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14. ABSTRACT

The angiogenic factor Cyr61 (also known as CCN1) plays a key role in both the maintenance and the enhancement of a malignant phenotype in breast cancer. Cyr61 is overexpressed in about 30% of triple negative breast carcinomas, whereas Cyr61 expression levels in normal breast tissues are negligible. Our recent studies showed that Cyr61 overexpression renders human breast cancer cells highly resistance to the microtubule-interfering agent paclitaxel (Taxol), a current drug of choice for the treatment of metastatic breast cancer. We have confirmed that expression of αvβ3, a Cyr61 receptor, is markedly up-regulated in breast cancer cells expressing Cyr61. Our most recent data demonstrate that functional blockade of αvβ3 with a synthetic chemical peptidomimetic based upon the αvβ3 the RGD (Arg-Gly-Asp) motif, is specifically cytotoxic towards Cyr61-overexpressing breast cancer. Pharmacological interference with the Cyr61/αvβ3 interaction restores Taxol efficacy, implying that a previously unrecognized Cyr61/ αvβ3 -driven cellular signaling actively modulates breast cancer cell growth, apoptosis and Chemosensitivity.

15. SUBJECT TERMS
Cyr61, integrin, alpha v beta 3, chemoresistance, taxane-based therapy, therapy, triple negative tumors

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INTRODUCTION

Epidemiological evidence strongly implicates ovarian involvement in the etiology of breast cancer. The greatest nonhereditary risk factor for breast cancer is being a normal female, with the risk in both women with primary ovarian failure and men being approximately 1% of that found in normal women (1).

Heregulin (HRG) is a growth factor that acts as an indirect activator for the *erbB*-2 oncogene product (3-10), and as a direct activator of the *erbB*-3 and *erbB*-4 signaling pathways (11-18). We demonstrated that MCF-7 cells transfected with HRG acquire a growth advantage in E2-depleted conditions and become E2-independent and antiestrogen-resistant (*in vitro* and *in vivo*), as opposed to the parental MCF-7/V cells, which do not exhibit any of these phenotypes. In the search for differentially expressed genes that may contribute to HRG induction of E2-independence and antiestrogen resistance, we have identified the human homologue of the murine Cyr61 gene product (19, 21).

Cyr61 belongs to the CCN family of proteins, which consists of connective tissue growth factor (CTGF), Cysteine-rich protein 61 (Cyr61), nephroblastoma overexpressed protein (NOV), WISP-1, WISP-2, and WISP-3 (24). These structurally conserved yet functionally diverse proteins share four modular domains, with sequence similarities to insulin-like growth factor-binding protein (IB), von Willebrand factor type C repeat (VWC), Thrombospondin type 1 repeat (TSP-1), and growth factor Cysteine knots (Cys-knot). The carboxyl terminal domain of Cyr61 is necessary for the adhesion of primary human fibroblasts, but not for cell migration or DNA synthesis (25). Furthermore, it has been suggested that one of the family members, CTGF, may be regulated by proteolytic cleavage (26, 27). Thus, distinct protein fragment(s) may elicit diverse functions of the CCN proteins.

It has been recently shown in smooth muscle that Cyr61, a secreted protein, can translocate to the nucleolus in response to mechanical stretch. The cellular localization of the Cyr61 protein or its cleaved products may account for some of the distinctive biological effects of Cyr61. However, the sequences responsible for the multiple biological effects mediated by Cyr61 remain to be elucidated. The presence of conserved structural modules suggests that the different domain of Cyr61 may mediate a variety of Cyr61 effects, and also that a given domain may be involved in various activities of Cyr61. Cyr61 is a ligand for the vascular αvβ3 (28) that plays an important role in angiogenesis and tumor invasion (29, 30). The αvβ3 integrin is highly expressed in angiogenic vessels and it is associated with breast cancer (31). Moreover, it has been demonstrated that αvβ3 is a prognostic indicator predictive of relapse-free survival in breast cancer (30, 31). Encoded by a growth factor-inducible immediate-early gene, Cyr61 is a cysteine-rich matricellular protein that supports cell adhesion and induces adhesion signaling (20). Furthermore, Cyr61 stimulates endothelial cell migration and enhances growth factor-induced DNA synthesis in culture (20) and induces angiogenesis *in vivo* (24). Consistent with the angiogenic activity of Cyr61, its expression is elevated during wound healing. Mechanistically, Cyr61 acts as a non-RGD-containing ligand of integrin receptors (25). Integrins are heterodimeric cell-surface receptors capable of transducing extracellular signals and regulating cell adhesion, motility, proliferation, survival, and differentiation. Recent studies indicate that Cyr61 promotes cell adhesion, migration, and proliferation, survival, and tubule formation through integrin αvβ3 (32). Therefore, identifying the avb3 binding site(s) of Cyr61 has provided new insights into how these matricellular proteins interact with their receptors, and will help to elucidate the αvβ3 -specific activities of Cyr61 in developmental and pathological angiogenesis. Using biochemical, functional, and mutational approaches, Lau et al (33) have shown that a novel 20-residue sequence, V2, in the VWC domain of Cyr61, acts as a functional binding site for integrin αvβ3. In addition, D125 in the V2 sequence is critical for interaction with αvβ3, and a single amino acid D1256A substitution in the context of full-length Cyr6 is sufficient to selectively impair αvβ3-mediated activities in endothelial cells, including stimulation of cell migration and enhancement of DNA synthesis.

We suggest that Cyr61 may be a critical regulator in breast cancer progression, possibly through its binding to αvβ3. Although these processes involve much more complex cellular responses than adhesion, they are completely consistent

with the capabilities of integrin to induce signaling events. The participation of integrin in modulation of growth factor-mediated signaling has also been established (33, 34). Angiogenesis requires the coordinated execution of a series of cellular processes, beginning with the degradation of the basement membrane and ECM surrounding the blood vessels, processes that have been attributed to HRG and/or Cyr61 in a variety of models (17-19, 35-37). However, we have not fully examined the potential role of Cyr61 in breast cancer progression and chemoresistance. Because we recently established that Cyr61 is a downstream effector of Heregulin (HRG) and mostly probably the mediator of chemomigration and metastasis (22), these effects probably occur through the interaction of Cyr61 with the αvβ3. Cyr61 stimulates chemotaxis in endothelial cells and induces neovascularization *in vivo* (23, 24). We have shown that forced expression of Cyr61 in MCF-7 cells markedly up-regulated the expression of αvβ3 (> 200 times). Small peptidomimetic αvβ3 integrin antagonists dramatically decreased cell viability of Cyr61-overexpressing MCF-7 cells, whereas control MCF-7/V remained insensitive. Mechanistically, functional blockade of αvβ3 specifically abolished Cyr61-induced hyperactivation of ERK1/ERK2 MAPK, whereas the activation status of AKT did not decrease. Moreover, Cyr61 overexpression rendered MCF-7 cells significantly resistant (> 10-fold) to Taxol-induced cytotoxicity and αvβ3 inhibition converted the Cyr61-induced, Taxol-resistant phenotype into a hypersensitive one. Thus, the augmentation of Taxol-induced apoptotic cell death in the presence of αvβ3 antagonists demonstrated a strong synergism (39a).

We have recently shown that Cyr61 overexpression in tumor cells enhances tumorigenicity by increasing tumor size and vascularization (23). Consistent with the notion that Cyr61 promotes an aggressive breast cancer phenotype, we have determined previously that Cyr61 gene expression is elevated in highly invasive and metastatic human breast cancer cells and tumor biopsies (22). Specifically, Cyr61 overexpression is correlated with a more advanced stage of malignancy in patient tumor biopsies (38a-b). Our studies suggest that expression of Cyr61 at the time of diagnosis may provide critical prognostic data indicating the responsiveness to therapy. However, the particular contribution of Cyr61 in cell survival and chemoresistance remains to be determined.

In addition to conferring aggressive malignant behavior, HRG overexpression has been observed to induce tumorigenesis of MCF-7 cells (MCF-7/HRG) and to affect the sensitivity of breast cancer cells to chemotherapy (39-41). Our previous studies demonstrated that HRG-transfected cells showed a marked increase in sensitivity to chemotherapeutic agents such as doxorubicin and etoposide (41, 42). Taxol, a promoter of microtubule polymerization commonly used in the treatment of advanced or metastatic breast cancer, also has antiangiogenic properties that are associated with down-regulation of Vascular Endothelial Growth Factor (VEGF) (43).

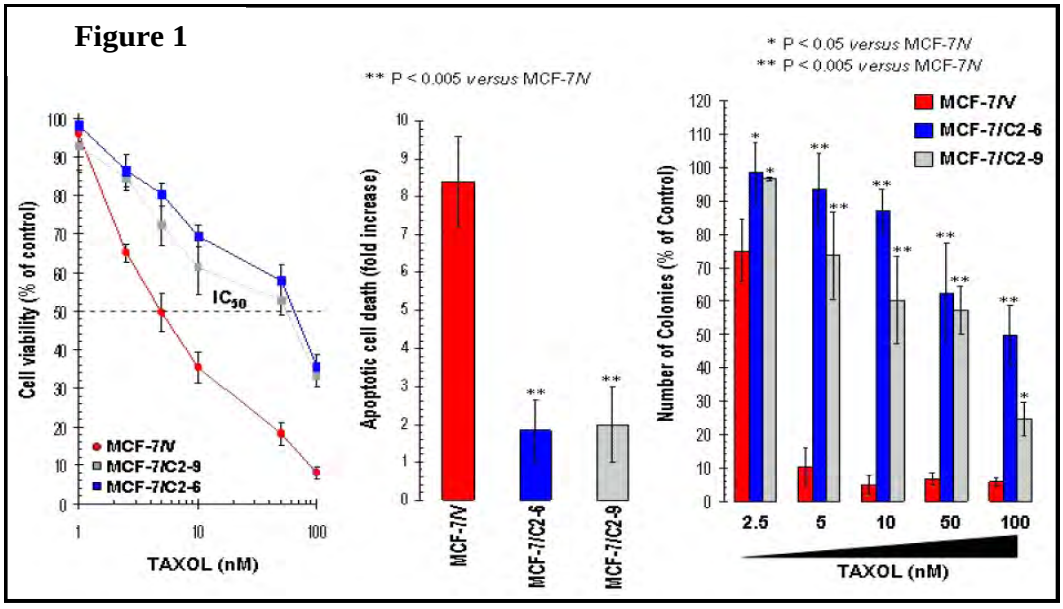
In summary, the tumor cell microenvironment has been found to have a significant bearing on the survival of tumor cells following exposure to a wide variety of anti-neoplastic agents, prior to the acquisition of drug resistance mechanisms (44-46). Our data from *in vitro* and *in vivo* breast cancer models suggest that some of the HRG-induced phenotypes are mediated indirectly via the up-regulation of other genes in an autocrine or a paracrine manner. For example, the expression of Cyr61 was significantly upregulated in the MCF-7/HRG-derived tumors (47, 48). Because of the pro-angiogenic abilities of Cyr61 through αvβ3 integrin signaling (49-53), we recently envisioned that this angiogenic factor could also act as a survival factor for both tumor and endothelial cells by modifying chemotherapeutic effectiveness.

BODY

1. Cyr61-INDUCED TAXOL RESISTANCE: 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. Integrin signaling has recently been shown to modulate cancer cell responses to chemotherapeutic agents. We evaluated whether Cyr61-induced up-regulation of αvβ3 would modulate breast cancer cell response to paclitaxel (Taxol™), an antimitotic drug commonly used in the treatment of advanced or metastatic breast cancer, by performing three independent sets of assays. *First, we determined that the transfected Cyr61 cells (MCF-7/C2-6 and MCF-7/C2-9) were*

exceptionally more resistant to Taxol-induced cytotoxicity (9 and 12-fold) when compared with matched control MCF-7/V cells (Fig. 1 left). We then hypothesized that the reduced sensitivity to Taxol seen in Cyr61-overexpressing MCF-7 cells was probably not simply the result of changes in cell proliferation, but could actually be due to a Cyr61-promoted decrease in apoptotic cell death following Taxol-induced cell damage. Thus, we exposed the Cyr61 expressing cells to 10 nM Taxol and found that these cells exhibited a negligible degree of cell death. In contrast, Taxol induced a significant degree of cell death in the control MCF-7/V cells (Fig. 1 center). Cell death was measured using an ELISA assay which is capable of detecting the DNA-histone fragmentation, and *x*–fold increase in apoptosis-related cell death was calculated by comparing the ELISA readings of treated samples with the values of the untreated cells as 1.0. We then tested the sensitivity of MCF-7/Cyr61cells to Taxol Assays were performed in a dose-dependent manner. Very low concentrations of Taxol (< 10 nM) suppressed colony formation of MCF-7/V cells. In contrast, significantly higher concentrations of Taxol (> 50 nM) were required to partially inhibit the clonogenic growth of MCF-7/C2-6 and MCF-7/C2-6 clones (Fig. 1 right).

Figure 1

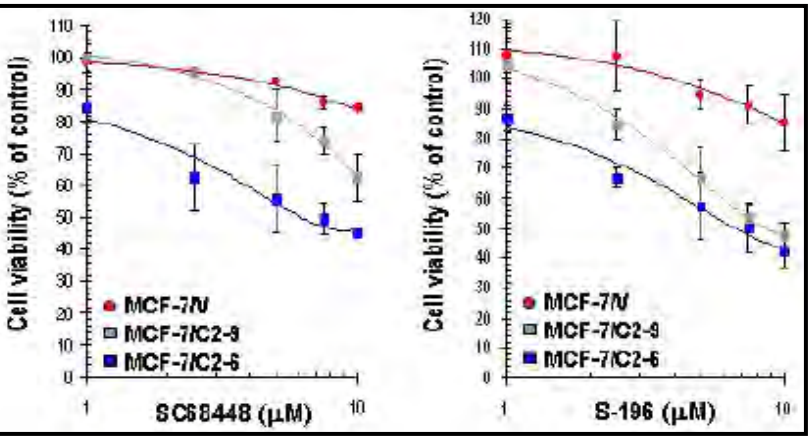


Thus, Cyr61 overexpression increases the resistance of breast cancer cells to Taxol 5 to 10 fold under anchorage-independent growth conditions. Together, these findings clearly demonstrate that Cyr61 expression dramatically decreases the efficacy of Taxol towards breast cancer cells.

2. Cyr61-INDUCED TAXOL RESISTANCE IS BLOCKED BY SPECIFIC RDG-**αvβ**3-COMPOUNDS: We next evaluated whether cell survival and proliferation in Cyr61-overexpressing MCF-7 cells was associated with an increased signaling through Cyr61/αvβ3, by examining the cytotoxic effects of specific RGD αvβ3 antagonists (provided by Dr. Griggs, at Pfizer) towards breast cancer cells that have been transfected with Cyr61. Comparison of IC_{50} values for each receptor determines compound selectivity between β3 integrins (αvβ3 and αIIbβ3). This evaluation was critical in order to further assess the most appropriate compound to further study *in vitro* and *in vivo*. We demonstrated a dose-dependent effect of SC56631, SC68448, S-247, S-197, and S-205 on the metabolic status of Cyr61- overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones and MCF-7/V control cells (Fig. 2).

A marked decrease in the cell viability was observed in Cyr61- and αvβ3-overexpressing MCF-7/C2-6 and MCF-7/C2-9 clones cultured in the presence of SC68448, S-247, and S-196 (55) The MCF-7/C2-6 and MCF-7/C2-9 cells were exquisitely sensitivity to the αvβ3 antagonist S-247, the most potent and selective αvβ3 antagonist, with IC_{50} values lower than 3 μM. Conversely, Cyr61- and αvβ3-negative MCF-7/V cells were unresponsive to the αvβ3 antagonist S-247. Similar results were obtained in anchorage-dependent growth assays. MCF-7/C2-6 and MCF-7/C2-6 were insensitive to very high concentrations of S-205, a highly potent and selective antagonist of the β3 integrin $\alpha_{IIb}\beta_3$, further showing the specificity of the Cyr61/ αvβ3 interaction.

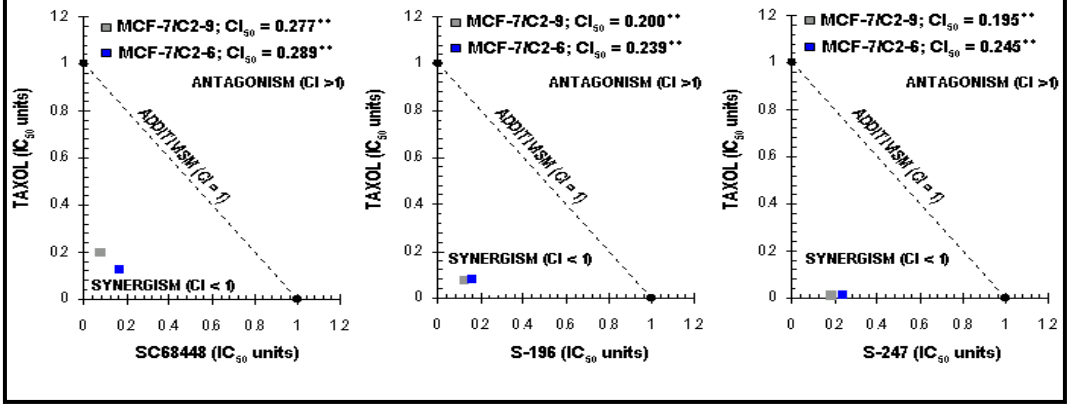
$\alpha_v\beta_3$ antagonist	IC_{50} ($\alpha_v\beta_3$)	IC_{50} ($\alpha_{IIb}\beta_3$)
SC56631	10	9 (1)
SC68448	0.8	407 (509)
S-247	0.6	1290 (2150)
S-196	1.19	54 (45)
S-205	> 100000	136 (-)



3. Cyr61 EXPRESSION PROMOTES A Cyr61-αvβ3 AUTOCRINE PRO-SURVIVAL LOOP IN MCF-7 BREAST CANCER CELLS: To investigate whether Cyr61-induced resistance to Taxol was specifically mediated through αvβ3, Cyr61-overexpressing and control cells were treated with Taxol in the absence or presence of αvβ3 antagonists. To measure the increase in Taxol sensitivity, a "sensitization factor" was determined by dividing Taxol IC_{50} values in the absence of αvβ3 antagonists by those in their presence. The results were that SC68448, S-196, and S-247 enhanced the cytotoxic activity of Taxol in a dose-dependent manner. As the concentration of αvβ3 antagonists increased, the efficacy of Taxol was significantly increased; moreover, the sensitization effects were dependent on the ability of the peptidomimetic antagonists to specifically interact with αvβ3. Thus, MCF-7/C2-6 and MCF-7/C2-9 cells in the presence of 0.5μM SC68448 (the αvβ3 antagonist with the lowest affinity and specificity for αvβ3) showed a 5 to 9-fold increase in Taxol sensitivity, while the S-247 (the αvβ3 antagonist with the highest affinity and specificity for αvβ3) increased Taxol-induced cytotoxicity up to 80 times. Taxol- induced cytotoxicity was not significantly changed when αvβ3-negative MCF-7/V cells were co-exposed to SC68448, S-196 or S-247 (Fig 3). These compounds as single agents significantly decreased cell viability of MCF- 7/Cyr61 expressing cells, suggesting the presence of a potentially significant synergic and/or antagonist component. Thus, possible synergistic interactions between αvβ3 antagonists and Taxol could not be accurately discriminated from additive or antagonistic effects on the basis of the above data alone. We performed a series of isobologram transformations of multiple dose-response analyses. The amount of the two agents together necessary to reduce MCF-7/C2-6 and MCF-7/C2-9 cell viability by 50% was only ~ 0.2 times as much as would be required if they demonstrated purely additive behavior ($P < 0.001$).

Figure 3

Cells	IC_{50} (nM)	Resistance (*x*-fold)	+ SC68448 IC_{50} (nM)	+ S-196 IC_{50} (nM)	+ S-247 IC_{50} (nM)
MCF-7/V	5	-	5.5 (1)	4.0 (1)	3.5 (1)
MCF-7/C2-9	45	9	9.0 (5)	3.5 (13)	0.6 (80)
MCF-7/C2-6	60	12	7.5 (8)	5.0 (12)	0.75 (80)



These results imply that Cyr61 regulates breast cancer cell sensitivity to chemotherapeutic agents such as Taxol mainly through its interaction with the **αvβ3** *integrin, and that therapies depriving Cyr61 cells of their* **αvβ3** *proliferative signaling dramatically increase Taxol efficacy.*

4. siRNA BLOCKAGE OF Cyr61 EXPRESSION RESULTS IN THE ANCHORAGE-INDEPENDENT GROWTH ARREST OF BREAST CANCER CELLS: One of the premises of our proposal is that Cyr61 is a marker for invasive breast carcinomas and that it induces antiestrogen and Taxol resistance, thereby becoming a good candidate for targeted therapy. Since the initial submission of the proposal we have worked to generate additional data to further support this hypothesis with putative specific Cyr61 targeted siRNA. Out of several siRNA tested, we have identified two specific siRNA that were introduced into stable retroviral vectors to generate stable cell lines that will no longer express Cyr61 or express a marked decrease in Cyr61 expression levels to further investigate the value and the significance of Cyr61 expression in the progression of breast carcinomas in vivo. We initially infected, the MDA-MB-231 cells, which express superior levels of Cyr61, and assessed the extent to which Cyr61 expression was diminished. After the analysis of the stable retroviral expressing cells, the expression of Cyr61 was significantly reduced (MDA-MB-231-shRNACyr61-1) and undetectable (MDA-MB-231- shRNACyr61-2) compared with the wild type cells MDA-MB-231WT or the siRNA control cell line MDA-MB-231-shRNA-Vector (Fig 4A). The initial biological effect of knocking down Cyr61 in MDA-MB-231 cells was assessed by the anchorage-independent growth assay. Rates of MDA-MB-231-siRNA vector cell cloning were as expected, almost 700 colonies in 6 days. In contrast, the MDA-MB-231-shRNA-CYR61 cell lines had a tremendous difficulty cloning even after 14 days of incubation. This was most significant in the MDA-MB-231-shRNA-Cyr61-2 cells that correlated highly with the low Cyr61 expression (Fig. 4B). To determine whether the expression of Cyr61 influences αvβ3 expression, we also measured the level of αvβ3 in the MDA-MB-231-shRNA-Cyr61 cell lines versus the control siRNA cell line. The level of αvβ3 expression was reduced to 50% of control, suggesting that indeed there is a balance between Cyr61 and αvβ3 expression (Fig 4C); similar results were obtained in HS578T cells.

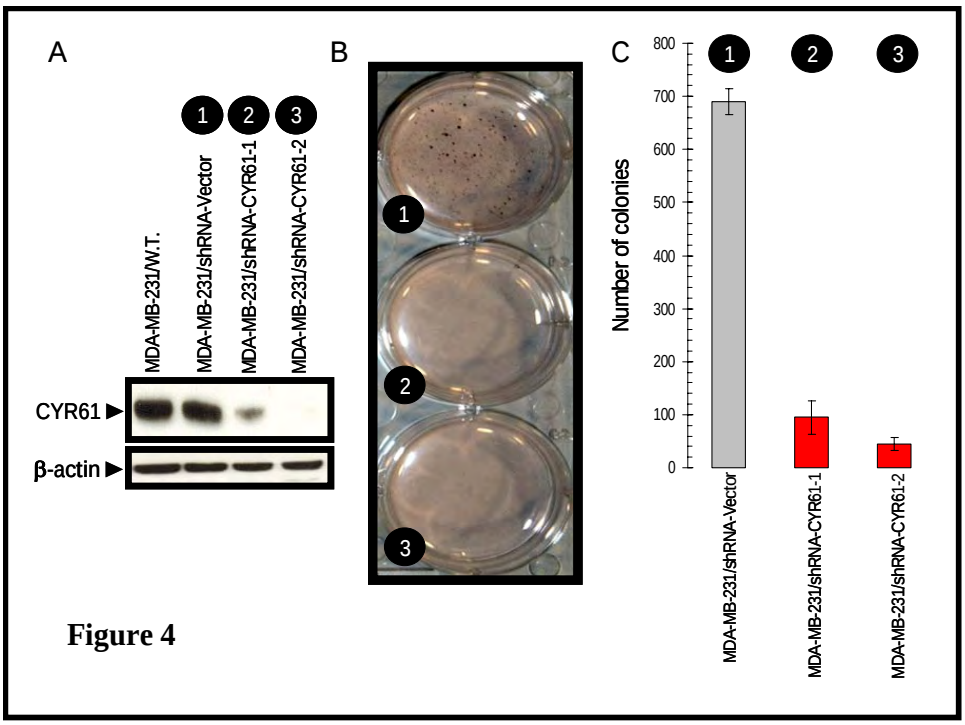


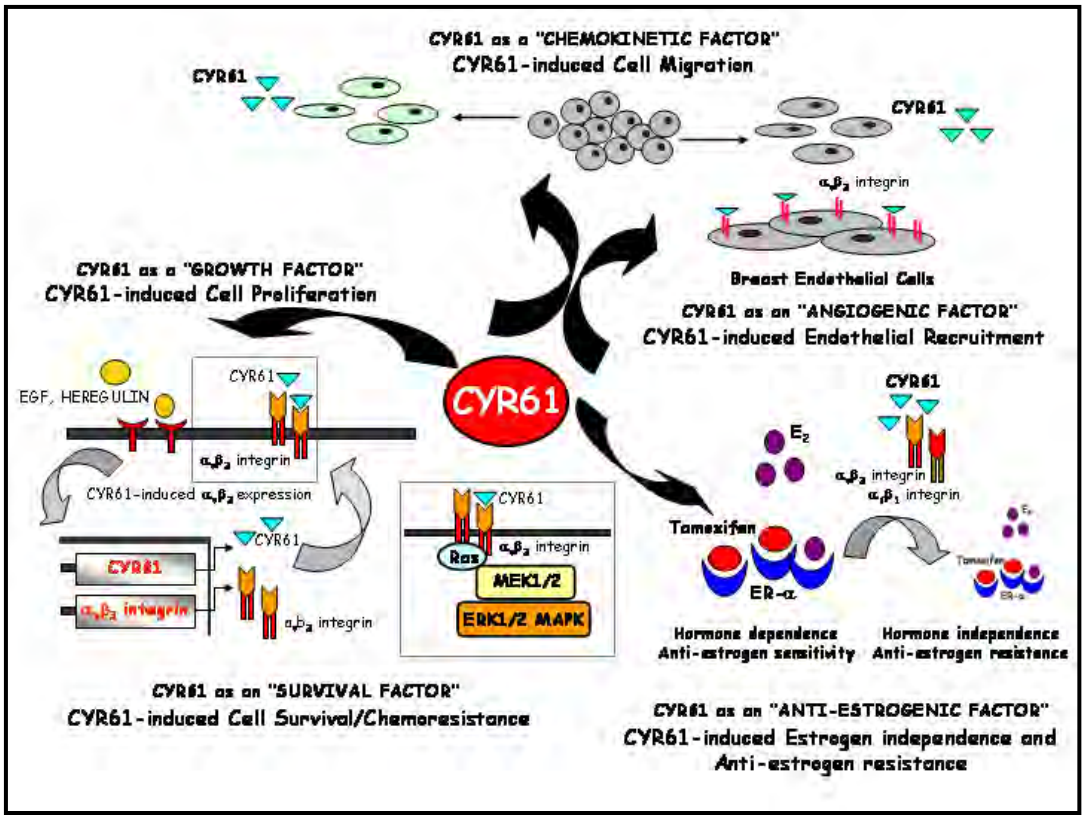
Figure 4

These results demonstrated that Cyr61 is a survival factor for invasive breast carcinomas and a key protein that could serve as a potential targeted therapy.

KEY RESEARCH ACCOMPLISHMENTS

We have discovered a novel role of Cyr61 in breast tumorigenesis and progression. Given that the angiogenic factor Cyr61 is also a growth-regulator, it is hypothesized that up-regulation of Cyr61 in breast cancer cells as we have observed may drive breast tumorigenesis and progression in several concerted modes as follows:

- *Promoting tumor cell proliferation in an autocrine manner augmenting growth factor bioactivity and/or emitting proliferative signals via the αvβ3 integrin receptor,*
- *Regulating endothelial recruitment tumor neovascularization in a paracrine fashion through an αvβ3-dependent mechanism,*
- *Directing tumor epithelial invasiveness and aggressiveness as a chemokinetic factor*
- *Specific αvβ3-RGD- peptidomimetic agents reverse Cyr61 induced taxol-resistance*
- *Specific αvβ3-RGD- peptidomimetic agents block Cyr61 induced anchorage-independent growth*
- *Specific αvβ3-RGD- peptidomimetic agents block Cyr61 induced anchorage-independent growth*
- *Specific αvβ3-RGD- peptidomimetic agents synergize with Taxol to induce apoptotic cell dead of Cyr61 overexpressing cells and promote inhibition anchorage-independent growth*



REPORTABLE OUTCOMES

- *Specific αvβ3-RGD- peptidomimetic agents reverse Cyr61 induced taxol-resistance*
- *Specific αvβ3-RGD- peptidomimetic agents block Cyr61 induced anchorage-independent growth*
- *Specific αvβ3-RGD- peptidomimetic agents block Cyr61 induced anchorage-independent growth*
- *Specific αvβ3-RGD- peptidomimetic agents synergize with Taxol to induce apoptotic cell dead of Cyr61 overexpressing cells and promote inhibition anchorage-independent growth*

CONCLUSION

Breast cancer progression is a multistep process, in which the main families of adhesion and pro-angiogenic molecules, including integrins, have a pivotal role in the development of recurrent, invasive and distant metastasis. In this regard, expression of αvβ3 is significantly higher in tumors of patients with metastasis than in those without metastasis. Accordingly, vascular αvβ3 overexpression in breast cancer has been correlated with a poor prognosis for patient survival (31). *Of particular therapeutic interest is the fact that in breast cancer, αvβ3 is expressed on both angiogenic endothelial cells and tumor cells. Interestingly, as a ligand for αvβ3, it is plausible that Cyr61 may emit its survival effects through integrin signal transduction. A therapeutic strategy directed against Cyr61 and/or αvβ3 signaling would allow, therefore, a dual antiangiogenic and antitumor strike with a single drug. SC68448 and S-247, a non-peptidomimetic compound such a Vitaxin, a humanized functional blocking antibody capable of blocking the integrin receptor αvβ3, are currently under clinical development. Therefore, our experimental approach will shed light on the understanding of a new mechanism-based strategy for the development of new therapies for the treatment of metastatic human breast cancer.*

*The experiments proposed in the current application are design to test these hypotheses and will characterize the new Cyr61/ **αvβ**3 autocrine loop (survival loop) in breast cancer and validate Cyr61 as a prognostic marker for breast cancer progression and drug resistance. Furthermore, our studies will be the first studies that will provide a rationale and proof-of-concept to develop a clinical trial based upon Cyr61/ **αvβ**3 target therapies alone or in combination with taxane-based therapies.*

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APPENDICES

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