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13. ABSTRACT (Maximum 200 Words)

Successful tumor growth requires angiogenesis, or the sprouting of new blood vessels from existing ones, to supply tumor cells with essential nutrients and oxygen. In addition, cancer cells must also establish a productive interaction with their neighboring stroma, which produces a matrix environment conducive to tumor growth and may also help shield tumor cells against systemic immune surveillance. A novel angiogenic factor essential for vascular development, Cyr61, has been recently identified as a marker for invasive breast carcinomas. Cyr61 is strongly induced by TGF-β1, and may play an important role in the interaction between tumor cells and stromal fibroblasts in tumor growth. In this study, we have demonstrated unique effects of Cyr61 upon normal breast epithelial cells and breast cancer cells. In addition to being able to support cell adhesion and DNA synthesis upon EGF stimulation, Cyr61 can also cooperate with the action of TGF-β, but only in normal breast epithelial cells and not in cancer cells. These results suggests the possibility that Cyr61, which is known to be induced by TGF-β, to participate in TGF-β promoted tumorigenesis. Understanding the precise role of Cyr61 in tumor growth is being investigated.

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Table of Contents

INTRODUCTION

Successful tumor growth requires angiogenesis, or the sprouting of new blood vessels from existing ones, to supply tumor cells with essential nutrients and oxygen. Thus, the growth of tumors beyond ~1 mm in diameter is absolutely dependent on neovascularization. Inhibition of tumor angiogenesis can dramatically restrict tumor growth, and remarkable efficacy of anti-angiogenic cancer therapy has been demonstrated in a number of animal studies. In addition, cancer cells must also establish a productive interaction with their neighboring stroma, which produces a matrix environment conducive to tumor growth and may also help shield tumor cells against systemic immune surveillance. Stromal fibroblasts, rather than the tumor cells themselves, are often the primary sources of proteolytic enzymes necessary for tumor invasion and metastasis. Thus, the ability of cancer cells to induce angiogenesis and to interact with stroma are two important processes in tumor development. Identification of factors that can play roles in both processes is therefore particularly exciting. A novel angiogenic factor essential for vascular development, Cyr61 (Babic et al., 1998; Mo et al., 2002), has been recently identified as a marker for invasive breast carcinomas (Xie et al., 2001). In this study, we examine the role Cyr61 plays in the interaction between tumor cells and stromal fibroblasts in tumor growth.

BODY

1. Cyr61 supports mammary epithelial and adenocarcinoma cell adhesion through integrin $\alpha_6\beta_1$.

During this first year of funding, we have demonstrated that Cyr61 can support adhesion of normal mammary epithelial and adenocarcinomas cells (Fig. 1). Immobilized, purified Cyr61 protein alone can support cell adhesion, with maximal adhesion occurring at about 1 μg/ml. Maximal adhesion occurs within 30 minutes after cell plating (data not shown). These results identified Cyr61 as a novel adhesion factor for mammary epithelial cells as well as cancer cells of breast epithelial origin.

Cell adhesion to Cyr61 can be completely inhibited by the presence of soluble heparin and monoclonal antibodies (mAbs) that block the activity of integrin α_6 (GoH3), and partially inhibited by antibodies against integrin β_1(JB1A)(Fig. 1C). These data indicate that Cyr61 utilizes cell surface HSPGs and integrin $\alpha_6\beta_1$ as adhesion receptors.

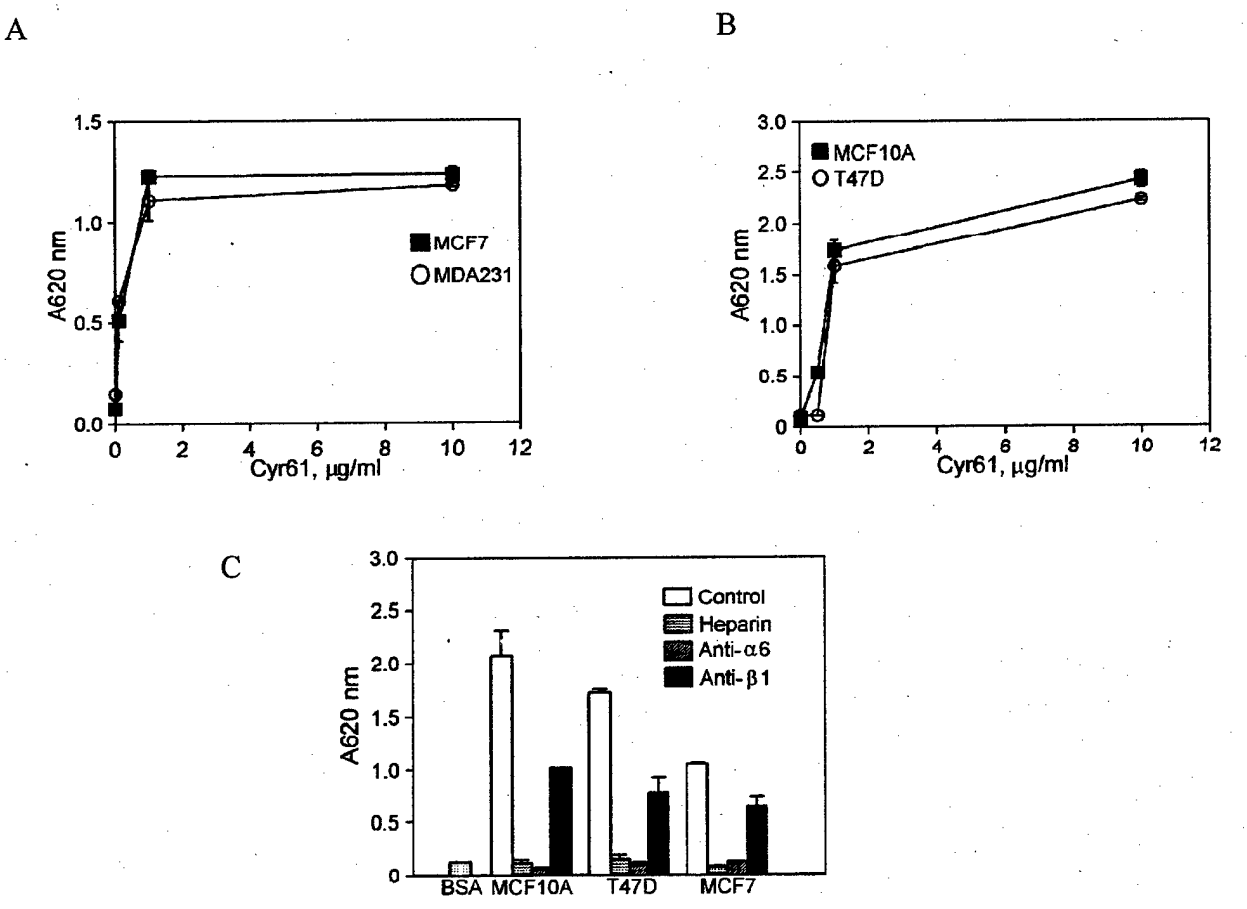

Fig. 1. Mammary epithelial and adenocarcinoma cells adhesion to Cyr61. (A) 96-well plates were coated with purified recombinant Cyr61 at varying concentrations from 0.2 to 10 μg/ml. Wells were blocked with 1% BSA before use. MCF7 and MDA231 cells were suspended in serum-free culture media, plated on Cyr61-coated plates and incubated at 37°C for 30 minutes. Adherent cells were quantified by dye extraction and 620 nm light absorption. (B) MCF10A and T47D cells were suspended in serum-free culture media and adhered to Cyr61-coated 96-well plates for 30 minutes. (C) Cells were suspended in serum-free culture media and mixed with blocking agents including heparin (2 μg/ml), anti-integrin α6 mAb (clone GoH3) 25 μg/ml, and anti-integrin β_1 mAb (clone JBS5) 25 μg/ml. Cells were kept in suspension by rocking at room temperature for 30 minutes to allow antibody to bind, and then plated on Cyr61-coated plates.

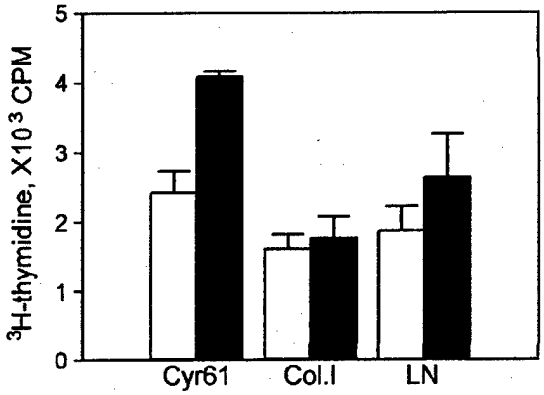

Fig. 2. Epidermal growth factor (EGF) promotes ^{3}H-thymidine incorporation in MCF7 cells adhered to Cyr61 and laminin, but not collagen. MCF7 cells were plated in serum-free condition on dishes pre-coated with various ECM proteins for 1 hour to allow for cell adhesion. These proteins include Cyr61 (10 μg/ml), native type I collagen (10 μg/ml) and laminin (20 μg/ml). EGF was added to adherent cells at final concentration of 10 ng/ml. Tritium-labeled thymidine was added together with EGF at 1 μCi/ml. Incubation was done at 37°C for 24 hours. TCA precipitation was done after incubation, and radioactivity of the TCA-insoluble material was counted using scintillation counting.

It is known that when epithelial cells adhere to basement membrane proteins like laminin, they respond to EGF by increasing DNA synthesis. However, when cells are adhered to type I collagen, a matrix protein that exists in the stroma of mammary gland outside the basement membrane, they become refractory to EGF stimulation. We tested if Cyr61, as an adhesive substrate, can support EGF induction of DNA synthesis by MCF7 cells. As shown in Fig. 2, Cyr61 can sustain EGF induction of thymidine incorporation, an activity similar to that of laminin. Type I collagen, however, was unable to support EGF activity (Fig. 2). Our previous results showed that conditioned medium of MCF7 cells can induce Cyr61 expression by fibroblasts, thus may provide a source of Cyr61 in stromal tissue. These data suggest that Cyr61 may be a stromal adhesion protein that promotes EGF activity on early stage cancer cells, when the cells first break open basement membrane barrier and start to invade the stroma.

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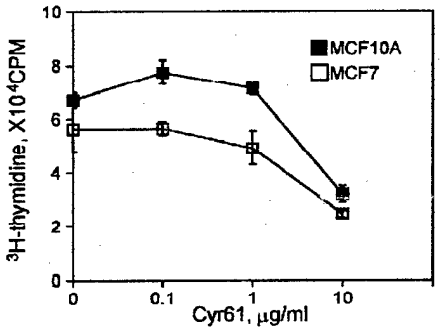

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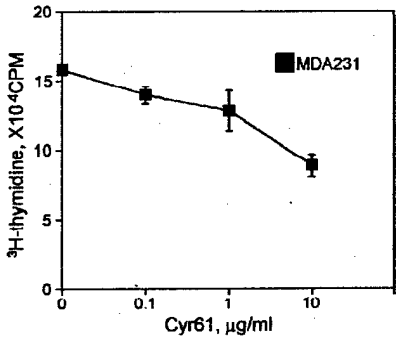

Fig. 3. Soluble Cyr61 suppresses 3H-thymidine incorporation in breast epithelial and cancer cells. (A) MCF10A and MCF7 cells were plated in 24-well culture plates at 5x104 cells per well in 10% FBS-containing growth media and cultured for 24 hours. Afterwards, the culture media were changed to serum-free media containing Cyr61 with concentrations as indicated, and 3H-thymidine at 1 μCi/ml. Cells were cultured for an additional 24 hours before TCA-precipitation and scintillation counting were performed. (B) MDA231 cells were tested for effects of Cyr61 on 3H-thymidine incorporation in the same way as described above.

2. Soluble Cyr61 suppresses DNA synthesis

Since Cyr61 is able to enhance growth factor-induced DNA synthesis in fibroblasts and endothelial cells (Kireeva *et al.*, 1996), it is somewhat surprising to us when we discovered that Cyr61 actually suppresses thymidine incorporation in breast cancer cells. Cyr61 added to serum-

free cell culture media can suppress thymidine-incorporation in tumoringenic breast cancer lines, including MCF7 (Fig. 3A) and MDA231 (Fig. 3B), as well as in normal mammary epithelial cell line MCF10A (Fig. 3A). The effect is dosage-dependent (Fig. 3). The number of live cells after Cyr61-treatment remains the same as judged by trypan blue exclusion assay (not shown). Thus, the lowered thymidine incorporation response is due to a suppression of DNA synthetic activity, but induced apoptosis.

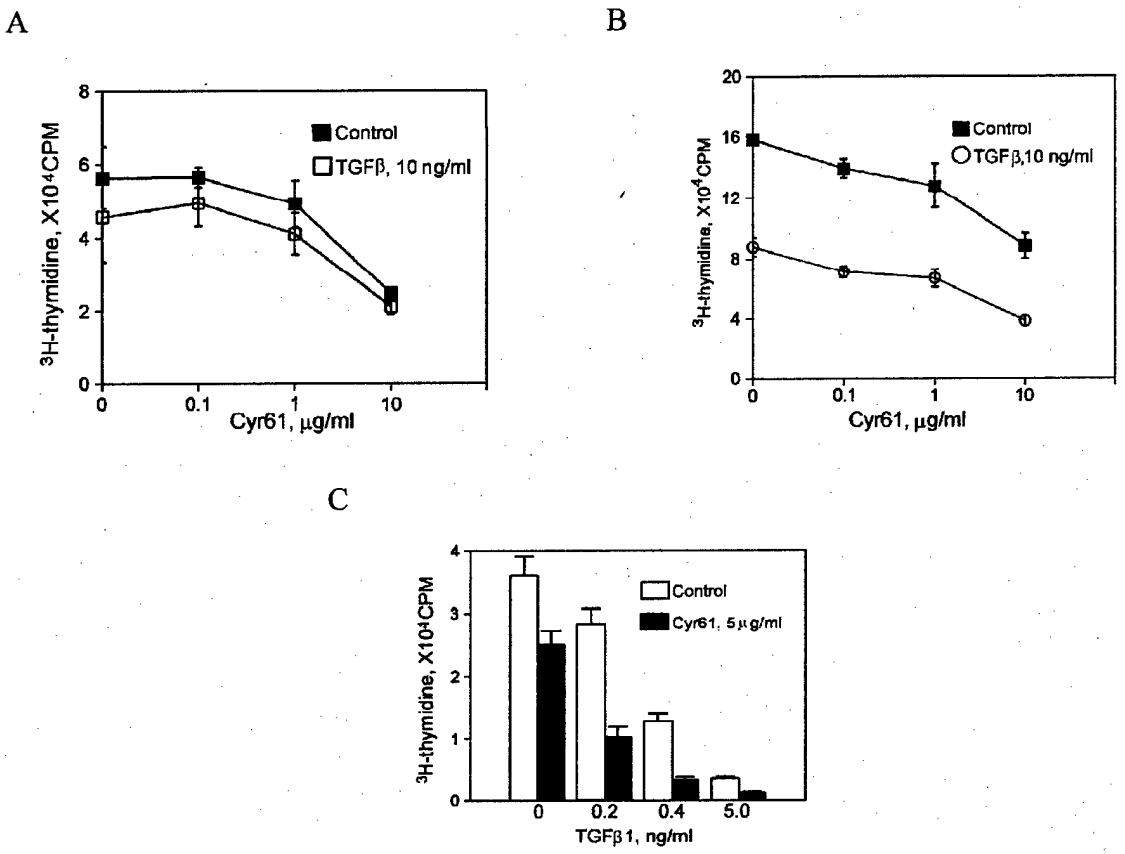

Fig. 4. TGFβ1 and Cyr61 act synergistically to suppress ^{3}H-thymidine incorporation in normal breast epithelial cells, but not breast cancer cells. (A) MCF7 cells were plated in 24-well plates at $5x10^4$ cells per well and cultured in growth medium for 24 hours. The growth medium was then replaced with serum-free medium containing varying concentrations of Cyr61, with or without 10 ng/ml of TGFβ1, and 1 µCi/ml of ^{3}H-thymidine was added at the same time. Cells were incubated for 24 hours before TCA-precipitation and radioactivity was measured by scintillation counting. (B) MDA231 cells were tested for the possible interaction of Cyr61 and TGFβ in the same way as described above. (C) MCF10A cells were plated at $5x10^4$ cells per well in 24-well plates and cultured for 24 hours. Cells were then treated with varying concentrations of TGFβ1 in serum-free media, with or without 5 µg/ml of Cyr61, together with 1 µCi/ml of ^{3}H-thymidine.

Among the growth factors that regulate mammary epithelial cell proliferation, TGFβ-1 stands out as a major factor that suppresses epithelial cell growth. It is known that breast cancer cells derived from higher grades tumors have down-regulation in TGFβ-1 signaling, due to reasons such as loss of receptor expression. Based on these findings it is hypothesized that TGFβ-1 plays dual roles in breast cancer development (Dumont and Arteaga, 2000). In early stages, cancerous epithelial cells can still respond to TGFβ-1 by slowing down proliferation and increasing apoptosis. Thus TGFβ-1 acts as a tumor suppressor. In later stages, cancer cells have accumulated mutations that disrupt TGFβ-1 signaling, thus cell proliferation is no longer suppressed by TGFβ-1. In such a situation, TGFβ-1 can promote tumor growth by enhancing angiogenesis and tumor/stromal cell interactions, thus acting as a tumor promoter.

We tested whether Cyr61 can cooperate with TGFβ-1 in regulating epithelial cell DNA synthesis. As shown in Fig. 4C, Cyr61 enhanced the effect of TGFβ in suppressing DNA synthesis at suboptimal doage in the normal breast epithelial cell line MCF10A. However, in tumorigenic cell lines such as MCF7 (Fig. 4A) and MDA231 (Fig. 4B), Cyr61 has no effect on TGFβ-1 activity. Thus, Cyr61 acts cooperatively with TGFβ-1 only in normal epithelial cells. We hypothesize that, as part of the process of natural selection of malignant cells, mutations that disrupt Cyr61 and TGFβ interaction may occur, and such mutation may confer growth advantages because it alleviates the growth suppressing effect of TGFβ-1 on cancer cells.

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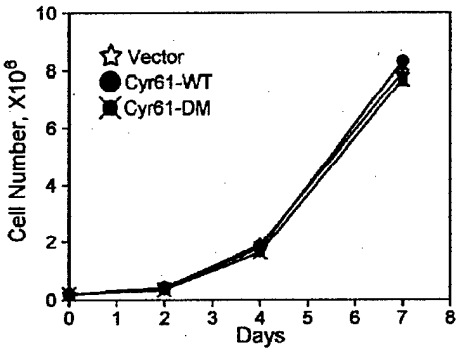

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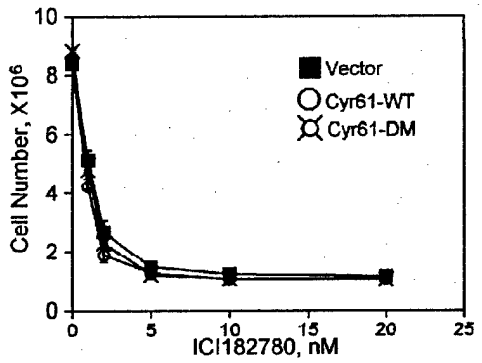

Fig. 6. Cell growth rate and estrogen-dependency are not affected by expressing Cyr61-WT or Cyr61-DM in MCF7 cells. (A) MCF7 cells transfected with empty vector, and vectors containing Cyr61-WT and Cyr61-DM cDNAs, were seeded at $2x10^5$ cells per well in 6-well plates. Cells were cultured in 10% FBS-containing growth medium with no ER-antagonist for various days as indicated. Cell number was counted by hamocytometer after trypan blue staining. (B) MCF7 cells transfected with various expression vectors were seed at $2x10^5$ cells per well in 6-well plates. Cells were cultured in the presence of various concentrations of ER-antagonist ICI182780 for 7 days, before cell number was counted.

3. Effects of Cyr61 expression in MCF7 cells on tumorigenicity.

Full-length human Cyr61 cDNA was cloned in mammalian expression vector pCMV-script. The vector is designed for high expression of cloned cDNAs, using a strong human CMV promoter. A Cyr61 cDNA carrying specific mutations that disrupt the heparin binding activity of the encoded protein was also used (Cyr61-DM) (Chen *et al.*, 2000). The cloned DNA was transfected into normal breast epithelial cell MDA10A, which has no endogenous Cyr61 expression, and breast cancer cell lines that have no or very low Cyr61 expression, including MCF7, T47D and BT20. After G418 selection, stable populations of G418 resistant cells were obtained in all cell lines. Expression of Cyr61 was verified, using Western blot analysis, only in MCF7 cells (data not shown). The other cell lines, although carrying G418-resistant marker, didn't express Cyr61 for unknown reason.

The growth curves of control, Cyr61-WT (wild type), and Cyr61-DM-expressing MCF7 cells are identical (Fig. 5), showing that Cyr61 expression has no effect on cell growth or death, at least under optimal *in vitro* culture condition. It is known that the growth of MCF7 cells is estrogen-dependent and that estrogen receptor antagonist, ICI128780, can inhibit MCF7 cell proliferation. As shown in figure 6A, both Cyr61-WT and Cyr61-DM expressing cells are sensitive to ICI128780 inhibition (10 nM), as are the control cells. A careful titration of ICI effective concentration showed that cells are equally sensitive to the drug (Fig. 5B). These results demonstrate that Cyr61 has no effect on estrogen-dependent cell growth.

Female nude mice carrying subcutaneous estrogen supplementing pellets were used as the recipients of Cyr61 expressing MCF7 cells. Five mice were used for each group. Cells were transplanted subcutaneously on hind legs. Each mouse received two transplants, vector control cells were on the left side, Cyr61-WT or -DM cells on the right. The size of tumor was measured about once a week. At the end of experiments, tumors were dissected from sacrificed animals, weighed to determine their size, and then processed for histology. As shown in Fig.6, the size of tumors vary widely even within the same groups to allow confident analysis. It is noticeable that the average size of tumors derived from Cyr61-WT and Cyr61-DM expressing cells is about 1/2 to that from control cells. In a subsequent repeat experiment (Fig.7), the difference in average tumor sizes was not reproduced, and the variability in tumor size is still too much for analysis. One possibility for the variability may be due to the fact that a pool of transfected cells were injected into nude mice, and the level and long-term consistency of Cyr61 expression may be different for different pools of transfected cells. We are now repeating these experiments using single cell clones of stably transfected MCF7 cells.

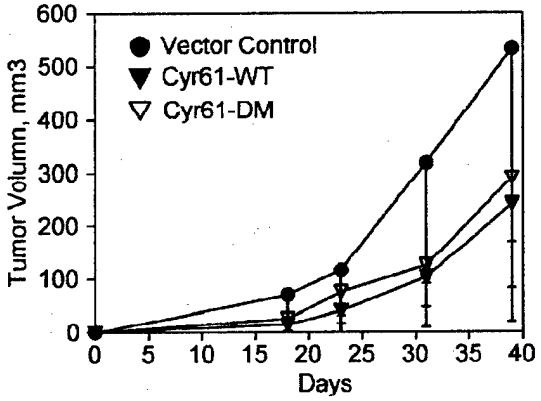

Fig. 7. MCF7 cell-based tumor growth in nude mice. Female athymic nude mice at 4-6 weeks old were used. 17β-estrodiol pellets were implanted subcutaneously in mice before tumor cell transplantation. MCF7 cells transfected with various expression vectors were selected by G418 and Cyr61 expression was verified by Western blot. Cells were suspended in serum-free medium at $1.5x10^7$ cells per ml, and 0.2 ml of suspension was injected subcutaneously above the hind legs, thus giving $3x10^6$ cells per transplant. The size of tumors was measured about once a week, and tumor volume was calculated according to the formula: $\pi/6$ x (length x width x height). At the end of the experiment, tumor weigh and volume were determined after mice were sacrificed.

4. Cyr61 induced cell signaling

To study the intracellular signaling response induced by Cyr61, serum-starved MCF7 cells were treated with Cyr61 added to serum-free media at 10 μg/ml. Cells were incubated for various times from 1 hour to 24 hours. Total cell lysates were collected and analyzed by Western blotting with various antibodies. The results showed that p70S6K and Cdc2 were both transiently activated by Cyr61 from 2 to 6 hours. p21 showed a brief up-regulation, while p27 had brief down-regulation (data not shown). These results are preliminary and are in the process of being confirmed.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that Cyr61 is a novel cell adhesive molecule for breast cancer cells
- Showed that breast cancer cells adhere to Cyr61 through integrin $\alpha_6\beta_1$
- Showed that breast epithelial cells adhered to Cyr61, but not to collagen, are able to support EGF-stimulated DNA synthesis.
- Showed that Cyr61 suppresses DNA synthesis in breast epithelial cells and synergize with TGFβ1
- Preliminary experiments indicated the Cyr61 expressing MCF7 cells engendered smaller tumors in nude mice than controls. However, results from these experiments are variable and must be repeated.
- Preliminary experiments indicated that p70S6K and Cdc2 are transiently activated by Cyr61. These results are being confirmed.

REPORTABLE OUTCOMES

Development of cell lines and reagents: MCF7 cells transfected with vectors expressing wild type and mutant Cyr61 have been developed.

Training: These experiments have further the training of a postdoctoral fellow, Dr. Chih-Chiun Chen, whose expertise in molecular biology is now complemented by experience in tumor biology.

Publication: None

CONCLUSIONS

In this study, we have demonstrated unique effects of CYR61 upon normal breast epithelial cells and breast cancer cells. In addition to being able to support cell adhesion and DNA synthesis upon EGF stimulation. CYR61 can also cooperate with the action of TGF-β, but only in normal breast epithelial cells and not in cancer cells. These results suggests the possibility that CYR61, which is known to be induced by TGF-β, to participate in TGF-β promoted tumorigenesis. Understanding the precise role of CYR61 in tumor growth still awaits further analysis.

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APPENDICES

None