Invasion and increased expression of S100A4 and CYR61 in mesenchymal transformed breast cancer cells is downregulated by GnRH

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Abstract. S100 calcium binding protein A4 (S100A4) and cysteine-rich angiogenic inducer 61 (CYR61) play important roles in epithelial-mesenchymal-transition (EMT), invasion and metastasis by promoting cancer cell motility. Recently we were able to show that invasion of GnRH receptor-positive breast cancer cells is time- and dose-dependently reduced by GnRH analogs. We have now analyzed whether GnRH treatment affects S100A4 and CYR61 in mesenchymal transformed breast cancer cells. S100A4 and CYR61 expression was analyzed using RT-PCR. Invasion was quantified by assessment of breast cancer cell migration rate through an artificial basement membrane. The role of S100A4 and CYR61 in invasion of breast cancer cells was analyzed by neutralizing their biological activity. Expression of S100A4, CYR61 and GnRH receptor in human breast cancers, normal and other non-malignant breast tissues was analyzed by immunohistochemistry. Invasion and expression of S100A4 and CYR61 in MDA-MB-231 breast cancer cells were significant higher as compared with MCF-7 breast cancer cells. Invasion and expression of S100A4 and CYR61 were significantly increased in mesenchymal transformed MCF-7 cells (MCF-7-EMT). The increased invasion of MCF-7-EMT cells could be reduced by anti-S100A4 and anti-CYR61 antibodies. In addition, invasion of MDA-MB-231 cells was decreased by anti-S100A4 and anti-CYR61 antibodies. Treatment of MCF-7-EMT and MDA-MB-231 cells with GnRH agonist Triptorelin resulted in a significant decrease of invasion and expression of S100A4 and CYR61. Both, S100A4 and CYR61 were found highly expressed in biopsy specimens of breast hyperplasia and malignant breast cancers. GnRH receptor expression was detectable in approximately 71% of malignant breast cancers. Our findings suggest that S100A4 and CYR61 play major roles in breast cancer invasion. Both, invasion and expression of S100A4 and CYR61 can be inhibited by GnRH treatment.

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Introduction

S100 calcium binding protein A4 (S100A4) is controlled directly by the Wnt/β-catenin signal pathway and is involved in a number of biological mechanisms, i.e. cell movement, surviving, differentiation, and organization of the cytoskeleton (1-5). In addition, S100A4 plays an important role in the biology of stem cells (2,4,5). In S100A4 deficient mice tumor development and metastasis are suppressed (6). S100A4 is overexpressed in tumor cells with metastatic phenotype, while inhibition of S100A4 expression reduces the metastatic potential of tumor cells (7,8).

Cysteine-rich angiogenic inducer 61 (CYR61, CCN1) is part of the CCN intercellular signaling protein family and has various physiological functions, which are strongly dependent on the respective tissue (9,10). In some tissues integrin-dependent proliferation, migration and adhesion is induced by CYR61 (11). In addition, CYR61 can cause directed migration as chemoattractant (12,13). Cyr61 has an important role during vascularization in embryonic development, angiogenesis, and wound healing (10,14,15). In addition, CYR61 plays an important role in skeleton formation and development of the neural system (14,15). Furthermore, CYR61 is involved in transformation processes of the extracellular membrane (16). CYR61-caused effects in tumor tissues depend also strongly on the respective kind of tissue and stage of the disease (17-23). In breast cancers CYR61 leads to increased vascularization of tumors, increased cell proliferation, increased migration and a general increase of tumorigenicity (18,24). A significant connection between CYR61 overexpression and stage of cancer, tumor size, lymph node infestation, and poor survival prognosis can be determined for cancers such as breast cancer, glioma and squamous cell carcinoma (18,20,24,25).

Between 50 and 60% of human breast cancers and in addition most breast cancer cell lines express receptors for gonadotropin-releasing hormone (GnRH) (26-29). Approximately 74% of triple-negative breast cancers express GnRH receptors (30). Previous work showed that invasion of breast cancer cells through an artificial basement membrane was increased when they were cocultured with human primary osteoblasts (hOB) (31). We demonstrated that invasion

in vitro (31) and metastasis *in vivo* (32) of GnRH receptor-positive breast cancer cells was time- and dose-dependently reduced by GnRH analogs. We have now analyzed whether GnRH treatment affects S100A4 and CYR61 in mesenchymal transformed breast cancer cells.

Materials and methods

Cell lines and culture conditions. Human breast cancer cell lines MDA-MB-231 and MCF-7 (wild-type) and osteoblast-like osteosarcoma cell line MG63 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). In order to guarantee the identity of the cell lines over the years, cells were expanded after purchase and aliquots were stored in liquid nitrogen. Every half year a new frozen stock was opened and expanded to carry out the experiments. Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in air in phenol red-free Dulbecco's minimal essential medium (DMEM, PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% charcoal-stripped fetal calf serum (cs-FCS) from Allgaeu BioTech Service (Görisried, Germany).

Human tissues. To analyze expression of GnRH receptor, S100A4, and CYR61 in specimens of human breast cancers we used human tissue arrays (US Biomax, Inc., Rockville, MD, USA) containing paraffin-embedded human normal and malignant breast tissue specimens, whose characteristics are outlined in Table I. Informed consent for the use of human tissues was obtained in accordance with the ethics guidelines that were effective at the time of collection and processing.

Immunohistochemistry. The tissue array slides were deparaffinized and rehydrated. Antigens were retrieved by incubation with 0.01 M citrate buffer (pH 6.0) in a microwave (700 W) for 5 min. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide solution for 6 min. After washing in PBS, the slides were treated with polyclonal rabbit anti-human GnRH receptor antiserum (33), polyclonal rabbit anti-human S100A4 antibody (Abcam, Cambridge, UK), or polyclonal rabbit anti-human CYR61 antibody (Abcam) in a 1:10,000, 1:1,000, or 1:1,000 dilution, respectively in 1% BSA in 10 mM Tris, pH 8.0, 500 mM NaCl and 0.1% Tween-20 (TBST) for 1 h and, after being washed, were detected with the ready-to-use secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody detection system according to the instructions of the supplier (Zymed Laboratories, San Francisco, CA, USA). Controls were performed by substitution of the primary antiserum with pre-immune serum of the same rabbit (anti-human GnRH receptor) or by omission of the primary antibody. Counterstaining was performed using Meyer's hematoxylin for 10 sec. The slides were then dehydrated, cleared, mounted with Permount, and studied by light microscopy.

Co-culture and microinvasion assay. Invasion was measured by assessment of the breast cancer cell migration rate through an artificial basement membrane in a modified Boyden chamber, where the breast cancer cells and the MG63 cells were grown without direct cell-to-cell contact. The membrane of the cell culture insert (upper well) consisted of polycarbonate (8-μm pore diameter, Millipore, Schwalbach, Germany) and was coated on ice with Matrigel® [extracellular matrix (ECM) gel; Becton Dickinson Biosciences, Heidelberg, Germany] diluted 1:2 in serum-free DMEM. Breast cancer cells were seeded into the upper wells (inserts) of the chamber, while the MG63 cells were seeded into the lower wells. The cells were cultured in DMEM supplemented with 10% charcoal-stripped fetal calf serum (cs-FCS) without phenol red for 12 h to allow the cells to attach. Thereafter the upper wells were placed on top of the lower wells (time point t_0) and the breast cancer cells were treated right away with increasing concentrations of GnRH agonist Triptorelin (10^{-11}-10^{-5} M) every 24 h for 96 h, with polyclonal rabbit anti-human S100A4 antibody (Abcam; 15 μg/ml) or with polyclonal rabbit anti-human CYR61 antibody (Abcam; 15 μg/ml) for 96 h. A non-specific polyclonal rabbit isotype control antibody was used as negative control (Abcam). After 96 h the invaded breast cancer cells under the membrane were counted. Controls were performed by omission of the MG63 cells.

Generation of aggressive MCF-7 cells. Mesenchymal transformed MCF-7 cells (MCF-7-EMT) were generated as described by Ziegler *et al* (34). Briefly, single-cell suspensions of wild-type MCF-7 cells (MCF-7 WT) were suspended at a density of 40,000 cells/ml in DMEM/F-12 containing 5 μg/ml insulin (Sanofi-Aventis, Frankfurt, Germany), 0.5 mg/ml hydrocortisone, 2% B27 supplement (Invitrogen, Darmstadt, Germany), 20 ng/ml epidermal growth factor (EGF; Sigma, Deisenhofen, Germany), 20 ng/ml fibroblast growth factor-2 (FGF-2; Sigma), and 1% penicillin/streptomycin (PAA Laboratories GmbH) before incubation on ultralow adherence six-well plates (2.5 ml per plate; Corning, Lowell, MA, USA). For prolonged mammosphere culture (5-6 weeks) the cells were passaged weekly. Mammospheres were harvested, incubated with trypsin for 3 min at 37˚C, and dissociated with a 21-gauge needle. After checking for single cells, the cells were pelleted and suspended in mammosphere culture medium to 40,000 cells/ml before culturing on ultralow adherence plates. To show changes in morphology, bright field images of living MCF-7 WT cells and MCF-7-EMT cells were taken.

Isolation of mRNA and cDNA synthesis. The cells were detached immediately with 0.5 g trypsin (Biochrom, Berlin, Germany) and 5 mmol EDTA in 1 liter PBS/bovine serum albumin. Total RNA was prepared by the RNeasy protocol (Qiagen, Hilden, Germany). The concentration of RNA in each sample was determined by photospectroscopy. First-strand cDNA was generated by reverse transcription of 1 mg total RNA, using p(dT)15 primers (Roche Diagnostics, Mannheim, Germany) with MMLV-reverse transcriptase, according to the instructions of the suppliers (Life Technologies, Karlsruhe, Germany). After determination of the concentrations of the cDNAs, the samples were used for PCR analysis. The integrity of the samples was tested by RT-PCR of the ribosomal housekeeping gene L7.

Semiquantitative PCR amplification. The cDNAs (2 ng) were amplified in a 50 μl reaction volume containing 10 mM Tris/HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM

Table I. Immunohistochemical detection of S100A4, CYR61 and GnRH receptor in human breast cancers, normal and other non-malignant breast tissues.

No.	Age	Path. diagnosis	Type	Grade	TNM	AR	ERα	PR	HER2	S100A4	CYR61	GnRH-R
1	50	Normal	Normal			+-++, 30%	+-++, 15%	-	-	-	-	+
2	43	Normal	Normal			+-++, 20%	+, 5%	++, 15%	++	-	-	+
3	50	Normal	Normal			+-++, 20%	+-++, 15%	+, 5%	-	-	-	+
4	30	Periductual mastitis	Hyperplasia			+-++, 20%	+-++, 5%	++, 5%	-	-	-	-
5	52	Hyperplasia	Hyperplasia			-	+-++, 10%	++, 10%	-	+	-	+
6	38	Hyperplasia	Hyperplasia			-	+-++, 5%	++, 5%	-	+	+	+
7	41	Hyperplasia	Hyperplasia			+, 20%	+, 15%	++, 20%	-	+	+	-
8	40	Fibrocystic changes	Hyperplasia			+-++, 20%	+-++, 15%	+-++, 20%	+	+	+	+
9	41	Fibrocystic changes	Hyperplasia			+, 20%	+-++, 15%	++-+++, 30%	+	++	+	+
10	39	Fibroadenoma	Benign			+, 5%	+-++, 5%	+-++, 10%	+	-	-	+
11	16	Fibroadenoma	Benign			+, 50%	++-+++, 80%	++-+++, 80%	+/-	-	-	+
12	34	Fibroadenoma	Benign			+-++, 30%	++-+++, 20%	++-+++, 20%	+	-	-	+
13	38	Phyllodes sarcoma	Sarcoma		TisN0M0	+-++, 10%	+-++, 10%	++-+++, 50%	+-++	+	-	-
14	52	Intraductal carcinoma	In Situ	I	TisN0M0	+/-	+++, 80%	-	++-+++	+	+	+
15	33	Intraductal carcinoma	In Situ	I	TisN0M0	+/-	++, 20%	++-+++, 50%	++-+++	+	+	+
16	37	Invasive ductal carcinoma (part. intraductal)	In Situ	I-II	T3N1M1	+-++, 20%	++-+++, 60%	++-+++, 50%	+	+	++	+
17	53	Ductal carcinoma *in situ*	In Situ	I	TisN0M0	+, 15%	-	-	+++	+	+	++
18	61	Ductal papillary adenocarcinoma	Malignant	I-II	T2N0M0	+, 15%	+-++, 20%	+-++, 10%	+	+++	++	++
19	41	Invasive ductal carcinoma	Malignant	II	T2N0M0	++, 5%	++, 5%	++-+++, 10%	+	++	++	+
20	55	Invasive ductal carcinoma	Malignant	III	T3N2M0	+-++, 5%	+, 50%	+-++, 5%	+	+++	++	+
21	44	Invasive ductal carcinoma	Malignant	II-III	T3N0M0	-	-	-	-	+++	+	++
22	72	Invasive ductal carcinoma	Malignant	II	T2N0M0	++, 20%	-	-	++-+++	++	+	++
23	41	Invasive ductal carcinoma	Malignant	II	T3N2M0	-	+, 5%	-	+++	++	+	++
24	18	Invasive ductal carcinoma	Malignant	II-III	T2N0M0	+/-	+-++, 5%	+-++, 5%	+	+	+	-
25	31	Invasive ductal carcinoma	Malignant	II-III	T2N0M0	+/-	-	-	+++	+++	+	++
26	54	Invasive ductal carcinoma	Malignant	II	T2N0M0	-	-	-	+++	++	+	-
27	75	Invasive ductal carcinoma	Malignant	III	T3N0M0	++, 30%	+, 10%	+-++, 10%	-	++	+	+
28	30	Invasive ductal carcinoma	Malignant	I-II	T3N0M0	-	-	+-++, 5%	-	+++	+	+
29	43	Invasive ductal carcinoma	Malignant	III	T3N0M0	-	-	-	-	++	+	++
30	37	Invasive ductal carcinoma	Malignant	III	T2N0M0	-	-	+-++, 5%	+++	++	-	+
31	42	Invasive ductal carcinoma	Malignant	II-III	T4N2MX	-	+, 50%	++-+++, 60%	+	++	++	-
32	30	Invasive ductal carcinoma	Malignant	II	T2N0M0	-	-	-	-	+++	+/-	++
33	39	Invasive ductal carcinoma	Malignant	I-II	T3N0M0	-	++-+++, 50%	+-++, 50%	+/-	++	+++	+
34	46	Invasive ductal carcinoma	Malignant	III	T2N0M0	+/-	+, 5%	-	+	++	+	-
35	40	Invasive ductal carcinoma	Malignant	II	T3N1M0	-	+, 10%	+-++, 10%	-	++	+	+
36	58	Invasive ductal carcinoma	Malignant	II	T3N0M0	-	-	-	++-+++	++	+	-

Table I. Continued.

No.	Age	Path. diagnosis	Type	Grade	TNM	AR	ERα	PR	HER2	S100A4	CYR61	GnRH-R
37	35	Invasive ductal carcinoma	Malignant	III	T2N0M0	++-+++, 60%	++-+++, 30%	+-++, 10%	+++	+++	+++	++
38	53	Invasive ductal carcinoma	Malignant	II-III	T2N0M0	++-+++, 10%	+-++, 10%	-	+++	++	++	-
39	50	Invasive ductal carcinoma	Malignant	II-III	T4N3M1	-	+, 5%	+, 5%	+	+++	+	+
40	48	Invasive ductal carcinoma	Malignant	II-III	T2N0M0	+, 5%	++-+++, 30%	++-+++, 50%	+/-	++	+++	+
41	51	Invasive ductal carcinoma	Malignant	II	T3N1M0	+/-	++, 50%	+-++, 15%	-	++	++	-
42	43	Invasive ductal carcinoma	Malignant	II-III	T4N2MX	-	-	-	+++	+++	+	-
43	43	Invasive ductal carcinoma	Malignant	II-III	T3N1M0	+/-	-	-	+-++	+++	+/-	++
44	40	Invasive ductal carcinoma	Malignant	III	T3N0M0	-	-	-	+++	+++	+	+
45	47	Invasive ductal carcinoma	Malignant	III	T3N1M0	-	-	-	++-+++	++	+	++
46	49	Invasive ductal carcinoma	Malignant	II-III	T3N1M0	+-++, 5%	++-+++, 20%	++-+++, 60%	+	+++	+++	-
47	58	Invasive mucinous adenocarcinoma	Malignant		T3N2M0	-	-	-	+-++	++	+/-	++
48	40	Invasive lobular carcinoma	Malignant	III	T2N0M0	+/-	++-+++, 50%	+-++, 15%	-	+	+++	+

Age of the patients, pathology diagnosis, tissue type, tumor grading, TNM classification as well as expression levels of androgen receptor (AR), estrogen receptor α (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) were supplied by the manufacturer. Expression of S100A4, CYR61 and GnRH receptor was analyzed in this study. No staining (-), borderline staining (+/-), weak staining (+), moderate staining (++), strong staining (+++), percentage of positive cells (%).

magnesium chloride, 200 mM of each of the dNTPs, 1 mM of the appropriate primers (S100A4 (239 bp): forward primer, 5'-TCT CTC CTC AGC GCT TCT TC-3'; backward primer, 5'-GCT GTC CAA GTT GCT CAT CA-3'; CYR61 (241 bp): forward primer, 5'-CTC CCT GTT TTT GGA ATG GA-3'; backward primer, 5'-TGG TCT TGC TGC ATT TCT TG-3'; L7 (357 bp), forward primer, 5'-AGA TGT ACA GAA CTG AAA TTC-3'; backward primer, 5'-ATT TAC CAA GAG ATC GAG CAA-3') and 1.25 U AmpliTaq Gold polymerase (Applied Biosystems, Weiterstadt, Germany) in an Applied Biosystems DNA thermal cycler 9600. Twenty-five (S100A4), 27 (CYR61) or 18 (L7) cycles of amplification representing the exponential phase of the PCR were carried out: Denaturation at 94˚C for 60 sec; annealing at 58˚C (S100A4), 55˚C (CYR61) or 54˚C (L7) for 60 sec; followed by extension at 72˚C for 60 sec. The PCR products were separated by gel electrophoresis in 1.5% agarose and visualized by ethidium bromide staining on a UV transilluminator. For correct densitometric analysis, the S100A4 or the CYR61 fragment and the L7 fragment were run on the same gel. For quantification the bands were analyzed using a Biometra BioDoc Analysis system (Biometra, Göttingen, Germany). Expression levels were calculated in comparison with the expression levels of the control (=100%). Expression levels of the housekeeping gene L7 were used for standardization.

Statistical analysis. All experiments were repeated at least three times with different passages of the respective cell lines. The data were tested for significant differences by un-paired two-tailed t-test or by one-way analysis of variance followed by Tukey's multiple comparisons test for comparison of individual groups, after a Bartlett test had shown that variances were homogeneous using GraphPad Prism 6.01 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Correlation of invasiveness and expression of S100A4 and CYR61. Invasion of MDA-MB-231 breast cancer cells measured by assessment of breast cancer cell migration rate through an artificial basement membrane in a modified Boyden chamber was 5-fold higher (588.8±63.2% vs. MCF-7; =100%; P<0.001) as compared with MCF-7 breast cancer cells (Fig. 1A). Expression of S100A4 mRNA in MDA-MB-231 breast cancer cells was 4.7-fold higher (470.0±120.9 vs. MCF-7; =100%; P<0.05) as compared with MCF-7 breast cancer cells (Fig. 1B). CYR61 mRNA expression in MDA-MB-231 breast cancer cells was 7.2-fold higher (719.5±169.7 vs. MCF-7; =100%; P<0.01) as compared with MCF-7 breast cancer cells (Fig. 1B).

Expression of S100A4 and effects of anti-S100A4 antibody treatment on invasion of MCF-7-EMT and MDA-MB-231 cells. Mesenchymal transformed MCF-7 cells (MCF-7-EMT) showing significantly increased invasion in contrast to wild-type MCF-7 (MCF-7 WT) cells were generated using prolonged mammosphere culture (34,35). Expression of S100A4 mRNA in MCF-7-EMT cells was significantly increased to 165.1±21.0% as compared with MCF-7 WT cells (=100%; P<0.05) (Fig. 2A).

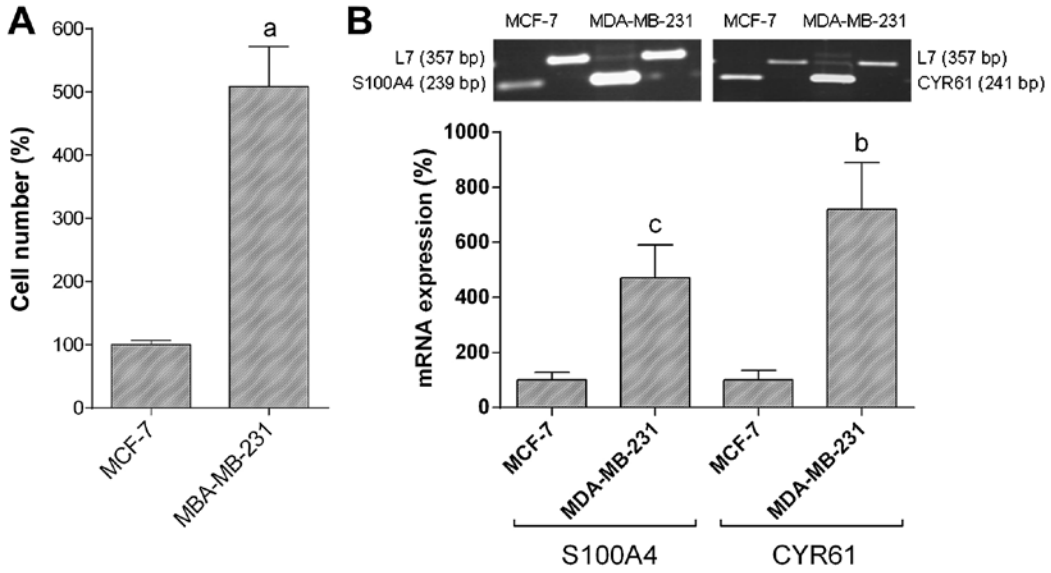

Figure 1. (A) Invasiveness of MCF-7 and MDA-MB-231 human breast cancer cells. Number of invaded MCF-7 and MDA-MB-231 breast cancer cells cultured in a modified Boyden chamber. Columns represent means ± SEM of data obtained from eight independent experiments in eight different passages of each cell line. a, P<0.001 vs. MDA-MB-231 (unpaired t-test, two-tailed). (B) Expression of S100A4 and CYR61 in MCF-7 and MDA-MB-231 human breast cancer cells. Quantification of S100A4 and CYR61 mRNA expression of MCF-7 and MDA-MB-231 human breast cancer cells. Expression levels of the housekeeping gene L7 were used for standardization. Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. b, P<0.01 vs. MDA-MB-231; c, P<0.05 vs. MDA-MB-231 (unpaired t-test, two-tailed).

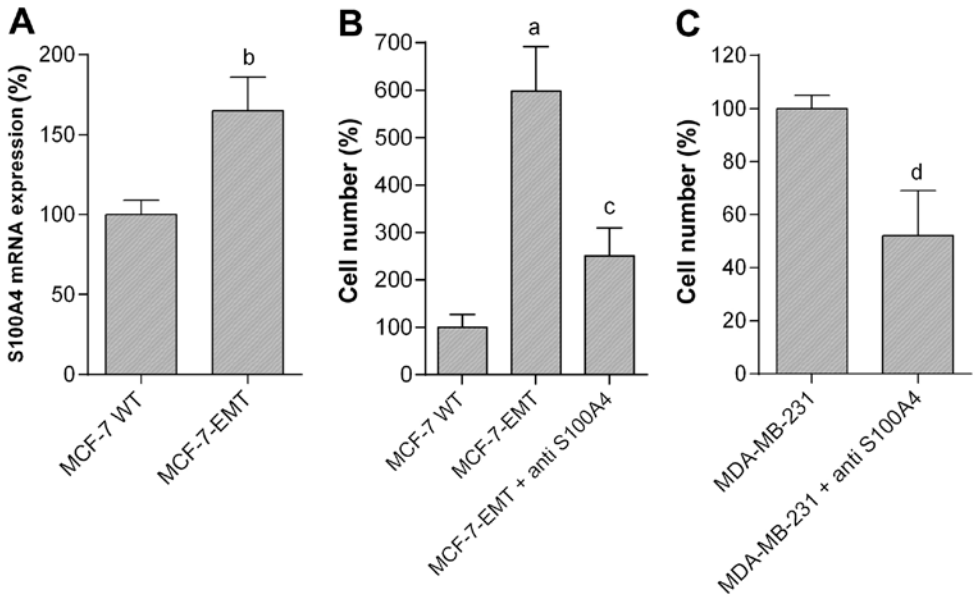

Figure 2. (A) Expression of S100A4 in MCF-7 WT and MCF-7-EMT human breast cancer cells. Quantification of S100A4 mRNA expression of wild-type MCF-7 (MCF-7 WT) and mesenchymal transformed MCF-7-EMT cells. Expression levels of the housekeeping gene L7 were used for standardization. Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. b, P<0.05 vs. MCF-7 WT (unpaired t-test, two-tailed). (B and C) Effects of anti-S100A4 antibody treatment on invasion of MCF-7-EMT and MDA-MB-231 cells. (B) Number of invaded MCF-7 WT and MCF-7-EMT cells cultured in a modified Boyden chamber without or with 96 h of treatment with anti-S100A4 antibody (15 μg/ml). (C) Number of invaded MDA-MB-231 cells cultured in a modified Boyden chamber without or with 96 h of treatment with anti-S100A4 antibody (15 μg/ml). Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. a, P<0.001 vs. MCF-7 WT; c, P<0.01 vs. MCF-7-EMT (ANOVA followed by Tukey's multiple comparisons test); d, P<0.05 vs. untreated MDA-MB-231 (unpaired t-test, two-tailed).

To emphasize the importance of S100A4 we observed the invasiveness of MCF-7-EMT and MDA-MB-231 breast cancer cells after treatment with anti-human S100A4 antibody (Fig. 2B and C). MCF-7-EMT cells cultured in a modified Boyden chamber showed significant increased invasion in contrast to the wild-type cells (Fig. 2B). Invasion of MCF-7-EMT cells was 6-fold higher (598.3±94.5% vs. MCF-7 WT; P<0.001) in comparison to MCF-7 WT cells. Treatment with S100A4 antibody led to a significant decrease of invaded MCF-7-EMT cells to 251.1±59.2% of control (P<0.01 vs. MCF-7-EMT) (Fig. 2B). The naturally high invasiveness of MDA-MB-231 cells was reduced to 52.4±17.2% of control

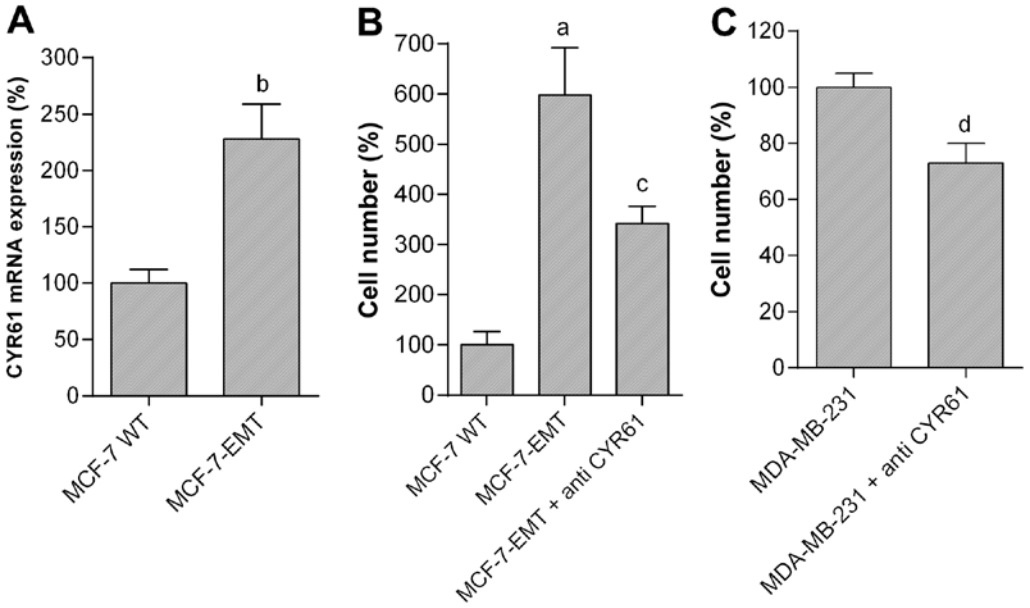

Figure 3. (A) Expression of CYR61 in MCF-7 WT and MCF-7-EMT human breast cancer cells. Quantification of CYR61 mRNA expression of wild-type MCF-7 (MCF-7 WT) and mesenchymal transformed MCF-7-EMT cells. Expression levels of the housekeeping gene L7 were used for standardization. Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. b, P<0.05 vs. MCF-7 WT (unpaired t-test, two-tailed). (B and C) Effects of anti-CYR61 antibody treatment on invasion of MCF-7-EMT and MDA-MB-231 cells. (B) Number of invaded MCF-7 WT and MCF-7-EMT cells cultured in a modified Boyden chamber without or with 96 h of treatment with anti-CYR61 antibody (15 μg/ml). (C) Number of invaded MDA-MB-231 cells cultured in a modified Boyden chamber without or with 96 h of treatment with anti-CYR61 antibody (15 μg/ml). Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. a, P<0.001 vs. MCF-7 WT; c, P<0.01 vs. MCF-7-EMT (ANOVA followed by Tukey's multiple comparisons test); d, P<0.05 vs. untreated MDA-MB-231 (unpaired t-test, two-tailed).

(=100%; P<0.001) after treatment with S100A4 antibody (Fig. 2C). Controls using a non-specific isotype control antibody showed no effects (not shown).

Expression of CYR61 and effects of anti-CYR61 antibody treatment on invasion of MCF-7-EMT and MDA-MB-231 cells. Expression of CYR61 mRNA in MCF-7-EMT cells was significantly increased to 228.2±30.9% as compared with MCF-7 WT cells (=100%; P<0.01) (Fig. 3A). To analyze the role of CYR61 we observed the invasiveness of MCF-7-EMT and MDA-MB-231 breast cancer cells after treatment with anti-human CYR61 antibody (Fig. 3B and C). The 6-fold increased invasion of MCF-7-EMT cells in comparison to MCF-7 WT cells (598.3±94.5% vs. MCF-7 WT; P<0.001) was significantly decreased to 341.4±35.3% of control (P<0.05 vs. MCF-7-EMT) after treatment with CYR61 antibody (Fig. 3B). The naturally high invasiveness of MDA-MB-231 cells was reduced to 73.1±7.0% of control (=100%; P<0.05) after treatment with CYR61 antibody (Fig. 3C). Controls using a non-specific isotype control antibody showed no effects (not shown).

Effects of GnRH agonist treatment on invasion and expression of S100A4 and CYR61. In the GnRH receptor-positive breast cancer cell lines MCF-7-EMT (Fig. 4A) and MDA-MB-231 (Fig. 4D), the number of invaded cells was dose-dependently reduced by 5 days of treatment with increasing concentrations (10^{-11}-10^{-5} M) of GnRH agonist Triptorelin.

At 10^{-11} M Triptorelin concentration a slight decrease in number of invaded MCF-7-EMT cells to 73.2±9.1% of control (=100%; not significant) was observed (Fig. 4A). At 10^{-9} M concentration of Triptorelin, the reduction in cell number was significant (55.8±10.9% of control; 100%; P<0.05). At 10^{-7} M Triptorelin concentration a significant decrease in number of invaded MCF-7-EMT cells to 42.0±7.9% of control (100%; P<0.01) was observed. Triptorelin at 10^{-5} M had almost the same inhibitory effects on cell invasion (38.7±12.5% of control (100%; P<0.01).

At 10^{-11} M Triptorelin concentration the number of invaded MDA-MB-231 cells was slightly decreased to 75.1±16.8% of control (100%; not significant) (Fig. 4D). At 10^{-9} M concentration of Triptorelin the number of invaded MDA-MB-231 cells was reduced to 67.2±5.4% of control (100%; not significant). The inhibitory effects were maximal at 10^{-7} M concentration of Triptorelin and corresponded to 53.1±5.8% of control (100%; P<0.05). Triptorelin at 10^{-5} M showed comparable inhibitory effects on cell invasion (58.9±10.7% of control (100%; P<0.05).

To analyze whether GnRH plays a role in S100A4 and CYR61 function, MCF-7-EMT (Fig. 4B and C) and MDA-MB-231 (Fig. 4E and F) cells were treated with GnRH agonist Triptorelin and mRNA expression of S100A4 and CYR61 was measured. After 48 h of treatment with Triptorelin (10^{-7} M) S100A4 mRNA expression in MCF-7-EMT cells was significantly reduced to 53.2±14.7% of control (100%; P<0.05) (Fig. 4B). Expression of CYR61 mRNA in MCF-7-EMT cells was significantly decreased to 33.1±10.8% of control (100%; P<0.01) (Fig. 4C). Treatment of MDA-MB-231 cells with 10^{-7} M Triptorelin for 48 h resulted in a significant decrease of S100A4 mRNA expression to 68.7±7.1% of control (100%; p<0.05) (Fig. 4E), whereas CYR61 mRNA was significantly reduced to 45.0±11.9% of control (100%; P<0.01) (Fig. 4F).

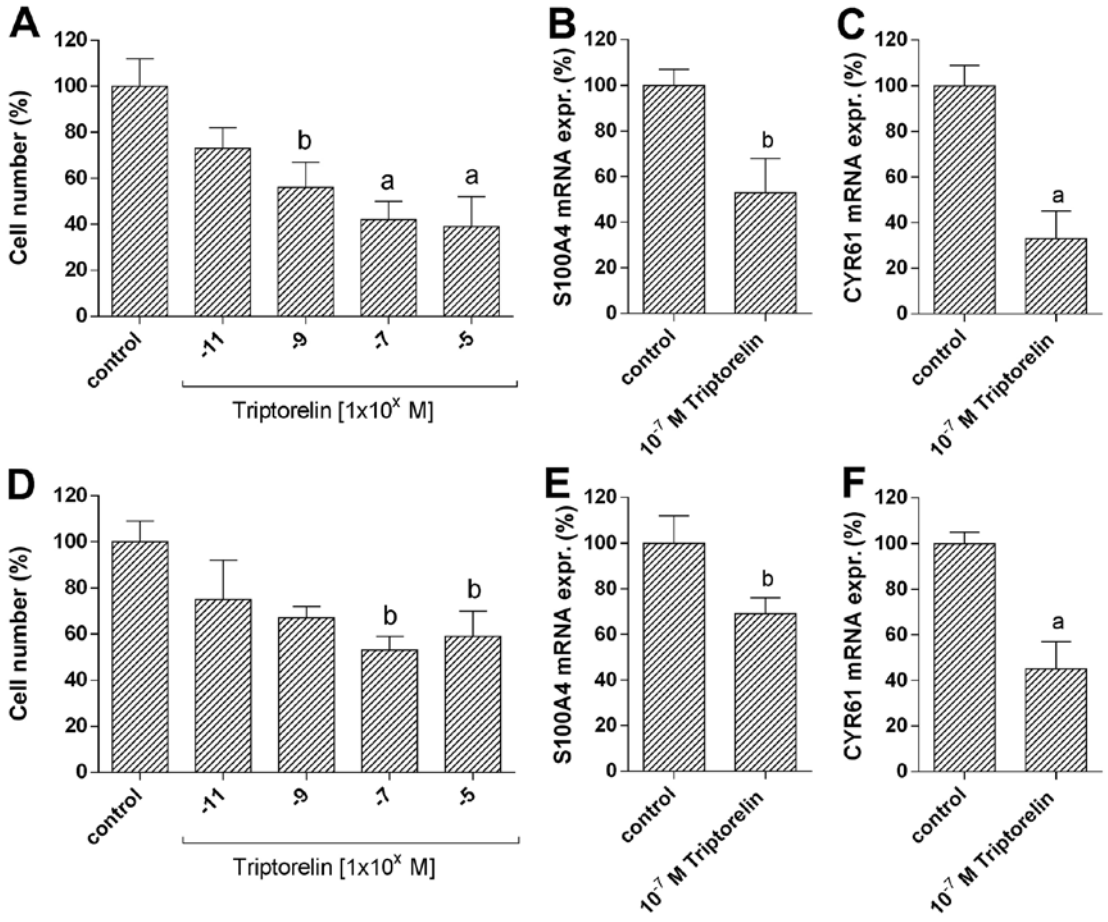

Figure 4. Effects of GnRH agonist Triptorelin treatment on invasion and expression of S100A4 and CYR61. Number of invaded MCF-7-EMT (A) and MDA-MB-231 (D) cells cultured in a modified boyden chamber after 5 days of treatment with increasing concentrations of GnRH agonist Triptorelin (10^{-11}-10^{-5} M). Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. a, P<0.01 vs. control; b, P<0.05 vs. control (ANOVA followed by Tukey's multiple comparisons test). Quantification of S100A4 mRNA expression of MCF-7-EMT (B) and MDA-MB-231 (E) cells after 48 h of treatment without or with GnRH agonist Triptorelin (10^{-7} M). Quantification of CYR61 mRNA expression of MCF-7-EMT (C) and MDA-MB-231 (F) cells after 48 h of treatment without or with GnRH agonist Triptorelin (10^{-7} M). Expression levels of the housekeeping gene L7 were used for standardization. Columns represent means ± SEM of data obtained from six independent experiments in six different passages of the cell lines. a, P<0.01 vs. control; b, P<0.05 vs. control (unpaired t-test, two-tailed).

Immunohistochemical detection of S100A4, CYR61 and GnRH receptor in human breast cancers, normal and other non-malignant breast tissues. Following data were given by the supplier of the tissue arrays (materials and methods section): Age of the patients, pathology diagnosis, tissue type, tumor grading, and TNM classification (Table I). Expression levels of androgen receptor (AR), estrogen receptor α (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) were known (Table I).

In biopsy specimens of breast hyperplasia and malignant breast cancers S100A4 and CYR61 were found highly expressed (Table I). Carcinoma *in situ* showed lower expression of S100A4 and CYR61, whereas normal breast tissues and benign fibroadenoma were S100A4 and CYR61 negative. In most cases, high CYR61 expression, but not high S100A4 expression was correlated with high ERα expression. Benign fibroadenoma with high ERα expression remained CYR61 negative. Correlations to expression of AR, PR and HER2 were not found. GnRH receptor expression was detectable in 4 of 6 cases of benign fibroadenoma (67%), in 3 of 3 cases of benign fibroadenoma (100%), in 4 of 4 cases of carcinoma *in situ* (100%), and in 22 of 31 cases of malignant breast cancers (71%).

Discussion

We have established a coculture model to mimic the *in vivo* invasion process and to analyze EMT (31,36,37). Using this model we can induce non invading MCF-7 breast cancer cells to substantial invasion and thus to markedly increase the number of cells undergoing EMT (31,34). By prolonged mammosphere culture we have generated a mesenchymal transformed MCF-7 cell line (MCF-7-EMT), which in contrast to wild-type MCF-7 cells (MCF-7 WT) exhibit significantly increased invasive behavior *in vitro* and *in vivo* as well as increased expression of EMT-related genes (34).

S100A4 and CYR61 play important roles in EMT, invasion and metastasis by promoting cancer cell motility (6,11,38,39). We found both genes highly expressed in high invasive MDA-MB-231 breast cancer cells. Jiang *et al* demonstrated a significant increase of CYR61 expression in breast cancers in comparison to normal breast tissue (40). This increase is correlated with poor prognosis, lymph node status as well as metastatic propagation (40,41). Jenkinson *et al* showed a clear influence of S100A4 on invasiveness of breast cancer cells (42). Invasion of breast cancer cells with S100A4 overexpression

was strongly increased as compared with the non-transfected controls.

MCF-7 cells have no invasive behavior. In addition, MCF-7 cells show very low expression of S100A4 and CYR61. After mesenchymal transition, invasion and expression of S100A4 and CYR61 of MCF-7 cells were found to be markedly increased. The increased invasion of MCF-7-EMT cells was reduced by anti-S100A4 and anti-CYR61 antibodies. In addition, invasion of naturally high invasive MDA-MB-231 cells was decreased by anti-S100A4 and anti-CYR61 antibodies showing the important role of these factors. Nguyen *et al* have attributed the migration-promoting effect of CYR61 to MMP-1 expression (43). In a fibroblast-directed migration assay, loss of CYR61 in breast cancer cells led to inhibition of MMP-1. In the same assay, absence of MMP-1 activity in the fibroblasts inhibited CYR61-permitted migration of the breast cancer cells (43).

Previously we showed that *in vitro* invasion and *in vivo* metastasis of GnRH receptor-positive breast cancer cells is time- and dose-dependently reduced by GnRH analogs (31,32). Now we have analyzed whether GnRH treatment affects increased invasion and expression of S100A4 and CYR61 in mesenchymal transformed breast cancer cells. Treatment of mesenchymal transformed MCF-7-EMT and naturally high invasive MDA-MB-231 cells with GnRH agonist Triptorelin resulted in a significant decrease of invasion and expression of S100A4 and CYR61. Lin *et al* reported that neutralizing of CYR61 using an anti-CYR61 antibody resulted in inhibition of breast cancer growth and metastasis *in vivo* (44). We used a non-toxic and easy to apply GnRH agonist to reduce CYR61 and S100A4 expression. In addition, our earlier studies showed anti-metastatic activities of GnRH agonists without undesirable side effects (31,32). However, overexpression of CYR61 in endometriosis could not be reduced after therapy with GnRH agonist Leuprorelin (45).

To further show the clinical significance of S100A4 and CYR6, we have analyzed their expression in biopsy specimens. Both, S100A4 and CYR61 were found highly expressed in biopsy specimens of malignant breast cancers, whereas their expression in carcinoma *in situ* was much lower. Normal breast tissues and benign fibroadenoma were S100A4 and CYR61 negative. Noteworthy, we found in breast hyperplasia a high expression as well, although its biological background and relevance remains uncertain. High CYR61 expression but not high S100A4 expression seems to be associated with high estrogen receptor α (ERα) expression. It is known that expression of CYR61 is induced by estrogen (18). Correlation of high CYR61 expression with high ERα expression was found in many studies (18,46-49). However, benign fibroadenoma with high ERα expression remained CYR61 negative, indicating that additional mechanisms regulate CYR61 expression.

To use GnRH agonists for treatment of invasive breast cancer it is essential that these tumors express GnRH receptors. We found GnRH receptor expression in approximately 71% of malignant breast cancers (n=31). In a recent study we were able to demonstrate GnRH receptor expression in approximately 74% of triple-negative breast cancer specimens (n=42) (30). Another study found GnRH receptor expression in all analyzed triple-negative breast cancers (n=16) (50).

In conclusion, our findings suggest that S100A4 and CYR61 play major roles in breast cancer invasion. Treatment with GnRH agonist Triptorelin decreases invasion and expression of S100A4 and CYR61. The precise mechanisms remain unclear and are part of our current research. The use of GnRH agonists or similar S100A4 and CYR61 blocking treatments might represent novel anti-metastatic therapeutic approaches and should be further explored.

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