Cancer Immunol Immunother (2012) 61:677–687
DOI 10.1007/s00262-011-1135-y

ORIGINAL ARTICLE

A novel anti-Cyr61 antibody inhibits breast cancer growth and metastasis in vivo

Jinpiao Lin · Rongfen Huo · Li Wang · Zhou Zhou · Yue Sun · Baihua Shen · Rongfang Wang · Ningli Li

Received: 13 June 2011 / Accepted: 18 October 2011 / Published online: 3 November 2011

Abstract Cysteine-rich protein 61(CCN1/Cyr61) has been implicated as an important mediator in proliferation and metastasis of breast cancer, which indicated that blockage of Cyr61 might be a potent target for breast cancer treatment. However, the antitumor effect of anti-Cyr61 antibodies on breast cancer in vivo has not been reported so far. In this study, we reported the effect and likely mechanism of generated anti-human Cyr61 monoclonal antibodies (mAb) on Cyr61 high expression line MDA-MB-231, known as a highly malignant and invasive human breast cancer cell line, at aspects of proliferation and migration in vitro and in vivo. We found the mAb, denoted as 093G9, revealed inhibitory effects on MDA-MB-231 cell proliferation, migration, and invasion through downregulation of both AKT and ERK phosphorylation in vitro compared with its isotype control. 093G9 also showed significant efficacy on suppressing primary tumor growth and spontaneous lymph node metastasis in in vivo mouse model. The specific epitope recognized by 093G9 was identified to be ^{140}LPNLGCP^{146}, adjacent to the VWC domain of Cyr61 by Ph.D.-C7C phage library display system. Our study provides direct evidence that Cyr61 can be a potent therapeutic target for patients who bear high Cyr61 expression breast cancer. Furthermore, the mAb, 093G9 developed in our laboratory, has shown a promising therapeutic characteristic in breast cancer.

Keywords Cysteine-rich protein 61 · Breast cancer · MDA-MB-231 cell line · Monoclonal antibody

Electronic supplementary material The online version of this article (doi:10.1007/s00262-011-1135-y) contains supplementary material, which is available to authorized users.

J. Lin · R. Huo · L. Wang · Z. Zhou · Y. Sun · B. Shen · N. Li
Shanghai Institute of Immunology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

J. Lin · R. Huo · Z. Zhou · Y. Sun · B. Shen · N. Li (✉)
Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China
e-mail: ninglixiaoxue57@yahoo.com.cn

L. Wang
Department of Immunology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

R. Wang
Diasys Diagnostic Systems (Shanghai) Co., Ltd, Shanghai International Medical Zone Building 16, Lane 588, Tianxiong Road, Shanghai 201318, China

Introduction

Cyr61 (CCN1), as the first cloned member of cysteine-rich protein (CCN) family, is a secreted, cysteine-rich, heparin-binding extracellular matrix-associated protein [1, 2]. To date, a number of reports describe that Cyr61 is involved in many cell biological functions. For example, Cyr61 has been identified to mediate cell adhesion, migration, proliferation, apoptosis, and angiogenesis [3–6].

As its widely spreading integrin receptors, Cyr61 located in different tissues and may play different roles [5, 7, 8]. Previous studies in tumors pathogenesis revealed that overexpression of Cyr61 is related to the development of neovascularization and tumor formation [9–13]. Among these studies, the expression profile of Cyr61 in breast cancer is reported intensively [5, 14–17]. For example, overexpression of Cyr61 was found in 70% of breast cancer patients with infiltrating ductal carcinomas [7]. In some

reports, Cyr61 was localized exclusively for hyperplastic ductal epithelial cells [5, 7, 14]. Moreover, some studies showed that overexpression of Cyr61 was related to lymph node metastasis [15, 17–20]. According to these findings, it is hypothesized that overexpressing Cyr61 might involved in breast cancer growth and metastasis. Consistently, Cyr61 high-expression breast cancer cell line MDA-MB-231 showed faster growth and more severe metastasis than Cyr61 low-expression cell line MCF-7 in vitro and in vivo, when Cyr61 overexpressed in MCF-7 increased the cells invasion, migration, and proliferation ability [7, 16, 21, 22]. These indicated that blockage of Cyr16 might be a potent target for breast cancer treatment.

Like other members of CCN family, Cyr61 is composed of an N-terminal secretary peptide followed by four structural domains that include (1) an insulin-like growth factor–binding protein homology domain (IGFBP domain), (2) a von Willebrand factor type C domain (VWC domain), (3) a thrombospondin type 1 repeat homology domain (TSP domain), and (4) a C-terminal domain (CT domain). It possesses heparin-binding motifs and sequence similarity to the C-termini of von Willebrand factor and mucin [3]. The structure and function of CCN family have been studied intensively [1, 23–26]. In breast cancer research, reports implicated that the domain 4 of Cyr61 was important for promoting tumor cell proliferation, migration, and invasion [27]. These results indicated that prominent expression of Cyr61 contributed to breast cancer development, where blocking or downregulation of Cyr61 might be benefit for breast cancer therapy [20–22].

Given its role in breast cancer cells' growth and metastasis, Cyr61 might be a candidate target for human breast cancer treatment. In this study, using anti-Cyr61 monoclonal antibody generated in our laboratory, we examined Cyr61 expression profile among Chinese breast cancer patients with different clinical stages, and the results showed that Cyr61 was overexpressed in patients as previous reports [7]. Further, we examined the role of Cyr61 in tumorigenesis of MDA-MB-231 cells using siRNA and anti-Cyr61 monoclonal antibody 093G9. Moreover, we showed that 093G9 could inhibit MDA-MB-231 migration and invasion in vitro consistent with upregulation of MMPs inhibitor TIMP1 and TIMP2. In addition, using nude mice tumor model, we showed 093G9 could arrest MDA-MB-231 growth and lymph node metastasis in vivo. Furthermore, peptide LPNLGCP located in VWC domain of Cyr61 was identified as an epitope recognized by 093G9 using Ph.D.-C7C phage library display analysis. This suggested that LPNLGCP of VWC domain in Cyr61 protein might be very important in breast cancer cells proliferation, migration, and invasion. Taken together, we provided a solid evidence of inhibition of growth and lymph node metastasis in breast cancer by blockade of Cyr61, which promised Cyr61 as a target for breast cancer treatment.

Materials and methods

Cell lines and animals

Two human breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC). Five-week-old female nude mice and BALB/c mice were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Science. Mice were housed in pathogen-free conditions and were treated in accordance with the guideline of the Committee on Animals of the Shanghai Jiaotong University School of Medicine.

Production of monoclonal antibody

The 093G9 and 096B7 hybridoma cell lines secreting mouse monoclonal antibody (mAb) against human Cyr61 were generated by the standard hybridoma technique in our laboratory [28]. The mouse mAbs, 093G9 (IgG1, κ) and 096B7 (IgG1, κ), were purified by Protein A affinity chromatograph. The purity of mAbs 093G9 and 096B7 was confirmed by HPLC.

Indirect ELISA

The recombinant human Cyr61 (hCyr61) was diluted to a concentration of 1 μg/ml with 0.05 M Tris–Hcl buffer (pH 8.0) and coated on a 96-well plate with 100 μl/well, 4°C overnight. After washing the plates three times with phosphate-buffered saline (PBS), 1% BSA containing 0.05% Tween 20 in PBS was added to the well for blocking for 2 h at 37°C to avoid non-specific binding. After washing the plates three times, the anti-Cyr61 mouse monoclonal antibody was added and incubated for 1 h at 37°C. After washing again, HRP-conjugated goat anti-mouse IgG (R&D) was added and incubated for 1 h at 37°C. The plate was washed again and incubated with 100 μl of tetramethyl benzidine (TMB) substrate at room temperature for 15 min. After stopping the reaction with 2 M H_2SO_4, the absorbance was measured at 450 nm.

Characteristics of mAbs

Titers of culture supernatant and ascites form of hybridoma cells were detected by indirect ELISA after serial dilutions. The isotypes of the immunoglobulins were analyzed using the Mouse Immunoglobulin Isotype Panel

kit (SouthernBiotech, AL, USA), according to the manufacturer's instructions. The concentrations of the obtained mAbs were measured by Mouse IgG ELISA Quantitation kit (BETHYL, USA), according to the manufacturer's instructions.

Biacore analysis

The kinetic parameters of anti-Cyr61 antibodies for Cyr61 were determined using the Biacore T100 instrument (Biacore AB, Uppsala, Sweden) according to the manufacturer's instructions. Briefly, covalent immobilization of purified recombinant human Cyr61 onto a Series S CM5 sensor chip (Biacore) was performed using an amine coupling kit (Biacore). Twofold serial dilutions of anti-Cyr61 monoclonal antibodies (mAbs) were injected over the sensor chip surface at a flow rate of 50 μl/min. Cyr61 irrelevant antibodies were used as controls. The constants of association (Ka) and dissociation (Kd) rate of anti-Cyr61 mAbs were determined with the Biacore T100 Evaluation software version 2.0 (Biacore).

Tissue microarray (TMA) and immunohistochemistry (IHC) assay

The breast cancer TMA was supplied by Xi'an Alena Biotechnology Ltd., Co. (Xi'an, China). IHC studies were performed with a standard EliVision™ method. In brief, TMA sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. For antigen retrieval, TMA slides were microwave-treated in 10 mM citrate buffer (pH 6.0) for 10 min. The slides were incubated with mouse anti-Cyr61 mAb (1:200 dilution) overnight at 4°C. The slides were then incubated with a HRP-conjugated goat anti-mouse polyclonal antibody for 60 min and subsequently reacted with DAB. The nucleus was counterstained with hematoxylin. Negative controls were performed by replacing the primary antibody with mouse IgG. To evaluate the IHC staining of Cyr61 in breast cancer TMA, a semi-quantitative scoring criteria for IHC of Cyr61 was used, in which both staining intensity and positive areas were recorded as − (negative), + (weak positive), ++ (moderate positive), and +++ (strong positive), respectively.

RNA extraction and real-time PCR

Real-time PCR was performed as previously reported [8]. The sequence of primers were as follows: GAPDH, forward 5′-GTGAAGGTCGGAGTCAACG-3′ and reverse 5′-TGA GGTCAATGAAGGGGTC-3′; Cyr61, forward 5′-TC CAGCCCAACTGTA AACATCA-3′ and reverse 5′-GGA CACAGAGGAATGCAGCC-3′; TIMP1, forward 5′-GGT GGGTGGATGAGTAATGCA-3′ and reverse 5′-AGCGG GTGCGG AAACC-3′; TIMP2, forward 5′-GGGCCAAA GCGGTCAGT-3′ and reverse 5′-TAGGGTTGCCATA AATGTCGTTT-3′.

Western blotting analysis

Both Cyr61 expression in MDA-MB-231, MCF-7 cells and the rhCyr61 protein were detected by Western blotting analysis with special anti-human Cyr61 mAbs 093G9, 096B7 and commercial anti-human Cyr61 mAb (R&D, MN, USA). The activation of AKT and ERK (ERK1/ERK2) was analyzed using specific phosphorylated antibodies (Cell Signaling Technology Inc, Beverly, MA, USA). In briefly, lysates were loaded and run electrophoresis followed by transferring to PVDF membranes (Millipore, MA, USA) at 60v for 2 h. The membranes were blocked with 5% non-fat milk, washed with PBST, and incubated with mAbs at 4°C overnight. Then, the membranes were incubated with HRP-conjugated goat anti-mouse IgG at room temperature for 45 min followed by washing with PBS. The target proteins were examined with ECL system (Millipore, MA, USA) and visualized with autoradiography film.

Immunofluorescence assay

A total of 1×10^5 cells seeded on glass coverslips and grown under standard conditions for 24 h were rinsed in PBS and fixed with 4% paraformaldehyde for 20 min. After permeabilizing with 0.1% Triton X-100 (Sigma, MO, USA) in PBS, cells were blocked with 10% normal goat serum for 1 h. The mouse anti-cyr61 mAb: 096B7 or 093G9 were applied at 1:400 dilutions at 4°C overnight. Alexa Fluor 488-conjugated anti-mouse IgG (ZyMed, CA, USA) was used as a secondary antibody at 1:1,000 dilution for 1 h. Nuclei were counterstained with DAPI (ZyMed, CA, USA) for 3 min. Coverslips with antifade fluorescent mounting medium were transferred onto glass slides and sealed with nail polish.

Cyr61 gene expression knockdown by RNAi

Cyr61 gene knockdown was performed as previously reported [8]. In brief, MDA-MB-231 cells were cultured in 12-well plates. A transfection mixture of siRNA oligonucleotides and Lipofectamine 2000 reagent (Invitrogen, CA, USA) in serum-free medium was added to medium-aspirated cells for 4 h. Then, the medium was replaced with complete DMEM containing 10% FCS for another 24 h incubation.

Cell division and proliferation analysis

MDA-MB-231 cells were labeled with CFSE, and cell division was detected by flow cytometry after Cyr61 gene knocked down. Proliferation was measured by thymidine (^{3}H-TdR) incorporation. In briefly, MDA-MB-231 cells (1.2×10^4 per well) were co-cultured with 093G9, 096B7, or IgG1 isotype control mAb (control IgG) at final concentration of 5, 10, 20 μg/ml, respectively. Cells were pulsed with 1 μCi thymidine (Amersham Biosciences, Piscataway, NJ, USA) for another 16 h. Incorporated radioactivity was measured as c.p.m (Count per min) using a β-plate counter.

Cell migration and invasion assay

Migration assay was performed in a 24-well Transwell tissue culture plate (Costar Corning, MA, USA) with a polycarbonate filter (pore size 5 μm). A total of 5×10^4 cells/well were seeded in upper chambers with 200 μl 0.1% BSA DMEM, and 800 μl supernatant from MDA-MB-231 cells containing secreted Cyr61 as chemoattractant was put at bottom chambers. Then, cells were allowed to migrate in the presence of 093G9 or control IgG at a concentration of 5, 10, and 20 μg/ml for 4 h, respectively, followed by fixed filters with 4% paraformaldehyde for 15 min at room temperature. The cells remained on fixed filters were observed under microscope and counted using 100 ocular grids to quantitative migrated cells. Total cells on six random fields per filter were photographed and counted as migrated cells. For invasion assay, similar for migration assay except (1) transwell insert chambers with 8-μm pore filters were coated with a final concentration of 1 mg/ml of Matrigel (Becton–Dickinson) and (2) cells in upper chambers were allowed to migrate for 24 h. Percent inhibition of migration or invasion = 1 − (cells on fixed filter divided total cells seeded in upper chambers) × 100%.

Animal tumor model and mAbs treatment

A total of 5×10^6 MDA-MB-231 cells were inoculated into 5-week-old female nude mice subcutaneously. Two days later, inoculated mice were randomly assigned to two groups receiving 093G9 and control IgG (5 mg/kg body weight) i.p., twice per week, respectively. Tumor size was measured with caliper once every 3 days until the mice of control group were evaluated as dead after tumors reached a size of 1 cm^3 as previous reports. For lymph node metastatic analysis, 2×10^6 MDA-MB-231 cells were injected into the claw pad followed by 093G9 and control IgG treatment same as described earlier. Thirty-five days later, the mice were killed and lymph node metastasis was observed. Tumor volume (mm^3) = 0.5 × length ×width^2. At the end of the experiment, the mice were killed and primary tumors were dissected, weighed, and fixed in 10% formalin for Cyr61 expression analysis with H&E staining and IHC assay.

Biopanning against anti-hCyr61 monoclonal antibody assay

For biopanning epitopes of two anti-Cyr61 monoclonal antibodies, Ph.D.-C7C or Ph.D.-12 phage library displaying assay kits (NEB, MA, USA) were used and panning performed according to the manufacture's instructions. In brief, microplate wells were coated with mAbs 093G9 or 096B7 (100 μg/well) and blocked with 0.5% BSA (bovine serum albumin) in PBS. The phage library (1.0×10^{11} plaque forming units) was added to the wells, incubated for 1 h, and washed with TBS-0.1% Tween 20. ER2738 *Escherichia coli* were added and cultured for phage propagation. Phages recovered from the culture supernatant with polyethylene glycol precipitation were used for the next round of panning. After 3 rounds of panning, the phages were cloned on the culture plates for the formation of phage plaques. The positive phage clones were subjected to DNA sequencing and epitope screening.

Statistical analysis

Group measures were shown as mean ± SEM. A Student's t test was used to analyze the differences between the groups. One-way ANOVA was initially performed to determine whether there was an overall statistically significant change followed by the two-tailed paired or unpaired Student's t test. P value of <0.05 was considered statistically significant.

Results

Cyr61 high expression in breast cancer tissue

Using TMA and IHC assays, we examined Cyr61 expression in breast cancer tissue obtained from 78 Chinese patients with different clinical stages and pathological types. In breast cancer tissues, Cyr61 was found strong expression in both breast cancer cells and ductal epidermal cells, while in normal breast tissues, Cyr61 was only expressed in ductal epidermal cells (Supplement Figure). Table 1 shows that of the breast cancers studied, 4 were negative or equivocal for Cyr61 expression. Cyr61 protein was expressed in all different clinical stages, including patients with infiltrating breast cancer, lymph node metastasis, and ovarian metastasis (Table 1). This result was consistent with previous reports: Cyr61 indeed

Table 1 Expression of Cyr61 in breast cancer tissues

Sample (no.)	Age	Clinical stage	Staining intensity					Positive straining (%)
			− (%)	± (%)	+ (%)	++ (%)	+++ (%)	
Infiltrating ductal carcinoma (54)	47 ± 6.8	IIA	0 (0)	0 (0)	2 (10)	7 (37)	10 (53)	100
		IIB	2 (33)	0 (0)	0 (0)	2 (33)	2 (33)	66
		IIIA	1 (5)	0 (0)	3 (15)	7 (35)	9 (45)	95
		IIIB	0 (0)	1 (11)	0 (0)	3 (33)	5 (56)	100
Ductal carcinoma in situ (2)	51		0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	100
Signet ring cell carcinoma (2)	60	IIB	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	100
Lymph node metastatic carcinoma (18)	46 ± 8		0 (0)	0 (0)	3 (16)	5 (28)	10 (56)	100
Ovarian metastatic carcinoma (2)*	37	IV	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	100

* The ovarian metastatic carcinoma specimens were from breast cancer patients

overexpressed in breast cancer regardless in primary or in metastatic breast cancer [7, 11, 15, 18].

Characterization of anti-Cyr61 mAbs 093G9 and 096B7

In a set of Biacore experiments, the affinity of 093G9 and of 096B7 to human Cyr61 was determined. Twofold dilutions of purified human Cyr61 (1–64 nM) were injected into the flow cell containing immobilized mAbs 093G9 or 096B7. Based on the experimental data, association and dissociation curves were calculated (black lines) and fitted to the recorded curves (colored lines). Association (Ka) and dissociation rate constants (Kd) were determined to be $Ka = 1.019 \times 10^6$ and $Kd = 0.00992$ for 096B7, and $Ka = 3.125 \times 10^5$ and $Kd = 0.001952$ for 093G9, respectively. The affinity constant Kd was calculated from Kd and Ka to be 9.7×10^{-9} and 6.2×10^{-9} (Fig. 1a). This result indicated that 093G9 and 096B7 showed high antigen-binding activity to Cyr61. The purity of mAbs 096B7 and 093G9 was more than 95% determined by HPLC (Fig. 1b). ELISA showed the high titers of 093G9 and 096B7 binding Cyr61, which were similar to a commercial available one (R&D) (Fig. 1c). The specificity of mAbs was analyzed by Western blot. As shown in Fig. 1d, mAbs 093G9 and 096B7 could bind to human Cyr61 extracted from MDA-MB-231 cells and rhCyr61 specifically as the R&D antibody did.

Knockdown Cyr61 reduces division of cell line MDA-MB-231

As known that Cyr61 high expressed breast cancer cell line MDA-MB-231 grows faster and increases metastasis compared with low-expressed Cyr61 cell line MCF-7, we first tested Cyr61 expression profiles with our anti-Cyr61 monoclonal antibodies (mAbs) 093G9 and 096B7. The results showed that both mAbs could bind to nature Cyr61 expressed in MDA-MB-231 by Western blot and immunofluorescence staining assay (Fig. 2a, b). Further, to address whether Cyr61 involved in proliferation of MDA-MB-231, Cyr61 expression was inhibited by RNAi technique. The results showed when Cyr61 expression was downregulated up to 60% by siRNA (Fig. 2c), the division of MDA-MB-231 was reduced more than 4 generations (Fig. 2d). These results demonstrated that Cyr61 was very important in MDA-MB-231 cells division.

Anti-Cry61 antibody 093G9 inhibits MDA-MB-231 proliferation in vitro and in vivo

To further address whether Cyr61 involved in MDA-MB-231 cell's growth, we examined proliferation of MDA-MB-231 treated with anti-Cyr61 mAbs 093G9 and 096B7, respectively. The results showed that only 093G9 could inhibit MDA-MB-231 cells proliferation significantly with a dose-dependent manner (Fig. 3a), whereas 096B7 did not show inhibitory capability. To understanding the signaling pathway involved in this activity, downstream protein phosphorylation profiles after Cyr61 mAb neutralization were analyzed. When Cyr61 on MDA-MB-231 was neutralized by 093G9, phosphorylated AKT and ERK decreased remarkably (Fig. 3b). Moreover, to address whether 093G9 could inhibit MDA-MB-231 growth in vivo, we treated nude mice bearing MDA-MB-231 with 093G9. The results showed that tumor volume in the mice received 093G9 was significantly smaller than those treated with control IgG (Fig. 3c, left panel), which consequently 093G9-treated mice had longer life span (Fig. 3c, right panel). Meanwhile, IHC analysis revealed that 093G9-treated tumor cells showed weak Cyr61 expression compared with its counterpart that received control IgG

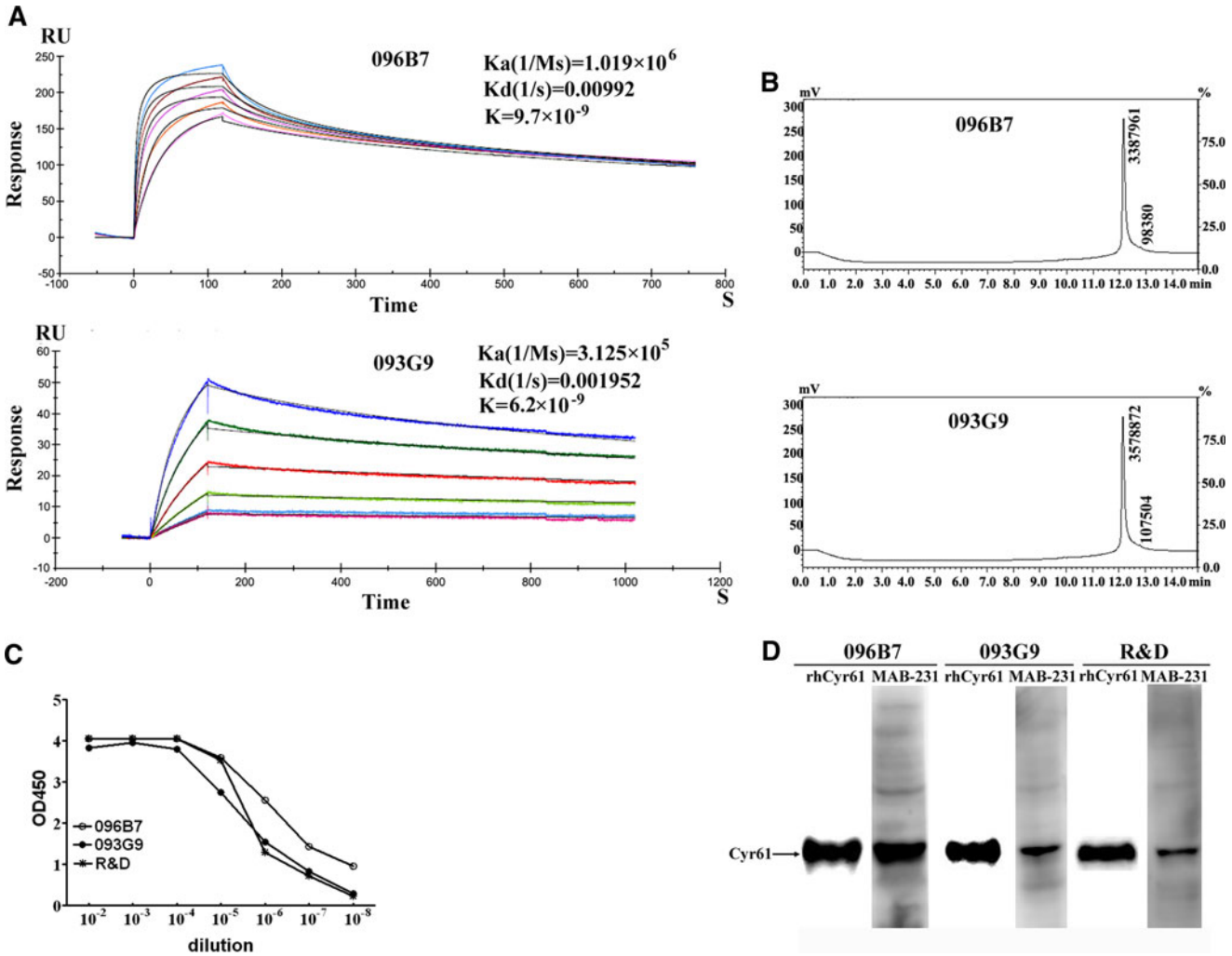

Fig. 1 Characterization of anti-Cyr61 mAbs 093G9 and 096B7. **a** Cyr61 was injected at twofold increasing concentrations from 1 to 64 nM (*curves bottom–top*) into the flow cell containing immobilized mAbs 093G9 or 096B7. Calculated *curves* (*black*) were superimposed on recorded curves (*colored*). The affinity constant Kd was calculated from Kd to Ka. **b** The purity of mAbs 096B7 and 093G9 was determined by HPLC. **c** ELISA. Serial log dilutions of 096B7, 093G9, or R&D antibodies were added to ELISA plates precoated with human Cyr61 and incubated. After washing, mAbs binding to Cyr61 were detected with HRP-conjugated goat anti-mouse IgG. **d** 096B7, 093G9, and R&D antibodies bind to rhCyr61 protein and human Cyr61 protein detected by Western blot assays

(Fig. 3d). These results implicated that 093G9 could block the biological activity of Cyr61 on cell proliferation in vitro and in vivo by downregulation of AKT and ERK phosphorylation.

093G9 inhibits MDA-MB-231 migration and invasion in vitro and in vivo

As many reports presumed that Cyr61 may mediate not only breast cancer cells' growth but also metastasis and invasion, whether 093G9 could inhibit MDA-MB-231 migration and invasion was analyzed using transwell system. The results indicated that 093G9 inhibited MDA-MB-231 migration and invasion in a dose-dependent manner (Fig. 4a). When treated MDA-MB-231 cells with 20 μg/ml 093G9, the inhibition percent of migration reached to 70% and that of invasion was 60%, respectively (Fig. 4b). Because previous reports implicated that MMPs involved in invasion and metastasis, we detected MMPs inhibitors, TIMP1 and TIMP2, expression in MDA-MB-231 cells treated with 093G9 or control IgG. The result showed that TIMP1 and TIMP2 transcription in 093G9-treated MDA-MB-231 cells was upregulated significantly (Fig. 4c). Furthermore, using nude mice footpad inoculation model, we found that events of lymph node metastasis were significantly less in 093G9-treated group (20%) compared with that received control IgG (60%), consistently, metastatic tumor volume was significantly larger in control group (Fig. 4d). In contrast, 096B7 did not inhibit migration and invasion of MDA-MB-231 cells both in

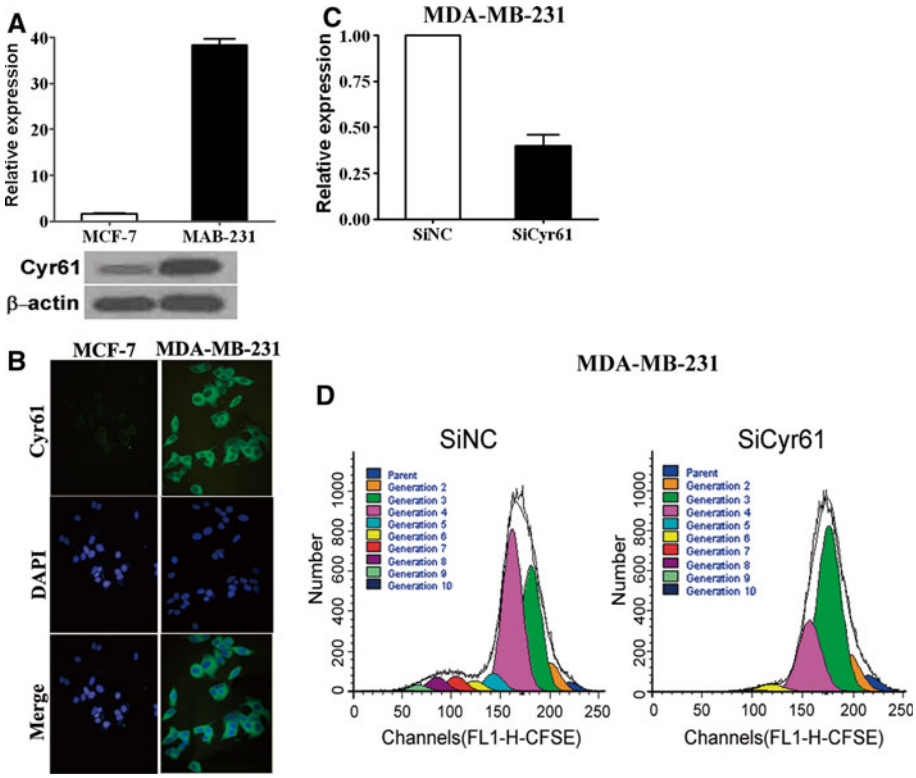

Fig. 2 Cyr61 gene knockdown reduced MDA-MB-231 cells division. **a** Cyr61 expression in MCF-7 and MDA-MB-231 cells. *Upper panel* real-time PCR evaluation of Cyr61 expression. *Lower panel* Western blot analysis of Cyr61 expression. **b** Immunofluorescence assay shows mAb 093G9 can bind to natural Cyr61 protein in cells. **c** Cyr61 mRNA was downregulated by special RNAi in MDA-MB-231 cells. **d** MDA-MB-231 cells division reduced remarkably after RNAi. The *peaks* in different *colors* represent the passages of MDA-MB-231 cells labeled with CFSE. SiNC: negative control. SiCyr61: Cyr61 gene was knockdown in MDA-MB-231 cells

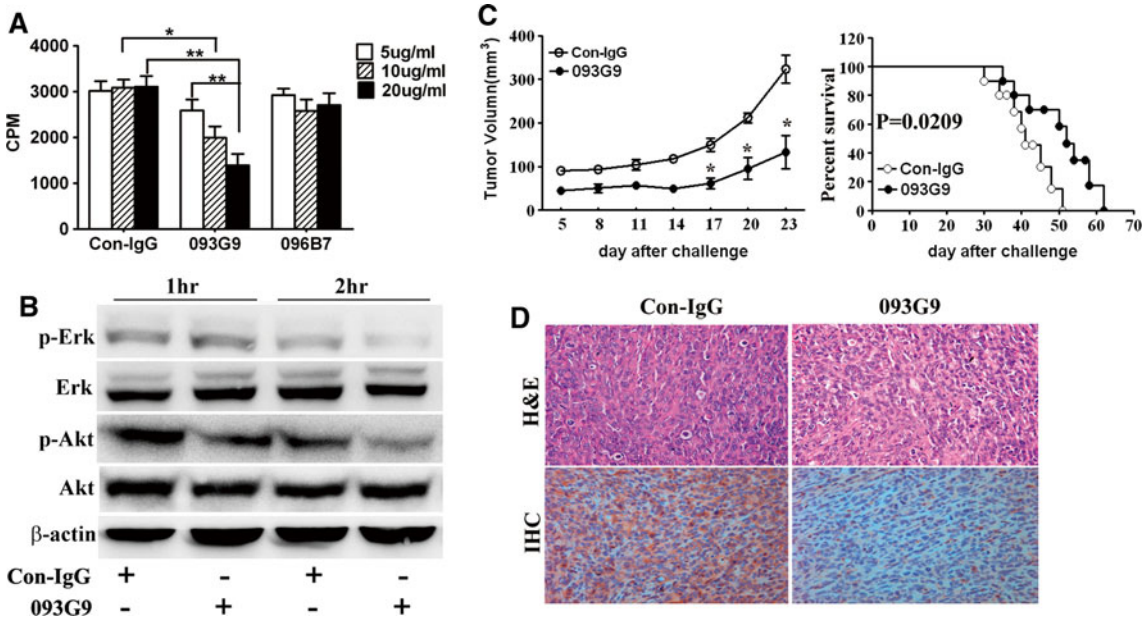

Fig. 3 mAb 093G9 inhibits MDA-MB-231 cells' growth in vitro and in vivo. **a** Decreased proliferation of MDA-MB-231 cells treated with mAbs labeled on *axis*. *Open bars* represents 5 μg/ml mAbs were added. *Shadow bars* mean 10 μg/ml mAbs were used. *Black bars* represents 20 μg/ml mAbs were added. **b** Phosphorylated AKT and ERK extracted from MDA-MB-231 cells co-cultured with mAb 093G9 or Con-IgG at different time. **c** Tumor volume (*left panel*) and percent survival (*right panel*) treated with mAb 093G9 and Con-IgG. *Open circle* represented mice treated with Con-IgG ($n = 10$). *Solid circle* represented the mice received 093G9 ($n = 10$). **d** H&E staining and immunohistochemical staining of Cyr61 expression in tumor tissue treated with Con-IgG or 093G9 at an original magnification ×200. The data represent mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$

vitro and in vivo (data not shown). These demonstrated that neutralizing Cyr61 by 093G9 could inhibit MDA-MB-231 cells migration and invasion effectively both in vitro and in vivo. Upregulation of TIMP1 and TIMP2 (MMPs inhibitors) might contribute to inhibitory activity of 093G9.

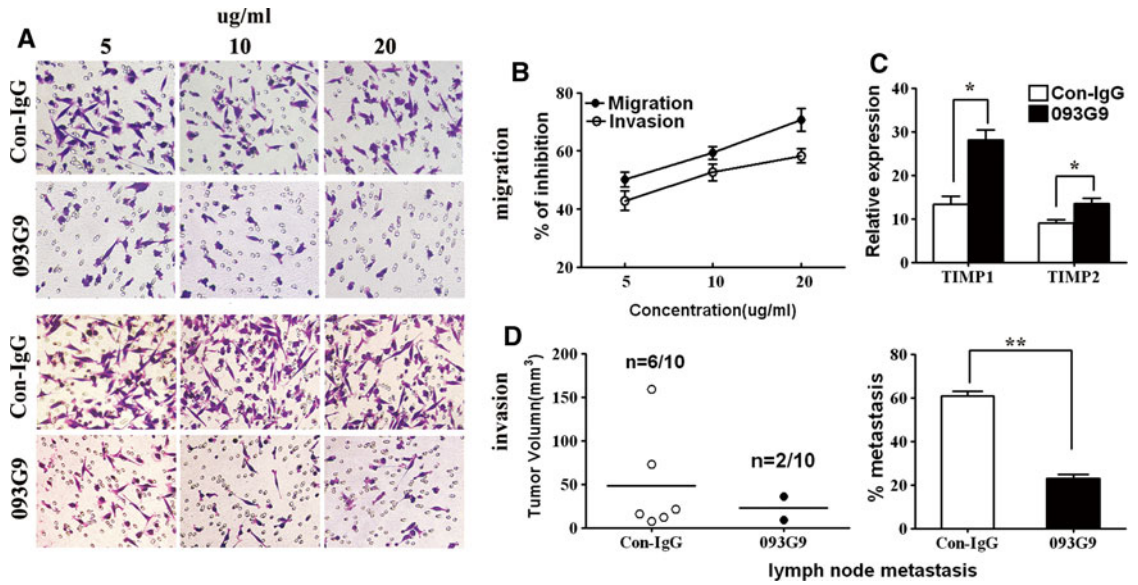

Fig. 4 mAb 093G9 inhibits MDA-MB-231 cells migration and invasion in vitro and lymph node metastasis in vivo. **a** Transwell system assay for MDA-MB-231 cells migration ability (*top panel*) and invasion ability (*bottom panel*). Cells on filters were fixed and stained with gentian *violet*, and then, six random fields per filter were photographed and counted. **b** Percent inhibition of migration (*solid circles*) and invasion (*open circles*) after treated with mAb 093G9 compared with control IgG. **c** The relative expression of TIMP1 and TIMP2. *Open bar* represented expression profile treated with Con-IgG, and *black bar* represented expression profile treated with 093G9. **d** Lymph node metastasis profile. Tumor volume (*left panel*) and percent metastasis (*right panel*) treated with mAb 093G9 or Con-IgG. *Open circle* or *bar* represented mice treated with Con-IgG ($n = 10$). *Solid circle* or *bar* represented the mice received 093G9 ($n = 10$). The data represent mean ± SEM of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$

Mapping of Cyr61 epitopes corresponding to mAbs 093G9 and 096B7

To account for the neutralization activity of mAb 093G9, the epitopes corresponding to mAbs 093G9 and 096B7 were further identified through screening of a phage library displaying random C7C-mer (for 093G9) and 12-mer (for 096B7) peptides simultaneously. In the third round of selection, phages that bound with higher affinity to mAb 093G9 and to mAb 096B7 were enriched. In this selection round, sequencing of the inserts of 25 clones recovered from the wells of mAb 093G9 and 32 clones recovered from the wells of mAb 096B7 revealed their difference. The consensus motif can be summarized as L(P/L)N(W/F/C)(N/T/L)P(H/Q/T) (amino acid separated by a solidus indicated the amino acid substitutions) for 093G9 and WHTRTNDQ for 096B7, respectively (Table 2). The selected consensus motif LPNLGCP showed 100% homology to the segment of human Cyr61 VWC domain (amino acid 140–146) and TR-VTND of human Cyr61 TSP-1 region (amino acid 249–254). The binding of mAb 093G9 and mAb 096B7 to the corresponding epitopes was also confirmed with ELISA analysis (data not shown). These results further demonstrated that VWC fragment was a key domain of human Cyr61 for cancer cell proliferation, migration, and tumor invasion. Blockade of Cyr61 VWC domain by mAb 093G9 (LPNLGCP) could therefore inhibit cancer cell proliferation, migration, and invasion in vitro and in vivo. In contrast, tickling to Cyr61 TSP-1 region by 096B7 (TR-VTND) did not show any such effects.

Discussion

Overexpression of Cyr61 has been reported in breast cancer patients with different clinical stage, which contribute to breast cancer tumorigenesis and cancer progression [5, 7, 14–18, 20, 29]. Cyr61-transfected MCF-7, a lower growth and migration breast cancer cell line, rendered more proliferation, migration, and invasion capability, resemble to its counterpart, MDA-MB-231 [7, 18, 20]. In this study, using MDA-MB-231 cell line model, we not only demonstrated that blockade of Cyr61 by neutralizing mAb would arrest breast cancer cell proliferation, migration, and invasion in vitro and in vivo but also identified VWC region of Cyr61 as a potential novel domain contributing to breast cancer development.

We found that Cyr61 was overexpressed among 66–100% breast cancer Chinese patients with different clinical stages and pathological types and lymph node metastasis as previous reports [15, 18, 19]. Interestingly, we also observed that Cyr61 was highly expressed in breast cancer patients with ovarian metastasis. Although lymph node metastasis is common and plays an important role in

Table 2 Identification of the epitopes recognized by anti-Cyr61 mAbs 093G9 and 096B7

	Binding peptide	
	093G9 (Ph.D.-C7C)	096B7 (Ph.D.-12)
Selected sequences	LLNWNPH, LPNWNPH, LLNWDPQ, LKNWNPH	HFFKWHTRTNDQ, HFFKWHTRTNDQ, HMFKWHTRTNDQ
	LPNWTPQ, LLNWTPH, LANFTPH, LMNWTPH	HYFKWHTRTNDQ, HFFKWHTRTNDQ, HFFKWHTRTNDQ
	LLNCNPQ, LFNFDPT, LLNFSPY, LFNFDPT	HFFKWHTRTNDQ
Consensus motif	LPNLGCP (aa140–146)	WHTRTNDQ (aa249–254)
Corresponding segment of human Cyr61	$L_1(P/L)_2N_3(W/F/C)_4(N/T/L)_5P_6(H/Q/T)_7$	TRVTND

clinical diagnosis and treatment of breast cancer, overexpression of Cyr61 found in ovarian metastasis is rather important for early diagnosis and treatment for patients [29, 30]. Thus, the finding of Cyr61 overexpression in ovarian metastasis may be not only as a potential therapeutic target but also as a potential risk factor for ovarian metastasis.

Our previous study has shown that Cyr61 plays a critical role in fibroblast synoviocyte (FLS) proliferation through IL-17 pathway [8]. In breast cancer research, whether the proliferation of MDA-MB-231 could be inhibited through Cyr61-specific blockade in vivo remains unknown. In current study, using RNAi technique, we successfully arrested proliferation of MDA-MB-231 cells. Moreover, we showed that anti-Cyr61 monoclonal antibody (093G9) could significantly inhibit proliferation, migration, and invasion of MDA-MB-231 cells. Similarly, this neutralizing mAb could inhibit MDA-MB-231 tumor growth and lymph node metastasis in vivo.

It is known that there are different integrin subunits expressed on cell surface as Cyr61 receptors [1, 3, 5, 7, 8, 25, 31]. Cyr61 interacting with special integrins will result in unique biological consequence via various signaling pathways. For example, on gastric cancer cell, AGS cell line, overexpressing Cyr61 may activate NF-κB via α3/COX2 pathway [10]. On breast cancer cell line, Cyr61 interacting with integrin $\alpha v\beta 3$ will trigger FAK-PI3 K signaling pathway to promote cells proliferation, in which AKT acts as a critical kinase. Blocking Cyr61–$\alpha v\beta 3$ interaction will decrease phosphorylation of AKT [1, 10, 18, 32]. Nevertheless, ERK1/ERK2 MAPK is reported to involve in Cyr61-transfecting MCF-7 activation through $\alpha v\beta 3$ receptor without change in AKT activation recently [25, 33]. However, using MDA-MB-231 cell line, we found that phosphorylated AKT and ERK1/ERK2 remarkably decreased when Cyr61 was blocked by mAb 093G9. Our study indicated that in MDA-MB-231 cells, Cyr61 could activate FAK-PI3 K and ERK1/ERK2 MAPK signaling pathways simultaneously as reported in ovarian carcinomas [34].

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent enzymes that cleave extracellular matrix (ECM) proteins during cancer invasion [35–37]. Previous reports implicated that MMP-1 was one of the most highly upregulated MMPs in breast cancer and involved in invasion and metastasis [38, 39]. Recently, MMP1 expressed by breast cancer cells is reported as a mediator of transendothelial migration, tumor seeding, and relapse to lungs and brain [31, 40, 41]. On the other hand, tumor-derived Cyr61 could induce stromal MMP-1 production and thereby mediate tumor cell migration and invasion [38]. Blockade of Cyr61 might be benefit for alleviating breast cancer invasion and metastasis. TIMP1 and TIMP2 are considered as main inhibitors of MMP-1 in breast cancer cells [35, 42–45]. In this study, using neutralizing antibody 093G9, we found that blockade of Cyr61 reduced cancer cell migration and invasion companied by TIMP1 and TIMP2 upregulated dramatically. Furthermore, in nude mice model, we found that lymph node metastasis was decreased remarkably in 093G9-administrated group. These data indicated that neutralizing Cyr61 could alleviate breast cancer cells invasion via upregulation of MMP1 inhibitors, TIMP1 and TIMP2.

Given that Cyr61 consists of four structural domains [1], reports implicated that the domain 4 of Cyr61 is important for promoting tumor cell proliferation, migration, and invasion in breast cancer research [27]. However, in current study, using Ph.D.-C7C phage library display system, we identified that peptide LPNLGCP located in domain 2, VWC domain (aa140–146), of Cyr61 was recognized by 093G9, while the epitope of mAb 096B7 was a linear consensus motif TR-VTND located in TSP-1 region (aa249–254). This demonstrated that the domain 2 of Cyr61 plays an important role in breast cancer cell tumorigenesis and cancer progression.

VWC domain, also referred to as chordin-like cysteine-rich (CR) repeats, is a common motif found in more than 500 ECM proteins [1, 23, 24, 26, 33]. In Cyr61 protein, this region with typically 70–100 amino acids in length can bind to $\alpha v\beta 3$ and contains ten conserved cysteine residues and a pair of cysteine-containing motifs [1]. The functional study shows that VWC repeats in CCN proteins regulate bone morphogenetic proteins (BMPs) and TGF-β signaling. Some reports implicated that the domain is the one to bind integrin and thereby mediate Cyr61 function. However, no evidence proved role of this domain directly involved in breast cancer development. Our data indicated for the first time that VWC domain might play very important role in proliferation, migration, and invasion of Cyr61 high-expression breast cancer cells.

Epitope recognized by 096B7 located in the TSP-1 domain, the third domain of Cyr61, was observed in our study. The TSP-1 domain is able to bind sulphated glycoconjugates and interacts with $\alpha 6\beta 6$, the same as the CT domain of Cyr61 [1]. Although it is suggested to play a role in adhesion or interactions with the ECM, the exact function of the TSP-1 domain remains unclear. Our data suggested that direct binding to this domain cannot abolish the function of Cyr61 in breast cancer cell proliferation, migration, and invasion.

Taken together, in this study, we found the inhibition of growth and lymph node metastasis of breast cancer cells by blockade circular functional epitope of VWC domain of Cyr61 using mAb 093G9, which might be a novel potential target for developing therapeutics to Cyr61 overexpressing breast cancer.

Acknowledgments We thank Dr. Guang Yang and Yaping Gao for discussion of data of phage library display. This work was supported by National Basic Research Program of China (2010CB529103), National Natural Science Foundation of China (30872990, 81072468, 81172856), Science and Technology Commission of Shanghai Municipality (10JC1408300), Shanghai Municipal Education J50207.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Holbourn KP, Acharya KR, Perbal B (2008) The CCN family of proteins: structure-function relationships. Trends Biochem Sci 33(10):461–473
2. Brigstock DR (1999) The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev 20(2):189–206
3. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF (1998) CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci USA 95(11):6355–6360
4. Brigstock DR (2003) The CCN family: a new stimulus package. J Endocrinol 178(2):169–175
5. Menendez JA, Mehmi I, Griggs DW, Lupu R (2003) The angiogenic factor CYR61 in breast cancer: molecular pathology and therapeutic perspectives. Endocr Relat Cancer 10(2):141–152
6. Leask A, Abraham DJ (2006) All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci 119(Pt 23):4803–4810
7. Sampath D, Winneker RC, Zhang Z (2001) Cyr61, a member of the CCN family, is required for MCF-7 cell proliferation: regulation by 17beta-estradiol and overexpression in human breast cancer. Endocrinology 142(6):2540–2548
8. Zhang Q, Wu J, Cao Q, Xiao L, Wang L, He D, Ouyang G, Lin J, Shen B, Shi Y, Zhang Y, Li D, Li N (2009) A critical role of Cyr61 in interleukin-17-dependent proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. Arthritis Rheum 60(12):3602–3612
9. Xie D, Yin D, Wang HJ, Liu GT, Elashoff R, Black K, Koeffler HP (2004) Levels of expression of CYR61 and CTGF are prognostic for tumor progression and survival of individuals with gliomas. Clin Cancer Res 10(6):2072–2081
10. Lin MT, Zuon CY, Chang CC, Chen ST, Chen CP, Lin BR, Wang MY, Jeng YM, Chang KJ, Lee PH, Chen WJ, Kuo ML (2005) Cyr61 induces gastric cancer cell motility/invasion via activation of the integrin/nuclear factor-kappaB/cyclooxygenase −2 signaling pathway. Clin Cancer Res 11(16):5809–5820
11. D'Antonio KB, Toubaji A, Albadine R, Mondul AM, Platz EA, Netto GJ, Getzenberg RH (2010) Extracellular matrix associated protein CYR61 is linked to prostate cancer development. J Urol 183(4):1604–1610
12. Goodwin CR, Lal B, Zhou X, Ho S, Xia S, Taeger A, Murray J, Laterra J (2010) Cyr61 mediates hepatocyte growth factor-dependent tumor cell growth, migration, and Akt activation. Cancer Res 70(7):2932–2941
13. Leask A (2010) A sticky situation: CCN1 promotes both proliferation and apoptosis of cancer cells. J Cell Commun Signal 4(1):71–72
14. Grzeszkiewicz TM, Kirschling DJ, Chen N, Lau LF (2001) CYR61 stimulates human skin fibroblast migration through integrin alpha vbeta 5 and enhances mitogenesis through integrin alphavbeta 3, independent of its carboxyl-terminal domain. J Biol Chem 276(24):21943–21950
15. Xie D, Nakachi K, Wang H, Elashoff R, Koeffler HP (2001) Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. Cancer Res 61(24):8917–8923
16. Tsai MS, Bogart DF, Castaneda JM, Li P, Lupu R (2002) Cyr61 promotes breast tumorigenesis and cancer progression. Oncogene 21(53):8178–8185
17. Hirschfeld M, zur Hausen A, Bettendorf H, Jager M, Stickeler E (2009) Alternative splicing of Cyr61 is regulated by hypoxia and significantly changed in breast cancer. Cancer Res 69(5):2082–2090
18. Lin MT, Chang CC, Chen ST, Chang HL, Su JL, Chau YP, Kuo ML (2004) Cyr61 expression confers resistance to apoptosis in breast cancer MCF-7 cells by a mechanism of NF-kappaB-dependent XIAP up-regulation. J Biol Chem 279(23):24015–24023
19. Monnier Y, Farmer P, Bieler G, Imaizumi N, Sengstag T, Alghisi GC, Stehle JC, Ciarloni L, Andrejevic-Blant S, Moeckli R, Mirimanoff RO, Goodman SL, Delorenzi M, Ruegg C (2008) CYR61 and alphavbeta5 integrin cooperate to promote invasion and metastasis of tumors growing in preirradiated stroma. Cancer Res 68(18):7323–7331
20. Troester MA, Lee MH, Carter M, Fan C, Cowan DW, Perez ER, Pirone JR, Perou CM, Jerry DJ, Schneider SS (2009) Activation

of host wound responses in breast cancer microenvironment. Clin Cancer Res 15(22):7020–7028
21. Jiang WG, Davies G, Martin TA, Parr C, Watkins G, Mason MD, Mokbel K, Mansel RE (2005) Targeting matrilysin and its impact on tumor growth in vivo: the potential implications in breast cancer therapy. Clin Cancer Res 11(16):6012–6019
22. Jin Y, Kim HP, Cao J, Zhang M, Ifedigbo E, Choi AM (2009) Caveolin-1 regulates the secretion and cytoprotection of Cyr61 in hyperoxic cell death. FASEB J 23(2):341–350
23. Bork P (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett 327(2):125–130
24. Schober JM, Lau LF, Ugarova TP, Lam SC (2003) Identification of a novel integrin alphaMbeta2 binding site in CCN1 (CYR61), a matricellular protein expressed in healing wounds and atherosclerotic lesions. J Biol Chem 278(28):25808–25815
25. Menendez JA, Vellon L, Mehmi I, Teng PK, Griggs DW, Lupu R (2005) A novel CYR61-triggered 'CYR61-alphavbeta3 integrin loop' regulates breast cancer cell survival and chemosensitivity through activation of ERK1/ERK2 MAPK signaling pathway. Oncogene 24(5):761–779
26. Perbal B (2009) Alternative splicing of CCN mRNAs…it has been upon us. J Cell Commun Signal 3(2):153–157
27. O'Kelly J, Chung A, Lemp N, Chumakova K, Yin D, Wang HJ, Said J, Gui D, Miller CW, Karlan BY, Koeffler HP (2008) Functional domains of CCN1 (Cyr61) regulate breast cancer progression. Int J Oncol 33(1):59–67
28. Yokoyama WM (2001) Production of monoclonal antibodies. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (eds) Current protocols in immunology. John Wiley & Sons, New York, pp 2.5.1–2.5.17
29. Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP (2008) Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Res 68(11):4311–4320
30. Mouridsen HT, Rose C, Brodie AH, Smith IE (2003) Challenges in the endocrine management of breast cancer. Breast 12(Suppl 2):S2–S19
31. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, Massague J (2009) Tumor self-seeding by circulating cancer cells. Cell 139(7):1315–1326
32. Xie D, Yin D, Tong X, O'Kelly J, Mori A, Miller C, Black K, Gui D, Said JW, Koeffler HP (2004) Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and beta-catenin-TCF/lef signaling pathways. Cancer Res 64(6): 1987–1996
33. Vellon L, Menendez JA, Lupu R (2005) AlphaVbeta3 integrin regulates heregulin (HRG)-induced cell proliferation and survival in breast cancer. Oncogene 24(23):3759–3773
34. Gery S, Xie D, Yin D, Gabra H, Miller C, Wang H, Scott D, Yi WS, Popoviciu ML, Said JW, Koeffler HP (2005) Ovarian carcinomas: CCN genes are aberrantly expressed and CCN1 promotes proliferation of these cells. Clin Cancer Res 11(20): 7243–7254
35. Denys H, De Wever O, Nusgens B, Kong Y, Sciot R, Le AT, Van Dam K, Jadidizadeh A, Tejpar S, Mareel M, Alman B, Cassiman JJ (2004) Invasion and MMP expression profile in desmoid tumours. Br J Cancer 90(7):1443–1449
36. Pendurthi UR, Tran TT, Post M, Rao LV (2005) Proteolysis of CCN1 by plasmin: functional implications. Cancer Res 65(21): 9705–9711
37. Pearce EG, Laxton RC, Pereira AC, Ye S (2007) Haplotype effects on matrix metalloproteinase-1 gene promoter activity in cancer cells. Mol Cancer Res 5(3):221–227
38. Nguyen N, Kuliopulos A, Graham RA, Covic L (2006) Tumor-derived Cyr61(CCN1) promotes stromal matrix metalloproteinase-1 production and protease-activated receptor 1-dependent migration of breast cancer cells. Cancer Res 66(5):2658–2665
39. Butler GS, Dean RA, Tam EM, Overall CM (2008) Pharmacoproteomics of a metalloproteinase hydroxamate inhibitor in breast cancer cells: dynamics of membrane type 1 matrix metalloproteinase-mediated membrane protein shedding. Mol Cell Biol 28(15):4896–4914
40. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL, Massague J (2005) Genes that mediate breast cancer metastasis to lung. Nature 436(7050):518–524
41. Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA, Massague J (2009) Genes that mediate breast cancer metastasis to the brain. Nature 459(7249):1005–1009
42. Fong KM, Kida Y, Zimmerman PV, Smith PJ (1996) TIMP1 and adverse prognosis in non-small cell lung cancer. Clin Cancer Res 2(8):1369–1372
43. Ree AH, Florenes VA, Berg JP, Maelandsmo GM, Nesland JM, Fodstad O (1997) High levels of messenger RNAs for tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in primary breast carcinomas are associated with development of distant metastases. Clin Cancer Res 3(9):1623–1628
44. Bhuvarahamurthy V, Kristiansen GO, Johannsen M, Loening SA, Schnorr D, Jung K, Staack A (2006) In situ gene expression and localization of metalloproteinases MMP1, MMP2, MMP3, MMP9, and their inhibitors TIMP1 and TIMP2 in human renal cell carcinoma. Oncol Rep 15(5):1379–1384
45. Sampieri CL, Nuttall RK, Young DA, Goldspink D, Clark IM, Edwards DR (2008) Activation of p38 and JNK MAPK pathways abrogates requirement for new protein synthesis for phorbol ester mediated induction of select MMP and TIMP genes. Matrix Biol 27(2):128–138