Cyr61 promotes breast tumorigenesis and cancer progression [1]

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Abstract

Cyr61, a member of the CCN family of genes, is an angiogenic factor. We have shown that it is overexpressed in invasive and metastatic human breast cancer cells and tissues. Here, we investigated whether Cyr61 is necessary and/or sufficient to bypass the "normal" estrogen (E2) requirements for breast cancer cell growth. Our results demonstrate that under E2-depleted condition, Cyr61 is sufficient to induce MCF-7 cells grow in the absence of E2. MCF-7 cells transfected with Cyr61 (MCF-7/Cyr61) became E2-independent but still E2-responsive. On the other hand, MCF-7/vector cells remain E2-dependent. MCF-7/Cyr61 cells acquire an antiestrogen-resistant phenotype, one of the most common clinical occurrences during breast cancer progression. MCF-7/Cyr61 cells are anchorage-independent and capable of forming Matrigel outgrowth patterns in the absence of E2. ERα expression in MCF-7/Cyr61 cells is decreased although still functional. Additionally, MCF-7/Cyr61 cells are tumorigenic in ovariectomized athymic nude mice. The tumors resemble human invasive carcinomas with increased vascularization and overexpression of vascular endothelial growth factor (VEGF). Our results demonstrate that Cyr61 is a tumor-promoting factor and a key regulator of breast cancer progression. This study provides evidence that Cyr61 is sufficient to induce E2-independence and anti-E2 resistance, and to promote invasiveness *in vitro*, and to induce tumorigenesis *in vivo*, all of which are characteristics of an aggressive breast cancer phenotype.

Main Text

About 60% of human breast carcinomas express estrogen receptor (ER), are dependent upon estrogen (E2) for growth, and thus respond to the treatment with an ER antagonist, tamoxifen (Tam). Many breast carcinomas, however, over time become less sensitive to estrogen, and thus more resistant to the endocrine treatment, developing into more aggressive tumors. Aggressiveness of breast cancer cells is commonly attributed to the ability of the cells to overcome the estrogen (E2) requirements for growth, and in most cases to acquire antiestrogen resistance. However, the mechanism by which breast cancer progresses from an E2-dependent and anti-E2-responsive phenotype to an E2-independent and anti-E2-resistant phenotype is not yet well established.

We have shown previously that expression of heregulin (HRG), a growth factor that activates the *erbB*-2/3/4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype (Cardillo *et al.,* 1995). We have further demonstrated that HRG induces breast cancer progression, as determined by the loss of estrogen receptor (ER) function and E2 response, tumorigenicity, invasion, and metastasis (Tang *et al.,* 1996; Lupu *et al.,* 1995, 1996; manuscript submitted for publication). We hypothesize that HRG induces activation of the *erbB* signaling pathways, leading to regulation of downstream genes that control cancer cell growth and tumor progression. We thus isolated and identified Cyr61, an angiogenic factor, which was differentially expressed in ER-negative, HRG-positive, invasive, and metastatic breast cancer cells, and in 30% of breast tumor biopsies (Tsai *et al.,* 2000). We showed that Cyr61 is important

for HRG-mediated chemomigration and invasiveness of breast cancer cells *in vitro* (Tsai *et al.*, 2000).

Cyr61 belongs to the CCN family of angiogenic regulators, which consists of Cyr61, CTFG, Nov, WISP-1, WISP-2, and WISP-3 (Lau and Lam, 1999). Cyr61 is a cysteine-rich, heparin-binding protein that is secreted and associated with the cell surface and the extracellular matrix (ECM) (Yang *et al*., 1991; Kireeva *et al*., 1997). It has been shown that Cyr61 binds to integrins, such as αvβ3, αIIbβ3 and α6β1 (Kireeva *et al*., 1996a; Jedsadayanmata *et al*., 1999; Chen *et al*., 2000). Cyr61 mediates cell adhesion, stimulates cellular migration, enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells, and increases chondrogenesis in mesenchymal cells (O'Brient *et al*., 1992; Kireeva *et al*., 1996b; Kireeva *et al*., 1997; Frazier *et al*., 1996; Kolesnikova and Lau, 1998). Moreover, Cyr61 stimulates an integrin αvβ3-dependent chemotaxis of endothelial cells (Babic *et al*., 1998). Most significantly, expression of Cyr61 enhances neovascularization and tumor formation of human tumor cells in immunodeficient mice (Babic *et al*., 1998, 1999; Xie *et al*., 2001).

To determine whether expression of Cyr61 is necessary and/or sufficient to promote breast cancer progression, Cyr61 was introduced into the MCF-7 breast cancer cells, which are ER-positive, E2-responsive *in vitro*, E2-dependent *in vivo*, and never metastasize *in vivo*. MCF-7 cells were stably transfected with a eukaryotic expression vector containing the full-length cDNA of the human Cyr61 gene (MCF-7/Cyr61), or with an empty vector (MCF-7/V) as a negative control. A number of Cyr61 (10 clones) and vector-transfected clones (4 clones) were

isolated and characterized for the expression of Cyr61 at both the mRNA and protein levels. Cyr61 was highly expressed in the Cyr61-transfected cells as determined by the RNase protection assay (Fig. 1A) and by Western blot analysis using conditioned media concentrated from the Cyr61 clones or vector control clones (Fig. 1B). Importantly, the expression level of Cyr61 in MCF-7/Cyr61 was comparable to that in MDA-MB-231, which is an aggressive breast cancer cell line and naturally expresses high levels of Cyr61. Our results demonstrated a 5- to 35-fold increase in Cyr61 mRNA and/or protein in the MCF-7/Cyr61 cells, as compared with the wild type or MCF-7/V cells in which Cyr61 expression was very low or nearly (Fig. 1). Since Cyr61 mRNA and protein expression did not vary significantly, and their cellular behavior was similar in most of the clones, we chose to present the data obtained in one vector clone (V2) and two representative Cyr61 clones (C6 and C20).

The MCF-7/Cyr61 cells showed a growth advantage in E2-depleted media, having a 3- to 5-fold increase in growth as compared with the MCF-7/V cells (Fig. 2A). The average doubling time in E2-free conditions for the MCF-7/Cyr61 cells is approximately 36 hours, in contrast with 72 hours for the MCF-7/V cells (data not shown). These results demonstrate that MCF-7/Cyr61 cells acquire an E2-independent phenotype. We thus postulate that overexpression of Cyr61 provides MCF-7 cells a growth advantage to bypass their “normal” estrogenic requirement for cellular proliferation. As expected, E2 stimulated the growth of MCF-7/V cells (Fig. 2A) because MCF-7 cells are E2-responsive (Pratt and Pollak, 1993). Although the MCF-7/Cyr61 cells acquire E2-independence, these cells are still responsive to E2 (Fig. 2A), one of the clinical

phenotypes found in women suffering from breast cancer????. We next tested the ability of antiestrogens to block the E2 induction of cell growth. Two distinct antiestrogens were used, Tamoxifen (Tam) and ICI 182,780 (ICI). Tam, a well-known antiestrogen, functions as an agonist and antagonist through both transcriptional activation domains (AF1 and AF2) of ER. ICI, a pure antiestrogen, acts solely as an antagonist through the AF1 domain (MacGregor and Jordan, 1998). When MCF-7/Cyr61 was treated with ICI (Fig. 2A), and Tam (data not shown), both antiestrogens were unable to block the E2-independent growth of the MCF-7/Cyr61 cells, suggesting that Cyr61 provides a true growth advantage that is not blocked by antiestrogens. ~~In anchorage-dependent growth assays, both antiestrogens were not effective in reducing colony formation of MCF-7/Cyr61 cells~~. Both antiestrogens only reduced the growth induced by E2. That is, the antiestrogen ICI inhibited only the anchorage-dependent growth of MCF-7/Cyr61 cells to the elevated basal level. ~~meaning the increase induced by E2. More importantly~~, ICI was not able to reduce the growth of MCF-7/Cyr61 to the same growth level observed in the wild type or MCF-7/V cells (Fig. 2A). The fact that both antiestrogens had similar effects supports the hypothesis that the growth stimulation by E2 is indeed mediated directly through ER and most probably not mediated through other secondary mechanisms.

In summary, our data demonstrate that overexpression of Cyr61 stimulate cell growth of E2-dependent cells in the absence E2, resulting in cells becoming estrogen-independent for growth. On the other hand, E2 further enhanced cellular proliferation of Cyr61-expressing cells, demonstrating that these cells, although independent of E2, are still responsive to E2. Consistent

with our previous findings that E2 induces Cyr61 expression, and Tam and ICI blocks E2-induced Cyr61 expression (Tsai *et al*., 2001), here, we demonstrated that both antiestrogens Tam and ICI decrease E2-stimulated growth in Cyr61-expressing cells above and beyond ???what was already stimulated by overexpression of Cyr61. However, Tam and ICI do not block the proliferation of Cyr61-expressing cells to the same basal level observed in vector or wild-type MCF-7 cells. Therefore, overexpression of Cyr61 alone, most probably, accounts for the growth advantage observed in the MCF-7 cells in E2-depleted media. Moreover, our *in vitro* studies represent a situation similar to what often occurs in human breast carcinomas *in vivo*, in which E2 can induce tumor growth (or not), in spite of the levels of ER expression are almost identical. Clinical trials have been conducted with E2 followed by chemotherapeutical drugs, based on the rationale that E2 stimulates tumor growth, allowing cells to enter the cell cycle, and thus provides a better environment for chemotherapeutic drugs to be more effective.

Cyr61-induced E2-independence became more evident when we further demonstrated that MCF-7/Cyr61 cells are anchorage-independent in the absence of E2 (Fig. 2B), that is, these cells form colonies in soft agar assays. 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. In general, the MCF-7/Cyr61 cells formed large colonies in the size range of 100-150 μm. E2 slightly stimulated the colony formation in the MCF-7/Cyr61 cells (Fig. 2B). ICI was not able to block the colony formation induced by E2 in MCF-7/Cyr61 (Fig. 2B). All other MCF-7/Cyr61 clones behave similarly (data not shown). Neither vector nor wild

type MCF-7 cells formed significant number of colonies in the absence of E2. Smaller colonies or single cells were seen in MCF-7/V cells in the presence of E2 (Fig. 2B). As expected, E2-induced anchorage-independent growth of MCF-7/V cells and was completely blocked by antiestrogens ICI (Fig. 2B) and Tam (data not shown).

To determine whether Cyr61 promotes an invasive phenotype, MCF-7/Cyr61 cells were tested in the Matrigel outgrowth assay, which has been employed frequently as a reliable system to assess *in vitro* invasiveness of breast cancer cells (Sommers *et al*., 1994; Hijazi *et al*., 2000). The MCF-7/Cyr61 clones C6 and C20 showed extensive outgrowth in Matrigel; the colonies appeared large and irregular in shape. In contrast, the MCF-7/V cells were not able to migrate through and proliferate in Matrigel matrix, remaining as single cells in the matrix even in the presence of E2 (Fig. 2C). Significantly, Cyr61 promotes outgrowth of MCF-7 cells in the Matrigel matrix in the absence of E2, suggesting that Cyr61 is capable of inducing a critical invasive phenotype of breast cancer cells in an E2-indpendent manner. These results strongly indicate that expression of Cyr61 enhances the *in vitro* invasiveness of breast cancer cells, which may thereby provide the appropriate milieu for these cells to migrate and perhaps metastasize *in vivo*. All other MCF-7/Cyr61 clones behave similarly (data not shown).

It has been shown previously that a possible mechanism to acquire E2-indepent and antiestrogen-resistant phenotype via the loss of ER expression and/or ER function. The ERα expression was determined by RNase Protection assays; the ERα function was determined as the ability of E2 to regulate the expression of E2-responsive genes (Govind and Thampan, 2001).

We first examined the basal level of ER expression in MCF-7/Cyr61, because the level of ERα expression is extremely low???? in MCF-7 cells (Vladusic *et al*., 1998). The basal level of ERα expression is defined as ERα expression in the absence of E2 in cells cultured in phenol red-free media containing 5% charcoal-treated fetal bovine serum. Our data indicate that the basal level of ER expression was markedly reduced (30-50%) in all of the MCF-7/Cyr61 clones, as compared with MCF-7/V cells (Fig. 3A, left and middle panels). These results indicate that Cyr61 expression is correlated 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). It has been shown recently that Cyr61 expression is frequently associated with diagnosis of advanced disease (Xie *et al*., 2001), however, the sample number tested was relatively small. Paradoxically, the same report showed that Cyr61 expression in human breast biopsies is correlated with ER positivity (Xie *et al*., 2001), even though ER expression is an indicator of good prognosis for breast cancer (Brower *et al*., 1999). Therefore, more studies are required to establish the mechanisms of Cyr61 action and its role in breast carcinomas.

We next examined whether Cyr61 promotes loss of ER function by assessing regulation of several well-documented E2-responsive genes. We tested ERα, shown to be downregulated by E2, and progesterone receptor (PgR), Cathepsin D and pS2, all of which have been shown to be upregulated by E2 in MCF-7 cells (Read *et al*., 1989; Nardulli *et al*., 1988; Cavailles *et al*.,

1988; Weaver *et al.*, 1988). We have previously demonstrated that the loss of PgR regulation by E2 attests for the loss of ER function when MCF-7 cells were transfected with HRG (Tang *et al.*, 1996; Saceda *et al.*, 1996). Our studies were then focused on E2 regulation of ERα and PgR expression as previously described (Tang *et al.*, 1996). Although the level of ERα expression is lowered, E2 still downregulates the expression of ERα in MCF-7/Cyr61 cells. This regulation is normally tightly controlled by E2 in the parental MCF-7 cells. Our results demonstrate that in the presence of E2, ERα expression is downregulated about 40-50% in the MCF-7/V cells, as compared with the untreated cells (Fig 3A, right panel). In contrast, E2 does not downregulate ERα expression in MCF-7/Cyr61 cells to the same extent as in the MCF-7/V cells. E2-induced downregulation of ERα in the MCF-7/Cyr61 cells was only about 10-25% as compared with the untreated cells (Fig.3A, left panel). The diminished effect of E2 on ERα is most probably because the basal level of the ERα expression in the MCF-7/Cyr61 cells is already markedly reduced (Fig.3A, middle panel), therefore, additional treatment with E2 cannot further induce a more significant reduction in ERα expression (Fig. 3A, right panel).

We then tested whether ERα was still a functional receptor for E2 to induce upregulation of PgR gene expression in the MCF-7/Cyr61 cells. Interestingly, even though ERα expression was significantly lowered in MCF-7/Cyr61 cells, E2 induced a marked upregulation in PgR mRNA expression. The increased in PgR expression was between 300 to 400%, as compared with the untreated MCF-7/Cyr61 cells (Fig. 3B). As expected, E2 upregulated the levels of PgR

in the MCF-7/V cell by about 200%. Similarly, we observed E2-induced upregulation of other E2-responsive genes, including Cathepsin D and pS2 (data not shown). These data substantiate 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. Our combined results demonstrate that Cyr61 is sufficient to confer E2-independence and anti-E2 resistance. Interestingly, the cells transfected with Cyr61 retained E2-responsiveness and the ERα function. On the other hand, we have clearly demonstrated that Cyr61 is capable of downregulating ER expression, and moreover, promotes an invasive phenotype. This is consistent with our previous data which demonstrates that in breast cancer cells, Cyr61 expression correlates with the loss of ER expression, increased tumorigenicity, and the ability of cells to metastasize (Tsai *et al*., 2000).

To assess the effect of Cyr61 expression *in vivo*, MCF-7/V and MCF-7/Cyr61 cells were inoculated into the mammary fat pads of 4-5 week old ovariectomized athymic nude mice. Tumors were spontaneously formed only in those mice injected with MCF-7/Cyr61 cells. These tumors grew independently of hormonal stimulation (Fig. 4A). MCF-7/V cells used as a control only developed tumors in the presence of E2 stimulation (Fig. 4A). These data indisputably demonstrates that transfection of MCF-7 cells with Cyr61 promotes tumorigenesis *in vivo* in the absence of hormonal stimulation in the complete absence of E2. These findings support our *in*

vitro data demonstrating that the MCF-7/Cyr61 cells had a growth advantage in the absence of E2. Significantly, the data from the our model system provides novel evidence which resembles many breast cancer clinical cases, showing the progression of the disease from an ERα-positive and E2-dependent phenotype to an E2-independent phenotype, yet frequently without the loss of ERα expression.

The tumors developed by the MCF-7/Cyr61 cells were excised and analyzed macroscopically. The vast majority of the tumors appeared as firm, poorly defined masses. The lesions measured between 1 and 1.5 cm in the greatest dimension. The MCF-7/Cyr61-derived tumors demonstrated fixation to the underlying soft tissues, as well as erosion of the overlying skin. Enlargement of axillary lymph nodes was detected in the same group, whereas no macroscopic metastatic foci to visceral organs were noticed (Fig. 4A). Lymph nodes involvement in the tumor was not observed. In all of the MCF-7/Cyr61 cell groups, the tumors were characterized by histological, tightly cohesive areas of large, pleiomorphic cells with irregular nuclei and numerous mitotic figures. Multinucleated cells were observed. Neovascular formation and areas of necrotic tissues were observed (Fig. 4B).

By immunohistochemical staining using an anti-Cyr61 antibody, we demonstrated clearly that the tumors developed from MCF-7/Cyr61 cells expressed very high levels of Cyr61 protein. We observed very high levels of Cyr61 expression in the tumors developed from MCF-7/Cyr61 cells. On the contrary, the levels of Cyr61 expression in tumors developed with the control MCF-7/V in the presence of E2 was very low or undetectable (Fig. 4C). These data demonstrate that

the expression of Cyr61 is maintained *in vivo*, and that the phenotypic changes are mediated most probably through the Cyr61 protein. It was obvious that the tumors developed from the Cyr61-expressing cells were extremely vascularized. We therefore tested the expression of another angiogenic factor, apart from Cyr61, the vascular endothelial growth factor (VEGF), a growth factor to stimulate neovascularization. A marked increased in VEGF expression was observed in the tumor sections derived from the MCF-7/Cyr61 cells, but not in sections derived from MCF-7/V tumors formed in the presence of E2 (Fig. 4D).

Overall, the role of Cyr61 in breast cancer is still under investigation and further studies are necessary to determine the mechanism by which Cyr61 promotes breast cancer progression. We have clearly established that Cyr61 promotes tumor growth of breast cancer cells, in accordance with earlier observations, showing that a gastric adenocarcinoma cell line RF1 becomes tumorigenic when Cyr61 is introduced in those cells (Babic *et al*., 1998). When this work and manuscript were in progress, a report described the ability of Cyr61 to induce tumorigenicity of breast cancer and breast normal cells (Xie *et al*., 2001). However, the study does not address ER expression or function, neither the extensive biological characterization presented here.

In this study, we demonstrate that expression of Cyr61 leads to E2-independence and anti-E2 resistance in MCF-7 cells for both anchorage-dependent and -independent growth. In the Matrigel matrix, Cyr61 promotes outgrowth of the MCF-7 cells in an E2-independent manner. Apparently, Cyr61 downregulates the expression of ERα yet does not disrupt its function. Thus,

Cyr61-expressing cells are still E2-responsive. We also demonstrate that overexpression of Cyr61 induces tumor formation in immunodeficient mice and promotes the expression of VEGF, an important regulator of neovascularization. Our current results further imply that Cyr61 is a downstream effector of HRG, because Cyr61 can bypass the effect of HRG and can induce similar phenotypic changes of breast cancer cells as promoted by HRG.

It has been shown that Cyr61 is an angiogenic ligand for αvβ3 (Babic *et al*., 1998, 1999). We have previously reported that a functional blocking antibody against αvβ3 is capable of blocking HRG induction of the aggressive phenotypes of the breast cancer cells (Tsai *et al*., 2000). We had proposed that Cyr61 mediates tumor growth and angiogenesis of breast cancer cells via either an autocrine or paracrine manner through its binding to the αvβ3 integrin receptor. This receptor is often expressed in endothelial as well as epithelial cells. It is known that integrin receptors mediate cellular adhesion to the extracellular matrix, which exerts profound control over the cells. Although Cyr61 binds to integrins αvβ3, αIIbβ3 and α6β1, so far, only the αvβ3 integrin has been shown to play a major role in breast cancer tumor vascularization and progression (Meyer *et al*., 1998). More importantly, it has been recently demonstrated that overexpression of αvβ3 is a marker for poor prognosis in breast cancer (Gasparini *et al*., 1998). We can then speculate that binding of Cyr61 to the angiogenic integrin receptor αvβ3 should provide new insights into the possible mechanism by which Cyr61

promotes breast tumorigenesis and cancer progression. We are currently investigating this avenue that will provide further molecular mechanisms of Cyr61 action.

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Figure Legends

Figure 1. Expression of Cyr61 in MCF-7/Cyr61 clones. (A) Total RNA was isolated from subconfluent MCF-7/V and MCF-7/Cyr61 cells, and 30 µg of RNA was analyzed for Cyr61

mRNA expression by RNAse protection assay as previously described (Tsai *et al.*,2000). The GAPDH probe was used as an internal control for RNA loading. Representative vector and Cyr61 clones were shown. (B) Subconfluent breast cancer cell lines were cultured in serum-free media for 48 hr. Conditioned media were collected, concentrated 100X, and analyzed by Western blotting with a rabbit polyclonal anti-Cyr61 antibody as previously described (Tsai *et al.*,2000).

Figure 2. Induction of E2-independency and anti-E2 resistance of MCF-7 cells by Cyr61. (A) Subconfluent MCF-7/V and MCF-7/Cyr61 clones were cultured in media containing charcoal stripped fetal bovine serum (FBS) for 4 days and plated (2,500 cells/well) in triplicate in 24-well plates for proliferation assay. Cells were incubated in the presence of solvent control (0.1% ethanol; C), E2 (1 nM; E), ICI (100 nM; I), and the combination of E2 and ICI (E/I). Cell number was counted at day 7. Data of two representative Cyr61 clones are shown from at least four independent experiments. Similar results were obtained from other Cyr61-overexpressing clones. (B) Subconfluent MCF-7/V and MCF-7/Cyr61 clones were cultured in E2-depleted media for 4 days and plated (20,000 cells/well) in triplicate in 6-well plates for anchorage-independent soft agar assay as previously described (Guerra-Vladusic *et al.*,1999). Cells were cultured in the presence of solvent control (C), E2 (E), ICI (I), and the combination of E2 and ICI (E/I) as described in Fig. 2A. Data of two representative clones are shown from at least four independent experiments. Similar results were obtained from other Cyr61-overexpressing clones. (C) Subconfluent MCF-7/V and MCF-7/Cyr61 clones were cultured in E2-depleted media for 4

days and plated (5,000 cells/well) in triplicate in 12-well plates in the presence or absence of E2 for the Matrigel. Outgrowth assay as previously described (Hijazi *et al*., 2000). The outgrowth pattern was developed and photographed after 10-day incubation. Two representative clones are shown with similar results from three independent experiments. Similar results were obtained from other Cyr61-overexpressing clones

Figure 3. E2-responsive gene expression in MCF-7/Cyr61 clones. Subconfluent MCF-7/V and MCF-7/Cyr61 cells were cultured in media containing charcoal-stripped FBS for 4 days and treated in the presence or absence of E2 for 6 hrs. Total RNA was isolated, and 20 μg and 30 μg of total RNA were analyzed respectively for the expression of (A) ERα and (B) PgR by the RNase protection assay as previously described (Tang *et al*., 1996). Expression of ERα or PgR was normalized by GAPDH.

Figure 4. Expression of Cyr61 and VEGF in implanted breast tumor sections from nude mice. (A) Photographs of nude mice bearing human breast tumors. Tumors were developed by implanting MCF-7/V and MCF-7/Cyr61 cells ($2x10^6$ per site) into the mammary fat pads of 4-5 week old ovariectomized athymic nude mice with or without E2 pellets (25 mg, 90-day slow releasing) as previously described (Tang *et al*., 1996). (B) H&E staining of human breast tumor sections derived from MCF-7/V (a-100x and b-200x) and MCF-7/Cyr61 (c-100x and d-200x). (C) Immunohistochemical analysis of Cyr61 expression in breast tumor sections developed from implanted MCF-7/V and MCF-7/Cyr61 cells in nude mice as previously described (Tsai *et al*.,

2000). (D) Immunohistochemical analysis of VEGF expression in breast tumor sections derived from implanted MCF-7/V and MCF-7/Cyr61 cells in nude mice. Microphotographs are shown at 200x magnifications, unless otherwise specified.

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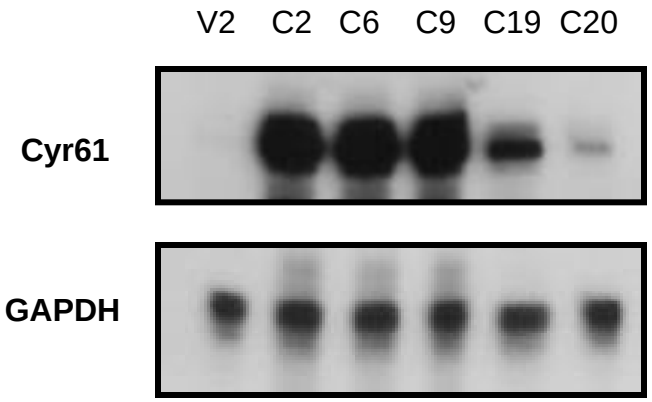

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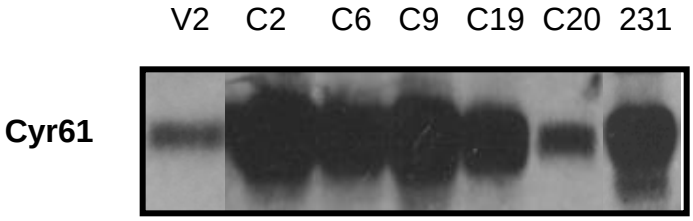

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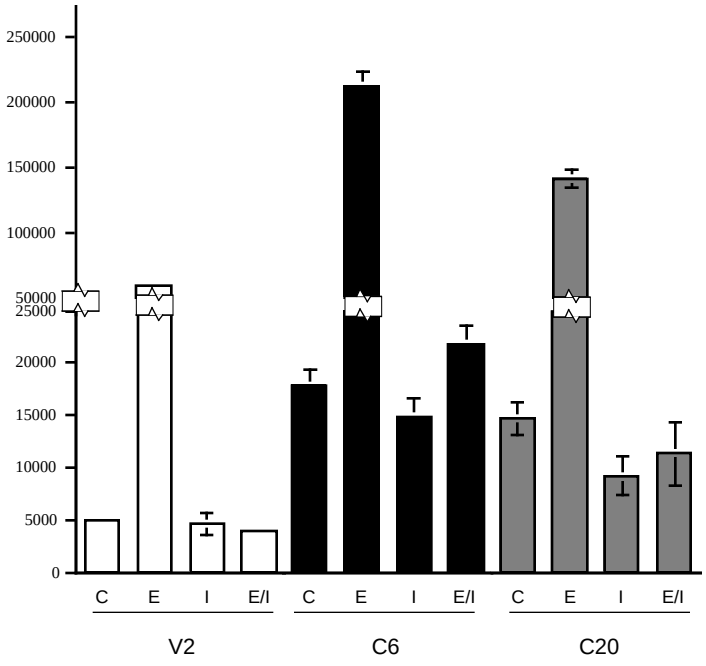
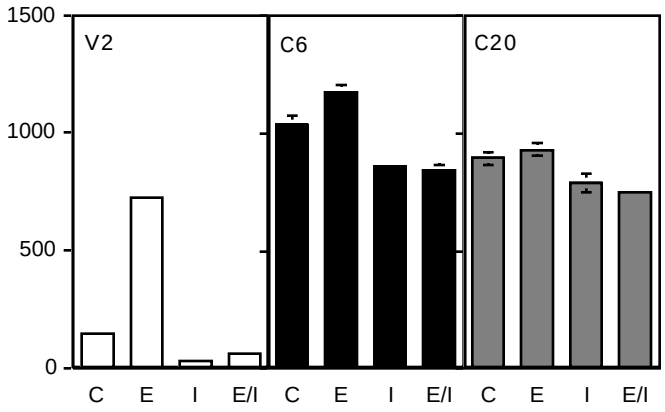
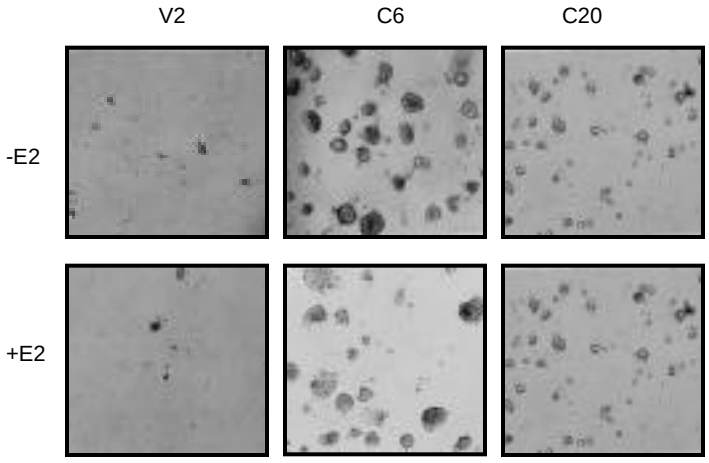

250000
200000
150000
100000
50000
25000
20000
15000
10000
5000
0
C
E
I
E/I
C
E
I
E/I
C
E
I
E/I
V2
C6
C20

B



C



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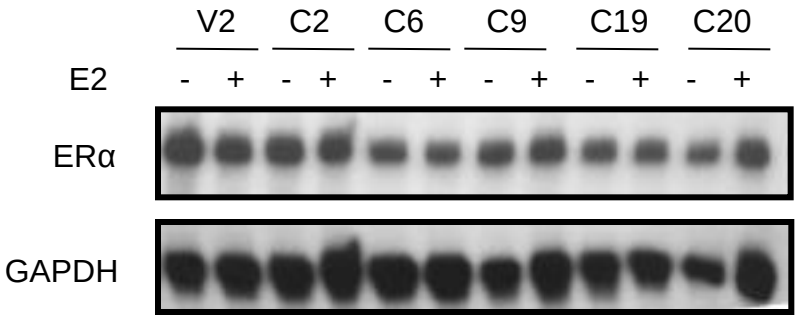
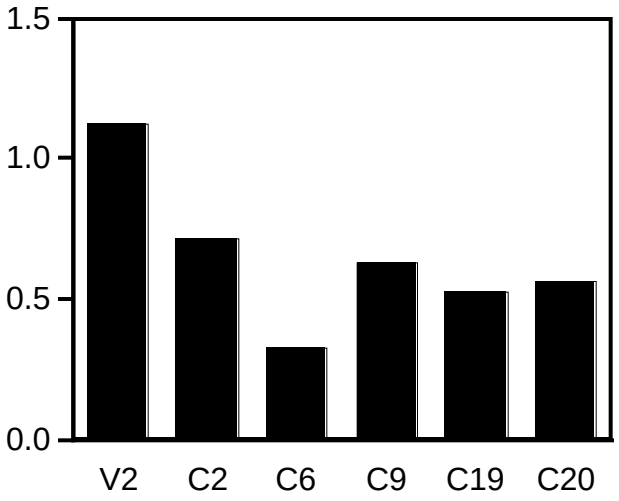
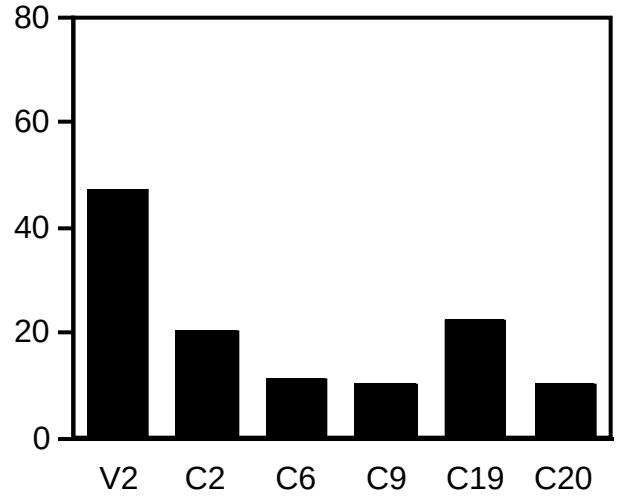
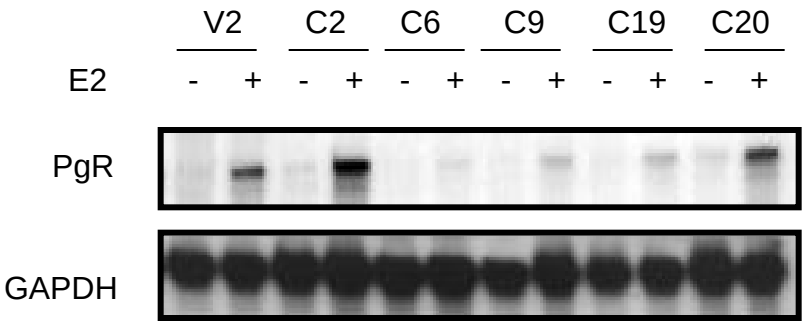
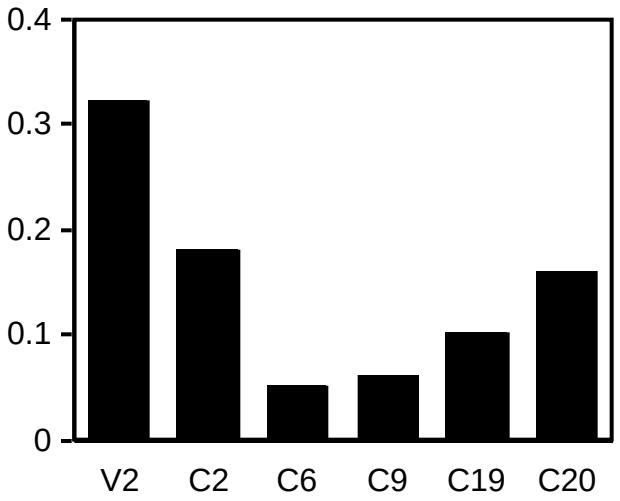

V2
C2
C6
C9
C19
C20
E2
- + - + - + - + - + - +
ERα
GAPDH


1.5
1.0
0.5
0.0
V2
C2
C6
C9
C19
C20


80
60
40
20
0
V2
C2
C6
C9
C19
C20

B


V2
C2
C6
C9
C19
C20
E2
- + - + - + - + - + - +
PgR
GAPDH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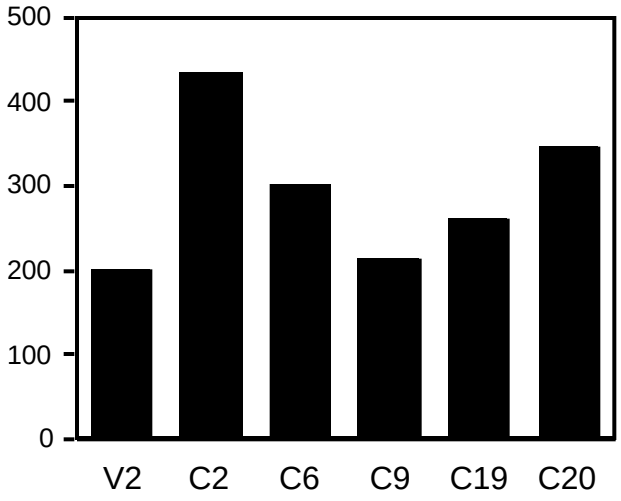
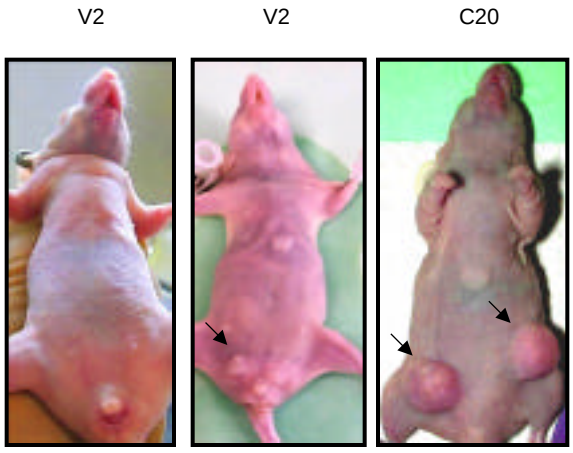
0.3
0.2
0.1
0
V2
C2
C6
C9
C19
C20


500
400
300
200
100
0
V2
C2
C6
C9
C19
C20

A



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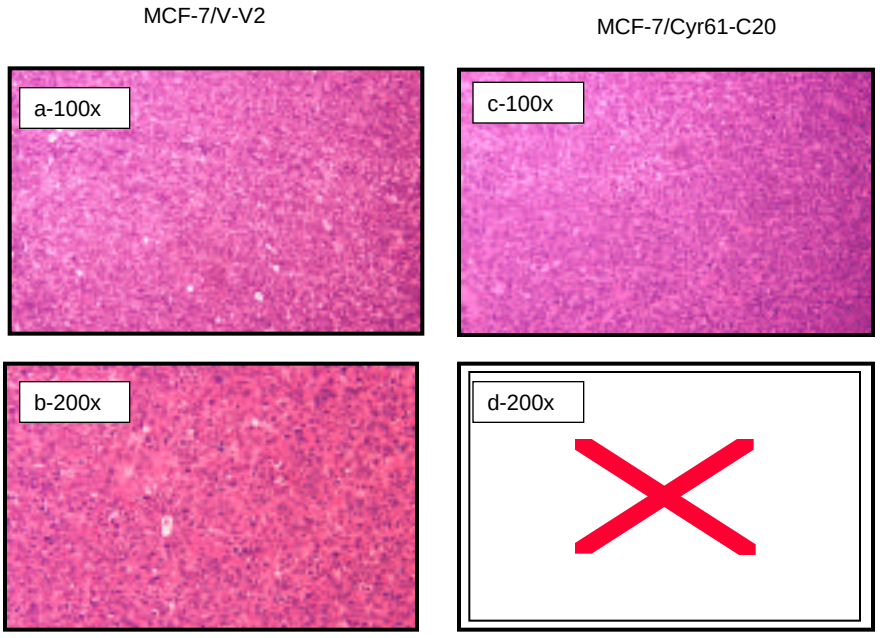
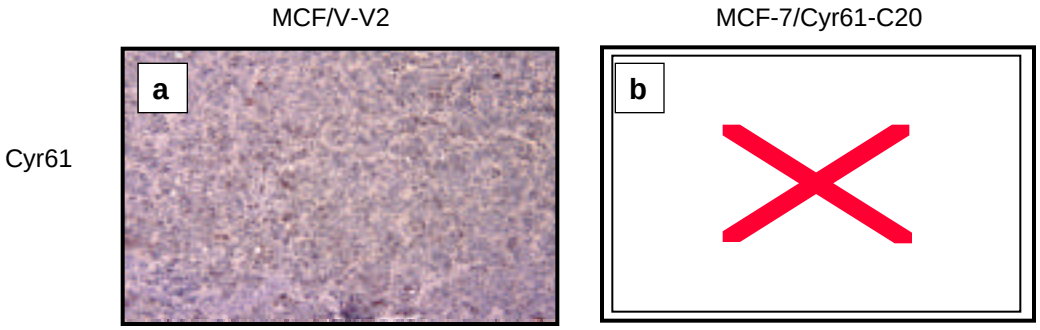


C



D