Self-renewal of CD133\textsuperscript{hi} cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer

Pasquale Sansone\textsuperscript{1}, Claudio Ceccarelli\textsuperscript{2}, Marjan Berishaj\textsuperscript{1}, Qing Chang\textsuperscript{1}, Vinagolu K. Rajasekhar\textsuperscript{1}, Fabiana Perna\textsuperscript{3}, Robert L. Bowman\textsuperscript{4}, Michele Vidone\textsuperscript{5}, Laura Daly\textsuperscript{1}, Jennifer Nnoli\textsuperscript{1}, Donatella Santini\textsuperscript{6}, Mario Taffurelli\textsuperscript{5}, Natalie N.C. Shih\textsuperscript{7}, Michael Feldman\textsuperscript{7}, Jun J. Mao\textsuperscript{8}, Christopher Colameco\textsuperscript{8}, Jinbo Chen\textsuperscript{8}, Angela DeMichele\textsuperscript{8,9}, Nicola Fabbri\textsuperscript{10}, John H. Healey\textsuperscript{10}, Monica Cricca\textsuperscript{2}, Giuseppe Gasparre\textsuperscript{5}, David Lyden\textsuperscript{11,12}, Massimiliano Bonafe\textsuperscript{2,13} & Jacqueline Bromberg\textsuperscript{1,14}

The mechanisms of metastatic progression from hormonal therapy (HT) are largely unknown in luminal breast cancer. Here we demonstrate the enrichment of CD133\textsuperscript{hi}/ER\textsuperscript{lo} cancer cells in clinical specimens following neoadjuvant endocrine therapy and in HT refractory metastatic disease. We develop experimental models of metastatic luminal breast cancer and demonstrate that HT can promote the generation of HT-resistant, self-renewing CD133\textsuperscript{hi}/ER\textsuperscript{lo}/IL6\textsuperscript{hi} cancer stem cells (CSCs). HT initially abrogates oxidative phosphorylation (OXPHOS) generating self-renewal-deficient cancer cells, CD133\textsuperscript{hi}/ER\textsuperscript{lo}/OXPHOS\textsuperscript{lo}. These cells exit metabolic dormancy via an IL6-driven feed-forward ER\textsuperscript{lo}-IL6\textsuperscript{hi}-Notch\textsuperscript{hi} loop, activating OXPHOS, in the absence of ER activity. The inhibition of IL6R/IL6-Notch pathways switches the self-renewal of CD133\textsuperscript{hi} CSCs, from an IL6/Notch-dependent one to an ER-dependent one, through the re-expression of ER. Thus, HT induces an OXPHOS metabolic editing of luminal breast cancers, paradoxically establishing HT-driven self-renewal of dormant CD133\textsuperscript{hi}/ER\textsuperscript{lo} cells mediating metastatic progression, which is sensitive to dual targeted therapy.

\textsuperscript{1}Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York 10021, USA. \textsuperscript{2}Department of Experimental, Diagnostic and Specialty Medicine, AlmaMater Studiorum, Universita' di Bologna, Bologna 40138, Italy. \textsuperscript{3}Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, New York, New York 10021, USA. \textsuperscript{4}Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, New York 10021, USA. \textsuperscript{5}Department of Medical and Surgical Sciences, AlmaMater Studiorum, Universita' di Bologna, Bologna 40138, Italy. \textsuperscript{6}Pathology Unit, Policlinico S.Orsola-Malpighi University Hospital, Bologna 40138, Italy. \textsuperscript{7}Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. \textsuperscript{8}Department of Biostatistics and Epidemiology, Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. \textsuperscript{9}Department of Medicine, Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. \textsuperscript{10}Orthopedics Service, Memorial Sloan Kettering Cancer Center, New York, New York 10021, USA. \textsuperscript{11}Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, New York 10021, USA. \textsuperscript{12}Department of Pediatrics, Cell and Developmental Biology, Children's Cancer and Blood Foundation Laboratories, Weill Cornell Medical College, New York, New York 10021, USA. \textsuperscript{13}CRBA Laboratory, Policlinico Universitario S. Orsola-Malpighi, Bologna 40138, Italy. \textsuperscript{14}Department of Medicine, Weill Cornell Medical College, New York, New York 10021, USA. Correspondence and requests for materials should be addressed to D.L. (email: dcl2001@med.cornell.edu) or to M.B. (email: massimiliano.bonafe@unibo.it) or to J.B. (email: bromberj@mskcc.org).
Canonic al cancer stem cell (CSC) phenotypes—CD44hi/CD24lo—have been documented to sustain tumour growth and resistance to conventional anticancer therapies (for example, anti-Her2 and chemotherapy/radiation therapy) in several tumour models. However, discrepancies in CSC phenotypes and abundance are quite variable in clinical specimens, suggesting that CSCs likely evolve with primary tumour growth, with metastatic progression and in response to therapies. Indeed, the acquisition of novel genetic changes, including gain of function mutations in the ESR1 gene, loss of PTEN and discordant expression of Her2 protein, has been observed in ~20% of metastases following conventional anticancer therapies. In addition, a reduction in oestrogen receptor alpha (ER) expression as well as a discrepancy in ER levels between primary tumours and metastatic disease are often observed with the development of tamoxifen resistance without changes in Her2 expression (~80% of cases).

Although decreased expression of ER, increased circulating interleukin 6 (IL6) levels and the presence of circulating CSCs have independently been associated with metastatic progression in breast cancer patients, no models have been proposed to explain their role in endocrine-resistant disease. In this manuscript, we developed the hypothesis that resistance to hormonal therapy (HT) occurs through a change in the self-renewal capacity of metastases, evolving from an ER-dependent to an ER-independent one. We generated experimental and patient-derived models of HT-resistant metastases and determined the evolution of a feed-forward ER–CD133–IL6R–IL6–Notch loop underlying the process of HT resistance. These observations led to therapeutic interventions reversing HT-resistant diseases.

**Results**

**Increased CD133 and IL6 expression in HT-resistant cancers.** We hypothesized that HT and resistance to HT would lead to the expansion of cells expressing the CSC marker CD133 in patients with ER+ breast cancer. Luminal (ER+) breast tumours were sampled before and after neoadjuvant HT; specifically aromatase inhibition (letrozole) and the expression of CD133 mRNA sampled before and after neoadjuvant HT; specifically aromatase inhibition (letrozole) and the expression of CD133 mRNA sampled before and after neoadjuvant HT. Increased levels of CD44hi cells were identified in HT-resistant (HTR) disease (MCF7, ZR75 xenografts with tamoxifen resistance without changes in Her2 expression (~80% of cases)).

**IL6R blockade re-sensitizes HTR metastasis to HT.** These findings led us to examine the consequences of perturbing ER/IL6 signalling on tumour growth and the development of HT resistance. Mice bearing established MCF7 xenografts were treated with HT (tamoxifen) and an IL6R-blocking antibody (tocilizumab), alone or in combination. In these experiments, single therapy alone (tamoxifen or tocilizumab) did not exert significant antitumorigenic effects in the preclinical xenograft trials. Tamoxifen either promoted (tamoxifen-resistant, TamR) or led to a partial reduction in tumour growth (tamoxifen partial resistant, TamR2) compared with controls, while the tamoxifen/tocilizumab regimen potently reduced tumour burden and/or prevented growth in the TamR tumours (Fig. 1g and Supplementary Fig. 2f,g). Overall, these data demonstrate fully tamoxifen-induced CD133hi cells (Fig. 1f), and the self-renewal potential (secondary MS formation) of these cells was further enhanced with exogenous IL6 (Supplementary Fig. 1j,k).

Because ER is a known repressor of IL6 gene expression, we determined whether HT-treated cells/tumour-bearing mice would lead to increased IL6 expression. Accordingly, secreted IL6 and IL6 mRNA expression was elevated in cultured cells derived from tumours and metastases as well as in the sera of mice bearing HT-resistant xenografts (treatment with tamoxifen or fulvestrant; Fig. 1e and Supplementary Fig. 1h,i). Importantly, IL6 mRNA expression was preferentially increased in xenograft-derived CD133hi cells (Fig. 1f), and the self-renewal potential (secondary MS formation) of these cells was further enhanced with exogenous IL6 (Supplementary Fig. 1j,k).

**CD44hi/CD133lo cells had reduced levels of CD24, ALDH1 and CD44 and increased expression of the HTR/CSC marker ABCG2 (ref. 17) as compared with CD44hi/CD133lo cells (Supplementary Fig. 1e and Supplementary Data 1). In agreement with previous investigations, CD44hi/CD133lo cells express low levels of CD24 compared with the non-stem cell population CD133lo/CD44lo (Supplementary Data 1). Thus, both CD133hi/CD44lo and CD44hi/CD133lo had tumorigenic potential on mammary fat pad (MFP) engraftment, as few as 50 orthotopically implanted CSCs, and were able to generate a tumour (Supplementary Fig. 1f) and could form tertiary mammosphere (MS) in vitro (Supplementary Fig. 1g). Although both CD44hi and CD133hi cells display CSC features, CD133hi CSCs are preferentially enriched following HT and promote the self-renewal of luminal metastases after the suppression of oestrogen receptor activity.

Moreover, CD133hi/CD44lo cells had reduced levels of CD24, ALDH1 and CD44 and increased expression of the HTR/CSC marker ABCG2 (ref. 17) as compared with CD44hi/CD133lo cells (Supplementary Fig. 1e and Supplementary Data 1). In agreement with previous investigations, CD44hi/CD133lo cells express low levels of CD24 compared with the non-stem cell population CD133lo/CD44lo (Supplementary Data 1). Thus, both CD133hi/CD44lo and CD44hi/CD133lo had tumorigenic potential on mammary fat pad (MFP) engraftment, as few as 50 orthotopically implanted CSCs, and were able to generate a tumour (Supplementary Fig. 1f) and could form tertiary mammosphere (MS) in vitro (Supplementary Fig. 1g). Although both CD44hi and CD133hi cells display CSC features, CD133hi CSCs are preferentially enriched following HT and promote the self-renewal of luminal metastases after the suppression of oestrogen receptor activity.

To determine the ER/CD133/IL6 interplay in the development of HT-resistant metastatic disease, we generated an in vivo model of metastatic progression. Briefly, tumour-bearing mice were treated with HT and, when primary tumours were ~1 cm, they were removed and the mice continued to receive HT (tamoxifen or
fulvestrant), and metastatic burden was followed by in vivo imaging (IVIS luciferase) and confirmed at necropsy. Although the tamoxifen treatment reduced the incidence of metastatic disease in tumour-bearing mice, 5% of the animals developed widely metastatic disease, recapitulating the canonical metastatic spread observed in humans (Supplementary Fig. 3a,b, TamR—lymph nodes, peritoneum and bones, Supplementary Table 2). Interestingly, TamR metastases were markedly enriched (~30-fold) for CD133<sup>hi</sup> cells and had an increased expression of secreted/autocrine IL6 compared with the primary tumours (from...
the same mouse) or from both primary tumours and metastases from placebo-treated mice (Fig. 1i–k and Supplementary Fig. 3c).

To determine whether the ER–IL6 feedback loop promoted the metastatic potential of HT-resistant disease, we first isolated tamoxifen- and fulvestrant-resistant (TamR/FulvR) metastatic lesions from different organs (n = 13, including bones and lymph nodes). Notably, these metastases were genotypically identical to their matched primary tumours (Supplementary Table 2). To determine whether IL6 blockade (tocilizumab) would influence sensitivity to HT, FulvR bone metastases (FulvR-Bone-Met) were isolated, passaged and re-injected into new cohorts of mice: once tumours reached ~1 cm, mice were randomized to receive fulvestrant with or without tocilizumab (Fig. 2a: Fulv, Fulv-Toci). The addition of tocilizumab to HT led to a dramatic reduction in both primary and spontaneous metastatic tumour burden (lungs and bones) with a concomitant elimination of CD133hi CSCs (Fig. 2b–d and Supplementary Fig. 3d).

Consistent with the experimental models, we isolated and cultured patient-derived HTR bone metastases (Supplementary Table 3, HTR-Bone-Met) and determined that these cells produced increased levels of IL6 mRNA in response to fulvestrant (Supplementary Fig. 3e). Importantly, fulvestrant enhanced the self-renewal (growth as secondary MS) of the HTR-Bone-Mets, which was abrogated with the addition of tocilizumab (Fig. 2e,f and Supplementary Fig. 3f).

\[ \text{IL6R blockade increases ER and reduces Stat3/Notch3/CD133.} \]

To determine whether HT-resistant metastases could re-acquire HT sensitivity \textit{in vitro}, individual FulvR metastases were cultured in the presence of HT (tamoxifen or fulvestrant) for 2 months. Some (2/25) of the metastases remained completely resistant to HT (HTR), while the majority exhibited partial sensitivity to fulvestrant but remained resistant to tamoxifen (Fig. 3a and Supplementary Fig. 4a, HTS).

Molecularly, HTR-Mets expressed higher levels of pStat3 and reduced levels of pHer2 and ER as compared with the HTS ones (Supplementary Fig. 4b). In addition, HTR-Mets had increased levels of the IL6-regulated component of Notch signalling—Notch3—together with a higher mRNA expression of IL6, Stat3 and CD133 (Fig. 3b,c). Finally, CD133hi tumour cells (enriched in areas with stromal infiltrates) also expressed Notch3 protein and, using gene set enrichment analysis, CD133hi cells compared with CD133lo cells had elevated mRNA levels of transcripts downstream of Notch signalling (Supplementary Fig. 4c,d, GSE69280).

To determine the functional role of Stat3 and Notch3 in HT resistance, we reduced their levels by stable short hairpin RNA (shRNAs; shS3/shN3) that restored fulvestrant and tamoxifen sensitivity to the HTR cells \textit{in vitro} (both two-dimensional and three-dimensional growth) without affecting growth in the absence of HT (Fig. 3d,e). Although the knockdown of Stat3 did not decrease CD133 mRNA expression, ShS3-HTR cells (cultured with fulvestrant) had reduced Notch3 mRNA levels, suggesting that Notch3 is downstream of Stat3 and re-expressed ER mRNA (Fig. 3f). These latter observations led us to hypothesize that ER re-expression could be a possible mechanism underlying restoration of endocrine sensitivity with the blockade of IL6 signalling.

To test this hypothesis, CD44hi and CD133hi cells were isolated from HT-resistant metastases (TamR and FulvR, from multiple
the presence/absence of Fulv (10 μM) for 10 days using CalceinAM fluorescent probe; partial (sensitive, HTS) or complete hormone-resistant features (HTR) were generated. Data are reported as mean (fluorescence) ± s.e.m. of the last time point of the growth curve (three biological replicates with three technical replicates each). (b) Fold increase in the expression of CD133, Stat3, IL6 and Notch3 mRNA using qPCR from HTR and HTS cells treated with Fulv (see a. HTS transcript as reference). (c) Representative images of Notch3 expression by IHC of lung metastases from placebo versus TamR mice (scale bar, 20 μm). (d) Proliferation (CalceinAM fluorescence) in Stat3 or Notch3 stably silenced HTR cells (shS3, shN3) derived from FulvR-Met and propagated (10 μM) from FulvR-metastatic cells (MetMS) expressing stable shS3 and shN3 cultures from FulvR-Met (MetMS) expressing stable shS3 and shN3 in vitro (10 μM). Data are reported as the mean (fluorescence) ± s.e.m. of each time point of the growth curve (three biological replicates with three technical replicates each). (e) Number-No. of II-MS (>100 μm) from FulvR-metastatic cells (MetMS) expressing stable shS3 and shN3 cultured in the presence/absence of Fulv (10 μM) and/or tamoxifen (1 μM, 14 days). (f) qPCR of CD133, Notch3 and ER mRNA (fold increase Fulv versus untreated cells) in shS3-FulvR-metastatic cells cultured in the presence/absence of Fulv in vitro (10 μM, 7 days). (g) Number of II-MS (diameter >100 μm) from FACS-isolated (10^5 cells per well) CD44lo and CD133lo cells from TamR-Met and FulvR-Met (Supplementary Table 2) treated with Tam (1 μM) or Fulv (10 μM) and tocilizumab (50 μg ml^{-1}), either alone or in combination (7 days). (h) Western blot analysis and IHC images for ER expression from HTR cancer cell lines (ZR751, BT474) and xenografts (MCF7) following treatment with Fulv (10 μM, 14 days) in presence/absence of tocilizumab (50 μg ml^{-1}, treatment for 14 days) and after shS3 (scale bar, 50 μm). Data are reported as mean ± s.d. of three independent experiments (n = 3; b, e, f, g). P values (*P<0.05, **P<0.001) refer to t-test (a, b, f), multiple comparisons corrected t-test after GLM ANOVA (e, g) and GLM for repeated measures (d).
Figure 4 | HT induces a dormant CD133hi/OXPHOSlo/Mitolo phenotype. (a) Graph showing the percentage of M5 with diameter >100 µm generated from FACS-isolated CD44lo/CD133lo MCF7 tumour cells treated with tamoxifen (1 µM, TamR cells) for 2 months, representative images of cultured cells in 3D (scale bar, 50 µm). (b) Quantification of mtDNA (see Methods) in tumour-derived tissue and cells isolated from TamR xenografts (from Fig. 1g). Representative immunofluorescence of MAg expression in TamR tumours (scale bar, 15 µm). (c) OCR and ECAR of cancer cells described in a and treated with mitochondrial stressors (oligomycin 1 µM, FCCP 300 nM, 2DG 50 mM, Rotenone 300 nM, see Methods). (d) MS growth (fold increase in the number of MS >100 µm; as a reference we used non-treated cells) in TamR-derived CD44lo/CD133lo and CD133lo/CD44lo cultured in IL6-containing conditioned media derived from tamoxifen-treated cells (CM-Tam) in the absence/presence oftocilizumab (50 µg ml⁻¹, 7 days). (e) OCR was measured in cells following treatment with Tam (1 µM, 2 weeks), IL6 (10 ng ml⁻¹, was added 24 h before analysis) and IL6/Tam in the absence or presence of mitochondrial stressors (0.5 µM oligomycin, 100 nM rotenone—doses leading to partial inhibition of OXPHOS potential) and in presence of high glucose (20 mM). Bar graph representing the median AUC ± s.d. area under the curve of OCR, pMol min⁻¹, see Methods (three biological replicates with three technical replicates each). Data are reported as mean ± s.d. of three independent experiments (n = 3; a,b,d). P values (*P < 0.05, **P < 0.0001) refer to t-test (a,b) and post hoc t-test after GLM ANOVA (d,e).

for 2 months, and the surviving TamR cells (DAPI-negative) were enriched 100-fold for CD133hi/CD44lo-expressing cells (tamoxifen-resistant cancer cells, TamR) and conversely were reduced in CD44hi-expressing cells compared with vehicle-treated (control) cells (Supplementary Fig. 5a–c). However, these CD133hi/CD44lo/TamR cells did not proliferate nor self-renew (Fig. 4a), lacked features of senescent cells (beta-galactosidase-negative) and were arrested in G2/M of the cell cycle (Supplementary Fig. 5d). In agreement with these functional observations, analysis of tissues from TamR xenografts revealed reduced mitochondrial DNA copy number (mtDNA) and reduced expression of mitochondrial antigen (MAg, a surrogate for mitochondrial mass; Fig. 4b)⁹. Further analysis demonstrated that CD133hi/ERlo-expressing cells also have reduced MAg expression (Supplementary Fig. 5e). In line with these observations, xenograft-derived fluorescent-activated cell sorting (FACS)-sorted CD133hi/CD44lo cells had reduced expression of enzymes regulating lipidogenesis and glycolysis using microarray analysis (PMK-2, LDHA, ACACA, FASN, ACLY, ALDOC and ENO2) as compared with the CD133lo/CD44lo cells (Supplementary Fig. 5f, GSE69280). Consistent with the loss of mtDNA/mass, TamR cells had reduced mitochondrial activity (Fig. 4c, as measured by reduced oxidative phosphorylation (OXPHOS) and extracellular media acidification potential). To demonstrate a functional link between the loss of mitochondrial bioenergetics and the generation of CD133hi CSCs, we cultured CD133lo/CD44lo cells from multiple luminal breast cancer models: T47D, BT474 and ZR751 in media containing ethidium bromide, which eliminates mitochondrial DNA (MitoS media, see Methods, Supplementary Fig. 6a). Similarly to what was observed with tamoxifen treatment, the growth of luminal breast cancer cells in MitoS media led to a 20- to 100-fold increase in CD133hi cells (Supplementary Fig. 6b). Likewise, xenograft-derived MCF7 CD133lo/CD44lo cells grown in the presence of MitoS media also demonstrated a reduction in CD44lo cells and a 100-fold increase in CD133hi/CD44lo cells, which could not self-renew (Supplementary Fig. 6c,d). Notably, MitoS cells not only expressed high levels of CD133 mRNA but also the IL6R and reduced levels of ER mRNA (Supplementary Fig. 6e). Overall, these data suggest that the suppression of OXPHOS activity (MitoS) generates self-renewal-deficient CD133hi/ERlo cells.

We then hypothesized that IL6 could drive the exit from HT-induced dormancy of these CD133hi/ERlo/MitoS cells by restoring mitochondrial activity in an ER-independent manner (for example, during or following chronic suppression of ER activity). IL6 from the conditioned media of tamoxifen-treated cancer cells (CM-IL6 + Tam) mediated the self-renewal capacity of TamR-FACS-isolated CD133hi cells, demonstrating the sufficiency of this cytokine in promoting exit from metabolic dormancy, while the same conditioned media promoted the growth arrest of CD133lo cells (Fig. 4d). We then demonstrated that IL6 administration of tamoxifen-treated cells selectively reversed tamoxifen-mediated inhibition of mitochondrial activity (OCR, oxygen consumption rate) including cells treated with mitochondrial stressors (Fig. 4e, rotenone and oligomycin).

Exit from metabolic dormancy and HTR via IL6/Notch3. Further analysis of cancer cells treated with mitochondrial stressors and/or tamoxifen demonstrated that IL6 treatment reduced cell death (Supplementary Fig. 7a) and led to an
HT leads to a reduction in ER model describing metastatic progression in luminal breast cancer. Primary tumour cancer stem cells rely on ER expression/signalling for their self-renewal, which occurs in an IL6 (autocrine)-dependent manner and an ER-independent manner. IL6/Stat3 blockade (tocilizumab) restores ER expression and decreased mitochondrial activity. Persistent loss of ER expression in tumour a dormant cancer stem cell phenotype (CD133hi/ERlo), characterized by increased IL6R expression in tumour dormancy (Supplementary Fig. 7d). We found that restoration of OCR by IL6 in ERlo/OXPHOSlo cells could be achieved by IL6 administration (Fig. 5b). In addition, by reducing Notch3 expression, the generation of CD133hi cells was virtually eliminated with concomitant suppression of ER (fulvestrant) and/or mitochondrial activity (MitoS media, see Methods), suggesting that Notch3 expression is crucial for the survival of CD133hi/Notch3lo-positive feed-forward loop, preventing the self-renewal of HTR CD133hi CSCs. Data are reported as mean ± s.d. of three independent experiments (n = 3; b,c,c). P values (*P < 0.05, **P < 0.001) refer to t-test (c) and t-test after GLM ANOVA (b).
respiration (HT). The IL6/Notch3 pathway promoted the self-renewal of HT-therapy-induced dormant CD133hi/ERlo/Mito cells in an ER-independent manner (Fig. 5e).

Discussion

Although adjuvant HT is aimed at targeting ER+ metastatic disease, HT is not curative20. Thus, an understanding of the molecular underpinnings of metastatic disease and HT resistance is essential to both prevent and treat this fatal disease. Because there are no metastatic models that encompass the pathological progression of ER+ breast cancer, we focus on inhibiting pathways that are critical for primary tumour growth rather than targeting the cell types/pathways that mediate hormone-resistant metastasis. Thus, development of novel therapies would be greatly facilitated by preclinical tumour models that recapitulate the disease process.

Here we established an experimental model of HT-resistant metastatic disease and demonstrated that chronic suppression of ER led to reduced expression of ERz and conversely increased expression of autocrine IL6, selectively in CD133hi cells, driving the self-renewal capacity of these cells in HT-resistant metastases. Importantly, these same phenomena were observed in patient-derived HT-resistant bone metastases. Specifically, HT enhanced MS-forming capacity of these cultured cells with concomitant increases in IL6 expression. In addition, CD133hi expression was increased in patient-derived HT-resistant metastases as compared with primary tumours. Importantly, combination HT and anti-IL6R (tocilizumab) therapy effectively restored HT sensitivity via the re-expression of ERz and abrogation of CD133hi self-renewal in both the preclinical models and in human specimens.

In addition, we identified the stepwise generation of a mitochondrial-defective/therapy-induced dormant population—CD133hi/ERlo/Mito—generated through the actions of HT via the suppression of OXPHOS, which reduces ER expression and conversely upregulates CD133hi-expressing cells. Chronic HT also induced high levels of paracrine IL6 expression. Mechanistically, we then demonstrated that these metabolically dormant cells (CD133hi/ERlo/Mito) exit quiescence via an IL6/Stat3/Notch3-mediated induction of mitochondrial activity.

Importantly, our results are the first to establish a truly representative spontaneous model of ER+ breast cancer, with powerful implications in furthering our understanding the mechanisms underlying the process of HT-mediated dormancy, resistance and metastatic progression to bone and other organs. Finally, our data support the use of targeted therapies against IL6 (anti-IL6R therapy) in patients who have developed HT-resistant disease, which we propose will block the IL6hi/ERlo feed-forward loop underlying the de novo expansion of CD133hi CSCs and metastatic progression (Fig. 5e).

Methods

Establishment of endocrine-resistance metastatic disease model.

Primary tumour and metastatic cells were collected from long-term xenografts generated following repeated orthotopic injection in the mammary fat pad of non-obese diabetic/sclerotic/severe combined immunodeficiency mice (NOD/SCID) mice. Cells were isolated and directly stained for FACS analysis or were sorted by green fluorescent protein (GFP) using antibodies against CD44 and CD133 (DAPI-negative). FACS-isolated tumour cells were re-transplanted into recipient mice. All the in vitro experiments were generated using tumour-derived cancer cells from xenografts and primary human ductal carcinoma tissues. Preclinical therapeutic trials were generated using xenografts from tumorigenic MCF7 clones administered with tamoxifen citrate implants, fulvestrant (Faslodex) and tocilizumab (ACTEMRA).

Cell lines and drugs and preclinical trials.

MCF7, ZR75, T47D and BT47 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC). All cancer cell lines (ATCC) were mycoplasma free, engineered to express a GFP-positive luciferase expression vector, maintained in MEM/RPMI media supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 units ml−1 penicillin and 1 mg ml−1 streptomycin (Media Core). Before use, cancer cells were FACs-sorted (for GFP) and injected bilaterally in the mammary fat pads of 5–7-week-old NOD/SCID (obtained from CNI Frederick, MD). For each in vivo experiment, cancer cells were mixed with an equal volume of Matrigel (BD Biosciences) in a total volume of 50 l. Bioluminescence (Xenogen, Xenogen/Ivanovo) was measured once per week. The bioluminescent signal was detected using a Xenogen Sterling IVIS200 imaging system. Tumour volume (in vivo) was calculated as (L × W × 0.5) mm3. For immunostaining assays, organs were collected and fixed overnight in 4% paraformaldehyde, washed, embedded in paraffin and sectioned (Histo-Serve Core). Haematoxylin and eosin staining was performed using standard protocols. For the detection of metastases at secondary sites, we performed perfused GFP and ER double immunofluorescence/immunohistochemistry staining (Supplementary Table 4). All the surgical procedures and protocol were checked in the institutional guidelines and an approved protocol from our Institutional Animal Care and Use Committees at MSKCC. For the in vitro studies we used tumour cells isolated from primary and metastatic lesions from xenografts using FACS (GFP). The following reagents, 4-hydroxytamoxifen, fulvestrant, rotenone, oligomycin, 2-deoxy-D-glucose (2DG) and IL6 and IL6R, were purchased from Sigma-Aldrich, and trastuzumab was purchased from Genentech. For the preclinical studies, we implanted tamoxifen citrate pellets (5 mg per pellet for mouse, Innovative Research of America) between the scapulas of mice bearing primary MFP tumours. Injectable fulvestrant (Faslodex, AstraZeneca) was given intravenously to mice for 4 weeks, and the other treatments were given twice a week for 2 months. Tocilizumab (Actemra, Roche Pharmaceuticals) was diluted in PBS at a final concentration of 20 mg ml−1. A dosage of 100 μg g−1 per mouse was administered intraperitoneal every week. Control mice received isotype control (placebo). The beta-galactosidase kit was purchased from Cell Signaling; cells were lysed according to the manufacturer’s protocol. Cell viability and PI staining was performed in the Flow Cytometry Core at our institution according to their protocol. Primary tumour and metastatic cells (from multiple sites, Supplementary Table 2) were isolated from xenografts, cultured and engineered ex vivo to express shRNAs for Notch3 and Stat3, which were described previously22,23.

FACS analysis.

Tumours were digested in sterile Epicupid media ( Stem Cell Technology), minced with sterile razor blades and incubated for 3 h in the presence of Collagenase/Hyaluronidase (1,000 units per sample). Cells were washed with sterile filtered PBS supplemented with 1% bovine serum albumin (PBS-BSA 1%) and filtered through a 40 μm nylon mesh (BD Biosciences). The CD44 and CD133 antigens, cells were stained in a volume of 100 μl (PBS-BSA 1%) with each antibody CD44-APC (Clone IM7, eBiosciences) and CD133/1-PE (Clone AC133, Miltenyl Biotech). Cells were labelled on ice for 30 min and analysed (BD FACs Aria III, Flow Core). Gene expression and genotypic analyses

For microarray gene expression and profiling and real-time PCR (QPCR), we extracted RNA using TRIzol (Invitrogen) from in vivo-derived tumour-derived cancer cells. RNA concentration and purity was determined using a NanoDrop 2000. The MSKCC Genomics Core Facility prepared the samples according to the standard protocols and hybridized them on Affymetrix HG-U133 Plus 2.0 microarrays. We processed the data using GC Robust multi array Average together with updated probe set definitions (hs133phsentrezg). Differentially expressed genes between groups were identified with fold-change and T-test cutoffs (GSE69280 array deposited in NCBI, Supplementary Fig. 4d and Supplementary Data 1). For microarray analysis of published data sets, normalized gene expression data were downloaded from the Gene Expression Omnibus. Each gene was mean-centred and scaled by s.d. All analyses were conducted in R. Normalized gene expression data were downloaded from the NCBi for data set GSE2841 (ref. 23; Fig. 1a). A paired, one-tailed, t-test was used to determine whether there was an upregulation of CD133 or ER in patients following Letrozole treatment (Fig. 1a). For QPCR, 1 μl of total RNA was reverse-transcribed to CDNA using Superscript III following the manufacturer’s protocol (Invitrogen). QPCR for CD133, ER (ESR1), IL6, IL6R, Notch3, CD44 and Stat3 was performed on 250 ng of CDNA using TaqMan pre-custom probes (Applied Biosystems, ESR1: Hs00334178_m1; CD133: Hs01009250_70; IL6R: Hs01075666_69; IL6: Hs00985639_66; Notch3: Hs01128541_81; CD44: Hs01075661_70; Stat3: Hs00377827_m1; B2m: Hs01081531_20; Gapdh: Hs00788774_20) and ABI Prism 7900HT sequence detection system (Applied Biosystems) in accordance with the manufacturers’ protocols. For the detection of CD133 expression (RT–PCR) analysis was performed using the following primers: IL6 Forward 5′-TCCACGTTGCTGCAGGTAATAAGTTGTAAG-3′, Reverse 5′-GCCGCAATGAGGATACG-3′; T47D Forward 5′-TATGTTTATGGTGTTGCAGAG-3′, Reverse 5′-CAGATCTTGCAGCAGCATTCA-3′; B2m Forward 5′-ACCCACCTGAAAAATGATG-3′, Reverse 5′-AATCTCAAACTACCTGAT-3′. PCR primers and
reagents for RT–PCR were purchased from Invitrogen. Genomic analysis of MCF7 variants is described in Supplementary Table 2 (from HT-resistant metastasis); it was performed by the University of Montreal (Oncology Core). DNA was extracted from FACS-isolated cancer cells (GFP +) and sequenced for 341 cancer genes (IMPACT analysis).

**Metabolic assays.** Glucose uptake was measured with radiolabelled glucose (Deoxy-D-glucose, 2-[1,2-3H]S, Perkin Elmer). Mitochondrial stress media (Mitos) was prepared by adding 2 mM glucose and 1% of serum to the media-specific recipe for generating cells lacking mitochondrial DNA (p0 media: 0.4 μg ml−1 ethidium bromide, 50 μg ml−1 uridine and 100 μg ml−1 pyruvate). Loss of mitochondrial respiration and OXPHOS potential in Mitos cells was assessed using the seahorse technology (www.seahorsebio.com)24. OCR extracellular acidification rate and glycolytic potential were determined using the seahorse extracellular flux analyser (XF-96, XF-24 Seahorse Bioscience). To allow comparison between experiments, data are presented as OCR (p Mol min−1) and extracellular acidification rate (ECAR) (μM pH min−1). Graphs showing the kinetic of OCR and extracellular acidification (ECAR) under basal condition followed by the sequential addition of oligomycin (1 μM), p-trifluoromethoxyphenyl-hydrazone (FCCP, 300 μM), 2DG (50 mM), rotenone (300 μM) and glucose (20 mM) are reported (cell mito and glycolysis stress kit from Seahorse Bioscience).

The progress curve is annotated to show the relative contribution of basal, ATP-linked (oligomycin) and maximal (FCCP) oxygen consumption and respiration capacity of the cells (after the addition of 2DG + rotenone). Furthermore, OXPHOS and glycolytic potentials were determined by measuring the area of the progress curve after the addition of glucose and rotenone/oligomycin. For in vitro labelling of mitochondria, Mitotracker dye was used (Invitrogen), whereas for in vivo studies Mito-Staining was performed. mtDNA was measured by slightly modifying the protocol described in the manuscript from ref. 24. In particular, 20–100 ng of DNA were used in a real-time PCR using the Kapa probe fast solution (Vector Laboratories Inc.) was conducted before overnight incubation of the primary day–3 samples with breast cancer cells. The primary day–3 samples were incubated overnight at RT in a humid chamber and processed with a non-biotin peroxidase-amplified system (Novolink, Novocastra Lab) according to the manufacturer’s instructions. Primary samples were used for immunohistochemical reaction with 3,3′–diaminobenzidine tetrahydrochlordide (DAB)/H2O2 solution. Sections were counterstained with Harris haematoxylin, dehydrated and mounted in Bio-Mount (Bio-Optica).

Cell populations were harvested and resuspended in 1 ml of PBS supplemented with complete PBS plus 1% FCS. Cells were transferred to 96-well U-bottom plates at a density of 2 × 103 per well. Depending on the cell line, overnight incubation was done at 37°C in a humidified atmosphere in 5% CO2 and 95% air with 100 μl of media per well. For the luciferase assay, cells were plated in six-well plates at a density of 2 × 105 per well. Plates were transfected with 0.1 μg of luciferase reporter (pGL3-Basic) and 0.1 μg of each of the reporter plasmids. pER and pERtk were a gift from Dr Andre’ Tremblay (University of Montreal); pIL6 was a gift from Dr William L. Farrar (The University of Pennsylvania cohort) and (2) from IRB protocol #97-094 (USA-MSKCC cohort), which includes the analysis of patient-derived specimens.

**Immunostaining analysis.** Immunohistochemical analysis was performed on paraffin sections from eight HTR-metastatic tissues and matched primary tumours (Supplementary Table 3, University Penn cohort), eight HTR breast cancer bone metastases (Supplementary Table 3, MSKCC cohort) and experimental xenografts (Figs 1–3). Sections were de-waxed, rehydrated and retrieved using a Tris-EDTA pH 9.0 solution (20 min at 98°C) for anti-Her2 and anti-ErbB2 antibodies. Endogenous peroxidase activity was quenched with a Methanol/H2O2 1.5% solution (20 min at room temperature (RT)). Primary antibodies were incubated overnight at RT in a humid chamber and processed with a non-biotin peroxidase-amplified system (Novolink, Novocastra Lab) according to the manufacturer’s instructions. Primary samples were used for immunohistochemical reaction with 3,3′–diaminobenzidine tetrahydrochlordide (DAB)/H2O2 solution. Sections were counterstained with Harris haematoxylin, dehydrated and mounted in Bio-Mount (Bio-Optica). For immunostaining analysis, cells were plated in 24-well plates at a density of 2 × 105 per well. Depending on the cell line, overnight incubation was done at 37°C in a humidified atmosphere in 5% CO2. After fixation in 4% paraformaldehyde (PFA) and permeabilisation with 0.1% Triton X-100, TUNEL (in vitro) or DAPI staining at flow cytometer (Dako Cytomation). IL6 enzyme-linked immunosorbent assay (ELISA) was performed using the conditioned medium collected from 5–day cultures of tumour-derived cells seeded at 200,000 cells per plate. Blood samples were drawn through the orbital vein just before killing the mice. Plasma separated from whole blood by centrifugation at 14,000 r.p.m. at 4°C. Plasma from tumour-bearing mice and the conditioned medium from in vivo cultures were then analysed with the human IL6 ELISA kit (BD Biosciences) according to the manufacturer’s instructions (SpectraMax plate platform).

**Statistical analysis.** Statistical analysis was performed using SPSS (SPSS Incorporation). Continuous variables were analysed by unequal variance t-test, paired t-test (samples, n = 2), general linear model (GLM) ANOVA or GLM for repeated measures (samples, n > 2). Mann–Whitney, Wilcoxon and Friedman tests were used to analyse ordinal variables. P values were adjusted for multiple comparisons according to Bonferroni correction. Association among quantitative variables was quantified by Pearson correlation coefficient. Categorical variables were analysed by Fisher’s exact test or Monte Carlo χ2-test. All the tests were two-sided. P < 0.05 was considered significant. The Elda software was used to measure the statistics of limiting dilution experiments (Supplementary Fig. 1f)27.
6. Arapantoni-Dadioti, P. et al. Discordant expression of hormone receptors and HER2 in breast cancer. A retrospective comparison of primary tumours with paired metachronous recurrences or metastases. J. BUON 17, 277–283 (2012).

7. Niikura, N. et al. Loss of human epidermal growth factor receptor 2 (HER2) expression in metastatic sites of HER2-overexpressing primary breast tumours. J. Clin. Oncol. 30, 593–599 (2012).

8. Toy, W. et al. ER Sl bindig-domain mutations in hormone-resistant breast cancer. Nat. Genet. 45, 1439–1445 (2013).

9. Osborne, C. K. & Schöff, R. Mechanisms of endocrine resistance in breast cancer. Annu. Rev. Med. 62, 233–247 (2011).

10. Idirisinghe, P. K. et al. Hormone sensitivity with differential effects on estrogen receptor signaling in clone models of human epidermal growth factor receptor 2-negative breast cancer with acquired endocrine resistance. Clin. Cancer Res. 16, 1486–1497 (2010).

11. Knupfer, H. & Preiss, R. Significance of interleukin-6 (IL-6) in breast cancer (review). Breast Cancer Res. Treat. 102, 129–135 (2007).

12. Nadal, R. et al. CD133 expression in circulating tumour cells from breast cancer patients: potential role in resistance to chemotherapy. Int. J. Cancer 133, 2398–2407 (2013).

13. Creighton, C. J. et al. Residual breast cancers after conventional therapy display mesenchymal as well as tumour-initiating features. Proc. Natl Acad. Sci. USA 106, 13820–13825 (2009).

14. Giordano, G., Gabrielli, M., Gnetti, L. & Ferri, T. Oncocytic carcinoma of parotid gland: a case report with clinical, immunohistochemical and ultrastructural features. World J. Surg. Oncol. 4, 54 (2006).

15. Rimawi, M. F. & Osborne, C. K. Breast Cancer: Blocking both driver and escape pathways improves outcomes. Nat. Rev. 9, 133–134 (2012).

16. Chang, Q. et al. The IL-6/JAK/Stat3 feed-forward loop drives tumourigenesis and metastasis. Neoplasia 15, 848–862 (2013).

17. Sassone, P. et al. p66Shc/Notch-3 interplay controls self-renewal and hypoxia survival in human stem/progenitor cells of the mammary gland expanded in vitro as mammospheres. Stem Cells 25, 807–815 (2007).

18. Creighton, C. J. A gene transcription signature associated with hormone independence in a subset of both breast and prostate cancers. BMC Genomics 8, 199 (2007).

19. Bai, R. K., Perng, C. L., Hsu, C. H. & Wong, L. J. Quantitative PCR analysis of CD133hi cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. Nat. Commun. 7:10442 doi: 10.1038/ncomms10442 (2016).

Acknowledgements
We are grateful to Mesra\% Turkc\%a\l, A\%sar Barl\%a, Sho Fujisawa, Romin Yevgenyi (Molecular Cytology Core) and Ashley Duane for advice and technical assistance. In addition, we thank Dr Andre\% Tremblay (University of Montreal) for his pER\%s and ER\%E constructs and Dr William L. Farrar (The National Cancer Institute, Frederick) for his pl\%L. P.S. was supported by the Department of Defense (Postdoctoral Award W81XWH-10-1-1013). Our work was also supported by grants from the National Institutes of Health (R01: CA87637 (J.B.)), Charles and Marjorie Holloway Foundation (J.B.), Susman Family Fund (J.B.), Lerner Foundation (J.B.), AstraZeneca (J.B.), Breast Cancer Alliance (J.B.), Manhasset Women’s Coalition Against Breast Cancer (J.B.), NYS Women’s Bowling Association (J.B.), The Beth C. Tortolani Foundation (J.B. and D.L.). J.B. has consulted for Roche, Medimmune and Bristol-Myers Squibb, and has received research support from AstraZeneca. This study was also supported by MSK Cancer Center Support Grant/Core Grant (P30 CA008748; J.B); National Institutes of Health (U01-CA169538) (D.L. and J.B.), The Manning Foundation (D.L.), The Hartwell Foundation (D.L.), Fundacao para a Ciencia e a Tecnologia (D.L.), The Nancy C and Daniel P Paduanio Foundation (D.L.), The Mary Kay Foundation (D.L.), Pediatric Oncology Experimental Therapeutic Investigator Consortium (POETIC, D.L.), James Paduanio Foundation (D.L.), Malcolm Hewitt Weiner Foundation (D.L.), Theodore A Rapp Foundation (D.L.) and American Hellenic Educational Progressive Association 5th District Cancer Research Foundation (D.L.). G.G.’s laboratory is funded by Associazione Italiana Ricerca sul Cancro (AIRC, grant IGI4242 JANEUTICS), Fondazione Umberto Veronesi (grant DISCO TRIP) and FFP EU ITN Marie Curie Action project MEET (G.A.317433). M.V. is supported by a triennial AIRC fellowship ‘Wanda Cantone e Alberto Rigillo’. M.B. is supported by the Cornelia and Roberto Pallotti Legacy. The Wellness after Breast Cancer study (U. Penn) was supported by the National Institutes of Health (R01: CA158243 (J.J.M.)) Susan G. Komen (KG110876; A.D.).

Author contributions
P.S. conceived and developed the study. P.S., C.Ce., M.B., D.S., J.N., G.G., F.P., R.L.B., M.C., L.D. and J.B. analysed data. C.C.E., D.S., N.N.C.S., M.F., C.G., J.C., J.M., A.D., N.F., J.H.H. and M.T. collected and provided patient samples. P.S., M.B. and J.B. wrote the paper with input from C.C.E. and D.L. J.B. oversaw the project. All the authors discussed the results and commented on the manuscript.

Additional information
Accession codes: GSE69286 array deposited in NCBI
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Sassone, P. et al. Self-renewal of CD133hi cells by IL6/Notch3 signalling regulates endocrine resistance in metastatic breast cancer. Nat. Commun. 7:10442 doi: 10.1038/ncomms10442 (2016).