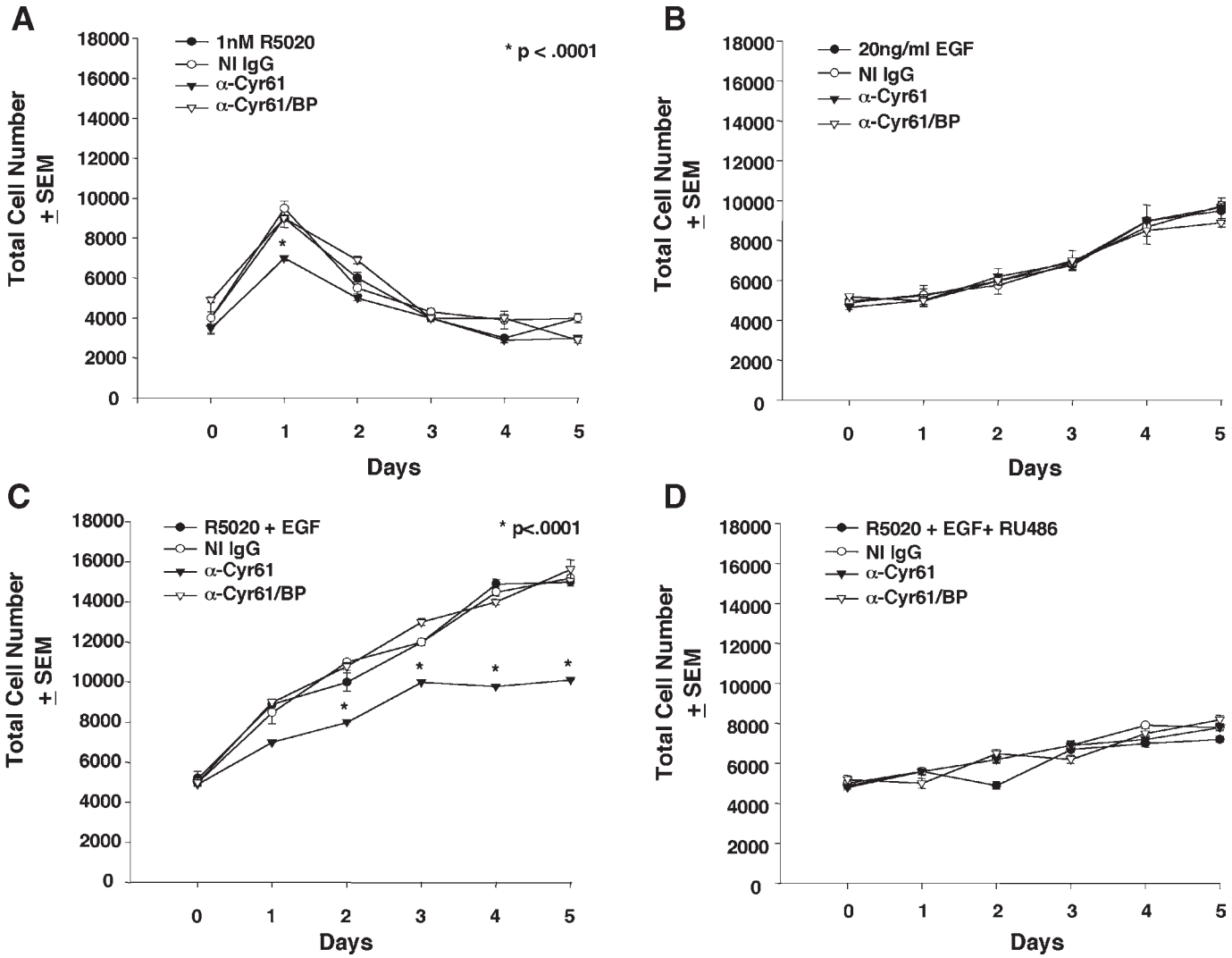

Fig. 6. Effects of anti-Cyr61 neutralizing antibodies on R5020 and EGF-driven cell proliferation in T47D cells. Cell proliferation assays were conducted over a period of 5 consecutive days in culture in the presence or absence of 1.0 n*M* R5020 (**A**), 20 ng/mL EGF (**B**), a combination of R5020 and EGF (**C**), or a combination of R5020, EGF, and 100 n*M* RU486 (**D**). In addition, T47D cells were coincubated with either 10 µg/mL α-Cyr60, 10 µg/mL NI IgG, or a combination of α-Cyr61 and 10 µg/mL peptide epitope (α-Cyr61/BP) as an antibody blocking reagent. Culture medium was changed every day containing the same treatments of steroids, antisteroids, growth factors, and antibodies. At the end of treatments, monolayers were trypsinized, combined with cells in suspension, and counted on a Coulter counter. Cell proliferation experiments were performed in quadruplicates and repeated three times. Numerical values represent total cell numbers ± SEM. *Significant decrease in cell proliferation compared to NI IgG controls. $p < 0.0001$.

of oncogenes and loss of tumor suppressor genes, (2) growth priming of tumor cells by sex steroid hormones via the induction of cell cycle regulatory genes, transcription factors, and cell-adhesion molecules, and (3) the autocrine and/or paracrine action of growth factors (reviewed in ref. *3*). In the mammary gland, ovarian sex steroids tightly regulate development. Moreover, the induction of estrogen and progesterone target genes in mammary epithelial cells that act as local mediators may be critical for neoplastic generation, and, subsequently, tumorigenesis. Given that, we provide evidence that the angiogenic factor Cyr61, a serum-inducible immediate–early gene, is a progesterone as well as growth factor target gene that is transcriptionally regulated and is necessary for hormone-dependent cell growth in human T47D mammary adenocarcinoma cells.

Although the proliferative nature of progesterone's action on normal ductal and lobular epithelial cells during mammary gland development and lactation is well defined, its role in breast tumorigenesis and progression remains controversial. Indeed, progestins such as MPA are often used as second-line therapy in breast cancer management and is supported by the observation that in vitro progesterone and progestins appear to act in a biphasic fashion to rapidly induce proliferation and then subsequently inhibit growth after consecutive treatments (reviewed in refs. *7* and *34*). Accordingly, T47D mammary adenocarcinoma cells that overexpress

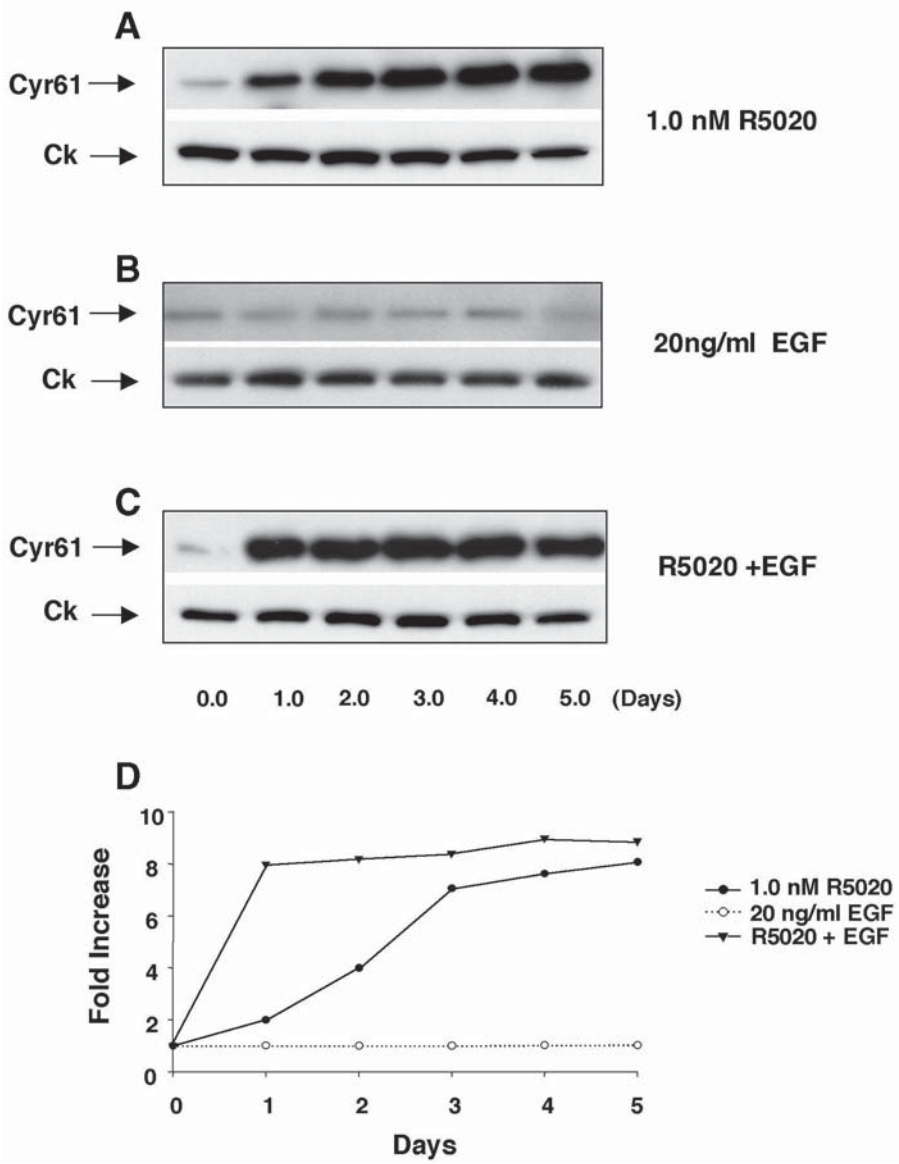

Fig. 7. Cyr61 expression in EGF- and R5020-treated T47D cells. Representative Western blots of T47D cells treated with 1 n*M* R5020 (**A**), 20 ng/mL EGF (**B**), or a combination of EGF and R5020 (**C**) for 0, 0.5, 1.0, 2.0, 4.0, 8.0, and 24.0 h. Detection of Cyr61 proteins was accomplished by using an affinity-purified anti-Cyr61 polyclonal antibody and enhanced chemilumenescent, as described in the Materials and Methods section. (**D**) Cyr61 protein levels were quantitated by densitometric analysis utilizing a Bio-Rad molecular imager. Numerical values are based on the relative OD of the band size and the total amount of Cyr61 was normalized to the level of cytokeratin. The fold expression of Cyr61 was calculated by dividing the ratio Cyr61/cytokeratin in treated cells by untreated cells. Values represent the mean ± SEM for three experiments.

PR represents a commonly employed model to identify progesterone effector pathways and target genes that modulate tumor cell growth following progestin treatment. In this model, cells can overcome progestin inhibition after cotreatment with EGF, whereas EGF treatment alone results in a minimal T47D growth response (reviewed in ref. *7*). The inhibitory action of progestins is, in part, the result of the upregulation of the cell cycle regulators and the cyclin-dependent kinase inhibitors p21 and p27 *(35,36)*. However, progesterone target genes such as EGFR, c-fos, c-jun, and cyclin D1 may "prime" mammary adenocarcinoma cells toward the proliferative actions of growth factors such as EGF during the earlier phases of tumor growth *(5,37)*. One mechanism in which progestins may sensitize breast cancer cells for growth signals is by potentiating EGF-stimulated p42/44 mitogen-activated protein (MAP) kinase, p38 MAP kinase, and JNK activities *(38)*. The latter suggests that crosstalk exists between progesterone and EGF signaling pathways in regulating breast tumor cell growth. In addition, upregulation of matrix metalloproteinases (MMP) and extracellular matrix proteins such as laminin by progesterone may assist in tumor extravasation into the stromal layer

as tumor cells become progressively invasive during metastasis (reviewed in ref. *39*). Therefore, it is conceivable that P_4 or progestins exert their proliferative actions by induction of target genes that modulate either breast tumor growth or invasion.

Interestingly, other steroid hormones such as E_2 also induce Cyr61 in mammary adenocarcinoma cell lines that overexpress ER, implying that this extracellular matrix protein may be a general mediator of ovarian-hormone-dependent tumor growth *(29–31)*. However, it is noteworthy that maximal levels of both Cyr61 mRNA and protein are observed between 8 and 48 h after a single progestin treatment compared to the immediate–early kinetics observed with other inducers such as serum, bFGF, EGF, HB-EGF, vitamin D_3, TNF-α and IL-1 *(8–12)*. The induction of Cyr61 paralleled in time the increase in T47D cell proliferation after progestin and growth factor cotreatment, suggesting that delay expression may have functional significance.

Based on previous works, the role of Cyr61 in mammalian cell development indicated that aberrant expression might predispose cells toward dysregulated growth, such as in tumorigenesis. Indeed, overexpression of Cyr61 has been observed in several human cell lines derived from human bladder papilloma, colon adenocarcinoma, melanoma, and meduloblastoma *(24)*. In some instances, Cyr61 is thought to promote tumorigenesis. For example, transfection of a Cyr61 expression vector into the gastric adenocarcinoma cell line RF-1, which does not express Cyr61, increases these cells' tumorigenicity in vivo *(24)*. In addition, a study by Xie et al. has demonstrated that mammary adenocarcinoma cells that overexpress Cyr61 are capable of anchorage-independent growth and migration in the absence of hormone or growth factor *(30)*. Conversely, Cyr61 is shown to be downregulated in uterine leiomyoma smooth muscle cells ex vivo, the epithelium of prostate cancer biopsies, and an embryonal rhabdomyosarcoma cell line, RD, suggesting that it may also function as a tumor suppressor or promote cellular differentiation *(28,40,41)*. Indeed, a recent report by Tong et al. have demonstrated that in non-small-cell lung cancer lines (NSCLC) NCI-H520 and NCI-H460, which have no endogenous Cyr61, formed 60–90% fewer colonies after stable transfection of Cyr61 expression vector when compared to the empty vector control *(42)*. The decrease in cell proliferation in the H520-Cyr61 and H560-Cyr61 NSCLC lines was associated with G1 arrest and upregulation of known tumor suppressors such as p53, $p21^{WAF1}$, and pRB2/p130 *(42)*. Interestingly, rCop1, a member of the CCN family and homolog of Cyr61, has been shown to be a negative regulator of cell transformation when overexpressed and thus behaves similarly to tumor suppressors *(43)*. Therefore, depending on the cell and tumor types, variable expression of Cyr61 may either play a positive or negative role in dysregulated cell growth.

Because our studies demonstrate that anti-Cyr61 neutralizing antibodies can inhibit progestin as well as growth-factor-dependent DNA synthesis and cell proliferation by greater than 30%, Cyr61 appears to play a novel role in T47D mammary adenocarcinoma cell growth in vitro. As a ligand for the integrin receptors $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_5$ and $\alpha_6\beta_1$ *(25, 44,45)*, it is plausible that Cyr61 may emit its growth effects through integrin signal transduction or through a yet to be identified cognate receptor that may be expressed on breast tumor epithelial cells. Indeed, a recent study by Chen et al. has demonstrated that in primary human fibroblasts, Cyr61 binds to $\alpha_6\beta_1$ and activates integrin-driven intracellular signaling components such as focal adhesion kinase, paxillin, and Rac in an immediate–early fashion, followed by sustained activation of p42/p44 MAPKs *(46)*. Given the modular structure of the Cyr61 protein, it is conceivable that different domains of Cyr61 can dictate multiple functions by differential binding to various integrin receptors, thereby coordinating cell proliferation, migration, and adhesion—critical pathways in tumor growth and progression *(47)*.

More relevant to breast tumor growth, Cyr61 has been shown to bind to $\alpha_v\beta_3$ receptors in MCF-7 cells and is necessary for heregulin-mediated chemomigration *(48)*. Furthermore, overexpression of Cyr61 in MCF-7 cells leads to activation of MAPK and AKT signal transduction pathways, which, in turn, induces anchorage-independent growth *(49)*. Moreover, Cyr61 overexpression led to increased MMP9 expression and activity, thereby inducing MCF-7 transformation in a Matrigel-outgrowth assay *(49)*. The hypothesis that Cyr61 may act as a growth factor in breast cancer cells is further strengthened by the observation that overexpression of Cyr61 in MCF-12A normal breast cells and MCF-7 adenocarcinoma cells induced tumor formation and promoted neovascularization in nude mice xenographs *(30, 50)*. Alternatively, because Cyr61 is a growth-factor-binding protein and augments growth-factor-dependent DNA synthesis *(51)*, it may promote mammary tumor cell growth by directly interacting with certain growth factors to enhance their bioactivity. Indeed, the observation in this study that Cyr61 inhibition effected EGF-driven T47D cell growth after progestin treatment suggests that its function may be codependent on growth factor activities. Taken together, Cyr61 overexpression in mammary adenocarcinoma cells leads to increased growth and migration both in vitro and in vivo. These events may be mediated via integrin receptors and subsequent activation of MAPK and AKT signal transduction pathways.

Invasive ductal carcinoma (IDC) is the most common type of breast cancer and accounts for 80% of known cases. Previous work in our laboratory had observed selective overexpression of Cyr61 in mammary tumor epithelial cells in 70% of a subset of ER+/EGFR+ IDC breast tumors *(29)*. Overexpression of Cyr61 protein in 50% of the IDC patients studied in this report whose tumors were PR+ further extends our hypothesis that Cyr61 may be an important and general mediator of hormone-dependent breast tumor development in vivo. Furthermore, recent reports by have demonstrated

that Cyr61 is overexpressed in IDC tumors at multiple stages that are ER+ but tends to be associated with more advanced disease *(30,48,50)*. As our in vitro results demonstrated, overexpression of Cyr61 in PR+ breast tumors may be the result of the ability of P_4 to synergize with growth factors such as EGF to sustain Cyr61 expression and prolong its mitogenic activity. Alternatively, mutations within progesterone or growth-factor-dependent activated elements that result in aberrant promoter activity, gene amplification, or increased mRNA stabilization may also lead to Cyr61 overexpression. Growth factor upregulation of Cyr61 in steroid-responsive breast tumors may also predispose a subset of tumor epithelial cells toward continued dysregulated proliferation, as their growth becomes hormone independent or metastatic in the malignancy process. Furthermore, upregulation of Cyr61 in PR+ breast tumors by P_4 or progestins may augment the bioactivity of growth factors that, through a positive feedback, would, in turn, result in waves of Cyr61 expression and continued cell proliferation.

To summarize, Cyr61 is regulated by the progestin R5020 and P_4 in a delayed fashion and is necessary for hormone-dependent DNA synthesis and EGF-driven cell proliferation in T47D breast adenocarcinoma cells in vitro. Similar to EGF, Cyr61 may be one of many progesterone-regulated genes that serves as a cofactor in sensitizing breast tumor cells to the proliferative actions of growth factors. Accordingly, Cyr61 expression in breast cancer may conceptually orchestrate multiple activities to (1) promote tumor cell proliferation in an autocrine and/or paracrine fashion by augmenting growth factor activity *(11,48)* or transmitting proliferative signals via intergrin-mediated signal transduction pathways *(45,47)*, (2) coordinate tumor epithelial migration and progression chemokinetically *(30,48)*, and (3) regulate tumor neovascularization in a paracrine fashion as a pro-angiogenic factor *(24–26,30)*. The modular domain structure of Cyr61 and its localization to the extracellular matrix may account for its ability to concomitantly dictate multiple functions during tumorigenesis, such as cell proliferation and migration. In addition to promoting angiogenesis and cell migration, overexpression of Cyr61 selectively in the ductal epithelium of breast tumors may provide a locally mediated growth advantage through an autocrine and/or paracrine fashion during hormone-driven tumorigenesis and in a paracrine mode during tumor progression.

Materials and Methods

Materials

17β-Estradiol (E_2), progesterone (P_4), 5α-dihydrotestosterone, (DHT), dexamethasone (DEX), cyclohexamide (Chx), and actinomycin D (Act D) were purchased from Sigma–Aldrich (St. Louis, MO). The progesterone receptor agonist R5020 was obtained from NEN Life Science Product, Inc. (Boston, MA). EGF and heparin-binding EGF (HB-EGF) were purchased from R & D Systems, Inc. (Minneapolis, MN). 2-Hydroxyflutamide (2-OH-Flu) was a kind donation from Ligand Pharmaceuticals Inc. (San Diego, CA.). RU486 was obtained from Shanghai Institute of Organic Chemistry (Shanghai, China).

Study Subjects and Tissue Procurement

Breast tumor biopsies and matched normal mammary tissue specimens were obtained from a biorepository (Clinomics Inc., Pittsfield, MA) following informed patient consent and internal review board approval at the Berkshire Hospital (Berkshire, MA). Healthy mammary tissue that served as positive controls were extracted from the surrounding breast tumors and were not contaminated with tumor tissue, as assessed by the lack of positive immunostaining for proliferation markers (PCNA) and intact mammary gland morphology. Patients ($n = 20$) were between the ages of 42 and 68 yr (average, 54 yr) and diagnosed with stage II invasive ductal carcinoma following histological examination. Although 4 out of 20 patients were postmenopausal, none were administered hormone replacement therapy nor were they treated with MPA or other progestins. In addition, tumors were classified as PR+ and ER– by immunohistochemical staining of formalin-fixed tumor biopsies using mono-specific anti-PR and anti-ER antibodies that did not crossreact with either receptor (Sigma Immunochemicals, St. Louis, MO). Tissue specimens were immediately snap-frozen in liquid nitrogen following surgical extraction and stored at –20ºC prior to protein isolation.

Cell Culture

T47D and MDA-MB-231 adenocarcinoma cell lines were obtained from ATCC (Rockville, MD) and propagated in DMEM/F12 Ham's-10 media containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 m*M* Glutamax (Gibco-BRL, Rockville, MD). MDA-MB-231 cells are progesterone receptor negative, whereas in comparison, T47D cells express 2.3×10^6 sites per cell based on whole-cell radiolabel-ligand-binding assays *(32)*. For steroid and/or growth factor treatments, cells were cultured in Phenol Red-free DMEM/F12 media containing 2% charcoal-stripped FBS (Hyclone Inc., Logan, UT).

Cyr61 Antibodies

Two anti-Cyr61 polyclonal antisera were generated at the Lousiana State University Medical Center Core Facilities (Baton Rouge, LA) to peptides corresponding to a.a. 163–229 or a.a. 371–381 of the human Cyr61 as antigens. Peptides were coupled to keyhole-limpet hemocyanin and injected into female New Zealand white rabbits. After preliminary screening of crude antisera by Western analysis of human uterine smooth muscle cell lysates, which contain high basal levels of Cyr61 protein, polyclonal antibodies were purified by affinity chromatography, utilizing their respective peptides as the immunoabsorbent.

Northern Blotting for Cyr61

Total cellular RNA was isolated from cultured adenocarcinoma cells by guanidium isothiocynate lysis followed by phenol/chloroform extraction. Subsequently, total cellular RNA (20 µg) was subjected to electrophoresis in an 1% agarose gel containing 1 *M* formaldehyde and transferred onto nylon membranes by capillary electrophoresis. A 0.41-kb human Cyr61 cDNA fragment was radiolabeled with [α-^{32}P]-dCTP (3,000 Ci/mmol) using the random-primer technique (Rediprime II, Amersham Inc.) and used as the hybridization probe. Relative levels of Cyr61 were normalized to glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) after reprobing membranes with a ^{32}P-radiolabeled oligonucleotide according to manufacturer's protocol (endlabeling kit, Gibco-BRL, Rockville, MD).

Protein Extraction and Immunoblotting for Cyr61

Tissue protein extracts were prepared from breast tumors and matched normal mammary tissue specimens by homogenization in 50 m*M* Tris-HCl, pH8.0, 250 m*M* NaCl, 1.0% Nonidet P-40, 1.0% Trtion-X 100, 2.0% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 1 m*M* EDTA, and a protease inhibitor cocktail containing 10 µg/mL pepstatin, aprotinin, and leupeptin (Sigma–Aldrich). Protein extracts (20 µg) were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions in 10% bis-acrylamide and electrophoretically transferred to polyvinyl difluoride membrane (Immobilon-P; Bio-Rad, Redding, CA). Membranes were blocked with 5% dry milk in TBS/0.1% Tween-20 (TBST), and incubated with anti-Cyr61 pAb (10 µg/mL). Primary antibody binding was detected using a Donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) and an enhanced chemiluminescence detection system (Amersham). All immunoblots were subsequently reprobed with 1 µg/mL of anti-pan-cytokeratin monoclonal antibodies (Sigma–Aldrich) to verify equivalent protein loading.

Cyr61 Neutralization Assays with Anti-Cyr61 Antibodies

Polyclonal antibodies were raised to a 65-a.a. peptide corresponding to the central domain of Cyr61, which was selected based on a lack of homology to other CCN family members and published effectiveness of the antibodies in neutralizing bFGF-mediated DNA synthesis in human microvascular endothelial cells *(51)*. The antibodies were affinity purified and assayed for specificity by Western blot analysis using E_2-treated MCF-7 whole-cell lysates. A major band was detected at 42 kDa, which is the molecular weight of Cyr61, along with a minor band at 77 kDa. For DNA synthesis, T47D and MDA-MB-231 cells were treated with either 1.0 nM R5020, 20 ng/mL EGF, or 20 ng/mL HB-EGF in the presence or absence of 10 µg/mL anti-Cyr61 antibodies for 18 h and bromodeoxyuridine (BrdU) incorporation was measured using the BioTrak enzyme-linked immunosorbent assay (ELISA) kit (Amersham, Arlington Heights, IL) with an horseradish peroxidase reporter enzyme according to the manufacturer's instructions. All treatments were performed in quadruplicates and each experiment was repeated at least three times. For cell proliferation assays, T47D cells were cultured in 0.1% charcoal-stripped FBS media containing 1.0 n*M* R5020, 20 ng/mL EGF, a combination of R5020 and EGF, or a combination of R5020, EGF, and 100 n*M* RU486 for 5 d in the presence or absence of 10 µg/mL of anti-Cyr61 neutralizing antibodies, nonimmune IgG, and anti-Cyr61 blocking peptide at 37^0C in 5% CO_2 with treatment changes every day. Following steroid or growth factor treatment, monolayers were trypsinized, combined with cells in the culture supernatant, and counted in a Coulter Multisizer II counter (Coulter Corporation, Miami, FL).

Densitometric and Statistical Analysis of Cyr61 Levels

Densitometric analysis of Cyr61 mRNA levels was accomplished with a Molecular Dynamics phosphorimager and image quantitation software**.** Numerical values are based on the relative volume of the radioactive band and the level of Cyr61 was normalized to the level of GAPDH. Cyr61 protein levels detected by Western analysis were quantitated by densitometric analysis utilizing a Bio-Rad molecular imager. Numerical values are based on the relative optical density of the band and the level of Cyr61 was normalized to the level of cytokeratin. Data derived from densitometric measurements of RNA and protein bands were analyzed using SAS statistical software (SAS Inc., Cary, NC) for significance using an one-way analysis of variance (ANOVA) for a factorial experimental design. Values derived from densitometric measurements of protein levels in breast tumor lysates and matched controls were analyzed using SAS statistical software for significance using the paired *t*-test method for two groups. The fold changes in the levels of Cyr61 protein and mRNA were considered significant if *p*-values were less than 0.05.

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