

RESEARCH ARTICLE

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The PDGFR β /ERK1/2 pathway regulates CDCP1 expression in triple-negative breast cancer

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Abstract

Background: CDCP1, a transmembrane protein with tumor pro-metastatic activity, was recently identified as a prognostic marker in TNBC, the most aggressive breast cancer subtype still lacking an effective molecular targeted therapy. The mechanisms driving CDCP1 over-expression are not fully understood, although several stimuli derived from tumor microenvironment, such as factors present in Wound Healing Fluids (WHFs), reportedly increase CDCP1 levels.

Methods: The expression of CDCP1, PDGFR β and ERK1/2 cell was tested by Western blot after stimulation of MDA-MB-231 cells with PDGF-BB and, similarly, in presence or not of ERK1/2 inhibitor in a panel of TNBC cell lines. Knock-down of PDGFR β was established in MDA-MB-231 cells to detect CDCP1 upon WHF treatment. Immunohistochemical staining was used to detect the expression of CDCP1 and PDGFR β in TNBC clinical samples.

Results: We discovered that PDGF-BB-mediated activation of PDGFR β increases CDCP1 protein expression through the downstream activation of ERK1/2. Inhibition of ERK1/2 activity reduced per se CDCP1 expression, evidence strengthening its role in CDCP1 expression regulation. Knock-down of PDGFR β in TNBC cells impaired CDCP1 increase induced by WHF treatment, highlighting the role of this receptor as a central player of the WHF-mediated CDCP1 induction. A significant association between CDCP1 and PDGFR β immunohistochemical staining was observed in TNBC specimens, independently of CDCP1 gene gain, thus corroborating the relevance of the PDGF-BB/PDGFR β axis in the modulation of CDCP1 expression.

Conclusion: We have identified PDGF-BB/PDGFR β -mediated pathway as a novel player in the regulation of CDCP1 in TNBCs through ERK1/2 activation. Our results provide the basis for the potential use of PDGFR β and ERK1/2 inhibitors in targeting the aggressive features of CDCP1-positive TNBCs.

Keywords: TNBC, CDCP1, PDGFR β , FISH, ERK1/2, PDGF-BB, IHC

Background

Triple-negative breast cancers (TNBCs) comprise mammary carcinomas that do not express estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor receptor-2 (HER-2). TNBCs are an aggressive tumor subtype, characterized by a high risk of recurrence within 5 years after diagnosis and a high

mortality rate [1, 2]. Due to the lack of specific molecular targets, chemotherapy remains the standard systemic therapy for TNBCs, but it has dissatisfactory long-term results. Recently, we proposed the transmembrane protein CUB domain-containing protein-1 (CDCP1), which is overexpressed in TNBCs and involved in tumor progression, as a new therapeutic target for TNBCs [3]. CDCP1 is a cleavable transmembrane protein that is overexpressed in several types of cancer cells [4–10]. CDCP1 encodes a 135-kDa protein that is proteolyzed into a cleaved 70-kDa form [11–13], which can homodimerize and initiate prometastatic activity [11, 14].

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CDCP1 increases the migration and invasiveness of cancer cells and anchorage-independent cell survival [15, 16] through its interaction with important signalling pathways in tumor aggressiveness, such as Akt [11], PKC δ [17], Src [12, 14, 16, 18–20], and Extracellular signal-regulated kinases 1–2 (ERK1/2) [21]. Accordingly, several studies have suggested that the overexpression of this protein in tumors is related to worse outcomes in lung cancer [4], pancreatic cancer [5], renal cell carcinoma [7], ovarian cancer [8], and hepatocellular carcinoma [9]. The mechanisms by which CDCP1 expression is regulated in TNBCs are unknown.

The correlation that we observed between a gain in *CDCP1* copy number and the number of cells that express CDCP1 in TNBCs supports that *CDCP1* polysomy is involved in CDCP1 overexpression in this breast cancer subtype. However, because approximately 50% of TNBC tumors overexpressing CDCP1 lack polysomy, the CDCP1 expression might be regulated by transcriptional and post-translational mechanisms, regardless of a genetic gain (e.g., by influencing the half-life of CDCP1 through EGFR-mediated inhibition of palmitoylation-dependent degradation of CDCP1 [22]). We demonstrate that activation of platelet-derived growth factor receptors beta (PDGFR β) by PDGF-BB upregulates CDCP1 expression and that ERK1/2 activation is crucial for this upmodulation. Consistently, a significant association between CDCP1 and PDGFR β expression was observed in TNBC specimens, independent of gains in *CDCP1*, confirming the link between these two molecules.

Methods

Cell lines, cultures, and treatments

The human breast cancer cell lines MDA-MB-231 (ATCC[®] HTB-26[™]), BT549 (ATCC[®] HTB122[™]), HCC1937 (ATCC[®] CRL 2336[™]), MDA-MB-468 (ATCC[®] HTB-132[™]), (American Type Culture Collection, Manassas, VA), SUM149, and SUM159 (Asterand Bioscience, Detroit, MI now acquired by BioreclamationIVT, Westbury, NY) were authenticated using a panel of microsatellite markers. Cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO₂ as previously described in Turdo et al. [3]. For stimulation experiments, MDA-MB-231 cells were starved in serum-free medium for 24 h and then treated for 48 h with a pool of 5 WHFs at a final concentration of 5% as described [23] or with PDGF-BB, Mib1b, MCP1, IP10, Il1ra, Il1b, G-CSF, Il8, Il6, EGF, FGF, Heregulin, PDGF-AA, PDGF-AB (PeproTech, Rocky Hill, NJ) at 50 ng/mL. Cells were treated in indicated experiments with *cycloheximide* (1 μ M) or UO126 (2 μ M), both of which were dissolved in DMSO (maximum concentration 0.1%) (Sigma-Aldrich).

Antibodies

FACS analysis was performed with Alexa Fluor[®] 647 anti-human CD318 (CDCP1) (BioLegend, San Diego, CA). Biochemical analyses were performed using rabbit polyclonal antibodies against CDCP1, phospho-CDCP1 (Tyr734), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (Cell Signaling, Danvers, MA), and PDGFR β (Santa Cruz Biotechnology, Dallas, TX) or mouse monoclonal anti-phospho-PDGFR β (Tyr751) (clone 88H8) (Cell Signaling); polyclonal anti-rabbit or -mouse IgG (GE Healthcare, Chicago, IL) was the secondary antibody. Actin was revealed by probing with peroxidase-linked mouse monoclonal anti-actin (Sigma-Aldrich).

Western blot

To prepare crude cell lysates, cells were processed as described [24]. Protein concentrations were determined by Coomassie Plus protein assay (Thermo Fisher Scientific, Waltham, MA). The samples were separated on NuPage SDS-Bis-Tris gels (Thermo Fisher Scientific) and transferred to PVDF membranes (Merck Millipore, Billerica, MA). Signals were detected using ECL reagent (GE Healthcare). Protein expression was normalized to that of actin, and densitometry was performed in Quantity One 4.6.6 (Bio-Rad, Hercules, CA).

Cytofluorimetric analysis

CDCP1 protein was detected by FACS analysis by staining cells with PE anti-human CD318 (CDCP1) Antibody (BioLegends). Cells not stained with antibody were used as controls. The gates were set based on light scatter properties after debris and doublet exclusion; a representative gating strategy is shown in Additional file 1: Figure S1. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

Knockdown of PDGFR β by siRNA transfection

To knock down PDGFR β , cells were transfected with 100 nM of specific silencer siRNA (ID s10242) or a N.1 negative control siRNA (Thermo Fisher Scientific) using RNAiMAX (Life Technologies), harvested at 48 h post-transfection, and examined for protein expression by western blot.

Patients

Samples from 65 TNBC patients diagnosed between August 2002 and February 2007 were collected in our institute (Fondazione IRCCS Istituto Nazionale dei Tumori) [3, 25].

Immunohistochemistry

Expression of CDCP1 and PDGFR β was analyzed by IHC in consecutive 2- μ m formalin-fixed, paraffin-embedded (FFPE) tumor sections, using rabbit polyclonal anti-CDCP1

(1:50) (PA5–17245, Thermo Fisher Scientific) and rabbit anti-human PDGFR β (1:200) (Y92, Abcam), respectively. Antigen retrieval was performed by heating the sections for 5 min at 96 °C in 10 mM citrate buffer, pH 6.0. Staining was visualized using streptavidin-biotin-peroxidase (Dako, Agilent Technology, Santa Clara, CA) and 3,3'-diaminobenzidine (DAB; brown signal) (Dako), and the sections were counterstained with hematoxylin. Images were acquired by ECLIPSE TE2000-S inverted microscope (Nikon Instruments, Melville, NY) at 20X and 40X magnification. The reactivity of anti-CDCP1 and anti-PDGFR β was considered to be positive per Turdo 2016 and D'Ippolito 2016 [3, 25]. Specifically, based on the intensity of PDGFR β staining in neoplastic cells, we assigned tumors a score of 0 (absence of signal) or 1 (weak to strong cytoplasmic signal and membrane signal). Reactivity of polyclonal anti-CDCP1 was defined as positive when $\geq 10\%$ of tumor cells showed membrane staining.

Fluorescence in situ hybridization (FISH)

All FISH analyses were performed in FFPE tissues in areas that were selected by the pathologist as being CDCP1-positive by IHC or, for IHC-negative cases, representative of the tumor. Tumors were classified as positive or negative per Turdo et al. [3].

Statistical analysis

Relationships between categorical variables were analyzed by Fisher's exact test. Differences were considered to be significant at $p \leq 0.05$. All analyses were performed using SAS 9.4 (SAS Institute Inc.).

Results

PDGFR β regulates CDCP1 expression in TNBC cells

To examine the molecules that regulate CDCP1 expression in TNBCs, the TNBC cell line MDA-MB-231 was stimulated for 48 h with various ligands, including growth factors, cytokines, and chemokines in wound healing fluids (WHFs) [26], that we found upregulate CDCP1 robustly [3]. This panel of molecules was chosen from small molecules that are involved in breast cancer progression. Plasma membrane CDCP1 levels were determined by cytofluorimetry, and the percentage of increase in expression following stimulation with each molecule was calculated respect to the maximum increase that was induced by WHE, used as a positive control.

Regarding growth factors, PDGF-BB was among the strongest inducers of CDCP1 in MDA-MB-231 cells (Fig. 1a). CDCP1 upregulation was also affected by EGF, FGF-basic, and HRG. Of the cytokines and chemokines, except for slight upregulation by MCP1, IL1RA, and IL-1b, none increased CDCP1 levels, suggesting that the regulation of CDCP1 in TNBC cells depends primarily

on growth factors. Thus, we focused on the PDGF-BB/PDGFR β pathway.

By western blot, we confirmed the robust upmodulation of CDCP1 after PDGF-BB stimulation in MDA-MB-231 cells (Fig. 1b). PDGF-BB-induced signaling was initiated by activation of its cognate receptor, PDGFR β , which results still phosphorylated after 48 h of treatment with PDGF-BB. Consistent with its stimulation, the total level of PDGFR β decreased compared with unstimulated cells, presumably due to its postactivation protein degradation [27]. Similarly, CDCP1 phosphorylation (Y734) rose after PDGF-BB treatment.

To confirm that the upregulation of CDCP1 protein on PDGF-BB stimulation was attributed to greater CDCP1 translation, MDA-MB-231 cells were stimulated with or without the protein neosynthesis inhibitor *cycloheximide* (CHX). By western blot, no PDGF-BB-induced CDCP1 upmodulation occurred in the presence of CHX, indicating that during PDGF-BB stimulation, the increase in CDCP1 protein is due to protein neosynthesis (Fig. 1c).

To verify the function of PDGFR β in the regulation of CDCP1 expression, PDGFR β was transiently knocked down in MDA-MB-231 cells for 24 h (Fig. 2a). By western blot, CDCP1 declined in PDGFR β siRNA-treated versus scramble siRNA-treated cells.

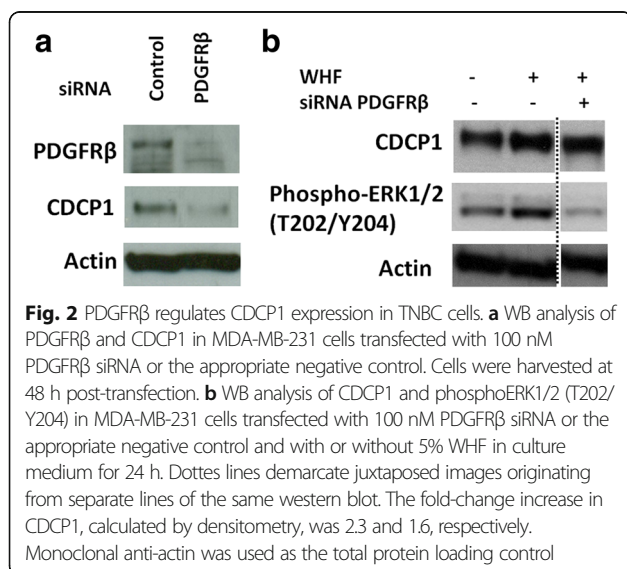
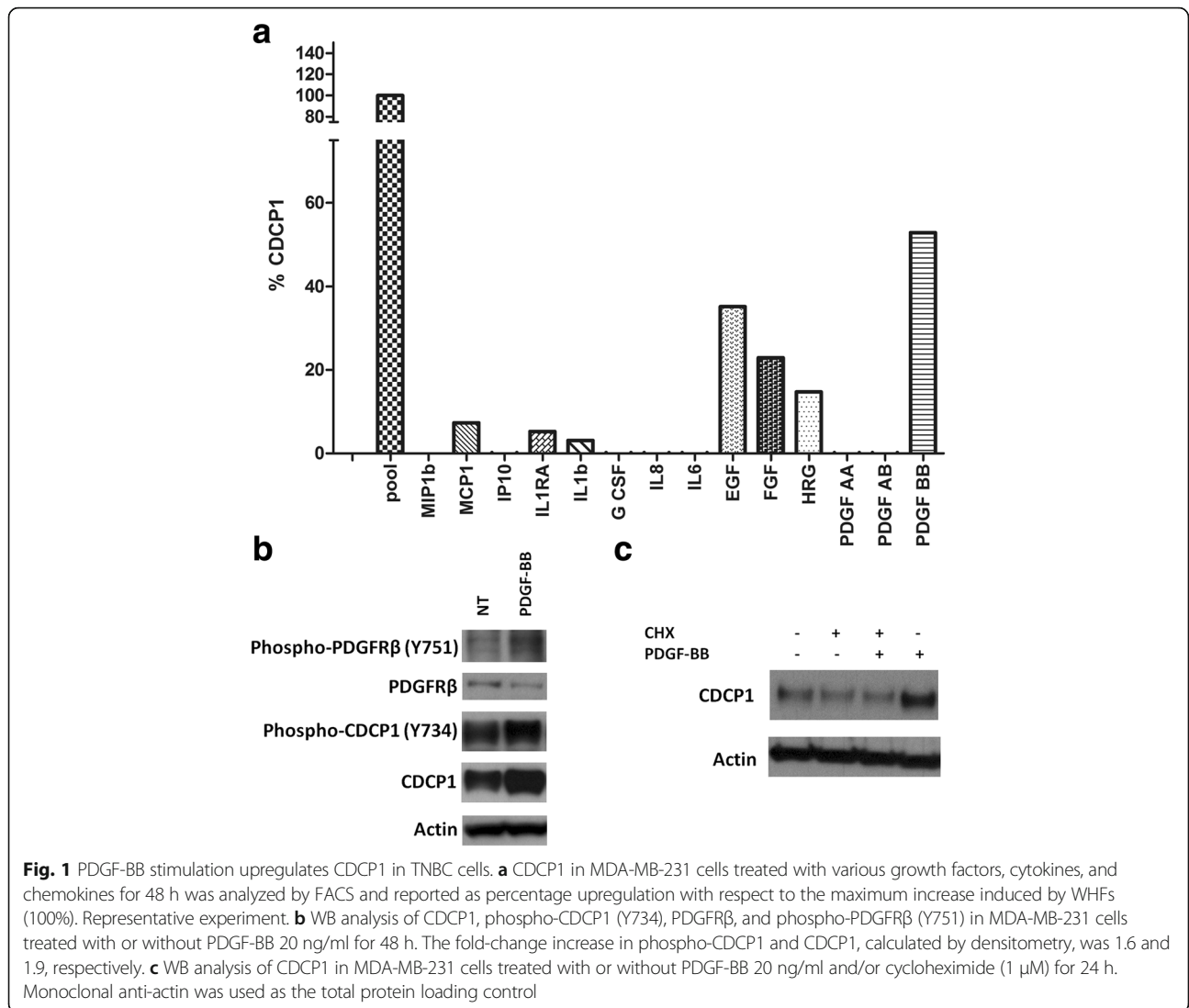
To determine whether PDGF-BB participates in the increase in CDCP1 expression after TNBC cell stimulation with WHFs, PDGFR β was transiently knocked down in MDA-MB-231 cells, which were then stimulated with WHFs for 24 h, 24 h after siRNA transfection (Fig. 2b). In knockdown cells, the increase of CDCP1 was partially impaired on WHF treatment.

PDGFR β activation triggers several transduction signals, such as the Ras-ERK pathway. ERK1/2 governs the upregulation in CDCP1 following stimulation with growth factors. Notably, ERK was less active in cells in which PDGFR β was knocked down (Fig. 2b).

PDGF-BB-induced CDCP1 upregulation depends on the PDGFR β /ERK axis

To determine whether ERK activation is crucial in PDGF-BB-induced CDCP1 in our cell model, ERK status was examined on short-term stimulation, wherein downstream pathways of RTK are usually activated. MDA-MB-231 cells were starved and then treated with PDGF-BB for 10 and 60 min, confirming that PDGF-BB-induced PDGFR β activation stimulated ERK1/2, starting from 10 min and persisting at 1 h (Fig. 3a).

To confirm that PDGF-BB stimulation induces CDCP1 expression through ERK1/2 activation, MDA-MB-231 cells were treated for 24 h with PDGF-BB with or without the ERK1/2 inhibitor UO126 (2 μ M). ERK1/2 inactivation, on PDGF-BB treatment, mitigated the upregulation of



CDCP1. CDCP1 levels fell in the presence of UO126 (Fig. 3b). Notably, UO126 diminished basal CDCP1 levels in unstimulated starved cells. CDCP1 upregulation upon the activation of the PDGFRβ/ERK axis was confirmed in two additionally triple negative breast cancer cell lines BT-549 and SUM149 (Additional file 2: Figure S2). These data indicate that PDGF-BB mediates the increase in CDCP1 through the ERK1/2 pathway.

Next, we starved MDA-MB-231 cells and treated them with or without a pool of 5 WHFs at a final concentration of 5% for 24 h, with or without UO126, to determine whether ERK1/2 activation is required for WHF-induced upmodulation of CDCP1 in TNBCs. By western blot, WHF increased CDCP1 levels only in the presence of functional ERK1/2 (Fig. 3c).

To confirm that ERK1/2 is necessary for CDCP1 expression in TNBC cells, a panel of CDCP1-positive TNBC cell lines [3] was treated with UO126 under standard culture

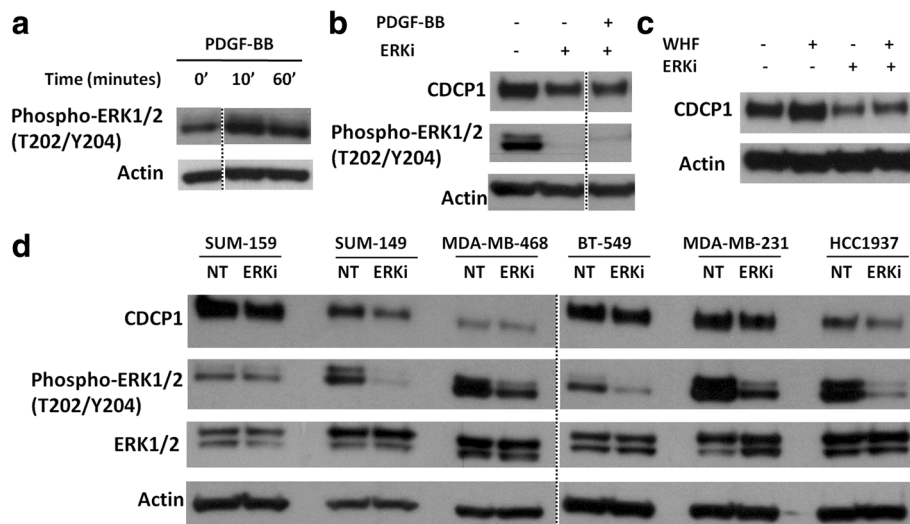


Fig. 3 ERK1/2 activity regulates CDCP1 expression in TNBC cells. **a** WB analysis of phospho-ERK1/2 (T202/Y204) in MDA-MB-231 cells treated with or without 20 ng/ml PDGF-BB for 10 and 60 min. **b** WB analysis of CDCP1 and phosphoERK1/2 (T202/Y204) in MDA-MB-231 cells treated with or without the ERK1/2 inhibitor UO126 (2 μ M) and stimulated with or without 20 ng/ml PDGF-BB for 24 h. **c** WB analysis of CDCP1 in MDA-MB-231 cells treated with or without UO126 (2 μ M) and stimulated with or without 5% WHF in culture medium for 24 h. Dotted lines demarcate juxtaposed images originating from separate lines of the same western blot. **d** WB analysis of CDCP1, phosphoERK1/2 (T202/Y204), and ERK1/2 in SUM149, SUM159, MDA-MB-468, BT-549, MDA-MB-231, and HCC1937 cells treated with or without UO126 (2 μ M) under standard medium conditions for 24 h. Monoclonal anti-actin was used as the total protein loading control

conditions for 24 h (Fig. 3d). CDCP1 levels decreased in 5 of the 6 cell lines on treatment with this ERK1/2 inhibitor; only in MDA-MB-468 cells CDCP1 expression was unaffected by ERK1/2 inactivation. These data confirm the implication of ERK1/2 in the regulation of CDCP1 expression in TNBCs. A schematic representation of CDCP1 up-regulation upon PDGF-BB, PDGFR β pathway activation, via ERK1/2 is shown in Fig. 4.

CDCP1 and PDGFR β expression associated in TNBC tissues

The association between PDGFR β and CDCP1 expression was examined in 65 formalin-fixed, paraffin-embedded (FFPE) primary TNBC specimens by immunohistochemistry (IHC) (Table 1). Of these samples, 41.5% was node (N)-positive, and 60.0% of tumors were stage T1. As expected for TNBCs, the tumors were primarily grade III (84.6%) and highly necrotic (75.4%). Multifocality was observed in 19.9% of cases, and 35.4% of patients had ductal carcinoma in situ (DCIS). Tumors were categorized as CDCP1-positive or -negative per Turdo et al. [3] and were divided according to the presence or absence of PDGFR β staining in association with tumor cells, as described in D'Ippolito et al. [25] (Fig. 5).

Because we have reported the function of PDGFR β in mediating tumor vasculogenic properties in TNBCs [28], and we also reported that CDCP1 knocked-down impairs vasculogenic mimicry in vitro, TNBC tumors were then analyzed, based on the presence or absence of PDGFR β

staining in association with tumor cells in vascular-like structures or organized into tumor nests. Regarding tumor nests, 57% (37/65) of cases were CDCP1-positive, 45% (29/65) was PDGFR β -positive, 32% (21/65) was positive for CDCP1 and PDGFR β (double-positive), and 31% (20/65) was negative for CDCP1 and PDGFR β (double-negative). CDCP1-positive cases did not differ significantly from their negative counterparts about clinical variables (Table 1), but PDGFR β positivity was significantly associated with higher grade and younger age. All cases with grade I and II tumors did not express PDGFR β , whereas nearly 50% of grade III tumors were PDGFR β -positive ($p = 0.0029$; Fisher's exact test); most PDGFR β -positive cases were younger than PDGFR β -negative cases ($p = 0.0040$; Fisher's exact test).

About PDGFR β expression in tumor and vascular lacunae according to CDCP1 levels, 72.4% (21/29) of PDGFR β -positive cases in tumor nests were CDCP1-positive (Table 2; $p = 0.0429$; Fisher's exact test). In our analysis of vascular lacunae, a trend of association was revealed between PDGFR β in tumor cells in vascular-like structures and CDCP1 expression (Table 2; $p = 0.0795$; Fisher's exact test).

Based on the relationship between CDCP1 expression and gains in *CDCP1*, we examined whether PDGFR β was differentially expressed, depending on *CDCP1* status. *CDCP1* status was analyzed in 53 of 65 available TNBC specimens by fluorescence in situ hybridization (FISH). As a result, we identified 4 genetic categories:

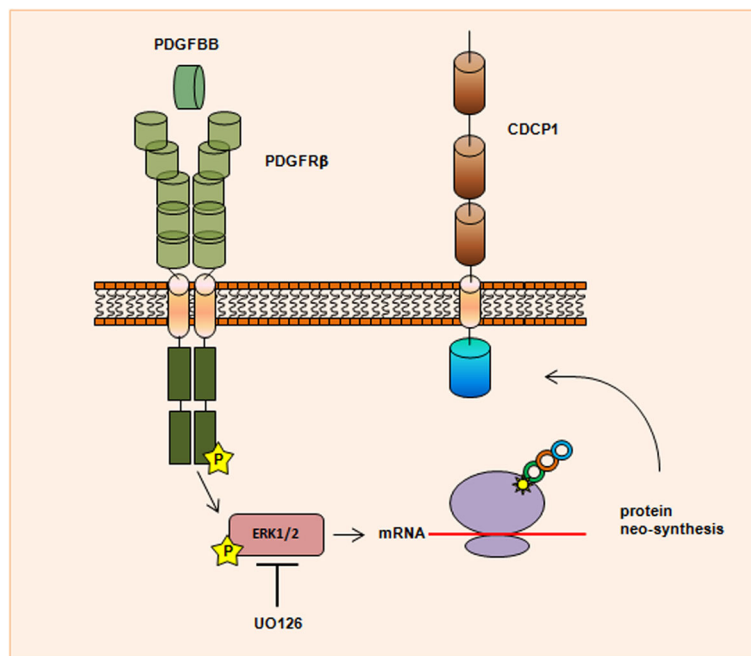


Fig. 4 Schematic representation of CDCP1 upregulation induced by PDGF-BB/PDGFR β pathway through ERK1/2 activation. PDGFR β dimerizes and is activated upon binding of the PDGF-BB ligand, causing the activation of the kinase domain, visualized as tyrosine phosphorylation (P) of the receptor molecules. In conjunction with dimerization and kinase activation, the receptor molecules undergoes a conformational changes, which allow a basal kinase activity, leading to full enzymatic activity directed toward downstream mediators such as ERK1/2. ERK1/2 activity is necessary for CDCP1 protein neo-synthesis, as demonstrated by the reduction of CDCP1 protein levels in presence of UO126, an inhibitor of ERK1/2

deleted ($n = 2$) and disomic ($n = 40$) cases were considered to be *CDCP1*-negative, and amplified ($n = 2$) and polysomic ($n = 9$) cases were *CDCP1*-positive (Fig. 6). Our FISH analysis did not reveal any imbalance in *CDCP1* gains in PDGFR β -positive or -negative TNBC cases (Table 3). Among FISH-positive cases, 7 of 10 *CDCP1*-positive cases expressed PDGFR β ; similarly, among FISH-negative cases, 10 of 19 *CDCP1*-positive cases expressed PDGFR β ($p = 0.4495$). These data demonstrate that *CDCP1* and PDGFR β expression is linked in TNBC specimens, independent of gains in *CDCP1*.

Discussion

Our study described for the first time the role of PDGFR β signalling in regulating *CDCP1* expression in TNBCs. We have demonstrated that the induction of *CDCP1* peaks on treatment with PDGF-BB, among various cytokines, chemokines, and growth factors, highlighting its significance in the regulation of this protein in the TNBC phenotype. Notably, the increase and maintenance of *CDCP1* phosphorylation after treatment with PDGF-BB implicate PDGFR β in the regulation of *CDCP1* activity. Thus, we speculate that signals downstream of PDGFR β are also crucial for the promotion of *CDCP1*-mediated prometastatic features through the activation of Src family kinases (SFKs). *CDCP1* Tyr734 is the primary SFK-mediated phosphorylation site [13, 29],

crucial for the recruitment of PKC δ and resulting in *CDCP1*-mediated invasiveness.

Growth factor receptors that are involved in tumor progression have been implicated in *CDCP1* overexpression. The EGF-EGFR axis promotes *CDCP1* expression in ovarian cancer models, and bone morphogenetic protein 4 induces *CDCP1* in pancreatic cancer cells [5, 30]. Our analyses suggest that growth factors other than PDGF-BB mediate the upregulation of *CDCP1*. For example, HRG (likely via EGFR/HER3 heterodimerization) and FGF upmodulate *CDCP1* on the cell membrane of TNBCs. The redundancy of signalling pathways that are downstream of these tyrosine kinase receptors suggests that the activation of several growth factor receptors converge at common mediators in *CDCP1* synthesis, the most important of which are members of the RAS/RAF/MEK/ERK pathway, which have been implicated in *CDCP1* mRNA and protein expression. Activation of Ras-ERK signalling alone induces *CDCP1* expression in NCSLC, likely through the transcription factor AP-1 [21].

The RAS/RAF/ERK pathway is stimulated in TNBC [31], leading cells to acquire an aggressive phenotype—i.e., promoting invasiveness and migration [32]. Thus, ERK1/2 might also promote these features through the regulation of *CDCP1* expression. Several stimuli, mediated by growth factors, converge to activate ERK1/2, paralleling the rise in *CDCP1* on the treatment of MDA-MB-231 cells with the

Table 1 Clinical characteristics of TNBC patients according to expression of PDGFR β and CDCP1

	Overall cohort (N = 65 ^b)	PDGFR β pos (N = 29 ^b)	PDGFR β neg (N = 36 ^b)	P value ^c	CDCP1 pos (N = 37 ^b)	CDCP1 neg (N = 28 ^b)	P value ^c
Age							
> = 50 years	42 (64.6%)	13 (44.8%)	29 (80.6%)	0,0040	22 (59.5%)	20 (71.4%)	0.4332
< 50 years	23 (35.4%)	16 (55.2%)	7 (19.4%)		15 (40.5%)	8 (28.6%)	
Grade							
I, II	9 (14.1%)	0 (0%)	9 (25.7%)	0,0029	5 (13.5%)	4 (14.8%)	1.0000
III	55 (85.9%)	29 (100.0%)	26 (74.3%)		32 (86.5%)	23 (85.2%)	
na	1		1			1	
Necrosis							
No	14 (22.2%)	6 (20.7%)	8 (23.5%)	1,0000	6 (16.7%)	8 (29.6%)	0.2395
Yes	49 (77.8%)	23 (79.3%)	26 (76.5%)		30 (83.3%)	19 (70.4%)	
na	2		2		1	1	
Multifocality							
No	52 (82.5%)	24 (82.8%)	28 (84.4%)	1,0000	28 (77.8%)	24 (88.9%)	0.3255
Yes	11 (17.5%)	5 (17.2%)	6 (17.6%)		8 (22.2%)	3 (11.1%)	
na	2		2		1	1	
N positivity							
No	38 (58.5%)	17 (58.6%)	21 (58.3%)	1,0000	19 (51.4%)	19 (67.9%)	0.2117
Yes	27 (41.5%)	12 (41.4%)	15 (41.7%)		18 (48.6%)	9 (32.1%)	
Size							
> 2 cm	39 (60.9%)	11 (39.3%)	15 (41.7%)	0,8035	15 (40.5%)	10 (37.0%)	0.8015
< = 2 cm	25 (39.1%)	18 (64.3%)	21 (58.3%)		22 (59.5%)	17 (63.0%)	
na	1	1				1	
DCIS ^a							
No	40 (63.5%)	20 (68.9%)	20 (58.9%)	0,4424	23 (63.9%)	17 (63.0%)	1.0000
Yes	23 (36.5%)	9 (31.0%)	14 (41.2%)		13 (36.1%)	10 (37.0%)	
na	2		2		1	1	

^aDCIS, ductal carcinoma in situ^bFrequency percentages were calculated on available cases^cFisher's exact test

various growth factors that we tested. As observed in a panel of TNBC models, treatment with an ERK1/2 inhibitor under standard culture conditions downregulated CDCP1, demonstrating that this pathway is crucial for CDCP1 expression in the TNBC phenotype. The MDA-MB-468 cell line was the only model that showed no variation in the CDCP1 expression on ERK1/2 inhibition; similarly, it was the TNBC line that upregulated CDCP1 RNA and protein the least on treatment with WHD [3]. These data suggest the existence of another regulatory mechanism in addition to RTK/ERK1/2 activation. That ERK is less active when the PDGFR β expression is abrogated in TNBC confirms the link between these two tyrosine kinases. CDCP1 expression regulation, upon PDGFR β /ERK1/2 pathway activation, was investigated also in non-breast cancer cells. CDCP1 expression did not increase upon PDGF-BB treatment in the human large cell lung cancer cells NCI-H460 (ATCC[®] HTB-177[™]), whereas it

was slightly up-regulated in the human esophageal adenocarcinoma cells OE19 (Sigma-Aldrich). Interestingly, in both cell lines, the presence of ERK1/2 inhibitor strongly reduces CDCP1 protein level (unpublished data). This data suggests that ERK1/2 could be a crucial hub for the regulation of CDCP1 expression, not only in breast cancer cells. As a support, it has been shown that CDCP1 expression is regulated through ERK1/2 recruitment in ovarian cancer cells stimulated with EGF [29]. On the contrary, the sensitivity to PDGFR β activation seems to be dependant by the cells.

The PDGFR β axis is involved in breast cancer because tumor tissue and the surrounding stroma express PDGFR β [33, 34]. Stromal PDGFR β expression is associated with a poor prognosis [35, 36]. Also, breast cancer cells and fibroblasts secreted PDGF-like factors that sustain the PDGFR pathway in tumor cells [37, 38]. Considering the low expression of PDGFR α in MDA-MB-231

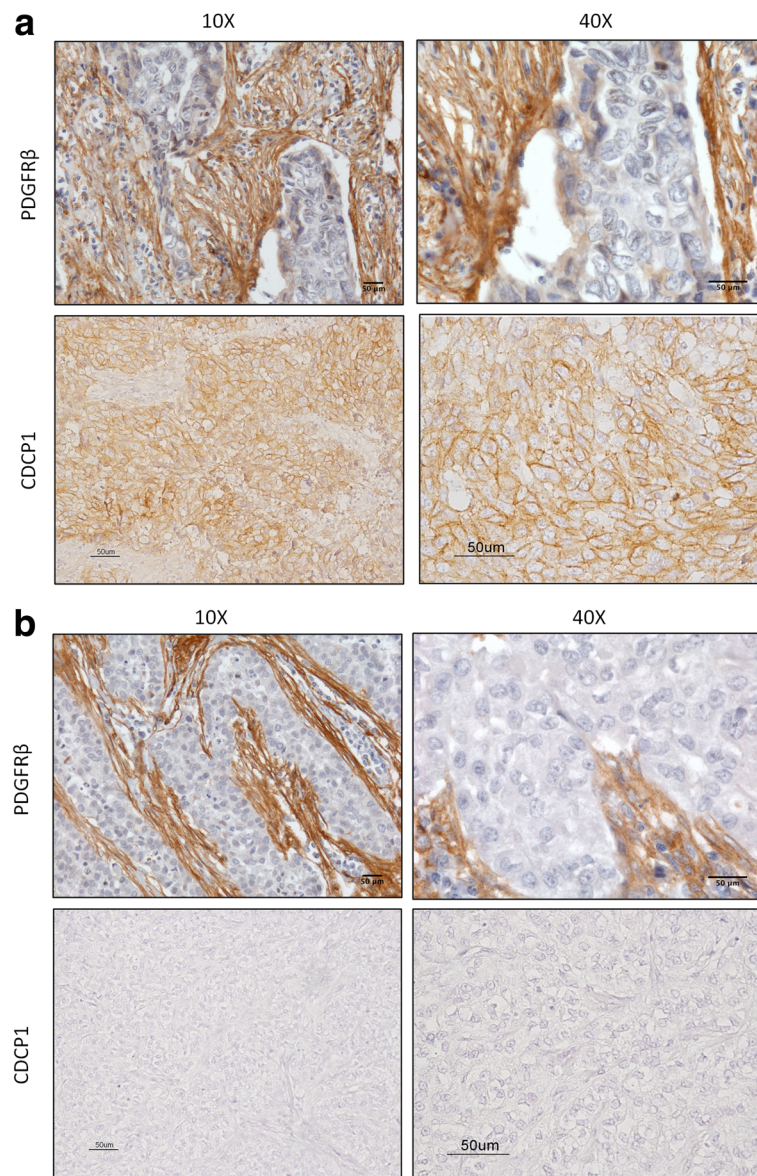


Fig. 5 IHC staining of PDGFRβ and CDCP1 in TNBC. FFPE sections of TNBC specimens were analyzed by IHC for PDGFRβ and CDCP1. **a** Representative image of a PDGFRβ- and CDCP1-positive case, with plasma membrane staining, at 10X and 40X magnification; **b** Representative image of a PDGFRβ- and CDCP1-negative case, at 10X and 40X magnification

Table 2 PDGFRβ expression in tumor and vascular lacunae according to expression of CDCP1 (IHC CDCP1)

	IHC CDCP1		P value ^a
	Pos	Neg	
PDGFRβ tumor nest			
Pos (N = 29)	21 (72.4%) ^b	8 (27.6%)	0,0429
Neg (N = 36)	16 (44.4%)	20 (55.5%)	
PDGFRβ vascular lacunae			
Pos (N = 27)	19 (70.4%)	8 (29.6%)	0,0795
Neg (N = 38)	18 (47.4%)	20 (52.6%)	

^aFisher's exact test

cells [39], we cannot exclude that other isoforms of PDGF receptors regulate CDCP1 expression. Notably, by immunohistochemistry, PDGFRβ and CDCP1 expression correlated significantly in a cohort of 65 TNBC specimens, confirming that the PDGF-BB/PDGFRβ axis governs CDCP1 expression in human tumors.

However, supporting that several growth factor receptors can regulate the expression of CDCP1, not all CDCP1-positive specimens expressed PDGFRβ in the tumor cells. We have reported that gains in *CDCP1* are significantly associated with CDCP1 expression but that nearly half of CDCP1-positive cases do not show such gains. In the

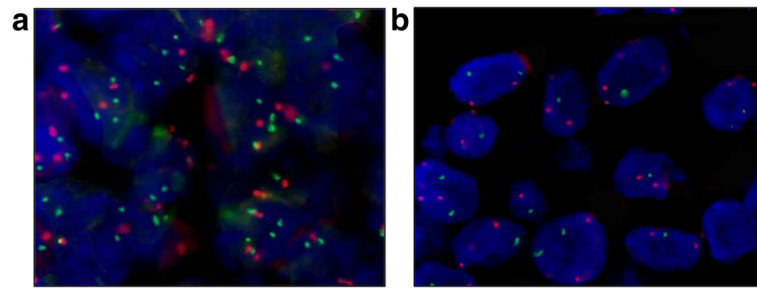


Fig. 6 Genetic alterations of CDCP1 in TNBC. FFPE sections of TNBC specimens were analyzed by dual-color FISH using for CDCP1 genetic alteration CDCP1/CEP3 probes on a FFPE sections of TNBC specimens FISH. **a** Representative image of TNBC specimen positive for CDCP1 IHC staining, showing tumor cells with > 3 red signals for CEP3 and > 3 green signals for the CDCP1 locus (polysomy); **b** Representative image of TNBC specimen negative for CDCP1 IHC staining, showing tumor cells with > 3 signals for CEP3 and < 3 green signals for the CDCP1 locus (deletion)

current study, in the absence of a gain in *CDCP1*, *CDCP1* was expressed at the same frequency in PDGFRβ-positive and -negative TNBC specimens, indicating that PDGFRβ supports *CDCP1* expression independently of a gain in *CDCP1*. Accordingly, *CDCP1* polysomy was observed in MDA-MB-231 cells (data not shown), in which PDGFRβ stimulation further increased basal *CDCP1* levels.

Further, our group has demonstrated that the ability of TNBCs to form vascular-like channels [28] is associated with increased tumor aggressiveness and that this phenotype is related strictly to the expression of PDGFRβ [25]. Considering that *CDCP1* also contributes to vasculogenic mimicry [3], we hypothesize that PDGFRβ mediated this peculiar TNBC phenotype by regulating the expression of *CDCP1*. Accordingly, the association between PDGFRβ and *CDCP1* was nearly significant—almost 70% of vascular lacunae that expressed PDGFRβ were positive for *CDCP1*.

In our previous paper on *CDCP1* role in TNBC [3], we showed that knock-down of *CDCP1* expression in TNBC cell lines did not affect their in vitro growth capability in 2D cultures and, accordingly, no association was found between *CDCP1* expression and proliferation rates in TNBC specimens, evaluated by Ki-67 marker. Regarding

the role of PDGFRβ, it is crucial for the vasculogenic properties of tumor cells, and therefore, its role in tumorigenesis mainly accounts for the activation of migration/invasion/angiogenesis pathways in cancer cells. Consistently, in a previous paper [27], we reported that inhibition of PDGFRβ pathways only slightly influences proliferation of TNBC, and that its role in TNBC aggressiveness mainly depends on the capacity to induce vasculogenic mimicry.

In conclusion, we have identified PDGF-BB/PDGFRβ as a new pathway that is involved in the regulation of *CDCP1* expression in TNCBs through ERK1/2 activation. Our results provide the basis for the potential use of PDGFRβ and ERK1/2 inhibitors in targeting the high aggressiveness of TNBCs.

Conclusions

We have identified PDGF-BB/PDGFRβ-mediated pathway as a novel player in the regulation of *CDCP1* in TNCBs through ERK1/2 activation. Our results provide the basis for the potential use of PDGFRβ and ERK1/2 inhibitors in targeting the aggressive features of *CDCP1*-positive TNBCs.

Additional files

Additional file 1: Figure S1. Gating strategy for *CDCP1* flow cytometric analysis. Flow cytometric analysis of MDA-MB-231 cells starved in serum-free medium for 24 h and then treated for 48 h with FGF 50 ng/mL. (PDF 470 kb)

Additional file 2: Figure S2. PDGFR-BB stimulation upregulates *CDCP1* in TNBC cells. Western blot analysis of *CDCP1* and Vinculin expression in SUM-149 and BT549 cells upon PDGF-BB and ERKi treatment. (PDF 249 kb)

Table 3 PDGFRβ expression in tumor according with expression of *CDCP1* protein (IHC *CDCP1*) and *CDCP1* genetic gain

		IHC <i>CDCP1</i>		<i>P</i> value ^a
		Pos	Neg	
<i>CDCP1</i> FISH neg	PDGFRβ tumor nest			
	Pos (<i>N</i> = 17)	10 (58.8%)	7 (41.2%)	0,2092
	Neg (<i>N</i> = 25)	9 (36.0%)	16 (64.0%)	
<i>CDCP1</i> FISH pos	PDGFRβ tumor nest			
	Pos (<i>N</i> = 7)	7 (100%)	0 (0%)	0,3636
	Neg (<i>N</i> = 4)	3 (75.0%)	1 (25.0%)	

^aFisher's exact test

Abbreviations

CDCP1: CUB domain-containing protein-1; ERK1/2: Extracellular signal-regulated kinases 1–2; FISH: Fluorescent in situ hybridization; IHC: Immunohistochemistry; PDGF-BB: Platelet-derived growth factor-BB; PDGFRβ: Platelet-derived growth factor receptors beta; TNBC: Triple-negative breast cancer; WHFs: Wound Healing Fluids

Acknowledgments

The Authors thank Mrs. Laura Mameli for secretarial assistance.

Funding

This work was supported by the Associazione Italiana Ricerca sul Cancro under Grant 11484 to Manuela Campiglio and fellowship from Fondazione Umberto Veronesi 2017, 2018 to Francesca Bianchi. The funding body had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

References number: 40.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Authors' contributions

LF performed the experiments and wrote the paper; FT performed the experiments and interpreted the data; CG cultured cell lines and provided technical support for the experiments; PC performed statistical analysis; PG and GS performed FISH analysis and interpreted the data; ED, BB and PA performed IHC analysis, discussed the data and critically revised the manuscript; MI discussed the data and contributed to the final version of the manuscript; RA drew up the human TNBC series including clinico-pathological characteristics; LS critically revised the manuscript; ET interpreted immunohistochemical data, discussed all the data and wrote the paper; MC conceived the study, planned and supervised the experiments; FB performed statistical analysis, processed and interpreted the experimental data, wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients gave written consent for use of their biological materials for future investigations and research purposes. Research involving human material and human data that are reported in the manuscript has been performed with the approval of an appropriate ethics committee "Institutional Independent Ethics Committee of the Fondazione IRCCS Istituto Nazionale dei Tumori, Milano" DI 0001336. Research was carried out in compliance with the Helsinki Declaration. None of the cell lines used in this paper required ethics approval for their use, since they are sale for research use.

Competing interests

The authors declare that they have no competing interests.

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Received: 4 October 2017 Accepted: 11 May 2018

Published online: 23 May 2018

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