PAK4 regulates stemness and progression in endocrine resistant ER-positive metastatic breast cancer

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ABSTRACT

Despite the effectiveness of endocrine therapies to treat estrogen receptor-positive (ER+) breast tumours, two thirds of patients will eventually relapse due to de novo or acquired resistance to these agents. Cancer Stem-like Cells (CSCs), a rare cell population within the tumour, accumulate after anti-estrogen treatments and are likely to contribute to their failure. Here we studied the role of p21-activated kinase 4 (PAK4) as a promising target to overcome endocrine resistance and disease progression in ER + breast cancers. PAK4 predicts for resistance to tamoxifen and poor prognosis in 2 independent cohorts of ER + tumours. We observed that PAK4 strongly correlates with CSC activity in metastatic patient-derived samples irrespective of breast cancer subtype. However, PAK4-driven mammosphere-forming CSC activity increases alongside progression only in ER + metastatic samples. PAK4 activity increases in ER + models of acquired resistance to endocrine therapies. Targeting PAK4 with either CRT PAKi, a small molecule inhibitor of PAK4, or with specific siRNAs abrogates CSC activity/self-renewal in clinical samples and endocrine-resistant cells. Together, our findings establish that PAK4 regulates stemness during disease progression and that its inhibition reverses endocrine resistance in ER + breast cancers.

1. Introduction

Endocrine resistance is a major problem for the treatment of Estrogen Receptor (ER)-positive breast tumours. Despite their un-doubted benefit in clinical practice, anti-estrogen therapies fail for at least two thirds of ER + breast cancer patients due to de novo or acquired resistance, which eventually lead to metastatic relapse [1]. Several studies have reported that Cancer Stem-like Cells (CSCs), a rare cell population within the tumour, accumulate after anti-estrogen treatments and are likely to contribute to their failure. Here we studied the role of p21-activated kinase 4 (PAK4) as a promising target to overcome endocrine resistance and disease progression in ER + breast cancers. PAK4 predicts for resistance to tamoxifen and poor prognosis in 2 independent cohorts of ER + tumours. We observed that PAK4 strongly correlates with CSC activity in metastatic patient-derived samples irrespective of breast cancer subtype. However, PAK4-driven mammosphere-forming CSC activity increases alongside progression only in ER + metastatic samples. PAK4 activity increases in ER + models of acquired resistance to endocrine therapies. Targeting PAK4 with either CRT PAKi, a small molecule inhibitor of PAK4, or with specific siRNAs abrogates CSC activity/self-renewal in clinical samples and endocrine-resistant cells. Together, our findings establish that PAK4 regulates stemness during disease progression and that its inhibition reverses endocrine resistance in ER + breast cancers.

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family of serine/threonine kinases, originally described as downstream effectors of small Rho GTPases, Rac and Cdc42, is crucial for cytoskeletal dynamics, survival, proliferation, metabolism and invasion. In mammals, six members have been identified and classified into two groups based on sequence and structure similarities: Group I, PAK1-3; and Group II, PAK4-6. PAK function is upregulated in many human cancers (including melanoma, hepatocellular carcinoma, pancreatic, ovarian, prostate and breast cancer) [20–25], and copy number aberrations have frequently been described in the chromosomal regions containing PAK1 and PAK4 genes [20,21,24,26–28]. Data supporting a role in breast cancer include oncogenic transformation of immortalised mouse mammary epithelial cells by PAK4 overexpression and PAK4 RNAi reversing the malignant phenotype of MDAMB231 breast cancer cells [29,30]. Moreover, 3 independent studies on the expression of PAK4 in breast clinical specimens at different disease stages showed that high protein levels correlate with larger tumour size, lymph node involvement and invasive disease [31–33]. Furthermore, PAK4 expression associates with poor clinical outcome in tamoxifen-treated patients and was demonstrated to positively regulate ER transcriptional activity in an endocrine resistant breast cancer cell line [34].

Here we show PAK4 predicts resistance to tamoxifen and poor prognosis in 2 cohorts of ER + breast cancer tumours. Using patient-derived breast tumour cells, we demonstrate that blockade of PAK4 signalling using a small molecule inhibitor reduces CSC activity and overcoming endocrine resistance. In metastatic patients, we show PAK4 expression is associated with endocrine resistant cancer progression. Our results indicate that PAK4 is essential for maintaining CSC features in patient-derived ER + metastatic breast cancers and in acquired resistance to endocrine therapies. We conclude that the use of anti-PAK4 therapies will help tackle resistance in ER + breast cancer patients.

2. Materials and methods

2.1. Identification and characterisation of CRT PAKi

Several compounds which inhibit PAK4 were identified out of a high-throughput screening on ~80,000 small molecules from the Cancer Research UK’s Commercial Partnerships Team (formerly known as Cancer Research Technology, CRT) compound collection. Exploration of the structural-activity relationship was carried out around novel ATP competitive chemotypes, with compounds being routinely tested against both PAK4 and PAK1 (Supp. Figure 1A). “Hit compounds” were selected to progress to a cellular pharmacodynamic biomarker assay, which measured the inhibition of phosphorylation of a direct substrate of PAK4, and also, to examine toxicity by looking at drug metabolism and pharmacokinetics (DMPK) in vitro. Among all, CRT PAKi showed greater potency, low microsomal intrinsic clearance and reduced colony formation in a dose-dependent manner in established cell lines of different origin (Tables 1 and 2). 1 μM of CRT compound was profiled against the kinase assay panel of 456 targets (LeadHunter Panels, DiscoverX), showing a promising off-target profile. In vivo pharmacokinetic studies showed that its bioavailability was 49%, and that high levels of the compound were detected in the muscle up to 7 h post-administration (Supp. Figure 1B) [23]. CRT PAKi was prepared in-house at Cancer Research UK Therapeutic Discovery Laboratories (purity >99% by LCMS and HNMR) and provided by Cancer Research UK’s Commercial Partnerships Team (London, UK).

2.2. Cell lines and reagents

Endocrine-resistant MCF-7 cell lines were kindly provided by Dr Julia M. Gee (University of Cardiff, Wales) [13,35]. Parental MCF-7 cells were cultured in phenol-red DMEM/F12 media containing 10% foetal bovine serum and 2 mM l-glutamine. Tamoxifen-resistant (TAMR) and Fulvestrant-resistant (FULVR) MCF-7 cells were routinely cultured in phenol red-free DMEM/F12 media supplemented with 5% charcoal-stripped serum and 2 mM l-glutamine in the presence of either 0.1 μM 4-OH-Tamoxifen or 0.1 μM Fulvestrant, respectively. 4-OH-Tamoxifen (Sigma-Aldrich, Cat. No.#H7904, purity ≥98% by HPLC) and Fulvestrant (TOCRIS Bioscience, Cat. No.#1047, purity ≥99% by HPLC) were purchased.

2.3. Metastatic patient-derived samples

Metastatic samples from breast cancer patients were collected at both The Christie NHS Foundation Trust and The University Hospital of South Manchester NHS Foundation Trust through the Manchester Cancer Research Centre Biobank (Manchester, UK). Patients were informed and consented according to local National Research Ethics Service guidelines (Ethical Approval Study No.: 05/Q1402/25 and 12/ROCL/01). Sample processing to isolate breast cancer cells from metastatic fluids (pleural effusions or ascites) was carried out as described elsewhere [36].

2.4. Cell proliferation

Cell proliferation was carried out using the Sulforhodamine B (SRB) assay [37]. Briefly, 1500 cells were seeded per well in 96-well plates. Plates were incubated at 37 °C in a humidified incubator with 5% CO2 until cell attached to the substrate. Then a plate was set aside for a no-growth control (day 0) and treatment was added to the rest. Cells were treated with either 0.5 μM CRT PAKi, 1 μM tamoxifen, 100 nM fulvestrant, combination of therapies or vehicle control. Treatment-containing media was refreshed every three days. At different time points, cells were fixed by adding 25 μl of cold 50% trichloroacetic acid (TCA) to each well and incubating the plates at 4 °C for at least 1 h. Plates were washed 5 times with water and left to air dry. Then cells were stained with 100 μl of 0.057% SRB in 1% acetic acid solution at room temperature for 30 min. Then unbound dye was washed away by rinsing quickly with 1% acetic acid for several times and left air dry. SRB was dissolved by adding 200 μl of 10 mM Tris pH 10.5 to each well for 5 min at room temperature and absorbance at 510 nm was measured using a Versa Max microplate reader (MolecularDevices).

2.5. Colony formation assay

The assay was performed as described elsewhere [38]. Briefly, 500 cells were seeded per well in 6-well plates in the presence of either 0.5 μM CRT PAKi, 1 μM tamoxifen, 100 nM fulvestrant, combination of therapies, vehicle control or left untreated (control). After 10–18 days, media was removed and cells washed once with PBS following by

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td><strong>In vitro cellular pharmacodynamics, drug metabolism and pharmacokinetics of CRT PAKi.</strong></td>
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<tr>
<td>CRT PAKi</td>
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<tr>
<td>PAK4</td>
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<tr>
<td>PAK1</td>
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<tr>
<td>PD Biomarker</td>
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<td>Microsomal G1 Int</td>
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pIC50 values in the presence of ATP, the ability of inhibiting phosphorylation of a direct substrate of PAK4 and the microsomal intrinsic clearance are shown.
Table 2

Effect of CRT PAKi on proliferation.

<table>
<thead>
<tr>
<th>CRT PAKi</th>
<th>Phenotypic pIC50 (anchorage independent growth)</th>
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<tr>
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<td>pancreatic</td>
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Anchorage independent growth was assessed in cancer cell lines of different origin and pIC50 values for the compound were calculated.

fixation with acetone/methanol (1:1) for 30 s. Fixative was removed and plates were allowed to air dry. Then plates were rinsed with distilled water and stained with Giemsa dye for 2–3 min or until the colour of colonies was strong. Rinse with distilled water twice and air dry. Colonies were counted using a GelCount colony counter (Oxford Optronix).

2.6. Quantitative real-time PCR

mRNA expression was detected using quantitative RT-PCR. Total RNA was extracted at different conditions and qRT-PCR reactions were performed as described in Ref. [2]. Applied Biosystems Taqman Gene Expression Assays used: PAK1, #Hs00094562_m1; PAK4, #Hs00110061_m1; GAPDH, #Hs99999905_m1; and ACTB, #Hs99999903_m1.

Expression levels were calculated using the ΔΔCt quantification method using GAPDH and ACTB as housekeeping genes.

2.7. Western blot

Cells lysates were prepared by resuspending cells in cell lysis buffer (25 mM HEPES, 50 mM NaCl, 10% glycerol, 1% Triton-X-100, 5 mM EDTA) containing proteases and phosphatases inhibitors (Roche MiniProtease Inhibitor cocktail; 1 μM PMSF; 50 mM sodium pyrophosphate; 50 mM sodium fluoride; 1 μM sodium orthovanadate), then cells were incubated on rotation for 1 h at 4 °C, and subsequently protein lysates were obtained by centrifugation at 12,000 g at 4 °C for 10 min. Protein concentration was determined using BCA Protein Assay Kit (Pierce). Samples were prepared under reducing conditions, subsequently loaded in pre-cast 10% gels (BioRad, #4561033) and run at 25 V for 15 min 1.3 A using the Transblot Turbo Transfer System (BioRad). Membranes were blocked in 5% bovine serum albumin (BSA)/PBS-0.001% Tween 20 (PBS-T) for 1 h at room temperature, followed by incubation with primary antibody diluted in 5% BSA/PBS-T at 4 °C overnight. Primary antibodies used: anti-PAK1 (Cell Signalling, #2602), anti-PAK4 (Cell Signalling, #3242), β-actin (Sigma, #A2228). After 3 washes with PBS-T, HRP-conjugated secondary antibodies (Dako) were incubated for 1 h at RT. Blots were developed using Luminata Classic (Millipore, Merck) and hyperfilm (Amersham GE Healthcare).

2.8. Mammosphere-forming assay

Cancer stem cell activity was assessed by the mammosphere-forming assay following the protocol described in Ref. [39]. When indicated, cells were directly treated in mammosphere culture with either 0.01–1 μM CRT PAKi (or control vehicle, DMSO); 1 μM 4-OH-Tamoxifen or 100 nM Fulvestrant (or control vehicle, ethanol).

2.9. PAK4 silencing

PAK4 expression was silenced in MCF-7 TAMR and FULVR cells using siRNA technology. Endocrine-resistant cells were transfected with either 10 nM control siRNA (Dharmacon, D-001810-01), siPAK4 #1 (Ambion, S20135) or siPAK4 #2 (Qiagen, SI04990000). Transfection was performed using DharmaFECT (Dharmacon) following the manufacturer’s instructions. Then transfected cells were harvested 48 h post-transfection and PAK4 downregulation was confirmed by Western blot and quantitative RT-PCR.

2.10. Gene expression meta-analyses of ER + primary breast tumours

The gene expression data on 669 ER + tamoxifen-treated tumours (GSE6532, GSE9195, GSE17705, and GSE12093) and 343 ER + untreated tumours (GSE2034 and GSE7390) was integrated from published Affymetrix microarray datasets with correction for batch effects as described previously [2]. Comprehensive survival analysis was conducted using the survivALL R package to examine Cox proportional hazards for all possible points-of-separation (low-high cut-off points) [40].
### 2.11. Statistics

Statistical significance was determined using GraphPad Prism software. Normal distribution of data was assessed using D’Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Normal Parametric tests including one-way ANOVA with Tukey’s HSD post hoc test were performed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Histology</th>
<th>Grade</th>
<th>Receptor status</th>
<th>Chemo</th>
<th>Hormone Therapy</th>
<th>Targeted Therapy</th>
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<td>ILC</td>
<td>3</td>
<td>+ + Neg</td>
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<td>Lapatinib</td>
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<td>+ + +</td>
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<td>NK</td>
<td>Neg Neg Neg</td>
<td>FEC-T Paclitaxel Bevacizumab Gemicitabine/Carboplatin Eribulin</td>
<td>Tamoxifen Letrozole Fulvastrant</td>
<td>Denosumab</td>
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<td>BB3RC84</td>
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<td>NK</td>
<td>Neg Neg Neg</td>
<td>AC Docetaxel Ixabepilone/Capcitabine /Bevacizumab Cisplatin/Gemicitabine</td>
<td>Tamoxifen</td>
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</table>

ILC, invasive lobular carcinoma; IDC, intraductal carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, Human epidermal growth factor receptor 2; Neg, negative; EOX, Epirubicin/Oxaliplatin/Capcitabine; FEC, 5Fluorouracil/Epirubicin/Cyclophosphamide; 5FU, 5Fluorouracil; CMF, Cyclophosphamide/Methotrexate/5Fluorouracil; ECF, Epirubicin/Cisplatin/5Fluorouracil; AC, cyclophosphamide/doxorubicin; NK, not known.
multiple comparisons test or two-tailed unpaired Student’s t-test were performed. When normality assumption was not possible, non-parametric Kruskal-Wallis with Dunn’s multiple comparisons test or non-parametric Mann-Whitney test were performed. Data are always expressed as mean ± SEM of at least 3 independent experiments. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. PAK4 predicts for tamoxifen resistance and poor prognosis in ER + breast cancer

Overexpression of PAK1 and 4 in ER + breast tumours that are refractory to endocrine therapy have previously been linked to tamoxifen resistance and poor prognosis [23,34,41,42]. However, PAK4 is the only family member that associates with clinical outcome data using relapse-free survival as endpoint [34]. Then we assessed whether PAK1/4 expression would predict for patient outcome to tamoxifen treatment using overall survival data from 2 independent ER + breast cancer patient cohorts. We carried out meta-analyses using four published Affymetrix gene expression datasets. Initially, a comprehensive survival analysis was performed on 669 pre-treated tumours of ER + breast cancer patients, who subsequently received tamoxifen as adjuvant therapy. After ranking gene expression data by PAK4 (low to high expression), all possible points-of-separation and their significance are shown in the survivALL plots (Supp. Figure 2A). The heatmap indicates PAK4 expression is independent of PAK1, ESR1, PGR, ERBB2 or the marker of proliferation AURKA (Fig. 1A and B). At most significant cut-point, the subsequent Kaplan-Meier survival analysis revealed that high levels of PAK4 were significantly correlated with metastatic relapse (Fig. 1A, bottom panel). In contrast, only very high or very low levels of PAK1 were associated with metastasis (Supp. Fig. 2C & E). However, elevated levels of both PAKs were associated with poor clinical outcome showing reduced overall survival in an independent cohort of untreated ER + breast cancer patients (n = 343; Fig. 1B, Supp. Fig. 2B, D, F). Thus, PAK4 could be used as a prognostic tool to identify ER + breast cancer patients with high risk of developing endocrine resistance and therefore benefit from the use of anti-PAK4 therapies in the adjuvant setting.

3.2. PAK4 expression correlates with CSC activity in metastatic breast cancer patients

PAK1/4 expression was measured in 18 patient-derived metastatic...
samples, including all clinically defined breast cancer subtypes (Table 3, Fig. 2A & B). We found that their expression was unrelated to subtype and that PAK4 was more frequently detected and more highly expressed than PAK1. In breast cancer cell lines, PAK4 but not PAK1 mRNA expression was significantly associated with luminal subtype (Suppl. Fig. 3A and B). In patient-derived samples, there was a highly significant correlation of PAK4 mRNA expression and cancer stem cell (CSC) activity measured using the mammosphere-forming assay (Fig. 2C, Pearson correlation coefficient = 0.810; p-value < 0.00005; Supp. Figure 3B, Pearson correlation coefficient = 0.104; p-value = 0.682). Next, we tested the effect of increasing concentrations of a PAK1/4-specific inhibitor (CRT PAKi) on the mammosphere-forming efficiency. This compound has an encouraging off-target profile indicating high selectivity for PAK1/4 (Fig. 2D). In 9 metastatic patient-derived samples PAK1/4 inhibition reduced cancer stem cell activity in a dose-dependent manner (Fig. 2E). Further sub-group analysis showed this effect was due to its activity in ER+ metastatic breast cancer samples, with PAK1/4 inhibition impairing breast CSC activity up to 60% (Fig. 2F); whereas the CSC activity of triple negative samples (n = 2) remained unaffected in the presence of the CRT compound (Supp. Fig. 3D and E). These data suggest that PAK4 expression is important in the maintenance of the CSC pool in metastatic ER+ breast cancer.

3.3. PAK1/4 expression is related to cancer progression

Next, we examined sequential metastatic samples of 2 ER+ breast cancer patients. The patients’ clinical treatment history is summarized in Fig. 3A and B. Our analyses showed that both PAK1/4 protein levels and CSC activity increased alongside with disease progression. We detected increased expression of both PAK family members in samples from patient BB3RC44 (~2 or 1.6-fold for PAK1/4, respectively, Fig. 3A), whereas a striking increase of PAK1 levels was observed in patient BB3RC81 (~65-fold, Fig. 3B). These results show that an increase in PAK1/4 expression is correlated with disease progression in ER+ breast cancers, establishing their involvement in the failure of endocrine therapies.

Fig. 3. PAK1/4 and CSC activity increases during cancer progression. Examination of sequential samples of 2 ER+ metastatic patients, BB3RC44 (A) and BB3RC81 (B). The clinical treatment history of each patient is summarized in the top panels. Colours: light blue, pink or green indicate hormonal, chemo- or bone-directed therapy, respectively. Orange arrows pointed when the samples were taken. PAK1/4 protein levels and CSC activity measured as mammosphere-forming efficiency (MFE) are shown in bottom panels. Denistometric values of the ratio PAK to β-actin are indicated below the blots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3.4. PAK4 downregulation restores endocrine sensitivity in resistant cells

These patient data suggest either PAK1 or -4 or both have a role in endocrine resistance. To test our hypothesis, we used in vitro ER + MCF-7 cell lines of acquired resistance after long-term exposure to either tamoxifen (TAMR) or fulvestrant (FULVR), respectively [13,35]. Initially, we assessed the expression of PAK1/4 in parental, TAMR and FULVR cells. Both PAK1 and PAK4 were overexpressed in resistant cells compared to parental cells (Fig. 4A). However, short-term treatment with tamoxifen or fulvestrant in MCF-7 cells did not induce a significant upregulation of PAK1/4 gene expression (data not shown). To further confirm the role of PAK1/4 in endocrine resistance and stemness, we evaluated CSC activity for endocrine resistant cells in the presence of CRT PAKi. PAK1/4 chemical inhibition abrogated CSC self-renewal (> 95% in TAMR and 80% in FULVR, respectively; Fig. 4B) but not primary mammosphere formation (Supp. Figure 4A). Similarly, PAK4 silencing in TAMR and FULVR cells not only impaired breast CSC activity (Fig. 4C & Supp. Fig. 4B-D), but also restored their sensitivity to tamoxifen and fulvestrant, respectively (Fig. 4D & Supp. Figure 4E). These findings indicate that breast CSC activity in endocrine resistant cells depends on PAK4, which can be targeted to overcome endocrine resistance. We hypothesized that PAK4 inhibition in combination with endocrine therapies will benefit ER + breast cancer patients. To test this, initially we assessed the effect of CRT PAKi on proliferation and

Fig. 4. PAK4 downregulation restores anti-estrogen sensitivity in resistant cells. (A) PAK1/4 expression was detected in endocrine resistant MCF-7 cells by Western Blot. Right panel shows densitometric analyses referred to β-actin expression as control of even protein loading. (B) Second generation mammospheres were plated to assess for mammosphere self-renewal (MSR) of cells treated in the first generation with 0.5 μM CRT PAKi or vehicle (DMSO) in resistant cells. (C) Effects of PAK4 silencing on CSC activity in TAMR cells. Two different siRNAs were used against PAK4 (siPAK4#1, siPAK4#2). Then CSC activity of siRNA-transfected TAMR cells was evaluated using the mammosphere-forming assay. The inset shows PAK1/4 mRNA expression in siRNA-transfected cells. In the right upper panels, PAK4 downregulation at protein level was observed by Western Blot. In the right lower panel, densitometric analyses referred to β-actin expression as control of even protein loading are shown. (D) PAK4-silenced TAMR cells were cultured with either 1 μM tamoxifen or vehicle control (ethanol) during the mammosphere-forming assay. Mammosphere-forming efficiency (MFE) is shown. (E) Combination of PAK4 inhibition and anti-estrogen therapies in ER + metastatic breast cancer. Mammosphere-forming efficiency of patient-derived samples treated with either 0.5 μM CRT PAKi, 100 nM fulvestrant or both therapies was assessed. Stats, p-values: * ≤ 0.05; ** < 0.01; *** < 0.001; **** < 0.0001.
colony formation in endocrine resistant cells (Fig. 5). We found that the compound specifically reduced proliferation and colony formation in TAMR and FULVR (Fig. 5A and B), but it had no impact on growth in parental cells (Fig. 5A). However, CRT PAKi interfered with cell attachment of parental cells as colony formation was significantly impaired (Fig. 5B). This phenotype may be due to the pivotal role of PAK signalling in adhesion, as main effector of Rac/Cdc42 during filopodia and lamellipodia formation [43,44]. (Fig. 5C and D). Moreover, the presence of CRT PAKi with either tamoxifen or fulvestrant had a significant cooperative effect reducing proliferation and colony formation of endocrine resistant cells even further (Fig. 5A, C, D).

To confirm our findings, we treated 4 ER + patient-derived breast cancer metastatic samples with either fulvestrant or CRT PAKi as single agents or in combination. CRT PAKi on its own did not have a significant effect on MFE but its combination with the standard of care fulvestrant had a synergistic effect reducing CSC activity more than half. When patient-derived samples were separated into responders versus non-responders, we identified that only ER + breast cancer patients with high levels of PAK4 benefit from the combination of therapies (Fig. 4E and Supp. Figure 4F), suggesting PAK4 expression is a predictive biomarker of response. These results confirm the importance of targeting PAK4 to potentiate endocrine therapy and overcome resistance.

4. Discussion

Despite the remarkable impact on survival caused by the introduction of endocrine therapies for the treatment of ER + breast cancers, late recurrences occur in some patients due to the development of resistance to these single agents. Several authors have shown that breast CSC activity and frequency are enhanced upon endocrine therapies such as tamoxifen and fulvestrant, suggesting that this drug-resistant population accounts for the eventual metastatic relapse [2,3]. Here we report for first time that PAK4 signalling is essential for maintaining CSC features in ER + metastatic breast cancers. Also, PAK4 can be used as a predictive biomarker of response to endocrine therapies, and furthermore, its inhibition reverses endocrine-driven resistance in ER + breast cancer patients.

The relationship between PAK4 and stemness has previously been described in pancreatic cancer cell lines [45,46]. In this study,
pancreatic CSCs express high levels of PAK4 and its silencing reduced not only sphere formation, but also stem cell-related markers [46]. In agreement with these findings, we found that PAK4 significantly correlated with mammosphere-forming ability, and treatment with CRT PAKi reduced breast CSC activity in a dose-dependent manner in metastatic samples of all subtypes. Using RNA-seq data from 10 breast cancer Patient-Derived Xenografts (PDXs), we observed that PAK4 expression correlated with DLI1, NOTCH1-4, PCTCH1 and GLI1 (data not shown). These genes are involved in NOTCH or Hedgehog signalling, both developmental pathways that regulate CSC homeostasis and self-renewal [47].

Most importantly, the effect of PAK4 inhibition on CSCs is restricted to ER + metastatic samples, as the presence of CRT PAKi did not alter CSC activity of the ER-negative subtype. In fact, PAK4 expression significantly correlated with stem cell-related genes such as SOX2, POU5F1 or ALDH1A3 only in metastatic ER + PDXs (data not shown). PAK4 is often amplified in basal-like cancers, which give rise to TNBC [26]; and silencing PAK4 or using inhibitors that induce protein destabilisation reduce proliferation and in vivo tumorigenesis in TNBC, but not in ER + or HER2+ cell lines [29,48]. This discrepancy in the role of PAK4 between breast cancer subtypes might be either associated with its additional kinase-independent functions [33,49], which are compromised upon reducing protein levels and therefore could drive tumorigenesis in TNBC; or, instead, with off-target activity of these inhibitors, e.g. affecting enzymes involved in NAD metabolism [50]. Mechanistically, differences among subtypes can be related to the presence of ER, as a positive feedback loop has been described where ER promotes PAK4 expression and, in turn, PAK4 regulates its transcriptional activity in endocrine resistant cells [34]. Further investigation is needed to fully understand the specific resistance mechanism in each breast cancer subtype.

In most adult tissues, PAK4 expression is low. However, its overexpression has not only been associated with oncogenic transformation [29,30], but also with disease stage in breast clinical specimens [31–33]. We found that PAK1/4-driven CSC activity increased as the disease progressed in sequential metastatic samples taken from 2 ER + breast cancer patients. However, PAK4 expression only increased during progression in patient BB3RC4, who received several lines of endocrine therapy after metastatic relapse, suggesting a resistant phenotype. Whereas patient BB3RC81 was treated with just chemotherapy after recurrence and progression seems to rely on a PAK1-dependent mechanism.

Then we confirmed overexpression of PAK4 in endocrine resistant MCF7 cells. Importantly, CRT PAKi abrogated almost completely CSC self-renewal and silencing of PAK4 not only reduced mammosphere formation, proliferation and colony formation, but also restored the effect of tamoxifen and fulvestrant in endocrine resistant cells. However, experiments with cell line-derived and/or patient-derived xenografts would be essential to demonstrate endocrine treatment response in vivo. Restoration of sensitivity has already been reported using GNE-2861, a group II PAK inhibitor, in tamoxifen-resistant in vivo xenografts would be essential to demonstrate endocrine treatment response in vivo. However, experiments with cell line-derived and/or patient-derived xenografts would be essential to demonstrate endocrine treatment response in vivo. Restoration of sensitivity has already been reported using GNE-2861, a group II PAK inhibitor, in tamoxifen-resistant in vivo xenografts would be essential to demonstrate endocrine treatment response in vivo. However, experiments with cell line-derived and/or patient-derived xenografts would be essential to demonstrate endocrine treatment response in vivo. Therefore, PAK4 not only has prognostic value as a marker of bad outcome in breast cancer, but the inhibition of PAK4 could have therapeutic potential. However, further studies are needed to fully understand the mechanism of action of PAK4 and to identify potential therapeutic targets.

In conclusion, we report for first time that PAK4 is a promising target to reduce CSC activity in ER + metastatic breast cancers and therefore its expression can be used as a prognostic and preventive tool for patient stratification to identify those who will benefit from complementary anti-PAK4 therapies.

Conflicts of interest

ID, RN, ET and VS are/were employees of Cancer Research UK's Commercial Partnership Team who own licensing rights for CRT PAKi used in this study.

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Appendix A. Supplementary data

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