Published in final edited form as:

Breast Cancer Res Treat. 2011 November ; 130(2): 377–385. doi:10.1007/s10549-010-1312-2.

Significance of ER–Src axis in hormonal therapy resistance

Sreeram Vallabhaneni, Binoj C. Nair, Valerie Cortez, Rambabu Challa, Dimple Chakravarty, Rajeshwar Rao Tekmal, and Ratna K. Vadlamudi

Department of Obstetrics and Gynecology and CTRC, The UT Health Science Center at San Antonio, 7703 Floyd Curl Drive, Mail Code 7836, San Antonio, TX 78229-3900, USA

Abstract

The estrogen receptor (ER) is implicated in the progression of breast cancer. Despite positive effects of hormonal therapy, initial or acquired resistance to endocrine therapies frequently occurs. Recent studies suggested ER α -coregulator PELP1 and growth factor receptor ErbB2/HER2 play an essential role in hormonal therapy responsiveness. Src axis couples ER α with HER2 and PELP1, thus representing a new pathway for targeted therapy resistance. To establish the significance of ER–Src axis in PELP1 and HER2 mediated therapy resistance, we have generated model cells that stably express Src-shRNA under conditions of PELP1, HER2 deregulation. Depletion of Src using shRNA substantially reduced E2 mediated activation of Src and MAPK activation in resistant model cells. Pharmacological inhibition of Src using dasatinib, an orally available inhibitor substantially inhibited the growth of therapy resistant MCF7–PELP1, MCF7–HER2, and MCF7–Tam model cells in proliferation assays. In post-menopausal xenograft based studies, treatment with dasatinib significantly inhibited the growth of therapy resistant cells. IHC analysis revealed that the tumors were ER α positive, and dasatinib treated tumors exhibited alterations in Src and MAPK signaling pathways. Combinatorial therapy of tamoxifen with dasatinib showed better therapeutic effect compared to single agent therapy on the growth of therapy resistant PELP1 driven tumors. The results from our study showed that ER–Src axis play an important role in promoting hormonal resistance by protooncogenes such as HER2, PELP1, and blocking this axis prevents the development of hormonal independence in vivo. Since PELP1, HER2, and Src kinase are commonly deregulated in breast cancers, combination therapies using both endocrine agents and dasatinib may have better therapeutic effect by delaying the development of hormonal resistance.

Keywords

Therapy resistance; Estrogen receptor; HER2; ER; Src; PELP1; Breast cancer; Extranuclear signaling

Introduction

The estrogen receptor (ER) is implicated in the progression of breast cancer [1]. Endocrine therapy using tamoxifen, a selective estrogen receptor modulator (SERM) improves relapse-free and overall survival [2]. More recently aromatase inhibitors, which deplete peripheral estrogen (E2) synthesis, are shown to substantially improve disease-free survival in postmenopausal women [3]. Endocrine therapy also has a positive effect on the treatment of

advanced metastatic disease. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs [4]. Although the mechanisms for hormonal therapy resistance remains elusive, and emerging evidence implicates human epidermal growth factor receptor-2 (HER2/ErbB2), mitogen-activated protein kinase (MAPK), and protein kinase B (AKT) pathways in development of therapy resistance [5, 6].

In addition to its well-studied nuclear functions, ER also participates in extranuclear signaling events in the cytoplasm and membrane [7]. Such signaling has been linked to rapid responses to estrogen which generally involves the stimulation of the Src kinase, MAPK, and AKT [8, 9]. Accumulating evidence suggests that ER α coregulators play an essential role in hormonal responsiveness and cancer progression [8, 10, 11]. Proline, glutamic-acid, and leucine-rich protein 1 (PELP1) is an ER α -coregulator that functions in nuclear as well as in extranuclear actions [12]. PELP1, a recently discovered proto-oncogene [13], exhibits aberrant expression in many hormone-related cancers [14] and is a prognostic indicator of shorter breast cancer-specific survival and disease-free intervals when over-expressed [15].

Proto-oncogene c-Src is a multifunctional intracellular tyrosine kinase implicated in the regulation of a variety of processes including proliferation, differentiation, survival, and motility [16]. Src interacts with multiple cellular factors including HER2, ER α , PELP1 and breast tumors frequently over-express Src kinase [17]. Dasatinib (Trade name SPRYCEL), an orally available inhibitor of Src family tyrosine kinases that is currently approved by FDA for leukemia, is now in phase I and II clinical trials for treatment of solid tumors [18, 19]. Recent evidence suggest that ER α -coregulator PELP1 plays an essential role in coupling ER α with Src kinases leading to hormonal resistance and that PELP1-Src interactions play an important role in PELP1 functions [14].

In this study, we examined whether ER α -Src axis constitutes a critical pathway used by breast cancer cells for developing resistance. Using Src-shRNA, Src inhibitor dasatinib and therapy resistant model cells, we demonstrate that a functional Src axis is essential for ER α -coregulator mediated extranuclear actions. Our results also suggest that ER α -PELP1 coregulator-Src axis plays an important role in promoting hormonal resistance by oncogenes and blocking this axis reduces growth of therapy resistant cells in vivo and that dasatinib may have a therapeutic potential of treating hormonal therapy resistance.

Materials and methods

Reagents

MCF7 cells were purchased from American-type culture collection (ATCC, Manassas, VA). 17 β estradiol, tamoxifen, and Actin antibody were purchased from Sigma Chemical Co (St. Louis, MO). PELP1 antibody was purchased from Bethyl laboratories (Montgomery, TX). Antibodies against phospho-AKT and total AKT, phospho-MAPK and total MAPK, phospho-Src and total c-Src were purchased from Cell Signaling (Beverly, MA). Dasatinib was obtained from LC Laboratories (Woburn, MA).

Model cells

MCF7-PELP1 cells [20], MCF7-HER2 [21], and MCF7-Tam cells [21] were earlier described. MCF7-PELP1 and MCF7-HER2 cells stably expressing Src-shRNA were generated using validated Src-shRNA lentiviral particles (SHCLMV) purchased from Sigma and using Puromycin selection (1 μ g/ml).

Western blotting

Model cells were cultured in RPMI Media containing 5% Dextran Charcoal treated Serum for at least 48 h prior to estrogen treatment (100 nM). Cells were washed with phosphate buffer saline (PBS) after 5 min of treatment and lysis was done using RIPA buffer containing phosphatase and protease inhibitors and samples were run on either 7% or 10% SDS-PAGE. Western blot analysis with either phospho antibodies or total antibodies was performed as previously described [22].

Cell proliferation assay

Cell proliferation rate was measured using a 96-well format with Cell Titer-Glo Luminescent Cell Viability Assay (Promega; G7572). 5×10^3 cells were plated in each well of a Corning® 96 well flat clear bottom, opaque wall microplates and cultured in RPMI Media containing 2.5% DCC treated serum for 24 h and followed by treatment with or without estrogen (100 nM/well) for another 72 h. Luminescence was recorded using automatic Fluoroskan Luminometer as per the manufacturer's recommendation.

In vivo tumorigenesis assays

For tumorigenesis studies, model cells (5×10^6 cells) were implanted subcutaneously into the flanks of 6- to 7-week-old female nude mice as described [23]. Nude mice (nu/nu) were injected with control MCF7 cells or MCF7 cells that over-express PELP1 by mixing them with equal volume of Matrigel. Because athymic mice were deficient in adrenal androgens, they were supplemented with sub-cutaneous injections of the aromatase substrate androstenedione (100 lg/day) for the duration of the experiment as described for the postmenopausal model [24]. Treatment was initiated after 3 weeks of inoculation and treatment with dasatinib (15 lg/mouse/day/oral), or combination with tamoxifen (100 lg/mouse/day/subcutaneous) or letrozole (15 lg/mouse/day) was continued for 6 weeks. Tumor volumes were measured with a vernier caliper at weekly intervals. After 6 weeks, mice were euthanized, and tumors were removed, weighed and processed for IHC staining. Tumor volume was calculated using a modified ellipsoidal formula: tumor volume = $1/2(L \times W^2)$, where L is the longitudinal diameter and W is the transverse diameter [25,26].

Immunohistochemistry

Immunohistochemical analysis was performed using a method as described [27]. PELP1 antibody (IHC-00013, 1:750) from Bethyl Lab, ER α (SC-7207; 1:50), and phospho-Tyr419-c-Src (sc-101802; 1:50) from Santa Cruz Biotech, Inc, phospho-MAPK (4376s; 1:100) from Cell Signaling, PCNA (VP-P980; 1:100) from Vector Lab were used in conjunction with proper controls, visualized by DAB substrate (Vector Lab) and counterstained with hematoxylin (Vector Lab, Inc. CA). TUNEL analysis was done using the In situ Cell Death Detection Kit (Roche; 11684 795910) as per the manufacturer's protocol. Apoptotic cells were identified by positive TUNEL staining and five randomly selected microscopic fields in each group were used to calculate the relative ratio of TUNEL-positive cells.

Statistical analysis

Statistical differences among groups were analyzed with either t test or ANOVA as appropriate using SPSS software.

Results

Functional Src kinase axis is needed for PELP1 dependent ER actions

Recent studies established PELP1 as an independent prognostic indicator of shorter breast cancer-specific survival and disease-free intervals [15]. In earlier studies, we have established breast cancer model cells with stable expression of PELP1 (MCF7–PELP1) to mimic the situation commonly seen in a subset of breast tumors. These model cells express two- to threefold more expression of PELP1 compared to endogenous levels of PELP1 and exhibit increased E2 mediated proliferation [28], therapy resistance [29], and tumorigenesis in xenograft models [13]. To study the in vivo significance of Src kinase in PELP1 mediated actions, we established MCF7–PELP1 model cells (pooled clones) stably expressing Src-shRNA using a lentivirus system with puromycin selection. Western blot analysis of total lysates from PELP1–Src-shRNA clones revealed that the Src-shRNA down regulated Src expression to ~75% of the level seen in the parental MCF7–PELP1 and the vector-transfected control clones (Fig. 1a). Src knockdown did not affect the expression of ER α in these clones (Fig. 1b). Since PELP1 participates in ER α -extranuclear actions, we examined the significance of endogenous Src in the activation of ER α -extranuclear signaling pathways. We measured the activation of signaling pathways including Src, and MAPK after treating cells with estrogen (E2) for 5 min. Estrogen addition uniquely promoted activation of Src and MAPK pathways in MCF7 cells. As observed before, MCF7–PELP1 cells showed further increase in activation of MAPK compared to MCF7 cells. Src-shRNA-expressing MCF7–PELP1 cells had significantly less Src, and MAPK activation (Fig. 1c). We then examined whether Src down regulation affected PELP1-mediated increase in E2 driven proliferation using a Cell Titer-Glo assay. PELP1 expression increased estrogen-mediated cellular proliferation compared to MCF7 cells, while Src downregulation in MCF7–PELP1 clones diminished its ability to increase cell proliferation (Fig. 1d).

HER2-mediated ER extranuclear actions requires Src kinase

Deregulation of HER2 expression/signaling has emerged as the most significant factor in the development of hormonal resistance [9, 20] and cross-talk between the ER and HER2 pathways has been shown to promotes endocrine therapy resistance [20, 21]. ER α -coregulator PELP1 interacts with HER2 and is implicated in facilitating the ER α crosstalk with HER2 signaling pathways [29]. To examine whether Src axis plays a role in HER2 mediated ER α extranuclear actions, we have down regulated Src kinase using shRNA delivery. We have established two pooled clones of Src-shRNA in a MCF7–HER2 background, a well-established model cell for HER2 deregulation [21]. Western blot analysis of HER2–Src-shRNA clones revealed 85-90% decrease in Src expression compared to the level seen in the parental MCF7–HER2–vector clone (Fig. 2a). Src knockdown did not significantly affect the expression of ER α in these clones (Fig. 2b). To examine the significance of endogenous Src in the HER2 mediated activation of ER α -extranuclear signaling, we measured the activation of Src, and MAPK after treating cells with estrogen for 5 min. Estrogen addition uniquely promoted activation of Src and MAPK pathways in MCF7 cells and MCF7–HER2 cells showed excessive activation of MAPK and AKT pathways. However, Src-shRNA-expressing MCF7–HER2 cells had significantly less AKT and MAPK activation (Fig. 2c). In cell proliferation assays, MCF7–HER2 cells showed significantly increased proliferation compared MCF7 cells, while Src-shRNA-expressing MCF7–HER2 clones showed decreased proliferation compared to parental MCF7–HER2 cells (Fig. 2d). Collectively, these results suggest that functional Src axis is necessary for HER2 mediated ER extranuclear actions and proliferation.

Dasatinib blocks estrogen-mediated ER extranuclear actions in therapy resistant cells

Earlier studies have shown that MCF7–PELP1 and MCF7–HER2 model cells exhibit hormonal therapy resistance. Since our findings suggested that Src kinase plays a key role in estrogen-mediated extranuclear signaling in these cells, we examined the effect of pharmacological inhibition of Src kinase using dasatinib, a well-established orally available inhibitor of Src family tyrosine kinases [19]. In addition to inhibiting SRC in the subnanomolar range, dasatinib also variably inhibits other SFKs, c-KIT, PDGFR, and ephrin A2 [30]. As a second model, we have used MCF7–Tam model cells, a well-studied model cells that exhibit acquired resistance to tamoxifen [21]. Short time estrogen treatment of MCF7 cells resulted in increased activation Src and MAPK pathways (Fig. 3a). Interestingly, all three resistant model cells have constitutively higher levels of Src activation and estrogen treatment substantially increased activation of MAPK in these model cells compared to therapy sensitive MCF7 cells (Fig. 3a). Dasatinib pretreatment abolished estrogen-mediated activation of Src and MAPK pathways in therapy sensitive MCF7 and also in all three therapy resistant models (Fig. 3a). In estrogen driven proliferation assays, dasatinib (200 nM) treatment substantially reduced PELP1 mediated increase in estrogen driven cell proliferation (Fig. 3b). Similarly, dasatinib (200 nM) treatment also reduced the proliferation of therapy resistant MCF-Tam and MCF7–HER2 cells. Collectively, these results suggest that Src signaling plays a role in proliferation of therapy resistant cells and dasatinib can potentially be used to reduce estrogen-mediated extranuclear signaling in therapy resistant cells.

Dasatinib decreases PELP1 oncogenic potential in vivo

PELP1 deregulation promotes in vivo tumorigenesis [13]. PELP1 also promotes local estrogen synthesis via Src kinase pathway facilitating growth of tumors in an autocrine manner [31]. Therefore, we hypothesized that PELP1 driven tumors can be therapeutically targeted using dasatinib and performed a proof of principle experiment using a post-menopausal xenograft model. Nude mice (nu/nu) were injected with control MCF7 cells or MCF7–PELP1 cells that overexpress PELP1 by mixing them with equal volume of Matrigel. Because athymic mice were deficient in adrenal androgens, they were supplemented daily with s.c. injections of the aromatase substrate androstenedione (100 lg/day) for the duration of the experiment. Under these conditions, injected MCF7 cells did not form tumors. As observed before, MCF7–PELP1 expressing cells formed tumors in the absence of exogenous estrogen supplementation suggesting local derived estrogen supported the growth of MCF7–PELP1 cells. When the tumor volume reached 100 mm³, mice ($n = 4$) were either treated with dasatinib (15 mg/kg/day/oral) or treated with vehicle (citrate buffer). Dasatinib treatment significantly reduced the PELP1-driven tumor volume (Fig. 4a) and tumor weight (Fig. 4b). Dasatinib treated tumors revealed decreased proliferation as evidenced by decreased nuclear PCNA staining and exhibited decreased Src and MAPK kinase activity seen by diminished phospho antibody staining (Fig. 4c). These results suggested that functional Src-MAPK axis is needed for PELP1 mediated tumorigenesis in vivo and dasatinib can be potentially used to reduce PELP1 mediated tumor growth.

Dasatinib in combination with antiestrogens reduces PELP1 mediated therapy resistance

PELP1 deregulation is known to promote tamoxifen resistance [29, 32]. We therefore examined whether dasatinib reduces PELP1-mediated therapy resistance. Xenografts established as described in earlier section were randomly assigned to groups ($n = 4$) and treated with tamoxifen, letrozole, dasatinib alone or in combination. As seen in previously published studies [32], tamoxifen did not affect the growth of PELP1 driven tumors, while dasatinib substantially inhibited tumor volume (Fig. 5a) and weight (Fig. 5b). Combination treatment of tamoxifen with dasatinib showed further decreases in tumor volume and growth compared dasatinib alone, however, the differences are not statistically significant.

Similarly, letrozole, an aromatase inhibitor, substantially reduced PELP1 driven tumor growth underscoring the importance of locally synthesized estrogen in PELP1 driven tumor growth, however, the combination of letrozole with dasatinib only slightly enhanced the therapy response. IHC examination of PCNA staining revealed that dasatinib treatment decreased proliferation of tumor cells, while TUNEL staining showed increased apoptosis in dasatinib treated tumor cells. Collectively, these results suggest that pharmacological inhibition of Src could be used to treat therapy resistance induced by deregulation of proto-oncogene PELP1.

Discussion

Estradiol (E2), ER, and ER coregulators have been implicated in the development and progression of breast cancer. Two thirds of breast tumors express ER α and women having ER α ve tumors are treated with endocrine therapy and tumors appear to use adaptive mechanisms for growth after the initiation of first-line endocrine therapy and hormonal therapy resistance is a major clinical problem [33]. In this study, we found that; (a) functional Src axis is needed for optimal activation of ER α extranuclear actions, (b) Src plays a key role in PELP1 and HER2 oncogene mediated ER α extranuclear actions and proliferation, (c) Excessive ER α extranuclear signaling in therapy resistant cells is inhibited by pharmacological inhibition of Src, (d) Functional Src axis is needed for PELP1 mediated oncogenic functions in vivo, and (e) Combination therapy of Src inhibitor with antiestrogens is more effective in blocking therapy resistance. Collectively, these results suggests that deregulation of PELP1 axis has the potential to contribute to breast cancer progression and therapy resistance by accelerating ER extranuclear actions.

It is increasingly clear that ER α also participates in cytoplasmic and membrane-mediated signaling events (extragenomic signaling) and generally involves cytosolic kinases including Src, MAPK, PI3K [7, 34]. Accumulating evidence strongly suggests that ER signaling requires coregulatory proteins and their composition in a given cell determine the magnitude and specificity of the ER α signaling [35, 36]. Some evidence suggests that the extranuclear effects of estrogen can regulate different cellular processes, such as proliferation, survival, and apoptosis. However, the pathological significance of ER extranuclear signaling and its role in therapy resistance remain unknown. Our results show that therapy resistant cells exhibit excessive activation of ER α extranuclear actions and blockage of endogenous Src axis either by Src specific shRNA or Src inhibitors significantly attenuated ER extranuclear actions and resulted in reduced proliferation. Collectively, our results suggest that ER α extranuclear action involves Src kinase and deregulation of Src kinase seen in breast tumors may have implications for potential activation of ER α extranuclear actions leading to therapy resistance.

PELP1, a recently discovered proto-oncogene [13], exhibits aberrant expression in many hormone-related cancers [14] and over-expression of PELP1 is a prognostic indicator of shorter breast cancer-specific survival and disease-free intervals [15]. In our studies, we found that PELP1-driven tumors are ER α positive and have excessive activation of Src and MAPK pathways. Inhibition of Src pathway using the orally available Src kinase inhibitor dasatinib substantially reduced PELP1-driven tumor growth with concurrent reduction in the activation of Src and MAPK pathways. These results further implicate that PELP1-Src axis mediated ER extranuclear actions may play role in breast tumorigenesis.

Src interacts with multiple cellular factors including HER2, EGFR, ER α , and breast tumors over-express Src kinase [17]. Emerging evidence suggests that PELP1 acts as a scaffolding protein coupling ER with Src kinase leading to activation of ER α -Src-MAPK pathway [14]. Mutational analysis of ER α and c-Src mutants revealed that PELP1 interacts with c-Src SH3

domain via its N-terminal PXXP motif. ER α interacts with Src's SH2 domain at phosphotyrosine 537, and the PELP1-ER interaction further stabilizes this complex [6]. Since breast tumors overexpress wild type Src kinase, deregulation of PELP1 seen in breast tumors can contribute to activation of Src kinase leading to excessive activation of ER-PELP1-Src signaling pathway.

HER2, an oncogene that is overexpressed, amplified, or both, in several human malignancies including breast tumors. ER α expression occurs in ~50% HER2 positive breast cancers and cross-talk between the ER and HER2 pathways promotes endocrine therapy resistance [37, 38]. ER α coregulators are also targeted by excessive ER α -HER2 crosstalk leading to hormonal resistance in a subset of breast tumors [39]. Further, HER2 overexpression can also promote ligand-independent recruitment of coactivator complexes to E2-responsive promoters and thus may play a role in the development of therapy resistance [40]. ER α -coregulator PELP1 interacts with HER2, and growth factor signaling to promote phosphorylation of PELP1 [29]. Deregulation of HER2 signaling is known to modulate PELP1 function leading to enhanced aromatase activation via Src kinase [31]. Our study suggests that endogenous Src plays an important role in HER2-ER crosstalk leading to activation of MAPK and AKT pathways.

While hormonal resistance can occur via multiple mechanisms, understanding the key pathways for resistance and targeting them is essential to extend or restore sensitivity of the therapeutic effect of tamoxifen or other SERMs. Since multiple signaling pathways in addition to hormones are involved in activating ER α [6, 37], combination therapies using both endocrine and non-endocrine agents that block different pathways may have a better therapeutic effect and may delay development of hormonal resistance. Our results suggest that the Src inhibitor dasatinib, represents a non-endocrine drug that could be used to block ER α extranuclear actions. Dasatinib efficiently blocked the activation of ER α -mediated extranuclear signals in three different models of therapy resistance. Further, dasatinib also inhibited ER-coregulator mediated tumorigenesis in vivo and dasatinib treatment sensitized PELP1 driven tumor to tamoxifen therapy. These findings suggest that dasatinib has the potential to block ER α -PELP1-mediated extranuclear signals and thus may serve as an alternative to non-endocrine drug for combinatorial therapy.

In summary, our data provide evidence demonstrating the significance of ER-PELP1-Src axis mediated extranuclear signaling to the therapy resistance. Our findings also identified Src as a novel therapeutic target for blocking of ER α -PELP1 signals and the Src inhibitor dasatinib represents a novel drug to prevent the emergence of therapy resistance in combination with endocrine therapy.

Acknowledgments

This study was supported by the grants NIH-CA0095681 and DOD-W81XWH-08-1-0604.

References

1. Ariazi EA, Ariazi JL, Cordera F, Jordan VC. Estrogen receptors as therapeutic targets in breast cancer. *Curr Top Med Chem*. 2006; 6:195–216.
2. Lewis-Wambi JS, Jordan VC. Treatment of postmeno-pausal breast cancer with selective estrogen receptor modulators (SERMs). *Breast Dis*. 2005; 24:93–105. [PubMed: 16917142]
3. Leary A, Dowsett M. Combination therapy with aromatase inhibitors: the next era of breast cancer treatment? *Br J Cancer*. 2006; 95:661–666. [PubMed: 16926831]
4. Musgrove EA, Sutherland RL. Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer*. 2009; 9:631–643. [PubMed: 19701242]

5. Gururaj AE, Rayala SK, Vadlamudi RK, Kumar R. Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations. *Clin Cancer Res.* 2006; 12:1001s–1007s. [PubMed: 16467116]
6. Schiff R, Massarweh SA, Shou J, Bharwani L, Arpino G, Rimawi M, Osborne CK. Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. *Cancer Chemother Pharmacol.* 2005; 56(Suppl 1):10–20. [PubMed: 16273359]
7. Losel R, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol.* 2003; 4:46–56. [PubMed: 12511868]
8. Acconcia F, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Lett.* 2005; 238:1–14. [PubMed: 16084012]
9. Song RX, Zhang Z, Santen RJ. Estrogen rapid action via protein complex formation involving ERalpha and Src. *Trends Endocrinol Metab.* 2005; 16:347–353. [PubMed: 16126407]
10. Hall JM, McDonnell DP. Coregulators in nuclear estrogen receptor action: from concept to therapeutic targeting. *Mol Interv.* 2005; 5:343–357. [PubMed: 16394250]
11. Smith CL, O'Malley BW. Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev.* 2004; 25:45–71. [PubMed: 14769827]
12. Vadlamudi RK, Wang RA, Mazumdar A, Kim Y, Shin J, Sahin A, Kumar R. Molecular cloning and characterization of PELP1, a novel human coregulator of estrogen receptor alpha. *J Biol Chem.* 2001; 276:38272–38279. [PubMed: 11481323]
13. Rajhans R, Nair S, Holden AH, Kumar R, Tekmal RR, Vadlamudi RK. Oncogenic potential of the nuclear receptor coregulator proline-, glutamic acid-, leucine-rich protein 1/modulator of the nongenomic actions of the estrogen receptor. *Cancer Res.* 2007; 67:5505–5512. [PubMed: 17545633]
14. Vadlamudi RK, Kumar R. Functional and biological properties of the nuclear receptor coregulator PELP1/MNAR. *Nucl Recept Signal.* 2007; 5:e004. [PubMed: 17525794]
15. Habashy HO, Powe DG, Rakha EA, Ball G, Macmillan RD, Green AR, Ellis IO. The prognostic significance of PELP1 expression in invasive breast cancer with emphasis on the ER-positive luminal-like subtype. *Breast Cancer Res Treat.* 2009; 120:603–612. [PubMed: 19495959]
16. Trevino JG, Summy JM, Gallick GE. SRC inhibitors as potential therapeutic agents for human cancers. *Mini Rev Med Chem.* 2006; 6:681–687. [PubMed: 16787379]
17. Russello SV, Shore SK. SRC in human carcinogenesis. *Front Biosci.* 2004; 9:139–144. [PubMed: 14766353]
18. Huang F, Reeves K, Han X, Fairchild C, Platero S, Wong TW, Lee F, Shaw P, Clark E. Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. *Cancer Res.* 2007; 67:2226–2238. [PubMed: 17332353]
19. Summy JM, Gallick GE. Treatment for advanced tumors: SRC reclaims center stage. *Clin Cancer Res.* 2006; 12:1398–1401. [PubMed: 16533761]
20. Nagpal JK, Nair S, Chakravarty D, Rajhans R, Pothana S, Brann DW, Tekmal RR, Vadlamudi RK. Growth factor regulation of estrogen receptor coregulator PELP1 functions via protein kinase A pathway. *Mol Cancer Res.* 2008; 6:851–861. [PubMed: 18505929]
21. Nabha SM, Glaros S, Hong M, Lykkesfeldt AE, Schiff R, Osborne K. Upregulation of PKC-delta contributes to antiestrogen resistance in mammary tumor cells. *Oncogene.* 2005; 24:3166–3176. [PubMed: 15735693]
22. Dimple C, Nair SS, Rajhans R, Pitcheswara PR, Liu J, Balasenthil S, Le XF, Burow ME, Auersperg N, Tekmal RR, Broaddus RR, Vadlamudi RK. Role of PELP1/MNAR signaling in ovarian tumorigenesis. *Cancer Res.* 2008; 68:4902–4909. [PubMed: 18559538]
23. Vadlamudi RK, Bagheri-Yarmand R, Yang Z, Balasenthil S, Nguyen D, Sahin AA, den Hollander P, Kumar R. Dynein light chain 1, a p21-activated kinase 1-interacting substrate, promotes cancerous phenotypes. *Cancer Cell.* 2004; 5:575–585. [PubMed: 15193260]
24. Long BJ, Jelovac D, Handratta V, Thiantanawat A, MacPherson N, Ragaz J, Goloubeva OG, Brodie AM. Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. *J Natl Cancer Inst.* 2004; 96:456–465. [PubMed: 15026471]

25. Jensen MM, Jorgensen JT, Binderup T, Kjaer A. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. *BMC Med Imaging*. 2008; 8:16. [PubMed: 18925932]
26. Euhus DM, Hudd C, LaRegina MC, Johnson FE. Tumor measurement in the nude mouse. *J Surg Oncol*. 1986; 31:229–234. [PubMed: 3724177]
27. Vadlamudi RK, Balasenthil S, Sahin AA, Kies M, Weber RS, Kumar R, El-Naggar AK. Novel estrogen receptor coactivator PELP1/MNAR gene and ERbeta expression in salivary duct adenocarcinoma: potential therapeutic targets. *Hum Pathol*. 2005; 36:670–675. [PubMed: 16021574]
28. Balasenthil S, Vadlamudi RK. Functional interactions between the estrogen receptor coactivator PELP1/MNAR and retinoblastoma protein. *J Biol Chem*. 2003; 278:22119–22127. [PubMed: 12682072]
29. Vadlamudi RK, Manavathi B, Balasenthil S, Nair SS, Yang Z, Sahin AA, Kumar R. Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res*. 2005; 65:7724–7732. [PubMed: 16140940]
30. Chang Q, Jorgensen C, Pawson T, Hedley DW. Effects of dasatinib on EphA2 receptor tyrosine kinase activity and downstream signalling in pancreatic cancer. *Br J Cancer*. 2008; 99:1074–1082. [PubMed: 18797457]
31. Rajhans R, Nair HB, Nair SS, Cortez V, Ikuko K, Kirma NB, Zhou D, Holden AE, Brann DW, Chen S, Tekmal RR, Vadlamudi RK. Modulation of in situ estrogen synthesis by PELP1: potential ER autocrine signaling loop in breast cancer cells. *Mol Endocrinol*. 2008; 22:649–664. [PubMed: 18079323]
32. Kumar R, Zhang H, Holm C, Vadlamudi RK, Landberg G, Rayala SK. Extranuclear coactivator signaling confers insensitivity to tamoxifen. *Clin Cancer Res*. 2009; 15:4123–4130. [PubMed: 19470742]
33. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer*. 2002; 2:101–112. [PubMed: 12635173]
34. Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol*. 2005; 19:833–842. [PubMed: 15695368]
35. Lonard DM, O'Malley BW. The expanding cosmos of nuclear receptor coactivators. *Cell*. 2006; 125:411–414. [PubMed: 16678083]
36. Collingwood TN, Urnov FD, Wolffe AP. Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. *J Mol Endocrinol*. 1999; 23:255–275. [PubMed: 10601972]
37. Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res*. 2003; 9:447S–454S. [PubMed: 12538499]
38. Marcom PK, Isaacs C, Harris L, Wong ZW, Kommarreddy A, Novielli N, Mann G, Tao Y, Ellis MJ. The combination of letrozole and trastuzumab as first or second-line biological therapy produces durable responses in a subset of HER2 positive and ER positive advanced breast cancers. *Breast Cancer Res Treat*. 2006; 102:43–49. [PubMed: 16897431]
39. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, Schiff R. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst*. 2004; 96:926–935. [PubMed: 15199112]
40. Shin I, Miller T, Arteaga CL. ErbB receptor signaling and therapeutic resistance to aromatase inhibitors. *Clin Cancer Res*. 2006; 12:1008s–1012s. [PubMed: 16467117]

Fig. 1.

Down regulation of Src kinase reduces PELP1 mediated ER extra-nuclear signaling. **a** MCF7-shRNA, MCF7-PELP1, and MCF7-PELP1-Src-shRNA cells were lysed and expression of Src was analyzed by western blotting. **b** Total lysates from MCF7-PELP1 and MCF7-PELP1-Src-shRNA model cells were analyzed for the expression of ERα by western blotting. **c** MCF7, MCF7-PELP1, and MCF7-PELP1-Src-shRNA cells were cultured in 5% DCC serum containing medium treated with or without estrogen. Activation of Src and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **d** Cell proliferation capacity of MCF7, MCF7-PELP1, and MCF7-PELP1-Src-shRNA stable cells were analyzed after treating the cells with or without E2 using Cell Titer-Glo assay. ** $P < 0.001$

Fig. 2.

Down regulation of Src kinase reduces HER2 mediated ER extra-nuclear signaling. **a** MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA cells were lysed and expression of Src was analyzed by western blotting. **b** Total lysates from MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA were analyzed for the expression of ERα, by western blotting. **c** MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA cells were cultured in 5% DCC serum containing medium treated with or without E2. The activation of Src, AKT, and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **d** Cell proliferation capacity of MCF7-shRNA, MCF7-HER2, and MCF7-HER2-Src-shRNA stable cells were analyzed using Cell Titer-Glo assay. ** $P < 0.001$

Fig. 3.

Dasatinib reduces E2 mediated ER extranuclear actions on therapy resistant cells. **a** MCF7, MCF7-HER2, MCF7-PELP1, and MCF7-Tam cells were cultured in 5% DCC serum containing medium treated with or without E2 in the presence or absence of dasatinib. The activation of Src, and MAPK signaling pathways was analyzed by western blotting of total protein lysates with phospho-specific antibodies. **b** Cell proliferation capacity of MCF7 and MCF7-PELP1 cells were analyzed after treating the cells with or without E2 in the presence or absence of dasatinib using Cell Titer-Glo assay. **c** Cell proliferation capacity of MCF7-Tam, and MCF7-HER2 cells were analyzed after treating the cells with or without dasatinib using Cell Titer-Glo assay. * $P < 0.05$; ** $P < 0.001$

Fig. 4.

Dasatinib reduces PELP1 driven cancer progression in vivo. **a** Nude mice were injected subcutaneously with MCF7 or MCF7-PELP1-WT. When the tumor reached $\sim 100 \text{ mm}^3$, it was treated with or without dasatinib ($n = 4$). Tumor growth was measured at weekly intervals. Tumor volume is shown in the graph. **b** The average tumor weight is shown in the graph. Representative images of tumors are shown. **c** Status of PELP1, ER, Src phosphorylation and PCNA expression as a marker of proliferation was analyzed by immunohistochemistry (IHC). * $P < 0.05$

Fig. 5.

Antitumor effects of dasatinib in combination with tamoxifen and letrozole. **a** Nude mice were injected subcutaneously with MCF7–PELP1–WT. When the tumor volume reached $\sim 100 \text{ mm}^3$, then dasatinib or combination treatment was started ($n = 4$) and tumor growth was measured at weekly intervals. Tumor volume is shown in the graph. **b** The average tumor weight is shown in the graph. **c** TUNEL staining as a marker of apoptosis and PCNA expression as a marker of proliferation was analyzed by immunohistochemistry (IHC). $*P < 0.05$. **d** Representative results and quantitative analysis of TUNEL staining. Columns, mean TUNEL labeling percentage based on five randomly selected high-power microscopic fields for each group. $**P < 0.001$. *NS* non significant