Cyr61 Suppresses Growth of Human Endometrial Cancer Cells*

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Abstract

Cyr61 (CCN1) is a member of the CCN protein family; these secreted proteins are involved in diverse biological processes such as cell adhesion, angiogenesis, apoptosis, and either growth arrest or growth stimulation depending on the cellular context. We studied the role of Cyr61 in endometrial tumorigenesis. Levels of Cyr61 were decreased in endometrial tumors compared with normal endometrium. Knockdown of Cyr61 expression by RNA interference in a well differentiated endometrial adenocarcinoma cell line (Ishikawa) stimulated its cellular growth. Conversely, overexpression of the protein in the undifferentiated AN3CA endometrial cancer cell line decreased their growth concurrently with increased apoptosis in liquid culture. These same cells had decreased clonogenic capacity and a nearly complete loss of tumorigenicity in vivo. Furthermore, partially purified Cyr61 suppressed growth of endometrial cancer cells. The increased apoptosis in these endometrial cancer cells with forced overexpression of Cyr61 was associated with elevated expression of the pro-apoptotic proteins Bax, Bad, and TRAIL (tumor necrosis factor receptor-associated ligand). Cyr61-induced caspase-3 activation and depolarization of mitochondrial membrane. In summary, endometrial cancer cells have decreased expression of Cyr61 compared with normal endometrium, and this lowered expression may provide the transformed cells a growth advantage over their normal counterpart.

Cyr61 (cysteine-rich 61) belongs to the CCN protein family, which includes Cyr61, CTGF (connective tissue growth factor), NOV (nephroblastoma-overexpressed), WISP-1, -2, and -3 (Wnt-1-induced secreted proteins) (1–4). Each member of this family shares several modules that have homology to other proteins: IGFBP (insulin-like growth factor-binding protein), VWC (von Willebrand type C domain), TSP-1 (thrombospondin-1), and CT (cystine knot). The CCN family of proteins has been implicated in diverse biological processes such as cell adhesion, migration, angiogenesis, wound healing, and tumorigenesis (5–7). Cyr61 can be rapidly induced in quiescent fibroblasts by a variety of stimuli such as serum and platelet-derived growth factor (1, 8). It is secreted and becomes associated with the extracellular matrix and cell surface. It can behave as a novel ligand for integrins and, thus, is involved in the integrin signaling pathway (9, 10).

Expression of the Cyr61 protein can be regulated by estrogen, and it is often highly expressed in human breast cancers (11, 12). The high levels of expression of Cyr61 are associated with a more advanced stage of breast cancer (13). Furthermore, overexpression of Cyr61 in MCF-7 breast cancer cells substantially increased their ability to form large tumors when grown in nude mice (12). Paradoxically, Cyr61 functions as a tumor suppressor in non-small cell lung cancers by up-regulation of p53 and p21 and decreased activity of cyclin-dependent kinase 2 (14, 15). Levels of Cyr61 are decreased in prostate cancers compared with the neighboring epithelium of the normal prostate; and Cyr61 probably plays a role in aberrant cell-cell interaction between the epithelial and stromal cells in prostate carcinoma (16). Using cDNA expression arrays and immunohistochemistry, Cyr61 was found to be down-regulated in papillary thyroid carcinoma compared with normal thyroid samples (17). High levels of Cyr61 expression were detected in uterine smooth muscle cells, while leiomyomas expressed significantly lower protein levels (18).

Endometrial adenocarcinoma is the most common gynecologic malignancy. Development of these cancers arises from a series of genetic alterations that transform the normal endometrium through the stages of hyperplasia, dysplasia, and finally overt carcinoma. In this study, we found that expression levels of Cyr61 were decreased in endometrial tumors and cancer cell lines compared with the normal endometrium. Forced expression of Cyr61 in undifferentiated AN3CA endometrial cancer cells inhibited cellular growth both in liquid culture and in clonogenic assays, decreased tumor forming potential in nude mice, and promoted endometrial cancer cell apoptosis associated with elevated levels of Bax, Bad, and TRAIL. Also, addition of partially purified Cyr61 to endometrial cancer cells inhibited their growth, increased caspase-3 activity and decreased mitochondrial membrane potential. On the other hand, suppression of endogenous Cyr61 mRNA in the well-differentiated Ishikawa endometrial adenocarcinoma cells increased their cell numbers. In summary, decreased expression of Cyr61 in transformed endometrial cells may represent a mechanism to provide these cells with a growth advantage over their normal counterpart.

EXPERIMENTAL PROCEDURES

RNA Analysis by Real-time PCR—Total RNA was extracted by TRIzol (Invitrogen) from a series of endometrial cancer cell lines as well...
As matched endometrial cancers and normal endometrium from the same patients after their informed consent. Quantitative real-time PCR was performed using TaqMan reagents (PE Biosystems) as described previously (12). The oligonucleotide primers and probe sequences used were: Cyr61: 5'-ACTTCTAGTGTTCACAGTCTC-3', 5'-AAATCGGGGGTTTCTT-CACA, and 5'-TTCATCGACAGGCTGCCAGGG; CTGF: 5'-AGT- ATGGCAGCATGCAAG, 5'-ATGTCAGCTGCTGCTTGCAG, 5'-GTG-TGCTGAGCCAGGGTG, and 5'-TACTCTACTGCTGAGGCTAG- GCCC; WISP: 5'-ATTAAACGGCTGTCTGTTGA, and 5'-AGGATGCA-GAACCAGGCT, and 5'-CGTTGGCAAGTTGGCTACGG; WISP: 5'-CCACACAGGGGTGTATG, 5'-GTTCAGCTGCTGCTTGCAG, and 5'-CAATGAGCAAGAAGTGTCTGC; TRAIL: 5'-GATCATTGCTCCTCCGAG, 5'-ACGCGATGTGCTGTCACCAC, and 5'-CAACAGAAGATCGGATGCA.

For normalization, β-actin probe was used. The sequence for the β-actin probe was 5'-CTCGTTGTCACCTCCCTCCGAGC-3', the β-actin-specific primers were 5'-GATCATTGCTCCTCCGAGC-3' and 5'-ACTCC-TGCTTGCAGTGCAAG-3'.

**Cell Culture and Transfection—**Endometrial cancer cell lines AN3CA, Ishikawa, HEC59, HEC1A, and HEC1B were maintained in HEC59 by Dr. Timothy J. Kinsella, Case Western Reserve University. American Type Culture Collection. Ishikawa was kindly provided by Dr. Kinsella. AN3CA, HEC1A, HEC1B, KLE, and RL95–2 were purchased from ATCC. Cyr61 cDNA was previously cloned into the pcDNA3.1 plasmid (Upstate Biotechnologies). Negative control FOPFLASH contains upstream of thymidine kinase minimal promoter in front of luciferase gene. pcDNA3.1 was used for transfections of cultured cells. pcDNA3.1 or empty vector pcDNA3.1 were used in cell transfection. Forty-eight hours after transfection, the cells were replaced in 600 μg/ml of G418 and isolated stable clones were selected for assay after 2 weeks. One G418-resistant clone selected from empty pcDNA3.1 transfection was designated as AN3CA-neo. Seven G418-resistant stable clones after Cyr61 transfection were screened for Cyr61 expression with real-time PCR and Western blot analysis. Only the two clones with the most prominent Cyr61 expression are shown and used for this study.

**Western Blot Analysis—**Cell lysates were prepared from cells harvested by lysis buffer containing 50 mM Tris, pH 7.6, 5 mM EDTA, 300 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 1 mM dithiothreitol, plus protease inhibitor mixture (Roche Applied Science). Equal amounts of lysates were separated by SDS-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene difluoride membranes. Anti-Cyr61 polyclonal rabbit antisera were prepared in this laboratory (12). Antibodies to glyceraldehyde-3-phosphate dehydrogenase, Bad, Bak, Bcl-2, and Bcl-xL were purchased from Santa Cruz Biotechnology.

**Cell Proliferation and Colony Formation Assay—**For cell growth analysis, cells (3 × 10⁴ cells/well) were seeded in 96-well plates and cultured for various durations. Cell numbers were measured by MTT assay according to the manufacturer’s protocol (Roche Applied Science). For colony formation, equal number of transfected cells were seeded in 24-well plastic plates and cultured under G418 selection (600 μg/ml). After 2 weeks, the colonies were stained with 0.1% crystal violet in 50% methanol and photographed with an inverted phase contrast microscope.

**Tumorigenicity Analysis—**AN3CA-neo and AN3CA-Cyr61#1 cells (1 × 10⁷ cells) were subcutaneously injected on different sides of the same mouse. A total of 5 nude mice were used for this study. Size of the tumors was measured every week for 4 weeks; and euthanasia was performed when the tumor reached 1 cm in diameter. The tumors were excised and measured. The results are shown and used for this study.

**RNA Interference—**Hairpin-expressing clones were made using primers designed by using the Web-based siRNA hairpin engine at Cold Spring Harbor Laboratories (karadinh.cshl.org:9131/RNAi/). A 17-nucleotide homology with a U6 promoter was placed at the 3’-end of the primer, a Pol III termination cordon was incorporated at the 5’-end, the center encompasses the stem and loop structure. For mapping convenience, the loop includes a HindIII site. For PCR reaction, a SP6 promoter was used as the second primer and a cloned U6 promoter, pCGU6, as template. The reaction was set up using Hot Master Taq, with 0.5 mM dNTPs, 3 μM oligonucleotides, and 10 ng template in the buffer provided by the manufacturer (Qiagen). After the reaction, the resulting 600-base pair fragment with the U6 promoter and the hairpin, was cloned into pCR2.1 using a TOPO TA cloning kit and confirmed by DNA sequencing. One mutant fragment, which lacks the hairpin sequence producing a non-functional RNA interference (RNAi), was used as control RNAi. Ishikawa cells were co-transfected with pcDNA3.1 and either Cyr61 RNAi or control RNA, and stable clones were selected with G418 (600 μg/ml). One stable clone from each “empty” vector, containing only the neo gene, was designated Ishikawa-neo. Several stable clones from Cyr61 RNAi transfected cells were screened for Cyr61 protein knockdown by Western blot analysis. The sequence for Cyr61 RNAi was 5'-GGCCACATCAACATGCTAGTCTGCAAGCTGAGTTGAC-3', and the sequence for the control RNAi was GGCCACATCAACATGCTAGTCTGCAAGCTGAGTTGAC.

**Conditioned Media Preparation—**Cyr61-pcDNA3.1- or pcDNA3.1-transfected cells were grown to about 80% confluency and replaced with serum-free medium for another 48 h. The conditioned medium were centrifuged at 3,000 × g for 10 min, and the supernatant was aliquoted and stored at −80 °C. Heparin-agarose beads (Sigma) were packed and equilibrated with serum-free medium, and incubated with conditioned medium at 4 °C overnight to remove Cyr61 from the conditioned medium.

**Cell Cycle and Apoptosis Analyses—**Cells were trypsinized, washed in phosphate-buffered saline, and fixed in 50% ice-cold methanol for 30 min. The distribution of the cells in G1, S, and G2/M cell cycle phases was determined by flow cytometry after staining with propidium iodine. Annexin V/PI double staining was employed to identify the apoptotic and viable cells following the manufacturer’s instructions. The percentage of early apoptotic (FITC-positive and PI-negative) cells was calculated from the data originated from flow cytometry.

**Reporter Assay—**Cells were plated at a subconfluent density and co-transfected with 1 μg of the reporter plasmid and the appropriate expression vectors. TOPFLASH construct contains 4 copies of TCF binding sites upstream of the luciferase gene. Renilla luciferase plasmid pRL-TK was co-transfected as an internal control for transfection efficiency. Luciferase 2000 and Opti-MEM were used for transfections following the manufacturer’s instructions (Invitrogen). Cell lysates were prepared 48 h after transfection, and the reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

**Measurement of Caspase-3 Activity and Mitochondrial Membrane Potential—**Ishikawa cells were incubated with conditioned medium for 24 or 48 h, and caspase-3 activity was measured using Caspase-Glo3/7 Assay (Promega) following the manufacturer’s protocol. Basically, cells were lysed and incubated with prolinesuminate substrate containing the caspase-3 recognition sequence. The resulting cleavage of the substrate by caspase-3 generates luminescent signal, which then is detected by a luminometer. Mitochondrial membrane potential was measured by using JC-1 (Molecular Probes) fluorescent dye. JC-1 aggregates in mitochondrial membrane and emits at a wavelength of 590 nm; upon depolarization of the membrane, JC-1 forms monomer and emits at 530 nm. The normalized JC-1 ratio is the difference between the fluorescence at 590 nm and 530 nm and normalized to a starting value of 1.

**RESULTS**

Cyr61 Expression Was Down-regulated in Endometrial Tumors Compared with Matched Normal Tissues—The expression of CCN genes (Cyr61, CTGF, NOV, WISP1, WISP2, and WISP3) in endometrial tissues was assessed by quantitative real-time reverse transcription-PCR. In a collection of randomly selected 8 pairs of matched samples from patients, statistically significant differences were found in the expression of Cyr61, NOV, and WISP2 (Fig. 1A, p < 0.05). Elevated levels of NOV and WISP2 were expressed in endometrial tumors, whereas Cyr61 expression was down-regulated. The expression of Cyr61 was quantified further in endometrial cancer cell lines and paired tissue samples. Human mammary adenocarcinoma cell line MDA-MB-231 was used as a positive control, which we previously have shown, had high levels of Cyr61 expression (12). In 7 of the 8 paired tissue samples, the expression of
Cyr61 was higher in the normal endometrial tissue compared with the matched tumors (Fig. 1B, p \(=\) 0.012, Student’s t test, one-tail, two-sample assuming unequal variances). In a series of endometrial cancer cell lines, the level of Cyr61 varied (Fig. 1B). The lowest Cyr61 expression was found in AN3CA cells, an undifferentiated endometrial adenocarcinoma (Fig. 1B). Compared with the AN3CA cells, a well-differentiated endometrial cell line Ishikawa showed higher expression of Cyr61 (Fig. 1B).

**Overexpression of Cyr61 in AN3CA Endometrial Cancer Cells Suppressed Colony Formation, Cell Growth in Liquid Culture, and Tumor Growth in Nude Mice**—Since Cyr61 transcripts were expressed at a higher level in the normal endometrium compared with matched tumors, we hypothesized that Cyr61 may be important in the regulation of cell growth of normal endometrium. AN3CA cells, which endogenously expressed low levels of Cyr61, were transiently transfected with empty vector (pcDNA3.1) and formed numerous colonies, while the number of colonies were dramatically reduced when these cells were transiently transfected with Cyr61-pcDNA3.1 (Fig. 2A). Furthermore, AN3CA cells were stably transfected with either a Cyr61 expression vector (Cyr61-pcDNA3.1) or an empty vector pcDNA3.1. Several G418-resistant clones were isolated from Cyr61-pcDNA3.1 transfection and screened for Cyr61 expression levels by both real-time RT-PCR and Western blot analysis. The protein levels of two stable clones overexpressing Cyr61 (AN3CA-Cyr61#1 and #2) are shown in Fig. 2B; and these two clones were used for further study. The effect of Cyr61 expression on cell growth in vitro was evaluated by MTT assay. Compared with AN3CA-neo control cells, the 2 stable clones (AN3CA-Cyr61#1 and #2) showed significant reduction.
Next, the effect of Cyr61 on tumor formation in vivo was examined. Cyr61-overexpressing cells (AN3CA-Cyr61#1) and vector control cells (AN3CA-neo) were injected on opposite flanks of nude mice, and tumor growth was monitored weekly. After 4 weeks, the tumors were removed and measured. In all five mice injected with tumor cells, the Cyr61-overexpressing cells (AN3CA-Cyr61#1) developed markedly smaller tumors than the control endometrial cancer cells (AN3CA-neo) in the nude mice ($p = 0.0088$, one-tail Student’s $t$ test, Fig. 2D).

Cell Growth Was Increased after Cyr61 Gene Silencing in Ishikawa Cells—To determine if endogenous Cyr61 plays a role in cell growth, we used a U6 promoter-driven RNAi method to silence the Cyr61 gene in the Ishikawa endometrial cancer cells, which constitutively express easily detectable amounts of endogenous Cyr61. These cells were co-transfected with pcDNA3.1 and either a Cyr61-specific RNAi or a control RNAi expression vector. Stable clones were selected with G418 and were screened for Cyr61 expression by Western blot analysis. Two stable clones AN3CA-Cyr61#1 and #2 had prominent expression of Cyr61. Glyceraldehyde-3-phosphate dehydrogenase was used as loading control. Cyr61 binds heparin and heparin affinity chromatography has been used in Cyr61 purification (19). We used heparin agarose beads to deplete Cyr61 from the conditioned medium (Fig. 4B), which resulted in the loss of the growth inhibitory activity (Fig. 4C).

Cyr61 Inhibits Growth of Endometrial Cancer Cells at Least in Part by Promoting Apoptosis—To explore the mechanism of growth suppression in Cyr61-overexpressing cells (AN3CA-Cyr61#1 and #2), cell cycle analysis was performed. The number of cells in S-phase was 45 and 42% for the AN3CA-neo and AN3CA-Cyr61#1 cells, respectively. In contrast, expression of Cyr61 caused a remarkable increase (from 7 to 28%) in the subdiploid DNA (sub-G0/G1) content in AN3CA-Cyr61#1 sug-
suggesting an increased cell death (Fig. 5A). Apoptosis was also assessed by annexin V-FITC staining to detect phosphatidylserine on the cell surface in conjunction with propidium iodide staining. As shown in Fig. 5B, Cyr61-expressing cells (AN3CA-Cyr61#1) showed an increase in annexin V-positive and PI-negative cells (26%) when compared with AN3CA-neo control cells (6%) indicating increased cell death via apoptosis. These results indicated the anti-growth activity of Cyr61 was associated at least in part with induction of cellular apoptosis.

Given that the relative expression of the Bcl-2 family of proteins is important in the regulation of apoptosis (20), the levels of several pro- and anti-apoptotic proteins were examined. Compared with control AN3CA-neo cells, a slight decrease in the levels of the anti-apoptotic protein Bcl-2 was observed, and no significant change in either Bcl-xL or the inactive phosphorylated Bad, was found in the Cyr61-overexpressing cells (AN3CA-Cyr61#1 and #2, Fig. 5C). In contrast, the levels of the pro-apoptotic proteins Bax and Bad were prominently increased in the AN3CA-Cyr61#1 and #2 cells (Fig. 5C). Furthermore, the expression of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was significantly increased in the AN3CA-Cyr61 cells compared with AN3CA-neo control cells as measured by quantitative real-time PCR (Fig. 5D).

Since we observed increased levels of Bax in endometrial cancer cells overexpressing Cyr61, we next examined the downstream targets of the Bax-mediated apoptosis pathway. We first measured the caspase-3 activity in endometrial cancer cells treated with Cyr61-conditioned medium. A substantial increase of caspase-3 activity occurred in the Ishikawa cells treated with Cyr61-conditioned medium compared with control (V)-conditioned medium-treated cells (Fig. 6A). Furthermore, we examined the mitochondrial potential in these cells. We found that Cyr61-conditioned medium treated cells exhibited a time-dependent decrease in their mitochondrial potential, while control (V)-conditioned medium-treated cells had a minimal drop in potential (Fig. 6B).

During apoptosis, c-myc has been reported to enhance activation of Bax (21). We examined expression of c-myc in our stably transfected endometrial cancer cells. Levels were in-
increased in Cyr61-overexpressing cells, AN3CA-Cyr61#1; conversely, expression was decreased in the Cyr61-knockdown, Ishikawa-i1 cells (Fig. 7A). c-myc is a known target gene of LEF/TCF transactivation. Therefore, we investigated the ability of Cyr61 to enhance TCF-dependent transcriptional activity as measured by reporter assays. TOPFLASH was co-transfected with either Cyr61-pcDNA3.1 or pcDNA3.1 empty vector in AN3CA cells. Stimulation of TCF reporter activity was found only in AN3CA cells co-transfected with the Cyr61 expression vector (Fig. 7B). No activity was observed in the negative

**FIG. 4.** Effect of Cyr61-conditioned medium on cell growth as measured by MTT assay. A, Western blot analysis of Cyr61 expression in conditioned medium. Conditioned medium was collected from either pcDNA3.1 (V)- or Cyr61-pcDNA3.1 (Cyr61)-transfected AN3CA cells after they were grown in serum-free medium for 48 h. B, Cyr61 depletion from conditioned medium by heparin-agarose beads. Conditioned medium were incubated with heparin beads overnight, and heparin-bound and unbound material were analyzed for Cyr61 expression. C, Ishikawa-i1 (endometrial cancer cells) and H460 (non-small cell lung cancer cells) were incubated with either control medium (V), Cyr61-conditioned medium (Cyr61), or Cyr61-depleted conditioned medium. Data are expressed as mean MTT activity ± S.E. from triplicate wells in two separate experiments.
FIG. 5. Increase in apoptosis and expression of the pro-apoptotic proteins Bax, Bad and TRAIL in Cyr61-overexpressing AN3CA endometrial cancer cells. A, cell cycle analysis. FACS histogram shows the DNA content of cells after propidium iodide staining of methanol-fixed cells. To the right of the dotted line is normal diploid DNA and to the left is sub-G₀/G₁ DNA content (degraded DNA originated from apoptotic cells). B, apoptosis analysis. Cells were stained with annexin-V-FITC and PI to differentiate apoptosis and necrosis. Viable cells are FITC-negative/PI-negative; late apoptosis or cell death is FITC-positive/PI-positive; and early apoptotic cells are FITC-negative/PI-negative (lower right box with % of population). Profiles show one experiment, which was similar to 2 additional independent experiments. Also, another clone (AN3CA-Cyr61#2) showed a similar profile as AN3CA-Cyr61#1 in both cell cycle analysis and annexin-V assays (data not shown). C, Western blot analysis. Expression levels of Bcl-2 family members including Bcl-2, Bcl-xL, Bad, and Bax. The anti-Bad antibody recognizes both phosphorylated and unphosphorylated Bad. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. D, real-time PCR for relative TRAIL mRNA levels in AN3CA-neo and AN3CA-Cyr61#1 cells. The relative gene expression levels were calculated by normalization to β-actin. Data represent the mean of triplicate measurements.
recently been shown to be important for neuronal cell death levels of cdk2 kinase activity (14, 15). Induction of Cyr61 has correlated with enhanced expression of p53 and p21 and decreased with lower levels of Cyr61 (24). Paradoxically, Cyr61 acts as an anti-apoptosis gene XIAP. Cells from glioblastoma multiforme expressed higher levels of Cyr61; and these brain tumors with high levels of Cyr61 had a worse prognosis than those with lower levels of Cyr61 (24). Paradoxically, Cyr61 acts as a tumor suppressor in non-small cell lung cancers and is associated with enhanced expression of p53 and p21 and decreased levels of cdk2 kinase activity (14, 15). Induction of Cyr61 has recently been shown to be important for neuronal cell death through c-Jun N-terminal kinase activation (25). Taken together, Cyr61 has disparate functions depending on the tissue of origin. This appears also to be the case for other members of the CCN family of proteins. Their biological properties are dependent upon their interacting molecules, be they either positive or negative effectors (5, 6). CTGF stimulates proliferation of fibroblasts and endothelial cells (7, 26), but induces apoptosis in human aortic smooth muscle cells (27). WISP-1 increases cell proliferation of rat kidney fibroblasts (28) but suppresses cell invasion and motility of lung cancer cells (29).

We initially examined the gene expression levels of all 6 CCN members (Cyr61, CTGF, NOV, WISP1, WISP2, and WISP3) in 8 pairs of matched normal and endometrial cancer samples from the same individuals. By quantitative real-time PCR, expression of Cyr61 was lower in tumors compared with normal samples. A similar decreased Cyr61 expression has been noted between matched normal tissue and leiomyomas (18), prostate cancers (16), embryonal-rhabdomyosarcomas (30), and non-small cell lung cancers (14). We found no significant change in the expression levels of CTGF, WISP1, and WISP3; while NOV and WISP2 were overexpressed in endometrial tumors compared with the normal endometrium. We further compared the levels of Cyr61 in several endometrial cancer cell lines as well. Ishikawa and KLE cells are well differentiated; RL95–2, HEC1A, HEC1B, and HEC59 are modestly well-differentiated; and AN3CA cells are undifferentiated. No correlation was noted between the levels of expression of Cyr61 and cell differentiation in the endometrial cancer cell lines that we examined.

Based on two independent approaches, we demonstrated that Cyr61 is a negative regulator of endometrial cell growth. Using RNAsi, we showed that reduction of Cyr61 expression in Ishikawa cells was associated with an increase in cell numbers and the forced expression of Cyr61 in AN3CA endometrial cancer cells resulted in their decreased cell growth. Colony formation assays in vitro and ability to form tumors in immunodeficient mice both showed decreased growth compared with wild-type cells. These results demonstrate the growth inhibitory effect of Cyr61 in endometrial cancer cells. Cyr61 is a secreted protein and can function as a novel ligand for cell surface integrin receptors. Conditioned medium collected from Cyr61-transfected cells inhibited cellular accumulation of Ishikawa endometrial cancer cells and also in control H460 non-small cell lung cancer cells, in which Cyr61 overexpression has previously been shown to cause growth suppression (14). After incubation with heparin-coated beads to deplete Cyr61 from the conditioned medium, the inhibitory effect was reversed. These results are consistent with growth inhibition of endometrial cancer cells potentially occurring by Cyr61 interacting with a cell surface protein, such as integrins.

Approximately 25% of both AN3CA-Cyr61#1 and #2 cells had a subdiploid DNA content compared with 7% of the control AN3CA-neo cells as observed by cell cycle analysis. These findings indicate degradation of DNA in Cyr61-expression cells. Annexin V assays verified that these cells were undergoing apoptosis. The tumor suppressor gene p53 plays an important role in apoptosis, and its mutational inactivation is common in human cancers including 15–25% of endometrial carcinomas (31). Forced expression of Cyr61 in the AN3CA endometrial cancer cells is sufficient to promote apoptosis. These cells have mutant p53, showing that Cyr61 can cause apoptosis independent of wild-type p53 activity.

To maintain equilibrium between proliferation and death of cells is vital for many biological processes, especially in reproductive tissues such as the mammary gland, ovary, and uterus (32, 33). Defects in this process predispose the tissue to a
variety of disorders including cancer. The family of Bcl-2 proteins is involved in the regulation of programmed cell death and the relative ratio between pro- (Bax and Bad) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) dictates the fate of the cells (34). In normal endometrium, the main site of apoptosis is the glandular epithelial cells. Both Bcl-2 and Bax are present in these cells in the proliferative phase. During the secretory phase, levels of Bax protein increase (35) and/or Bcl-2 decrease (36) resulting in apoptosis and shedding of the endometrial lining. Our results showed that overexpression of Cyr61 in AN3CA cells increased the levels of Bax. Activation of Bax can cause the change of mitochondrial membrane potential and release of cytochrome c resulting in apoptosis (37, 38). Our results showed that Cyr61 induced depolarization of the mitochondrial membrane. Our data also found an increased level of Bad in AN3CA cells overexpressing Cyr61. Up-regulation of unphosphorylated Bad in AN3CA-Cyr61#1 cells enhances its ability to interact with Bcl-2 and Bcl-xL and to inhibit their anti-apoptotic activity (39). A second pathway involved in mediating apoptosis is through the tumor necrosis factor (TNF) family of proteins (40). Apoptosis is mediated by the interaction between TNF receptors and their ligands, which leads to a cascade of activation of caspases. Using real-time PCR, we found that AN3CA cells transfected with Cyr61 showed increases in TRAIL mRNA. TRAIL interacts with the TNF receptors known as DR-4 and -5 to activate caspase-8 (41, 42). In apoptosis, both the membrane-dependent extrinsic pathway involving TNF receptors and the intrinsic pathway involving the mitochondrial, converge on the downstream effector caspase-3 (43). Our data suggest that Cyr61 induced activation of caspase-3 through both signaling pathways.

c-myc is implicated in a wide range of biological processes including oncogenic transformation and apoptosis (44). Cyr61-dependent inhibition of cell growth is associated with c-myc induction in non-small cell lung cancer (NSCLC) cell lines H460 and H520 (14, 15). Our results showed that in endometrial cancer cell lines AN3CA and Ishikawa, the expression levels of Cyr61 correlated with levels of c-myc. c-Myc can induce apoptosis, possibly through activation of the pro-apoptotic Bax (45), which was induced in our Cyr61-transfected cells. c-Myc is a target gene of the LEF/TCF transcriptional factor (15, 46). Our TCF reporter assays showed that AN3CA cells co-transfected with the Cyr61 expression vector can activate the TCF reporter. Further studies are needed to determine how forced expression of Cyr61 activates the LEF/TCF transcriptional factor. In both glioma cells and non-small cell lung cancer cells, we found that Cyr61 triggered nuclear translocation of β-catenin and activated the β-cate-
nin/TCF signaling, which up-regulated expression of c-Myc (15, 24). In the lung cancers, this was associated with increased expression of p53 and p21 (15).

We have not studied the mechanisms causing the decrease in Cyr61 expression in endometrial cancer. Our data show that Cyr61 transcripts are decreased; and Cyr61 mRNA expression was not inducible by either 17β-estradiol or tamoxifen treatment in endometrial cancer cell lines (data not shown). The lower levels of Cyr61 could occur by decreased stability of the transcript or decreased transcription of the gene, perhaps by methylation of the promoter region or aberrant expression levels of the necessary transcription factors. During carcinogenesis of endometrial adenocarcinomas, multiple molecular alterations have been identified including microsatellite instability (20–30%), frequent mutations of PTEN (30–60%), K-ras (10–30%), as well as β-catenin (28–35%) (47). One or several of these alterations may affect levels of Cyr61. In this study, we provide evidence that Cyr61 is able to suppress endometrial cancer cell growth. Cyr61 promotes apoptosis of endometrial carcinoma cells probably by activation of the pro-apoptotic proteins Bax and Bad, depolarization of mitochondrial membrane potential, and caspase-3 activation, which results in the inhibition of clonogenic growth and loss of tumorigenicity of the endometrial cancer cells probably by silencing expression of Cyr61.

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