Etk/Bmx Regulates Proteinase-Activated-Receptor1 (PAR1) in Breast Cancer Invasion: Signaling Partners, Hierarchy and Physiological Significance

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Abstract

Background: While protease-activated-receptor 1 (PAR1) plays a central role in tumor progression, little is known about the cell signaling involved.

Methodology/Principal Findings: We show here the impact of PAR1 cellular activities using both an orthotopic mouse mammary xenograft and a colorectal-liver metastasis model in vivo, with biochemical analyses in vitro. Large and highly vascularized tumors were generated by cells over-expressing wt hPar1, Y397Z hPar1, with persistent signaling, or Y381A hPar1 mutant constructs. In contrast, cells over-expressing the truncated form of hPar1, which lacks the cytoplasmic tail, developed small or no tumors, similar to cells expressing empty vector or control untreated cells. Antibody array membranes revealed essential hPar1 partners including Etk/Bmx and Shc. PAR1 activation induces Etk/Bmx and Shc binding to the receptor C-tail to form a complex. Y/A mutations in the PAR1 C-tail did not prevent Shc-PAR1 association, but enhanced the number of liver metastases compared with the already increased metastases obtained with wt hPar1. We found that Etk/Bmx first binds via the PH domain to a region of seven residues, located between C378-S384 in PAR1 C-tail, enabling subsequent Shc association. Importantly, expression of the hPar1-7A mutant form (substituted A, residues 378-384), which is incapable of binding Etk/Bmx, resulted in inhibition of invasion through Matrigel-coated membranes. Similarly, knocking down Etk/Bmx inhibited PAR1-induced MDA-MB-435 cell migration. In addition, intact spheroid morphogenesis of MCF10A cells is markedly disrupted by the ectopic expression of wt hPar1. In contrast, the forced expression of the hPar1-7A mutant results in normal ball-shaped spheroids. Thus, by preventing binding of Etk/Bmx to PAR1 C-tail, hPar1 oncogenic properties are abrogated.

Conclusions/Significance: This is the first demonstration that a cytoplasmic portion of the PAR1 C-tail functions as a scaffold site. We identify here essential signaling partners, determine the hierarchy of binding and provide a platform for therapeutic vehicles via definition of the critical PAR1-associated region in the breast cancer signaling niche.

Introduction

Protease-activated receptor-1 (PAR1), a G protein-coupled receptor (GPCR), is the first and prototype member of the mammalian PAR family, which comprises four genes. The activation of PAR1 involves the release of an N-terminal peptide and the exposure of an otherwise hindered ligand, resulting in an exclusive mode of activation and a general paradigm for the entire PAR family (1–3). While a well-known classical observation points to a tight link between hyper-activation of the coagulation system and cancer malignancies, the molecular mechanism that governs pro-coagulant tumor progression remains poorly defined [1,2,3]. Surprisingly, the zinc-dependent matrix-metalloprotease 1 (MMP-1), a collagenase that efficiently cleaves extracellular matrix (ECM) and basement membrane components, has been shown to specifically activate PAR1 [4]. The PAR1 -MMP1 axis may thus provide a direct mechanistic link between PAR1 and tumor metastasis.

Levels of hPar1 expression and epithelial tumor progression are correlated in both clinically obtained biopsy specimens and a wide spectrum of differentially metastatic cell lines [5,6]. PAR1 also plays a role in the physiological invasion process of placental cytotoxophoblasts during implantation into the uterus decidua [7]. Trophoblast invasion shares many features with the tumor cell invasion process; it differs, however, by the time-limited hPar1 expression, which is confined to the trophoblast-invasive period, and is shut off immediately thereafter, when the need to invade disappears [7]. This provides strong support for the idea that the hPar1 gene is part of an invasive gene program.
Importantly, PAR₁ cellular trafficking and signal termination appear to occur in a different mode than other GPCRs. Instead of recycling back to the cell surface after ligand stimulation, activated PAR₁ is sorted to the lysosomes and degraded [8,9]. Aberrant PAR₁ trafficking, resulting in receptor-populated cell surfaces and causing prolonged and persistent signals, has been found in breast cancer [10]. While cellular trafficking of PAR₁ impinges on the extent and mode of signaling, identification of individual PAR₁ signaling partners and their contribution to breast cancer progression remain to be elucidated.

In the present study, we have identified PAR₁ C-tail as a scaffold site for the immobilization of signaling partners. In addition to identifying key partners, we have determined the hierarchy of binding and established a region in PAR₁ C-tail critical for breast cancer signaling. The association of Etk/Bmx and Shc to form a physical complex with PAR₁ C-tail is demonstrated. The prime link of Etk/Bmx to PAR₁ is mediated via its PH domain, enabling the subsequent immobilization of Shc. The physiological significance of PAR₁-Etk/Bmx binding is emphasized by the inhibition of Matrigel invasion and appearance of nearly intact acini morphogenesis of polarized cell architecture when this site is mutated. The use of consecutive A residues inserted into the proposed Etk/Bmx binding region of PAR₁ C-tail (e.g., hPar1-1A) abolished PAR₁ -induced pro-oncogenic properties. Thus, by preventing the binding of a key signaling partner to PAR₁ C-tail, efficient inhibition of PAR₁ -induced tumor-associated functions, including loss of epithelial cell polarity, migration and invasion through basement membranes, is obtained. Elucidation of the PAR₁ C-tail binding domain may provide a platform for new therapeutic vehicles in treating breast cancer.

**Results**

**PAR₁-enhanced tumor growth and angiogenesis in vivo** is abrogated in the presence of a truncated PAR₁ form

To investigate the role of PAR₁ signaling in breast tumor growth and vascularization in vivo, we over-expressed wt hPar1 and deletion constructs [e.g., L369Z] which lacks the entire cytoplasmic tail, and Y397Z, which exhibits persistent signaling due to impaired internalization [11,12] in MCF7 cells. The functional outcome of MCF7 cells over-expressing various hPar1 constructs in vivo was assessed by orthotopic mammary fat pad tumor development (proper expression and characterization of the plasmids are shown in Figure S1). MCF7 cells over-expressing either Y397Z or wt hPar1 constructs (e.g., MCF7/Y397Z hPar1; MCF7/wt hPar1) markedly enhanced tumor growth in vivo following implantation into the mammary glands (Fig. 1C and D), whereas MCF7 cells over-expressing truncated hPar1 behaved similar to control MCF7 cells in vector-injected mice, which developed only very small tumors (Fig. 1C). The tumors obtained with MCF7/wt hPar1 and MCF7/Y397Z hPar1 were 5 and 5.8 times larger, respectively, than tumors produced by the MCF7/empty vector-transfected cells. Histological examination (H&E staining) showed that while both MCF7/wt hPar1 and MCF7/Y397Z hPar1 tumors infiltrated into the fat pad tissues of the breast, the MCF7/Y397Z hPar1 tumors further infiltrated the abdominal muscle (Fig. 1D). In contrast, tumors produced by empty vector or truncated hPar1-transfected cells were capsulated, with no obvious cell invasion. Proliferation levels were evaluated by immunostaining with Ki-67 and were 3 times higher in Y397Z hPar1 or wt hPar1 tumors (Fig. 2) than in the small tumors produced by either empty vector or truncated hPar1-transfected cells (p<0.0001, Fig. 2B). Tumor growth can also be attributed to blood vessel formation [13,14]. The hPar1-induced breast tumor vascularization was assessed by immunostaining with anti-lectin- and anti-CD31 antibodies. Both MCF7/Y397Z and MCF7/wt hPar1 tumors were intensely stained (Fig. 2A; Bi and iii). In contrast, only a few blood vessels were found in the small tumors of empty vector or truncated hPar1 [Fig. 2A; Bi and iii]. Thus, both MCF7/wt hPar1 and MCF7/Y397Z hPar1 cells were shown to effectively induce breast tumor growth, proliferation and angiogenesis, while the MCF7/truncated hPar1 and MCF7/empty vector-expressing cells had no significant effect.

**PAR₁ C-tail binds the Shc adaptor protein**

To identify proteins that associate with the PAR₁ C-terminus and participate in the tumor signaling pathway, we fused the cytoplasmic tail of hPar1 to a GST protein and used the construct as “bait” to specifically detect associated proteins. Lysates obtained from a highly metastatic breast carcinoma line (e.g., MDA-MB-435 cells) were assessed for binding to the GST-PAR₁ C-tail column. Amino acid sequence analysis of proteins bound to the column repeatedly indicated the presence of the Shc adapter protein. Indeed, application of MDA-MB-435 cell lysates onto a GST- PAR₁ C-tail column or a GST control column showed the three Shc isoforms specifically bound to the GST-PAR₁ C-tail column, but not to the GST control column (Fig. 3A). Shc isoforms refer to a series of proteins (e.g., 66, 52 and 46 kDa) termed Shc (Shc homology 2/α-collagen-related) [15,16]. cDNA analyses of the family proteins has demonstrated that the 46- and 52-kDa species arise from alternative translation initiation sites within the same transcript, giving rise to a 59-amino acid terminal truncation of the 46-kDa isoform compared to the 52-kDa isoform. In contrast, the 66-kDa species most likely arises from an alternatively spliced message since there is only one Shc gene and the carboxy terminal antibodies cross react with all three molecular weight species. Co-immunoprecipitation studies using either PAR₁ (Fig. 3B) or Shc antibodies (Fig. 3C) confirmed the PAR₁ -Shc association 5 min after TFLLRNPDK activation; this association remained high during the 30 min of analysis (Fig. 3B and C). The Shc protein comprises multiple protein docking sites, including SH2, phospho-tyrosine binding site (PTB) and collagen homology domains 1 and 2 (CH1, CH2). When a GST-Shc-SH2 domain pull-down assay was used following loading with PAR₁ activated MDA-MB-435 cell lysates, we obtained PAR₁ -specific binding to the Shc-SH2 domain. In contrast, when the tandem SH2 domain from an irrelevant protein was used as a control, no binding of PAR₁ was observed (Fig. 3D).

While searching for the PAR₁ C-tail putative tyrosine residues capable of undergoing phosphorylation and serving as possible binding sites for the Shc protein (NetPhos 2.0 server), we found four candidates: Y381, Y383, Y397, and Y420 (Fig. 3E and i). By these, only three were predicted to undergo phosphorylation: Y381, Y397 and Y420 (Fig. 3Eii). Since our preliminary data showed that Y397Z hPar1 was potent in signaling (Figs 1 and 2) and able to associate with Shc when transiently expressed in COS-1 cells (data not shown), we postulated that the Shc binding site(s) in the PAR₁ C-tail is/are located upstream of tyrosine 397. Indeed, sequence alignment of PAR₁ C-tail in nine different species demonstrates several highly conserved regions (data not shown), among which are the Y381/VAFY383 residues. Replacement of the relevant tyrosine (Y) residues upstream to Y397Z hPar1 with alanine (Ala, A) (e.g., Y381A or Y383A and the double mutant Y381A & Y383A) did not prevent the recruitment and physical association between Shc and PAR₁ (for more details see section of “Hierarchy of binding”, below).
Figure 1. PAR1 enhances tumor growth and angiogenesis in a xenograft mouse model. A. Schematic representation of the hPar1 constructs. wt hPar1, truncated hPar1 (devoid of the cytoplasmic tail), Y397Z hPar1 (shorter C-tail of persistent signaling). Aii. Semi-quantitative RT-PCR analysis of cells transfected with wt hPar1, Y397Z hPar1, truncated hPar1 or empty vector using primers to PAR1 N-terminus (upper panel), C-terminus (middle panel), or GAPDH (lower panel). PAR1 N-terminus primers were as follows: hPar1- sense: 5′-CTCGTCTCCTAGGAGCAAGC-3′, antisense orientation: 5′-TTGATCCTGAACTTTTCTTTG-3′ (564-bp PCR product). PAR1 C-tail primers – sense: 5′-TAC TAT TAC GCT GGA TCC TCT GAG-3′ and antisense: 5′-CTT GAA TTC CTA AGT TAA CAGCT-3′. These primers give rise to a 181-bp product corresponding to the entire PAR1 C-tail site, as follows: Y381A – YASSECQRYVYSILCCKESSDPSYNSSGQLMASKMDTCSSNLNNSIYKKLLT.

Y381A-hPar1 exhibits high metastatic potential

We further demonstrated the functionality of the Y381A hPar1 mutant in vivo using a colorectal-liver metastasis model (18), which provides a rapid metastatic model of liver foci formation. Mouse CT-26 colon carcinoma cells were genetically engineered to over-express wt hPar1, Y381A hPar1 or empty vector constructs. These over-expressing cells were injected intra-splenically into CB6F1 mice (either PAR1-activated or not) to generate liver metastases. Tumor growth kinetics and liver metastatic foci appearance were monitored twice a week by MRI. Both wt hPar1 and Y381A hPar1 enhanced liver metastatic foci formation, compared to control CT-26 cells. Furthermore, mice inoculated with cells expressing Y381A hPar1 showed especially extensive and rapid appearance of liver metastasis as compared to mice inoculated with cells over-expressing wt hPar1 (Fig. 4). Representative MRI images (Fig. 4A) of excised livers and histological sections (Fig. 4B), obtained on day 16, demonstrated high metastatic potential of both activated Y381A hPar1 and wt hPar1. An elevated number of metastatic foci were observed with the wt hPar1 after PAR1 activation (Fig. 4A), and an even more dramatic increase was obtained with the activated Y381A hPar1 construct (Fig. 4A, C). Quantification of liver metastasis as a function of time is shown in Figure 4C. These results emphasized that the Y381A hPar1 mutated construct is at least as functional as the wt hPar1, and the substitution of Y to A did not impair the ability of hPar1 to initiate signaling and therefore result in metastasis. The results may further suggest that PAR1, C-tail, since replacement of a key tyrosine residue by alanine (Y381A) does not impair PAR1 function as manifested by metastatic foci formation. It is thus postulated that whereas Shc is not associated with PAR1 via the traditional tyrosine-phosphorylated-SH2 complex formation, it probably involves a third mediator connecting with PAR1. The molecular mechanism of Y381A hPar1-enhanced liver metastasis remains to be fully elucidated.

Antibody-array for protein-protein interactions reveals signaling candidates

To detect the putative mediator(s) linking PAR1 to potential signaling proteins, we examined custom-made antibody-array membranes. For this purpose, aggressive breast carcinoma MDA-MB-435 cells (with high hPar1 levels) were incubated with the antibody-array membranes before and after PAR1 activation (15 minutes). This identified several activation-dependent proteins which interact with PAR1, including IGAM, c-Yes, Shc and Etk/
Bmx (see Figure S2). Of these proteins, we chose to focus here on Etk/Bmx and Shc.

The epithelial tyrosine kinase (Etk), also known as Bmx, is a non-receptor tyrosine kinase that is unique by virtue of being able to interact with both tyrosine kinase receptors and GPCRs [17]. This type of interaction is mainly attributed to the pleckstrin homology (PH) which is followed by the Src homology SH3 and SH2 domains and a tyrosine kinase site [18]. Etk/Bmx- PAR1 interactions were characterized by binding lysates exhibiting various hPar1 forms to GST-Etk/Bmx. While Y397Z hPar1 and wt hPar1 showed specific association with Etk/Bmx, lysates of truncated hPar1 or JAR cells (lacking PAR1) exhibited no binding (Fig. 5A). In order to substantiate the physical association between PAR1 C-tail and Etk/Bmx-PH domain we proteolytically cleaved the C-tail portion of both wt- and Y381A hPar1-modified tail and applied the purified fragments onto a GST-PH Etk/Bmx column. Specific binding was observed with both the wt hPar1 and Y383A hPar1 purified C-tails (Fig. 5B). Next, we analyzed various modified PAR1-GST-C tail constructs (e.g., wt hPar1, Y381A hPar1 and Y383A hPar1) for binding to either wt- or kinase-inactive Etk/Bmx (KQ) cell lysates [18,19]. A tight association between the PAR1 C-tail and Etk/Bmx was obtained, independent of whether wt hPar1 or the Y/A mutant forms of PAR1 C-tail were examined. This was true for both wt- and KQ- Etk mutant (Fig. 5C).

Differential expression of Etk/Bmx in breast biopsies

PAR1 is highly expressed in breast carcinoma specimens, but not in normal breast tissue, as evidenced by in situ hybridization analyses [5,20]. Immunohistochemical staining of PAR1 tissue sections confirms the earlier described RNA riboprobe analysis for hPar1. Invasive carcinoma specimens were selected from infiltrating ductal carcinoma (IDC) of high nuclear grade and with evidence of vascular invasion and lymph node metastases. Immunohistological analyses of both PAR1 and Etk/Bmx showed little staining in comedo DCIS and ductal carcinoma in situ, but high levels of staining in IDC and lobular carcinoma (Fig. 5D;
Table 1). These results further suggest a direct correlation between PAR1 and Etk/Bmx expression in malignant breast cancer progression. PAR1-Etk/Bmx association was also demonstrated by co-immunoprecipitation analysis of MDA-MB-435 cells (expressing both endogenous PAR1 and Etk/Bmx) (Figure S3B).

Hierarchy of binding

Next, we wished to determine the chain of events mediating the signaling of PAR1 C-tail-Shc and Etk/Bmx. To this end, analysis of MCF7 cells that express little to no hPar1 were ectopically forced to over-express hPar1. When co-immunoprecipitation with anti-PAR1 antibodies following PAR1 activation was performed, surprisingly, no Shc was detected in the PAR1 immunocomplex (Fig. 6A; MCF7/hPar1; right panel). Shc association with PAR1 was fully rescued when MCF7 cells were initially co-transfected with Etk/Bmx (Fig. 6A), with abundant assembly of Shc in the immunocomplex. Thus, Etk/Bmx is a critical component that binds to activated PAR1 C-tail and enables subsequent binding of Shc. Shc may bind either to phosphorylated Etk/Bmx, via the SH2 domain, or in an unknown manner to the PAR1 C-tail, provided that Etk/Bmx is present and is PAR1-bound. One cannot, however, exclude the possibility that Bmx binds first to Shc and only then does the complex of Etk/Bmx-Shc bind to PAR1.

Identification of PAR1-Etk/Bmx binding region: functional consequences

Peptides (representing various regions in PAR1 C-tail) were used in a competition analysis assay for the binding of PAR1 cell lysates to GST-PH-Etk/Bmx. An 18-amino-acid peptide encompassing residues 375–392 of PAR1 C-tail (e.g., termed peptide 4) yielded a dose-dependent inhibition within the range of 1–1000 nM applied peptide (Fig. 6B). Two other peptides, representing PAR1 C-tail 387–400 (Fig. 6Bc; termed peptide 5) or residues 393–412 (data not shown), did not compete.

Based on the competition assay we prepared mutated hPar1 constructs with successive replacement of seven residues (378–384;
CQRYVYS). MCF7 clones expressing either HA-hPar1-7A or HA-wt hPar1 showed the following outcome (characterization and proper expression of the mutant is shown in Figure S4): HA-wt following activation (Fig. 7A). We thus conclude that the critical region for Etk/Bmx binding to PAR1 C-tail resides in the vicinity of CQRYVYS.

Activated MCF7 hPar1-7A mutant cells (also expressing Etk/Bmx) failed to invade Matrigel-coated membranes, as compared to a potent invasion level obtained by activated wt hPar1 or Y381A hPar1 CT-26-transfected cells, obtained at day 16, in the absence (left panel) or presence (right panel) of SFLRN, are seen. Liver margins are marked with a dashed green line; yellow lines mark tumor foci; scale bar represents a size of 1 cm and applies to all the images in A. B. Activated (SFLRN) day 16

In a parallel experiment, a wound assay for the rate of cell migration, showing the ability of the cells to fill in gaps in an MDA-MB-435 cell monolayer, was performed. Rapid closure of the hPar1, but not HA-hPar1-7A, exhibited binding association with Etk/Bmx wound was observed following TFLLRNPNDK PAR1 activation. This PAR1-induced cell migration was markedly inhibited when the cells were infected with siRNA-Etk/Bmx construct to knock down the endogenous Etk/Bmx levels present in MDA-MB-435 cells (Fig. 7C, top panel). Similar inhibition was obtained in the presence of the PAR1 antagonist, SCH 79797 (Fig. 7C, bottom panel), pointing to the important role of both PAR1 and Etk/Bmx in wound closure/migration of PAR1-activated MDA-MB-435 cells. The highly ordered tissue organization of normal epithelia is aggressively disrupted in pathological conditions. This is well recapitulated in the MCF10A cell-growth model, mimicking epithelia apico-basal polarity (24). We examined the morphogenesis of MCF10A mammary acini in three-dimensional (3-D) basement membrane cultures. Normal-appearing intact spheroids are formed in the presence of control Etk/Bmx-expressing MCF10A cells and activation by SFLRN, a PAR1 agonist peptide (Fig. 8A; a, b). In contrast, in the presence of ectopically forced hPar1 (expressing also Etk/Bmx) and following PAR1 activation, an oncogene-like, migratory morpho-

Figure 4. Y381A hPar1 construct enhances liver metastasis formation. A. MRI analysis. CT-26 mouse carcinoma cells (that do not express endogenous hPar1), ectopically forced to express either wt hPar1 or Y381A hPar1 constructs, were injected intra-splenically into CB6F1 mice to generate liver metastases. Tumor assessment was performed using T2W fast SE images (TR/TE = 2000/40 ms). Representative axial liver sections of wt hPar1 or Y381A hPar1 CT-26-transfected cells, obtained at day 16, in the absence (left panel) or presence (right panel) of SFLRN, are seen. Liver margins are marked with a dashed green line; yellow lines mark tumor foci; scale bar represents a size of 1 cm and applies to all the images in A. B. Anatomical and histological examination. Gross anatomical photos (Top) and H&E staining of liver sections (harvested on day 16) of activated wt hPar1 or Y381A hPar1 CT-26 cells. T = tumor and N = normal; yellow lines mark tumor foci; original magnification ×100. C. Histogram of the number of liver metastases per mouse as measured by MRI. The experiments were performed in the presence or absence of the PAR1 agonist peptide (n = 3–5 mice/group). Activation of PAR1 accelerated both tumor size and the number of detectable foci as well as their time of appearance. X stands for sacrificed mice with overloaded liver tumors.

doi:10.1371/journal.pone.0011135.g004
 genesis was obtained which was characterized by a complete loss of the cell-cell tight junction contacts and the invaded basement membrane architecture (Fig. 8A; c and g). Significantly, when the MCF10A cells (in the presence of endogenously expressed Etk/Bmx) were infected with the mutant cytoplasmic form of hPar1 (hPar1-7A) and SFLLRNPDK PAR1-activated, nearly normal-appearing spheroid morphology was obtained (Fig. 8A; d, f). This outcome highlights the fact that by preventing immobilization of Etk/Bmx on PAR1 C-tail, inhibition of invasion and lack of apico-basal polarity morphogenesis of an oncogene-like phenotype in MCF10A cells are observed.

Discussion

In the present study we characterized the contribution of PAR1 signaling events in breast cancer progression. We utilized constructs of hPar1, including wt hPar1 and either deleted or mutant hPar1 forms, and analyzed them for their ability to induce tumor development in mouse mammary gland and colorectal-liver metastasis models in vivo, as well as characterizing them biochemically in vitro. Injection of cells expressing wt hPar1 or Y397Z hPar1 into mouse mammary glands resulted in pronounced mammary tumor growth and angiogenesis. The
truncated PAR1 construct, however, which lacked the cytoplasmic tail, was incapable of promoting PAR1-induced tumors in vivo. These findings emphasize the pivotal role of the cytoplasmic tail in PAR1 function. Antibody-array analyses for the detection of signaling partners that bind to PAR1 revealed the involvement of Shc and Etk/Bmx, among others. We describe herein a novel signaling complex formed between Etk/Bmx and Shc assembled onto the PAR1 C-tail, thus providing a

Table 1. Expression of PAR1 and Etk/Bmx in breast cancer biopsy specimens (representing Fig. 5D).

| Histological subtype | Cases (N = 36) | PAR1 | Etk/Bmx | PAR1 | Etk/Bmx | PAR1 | Etk/Bmx |
|----------------------|---------------|------|---------|------|---------|------|---------|
| Normal               | 12            | 0.8 ± 0.2 | 1.2 ± 0.2 | 0     | 1       | 0    | 0       |
| DCIS                 | 8             | 12.5 ± 3.7 | 14 ± 3.0 | 2 (25%) | 1 (12.5%) | 6 (75%) | 7 (87.5%) |
| IDC                  | 9             | 40 ± 10.5 | 42 ± 12.3 | 2 (22%) | 1 (11%) | 1 (11%) | 0       |
| Lobular carcinoma    | 7             | 45 ± 7.3 | 43 ± 11.6 | 1 (14%) | 0       | 1 (14%) | 1 (14%) |

Histological scoring of (N) cases: +1 less than 25% positive cells (weak positive); +2 between 25–75% positive cells (moderate); +3 more than 75% of positive cells (strong). All controls were negative (0–5% positive cells). Extent of expression classified by score (1–3), number of positive cells/field (x = 8).

doi:10.1371/journal.pone.0011135.t001

![Figure 6. Hierarchy of Shc and Etk/Bmx binding and identification of binding region on PAR1 C-tail. A. Etk/Bmx is needed for Shc association with PAR1 C-tail. MCF7 cells before (MCF7/hPar1) and after (MCF7/hPar1 & Etk/Bmx) forced Etk/Bmx and hPar1 co-immunoprecipitation with Shc. No Shc is immunoprecipitated with activated PAR1 when Etk/Bmx is absent (right panel: MCF7/hPar1; wt hPar1). In contrast, extensive co-immunoprecipitation is seen in the presence of forced Etk/Bmx (MCF7/hPar1 & Etk/Bmx). This is true regardless of the various constructs of hPar1 expressed: Y383A&Y381A hPar1, Y397Z hPar1, Y381A hPar1 or wt hPar1. The IP was performed using anti-Par1 (ATAP, Santa Cruz, CA). B. Peptide competition for PAR1 binding to GST-PH-Etk/Bmx. i. Peptide 4, encompassing residues 375–396 of PAR1 C-tail, competes with PAR1 for binding to the GST-PH-Etk/Bmx. Specific binding of PAR1 to the GST-PH-Etk/Bmx is inhibited in the presence of increased peptide concentration (200 nM-1 μM). Lower panel: Representative histograms show the relative intensities of the bands expressed as a ratio of PAR1 to GST. Bii. Peptide 5, representing another region (e.g., 379–402), does not compete at a concentration of 1 μM. GST protein serves as a loading control. Lower panel: Representative histograms show the relative intensities of the bands expressed as a ratio of PAR1 to GST. doi:10.1371/journal.pone.0011135.g006]
central docking site to facilitate tumor progression, invasion and angiogenesis. Studies of Par1-deficient mice revealed a critical role for PAR1 in blood vessel formation [13]. PAR1 induces tumor angiogenesis via the up-regulation of at least VEGF and Gro oncogenes (27,28). Furthermore, hPar1 gene withdrawal leads to the selective regression of immature but not mature blood vessels [21]. We show here that tumors generated by either wt hPar1 or persistent Y397Z hPar1 are capable of inducing marked vascularization, as compared to only a few blood vessels formed by the truncated hPar1-induced tumors. Together, these results emphasize a central role for PAR1 expression and signaling in tumor progression.

A critical role for Etk/Bmx and Shc was demonstrated by their selective recruitment to activated PAR1 C-tail. Moreover, the prime event of Etk/Bmx binding is a prerequisite for further PAR1-Shc association. We demonstrate herewith the hierarchy and sequence of events during PAR1 signaling in breast cancer progression. While PAR1-induced Shc phosphorylation has been previously reported [14,22,23], we provide here the first evidence for a physical association between Shc and PAR1. Substitution of tyrosine residues in PAR1 C-tail by alanine did not abrogate the recruitment of Shc to PAR1 tail, indicating that PAR1 tyrosine residues are not involved in the direct association between PAR1 and Shc. Furthermore, the mutant Y381A showed enhanced metastatic capabilities when compared to wt hPar1 in a colorectal-
liver metastasis animal model. This implies that the Y/A substitution (Y381A) most likely endows PAR1 with better accessibility to essential signaling proteins and thereby enables enhanced metastasis (a hypothesis that is currently under investigation). The fact that the tyrosine residues in PAR1 C-tail do not play a role in the binding of Shc implies the requirement of a third mediating partner. Indeed, Etk/Bmx binding to PAR1 C-tail through the PH-domain is required for PAR1-Shc association. One cannot, however, exclude the possibility that Bmx binds first to Shc and only then does the complex of Etk/Bmx-Shc bind to PAR1.

The fact that we have obtained tumors in vivo following implantation of either MCF7 cells over-expressing wt hPar1, or mutant hPar1-7A in the mammary fat pads of mice (Fig. 1), raises the issue as to the significance of Etk/Bmx. When we have immunostained for Etk/Bmx, xenograft sections derived from the tumors obtained of MCF7 cells over-expressing hPar1, in vivo in a manner that remains to be fully explored. One attractive possibility is that environmental cues in-vivo are responsible for the expression of Etk/Bmx in PAR1-derived tumors. It is plausible that the very low endogenous levels of Etk/Bmx originally present in MCF7 cells are markedly induced in vivo, due to micro environmental signals (in a yet unknown manner).

The recruitment of Etk/Bmx and Shc to PAR1 C-tail provides a bridge linking PAR1 with receptor tyrosine kinases (RTKs) and associated downstream signaling pathways. For example, the proto-oncogene Src plays a pivotal role in integrin activation during tumor progression [24]. While the C-terminal Src kinase consists of SH3, SH2 and kinase domains, it may be recruited to a phosphorylated site in Etk/Bmx (following PAR1 activation; see Figure 8. Morphogenesis of MCF10A spheroids infected with either wt hPar1, mutant hPar1-7A or with Etk/Bmx maintained in 3-D Matrigel cultures. A. Upper panel. Representative phase-contrast microscopic images of MCF10A cells under the following conditions: a. control untreated MCF10A. b. MCF10A cells infected with Etk/Bmx and SFLLRNPNDK PAR1-activated. c. MCF10A cells infected with both wt hPar1 and Etk/Bmx and SFLLRNPNDK-activated. d. MCF10A cells infected with both the mutant hPar1-7A and Etk/Bmx and SFLLRNPNDK-activated. e. Control untreated MCF10A, DAPI staining. f. hPar1-7A Etk/Bmx stained with SFLLRNPNDK, DAPI staining. g. hPar1 Etk/Bmx stained with SFLLRNPNDK, DAPI staining. h. Anti E-Cadherin, hPar1-7A+SFLLRNPNDK+Etk/Bmx.

B. Scheme taken from Debathn et al., Methods, 30 (2003) 256-268

C. RT-PCR analyses for the different MCF10A-infected cells. Levels of expression as compared to those of a house-keeping gene (GAPDH). A. MCF10A-infected wt hPar1 B. MCF10A infected with mutant hPar1-7A. C. Empty vector infected-MCF10A cells. D. Control, untreated MCF10A cells. E. MCF10A cells infected with Etk/Bmx.
doi:10.1371/journal.pone.0011135.g008
Whereas Etk has been shown to mediate integrin signaling by binding to the FAK FERM domain through the Etk PH domain [25], activation of PAR1 has also been shown to induce FAK phosphorylation and integrin signaling via cross talk with αβ3 integrin [26]. However, it is not known how PAR1 activation leads to αβ3 integrin activation and FAK phosphorylation. While, in breast cancer, activated PAR1 is engaged in binding Etk/Bmx via the PH domain, thus occupying this site, it is plausible that the Pro-712-713 proline-rich region of FAK (e.g., Pro-X-X-Pro-X-Arg) associates with the SH3 domain present in Etk/Bmx, connecting PAR1 to integrins via FAK association to Etk/Bmx. This possibility needs to be fully explored. It has also been demonstrated that the docking protein p130Cas forms a complex with Etk/Bmx. This protein plays a role in the formation of focal adhesion complex sites, the regulation of actin cytoskeleton reorganization and cell migration. Since Cas undergoes tyrosine phosphorylation prior to complex formation, it is feasible that phosphorylated p130Cas associates with the SH2 domain of PAR1-C-tail immobilized Etk/Bmx [27].

PAR1 induces activation of the small GTPase RhoA, which participates in formation of focal adhesions and in regulating the reorganization of the actin cytoskeleton. It was suggested that Etk/Bmx activates Rho A by releasing the GDI from the inactive RhoA-GDI complex through the interaction with Etk/Bmx PH domain [28]. Perhaps a balance is obtained between the free Etk/Bmx-PH domain in activating RhoA and the PAR1-C-tail immobilized Etk/Bmx-PH domain, in the delicate control of cytoskeletal reorganization and actin stress fiber formation during breast cancer progression.

Akt/PKB, first identified as the cellular homologue of the transforming oncogene v-Akt [29], is a core component of the phosphoinositide 3-kinase (PI3K) signaling pathway and effectively promotes cancer cell survival and proliferation. Activation of PI3K may be mediated via association of the SH2 domain. This scaffold assembly of PAR1-Etk/Bmx in Breast Cancer

Materials and Methods

Cell culture

MCF7 and MDA-MB-435 human breast carcinoma, CT-26 mouse colon carcinoma, HEK-293 cells and the African green monkey kidney fibroblast cell line COS1 (these cell lines were obtained from the ATCC, VA, USA) were maintained in DMEM with 10% fetal calf serum. Stable clonal cell lines over-expressing wt hPar1, Y381A&383A hPar1, G418 resistance (800 μg/ml) to PLoS ONE|www.plosone.org 11 June 2010 | Volume 5 | Issue 6 | e11135

Plasmons and transfection

MCF7 cells were transfected with 1–2 μg of either wt human hPar1 or truncated hPar1 or Y397Z hPar1 and Y/A mutants; T383A/Q384A hPar1 or the wt-Etk and Etk-KQ were selected for G418 resistance (800 μg/ml).

Previous research suggested the importance of this region in protein-protein interaction and signaling. Stable MCF7 clones expressing both wt hPar1 and Etk/Bmx exhibit enhanced invasion properties potently inhibited by a PAR1-specific antagonist. In contrast, low levels of invasion are shown by stable MCF7 clones of Etk/Bmx and hPar1-7A mutants. Similar data, further supporting the importance of Etk/Bmx in PAR1-induced migration, is demonstrated by wound-gap closure in cell monolayers. The key role of Etk/Bmx is also shown in the nearly normal acinar structure of MCF10A cell cultures maintained in a three-dimensional (3-D) environment in vivo. This 3-D cell culture mimics large, epithelial tissues in vivo, in the context of apico-basal polarized structure. The ectopic forcing of wt hPar1 in MCF10A cells, followed by activation, results in dramatic alterations of disrupted tight-junction contacts, complete loss of cell polarity and extensive protrusions of invaded basement membranes. Blocking Etk/Bmx binding to PAR1 by over-expressing hPar1-7A mutant in MCF10A cells maintained in 3-D basement membrane cultures abrogates the oncogene-like phenotype. Instead, nearly ball-shaped spheroids of apico-basal polarization are obtained. This outcome strongly supports a fundamental role for Etk/Bmx in PAR1-induced invasion. Our data contribute to a list of Etk/Bmx counter-partners, such as TNF, that mediate angiogenesis via binding to a 16-amino-acid sequence of TNFR2, which binds to multiple Etk/Bmx binding domains (PH, TEC and SH2) [37]. Similarly, the Etk-PH-domain has been shown to regulate integrin signaling, and promote cell migration through the specific association of FAK via the FERM domain [25]. In addition, Etk/Bmx immobilized to PAR1-C-tail may provide a central junction site for possible cross-talk between PAR1 and EGFR, as well as ErbB2/HER2, following PAR1 activation [38].

Our study identifies essential signaling partners in PAR1-mediated breast cancer progression, determines the hierarchy of binding and identifies a critical associating region in the PAR1 C-tail. This is the first demonstration of the PAR1 C-terminus serving as a scaffold tail. The identification of a PAR1 C-tail binding domain may provide a platform for new therapeutic vehicles in the treatment of breast cancer.
RNA isolation and RT-PCR

RNA was isolated with Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer’s instructions. After reverse transcription of 1 μg total RNA by oligo (dT) priming, cDNA was amplified using Taq DNA polymerase (Promega, Madison, WI). Comparative semi-quantitative PCR was performed using the following primers: GAPDH sense: 5'-GCC CCC ATG GCA AAT TCC ATG GC-3' and antisense: 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'; PAR1 N-terminus primers were as follows: hPar1-sense: 5'-CTCGTCCTCAAGGAGACAAGCGGTTTTG-3' and antisense orientation: 5'-TGGGATCGGAACTTTCATTCTTTG-3'.

Histological evaluation and scoring

The combined histological results were assessed and scored as previously described[40,41]. The measurements per slide section was carried out using anatomical compartments, using an ocular micrometer (WHIOX2, Olympus, New Jersey, USA). Slides review was independently performed by two investigators (BM and RB). Discrepancies were resolved by simultaneous re-examination of the slides by both investigators using a double-headed microscope. The microscope was calibrated with a micrometer slide before each measurement. All measurements were performed on the monitor screen using a x40 objective. On examining the sections for selection of fields tumor cells from the most cellular area at the center of the tumor were selected. Necrotic and inflammatory area were avoided. Eight microscopic fields were screened, 10 cells/field were selected and no less than 50 cells/tumor case were assessed. The positive rate of staining is expressed as a mean ± SD per tumor histological subtype from selected cases.

Immunohistochemistry

Sections were subjected to inactivation of endogenous peroxidase (3% H2O2 in DDW), antigen retrieval by microwave oven (3 min) in citrate buffer (0.01 M, pH 6.0), and blocking with 10% goat serum in PBS. Sections were then incubated with antibodies directed against Von-Willebrand factor (anti-factor VIII, DAKO, Carpinteria, CA), Ki-67 (Clone SP6, Lab Vision-NeoMarkers, Fremont, CA), or an endothelial cell-specific lectin (Banisteria simplicifolia BS-1 isolation) [42], followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (DAKO, Carpinteria, CA). Color was developed by incubation (10 min) with the Zymed AEC substrate kit (Zymed Laboratories, South San Francisco, CA), and counterstained with Mayer’s hematoxylin.

Preparation of hPar1 constructs: truncated hPar1, Y397Z hPar1, Y383A hPar1 and Y381A hPar1

Detection of hPar1 was carried out using primers: sense orientation: 5' - CTGGTCCTCAAGGAGACAAGCGGTTTTG-3', antisense orientation: 5'-TGGGATCGGAACTTTCATTCTTTG-3'. For the PAR1, C-tail primers: sense orientation: 5' - TACTATTACGCTGGATCCTCTGAG-3', antisense: 5'- CTGGGATCGGAACTTTCATTCTTTG-3'. These primers give rise to a 181-bp product corresponding to the entire PAR1, C-tail site, as follows:

YY - YASSECQRYVYSILCCKESSDPSYNSSQGLMASKMDTCSNLSNNSIYKYLKL

Liver metastasis model

CT-26 mouse colon carcinoma cells were stably transfected with either wt hPar1 or hPar1-Y381A constructs. The activation of PAR1 (using the peptide SFLLRN) was performed prior to injection into the mice. CB6F1 mice were anesthetized (75 mg/kg ketamine + 3 mg/kg xylazine, i.p.), and the spleen was exteriorized through an incision (1.0 cm) on the left side of the mouse. CT-26 cells (10⁴ cells/mouse) transfected with the different constructs (e.g., hPar1 wt, hPar1 Y381A, mock-transfected vector) were injected into the spleen using a 30-gauge needle. The cell suspension was allowed to settle in the spleen for 10 min. The wound was sutured and the animal was allowed to recover. MRI imaging was monitored every 2-3 days on a 4.7T Bruker Biospec spectrometer using a bird-cage coil. Tumor assessment was made by serial coronal and axial T2W fast SE images (TR/TE = 2000/40 ms). All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Hebrew University, Jerusalem, Israel (MD-107.05-4).

Histology

Tissue samples derived from the primary tumors were fixed with 4% formaldehyde in PBS, embedded in paraffin and sectioned (5-μm sections). After de-paraffinization and re hydration, sections were stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry using specific antibodies.

Animal studies: Mammary gland model

Female athymic nude mice at 6-8 weeks of age were preimplanted subcutaneously with pellets containing 1.7 mg β-estradiol (60-day release, Innovative Research of America, Sarasota, FL). Mouse mammary pads were then injected with 1×10⁷ MCF-7 cells stably transfected with hPar1 wt and mutant constructs (e.g., wt, Y397Z and truncated) or pcDNA3 control plasmid. Mice were monitored for tumor size by external caliber measurements (length and truncated) or pcDNA3 control plasmid. Mice were monitored every 2-3 days on a 4.7T Bruker Biospec spectrometer using a bird-cage coil. Tumor assessment was made by serial coronal and axial T2W fast SE images (TR/TE = 2000/40 ms). All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Hebrew University, Jerusalem, Israel (MD-107.05-4).

PAR1 activation

PAR1 was activated by the SFLLRN (H-Ser-Phe-Leu-Leu-Arg-Asn-NH2) peptide, the TFLLRNPNDK peptide, a selective PAR1 agonist, or thrombin (1 U/ml).

RNA isolation and RT-PCR

RNA was isolated with Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer’s instructions. After reverse transcription of 1 μg total RNA by oligo (dT) priming, cDNA was amplified using Taq DNA polymerase (Promega, Madison, WI). Comparative semi-quantitative PCR was performed using the following primers: GAPDH sense: 5'-GCC CCC ATG GCA AAT TCC ATG GC-3' and antisense: 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'; PAR1 N-terminus primers were as follows: hPar1-sense: 5'-CTCGTCCTCAAGGAGACAAGCGGTTTTG-3' and antisense orientation: 5'-TGGGATCGGAACTTTCATTCTTTG-3'.

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PAR1 activation

PAR1 was activated by the SFLLRN (H-Ser-Phe-Leu-Leu-Arg-Asn-NH2) peptide, the TFLLRNPNDK peptide, a selective PAR1 agonist, or thrombin (1 U/ml).

Histology

Tissue samples derived from the primary tumors were fixed with 4% formaldehyde in PBS, embedded in paraffin and sectioned (5-μm sections). After de-paraffinization and re hydration, sections were stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry using specific antibodies.
MDA-MB-435 cell lysates were applied to GST-PAR1 C-tail or immobilized on glutathione Sepharose beads (Pharmacia). Briefly, were lysed according to published procedures, and then serine 369 to residue 425, was prepared using RT-PCR (5'- TAT TAC GCT GGA TCC TCT GAG-3'). The amplified DNA fragment was digested with HindIII from PAR1 cDNA and cloned into a pcDNA3 plasmid, followed by DNA sequencing. To confirm the functional integrity of the DNA constructs, wt and mutant cDNAs were transiently expressed in COS-1 cells that were subsequently subjected to FACS analysis with a PAR1-specific antibody (WEDE15-PE, Immunotech, Cedex, France).

**HA-tag wt hPar1 and HA-mutant hPar1-7A C-tail constructs**

The mutants were designed for insertion of A at the carboxy terminus of PAR1 residues 378–384: SSE QRYYVSILCC (named hPar1-7A mutant). For HA-tag wt hPar1 construct PCR primers were designed and added downstream to the ATG start codon. Primers for the HA-tag are as follows: sense: 5'- TAC CCA TAC GAT GTT CCA GAT TAC GCT-3' and anti-sense: 5'-AGC GTG ATC TGG AAC ATC TA TGG GTA-3'. Replacement of seven residues with Ala A at positions 378–384 was made by synthesis of oligos containing the mutation. Primer sequences were as follows: hPar1 7A mutant: sense: 5'-TCT GAG GCT GGT GCT GCT GTA ATC TTA GAG TAC AGC TT-3' and anti-sense: 5'-TAA GAT AGC TGG AGC AGC AGC AGC CTC AGA -3'. PCR products were then used as primers on an hPar1 cDNA plasmid to create an extended product of introduced mutations into the full-length sequence. The amplified DNA fragment was digested with PinI and XhoI from PAR1 cDNA and cloned into pcDNA3-hPar1 plasmid followed by DNA sequencing.

**GST-C-tail cloning**

GST-C-tail of PAR1 fragment, containing 54 amino acids from serine 369 to residue 425, was prepared using RT-PCR (5'-TAC TAT TAC GCT GGA TCC TCT GAG-3' and 5'-CTG AAT TCC TAA GGT AAC AGC TT-3'). The resulting DNA fragment was further cut with the appropriate restriction enzymes (BanHI and EcoRI) and ligated into pGEX2T vector. The GST-C-tail was separated by SDS-PAGE, which indicated that the fusion protein of the C-tail was adequately prepared. The molecular weight of GST protein is 27 kD and the GST-C-tail fusion protein is 32 kD. GST-Shc-SH2 and tandem SH2 were kindly provided by S. Katzav, Hubert H. Humphrey for Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem.

**GST fusion protein columns**

Fusion proteins were purified from transformed Escherichia coli bacteria that had been stimulated with isopropyl-ß-D-thiogalactopyranoside (IPTG) at a concentration of 0.3 µM. Bacteria were lysed according to published procedures, and then immobilized on glutathione Sepharose beads (Pharmacia). Briefly, MDA-MB-435 cell lysates were applied to GST-PAR1 C-tail or GST control columns. After 2 h binding periods to the designated protein/s cell lysates to the columns, a washing step was performed. The washes [x3] were carried out using a "wash buffer" including: 100 mM NaCl, 20 mM EDTA, 10 mM Tris, pH 8.0 and 1% Triton X100. This step was performed in order to wash out non-specific proteins, leaving the GST-PAR1 C-tail column firmly bound to targeted cell lysate proteins. Next, elution of bound proteins was performed via the addition of gel “sample buffer” and appropriate boiling. The samples were run electrophoretically on SDS-PAGE gels, followed by immunoblotting with the indicated antibodies and ECL detection. The PH domain in Etk/Bmx was bound to GST column as previously described[25].

**Purification of PAR1 C-tail fragments**

PAR1 C-tail fragments were generated using a “thrombin cleavage capture kit” (Novagen, Madison, WI; Cat no. 69022-3). The enzyme used for the cleavage was bioynlated human thrombin. Briefly, the cleavage was performed according to the manufacturer instructions. Biotinylated thrombin was removed from the cleavage reaction using streptavidin agarose beads, and the cleaved peptides (e.g., wt PAR1 -C-tail and Y381A C-tail) were isolated and loaded on a GST-Etk-PH column. After incubation for 4 h the purified fragments were applied onto the GST-PH-Etk/Bmx column and detected following gel separation and western blotting analysis using anti-PAR1 antibodies (ATAP, Santa Cruz, CA).

**Flow Cytometry Analysis**

To activate PAR1, thrombin (1 U/ml) was added for 5 min. The cells were detached from the plates with 0.5 mM EDTA in 0.1 M sodium phosphate at pH 7.4 (Biological Industries), washed and re-suspended in PBS. The cells were analyzed by FACS after incubation for 60 min at 4°C with 10 µg/ml anti-PAR1-wede-PE antibodies.

**Western blot and immunoprecipitation analysis**

Cells were activated with agonist peptide TFLLRNPDNK for the indicated periods of time and solubilized in lysis buffer containing10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, and protease inhibitors (5 mg/ml aprotinin, 1 mM phenylmethlysulphonylfluoride, and 10 mg/ml leupeptin) at 4°C for 30 min. The cell lysates were subjected to centrifugation at 12,000 rpm at 4°C for 20 min. We used 400 µg of the supernatants with anti-PAR1 (ATAP, Santa Cruz, CA 1 µg), anti-HA (anti-HA sc-7392; Santa Cruz, CA), anti-Shc or Etk/Bmx antibodies (10 µg/ml). After overnight incubation, Protein A-Sepharose beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) were added to the suspension (50 µl), which was rotated at 4°C for 1 h. The immunocomplexes were eluted and run electrophoretically on a 10% SDS-PAGE gel, followed by transfer to an Immobilon-P membrane (Millipore). Membranes were blocked and probed with 1 µg/ml amounts of the appropriate antibodies as follows: anti-PAR1 thrombin receptor mAb, (ATAP, from Santa Cruz, 1:1000); anti-Shc (BD, 1:2000); anti-Bmx (Transduction Laboratories, 1:1000) or anti-PY (Upstate 4G10, 1:2500), suspended in 3% BSA in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5% Tween-20. After washes the blots were incubated with secondary antibodies conjugated to horseradish-peroxidase. Immunoreactive bands were detected by enhanced chemiluminescence (ECL). Membranes were stripped and incubated with anti-IP antibodies to ensure equal protein load.

Anti- PAR1 polyclonal antibodies were generated using two regions of the N-terminus portion R/SFFLRN by the synthetic peptides NH2-CQRYVYSILCC-CLLRNPNDKYEPFWED-COOH and NH2-KAAAAAAA-AAAAAAAA-A. Antibody array

A custom-made antibody array (hypromatrix) containing thirty antibodies against suspected proteins was prepared (see Fig. S2). The antibodies were immobilized on a membrane, each at a predetermined position, and they retained their capabilities of...
recognizing and capturing antigens as well as antigen-associated proteins. MDA-MB-453 cells were activated for 10 min with thrombin (1 U/ml). Untreated or activated cells were lysed with Triton extraction solution: 15 mM Tris, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, 0.5% Triton X-100, 10 μg/ml leupeptin and 0.5 mM PMSF. Protein extract was incubated on pre-blocked membrane for 2 h at room temperature. The antibody array was washed with TBST and incubated with biotinylated PAR1 antibody (ATAP) for 2 h at room temperature. The antibody array was washed again with TBST, and the membrane was incubated with HRP-conjugated streptavidin for 1 h. Protein-protein interactions were detected by ECL and exposure to X-ray film.

Matrigel invasion assay

Blind-well chemotaxis chambers with 13-mm diameter filters were used for this assay. Polyvinylpyrrolidone-free polycarbonate filters, 8 mm pore size (Costar Scientific Co., Cambridge, MA), were coated with basement membrane Matrigel (25 μg/filter), as previously described [45]. Briefly, the Matrigel was diluted to the desired final concentration with cold distilled water, applied to the filters, and dried under a hood. Cells (2×10^5) suspended in assay medium containing 4% Matrigel and 10 ng/ml EGF was coated eight-chambered slide. Assay medium containing 4% Matrigel per well and left to solidify for 15 min. The cells were resuspended at a concentration of 10^5 cells per 4.0 ml. Eight filters at 37°C for 18 h on the lower surface of the filter and cells within each field were removed by wiping with a cotton swab. The filters were fixed and stained with DifQuick ECL and exposure to X-ray film.

MCF10A morphogenesis assay

The assay was performed as previously described (24). In brief, while the MCF10A cells were maintained in DMEM/F12 medium with 20% donor horse serum, the cells for spheroid assay (DMEM/F12 supplemented with 2% donor horse serum, 10 μg/ml insulin, 1 ng/ml cholaera-toxin, 100 μg/ml hydrocortisone, 50 μ/ml penicillin and 50 μg/ml streptomycin) were resuspended at a concentration of 10^5 cells per 4.0 ml. Eight-chambered RS glass slides (Nalgene) were coated with 35 μl Matrigel per well and left to solidify for 15 min. The cells were mixed 1:1 with assay medium containing 4% Matrigel and 10 ng/ml EGF, and 400 μl were added to each chamber of the Matrigel-coated eight-chambered slide. Assay medium containing SFLLRNPNDK PAR1 activation peptide and 5 ng/ml EGF was replaced every 4 days. The images were taken between days 8–12. In the representative experiment shown images were taken on day 10. The media and supplements were replaced every 4 days and thus, the activating peptide was added fresh to the medium every 4 days.

Supporting Information

**Figure S1** Surface expression of various hPar1 constructs (e.g., deletion constructs and Y/A mutations) following ectopic insertion to MCF7 cells. A. PAR1 constructs (e.g., wt, deleted constructs and Y/A mutations). Schematic representation of hPar1 deletion constructs derived from human PAR1 cDNA. Mutations of Y/A insertions of the functional relevant Y residues in PAR1 C-tail (e.g., Y381A hPar1 and the double mutant Y381A & Y383A hPar1) whereby replacement of Y/A at positions 381 and 383 of PAR1 C-tail was performed. B. FACS (fluorescent activated cell sorter) analysis. Flow cytometric analysis of surface-expressed wt hPar1, hPar1 deletion constructs, Y381A hPar1 and the double mutant Y381A & Y383A hPar1. Constructs were transiently expressed in COS-1 cells and surface expression was determined by flow cytometry analysis using anti-PAR1 abs (WEDE-PE 2584, Immunotech), directed to detect cell surface levels of PAR1 (analyses performed on intact cells). Empty peak - represents the isotype control antibody alone; black peak - represents PAR1 antibody. C. Histograms representing the surface expression of wt, deleted and mutant hPar1 constructs before and after activation. Surface expression levels of the various hPar1 constructs (e.g., wt hPar1, truncated hPar1, Y381Y hPar1, Y381A hPar1 and Y381A & Y383A hPar1) transfected into COS-1 cells were determined. The various COS-1 transfected cells were evaluated by FACS analysis before (open bars) and after (black bars) a 30-minute activation with thrombin. Similar results were obtained in MCF7 cells expressing the hPar1 various constructs (data not shown). D. PAR1 expression levels in MCF7 cells transfected with various hPar1 constructs. Western blot analysis of MCF7 cells expressing either empty vector (A) or Y381Y hPar1 (B), truncated hPar1 (C), the double mutant Y381A & Y383A hPar1/D, Y381A hPar1 (E), as also as hPar1 (F). Levels of protein loading were evaluated by b-actin.

**Figure S2** Antibody-array of protein-protein interactions and physical association between PAR1 and the signaling partner Etk/Bmx. A. Custom-made antibody array. Table lists antibodies embedded on membranes showing the orientation map to create the custom array, as described in Materials and Methods. B. Lysates of MDA-453 cells before (i) and after (ii)thrombin (1 U/ml, 15 min) activation were applied to the membranes. Specific PAR-1 binding to the array was detected via incubation with biotinylated anti-PAR1 antibodies.

**Figure S3** The phosphorylation status of Etk/Bmx associated PAR1 following PAR1 activation. Ai. HEK-293 cells were transfected with either wt Etk/Bmx or inactive KQ kinase -Etk/ Bmx. Lysates were immunoprecipitated with anti Bmx and western blotted with 4G10 abs to detect levels of phosphorylation. Western blot analysis shows the levels of either endogenous Etk/ Bmx (lanes c & d) or ectopically enforced Etk/Bmx (a & b) as compared to a house keeping gene b-actin. Aii. Endogenous levels of Etk/Bmx in HEK-293 cells. Western blot analysis was performed in lysates of HEK-293 cells before (c,d) and after (a,b) transfection with Etk/Bmx constructs. The equal loading levels were determined by a house keeping b-actin protein levels. B. PAR1-Bmx association. MDA-435 cells were TFLLRNPNDK-activated. Lysates were co-immunoprecipitated with anti-PAR1 antibodies, and eluted proteins were detected with Bmx antibodies. A strong association between PAR1 and Etk/Bmx was observed as early as 1 minute after activation reaching maximal levels after 10 minutes.

**Figure S4** Characterization of MCF7 clones of HA-tagged wt and mutants of hPar1 constructs. A. FACS analysis of MCF7 clones. Surface expression of HA-wt hPar1 and HA-hPar1-7A, was determined by using anti-PAR1 abs (WEDE-PE 2584, Immunotech). Empty peak - represents the isotype control antibody alone; black peak - represents PAR1 antibody. B. Histogram representing surface levels of the constructs (e.g., HA-wt hPar1 and HA-hPar1 7A) as determined by FACS analysis. C. Western blot analysis of MCF7 cells transfected with empty vector and representative
MCF7 clones (e.g., HA-wt hPar1 and HA-hPar1-7N). The protein levels are compared to a house keeping of b-actin protein levels. Found at: doi:10.1371/journal.pone.0011135.s004 (2.05 MB TIF)

**Figure S5** Immuno histological staining of Etk/Bmx in sections of mouse mammary tumor xenografts generated following implantation of MCF7 cells expressing wt hPar1 and variant constructs. MCF7 cells expressing various hPar1 forms (e.g., wt hPar1, truncated hPar1, Y597Z hPar1 and empty vector) were inoculated into the mammary fat pads of mice. After 45 days the tumors were excised and embedded with paraffine. Antibodies directed against Etk/Bmx (Transduction Laboratories; BD Biosciences, California) were applied on sections derived from each of the designated treatment. The right panel represents staining in the absence of anti Etk/Bmx antibodies and presence of a secondary antibody - only (for controls). As one can note, specific Etk/Bmx staining is observed in wt hPar1 and particularly strong staining is noticed in Y597Z hPar1 (Mag ×200). No staining is observed in either the truncated hPar1 or empty vector sections. This staining is a representative experiment of three times staining experiments performed on these mice mammary xenograft sections.

Found at: doi:10.1371/journal.pone.0011135.s005 (2.05 MB TIF)

**Acknowledgments**

We thank members of the lab for stimulating and helpful discussions. We would also like to thank Cheryl Balbshay for assisting in manuscript editing and the team of MRI analysis group at Hadassah-Hebrew University Hospital for excellent technical work and data processing analysis.

**Conceived and designed the experiments:** IC HT SGG RBS. Performed the experiments: IC MM EW RA EG OB. Analyzed the data: IC MM HT BM BU RA RBS. Contributed reagents/materials/analysis tools: YQ. Wrote the paper: IC RA RBS.

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