CDC2 Mediates Progestin Initiated Endometrial Stromal Cell Proliferation: A PR Signaling to Gene Expression Independently of Its Binding to Chromatin

Griselda Vallejo1, Alejandro D. La Greca1,3, Inti C. Tarifa-Reischle1,3, Ana C. Mestre-Citrinovitz1, Cecilia Ballaré2, Miguel Beato2,3, Patricia Saragüeta1*

1 Instituto de Biología y Medicina Experimental, IByME-Conicet, Buenos Aires, Argentina, 2 Centre de Regulació Genòmica, (CRG), Barcelona, Spain, 3 University Pompeu Fabra (UPF), Barcelona, Spain

Abstract

Although non-genomic steroid receptor pathways have been studied over the past decade, little is known about the direct gene expression changes that take place as a consequence of their activation. Progestosterone controls proliferation of rat endometrial stromal cells during the peri-implantation phase of pregnancy. We showed that picomolar concentration of progestin R5020 mimics this control in UIII endometrial stromal cells via ERK1-2 and AKT activation mediated by interaction of Progesterone Receptor (PR) with Estrogen Receptor beta (ERb) and without transcriptional activity of endogenous PR and ER. Here we identify early downstream targets of cytoplasmic PR signaling and their possible role in endometrial stromal cell proliferation. Microarray analysis of global gene expression changes in UIII cells treated for 45 min with progestin identified 97 up- and 341 down-regulated genes. The most over-represented molecular functions were transcription factors and regulatory factors associated with cell proliferation and cell cycle, a large fraction of which were repressors down-regulated by hormone. Further analysis verified that progestins regulate CcnD1, JunD, Usf1, Gfi1, Cyr61, and Cdkn1b through PR-mediated activation of ligand-free ER, ERK1-2 or AKT, in the absence of genomic PR binding. ChIP experiments show that progestin promoted the interaction of USF1 with the proximal promoter of the Cdc2 gene. Usf1 knockdown abolished Cdc2 progestin-dependent transcriptional regulation and cell proliferation, which also blocked Cdc2 knockdown. We conclude that progestin-induced proliferation of endometrial stromal cells is mediated by ERK1-2 and AKT dependent early regulation of USF1, which directly induces Cdc2. To our knowledge, this is the first description of early target genes of progestin-activated classical PR via crosstalk with protein kinases and independently of hormone receptor binding to the genomic targets.

Citation: Vallejo G, La Greca AD, Tarifa-Reischle IC, Mestre-Citrinovitz AC, Ballaré C, et al. (2014) CDC2 Mediates Progestin Initiated Endometrial Stromal Cell Proliferation: A PR Signaling to Gene Expression Independently of Its Binding to Chromatin. PLoS ONE 9(5): e97311. doi:10.1371/journal.pone.0097311

Editor: John P. Lyndon, Baylor college of Medicine, United States of America

Received November 11, 2013; Accepted April 17, 2014; Published May 23, 2014

Copyright: © 2014 Vallejo et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants to P.S. from Agencia Nacional de Promoción Científica y Tecnológica (PICT 5-34086 and PID 88) Argentina and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 2005-2006); and to M.B. from the Ministerio de Educación y Ciencia (MEC) BMC 2003-02902, Consolider (CD2006-00049). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sarag@dna.uba.ar

These authors contributed equally to this work.

Introduction

Ovarian steroids are considered to act mainly through direct regulation of transcription via interaction of their receptors with target genes [1], but rapid effects of steroids independent of transcriptional responses have been reported in different tissues and cellular types [2–4]. Steroid hormones are able to rapidly and transiently activate the SRC/RAS/ERK kinases cascade through a direct interaction between cytoplasmic steroid receptors with SRC [3,5–6]. This activation is essential for some physiologic responses to hormones, such as cell proliferation or inhibition of apoptosis [7]. Moreover, estrogen receptor alpha (ERa) also interacts with the regulatory subunit of the phosphoinositol-3-kinase, leading to the ER activation as well as the activation of AKT [8]. Activation of cytoplasmic cascades could be involved in the transcriptional regulation of some ovarian hormone target genes. RSK2 kinase, which interacts with the hormone binding domain of ERa and phosphorylates it at Ser 167 [9], can also phosphorylate histone H3 at S10 and thus participate in gene activation. A direct connection between rapid kinase activation and gene induction by steroid hormones has been reported in breast cancer cells. The activation of ERK and MSK1 and their recruitment, along with phosphorylated PR (pPR) to the MMTV promoter leads to phosphorylation of histone H3 at S10, displacement of HP1g, and recruitment of ATP-dependent remodeling complexes, coactivators, and RNA polymerase II [10]. These results show that the cytoplasmic and nuclear pathways activated by steroid hormones converge on chromatin to enable gene regulation in T47D cells.

The uterine endometrium undergoes cyclic stages of proliferation, differentiation and remodelling under the control of steroid hormones. Even though proliferation of endometrial stromal cells followed by differentiation into decidual cells is dependent on progesterone and estradiol [11,12], a dominant role of progesterone during decidualization has been demonstrated using anti-
Progesterone alone is able to induce uterine stromal proliferation before decidualization, an effect potentiated by estrogens [15]. Evidence that progesterone receptor (PR) is crucial for decidualization comes from PR-deficient mice [16]. Our previous studies in the UIII stromal cell derived from rat endometrium concluded that induction of proliferation upon the addition of progestins requires both progesterone and estrogen receptor beta (ERβ) [17]. The interaction of both receptors in the cytoplasm is needed to activate the extracellular signal-regulated kinases 1 and 2 (ERK1-2) as well as the AKT signaling pathway. UIII cells do not express ERalpha but express PR and ERbeta though at levels insufficient for hormonal transactivation of their respective target genes via binding to genomic target sequences [17]. However, whether the progestin action via kinase signaling can regulate genes independently of PR binding to chromatin remains an open question. In this study we used UIII cell line to explore the cohort of early regulated genes by the cytoplasmic component of progesterone-PR pathway independent of PR binding to genomic targets. We describe early downstream targets of progestin-dependent ERK and AKT activation via PR and ER, and study their role in stromal endometrial proliferation in cultured UIII cells.

Materials and Methods

Materials
Available at SI M&M.

Cell Culture and Hormone Treatment Experiments
UIII rat normal uterine stromal cells were kindly provided by Dr. Cohen and maintained in M199 medium supplemented with 10% fetal bovine serum (FBS) and gentamycin (100 μg/ml) at 37°C in humidified 95% air with 5% CO as the authors first described them [18]. Culture media were changed every 2 days.

For hormone treatment experiments in absence of serum, cells were cultured in FBS and, 48 h later, media were replaced by fresh M199 without serum. After three days in serum-free conditions, media were replaced by either vehicle or hormones.

RNA Extraction, sqPCR and qPCR Analysis
In all cases total RNA isolation and cDNA synthesis were performed as described [17].

SQPCR and qPCR: JunD, Usf1, Cyr61, Pten, Cdkn1b, Crebbp, Gfi1 and β-actin mRNA levels were quantified as described [19]. The primers used are detailed in Table S1. Find details of these protocols in SI M&M.

Microarray Analysis
Serum starved UIII cells were treated with ethanol or R5020 10−10 M during 45 minutes. Isolated RNA was hybridized to an oligo microarray (60 mer) from Agilent (G4130). cDNA was synthesized according to manufacturer’s instructions (Agilent). Detailed protocols are available at www.agilent.com/chem/dnmanuals-protocols. Briefly, the cDNA was used as a template for synthesis, amplification and staining of cRNA. The dCTP conjugated to cy3 or conjugated to cy5 was incorporated by T7 RNA polymerase to obtain cRNA-cy3 or cRNA-cy5 from the cDNA vehicle or progestin treated cells respectively. The first experiment was performed with an inverted dye swap staining (indicated as DS in figure legend). The cRNA-cy3 and cRNA-cy5 were purified before chip hybridization. The images of competitive hybridization were scanned and data from images were extracted to quantify gene expression on each spot. The data analysis was performed with ACFM 4.0 [20]. Microarray analysis was performed at the Microarray unit from the Centre de Regulació Genómica, Barcelona, Spain. The dataset was reported to GEO databank under GSE55992 accession number.

Statistical Analysis for Microarrays Data
The details of experimental design, transformation and statistical treatment of microarray data protocols are available at SI M&M.

In Silico Analysis
In silico analysis was performed using GO Tree Machine and OnoExpress softwares. Details of the analysis in SI M&M.

siRNA and Transfection
For knockdown with siRNA and hormone treatment experiments in absence of serum, UIII cells were cultured in FBS and, 24 h later, media were replaced by white M199 with 10% dextran-coated charcoal-foetal bovine serum (DCC-FBS) and without antibiotics, in this conditions the cells were transfected. Cdc2 siRNA (sc-29253, Santa Cruz Biotechnologies, California, USA), USF1 siRNA (sc-270501, Santa Cruz Biotechnologies, California, USA) or scramble siRNA (Negative control siRNA, Qiagen, Gene Glove) were used in 100 nM. Lipo 2000 (Lipofectamin 2000, Invitrogen) was used as the vehicle of transfection. Forty-eight hours later media were replaced by fresh M199 without serum and the cells were starved overnight. After one night in serum-free conditions, media were replaced by either vehicle or hormones.

Western Blots
Protein samples were analyzed as described [17]. Quantification of blot intensities were performed with data obtained within a linear range of exposure (G:Box-Syngene). Details of these protocols in SI M&M.

Chromatin Immunoprecipitation Experiments
ChIP experiments were performed as described [22]. UIII cells were seeded in 145 mm culture dishes and after hormonal treatments, chromatin was collected. The antibodies used for the immunoprecipitations were USF1 (Santa Cruz Bio. H-86), PR (Santa Cruz Bio. H-190) and normal rabbit IgG (Cell Signaling). The primers used for qPCR performed on immunoprecipitated (IP) and non-immunoprecipitated (input) DNA are detailed in Table S1 and S2. USF1 enrichment was expressed as percentage of input relative to T0 according to Comparative Ct method. Ct values were acquired with Bio Rad CFX Manager. PR enrichment was detailed in Figure 5C.

Statistical Analysis
Analysis of variance followed by Tukey Multiple Comparison Test was used for statistical testing in all figures unless otherwise indicated. t-Test was performed to compare mRNA expression Fig. 2B and Fig. 5E, and cell number in Fig. 4E and Fig. 5F. Differences were considered significant if P<0.05. Statistical analysis was carried out with GraphPad Prim 4.0 (GraphPad Software Inc., La Jolla, CA, USA).
Results

R5020 Modulates Early Genes Expression in Stromal Endometrial UIII Cells

We initiated the search for early downstream targets of the R5020 signaling pathway by exploring the optimal concentration and time point for gene expression profiling. Cyclin D1 (Ccnd1) was used as a well-known cell cycle regulator involved in progestin-dependent proliferation [23–24]. Two progestin concentrations were used: 10^{-10} M, known to activate the cytoplasmic initiated effects in UIII [17], and 10^{-8} M, usually associated to steroid transcriptional activation. Both concentrations transiently induced Ccnd1 mRNA levels with a maximum at 45 minutes, but the lower concentration was more effective (Fig. 1A). When a larger range of R5020 concentrations was tested at 45 minutes, the optimal concentration was 10^{-10} to 10^{-8} M (Fig. 1B) shows the statistical quantification of 3 experiments). Pre-treatment of the cells with the PR antagonist RU486 abolished the Ccnd1 mRNA induction (Fig. 1C), indicating that it is mediated by the classical PR. The ER antagonist ICI 182780 had a similar effect revealing that Ccnd1 regulation by R5020 requires ligand-free ER (Fig. 1C).

Pre-treatment with PD 98059 alone increased Ccnd1 transcript levels (Fig. 1C) and diminished the increase in transcript levels induced by R5020, indicating that activation of ERK1-2 plays a complex role in controlling basal and hormone regulated Ccnd1 expression. Instead, PI3K/AKT inhibitor LY 294,002 (LY) blocked progestin induction of Ccnd1, suggesting that activated AKT is involved in Ccnd1 progestin-dependent regulation (Fig. 1C). Another progesterone target gene c-Myc was also induced by 10^{-10} M R5020 in these conditions (Fig. 1D); Cdkn1a/p21 mRNA was only slightly induced in early response to 10^{-10} M R5020 (Fig. 1D), but this gene is known to be regulated at later time points after progesterin treatment [25].

Transcription Factors and Cell Cycle Regulators are the Main Functional Categories of the Progestin-dependent Gene Network in Endometrial Stromal Cells

RNA analysis with oligonucleotide micro-arrays (Agilent 44 K arrays) from cells cultured for 45 minutes with vehicle or 10^{-10} M R5020 showed that 97 genes are significantly up-regulated (over 1.40-fold, B Rank $\geq$85B), and 341 genes are significantly down-regulated (more than −1.40 fold; B Rank $\geq$85B) (Tables S3 and S4, respectively; the fold change (FC) numbers represent the average from three biological replicates and a dye swap data set).

GOTM software (Gene Ontology Tree Machine) [26] analysis of the 438 progestin-regulated genes showed that the most significant differentially over-represented ontology terms were related mainly to transcription regulation: Cellular Component Categories, nucleus (44 genes, with P = 0.001) and chromatin (5 genes with P = 0.004); Functional Component Categories, transcription factor activity (18 genes, 5 of which were up-regulated, JunD, MafB, Klf4, Usf1, Crebbp, and 13 were down-regulated RGD1508861, Egr3, Nfix, Che1, Runc3, E2f1, Tcf3, Caskn1, Klf1, Gfi1, Tcf12, And4a, Mllt10, with P = 0.002) (Fig. 2B, TF), chromosome organization and biogenesis (3 genes, 3 of which were up-regulated, Rbbp4, Crebbp, Plcda3, and the remaining 5 were down-regulated, Smarce1, Ssehd1, Tusk1bp1, Tcf3, Klf1, with P = 0.006), ubiquitination-dependent protein catabolism (2 up-regulated genes, Ube3a, Ube2a, and 3 down-regulated genes, Atc1, AnkB, Usp7, with P = 0.006), and central nervous system development (2 up-regulated genes, Klf4, Ube3a, and 7 down-regulated genes, B3gl5, Odkz2, Pitx3, E2f1, Atc1, Sep4, Pitpm1, with P = 0.007) (Fig. 2A).

Figure 1. Low concentration of progestin increases Ccnd1 transcript levels in UIII endometrial stromal cells. A) UIII cells were treated with vehicle (−), 10^{-10} M R5020 (−10) or 10^{-8} M R5020 (−8) during 30, 45 and 60 minutes in serum-free culture medium and total RNA was isolated and subjected to sq-PCR. Figure shows sybr green-stained gels of sq-PCR products for Ccnd1 and β-Actin of a representative experiment selected from three independent experiments with similar results. B) UIII cells were treated with vehicle (0) or with increasing 10^{-11} M (−11) to 10^{-8} M (−8) concentrations of R5020 (R5020 (logM)) for 45 minutes. The graph represents the values for Ccnd1 fold change relative to β-Actin were divided by the vehicle-treated value (control). Data represent average ± SEM from 5 to 9 independent experiments. **P<0.01, ***P<0.001 v. vehicle. C) Anti-progestin RU486, antiestrogen ICI and inhibitors of ERK1-2 and AKT pathways effects on Ccnd1 mRNA expression. Cells were pre-treated for 30 minutes with 10^{-6}M RU486 (RU), 10^{-7}M ICI 182.780 (ICI), 50 μM PD 98.059 (PD) or 50 μM LY 294,002 (LY) followed by a 45 minute treatment with vehicle (−) or 10^{-10} M R5020 (−) as indicated. Figure shows sybr green-stained gels of sq-PCR products for Ccnd1 and β-Actin of a representative experiment selected from three independent experiments with similar results. D) Ccnd1, c-Myc and Cdkn1a/p21 transcript expression was analysed in UIII cells treated as described in B.

doi:10.1371/journal.pone.0097311.g001

The search for the ontology terms Cellular Proliferation and Cell Cycle yielded 23 genes (Fig. 2B, CCR-Cell Cycle Regulators). Seven of them are transcription factors (Crebbp, Che1, Runc3 E2f1, Tcf3, Gfi1 and Tcf12) and 3 are chromatin modifiers (Rbbp4, Tcf3 and Crebbp). Eight genes were up-regulated and 15 were down-
regulated are shown in green.

Figure 2. Transcription Factors and Cell Cycle Regulators are the main early progestin-regulated genes. A) The categories of over-represented Gene Ontology terms (GO terms) are shown by their decreasing p-values. The categories were identified by GOTM (Gene Ontology Tree Machine) software over the statistical regulated genes as indicated in supplementary Materials and Methods. Up-regulated genes percentages are shown in red, and down-regulated genes are shown in green. B) Venn diagram shows the set of TF and CCR regulated genes within a given signalling pathway is shown in red, and down-regulated genes are shown in green. C) The signaling pathways associated to the differential gene expression pattern are shown by their decreasing p-value. Pathways identified by Pathway Express Software containing at least four progestin-dependent regulated genes included in a given Signalling Pathway (SP), with a p-value≤0.05. The percentage of up-regulated genes within a given signalling pathway is shown in red, and down-regulated genes are shown in green. Statistical details are described in M&M. D) The table shows individual fold changes of three independent biological samples (1,2,3) and one dye swap data set (1DS) analyzed by microarray, and the mean fold change of all 4 values (Media). Fold changes over vehicle treated cell values were calculated as described in SI M&M. E) q real time PCR validation for JunD, Usf1, Crebbp, Gfi1, Cyr61, Pten and Cdkn1b mRNA relative to β-Actin. The figure shows media ± SEM from three to six independent experiments. *p<0.05, **p<0.01, ***p<0.001 v. vehicle treated cells. F) sq-PCR validation for transcription factors (TF) JunD, Usf1, transcription factors and cell cycle regulators (TF+CCR) Crebbp, Gfi1, Cyr61, and cell cycle regulators (CCR) Pten, Cdkn1b and β-Actin.

doi:10.1371/journal.pone.0097311.g002

Role of Hormone Receptors and Kinases on Progestin Gene Regulation

We used hormone receptor antagonists and kinase specific inhibitors to study the involvement of PR (RU), ER (ICI), ERK1-2 (PD) and AKT (LY) on progestin regulation of the validated target genes including JunD, Usf1, Crebbp, Gfi1, Cyr61, Pten and Cdkn1b. Figure 3 shows the values of changes in gene expression of the tested genes in cells treated with 10^{-10} M R5020 for 45 min after preincubation for 30 min with vehicle or with RU486, ICI182780, PD98059 and LY294002. PR antagonist RU486 blocked up-regulation of JunD, Cyr61 and Usf1 (Fig. 3A and 3C), as well as down-regulation of Gfi1 and Cdkn1b (Fig. 3B and 3C). In absence of progestin, the antagonist treatment down-regulated Gfi1 transcript expression, indicating that basal Gfi1 transcription requires ligand-free PR (Fig. 3B). These results show that progestin regulates JunD, Cyr61, Usf1, Gfi1 and Cdkn1b through classic PR.

ER antagonist ICI 182780 revealed that Usf1, Gfi1, Cyr61, and Cdkn1b regulation by R5020 requires ligand-free ER (Fig. 3A, 3B and 3C). JunD mRNA expression level in the presence of ICI alone was similar to its expression in the presence of ICI+R5020 and was higher than in control conditions (Fig. 3A). However, the level of JunD mRNA in presence of both R5020 and ICI was lower than with R5020 alone. Additionally, ICI treatment compromised the response of JunD and Cdkn1b genes to R5020 due to a differential effect on the basal activity of the two genes: whereas it increased basal activity of JunD, it decreased basal activity of Cdkn1b. We conclude that, in addition to PR, ER is partially involved in the induction of JunD by progestin (Fig. 3A). Pre-treatment with ICI blocks the progestin down-regulation of Gfi1 and Cdkn1b,
suggesting that the ligand-free ER is required for progestin gene repression.

Pre-treatment with PD 098.059 abolished R5020 induction of JunD, Usf1, and Cyr61 (Fig. 3A and 3C), as well as down-regulation of Gfi1 and Cdkn1b (Fig. 3B and 3C).

PI-3K/AKT inhibitor LY 294.002 (LY) precludes progestin induction of JunD, Usf1 and Cyr61 as well as repression of Gfi1 and Cdkn1b (Fig. 3A, 3B and 3C). In the presence of LY, R5020 reduced Pten mRNA levels to a lower extent than in its absence. Although the statistical significance of this finding remains to be established, the data suggest that progestin-dependent inhibition of Pten transcript expression requires ERK1-2 activation and is independent of AKT activation.

Although individual genes show more complex behaviours, such as Gfi1 in response to RU and Cdkn1b in response to ICI, the inhibitory effects of RU, ICI, PD and LY suggest that regulation by progestin R5020 of these genes needs classical PR, ER, ERK1-2 and/or AKT activation. Thus at least two different signaling pathways are involved in the short-time progestin control of the expression of these transcription factors and cell cycle regulators.

Since the effects of ICI indicate a signaling pathway that involves ER activation in the absence of estrogens, we have tested the effect of estrogens on the expression of validated R5020-regulated genes. Except in the case of Gfi1 repression, estradiol (10^{-8} M) did not show the same regulation pattern as observed with R5020 (Fig. S1), suggesting that the outcome of activation of the PR-ERbeta pathway does not depend on estradiol as a ligand.

**Figure 3. PR, ER, ERK1-2 and AKT activation on progestin-regulated transcription factors and cell cycle regulators mRNAs.** UIII cells were pre-treated for 30 minutes with 10^{-8} M RU486 (RU), 10^{-7} M ICI 182.780 (ICI), 50 μM PD 98.059 (PD) or 50 μM LY 294.002 (LY) followed by a 45 minute treatment with vehicle (−) or 10^{-10} M R5020 (+) when indicated. A) q-PCR of mRNAs from JunD and Usf1 relative to β-Actin mRNA. B) q-PCR of Cebbp and Gfi1 mRNA relative to β-Actin mRNA. C) q-PCR of Cyr61, Cdkn1b and Pten mRNA relative to β-Actin mRNA. The figures show media ± SEM from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs vehicle-treated cells. doi:10.1371/journal.pone.0097311.g003

**Downstream Targets of Progestin-regulated Transcription Factors Usf1 and Cebbp**

We next investigated the involvement of downstream target genes of regulated transcription factors in progestin-dependent proliferation. Cdc2 and cyclin b1 (Ccnb1) have been described as USF1 target genes [28–29], while p21 (Cdkn1a) and c-Myc are
regulated by GF1[30], and Cdc6 and cyclin E (CcnE) are targets of CREBBP[31].

Time-course experiments of response to R5020 showed that the levels of mRNA for Usf1 increased at 45 min, decreased slightly at 2 and 6 hours and reached a maximum at 12 hours (Fig. 4A). This pattern gathers strength for the Cdc2 mRNA levels that were up-regulated at 45 min, returned to control levels at 2 and 6 hours, and increased again at 12–24 hours (Fig. 4A). Ccnb1 mRNA was also transiently up-regulated at 45 min and at 12 hours (Fig. 4A). These effects are all mediated by PR as they were blocked by the antiprogestin RU486 at 12 hours treatment (Fig. 4B).

Through western blot, we analyzed the protein levels of USF1, its target CDC2/CDK1, the S-phase marker PCNA, and total ERK2 in cells treated with vehicle or $10^{-10}$ M R5020 during 6, 12 and 24 hours (Fig. 4C). USF1 and CDC2 were increased at 6 and 24 hours, and showed basal levels at 12 hours (Fig. 4C). On the other hand, PCNA increased only at 12 hours, suggesting that DNA synthesis occurs as a consequence of the initial rise in USF1 and its target CDC2 at 6 hours (Fig. 4C).

In addition to USF1 targets, we also looked at CREBBP target CcnE. Although significant Cebbp mRNA induction by progestin was not consistently observed, the mRNA levels of CcnE, a target gene of CREBBP (Fig. S2A), increased gradually from 45 min to 12 hours in response to progestin treatment. CcnE progestin dependent induction after a 12 hours treatment was abolished by
RU486 pretreatment (Fig. S2B), indicating the need for the classic PR.

**USF1-induced CDC2 is Required for Progestin-dependent Proliferation**

After 24 hours of progestin treatment, the cell number increased 2 fold (Fig. 4D). A 60 percent depletion of CDC2 with specific siRNA (Fig. 4E insert) precluded progestin dependent cell proliferation while cell proliferation was maintained in cells transfected with scramble siRNA (Fig. 4E), demonstrating that CDC2 mediates progestin-dependent proliferation.

To explore the mechanism of USF1 transcription factor regulation of Cdc2 expression, we performed ChIP experiments over a region of Cdc2 promoter (see Cdc2 promoter pattern at Figure 5A), which contains two specific nucleotide heptamers known to function as binding sites for USFs and to be highly conserved in different species [26]. This ChIP showed that after 30 min of treatment with R5020 USF1 binds to both Cdc2 promoter sequences (Figure 5B, regions 1 and 3) while two other nearby regions were negative for USF1 binding (Figure 5B, regions 2 and 4). These results are consistent with binding of USF1 to its target Cdc2 mediating the regulated expression of this cell cycle kinase in UIII cells.

We have shown that endogenous PR is not able to induce transcription of an exogenous PRE construction in UIII cells [17]. Therefore, we did not expect to find PR binding to DNA target sequences. Nevertheless, to exclude PR direct interaction with Cdc2 promoter, prior to hormone treatment (T0) and 60 min after addition of $10^{-10}$ M R5020 (R60), we tested PR binding to Cdc2 proximal promoter, $-0.75$ Kb to +0.5 Kb relative to the transcription start site (TSS) using 3 of the USF1 ChIP pairs of primers (regions 2, 3 and 4 in Fig. 5B) and an extra pair which partially overlaps with region 1 (region 1 bis in Figure 5C). The results confirmed the absence of PR binding to Cdc2 DNA proximal promoter sequence (Fig. 5C) although we do not exclude binding to other distant regions of the gene. PR is recruited on a distal position ($-10.9$ Kb to $-10.7$ Kb) of Cdc2 promoter in T47D human mammary epithelial cells under progestin treatment [21] and this region contain several potential PREs (TGTYCYC), but this region is not conserved in mouse or rat genomes (NCBI/BLAST/blast suite).

To compare Cdc2 regulation by USF1 and PR, we analyzed USF1 recruitment in ENCODE human cell lines (http://genome.ucsc.edu/) [32] and PR recruitment in human Ishikawa endometrial cells (La Greca A et al., unpublished data) and T47D cells [21]. The USF1 element in Cdc2 described in A549 epithelial cell line derived from a lung carcinoma tissue-, H1-hESC embryonic stem cells and HepG2 hepatocellular carcinoma was in the region +1044 to +1300 from the TSS, differently positioned from what we found for USF1 recruitment (Fig. 5D). PR recruitment in Cdc2 of human Ishikawa endometrial cells was negative (La Greca et al., unpublished data), while it was positive in T47D cells [21], hinting at possible tissue specificity.

Progestin-dependent regulation of Cdc2 is evident from the fact that after 12 hours of progestin treatment, the Cdc2 transcript expression increased around four times over vehicle treated cells (Fig. 4A). To confirm that progestin regulation of Cdc2 was mediated by USF1, we knocked down USF1 and measured Cdc2 mRNA after 12 hours of progestin and vehicle treatment. A 60 percent depletion of USF1 with specific siRNA (see Western blot in Fig. 5E insert) abolished progestin-dependent Cdc2 transcript upregulation, while Cdc2 mRNA expression was not modified in cells transfected with scramble siRNA (Fig. 5E). This finding demonstrates that Cdc2 direct transcriptional regulation is mediated by progestin-dependent USF1. Also, USF1 specific siRNA abrogated progestin-dependent proliferation (Fig. 5F). Taken together, these results point to Cdc2 as a possible cell cycle target of early transcription factor USF1 whose expression is selectively induced by progestin via the interaction of PR-ERbeta and ERK and AKT activation independently of PR binding to genomic targets (Fig. 5G).

**Discussion**

In this study we explored the possibility that steroid hormones can regulate gene expression via activation of kinase signalling pathways without requiring a direct interaction of their receptors with the target genes in chromatin. As we have shown previously, UIII cells are a good model system to study cytoplasmic initiated effects of low concentration of progestin in the presence of PR and ERb but not ERa and without transcriptional activity of both endogenous receptors [17]. Here we identify transcription factors as downstream molecular targets of progestin activation of ERK1-2 and AKT involved in proliferation of UIII cells and specifically explore Cdc2—a USF1 transcription factor target—involved in proliferation.

We first analyzed the expression of cyclinD1, the regulatory component of the complex CyclinD1-CDK4 that, together with cyclinE-CDK2, promotes S phase entry through the phosphorylation of pRb [33]. Even though there are no progesterone response elements (PRE) on Cnd1, progestin regulates its transcription through a proximal promoter c-Ets-2 binding site [34–35]. We found a rapid and transient induction of Cnd1 transcripts in progestin treated UIII cells mediated by classical PR. Unexpectedly, optimal transcriptional activation was observed in response to low progestin concentrations, $10^{-10}$ M and $10^{-9}$ M R5020. This observation is consistent with our previous results showing that the optimal concentration of progestin inducing UIII cell proliferation as well as ERK1-2 and Akt activation is in the subnanomolar range, and suggests that Cnd1 is a mediator of the proliferative response. Therefore we used a low concentration of progesterin ($R5020\ \text{10}^{-10}\ \text{M}$) and a short time treatment (45 minutes) to identify the global set of target genes involved in the initiation of progestin-dependent proliferation in endometrial cells.

Using oligonucleotide microarrays, we identified 438 regulated genes, of which 78 percent were significantly down-regulated, suggesting that repression could be a relevant molecular mechanism by which progestrone regulates stromal proliferation in endometrium. The over-represented ontology terms revealed that progestin downstream genes are mainly involved in the regulation of transcription, notably transcription factors, steroid receptor coregulators and chromatin remodelling molecules/regulators/modifiers. We detected a set of 23 genes encompassed in the Cellular Proliferation and Cell Cycle ontology terms and found that 9 of them were also present in the overrepresented set of transcription factors and chromatin remodelling genes. We validated the expression of genes described as transcription factors (JunD, Usf1), as transcription factors and cell cycle regulators (Cebbp and Gfi1), and as cell cycle regulators (CyclinB1 and Cdkn1b).

Usf1 (up-regulated 1.42-fold) encodes a ubiquitous transcription factor that regulates gene networks involved in stress and immune response, cell cycle and cell proliferation. USF1 transcription factors have been shown to be targets of ERK1-2 in epidermal keratinocytes [36]. USF1 controls cell proliferation by targeting cell cycle genes such as p23 [37], Cdc2 [28] and cyclin b1 [29]. CDC2 or cyclin dependent kinase1 (CDK1) forms a complex with CyclinB1, whose activation by phosphorylation promotes the entry
Figure 5. Progestin directs USF1 binding to Cdc2 promoter. A) Cdc2 proximal promoter (–0.75 Kb to +0.5 Kb) contains two specific heptamers binding sites for USFs and two nearby unrelated regions. B) USF1 is recruited to the Cdc2 proximal promoter sequence. U111 cells were treated with 10–10 M R5020 for 30 min and subjected to ChIP with IgG as control, or a USF1 antibody and the purified DNA was used for q-PCR. The values represent average ± SEM fold changes relative to inputs over T0 from 3 independent experiments for each of the four regions (1–4) shown in the upper Cdc2 promoter scheme. *P<0.05, **P<0.01 v. vehicle. C) PR is not recruited to the Cdc2 proximal promoter sequence. After performing PR ChIP in untreated (–) and R5020 treated cells (R), we analyzed the recruitment to Cdc2 proximal promoter sequence by qPCR using 4 pairs of primers, 3 of which were used for USF1 ChIP analysis (Regions 2, 3, and 4) and a pair (1 bis), which covers the corresponding region (Figure 5C). Ct: Crossing threshold, N/D: not detected. D) USF1, PR and phospho Pol II recruitment to Cdc2 promoter (– 10 Kb to +1 Kb) in U111 rat endometrial cells, in human cell lines (ENCODE: A549 epithelial cell line derived from a lung carcinoma tissue, H1-hESC embryonic stem cells, HepG2 hepatocellular carcinoma), Ishikawa human endometrial cells [La Greca et al, unpublished data], and T47D epithelial mammary cells [30]. E) USF1 mediates progestin regulation of Cdc2. The graph shows Cdc2 mRNA expression determined by q-PCR after 12 h of treatment with vehicle and progestin of cells transfected with scramble siRNA and with Usf1 siRNA 48 h before. ***P<0.001, NS: Non Significant. Lines indicate statistical comparison; standard deviation is indicated. Inset E) Picture shows western blots for USF1 and GAPDH of cells transfected with Usf1 and scramble siRNAs. F) Number of cells transfected without siRNA, with scramble siRNA and with Usf1 siRNA 24 h before treatment with vehicle and progestin. **P<0.01, ***P<0.001, NS: not significant. Lines indicate statistical comparison; standard deviation is indicated. G) Model depicting PR-ER, ERK and AKT activation necessary for
PR-ER preformed complex activates ERK and AKT at the cytoplasm. The progestin cytoplasmic signaling differentially regulates early gene expression, mainly of cell cycle regulators and transcription factors. The transcription factor USF1 is recruited to Cdc2 proximal promoter, and Cdc2 induction under hormone treatment is required for progestin-dependent endometrial stromal cells proliferation.

doi:10.1371/journal.pone.0097311.g005

in the mitotic phase of the cell cycle [38]. USF1 specifically mediates transcriptional activation of Ccnb1 (Cyclin B1) just before and during mitosis in HeLa cells [29].

We found that Usf1 and its targets Cdc2 and Ccnb1 were transiently induced at 45 min and at a later time (12 hours), USF1 and Cdc2 proteins also showed progestin-dependent regulation. Cdc2 hormone-dependent regulation preceded PCNA increase, supporting recent evidence that Cdc2 regulates G1 progress and G1-S phase transition [39]. It is to be noted that the reduction of Cdc2 by means of siRNA UIII cells did not respond to progestin effects.

To test USF1 regulation of Cdc2 at a transcriptional level we performed ChIP and tested USF1 recruitment over Cdc2 proximal promoter. Our results show a USF1 progestin-dependent recruitment to two specific heptamers in Cdc2 promoter region, which were described as USF binding sites [28]. The genomic position of these bindings was different from the one described for several human cell lines in ENCONDE project (http://genome.ucsc.edu/). We confirmed the absence of PR binding to Cdc2 DNA proximal promoter sequence in accordance with the absence of PR binding in Chip seq experiments performed in Ishikawa cells treated with R5020 (data not shown) and in T47D cells treated with R5020 [21]. This encourages the study of Cdc2 gene expression regulation mediated by cytoplasmic PR pathway. A functional approach using siRNA showed that depletion of USF1 abolished Cdc2 progestin-dependent transcriptional regulation and progestin-dependent proliferation, confirming that USF1 mediates the progestin transcriptional regulation of Cdc2 and R5020 induced proliferation.

To explore the repertoire of the described transcription factors binding sites to the complete set of TF regulated by R5020 we performed an in silico search using TRANSFAC software (www.gene-regulation.com) over the set of 32 progestin regulated genes shown in Fig. 2D. This analysis in the absence of PR elements (PRE), and in the presence of steroid hormone response elements (SHRE) in 3 genes, of USF1 elements in 15 genes, of ELK1 elements in 14 genes and of other non-SHRE in the remaining 13 genes. This pattern is consistent with non-direct binding of PR to this set of kinase regulated genes.

Our finding suggests that the progestin regulated interaction between USF1 and its target Cdc2 could regulate the expression of this cell cycle kinase in UIII cells in the absence of genomic PR binding.

Our present results identify for the first time in endometrial stromal cells a set of early target genes of progesterin-activated classical PR via ERβ and protein kinases. Progestin regulation of JunD, Usf1, Gfi1, Pten, Cdkn1b and Cyr61 required ERK1-2 or AKT activation and ligand-free ERβ, while regulation of Ccnd1 was mediated by activation of AKT but not ERK1-2. This study suggests that the downstream targets of cytoplasmic kinases activated by PR in UIII cells are predominantly early induced transcription factors and repressors or inhibitors of cell proliferation that are down-regulated by progestins, as well as activators of cell cycle regulators. One of them, CDC2, is up-regulated by USF1 and required for progestin-induced proliferation in a pathway that does not bind PR to Cdc2 proximal promoter.

### Supporting Information

**Figure S1** Estradiol effects on validated Transcription Factors and Cell Cycle Regulators. UIII cells were treated as described in Figure 1B and treated with vehicle (OH), R5020 $10^{-10}$ M (R10) or Estradiol $10^{-8}$ M (E3) for 45 minutes in a serum-free culture medium. Graphs show JunD, Usf1, Gyr61, Cdkn1b mRNAs expression determined by q-PCR. Gfi1, Ccnd1 and c-Myc mRNAs expression was analyzed by sq-PCR and representative electrophoresis gels stained with sybr-green are shown in the insets. In all cases values for gene fold change relative to β-Actin were divided by the vehicle-treated value. Data represent average ± SEM for 3–5 independent experiments. *P<0.05, **P<0.01, ***P<0.001.

(TIF)

**Figure S2** Progestin regulation of Crebbp transcription factor targets. UIII cells were treated as indicated in Fig. 1D. The values for mRNAs fold change relative to β-Actin were divided by the vehicle-treated value for each time point tested. A) Crebbp, Ccnd1 and β-Actin at 45 minutes, 2, 6, 12 and 24 h of $10^{-10}$ M R5020. Data represent average ± SEM from 3 to 5 independent experiments. **P<0.01 vs vehicle. B) Representative products of sq-PCR of these genes in 30 minutes RU486 pre-treated cells from three independent experiments with similar results.

(TIF)

**Table S1** PCR primer sequences designed by Oligo Primer Analysis Software (Molecular Biology Insights, Inc.).

(DOC)

**Table S2** PCR primers position relative to Cdc2 Transcription Start Site (TSS). Primers Ubs 1 and 3 correspond to region 1 and 3 respectively, while primers nUbs 2 and 4 correspond to regions 2 and 4 respectively in figure 5C. Primers Ubs 1 bis are located just upstream of the Ubs 1 pair and cover a region which partially overlaps with region 1, namely 1 bis.

(DOC)

**Table S3** Progestin-dependent up-regulated gene expression pattern. The table shows individual fold changes of up-regulated genes after 45 min treatment with R5020 $10^{-10}$ M related to vehicle. Data were taken from three independent samples (E1, E2, E3) and one dye swap experiment (1DS) analyzed by microarray and expressed by mean fold change of all 4 values (FC). Colour scale for up (red), non (black) and down (green) regulated genes is shown.

(DOC)

**Table S4** Progestin-dependent down-regulated gene expression pattern. The table shows individual fold changes of statistical down-regulated genes after 45 min treatment with R5020 $10^{-10}$ M related to vehicle. Down (green) regulated genes are ordered by increasing mean fold change. Data shown as indicated in Table S3.

(DOC)

**Acknowledgments**

The authors thank Guillermo Vicent for helpful discussions and suggestions and Gabriel Rabínovich for critical reading of the manuscript. Griselda
Vallejo is an assistant researcher from CONICET, Ana C, Mestre-Citrinovitz and Alejandro D. La Greca are doctoral fellows from CONICET.

References

1. Beato M, Herrlich P, Schutz G (1995) Steroid hormone receptors: many actors in search of a plot. Cell 83, 851–7.
2. Migliaccio A, Di Domenico M, Castoria G, di Falco A, Bontempo P, et al. (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 15(6), 1292–300.
3. Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, et al. (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J. 17(7), 2008–18.
4. Castoria G, Barone MV, Di Domenico M, Bilancio A, Ameraneto D, et al. (1999) Non-transcriptional action of oestradiol and progesterin triggers DNA synthesis. EMBO J. 18(9), 2500–10.
5. Migliaccio A, Castoria G, Di Domenico M, di Falco A, Bilancio A, et al. (2000) Steroid-induced androgen receptor-oestradiol receptor complex triggers prostate cancer cell proliferation. EMBO J 19, 5406–5417.
6. Boonyaratanakornkit V, Scott MP, Ribon V, Sherman L, Anderson SM, et al. (2000) Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol Cell. 2(12), 269–80.
7. Kousteni S, Bellido T, Plogicl L, O'Brien CA, Bodenner DL, et al. (2001) Nongenotoxic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104(5), 719–30.
8. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, et al. (2000) Interaction of oestrogen receptor with the regulatory subsite of phospholipid-linositol-3-OH kinase. Nature. 407(6803), 538–41.
9. Clark DE, Pooten-Smith CE, Smith JA, Lunnigan DA. (2001) Rsk2 allosterically activates estrogen receptor alpha by docking to the hormone-binding domain. EMBO J. 20(3), 4304–94.
10. Vicent GP, Ballaré C, Nacht AS, Classell J, Subtil-Rodríguez A, et al. (2006) Induction of progesterone target genes requires activation of Erk and Mdk kinases and phosphorylation of histone H3. Mol Cell. 24(3), 367–81.
11. Psychopoulo A (1973) Hormonal control of ovulation. Vitam Horm 3, 201–256.
12. Parr MB, Parr EL (1989) Immunohistochemical investigation of secretory component and immunoglobulin A in the genital tract of the female rat. J Reprod Fertil 85, 105–113.
13. Grummer R, Chwalisz K, Mulholland J, Traub O, Winterhager E (1994) Regulation of connexin26 and connexin43 expression in rat endometrium by ovarian steroid hormones. Biol Reprod 51, 1109–1116.
14. Zhang Z, Funk C, Glasser SR, Mulholland J (1994) Progesterone regulation of heparin-binding epidermal growth factor-like growth factor gene expression during sensitization and decidualization in the rat uterus: effects of the antiprogestin, ZK 90.299. Endocrinology 133, 1256–1263.
15. Hurt-Hudson YM, Andrews GK, Dey SK (1998) Cell-type specific localization of c-Myb protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. Endocrinology 125, 1683–1690.
16. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, et al. (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 9, 2256–2278.
17. Vallego G, Ballaré C, Baranao JL, Beato M, Saraguet A (2005) Progesterin activation of nongenomic pathways via cross-talk of progesterone receptor with estrogen receptor beta induces proliferation of endometrial stromal cells. Mol Endocrinol 19, 3023–3037.
18. Cohern H, Pageaux JF, Méminand C, Fayard JM, Lauger C (1993) Normal rat uterine stromal cells in continuous culture: characterization and progesterin regulation of growth. Eur J Cell Biol 61, 116–125.
19. Vallego G, Maschi D, Mestre-Citrinovitz AC, Alba K, Maros-R, et al. (2010) Changes in global gene expression during in vitro decidualization of rat endometrial stromal cells. J Cell Physiol. 222, 127–37.
20. Breitkreutz BJ, Jorgensen P, Breitkreutz A, Tyers M (2001) AFM 4.0: a toolbox for DNA microarray analysis. Genome Biol. 2(9), SOFTWARE001.
21. Ballart C, Castellano G, Gavezig L, Althamme S, Gonzalez-Vallinas J, et al. (2013) Nuclearosome-driven transcription factor binding and gene regulation. Mol Cell. 10, 49(1): 67–79.
22. Strutt H, Paro R (1999) Mapping DNA target sites of chromatin proteins in vivo by formaldehyde crosslinking. Methods Mol Biol 115, 45–67.23. Said TK, Conneely OM, Medina D, O’Malley BW, Lydon JP (1997) Progesterone, in addition to estrogen, induces cyclin D1 expression in the murine mammary epithelial cell, in vivo. Endocrinology 138(9), 3933–9.
23. Ciacoli L, Addis R, Sasso A, Alnuci I, Petrizzi VB, et al. (2004) Estrogen and progesterone promote persistent CCND1 gene activation during G1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol Cell Biol 24(16): 7260–74.
24. Groshong SD, Owen GI, Grimsson B, Schauer IE, Todd MC, et al. (1997) Biphasic regulation of breast cancer cell growth by progesterone: role of the cyclin-dependent kinase inhibitors, p21 and p27(Kip1). Mol Endocrinol 11(11): 1593–607.
25. Zhang H, Schmoyer D, Kiyov S, Snoddy J (2004) GOTOee Machine (GOTM): a web-based platform for interpreting sets of interesting genes using Gene Ontology hierarchies. BMC Bioinformatics 5: 16.
26. Draughn S, Khatri P, Martins RP, Ostermeier GC, Krawetz SA (2003) Global functional profiling of gene expression. Genomics 81(2): 90–104.
27. North S, Espanel F, Barignies F, Vieder B, Vallet V, et al. (1999) Regulation of ccD2 gene expression by the upstream stimulatory factors (USFs). Oncogene 18: 1945–1953.
28. Cogwell JP, Godleviski MM, Bouman M, Bisi J, Babios L (1995) Upstream stimulatory factor regulates expression of the cell cycle-dependent cyclin B1 gene promoter. Mol Cell Biol 15(5): 2782–2790.
29. Duan Z, Horwitz M (2003) Targets of the transcriptional repressor oncoprotein Gfi-1. Proc Natl Acad Sci U S A 100(10): 5032–7.
30. Dimova DK, Dyson NJ (2005) The E2F transcriptional network: old acquaintances with new faces. Oncogene 24(17): 2810–2826.
31. Gerstein M (2012) Genomics: ENCODE leads the way on big data. Nature 489(7415): 208.
32. Fu M, Wang C, Li Z, Sakamaki T, Pestell RG (2004) Minireview: Cyclin D1: Normal and Abnormal Functions. Endocrinology 145(12): 5439–5447.
33. Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, et al. (1995) Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions. J Biol Chem 270(40): 23589–97.
34. Vicent GP, Ballaré C, Nacht AS, Classell J, Subtil-Rodríguez A, et al. (2006) Induction of progesterone target genes requires activation of ERK and Mdk kinases and phosphorylation of histone H3. Mol Cell. 24(3), 367–81.
35. Garel A, Mijaawan RI, Walsh R, Blumenberg M (2008) Transcriptional profiling defines the roles of ERK and p38 kinases in epidermalkeratinocytes. J Cell Physiol 215(2): 292–308.
36. Reisman D, Rotter V (1995) The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the p53 tumor suppressor gene. Nucleic Acids Res. 23, 349–350.
37. Lindqvist A, von Zou W, Karlsson Rosenthal C, Wolthuis RM (2007) Cyclin B1-Cdk1 activation continues after centrosome separation to control mitotic progression. PLoS Biol 5(5): e123.
38. Hu X, Mocinski L (2011) Cdc2: a monopotent or pluripotent CDK? Cell Profil 44(5): 205–11.

Author Contributions

Conceived and designed the experiments: GV ADL MB PS. Performed the experiments: GV ADL MB-AC. Analyzed the data: GV ADL MB-AC CJB. MB PS. Contributed reagents/materials/analysis tools: ACM-C CJB. Wrote the paper: MB PS.