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Progestins activate 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) in breast cancer cells

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PFKFB (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase) catalyses the synthesis and degradation of Fru-2,6-P_2 (fructose-2,6-bisphosphate), a key modulator of glycolysis and gluconeogenesis. The *PFKFB3* gene is extensively involved in cell proliferation owing to its key role in carbohydrate metabolism. In the present study we analyse its mechanism of regulation by progestins in breast cancer cells. We report that exposure of T47D cells to synthetic progestins (ORG2058 or norgestrel) leads to a rapid increase in Fru-2,6-P_2 concentration. Our Western blot results are compatible with a short-term activation due to PFKFB3 isoenzyme phosphorylation and a long-term sustained action due to increased PFKFB3 protein levels. Transient transfection of T47D cells with deleted gene promoter constructs allowed us to identify a PRE (progesterone-response element) to which PR (progesterone receptor) binds and thus transactivates *PFKFB3* gene transcription. PR expression in the PR-negative cell line MDA-MB-231 induces endogenous *PFKFB3* expression in response to norgestrel. Direct binding of PR to the PRE box (−3490 nt) was confirmed by ChIP (chromatin immunoprecipiation) experiments. A dual mechanism affecting PFKFB3 protein and gene regulation operates in order to assure glycolysis in breast cancer cells. An immediate early response through the ERK (extracellular-signal-regulated kinase)/RSK (ribosomal S6 kinase) pathway leading to phosphorylation of PFKFB3 on Ser^{461} is followed by activation of mRNA transcription via *cis*-acting sequences on the *PFKFB3* promoter.

Key words: breast cancer, gene regulation, glycolysis, metabolism, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3), progestins.

INTRODUCTION

Oestrogens and progestins regulate female reproductive activity via receptor binding [1]. Three PR (progesterone receptor) isoforms arise from a single gene on chromosome 11, at q22-q23. PR isoforms include the full-length PRB (116 kDa), N-terminally truncated PRA (94 kDa) and PRC (60 kDa) [2]. The expression of PRA and PRB is conserved from rodents to humans, and both bind progesterone and progesterone agonists and antagonists. PRB is responsible for the normal proliferative and differentiative responses of the mammary gland to progesterone, as shown using PRA knockout mice [1]. Upon ligand binding, PR dimerization allows the PR dimer to interact with specific PREs (progesterone-response elements) within target gene promoters [3,4]. The binding of PR to PREs is followed by the recruitment of co-activators and the basal transcription machinery, which finally up-regulates target gene transcription [1].

In addition to this classical PR nuclear action, new PR mechanisms have been described. For instance, PR affects gene expression either when the receptor binds to other transcription factors such as Sp1 (specificity protein 1), AP-1 (activator protein 1) or STAT (signal transducer and activator of transcription) [5–8], or when it initiates kinase cascades [c-Src (cellular Src) and MAPK (mitogen-activated protein kinase)] to activate transcription factors or modify chromatin [3,9,10], which are known as non-genomic or extranuclear PR actions. Moreover, similar to other SHR (steroid hormone receptor) family members, PR can be phosphorylated and dephosphorylated, which adds more complexity to the action of PR. PR contains 14 serine residues that are phosphorylated by mitogenic protein kinases [11], and these post-translational modifications may co-ordinate responses to growth factors and steroid hormones [2].

Several mammalian genes are directly regulated by progestins, for example *c-jun*, c-*fos*, *FASN* (fatty acid synthase) [12], *ALP* (alkaline phosphatase), *LDH* (lactate dehydrogenase), *CCND1* (cyclin D1), *p21* [13] *HSD11B2* [hydroxysteroid (11-β) dehydrogenase 2] [14] and *E2F1* (E2F transcription factor 1) [15]. However, the genes induced as an acute response to progestin treatment that mediate effects on cell proliferation and differentiation are poorly defined. Expression of functional mutants of PR is useful to delineate sets of genes regulated by different mechanisms [16].

Hamilton et al. [17], using the differential display technique, identified a progestin-responsive gene with a sequence similar to PFKFB (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), which has been cloned from different tissues [18–20]. This gene encodes the ubiquitous bifunctional isoenzyme PFKFB3, which catalyses the synthesis and

Abbreviations used: AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipiation; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; ER, oestrogen receptor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; Fru-2,6-P_2, fructose 2,6-bisphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEO, Gene Expression Omnibus; HA, haemagglutinin; HSD11B2, hydroxysteroid (11-β) dehydrogenase 2; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK/ERK kinase; MMTV, murine mammary tumour virus; MSK, mitogen- and stress-activated kinase; NG, norgestrel; PFK-1, 6-phosphofructo-1-kinase; PFKFB, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PR, progesterone receptor; PRE, progesterone-response element; RSK, ribosomal S6 kinase; RT-qPCR, quantitative real-time PCR; STAT, signal transducer and activator of transcription; WT, wild-type.

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degradation of Fru-2,6-P_2 (fructose 2,6-bisphosphate), the most powerful allosteric activator of PFK-1 (6-phosphofructo-1-kinase) and inhibitor of fructose 1,6-bisphosphatase, and hence critically regulates carbohydrate metabolism [21,22]. *PFKFB3* is one of the four independent genes (*PFKFB1–PFKFB4*) which encode the different isoenzymes of the PFKFB family [23,24]. The PFKFB3 isoenzyme has the highest kinase/bisphosphatase ratio, favouring net synthesis of Fru-2,6-P_2 [25]. Its expression is induced in response to other stimuli such as insulin [26], pro-inflammatory molecules [20] or hypoxia through HIF (hypoxia-inducible factor) interaction with the consensus HRE (hypoxia-response element) sites in its promoter region [27]. PFKFB3 is expressed ubiquitously and is present in proliferating tissues [19,28–31], transformed cells [17,20,26,27,32] and in various solid tumours and astrocytomas [33,34].

To gain insight into the molecular mechanism behind progestin regulation of PFKFB3 expression, we have characterized the human *PFKFB3* gene response to progestin in the T47D and MCF7 breast cancer cell lines. The results show that PR utilizes two separate mechanisms to achieve PFKFB3 isoenzyme regulation. First, an immediate early response phosphorylation mechanism involving MAPK signalling and, secondly, the direct classical PR-activated DNA-binding action leading to PFKFB3 expression owing to promoter transcriptional regulation.

EXPERIMENTAL

Reagents and antibodies

Organon 2058 was purchased from Organon. D-(−)-Norgestrel, H89 and the PR antagonist mifepristone (RU486) were obtained from Sigma. R5020 was purchased from PerkinElmer Life Sciences. ICI182780 was from Tocris. The MAPK inhibitors PD98059 and UO126 were from Calbiochem, and BI-D1870 was from Enzo Life Sciences. The MAPK inhibitors RU486 and ICI182080 were added to cell culture medium 30 min prior to the addition of progestins. Cycloheximide (Calbiochem) was dissolved at a concentration of 20 mg/ml in water and filter sterilized. Actinomycin D (Merck) was dissolved at a concentration of 0.5 mg/ml in sterile water. Antibodies against phospho-ERK (extracellular-signal-regulated kinase) 1/2, total ERK and phospho-[RSK (ribosomal S6 kinase) 1 (Ser^{380})] (Cell Signaling Technology) were used at a dilution of 1:1000; α-tubulin (1:4000 dilution) and HA (haemagglutinin) (Sigma) (1:1000 dilution); PRA and PRB (1:200 dilution) and RSK1 (C-21) (1:1000 dilution) were from Santa Cruz Biotechnology and specific polyclonal antibody against the C-terminus of PFKFB3 (1:600 dilution) was obtained as described by Riera et al. [26]. Two rabbit polyclonal anti-[phospho-PFKFB3 (Ser^{461})] and anti-[phospho-PFKFB3 (Ser^{478})] antibodies were raised against the phosphorylated peptide RRN(Sp)VTP (corresponding to residues 458–464 of human PFKFB3 protein in which Ser^{461} was phosphorylated) and RIN(Sp)FEE (corresponding to residues 475–481 of human PFKFB3 protein in which Ser^{478} was phosphorylated) (1:200 dilution). Both antibodies were generated by subcutaneous immunization of white New Zealand rabbits with the indicated PFKFB3 C-terminal peptides conjugated to keyhole limpet haemocyanin.

Cell culture

The T47D, MCF7, MDA-MB-231 and TYML cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) (Biological Industries) supplemented with 10 % heat-inactivated FBS (fetal bovine serum) (Biological Industries), 100 units/ml penicillin/streptomycin, 0.1 mM sodium pyruvate and 0.2 mM L-glutamine and incubated in a humidified atmosphere of 5 % CO_2 at 37 °C.

TYML cells [a PR-negative clonal derivative cell line of T47D harbouring one integrated copy of the progestin-responsive MMTV (murine mammary tumour virus)-luciferase reporter] expressing the WT (wild-type) PRB, or a PRB mutant in the DBD (DNA-binding domain) from the retroviral vector pRAV-FLAG, or containing the empty vector (control, Ø) have been described previously [16]. Briefly, the PRB-DBD mutant carried the triple mutation G584E/S585G/V589A in the P-box at the first PR zinc-finger involved in direct contact with DNA specific PRE sequences. ChIP (chromatin immunoprecipiation) experiments in TYML-derived cell lines have shown that PRB-DBD was drastically affected with regard to its ability to interact with the integrated MMTV promoter, as the binding region to the PRE promoter sequences was mutated [16]. From now on we have named these cells WT-PRB, mutant-DBD and empty.

For experiments involving progestins, cells were starved for 24 h in serum-free DMEM supplemented with penicillin/streptomycin, sodium pyruvate and L-glutamine prior to hormone treatment. Then cells were treated with ORG2058 (10 nM), NG (norgestrel; 100 nM) or vehicle (ethanol for ORG2058 and chloroform for NG) for different times. Where indicated, RU486 (100 nM), ICI182780 (10 μM), PD98059 (50 μM), U0126 (5 μM) and BI-D1870 (10 μM) were also added 30 min before hormone induction.

Western blot analysis

Cells were washed twice in ice-cold PBS and lysed with 0.3 % CHAPS, 10 mM Tris/HCl, pH 7.5, and 100 mM NaCl supplemented with protease [1 mM PMSF (Roche), 0.1 μg/ml benzamidine (Sigma), 5 μg/ml leupeptin (Sigma) and 1 μg/ml pepstatin A (Sigma)] and phosphatase [1 mM NaF (Sigma), 0.01 M 2-glycerophosphate (Sigma) and 0.2 mM sodium orthovanadate (Calbiochem)] inhibitors. Cells were scraped and heated to 95 °C for 10 min. Protein extracts were resolved on SDS/PAGE (10 % gels) under reducing conditions and then transferred on to nitrocellulose membranes (Millipore) and subjected to Western blotting using the above indicated antibodies at 1:1000 dilution {except anti-PFKFB3 at 1:600, anti-PR at 1:200, anti-[phospho-PFKFB3(Ser^{461})] and anti-[phospho-PFKFB3(Ser^{478})] at 1:200, and anti-α-tubulin at 1:4000}. Immunocomplexes were visualized with a horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibody (1:10 000 dilution) followed by incubation with ECL (enhanced chemiluminescent) Western blot reagent (Biological Industries).

Fru-2,6-P_2 determination

Cells were washed twice in ice-cold PBS and lysed with 100 mM NaOH plus 0.1 % Triton X-100. Fru-2,6-P_2 was determined following the method described previously [21,35]. Protein concentration was determined by the Bradford-based Bio-Rad Laboratories Assay. In all Figures, data are expressed as the fold change against basal conditions (means $\pm$ S.E.M., $n = 3$ for each condition) ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, determined by Student's t test).

Lactate determination

Lactate was measured spectrophotometrically from 1 ml of supernatant from cells treated for 8 h with the correspondant treatment by using standard enzymatic methods [36].

RT-qPCR (quantitative real-time PCR)

RT-qPCR was performed on RNA extracts from T47D, MCF7, MDA-MB-231 and TYML cells. Total RNA was prepared from cultured cells under progestin stimulation for different times. RNA was isolated according to the manufacturer's protocol (Ultraspec RNA Biotecx Laboratories). The concentration and purity of all RNA samples was determined using NanoDrop and formaldehyde gel electrophoresis. Total RNA (2 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with random primers. *PFKFB3* was specifically amplified by RT-qPCR using the probe/primer set (Hs00190079_m1) for human *PFKFB3* (Applied Biosystems). The threshold cycle number (C_t) was obtained. The relative expression of each gene was normalized to that of the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (probe/primer: Hs99999905_m1), and gene expression in each sample was then compared with expression in basal conditions (each cell line under progestin treatment for 0 h).

Microarray hybridization

For the microarray experiment, T47D derivative cells were grown in RPMI 1640 medium, supplemented with 10 % (v/v) FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Serum-containing medium was replaced with serum-free medium 48 h before the addition of hormone. Cells were untreated or treated with 10 nM R5020 for 6 h, in duplicate, and RNA was extracted. Procedures for microarray hybridization and data analysis have been described previously [36a]. Microarray data are available at GEO (Gene Expression Omnibus) with accession number GSE25077 (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi).

Plasmid constructs

Constitutively active 3× FLAG–RSK1 was a gift from Dr J.L. Maller (Department of Pharmacology, University of Colorado Denver, Aurora, CO, U.S.A.) [37].

To study the promoter regions, we used the recombinant plasmids PFKFB3/−3566 and the deleted constructs PFKFB3/−2494 and PFKFB3/−938 of the *PFKFB3* gene promoter cloned in pGL2basic (Promega) using the luciferase gene as reporter. PRB plasmid was provided by Dr M. Beato (Centre for Genomic Regulation, Pompeu Fabra University, Barcelona, Spain). A pGL2-basic vector with the c-*fos* minimal promoter unit served as the basis for reporter constructs PRE-WT-cfos and PRE-Mut-cfos. The fragment of 29 nt of *PFKFB3* promoter (from −3514 to −3486) with the sequence 5′-AACCACTCCAC**TGTCCT**-CTCAAGGCTGGA-3′ (containing the putative PRE marked in bold type) was subcloned to obtain the PRE-WT-cfos construct. The same 29 nt fragment with a mutation in three base pairs of the PRE-binding site (underlined) (5′-AACCACTCCAC-GGGACTCTCAAGGCTGGA-3′) was used for the PRE-Mut-cfos construct. The identity of cloned products was confirmed by nucleotide sequence analysis. A plasmid that codes for green fluorescent protein was used to monitor transfection efficiency. HA-tagged PFKFB3 plasmid expression vector was generated to monitor PFKFB3 expression using the anti-HA antibody. Myc-tagged PFKFB3 plasmid expression vector was generated to immunoprecipitate PFKFB3 using anti-Myc antibody. Expression vectors coding for isoform A of PR, WT isoform B of PR and mutant DBD isoform B of PR were used to transiently transfect cells.

Immunoprecipitation and kinase assay

For immunoprecipitation assays, HEK (human embryonic kidney)-293 cells were transiently transfected with 3× FLAG–RSK1 or Myc–PFKFB3. Cells were washed twice in ice-cold PBS and lysed on ice with 1 ml per 10 cm dish of ice-cold buffer (10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.3 % CHAPS, 50 mM NaF, 50 mM 2-glycerophosphate, 200 μM Na_3VO_4, 100 μM PMSF, 1 μM pepstatin A and 1 μg/ml leupeptin). Lysates were pre-cleared by centrifugation at 15000 ***g*** for 10 min at 4 °C and equivalent protein amounts (500–700 μg) wer incubated overnight at 4 °C with anti-FLAG or anti-Myc monoclonal antibodies. Immune complexes were collected with Protein G–Sepharose (GE Healthcare) and washed four times in lysis buffer and twice in kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol and 5 % glycerol). Washed immunoprecipitates were used for *in vitro* kinase assays in kinase buffer containing 50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol and 200 μM ATP supplemented with 1 μM ATP and 3 pmol of [γ-^{32}P]ATP at 30 °C for 40 min. Reactions were stopped by addition of SDS sample buffer and boiled for 5 min. The reaction mixture was separated by SDS/PAGE and analysed by autoradiography.

Transfections and luciferase assays

Transfections were performed using Lipofectamine™ 2000 (Invitrogen) following the supplier's protocol. The different promoter/reporter fusion plasmids (6 μg) and 0.5 μg of the pSV40-β-galactosidase control vector (Promega) were co-transfected into 10-mm-diameter dishes containing 80 % confluent cells. At 4 h later, the cells were washed twice in PBS and maintained in complete medium. At 4 h later, the cells were distributed into 24-well plates and maintained overnight in complete medium. After 24 h of starvation, cells were treated with progestins. At the times indicated, the cells were washed twice in PBS and lysed in buffer. Luciferase activity was measured in supernatant extracts 16 h later. Co-transfection with pSV40-β-galactosidase plasmid DNA was carried out to normalize transfection efficiencies in the transfectants. Transfections were performed at least in triplicate, and the individual values were averaged to give the result of one experiment. Luciferase activity was measured in a TD 20/20 luminometer (Turner Designs). β-Galactosidase activity was determined in 6 μl of cell extract using the luminescent β-galactosidase detection kit II (Clontech). The data are presented as the means ± S.E.M. At least three separate experiments were performed with each plasmid DNA preparation.

Co-transfection experiments using PFKFB3/−3566 and PR plasmid expression vector constructs were performed in MDA-MB-231 cells using various amounts of each plasmid, such that all cells received a total of 12 μg plasmid DNA.

ChIP

ChIP assays were performed as described previously [16]. Briefly, chromatin from T47D and TYML cells expressing FLAG-tagged WT or mutant PRB, cultured and treated as described previously [16], was used. Chromatin was routinely sonicated to an average fragment size of 400–500 bp, in a Branson Sonifier (eight cycles of 15 s sonication with 30 s rest time at 4 °C). Rabbit or mouse IgG (Sigma) was used as a control for non-specific interaction of DNA. To determine the linear range of the amplification, different numbers of cycles and dilution series of input DNA were used for PCR analysis of each amplicon. Input was prepared with 10 % of the chromatin material used for an immunoprecipitation.

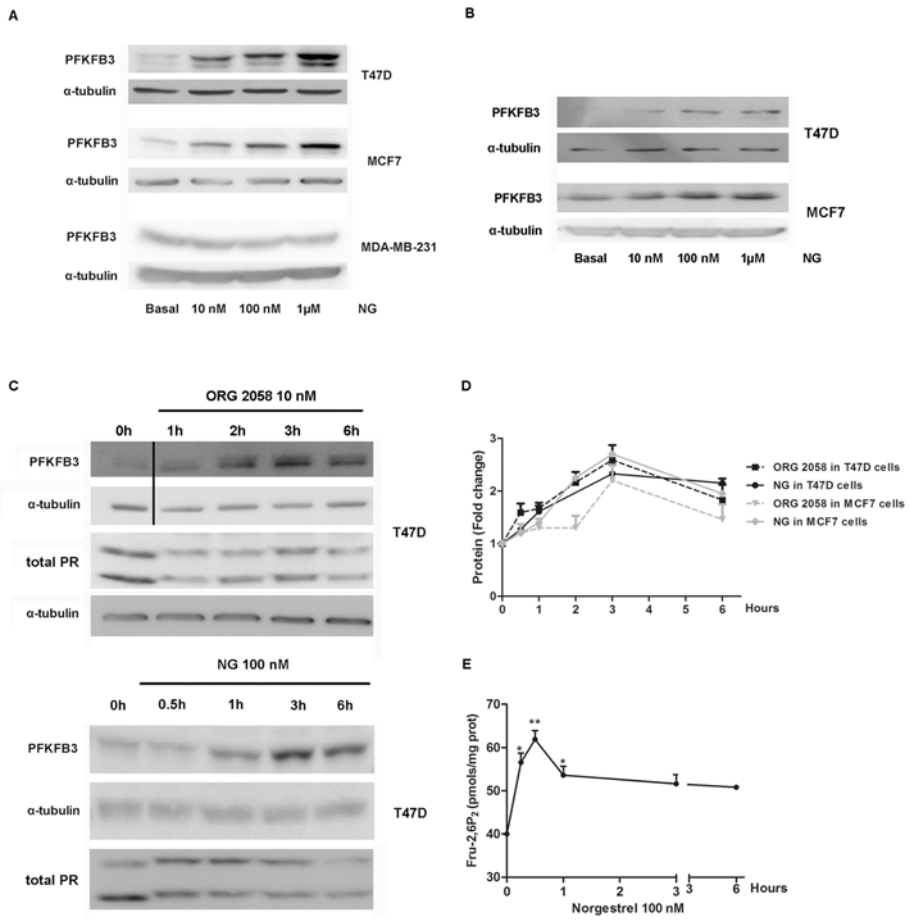

Figure 1 Dose- and time-response treatment experiments with progestins in T47D, MCF7 and MDA-MB-231 breast cancer cell lines

(**A**) T47D, MCF7 and MDA-MB-231 cells were treated with different concentrations of ORG 2058 or vehicle for 5 h. (**B**) T47D and MCF7 cells were treated with different concentrations of NG or vehicle for 5 h. (**C**) Serum-starved T47D cells were stimulated with ORG 2058 (10 nM) and NG (100 nM) or vehicle for the indicated time periods. Antibodies directed against total PFKFB3, total PR and α-tubulin (loading control) were used as indicated. A black line represents separate blots. (**D**) Summary of Western blot densitometries in T47D and MCF7 cell lines treated with ORG 2058 and NG or vehicle for 0, 0.5, 1, 2, 3 and 6 h. The charge was normalized with monoclonal α-tubulin antibody. (**E**) Fru-2,6-P_2 concentration in T47D cells treated for 0, 15, 30, 60 and 180 min with NG 100 nM or vehicle. Data represent the fold change against 0 h (basal conditions) (means $\pm$ S.E.M., $n = 3$ for each condition) ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, determined by Student's *t* test).

Later, a 1:10 dilution of input material was performed before PCR amplification. The human β-globin gene amplicon was used as a loading control, detecting non-specific binding of genomic DNA to beads or IgGs. The number of PCR amplification cycles was 39 for *PFKFB3*, 33 for *HSD11B2* (11β-distal A) and 36 for β-globin. PCR products were resolved in agarose gel. Primer sequences and specific PCR conditions are available on request.

RESULTS

Progestin induction of PFKFB3 expression in breast cancer cell lines

Three different breast cancer cell lines were examined: T47D (containing high levels of PR), MCF7 (containing average PR levels) and MDA-MB-231 cells (without PR) [38]. Cultured cells were incubated with a range of concentrations of the synthetic progestin ORG 2058 for 5 h. PFKFB3 protein induction was detected at 10 nM ORG 2058 treatment in T47D and MCF7 cell lines. In contrast, we did not detect PFKFB3 progestin stimulation in the PR-negative MDA-MB-231 cells at any of the ORG 2058 doses tested (Figure 1A). NG, another synthetic progestin, had the same effect on PFKFB3 protein induction, as shown in Figure 1(B). To determine the time dependency of progestin stimulation of PFKFB3 protein, cells were incubated at various time points after ORG 2058 and NG treatments. A significant increase in total PFKFB3 protein was observed at 3 h after treatment with ORG2058 or NG in T47D cells (Figure 1C). Similar results were obtained with both treatments in the MCF7 cell line. Figure 1(D) shows a summary of Western blot densitometries in T47D and MCF7 cell lines treated with either of these progestins. To confirm the physiological role of PFKFB3 induction, we analysed whether the Fru-2,6-P_2 concentration changed accordingly (Figure 1E). Surprisingly, an early peak of Fru-2,6-P_2 was observed at 30 min of progestin treatment, which persisted for 6 h. Since the increase in PFKFB3 protein content was undetectable at 30 min after treatment, an early event must be responsible for the effect of progestin on Fru-2,6-P_2 concentration.

PFKFB3 is phosphorylated by progestin signalling pathway activation

To investigate the mechanisms underlying the short-term effects of progesterone on Fru-2,6-P_2 concentration, we first analysed the activation of the PR signalling pathway. MAPK activation by progestin in T47D cells can be detected experimentally by

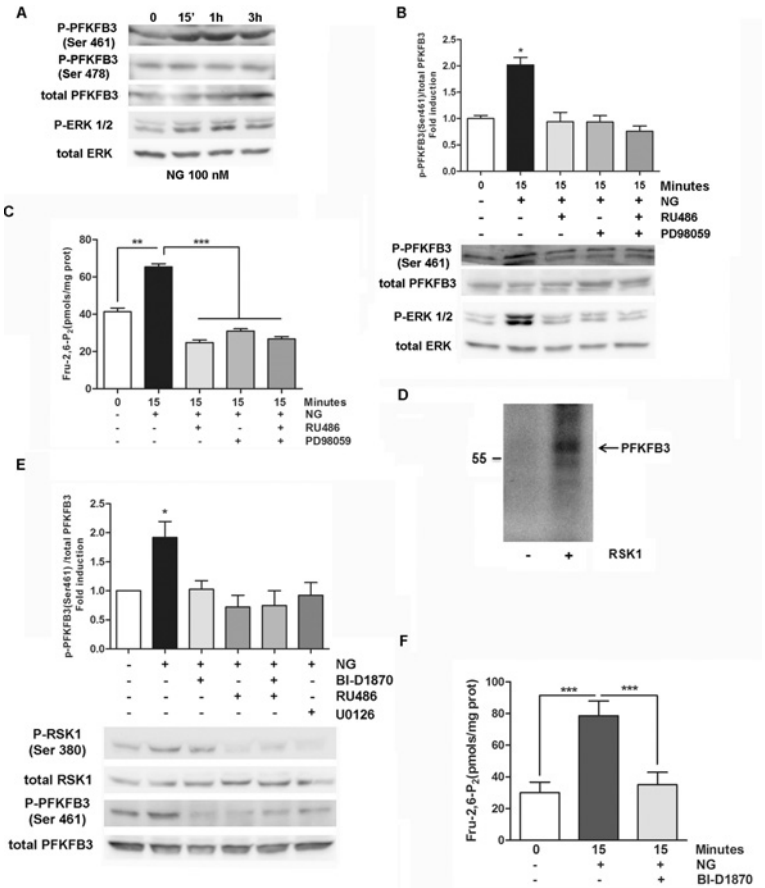

Figure 2 Short-term regulation of PFKFB3 by progestins

(**A**) Time-course analysis of PFKFB3 phosphorylation at Ser^{461} in response to NG. Cells cultured in serum-free medium for 24 h were left untreated (0) or treated with 100 nM NG for the indicated time periods. (**B**) As indicated, serum-starved T47D cells were stimulated for 15 min with NG 100 nM or treated with RU486 and PD98059 or vehicle for 30 min before the stimulation of cells with the hormone. The graph represents the densitometry of three independent experiments expressed as the fold change against basal conditions (means ± S.E.M., $n = 3$ for each condition) ($^{*}P < 0.01$, determined by Student's t test) (**C**) Fru-2,6-P_2 concentration at 0 or 15 min. Results represent the fold change against 0 h (basal conditions) (means ± S.E.M., $n = 3$ for each condition) ($^{**}P < 0.01$, $^{***}P < 0.001$, determined by Student's t test). (**D**) *In vitro* kinase assay using immunoprecipitated PFKFB3 which was incubated with constitutively active RSK1 in the presence of [γ-^{32}P]ATP and visualized by SDS/PAGE and autoradiography. The arrow indicates the band corresponding to PFKFB3 phosphorylation. (**E**) As indicated, serum-starved T47D cells were stimulated for 15 min with 100 nM NG or treated with BI-D1870, RU486 and U0126 or vehicle for 30 min before the stimulation of cells with the hormone. Antibodies against phospho (P)-RSK1 (Ser^{380}), total RSK1, phospho-PFKFB3 (Ser^{461}), total PFKFB3, phospho-ERK and total ERK were used as indicated. In all cases, the graph represents the densitometry of three independent experiments expressed as the fold change against basal conditions (means ± S.E.M., $n = 3$ for each condition) ($^{*}P < 0.01$, determined by Student's t test). (**F**) Fru-2,6-P_2 concentration at 0 or 15 min in T47D treated as indicated in Figure 2(E). The results represent the fold change against 0 h (basal conditions) (means ± S.E.M, $n = 3$ for each condition) ($^{***}P < 0.001$, determined by Student's t test). The representative Western blot (**A**) is a composite of a larger gel in which all of the samples were run simultaneously together. The composite was made by splicing complete lanes together for presentation purposes.

examining phosphorylation of ERK1/2 shortly after hormone treatment [3,9]. We confirmed ERK phosphorylation in T47D cells by Western blot analysis 15 min after NG administration (Figure 2A). At the same time, we analysed PFKFB3 phosphorylation using two phospho-specific antibodies that recognize PFKFB3 when it is phosphorylated at Ser^{461} or Ser^{478}. As shown in Figure 2(A), PFKFB3 is also robustly phosphorylated at Ser^{461}, whereas phosphorylation at Ser^{478} remains unaffected. If the covalent modification of PFKFB3 is a progesterone-receptor mediated event, it would be predicted to be inhibited by an antiprogestin, RU486, a competitive inhibitor of PR. To test this prediction, T47D cells were pretreated with RU486 and then stimulated with NG. Under these conditions, the hormone treatment did not promote Ser^{461} PFKFB3 phosphorylation (Figure 2B). As expected, ERK phosphorylation was also abolished by RU486 (Figure 2B). To confirm the involvement of the ERK signalling pathway in PFKFB-Ser^{461} phosphorylation, T47D cells were pretreated with the MEK1/2 (MAPK/ERK kinase) inhibitor PD98059 or U0126 before stimulation with NG. Ser^{461} phosphorylation was strongly reduced by PD98059 (Figure 2B) and U0126 (Figure 2E) at a concentration that blocked ERK1/2 phosphorylation (Thr^{202}/Tyr^{204}).

To further explore the phosphorylation of Ser^{461} of PFKFB3 by the ERK signalling pathway, we attempted to discern the kinase responsible for the covalent modification of PFKFB3. As Ser^{461} of PFKFB3 is not an ERK site (SP/TP), we searched for kinases downstream of ERK which could explain the phosphorylation of the enzyme. P90RSK is a protein kinase activated by ERK in response to mitogens and growth factors. The region around the residue corresponding to Ser^{461} of PFKFB3 has a suitable consensus site for phosphorylation by p90RSK (Arg/Lys-X-Arg-X-X-Ser/Thr or Arg-Arg-X-Ser/Thr [39]). Keeping this in mind, we examined whether PFKFB3 was a direct substrate of RSK by *in vitro* phosphorylation assays. PFKFB3 was phosphorylated by RSK1 (Figure 2D). Next, we explored whether p90RSK was directly related with endogenous PFKFB3 phosphorylation

at Ser[461] after NG stimulation. To test this, T47D cells were pretreated with the ATP-competitive inhibitor of the N-terminal AGC kinase domain of RSK, BI-D1870 [40], and then stimulated with NG. In these conditions, progestin treatment did not promote Ser[461] PFKFB3 phosphorylation (Figure 2E). Progestin increase of Fru-2,6-P_2 concentration after 15 min of hormone treatment was also abolished by BI-D1870 (Figure 2F). Altogether, these results suggest that the kinase downstream of ERK signalling pathway, p90RSK, is involved in the phosphorylation of PFKFB3 at Ser[461] in this system.

Progestin activation of *PFKFB3* transcription is mediated by PR and requires the distal −3566/−2494 *PFKFB3* promoter region

To examine whether PR was also directly involved in increasing PFKFB3 protein levels at later times, the PR antagonist RU486 was used. T47D or MCF7 cells were treated in serum-free medium for 3 h with 10 nM ORG 2058 or 100 nM NG and 100 nM RU486 or 1 μM RU486 respectively, either alone or in the presence of the inhibitor. The enrichment of total PFKFB3 protein levels due to progestin treatments was almost completely abolished when cells were pretreated with RU486 (Figure 3A). Fru-2,6-P_2 concentration was significantly increased after 3 h of progestin treatment, but remained at basal levels in the presence of RU486. In addition, the involvement of ERK1/2 in this delayed progestin response of PFKFB3 was tested by pretreating cells with the inhibitor PD98059 before the stimulation with either ORG2058 or NG. As shown in Figure 3(B), PD98059 maintained Fru-2,6-P_2 at basal levels in both T47D and MCF7 cells.

As shown in Figure 3(C), lactate measurements in T47D cells after NG treatment increased significantly (2-fold induction). Similar results were obtained in measurements performed in a cell system overexpressing PRB (PRB WT TYML cells), whereas no changes compared with basal levels were detected after NG stimulation when a PR-negative system was used (empty TYML cells). Furthermore, pre-treatment of cells with RU846 and PD98059 abrogated the increase in lactate concentration due to progestin stimulation in T47D and PRB WT TYML cell lines.

To explore whether this increase in total PFKFB3 protein concentration, seen after 3 h of progestin treatment, was due to modifications in *PFKFB3* mRNA levels, we performed reverse transcription followed by RT-qPCR using a specific probe against human *PFKFB3* cDNA. *PFKFB3* transcript accumulation in T47D and MCF7 cells was examined at different time points for up to 12 h after NG addition (Figure 4A). In T47D cells, *PFKFB3* mRNA increased more than 2-fold within 1 h and a similar pattern was seen in MCF7 cells (Figure 4A). In addition, in a microarray data set generated by treatment of T47D cells with the progestin R5020, PFKFB3 expression was induced 3.5-fold by the hormone (Figure 4B) (Microarray data are available at GEO with accession number GSE25077; M. Sancho and A. Jordan, unpublished results). Under the same conditions, PFKFB1 and PFKFB2 expression was not induced and PFKFB4 was only slightly induced (1.45-fold) by hormonal treatment.

We next investigated the mechanism by which progestin regulates *PFKFB3* mRNA expression. First, T47D cells were treated with NG in the presence of the protein synthesis inhibitor cycloheximide. As shown in Figure 4(C), cycloheximide did not block the progestin-mediated induction of *PFKFB3* mRNA. Therefore it seems that *PFKFB3* gene transcription induction is due to the direct effects of PR and does not require *de novo* protein synthesis. Treatment of cells with actinomycin D abolished *PFKFB3* mRNA NG induction (Figure 4C).

PFKFB3 transcription was also analysed in the presence of the PR antagonist RU486 before NG stimulation. Figure 4(D) shows that RU486 blocked *PFKFB3* progestin-induced transcription, whereas treatment of the cells with this inhibitor did not influence basal *PFKFB3* transcription (results not shown).

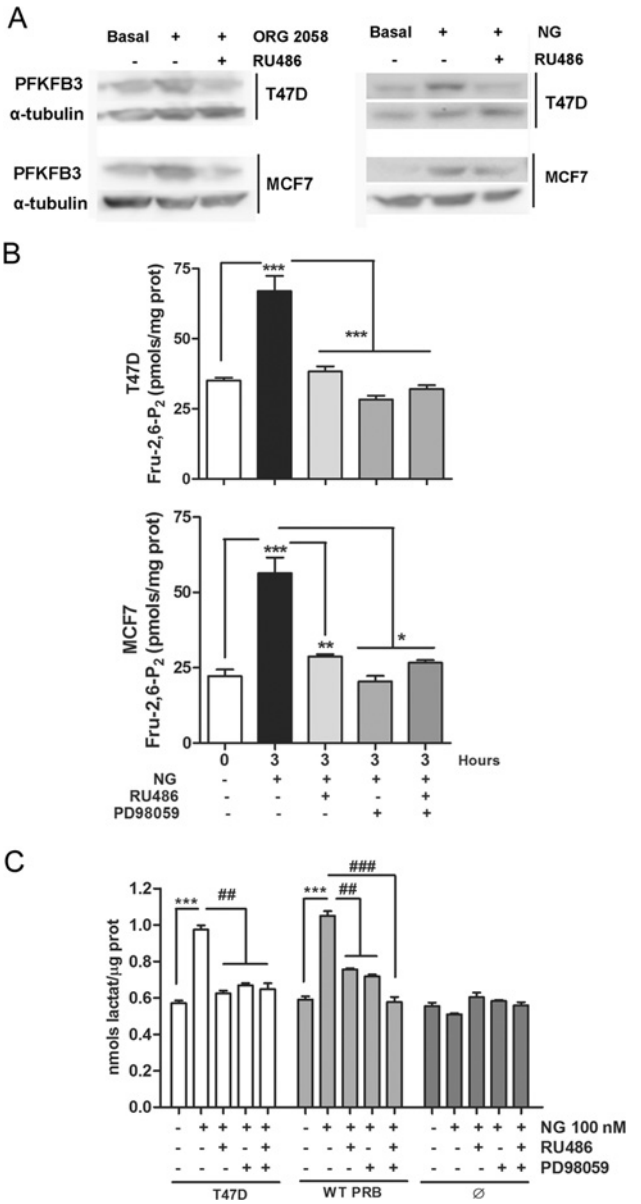

Figure 3 Long-term effects of progestin induction of PFKFB3 are mediated by PR

(**A**) Immunoblot analysis against total PFKFB3 in T47D and MCF7 cells at 3 h after the indicated treatments. α-Tubulin antibody was used as a loading control. (**B**) Fru-2,6-P_2 concentration determination at 3 h after the indicated treatments. The results were normalized to protein concentration and expressed as pmols of Fru-2,6-P_2/mg of protein. The results represent the fold change against 0 h (basal conditions) (means ± S.E.M., $n = 3$ for each condition) ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, determined by Student's t test). (**C**) Lactate measurements in T47D, WT-PRB TYML and empty TYML (Ø) cells. Lactate concentration was determined enzymatically 8 h after treatment with NG or vehicle and RU486 or PD98059 were added 30 min before the hormone treatment where indicated. The results were normalized to protein concentration and expressed as nmols of lactate/μg of protein. The results represent the fold change against 0 h (basal conditions) (means ± S.E.M., $n = 3$ for each condition) ($^{***}P < 0.001$, for NG-treated cells with respect to basal conditions and $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, for NG plus inhibitor-treated compared with NG-treated cells determined by Student's t test).

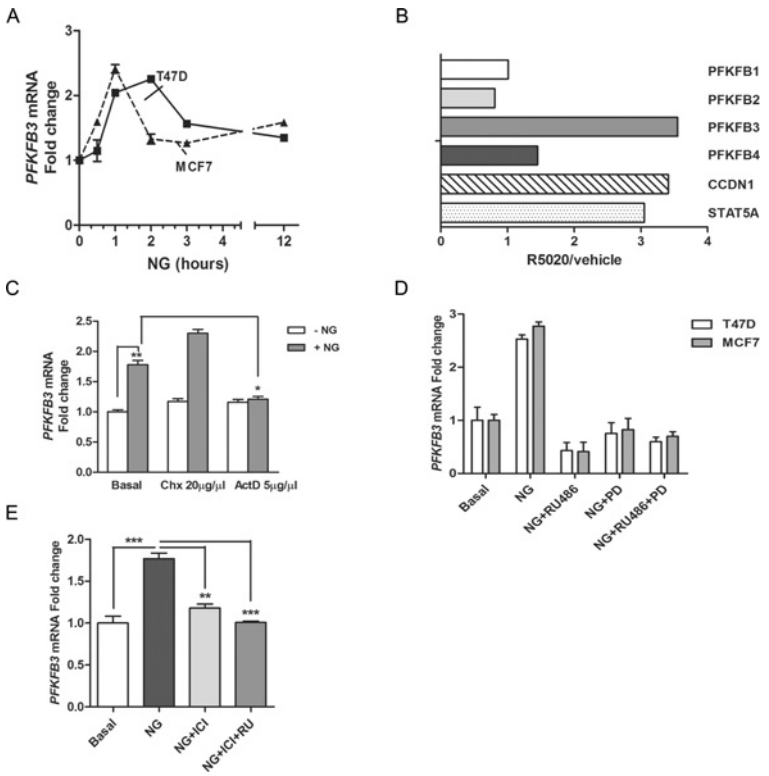

Figure 4 Effect of progestin treatment on the transcription of *PFKFB3* gene and its dependence on PR

(**A**) RT-qPCR with human *PFKFB3*-specific primers at different time points in T47D and MCF cells stimulated with NG (100 nM) or vehicle. (**B**) Induction of PFKFB isoform expression in response to progestin (10 nM R5020) treatment for 6 h of serum-starved T47D cells, extracted from a genome-wide microarray (Illumina 22k probes) data set (GSE25077). *CCDN1* and *STAT5A* gene induction is used for comparison. (**C**) RT-qPCR with human *PFKFB3*-specific primers of serum-starved T47D cells treated with cycloheximide (Chx; 20 μg/μl) or actinomycin D (ActD; 5 μg/μl) for 3 h and treated 30 min later with NG (100 nM) where indicated. (**D**) RT-qPCR with human *PFKFB3*-specific primers of serum-deprived T47D and MCF7 treated with NG (100 nM) or vehicle for 1 h. As indicated, RU486 and PD98059 (PD) were added 30 min before progestin addition. (**E**) RT-qPCR with human *PFKFB3*-specific primers of serum-starved T47D cells were treated with NG (100 nM) or vehicle for 1 h. When indicated, RU486 (RU; 100 nM) and ICI182780 (ICI; 10 μM) were added 30 min prior to hormone addition. In all conditions, *GAPDH* expression was measured for normalization and fold induction of *PFKFB3* mRNA of NG-treated cells with respect to vehicle is represented. All values represent means $\pm$ S.E.M. of three experiments (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, determined by Student's *t* test).

As has been described previously, induction of progesterone target genes in the T47D cell model depends on a transient activation of Src/p21Ras/ERK pathway that requires not only PR but also ER (oestrogen receptor) [3,10]. Thus, to probe whether progesterone-induced *PFKFB3* mRNA activation is dependent on ERK activation, we assayed the MEK1/2 inhibitor, PD98059, before NG stimulation. As shown in Figure 4(D), PD98059 inhibited the stimulatory effect of the agonist treatment. Similar behaviour was observed in cells treated with the oestrogen-specific antagonist ICI 182780 (Figure 4E), suggesting that the well-established ERK1/2 activation pathway by progestins also participates in *PFKFB3* mRNA induction.

To investigate further progestin activation of *PFKFB3* gene transcription, we attempted to discern the minimal promoter region required for hormone response. Different fragments of the *PFKFB3* human promoter, cloned in the pGL2 basic luciferase expression vector [27], were transiently transfected in T47D cells. The β-galactosidase expression vector was used to normalize transfection efficiencies. Luciferase activity was measured after 24 h of progestin treatment in serum-free medium. As shown in Figure 5(A), luciferase activity from cells transfected with the *PFKFB3*/–3566 construct and treated with progestin was significantly higher than that obtained with the shorter construct *PFKFB3*/–2494. These data suggest that the sequence from -3566 to -2494 of the human *PFKFB3* gene may contain a PRE. Detailed analysis with MatInspector program revealed a putative half-site PRE (TGTCCT) located near the position -3490 relative to the start site in the 5′-flanking region of the human *PFKFB3* promoter. Moreover, we assayed the effect of RU486 and PD98059 on the transient transfection of the *PFKFB3*/–3566 construct after NG treatments. As shown in Figure 5(B), both treatments with RU486 and PD98059 inhibitors maintained the luciferase activity of *PFKFB3* promoter at basal levels.

To determine unequivocally whether progesterone-mediated activation of the *PFKFB3* promoter is dependent on a putative PRE-binding site at position -3490 of the *PFKFB3* gene, we subcloned a 29-nt fragment around this promoter region into a c-*fos* minimal promoter unit in a luciferase reporter vector. The same fragment encompassing -3514 to -3486, relative to the transcription start site, was used to create the reporter constructs PRE-WT-cfos and PRE-Mut-cfos. The two constructs are identical except that the latter contains a mutation in three base pairs of the PR-binding site. To study enhancer activity, we measured relative luciferase units of T47D cells transfected with PRE-WT-cfos and PRE-Mut-cfos constructs; β-galactosidase expression vector was used to normalize transfection efficiencies, in the presence or absence of NG. As shown in Figure 5(C), a 7-fold increase was detected in the PRE-WT-cfos construct, which was inhibited by RU486, whereas the PRE-Mut-cfos had no effect following hormone treatment. WT and mutated constructs displayed similar basal reporter activities (results not shown).

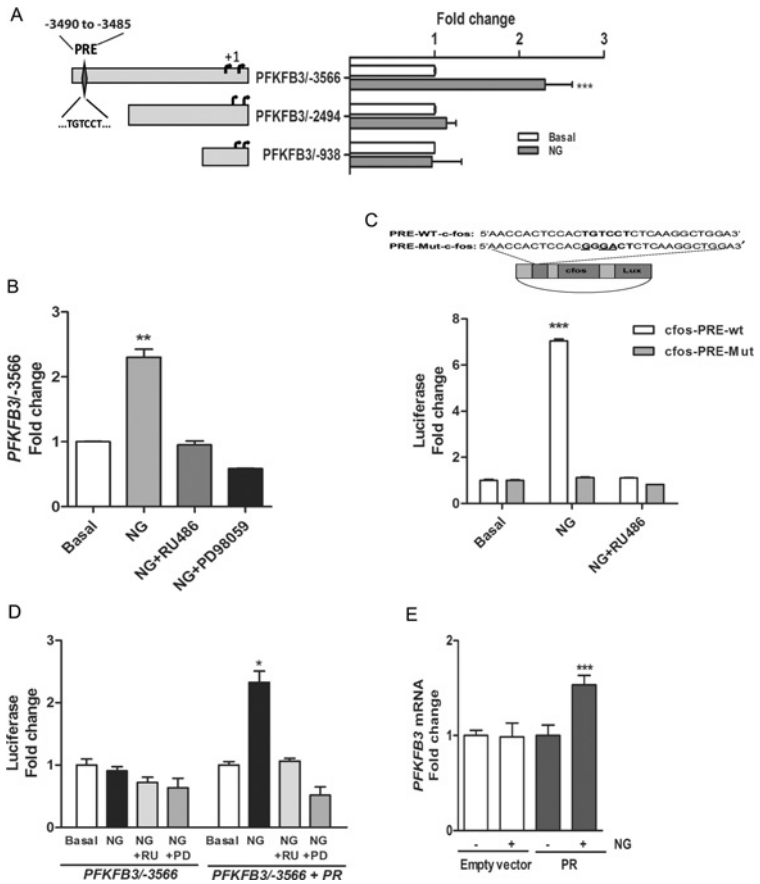

Figure 5 Promoter analysis of *PFKFB3*

(**A**) Luciferase expression plasmid constructs of the *PFKFB3* promoter transiently transfected in T47D cells after NG (100 nM) or vehicle treatment. (**B**) Luciferase expression of the promoter construct *PFKFB3*/–3566 in T47D after the indicated treatments with NG and inhibitors. (**C**) Scheme of the WT and mutated 29 nt region (–3514/–3486) subcloned as an enhancer in a luciferase reporter pGL2-basic containing the c-*fos* minimal promoter unit (pGL2-basic-cfos). PRE-WT-cfos and PRE-Mut-cfos resulting constructs are identical except that the latter contains a mutation in three base pairs of the PR-binding site (nucleotides underlined). The PRE sequence is indicated in bold. (**D**) MDA-MB-231 cells were co-transfected with the *PFKFB3*/ – 3566 promoter construct and PRB plasmid expression or empty vector. Luciferase expression was assayed after treatments with NG (100 nM), vehicle and/or inhibitors when indicated. PD, PD98059; RU, RU486. In all cases (**A**, **B**, **C** and **D**), luciferase activity and β-galactosidase activities were measured in cell extracts 16 h later. Fold induction of luciferase activity of NG-treated cells with respect to vehicle is represented. The values represent the means $\pm$ S.D. of three experiments performed in triplicate. (**E**) Gene expression measured by RT-qPCR with human *PFKFB3* specific primers in MDA-MB-231 cells after transfection with PRB plasmid expression or empty vector. *GAPDH* expression was measured for normalization and fold induction of *PFKFB3* mRNA of NG-treated cells with respect to vehicle is represented. In all cases, the values represent means $\pm$ S.E.M. of three experiments performed in triplicate. ($^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, determined by Student's *t*-test).

Moreover, after transfecting the *PFKFB3*/–3566 promoter fragment in MDA-MB-231 cells, which are characterized by the absence of PR, we did not observe any increase in the luciferase levels after NG treatment (Figure 5D). In contrast, co-transfection of the *PFKFB3* promoter (*PFKFB3*/–3566) together with a PR expression vector encoding PRB in MDA-MB-231 cells resulted in a 2.5-fold induction in luciferase activities after NG treatment. This activation was abolished by RU486 and also by PD inhibitors (Figure 5D). As expected, RT-qPCR analysis showed an increase of the relative amount of endogenous *PFKFB3* mRNA after NG stimulation in MDA-MB-231 cells containing the PR expression plasmid (Figure 5E).

Participation of PR in *PFKFB3* gene induction

To examine the role of the PR-mediated response in *PFKFB3* transcription, we used three stable TYML-derived cell lines, as described in the Experimental section, expressing WT-PRB, mutant-DBD and empty vector control [16]. First, we analysed the basal expression of *PFKFB3* in the three TYML cell lines by RT-qPCR. The results showed no differences between untreated cells (Figure 6A, first column). Using RT-qPCR, we observed a 6-fold increase in the expression of the *PFKFB3* gene 1 h after the addition of NG in WT-PRB cells, whereas no induction was observed in cells containing the empty vector (Figure 6A). Induction of *PFKFB3* mRNA by NG was significantly decreased in cells expressing the mutant-DBD cell line compared with WT-PRB cells. These results indicate that progestin-induced expression of *PFKFB3* depends on PRB. Treatment of WT-PRB and mutant-DBD cells with PR antagonist (RU486) before the stimulation with NG significantly reduced *PFKFB3* mRNA transcription (Figure 6A). A high concentration of RU486 (1 μM) completely abolished NG induction in WT-PRB cells (Figure 6B). As mutant-DBD responds to NG (2-fold increase) and this is partially abolished by RU486, some PR DBD-independent recruitment in a close region or the indirect participation of other stimulated transcription factor cannot be discarded. Additional support to these findings was provided by co-transfecting MDA-MB-231 cells with the *PFKFB3* reporter promoter (*PFKFB3*/–3566), together with different expression vectors encoding isoform A of PR (PRA), isoform B of PR (WT-PRB),

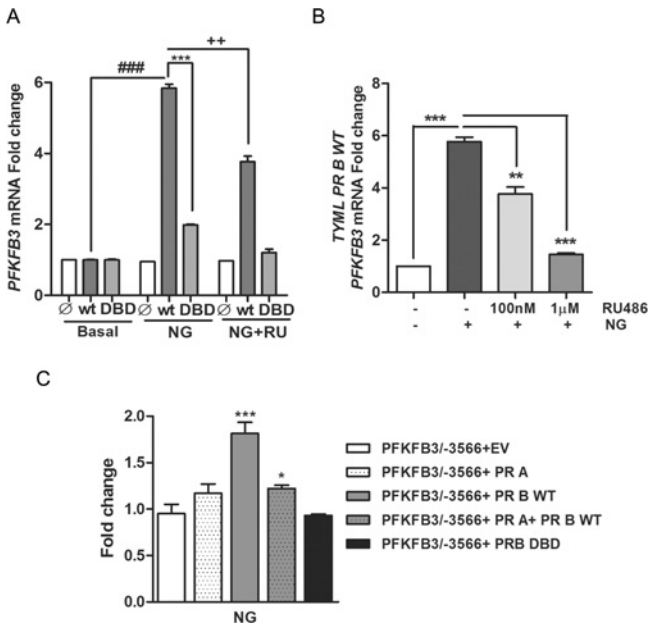

Figure 6 Study of NG response in breast cancer cell lines stably expressing human PRB WT and DBD mutant

(**A**) Serum-deprived TYML derived cells were stimulated for 1 h with NG (100 nM) or vehicle (∅). Where indicated, RU486 (RU) was added 30 min before hormone induction. Cells were harvested, RNA was extracted, and gene expression was measured by RT-qPCR with human *PFKFB3*-specific primers. *GAPDH* expression was measured for normalization. Fold induction of *PFKFB3* mRNA of NG-treated cells with respect to vehicle is represented. The values represent means ± S.E.M. of at least three independent experiments ($^{\#\#}P < 0.001$, for WT PRB NG-treated with respect to WT PRB basal; $^{***}P < 0.001$, for mutant DBD PRB NG-treated compared with WT PRB NG-treated and $^{++}P < 0.01$, for WT PRB NG plus RU486-treated relative to WT PRB NG-treated cells, determined by Student's *t* test). (**B**) Serum-starved WT PRB TYML cells were stimulated for 1 h with NG (100 nM) or vehicle. Where indicated, RU486 (100 nM or 1 μM) was added 30 min before progestin treatment. Cells were lysed, total RNA was extracted and mRNA levels of *PFKFB3* were measured by RT-qPCR and normalized to *GAPDH*. Fold induction of *PFKFB3* mRNA of NG-treated cells with respect to vehicle is represented. The results represent the average of three independent experiments ± S.E.M. ($^{***}P < 0.001$ and $^{*}P < 0.05$, determined by Student's *t* test). (**C**) MDA-MB-231 cells were transiently co-transfected with the promoter construct *PFKFB3*/–3566 and PRA, wt PRB, mutant DBD PRB, PRA and PRB together or empty vector (EV) expression vectors. Cells were cultured in serum-free medium and 24 h later were stimulated with NG (100 nM) or vehicle. Luciferase activity was measured in cell extracts 16 h later. Fold induction of luciferase activity of NG-treated cells with respect to vehicle is represented. The values represent the means ± S.E.M. of at least three experiments performed in triplicate ($^{***}P < 0.001$ and $^{*}P < 0.05$, determined by Student's *t* test).

mutant-DBD (DBD-PRB) or empty vectors. As shown in Figure 6(C), only the WT-PRB cotransfection resulted in significant luciferase increase (nearly 2-fold). Surprisingly, PRA did not enhance luciferase activity, nor did PRA and WT-PRB together. It has been described that PRA can act as an inhibitor of progesterone response [41]. The present results also support this hypothesis and may explain the finding that in WT-PRB TYML-derived cells we observed nearly 6-fold increase in *PFKFB3* mRNA levels after NG treatment, whereas in original T47D cells we only obtained a 2.5-fold increase. Furthermore, co-transfection of the PFKFB3/–3566 promoter construct with the mutant-DBD PRB did not induce luciferase activity (Figure 6C), which indicates that the contribution in the progestin responsiveness of each PR isoform is basically attributable to PRB and that the DBD is necessary for the progestin promoter responsiveness of *PFKFB3* gene.

PR binds to the PRE sequence located around the −3490 position in the *PFKFB3* promoter

To further characterize the regulation of *PFKFB3* gene expression by progestins, PR recruitment to the endogenous promoter was analysed by ChIP using an anti-PR antibody. ChIP analysis demonstrated PR recruitment at the region flanking the PRE (−3514 to −3462) of the *PFKFB3* promoter in response to R5020 treatment in T47D cells (Figure 7A). Similarly, Figure 7(B) shows ChIP analysis results using WT PRB and mutant DBD. As β-globin was used to normalize DNA amplification, a densitometric analysis was performed to quantify differences among samples after different times of hormone treatment (Figure 7C). The results showed increased recruitment of PR after 10 and 30 min of hormone treatment to the *PFKFB3* promoter in both T47D and WT-PRB cells, whereas the equivalent samples with the mutant DBD showed reduced recruitment. To confirm these results, ChIP experiments with two regions of the HSD11B2 promoter described previously as PR responsive were also analysed [14]. PFKFB3 behaviour is similar to that showed by the proximal region of *HSD11B2*, which is DBD-dependent. On the contrary, the distal region of *HSD11B2*, which has been probed as DBD-independent, presents similar levels of recruitment in WT PRB and mutant DBD cells. These results indicate that the ability of PR to bind DNA is required for its association with the *PFKFB3* promoter region flanking the suggested PRE.

DISCUSSION

Several years ago, *PFKFB3* was identified as a progestin-responsive gene [17] using a differential display technique in T47D breast cancer cells. We now report evidence that the *PFKFB3* gene is an early molecular target of progestins. Progestins utilize two separate mechanisms to regulate the PFKFB3 isoenzyme: an immediate early response through MAPK-dependent phosphorylation of PFKFB3 protein, and a second effect activating mRNA transcription via *cis*-acting sequences on the *PFKFB3* promoter.

We show that progestins upregulate *PFKFB3* in T47D and MCF7 PR-positive cell lines, whereas no response to these hormones was observed in the PR-negative MDA-MB-231 cell line, which shows that induction of *PFKFB3* depends on PR. Interestingly, an unexpected fast increase in Fru-2,6-P_2 concentration was observed 15 min after progestin addition. This early response correlates with the phosphorylation of the PFKFB3 isoenzyme. It has been described that PR ligand binding induces rapid and transient (5–10 min) activation of ERK1/2 MAPK signalling that is independent of PR functioning as a transcription factor [9]. The results from the present study show that ERK phosphorylation in T47D after NG treatment is accompanied by PFKFB3 phosphorylation on Ser^{461} and the consequent increase in Fru-2,6-P_2 concentration, which leads to an allosteric activation of PFK1 and a subsequent increase in the glycolytic flux [42]. Consistently, both effects are totally abrogated by the presence of PR- or ERK-specific inhibitors (RU486 or PD98059 and U0126 respectively). Furthermore, phosphorylation of PFKFB3 at Ser^{461} by AMPK (AMP-activated protein kinase) has been associated with an increase in the V_{max} value of the kinase activity [43], leading to an increased glycolytic rate and enhanced proliferation [42].

The C-terminal region around Ser^{461} of PFKFB3, also highly conserved in the PFKFB2 isoenzyme, contains two putative phosphorylation sites (at Ser^{461} and Ser^{478}) for multiple kinases [22]. The first serine residue can be phosphorylated by AMPK

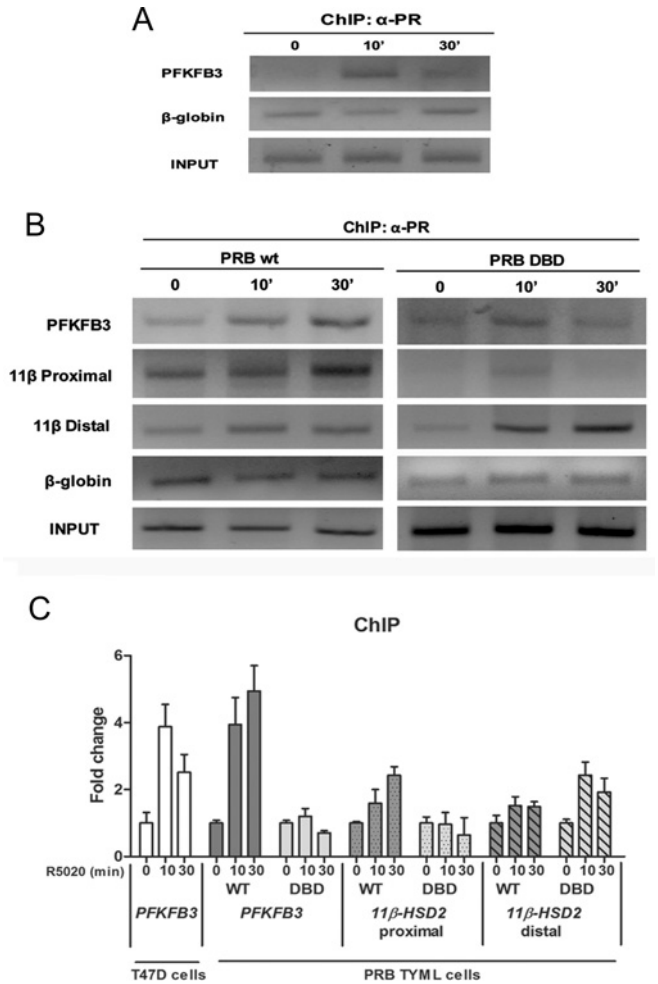

Figure 7 PR recruitment to the *PFKFB3* promoter after progestin induction

(**A**) ChIP analysis of PR recruitment to the *PFKFB3* promoter in response to progestin in T47D cells treated with R5020 at indicated times before chromatin preparation for ChIP. (**B**) ChIP analysis of PR recruitment to the *PFKFB3* promoter in response to progestin in WT PRB and mutant DBD PRB TYML cells treated with R5020 (10 nM) at indicated times before chromatin preparation for ChIP. In all cases, PR-containing chromatin fragments were immunoprecipitated with anti-PR antibody. The precipitated DNA fragments were subjected to PCR with primers for *PFKFB3* (to specifically amplify the flanking sequence region around the PRE at -3514 in *PFKFB3* promoter); proximal and distal-PRE flanking sequence of *HSD11B2*, and β-globin were used as controls followed by ethidium bromide-stained 2 % agarose gel loading. Amplification of input DNA (representing 1 % of immunoprecipitated material) is shown for comparison. (**C**) The level of amplified DNA was quantified by densitometric scanning of stained gels and corrected for the amount of β-globin DNA amplification. Values are expressed as fold change with respect to basal condition (at zero time). The results represent means $\pm$ S.E.M. from three independent experiments.

in both isoenzymes [42–44]. Other kinases can phosphorylate PFKFB2, such as PKB (protein kinase B) [45] and PKC (protein kinase C) [46], and it is also phosphorylated in response to insulin [22,47]. However, PFKFB3 phosphorylation by kinases other than AMPK has not been described [22]. In the present study we provide a mechanism by which PR signalling through the ERK/RSK pathway could activate glycolysis by direct phosphorylation of PFKFB3 at Ser^{461} as an early event. Although AMPK phosphorylation of PFKFB3 in hypoxic or ischaemic microenvironments would represent a protective mechanism for metabolically depressed or ATP-deficient cells, the PFKFB3 phosphorylation induced by progestins would reinforce the activation of glycolysis.

The blocking of *PFKFB3* mRNA after progesterone induction with the PR antagonist (RU486) or steroid inhibitor (ICI182780) and MEK-specific inhibitor (PD98059) reveals a similar behaviour that it has been described previously for the MMTV promoter gene. Vicent et al. [3,10] demonstrated that binding of progestin to PR/ER complexes activates the Src/Ras/ERK pathway, leading to the accumulation of phosphorylated ERK in the nucleus. As demonstrated for the MMTV promoter, only the complex composed of PR phosphorylated by phospho-ERK and phospho-MSK1 (mitogen- and stress-activated kinase 1; also phosphorylated by phospho-ERK) is recruited to the promoter, activating gene transcription. Additionally, a non-productive complex has been observed in the absence of activation of ERK and MSK due to the binding of non-phosphorylated PR homodimers to the corresponding hormone-responsive elements [3,10].

PFKFB3 is a gene that depends on both PR action mechanisms, i.e. it requires activation of a signalling pathway initiated by the interaction between PR and ER and direct binding of PR to DNA, as occurs for the MMTV model promoter. The majority of progestin-induced genes fall into this category [16].

The *PFKFB3* gene contains multiple copies of the AUUUA sequence in the 3′-untranslated region. The AUUUA motif is typical of proto-oncogenes and pro-inflamatory cytokines and confers instability and enhanced translational activity [20]. In the light of these data, we studied changes in *PFKFB3* mRNA in T47D cells in the presence of actinomycin D. The use of this transcription inhibitor did not affect the mRNA levels after long incubations (results not shown), and no changes in mRNA levels were detected with actinomycin D in the presence or absence of NG. Given that NG increases *PFKFB3* mRNA levels as early as 1 h after treatment, and the inability of cycloheximide to block this increment, we suggest that the *PFKFB3* gene might be a direct transcriptional target of PR. Bearing in mind that the effects of progestin on *PFKFB3* transcription are independent of mRNA stability and *de novo* protein synthesis, we focused on the gene promoter. Sequence analysis revealed a putative half-site PRE (TGTCCT) located near to the position -3490 relative to the transcription start site in the 5′-flanking region of the human *PFKFB3* promoter. In promoter assays we observed a significant increase in luciferase activity with transient transfections of *PFKFB3*/–3566 promoter fragment in T47D, not observed in PR-negative MDA-MB-231, which emphasizes the role of PR in the transcriptional regulation of the gene. Co-transfection of MDA-MB-231 with a PRB plasmid expression together with *PFKFB3*/–3566 increased *PFKFB3* promoter response. Confirmation of the direct implication of the PRE consensus binding site at position -3490 comes from transfection experiments using PRE-WT-cfos and PRE-Mut-cfos constructs of the human *PFKFB3* promoter in the T47D cell line, showing progestin response in the WT construct, whereas PRE-Mut-cfos had no effect. This is also the case for other genes for which progestins act as ligand-activated transcription factors, such as c-*myc* [48]. Additional evidence of the progestin effects was seen by microarray hybridization in T47D derivative cells. The *PFKFB3* gene was induced by the hormone, similar to other typical progestin-responsive genes. To study further the implication of PR in the progestin response of *PFKFB3*, we used TYML cell lines stably expressing FLAG-tagged WT-PR and the mutant-DBD PRB. Progestin treatment of TYML cells expressing the mutant-DBD failed to activate *PFKFB3* expression to the levels observed in WT-PRB. Luciferase reporter assays performed in MDA-MB-231 cells with *PFKFB3* promoter together with WT-PRB, PRA and mutant-DBD showed that PR binding to the promoter is essential to positive gene regulation. Finally, PRA seems to exert an inhibitory effect over the action of PRB in MDA-MB-231, as previously

reported, showing that the two PR isoforms regulate the same gene in a different way [7]. ChIP analysis demonstrated the binding of PR to the identified PRE sequence present in the *PFKFB3* promoter.

The specific role of induction or activation of PFKFB3 isoenzyme by progestins must be related with its key function on PFK-1 stimulation. PFK-1 is mainly inactive in the absence of allosteric modulators, and the main role of Fru-2,6-P_2 is to relieve its ATP inhibition, allowing glycolysis to proceed [21]. The results now presented show that Fru-2,6-P_2 as well as lactate concentrations increase after progestin treatment. These results suggest PFKFB3 as being the link of progestin action with the glycolytic pathway. The *PFKFB3* gene product is present in proliferating cells [19,28–31], transformed cells [17,20,26,27,32] and in various solid tumours and leukaemias [33,34], and the inhibition of its expression reduces cell proliferation and tumour growth in animal models [30,32]. The PFKFB3 isoenzyme is degraded through the ubiquitin-proteasome proteolytic pathway [49], through the E3 ubiquitin ligase APC/Cdh1 (anaphase-promoting complex/cyclosome) [31], linking cell-cycle progression and the metabolic response that underpins it. Furthermore, the PFKFB3 isoenzyme was found to be regulated through the PI3K (phosphoinositide 3-kinase)/Akt/mTOR (mammalian target of rapamycin) pathway [29] and to be localized to the nucleus of several transformed cells, where it regulates proliferation via cyclin-dependent kinases [50]. Taken together, these results indicate that Fru-2,6-P_2 is essential to the maintenance of glycolytic flux necessary for providing energy and biosynthetic precursors to dividing cells.

The finding that the *PFKFB3* gene is overexpressed in different transformed cells and tumours, and activated by hypoxia, progestins and/or oncogenes, indicates that it has an essential role in the glycolytic phenotype of cancer cells, facilitating the adaptation and survival of tumour cells in their hypoxic or energy-deficient microenvironment [17,24,30].

The dependence of cancer cells on survival mechanisms that are coupled to energy mechanisms suggests several potential therapeutic avenues. Transformed cells may be particularly sensitive to inhibition of glycolytic flux when oxygen and glucose limitations are imposed by surrounding cells. This has been revealed in experiments using PFKFB3 inhibitors [30] or inhibiting *PFKFB3* gene expression [30,32]. Importantly, these studies suggest that selective depletion of Fru-2,6-P_2 in cancer cells may suppress glycolytic flux and decrease their survival and growth.

In conclusion, the results from the present study are compatible with a model of high maintenance of glycolysis in breast cancer cells where the participation of *PFKFB3* plays a key role. First, PFKFB3 phosphorylation is an immediate early response mechanism. This precedes a later effect on mRNA transcription via the classical role of PR as a transcription factor, which finally leads to an increment in total PFKFB3 protein.

AUTHOR CONTRIBUTION

Laura Novellasdemunt performed the experiments and analysed the data. Mercè Obach performed Organon experiments. Lluís Millán-Ariño and Albert Jordan provided TYML cell lines and contributed to experiments involving ChIP. Anna Manzano, Francesc Ventura and José Luis Rosa revised the paper and participated in the project. Àurea Navarro-Sabaté and Ramon Bartrons designed the experiments, interpreted the results and wrote the paper.

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REFERENCES

1 Gao, X. and Nawaz, Z. (2002) Progesterone receptors – animal models and cell signaling in breast cancer: role of steroid receptor coactivators and corepressors of progesterone receptors in breast cancer. Breast Cancer Res. **4**, 182–186
2 Lange, C. A., Gioeli, D., Hammes, S. R. and Marker, P. C. (2007) Integration of rapid signaling events with steroid hormone receptor action in breast and prostate cancer. Annu. Rev. Physiol. **69**, 171–199
3 Vicent, G. P., Ballaré, C., Nacht, A. S., Clausell, J., Subtil-Rodríguez, A., Quiles, I., Jordan, A. and Beato, M. (2006) Induction of progesterone target genes requires activation of Erk and Msk kinases and phosphorylation of histone H3. Mol. Cell **24**, 367–381
4 Daniel, A. R., Knutson, T. P. and Lange, C. A. (2009) Signaling inputs to progesterone receptor gene regulation and promoter selectivity. Mol. Cell. Endocrinol. **308**, 47–52
5 Owen, G. I., Richer, J. K., Tung, L., Takimoto, G. and Horwitz, K. B. (1998) Progesterone regulates transcription of the p21 (WAF1) cyclin-dependent kinase inhibitor gene through Sp1 and CBP/p300. J. Biol. Chem. **273**, 10696–10701
6 Proietti, C., Salatino, M., Rosemblit, C., Carnevale, R., Pecci, A., Kornblihtt, A. R., Molinolo, A. A., Frahm, I., Charreau, E. H., Schillaci, R. et al. (2005) Progestins induce transcriptional activation of signal transducer and activator of transcription 3 (Stat3) via a Jak- and Src-dependent mechanism in breast cancer cells. Mol. Cell. Biol. **25**, 4826–4840
7 Richer, J. K., Jacobsen, B. M., Manning, N. G., Abel, M. G., Wolf, D. M. and Horwitz, K. B. (2002) Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J. Biol. Chem. **277**, 5209–5218
8 Tseng, L., Tang, M., Wang, Z. and Mazella, J. (2003) Progesterone receptor (hPR) upregulates the fibronectin promoter activity in human decidual fibroblasts. DNA Cell Biol. **22**, 633–640
9 Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M. and Auricchio, F. (1998) Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J. **17**, 2008–2018
10 Vicent, G. P., Nacht, A. S., Zaurín, R., Ballaré, C., Clausell, J. and Beato, M. (2010) Minireview: role of kinases and chromatin remodeling in progesterone signaling to chromatin. Mol. Endocrinol. **24**, 2088–2098
11 Moore, N. L., Narayanan, R. and Weigel, N. L. (2007) Cyclin dependent kinase 2 and the regulation of human progesterone receptor activity. Steroids **72**, 202–209
12 Chalbos, D., Haagensen, D., Parish, T. and Rochefort, H. (1987) Identification and androgen regulation of two proteins released by T47D human breast cancer cells. Cancer Res. **47**, 2787–2792
13 Faivre, E., Skildum, A., Pierson-Mullany, L. and Lange, C. A. (2005) Integration of progesterone receptor mediated rapid signaling and nuclear actions in breast cancer cell models: role of mitogen-activated protein kinases and cell cycle regulators. Steroids **70**, 418–426
14 Subtil-Rodriguez, A., Millán-Ariño, L., Quiles, I., Ballaré, C., Beato, M. and Jordan, A. (2008) Progesterone induction of the 11β-hydroxysteroid dehydrogenase type 2 promoter in breast cancer cells involves coordinated recruitment of STAT5A and progesterone receptor to a distal enhancer and polymerase tracking. Mol. Cell. Biol. **28**, 3830–3849
15 Wade, H. E., Kobayashi, S., Eaton, M. L., Jansen, M. S., Lobenhofer, E. K., Lupien, M., Geistlinger, T. R., Zhu, W., Nevins, J. R., Brown, M. et al. (2010) Multimodal regulation of E2F1 gene expression by progestins. Mol. Cell. Biol. **30**, 1866–1877
16 Quiles, I., Millán-Ariño, L., Subtil-Rodríguez, A., Miñana, B., Spinedi, N., Ballaré, C., Beato, M. and Jordan, A. (2009) Mutational analysis of progesterone receptor functional domains in stable cell lines delineates sets of genes regulated by different mechanisms. Mol. Endocrinol. **23**, 809–826

17 Hamilton, J. A., Callaghan, M. J., Sutherland, R. L. and Watts, C. K. (1997) Identification of PRG1, a novel progestin-responsive gene with sequence homology to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Mol. Endocrinol. **11**, 490–502
18 Ventura, F., Ambrosio, S., Bartrons, R., el-Maghrabi, M. R., Lange, A. J. and Pilkis, S. J. (1995) Cloning and expression of a catalytic core bovine brain 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Biochem. Biophys. Res. Commun. **209**, 1140–1148
19 Manzano, A., Rosa, J. L., Ventura, F., Pérez, J. X., Nadal, M., Estivill, X., Ambrosio, S., Gil, J. and Bartrons, R. (1998) Molecular cloning, expression, and chromosomal localization of a ubiquitously expressed human 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene (PFKFB3). Cytogenet. Cell Genet. **83**, 214–217
20 Chesney, J., Mitchell, R., Benigni, F., Bacher, M., Spiege, L., Al-Abed, Y., Han, J. H., Metz, C. and Bucala, R. (1999) An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect. Proc. Natl. Acad. Sci. U.S.A. **96**, 3047–3052
21 Van Schaftingen, E. (1987) Fructose-2,6-bisphosphate. Adv. Enzymol. Relat. Areas Mol. Biol. **59**, 315–395
22 Rider, M. H., Bertrand, L., Vertommen, D., Michels, P. A., Rousseau, G. G. and Hue, L. (2004) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis. Biochem. J. **381**, 561–579
23 Okar, D. A., Manzano, A., Navarro-Sabaté, A., Riera, L., Bartrons, R. and Lange, A. J. (2001) PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. Trends Biochem. Sci. **26**, 30–35
24 Bartrons, R. and Caro, J. (2007) Hypoxia, glucose metabolism and the Warburg's effect. J. Bioenerg. Biomembr. **39**, 223–229
25 Sakakibara, R., Uemura, M., Hirata, T., Okamura, N. and Kato, M. (1997) Human placental fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase: its isozymic form, expression and characterization. Biosci. Biotechnol. Biochem. **61**, 1949–1952
26 Riera, L., Manzano, A., Navarro-Sabaté, A., Perales, J. C. and Bartrons, R. (2002) Insulin induces PFKFB3 gene expression in HT29 human colon adenocarcinoma cells. Biochim. Biophys. Acta **1589**, 89–92
27 Obach, M., Navarro-Sabaté, A., Caro, J., Kong, X., Duran, J., Gómez, M., Perales, J. C., Ventura, F., Rosa, J. L. and Bartrons, R. (2004) 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. J. Biol. Chem. **279**, 53562–53570
28 Duran, J., Navarro-Sabaté, A., Pujol, A., Perales, J. C., Manzano, A., Obach, M., Gómez, M. and Bartrons, R. (2008) Overexpression of ubiquitous 6-phosphofructo-2-kinase in the liver of transgenic mice results in weight gain. Biochem. Biophys. Res. Commun. **365**, 291–297
29 Duran, J., Obach, M., Navarro-Sabaté, A., Manzano, A., Gómez, M., Rosa, J. L., Ventura, F., Perales, J. C. and Bartrons, R. (2009) Pfkfb3 is transcriptionally upregulated in diabetic mouse liver through proliferative signals. FEBS J. **276**, 4555–4568
30 Yalcin, A., Telang, S., Clem, B. and Chesney, J. (2009) Regulation of glucose metabolism by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases in cancer. Exp. Mol. Pathol. **86**, 174–179
31 Almeida, A., Bolaños, J. P. and Moncada, S. (2010) E3 ubiquitin ligase APC/C-Cdh1 accounts for the Warburg effect by linking glycolysis to cell proliferation. Proc. Natl. Acad. Sci. U.S.A. **107**, 738–741
32 Calvo, M. N., Bartrons, R., Castaño, E., Perales, J. C., Navarro-Sabaté, A. and Manzano, A. (2006) PFKFB3 gene silencing decreases glycolysis, induces cell-cycle delay and inhibits anchorage-independent growth in HeLa cells. FEBS Lett. **580**, 3308–3314
33 Atsumi, T., Chesney, J., Metz, C., Leng, L., Donnelly, S., Makita, Z., Mitchell, R. and Bucala, R. (2002) High expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (iPFK-2; PFKFB3) in human cancers. Cancer Res. **62**, 5881–5887
34 Kessler, R., Bleichert, F., Warnke, J. P. and Eschrich, K. (2008) 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3) is up-regulated in high-grade astrocytomas. J. Neurooncol. **86**, 257–264
35 Van Schaftingen, E., Lederer, B., Bartrons, R. and Hers, H. G. (1982) A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose 2,6-bisphosphate. Eur. J. Biochem. **129**, 191–195
36 Gutmann, I. and Wahlefeld, A. W. (1974) l-(+)-Lactate. In Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 1464–1468, Academic Press, New York
36a Sancho, M., Diani, E., Beato, M. and Jordan, A. (2008) Depletion of human histone H1 variants uncovers specific roles in gene expression and cell growth. PLoS Genet. **4**, e1000227
37 Silverman, E., Frödin, M., Gammeltoft, S. and Maller, J. L. (2004) Activation of p90 Rsk1 is sufficient for differentiation of PC12 cells. Mol. Cell. Biol. **24**, 10573–10583
38 Horwitz, K. B. and McGuire, W. L. (1978) Nuclear mechanisms of estrogen action. Effects of estradiol and anti-estrogens on estrogen receptors and nuclear receptor processing. J. Biol. Chem. **253**, 8185–8191
39 Leighton, I. A., Dalby, K. N., Caudwell, F. B., Cohen, P. T. and Cohen, P. (1995) Comparison of the specificities of p70 S6 kinase and MAPKAP kinase-1 identifies a relatively specific substrate for p70 S6 kinase: the N-terminal kinase domain of MAPKAP kinase-1 is essential for peptide phosphorylation. FEBS Lett. **375**, 289–293
40 Sapkota, G. P., Cummings, L., Newell, F. S., Armstrong, C., Bain, J., Frodin, M., Grauert, M., Hoffmann, M., Schnapp, G., Steegmaier, M. et al. (2007) BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms *in vitro* and *in vivo*. Biochem. J. **401**, 29–38
41 Graham, J. D., Yeates, C., Balleine, R. L., Harvey, S. S., Milliken, J. S., Bilous, A. M. and Clarke, C. L. (1995) Characterization of progesterone receptor A and B expression in human breast cancer. Cancer Res. **55**, 5063–5068
42 Bando, H., Atsumi, T., Nishio, T., Niwa, H., Mishima, S., Shimizu, C., Yoshioka, N., Bucala, R. and Koike, T. (2005) Phosphorylation of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase/PFKFB3 family of glycolytic regulators in human cancer. Clin. Cancer Res. **11**, 5784–5792
43 Marsin, A. S., Bouzin, C., Bertrand, L. and Hue, L. (2002) The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. J. Biol. Chem. **277**, 30778–30783
44 Marsin, A. S., Bertrand, L., Rider, M. H., Deprez, J., Beauloye, C., Vincent, M. F., Van der Berche, G., Carling, D. and Hue, L. (2000) Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Curr. Biol. **10**, 1247–1255
45 Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. and Rider, M. H. (1997) Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J. Biol. Chem. **272**, 17269–17275
46 Rider, M. H., van Damme, J., Vertommen, D., Michel, A., Vandekerckhove, J. and Hue, L. (1992) Evidence for new phosphorylation sites for protein kinase C and cyclic AMP-dependent protein kinase in bovine heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. FEBS Lett. **310**, 139–142
47 Bertrand, L., Alessi, D. R., Deprez, J., Deak, M., Viaene, E., Rider, M. H. and Hue, L. (1999) Heart 6-phosphofructo-2-kinase activation by insulin results from Ser-466 and Ser-483 phosphorylation and requires 3-phosphoinositide-dependent kinase-1, but not protein kinase B. J. Biol. Chem. **274**, 30927–30933
48 Moore, M. R., Zhou, J. L., Blankenship, K. A., Strobl, J. S., Edwards, D. P. and Gentry, R. N. (1997) A sequence in the 5′ flanking region confers progestin responsiveness on the human c-myc gene. J. Steroid Biochem. Mol. Biol. **62**, 243–252
49 Riera, L., Obach, M., Navarro-Sabaté, A., Duran, J., Perales, J. C., Viñals, F., Rosa, J. L., Ventura, F. and Bartrons, R. (2003) Regulation of ubiquitous 6-phosphofructo-2-kinase by the ubiquitin-proteasome proteolytic pathway during myogenic C2C12 cell differentiation. FEBS Lett. **550**, 23–29
50 Yalcin, A., Clem, B. F., Simmons, A., Lane, A., Nelson, K., Clem, A. L., Brock, E., Siow, D., Wattenberg, B., Telang, S. et al. (2009) Nuclear targeting of 6-phosphofructo-2-kinase (PFKFB3) increases proliferation via cyclin-dependent kinases. J. Biol. Chem. **284**, 24223–24232

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