ERBB2 Increases Metastatic Potentials Specifically in Androgen-Insensitive Prostate Cancer Cells

Jessica Tome-Garcia¹, Dan Li¹, Seda Ghazaryan¹, Limin Shu¹, Lizhao Wu¹,²*

¹ Rutgers New Jersey Medical School-Cancer Center, Newark, New Jersey, United States of America, ² Department of Microbiology and Molecular Genetics, Rutgers New Jersey Medical School, Newark, New Jersey, United States of America

Abstract

Despite all the blood-based biomarkers used to monitor prostate cancer patients, prostate cancer remains as the second common cause of cancer mortality in men in the United States. This is largely due to a lack of understanding of the molecular pathways that are responsible for the aggressive forms of prostate cancers, the castrate-resistant prostate cancer and the metastatic prostate cancer. Cell signaling pathways activated by the ERBB2 oncogene or the RAS oncogene are frequently found to be altered in metastatic prostate cancers. To evaluate and define the role of the ERBB2/RAS pathway in prostate cancer metastasis, we have evaluated the impact of ERBB2- or RAS-overexpression on the metastatic potentials for four prostate cancer cell lines derived from tumors with different androgen sensitivities. To do so, we transfected the human DU145, LnCaP, and PC3 prostate cancer cells and the murine Myc-CaP prostate cancer cells with the activated form of ERBB2 or H-RAS and assessed their metastatic potentials by three complementary assays, a wound healing assay, a transwell motility assay, and a transwell invasion assay. We showed that while overexpression of ERBB2 increased the metastatic potential of the androgen-insensitive prostate cancer cells (i.e. PC3 and DU145), it did not affect metastatic potentials of the androgen-sensitive prostate cancer cells (i.e. LnCaP and Myc-CaP). In contrast, overexpression of H-RAS only increased the cell motility of Myc-CaP cells, which overexpress the human c-MYC oncogene. Our data suggest that ERBB2 collaborates with androgen signaling to promote prostate cancer metastasis, and that although RAS is one of the critical downstream effectors of ERBB2, it does not phenocopy ERBB2 for its impact on the metastatic potentials of prostate cancer cell lines.

Citation: Tome-Garcia J, Li D, Ghazaryan S, Shu L, Wu L (2014) ERBB2 Increases Metastatic Potentials Specifically in Androgen-Insensitive Prostate Cancer Cells. PLoS ONE 9(6): e99525. doi:10.1371/journal.pone.0099525

Editor: Wei-Guo Zhu, Peking University Health Science Center, China

Received April 11, 2014; Accepted May 15, 2014; Published June 17, 2014

Copyright: © 2014 Tome-Garcia et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are available in the paper.

Funding: Start-up funds from Rutgers New Jersey Medical School. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wuli@njms.rutgers.edu

Introduction

Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer mortality in men in the United States [1]. Despite increased screening for early detection and monitoring, prostate cancer-specific mortality has remained at the same level [2]. This is likely due to both the inability to diagnostically distinguish between the non-invasive, indolent localized prostate cancers and the very aggressive localized cancers with high metastatic potentials, and the poor understanding of the cellular and molecular basis for metastatic prostate cancers [3].

One of the best studied genes in human malignancies, including prostate cancer, is the ERBB2 or HER2 or NEU oncogene. ERBB2 is a member of the epidermal growth factor receptor (EGFR) family, which consists of four members (EGFR, ERBB2, ERBB3 and ERBB4) that act as tyrosine kinase receptors [4–7]. They are considered as potent mediators of cell growth and cancer development [8–10]. In breast cancer, amplification or overexpression of ERBB2 is a common event that appears in 15–30% of all specimens [11], and ERBB2 gene amplification and/or overexpression have been associated with a poor clinical outcome [12,13]. Consistent with an important role of ERBB2 in breast cancer metastasis, overexpression of a constitutively activated form of ERBB2 [i.e. NeuT] [14] in mice is sufficient to trigger metastatic mammary tumors [15]. However, the potential role of ERBB2 in the development of metastatic prostate cancer is unclear partly because various attempts to assess frequencies of ERBB2 amplification/overexpression in human prostate cancer samples yielded inconsistent results [16–25]. Interestingly, ERBB2 overexpression has been implicated in androgen-resistant metastatic prostate cancers [26], suggesting a possible role for ERBB2 in the acquisition of metastatic potentials of prostate cancer cells.

Overexpression of ERBB2 results in the induction of several signaling pathways, such as the phosphoinositide-3-kinase/protein kinase B (PI3K/AKT) pathway and the mitogen-activated protein kinase (MAPK) pathway [27]. Both the PI3K/AKT pathway and the MAPK pathway regulate cellular proliferation and cell survival, and have been implicated in cancer metastasis [28–30]. The principal downstream effector of ERBB2 that regulates these two kinase pathways is the oncogenic RAS, although ERBB2 is also able to activate PI3K/AKT independent of the RAS activation [31]. Importantly, PI3K/AKT and MAPK are the only RAS-effector pathways commonly mutated in human cancers [32].

RAS oncopgenes encode three monomeric GTPases, H-RAS, N-RAS, and K-RAS, which are activated when bound to GTP. While inhibition of RAS in androgen-independent PC3 prostate...
cancer cells and androgen-dependent LnCaP prostate cancer cells led to growth arrest and apoptosis [33], constitutive activation of the RAS/MAPK pathway in LnCaP prostate cancer cells promoted androgen hypersensitivity [34]. In addition, immuno- histochemical analysis of hormone-sensitive and hormone-refrac-
tory prostate cancer specimens showed that increased expression of N-RAS was associated with hormone-refractory prostate cancers, and was correlated with shorter time to tumor relapse and reduced disease-specific survival [35]. In a xenograph mouse model, activation of two RAS effector pathways, Raf/ERK and Raf/GEF, in the moderately metastatic DU145 prostate cancer cell line promoted metastasis to the brain and bone, respectively [36]. These data suggest a possible role of RAS in promoting metastasis in human prostate cancers.

To further define the potential roles of the ERBB2/RAS pathway in promoting prostate cancer metastasis, we have examined the effects of overexpression of ERBB2 or RAS on the metastatic properties of three human prostate cancer cell lines and one murine prostate cancer cell line with various levels of androgen sensitivities and different metastatic potentials. To do so, we first transfected three commonly used human prostate cancer cell lines (DU145, LnCaP, and PC3) and one murine prostate cancer cell line (Myc-CaP) with the activated form of ERBB2 or H-RAS. We then evaluated the metastatic potentials of the genetically modified cells by three different complementary assays, a wound healing assay, a transwell motility assay, and an invasion assay. We found that while overexpression of ERBB2 increased metastatic potentials specifically for androgen-insensitive human prostate cancer cells, overexpression of RAS did not have similar impacts on metastatic potentials but specifically increased cell motility of c-MYC-overexpressing murine Myc-CaP cells.

Methods

Cell Lines and Culture

Myc-Cap is a non-metastatic, androgen-sensitive murine prostate cancer cell line that was established from primary prostate tumors isolated from the P0-Hi-Myc mice [37]. LnCaP [38], DU145 [39], and PC3 [40] are three human metastatic prostate cancer cell lines with different androgen sensitivities and different metastatic properties (Table 1). LnCaP and PC3 cell lines were maintained in RPMI 1640 medium, and Myc-CaP cells were grown in DMEM. Both media were supplemented with 10% fetal bovine serum (FBS). DU145 cells were maintained in DMEM:Ham’s F12 medium (1:1) supplemented with 10% newborn calf serum. Amphotropic Phoenix cells were used for retroviral transfection and were maintained in DMEM supplemented with 12.5% FBS. Senescent B cell human skin fibroblast cells were generated by replicative senescence [41] and were used as a positive control for β-galactosidase activity assay. They were maintained in DMEM supplemented with 10% FBS. All cells were cultured in a humidified incubator at 37°C with 5% CO2.

Retroviral Transfection

Retroviral overexpression vectors pBabe-Puroycin-H-Ras and pBabe-Puroycin-ERBB2, which overexpress a mutated form of human H-RAS gene (H-RASG12V) and a constitutive activated form of human ERBB2 gene (Neat), respectively, were gifts from Dr. Gustavo Leone. High-titer viruses were produced by calcium phosphate transient transfection of retroviral constructs into amphotropic Phoenix packaging cells as previously described [42]. We infected prostate cancer cells with fresh retroviruses using standard methods in the presence of polybrene (4 μg/ml). Infected cells were subjected to selection with puromycin (2.5 μg/ml) for five days. Puromycin-resistant cells were cultured in fresh DMEM without puromycin for one day before being either harvested to prepare cell lysates for Western blotting analysis, or re-plated for experiments. All data presented were collected by using cells from at least two independent retroviral transfections that yielded similar levels of ERBB2 and RAS overexpression.

Western Blot

Cell lysates with equal amounts of proteins (30 μg) were separated in 8% SDS-PAGE, except for ERK and pERK, for which cell lysates were separated in 10% SDS-PAGE. Separated proteins were then electrophoretically transferred to a 0.45 μM nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK), which was subsequently blocked at 4°C for 1 hr with 5% nonfat dry milk in TBST (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween 20 (w/v)). The blots were then incubated with appropriate dilutions of primary antibodies overnight at 4°C in TBST containing 3% nonfat dry milk. Primary antibodies used for Western blot analysis include polyclonal antibodies for H-RAS (sc-520), E2F1 (sc-193), E2F2 (sc-633), ERK1/2 (sc-135900), and p-ERK1/2 (sc-81492) from Santa Cruz Biotechnology (Dallas, TX); actin (A2060) from Sigma (St Louis, MO); ERBB2 (MS-730-P) from Thermo Fisher Scientific (Fremont, CA); AKT (4061S), p-AKT (4060S), p38 (9212S), and p-p38 (9211S) from Cell Signaling (Beverly, MA). After washing 10 min for three times in TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies from Perkin Elmer (Boston, MA) either against rabbit (NEF 812001EA) or against mouse (NEF 822001EA) with a dilution of 1:3000 in TBST with 3% milk. After three washes with TBST for 10 min each, the blots were incubated at room temperature for 1 hr with ECL from Thermo Fisher Scientific (Rockford, IL), and exposed to an X-ray film for 2 min.

Wound Healing Assay

The migration ability of the cells was evaluated by using a wound healing assay as previously described with slight modifi-

| Cell line   | Origin                        | Metastasis level | Androgen sensitivity | References |
|-------------|-------------------------------|------------------|----------------------|------------|
| PC3         | Human bone metastasis         | High             | Insensitive          | [40,61,62] |
| DU145       | Human brain metastasis        | Moderate         | Insensitive          | [39,61,62] |
| LnCaP       | Human lymph node metastasis   | Low              | Sensitive            | [38,61,62] |
| Myc-CaP     | Mouse primary prostatecarcinoma | Non-metastatic   | Sensitive            | [37]       |

Table 1. Summary of the principal characteristics of the prostate cancer cell lines included in the study.
Cells were plated in 35 mm dishes and left to grow until reaching 100% confluence. Confluent cells were maintained under the same culture conditions for 48 hr to induce density arrest and to minimize cellular proliferation. Confluent plates were scratched down three times with a 200 μl pipette tip, creating left, center, and right scratches/wounds that crossed with two horizontal lines previously drawn as landmarks for quantifications. After the scratching, plates were washed once with medium to remove floating cells, and were replaced with fresh medium. Cells were allowed to migrate across the wounds and pictures were taken at various time points using a phase-contrast microscope until the wound was completely closed. The time points at which the pictures were taken depended on the migratory ability of different cell lines studied. Each experiment was performed in triplicates and was repeated at least once to validate the initial data.

Motility Assay (Boyden Chamber Assay)

The motility of the cells was also evaluated using cell culture inserts from BD Falcon (Franklin Lakes, NJ), and following the protocol previously established with minor modifications [44]. Cells that were previously maintained in a starvation medium with 0.2% FBS for 24 hr to minimize cellular proliferation, were resuspended at a concentration of 2 × 10^5 cells/ml in medium containing 0.2% FBS. 1 × 10^5 cells or 500 μl of cell suspensions were plated onto each insert, which was previously coated with 3 μg/ml of rat tail collagen solution from BD (Bedford, MA) overnight at room temperature. The lower chamber contained medium with 10% FBS as a chemo-attractant. Cells were allowed to pass through the porous membrane and were collected at different time points that were empirically determined based on the migratory ability of each cell line. Non-migratory cells were then removed from the surface of the membranes using cotton swabs. Cells that passed through the pores of the membrane were fixed and stained using the Diff-Quick nuclei and cytoplasm staining kit (Dade Behring, Newark, DE). Stained migratory cells were counted under a microscope. Each experiment was performed in triplicates and was repeated at least once to validate the initial data.

Invasion Assay

The invasiveness of the cells was measured using cell culture inserts from BD Falcon (Franklin Lakes, NJ) by following a previously described protocol with minor modifications [45]. As in the transwell-based motility assay described above, cells were maintained in a starvation medium with 0.2% FBS for 24 hr before seeding to minimize cellular proliferation. Inserts were coated with 50 μg/ml of rat tail collagen solution from BD (Bedford, MA) for 5 hr at room temperature. After the incubation, inserts were washed three times with serum-free medium and were allowed to dry at 37°C overnight. Once dried, membranes were covered with 100 μl of collagen solution at a final concentration of 1.3 mg/ml (for all cells) or with 100 μl of Matrigel (Cat. #356231) from BD (Bedford, MA) at a final concentration of 300 μg/ml (for Myc-CaP cells only), and were allowed to solidify at 37°C. Cells were resuspended at a concentration of 2 × 10^5 cells/ml in medium containing 0.2% FBS. 1 × 10^5 cells or 500 μl of cell suspensions were plated onto each insert. The remaining procedure was performed as described above in the motility assay section. Each experiment was performed in triplicates and was repeated at least once to validate the initial data.

Assessment of Senescence-associated β-galactosidase Activity

The endogenous β-galactosidase activity of prostate cancer cells was assessed by X-gal staining as previously described [46]. Cells seeded in 35 mm dishes in triplicates were fixed for three min at room temperature in 1X PBS containing, 2% formaldehyde and 0.2% glutaraldehyde. After two consecutive washes with 1X PBS, cells were incubated for 1 hr at 37°C with staining solution (2 ml per dish) that consists of X-gal at a final concentration of 1 mg/ml in dimethylformamide, 40 mM citric acid/sodium phosphate buffer pH 5.7 (0.1 M citric acid/0.2 M sodium phosphate), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, and 2 mM magnesium chloride. Pictures were taken under a phase-contrast microscope. Positive and negative cells were counted from at least three different fields.

Cell Growth Rate Assessment

Prostate cancer cells were seeded in 6-well plates in triplicates. The density of the initial seeding was empirically determined to allow us to count at least four time points before cells reached 100% confluence. Thus, cells were seeded as follows: 70000 cells for PC3, 35000 cells for LnCaP, 120000 cells for DU145, and 70000 cells for Myc-CaP. Twelve hours after seeding, cells were counted using a hemocytometer (Hauser Scientific, Horsham, PA) to be normalized as the corresponding seeding cell number and to be used as the first time point. Cells were then counted every 12 hr for PC3 and Myc-CaP cell lines or every 24 hr for DU145 and LnCaP cell lines. Growth rates were estimated by calculating and comparing the linear slope for each growth curve.

Statistical Analysis

Values are presented as mean ± SD. Statistical significance was determined by Student’s t-test with a significance threshold of P< 0.05. In all figures, statistical significances were denoted as *P< 0.05, **P<0.01, and ***P<0.001.

Results

Overexpression of ERBB2 and RAS Oncogenes in Prostate Cancer Cell Lines by Retroviral Infection

To overexpress ERBB2 and RAS oncogenes in prostate cancer cell lines, we transduced prostate cancer cells with pBabe-Puroycin- (PBP) based retroviruses overexpressing an activated form of ERBB2 (PBP-ERBB2) or a mutated form of H-RAS (PBP-RAS). As shown in Figure 1, Western blotting analysis indicated that transfection of cells with PBP-RAS retroviruses led to moderate up-regulations of RAS ranging from 1.5 fold (for LuCaP) to 4.7 fold (for PC3), and that transfection of cells with PBP-ERBB2 retroviruses led to moderate up-regulations of ERBB2 ranging from 2.5 fold (for DU145) to 4.0 fold (for Myc-CaP). Interestingly, although RAS overexpression did not change protein levels of ERBB2, ERBB2 overexpression elevated protein levels of RAS (2.9 fold) specifically in the Myc-CaP cells (Figure 1), which overexpresses the human c-MYC oncogene [37].

Overexpression of ERBB2 Leads to Moderate Increases in Cell Growth in LnCaP, DU145 and PC3 Cells

Considering that activation of the ERBB2/RAS signaling pathway in prostate cancer cells may affect their growth rates, which could influence the analysis to assess their metastatic potentials, we carried out a growth curve assay using asynchronous cells. As shown in Figure 2, ERBB2 overexpression led to moderate increases in growth rates for all of the three human prostate cancer
cell lines: an average of 43% increase for LnCaP cells, 33% increase for DU145 cells, and 25% increase for PC3 cells. However, overexpression of ERBB2 did not affect the growth rate of the murine prostate cancer cell line, Myc-CaP. In contrast, RAS overexpression did not have significant effects on the growth rates of any of the four cell lines (Figure 2).

Overexpression of RAS Reduces Cell Migration Rates of LnCaP and DU145 Cell Lines

The effects of ERBB2 and RAS overexpression on the metastatic potentials of LnCaP, DU145, PC3, and Myc-CaP prostate cancer cell lines were first assessed by performing a wound healing assay. To overcome potential effects of increased cellular proliferation in ERBB2-overexpressing cells (Figure 2) on the wound healing assay, we induced cell density arrest by maintaining confluent plates for 48 hours before making scratches/wounds. As shown in Figure 3, overexpression of ERBB2 and RAS showed various effects on the three human prostate cancer cell lines. Specifically, while overexpression of ERBB2 had no significant impact on the migration rates of all of the three human prostate cancer cell lines, overexpression of RAS significantly decreased the migration rates of LnCaP and DU145 cell lines. Compared to the human prostate cancer cell lines, the murine prostate cancer cell line Myc-CaP seemed to have lower migration rates, as evidenced by the fact that they failed to completely close the wound before they started to grow again around the 32 hour time point (data not shown), regardless of the status of ERBB2- or RAS-overexpression (Figure 3). Nevertheless, ERBB2- or RAS-overexpressing Myc-CaP cells had a moderate but statistically insignificant increase in migration rates as compared to control cells (Figure 3).

ERBB2 Overexpression Increases Cell Motilities of the Androgen-insensitive DU145 and PC3 Cells, and RAS Overexpression Increases the Cell Motility of the Myc-CaP Cells

To complement the wound healing data presented above, we also assessed the effects of overexpression of ERBB2 and RAS on the cell motility of the prostate cancer cell lines by a transwell-based cell motility assay using porous membrane inserts in transwells. While wound healing assay measures lateral cell motility resulted from the disruption of cell-cell interactions [47], Boyden chambers transwell assay measures chemo-attractive migration, which unlike wound healing assay, is independent of breakages in cell-cell junctions [48]. Therefore, these two assays are complementary and are often used in parallel to gain biological insights on different types of cell migration. To minimize the impact of differential cell growth rates of ERBB2 overexpression on the motility assay, we induced cell cycle arrest by maintaining cells under serum-starvation conditions for 24 hours before performing the motility assay. As shown in Figure 4, overexpression of ERBB2 significantly increased cell motilities of the two more metastatic, androgen-insensitive cell lines, DU145 cells and PC3 cells, with a 30% increase and a 6-fold increase, respectively. In contrast, ERBB2 overexpression significantly reduced the motilities of the two lowly or non-metastatic, androgen-sensitive prostate cancer cell lines, LnCaP and Myc-CaP. In addition, while RAS overexpression significantly reduced the motilities of LnCaP cells and PC3 cells, it substantially increased the motility of Myc-CaP cells, which overexpress the c-MYC oncogene.

ERBB2 Overexpression Promotes Cell Invasiveness in the Androgen-insensitive DU145 and PC3 Cells

The metastatic potentials of ERBB2- or RAS-overexpression in various prostate cancer cell lines were further assessed by evaluating their relative invasiveness by a transwell-based invasion assay.
ERBB2 Increases Metastatic Potentials of Prostate Cancer Cells

Moderate Levels of RAS Overexpression does not Promote Cellular Senescence in Prostate Cancer Cells

Previous studies have shown that prolonged expression of oncogenic RAS in human and rodent primary fibroblast cells in vitro [49] or high levels of overexpression of oncogenic RAS in mammary epithelial cells in vivo [50] led to increased cellular senescence. Since RAS-overexpressing LnCaP cells and DU145 cells showed reduced abilities to close a provoked wound (Figure 3), and since RAS-overexpressing PC3 cells showed decreased cell motilities in the transwell mobility assay (Figure 4), we sought to determine whether the decreased mobility in these RAS-overexpressing human prostate cancer cells can be explained by a potential increase in cellular senescence in those cells. To this end, we used in vitro X-gal staining to assess the senescence-associated β-galactosidase activities, a commonly used biomarker for cellular senescence [51]. As shown in Figure 6A and Figure 6B, moderate levels of RAS overexpression in our experimental setting (Figure 1) did not significantly increase cellular senescence in LnCaP, PC3, and DU145 cells, as evidenced by the fact that RAS-overexpressing cells showed similar percentages of X-gal-positive cells as their corresponding PBP control cells. In contrast, PC3 cells with a much higher level of RAS overexpression (i.e. 13.6 fold; Figure 6C, “Hi-Ras”) showed a significant increase in the percentage of X-gal-positive cells than either the PC3 cells infected with the control vector (Figure 6C, “PBP”) or the PC3 cells with a moderate expression of RAS (i.e. 4.2 fold; Figure 6C, “RAS”) (Figure 6A and 6B). The increased percentage of X-gal positive PC3 cells with a higher level of RAS overexpression is consistent both with their senescence-like morphology (i.e. big and

**Figure 2. Overexpression of ERBB2 led to moderate increases in cell growth of human prostate cancer cells.** Cell growth rates were assessed by cell counting every 12 hours or 24 hours for various prostate cancer cells that were transfected with control retroviruses (PBP), or retroviruses overexpressing either PBP-H-RAS (RAS) or PBP-ERBB2 (ERBB2). doi:10.1371/journal.pone.0099525.g002

- **Erbb2** Increases Metastatic Potentials of Prostate Cancer Cells

assay using cell inserts coated with a collagen matrix. This invasion assay allows cells to go through a 100 μM-thick collagen matrix from a low-serum-containing medium to a serum-enriched environment. As shown in Figure 5, the invasiveness of the androgen-insensitive DU145 cells and PC3 cells was substantially augmented in the presence of ERBB2 overexpression but not in the presence of RAS overexpression. These data are consistent with the data from the transwell-based motility assay showing that overexpression of ERBB2 in DU145 cells and PC3 cells led to increased cell motility (Figure 4). Consistent with their low or no metastatic potentials, neither Myc-CaP cells nor LnCaP cells appeared to be able to penetrate the collagen matrix even after 96 hours of incubation (data not shown). Importantly, overexpression of ERBB2 or RAS was insufficient to enable either Myc-CaP cells or LnCaP cells to pass through the collagen matrix (data not shown).

Since overexpression of RAS increased the cell motility specifically in the Myc-CaP cells (Figure 4), we performed a transwell-based invasion assay on Myc-CaP cells using Matrigel to replace collagen. Compared to the collagen matrix, the Matrigel matrix is a reconstituted basement membrane prepared from a mouse sarcoma that better mimics the extracellular environment of a cancer. As in the case of collagen matrix, no cell was seen to pass through the Matrigel matrix either in control cells or in the RAS-overexpressing cells (data not shown).
Figure 3. Overexpression of **RAS** or **ERBB2** did not increase lateral cell migration rates. Cell migration rates were estimated by a wound healing assay for prostate cancer cells that were transfected with either control retroviruses (**PBP**), or retroviruses overexpressing **PBP-H-RAS** (**RAS**) or **PBP-ERBB2** (**ERBB2**). Left panels showed percentages of wounds remained at different time points. The percentages of wounds were estimated based on the average of 12 measurements on each plate reflecting measurements of four evenly distributed sections on each of the three wounds/scratches on each plate. Data were presented as means ± SD from three replicates. Right panels showed representative images taken at different time points. All images were taken at the same scale with a scale bar of 200 μM displayed in the first image.

doi:10.1371/journal.pone.0099525.g003
flat) (Figure 6A) and with a previous report that high levels of Ras overexpression led to increased cellular senescence [50]. Taken together, our data suggest that the inability of RAS to promote cell motility or invasiveness in human prostate cancer cell lines is not due to premature cellular senescence.

**MAPK and/or PI3K-AKT Pathways are Activated as a Consequence of ERBB2 or H-RAS Overexpression**

The inability of RAS overexpression to promote metastatic potentials of prostate cancer cells may be due to a failure to activate its downstream signaling pathways upon moderate RAS overexpression. To explore this possibility, we assessed the various downstream effector kinases of the ERBB2/RAS signaling pathway by carrying out Western blot analyses using antibodies against ERK, AKT, and p38 kinases as well as their phosphorylated or activated forms. In parallel, we also carried out the same analysis on ERBB2-overexpressing cells. As shown in Figure 7, although overexpression of ERBB2 or RAS did not significantly alter the levels of total ERK, total AKT, or total p38 kinases, they activated the phosphorylated forms of those kinases in most cell lines. Specifically, RAS overexpression activates the ERK pathway in all four cell lines as it significantly elevated protein levels of p-ERK, ranging from a 1.7 fold increase in PC3 cells and an 18.4 fold increase in Myc-CaP cells (Figure 7). In addition, overexpression of RAS led to significant increases in protein levels of p-AKT in LncCaP cells (by 4.7 fold) and DU145 cells (by 27.1 fold). On the other hand, ERBB2 overexpression led to significant increases in protein levels of p-ERK in LncCaP cells (by 2.3 fold) and Myc-CaP cells (by 3.2 fold), p-AKT in LncCaP cells (by 4.3 fold), DU145 cells (by 12.5 fold), and Myc-CaP cells (by 3.7 fold).
as well as moderate increases in protein levels of p-p38 in LnCaP cells (by 1.5 fold), DU145 cells (by 2.0 fold), and PC3 cells (by 1.9 fold) (Figure 7). Overall, RAS overexpression led to the activation of more than one kinase in all cell lines except the Myc-CaP cell line, and ERBB2 overexpression activated more than one kinase in all cell lines except the PC3 cell line.

**Discussion**

Activation of ERBB2 and RAS oncogene is known to trigger cell signaling pathways commonly mutated in human cancers. Therefore, various attempts have been made to determine whether these two oncogenes are involved in the metastatic transformation of prostate cancer cells [36,52–54]. However, up to now, there is no conclusive evidence that links ERBB2 or RAS activation to the invasive behavior in prostate tumors. In the present study, we used three complementary approaches to assess the effect of ERBB2- and RAS-overexpression on the metastatic potentials of three metastatic human prostate cancer cell lines and one non-metastatic mouse prostate cancer cell line that have different androgen-sensitivities. All three human prostate cancer cell lines were derived from metastatic prostate cancer patients, with PC3 cells being highly metastatic, DU145 cells moderately metastatic, and LnCaP cells poorly metastatic (Table 1). On the other hand, the murine prostate cancer cell line Myc-CaP was derived from non-metastatic primary prostate carcinoma resulted from c-MYC overexpression. In addition, while PC3 cells and DU145 cells are androgen insensitive, LnCaP cells and Myc-CaP cells are androgen sensitive (Table 1). We showed that overexpression of ERBB2 increased metastatic potentials in androgen-insensitive PC3 cells and DU145 cells, as evidenced by increased cell motility and increased invasiveness in those cells. However, ERBB2 overexpression did not have any significant impact on androgen-sensitive LnCaP cells and Myc-CaP cells. These data suggest that ERBB2 increases metastatic potentials specifically in androgen-sensitive prostate cancer cells.

It has been previously shown that PC3 cells transfected with activated ERBB2 acquired the potential to metastasize from primary tumor to neighboring soft tissues and skeletons [54], suggesting a potential role of ERBB2 activation to promote prostate cancer metastasis. In addition, Chung and colleagues showed that one specific single-cell clone (N35) resulted from ERBB2 overexpression in PC3 cells was found to disseminate widely to the lymph nodes and distant organs upon orthotopic administration [55]. However, subcutaneous administration of the same single-cell clone (N35) and the other ERBB2-overexpressing single-cell clone into athymic nude mice did not induce metastasis, suggesting that the ability of ERBB2 to induce prostate cancer metastasis depends on an appropriate host microenvironment [55]. Furthermore, an EGFR tyrosine kinase inhibitor suppressed EGF-induced invasion in hormone-refractory DU145 cell line and PC3 cell line [56]. The fact that ERBB2 overexpression increased metastatic potentials specifically in the two androgen-insensitive prostate cancer cell lines (but not in the two androgen-sensitive prostate cancer cell lines) suggests that ERBB2 promotes prostate cancer metastasis by collaborating with androgen/androgen receptor signaling. Interestingly, overexpression of ERBB2 in PC3 cells and DU145 cells led to moderate up-regulations (i.e. a 1.9 fold increase for both cell lines) of activated p38 kinases, an event that is accompanied by moderate down-regulations (i.e. 40% for PC3 cells and 60% for DU145 cells) of activated ERK (Figure 7). It would be interesting to know whether the ability of ERBB2 to increase prostate cancer metastatic potentials depends on the activation of the p38 kinase signaling pathway and the down-regulation of the ERK signaling pathway.

It is worthwhile noting that the two androgen-insensitive prostate cancer cell lines (i.e. PC3 and DU1450) also possess higher metastatic properties than the two androgen-insensitive prostate cancer cell lines (i.e. LnCaP and Myc-CaP) (Table 1). Therefore, our data does not rule out the possibility that the relatively high metastatic property of the PC3 cells and DU145 cells contributes to the ability of ERBB2 to promote prostate cancer metastasis in those cells. It is also worthwhile noting that although overexpression of ERBB2 increased cell growth in the three human prostate cancer cell lines under growth conditions (Figure 2), such moderate increases in growth rates likely did not

---

**Figure 5.** ERBB2 overexpression increased the invasiveness of DU145 cells and PC3 cells. Cell invasiveness was assessed by a transwell-based invasion assay for prostate cancer cells that were transfected with either control retroviruses (PBP), or retroviruses overexpressing PBP-H-RAS (RAS) or PBP-ERRB2 (ERBB2). Each bar graph showed the numbers of cells that have passed through the collagen matrix either 72 hours (for PC3 cells) or 96 hours (for DU145 cells) after plating. Transwell inserts were stained and invading cells were counted for the entire inserts. Data were presented as means ± SD from three replicates. Representative images were shown underneath each bar graph. All images were taken at the same scale with a scale bar of 200 μM displayed in the first image. doi:10.1371/journal.pone.0099525.g005
make any significant contributions to the ability of ERBB2 to increase metastatic potentials in the PC3 cells and the DU145 cells. This is because all three assays used to assess metastatic potentials were set up under growth arrest conditions, thereby minimizing the potential effects of differential growth rates on our ability to evaluate metastatic potentials. In addition, the moderate
increases in growth rates in DU145 cells (a 33% increase) and PC3 cells (a 25% increase) (Figure 2) do not correlate with the much bigger differences in cell motilities and cell invasiveness assessed from the transwell-based motility assay (Figure 4) and the invasion assay (Figure 5). Furthermore, overexpression of ERBB2 did not increase cell motility or invasiveness in LnCaP cells (Figure 4 and data not shown) despite the fact that it increased growth rate (43%) in LnCaP cells even more than in PC3 cells and DU145 cells (Figure 2).

As one of the critical downstream effectors of ERBB2 pathway, RAS oncogene has been previously implicated in prostate cancer metastasis [36]. In the present study, we have shown that overexpression of H-RAS and overexpression of ERBB2 had different impacts on the metastatic potentials of various prostate cancer cell lines. Although overexpression of ERBB2 led to increased metastatic potentials in PC3 cells and DU145 cells, overexpression of H-RAS did not have similar effects on these two cell lines or the LnCaP cell line (Figure 4 and Figure 5), despite the fact that RAS overexpression did elevate p-ERK (particularly p-ERK1) as well as p-AKT and/or p-p38 in all of the three human prostate cancer cell lines (Figure 7). These data suggest that RAS overexpression does not recapitulate the effect of ERBB2 overexpression on metastatic potentials of prostate cancer cells and that ERBB2 increases metastatic potentials independent of H-RAS activation. Consistent with the latter notion, overexpression of H-RAS in the two androgen-insensitive cell lines did not activate H-RAS (Figure 1). Interestingly, RAS overexpression increased cell motility specifically in the MYC-overexpressing Myc-CaP cells (Figure 4), suggesting a collaborative role of MYC and RAS in promoting cell motility.

Conclusions

We have shown that overexpression of the constitutively activated form of ERBB2 (NeuT) increases the metastatic potential of the two androgen-insensitive human prostate cancer cell lines, DU145 and PC3, but not that of the two androgen-sensitive prostate cancer cell lines, LnCaP and Myc-CaP. These findings, coupled with previous xenograph data implicating a potential role of ERBB2 in the promotion of prostate cancer invasiveness or metastasis, strongly suggest a potential crosstalk between the ERBB2 signaling pathway and the androgen/androgen receptor signaling pathway in promoting prostate cancer metastasis. We also showed that overexpression of H-RAS in the same four cell lines specifically increased the cell motility of MYC-overexpressing Myc-CaP cells, suggesting that MYC collaborates with RAS to promote cell motility.

Acknowledgments

We thank Drs. Philip Watson and Charles Sawyers for providing the Myc-Cap cell line, Dr. Beverly E. Barton for providing DU145 and LnCaP cell lines, Dr. Joseph Bertino for providing the PC3 cell line, Dr. Garry Nolan for providing the amphotropic Phoenix cell line, Dr. Utz Herbig for providing the senescent BJ human skin fibroblast cell line, and Dr. Teresa Wood for providing antibodies against AKT, p-AKT, p38, and p-p38. We also thank Josephine Ng, Jill Deutsch, and Rahul Dutta for technical assistance.
Author Contributions
Conceived and designed the experiments: JTG LS DL SG. Contributed reagents/materials/analysis tools: JTG DL. Contributed to the writing of the manuscript: JTG LW.

References
1. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 11–30.
2. Ilie D, Neuberger MM, Djulbegovic M, Dahm P (2013) Screening for prostate cancer. Cochrane Database Syst Rev 1: CD004728.
3. Semanas J, Allegucci C, Boorjian SA, Mongan NP, Person JL (2012) Overcoming drug resistance and treating advanced prostate cancer. Curr Drug Targets 13: 1308–1323.
4. Ushiro H, Cohen S (1980) Identification of phosphotyrosine as a product of equine platelet factor-activated protein kinase in A-431 cell membranes. J Biol Chem 255: 8363–8365.
5. Toyoshima K, Semb Ka, Akiyama T, Ikawa S, Yamamoto T (1986) The c-erb-B-2 gene encodes a receptor-like protein with tyrosine kinase activity. Cold Spring Harb Symp Quant Biol 51 Pt 1: 977–982.
6. Katoh M, Yazaki Y, Sugimura T, Terada M (1993) c-erbB3 gene encodes secreted as well as transmembrane receptor tyrosine kinase. Biochem Biophys Res Commun 192: 1189–1197.
7. Pisani GM, Goloumou JM, Whitney GS, Green JM, Carlsson GW, et al. (1993) Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci U S A 90: 1746–1750.
8. Hynes NE, Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 5: 341–354.
9. Reddy KB, Nabh SM, Aumakswa N (2005) Role of MAP kinase in tumor progression and invasion. Cancer Metastasis Rev 22: 393–403.
10. Scabrit B, Baslaja G (2006) The epidermal growth factor receptor pathway: a model for targeted therapy. Clin Cancer Res 12: 3268–3272.
11. Lass KS, Haags KM (2005) Non-redundancy within the RAS oncogene family: insights into mutational disparities in cancer. Mol Cells 28: 315–320.
12. Erlich S, Tal-Or P, Liebling R, Blum R, Karanagaran D, et al. (2006) Ras inhibition results in growth arrest and death of androgen-dependent and androgen-independent prostate cancer cells. Biochem Pharmacol 72: 427–436.
13. Traynor P, McGlynn LM, Mukherjee R, Grimsley SJ, Bartlett JM, et al. (2008) An increase in N-Ras expression is associated with development of hormone refractory prostate cancer in a subset of patients. Dis Markers 27: 157–163.
14. Tung J, Pollock C, Tracy K, Check M, Martin P, et al. (2007) Activation of the Raf/MEK-Raf pathway promotes prostate cancer metastasis to bone. Mol Cell Biol 27: 7358–7350.
15. Watson PA, Elwood-Yen K, King JC, Wongpajit J, Lebeau CM, et al. (2005) Context-dependent hormone-refractory progression revealed through characterization of a novel murine prostate cancer cell line. Cancer Res 65: 11565–11571.
16. Horoszewicz JS, Leong SS, Chu TM, Wajsmann ZL, Friedman M, et al. (1980) The LNCaP cell line—a new model for studies on human prostatic carcinoma. J Natl Cancer Inst 67: 169–177.
17. Stone KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF (1978) Isolation of a human prostate cancer cell line (DU 145). Int J Cancer 21: 274–281.
18. Kaina B, Moreau K, Lehnert A, Kaina B, et al. (1998) Extension of life-span by introduction of telomerase into normal human cells. Science 279: 341–345.
19. Pear WS, Nolan GP, Scott ML, Baltimore D (1995) Production of high-titer helper-free retroviruses by transient transfection. Proc Natl Acad Sci U S A 90: 8982–8986.
20. Denker SP, Barber DL (2002) Cell migration requires both ion translocation and cytoskeletal anchoring by the Na-H exchanger NHE1. J Cell Biol 159: 1087–1096.
21. Boyd SN (1962) The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leukocytes. J Exp Med 115: 453–466.
22. Hujanen ES, Terranova VP (1985) Migration of tumor cells to organ-derived basement membranes in organ culture. Tumour Biol 6: 463–468.
23. Schwartz S, Jr., Morgan R, Jennings S, Austenfeld M, Van Veldhuizen P, et al. (1997) Overexpression of Her-2/neu may be an indicator of poor prognosis in prostate cancer. J Urol 158: 126–131.
24. Lau KS, Haigis KM (2009) Non-redundancy within the RAS oncogene family: insights into mutational disparities in cancer. Mol Cells 28: 315–320.
25. Rosell C, Ballet P, Plumas E, Traquici P (2004) Model driven quantification of individual and collective cell migration. Acta Biotheor 52: 343–363.
26. Chen HC (2005) Boyden chamber assay. Methods Mol Biol 294: 15–22.
27. Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature senescence associated with accumulation of p53 and p16INK4a. Cell 88: 593–602.
28. Sarkisian GJ, Krister RA, Stairs DB, Boxer RB, Moody SE, et al. (2007) Deere-depended oncogene-induced senescence in vivo and its evasion during mammmary tumorgenisis. Nat Cell Biol 9: 493–505.
29. Iwashina K, Campisi J, Dinini GP (2007) Methods to detect biomarkers of cellular senescence: the senescence-associated beta-galactosidase assay. Methods Mol Biol 371: 21–31.
30. Osman I, Mikhail M, Shuch B, Clute M, Cheli CD, et al. (2005) Serum levels of shed Her2/neu protein in men with prostate cancer correlate with disease progression. J Urol 174: 2167–2174.
53. Sikes RA, Chung LW (1992) Acquisition of a tumorigenic phenotype by a rat ventral prostate epithelial cell line expressing a transfected activated neu oncogene. Cancer Res 52: 3174–3181.
54. Zhou HE, Pisters LL, Hall MC, Zhao LS, Troncoso P, et al. (1994) Biomarkers associated with prostate cancer progression. J Cell Biochem Suppl 19: 208–216.
55. Zhou HY, Zhou J, Symmans WF, Chen BQ, Chang SM, et al. (1996) Transfected neu oncogene induces human prostate cancer metastasis. Prostate 28: 73–83.
56. Bonaccorsi L, Marchiani S, Muratori M, Forti G, Baldi E. (2004) Gefitinib ('IRESSA', ZD1839) inhibits EGF-induced invasion in prostate cancer cells by suppressing PI3 K/ AKT activation. J Cancer Res Clin Oncol 130: 604–614.
57. Leone G, DeGregori J, Sears R, Jakoi L, Nevins JR. (1997) Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. Nature 387: 422-428.
58. Tran PT, Fan AC, Bendapudi PK, Koh S, Komatsubara K, et al. (2008) Combined Inactivation of MYC and K-Ras oncogenes reverses tumorigenesis in lung adenocarcinomas and lymphomas. PLoS One 3: e2125.
59. D'Cruz CM, Gunther EJ, Boxer RB, Hartman JL, Sintasath L, et al. (2001) c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. Nat Med 7: 235–239.
60. Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, et al. (2000) Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev 14: 2501–2514.
61. Nemeth JA, Harb JF, Barroso U, Jr., He Z, Grignon DJ, et al. (1999) Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. Cancer Res 59: 1987–1993.
62. Tilley WD, Wilson CM, Marcelli M, McPhaul MJ (1990) Androgen receptor gene expression in human prostate carcinoma cell lines. Cancer Res 50: 5302–5316.