Progesterone inhibits human breast cancer cell growth through transcriptional upregulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ gene

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Abstract The effects of progesterone derivatives on breast cancer development are still controversial, probably accounting for their biphasic, opposed effects on mammary cell-cycle regulation. Here, we demonstrate in vitro that the growth-inhibitory effects of progesterone on breast cancer T-47D cells require the transcriptional upregulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (p27) gene. A statistical analysis of human tumor biopsies further indicates that p27 mRNA levels correlate to progesterone receptor (PR) levels. Moreover, p27 gene expression is inversely associated with tumor aggressiveness, and is a prognostic factor of favorable disease outcome. Thus, progesterone derivatives selectively activating the p27 gene promoter could be promising drugs against breast cancer progression.

Keywords: Breast cancer; Gene expression; Progesterone receptor; $p27^{Kip1}$; Pilot clinical study; Tumor suppressor

1. Introduction

The steroid hormone progesterone is required for the terminal growth and differentiation of the mammary gland [1]. In clinical practice, the expression of progesterone receptor (PR) with the estrogen receptor (ER) is currently assessed as a predictive marker for favorable disease prognosis and for response to hormonal therapy [2]. Moreover, prolonged synthetic progestin administration is recommended in the treatment of certain breast tumors [3]. For instance, second-line high-dose therapy with certain synthetic progestins effectively suppresses the growth of metastatic breast cancers that are ER- and PR-positive but display acquired resistance to the anti-estrogen tamoxifen [3]. Nevertheless, the effects of progesterone and its derivatives – notably when administrated in hormone replacement therapies (HRTs) or in oral contraceptives – on breast cancer risk are still controversial in epidemiological studies [4,5].

Control of cell-cycle progression appears to occur primarily through the regulation of the retinoblastoma gene product (pRB) phosphorylation – which represents the critical checkpoint of the $G_1 \rightarrow S$ transition – by specific "cyclin-dependent kinases" (CDKs) [6]. CDKs are constitutively expressed, and interactions with the transiently expressed cyclins and "CDK inhibitors" (CDKIs) activate and inhibit their activity, respectively. Thus, regulation of the expression of these cyclins and CDKIs acting in G_1 is a key event in the control of cell proliferation by a variety of growth signalling factors including steroid hormones [6]. Notably, progestin-induced growth arrest of T-47D breast cancer cells is related to an increased protein abundance of the CDKIs $p21^{Cip1/Waf1}$ (p21), $p27^{Kip1}$ (p27) as well as $p18^{INK4C}$ and changes in the associations formed with the different G_1 CDK complexes leading to the downregulation of their activity [7–9].

Induction of p27 protein expression has been demonstrated both to mediate G_1 cell-cycle arrest through inhibition of cyclin E/CDK2 complexes and to constitute a molecular switch that facilitates differentiation [9–12]. Furthermore, recent in vitro reports have associated the ability of progestins to inhibit mammary cancer cell proliferation and to induce the cell differentiation program [8,13,14]. However, whereas it has previously been demonstrated that progesterone-activated PR regulates transcription of the p21 gene by interacting with Sp1 and p300 at proximal Sp1-binding sites [15], the mechanisms involved in the progesterone-dependent p27 upregulation has not yet been investigated. In the present study, we first verified using a siRNA approach that p27 plays a key role in mediating growth-inhibitory action of progesterone on mammary breast carcinoma T-47D cells and demonstrated that progesterone up-regulates p27 expression at transcriptional level. In addition, we demonstrated that p27 mRNA levels are positively associated with PR protein expression levels and appeared as a prognostic indicator of favourable patient outcome. Taken together, our results indicate that the control of p27 gene expression may play a key role in the regulation of breast cancer cell growth, notably by the progesterone.

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Abbreviations: ER, estrogen receptor; PR, progesterone receptor; p21, $p21^{Cip1/WAF1}$; p27, $p27^{Kip1}$; HPG, histoprognostic grading; HRT, hormone replacement therapy; OS, overall survival; PRE, progesterone responsive element

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2. Materials and methods

2.1. Cell culture assays and molecular analyses

Human T-47D breast cancer cells were obtained from American Type Culture Collection (ATCC) and cultured as recommended in a medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), and antibiotic gentamycin (1%). Progesterone was added daily to the medium at a concentration of 30 nM (i.e., ~10-fold EC50 value for PR [16]) as previously performed [7].

Small interfering RNA (siRNA) assays were performed as described previously [17] with p27 (Ambion, siRNAs nos. 14072 and 14165) or control (Qiagen) siRNAs (10 nM). Experiments shown in the manuscript have been performed with the p27 siRNA no. 14072. p27 expression knockdown using the siRNA no. 14165 led to a ~42% decrease in p27 mRNA levels and comparable effects on cell proliferation as well as pRB phosphorylation as obtained with siRNA-no. 14072.

Reverse transcription and quantitative PCR were performed as described in [17] from total RNA from T-47D cells or breast cancer tissues (see below). mRNA levels were normalized to 28S RNA levels because of its stability during cell-cycle progression.

Cellular DNA content was analyzed following nuclear DNA staining using the fluorochrome 3,5-diaminobenzoicacid free acid (DABA) as described previously [17].

Transient transfection assays. T-47D cells were seeded for 24 h in 24-well plates at a density of 1×10^5 cells per well. Cells were transfected with FuGENE 6 (Roche Molecular Biochemicals) at a ratio of 3:1 FuGENE:DNA. The p27Luc reporter plasmids (100 ng) [18], comprising the indicated 5′-flanking DNA regions of the p27 gene subcloned in the luciferase reporter vector pGL2, were cotransfected with the indicated amounts of expression vectors and the internal control *Renilla* luciferase reporter plasmid pRL-Null (10 ng). The quantity of plasmids in the transfection mixture was normalized with empty pcDNA3 vector. 12 h after transfection, medium was changed and cells were incubated for an additional 34 h with progesterone or vehicle. Cells were then harvested and cell lysates (20 μl) were assayed for luciferase activity.

Western blot analyses were achieved from 30 μg of whole cell protein extracts. The anti-ppRB-Ser807/811 (9308, Cell Signalling) – raised against a pRB peptide phosphorylated on the Ser807/811 residue – anti-p27 (K25020, Transduction Laboratories) and anti-β-actin (sc-7210, Santa Cruz) – for normalization of protein levels – antibodies were used. Relative abundances were quantified with SCANWISE and Perfect-IMAGE V-5.3 (CLARA VISION, France) softwares. The blots represent typical results of three independent experiments.

Chromatin immunoprecipitation (ChIP) assays were performed as described in [17]. T-47D cells were incubated with either progesterone or with ethanol (vehicle) in 0.2% BSA-RPMI during 2 h 30 min before lysis.

Statistical analyses were performed using the non-parametric Kruskal–Wallis test. Values are expressed as means ± S.D. of a representative experiment performed in triplicate ($n = 3$) which was repeated at least three times.

The pilot statistical study on human breast cancer biopsies was performed as described in [17]. Among the tumor samples, 72% and 71% were ER and PR positive, respectively, and the classical correlation between ER and PR expression ($P < 0.001$, $r = 0.621$) was observed. Overall survival (OS) and relapse free survival were studied by Kaplan Meier method analysis. Comparison between curves was carried out by the log rank test. The RNA from the normal human mammary gland was obtained from BD Biosciences, France.

3. Results

3.1. Progesterone inhibits breast cancer $G_1 \rightarrow S$ cell-cycle transition by inducing p27 gene expression

To firstly decipher the contribution of p27 in mediating the effect of progesterone on mammary cell growth, a siRNA approach which targets p27 was used (Fig. 1). In agreement with previous studies [7,8], a decrease of cell proliferation associated with a decrease of pRB phosphorylation were provoked by progesterone in control-transfected cells. Strikingly, these effects were nearly entirely eliminated in cells transfected with

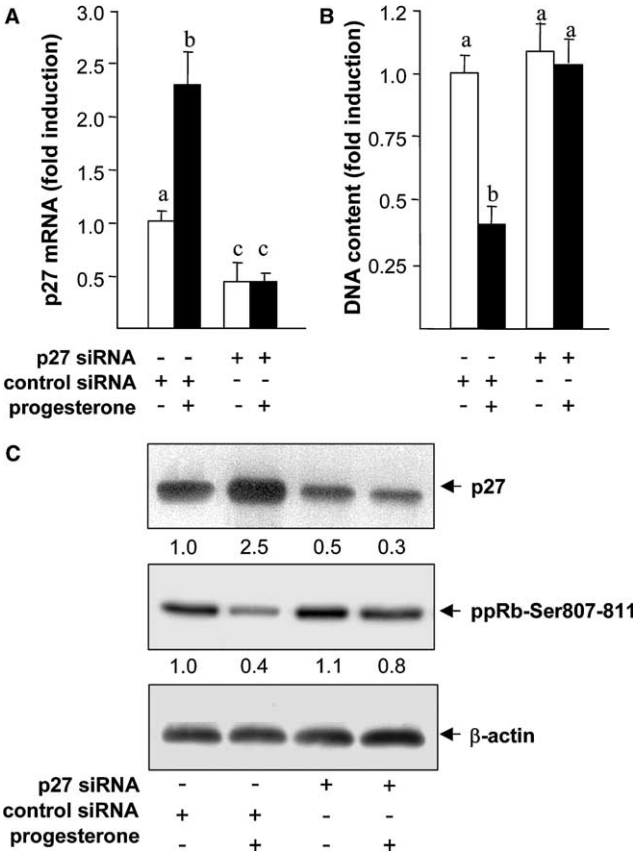

Fig. 1. Inhibition of T-47D cell proliferation by progesterone requires p27 gene expression. p27 mRNA levels (A), DNA content (B) as well as protein abundance of p27 and pRB phosphorylated at Ser807/811 (C) were determined 4 days after siRNA transfection. All values are expressed relative to vehicle-incubated control siRNA-transfected cells arbitrarily set as 1. Cellular DNA content and mRNA levels are means ± S.D. ($n = 3$). Different letters indicate statistically significant differences between values. p27 and ppRB-Ser807/811 levels normalized to β-actin levels are indicated below the blots.

p27 siRNA, demonstrating that p27 expression is required for the negative control of progesterone on mammary cell growth.

To analyze the molecular mechanisms involved in the effects of progesterone, p27 gene expression was measured in T-47D cells (Fig. 2A). Treatment with progesterone increased p27 mRNA in a time-dependent manner as early as 6 h after initiation of the treatment (Fig. 2A). In correlation, p27 protein levels increased following progesterone treatment and reached their maximum as early as after 12 h, a time-point preceding the cell-cycle arrest observed after 36 h. By contrast, progesterone treatment did not affect either p27 mRNA levels or the cell proliferation rate of MDA-MB-231 cells, which do not express PR [19] (Fig. 2B). Thus, progesterone may induce p27 protein expression, at least in part, through PR-dependent upregulation of p27 gene transcription.

3.2. Progesterone-activated PR enhances p27 promoter activity by binding to a multiprotein complex formed with Sp1 and p300 on the −549/−511 proximal p27 promoter

To further decipher whether p27 is a target gene of progesterone-activated PR, luciferase reporter constructs controlled by either the full-length −3568 bp promoter or 5′-deleted

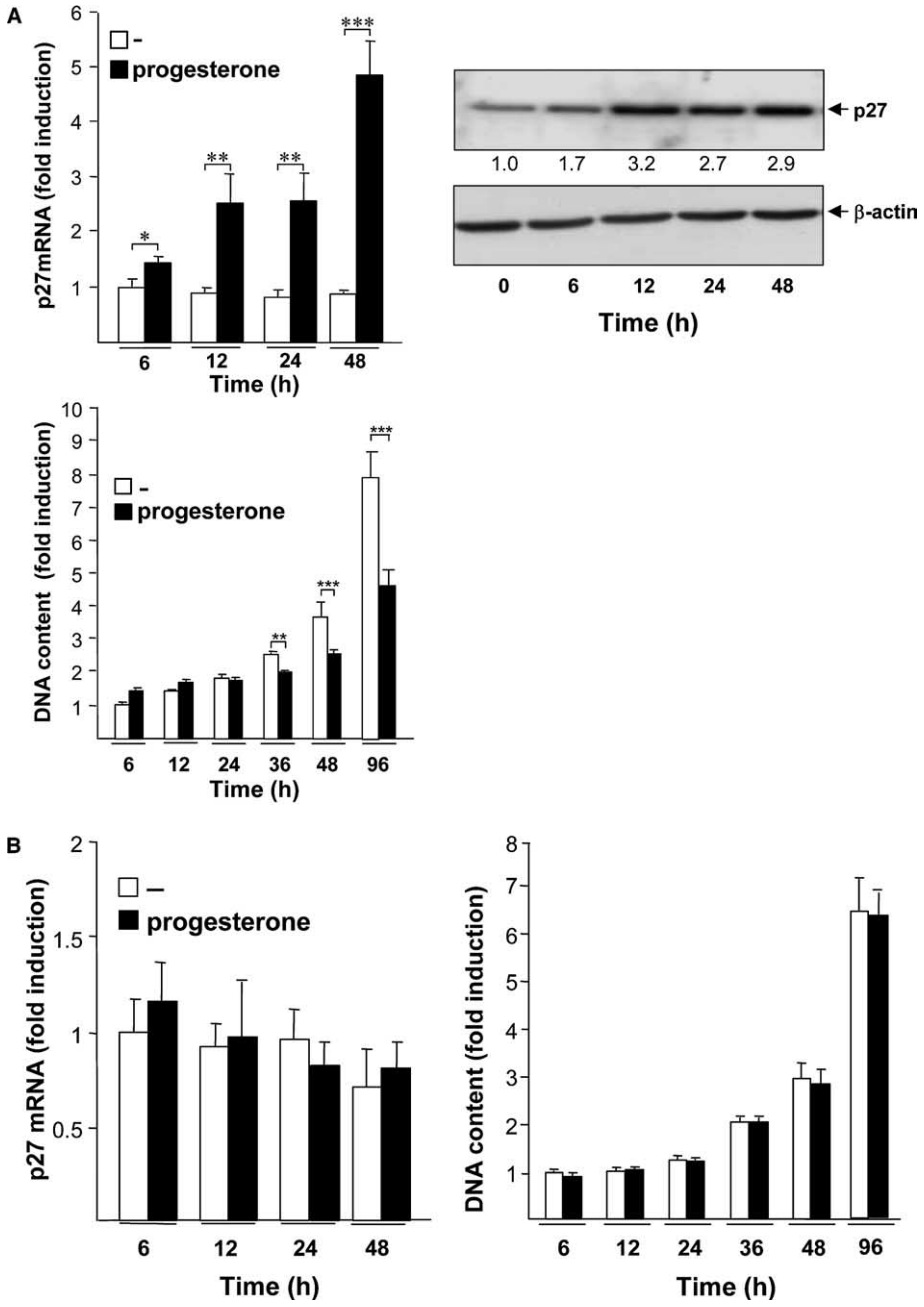

Fig. 2. Progesterone induces p27 gene expression and inhibits cell proliferation in T-47D (A), but not in MDA-MB-231 (B) cells. Cells treated for the indicated periods of time with progesterone (30 nM) or vehicle were harvested for quantitative RT-PCR, DNA content and Western-blot assays. p27/28S RNA levels and cellular DNA content are expressed relative to time 6 h, arbitrarily set as 1. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$ vs. vehicle-incubated cells. Numbers under the Western-blot of p27 indicate the amounts of p27/β-actin protein expressed relative to the levels in cells at time 0 h, arbitrarily set as 1. In B, no significant difference was found between progesterone-treated and untreated cells.

and/or mutated p27 promoter regions were next transfected into T-47D cells treated or not with progesterone (Fig. 3A). Progesterone treatment significantly induced (~3-fold) −3568 p27 promoter activity, demonstrating that progesterone regulates p27 gene expression at the transcriptional level. Moreover, progesterone-dependent p27 promoter activation was maintained upon 5′-deletion to -549bp, but not upon further deletion to −511 bp. The −549/−511 DNA region contains two Sp1-binding sites (denoted Sp1-1 and Sp1-2, respectively) as well as a CCAAT box, which have been previously identified as crucial elements for the regulation of this gene [18,20,21]. Interestingly, the induction of p27 promoter activity following progesterone treatment was either completely eliminated or reduced by mutation of Sp1-1 and Sp1-2 sites, respectively, but it was not affected by mutation of the CCAAT box.

Since Owen et al. have previously demonstrated that progesterone-activated PR interacts with Sp1 and p300 at proximal Sp1-binding sites to regulate transcription of the p21 gene [15], we further analyzed the putative PR-dependent p27 promoter activation by p300 (Fig. 3B). In fact, transfection of p300 enhanced progesterone-induced p27 promoter activity through these proximal Sp1-binding sites (Fig. 3B).

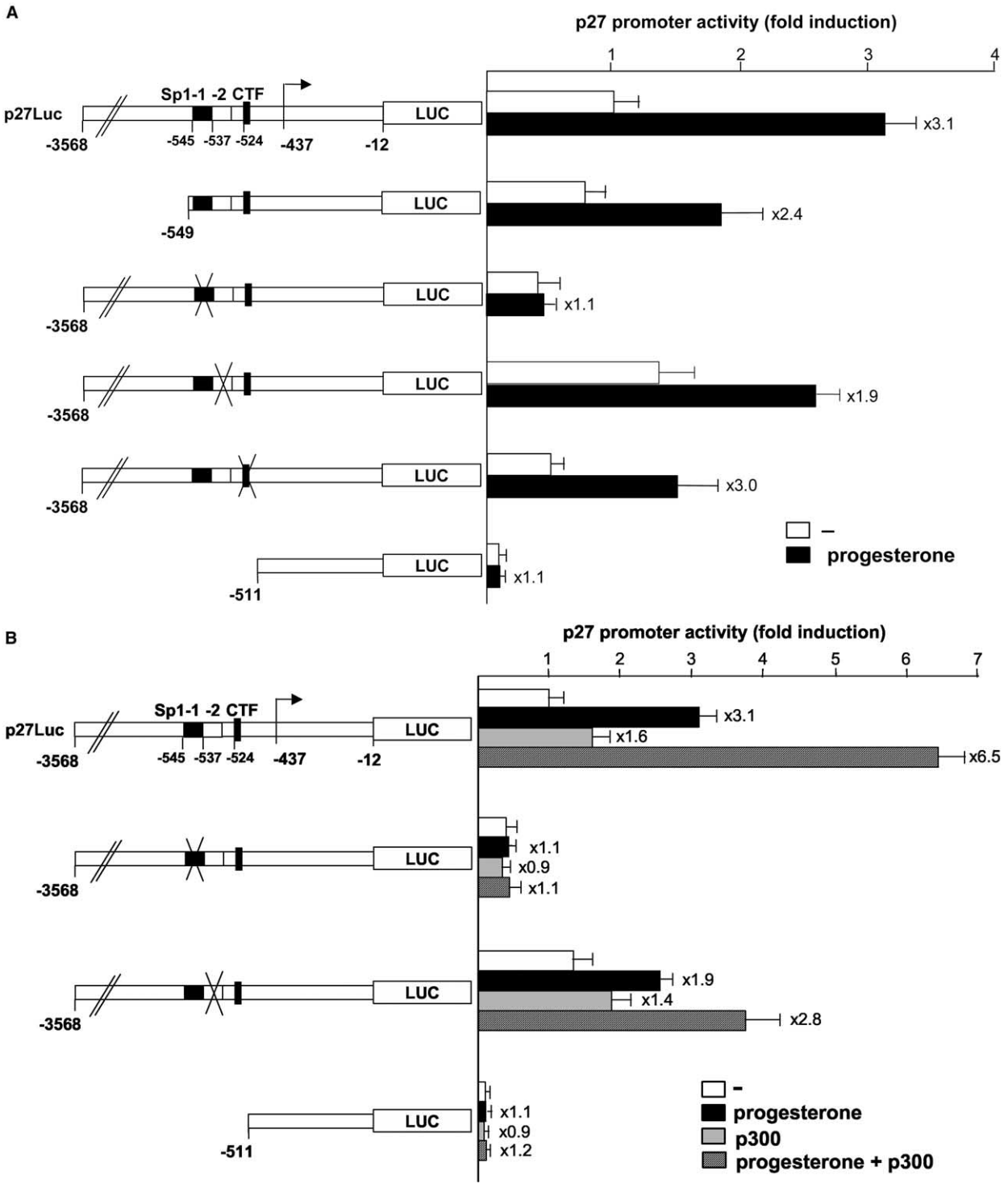

Fig. 3. Progesterone-activated PR enhances p27 promoter activity through interaction with p300 at proximal Sp1-binding sites in T-47D cells. T-47D cells were transiently co-transfected with the luciferase reporter constructs driven by the indicated p27 promoter deletion or mutation fragments without (A) or with (B) the p300 expression vector. Results are represented as fold increase (means ± S.D.), with the luciferase activity levels of the −3558p27Luc reporter construct arbitrarily set as 1. The arrow indicates the transcription start site.

To further investigate the *in cell* occupancy of these Sp1-binding elements by progesterone-activated PR, ChIP experiments were performed (Fig. 4). In good agreement with previous findings [18], the genomic DNA region encompassing the Sp1-1 and -2 binding sites was immunoprecipitated by the anti-Sp1 antibody. Moreover, in accordance with the results of the promoter reporter assays, the proximal Sp1-binding DNA element was immunoprecipitated by the anti-PR and anti-p300 antibodies.

Overall, these results indicate that progesterone-activated PR may cooperate with Sp1 and p300 to increase p27 gene expression through the proximal Sp1-binding sites, with the Sp1-1 site mediating the major response (Fig. 5).

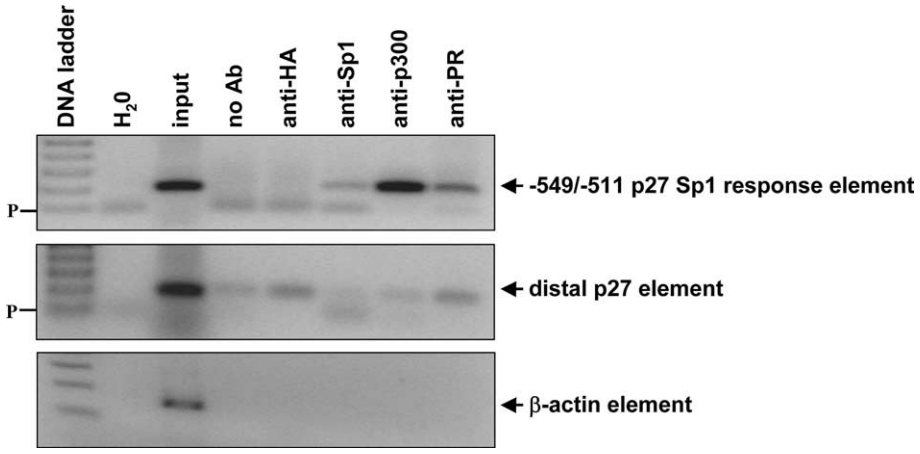

Fig. 4. PR occupies *in cell* the p27 gene promoter at the proximal element that contains the Sp1-binding sites. Chromatin was immunoprecipitated from T-47D cells incubated with progesterone. Immunoprecipitations were performed using antibodies directed against Sp1, p300 or PR. Controls include PCRs done without DNA (H_2O) or with non-precipitated genomic DNA (*input*), and immunoprecipitations performed without antibody (*no Ab*) or with an irrelevant antibody (*anti-HA*). P: low background signal attributable to ethidium bromide staining of the PCR primers.

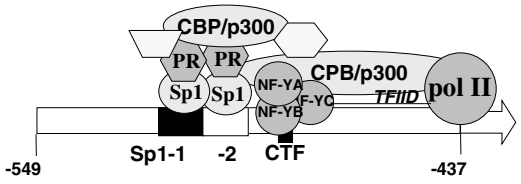

Fig. 5. Model of progesterone regulation of the p27 promoter. Data are consistent with a model in which progesterone-bound PR is indirectly tethered to the promoter through Sp1 protein(s) bound to both proximal Sp1 sites in a multiprotein complex that also includes CBP/p300. The Sp1 site 1 is necessary for basal p27 transcription, and both Sp1 sites are required for transcriptional upregulation by progesterone. The binding of Sp1 to the Sp1-1 site has previously been demonstrated by Inoue et al. [18], whereas the interaction of progesterone-bound PR with Sp1 and CBP/p300 has been shown by Owen et al. [15] by co-immunoprecipitation. In addition to PR, p300 can bind Sp1 as well as components of the basal transcription machinery through distinct regions [15,32].

3.3. p27 Gene expression in human breast tumors correlates with PR levels and is a prognostic indicator of lower tumor aggressiveness

To start assessing the putative patho-physiological relevance of these in vitro data, we analyzed possible correlations between p27 mRNA levels and classical clinical, histological and biological prognostic factors in 91 primary human breast cancer biopsies. Interestingly, the expression level of p27 did not follow a Gaussian curve, since the median value of p27 mRNA levels (22%) was lower than the mean value (30%), suggesting that tumor incidence is inversely related to p27 gene expression levels (Fig. 6A). Moreover, consistent with the ability of PR to *trans*-activate p27 in vitro, p27 transcript levels correlated positively with PR levels ($P = 0.01$, $r = 0.273$, Table 1). p27 Gene expression also correlated positively to the expression status of ER which is also a predictive marker of good recovery [2], whereas it was negatively associated with

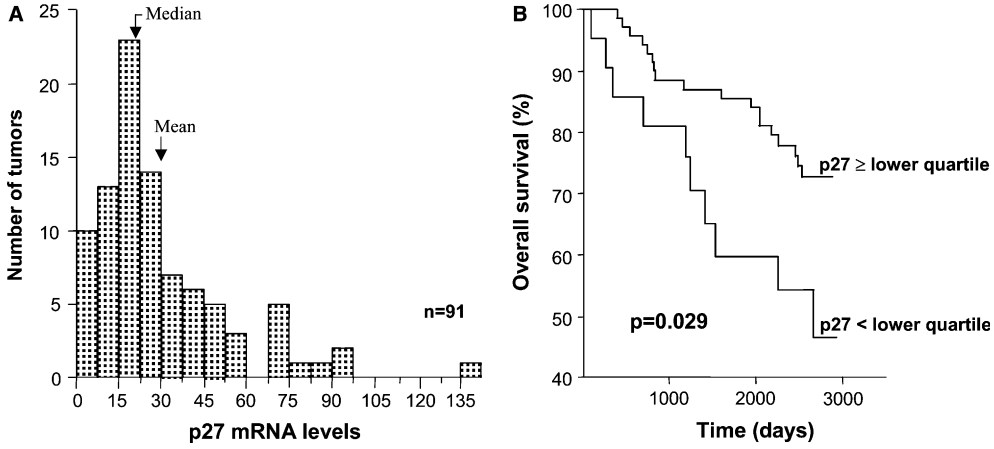

Fig. 6. Loss of p27 predicts poor outcome in breast cancer patients. (A) Analysis of CDKI p27 mRNA expression in human breast cancer biopsies. p27 mRNA levels were measured by quantitative RT-PCR in 91 breast tumor biopsies and were arbitrarily expressed as percentage relative to the levels obtained in T-47D cells. The number of tumor samples with a given level of p27 mRNA is shown in the *y*-axis. p27 mRNA levels in the normal mammary gland were 287% of its levels in T-47D cells (100%). (B) OS according to the presence of p27 mRNA. The Kaplan–Meier plots of OS relative to the p27 normalized mRNA expression are shown, with the lower quartile as clinical positive threshold. A longer OS (overall survival) is found in p27 mRNA-positive patients.

Table 1
Correlation (Spearman test) between normalized expression of p27 or p21 and clinical, histological and biological parameters

	p27		p21	
Parameters	*P*	*r*	*P*	*r*
HPG	0.041	−0.226	NS	
Node involvement	NS		NS	
Tumor size	NS		NS	
ER	<0.001	0.36	NS	
PR	0.01	0.273	NS	

histoprognostic grading (HPG) – a factor based on histological criteria such as cell differentiation, mitotic activity and the degree of nuclear polymorphism, allowing classification of tumors from best (HPG 1) to worst prognosis (HPG 3) [22] (Table 1). These data indicate that p27 gene expression may negatively influence tumor formation and development in humans. Correlating with this, survival curves achieved by the Kaplan–Meier method revealed that, as with PR [2], high p27 mRNA levels (i.e., above the lower quartile) are associated with a longer OS (overall survival) ($P = 0.029$) (Fig. 6B).

Since the growth-inhibitory effects of progesterone on T-47D cells is also related to the upregulation of protein expression of the CDKI p21 [7,8] through a transcriptional mechanism [15], we further analyzed whether p21 mRNA levels are also related to a good recovery. However, p21 gene expression was not correlated with any of the classic prognostic parameters analyzed, including PR (Table 1), and did not have a prognostic value in primary breast cancer (data not shown).

4. Discussion

In the present study, we firstly demonstrated by studying the mammary carcinoma PR-positive T-47D cell line – which represents a relevant model to evaluate in vitro the progesterone signalling in breast cancer cells [7,8,19] – that progesterone upregulates p27 expression at the transcriptional level and that this induction of p27 is required for the growth-inhibitory action of progesterone. Interestingly, silencing of p27 expression in T-47D cells did not significantly affect the basal cell growth measured 4-days after the initiation of the siRNA treatment (Fig. 1). This is possibly due to the induction of compensatory mechanisms and/or the fact that in these untreated cancer cells, basal levels of Cip/Kip proteins do not effectively inhibit cell-cycle progression [23]. By contrast, the response to progesterone requires the induction of p27 levels which may reach the threshold necessary for the inhibition of cyclin E/CDK2 complexes. Further, in vitro analyses of the molecular mechanisms involved indicated that, similarly as for p21 [15], progesterone-activated PR induces the p27 gene promoter activity by interacting with CBP/p300 and the transcription factor Sp1 at proximal Sp1-binding sites. Finally, we demonstrated in a pilot study using human breast cancer biopsies that p27, but not p21, gene expression levels are significantly associated with PR levels. This concurred with the requirement of p27 in the response to progesterone observed in vitro. Interestingly, proximal Sp1-binding sites have been previously shown to mediate the p27-induction by other factors and steroid hormones [18,20,21]. Thus, interactions formed between Sp1 and nuclear receptors at this proximal region of the p27 promoter could play a critical role in the convergence and integration of multiple signals to regulate p27 promoter activity (e.g., by modulating Sp1 tethering to the p27 gene promoter) and thus determine breast cancer cell fate.

p27 protein levels assessed by immunohistochemical or immunocytological staining in primary breast cancer tissues are associated with a favorable outcome, and their analysis as a diagnostic complement and prognostic marker has been thoroughly evaluated [24–26]. In clinical practice, considering that hormone receptor-positive tumors with low p27 levels respond poorly to anti-estrogen therapy, the scoring of p27 has been suggested to better determine therapeutic strategies [27]. To date, post-translational modifications of p27 have been proposed as major mechanisms underlying the regulation of p27 levels in human tumors [27]. In fact, mitogen-induced downregulation of p27 through ubiquitin-mediated degradation and proteolytic processing during the G_1 phase has been shown to regulate p27 protein levels and consequently result in the activation of cyclin/CDK complexes [28,29]. Furthermore, alterations in the proteasome-mediated degradation pathways which govern p27 protein levels and activity have been involved in the loss of p27 protein expression in certain breast tumors [25,30]. Interestingly, our results from the pilot study further demonstrate that p27 (and not p21) mRNA levels also have an intrinsic prognostic value in breast cancer. If confirmed by larger prospective studies, this observation suggests that, although the p27 gene is rarely mutated [27], (dys)regulation of p27 promoter activity is associated with breast cancer development. It would now be relevant to analyze whether methylation of CpG island(s) in the p27 promoter is related to the loss of p27 gene expression and the genesis of breast cancer, as previously shown in malignant melanomas [31]. On the other hand, considering the positive correlation between PR protein and p27 gene expression in human breast tumors, it appears that an alteration of activity of certain transcriptional factors – such as PR – regulating the p27 promoter, could also play a role in mammary tumorigenesis.

In conclusion, the present data identify a role for PR in the transcriptional control of the p27 gene which is corroborated by the correlation between PR protein and p27 mRNA levels in human mammary tumors. PR does not directly bind to a canonical PRE (progesterone responsive element), but interacts instead with the transcription factor Sp1 and general co-regulators (e.g., CBP/p300) resulting in the formation of a multiprotein complex at proximal Sp1-binding sites of the p27 promoter (Fig. 5). Moreover, despite the transcriptional regulation of both CDKIs p27 and p21 by progesterone, p21 mRNA levels did not correlate with PR expression in breast tumors. Finally, p27, but not p21, gene expression levels were significantly associated with a lower aggressiveness of breast tumors and with a good prognosis. Therefore, molecules selectively inducing p27 gene expression (e.g., selective PR modulators characterized by their ability to induce PR/p300 recruitment to the p27 promoter) could be a promising way of identifying new drugs for HRTs, oral contraceptives and/or treatment of breast cancer.

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