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Progestin drives breast cancer growth by inducing p21^{CIP1} expression through the assembly of a transcriptional complex among Stat3, progesterone receptor and ErbB-2

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ABSTRACT

Cell cycle regulator p21^{CIP1} has controversial biological effects in breast cancer since in spite of its role as cell cycle inhibitor and promoter of cellular senescence, it also induces cell proliferation and chemotherapy resistance. We here explored the molecular mechanisms involved in progestin regulation of p21^{CIP1} expression. We also investigated the biological effects of p21^{CIP1} in breast cancer cells. We found that the synthetic progestin medroxyprogesterone acetate (MPA) upregulates p21^{CIP1} protein expression via c-Src, signal transducer and activator of transcription 3 (Stat3) and ErbB-2 phosphorylation. Notably, we also found that ErbB-2 nuclear function plays a key role in MPA-induction of p21^{CIP1} expression. Interestingly, we determined that progestin drives p21^{CIP1} transcriptional activation via a novel nonclassical transcriptional mechanism in which progesterone receptor is recruited along with Stat3 and ErbB-2 to a Stat3 binding site at p21^{CIP1} promoter. Our findings revealed that ErbB-2 functions as a coactivator of Stat3 in progestin induction of p21^{CIP1} transcriptional activation. Furthermore, we demonstrated that blockage of p21^{CIP1} expression strongly inhibited *in vitro* and *in vivo* progestin-induced breast cancer cell proliferation. These results further support the hypothesis that according to cell context and type of stimulus, p21^{CIP1} is capable of inducing cell cycle progression. Moreover, we provided evidence that Stat3 and nuclear ErbB-2 are key players in progestin-induced p21^{CIP1} regulation.

1. Introduction

Cell cycle regulator p21^{CIP1} was first identified as a cyclin-dependent kinase (Cdk) inhibitor with the ability to bind and inhibit the kinase activity of cyclin E/CdK2, thus preventing progression through cell cycle [1]. In addition, by binding to proliferating cell nuclear antigen (PCNA), p21^{CIP1} was found to inhibit DNA replication [2]. However, p21^{CIP1} overexpression in different types of cancers, including breast tumors, has been associated with resistance to apoptosis induced by chemotherapeutic agents [3,4]. Despite its profound role in halting cellular proliferation, recent studies suggest that under certain conditions, p21^{CIP1} can in fact promote cellular proliferation and oncogenicity [5,6].

Progesterone plays a key role in the regulation of cell proliferation and differentiation in the mammary gland [7,8]. The physiological effects of progesterone occur mainly via the interaction with specific intracellular progesterone receptors (PR). In its classical mechanism of action, PR acts as a ligand-activated transcription factor on promoters containing progesterone response elements (PREs) [9]. Alternatively, PR may alter gene expression non-classically, where the receptor tethers to other transcription factors bound to DNA [10–14].

On the other hand, the ErbB family of receptor tyrosine kinases and the signal transducer and activator of transcription 3 (Stat3) are major players in the breast cancer scenario. Indeed, we have shown that in breast cancer the synthetic progestin medroxyprogesterone acetate (MPA) activates Stat3 via Janus (Jak) and the c-Src kinase protein families, and that Stat3 activation is an essential requirement for progestin-induced breast tumor growth [15]. We have also described that MPA induces ErbB-2 activation and nuclear translocation, functioning in the nucleus as a coactivator of Stat3, driving progestin-induced breast cancer growth [13].

Abbreviations: PR, progesterone receptor; MPA, medroxy progesterone acetate; CdK, cyclin dependent kinase; PREs, progesterone response elements; Stat3, signal transducer and activator of transcription 3; GAS, gamma interferon-activated sequence; siRNAs, small interference RNAs; ChIP, chromatin immunoprecipitation; HASs, HER-2-associated sequences; PR-B, isoform B of PR; WB, Western blot.

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Progestin has also been found to induce upregulation of $p21^{CIP1}$ protein expression in breast cancer cells [11,16]. Although $p21^{CIP1}$ promoter lacks canonical PREs, PR interacts with the transcription factor Specific protein 1 (Sp1) at Sp1 sites located in the $p21^{CIP1}$ proximal promoter to increase its protein levels [11,16]. Moreover, we have recently disclosed that progestin regulates $p21^{CIP1}$ expression non-classically by inducing an interaction between Stat3 and PR tethered to Sp1 in the promoter of $p21^{CIP1}$ in human breast cancer cells [14].

Notwithstanding all these data, and given the controversial role of $p21^{CIP1}$ in breast cancer, we set out to explore the molecular mechanism of progestin regulation of $p21^{CIP1}$ expression and the role of $p21^{CIP1}$ in progestin-induced breast cancer cell proliferation.

We demonstrated that MPA induces the recruitment of a transcriptional complex composed by Stat3, PR and ErbB-2 at a Stat3 binding site in the $p21^{CIP1}$ proximal promoter. ErbB-2 nuclear localization and its function as a coactivator of Stat3 are essential for progestin induction of $p21^{CIP1}$ expression. Finally, we also found that $p21^{CIP1}$ is required for progestin-driven breast cancer growth.

2. Experimental

2.1. 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. All animal studies were conducted as described previously [14], in accordance with the highest standards of animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IBYME Animal Research Committee. The C4HD tumor line displays high levels of estrogen receptor and PR, overexpresses ErbB-2 and ErbB-3, exhibits low ErbB-4 levels and lacks epidermal growth factor receptor expression [17]. This tumor line does not express glucocorticoid receptor or androgen receptor [17].

2.2. Reagents

Medroxyprogesterone acetate (MPA) and RU486 were purchased from Sigma–Aldrich (Saint Louis, MI). 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine (PP2), tyrphostin AG825, and Jak inhibitor I were purchased from Calbiochem (San Diego, CA).

2.3. Antibodies

The following antibodies were used for Western blots: phospho (p)-Stat3 (p-Stat3) (Tyr 705) (B-7), total Stat3 (C-20), ErbB-2 (C-18), $p21^{CIP1}$ (C-19) all purchased from Santa Cruz Biotechnology (Santa Cruz, CA); p-ErbB-2 (Tyr 1221/1222), p-ErbB-2 (Tyr 877), c-Src and p-Src (Tyr 416) all from Cell Signaling (Beverly, MA); actin (clone ACTN05) from Neomarkers (Freemont, CA) and β-tubulin from Sigma–Aldrich. The antibodies used for the chromatin immunoprecipitation (ChIP) assays were rabbit polyclonal anti-ErbB-2, anti-Stat3 and anti-PR antibodies (C-18, C-20, and H-190, respectively; from Santa Cruz Biotechnology). Rabbit IgG (Sigma–Aldrich) was used as a negative control.

2.4. Cell cultures and treatments

Primary cultures of epithelial cells from C4HD tumors were performed as previously described (2). T47D cells were obtained from American Type Culture Collection and PR-null T47D-Y cells were a generous gift from Horwitz (University of Colorado Health Sciences Center, Denver, CO). To evaluate the effects of the pharmacological inhibitors on MPA-induced $p21^{CIP1}$ protein expression, cells were starved in serum-free medium for 48 h and then preincubated in serum free medium for 90 min with RU486, PP2, tyrphostin AG825 or Jak Inhibitor I before the addition of MPA. Cell proliferation was evaluated by [^{3}H]thymidine incorporation assays, as previously described [18], after a 48 h treatment in serum-free medium.

2.5. Western blot analysis and immunoprecipitation assays

Lysates were prepared from cells subjected to the different treatments and proteins were subjected to SDS–PAGE as previously described (5). Membranes were immunoblotted with the antibodies detailed in each experiment. When phosphoprotein antibodies were used, filters were reprobed with total protein antibodies. Signal intensities of p-ErbB-2, p-Stat3 and p-c-Src bands were analyzed by densitometry and normalized to total protein bands. Similarly, signal intensities of $p21^{CIP1}$, Stat3 and ErbB-2 bands were normalized to actin bands. Data analysis showed a significant increase in $p21^{CIP1}$ levels by MPA treatment compared to non-treated cells, and a significant inhibition of MPA-induced $p21^{CIP1}$ protein expression when Stat3 small interfering RNAs (siRNAs), ErbB-2 siRNA and the pharmacological inhibitors were used ($P < 0.001$). A similar analysis showed a significant increase in p-ErbB-2, p-Stat3 and p-c-Src levels by MPA treatment in comparison with untreated cells. We also observed a significant inhibition of MPA-induced protein phosphorylation when using the pharmacological inhibitors of the activation of ErbB-2, Stat3 and c-Src, and a significant inhibition of ErbB-2, Stat3 and $p21^{CIP1}$ expression when ErbB-2, Stat3 and $p21^{CIP1}$ siRNAs were respectively used ($P < 0.001$).

2.6. Plasmids and transient transfections

The luciferase reporter plasmid downstream of the $p21^{CIP1}$ human promoter region (pGL3-$p21^{CIP1}$-2400) and the construct with GAS mutations (pGL3-$p21^{CIP1}$mGAS) were kindly provided by Dihua Yu, Department of Molecular and Cellular Oncology, The University of Texas, MD Anderson Cancer Center, TX. The Renilla luciferase expression plasmid RL-CMV was obtained from Promega (Madison, WI). The human wild-type ErbB-2 expression vector (hErbB-2WT) was a gift from Yamamoto (University of Tokyo, Japan) [19]. The GFP-tagged human ErbB-2 mutant which lacks the putative nuclear localization signal sequence (aa 676-KRRQQKIRKYTMRR-689), resulting in the sequence of KLM at the deletion junction (hErbB-2DNLS), was generously provided by Hung (The University of Texas M.D. Anderson Cancer Center, Houston, TX) [20]. The empty pEGFP-N1 vector was obtained from BD Biosciences Clontech. The plasmid encoding the human wild-type hPR-B was kindly provided by Horwitz.

In experiments assessing MPA capacity to induce the transcriptional activation of Stat3, T47D cells were transiently transfected for 48 h with 0.5 μg of pGL3-$p21^{CIP1}$-2400 or pGL3-$p21^{CIP1}$mGAS and 10 ng of RL-CMV used to correct variations in transfection efficiency. Total amount of transfected DNA was standardized by adding the pA3 Luc reporter vector. In experiments assessing the role of ErbB-2 in Stat3 transcriptional activation, cells were cotransfected with 1.5 μg of hErbB-2WT or hErbB-2ΔNLS or the empty vectors pMe18SM and pEGFP-N1. Fugene 6 transfection reagent (Roche Biochemicals, Indianapolis, IN) was used as described previously [13]. Cells were then starved for 24 h and treated with MPA during 24 h, or were left untreated. Transfection efficiencies, evaluated using the pEGFP-N1 vector and determined by the percentage of cells that exhibited GFP 4 days after transfection, varied between 60% and 70%. Transfected cells were lysed and luciferase assays carried out using the Dual-Luciferase Reporter Assay System (Promega) in accordance with manufacturer's instructions, and

luminescence was measured with the 20/20^{n}Luminometer (Turner Biosystems). Triplicate samples were analyzed for each datum point. Differences between experimental groups were analyzed by ANOVA followed by Tukey test between groups.

2.7. siRNA transfections

siRNAs targeting ErbB-2, Stat3 and p21^{CIP1} (CDKN1A) and control nonsilencing siRNA were synthesized by Dharmacon Inc. (Lafayette, CO). The sequences of the siRNAs were: ErbB-2 siRNA: 5′GAUGGUGCUUACUCAUUGA3′, which specifically knocks down mouse ErbB-2 but not human ErbB-2; Stat3 siRNA: 5′GGUCAAAUUUCCUGAGUUGUU3′ for mouse Stat3. The siRNA SMART pools were used for human and mouse p21^{CIP1}. The non silencing siRNA oligonucleotide used as a negative control does not target any known mammalian gene. Transfection of siRNAs was performed for 3 days by using DharmaFECT transfection reagent (Dharmacon) according to the manufacturer's directions.

2.8. ChIP assay

ChIP was performed as previously describe elsewhere [3] with minor modifications. Briefly, chromatin was sonicated to an average of about 500 bp. Sonicated chromatin was then immunoprecipitated using 4 μg of either an anti-ErbB-2, anti-Stat3, or anti-PR antibody and rabbit IgG as control. The IP was collected using Protein A (Millipore, Temecula, CA), which were washed repeatedly to remove nonspecific DNA binding. The chromatin was eluted from the beads, and cross-links were removed overnight at 65 °C. DNA was then purified and quantified by using real-time PCR.

2.9. Real-time quantitative PCR

The primers used for real-time PCR were as follows: 5′-GGTTGCCCTTTTTGGTAGTCT-3′ and 5′-ATTTTTCAGTCCCGTTTATTTCACA-3′ designed to amplify a region of the human p21^{CIP1} promoter containing one GAS site (-669). These primers were designed with "Primer Express" real-time PCR primer design software (Applied Biosystems). The real-time PCR program was: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.10. In vivo inhibition of p21^{CIP1} expression

C4HD cells were transfected with the mouse p21^{CIP1} siRNA SMART Pool. After transfection 10^6 cells from each experimental group were inoculated subcutaneously (s.c.) into animals treated with a 40-mg MPA depot in the flank opposite of the cell inoculum. Tumor volume, growth rate and growth delay were determined as previously described [15]. Comparison of tumor volumes between the two groups at specific times was done by analysis of variance followed by *t*-test between groups. Linear regression analysis was performed on tumor growth curves, and the slopes were compared by using analysis of variance followed by a parallelism test to evaluate the statistical significance of differences.

3. Results

3.1. MPA induces p21^{CIP1} protein expression via Stat3 and via ErbB-2 signaling and nuclear functions

In this study we used primary cultures of C4HD epithelial cells from the model of mammary carcinogenesis induced by MPA in female BALB/c mice [17] and the PR-positive human breast cancer cell line T47D. C4HD cells display high levels of estrogen receptor and PR, overexpress ErbB-2 and ErbB-3 and lack epidermal growth factor receptor [17]. As previously reported in T47D [11,16] and C4HD cells [14], MPA treatment increased the levels of p21^{CIP1} protein expression (Fig. 1A and B). This effect was completely abolished by the progestin antagonist RU486 (Fig. 1A and B). MPA treatment did not induce p21^{CIP1} protein expression in human PR-null T47D-Y cells (Fig. 1C). Transfection of these cells with the B isoform of PR (T47D-Y-PR-B) restored MPA ability to increase p21^{CIP1} protein levels (Fig. 1C). These results indicate that MPA positively regulates p21^{CIP1} protein expression through the classical PR. We next investigated the signaling pathways involved in MPA-induced p21^{CIP1} expression. We previously demonstrated that MPA induces Stat3 Tyr 705 phosphorylation via a Jak and c-Src-dependent pathway in breast cancer cells [15]. In addition, we recently found that MPA induces ErbB-2 phosphorylation at Tyr 1272, a major autophosphorylation site and at Tyr 877, a site other than the autophosphorylation ones, both of which are phosphorylated via c-Src [13]. Interestingly, we also found that MPA causes ErbB-2 translocation to the nucleus of breast tumor cells where it functions as a coactivator of Stat3 [13]. We therefore decided to explore whether the regulation of p21^{CIP1} protein levels was dependent on Stat3 and ErbB-2 activation. We found that inhibition of the activation of ErbB-2 with the tyrosine kinase inhibitor, AG825, and of Stat3 using the Jak inhibitor I, abrogated MPA-induced p21^{CIP1} protein expression in T47D cells (Fig 2A). Since we previously showed that MPA rapidly activates Stat3 and ErbB-2 via c-Src [13,14], we tested the role of c-Src in the effects of MPA on p21^{CIP1} expression. Preincubation of the cells with the c-Src family kinase inhibitor PP2, abrogated the capacity of MPA to induce p21^{CIP1} expression (Fig. 2A). Knockdown of ErbB-2 and Stat3 expression with siRNAs also inhibited the capacity of MPA to induce p21^{CIP1} expression in C4HD cells (Fig 2B). These findings demonstrate that ErbB-2 and Stat3 are key players in the mechanism of MPA-induced p21^{CIP1} expression. The dogma that ErbB-2 acts only at the plasma membrane level has been challenged by Wang et al. demonstrating that ErbB-2 migrates to the nuclear compartment, where it binds DNA at specific sequences, named HER-2-associated sequences (HASs) [21]. Furthermore, we have recently demonstrated ErbB-2 function as coactivator of Stat3 in the mechanism of progestin induction of cyclin D1 transcription [13]. Through database (MatInspector [http://www.genomatix.de]) searches we first identified that p21^{CIP1} promoter lacks HAS sites. In order to study the participation of nuclear ErbB-2 on MPA-induced p21^{CIP1} expression, we prevented ErbB-2 nuclear localization by transfecting T47D cells with an ErbB-2 nuclear localization domain mutant (hErbB-2-ΔNLS), which is unable to translocate to the nucleus [20]. We have also demonstrated that this mutant acts as a dominant negative inhibitor of endogenous ErbB-2 nuclear migration [13]. In addition, seminal findings and our own work demonstrated that ErbB-2ΔNLS retains its intrinsic tyrosine kinase activity as well as the capacity to activate classical ErbB-2 cascades, and does not affect endogenous ErbB-2 signaling [13,20]. As shown in Fig. 2C, MPA did not increase p21^{CIP1} protein levels in cells transfected with the hErbB-2ΔNLS. These results for the first time show that nuclear ErbB-2 is required for MPA-induction of p21^{CIP1} expression (Fig. 2C).

3.2. ErbB-2 acts as a coactivator of Stat3 in MPA-induced p21^{CIP1} promoter activation

Progestin has been found to induce the upregulation of p21^{CIP1} via an interaction between PR and Sp1 at Sp1 sites which map at −83 nt and −70 nt relative to the transcription start site of p21^{CIP1} promoter [11]. Interestingly, our own findings recently identified that Stat3 functions as a coactivator of PR in said regula-

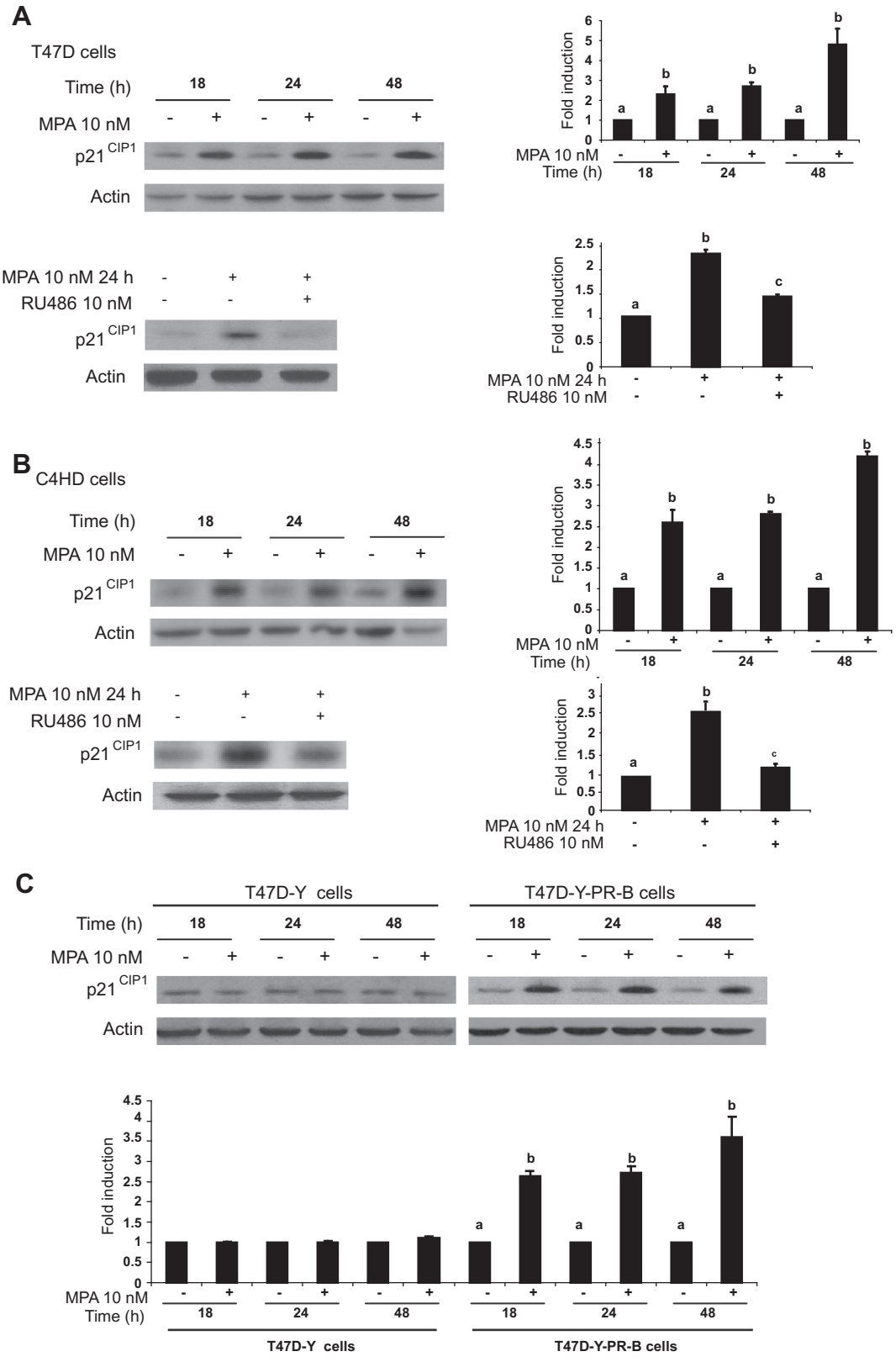

Fig. 1. MPA induces $p21^{CIP1}$ protein expression via the classical PR. T47D (A), C4HD (B), T47D-Y and T47D-Y-PR-B (C) cells were treated with MPA 10 nM for the time points indicated. (A and B) (lower panels), cells were preincubated with RU486 10 nM for 90 min and then treated with MPA 10 nM for 24 h. $p21^{CIP1}$ protein levels were analyzed by Western blot (WB). Signal intensities of $p21^{CIP1}$ bands in the WB were analyzed by densitometry and normalized to actin bands and are graphically represented in a bar plot. Data analysis showed that increase in $p21^{CIP1}$ in cells treated with MPA compared to untreated cells, and inhibition of MPA-induced $p21^{CIP1}$ expression levels caused by RU486 was significant ($P < 0.001$ for b versus a and for b versus c). The experiments for which the results are shown were repeated three times with similar results.

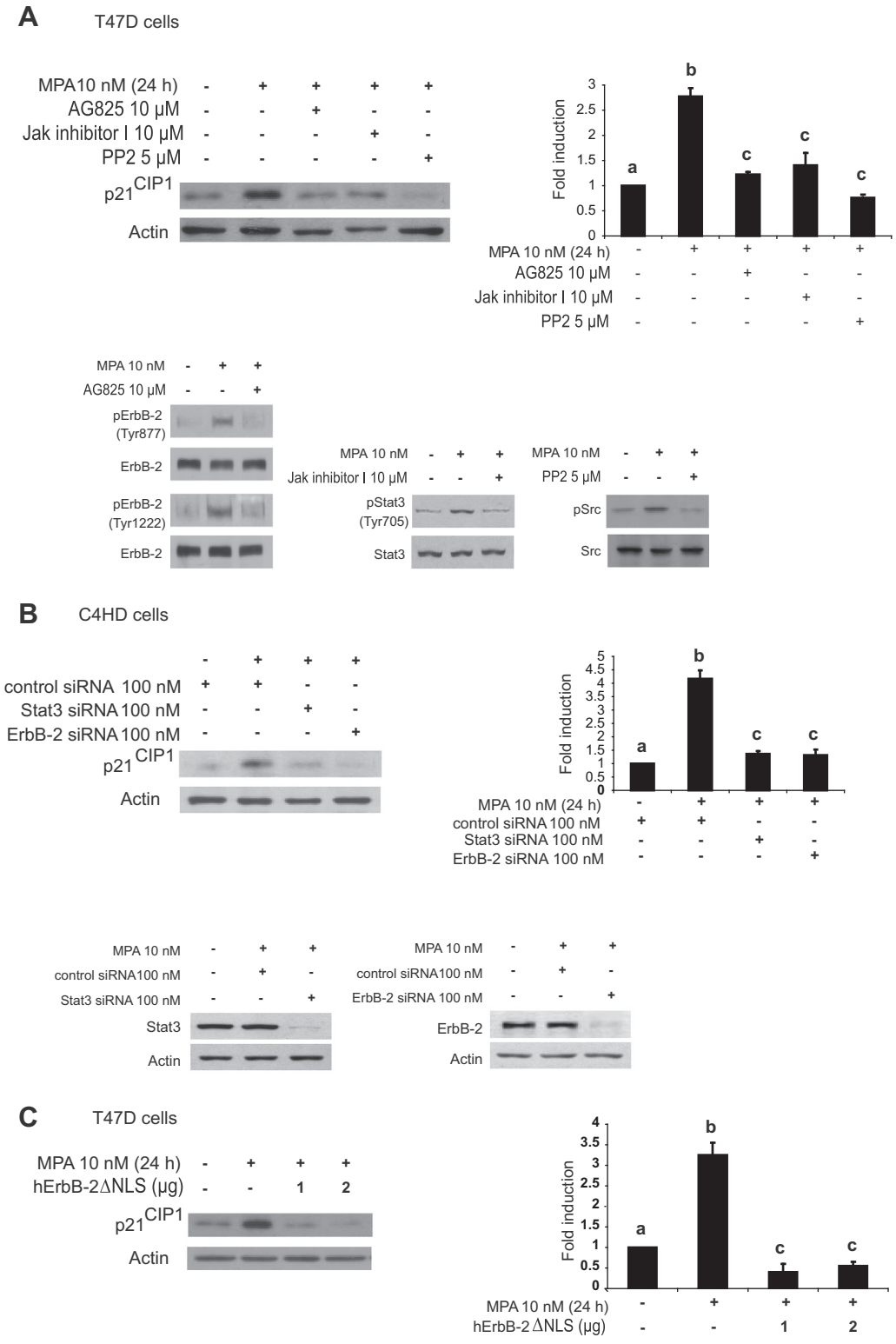

Fig. 2. MPA induces p21^{CIP1} expression via ErbB-2 and Stat3 in breast cancer cells. (A) T47D cells were preincubated with the indicated pharmacological inhibitors for 90 min and were treated with MPA 10 nM for 24 h. p21^{CIP1} levels were studied by WB. Signal intensities of p-ErbB-2, p-Stat3 and p-Src bands in the WB were analyzed by densitometry and normalized to total ErbB-2, total Stat3 and total Src bands, respectively. Data analysis showed that increase in p21^{CIP1} expression in cells treated with MPA, compared to untreated cells, and inhibition of MPA-induced p21^{CIP1} expression caused by AG825, JAK inhibitor I, and PP2 were significant ($P < 0.001$). The experiment was repeated three times with similar results. Controls of Stat3, ErbB-2 and c-Src inhibition of phosphorylation are shown (lower panels). (B) C4HD cells were transfected with siRNAs targeting Stat3, ErbB-2 or control siRNAs and treated with MPA 10 nM for 24 h. Controls of inhibition of ErbB-2 and Stat3 expression by siRNAs are shown (lower panels). Data analysis showed that Stat3 and ErbB-2 siRNAs resulted in 75–80% knockdown of Stat3 and ErbB-2 respectively ($P < 0.001$). (C) T47D cells were transfected with 1 μg or 2 μg of the hErbB-2ΔNLS expression vector and then treated with MPA 10 nM for 24 h. p21^{CIP1} levels were studied by WB. Data analysis showed that compared to control cells; the increase in p21^{CIP1} levels by MPA treatment was significant, as was the inhibition of MPA effects by ErbB-2ΔNLS. Signal intensities of p21^{CIP1} bands in the WB were analyzed by densitometry and normalized to actin bands and are graphically represented in a bar plot ($P < 0.001$ for b versus a and for b versus c). The experiments shown were repeated three times with similar results.

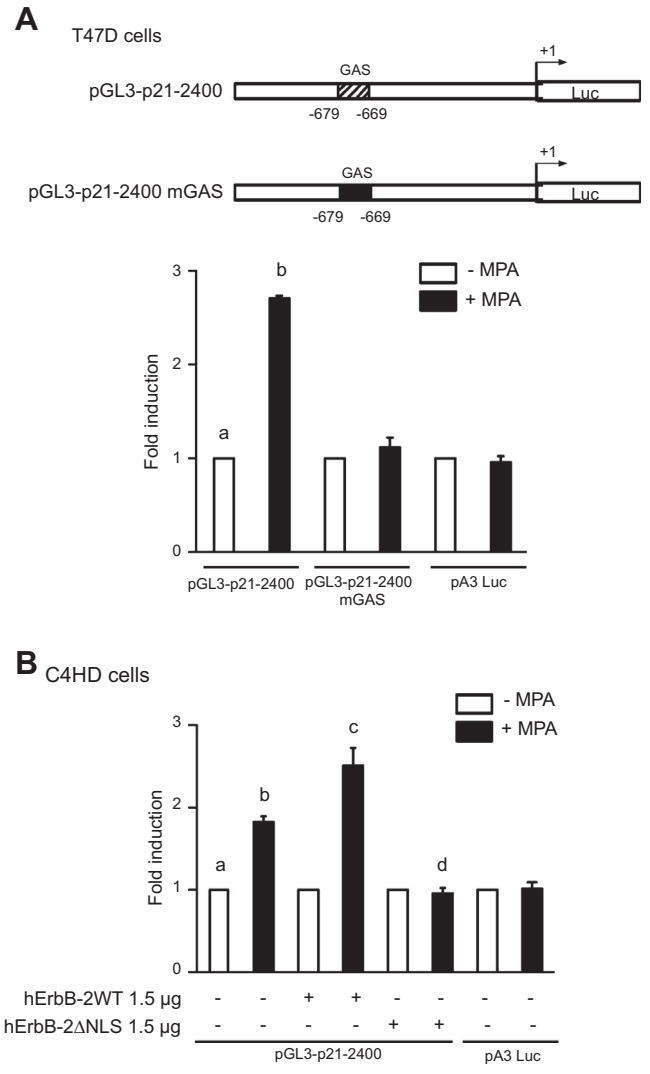

Fig. 3. ErbB-2 acts as a Stat3 coactivator in MPA-induced p21^{CIP1} promoter activation. (A) MPA induces p21^{CIP1} promoter activation via Stat3 binding to its GAS site. Top panel: schematic diagram of the 5′ promoter region of p21^{CIP1} gene. Dashed box, Stat3 binding site (GAS site) location on the promoter; black box, mutated GAS. T47D cells were transfected with a 2400-bp-length human p21^{CIP1} promoter luciferase construct containing the GAS site indicated in the top panel. T47D cells were also transfected with a 2400-bp-length human p21^{CIP1} promoter with mutations in the GAS site as indicated in the diagram. After transfections, cells were treated or not with 10 nM of MPA for 24 h. Results are presented as the fold induction of luciferase activity with respect to control cells. The data shown represent the means of data from three independent experiments ±SEM (for b versus a: $P < 0.001$). (B) ErbB-2 acts as a Stat3 coactivator. C4HD cells were transfected with the 2400-bp-length p21^{CIP1} promoter construct and were also cotransfected with the hErbB-2WT or hErbB-2ΔNLS vectors when indicated and treated with MPA 10 nM as described above for Fig 3A. Results are presented as the fold induction of luciferase activity with respect to control cells. The data shown represent the means of data from three independent experiments ±SEM (for b versus a, c versus b, and d versus b: $P < 0.001$).

tion [14]. However, the role of Stat3 as a transcription factor capable of binding directly to its GAS (gamma interferon-activated sequence) response elements at position −679 bp in p21^{CIP1} promoter has not yet been explored in the mechanism of PR regulation of p21^{CIP1} transcription. In order to study the participation of this GAS site, T47D cells were transiently cotransfected with a 2400-bp human p21^{CIP1} promoter luciferase construct containing the GAS site and with a plasmid encoding Renilla luciferase as an internal control. As previously described [16,22] 24 h of MPA treatment resulted in an increase in luciferase activity in cells transfected with the reporter construct (Fig. 3A). However, when cells were transfected with a reporter construct containing a mutated GAS sequence (pGL3-p21^{CIP1}-2400mGAS), MPA was unable to induce p21^{CIP1} promoter activation (Fig. 3A). These results indicate that MPA regulates p21^{CIP1} transcriptional activation via Stat3 binding to its GAS site at the proximal promoter.

We then specifically evaluated whether ErbB-2 acts as a transcriptional coactivator of Stat3 in the mechanism of MPA-induced p21^{CIP1} promoter activation. We found that overexpression of h-ErbB-2-WT enhanced MPA-induced p21^{CIP1} promoter activation in C4HD cells (Fig. 3B). On the contrary, transfection of C4HD cells with hErbB-2-ΔNLS resulted in complete abrogation of the effect of MPA on p21^{CIP1} promoter (Fig. 3B). Interestingly, we have recently described that the nuclear import of Stat3 mediated by MPA occurs independently of ErbB-2 nuclear localization [13]. Consequently, these results show that despite the nuclear presence of Stat3 in hErbB-2-ΔNLS-transfected cells [13], ErbB-2 function as a coactivator of Stat3 is mandatory for MPA stimulation of p21^{CIP1} transcription.

3.3. MPA induces the recruitment of a transcriptional complex composed by Stat3, ErbB-2 and PR to the p21^{CIP1} promoter

To assess the specific association of Stat3 and ErbB-2 in the context of living cells, we performed chromatin immunoprecipitation (ChIP) assays. T47D cells were treated with MPA for 5 min and chromatin was immunoprecipitated with Stat3 and ErbB-2 specific antibodies. The immunoprecipitates were analyzed by real time quantitative PCR using primers spanning the Stat3 binding site (Fig. 4). We found that MPA treatment induced Stat3 binding to its GAS site in p21^{CIP1} promoter (Fig. 4). ErbB-2 was also recruited

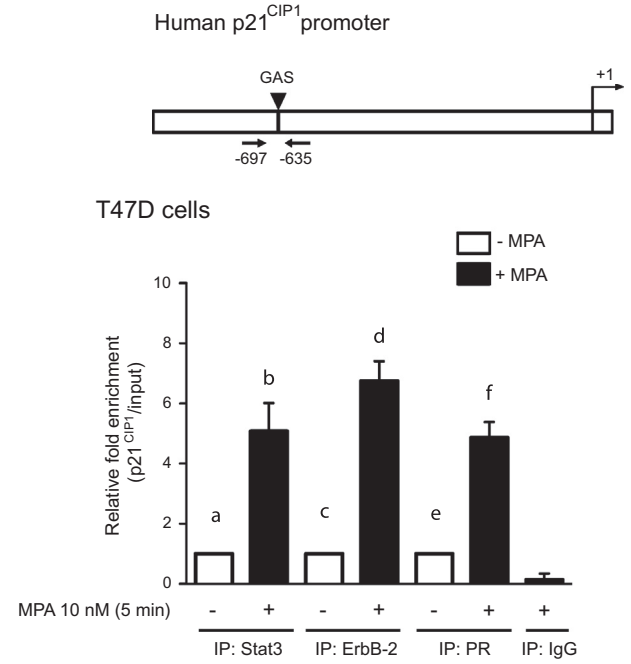

Fig. 4. MPA induces *in vivo* binding of Stat3, ErbB-2 and PR to the p21^{CIP1} promoter. Recruitment of Stat3, ErbB-2 and PR to p21^{CIP1} promoter was analyzed by ChIP assays in T47D cells treated with MPA 10 nM for 5 min. Immunoprecipitated DNA was amplified by real-time PCR (qPCR) using primers flanking the GAS sites indicated in the top panel. The arbitrary qPCR number obtained for each sample was normalized to the input, setting the value of the untreated sample as 1. Data are expressed as fold chromatin enrichment over untreated cells. (For b versus a, d versus c and f versus e: $P < 0.001$). The data shown represent the mean ± SEM from three independent experiments. Immunoprecipitation with IgG was used as a negative control.

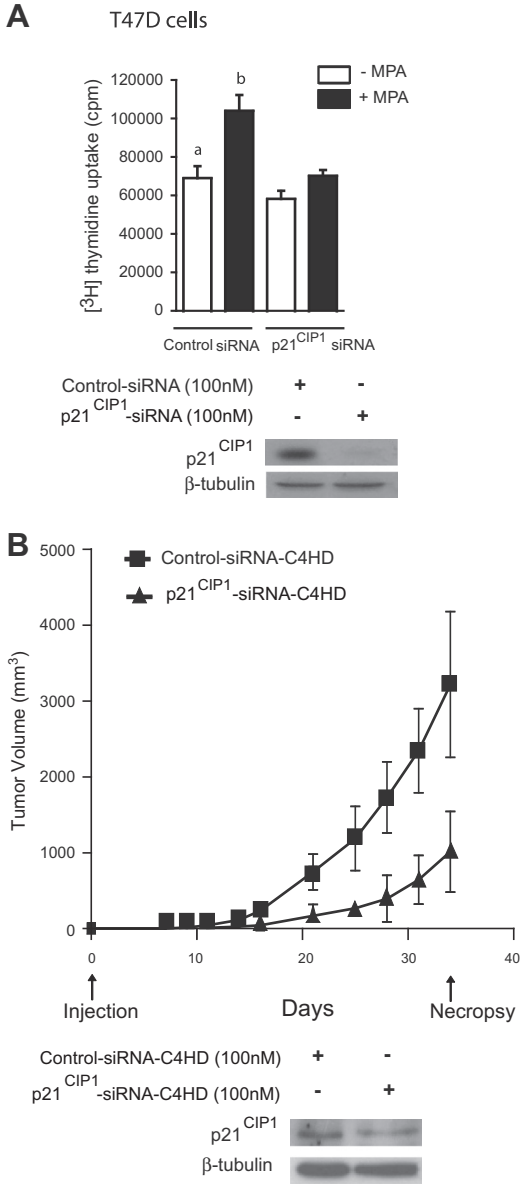

Fig. 5. $p21^{CIP1}$ is necessary for *in vitro* and *in vivo* breast cancer growth in response to MPA. (A) $p21^{CIP1}$ expression was silenced by transfection with $p21^{CIP1}$ siRNA. T47D cells were treated with MPA for 24 h and the incorporation of [^{3}H]thymidine was used as a measure of DNA synthesis. Data are presented as means ± SEM (for b versus a: $P < 0.001$). (B) C4HD cells (10^6) were transfected with $p21^{CIP1}$ siRNA or with control siRNA and inoculated subcutaneously (s.c.) into mice treated with MPA. Tumor volume was calculated as described in Materials and Methods. Each point represents the mean volume ± SEM for six independent tumors for all experimental groups. (A) and (B) Controls of inhibition of $p21^{CIP1}$ expression by siRNAs of T47D and C4HD cells treated with MPA for 24 h and 48 h, respectively are shown (lower panels). The experiments shown were repeated three times with similar results.

in the presence of MPA (Fig. 4). This finding, together with the luciferase assays results shown in Fig. 3B revealing that increased expression of ErbB-2 enhances MPA-induced $p21^{CIP1}$ transcriptional activation, demonstrate that ErbB-2 acts as a coactivator of Stat3. We also studied whether PR is also recruited to the GAS site. Interestingly, our findings revealed that MPA treatment resulted in a fivefold recruitment of PR to the GAS site (Fig. 4). Assembly of the Stat3/ErbB-2/PR multimeric complex and binding to the GAS site induced by MPA was detected at up to 30 min of MPA treatment (not shown), showing 5 min the strongest recruitment to DNA (Fig. 4). These results indicate that MPA induces the assembly of a transcriptional complex where Stat3 is bound to its responsive element, ErbB-2 functions as a coactivator, and PR is tethered to Stat3/ErbB-2.

Table 1
Tumor growth rates.[a]

Treatment	Mean tumor vol (mm^3) ± SEM	Growth rate (mm^3/day)	% Growth inhibition
Control-siRNA-C4HD cells	3215.25 ± 959.96[b]	93.47 ± 8.28[b]	
$p21^{CIP1}$-siRNA-C4HD cells	1013.83 ± 531.90[c]	26.76 ± 4.15[c]	68.46[d]

a: Growth rates were calculated as the slopes of growth curves. Volume and percentage of growth inhibition in tumors from mice injected with $p21^{CIP1}$-siRNA-C4HD cells with respect to mice injected with control-siRNA-C4HD-cells were calculated at day 34, as described in Materials and Methods.
b vs c: $P < 0.001$.
d: With respect to control-siRNA-C4HD cells. Delay in tumor latency compared with tumors from control group: 0 days. Delay in tumor growth in mice with $p21^{CIP1}$-siRNA-C4HD cells with respect to mice with control-siRNA-C4HD cells at day 34: 11 days.

3.4. $p21^{CIP1}$ is required for in vitro and in vivo MPA-induced cell growth of breast cancer cells

We explored the role of $p21^{CIP1}$ in breast cancer proliferation induced by progestin. As shown in Fig. 5A, knockdown of $p21^{CIP1}$ expression by specific siRNAs completely abrogated MPA proliferative effects in T47D cells. These results for the first time show that $p21^{CIP1}$ is required for MPA-induced breast cancer *in vitro* growth.

We then evaluated whether blockade of $p21^{CIP1}$ expression could modulate the *in vivo* proliferative response to MPA. For this purpose, we developed a pre-clinical model where C4HD cells were transiently transfected with siRNA targeting $p21^{CIP1}$ or with control siRNAs, and were then inoculated s.c. into mice treated with MPA. As shown in Fig. 5B, knockdown of $p21^{CIP1}$ expression strongly inhibited MPA-induced tumor growth. Mean volumes (Fig. 5B and Table 1) and growth rates (Table 1) of tumors that developed from $p21^{CIP1}$-siRNA-C4HD cells were significantly lower than those of control-siRNA-C4HD cells.

4. Discussion

The present study provides insight into the molecular mechanism involved in progestin regulation of $p21^{CIP1}$ expression and into the role of $p21^{CIP1}$ in progestin-induced breast cancer growth. Several reports, as well as our own findings, showed that progestin increases $p21^{CIP1}$ expression [11,14]. In this work we have for the first time shown that Stat3 and ErbB-2 are necessary for progestin-induced upregulation of $p21^{CIP1}$ levels in T47D and C4HD cells. Previously, Hawthorne et al. reported that Stat3 activation through ErbB-2 and c-Src led to transcriptional upregulation of $p21^{CIP1}$ [3]. Notably, our data demonstrated that MPA-induced phosphorylation of c-Src, Stat3 and ErbB-2 is mandatory for the regulation of $p21^{CIP1}$ levels. In addition, our results highlight the importance of ErbB-2 nuclear localization and function on progestin-induced $p21^{CIP1}$ expression in breast cancer cells. Our present findings demonstrate that progestins upregulate $p21^{CIP1}$ expression via the assembly of a transcriptional complex among ErbB-2, Stat3 and PR where Stat3 binds to its GAS site in $p21^{CIP1}$ proximal promoter while both ErbB-2 and PR function as coactivators. Importantly, we found that $p21^{CIP1}$ expression is required for MPA-induced breast cancer growth.

The human proximal promoter of $p21^{CIP1}$ gene contains several key regulatory regions and transcription factor binding sites. However, interactions between PR and other transcription factors at

said promoter have been poorly explored. A seminal study showed that progestin modulates $p21^{CIP1}$ expression via PR tethering to Sp1 [11]. We have previously found that progestin induces Stat3 recruitment as a coactivator of PR in the Sp1/PR complex at the Sp1 site on $p21^{CIP1}$ promoter [14]. Interestingly, epidermal growth factor or interferon gamma stimulation in A431 cells was reported to be mediated by Stat3 binding to its GAS sites on $p21^{CIP1}$ promoter [23]. However, the relevance of the GAS site on $p21^{CIP1}$ expression mediated by progestin remains unexplored. Here, we determined that the GAS site located at position −679 bp in $p21^{CIP1}$ promoter is necessary for progestin-induced transcriptional activation of $p21^{CIP1}$ gene in T47D cells. Through ChIP assays we found that Stat3 was rapidly recruited to the promoter of $p21^{CIP1}$ gene along with ErbB-2 and PR after progestin stimulation. A previous report using HepG2 cells has identified CBP/p300 and NcoA/SRC1a as transcriptional coactivators of Stat3 on $p21^{CIP1}$ promoter after IL-6 stimulation [24]. Interestingly, we here provided evidence that ErbB-2 acts not only as a receptor tyrosine kinase which activates signaling cascades but acts also as a transcriptional coactivator of Stat3 on $p21^{CIP1}$ promoter. Remarkably, our previous findings [13] showed that a Stat3/PR/ErbB-2 transcriptional complex is recruited at cyclin D1 promoter, suggesting that other key genes which are involved in progesterone-induced proliferation as well as $p21^{CIP1}$, might also be under the regulation of said complex. It is worth noting that in our studies we have specifically analyzed the proximal promoter of $p21^{CIP1}$ gene. Recent findings on steroid hormone receptor modulation of target genes raise the most exciting possibility that progestin may also induce long-range transcriptional control of $p21^{CIP1}$ gene expression via PR binding to cis-regulatory elements (PREs or half PREs) located far upstream or downstream from its transcriptional start site [25].

In spite of its already known role as an inhibitor of cell proliferation and an inducer of senescence [26], $p21^{CIP1}$ was demonstrated to be a critical mediator of anti-apoptotic effects [27,28] and promoter of cell proliferation and oncogenesis [5,6]. According to the latter findings, in the present work we found that blockage of $p21^{CIP1}$ expression in T47D and C4HD cells strongly inhibits *in vitro* and *in vivo* progestin-induced cell proliferation. In line with our own findings, Yang et al. have demonstrated that heregulin-induced proliferation was markedly reduced in T47D cells upon $p21^{CIP1}$ siRNA transfection, suggesting that upregulation of $p21^{CIP1}$ was required for the mitogenic activity of heregulin in breast cancer cells [29].

$p21^{CIP1}$ was reported to be essential for promoting the assembly of the cyclin D1–CDK4 complex in mammary epithelial cells [30], murine fibroblasts [31] and vascular smooth muscle cells, which promotes Rb phosphorylation and favors progression through the G1/S phase together with the mitogenic response [32]. This proposed model is consistent with $p21^{CIP1}$ cytoplasmic localization and function, whereas nuclear $p21^{CIP1}$ was reported be involved in the pro-differentiating and senescence-promoting effects of $p21^{CIP1}$ [26–28]. Cheng et al. demonstrated that overexpression of Akt-activated $p21^{CIP1}$ accelerated tumor onset and promoted lung metastasis in MMTV/neu mice, providing evidence that cytoplasmic phosphorylated $p21^{CIP1}$ has an oncogenic role in promoting mammary tumorigenesis and metastasis [6]. In addition, high levels of cytoplasmic $p21^{CIP1}$ expression levels determined cisplatin resistance in testicular cancer [33]. On the other hand, Winters et al. discuss that the role of ErbB-2 as a predictor of poor prognosis may partly be related to its ability to influence the relocalization of $p21^{CIP1}$ from the nucleus to the cytoplasm, resulting in a loss of $p21^{CIP1}$ tumour suppressor functions. Consequently, they conclude that cytoplasmic $p21^{CIP1}$ may be a surrogate marker of functional HER-2 *in vivo* [34]. In this sense, we are currently trying to determine whether MPA not only increases $p21^{CIP1}$ expression but also induces $p21^{CIP1}$ cytoplasmic localization in T47D and C4HD breast cancer cells, therefore promoting cell proliferation and progression through the cell cycle.

The clinical relevance of p21 was demonstrated by the association of $p21^{CIP1}$ overexpression to larger tumor size and worse prognosis [35], and by its involvement in chemoteraphy resistance [3,4]. Consistently, Yang et al. found that ErbB-2 overexpression was positively correlated with $p21^{CIP1}$ expression in breast tumors and that there was a highly significant correlation of $p21^{CIP1}$ positivity with worse disease free survival [36]. In addition, $p21^{CIP1}$ was recently identified as a key regulator of transforming growth factor β-mediated breast cancer cell migration and invasion in triple negative breast cancer cell lines [37]. These results add further support to the hypothesis that according to cell context and nature of stimulus, $p21^{CIP1}$ is capable of positively regulating cell cycle progression.

In our present work we demonstrated the assembly of a nuclear complex where PR is tethered to Stat3 bound to a GAS site at the $p21^{CIP1}$ proximal promoter. Interestingly, we found that ErbB-2 is also recruited to the Stat3/PR complex and that it functions as a coactivator. The assembly of this complex allows the expression of $p21^{CIP1}$ by progestin and leads to progestin-induced breast cancer growth. This mechanism reveals a new feature of the integration between genomic and nongenomic PR actions, having found that both Stat3 and ErbB-2 are rapidly phosphorylated by PR-induced signaling cascades.

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