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Effect of the tyrosine kinase inhibitor lapatinib on CUB-domain containing protein (CDCP1)-mediated breast cancer cell survival and migration

Jeanette Seidel, Klaudia Kunc, Kurt Possinger, Christian Jehn, Diana Lüftner *

Charité Campus Mitte, Medizinische Klinik mit Schwerpunkt Onkologie und Hämatologie, Humboldt Universität zu Berlin, Germany

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ABSTRACT

The surface receptor CUB domain-containing protein 1 (CDCP1) is highly expressed in several adenocarcinomas and speculated to participate in anchorage-independent cell survival and cell motility. Tyrosine kinase phosphorylation seems to be crucial for intracellular signaling of CDCP1. Lapatinib, a tyrosine kinase inhibitor (TKI), is approved for treatment of HER-2/neu overexpressing metastatic breast cancer and functions by preventing autophosphorylation following HER-2/neu receptor activation. This study aimed to investigate the effect of CDCP1 expression on anchorage-independent growth and cell motility of breast cancer cells. Moreover, studies were performed to examine if lapatinib provided any beneficial effect on HER-2/neu$^{(+)/-}$/CDCP1^{+} breast cancer cell lines. In our studies, we affirmed that CDCP1 prevents cells from undergoing apoptosis when cultured in the absence of cell–substratum anchorage and that migratory and invasive properties of these cells were decreased when CDCP1 was down-regulated. However, only HER-2/neu^{+}, but not HER-2/neu$^{(+)/-}$ cells showed decreased proliferation and invasion and an enhanced level of apoptosis towards loss of anchorage when treated with lapatinib. Therefore, we conclude that CDCP1 might be involved in regulating adhesion and motility of breast cancer cells but that lapatinib has no effect on tyrosine kinases regulating CDCP1. Nonetheless, other TKIs might offer therapeutic approaches for CDCP1-targeted breast cancer therapy and should be studied considering this aspect.

1. Introduction

During the last decades, tremendous efforts have gone into identifying new surface marker proteins in order to distinguish between malignant and healthy tissues, with the hope that they will serve as potential therapeutic targets. CUB domain containing protein 1 (CDCP1), a type I transmembrane protein, may represent such a target. It is overexpressed by various tumors of epithelial origin while its expression in corresponding healthy tissues is only moderate to weak [1–3]. It is absent on tissues of mesenchymal origin and normal peripheral blood populations [2,4] with the exception of hematopoietic or neuronal progenitors [4,5]. Its impact as a prognostic marker has been investigated in studies. The level of expression in human tumor specimens correlated with clinical prognosis and overall survival in colorectal, lung, endometrial and pancreatic adenocarcinomas [4,6–9]. Interestingly, CDCP1 shows no similarity to any other known protein family and no potential extracellular ligand has been identified. Thus, its function and intracellular signaling remains elusive. Some findings indicate that CDCP1 might be involved in processes of adhesion and/or deadhesion [10–12] while tyrosine phosphorylation might be crucial for outside-in-signaling of CDCP1 [12]. Some of its five conserved tyrosine residues at the cytoplasmatic tail were shown to be phosphorylated by src-family kinases (SFKs) [2,13] including Src, Yes or Fyn [10,14,15]. These findings are consistent with the observation that CDCP1-phosphorylation is tightly regulated in normal epithelial tissues, but not in a variety of adenocarcinomas [12]. Among other proteins, PKC delta seems to be a target protein of tyrosine phosphorylation via CDCP1 [8,14,16].

Due its prognostic value in distinct types of tumors and its potential participation in crucial steps of cancer progression such as anchorage independency, migration and cell invasiveness, our studies aimed to investigate the influence of CDCP1 protein expression in breast cancer, one of the most common malignancies in women. Since CDCP1 overexpression has been speculated to promote cell migration and to modulate invasive capacities of distinct adenocarcinomas [3,8,15], we conducted *in vitro* studies to determine the influence of CDCP1 expression on the migratory and invasive properties of breast cancer cell lines.

Abbreviations: CDCP1, CUB domain containing protein 1; SFK, Src family kinase; TKI, tyrosine kinase inhibitor; ECM, extracellular matrix; siRNA, small interfering RNA; FACS, fluorescence activated cell sorting.

* Corresponding author. Address: Medizinische Klinik und Poliklinik m. S. Onkologie & Hämatologie, Charite Campus Mitte, Chariteplatz 1, 10117 Berlin, Germany. Fax: +49 30 450 513919.

E-mail address: diana.lueftner@charite.de (D. Lüftner).

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Great achievements have been made in breast cancer therapy since the description of the growth factor receptor HER-2/neu which is overexpressed in about 20% of breast cancers. Monoclonal anti HER-2/neu antibodies (e.g. trastuzumab) and tyrosine kinase inhibitors (TKIs, e.g. lapatinib) which decrease intracellular phosphorylation of dimerized HER-2/neu receptors are used in clinical routine. Lapatinib was first approved as a second-line agent in combination with capecitabine in HER-2/neu overexpressing breast cancer patients [17–19]. Since CDCP1 signaling is speculated as being regulated via tyrosine kinase phosphorylation, the presented study investigated the proliferation and motility of HER-2/neu$^{+/-}$/CDCP1^{+} breast cancer cell lines in the presence of lapatinib. An inhibitory effect of lapatinib on the proliferative and migratory abilities of HER-2/neu-negative but CDCP1-positive tumor cells might then probably offer advantages in the individualized therapy of CDCP1-positive breast cancer.

2. Materials and methods

2.1. Chemicals

Culture media, fetal bovine serum (FBS), and antibiotics were obtained from GIBCO/PAA, Marburg, Germany. For FACS analysis, FITC-conjugated anit-CDCP1 antibody (MBL, Naka-ku Nagoya, Japan) and corresponding istotype control (BD Biosciences, Erembodegem, Belgium) were used. Anti-CDCP1-, anti-ß-actin- and corresponding secondary antibodies for western blotting were purchased from Cell Signaling Technology®, Massachusetts, USA. Ultra-low adhesion culture plates (HydroCell™; Nunc) and protein extraction reagent M-Per® were purchased from Thermo Fisher Scientific, Langenselbold, Germany. Protein concentration was determined using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Munich, Germany). Anchorage independency and cell proliferation were determined using 'Cell Death Detection ELISA Plus' and 'Cell Proliferation Reagent WST-1', respectively (Roche Diagnostics GmbH, Mannheim, Germany). 'ECL Plus Advanced Western Blotting Detection System' was a product of Amersham Biosciences (GE Healthcare Life Sciences, Munich, Germany). Unless otherwise stated, all basic chemicals, protease inhibitor cocktail, Benzonase and Boyden-chamber assays to investigate migration and invasion (InnoCyte™, Calbiochem) were products of MerckBiosciences (Schwalbach, Germany). All oligonucleotides for siRNA-mediated knockdown (Stealth™-siRNA) including control nonsense oligonucleotides and all products for western blotting including markers that are not separately listed above were purchased from Invitrogen Life Technologies (Karlsruhe, Germany). Transfection was performed using an AMAXA™ Nucleofector and appropriate solutions (Lonza, Verviers, Belgium). Lapatinib was purchased from AKScientific, California, USA.

2.2. Cell culture and transfection

Human breast cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). CDCP1-expression was screened via FACS analysis (data not shown). The mentioned Her-2/neu status of the cell lines originate from the MD Anderson Breast Cancer Cell Line Database, was confirmed by Western blotting. For the described experiments, the cell lines MDA-MB-231 (CDCP1^{+}/HER-2/neu^{-}), T47D (CDP1^{+}/HER-2/neu$^{(+)}$), SKBR3 (CDCP1$^{(+)}$/HER-2/neu^{+}) and MCF-7 (CDCP1^{-}/HER-2/neu$^{(+)}$) were used ($^{-}$/$^{(+)}$/$^{+}$: no/low to moderate (10^1–10^2)/moderate to high ($\geqslant 10^2$) fluorescence intensity in FACS-analysis). Cells were cultivated using Dulbecco's Modified Eagle Medium (DMEM) or RPMI, respectively, containing 10% FBS and 1% penicillin/streptomycin, in a humidified incubator at 37 °C with 5% CO_2. Culture medium was changed three times a week and cells were passaged using 2 mM EDTA/HBSS.

For transfection, 1×10^6 subconfluent cells were harvested, washed and resuspended in 500 μl of appropriate transfection solution (AMAXA™, Lonza). Two sets of CDCP1 stealth™-siRNA (Invtirogen) were mixed (1:1) and added to the suspension in a final concentration of 3 μM (CDCP1/HSS185608: CACGAGAAAG CAACAUUACAGUUCU; CDCP1/HSS185609: UAUAGAUGAGCGGUU UGCAAUGCUG). Cells were transfected via nucleofection (AMAXA™, Lonza) according to the manufacturer's instructions. Subsequently, cells were cultured in medium containing 10% FBS without antibiotics for 24 h in a 37 °C/5% CO_2 tissue incubator. *Knockdown* was evaluated using a FITC-conjugated anti-CDCP1 antibody via FACS measurement.

2.3. Western-blot analysis

Western-blot analyses were performed according to standard conditions. Briefly, 1×10^6 cells were solubilized using lysis buffer containing proteinase inhibitor cocktail (1:100) and Benzonase (1:1000). Protein (25 μg) were used for SDS–polyacrylamid-gel separation. Nitrocellulose membrane was probed using appropriate primary and secondary antibodies and enhanced chemiluminescence visualization (Fluor-S-MultiImager; Bio-Rad). QuantityOne software (Bio-Rad) was used for analysis. Membranes were stripped in stripping buffer (AppliChem) (45 min at room temperature; gently shaking) and then reprobed with b-actin as a loading control. Western blots were performed at least three times for each experiment.

2.4. Cell proliferation

Twenty-four hours after transfection, 3×10^4 mock-transfected or CDCP1-siRNA transfected cells, respectively, were seeded at least in triplicate into a normal or ultra-low adhesion 96-well culture plate (HydroCell™) and incubated for 48 h in medium containing 5% FBS. Proliferation was determined using WST-1 according to manufacturer's instructions via ELISA reader (model 200, Bio-Rad) Data were normalized to untreated, untransfected cells.

To test the influence of lapatinib on proliferation 0.5 μM or 1 μM lapatinib or 0.1% DMSO (negative control) was added. Proliferation was determined after 48 h of incubation.

2.5. Apoptosis assay

Cells (1×10^4) were seeded at least in triplicate into normal or HydroCell™ culture plates, respectively. To determine the level of apoptosis, the presence of free nucleosomes was measured using the 'Cell Death Detection ELISA Kit Plus' (Roche) according to the manufacturer's instructions. After 48 h of cultivation, the measured value of mock-transfected cells of each cell line cultured as adhesion culture was adjusted to 1 and the ratio of apoptosis in adherent and suspension cultures was calculated. To investigate the influence of lapatinib, cells were incubated in presence of 0.75 μM lapatinib for 48 h. For analysis, the level of apoptosis in mock-transfected cells cultured in adhesion cultures was adjusted to 1 to calculate relative levels of apoptosis.

2.6. Migration and invasion assays

In vitro cell migration was determined using the InnoCyte™ Cell Migration Kit (Merck Biosciences) according to the manufacturer's instruction. Briefly, 24 h after transfection, 1.5×10^4 CDCP1-siRNA-transfected or mock-transfected cells, diluted in serum-free medium, were seeded into a chamber that was placed upon a reservoir

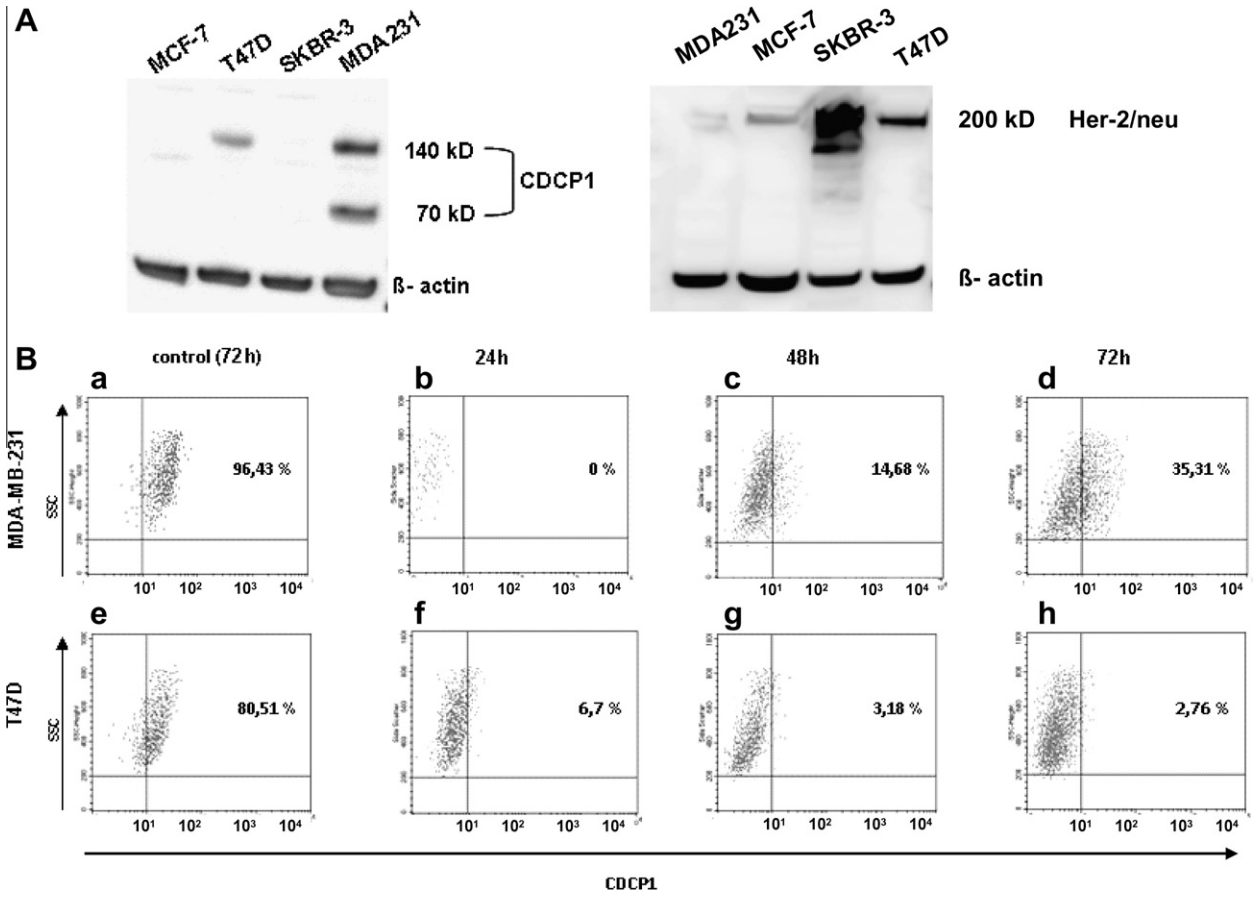

Fig. 1. (A) Western Blot analysis of parental cell lines. Detection of CDCP1 and Her-2/neu protein of investigated cell lines. Anti-CDCP1 antibody detects both, the 140 kDa and the 70 kDa form of CDCP1 protein. (B) FACS analysis of siRNA-transfected cell lines at various time points after transfection. Data show one representative of two independently performed experiments with similar results. (a) + (e) mock-transfected cells 72 h after reseeding to a microtiter plate. Cells at 24 h (b) + (f), 48 h (c) + (g) and 72 h (d) + (h) after transfection with CDCP1-siRNA.

filled with medium containing 10% FBS as a chemo-attractant and incubated for 24 h. Subsequently, migrated cells were dislodged from the underside of the upper chamber and stained with Calcein-AM (1:300) (37 °C, 30 min). After removing the insert, cells were measured in duplicate in a fluorescence ELISA-reader (Tecan Infinite 200 PRO; software: Magellan™ V 6.3) (excitation wavelength: 485 nm/ emission wavelength: 520 nm). In invasion assays, the upper chamber was coated with basement membrane matrix (BMM) solution (InnoCyte™ Invasion Assay). In principle, the assay was performed similarly to migration assays, but a total of 1.5×10^5 cells were seeded into the upper chamber. When invasion assays were performed in presence of the SFK inhibitor lapatinib, 2 μM lapatinib was used in both, the upper and lower chamber of the assay. Prior to application in any assay, cell viability was determined by trypan blue staining. All experiments were performed independently at least three times.

2.7. Statistical analysis

Unless otherwise stated, data are expressed as mean ± SD. Where applicable, the results were compared by using the unpaired, two-tailed Students *t*-test taking $p \leqslant 0.05$ as the level of significance.

3. Results

The ability to grow independently of contact to surrounding cells or components of the extracellular matrix is a common feature of cells with a highly malignant phenotype. Moreover, anchorage independency is a crucial prerequisite for the ability of tumor cells to migrate and invade distinct tissues or blood vessels. Therefore, we started investigating whether a decreased CDCP1 expression influences the survival of breast cancer cell lines cultured in suspension. Transient CDCP1 knockdown was achieved via transfection of CDCP1-siRNA, and FACS analysis identified a time span between 24 and 72 h after transfection that guaranteed a sufficient knock-down (>70% CDCP1-negative cells) for further studies (Fig. 1B). The anti-CDCP1-antibody used detects both, the 140 kDa and the cleaved 70 kDa isoform of the protein. MCF-7, a cell line without detectable CDCP1 expression in Western blotting and FACS, served as a negative control (Fig. 1A).

3.1. Reduced CDCP1 expression does not affect cell proliferation in vitro, whereas lapatinib only affects proliferation in HER-2/neu$^+$ cell lines

To investigate if CDCP1 influences the proliferation of breast cancer patient derived cell lines, mock-transfected and CDCP1-siRNA-transfected cells were cultured in the presence of 0.5 μM or 1 μM lapatinib for 2 or 4 days, respectively. CDCP1$^+$/HER-2/neu$^-$ MDA231 cells are rarely affected by lapatinib treatment, either when mock-transfected or after CDCP1-downregulation (Fig. 2). Interestingly, T47D cells (CDCP$^+$/HER-2/neu$^{(+)}$) show a variable behavior when being treated with lapatinib. With regard to the T47D cell line, lapatinib seemed to significantly increase proliferation when CDCP1 was still expressed ($p \leqslant 0.05$) (Fig. 2, left). In con-

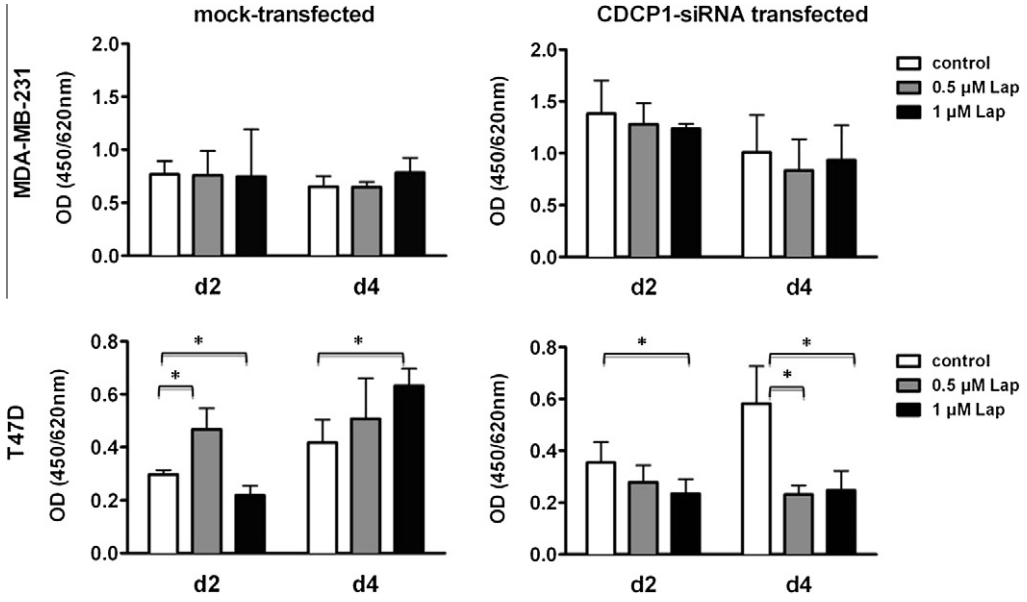

Fig. 2. Cell proliferation after treatment with lapatinib. Cells (3×10^4) were cultivated in the presence of 0.5 μM or 1 μM lapatinib (or 0.1% DMSO as a negative control) for 2 or 4 days and proliferation was determined using WST-1. Bars represent means and SD of 3 independent replicates (*indicates significant differences ($p \leqslant 0.05$)).

trast, CDCP1 downregulation made the cells more sensitive towards lapatinib, and proliferation was significantly decreased ($p \leqslant 0.05$). However, a time or dose dependent relation to lapatinib was not observed in the proliferation behavior of both, MDA231 and T47D cell lines.

3.2. CDCP1 is involved in anchorage independent growth

The membrane protein CDCP1 is suspected to participate in mechanisms mediating anchorage independency in adenocarcinomas of lung [15]. Moreover, CDCP1 was shown to be phosphorylated during mitosis-mediated detachment of breast cancer cells from tissue culture plates [11]. Thus we studied its impact in cell survival of breast cancer cells in absence of cell–matrix interactions using ultra-low adhesion culture plates that prevent any adhesion of the cultured cells.

All cell lines tested, whether CDCP1-downregulated or not, showed an increased level of apoptosis when cultured in suspension, but the sensitivity of CDCP1-siRNA transfected cells was slightly increased compared to mock-transfected cells. However, only CDCP1-siRNA transfected T47D cells showed a significantly increased level of apoptosis (5.8 ± 1.9) after being cultured in suspension compared to the mock-transfected cells (2.9 ± 1) ($p \leqslant 0.05$). It is noteworthy that the CDCP1-negative MCF-7 cells revealed a very low level of apoptosis (1.2 ± 0.6) although no expression of CDCP1 was detectable in Western blotting (Fig. 1A). Interestingly, MCF-7 cells formed grape-like clusters (mammospheres) of $\geqslant$20 cells when cultured in suspension. Both, mechanical and trypsin-mediated separation failed to disrupt these clusters (data not shown). In contrast, MDA231 cells and SKBR3 cells remained as single cells when being cultured in suspension, and T47D cells formed small clusters of only a few ($\leqslant$10) cells.

When lapatinib (0.75 μM) was added to cell culture, HER-2/neu$^-$ MDA231 cells were only slightly affected, even when being CDCP1-downregulated (Fig. 3B, left). On the other hand, SKBR3 cells, which highly express HER-2/neu but only weakly express CDCP1, show a significantly increased level of apoptosis either when being cultured in suspension or as adhesion culture (Fig. 3B, right). In contrast, T47D cells, expressing both, the HER-2/neu receptor and CDCP1, seemed to have an increased effect in the presence of lapatinib and showed a significantly reduced level of apoptosis compared to untreated cells (Fig. 3B, middle), especially when CDCP1-downregulated.

3.3. Downregulation of CDCP1 results in decreased migratory and invasive properties in highly metastatic CDCP1-positive breast cancer cell lines and lapatinib reveals a similar effect on in vitro-invasion

Aside from anchorage independent growth, tumor cell migration is an important prerequisite of a malignant phenotype. To prove whether CDCP1 is functionally involved in breast cancer cell migration, we investigated the migration of CDCP1-siRNA-transfected and mock-transfected cells over 24 h in a boyden chamber assay using serum as chemo-attractant. Latrunculin (LA), an inhibitor of actin polymerization as well as medium without serum served as negative controls. The migratory activity of mock-transfected cells was normalized to 100% for further analysis. After *knockdown* of CDCP1, the percentage of MDA231 cells migrating through the membrane was reduced by 30% (70.2 ± 21.5% migrating cells). In T47D transfected cells, the rate of migrating cells was significantly reduced to about half (52 ± 16.1%) of that of mock-transfected cells ($p \leqslant 0.05$). However, the CDCP1$^{(+)}$ cell line SKBR3 was only slightly affected by CDCP1-*knockdown* (96.2 ± 6.8% migrating cells). Lapatinib (2 μM), however, reduces the migratory activity of both, mock-transfected and CDCP1-siRNA-transfected MDA231 and T47D to the level of migration of untreated CDCP1-downregulated cells (Fig. 4A).

To simulate cell invasion under *in vitro* conditions, we used an upper chamber coated with extracellular matrix (ECM) proteins to mimic a native tissue barrier. Crossing this porous membrane requires cleavage of ECM proteins by cellular membrane proteins such as e.g. matrix metalloproteinases. *Knockdown* of CDCP1-expression completely blocked cell invasiveness of T47D cells (0.5% invasive cells compared to mock-transfected cells ($p \leqslant 0.01$)) and diminished the invasive potential of MDA231 cells to only 60% compared to mock-transfected control cells (Fig. 4B, a). No significant difference was observed between mock-transfected and siRNA-transfected MDA231 and T47D cells after treatment with 2 μM lapatinib (Fig. 4B, b).

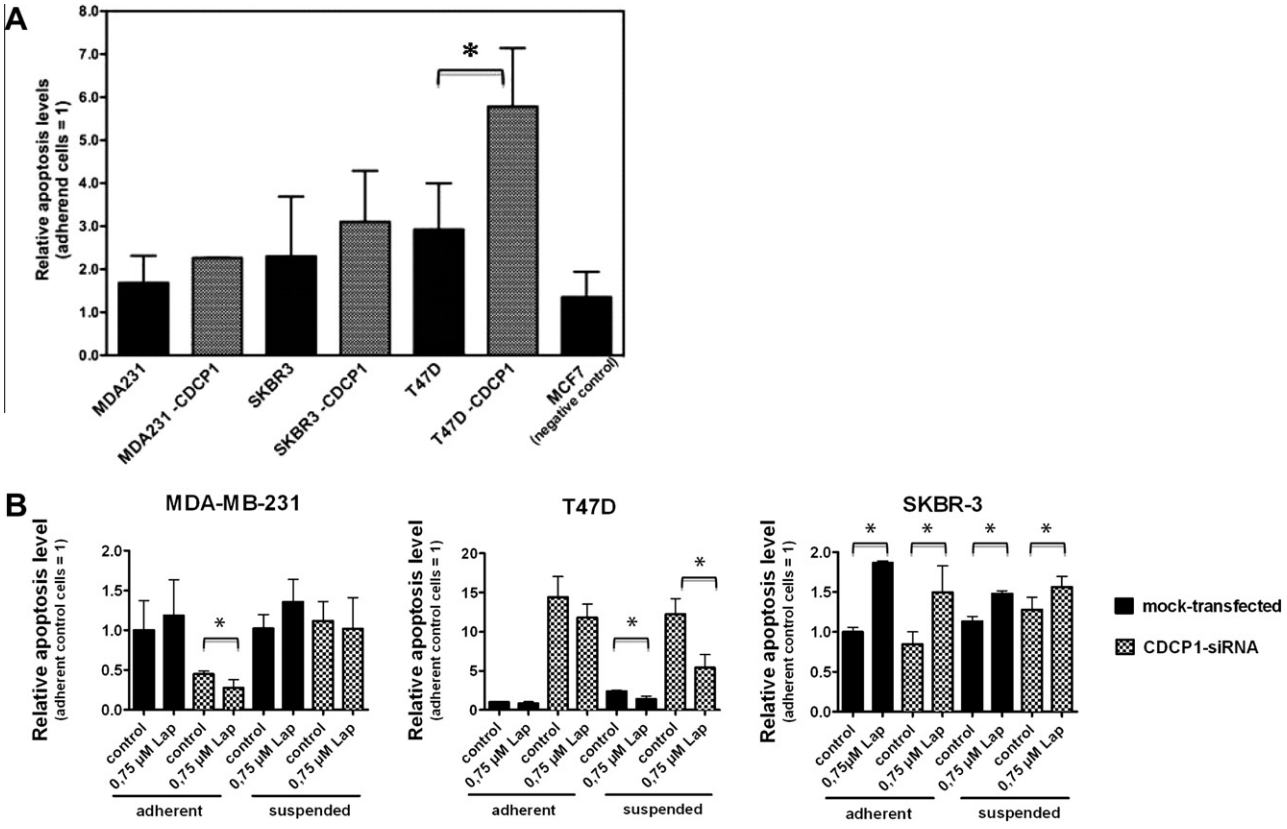

Fig. 3. Level of apoptosis in absence of anchorage to an extracellular matrix. (A) Cells (1×10^4) were seeded to normal or ultra-low adhesion culture plates and cultivated for 48 h. Cell death was evaluated via ELISA by measuring the enrichment of free nucleosomes at 405 nm. The bars represent the ratios (apoptosis (adherent cultured cells)/apoptosis (cells from suspension culture)) ± SD. (B) Cells were cultured as described above but in absence or presence of 0.75 μM lapatinib. Data of adherent untreated cells were normalized to an initial value of 1 to calculate relative apoptosis levels. Bars represent means and SD of three independent experiments (*indicates significant differences ($p \leqslant 0.05$) between untreated and lapatinib treated cells; CDCP1-: cells transfected with 3 μM CDCP1-siRNA.)

4. Discussion

Great efforts have been achieved in breast cancer therapy since the growth factor receptors Erb1 (EGFR) and Erb2 (HER-2/neu) were identified and therefore offer specific targets for targeted therapy. Among other approaches (e.g. inhibition with monoclonal antibodies such as trastuzumab), tyrosine kinase inhibitors such as lapatinib which inhibit EGFR and HER-2/neu derived receptor phosphorylation were applied in combination with chemotherapeutic agents [18,19]. However, to further improve breast cancer therapy, the identification of new potential biomarkers is required to offer new therapeutic targets. One potential candidate is CDCP1, a transmembrane protein that is found to be overexpressed and tyrosine-hyper-phosphorylated in various tumor tissues and which has been speculated to be part of mechanisms mediating anchorage independency and migratory activity in distinct adenocarcinomas than breast tumors [3,8,15].

Our studies clearly demonstrate that patient derived CDCP1-positive breast cancer cell lines show a reduction in *in vitro* migration and invasion and are more sensitive towards apoptosis when cultured in absence of anchorage, when CDCP1 expression is downregulated. In contrast, proliferation is not affected by a CDCP1-*knockdown*. These results recapitulate data from preliminary studies of adenocarcinomas derived from lung, pancreas and prostate [3,8,15].

CDCP1 signaling is triggered via phosphorylation by tyrosine-kinases [2,10,11,13]. In lung adenocarcinoma, the tyrosine kinases c-Fyn and c-Yes have been identified to mediate internal CDCP1-signaling but this had not been shown for breast tumors [15]. Therefore, we aimed to investigate if lapatinib could inhibit phosphorylation of CDCP1 in addition to HER-2/neu. The analyzed cell lines differed in their expression patterns of CDCP1 and HER-2/neu, and there appeared to be no direct correlation between CDCP1 signaling and tyrosine kinase inhibition by lapatinib. For instance, the HER-2/neu$^-$ MDA MB 231 cell line remained unaffected by lapatinib treatment when cells were tested for anchorage-free survival, either as adherent culture or when cultured in suspension. In contrast, HER-2/neu$^{(+)/+}$ T47D and SKBR3 cells show significant differences when cultured in presence of lapatinib. Interestingly, only SKBR3 cells were negatively influenced by lapatinib with increased apoptosis whereas T47D cells seem to be advantaged in the presence of lapatinib even with downregulated CDCP1. This might be due to the fact that SKBR3 cells are rarely CDCP1 positive and might loose their proliferative stimuli mediated via EGF receptors, and therefore the balance between cell death and proliferation favors enhanced apoptosis. However, proliferation and apoptosis in the absence of anchorage are most likely not only regulated by the two receptors HER-2/neu and CDCP1. Other survival mediating or proliferatory mechanisms may result in a decreased sensitivity towards cell death in T47D cells. Moreover, the large standard deviations indicate variability among the repeated experiments of each cell line. We observed in a series of FACS measurements that CDCP1 expression was not stable within the single cell lines in repeated measurements, although we always prepared the cells for analysis at the same time after the last passage. Furthermore, variation of culture conditions, e.g. serum concentrations, sort of medium and cultivation period from the last reseeding to culture plates revealed no correlation with the expression of CDCP1 which

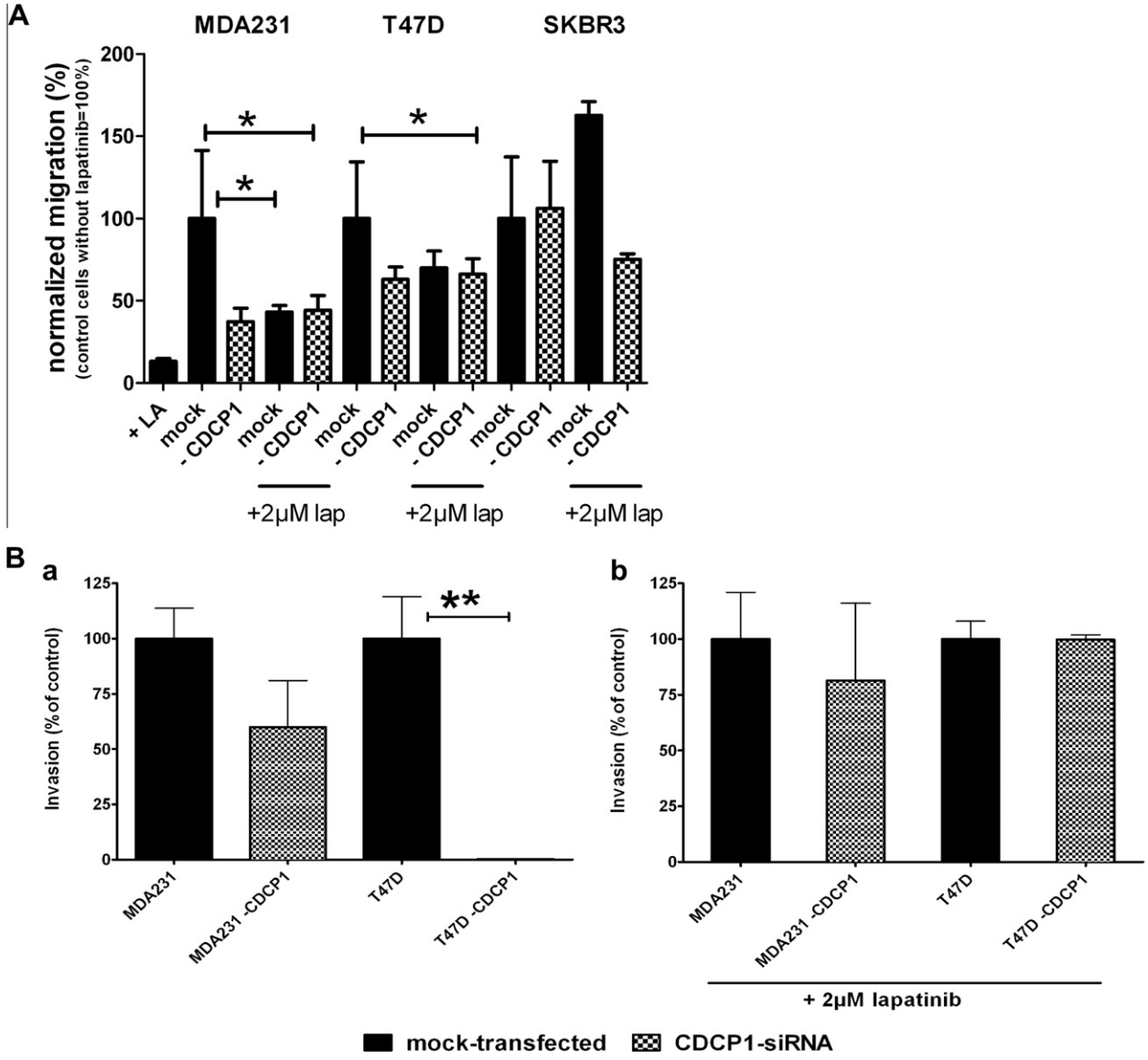

Fig. 4. (A) CDCP1-downregulation inhibits migration of breast cancer cells *in vitro*. CDCP1-siRNA transfected (3×10^4) or mock-transfected cells were seeded into a migration assay in absence or presence of 2 μM lapatinib and allowed to migrate through the porous for 24 h (10% FBS was used as a chemo attractant). Fluorescence of mock-transfected cells was adjusted to 100% migration for further calculations. LA: latrunculin (inhibitor of actin polymerization); -serum: no supplementation of serum as a chemoattractant. (B) CDCP1-knockdown as well as cultivation with tyrosine kinase inhibitor lapatinib results in a decreased invasiveness *in vitro*. (a) Cells (1.5×10^5) were seeded in invasion chambers as described above and were allowed to migrate for 24 h. Relative fluorescence of mock-transfected cells was set to 100%. (b) Invasiveness after 24 h of cultivation in presence of 2 μM lapatinib. Relative fluorescence of mock-transfected cells cultivated with lapatinib was set to 100%. Bars represent means of three replicates and SD (CDCP1- : cells transfected with CDCP1-siRNA (3 μM)). (*Indicates significant differences ($p \leqslant 0.05$); **indicates significant differences ($p \leqslant 0.01$)).

differed from measurement to measurement (data not shown). These observations might explain some intraindividual differences among the single experiments for each cell line. However, CDCP1 was clearly shown to have an impact on anchorage free survival and cell motility in CDCP1 positive breast cancer cell lines, and CDCP1 signaling is mediated by tyrosine kinases other than the kinase inhibited by lapatinib.

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