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The angiogenic factor CYR61 in breast cancer: molecular pathology and therapeutic perspectives

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Abstract

CYR61 (CNN1), a member of the cysteine rich 61/connective tissue growth factor/nephroblastoma overexpressed (CYR61/CTFG/NOV) family of growth regulators (CNN), is a pro-angiogenic factor that mediates diverse roles in development, cell proliferation, and tumorigenesis. We have recently shown that CYR61 is overexpressed in invasive and metastatic human breast cancer cells. Accordingly, elevated levels of CYR61 in breast cancer are associated with more advanced disease. Unfortunately, the exact mechanisms by which CYR61 promotes an aggressive breast cancer phenotype are still largely unknown. This review examines the functional role of CYR61 in breast cancer disease, presenting evidence that CYR61 signaling may play a major role in estrogen- as well as growth factor-dependent breast cancer progression. We also emphasize the functional significance of the molecular connection of CYR61 and its integrin receptor \( \alpha_v \beta_3 \) enhancing breast cancer aggressiveness. Moreover, we describe experimental evidence that establishes a novel role for CYR61 determining the protection of human breast cancer cells against chemotherapy-induced apoptosis through its interactions with the integrin receptor \( \alpha_v \beta_3 \). All these findings delineate a new noteworthy function of a CYR61/\( \alpha_v \beta_3 \) autocrine–paracrine signaling pathway within both angiogenesis and breast cancer progression, which would allow a dual anti-angiogenic and anti-tumor benefit with a single drug.

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Introduction

Steroid hormones closely regulate the growth of human breast cancer cells. Thus, anti-estrogen therapy is widely considered as the first-line therapeutic choice for the management of estrogen receptor (ER)-positive breast cancer. However, aggressiveness of human breast cancer is often related to the ability of the cells to overcome estrogen (E2) requirements for growth, and in most cases to acquire anti-estrogen resistance (Nicholson & Gee 2000). Thus, after the initial stages of breast cancer progression, tumors frequently acquire resistance to E2 with concurrent amplification and/or dysregulation of growth factors/growth factor receptor. The mechanisms that enable the progression from E2 dependence to E2 resistance remain poorly defined.

Within this context, we have shown previously that expression of heregulin (HRG), a growth factor that activates the erbB-2/3/4 receptor network signaling, is closely associated with an invasive breast cancer phenotype (Cardillo et al. 1995, Atlas et al. 2003). We have further demonstrated that HRG induces breast cancer progression, as determined by the loss of ER function and E2 response, tumorigenicity, invasion, and metastasis (Lupu et al. 1995, 1996, Tang et al. 1996, Atlas et al. 2003). As a part of our efforts to describe gene(s) directly involved in HRG-induced breast cancer aggressiveness, we recently isolated and identified CYR61, an angiogenic factor that is differentially expressed in ER-negative, HRG-positive breast cancer cells (Tsai et al. 2000). CYR61 (CCN1), an extracellular matrix-associated protein of the cysteine rich 61/connective tissue growth factor/nephroblastoma overexpressed (CCN) family which also includes CCN2 (CTFG), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3), is a product of an immediate early gene that mediates cell adhesion, induces

CYR61 is a novel ligand for integrins, and signaling through integrin receptors such as αvβ3, αvβ6, αvββ6, and αvβ5, may explain many, if not all, of the known diverse functions of CYR61 (Kireeva et al. 1998, Jedsadayanmata et al. 1999, Chen et al. 2000, Grzeszkiewicz et al. 2001). Although CYR61 binds to several integrins, so far only the integrin αvβ3 has been shown to play a major role in breast cancer tumor neoangiogenesis and progression (Meyer et al. 1998, R Lupu et al. unpublished data). More importantly, it has been demonstrated that overexpression of αvβ3 is a marker for poor prognosis in breast cancer (Gasparini et al. 1998). In this regard, we have previously reported that a functional blocking antibody against αvβ3 is capable of inhibiting HRG induction of the aggressive phenotypes of breast cancer cells (Tsai et al. 2000). Excitingly, we have recently found that forced expression of CYR61 in HRG-negative MCF-7 human breast cancer cells led to the up-regulation of the integrin receptor αvβ3 (Menéndez et al. 2002, 2003). Furthermore, our more recent experimental data have established a novel role for CYR61 overexpression in determining protection of human breast cancer cells against paclitaxel (Taxol) (Menéndez et al. 2002, 2003), a microtubule-targeting drug that is among the most effective agents in the treatment of advanced breast cancer, refractory or non-responsive to endocrine manipulation (Seidman et al. 1995, Perez 1998). In addition to its effect on angiogenesis, CYR61 may play, therefore, an important role transmitting survival signals in either an autocrine or paracrine manner through a CYR61/αvβ3-maximized regulatory loop in the absence of HRG over-expression.

This article provides a body of evidence demonstrating that aberrant expression of CYR61 promotes breast tumorigenesis and cancer progression by participating in the escape from anti-hormone control of cell growth. Also, we emphasize the functional significance of CYR61 and its mechanisms of action through the integrin receptor αvβ3 in human breast cancer cells. Ultimately, we speculate that targeting the CYR61/αvβ3-enhanced cell survival mechanism may prove therapeutically efficacious in the prevention or treatment of breast cancer.

Molecular pathology of CYR61 in breast cancer

CYR61, a molecular definition

CYR61 is a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (Yang & Lau 1991), biochemical features that resemble the Wnt-1 proto-oncogene and a number of known growth factors (Yang & Lau 1991). The human CYR61 cDNA encodes a protein 379 amino acids in length with a molecular mass of 42 kDa, and the gene is located on the short arm of chromosome 1 (1p22–31) (Charles et al. 1991, Jay et al. 1997). Of note, abnormalities of chromosome 1p have correlated with ER negativity and a poor prognosis in breast cancer (Hainsworth et al. 1992), and other malignancies (Simon et al. 1991, Shin et al. 1993, Gehring et al. 1995). CYR61 was originally identified by differential hybridization screening of a cDNA library of serum-stimulated BALB/c 3T3 fibroblasts (O’Brien et al. 1990). CYR61 is not expressed in quiescent fibroblasts, but it is transcriptionally activated within minutes after stimulation by serum, epidermal growth factor, basic fibroblastic growth factor (bFGF), platelet-derived growth factor, transforming growth factor-β, and 12-O-tetradecanoylphorbol 13-acetate (TPA) (Lau & Nathans 1985, 1987, Nathans et al. 1988, O’Brien et al. 1990, Tsai et al. 2002b).

CYR61 belongs to the CCN gene family of angiogenic regulators, which consists of CCN1 (CYR61), CCN2 (CTFG), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) (Lau & Lam 1999, Briggstock et al. 2003). All CCN proteins, including CYR61: (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 with similar sequence homologies to insulin-like growth factor-binding protein, to von Willebrand factor type C repeat, to thrombospondin type 1 repeat, and to growth factor cysteine knots; and (4) have been shown to mediate functions as diverse as cell proliferation, migration, adhesion, cell survival, differentiation, and extracellular matrix formation. They also regulate more complex processes, such as angiogenesis and tumorigenesis (for reviews see Briggstock 1999, Lau & Lam 1999, Perbal 2001).

Clinical relevance of CYR61 in human breast cancer

Increasing efforts have been devoted to defining the clinical relevance of cellular proteins that play a role in breast cancer progression. This interest has been particularly focused on proteins that are potential clinical indicators of disease prog-
nosis. To determine whether expression of CYR61 may have any clinical relevance in breast cancer, we performed a pilot study using Western blot analysis on proteins extracted from paraffin sections of breast tumor biopsies. In about 40% of the tumor specimens, all of which were ER-negative invasive breast carcinomas, we found high expression of the CYR61 protein (Tsai et al. 2000). Additional studies revealed that CYR61 was present in about 30% of breast tumor specimens as determined by immunohistochemistry, while no staining was observed in normal components of the biopsies (Tsai et al. 2000). Using Northern blot analysis, Xie et al. (2001b) also found CYR61 to be highly expressed in 36% of primary breast tumors. Sampath et al. (2001) showed that CYR61 was overexpressed in 70% of breast cancer patients with infiltrating ductal carcinomas of the breast and CYR61 was localized exclusively to hyperplastic ductal epithelial cells. More recently, overexpression of CYR61 was found in 39% of primary breast cancers using quantitative real-time PCR assay (Xie et al. 2001b). In this study, a significant correlation was found between elevated levels of CYR61 and advance stage and size of the primary tumor and lymph node involvement at the time of removal of the primary tumor.

All these results, taken together, indicate that prominent expression of CYR61 may play a role in the process of breast cancer development and might serve as a valuable tool for monitoring the tumor status of breast cancer patients. Further studies, however, should attempt to determine whether measurement of CYR61 at diagnosis could provide prognostic data and suggest those tumors that might be responsive to therapy. Incidentally, our group has extensively analyzed the aberrant signaling pathways that are activated by CYR61 overexpression which, hopefully, may offer new valuable therapeutic targets in breast cancer.

**Expression and regulation of CYR61 in human breast cancer cell lines**

We have demonstrated that expression of the growth factor HRG, a growth factor that activates the erbB-2/3/4 receptor network signaling, is highly associated with an aggressive progression of breast cancers to hormone independence, anti-estrogen resistance, invasion, and metastasis (Lupu et al. 1995, 1996, Tang et al. 1996, Hijazi et al. 2000). To identify genes that are involved in the HRG induction of breast cancer progression, a number of genes were isolated and cloned by differential expression in the MDA-MB-231 model, a human breast cancer cell line that naturally overexpresses HRG. Sequence and homology analyses evidenced that one of these genes was the human homologue of a mouse immediate early response gene, Cyr61. CYR61 was highly and selectively expressed (a 5- to 25-fold increase in the CYR61 mRNA level) in MCF-7 human breast cancer cells engineered to overexpress HRG (HRG-transfected MCF-7 clones T2, T6, T7, and T8) (Tang et al. 1996, Harris et al. 1998), but was almost undetectable in vector-transfected MCF-7 cells (Tsai et al. 2000). HRG-positive MDA-MB-231 cells also expressed high levels of CYR61 mRNA. Western blot and immunohistochemical analyses using an anti-CYR61 polyclonal antibody demonstrated that the CYR61 protein was selectively up-regulated in human breast cancer cells forced to overexpress HRG. Moreover, when the basal level of CYR61 expression was examined in a panel of human breast cell lines expressing varying levels of HRG and ER, a significant correlation was observed between HRG and CYR61 expression (Sampath 2000). Using Northern blot analysis, Xie et al. (2001b) showed that CYR61 was overexpressed in 70% of breast cancer patients with infiltrating ductal carcinomas of the breast and CYR61 was localized exclusively to hyperplastic ductal epithelial cells. More recently, overexpression of CYR61 was found in 39% of primary breast cancers using quantitative real-time PCR assay (Xie et al. 2001b). In this study, a significant correlation was found between elevated levels of CYR61 and advance stage and size of the primary tumor and lymph node involvement at the time of removal of the primary tumor.

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**Table 1 CYR61 overexpression is associated with ER-negativity and aggressiveness of human breast cancer cell lines.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CYR61</th>
<th>HRG</th>
<th>ER</th>
<th>Invasive in vitro</th>
<th>Metastatic in vivo</th>
<th>α,β2&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
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<td>−&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>BT-474</td>
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<tr>
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<td>+</td>
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</tr>
<tr>
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<td>ND</td>
<td>−/+</td>
<td>+++</td>
<td>−</td>
<td>++</td>
</tr>
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</table>

<sup>a</sup>indicates no expression; the number of plus signs (+) indicates the increase in expression.

<sup>b</sup>The integrin α,β2 expression is based on results from Kireeva et al. (1996), and our preliminary data. ND, not determined.

<sup>c</sup>Cells require E2 for invasion in vitro and growth in vivo and never metastasize in vivo.

<sup>d</sup>E2 induces expression of HRG.

<sup>e</sup>Cells require EGF or HRG to invade but never metastasize in vivo.
Menéndez et al.: Role of CYR61 in breast cancer progression

cancer cell lines, we found that CYR61 mRNA and protein were highly expressed in MDA-MB-231, HS578T, BT549, and MCF-7/HRG cells, all of which are HRG-overexpressing and ER-negative cells (Tsai et al. 2000). Conversely, CYR61 levels were low or undetectable in cells that do not express HRG and are ER negative, including MCF-7, ZR75B, T47D, and BT-474 cells (Tsai et al. 2000). These data are summarized in Table 1. Our data undoubtedly demonstrated that high level of CYR61 expression tightly correlates with HRG overexpression and inversely correlates with ER expression. Moreover, the expression of CYR61 strongly correlates with vimentin expression, a known marker for invasiveness (Thompson et al. 1992), and is associated with the ability of breast cancer cells to invade in vitro and metastasize in vivo. Hence, there exists a tight correlation between HRG overexpression, CYR61 up-regulation, and breast cancer progression.

In our evaluation of CYR61 in breast tumor biopsies (Tsai et al. 2000), CYR61 expression was closely correlated with tumor progression and ER negativity. It has been recently shown that CYR61 overexpression is associated with more advanced breast cancer disease. However, although the sample number was relatively small, Xie et al. (2001a) showed a significant correlation between CYR61 expression and ER positivity, even though ER expression is known to be an indicator of good prognosis for breast cancer (Brown et al. 2000). Similarly, Sampath et al. (2001) suggested that increased levels of CYR61 in ER-positive breast tumors might contribute to E2-driven tumorigenesis in vivo. Some of these conflicting observations may be due to different methodologies used to quantify CYR61 expression, such as moderately sensitive Northern blots detecting CYR61 expression in malignant and non-malignant breast epithelium (Xie et al. 2001a) versus highly sensitive and breast epithelium-specific immunohistochemical analysis (Tsai et al. 2000). These somewhat mystifying results led us to use a systematic in vitro and in vivo approach assessing the actual relationship of CYR61 and ER-signaling pathways in human breast cancer cell growth and progression.

Previous studies showed that murine Cyr61 is inducible by E2 and tamoxifen in the uterus of ovariectomized rats (Rivera-Gonzalez et al. 1998). However, it is not clear how CYR61 is regulated in human breast cancer, except that it has been indicated that CYR61 is E2 inducible, and that tamoxifen inhibited its mRNA expression in human breast cancer cells (Xie et al. 2001a). Therefore, we investigated in more detail how CYR61 expression is regulated in both ER-positive and ER-negative breast cancer cells (Tsai et al. 2002b). We were the first group to establish that expression of CYR61 at both the mRNA and protein levels was inducible by E2 in E2-dependent breast cancer cells (Tsai et al. 2002b). Moreover, we demonstrated that treatment of E2-depleted cells with the anti-estrogens tamoxifen andICI 182 780 caused a marked up-regulation of CYR61 mRNA.

Tamoxifen, a well-known anti-estrogen, functions as an agonist and antagonist through both transcriptional activation domains (AF1 and AF2) of ER. ICI 182 780, a pure anti-estrogen, acts solely as an antagonist through the AF1 domain (McGregor & Jordan 1998). Interestingly, the combination of E2 with either anti-estrogen abrogated the activation of CYR61 gene expression. We observed that CYR61 protein expression was also up-regulated (over 10- to 20-fold) by E2, tamoxifen and ICI 182 780 in MCF-7 cells. Other important regulators of CYR61 expression in breast cancer cells that we found were the phorbol ester TPA, vitamin D, and retinoic acid. TPA caused positive regulation of CYR61 expression in ER-positive MCF-7 cells. Vitamin D induced a transient stimulatory effect on CYR61 gene expression. Finally, the differentiating agent, retinoic acid, down-regulated CYR61 expression in MCF-7 breast cancer cells. In cells that acquire E2 independence but still express ER, such as MCF-7 cells transfected with HRG (MCF-7/HRG), E2 induced CYR61 expression to a much lower extent; conversely, these agents no longer mediated the expression of CYR61 in MDA-MB-231 cells, which express high levels of endogenous CYR61. This finding was consistent with the phenotype of the MDA-MB-231 cell line, which is ER negative, E2 independent, anti-estrogen resistant, highly invasive, and metastatic in vivo.

Together, our results proved that the fold induction of CYR61 by E2 and/or anti-estrogen agents coincides with the endogenous levels of CYR61 expression and is inversely correlated with the levels of ER expression in human breast cancer cells. Moreover, our results were in agreement with our knowledge that CYR61 promotes tumor growth, and that anti-estrogen agents have a positive impact on breast cancer cells expressing low levels of CYR61 (ER-positive breast cancer cells); conversely, these agents have no significant effect on cells that express high levels of CYR61 (ER-negative breast cancer cells).

CYR61 is sufficient to promote acquisition of E2 independence and anti-estrogen resistance in human breast cancer cells

CYR61 is differentially expressed in HRG-positive, invasive, and metastatic human breast cancer cells (Tsai et al. 2000). Moreover, enhanced expression of CYR61 correlates with lack of ER expression. To determine whether ectopic expression of CYR61 alone, in HRG-negative breast cancer cells, is necessary and/or sufficient to confer some biological activities induced by HRG, such as loss of E2 response and acquisition of anti-estrogen resistance, MCF-7 human breast cancer cells, which are ER positive, E2 responsive in vitro, and are growth inhibited by many anti-estrogen drugs (Nicholson et al. 1995), were engineered to overexpress CYR61. In anchorage-dependent assays, our breast cancer models of CYR61 overexpression showed a growth advan-
tage in E2-depleted media, having a three- to fivefold increase in growth as compared with control cells (Tsai et al. 2002). These results illustrate that overexpression of CYR61 provides a growth advantage to bypass the ‘normal’ E2 requirement for the proliferation of MCF-7 human breast cancer cells (Pratt & Pollak 1993, Tsai et al. 2002). Interestingly, CYR61-overexpressing MCF-7 cells were still responsive to E2, resembling one of the clinical phenotypes found in women suffering from breast cancer. When MCF-7/CYR61 cells were treated with tamoxifen or ICI 182 780, both anti-estrogens reduced only the growth induced by E2 in MCF-7/CYR61 cells. However, they were unable to block the E2-independent growth of the MCF-7/CYR61 cells, indicating that CYR61 provides a true growth advantage that cannot be inhibited by anti-estrogen agents. In other words, both anti-estrogens inhibited the growth of the cells only to the basal levels promoted by overexpression of CYR61, whereas they were not able to reduce cell growth to the same level achieved in the wild-type MCF-7 cells in the absence of E2. These in vitro experiments revealed, therefore, that CYR61 overexpression can stimulate cell growth of E2-dependent cells in the absence of E2s resulting in cells becoming E2 independent. On the other hand, E2s further enhances cell proliferation of CYR61-overexpressing cells, indicating that these cells, although independent of E2, are still responsive to E2. Therefore, it is likely that overexpression of CYR61 alone most probably accounts for the growth advantage observed in MCF-7/CYR61 cells in E2-depleted culture conditions.

A possible mechanism to acquire E2-independent and anti-estrogen-resistant phenotypes acts via the loss of ER expression and/or ER function. Within this context, we have observed that the basal level of ERα expression is markedly reduced (30–50%) in MCF-7/CYR61 cells (Tsai et al. 2002a). These results indicate that CYR61 expression correlates with the loss of ER expression, consistent with our previous finding that CYR61 expression is closely associated with tumor progression and ER negativity in tumor biopsies (Tsai et al. 2000). We next examined whether CYR61 promotes loss of ER function by assessing the regulation of several well-documented E2-responsive genes. Since we previously demonstrated that the loss of progesterone receptor (PgR) regulation by E2 attests for the loss of ER function (Saceda et al. 1996, Tang et al. 1996), our studies in MCF-7/CYR61 cells were focused on E2 regulation of ERα and PgR expression. Although the level of ERα expression was lowered, E2 exposure further down-regulated the expression of ERα in CYR61-overexpressing MCF-7 cells, and induced a marked up-regulation in PgR mRNA expression. Similarly, we observed E2-induced up-regulation of cathepsin D and trefoil factor 1 (TFF1, formerly pS2), which have been shown to be up-regulated by E2 in MCF-7 cells (Cavailles et al. 1988, Weaver et al. 1988). These data support the notion that ERα is still a functional receptor in MCF-7/CYR61 cells, although these cells are E2 independent and the level of ERα expression is lower than that in the parental cells.

**CYR61 enhances a metastatic phenotype by promoting cell proliferation in soft agar, cell migration and invasion, and Matrigel outgrowth of breast cancer cells**

Acquisition of a transforming phenotype is often correlated with the ability of cells to grow in an anchorage-independent fashion. It is well established that MCF-7 cells are not anchorage independent in the absence of E2. Colonies observed, if any, represent the background level for the colony formation assay. We and others observed that MCF-7 engineered to overexpress CYR61, in the absence of E2, formed large colonies in soft agar assays (Xie et al. 2001a, Tsai et al. 2002a). As expected, E2 exposure induced anchorage-independent growth of control MCF-7 cells, which was completely blocked by anti-estrogen. Interestingly, E2 exposure also slightly enhanced the colony formation in the CYR61-overexpressing MCF-7 cells, an E2-driven effect that was not reversed in the presence of the pure anti-estrogen ICI 182 780 (Tsai et al. 2002a). Xie et al. (2001a) noted that tamoxifen blocked the E2-stimulated colony formation in MCF-7/CYR61 cells. In contrast, we demonstrated that both tamoxifen and ICI 182 780 enhanced colony formation of MCF-7/CYR61 cells (Tsai et al. 2002a, R Lupu and M-S Tsai unpublished data). Nonetheless, all these studies clearly indicate that forced expression of CYR61 promotes anchorage-independent clonogenic growth of MCF-7 cells.

Using vitronectin-coated Boyden chambers, an in vitro assay to quantitate the invasive potential of tumor cells (Albini et al. 1987), Xie et al. (2001a) established that CYR61 stably transfected cell lines (MCF-12A and MCF-7) show significantly increased migration compared with the empty vector-transfected cells. In our experiments, we addressed the question whether CYR61 is a direct downstream regulation of HRG-induced metastatic properties in human breast cancer cells. For these studies we used HRG-transfected MCF-7 cells (Tang et al. 1996, Atlas et al. 2003), which have been shown to migrate through collagen in a Boyden chamber assay, and a CYR61-neutralizing antibody (Tsai et al. 2000). The anti-CYR61-neutralizing antibody inhibited migration of MCF-7/HRG cells in a dose-dependent manner. Similar results were observed in other invasive, HRG-overexpressing breast cancer cells, such as MDA-MB-231, Hs578T, and BT549. These studies demonstrated that CYR61 overexpression influences cell migration of MCF-7 cells, and suggest an association between the activation of CYR61 expression in human breast cancer and the invasive potential triggered by HRG. Recently, we established that MCF-7/CYR61 cells show extensive outgrowth in Matrigel (Tsai et al. 2002a), an in vitro assay that is frequently
employed as a reliable system to assess in vitro invasiveness of breast cancer cells (Sommer et al. 1994, Hijazi et al. 2000). Significantly, CYR61 overexpression promoted outgrowth of MCF-7 cells in the Matrigel matrix in the absence of E2, the colonies appearing large and irregular in shape. In contrast, control cells were not able to migrate through and proliferate in the Matrigel matrix even in the presence of E2, suggesting that CYR61 can induce an invasive phenotype of breast cancer cells in an E2-independent manner (Tsai et al. 2002a).

**CYR61 promotes tumorigenesis and neovascularization**

On the basis of our in vitro studies indicating that overexpression of CYR61 promotes anchorage-independent clonogenic proliferation in soft agar as well as cell migration and invasion, all of which are characteristics of an aggressive breast cancer phenotype, we envisioned that CYR61 overexpression should promote tumor development and neovascularization. Recently, we reported that MCF-7 cells transfected with CYR61 developed large and more vascularized tumors in ovariectomized nude mice (Tsai et al. 2002a). These tumors grew independently of hormonal stimulation, supporting our in vitro data suggesting that the MCF-7/CYR61 cells had a growth advantage in the absence of E2. The tumors developed for CYR61-overexpressing cells were highly vascularized. In agreement, overexpression of CYR61 promoted the expression of another important regulator of neovascularization, vascular endothelial growth factor (VEGF). Xie et al. (2001a) also described that MCF-7 breast cancer cells forced to overexpress CYR61 developed larger and more vascularized tumors in nude mice. Another interesting finding of this study is that overexpression of stably transfected CYR61 in the normal breast epithelial cell line MCF-12A, which does not normally express CYR61, resulted in tumor formation in nude mice. Taken together, these in vitro and in vivo findings suggest that prominent expression of CYR61 may facilitate transformation of breast tissue, and indisputably demonstrate that overexpression of CYR61 in MCF-7 cells promotes tumorigenesis in the absence of hormonal stimulation.

**Therapeutic perspectives of CYR61 in breast cancer**

**CYR61 overexpression promotes breast cancer cell resistance to Taxol-induced cell death: involvement of the phosphatidylinositol 3'-kinase (PI3'-kinase)/protein kinase B (AKT) pro-survival pathway**

We have previously demonstrated that HRG-overexpressing cells showed a marked increase in sensitivity to the topo-isomerase II inhibitors doxorubicin and etoposide (Harris et al. 1998). Interestingly, virtually every conventional cytotoxic anti-cancer drug has been ‘accidentally’ discovered to have anti-angiogenic effects in various in vivo models (Kerbel et al. 2000, Miller et al. 2001). Recently, the tumor cell microenvironment has been found to have a significant bearing on the survival of tumor cells following exposure to a wide variety of anti-neoplastic agents, prior to the acquisition of known drug resistance mechanisms. Accordingly, it has been recently demonstrated that some angiogenic factors such as VEGF and bFGF significantly reduced the potency of chemotherapy (Lissoni et al. 2000, Zhang et al. 2001, Tran et al. 2002). Because of the pro-angiogenic abilities of HRG and CYR61, we hypothesized that HRG could also act directly – or indirectly through CYR61 – as a survival factor for breast carcinoma cells modifying chemotherapy effectiveness. In this regard, we recently examined whether CYR61, in the absence of HRG overexpression, may play a role in the breast cancer cell responses to chemotherapy-induced damage. Significantly, we observed that CYR61 overexpression rendered MCF-7 cells resistant to paclitaxel (Taxol) in both anchorage-dependent and soft agarose colony-formation assays (Menéndez et al. 2002, 2003). Because apoptosis is the predominant mechanism of cytotoxicity induced by chemotherapeutic agents, we analyzed whether the failure of CYR61-overexpressing breast cancer cells to activate apoptosis may account for CYR61-promoted Taxol resistance. When MCF-7/CYR61 cells were examined for apoptosis-related parameters after Taxol exposure, no signs of the classical DNA laddering formation were observed in CYR61 transfectants compared with control cells. Accordingly, CYR61 overexpression induced a dramatic decrease in the number of TUNEL-positive cells compared with Taxol-treated control cells (Menéndez et al. 2002, 2003). It has been shown that Taxol-induced apoptosis involves a dose- and time-dependent accumulation of the tumor suppressor gene p53 and the inhibitor of cyclin-dependent kinases, p21WAF1/CIP1 (Blasgosklo´nny et al. 1995, Giannakakou et al. 2001). Our preliminary experiments, however, noted a reduced ability of the MCF-7/CYR61 cells to induce p53 expression in response to Taxol exposure, suggesting that CYR61 overexpression could suppress Taxol-induced apoptosis by interfering with the function of wild-type p53 in human breast cancer cells (Menéndez et al. 2002, 2003).

Simultaneously to Taxol resistance, MCF-7/CYR61 cells showed cross-resistance to wortmannin and LY294002, two pharmacological inhibitors of the PI3'-kinase activity. Interestingly, we have demonstrated that CYR61-overexpressing MCF-7 cells undergo up-regulation of the PI3'-kinase/AKT kinase pro-survival pathway (Tsai et al. 2002c, Menéndez et al. 2002, 2003). Because several studies have recently indicated that alterations in the PI3'-
kinase/AKT signal transduction pathway can modulate cell sensitivity to Taxol (Mitsuuchi et al. 2000, Hu et al. 2002, MacKeigan et al. 2002), it is likely that a new important function of CYR61 in human breast cancer is the promotion of cell survival by activating anti-apoptotic signaling pathways such as those composed of PI3'-kinase and the serine/threonine kinase AKT. Accordingly, the protective effect of CYR61 against Taxol-induced cytotoxicity was abolished under culture conditions inhibiting PI3'-kinase activity (Menéndez et al. 2002, 2003). These findings establish a novel role for CYR61 in determining protection of human breast cancer cells against Taxol-induced apoptosis through the activation of the PI3'-kinase/AKT pro-survival pathway.

The 'CYR61–αvβ3 integrin connection': a new molecular therapeutic target in human breast cancer

Depending on the biological context and model system, CYR61 can induce disparate functions. However, most of the CYR61-promoted effects are mediated via its direct binding with the integrin receptor αvβ3. Thus, we speculated whether CYR61-enhanced breast cancer progression requires expression of the integrin receptor αvβ3 for its actions. Indeed, we observed that the level of αvβ3 was significantly augmented in MCF-7 engineered to overexpress HRG compared with control cells (Tsai et al. 2000). Moreover, we determined that blockade of this integrin receptor using LM609, a monoclonal antibody directed against αvβ3, dramatically blocked the Matrigel outgrowth of HRG-overexpressing breast cancer cells in a dose-dependent fashion (Tsai et al. 2000). These results indicate, for the first time, that the functional αvβ3 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, through the interaction of CYR61 with integrin αvβ3.

Recently, we assessed whether CYR61, independent from HRG expression, affected the levels of the integrin receptor αvβ3. Use of a monoclonal anti-αvβ3 antibody demonstrated that, similarly to MCF-7/HRG cells, HRG-negative CYR61-overexpressing MCF-7 cells stained positively for αvβ3, whereas no significant staining was observed in control cells (Menéndez et al. 2002, 2003). To the best of our knowledge, this is the first indication showing that activation of αvβ3 expression in human breast cancer epithelial cells can be achieved solely by CYR61 overexpression, irrespective of HRG status. More importantly, the most recent data from our laboratory indicate that CYR61-promoted breast cancer cell survival and Taxol resistance are phenotypes associated with an increased αvβ3 integrin signaling. Using SC56631, a previously characterized synthetic chemical peptide mimetic based upon the αvβ3 ligand, Arg-Gly-Asp (RGD motif) (Engleman et al. 1997), we observed a marked decrease in the cell viability of HRG- and CYR61-overexpressing breast cancer cells but not in HRG- and CYR61-negative control cells. Furthermore, sub-optimal doses of SC56631 completely abolished Taxol resistance in MCF-7/CYR61 cells, as Taxol-induced cytotoxicity returned to the basal level observed in CYR61-negative control cells. Moreover, the nature of the interaction between SC56631 and Taxol was shown to be synergistic in CYR61-overexpressing breast cancer cells (Menéndez et al. 2002, 2003). These results, together, strongly suggest that therapies depriving CYR61-overexpressing cells of their αvβ3 signaling dramatically decrease cell survival and chemoresistance.

Integrin αvβ3 has been implicated in the pathophysiology of malignant tumors. In addition to its expression on the surface of angiogenic endothelial cells, integrin αvβ3 is expressed on the surface of tumor cells in a variety of cancers. In breast cancer, the integrin αvβ3 characterizes the metastatic phenotype, as its expression is up-regulated in invasive tumors and distant metastases (Gasparini et al. 1998). Thus, independent of its role in tumor angiogenesis, the integrin αvβ3 is functionally implicated in the pathogenesis of breast cancer. Integrin signals are involved in diverse biological responses, including angiogenesis and tumor progression, as well as in a variety of cellular activities, including cell migration, proliferation, and survival. Of interest, integrin signaling has recently been shown to modulate cancer cell responses to chemotherapeutic agents (Aoudjit & Vuori 2001). Specifically, interactions between cell surface integrins and extracellular matrix components have been shown to be responsible for this phenomenon of innate drug resistance, which we have termed cell adhesion-mediated drug resistance (Damiano et al. 1999, Gilmore et al. 2000). Thus, signal transduction pathways initiated by integrin ligandation may be potential bridge points for inhibiting cell survival during cytotoxic drug exposure. Among the signaling molecules involved in integrin-mediated cell survival is focal adhesion kinase (FAK), which becomes activated following integrin ligation and may in turn activate downstream survival pathways such as those composed of PI3'-kinase and the serine/threonine kinase AKT (King et al. 1997, Gilmore et al. 2000, Lee & Juliano 2000). Likewise, we have recently demonstrated that CYR61 overexpression in MCF-7 human breast cancer cells induces a significant increase in the expression of FAK (Menéndez et al. 2002, 2003). On the basis of these studies it is tempting to postulate that up-regulation of CYR61 in breast cancer cells may drive breast cancer cell survival and chemoresistance by emitting a proliferative and/or survival input via the integrin receptor αvβ3 and FAK, which might integrate signals from CYR61 to the PI3'-kinase/AKT pro-survival pathway. Importantly, CYR61 can also promote cell survival of angiogenic endothelial cells through interaction with integrin αvβ3 (Leu et al. 2002).
HRG-induced up-regulation of CYR61 may predispose breast tumor epithelial cells toward continued dysregulated proliferation and chemoresistance. The functional blocking of αvβ3 integrin in HRG- and CYR61-overexpressing cells induces cytotoxicity, suggesting that CYR61-activated αvβ3 integrin signaling is involved in breast cancer cell survival. Since CYR61 overexpression by itself activates the expression of the CYR61 receptor integrin αvβ3, up-regulation of CYR61 in human breast cancer may co-ordinate a metastatic phenotype in an autocrine/paracrine manner by activating an αvβ3/FAK/PI3'-kinase/AKT kinase signaling.

The ‘CYR61/αvβ3 connection’ thus appears to provide a promising molecular target for breast cancer disease that should permit a potentially synergistic strike against the tumor and its supporting vasculature (Fig. 1).

Conclusions

Breast cancer often progresses from an E2-dependent, non-metastatic, anti-estrogen-sensitive phenotype to an E2-independent, anti-estrogen-resistant, highly invasive and metastatic phenotype. Our results have demonstrated that CYR61 is a tumor-promoting factor that also acts as a key regulator of breast cancer progression. Significantly, in our current studies we have demonstrated that the angiogenic factor CYR61 is sufficient (1) to induce E2 independence and anti-estrogen resistance, (2) to promote invasiveness in vitro, and (3) to induce tumorigenesis and neovascularization in vivo. Our results further suggest that signaling through αvβ3 integrin allows for the maintenance of the cell viability of human breast cancer cells treated with Taxol. Although the exact mechanism through which the angiogenic factor CYR61 promotes cell survival and Taxol resistance in breast cancer cells is still unknown, it is tempting to postulate that CYR61-induced activation of the αvβ3/FAK/PI3'-kinase/ AKT pro-survival signaling could be a/the pathway involved in this phenotype. New anti-CYR61 and/or anti-αvβ3 therapeutic strategies may prevent vessel growth simultaneously rendering breast cancer cells more sensitive to Taxol-based chemotherapy.

In summary, our current approach indicates that activation of the CYR61/αvβ3 signaling network could drive breast cancer cells to escape hormonal requirements providing compensatory survival pathways that ultimately allow the acquisition of breast cancer chemoresistance. As knowledge of CYR61 involvement in breast cancer processes increases, the ‘CYR61/αvβ3’ connection in both angiogenic endothelial cells and tumor cells appears to be an increasingly attractive target for drug development (Fig. 2). Our data provide a starting point to exploit the potential anti-angiogenic and anti-tumor effects of highly specific αvβ3 inhibitors.
Figure 2 A new hypothetical model for the role of CYR61 in breast tumorigenesis and progression. Given that the angiogenic factor CYR61 is also a growth regulator, it is hypothesized that HRG-induced up-regulation of CYR61 in tumor epithelial cells may drive breast tumorigenesis and progression in several concerted modes: (1) by promoting tumor cell proliferation in an autocrine/paracrine fashion either augmenting growth factor bioactivity or emitting proliferative signals via αvβ3 integrin receptor, (2) by regulating endothelial recruitment tumor neovascularization in a paracrine fashion through an αvβ3-dependent mechanism, (3) by co-ordinating tumor epithelial cell migration as a chemokinetic factor, and (4) by increasing chemoresistance activating αvβ3/FAK/PI3′-kinase/AKT kinase pro-survival signaling.

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