Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;44:66-84
DOI: 10.1159/000484584
Published online: November 03, 2017
Accepted: September 14, 2017

Original Paper

Androgen Triggers the Pro-Migratory CXCL12/CXCR4 Axis in AR-Positive Breast Cancer Cell Lines: Underlying Mechanism and Possible Implications for the Use of Aromatase Inhibitors in Breast Cancer

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Key Words
Androgen • Androgen Receptor • CXCL12 • NCOA1 • CXCR4 • P53

Abstract
Background/Aims: Reports regarding the role of androgen in breast cancer (BC) are conflicting. Some studies suggest that androgen could lead to undesirable responses in the presence of certain BC tumor characteristics. We have shown that androgen induces C-X-C motif chemokine 12 (CXCL12) in BC cell lines. Our aim was to identify the mechanisms regulating the phenotypic effects of androgen-induced CXCL12 on Androgen Receptor (AR) positive BC cell lines. ***Methods:*** We analyzed the expression of CXCL12 and its receptors with qPCR and ELISA and the role of Nuclear Receptor Coactivator 1 (NCOA1) in this effect. AR effects on the CXCL12 promoter was studied via Chromatin-immunoprecipitation. We also analyzed publically available data from The Cancer Genome Atlas to verify AR-CXCL12 interactions and to identify the effect or Aromatase Inhibitors (AI) therapy on CXCL12 expression and disease progression in AR positive cases. ***Results:*** CXCL12 induction occurs only in AR-positive BC cell lines, possibly via an Androgen Response Element, upstream of the CXCL12 promoter. The steroid receptor co-regulator NCOA1 is critical for this effect. Androgen only induced the motility of p53-mutant BC cells T47D cells via upregulation of CXCR4 expression while they had no effect on wild-type p53 MCF-7 cells. Loss of CXCR4 expression and depletion of CXCL12 abolished the effect of androgen in T47D cells while inhibition of p53 expression in MCF-7 cells made them responsive to androgen and increased their motility in the presence to androgen. Patients with estrogen receptor positive (ER+)/AR+ BC treated with AIs were at increased risk of disease progression compared to ER+/AR+ non-AI treated and ER+/AR- AI treated cases. ***Conclusion:*** AIs may lead to unfavorable responses in some ER/AR positive BC cases, especially in patients with AR+, p53 mutant tumors.

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Introduction

Aromatase (CYP19) transforms androgen from the adrenals and the ovaries into estrogen that can induce the growth of estrogen-dependent tumors. This knowledge has led to the use of Aromatase Inhibitors (AIs) as therapeutic agents for breast cancer. AIs are replacing selective estrogen receptor modulators (SERMs) as first-line therapy for estrogen receptor (ER) positive breast cancer, especially in postmenopausal women. However, the fate and the effects of the accumulating androgen in the breast following AI administration are unknown. It is therefore unclear whether, in some cases of breast cancer, local increase of androgen may influence the evolution and outcome of the disease.

Several lines of evidence suggest that androgen might have direct effects on breast cancer cells, by binding to their selective androgen receptors (ARs), present in a large number of breast cancer cases [1]. The exact role of androgen in breast cancer development, prognosis and therapy has been in the center of significant controversies; this is partly due to different applied methodologies, with their focus on different androgen molecules and different groups of patients [2]. Some studies have identified significant correlations between high testosterone levels and breast cancer development in pre-menopausal women. Others have reported low DHEA-S as a risk factor for breast cancer [3-7], while negative studies, unable to correlate androgen levels with breast cancer risk, also exist [8]. Yet, elevated blood androgen levels have been reliably linked to increased breast cancer risk in postmenopausal women [3, 9, 10]. These controversies are also extended to the translational relevance of ARs as a biomarker in breast cancer. Surprisingly, ARs are more frequently present in breast cancer than ERs or PRs [11]. Presence of ARs in ER negative tumors has been related to better disease free survival [1, 12] but high AR expression has also been related to an increased likelihood of axillary metastasis [13], implying that AR expression triggers increased tumor capacity to metastasize, at least in lymph nodes. It is to note, however, that our current knowledge of AR expression and identification is limited to the nuclear form of the classical wild-type AR, and does not take into consideration any extranuclear action or isoform of the ARs. Moreover, most of the antibodies currently available for AR detection are polyclonal, limiting the development of companion diagnostics. Finally, AR splice variants like ARV7 have been recently related to a therapeutic failure in castration resistant prostate [14]. These splice variants lack the ligand binding domain but maintain their ability to bind DNA and activate AR-related actions in the prostate. These findings stress the need for further understanding of the role of ARs in breast cancer.

CXCL12 and its receptors CXCR4 and CXCR7 were first recognized in immune cells, regulating cell migration. We now know, through the widespread identification of CXCR4 on different solid tumors, including breast cancer, that this system controls several critical processes, related to primary tumor development and metastatic potential [15]. CXCR4 is essential for breast cancer cell migration to the lung, bone, and lymph nodes, that express CXCL12 [15]. It has therefore been suggested that CXCR4 could be a novel molecular diagnostic and therapeutic target in breast cancer patients [16]. The relation of estrogen and ERs with CXCL12 and CXCR4 in breast cancer cell proliferation and metastatic potential has been examined in depth during the last decade [17-22]. However, only a few reports have investigated the possible interaction between androgen/AR and CXCL12 and its receptors, mostly describing pro-migratory effects in prostate cancer [23-26].

In a previous work, studying the early effect of androgen on gene transcription in breast cancer cells we reported that testosterone triggered the expression of a number of immune-related genes, including CXCL12 [27]. In this study, we expand the investigation of the effect of androgen on CXCL12 expression in breast cancer cell lines, reporting that they may induce its expression, possibly via a direct binding to an Androgen Response Element (ARE) on the CXCL12 promoter, interacting with Sp1 sites and having an absolute requirement for the presence of NCOA1. We further report that androgen can induce both CXCL12 and its receptor CXCR4, leading to the increased motility of breast cancer cell lines, depending on the presence of wild type or mutated p53. We also analyze AR and CXCL12 tissue expression

in several publicly available data series of breast cancer patients. We report that there is a correlation between AR and CXCL12 expression and that AIs increase CXCL12 expression in breast cancer tissue. Finally, we report that in a small patient series, AIs administration in ER positive tumors co-expressing AR, potentially increased the risk of disease progression compared to non-AI treated cases, suggesting that AR and CXCL12 expression could be possible biomarkers for AI administration.

Materials and Methods

Cell cultures and Chemicals

The T47D, MCF-7, and MDA-MB-231 cell lines were purchased from DSMZ (Braunschweig, Germany), while SK-BR-3 cells were from ATCC-LGC Standards (Wesel, Germany). T47D, MCF-7, and MDA-MB-231 cells were cultured in RPMI and SK-BR-3 in McCoy's 5A medium, all supplemented with 10% fetal bovine serum (FBS), at 37 °C, 5% CO_2. All media were purchased from Invitrogen (Carlsbad, USA) and all chemicals from Sigma (St. Louis, USA) unless otherwise indicated.

RNA extraction and qRT-PCR

Cell lines were serum starved for 24 hours and were then treated with testosterone (10^{-7}M) for 3 to 24 hours. Where stated, cyproterone acetate (10^{-6} M) was added 1 hour before testosterone. Then cells were lysed to obtain mRNA, using a Nucleospin RNA II isolation kit, (Macherey-Nagel, Duren, Germany). RT-PCR and qRT-PCR were performed as described previously [28, 29]. Positive controls were run in parallel with samples. Changes were normalized according to 18S RNA expression. Experiments on individual samples were performed on different days, in duplicates. All primers were selected from qPrimer Depot (qPrimerDepot, http://primerdepot.nci.nih.gov) and synthesized by VBC Biotech (Vienna, Austria). The primers used were: *CXCL12* forward-TGG GCT CCT ACT GTA AGG GTT, reverse-TTG ACC CGA AGC TAA AGT GG; *CXCR4* forward-AGG TGC TGA AAT CAA CCC AC, reverse-CGT GGA ACG TTT TTC CTG TT; *CXCR7* forward-ATC CAT CGT TCT GAG GCG, reverse-CTC AGC ACT AAG GGA GCC AG; *18sRNA* forward-CTC AGC ACT AAG GGA GCC AG, reverse-CTC AGC ACT AAG GGA GCC AG.

Transfection of breast cancer cells with shRNA or siRNA

Short hairpin RNA against NCOA1. Short hairpin RNA (shRNA) against NCOA1 was prepared with the use of the psiRNA-h7SKGFPzeo Kit (Invivogen, San Diego, CA), according to the manufacturer's instructions, as described previously [30]. Briefly, the psiRNA-h7SKGFPzeo plasmid was digested with BbsI (New England Biolabs, Ipswitch, MA) and was gel-purified with Extract II columns (Macherey-Nagel, Duren, Germany). The following oligonucleotides were used: *NCOA1 oligo1*: 5′-ACC TCG CTG AGT CCA AAG ATA ACA AAC TCG -3′; *NCOA1 oligo2*: 5′- CAA AAA GCT GAG TCC AAA GTA AAC AAA CTC -3′; *non-template shRNA oligo-1*: 5′-ACC TCG GGT ATT TAG GCT ACG ATA GTT CAA GAG ACT ATC GTA GCC TAA ATA CCC TT-3′; *non-template shRNA oligo-2*: 5′-CAA AAA GGG TAT TTA GGC TAC GAT AGT CTC TTG AAC TAT CGT AGC CTA AAT ACC CG-3′. Annealed oligonucleotides (95°C for 5 min and left to cool slowly to 35°C) were ligated with T4-DNA ligase (TAKARA, Otsu, Shiga, Japan) with the digested psiRNA-h7SKGFPzeo plasmid and used for the transformation of LyoComp GT116 cells, plated on Fast-Media Zeo X–Gal plates. After 24 h incubation at 37°C, white colonies were picked, and minipreps, positive for the insert, were prepared by incubation of isolated DNA and digested with SpeI. A single positive miniprep for each shRNA was selected for expansion, and the presence of the proper insert was verified by sequencing.

T47D and MCF-7 cells were transfected with the shRNA plasmid using Lipofectamine 2000 according to standard protocols and after 24 hours, zeocin was added to the culture medium. Transfection efficiency was verified by microscopy, by studying GFP positivity (>95% after day 3) and by RT-PCR. All mRNA and protein expression experiments were initiated at day 3 post-transfection.

siRNA against TP53 and CXCR4. The oligonucleotides used for the short interfering RNA (siRNA) against TP53 were: oligo1: 5'-AAAAUUCUUUGUUUAGAACAA-3' and oligo2: 5'-GUU CUA AAC AAA GAA UUU UGU-3' while the oligonucleotides used for the siRNA against CXCR4 were oligo1: 5'-AUA UAC AAG AGA UGA AAU CCU-3' and oligo2: 5'-GAU UUC AUC UCU UGU AUA UGA-3'. The oligonucleotides: 5'-GUU AAA UUU CGA AUA UUA CAA-3' and 5'-GUA AUA UUC GAA AUU UGA CGU-3' were used as scrambled siRNA. The oligonucleotides were provided by VBC Biotech (Vienna, Austria). T47D and MCF-7 cells were transfected

with the siRNAs using Lipofectamine 2000 according to standard protocols. Experiments were performed 72 hours after transfection. Transfection efficiency was verified by qPCR.

ELISA

Centrifugation-cleared supernatants from cells, incubated for 24 hours in 24-well plates, were collected for CXCL12 analysis, while the cells were lysed and used for protein determination-normalization. CXCL12 was assayed on supernatant using a commercially available ELISA kit (Abnova® SDF-1alpha Human ELISA kit, Taoyuan City, Taiwan), according to the manufacturer's instructions. The effect of the medium was systematically subtracted. Results are expressed as picograms per milligram of total cellular proteins (measured with the Bradford Assay) per 24 h.

Chromatin Immunoprecipitation

T47D cells were grown in 75cm^2 flasks and treated with Testosterone (10^{-7}M) for 1 h. Cells were treated with 1% formaldehyde and the cross-linking reaction was stopped by incubation with glycine 0.125M. Cells were then washed with PBS/1mM PMSF, scrapped in the same solution, washed twice and lysed with 2ml Lysis buffer (1%SDS/10mM EDTA/50mM Tris (pH 8, 0)/1mM PMSF). Chromatin was sonicated (5 times, 45 sec intervals at 50% intensity (Vibra Cell sonicator, Sonics & Materials Co, Danbury, CT, USA), samples were centrifuged (12000rpm, 4^{o}C, 20 minutes) and the supernatant was aliquoted for further use. Chromatin was then incubated with 1X RIPA buffer, 1mM PMSF, and 5μg anti-Androgen Receptor antibody (C-19, sc-815, Santa Cruz, Dallas, TX, USA), or normal rabbit IgG overnight at 4^{o}C. Sepharose-G beads were preincubated with BSA and salmon sperm, chromatin samples were added and reactions continued for 3 h. After 3 washes, samples were treated overnight with 10mM TE/20% SDS/0.1mg/ml proteinase K and DNA was isolated with isoamyl alcohol:chloroform. Two sets of primer pairs were designed for qPCR to flank (a) the ARE sequence (forward: 5'-ACT GGG CTT GGA GCC GGG AA-3', reverse: 5'-TGC GCA GGA ATG GAG CTG GC-3', product 224bp) and (b) the Sp1 sites (forward: 5'- CTGA CGG AGA GTG AAA GTG C-3', reverse: 5'- AGA AGG TCA AAG GCC GGA G-3', product 235bp) in the CXCL12 promoter. Primers for the predicted ARE in the Runx2 promoter region (forward: 5'-AGG CAA GCC TCA GAG GGA CAA TTT-3' reverse: 5'GCT ACA GAG ATA AGA AGC CAC ATA CCT CCC-3', product 129bp) were used as further positive controls [31]. ChIP experiments were performed three times. ChIP-qPCR data were normalized with the Percent Input Method. Signals obtained from the ChIP were divided by signals obtained from a 1% input sample, representing the amount of chromatin used in the ChIP.

Proliferation assay

T47D and MCF-7 cells were plated at a density of 2×10^4 cells/ml in 24-well plates. They were grown for a total of 6 days, with a change of the medium containing fresh testosterone (10^{-7}M) on day 3. The 6-day period was chosen in order to be able to assay the effect of testosterone on at least two cell cycles. Growth and viability were measured by a modification of the tetrazolium salt assay [29].

Motility assay

In vitro scratch motility/migration assay was performed as described previously [32]. Briefly, cells were seeded in six-well plates and allowed to adhere for 24 h. The cells were treated with 10 μg/ml mitomycin C (Sigma) for 3 h (in order to block the effect of cell proliferation [33]) and washed with PBS. A 1-mm-wide scratch was made across the cell layer using a sterile pipette tip. Fresh, full medium containing testosterone (10^{-7} M) was added. All experiments were performed with medium prepared containing the same serum. Photographs were taken every 24 h at the same position of the scratch.

Boyden dual chamber migration assay

Cultures were detached with 0.25% Trypsin-EDTA and $2X10^4$ cells were plated in 100μl serum free medium on top of the filter membrane in a transwell insert (Corning® Transwell® polycarbonate membrane inserts, 6.5 mm with 8.0 μm pores). The plate was incubated for 10 minutes at 37^{o}C and 5% CO_2 to allow the cells to settle. Next, 600μl of serum supplemented medium (control) or 600μl consisting of 300μl serum supplemented medium and 300μl conditioned medium was added to the bottom chamber. Conditioned medium was prepared from the supernatant of a confluent T47D or MCF-7 cells culture in a 75cm^2 flask after 48 hours of culture (10ml). The supernatant was filtered through a 0.2μm syringe filter and half of it

was incubated for 30 minutes at RT with shaking with 5µl of an antibody against CXCL12 (Goat anti-human SDF-1 polyclonal antibody, sc-6193) while the other half was incubated with isotype control IgG (normal goat IgG, sc-2028) both from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Five µl of isotype IgG was also added in the medium used for the lower chamber of control inserts. After 16 hours, the transwell insert was removed and cells were incubated with 70% ethanol for 10 minutes and then left to dry. The insert was then incubated with 0.2% crystal violet at room temperature for 5-10 minutes, washed with distilled water and left to dry. Cells that migrated through the membrane toward the conditioned medium from 10 high power fields were counted and the average number of cells attached to the underside of the membrane were expressed relative to control (scrambled siRNA transfected cells, 600µl of serum supplemented medium in the lower chamber, isotype IgG).

Public microarray data analysis

Co-expression of AR and CXCL12 was studied in the METABRIC breast cancer data [34, 35] through the online tool cbioportal (http://www.cbioportal.org/) [36, 37].

Furthermore, 1448 TCGA normalized gene expression (based on RNAseq data) cases from 12 different pathologies were downloaded from http://compgenome.org/TCGA/software.html. These cases have been described elsewhere [38]. From these data, 151 breast cancer patients were extracted. AR status was assigned positive or negative based on the retrieval of a positive or negative value after z-transformation. Clinical data of breast cancer cases, including treatment applied, ER, PR, Her2 status, tumor status and disease relapse were retrieved from TCGA by the use of the TCGA assembler software in R [39] v1.0.3 and manually curated to match the Zodiac identified breast cancer cases.

Finally, we extracted data from the GEO-deposited GDS3116 study [40]. In this series of breast cancer patients, microarray analysis was performed on samples obtained before and after 14 days of treatment with letrozole. Normalized matrix data were downloaded from the GEO-dataset site and all probe sets related to the AR, CXCL12, CXCR4, CXCR7, and ESR1 were isolated. In case of multiple probe sets, the maximal value was retained for further analysis.

Statistical analysis

Statistics were performed with the SPSS v 21.0 (SPSS, Chicago, IL) program. Student's t-test, chi-square, one-way ANOVA were used where appropriate. A statistical threshold of 0.05 was retained for significance.

Results

Androgen induces CXCL12 gene expression and protein production from androgen receptor (AR) positive breast cancer cell lines

In a previous work [GSE18146, 27], we have reported that T47D and MDA-MB-231 cells express CXCL12 (probe sets 203666_at and 209687_at). In an ongoing work, we have also confirmed the expression of CXCL12 by MCF-7 and SKBR3 cells (Fig. 1A). We have previously reported [GSE18146, 27] that testosterone activated an early (3h) induction of transcripts and subsequently pathways related to cytokine/chemokine signaling and immune-related genes, including CXCL12, in T47D but not in MDA-MB-231 cells. Here, we report that testosterone induces CXCL12 in ER-positive MCF-7 and T47D cells, but not in the SKBR3 or the MDA-MB-231 cell line by qRT-PCR (Fig. 1B). As this differential expression might be related to the presence of active androgen receptors, we studied their expression in these four breast cancer cell lines. As reported in previous studies [41, 42] and verified here, AR mRNA was present only in T47D and MCF-7 cells (Fig. 1B inset), suggesting that the increase of CXCL12 by testosterone might be related to the presence of AR in these cell lines.

The implication of AR in the effect of androgen on CXCL12 expression was verified by qPCR for CXCL12, in testosterone (10^{-7}M) treated T47D, MCF-7, MDA-MB-231 and SKBR3 cells. Only AR positive breast cancer cell lines MCF-7 and T47D increased the expression of CXCL12 in response to testosterone, while AR negative cell lines MDA-MB-231 and SKBR3 did not show any induction (Fig. 1B). A time-course (3-24 hours) study of CXCL12 expression in response to testosterone of MCF-7 and T47D cells revealed that both cell lines respond

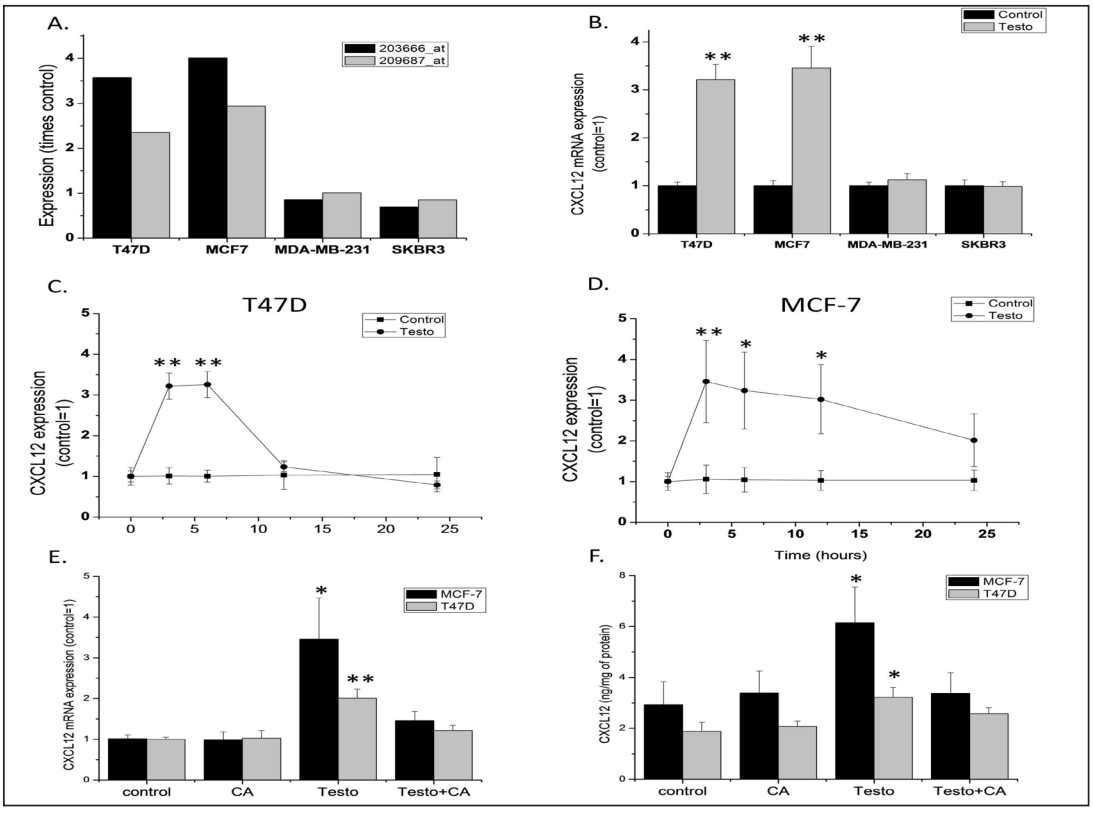

Fig. 1. A. Graphical presentation of transcripts 203666_at and 209687_at, both mapping CXCL12 in Affymetrix U133 plus2 human gene expression array. B. Real-time PCR analysis of CXCL12 expression in breast cancer cell lines verified that only AR positive cell lines increase its expression in response to Testosterone. Inset. RT-PCR for androgen Receptor Expression in T47D, MCF-7, MDA-MB-231 and SKBR3 cells. Only the first two cell lines express AR. All experiments were repeated in three independent duplicates and results were compared with Student's t-test. C and D. Time-course of CXCL12 expression in T47D and MCF-7 cells with qPCR. All experiments were repeated in three independent duplicates and results were compared with ANOVA. E and F. mRNA expression (E) and protein secretion (F) of CXCL12 in T47D and MCF-7 cells. Cells were treated with testosterone 10^{-7}M in the presence or absence of cyproterone acetate (CA, 10^{-6}M) for 3 or 24 hours for mRNA and protein studies respectively. Result from three independent experiments (mean±SE) repeated in duplicates compared with student's t-test (A, B, E, F) and ANOVA (C, D), * $p<0.05$, ** $p<0.01$ vs control or time 0 hours.

by increasing CXCL12 expression. However, MCF-7 cells displayed a sustained increased transcription over time, while T47D cells showed an initial induction at 3 and 6 hours followed by a return towards basal levels at 12 and 24 hours (Fig, 1C and D). Furthermore, the presence of the AR-antagonist cyproterone acetate during treatment resulted in a reversion of testosterone effect on CXCL12 transcription and secretion (Fig. 1E and F respectively), confirming the implication of AR in this phenomenon.

AR activation induces CXCL12 expression via an ARE upstream its promoter in cooperation with Sp1 sites within the promoter.

In order to identify whether the effect of androgen on CXCL12 expression is mediated via a direct genomic effect of AR, we examined the promoter of CXCL12 for the presence of an ARE. We identified with the ECR browser (http://ecrbrowser.dcode.org/) a putative ARE at position -1627 to -1613 upstream of the CXCL12 promoter (Fig. 2A). This *in silico* information was verified with chromatin immunoprecipitation (CHIP) followed by qPCR with specific primers flanking this region, in testosterone-treated T47D cells. Primers targeting a

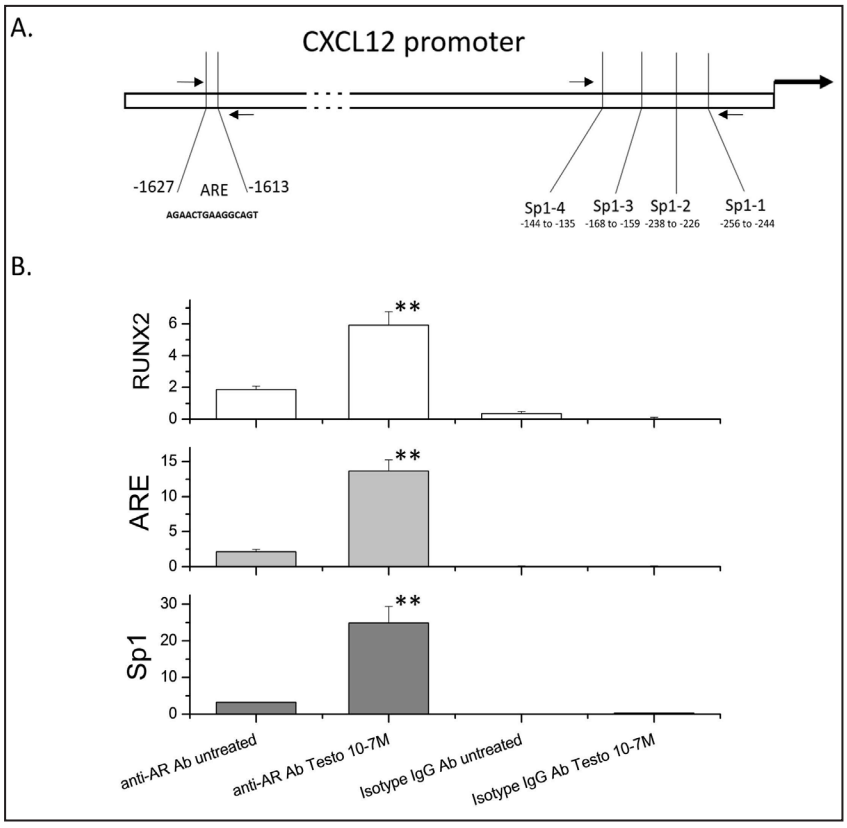

Fig. 2. A. graphical presentation of the CXCL12 promoter. The position of the putative Androgen Response Element and of the 4 Sp1 sites in the proximal promoter region are denoted. Arrows present graphically the position of the primers used for the Chromatin Immunoprecipitation assay presented in B. B. Chromatin Immunoprecipitation Assay with qPCR in T47D cells with primers targeting the ARE region of the RUNX gene (control-top), the ARE (middle) and the Sp1 (down) region of the CXCL12 promoter. In testosterone (10^{-7}M) treated T47D cells, the AR was bound to the amplified sequences. Result from three independent experiments (mean±SE) compared with student's t-test, ** $p<0.01$ vs anti-AR antibody untreated cells.

predicted ARE in the Runx2 promoter region (a promoter that has been shown previously to be an AR target [31]) were used as a positive control (Fig. 2B top panel). Our data show that the addition of testosterone results in the precipitation, by the anti-AR antibody, of the DNA sequence that includes the ARE upstream of the CXCL12 promoter (Fig. 2B middle panel), indicating that AR binds to this sequence and thus that it is a functional ARE.

The *in silico* analysis of the promoter region of CXCL12 further evidenced the presence of multiple Sp1 binding sites in the proximal region of the promoter (Sp1-1 -144 to -135, Sp1-2 -168 to -159, Sp1-3 -238 to -226 and Sp1-4 -256 to -244). Interaction of AR with Sp1 via DNA loop regulated by coactivators and other transcription factors has been reported previously to be important for the regulation of several genes [43-45]. We, therefore, assayed whether the sequence including the Sp1 sites in the promoter of CXCL12 was co-immunoprecipitated with AR. Again, in testosterone treated cells, chromatin immunoprecipitation was positive for the Sp1 containing sequence of the CXCL12 proximal promoter region (Fig. 2B, lower panel). This suggests that AR induces CXCL12 expression through a direct effect on its promoter, with a concomitant interaction with the proximal sequence of the promoter that contains the Sp1 sites.

NCOA1 is required for the androgen-induced CXCL12 expression

The DNA binding and transcriptional action of nuclear steroid receptors relays on the recruitment and physical interaction with receptor co-regulators [see 46, for a recent review]. Several such co-regulators have emerged as potential novel pharmaceutical targets, due to their ability to regulate the function of their relevant nuclear receptor, including the androgen receptor [47]. Among them, the family of Steroid/Nuclear receptor coactivator

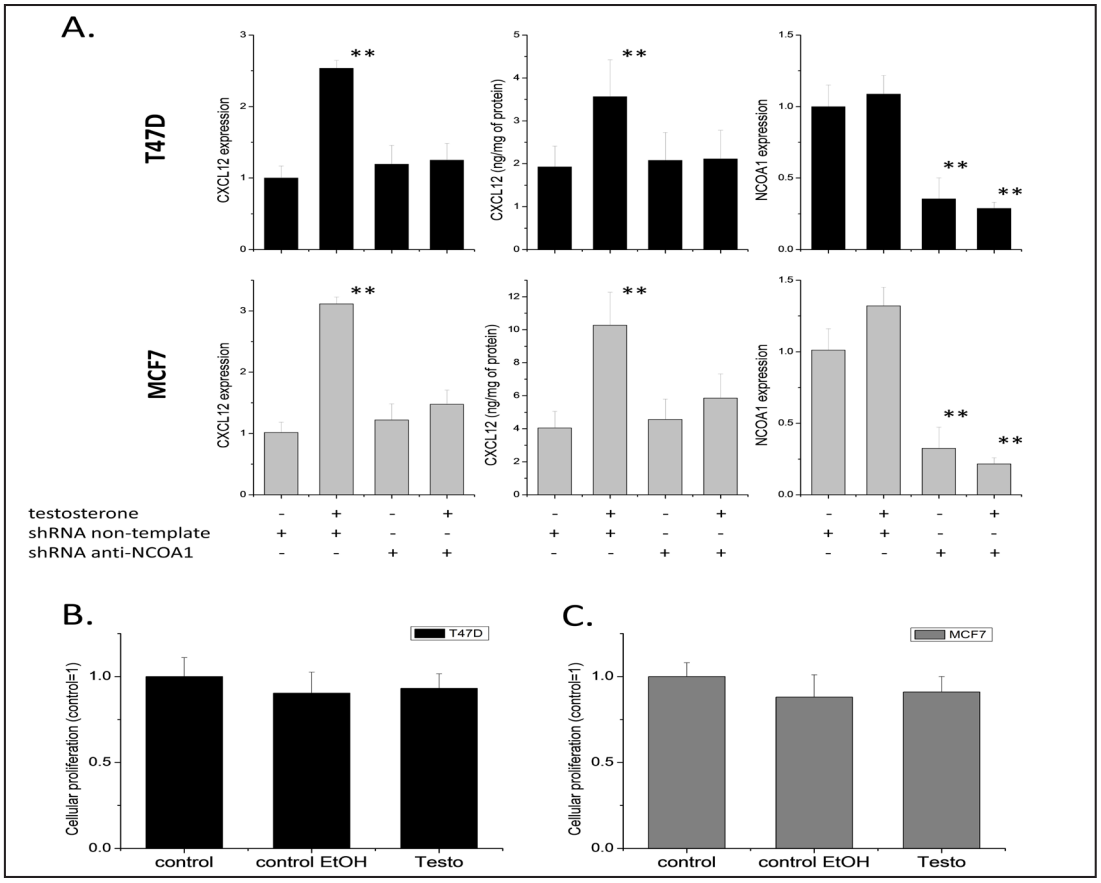

Fig. 3. A. Effect of NCOA1 downregulation on the mRNA expression (left panels) and protein production (middle panels) of CXCL12 in T47D (top) and MCF-7 (bottom) cells. Cells transfected with a non-template plasmid or with a plasmid containing shRNA against NCOA1 were treated with testosterone 10^{-7}M for 3 or 24 hours for mRNA and protein studies respectively. NCOA1 down-regulation was verified with qPCR (right panels). All experiments were repeated in three independent duplicates (mean±SE) and results were compared with ANOVA ** $p<0.01$. B and C. Effect of testosterone (10^{-7}M) on the proliferation of T47D (B) and MCF-7 (C) cells.

(SRC or NCOA) and especially its first member (NCOA1 or SRC-1) has been reported to mediate estrogen-related CXCL12 enhanced transcription in MCF-7 cells [48], while the role of NCOA2 in breast cancer is less well characterized. We investigated the possible implication of this co-activator on the AR-mediated transcription and translation/secretion of CXCR12 in the two cell lines that responded to androgen. We transfected T47D and MCF-7 cells with a specific shRNA against NCOA1 and then stimulated them with testosterone. We show that non-template RNA transfection had no significant effect on CXCL12 mRNA or protein. However, in both MCF-7 and T47D cells, NCOA1 shRNA reverted completely the effect of testosterone on CXCL12 transcription and protein synthesis/secretion, suggesting that this AR action is an absolute requirement of NCOA1 in order to proceed (Fig. 3A).

The effect of androgen on breast cancer cell lines motility depends on the enhanced expression of CXCL12 and its receptor CXCR4

CXCL12 actions in breast cancer are associated with cell proliferation, cell migration and the formation of breast cancer metastases [15]. Here, in order to investigate whether enhanced CXCL12 expression, under testosterone treatment, is implicated in an increased metastatic potential of MCF-7 and T47D cells, we treated the two cell lines with testosterone and investigated their proliferation and their motility potential. Testosterone had no effect on

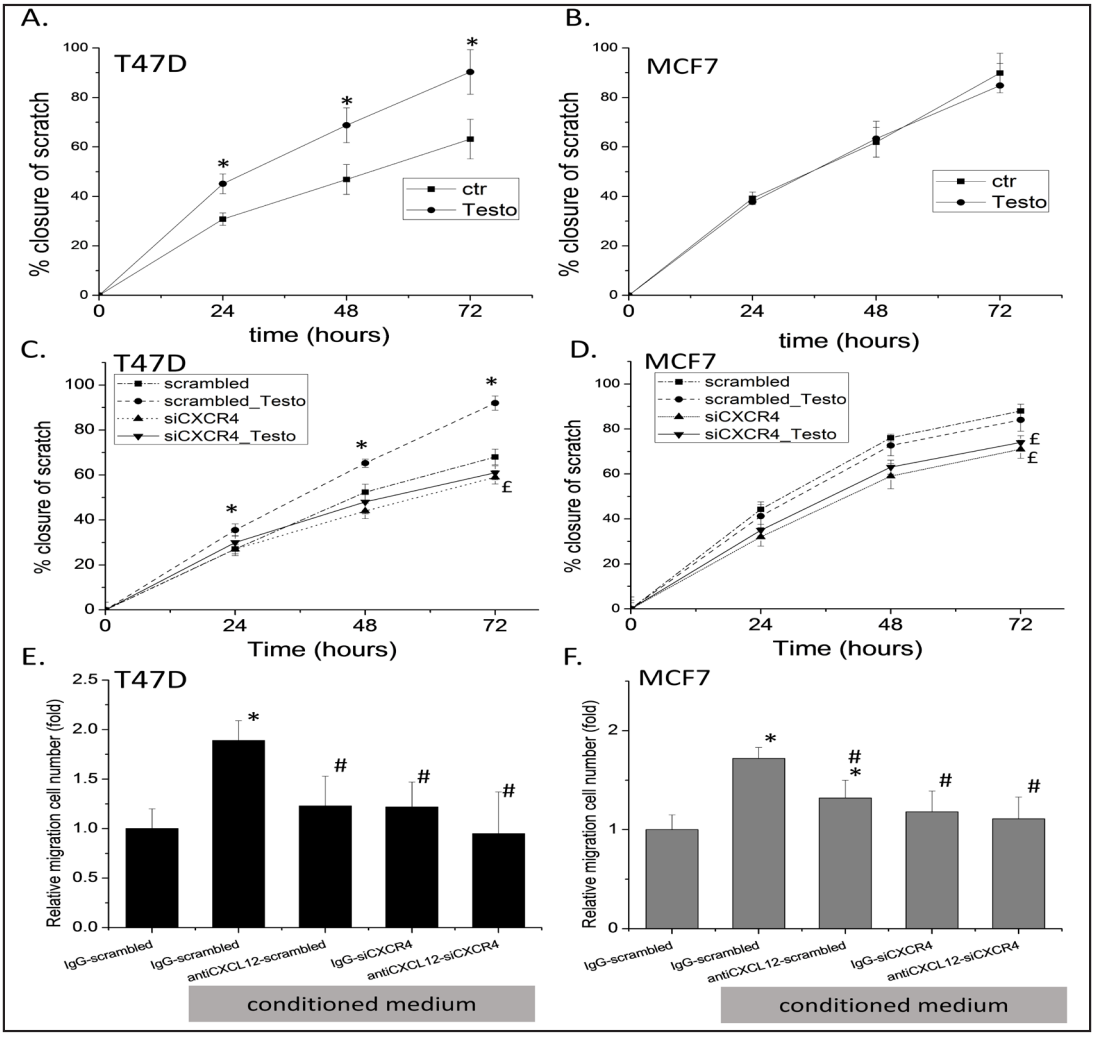

Fig. 4. A and B. Testosterone 10^{-7}M induced the motility of T47D cells (A) while it had no effect on MCF-7 cells (B), a result compatible with the induction of the first and inhibition in the latter of CXCR4. C and D. Inhibition of CXCR4 expression in T47D cells blocked the increased capacity for wound closure in the presence of androgen, while it caused an inhibition of the motility of MCF-7 cells regardless of the presence of testosterone. E and F. Boyden dual chamber migration assay showed that both T47D and MCF7 cells respond to the CXCL12 presence in the lower chamber and that loss of CXCR4 can inhibit their capacity to migrate towards it. All experiments were repeated in at least three independent duplicates (mean±SE) and results were compared with ANOVA,* $p<0.05$ and ** $p<0.01$ vs control, £ $p<0.01$ vs respective scrambled siRNA treated cells, # $p<0.01$ vs cells transfected with scrambled siRNA assayed in the presence of isotype IgG treated conditioned medium.

the proliferation of both T47D and MCF-7 cells (Fig. 3B and 3C). In the wound repair/scratch assay, our data show that testosterone, as expected [49], increased the motility of T47D cells (Fig. 4A). Unexpectedly, however, testosterone did not have any effect on the motility of MCF-7 cells (Fig. 4B), in spite of the increased and sustained expression of CXCL12 (see Fig. 1D).

In different systems, including breast cancer [17-22], it has been reported that CXCL12 can act in an autocrine manner, through its two cognitive receptors CXCR4 and CXCR7. Previous work has shown that estrogen induces both CXCL12 and its receptor CXCR4 in breast cancer and this mechanism is critical for their pro-proliferative and pro-migratory effect in breast cancer [17-22]. In order to investigate whether the differential effect of androgen on T47D and MCF-7 cells motility relies on differences in the expression of the CXCL12 receptors, we

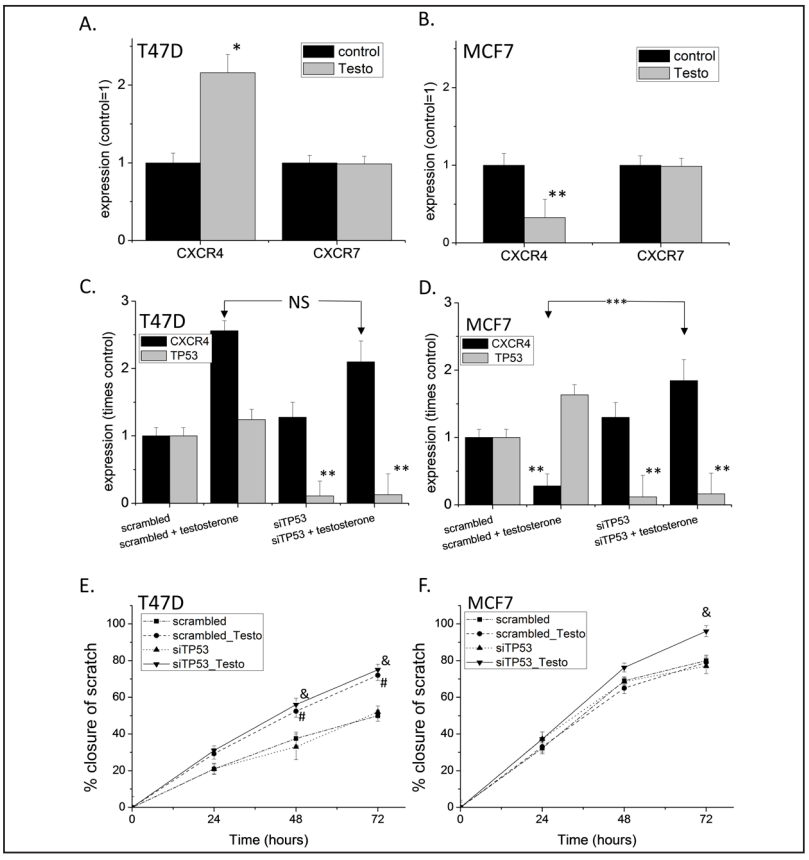

Fig. 5. Effect of testosterone (10^{-7}M) on the expression of CXCR4 and CXCR7 in T47D (A) and MCF-7 (B) cells. Effect of p53 inhibition on the induction of CXCR4 expression by testosterone in T47D (C) and MCF-7 cells (D). Effect of p53 inhibition on the motility of T47D (E) and MCF-7 (F) cells in response to testosterone. All experiments were repeated in three independent triplicates and results were compared with Student's t-test. * $p<0.05$, ** $p<0.01$ and *** $p<0.0001$ vs respective control, # $p<0.01$ vs scrambled siRNA transfected untreated cells, & $p<0.01$ vs siTP53 transfected untreated cells.

measured by qRT-PCR their expression profile in cells treated with testosterone (Fig. 5 A and B). No effect of testosterone was observed in CXCR7 expression. Contrariwise, testosterone induced the expression of CXCR4 in T47D cells, while it inhibited its expression in MCF-7 cells. It should be noted that MCF-7 cells express 40% more CXCR4 compared to T47D cells. siRNA inhibition of CXCR4 (Fig. 6A), blocked the effect of testosterone on cellular migration in T47D cells (Fig. 4C), while in MCF-7 cells, it caused a slight inhibition of cell migration that was independent of testosterone (Fig. 4D).

This critical role of the CXCL12/CXCR4 system in cell migration was further verified with Boyden dual chamber migration assay (Fig. 4E and F). The presence of CXCL12 in the conditioned medium of T47D or MCF-7 cells significantly increased cell migration. This increased migration was inhibited by either by antiCXCR4 siRNA transfection or by CXCL12 protein depletion of the medium by a neutralizing antibody against CXCL12. As expected, the effect of both inhibitory mechanisms was not additive.

The fact that CXCR4 displays such a different response to androgen in these two cell lines suggests that the regulation of its expression is under the control of more complicated mechanisms that are dependent on the intracellular machinery of each cell line. One of them could be p53 status which is unaltered in MCF-7 and mutated in T47D cells [50, 51]. In a previous study, it was reported that in MCF-7 breast cancer cells CXCR4 expression is under the negative feedback control of the non-mutated form of p53 [50]. Here we verified that inhibition of wild-type p53 expression in MCF7 cells by siRNA results in an enhanced expression of CXCR4 (Fig. 5D). In addition, p53 expression inhibition reverted the inhibitory effect of testosterone on CXCR4 expression. In T47D cells bearing a non-functional p53 mutation [52, 53] the expression of CXCR4 showed a slight reduction after p53 inhibition that was not significant but is in accordance with the finding that dominant negative and cancer-specific p53 mutants can induce CXCR4 expression [50]. Hence, loss of p53 expression

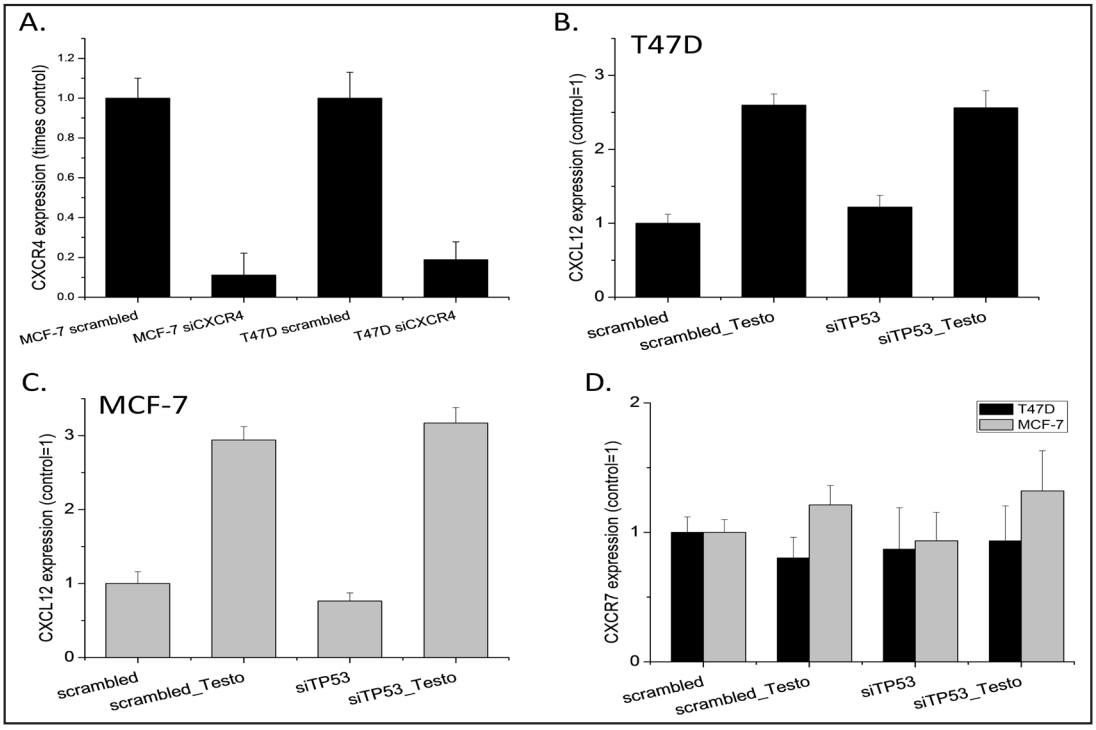

Fig. 6. A. Verification of siRNA induced CXCR4 silencing. B. Effect of p53 silencing on the expression of CXCL12 in T47D cells. C. effect of p53 silencing on the expression of CXCL12 in MCF-7 cells. D. Effect of p53 silencing on the expression of CXCR7 in T47D and MCF-7.

and the subsequent increased expression of CXCR4 might induce an increased migratory capacity of MCF7 cells. Indeed, when we repeated the motility assays using anti-TP53 siRNA treated MCF7 cells, we found that testosterone induced an increased migratory capacity. In contrast, no effect of TP53 inhibition was observed in T47D (Fig. 5E and F). It is to note that p53 inhibition had no effect on CXCL12 and CXCR7 expression in both cell lines (Fig. 6 B, C, and D).

AR expression is correlated with CXCL12 expression in breast cancer and AI therapy in ERα/AR positive tumors is related to increased CXCL12 expression and increased recurrence compared to non-AI treated ERα positive cases

Our *in vitro* data show that AR stimulation by testosterone increases CXCL12 expression and secretion together with CXCR4 expression, in a NCOA1 and p53-related manner, and this enhancement of expression increases cell migration. In order to investigate whether a similar situation occurs *in vivo,* we investigated the expression of CXCL12 and its relation to AR expression in several publicly available datasets.

We first analyzed data from the 2509 breast cancer cases of the METABRIC study [34, 35] and found a clear correlation between the expression of AR and CXCL12 ($p<0.001$). We then analyzed data from a series of 52 ER positive breast cancer patients that received 2 doses of the AI letrozole before surgical removal of their cancers [40], since in this setting positivity for AR is expected to be more than 90%. Thirty-seven of these patients displayed an increase in CXCL12 expression compared to 15 that displayed a decrease. Overall there was a 44% increase in the expression of CXCL12 in the tissue samples obtained before and after treatment (paired t-test, $p=0.0012$). These results are in accordance with our findings regarding the induction of CXCL12 by androgen.

In another approach we used the web-resource zodiac (http://compgenome.org/zodiac) [38], integrating and analyzing genomic and proteomic data of 1448 cases of 11 malignant pathologies, including 151 cases of breast cancer. As shown in Fig. 7A, in the

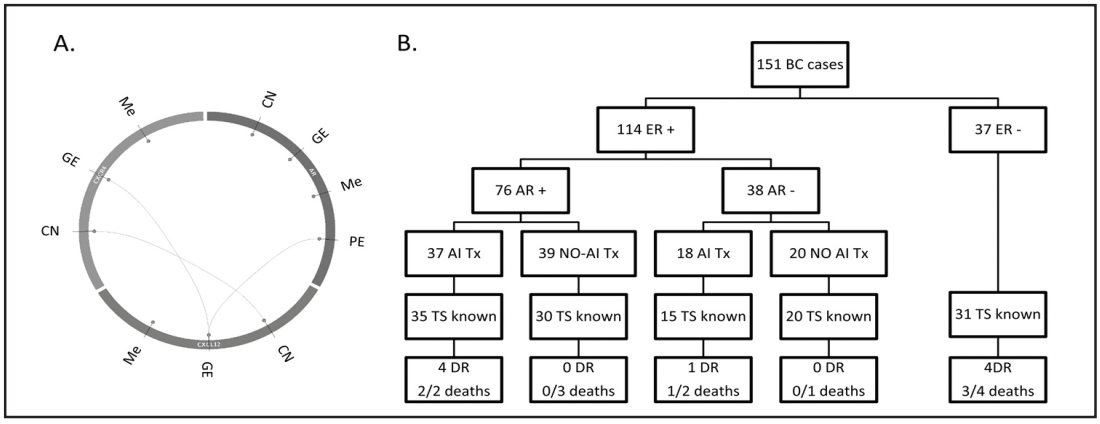

Fig. 7. A. The interaction between AR, CXCL12 and CXCR4 Gene Expression (GE), Copy Numbers (CN), Methylation (Me) and Protein Expression (PE), derived from the analysis of 1448 TCGA cases with the use of the Zodiac software as described in the Material and Methods section [38, 39]. B. Flowchart of the analysis of the 151 Cancer Genome Atlas breast cancer cases. AI treated ER+/AR+ cases displayed similar relapse rate with ER- cases. ER: estrogen receptor status, AR androgen receptor status, Tx: therapy, TS: tumor status, DR: Disease Relapse. Deaths in the lowest boxes are noted as breast cancer relapse related in the numerator and total in the denominator.

whole cohort of patients (n=1448), CXCL12 and CXCR4 expression are positively correlated (β=4.75, FDR<0.0001) and additionally AR protein expression is positively correlated with CXCL12 gene expression (β=4.92, FDR<0.0001), verifying our *in vitro* data and extending them to pathologies beyond breast cancer.

Finally, we downloaded transcription and proteomic data from the Zodiac site and extracted normalized data values only for the breast cancer patients (n=151), together with their clinical data, as described in Material and Methods. Analysis of these 151 cases identified 37 ER+/AR+ and 18 ER+/AR- cases that received AI therapy, alone or in combination with SERMs and/or chemotherapy. Analysis of the cases with known tumor status (Fig. 7B) revealed that in the AR+ cases there were 4 disease recurrences (2 cancer-related deaths, both in the disease recurrence cases) in the AI treated group and 0 recurrences (3 cancer unrelated deaths) in the AI non-treated group (one tailed chi square p=0.028). In the AR– cases there was only 1 recurrence (2 deaths, only one related to disease recurrence) in the AI treated group and 0 recurrences (1 cancer unrelated death) in the AI non-treated group. Finally, in the 31 ER- cases that tumor status was known there were 3 recurrences (4 deaths, 3 of them in the recurrences). Although data are limited and further investigations are required, disease recurrence in the ER+/AR+ AI treated group appears to be similar to that of the ER- group (22 out of 31 of whom where Triple Negative Breast Cancer cases (TNBC)), that is well known for its worse prognosis. This effect was not observed in AR negative AI treated cases.

Discussion

In spite of the tremendous progress in our understanding of the cellular and molecular biology of breast cancer and the widespread use of anti-hormonal (SERMs, aromatase inhibitors), chemotherapy and personalized biological therapy, its incidence rate remains relatively stable (0.1% increase between 2003-2012), while its mortality decreased only by 1.9% over the same period [54]. This rather alarming result reveals our still incomplete understanding of breast cancer biology, especially in cases that fail treatment, and the need for further research that can translate into more effective therapies. The present study subscribes in this direction, by reporting a direct effect of androgen, through functional androgen receptors, on the production and secretion of CXCL12 and its receptor CXCR4 by

breast cancer cells. We further describe that the underlying mechanism of this effect possibly includes AR binding on an ARE element upstream of the CXCL12 promoter, interaction with Sp1 elements and the absolute requirement of NCOA1. This CXCL12/CXCR4 enhancement leads to the increased motility of breast cancer cells. Translating these elements to a clinical context, we provide a limited clinical proof of principle from a group of AI treated patients. Although it is clear that further clinical data analysis is required, it is possible that there is a group of breast cancer patients with AR-positive breast tumors where AIs may have an undesirable effect.

CXCL12 and its receptors CXCR4 and CXCR7 are major elements implicated in breast cancer development and metastasis [15, 16]. In ER positive tumors, estrogen induces enhanced CXCL12/CXCR4&7 production/secretion, leading to cellular proliferation and tumor invasion/metastasis [17-22]. However, the interaction of androgen with the CXCL12/CXCR4/CXCR7 system in the breast had not been studied until now. Androgen has been implicated in the regulation of breast cancer cell proliferation [55] and data from prostate cancer show that androgen modulates CXCR4 and stimulate cellular migration [23, 26], a result we extend to breast cancer in this investigation. In addition, castrated male rats have lower CXCL12 levels compared to control rats and after induced myocardial infarction, they display reduced ability to recruit $CD34^+$ bone marrow cells to ischemic areas of the myocardium [25]. Our data of direct androgen effects on CXCL12 expression provide a molecular mechanism that explains this finding. We also report that, similarly to estrogen, NCOA1 is essential for the effective induction of CXCL12 by androgen [48]. Interestingly enough NCOA1 is also overexpressed in aromatase inhibitor resistant breast tumors [56]. Finally, our findings suggest that CXCL12 may belong to the list of genes that display interaction of a distal AR/ARE site with several proximal Sp1 sites in their promoter regions along with the genes of Cyclin-Dependent Kinase Inhibitor p21 [43], Monoamine oxidase A [44] and the mouse vas deferens protein (MVDP) [45].

Our findings suggest that androgen (circulating or locally produced/accumulated) may have an unfavorable effect in some breast cancer patients, mainly through a pro-metastatic action. A prerequisite for this would be the expression in these cases of CXCL12 receptors CXCR4 and/or CXCR7. To our surprise, androgen could induce CXCR4 expression only in T47D cells while they inhibited its expression in MCF-7 cells, leading to a clear phenotypic difference, with T47D cells having increased motility in response to androgen in contrast to MCF-7 cells, which remain insensitive to androgen treatment. One of the major differences between these cell lines is their different p53 status with MCF-7 bearing the intact, while T47D cells expressing mutant p53 (http://p53.free.fr/Database/Cancer_cell_lines/p53_cell_lines.html), although whether mutant p53 in these cells is dominant-negative or has distinct pro-survival effects is still under investigation [57]. Wild-type p53 has been found to suppress CXCR4 expression in MCF-7 cells [50]. Our finding of reversal of CXCR4 inhibition by androgen in this cell line, cells via the inhibition of p53, is in accordance with this previous result. Furthermore, the fact that p53 loss or non-functional mutation can revert action on CXCR4 expression from inhibitory to stimulatory has been reported previously in MCF-7 cells that were transfected with dominant-negative V143A p53 mutant or cancer-specific p53 mutants, R175H or R280K [50]. Our expression results and the specific stimulatory effect of testosterone on CXCR4 expression after p53 inhibition were also in accordance with our motility studies, with MCF7 cells becoming responsive to testosterone once p53 expression was blocked. Therefore, a non-functional p53 may be an important element regulating the response of breast cancer cells to increased androgen in the tumor microenvironment.

Another question raised by our findings is whether androgen has a direct effect on CXCR4 expression. Our data suggest that the effect of androgen on CXCR4 is indirect. Although we identified an ARE in the CXCR4 promoter region (data not shown) the fact that a functional p53 is pivotal for the inhibitory effect of androgen on CXCR4 expression suggests a more complicated series of events. Some of the possible mechanisms could include (a) a direct inhibitory effect of AR on the CXCR4 promoter that is mediated via a tethered interaction with p53 (b) an inhibitory effect mediated by a wild-type p53 effect

on the CXCR4 promoter with or without a tethered interaction with AR (c) a p53 mediated activation of a signaling pathway that mediates this inhibitory effect (d) an AR mediated activation of a signaling pathway that mediates this inhibitory effect etc. Furthermore, the protein levels and the activation status of other nuclear steroid receptors such as AR, ERα, ERβ, PR, other transcription factors/protein kinases and coregulators may also be involved. Further studies in the CXCR4 promoter region will help clarify these events.

The role of androgen in the breast remains controversial. In *in vitro* studies and animal models, androgen was found to be antiproliferative and pro-apoptotic [6], while other studies suggest the development of an aggressive pro-proliferative phenotype [58] depending on the cell type, androgenic agents administered and the type of the receptor protein involved (nuclear, extranuclear/membrane). Opposing proliferative responses of human breast cancer cell lines to androgen suggest that this action may not be solely mediated by binding to classical AR [59]. It has also been suggested that estrogen play a permissive role in androgen actions, since in their presence in culture media, DHEAS and androstenediol have antiproliferative effects in breast cancer, while in their absence the same molecules stimulate growth, an effect not observed with testosterone or DHT, that both inhibit cell proliferation independently of the presence of estrogen [60]. Most importantly, we have previously shown in T47D cells, that this effect is also dependent on non-genomic, membrane-initiated actions of androgen, triggering specific signaling cascades [61] since it can be reproduced with the use of membrane-impermeable BSA bound testosterone [62]. Therefore, the results of the *in vitro* androgen studies in the breast are highly dependent on the cell lines used, their ER status, the type/dose of the androgen used and factors yet unknown, due to our limited understanding of the mechanisms utilized by androgen in order to induce their effects.

ARs have been reported to be present in about 90% of ER+ tumors, 60% of ER−/HER2+ tumors and 20-40% of TNBCs [12, 63]. Regarding the role of AR expression on the clinical biology of breast cancer, reports have failed to identify a constant pattern: AR expression in ER negative breast cancer has been linked to increased disease free survival [1], but high AR expression in unselected breast cancer cases has been correlated to increased (axillary) metastasis [13]. AR positivity has been associated with better outcomes in ER+ breast cancer, possibly because it suggests a more well-differentiated state [64]. Other studies implicate AR expression to ER resistance, as AR overexpression induces SERM and AI resistance in ER positive cells, suggesting that patients with such tumors could benefit from combined anti-ER and anti-AR therapy [65]. Especially in TNBC, a recent meta-analysis of thirteen studies with 2826 patients reported that AR+ patients had lower tumor grade and more lymph node metastases, while AR positivity was related to prolonged disease free survival, but had no effect on overall survival [12]. Therefore, until now, we are far from understanding the role of AR in breast cancer.

Long before anti-estrogen and anti-HER2 were available and before the existence of studies regarding the expression of ARs in breast cancer, it was suggested that androgen may serve as molecules counteracting estrogen actions in breast cancer, in a yin-yang manner [66]. Testosterone was thus tested as a therapy for unselected breast cancer patients with response rates reported being around 20–25 % [63, 67]. These treatments never gained momentum and were quickly abandoned, especially when tamoxifen, the archetype of SERMs, proved to be effective in ER+ breast cancer. Apart from this initial use of androgen in the mid-twentieth century, only small clinical studies can be found in the literature. The anabolic androgen fluoxymesterone has been reported to increase tamoxifen remission rates from 15% to 38% in advanced ER+ breast cancer [68], while testosterone propionate led to 17% disease remission and 41.5% disease stabilization in patients with metastatic breast cancer, refractory to hormone treatment [69]. Clinical trials that are currently under way, and have focused on non-steroidal anti-androgen (enzalutamide, bicalutamide, darolutamide), selective androgen receptor modulators (Enobosarm), or anabolic steroid (Fluoxymesterone, CR1447) therapy in AR positive breast cancer cases, will probably shed more light in this field (data from www.clinicaltrials.gov). Interestingly, although most studies involve antiandrogen therapy in TNBC and metastatic breast cancer, androgen

anabolic treatments are also being studied in TNBC, but also in ER positive breast cancer cases, as well as in metastatic cases. Our work provides another element of androgen action in the breast, namely the enhanced production/secretion of the chemoattractant chemokine CXCL12 and the autocrine stimulation of its receptor CXCR4, through the activation of AR, findings that could be of value in the analysis of the abovementioned studies or in the design of new ones.

Our analysis of the public GDS3116 study and TCGA data shows that AR status could modify the effectiveness of AI therapy in breast cancer. AIs block the conversion of androgen to estrogen, thus increasing free testosterone and DHEA-S [70]. Previous studies have shown that patients with ER positive tumors that are responders to neoadjuvant AI therapy, display decreased AR mRNA and nuclear protein, while non-responders continue to have elevated AR expression [71, 72]. Our data are in accordance with previous reports that AR overexpression in breast cancer cell lines leads to tamoxifen and AI resistance [65, 73]. On a molecular basis, it has been shown that ER activation induces AR DNA binding in sites enriched for estrogen response elements, suggesting a collaboration of the two receptors when both of them are present in the nucleus [74]. Thus, inhibition of AR nuclear localization decreases E2-induced tumor growth of ER+/AR+ cell lines [74]. Therefore, increased AR expression may have unfavorable effects in AI treated patients and anti-androgen therapies may benefit a subgroup of AR positive patients. The opposite approach, androgen therapy in breast cancer has already been studied before as stated above and the combination of androgen and anti-estrogen as a potential strategy is a concept that has been also discussed in the literature. There seems to be a missing key, possibly a molecular signature that defines the pro- or anti-oncogenic response of breast cancer cells to androgen. A phase II study (NCT02000375) investigating the safety and activity of a combination of DHEA and an aromatase inhibitor in pre-treated ER+/AR+ postmenopausal patients was terminated early, before reaching its recruitment target, but its data have not been reported.

Personalized therapy for breast cancer has always been an important target since such therapies can be less toxic and more effective than chemotherapy. It relays on the precise measurement of a number of biological markers/elements of the tumor, in order to identify the optimal combination of drugs, beneficial for an individual patient. Our data show that, in this context, measurement of AR expression and p53 mutations as well as a CXCL12/CXCR4 presence on tumor cells, may be important biomarkers before the clinical decision for aromatase inhibitor administration is made. Although the retrospective character of our clinical data and the small number of patients with recurrence in this cohort are significant limitations of this study, our results suggest that more extensive investigations, directly addressing this issue should be performed, since they could identify a subgroup of ER positive breast cancer patients that may not benefit from AI therapy.

Abbreviations

AI (Aromatase Inhibitors); SERM (selective estrogen receptor modulator); ER (estrogen receptor); CXCL12 (C-X-C motif chemokine 12); CXCR4 (C-X-C chemokine receptor type 4); CXCR7 (C-X-C chemokine receptor type 7); AR (androgen receptor); TCGA (The Cancer Genome Atlas); NCOA1 (Nuclear Receptor Coactivator 1); ARE (Androgen Response Element); CHIP (chromatin immunoprecipitation); TNBC (Triple Negative Breast Cancer); DHEAS (dehydroepiandrosterone sulfate); HER2 (human epidermal growth factor receptor 2).

Acknowledgements

This work was supported by the European Union Programs Regional Potential/Translational Potential Grant 285948 (to GN and EC) and by the University of Crete Special account for Research grant No 3505 (to GN) and by the European Union-FP7 Marie Curie Actions-Career Reintegration Grants PCIG-GA-2011-303723 (to VP).

Disclosure Statement

All authors declare that they have no conflict of interests.

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