EphA2 induces metastatic growth regulating amoeboid motility and clonogenic potential in prostate carcinoma cells

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ABSTRACT

EphA2 kinase regulates cell shape, adhesion and motility and is frequently overexpressed in several cancers, including melanoma, prostate, breast, colon and lung carcinoma. Although a function in both tumour onset and metastasis has been proposed, the role played by EphA2 in tumour progression is still debated. In melanoma EphA2 has been reported to affect cell migration and invasiveness allowing cells to move by a proteolysis-independent strategy, commonly referred as amoeboid motility. With the aim to understand the role of EphA2 in prostate cancer metastatic spreading, we stably silenced EphA2 expression in a model of aggressive metastatic prostate carcinoma. Our results show that EphA2 drives the metastatic programme of prostate carcinoma, although its involvement greatly differs among metastatic steps. Indeed, EphA2 expression: i) greatly affects prostate carcinoma cell motility style, guiding an amoeboid movement based on Rho-mediated cell rounding and independent from metalloprotases, ii) is ineffective on transendothelial migration, adhesion onto extracellular matrix proteins, as well as on resistance to anoikis, iii) regulates clonogenic potential of prostate carcinoma, thereby increasing anchorage independent growth and self renewal, prostasphere formation, tumour onset, dissemination to bone and growth of metastatic colonies. Our finding indicate that EphA2 overexpressing prostate carcinoma cells gain an invasive benefit from their amoeboid motility style to escape from primary tumours and then, enhancing their clonogenic potential successfully target bone and grow metastases, thereby acknowledging EphA2 as a target for anti metastatic therapy of aggressive prostate cancers.
INTRODUCTION

Among members of the unique family of receptor tyrosine kinases, EphA2 is frequently up-regulated in a variety of cancers and tumour cell lines, including breast, prostate, liver, non-small cell lung and colon cancers, melanoma, ovarian cancer and neuroblastoma (1). Unlike other families of receptor tyrosine kinases which bind to soluble ligands, Eph receptors interact with cell surface-bound ephrin ligands, thus Eph-ephrin interaction stimulates a bi-directional signalling. Despite other classical oncogenes, Eph receptor signalling does not appear to convey a proliferative signal in many cell types, rather it can affect cell survival, cell-cell and cell-matrix attachment modulating tumour cell motility, invasion and metastasis (2). Even if a clear correlation between EphA2 and carcinogenesis has been underlined, the role of EphA2 in in vivo tumour growth and metastasis spreading is still a critical question. First of all, EphA2 expression is up-regulated during tumour-associated angiogenesis (3, 4) and accordingly, soluble EphA2-Fc receptor treatment results in decreased tumour vascular density, tumour volume and cell proliferation in the 4T1 model of metastatic mammary adenocarcinoma (3, 5). Beside the effect on tumour vasculature, EphA2 retains a role in tumour progression. First, the level of EphA2 expression in human prostate cancer cell lines relates to their metastatic potential in vivo (6) and EphA2 overexpression confers malignant transformation and tumourigenic potential in MCF10A normal epithelial cells (7) as well as increases invasiveness of pancreatic adenocarcinoma-cells (8, 9). Furthermore, overexpression of dominant negative EphA2 mutant or a kinase inactive form results in decreased tumour volume and increased tumour apoptosis (10, 11). Recently, Nasreen et al demonstrated that silencing EphA2 expression by using small-interfering RNA inhibits the proliferation and haptotaxis of malignant mesothelioma cells (12). In agreement, Landen et al demonstrated that the therapeutic delivery of EphA2 siRNA into an orthotopic mouse model of ovarian cancer reduced tumour growth when compared with a non silencing siRNA (13). Moreover, Brantley-Sieders et al., showed that EphA2
has a positive role during mammary tumour onset and growth in the MMTV-Neu transgenic mice model but not in mice overexpressing the polyomavirus middle T antigen, suggesting that, at least in breast carcinoma, EphA2 role in tumour progression depend on oncogene/tumour suppressor context (14). Recently, Parri et al., demonstrated that in prostate carcinoma cells EphA2 stimulation causes the retraction of the cell body and the re-direction of cell migration by activating the Src·FAK complex, leading to a Rho-dependent acto/myosin contractility response (15). Moreover, in a model of in vivo prostate carcinoma metastasis, we observed that the disruption of EphA2 kinase activity strongly inhibits PC3 cell motility and formation of metastatic colonies (11). Furthermore, we reported that PC3 cells expressing EphA2 display an amoeboid motility style, while cells expressing kinase deficient EphA2 mutants lose this particular ability to move without proteolitically destroying extracellular matrix (ECM) (11, 15). Besides, we have shown that in melanoma cells EphA2 re-expression converts the migratory style from mesenchymal to amoeboid-like, conferring them a strong invasive advantage leading to a successful metastatic programme (16).

In this background, we report here a central role of EphA2 expression for prostate carcinoma and metastatic spreading. Indeed, we show that the silencing of EphA2 is able to eliminate the skill of PC3 cells to move through an amoeboid motility style. This event is associated with a decrease in the stem cell markers, finally leading to an impairment of tumour growth and metastatic dissemination.
MATERIALS AND METHODS

Materials. Unless otherwise specified all reagents were obtained from Sigma. Anti EphA2 antibodies were from Upstate Biotechnology Inc. The invasion chambers were from Corning Costar. The Matrigel Matrix, anti Rac1 antibody and monoclonal anti-human chemokine (C-X-C motif) receptor 4 (CXCR4) antibody were from RD System. Cell Trace CFSE and Calcein were from Invitrogen. Ilomastat was from Chemicon International. Type I collagen, the FITC mouse anti human CD44 (clone G44-26) and PE mouse anti human CD24 (clone ML5) antibodies were from BD Bioscience (San Jose, CA, USA). The Amplite Universal Fluorimetric MMP Activity Assay Kit was from AAT Bioquest Inc, (Sunnyvale, CA, USA). Anti mouse Alexa 488 antibody was from Molecular Probes.

Cell culture. Prostate cancer cell lines (PC3) were purchased from ATCC, human umbelical vein endothelial cells (HUVECs) were obtained from BioWhittaker and cultured according to BioWhittaker instruction. PC3 cells were cultured in Ham’s F12 medium and maintained in 5% CO2 humidified atmosphere.

Short interfering RNA (siRNA) Transfection. PC3 cells were transfected with empty vector (wtPC3) or four different shRNA plasmids (SureSilencing™ shRNA Plasmids from SuperArray) validated specifically to knock down the expression of EphA2. Cells were selected by G418 treatment and a pool of stably transfected cells was generated in order to avoid clonal selection. Between the four different constructs provided by the kit, one of them caused the almost complete knock down expression of EphA2, as evaluated by western blot and FACScan analyses. To exclude off target effects due to the use of a single plasmid, a second construct provided by the same kit has been used to generate an additional EphA2 knockdown pool of stably transfected cells (EphA2-silenced PC3 pool 2, see Supplementary figure).
**Western blot analysis.** 1 x 10^6 cells were lysed for 20 min on ice in 500 µl of complete radioimmunoprecipitation assay (RIPA) lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin]. Lysates were clarified by centrifuging, separated by SDS-PAGE, and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.1% Tween 20 for 1 h at room temperature and were probed first with specific antibodies and then with secondary antibodies.

**Invasion assay.** PC3 cells were serum starved for 24 h and then 3 x 10^5 cells were seeded onto Matrigel-precoated Boyden chamber (8 mm pore size, 6.5 mm diameter, 12.5 μg Matrigel/filter) with or without 50 μM ilomastat. In the lower chamber, complete medium was added as chemoattractant. Following 24 h of incubation, the inserts were removed and the non invading cells on the upper surface were removed with a cotton swab. The filters were then stained using the Diff-Quik kit (BD Biosciences) and photographs of randomly chosen fields are taken.

**Colony formation in soft agar.** To examine colony growth in soft agar, 2 x 10^4 PC3 cells were plated per 35 mm dish in growth medium supplemented with 0.3% agar. This suspension was layered over 0.9% agar in growth medium. Colony growth was scored after 3 weeks under an inverted microscope.

**Gelatin zymography.** Serum free medium from confluent monolayer of cells was collected and 20 µl were added to sample buffer (SDS 0.4%, 2% glycerol, 10 mM Tris-HCl, pH 6.8, 0.001% bromphenol blue). The sample were run on a 10% SDS gel containing 0.1% gelatin. After electrophoresis the gel was washed twice with 2.5% Triton X-100 and once with reaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂). The gel was incubated overnight at 37°C with freshly added reaction buffer and stained with Laemli Comassie blue solution. Areas of gelatinase activity appear as clear bands against a dark background.
Quantitative MMP activity assay. MMP activity was measured with Amplite Universal Fluorimetric MMP Activity Assay Kit according to manufacturer instructions. Briefly, serum free medium from confluent monolayer of cells was collected and 5 µl were added to APMA (1mM) at 37°C for 1 h to detect MMP-2 activity and at 37°C for 2 h to detect MMP-9 activity. 50µl of the mixture was then added to 50 µl of MMP Red™ substrate solution. After 60 min of incubation the signal was read by fluorescence microplate reader with Ex/Em=540nm/590nm.

Cell adhesion Assay. Confluent monolayer of cells serum starved for 24 h were washed twice with PBS and then incubated for 10 min at 37°C with 0.25% trypsin. Trypsin was blocked with 0.5 mg/ml soybean trypsin inhibitor. 10^6 cells were then resuspended in 1 ml of fresh serum free medium and seeded onto dishes precoated with 10 µg/ml fibronectin.

Transendothelial cell migration. HUVECs were grown to confluence on the separating filter of a Boyden chamber (8 mm pore size, 6.5 mm diameter). HUVECs were activated with 10 ng/ml TNFα for 90 min. Thereafter, culture media were changed for fresh media and cells incubated for an additional 2.5 h. 3x10^5 PC3 cells, serum starved for 24 h and treated with calcein AM, were seeded onto HUVEC cells monolayer. In the lower chamber, complete medium was added as chemoattractant. Following 16 h of incubation, the insert was removed and the non invading cells on the upper surface were removed with a cotton swab. The number of cells that have migrated to the lower face of the filter was evaluated by counting the green cells using an inverted fluorescent microscope.

Real time PCR. Total RNA from wtPC3 and EphA2-silenced PC3 cells was extracted using RNeasy (Qiagen) according to the manufacturer instructions. Strands of cDNA were synthesized using a high capacity cDNA reverse transcription kit (Applied Biosystem) using 1 µg of total RNA. For quantification of CXCR4 mRNA, Real-Time PCR, using Power SYBR green dye (Applied Biosystem) was done on a 7500 Fast Real Time PCR system (Applied Biosystem). The primers for
CXCR4 were: 5'-AGCATGACGGACAAGTACAGG-3' (forward), 5’GATGAAGTCGGGAATAGTCAGC-3’ (reverse). Data are normalised to those obtained with Glyceraldehyde-3-phosphate dehydrogenase primers. Results (mean ± SD) are the mean of three different experiments.

**RhoA or Rac1 Activity Assays.** Cells were directly lysed in RIPA buffer, the lysates were clarified by centrifugation, and RhoA-GTP or Rac-GTP levels were quantified. Briefly, lysates were incubated with 10 µg of Rhotekin-Glutathione S-transferase (GST) fusion protein (Becton Dickinson) or p21-activated kinase-GST fusion protein, both absorbed on glutathione-Sepharose beads for 1 h at 4°C. GST-pulled down immunoreactive RhoA or Rac1 were then quantified by Western blot analysis. Lysates were normalized for RhoA or Rac1 content by immunoblot.

**Flow cytometer analysis.** The percentage of cells undergoing apoptosis after a 24 h or 72 h suspension treatment was assayed by the Annexin-VFLUOS Staining Kit (Roche Applied Sciences (Indianapolis, IN, USA) according to the manufacturer’s instructions. Briefly, 5x10^5 PC3 cells were washed with ice-cold phosphate-buffered saline and resuspended in 100 µl of binding buffer (HEPES buffered saline solution with 2.5 mM CaCl2). Fluorescein isothiocyanate-labelled annexin V and 10 ng/ml propidium iodide (PI) were added. Cells were then incubated for 10 min in the dark at room temperature. After the addition of 400 µl of binding buffer and agitation, flow cytometry was performed using a BD FACS Canto (BD Biosciences, San Jose, CA, USA). The analyser threshold was adjusted on the flow cytometer channel to exclude most of the subcellular debris to reduce the background noise. The totality of Annexin V+/PI- (early apoptotic) and Annexin V+/PI- cells (late apoptotic) were considered apoptotic.

To determine the surface expression of CXCR4, EphA2, CD44 and CD24 10^6 cells were detached non-enzymatically with 2.5 mM EDTA and incubated with the antibodies according to manufacturer instructions in PBS containing 1% BSA for 1 h at 4°C. After washing with PBS/1%
BSA cells were incubated with Alexa 488 labelled anti mouse antibodies for 30 min at 4°C. Upon washing, a flow cytometer analysis was performed.

**Cell migration in three-dimensional collagen matrices.** Reconstruction by time-lapse video microscopy and confocal microscopy was performed. Cells were detached by EDTA (2 mM), washed, incorporated into three-dimensional collagen/matrigel lattice (0.55 mg/ml Matrigel and 1.11 mg/ml type I collagen) and monitored by time-lapse video microscopy according to Friedl et al., (17). For three-dimensional time-lapse confocal microscopy (Leica-SP5 system), cells within the lattice were labeled by 5 μM Cell Trace CFSE, scanned at 3 min time intervals for simultaneous fluorescence and back scatter signal (reflection) and reconstructed. Three-dimensional motility of cells is shown by time lapse of xyzt analysis (three-dimensional analysis during time). Movies are a two-dimensional projection (xy) of all stacks during a time course. xy two-dimensional migration of cells has been excluded by analysis of zx axis movements during the same time course. For speed quantification, locomotor parameters of cells incorporated into three-dimensional collagen lattice were obtained by computer-assisted cell tracking and reconstruction of the x and y coordinates of the cell paths. The population speed is the ratio between the total length of the path of single cells and the time.

**Prostaspheres formation.** Cells were detached using Accutase (Sigma). For prostaspheres formation, single cells were plated at 150 cells/cm² on low attachment 100 mm plate (Corning) in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with B27 and N2 (Invitrogen), 5 μg/ml insulin, 20 ng/ml bFGF and 20 ng/ml EGF. Cells were grown under these conditions for 10 days and then prostaspheres were photographed.

**In Vivo Experiments. A)** Xenograft Experiments: experiments were performed in agreement with national guidelines and approved by the ethical committee of Animal Welfare Office of Italian Work Ministry and conform to the legal mandates and Italian guidelines for the care and
maintenance of laboratory animals. 6–8 week old male SCID-bg/bg mice (Charles River Laboratories International, Inc., Wilmington, MA, USA) were injected subcutaneously (s.c.), both in the right and left lateral flanks, with cells mixed in a 1:1 volume ratio with Matrigel, in a final volume of 200 µl. Animals were monitored, tumour size was measured by a caliper and tumour volumes determined by the length ($L$) and the width ($W$): $V = \frac{LW^2}{2}$.

**B) Bone Metastasis:** male CD1 nude mice were purchased from Charles River (Milan, Italy). Mice were maintained under the guidelines established by our institution (University of L’Aquila, Medical School and Science and Technology School Board Regulations, complying with the Italian Government Regulation n.116, 27/1/1992). The procedure of heart injection of prostate cancer cells in nude mice has been previously described (18). Briefly, $1 \times 10^5$ cells in 0.1 ml of saline solution, were injected in the left ventricle of 4-week-old nude mice previously anesthetized with a mixture of ketamine (25 mg/ml)/xylazine (5 mg/ml). The number of mice analysed was 8 per group. The development of tumour colonies in the whole skeletal apparatus was monitored at times by radiography using a Faxitron cabinet X-ray system (Faxitron X-ray Corp., Wheeling, IL). All animals were subjected to accurate necroscopy for the evaluation of the presence of tumour colonies in other anatomical sites.
RESULTS

Role of EphA2 in proliferation, adhesion and survival of prostate cancer cells. To deeply investigate the role of EphA2 overexpression in prostate cancer and its role in cell motility and tissue invasion, we silenced EphA2 expression by RNA interference. Thus, we generated a pool of PC3 cells in which EphA2 is stably knocked down (EphA2-silenced PC3 cells) with respect to control cells (wtPC3 cells), as indicated by western blot and flow cytometry analyses (Fig 1A,B).

The long route of prostate cancer cells to metastasize a distant organ is composed of a series of sequential steps (19, 20), involving entry into the systemic vasculature through the activation of trans-endothelial migration, sustain of cell survival, homing of cancer cells to the target tissue, adhesion to the new localization and growth of the colony to generate metastases. In order to understand the importance of EphA2 expression to produce successful metastases, we analysed EphA2 involvement in each step of the metastatic progression. The activation of trans-endothelial migration, a step crucial for both extravasation and intravasation of migrating metastatic cells, has been analysed by a trans-endothelial motility assay to test the ability of tumour cells to penetrate a monolayer of endothelial cells. As shown in Fig 1C the efficiency of cells to migrate through an endothelial cell barrier is very similar between EphA2-silenced PC3 cells and wtPC3.

In some cellular models EphA2 expression represents a way for cells to escape from anoikis as demonstrated in pancreatic adenocarcinoma cells (9), or to avoid caspase-9-mediated apoptosis as evidentiated in malignant mesothelioma cells (12). To test whether in PC3 cells the expression of EphA2 could confer resistance to anoikis, we analysed cell survival to 24 h and 72 h suspension by flow cytometry analysis. As shown in Fig 1D in this cellular model EphA2 expression is not responsible for any difference in cell survival between EphA2-silenced PC3 cells and wtPC3 cells.

In order to generate a metastatic tumour, colony prostate carcinoma cells must adhere to the new niches. We therefore investigated the adhesive properties of wtPC3 and EphA2-silenced PC3 cells
in a cell adhesion assay on fibronectin-coated plate. As shown in Fig 1E no remarkable differences have been observed between these two populations, suggesting that in this context, EphA2 is not involved in regulating the adhesive properties of prostate cancer cells.

**EphA2-silenced prostate cancer cells lose their ability to invade through an amoeboid motility style.** Recent results from our laboratory evidentiated a crucial role of EphA2 in the switch between mesenchymal to amoeboid motility style in cancer cells (16). In particular, we demonstrated that the re-expression of EphA2 in melanoma cells, through a mesenchymal-amoeboid transition (MAT), causes the acquisition of a MMP-independent/RhoA-dependent amoeboid strategy, conferring an increased motility and invasion to cells. Finally, this shift allows melanoma cells to successfully colonize lungs and peritoneal lymph nodes (16). In addition, we already reported that PC3 cells are able to invade through a Rho-dependent amoeboid motility style (11). In this light, we investigated the role of EphA2 in determining the style of prostate cancer cell motility. First, we analysed the invasive properties of wtPC3 and EphA2-silenced PC3 cells across a Matrigel barrier in the presence of the MMP inhibitor ilomastat. As shown in Fig 2A ilomastat greatly inhibits EphA2-silenced PC3 cells invasion, while wtPC3 motility is only marginally affected by this inhibitor. These data suggest a MMP-dependent motility style for EphA2-silenced PC3 cells, while EphA2 expression is associated with a MMP-independent motility style. Accordingly, MMP analysis by gelatine zymography reveals an up-regulation of MMP expression in EphA2-silenced PC3 cells (Fig 2B). Furthermore, a quantitative measure of MMP activity confirmed data obtained by gelatin zymography (Fig 2C). Mesenchymal and amoeboid motilities are differently regulated by the small GTPases RhoA and Rac1, being amoeboid motility correlated with RhoA activation and Rac1 inhibition and mesenchymal motility to the opposite regulation of these GTPases (21). We therefore analysed RhoA and Rac1 activation and, as shown in Fig 2D we observed that EphA2 silencing induces inhibition of RhoA and activation of Rac1, in line with a *de novo* acquired mesenchymal
motility strategy of these cells. In keeping with these data, confocal fluorescence-reflection video microscopy analysis, evidentiates a slow moving spindle shaped phenotype of EphA2-silenced PC3 cells across collagen I lattice with respect to wtPC3 cells (Fig 3A). Indeed, in keeping with previous data (22, 23), amoeboid wtPC3 cells move faster with respect to mesenchymal EphA2 silenced PC3 cells (Fig 3B). In addition, in three-dimensional Matrigel lattice, wtPC3 cells show a round shape morphology typical of amoeboid motility, while EphA2-silenced PC3 cells have an elongated, spindle-like shape, characteristic of mesenchymal motility (Fig 3C).

As one, these findings underline a strong correlation between EphA2 expression and a protease-independent migration strategy, commonly referred as amoeboid motility style. Indeed, the silencing of EphA2 impairs prostate carcinoma cells to invade using their amoeboid motility suggesting a limitation of their metastatic potential.

EphA2 receptor increases clonogenic potential and is required for tumour growth of PC3 cells in in vivo experiment. Recently, in both breast and prostate cancers, epithelial mesenchymal transition (EMT) has been reported to generate cells with stem-like properties (24) and a correlation between EphA2 expression and stem cell markers has been proposed (25). Tumour cell plasticity, i.e. the ability of cells to adapt to environmental changes through activation of ad hoc epigenetic programmes, includes EMT and MAT. Both EMT and MAT have reported to grant to cancer cells an increased metastatic potential (26-31). We hypothesized that, beside EMT, MAT may be associated with cancer stemness as well. Indeed, previous evidence from our laboratory showed that EphA2 drives melanoma cells towards a conversion of their mesenchymal style to an amoeboid-like behaviour, finally leading to a successful lung and lymph node peritoneal metastasis (16). Several reports have acknowledged the CD44\textsuperscript{high}/CD24\textsuperscript{low} ratio as a reliable indicator of stemness in prostate and breast carcinoma cells (32, 33). In this light, we analysed the expression of these two markers in EphA2 silenced PC3 cells. Flow cytometry analysis reveals that
EphA2 silenced cells show a decrease in the number of CD44\textsuperscript{high}/CD24\textsuperscript{low} positive cells with respect to wtPC3 cells (Fig 4C). In addition, the analysis of both tumour cell growth in soft agar and the PC3 clonogenic potential reveals that EphA2 is crucial for tumour cell proliferation. Indeed, wtPC3 cells are able to growth in soft agar, forming large and numerous colonies in anchorage-independent conditions (Fig 4A-B), while, silenced-EphA2 cells completely lose their independence from anchorage. This assay allows to test cell survival at longer time with respect to analysis of suspension culture, thus revealing differences that have not been previously detected by anoikis quantification. In agreement, only EphA2 expressing PC3 cells are able to form large and tightly packed prostasphere (Fig 4D), resembling the CD44 positive colonies containing stem cells described by Li et al. These clones are defined holoclones and are the only ones able to sustain prostate cancer development and serial tumour transplantation in NOD/SCID mice (34). We then compared the tumour-initiating capacities of wtPC3 and EphA2 silenced PC3 cells when injected subcutaneously into the flanks of SCID-bg/bg mice. We evidintiated that at lower concentrations (2x10\textsuperscript{4} and 2x10\textsuperscript{3} cells), EphA2 positively influences both latency and rate of tumour growth (Fig 4E). These results show that EphA2 level correlates with an enrichment in stem cell markers leading to a faster development of cancer. Histological examination of lungs showed the presence of spontaneous micrometastases only in mice injected with wtPC3 cells (Fig. 4F), thereby confirming that the expression of EphA2 is essential not only for tumour onset but also for the dissemination of cancer cells.

The cancer stem cell (CSC) hypothesis proposes that tumour progression is sustained by CSCs. Indeed, the ability of cancer cells to generate metastases depends on the dissemination of CSCs that have acquired invasive capabilities. These new properties allow the escape of CSCs from primary tumour mass to migrate towards and colonize distant organs. Prostate cancer has a remarkable tendency to metastasize to bone (35). For metastases to occur, the malignant cells must escape the
primary tumour, penetrate and circulate through the bloodstream, and then arrest and proliferate in
target tissues. The mechanisms that account for bone homing of prostate carcinoma cells have been
in part elucidated and the chemokine receptor CXCR4 has been involved. CXCR4 activation affects
prostate cancer cell metastatic behaviour by increasing both cell adhesiveness and invasiveness (36-
38). In order to evaluate whether the level of CXCR4 is under the control of EphA2, we measured,
in wtPC3 and EphA2-silenced PC3 cells, CXCR4 expression by Real Time PCR analysis and its
surface level by flow cytometry. EphA2-silenced PC3 cells show a decrease in both CXCR4 mRNA
and in the cell surface protein expression with respect to wtPC3 cells (Fig 5A,B), thereby sustaining
the involvement of EphA2 in CXCR4 expression.

In order to avoid that the differences observed between EphA2-silenced cells and wtPC3 cells may
be off targets effects due to the use of a single shRNA vector to deplete EphA2, we generated an
additional pool of EphA2 knockdown cells (EphA2-silenced PC3 pool 2) to validate our results (Fig
Suppl 1 A). We then performed some of the most representative experiments in order to exclude
that the observed effects may be a unique feature of the pool of cells under consideration. As shown
in Fig Suppl 1, we confirmed the differences between wtPC3 and EphA2-silenced cells for what
concerns the quantitative measure of MMPs activity (Fig Suppl 1 B), the tumour cell growth in soft
agar (Fig Suppl 1 C and D) and the cell surface expression of CXCR4 (Fig Suppl 1E). Overall,
these results prove that the differences observed between wtPC3 and EphA2-silenced PC3 cells are
actually due to the expression of EphA2 and are not a specific effect characteristic of a single pool
of cells.

Therefore, to evaluate the role of EphA2 expression in the regulation of in vivo metastatic program
in particular towards bone, both wtPC3 and EphA2-silenced PC3 cells were used in a rodent model
of bone metastasis assay (18). Eight mice were subjected to intracardiac injection of wtPC3 and
EphA2 silenced PC3 cells as already described (11). Fifty-two days after heart injection (end-point)
mice were sacrificed and subjected to a digital scan of total body radiography and to an accurate necroscopic analysis to confirm the presence of both osteolytic and visceral metastases. It is worth of notice that silencing of EphA2 kinase reduces the ability of PC3 carcinoma cells to produce osteolytic metastases up to 50% (Fig 5C, left). For what visceral metastases are concerned, the effect of silencing EphA2 expression is even more striking, as indicated by the complete abrogation of visceral metastases outgrowth in EphA2-silenced PC3 cells (Fig 5C, right). We also analysed the timing of bone metastases outgrowth by a time course radiographic assay, before the experiment end point. The onset of bone metastases in mice injected with EphA2-silenced PC3 cells is strongly delayed with respect to control cells. Indeed, while wtPC3 cells developed osteolytic lesions at 36 days (mean value) after intracardiac injection, EphA2-silenced PC3 cells begin the bone metastatic process significantly later (44 days) (Fig 5D-E). Altogether, these data enforce the idea that EphA2 kinase expression is a key determinant for the generation of cells with stem-cell properties endowed with ability to target secondary organs, such as lungs and bone.
DISCUSSION

Data presented herein show a key role of EphA2 in determining a productive metastatic programme mainly dependent on the activation of an amoeboid motility style and the maintenance of stemness. In particular, we disclose that EphA2 is crucial for: A) regulation of an amoeboid-based successful invasive strategy, B) increase of stem cell markers and regulation of tumour onset, C) dissemination to bone and growth of metastatic tumours.

Several papers clearly show that EphA2 is able to induce tumourigenesis such as in mammary epithelial cells (7), prostate, ovarian and breast cancer cells (6, 10, 39). The ability of EphA2 to induce tumourigenesis and metastatic dissemination has been correlated with the increased skill of EphA2 overexpressing cells to grow in soft agar, to invade into matrigel (7) and to increase the resistance to anoikis (9, 12). Among the previously mentioned characteristics concurring to determine a more aggressive phenotype, we would like to introduce two new features: the acquisition of an amoeboid motility style due to EphA2 overexpression and the increase in clonogenic potential. Indeed, EphA2 has a crucial role as motility and adhesive factor mainly affecting the balance of Rho, Rac, Cdc42 activities and protein involved in integrin signalling (2).

Recently, it has been shown that cancer cells are able to achieve different strategies to evade the primary tumour site and metastasize to distant organs (40). For the invasive migration of cancer cells, at least two devices are currently involved: (1) the mesenchymal style depending on extracellular proteolysis and (2) the proteolysis-independent amoeboid mode depending on the activity of Rho and the Rho-associated coiled-coil-forming protein kinase (ROCK). Indeed, Rho-GTPase is frequently over-expressed in several cancers, whereby increased activity correlates with tumour progression and underlines the different modes of tumour cell motility during invasion and...
metastasis. The skill of tumour cells to switch between different modes of motility has been shown to limit the efficiency of agents aimed to reduce invasion (41). Recently, beside the well known importance of mesenchymal motility in cancer cells invasion, growing evidence show the importance of proteolysis-independent movements in neoplastic cells (27, 42, 43). Torka R et al., showed that human mammary tumour cells displayed an increased activity of ROCK and its downstream effectors, leading to an invasive strategy based on a ROCK-dependent amoeboid motility model (44). Furthermore, mesenchymally migrating tumour cells, such as HT1080 fibrosarcoma and MDA-MB-231 mammary carcinoma cells, arrest their proteolytic dependent migration after addition of protease inhibitors and switch to amoeboid behaviour, involving vigorous shape change and the ability to squeeze through narrow regions of ECM, thereby rescuing their migration independently from pericellular proteolysis (45). Similarly, Sahai et al., demonstrated that ROCK activation causes a transition from mesenchymal to amoeboid movement in HT1080 leading to cortical actin polymerization and cell rounding; finally, this activation of Rho/ROCK signalling allows HT1080 to penetrate thick 3D matrigel layers (46). In our cellular model, we observed that EphA2 is a key molecule able to drive prostate carcinoma cells towards an amoeboid motility style. This aptitude, together with the acquisition of an increased clonogenic potential, seems to be crucial for a successful colonization of bone, lung and visceral metastatic sites. Indeed, EphA2 silenced cells, although showing an increased production of MMPs and invasion through Matrigel by mesenchymal motility, are completely unable to produce lung and visceral metastases and their ability to colonize bones is greatly reduced. Maybe, in the micro-environmental condition that PC3 cells find on their way to give in vivo metastases, the ability to move through an amoeboid motility is more useful rather than the ability to degrade the extracellular matrix. In addition, the increase in bone colonization evidenced in mice injected with wtPC3 cells is in agreement with our data showing that EphA2 exerts a positive control on
CXCR4 expression. CXCR4 is fundamental for the acceleration of the metastatic process of prostate tumours (36) and it is a marker of poor cancer-specific survival (47). We may therefore assume that the EphA2 is involved, through the enhancement of CXCR4 expression, in the development of pro-metastatic signals. In conclusion, we believe that, in keeping with data on melanoma and sarcoma, in prostate cancers a RhoA-dependent, MMP-independent motility style is crucial to successfully invade distant organs. Indeed, we evidenced a more motile phenotype of wtPC3 cells with respect to EphA2-silenced PC3 cells, as shown by speed measurement. This aptitude, typical of amoeboid movements, allows cells to squeeze between ECM gaps and concur to achieve a successful migratory strategy. In keeping with these data we have already disclosed that EphA2 in PC3 cells leads to a RhoA-dependent acto-myosin contractility dependent on Src/FAK activation, suggesting that the FAK/RhoA signalling pathway is mainly responsible for the EphA2 dependent increased motility (15). Accordingly, disruption of EphA2 activation by means of kinase deficient EphA2 mutants leads to abrogation of ephrinA1-induced cell rounding, inhibition of FAK mediated motility response and inhibition of EphA2-mediated invasion through a Rho-dependent and MMP-independent mechanism (11, 48). Besides, Parri et al., have recently demonstrated that EphA2 re-expression in murine melanoma cells, which use a mesenchymal motility, converts this motility style into the amoeboid one, conferring an invasive advantage to lung and lymph node metastases (16). Hence, the data here presented enlarge the emerging idea of EphA2 has an amoeboid promoting factor to prostate carcinoma, able to ensure an invasive gain adapting cells to environmental changes.

Together with a positive role of EphA2 in warranting an amoeboid motility style, we highlighted a function of EphA2 in the induction of clonogenic potential. Indeed, the CSC hypothesis proposes that tumour growth is sustained by CSCs able to self-renew, giving rise to differentiated progeny and able to reconstitute the whole tumour. CSCs have been characterised in several solid tumours
and among these, also in prostate cancers (49). Recently, it has been demonstrated that EMT is able to generate cells with cancer stem cells properties in breast and prostate cancers as well as in non transformed cells (24). Both EMT and MAT are examples of adaptation reactions, which can modify the cell’s shape, pattern, and migration mechanism. EMT is a process which involves the loss of cell–cell junctions and increased cell motility, finally leading to the acquisition of mesenchymal features. Conversely, MAT consists mainly of the abrogation of pericellular proteolysis and strengthening of Rho/ROCK signal pathways, allowing cells to fast moving among ECM gaps without destroying it. Tumour cells can switch between different motility styles, thereby adapting migration to the context, finally facilitating a sustained dissemination of single cells. These phenomena are defined “plasticity” and results in migratory ”escape” strategies to allow cancer cell dissemination in tissues. Both EMT and MAT are considered as key features for tumour progression (27, 30, 42-45). We now propose that the induction of stemness is a general phenomenon associated with shifts in motility styles and therefore, beside EMT, sustained also by MAT. First of all, we observed that EphA2 silencing reduces both anchorage-independent cell growth and the expression of stem cell markers. Indeed, we observed that wtPC3 cells, with respect to EphA2-silenced PC3 cells, show: i) higher CD44\(^{high}/CD24^{low}\)-ratio, ii) greater capacity to form round colonies with tightly packed cells; iii) acceleration of the onset of primary tumours; iv) capacity to form spontaneous metastases from s.c. primary tumours; v) higher bone osteolytic and visceral metastases. Our data show a small but significant increase of CD44\(^{high}/CD24^{low}\)-ratio in agreement with evidence reporting a dramatic increase in tumourigenicity of CD44\(^{+}\) with respect to CD44\(^{-}\) prostate cancer cells (50). In keeping, the colonies formed by wtPC3 cells resemble the holoclones observed by Li H et al (34), who demonstrated that only holoclones expressing high levels of stem and progenitor cell markers contain self-renewing cancer cells that can sustain long-term propagation in culture and tumour development. Altogether these data suggest that EphA2 is
crucial for the onset and growth of tumour colonies, as well as for metastases to occur, with a clear correlation with an increase in the clonogenic potential. Indeed, the decrease in stem cell markers and in cell plasticity observed in EphA2 silenced cells severely impairs the ability of prostate cancer cells to generate distant metastases.

We believe that both the ability of EphA2 to instruct cells to move by amoeboid motility and to generate cells endowed with self renewal properties, are key features of its function in cancer progression. In conclusion, these data on one side support the idea that MAT and clonogenic potential are inter-related phenomena and, on the other side, reinforce the hypothesis that cell plasticity and the achievement of stem-cell properties are crucial for the generation of prostate carcinoma metastases and thus for tumour spreading. Really, EphA2 expressing cells gain an invasive advantage from their amoeboid motility style thus escaping from the primary tumour and then, by means of the concomitant acquisition of clonogenic potential, can successfully develop metastases.
Reference List


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FIGURE LEGENDS

Figure 1: Role of EphA2 in the adhesive and survival properties of prostate cancer cells: A) Analysis of EphA2 expression in wtPC3 cells and in PC3 cells transfected with SureSilencing™ shRNA Plasmid validated specifically to knock down the expression of EphA2. Lysates of wtPC3 and EphA2-silenced PC3 cells were subjected to EphA2 immunoblot analysis. B) Flow cytometry analysis of cell surface EphA2 was performed with anti-EphA2 mAb in wtPC3 and EphA2-silenced PC3 cells. C) Transendothelial cell migration. Huvecs were grown to confluence on the membrane separating the two compartment of a Boyden chamber. Calcein loaded wtPC3 and EphA2-silenced PC3 cells were added in the upper chamber and allowed to migrate for 24 h. Cells that have invaded to the underside of the insert were then visualized using an inverted fluorescence microscope. The bar graphs represent the number of cell migrated to the lower filter in six randomly chosen fields of triplicate experiments. D) Survival properties of wtPC3 and EphA2-silenced PC3 cells. wtPC3 and EphA2-silenced PC3 cells were detached, suspended in serum-free medium and kept in suspension for 24 h or 72 h. The percentage of apoptotic cells was evaluated using the Annexin-VFLUOS Staining Kit. The bar graphs represent data of triplicate experiments. E) Adhesion assay: wtPC3 and EphA2-silenced PC3 cells are serum starved for 24h, detached, kept in suspension for 30’ and then plated onto fibronectin coated plates. After 1 h cells are fixed with formaldehyde. The bar graphs represent the number of adhering cells in six randomly chosen fields of triplicate experiments.

Figure 2: EphA2-silenced PC3 cells acquire a Rac1/MMPs dependent motility style: A) Boyden cell invasion assay. wtPC3 and EphA2 silenced PC3 cells, after 24 h of serum starvation were seeded into the upper compartment of Boyden chamber with or without 50 μM ilomastat. Cells were allowed to migrate through the filter coated with Matrigel toward the lower compartment filled with complete medium. Cell invasion was evaluated after Diff-Quick staining by counting cells in six randomly chosen fields. The results are representative of five experiments with similar
results. ∗p<0.005 samples vs untreated. B) Analysis of MMP activity. Confluent monolayers of wtPC3 and EphA2-silenced PC3 cells were serum deprived for 24 h. Media are then collected and analysed by gelatine zymography. The clear bands represent areas of gelatinase activity. The results shown are representative of four experiments. C) Quantitative analysis of MMP activity: confluent monolayers of wtPC3 and EphA2-silenced PC3 cells were treated as in A. 5 μl of medium was collected and added to APMA to activate pro-MMP2 and pro-MMP9 and then to MMP Red™ substrate solution according to manufacturer instructions. The signal was read by fluorescence microplate reader with Ex/Em=540nm/590nm. Data are normalized on the basis of protein content. The results shown are representative of three experiments. ∗p<0.005 EphA2-silenced PC3 cells vs wtPC3 cells. D) RhoA and Rac1 activity assay: RhoAGTP and Rac1GTP were analysed by pull-down assay from wtPC3 and EphA2-silenced PC3 cell lysates. The total amount of RhoA and Rac1 were quantified by anti-Rho and anti-Rac1 immunoblot. The results are representative of three experiments.

Figure 3: EphA2-silencing induces the loss of PC3 amoeboid-like motility strategy: A) Cell Trace CFSE loaded wtPC3 and EphA2-silenced PC3 cells were incorporated into three-dimensional collagen I lattice and monitored by confocal fluorescence reflection video microscopy. Bar, 10 μm. B) wtPC3 and EphA2-silenced PC3 speed was calculated dividing the total length of the path of each cells by time. The bar graphs represent the mean of cell speed in four randomly chosen fields of triplicate experiments. ∗p<0.005 EphA2-silenced PC3 cells vs wtPC3 cells. C) Cell Trace CFSE loaded wtPC3 and EphA2-silenced PC3 cells were incorporated into three-dimensional Matrigel lattice and cell morphology was observed by confocal microscopy. Bar, 20 μm

Figure 4: EphA2-silencing induces a decrease in stem cell markers and impairs tumour growth: A) Anchorage independent cell growth. 2x10^4 wtPC3 or EphA2-silenced PC3 cells were suspended in soft agar. After 21 days colony formation was scored microscopically and...
photographs are taken in six randomly chosen fields. Bar, 150 μm. B) Colonies of cells growing in soft agar are counted. Clusters containing at least 3 cells were defined as a positive colony. *p<0.005 EphA2-silenced PC3 cells vs wtPC3 cells. C) wtPC3 cells and EphA2-silenced PC3 cells were serum-starved and analysed for expression of the cell-surface marker FITC-CD44 and PE-CD24 by means of cytometry. The CD44$^{\text{high}}$/CD24$^{\text{low}}$ populations were plotted. *p < 0.005 EphA2-silenced PC3 cells vs wtPC3 cells. D) Representative images of clones obtained from wtPC3 cells or EphA2-silenced PC3 cells after 10 days of culturing at clonal densities. E) Xenograft growth in SCID bg/bg mice. wtPC3 cells or EphA2-silenced PC3 cells at the indicated numbers were subcutaneously injected with Matrigel both in the right and left lateral flanks of mice. The onset and the primary tumour growth are reported. *p<0.005 EphA2-silenced PC3 cells vs wtPC3 cells; # p<0.05 EphA2-silenced PC3 cells vs wtPC3 cells. F) Paraffin-embedded tissue sections from lung micrometastases (original magnification 40x) obtained by wtPC3 or EphA2-silenced PC3 cells injected in SCID bg/bg-mice were stained with hematoxylin and eosin.

**Figure 5:** EphA2 silencing impairs the formation of metastases in the in vivo experimental model for bone metastases: A) CXCR4 expression. Quantitative real time reverse transcription PCR of RNA extracted from wtPC3 and EphA2-silenced PC3 cells using primers for human CXCR4 and GAPDH gene. Results were normalized first to GADPH expression levels and then displayed relative to level in wtPC3 cells. Data are representative of three independent experiments.*p<0.05 wtPC3 vs EphA2-silenced PC3 cell. B) Flow cytometry analysis of CXCR4 expression in wtPC3 and EphA2-silenced PC3 cells. Cells were incubated with anti IgG (H+L) as negative control or anti CXCR4 antibodies. Data are representative of three experiments with similar results. C) Percentage of mice hearth injected with wtPC3 or EphA2-silenced P3 cells with at least one bone osteolytic lesions (left panel) and percentage of mice with evidence of at least one non-bone metastatic lesion as determined by necroscopic analysis (right panel). *p<0.005 EphA2-
silenced PC3 cells vs wtPC3 cells. D) The timing of the first detection of bone lytic lesions in mice injected with wtPC3 or EphA2-silenced PC3 cells. The radiographs were performed at days 36, 44 and 52. All the points shown are statistical relevant (p<0.005 EphA2-silenced PC3 cells vs wtPC3 cells). E) The images show a representative radiography of mice with (down) or without (upper) bone lytic lesions (indicated by white arrows).
Fig 2

A

![Bar graph showing number of cells migrated through Matrigel.](image)

- wtPC3
- EphA2 silenced PC3
- EphA2 silenced PC3 with ILOMASTAT

B

![Western blot showing MMP-9 and MMP-2.](image)

- wtPC3
- EphA2 silenced PC3

C

![Graph showing MMP-9 and MMP-2 activities.](image)

- wtPC3
- EphA2 silenced PC3

D

![Western blots for Rac1 GTP and RhoA GTP.](image)

- wtPC3
- EphA2 silenced PC3

- Rac1 GTP
- RhoA GTP

- Rac1 tot
- RhoA tot
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EphA2 induces metastatic growth regulating amoeboid motility and clonogenic potential in prostate carcinoma cells

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