p42/p44 MAPK-mediated Stat3Ser727 phosphorylation is required for progestin-induced full activation of Stat3 and breast cancer growth

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Abstract

Stat3 is a signaling node for multiple oncogenic pathways and is therefore frequently active in breast cancer. As experimental and clinical evidence reveals that progestins are key players in controlling mammary gland tumorigenesis, we studied Stat3 participation in this event. We have previously shown that progestins induce Stat3Tyr705 phosphorylation and its transcriptional activation in breast cancer cells. In this study, we demonstrate that progestins also induce Stat3 phosphorylation at Ser727 residue, which occurs via activation of c-Src/p42/p44 MAPK pathways in murine progestin-dependent C4HD cells and in T-47D cells. Expression of a Stat3Ser727A vector, which carries a serine-to-alanine substitution at codon 727, shows that Stat3Ser727 phosphorylation is required for full transcriptional activation of cyclin D1 gene expression by progestins and for in vivo Stat3 recruitment on cyclin D1 promoter. Transfection of Stat3Ser727A in murine and human breast cancer cells abolished progestin-induced in vitro and in vivo growth. Moreover, we found a positive correlation between progesterone receptor expression and nuclear localization of Stat3Ser727 phosphorylation in breast cancer biopsies. These data highlight Stat3 phosphorylation in Ser727 residue as a nongenomic action by progestins, necessary to promote breast cancer growth.

Key Words
- Stat3
- p42/p44 MAPK
- progestin
- breast cancer

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Stat3 belongs to a family of proteins that act as cytoplasmic signaling molecules and transcription factors following nuclear translocation (Bowman et al. 2000, Yu & Jove 2004). Under physiological conditions, cytoplasmic Stats are phosphorylated (p) on tyrosine residues by tyrosine kinase receptors after binding of growth factors (Silvennoinen et al. 1993, Olaiyioye et al. 1999) or by soluble tyrosine kinases of the Janus (Jak) and Src kinase families, in the case of cytokine receptor activation (Darnell et al. 1994, Heinrich et al. 1998). pStats form dimers, translocate to the nuclear compartment, bind to specific DNA response elements (i.e. γ interferon-activated sequence (GAS) sites), and activate transcription. In normal cells, Stat3 activation is tightly controlled; however, constitutive Stat3 phosphorylation on tyrosine residues has been found in a wide variety of human tumors (Bowman et al. 2000). In particular, Stat3 plays a key role in mammary cancer by promoting breast tumorigenesis (Yu & Jove 2004) and conferring resistance to apoptosis (Gritsko et al. 2006) and to chemotherapy (Real et al. 2002).

Accumulated evidence indicates that progestins are implicated in the etiology and pathogenesis of human breast cancer. Clinical observations as well as the recent extensive, randomized, and controlled Women's Health Initiative trial revealed that postmenopausal women who undergo a combined estrogen and progesterone hormone replacement therapy suffer a higher incidence of breast cancer than women who take estrogen alone (Beral 2003). In its classical mechanism of action, the progesterone receptor (PR) acts as a ligand-activated transcription factor on promoters containing progesterone response elements (PREs) (Tsai & O'Malley 1994). In addition to this direct transcriptional effect, progestins are able to mediate the activation of signal transduction pathways through a rapid or nongenomic mechanism (Migliaccio et al. 1991). Several kinases activated by a large number of ligands have been implicated in serine phosphorylation, involving an interaction between Stat3 and serine kinase signaling pathways (Decker & Kovarik 2000). However, Stat3Ser727 phosphorylation induced by progestins and its biological significance remain unexplored.

In this study, we found that progestin treatment of human breast cancer cell line T-47D and of murine progestin-dependent breast cancer C4HD cells induces phosphorylation of Stat3 at Ser727 residue. We also showed that progestin activation of the c-Src/p42/p44 MAPK signaling pathway is directly involved in Stat3-Ser727 phosphorylation and contributes to the recruitment of Stat3 to a GAS site in the cyclin D1 promoter. Prevention of Stat3 phosphorylation at Ser727 residue with a Stat3S727A expression vector reveals the importance of this residue in mediating progestin-induced breast cancer cell growth. We also examined 39 primary tumor samples obtained from patients with invasive ductal breast carcinoma and observed a positive correlation between Stat3Ser727 phosphorylation and PR (PGR) expression. As a whole, our data provide evidence that phosphorylation at Ser727 residue confers full transcriptional activity of Stat3 and that it is a requisite for progestin upregulation of cyclin D1 and in vivo and in vitro breast cancer growth.
Materials and methods

Animals and tumors

Experiments were carried out with virgin female BALB/c mice raised at the Institute of Biology and Experimental Medicine (IBYME) of Buenos Aires, Argentina and were maintained in pathogen-free conditions. All animal studies were conducted as described previously (Proietti et al. 2005) in accordance with the highest standards of animal care as outlined by the NIH Guide for the Care and Use of Laboratory Animals (Guide for the Care and Use of Laboratory Animals 1996) and were approved by the IBYME Animal Research Committee. The hormone-dependent ductal tumor line C4HD was originated in mice treated with 40 mg medroxyprogesterone acetate (MPA; Craveri, Buenos Aires, Argentina) every 3 months for 1 year and has been maintained by serial transplantation in animals treated with 40 mg s.c. MPA depot in the opposite flank to tumor inoculum (Balana et al. 1999). C4HD tumor line expresses PR and estrogen receptor (ER) and lacks glucocorticoid receptor expression (Balana et al. 1999, Lanari et al. 1999).

Cell culture, treatments, and proliferation assays

Primary culture of epithelial cells from C4HD tumors was performed as described previously (Rivas et al. 2008). Cells were incubated in DMEM–Ham F12 (DMEM:F12) 1:1 v/v (without phenol red) supplemented with 0.1% (v/v) charcoalized FCS (ChFCS) in the presence or absence of 10 nM MPA (Sigma) and 10 nM RU486 (Sigma). T-47D cells were obtained from the American Type Culture Collection and maintained in DMEM:F12 +10% FCS. T-47D-Y cells were a generous gift from K Horwitz (University of Colorado Health Sciences Center, Denver, CO, USA; Sartorius et al. 1994). When indicated, cells were pretreated for 90 min with 10 μM U0126 (Sigma) or with 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) 10 μM (Calbiochem, San Diego, CA, USA) dissolved in 1:2000 dimethyl sulfoxide, or dasatinib 10 μM (LC Laboratories, Woburn, MA, USA), to block p42/p44 MAPK signaling pathway or c-Src activity respectively before the addition of MPA. Controls were performed in order to verify that dimethyl sulfoxide (1:2000) did not modify MPA-modulated c-Src tyrosine phosphorylation, p42/p44 MAPK, or Ser727Stat3 phosphorylation. Cell proliferation was evaluated by a [3H]thymidine incorporation assay as described previously (Proietti et al. 2005). Assays were performed in octuplicate.

In former experiments, we have demonstrated that thymidine uptake correlates with the number of cells per well (Rivas et al. 2008). Proliferation was also evaluated by propidium iodide staining and flow cytometry analysis, as described previously (Rivas et al. 2010). Cell cycle analysis was performed using a FACScalibur flow cytometer (Becton-Dickinson, La Jolla, CA, USA) and Modfit LT Software.

Western blot analysis

Lysates were prepared from cells subjected to the different treatments described, and proteins were subjected to SDS–PAGE as described previously (Schillaci et al. 2006). Membranes were immunoblotted with the following antibodies: total Stat3 (C-20), pp42/p44 MAPK (E-4), total p42/p44 MAPK (C-14), all from Santa Cruz Biotechnology; pStat3Ser727 (GE4), phosphotyrosine c-Src (Tyr 416), and c-Src (36D10) from Cell Signaling (Beverly, MA, USA); PR (clone hPRa7), actin (clone ACTN05), and β-tubulin from Sigma. The specificity of pStat3Ser727 (GE4) antibody for western blot (WB) assays was previously reported (Sud et al. 2009, Turner et al. 2009). Human and mouse Stat3 homologies are completely identical, except for a single amino acid change at position 760 (Pietra et al. 1998).

The NE-PER nuclear and cytoplasmic extraction reagent technique (Thermo Fisher Scientific, Inc., Rockford, IL, USA) was performed according to manufacturer’s instructions and the subcellular extracts were subjected to SDS–PAGE and immunoblotting.

Transient transfections

C4HD or T-47D cells were transiently transfected for 48 h with 2 μg of the expression vectors for Stat3S727A (pcDNAs/FRT vector encoding a GFP–Stat3 fusion protein that carries a serine-to-alanine substitution at codon 727-GFP–Stat3S727A) and WT Stat3 (pcDNAs/FRT vector encoding a GFP–Stat3 fusion protein) (Lee et al. 2009). As control, cells were transfected with 2 μg of the empty vector (pcDNAs/FRT). All these vectors were kindly provided by H Yu (Beckman Research Institute, Duarte, CA, USA). Transfection efficiencies, evaluated by the percentage of cells that exhibited GFP 48 h after transfection, varied between 60 and 70%. C4HD cells were transfected in DMEM–F12 supplemented with 10 nM MPA and 2.5% ChFCS, and T-47D cells were transfected in DMEM with 10% ChFCS without antibiotics with
Fugene HD transfection reagent (Roche Biochemicals). The plasmid encoding human WT hPR-B was kindly provided by K Horwitz. Mutant PR-B engineered to convert three key prolines (P422A, P423A, and P426A) to alanines (PR-BmPro), thus abolishing PR binding to all the SH3 domains and inhibiting activation of the Src family tyrosine kinases (Boonyaratanakornkit et al. 2001), was generously provided by D Edwards (Baylor College of Medicine, Houston, TX, USA). After transfection, cells were washed and cultured for 24 h in 0.1% ChFCS before treatment with MPA for the indicated times. Total cell lysates were then prepared as described earlier for use in WB assays. To investigate the capacity of MPA to induce the transcriptional activation of Stat3, C4HD and T-47D cells were transiently transfected for 48 h with 2 μg of a luciferase reporter plasmid containing four copies of the m67 high-affinity binding site (Bromberg et al. 1999, Zhang et al. 2000) or with a luciferase reporter plasmid containing the cyclin D1 human promoter region (1745 cyclin D1-luc), kindly provided by R Pestell (Northwestern University Medical School, Chicago, IL, USA). Cells were co-transfected with 10 ng Renilla luciferase expression vector CMV-pRL (Promega) to correct variations in transfection efficiency. Transfected cells were lysed and luciferase assays carried out using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChiP) was performed as we described previously (Beguelin et al. 2010). Briefly, chromatin was sonicated to an average of about 500 bp. Sonicated chromatin was then immunoprecipitated using 4 μg Stat3 antibody and IgG as control. The immuno-precipitate was collected using protein A beads (Millipore, Temecula, CA, USA), which were washed repeatedly to remove nonspecific DNA binding. Chromatin was eluted from the beads, and cross-links were removed overnight at 65 °C. DNA was then purified and quantified using real-time PCR performed with an ABI Prism 7500 sequence detector, using SYBR green PCR master mix (Applied Biosystems). Primers used were as described previously (Beguelin et al. 2010). PCR was performed for 40 cycles with 15 s of denaturing at 95 °C and annealing and extension at 60 °C for 1 min.

In vitro cold phosphorylation assay

T-47D cells were treated with 10 nM MPA for 2 min or preincubated for 90 min with 10 μM U0126 before MPA stimulation. Cells were lysed in kinase lysis buffer (20 mM HEPES, pH 7.5, 10 mM EGTA, 1% NP-40, and 2.5 mM MgCl2) and MAPK was immunoprecipitated using anti-total p42/p44 MAPK antibody (C-14; Santa Cruz) from 500 μg protein extracts. Unphosphorylated Stat3 was immunoprecipitated from 500 μg protein extract from nontreated C4HD cells using the Stat3 antibody (C-20, Santa Cruz). The immunoprecipitated Stat3 was then subjected to an in vitro phosphorylation assay with p42/p44 MAPKs immunoprecipitated from cells subjected to each of the treatments described, following the procedure previously described (Rivas et al. 2010). Gels were transferred onto nitrocellulose. Their upper part was immunoblotted with pStatSer727 antibody while their lower part was revealed with an anti-pp42/p44 MAPK MAB. Filters were then stripped and hybridized with anti-total Stat3 or anti-total p42/p44 MAPK antibodies respectively.

siRNAs transfections

siRNAs targeting PR were synthesized by Dharmacon, Inc. (Lafayette, CO, USA; PR siRNA 5'-AUAGGCGAGACUACAGCGUU-3'). A nonsilencing siRNA oligonucleotide from Dharmacon, Inc. that does not target any known mammalian gene was used as a negative control. The transfection of siRNA duplex was performed for 3 days using DharmaFECT transfection reagent according to manufacturer’s directions.

Immunofluorescence and confocal microscopy

T-47D cells grown on glass coverslips were fixed and permeabilized in ice-cold methanol and were then blocked with PBS with 1% BSA. pStat3Ser727 was localized using a mouse monoclonal (6E4) antibody (Cell Signaling) followed by incubation with a goat anti-mouse IgG-Alexa 488 (Molecular Probes, Eugene, OR, USA) secondary antibody. Negative controls were carried out using PBS instead of primary antibodies. Approximately 100–200 cells were analyzed for each treatment, of which around 80% showed the same pattern of Stat3 phosphorylation and cellular localization. Cells were analyzed using a Nikon Eclipse E800 confocal laser microscopy system (Beguelin et al. 2010). Nuclei were stained with propidium iodide.

In vivo inhibition of Stat3Ser727 phosphorylation

C4HD cells were transiently transfected with the Stat3Ser727A expression vector or with the empty vector. After transfection, 105 cells from each experimental group

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were inoculated s.c. into animals treated with a 40 mg MPA depot in the flank opposite of the cell inoculum (n = 5). Tumor growth was measured three times a week with a vernier caliper. Tumor volume (mm$^3$) was calculated as $(L \times W^2)/2$, where $L$, length (mm) and $W$, width (mm). Tumor growth rates were determined as the slopes of growth curves. Linear regression analysis was performed on tumor growth curves, and the slopes were compared using ANOVA followed by a parallelism test to evaluate the statistical significance of differences.

**Patients and tissue microarrays**

Paraffin-embedded tissue samples from 48 archived invasive breast carcinomas were selected for construction of tissue microarray (TMA) blocks from the files of the Instituto de Oncología Henry Moore (Buenos Aires, Argentina), from 2001 to 2008. From nine tumor samples, immunohistochemical analysis of nuclear pStat3Ser727 levels was uninformative because of missing or unrepresentative samples in the array sections analyzed. All patients were treated with surgery. This study was conducted with the approval of ethics committees of IBYME and the Instituto de Oncología Henry Moore and informed written consents were obtained from all patients before inclusion. Pretreatment patient staging was classified according to the system of the American Joint Committee on Cancer (AJCC; Singleterry et al. 2002) through the Elston and Ellis histological grading (Page et al. 1995). TMAs were constructed as described earlier (Schillaci et al. 2012).

**Immunofluorescence and immunohistochemistry analysis of TMAs**

Immunofluorescence was performed as described previously (Schillaci et al. 2012), using the mouse monoclonal anti-pStat3Ser727 (6E4) antibody (dilution 1:50, overnight at 4 °C; Cell Signaling). Slides were then incubated with an anti-mouse Alexa 488-conjugated antibody (1:1000, Molecular Probes). Nuclei were stained with propidium iodide. Slides were analyzed by Nikon Eclipse E800 confocal laser microscopy system. Negative controls were carried out in the absence of primary antibodies. Staining intensity was graded on the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining, as previously used for nuclear pTy705Stat3 classification by other authors (Dolled-Filhart et al. 2003, Sato et al. 2011). Scoring of the TMA was completed by two independent observers (E Maronna and P Guzmán), with very high correlation between scorers. Discrepancy between them was averaged to get a single final score. A score of one or more was required for tumor sample to be considered positive for nuclear pStat3Ser727 expression (Sato et al. 2011). ER and PR were evaluated by immunohistochemistry (IHC) with clone 6F11 (Novocastra Laboratories, UK) and clone hPRa2 + hPRa3 (NeoMarkers) respectively and scored as described (Schillaci et al. 2012).

**Statistical analysis**

WB bands were quantified using Image J (NIH, Bethesda, MA, USA), phosphorylated protein band values were normalized to total protein bands, and cyclin D1 bands were normalized to β-tubulin bands. Differences between control and experimental groups along this work were analyzed by ANOVA followed by Tukey’s t-test between groups. A $P<0.05$ was accepted as statistically significant. Analyses of clinical data were conducted using SPSS Software version 17.0 (SPSS, Inc., Chicago, IL, USA). Correlations between categorical variables were performed using the Fisher’s exact test.

**Results**

**MPA induces Stat3Ser727 phosphorylation acting through the classical PR**

In this study, we used the progestin-dependent C4HD tumor from an experimental model of hormonal carcinogenesis in which the synthetic progestin MPA induces mammary adenocarcinomas in female BALB/c mice (Proietti et al. 2005). We have long demonstrated that MPA is able to induce tyrosine phosphorylation of Stat3 in C4HD cells and in the human breast cancer cell line T-47D (Proietti et al. 2005). We here evaluated Ser727 phosphorylation in C4HD and T-47D cells in response to MPA. As shown in Fig. 1A, MPA induced Stat3Ser727 phosphorylation within 5 min of treatment and remained elevated at 30 min in C4HD and T-47D cells. This effect was completely abolished by the progestin antagonist RU-486 or by knockdown of PR gene expression with PR siRNAs in C4HD cells (Fig. 1B). Moreover, in human PR null T-47D-Y cells, MPA treatment did not induce Stat3Ser727 phosphorylation (Fig. 1C). On the other hand, transfection of these cells with PR-B (T-47D-Y-PR-B) restores MPA ability to phosphorylate Stat3 at Ser727 residue (Fig. 1C). These results indicate that MPA regulates the rapid phosphorylation of Stat3Ser727 residue through the classical PR.
MPA induces Stat3Ser727 phosphorylation through the classical PR.

(A) MPA induces Stat3 phosphorylation on Ser727. C4HD cells (upper panel) or T-47D cells (lower panel) were treated with MPA for different time points. Western blots (WB) were performed with pStat3Ser727 or pp42/44 MAPK antibodies, and filters were reprobed with total Stat3 and pp42/44 MAPK antibodies. β-Tubulin is shown as loading control. Data analysis showed that the increases in pp42/44 MAPK phosphorylation in cells treated with MPA compared with the levels of nontreated cells were significant (P < 0.001). (B and C) MPA-induced Stat3Ser727 phosphorylation is mediated via the classical PR. (B) C4HD cells were treated with MPA for 10 min or pretreated with RU486 for 90 min before MPA treatment (left). C4HD cells were transfected with PR siRNA or control siRNA before MPA stimulation (10 min; right). WB were performed with pStat3Ser727 antibodies, and filters were reprobed with total Stat3 antibodies. WB shows the effects of siRNAs on PR expression. Data analysis showed that the inhibition of MPA-induced Stat3 phosphorylation levels caused by PR siRNA was significant (P < 0.001). (C) T-47D-Y cells were treated with MPA or were transfected with the PR-B isoform before MPA treatment. In all cases, bands were quantified using Image J and phospho-protein band values were normalized to total protein bands. Nontreated cell samples were set as 1.0. (A, B and C) Signal intensities of phospho-Stat3Ser727 bands normalized to total Stat3 bands are graphically represented in bar plots (*P < 0.05, **P < 0.01, and ***P < 0.001). Data are presented as mean ± S.E.M. of three experiments.

MPA induces Stat3Ser727 phosphorylation through the activation of c-Src/p42/p44 MAPK signaling pathway

It is known that progestins induce rapid c-Src activation in mammary tumor cells, including our C4HD tumor model (Migliaccio et al. 1998, Boonyaratanakornkit et al. 2001, Proietti et al. 2005). Pioneering works defined the proline-rich domain of human PR as an absolute requirement for interaction with c-Src (Boonyaratanakornkit et al. 2001) and consequent c-Src activation of signaling cascades in response to progestins (Migliaccio et al. 1998, Boonyaratanakornkit et al. 2001). To explore whether c-Src was involved in progestin-mediated Stat3Ser727 phosphorylation, we transfected T-47D-Y cells with the PR-BmPro mutant, in which three prolines (P422A, P423A, and P427A) were converted to alanines (T-47D-Y-PR-BmPro cells). Figure 2A shows that T-47D-Y-PR-BmPro cells lacked the ability to phosphorylate Stat3 at Ser727 residue in response to MPA, suggesting that progestin-activated c-Src acts as an upstream activator of Stat3. Moreover, the addition of the c-Src inhibitors PP2 or...
MPA induces Stat3Ser727 phosphorylation through Src/p42/p44 MAPK activation pathway. (A and B) c-Src mediates MPA-induced p42/p44 MAPK activation that leads to Stat3Ser727 phosphorylation. (A) T-47D-Y cells were transfected with the PR-BmPro mutant and were then treated with MPA. T-47D cells are shown as Stat3Ser727 phosphorylation control. (B) T-47D cells were treated with MPA for 15 min (pStat3Ser727) or 2 min (pSrc and p42/p44 MAPK) or preincubated for 90 min with PP2 or dasatinib before MPA treatment (left panel). C4HD cells were treated with MPA for 10 min or preincubated for 90 min with PP2 before MPA treatment (right panel). WB were performed with phospho-antibodies, and filters were reprobed with the respective total protein antibody. (C and D) p42/p44 MAPK mediates MPA-induced Stat3Ser727 phosphorylation. (C) C4HD cells were treated with MPA or preincubated for 90 min with U0126 before MPA treatment. WB were performed against pStat3Ser727 (upper panel) and pp42/p44 MAPK (lower panel) and filters were reprobed with the respective total protein antibody. (A, B and C) In all cases, bands were quantified using Image J and phospho-protein bands values were normalized to total protein bands. Nontreated cell samples were set as 1.0. Signal intensities of phospho-Stat3Ser727 bands normalized to total Stat3 bands are graphically represented in bar plots (**P<0.01 and ***P<0.001). Data are presented as mean ± s.e.m. of three experiments. Data analysis showed that the increases in p42/p44 MAPK and c-Src phosphorylation in cells treated with MPA compared with the levels of nontreated cells were significant (P<0.001). (D) A cold in vitro phosphorylation assay was performed with T-47D cells preincubated or not with U0126 and then treated with MPA for 2 min. p42/p44 MAPK was immunoprecipitated from each treatment and Stat3 immunoprecipitated from nontreated T-47D cells was used as substrate. Shown are WBs of Stat3, anti-phospho-Stat3Ser727, anti-phospho-p42/p44 MAPK, and p42/p44 MAPK. Signal intensities of phospho-Stat3Ser727 bands were analyzed by densitometry and normalized to total immunoprecipitated Stat3 bands. Nontreated cell samples were set as 1.0. This experiment was repeated three times with similar results. IP, immunoprecipitation.

Figure 2

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Stat3Ser727 phosphorylation by progestin

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dasatinib effectively inhibited Stat3Ser727 phosphorylation by MPA in T-47D cells (Fig. 2B). Although it is known that murine PR lacks the polyproline sequence known to interact with c-Src, we have previously demonstrated that MPA treatment for 2–10 min of murine C4HD cells induced strong c-Src tyrosine phosphorylation (Proietti et al. 2005). Interestingly, blockage of MPA-induced c-Src activation by PP2 treatment in C4HD cells leads to inhibition of Stat3Ser727 phosphorylation in these murine cells (Fig. 2B).

p42/p44 MAPK are serine/threonine kinases whose activation by progestin-induced c-Src signaling is well acknowledged (Migliaccio et al. 1998, Ballare et al. 2003). However, the role of p42/p44 MAPK in progestin-induced Stat3 activation has never been addressed. Here, we confirmed that MPA induces a rapid phosphorylation of p42/p44 MAPK in C4HD and T-47D cells. MPA-induced activation was observed as early as 2–5 min after treatment and preceded Stat3 serine phosphorylation (Fig. 1A). Pretreatment of C4HD cells with 10 µM U0126, a p42/p44
MAPK pathway inhibitor, suppressed phosphorylation of Stat3 at Ser727 residue (Fig. 2C). Blockade of c-Src activation by addition of dasatinib or PP2 abolished p42/p44 MAPK in T-47D cells. Similar results were obtained in C4HD cells treated with PP2, suggesting that MAPK phosphorylation is dependent on c-Src activation also in murine cells (Fig. 2B).

To further support our finding that the serine phosphorylation of Stat3 proceeds through the activation of p42/p44 MAPK-dependent pathway, we performed a cold in vitro phosphorylation assay. For this purpose, we immunoprecipitated p42/p44 MAPK from T-47D cells treated or not with MPA for 2 min and from T-47D cells pretreated with U0126 for 90 min before MPA treatment. We also immunoprecipitated Stat3 from unstimulated cells and used it as a source of unphosphorylated Stat3 in the assay. As shown in Fig. 2D, incubation of p42/p44 MAPK immunoprecipitated from T-47D cells treated with MPA with the unphosphorylated Stat3 induced phosphorylation of Stat3 at Ser727 residue. Neither p42/p44

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**Figure 3**

MPA effects on Stat3Ser727 cellular localization. (A) C4HD cells were treated with MPA for 10 and 15 min, and nuclear and cytosolic protein extracts were analyzed by WB. pStat3Ser727 blot was reprobed with a Stat3 antibody. Total cell lysates treated with MPA were blotted in parallel. β-Tubulin was used to control cellular fractionation efficiency. Bands were quantified using Image J and values of nuclear and cytosolic protein bands were normalized to actin. Fold changes in nuclear and cytosolic phospho-Stat3Ser727 and total Stat3 are graphically represented in bar plots (*P < 0.05, **P < 0.01, and ***P < 0.001). Data are presented as mean ± S.E.M. of three experiments. (B) T-47D cells were treated with 10 nM MPA for 10 and 15 min and pStat3Ser727 (green) was localized by immunofluorescence and confocal microscopy. Nuclei were stained with propidium iodide (red). Merged images show nuclear localization of pStat3Ser727 at 10 and 15 min of MPA treatment. The experiments were repeated five times, with similar results.
MAPK obtained from control cells nor p42/p44 MAPK inactivated by U0126 increased Stat3 phosphorylation (Fig. 2D). As a whole, these results strongly suggest that p42/p44 MAPK are the kinases activated by MPA responsible for the induction of Stat3 phosphorylation at Ser727 residue.

**MPA promotes nuclear localization of Stat3 phosphorylated at Ser727**

We have already reported that MPA induces Stat3Tyr705 phosphorylation and its nuclear translocation (Proietti et al. 2005). In order to investigate the localization of Stat3 phosphorylated in Ser727 induced by MPA, nuclear and cytoplasmic extracts from C4HD were prepared and evaluated by WB. Figure 3A shows that MPA treatment and cytoplasmic extracts from C4HD were prepared and evaluated by WB. Figure 3A shows that MPA treatment induced Stat3 phosphorylation in the cytoplasmic fraction and an increased translocation to the nuclear compartment (Fig. 3A). In addition, immunofluorescence staining and confocal microscopy studies in T-47D revealed that Stat3 phosphorylated at Ser727 is rarely detected in control cells. MPA treatment for 10 or 15 min resulted in strong staining of pStat3Ser727 both in the cytosol and in the nuclear compartment (Fig. 3B).

**MPA requires Stat3Ser727 phosphorylation to achieve maximal transcriptional activation of Stat3**

To address the question of whether serine phosphorylation influences the transcriptional activity of Stat3, C4HD cells were transiently co-transfected with Stat3Ser727A expression vector, which carries a serine-to-alanine substitution at codon 727, together with a luciferase reporter plasmid containing four copies of the m67 high-affinity binding site (4x67-7k-luc; Bromberg et al. 1999). As controls, cells were co-transfected with WT Stat3 expression vector or with an empty vector, together with the reporter plasmid. As previously described, MPA stimulation induced Stat3 transcriptional activation of Stat3 (Proietti et al. 2005; Fig. 4A). Transfection of C4HD cells with Stat3Ser727A inhibited the capacity of MPA to activate the m67-Luc reporter plasmid (Fig. 4A). These data indicate that Stat3Ser727 phosphorylation is required for MPA-induced maximal Stat3 activation. WB studies demonstrated similar expression of Stat3WT and Stat3Ser727A in C4HD transfected cells (Fig. 4A, right panel).

To explore the biological relevance of Stat3Ser727 phosphorylation, we studied cyclin D1 promoter activation, as it is a key breast cancer cell cycle regulator, whose promoter has Stat3 binding sites (Leslie et al. 2006) and lacks a PRE in its 1-kb promoter-proximal region (Skildum et al. 2005). C4HD and T-47D cells were transiently transfected with a 1745 bp human cyclin D1 promoter luciferase construct containing Stat3 binding sites, named GAS sites, at positions −984, −568, −475, −239, −68, and −27. Cells were co-transfected either with an empty vector, or a Stat3WT or a Stat3Ser727A expression vector. MPA treatment of both cell types resulted in an increase in cyclin D1 promoter activity in cells transfected with an empty vector or with Stat3WT vector (Fig. 4B). On the other hand, transfection of cells with Stat3Ser727A absolutely inhibited the effects of MPA on cyclin D1 promoter activation in both cell types (Fig. 4B). The reporter assays shown in Fig. 4A and B suggest that Stat3Ser727A vector is acting as a dominant negative of endogenous Stat3.

Finally, we sought to determine the participation of Stat3Ser727 phosphorylation in the upregulation of cyclin D1 protein expression by MPA. Abolishment of Stat3-Ser727 phosphorylation by transfection with Stat3Ser727A abrogated MPA-induced cyclin D1 expression in C4HD and T-47D cells (Fig. 4C). As a whole, our results indicate that Stat3 requires Ser727 phosphorylation to achieve full transcriptional activity on cyclin D1 promoter and protein expression upon MPA stimulation in breast cancer cells.

**Stat3Ser727 phosphorylation is required for in vivo binding of Stat3 to the promoter of cyclin D1**

To assess the participation of Stat3Ser727 phosphorylation on the specific association of Stat3 to its binding sites in the context of living cells, we used a ChIP assay. After 30 min of MPA treatment, chromatin were immunoprecipitated using a total Stat3 antibody. Our findings with T-47D cells transfected with an empty vector or with Stat3WT, using primers spanning the GAS site at position −948 of the human cyclin D1 promoter, showed a significant and specific MPA-induced binding of Stat3 (Fig. 4D, as was previously reported by our laboratory (Beguelin et al. 2010). We then questioned whether Stat3 phosphorylation in Ser727 is mandatory for Stat3 recruitment to the GAS sites of the cyclin D1 promoter. To address this issue, we transfected T-47D cells with a Stat3Ser727A expression vector. We observed that the absence of Stat3Ser727 phosphorylation blocked Stat3 occupancy of the GAS sites of cyclin D1 promoter. These results reveal that Stat3 phosphorylation in serine 727 residue is necessary for in vivo Stat3 recruitment to the cyclin D1 promoter after MPA treatment.
Serine 727 phosphorylation of Stat3 is a requirement for MPA-induced Stat3 full transcriptional activation. (A) C4HD cells were transfected with a luciferase reporter plasmid containing four copies of the m67 high-affinity binding site (4xm67-tk-luc) and a Renilla expression vector as an internal control. Cells were co-transfected with the empty vector (pcDNA 5FRT) or Stat3WT or Stat3S727A expression vectors. After transfection, cells were treated with MPA for 24 h. Results are presented as the fold induction of luciferase activity with respect to control cells not treated with MPA. Data shown represent the mean data from three independent experiments for each cell type ± S.E.M. Results are presented as the fold induction of luciferase activity with respect to control cells without MPA treatment. Statistical significances are calculated against cells without MPA treatment (*P < 0.05 and **P < 0.01). (B) MPA induces cyclin D1 promoter activation via Stat3Ser727 phosphorylation. Cells were transfected with a 1745 bp length human cyclin D1 promoter luciferase construct, which contains Stat3 binding sites (GAS) and lacks progesterone-responsive elements (PRE) (cyclin D1-luc). Cells were co-transfected with the empty vector or Stat3WT or Stat3S727A expression vectors. After transfection, cells were treated with MPA for 24 h. Results are presented as the fold induction of luciferase activity with respect to control cells not treated with MPA. Data shown represent the mean data from three independent experiments for each cell type ± S.E.M. Statistical significances are calculated against control cells.

**Figure 4**

Blockade of Stat3Ser727 phosphorylation inhibits *in vitro* and *in vivo* progesterin-induced breast cancer growth

These results led us to investigate the correlation between MPA-induced Stat3Ser727 phosphorylation and cell growth. Thus, C4HD and T-47D cells were transiently transfected with Stat3WT or Stat3S727A expression vectors or with the corresponding empty vector, and proliferation was evaluated by [3H]-thymidine incorporation at 48 or 24 h of MPA treatment respectively. As shown in Fig. 5A, expression of the Stat3S727A mutant had an inhibitory effect on MPA-induced proliferation of both human and murine breast cancer cells, compared with MPA-stimulated cells transfected with Stat3WT or an empty vector. Proliferation of transfected C4HD and T-47D cells was also evaluated by propidium iodide staining and flow cytometry analysis, with similar results. Expression of Stat3S727A had an inhibitory effect on MPA-induced growth of T-47D cells, reflected in a cell cycle arrest in phase G1 compared with Stat3WT-transfected T-47D cells in the presence of MPA (Fig. 5B).

Furthermore, we wanted to explore Stat3Ser727 requirement for *in vivo* progesterin-driven breast cancer growth, for which we took advantage of the well-described model of murine breast cancer tumor C4HD that requires progestin-driven breast cancer growth, pan-sarcoma irradiation, and flow cytometry analysis, with similar results. Expression of Stat3S727A had an inhibitory effect on MPA-induced growth of T-47D cells, reflected in a cell cycle arrest in phase G1 compared with Stat3WT-transfected T-47D cells in the presence of MPA (Fig. 5B).

Furthermore, we wanted to explore Stat3Ser727 requirement for *in vivo* progesterin-driven breast cancer growth, for which we took advantage of the well-described model of murine breast cancer tumor C4HD that requires progestin for *in vivo* growth in BALB/c mice (Beguelin et al. 2010). C4HD cells growing in 10 nM MPA were transfected with Stat3S727A expression vector or with an empty vector. After 48 h of transfection, 10⁶ cells from each...
experimental group were inoculated s.c. into animals treated with a 40 mg MPA depot in the flank opposite to the cell inoculum, and tumor width and length were measured three times a week for 35 days in order to calculate volume. As shown in Fig. 5C, the expression of Stat3S727A in C4HD cells strongly inhibited MPA-induced tumor growth. The mean volume and growth rates of tumors that developed from Stat3S727A–C4HD cells were significantly lower than the tumors from the control group (Supplementary Table 1, see section on T-47D Empty vector Stat3WT Stat3S727A

[3H]thymidine uptake (c.p.m.)

0
10000
20000

Empty vector Stat3WT Stat3S727A

[3H]thymidine uptake (c.p.m.)

0
10000
20000

Figure 5

Serine 727 phosphorylation is required for in vitro and in vivo breast cancer proliferation. (A) T-47D and C4HD were transfected with either the empty vector (pcDNA5/FRT) or Stat3WT or Stat3S727A expression vectors and were treated with MPA for 24 or 48 h respectively. Incorporation of [3H]thymidine was used as a measure of DNA synthesis. Data are presented as mean ± S.D. of octuplicates. Statistical significances are calculated against the MPA-treated empty vector or Stat3WT-transfected cells (***P<0.001). The experiments were repeated three times with similar results. (B) T-47D cells were transfected with Stat3WT or Stat3S727A expression vectors before MPA stimulation for 24 h and were then stained with propidium iodide and analyzed for cell cycle distribution by flow cytometry. Data from the experiments shown are representative of those from a total of four experiments. (C) Effect of blockade of Stat3Ser727 phosphorylation on C4HD in vivo growth. C4HD cells (10^6) from each experimental group (empty vector-C4HD or Stat3S727A-C4HD) were inoculated s.c. into mice treated with MPA. Each point represents the mean volume of five tumors ± S.D. The experiment was repeated twice with similar results (*P<0.05). (D) Stat3Ser727 phosphorylation and cyclin D1 expression in C4HD tumors. Tumor lysates were analyzed by WB with pStat3S727 or cyclin D1 antibodies. Shown are two representative samples of mice injected with empty vector-C4HD cells (lanes 1 and 2) and with Stat3S727A-C4HD cells (lanes 3 and 4). Membranes were then stripped and hybridized with an anti-Stat3 or anti-actin antibodies. In all cases, bands were quantified using Image J, phospho-Stat3 protein band values were normalized to total Stat3 protein bands, and cyclin D1 bands were normalized to actin bands. The first sample of empty vector-C4HD cells was set as 1.0. There was significant inhibition of cyclin D1 and Stat3Ser727 phosphorylation in mice injected with C4HD-Stat3S727A cells with respect to mice injected with empty vector C4HD cells (P<0.01).
supplementary data given at the end of this article). At the end of the experiment, we prepared tumor extracts and explored levels of Stat3Ser727 phosphorylation and cyclin D1 expression. As shown in Fig. 5D, significantly lower levels of Stat3Ser727 phosphorylation and cyclin D1 were found in tumors developed in mice injected with Stat3S727A–C4HD cells than in tumors of mice injected with empty vector-C4HD cells. The expression of the Stat3S727A–GFP fusion protein was not detected by WB at the end of the experiment (day 35). Histopathological analysis of tumors from Stat3S727A–C4HD cells showed extensive fibrotic areas and also displayed a marked decrease in mitotic figures when compared with tumors from empty vector-C4HD cells (Supplementary Table 1). As a whole, these results further support the direct relevance of Stat3Ser727 phosphorylation in progestin-induced in vitro an in vivo breast cancer proliferation.

Ser727 phosphorylation of Stat3 is associated with PR expression in invasive ductal carcinomas

Because the above-described in vitro and in vivo assays provided evidence that progestins induce Stat3Ser727 phosphorylation, which is essential for up-regulating cyclin D1 expression and cell proliferation, we explored whether Stat3Ser727 phosphorylation correlates with various clinicopathological parameters in patients with invasive ductal carcinomas. TMAs from 48 tumor samples from our cohort, obtained from patients before therapy, were analyzed for nuclear expression of pStat3Ser727 by immunofluorescence using a specific antibody. Expressions of ER and PR were performed by IHC using the corresponding antibodies. Of the 48 tumor samples on the TMAs, 39 tumor cores (82%) were interpretable for the nuclear localization of pStat3Ser727 significantly with the presence of PR ($P=0.027$, Table 1). In fact, among the PR-positive tumors, 22 out of 27 (81%) presented pStat3Ser727 nuclear localization. By contrast, among the PR-negative tumors, only 37% presented pStat3Ser727 nuclear localization (three out of eight tumors). We observed a trend for nuclear localization of pStat3Ser727 and ER expression, but the difference was not statistically significant ($P=0.061$).

Discussion

In this study, we have shown that progestins are able to induce Stat3Ser727 phosphorylation and that the c-Src/p42/p44 MAPK signaling pathway is involved in this phosphorylation event. In addition, we have shown that Ser727 phosphorylation of Stat3 is required to induce cyclin D1 expression and to promote in vivo and in vitro breast cancer cell proliferation. These findings contribute to a better understanding of the participation of non-genomic progestins effect on breast cancer growth,
showing that phosphorylation of Stat3 in serine 727 is an essential event.

A wide variety of kinases participating in Ser727 phosphorylation of Stat3 have been described in diverse cell types and stimuli such as MAPK, including p42/p44 MAPK, c-Jun kinases (JNK), p38 MAPK, PKCε, and mTOR kinases (Decker & Kovarik 2000). Recently, PKCε onco-genic activity was disclosed to proceed through the activation of Raf-1, MEK-1, and p42/p44 MAPK to phosphorylate Stat3 at Ser727 residue. The event of Stat3Ser727 phosphorylation was recognized to be essential for PKCε-induced invasion in various human cancer (Aziz et al. 2010). With respect to the rapid signaling of PR, Auricchio and coworkers described a nongenomic effect of progestins that accounts for the activation of the c-Src/p21ras/p42/p44 MAPK signaling pathway in human breast cancer cell line T-47D (Migliaccio et al. 1998, Ballare et al. 2003). Moreover, our own previous findings demonstrate that progestins induce Stat3Tyr705 phosphorylation through the activation of the c-Src/Jak signaling cascade (Proietti et al. 2005). These effects of progestins on cell signaling in the absence of transcription are dependent on classical PR. Indeed, human PR has, in the amino terminal domain, a polyproline motif (amino acids 421–428) that mediates direct interaction of PR with the SH3 domain of the nonreceptor tyrosine kinase c-Src and activates this kinase by an SH3 domain displacement mechanism (Boonyaratanakornkit et al. 2001). Evidence confirms that c-Src activation is mediated by PR outside the nucleus, supporting the fact that only PR-B can stimulate c-Src and not PR-A, which is mostly nuclear (Boonyaratanakornkit et al. 2007). In our reconstitution experiments in human PR null T-47D-Y breast cancer cells with the PR-BmPro, that lacks the polyproline motifs necessary for PR activation of c-Src, MPA treatment was not able to phosphorylate Stat3 at Ser727 residue. Interestingly, we found that MPA induces p42/p44 MAPK activation via c-Src in C4HD cells. Although mouse PR lacks the polyproline motif, we have already demonstrated that MPA induces c-Src activation and p42/p44 MAPK in C4HD murine cells (Proietti et al. 2005, Carnevale et al. 2007). Moreover, we recently

Figure 6
Cellular localization of pStat3Ser727 in invasive breast carcinomas. (A) Nuclear pStat3Ser727 score. Immunofluorescence staining of breast cancer specimens with anti-pStat3Ser727 antibody (green) and confocal analysis (see Materials and Methods section for antibody specifications). Nuclei were stained with propidium iodide (red). Merged images show nuclear localization of pStat3Ser727 in tumor samples. (B) Distribution of nuclear pStat3Ser727 immunofluorescence staining scores in invasive breast carcinomas (0–3).
described the importance of phosphorylation on Ser 294 in human and mouse PR regulation of c-Src activity in response to hereregulin (Proietti et al. 2009). Taking into account that MPA induces phosphorylation of PR on Ser 294 residue (Shen et al. 2001), this activated receptor could in turn also induce c-Src activation.

The evidence provided here supports a direct link between c-Src/p42/p44 MAPK and Stat3Ser727 phosphorylation. First, addition of the p42/p44 MAPK pathway inhibitor, U0126, resulted in abolishment of MPA capacity to phosphorylate Stat3Ser727. Secondly, inhibition of c-Src activity by pharmacological inhibitors (PP2 and dasatinib) prevented MPA-induced p42/p44 MAPK activation and Stat3Ser727 phosphorylation. Finally, the in vitro phosphorylation assay indicated that MPA-activated p42/p44 MAPK can phosphorylate Stat3 at Ser727 residue. Together, all these data strongly suggest that MPA triggers a signaling cascade inducing the sequential activation of c-Src, p42/p44 MAPK that leads to Stat3Ser727 phosphorylation both in human and in mouse breast cancer cells. There are several reports supporting the fact that the biological involvement of Stat3Ser727 phosphorylation is to achieve the full transcriptional activation of Stat3 (Wen et al. 1995, Decker & Kovarik 2000). Indeed, Shen et al. (2004) observed a marked reduction in Stat3 transcriptional activation in vivo when expressing the Stat3S727A mutant. As described earlier, the dominant negative mechanism of Stat3S727A relies on the ability to form homo- or heterodimers with the endogenous WT protein, resulting in poor transcriptional activation (Zhang et al. 1996, Bromberg et al. 1998). On the other hand, Ser727-phosphorylated Stat3 has also been suggested to mediate activation of transcription without detectable Tyr705 phosphorylation, as recently reported in chronic lymphocytic leukemia (Hazar-Haleyv et al. 2010). Collectively, the available data indicate that the effect of Stat3Ser727 phosphorylation probably depends on the type of extracellular stimulus, cell type, and the activation status of the cell studied. Our results indicate that the presence of the Stat3S727A mutant abolished the transcriptional activity of the m67 reporter and of cyclin D1 promoter. Moreover, our ChIP results on the cyclin D1 GAS site confirmed that Stat3 was not recruited to the promoter in the presence of Stat3S727A.

It is well acknowledged that cyclin D1 is a requirement for breast carcinogenesis (Yu et al. 2001). Here, we showed that progestin induction of cyclin D1 gene expression is dependent on the c-Src/p42/p44 MAPK/pStat3Ser727 signaling cascade. Moreover, in our present study, we found that this phosphorylation event is essential for progestin-induced breast cancer proliferation. We found that the presence of phosphorylated Stat3 at Ser727 is a requisite for progestin stimulation of in vitro and in vivo breast cancer growth. These findings support new avenues for therapeutic approaches targeting p42/p44 MAPK signaling, like the MEK inhibitor AZD6244 that is now in phase II trials for several malignancies (O’Neil et al. 2011, Patel & Kim 2012).

Interestingly, our clinical data show for the first time that there is an association between increased levels of pStat3Ser727 and PR expression in invasive ductal breast carcinomas. Indeed, we observed that 81% of PR-positive tumors also express nuclear pStat3Ser727, which provides support to accumulating evidence showing Stat3 activation in breast tumor samples (Dolled-Filhart et al. 2003, Sato et al. 2011). Moreover, our data suggest that phosphorylation in Ser727 residue may be attributable to the presence of PR and consequently to progestin action in breast cancer patients.

In conclusion, our present findings reveal that acting through a nongenomic signaling cascade, progestin is able to phosphorylate Stat3 in Ser727 residue leading to in vivo cyclin D1 upregulation and breast cancer growth. Thus, our results presented here encourage further exploration of the potential therapeutic value of targeting Stat3 through inhibition of upstream p42/p44 MAPK signaling in PR-positive breast tumors.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-12-0194.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Endocrine-Related Cancer

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